Journal of Bioscience and Bioengineering
VOL. 128 No. 5, 511–517, 2019

ELSEVIER

www.elsevier.com/locate/jbiosc

Bicarbonate and unsaturated fatty acids enhance capsular polysaccharide synthesis gene expression in oral streptococci, *Streptococcus anginosus*

Yuko Matsumoto,[2] Katsuhide Miyake,[1,*] Kento Ozawa,[2] Yasunori Baba,[2] and Takasei Kusube[2]

Department of Environmental Science and Technology, Faculty of Science and Technology, Meijo University, 1-501 Shiogamaguchi, Tenpaku, Nagoya, Aichi 468-8502, Japan[1] *and Research Institute for Bioresources and Biotechnology, Ishikawa Prefectural University, 1-308 Suematsu, Nonoichi, Ishikawa 921-8836, Japan*[2]

Received 21 January 2019; accepted 12 April 2019
Available online 23 May 2019

We recently reported on the capsular polysaccharide (CP) synthesis (*cps*) genes of the oral streptococci, *Streptococcus anginosus*. In this study, we investigate the effects of carbon dioxide (CO_2), bicarbonate (HCO_3^-) and unsaturated fatty acids (UFAs) on CP synthesis of *S. anginosus*. We found that CP production increased when bacteria were exposed to high concentrations of CO_2. This increase was similarly observed in the presence of sodium bicarbonate ($NaHCO_3$) under atmospheric condition. Since ectopic expression of carbonic anhydrase, which converts CO_2 to HCO_3^-, eliminated the requirement for CO_2 in CP production and growth of *S. anginosus* lacking this enzyme, it seemed that HCO_3^- is an essential factor for CP production. Furthermore, UFAs also stimulated the CP production. Promoter-reporter assay and quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis confirmed that stimulation of CP production occurs at the transcription level. The results of the promoter assays suggest that the expression and stimulation of *cps* genes by HCO_3^- or UFAs require the *cpsA* gene, which is located in the first position of the *cps* operon. With respect to the relationship between HCO_3^- and UFAs, HCO_3^- may stimulate UFA synthesis pathway at transcription level. Therefore, it is possible that UFAs are definitive signals for the CP production in *S. anginosus*.

[**Key words:** *Streptococcus anginosus*; Capsular polysaccharide; *cps* genes; Unsaturated fatty acids; CO_2; HCO_3^-]

Oral streptococci, *Streptococcus anginosus* and *Streptococcus intermedius,* are gram-positive bacteria well known as dental plaque forming bacteria (1). These bacteria require a CO_2-rich atmosphere for growth similar to other pathogenic bacteria such as strains of *Streptococcus pneumoniae* and *Haemophilus influenzae* (2). This phenomenon was discovered in the early 20th century (3). However, the reason why these bacteria require high CO_2 levels for growth has been obscure. Recently, Burghout et al. (4) reported that the CO_2 requirement of a certain strain of *S. pneumoniae* is linked to carbonic anhydrase, which catalyzes the reversible hydration of CO_2 to HCO_3^-. They suggested that HCO_3^- may be required for the biosynthesis of unsaturated fatty acids (UFAs), because the addition of either HCO_3^- or UFA partially compensated for the growth inhibition under CO_2-poor conditions. Furthermore, they proposed that long-chain polyglutamyl folate biosynthesis also plays a significant role in the growth of *S. pneumoniae* under CO_2-poor conditions (5). Thus, CO_2 and HCO_3^- appear to be important environmental factors for growth of some pathogenic bacteria, and carbonic anhydrases are considered novel drug targets (6). However, the relation between the expression of pathogenic factors such as capsular polysaccharides (CPs) and toxins, characteristic for these pathogenic bacteria and CO_2 requirement is unknown.

Bacterial CPs are major components of the biofilms that protect bacterial cells from environmental stresses. They also play an important role in pathogenicity. Oral streptococci also utilize CPs for forming dental plaques (1). Biosynthetic pathways of CPs and CP synthesis (*cps*) genes have been studied extensively in many streptococci species containing oral streptococci, as well as in other bacteria (7–11). CPs of many streptococcal species are synthesized by the Wzx/Wzy-dependent pathway, and the *cps* gene clusters for the pathway consist of four regulatory genes, *cpsA-D*, and downstream capsule biosynthesis genes encoding glycosyltransferases, the Wzx flippase and the Wzy polymerase. Among the regulatory genes, *cpsA* codes for a membrane bound transcription factor and is required for transcription of the *cps* operon (12). CpsB–D proteins sense the presence of oxygen (O_2) and directly influence CP biosynthetic pathways through tyrosine phosphorylation (13). To date, several environmental factors, such as O_2 and glucose levels, have been reported to regulate CP production (13,14). However, environmental factors directly affecting the transcription of the *cps* genes are not well elucidated.

The CO_2-dependent mechanism of growth and CP synthesis of oral streptococci is not well understood. In this study, we analyzed the influence of CO_2, HCO_3^- and UFAs on CP synthesis in the oral streptococcus, *S. anginosus*. We demonstrate that CO_2, HCO_3^-, and UFAs transcriptionally regulate CP synthesis in this bacterium.

MATERIALS AND METHODS

Bacterial strains, media and plasmids *S. anginosus* ATCC 33397 was cultured in Brain Heart Infusion Broth (BHI) (Nissui, Tokyo, Japan) under 5% CO_2 at 37°C. $NaHCO_3$ was added at 10 mM or 50 mM at atmospheric condition. Fatty acids were added at 10 μM in the presence of 0.1% Tween 40 as described previously (4).

* Corresponding author. Tel.: +81 52 838 2365; fax: +81 52 832 1179.
E-mail address: miyake@meijo-u.ac.jp (K. Miyake).

1389-1723/$ – see front matter
https://doi.org/10.1016/j.jbiosc.2019.04.010

Bacterial strains harboring pLSE1-derived plasmids were cultured in the presence of erythromycin (2 μg/ml). *Streptococcus agalactiae* type Ia strain was cultured in Todd-Hewitt broth as described previously (7). *E. coli* C600 and DH5α strains were used as plasmid hosts for constructing the overexpression plasmids. All *E. coli* clones were cultured in Luria–Bertani broth containing ampicillin (100 μg/ml) or tetracycline (14 μg/ml).

DNA manipulation Most DNA manipulations were performed using standard molecular biological methods. *S. anginosus* and *S. agalactiae* chromosomal DNAs were isolated as reported previously (7,10). Chromosomal DNA of *Lactococcus lactis* was a kind gift from Dr. Katayama at Ishikawa Prefectural University. PCR was performed with KOD -Plus- Neo (Toyobo, Osaka, Japan) and DNA fragments were prepared using MagExtractor–PCR & Gel Clean up (Toyobo). PCR cycling parameters were as follows: initial denaturation at 96°C for 2 min; 35 cycles at 98°C for 10 s, 50°C for 30 s, 68°C for time set to 1 min per 2 kb of target length. DNA sequences were determined by Eurofins Genomics sequencing service (Tokyo, Japan).

Construction of a plasmid used for protein expression To construct a plasmid for the expression of carbonic anhydrase, we amplified the gene from *S. agalactiae* type Ia chromosomal DNA using primers (5′-CATAGATCTAGGAGGATG**GACTACAAGGATGACGATGACAAG**ACCACTTATTTTGATAACTT-3′ and 5′-CATGCTAGCCTATTTAACTTCCACCATAC-3′). Restriction enzyme sites are underlined and FLAG tag sequence is indicated by bold letters. For ectopic expression of the carbonic anhydrase gene, we used the P23 promoter of *L. lactis*. The P23 promoter region was amplified from genomic DNA of *L. lactis* using primers (5′-CATGCGGCCGCGAAAAGCCCTGACAACCCT-3′ and 5′-CATAGATCTTGTAATTCTCCAATACTTA-3′). The amplified P23 promoter DNA was digested with *Not*I and *Bgl*II, and introduced into the pLSE1 vector (15) using T4 DNA ligase (pLSE1/P23). The amplified carbonic anhydrase gene was digested with *Bgl*II and *Nhe*I, and introduced into the downstream of the P23 promoter in the pLSE1/P23. The constructed plasmid was then transformed into *S. anginosus* by electroporation as described previously (16).

Construction of promoter reporter vectors To construct promoter reporter vectors for the *cps* gene cluster, promoter regions were amplified from *S. anginosus* ATCC 33397 and *S. agalactiae* type Ia chromosomal DNAs using the following primers: 5′-CATGCGGCCGCTGAATGATGGCACAGAAAGT-3′ and 5′-CATGGATCCTTAAAACTCCTATGCAAAAAT-3′ for the P1 promoter of *S anginosus*, 5′-CATGCGGCCGCTTGTCGCGCATATGACAGAA-3′ and 5′-CATGGATCCTGTAATTCTCCAATACTTA-3′ for the P1 promoter of *S agalactiae*, 5′-CATGCGGCCGCTGAATGATGGCACAGAAAGT-3′ and 5′-CTTAGATCTTTATTTCCCTTCCATCACTTT-3′ for the DNA region containing the P1 promoter and *cpsA* of *S anginosus*, 5′-CATGCGGCCGCCCATCTCAGTCTCAAGGTTCC-3′ and 5′-CATGGATCCTTATTCCTCCATTGTGTTCTTAAT-3′ for the DNA region containing the P1 promoter and *cpsA* of *S agalactiae*, 5′-CATGCGGCCGCTGAATGATGGCACAGAAAGT-3′ and 5′-CTTAGATCTCCCTACTCCTAAATGCTAATCTC-3′ for the DNA region between the P1 promoter and the P2 promoter of *S. anginosus*, 5′-CATGCGGCCGCCCATCTCAGTCTCAAGGTTCC-3′ and 5′-CTTGGATCTTTTAAACTCTTCAAGATAGCCACG-3′ for the DNA region between the P1 promoter and the P2 promoter of *S. agalactiae*, 5′-CATGCGGCCGCACAAGTCTCACCCAATCCAAC-3′ and 5′-CTTAGATCTCCCTACTCCTAAATGCTAATCTC-3′ for the P2 promoter of *S. anginosus*, and 5′-CATGCGGCCGCTATTCCTTGTTAATAATG-3′ and 5′-CATGCGGCCGCGATTTCCTCCTTATGAAA-3′ for promoter of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene of *S. anginosus*. Restriction enzyme sites are underlined. The DNA target sequences were ligated into pLSE1 plasmids. To introduce the Green Fluorescent Protein (GFP) gene downstream of the promoter DNA sequences, the GFP gene was amplified from pGFPuv (Takara, Shiga, Japan) using primers (5′-CATAGATCTAGGAGGATGAGTAAAGGAGAAGAACT-3′ and 5′-CATGCTAGCTTATTTGTAGAGCTCATCCAT-3′).

Western blotting Western blot analysis was used to examine exogenous protein expression and the results of the promoter reporter assays. Protein samples were prepared by sonicating bacterial cells in 8 M Urea buffer (8 M Urea, 30 mM Tris, pH 8.5). Bacterial cells were disrupted using the Q Sonica 500 sonicator (Waken, Tokyo, Japan) with 1.6 mm tip under 40% amplitude and 50% pulse for 1 min. To confirm exogenous protein expression of carbonic anhydrase, anti-DYKDDDDK (FLAG) tag monoclonal antibody (1E6, Wako, Osaka, Japan) was used at a dilution ratio of 1:2000. For the promoter reporter assay, GFP expression was analyzed by using anti-GFP monoclonal antibody (mFX75, Wako) at a dilution ratio of 1:1500. Goat anti-mouse IgG-HRP (sc-2005, Santa Cruz Biotechnology Inc., Dallas, TX, USA) was used as a secondary antibody at a dilution ratio of 1:2000. Antibodies were diluted with Can Get Signal Immunoreaction Enhancer Solution (Toyobo). Protein concentrations were determined using the TaKaRa BCA Protein Assay Kit (Takara).

ELISA analysis for CP production To estimate CP production, the ELISA method was used. *S. anginosus* cells were harvested in the late exponential growth phase at an optical density (OD_{600}) of around 1.0. Cell cultures were diluted to 1: 500 with phosphate buffered saline (PBS) and 50 μl of the diluted samples were added to ELISA plates pre-coated with 10 μg/ml of poly-D-lysine. After drying, 200 μl of 5% acetic anhydride in saturated sodium carbonate was added to each well to remove extra poly-D-lysine amino groups. Samples were incubated for 1 h at room temperature. After washing the wells with 200 μl of PBS 3 times, the wells were blocked with 5% skim milk in 200 μl of PBS containing 0.05% Tween 20 (PBST) at 4°C for 12 h, and then washed again with 200 μl of PBST 3 times. Rabbit antiserum against *S. anginosus* ATCC 33397 was added (1: 50,000 dilution in 200 μl of PBS) (10). The ELISA plates were kept at room temperature for 1 h before adding secondary antibody; goat anti-rabbit IgG-HRP (sc-2004, Santa Cruz Biotechnology Inc.) was used at a dilution of 1: 5000 in 200 μl of PBS. After washing with 200 μl of PBST 3 times, the reaction was developed using 200 μl of 10 μg/ml of *o*-phenylenediamine as a substrate. Once developed, the reaction was stopped using 50 μl of 8 M sulfuric acid and absorbance readings were determined at 490 nm. The CP amount was standardized with protein concentration or bacterial amount (OD_{600}). *S. anginosus* cultures were divided to two aliquots for measuring ELISA and protein concentration, respectively. To determine protein concentration, 6 ml of *S. anginosus* culture was centrifuged at 10,000 ×*g* for 10 min and harvested bacterial cells were dissolved in 2 ml of PBS. Then, cells were disrupted using the Q Sonica 500 sonicator (Waken) with 1.6 mm tip under 30% amplitude and 50% pulse for 2 min. After removing cell debris by centrifugation at 10,000 ×*g* for 5 min, supernatants were prepared for analyzing protein concentration. Protein concentrations of the samples were measured using optical density at 280 nm.

Quantitative reverse transcription PCR For RNA isolation, *S. anginosus* cultures were grown to OD_{600} of 1. After treatment with RNAprotect Bacteria Reagent (Qiagen, Tokyo, Japan), RNA extraction was performed with RNeasy Midi Kit (Qiagen). RNAs (1 μg) were reverse transcribed using ReverTra Ace (Toyobo) and 9-mer random primers. From the cDNA obtained, quantitative gene expression was analyzed using KOD-SYBR qPCR Mix (Toyobo). Specific primers were designed with the Primer Express 2.0 program to amplify internal regions of *cpsE* (5′-ATTGGTTCTCTCTTTGGACTTCTT-3′ and 5′-TCCGCCATCTTTACGAATCAT-3′), *fabK* (5′-CGGGTGCTCTGGCAAATG-3′ and 5′-CAAGCCCCGCAATTTGTC-3′) and *fabM* (5′-TCTAAGCCGGTCGTTATGAGTGT-3′ and 5′-CCGCGGCGACTACCATATTA-3′) genes. The expression level of an endogenous control gene (16s rRNA) was assayed with primers (5′-GGGACTTAGTGCCGCAGCTA-3′ and 5′-TTTCAACCTTGCGGTCGTACT-3′). Amplification was performed in triplicate using a 7300 Real Time PCR system (Applied Biosystems, Tokyo, Japan). PCR cycling parameters were as follows: initial denaturation at 96°C for 2 min; 40 cycles at 98°C for 10 s, 60°C for 10 s, 68°C for 30 s. To measure gene expression, relative expression of the 16s rRNA gene was compared with that of the target genes. Relative expression levels were calculated using the comparative C_T method ($\Delta\Delta C_T$ method) (17).

RESULTS

CO_2 enhances CP production *S. anginosus* requires 5% CO_2 for sufficient growth (18). To analyze the effects of CO_2 on CP production, *S. anginosus* was cultured under 5% and 0% (atmospheric) CO_2 conditions. Bacterial growth was measured with optical density readings at OD_{600}. CP production was analyzed by ELISA using anti-*S. anginosus* ATCC 33397 rabbit antiserum. The quantity of CP was standardized with protein concentration or OD_{600} of each sample. Since well-encapsulated and poorly-encapsulated bacteria may not be destroyed equally by the same sonication condition, standardization with OD_{600} was also performed in several assays (Fig. S1). The value of CP was expressed with dividing ELISA value by absorbance at 280 nm or OD_{600}. As shown in Fig. 1A, growth of *S. anginosus* was significantly enhanced by the presence of CO_2. Fig. 1B shows that CO_2 also enhanced CP production. These results demonstrate that CO_2 is also required for CP production as well as growth. When the CO_2 concentration was raised to 10%, no further enhancement was observed in bacterial growth and CP production (Figs. S1A and S1B). On the other hand, low concentration of CO_2 (0.1%) showed clear enhancement (Figs. S1A and S1B). Even 0.1% CO_2 seemed to be enough for sufficient bacterial growth and CP production.

HCO_3^- can substitute for CO_2 To investigate whether the effects of CO_2 are caused by HCO_3^-, which is a water-soluble form of CO_2, $NaHCO_3$ was added to *S. anginosus* cultures at 10 mM and 50 mM under atmospheric conditions. As shown in Fig. 2A and B, $NaHCO_3$ was able to stimulate both growth and CP production in the absence of rich CO_2. The increase in these levels was similar to that at 5% CO_2. Our results suggest that the CO_2 effects observed may be mostly due to the presence of HCO_3^-. CO_2 is

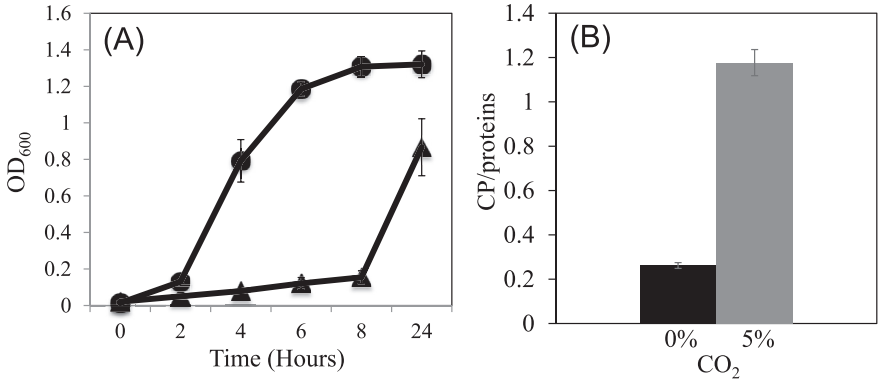

FIG. 1. The effects of CO_2 abundance on the growth and CP production of *S. anginosus*. (A) Growth of *S. anginosus* in BHI medium under rich CO_2 (5%) (closed circles) and atmospheric CO_2 (0%) (closed triangles). (B) CP production of *S. anginosus* under rich CO_2 (5%) and atmospheric CO_2 (0%). The amount of CP was measured by ELISA and normalized against the protein concentration. The value of CP was expressed with dividing ELISA value by absorbance at 280 nm. The data are plotted as averages with standard deviations from three independent assays.

normally present as gaseous form in water and conversion to HCO_3^- rarely occurs because equilibrium strongly favors CO_2 over HCO_3^-. We focused on the enzyme, carbonic anhydrase, which catalyzes the conversion between the two forms. It was observed that there is no gene similar to that of carbonic anhydrase in the genome of *S. anginosus* (NC_022239). However, the gene is present in the genome of other streptococci, *S. agalactiae* (HG939456), and we isolated this gene from *S. agalactiae* type Ia strain (7). After amplifying the carbonic anhydrase gene from chromosomal DNA of *S. agalactiae*, the gene was fused with the P23 promoter of *L. lactis*, which is known to function well in *Streptococcus* species (19). A pLSE1 plasmid containing the P23 promoter and the carbonic anhydrase gene was introduced into *S. anginosus*. As shown in Fig. 2C, *S. anginosus* harboring this plasmid expressed carbonic anhydrase (18 kD), which was confirmed by Western blot analysis using anti-DYKDDDDK tag antibody. The recombinant strain showed rapid growth (Fig. 2D) and high CP production under atmospheric conditions (Fig. 2E). This result also suggests that CO_2 functions to enhance growth and CP production after converting to HCO_3^- in bacterial cells.

UFAs can also substitute for CO_2 We were subsequently interested in how the presence of HCO_3^- enhances growth and CP production. Other study has demonstrated that UFAs can support bacterial growth of *S. pneumoniae* strains requiring CO_2 under low CO_2 conditions (4). Therefore, we also investigated the effect of UFAs on the growth and CP production of *S. anginosus*. The addition of fatty acids was performed in the presence of Tween 40 to improve fatty acid solubility. Our results show that UFAs such as oleic acid, linoleic acid and linolenic acid significantly

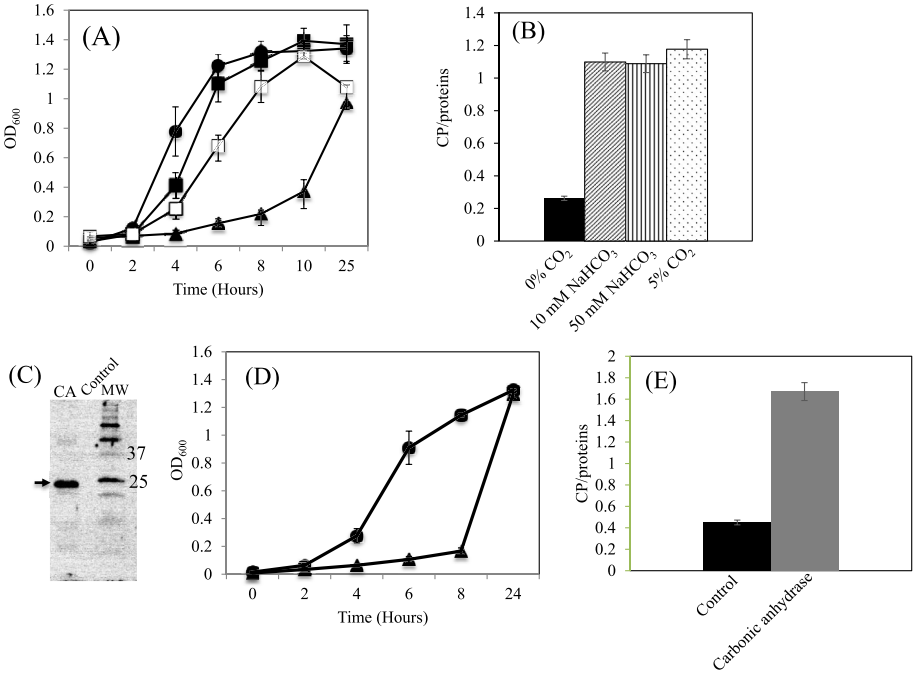

FIG. 2. The effects of the addition of $NaHCO_3$ on the growth and CP production of *S. anginosus*. (A) Growth of *S. anginosus* in BHI medium complemented with 10 mM (closed squares) or 50 mM $NaHCO_3$ (open squares) under atmospheric conditions. The results were compared with those of 5% (closed circles) and 0% (closed triangles) CO_2. (B) CP production of *S. anginosus* in BHI medium complemented with 10 mM or 50 mM $NaHCO_3$. (C) Ectopic expression of carbonic anhydrase in *S. anginosus*. *S. anginosus* containing expression (CA) and blank (control) plasmids were analysed for carbonic anhydrase expression by Western blot using anti-DYKDDDDK tag antibody. Loaded protein samples were adjusted to 5 μg/lane. Precision plus Protein standard (Bio-Rad, Hercules, CA, USA) was used as a molecular weight marker. (D) Growth of *S. anginosus* containing the plasmid for forced expression of carbonic anhydrase (closed circles) and control plasmid (closed triangles) under atmospheric condition. (E) CP production of *S. anginosus* containing the plasmid expressing carbonic anhydrase. The data are plotted as averages with standard deviations from three independent assays.

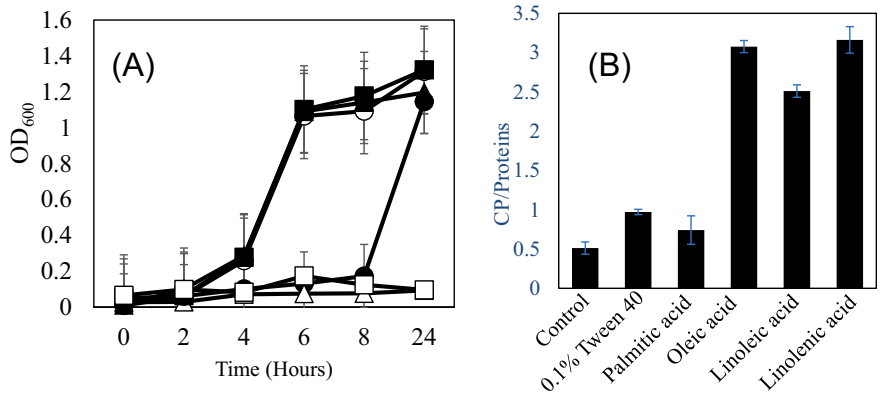

FIG. 3. The effects of the addition of UFAs on the growth and CP production of *S. anginosus*. (A) Growth of *S. anginosus* in BHI medium complemented with 10 μM oleic acid (open circles), linoleic acid (closed triangles), linolenic acid (closed squares), palmitic acid (open triangles) and stearic acid (open squares) under atmospheric conditions. *S. anginosus* was also cultured without any addition under atmospheric condition (closed circles). (B) CP production of *S. anginosus* in BHI medium complemented with 10 μM FAs. Tween 40 (0.1%) was added to all FA samples. The data are plotted as averages with standard deviations from three independent assays.

stimulated bacterial growth and CP production (Figs. 3 and S1C). Conversely, saturated fatty acids (SFAs) such as palmitic acid and stearic acid showed inhibitory effect on bacterial growth (Fig. 3A). Furthermore, palmitic acid did not show any effect on CP production of *S. anginosus* (Fig. 3B). The reason why SFAs exhibited growth inhibitory effect is still obscure. These results suggested that UFAs can function for growth and CP production instead of CO_2 and HCO_3^-.

CO_2, $NaHCO_3$ and UFAs enhance transcription of *cps* genes in *S. anginosus* We previously reported on the *cps* gene cluster of *S. anginosus* and its contribution to CP production (Fig. 4A) (10). To investigate whether the effects of CO_2, $NaHCO_3$ and UFAs on CP production occur at transcription level, we analyzed the transcription of the *cps* operon by quantitative reverse transcription PCR (RT-qPCR). The expression of the *cpsE* gene coding for glucosyltransferase was analyzed. As shown in Fig. 4B and C, transcription of the gene was significantly enhanced by the presence of CO_2, $NaHCO_3$ and UFAs. Overexpression of carbonic anhydrase also enhanced the *cpsE* gene transcription (Fig. 4D). The data indicates that the response of the bacteria to CO_2, $NaHCO_3$ and UFAs occurs at transcription level.

Promoter activity of the *cps* gene cluster was enhanced by CO_2, $NaHCO_3$ and UFA We investigated the effects of CO_2, HCO_3^- and UFA on promoter activity of the *cps* operon to determine if the environmental factors affect *cps* transcription by regulating promoter activity. To evaluate the promoter activities of the *cps* operon, we constructed 4 different promoter-reporter vectors (Fig. 4A). The first, cpsP1, contains only the promoter region of the entire *cps* gene cluster existing in front of the operon. The second, cpsP1-A, contains the promoter and *cpsA* gene, the first gene of the operon. The third, cpsP1-P2, contains the DNA region from the start of the first promoter through the end of the second promoter. The second promoter is located at the start of the *cpsE* gene. The third promoter construct also includes *cpsA*, *cpsB*, *cpsC* and *cpsD*. CpsA is believed to be a membrane bound transcription factor contributing to *cps* expression (12). CpsB–D are thought to be regulators that may control CP length or production through tyrosine phosphorylation sensing O_2 concentration (13). The fourth, cpsP2, contains only the putative second promoter. As a control promoter, we used the promoter for *S. anginosus* GAPDH gene, which is a house keeping gene. These promoter regions were introduced in upstream of the GFPuv gene. We analyzed promoter activities by studying the expression of the GFP gene. *S. anginosus* containing these promoter reporter vectors were cultured under various conditions. Western blot analyses using anti-GFP antibody showed that expression of the *cps* operon requires not only the promoter region but also the *cpsA* gene. As shown in Fig. 4E, constructs containing only *cps* promoters did not show any GFP production. On the other hand, the cpsP1-A construct showed GFP expression, and the expression was enhanced with addition of CO_2, HCO_3^- and UFA. This result indicated that *cpsA* is required for the transcription from the *cps* promoter as described previously (12). The membrane bound transcription factor may sense these environmental factors. Furthermore, the cpsP1-P2 construct exhibited more expression than the cpsP1-A construct. This suggested that *cpsB–D* enhances the transcription from the *cps* promoter by affecting CpsA function. The GAPDH promoter construct showed constant GFP expression regardless of conditions. These results were consistent with those from the transcriptional analyses of *cpsE*. CO_2, HCO_3^- and UFA seem to affect *cps* transcription by regulating its promoter activity.

HCO_3^- regulates UFA synthesis-related genes To investigate the possible relationship between the presence of HCO_3^- and UFA synthesis, we performed RT-qPCR to study the influences of HCO_3^- on the expression of UFA synthesis-related genes by RT-qPCR. Marrakchi et al. (20) previously reported that FabK and FabM could function on *trans*-2-decenoyl ACP for SFA synthesis and UFA synthesis in *S. pneumoniae*, respectively (Fig. 5A). In the present study, expression of *fabK* and *fabM* was compared under variable conditions. As shown in Fig. 5B, HCO_3^- activated the transcription of the *fabM* gene. Conversely, expression of the *fabK* gene was reduced in the presence of HCO_3^-. Because these two enzymes function at a branch point in the fatty acid synthetic pathway, the increase of *fabM* expression and the reduction of *fabK* expression might result in enhancement of UFA synthesis. These results suggest that CO_2 is converted to HCO_3^-, and HCO_3^- stimulates UFA synthesis. The marked reduction in transcription levels of both genes in the presence of linoleic acid appears to be caused by feedback inhibition.

DISCUSSION

In the present study, we analysed the relationship between CO_2 concentration and CP production in oral streptococci, *S. anginosus*. We found that CO_2 enhanced not only bacterial growth but also CP production. Interestingly, this phenomenon also occurs with the addition of HCO_3^- and UFAs in the place of 5% CO_2. As overexpression of carbonic anhydrase also stimulated bacterial growth and CP production under low CO_2 conditions, in an atmospheric

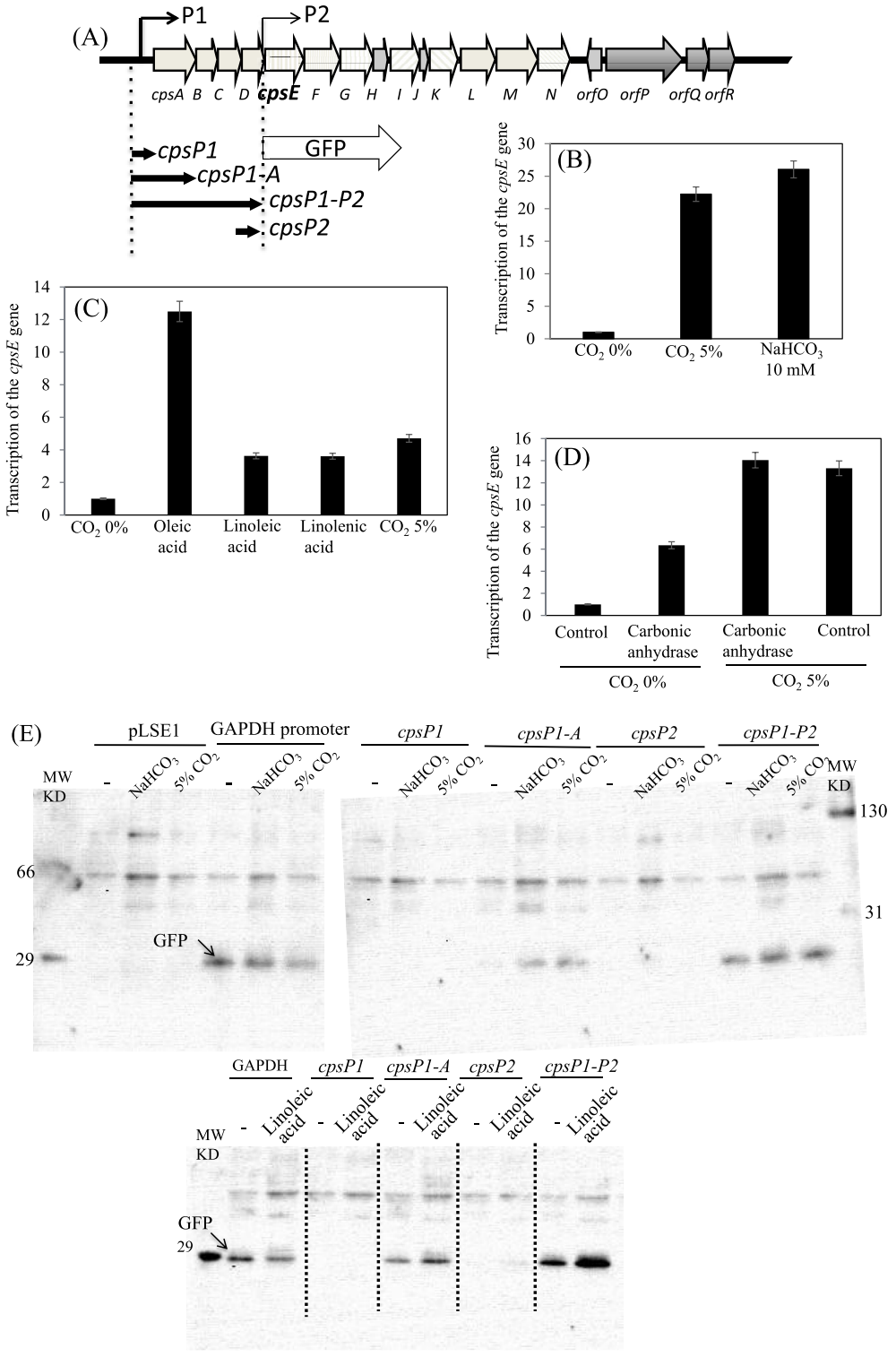

FIG. 4. Transcription of the *cps* operon under various conditions. (A) Structures of the *cps* operon of *S. anginosus* and promoter reporter vectors. (B) Transcription of the *cpsE* gene under atmospheric condition (0% CO_2), 5% CO_2 and 10 mM $NaHCO_3$ (atmospheric condition) was measured by RT-qPCR. The expression level of the control (0% CO_2) was defined as 1. (C) Transcription of the *cpsE* gene under atmospheric condition with 10 μM UFAs was measured by RT-qPCR. The expression level of the control (0% CO_2) was defined as 1. (D) Transcription of the *cpsE* gene of *S. anginosus* ectopically expressing carbonic anhydrase measured by RT-qPCR. The expression level of the control (0% CO_2, empty vector) was defined as 1. (E) Promoter activities of the *cps* operon under various conditions. Western blotting was performed to analyze GFP expression by using anti-GFP antibody. Loaded protein samples were adjusted to 8 μg/lane. Precision plus Protein standard (Bio-Rad) was used as a molecular weight marker.

environment, the effects of CO_2 seem to be caused by HCO_3^- converted from CO_2. The effect of these factors on bacterial growth has been reported in *S. pneumoniae* strains lacking carbonic anhydrase (4,5). Our study is the first to report the influence of these factors on CP production in an oral streptococcus. In this study, we standardized CP amount with protein concentration, because *S. anginosus* tends to agglutinate under poor CO_2 condition. Therefore, standardization with bacterial amount (OD_{600}) may be

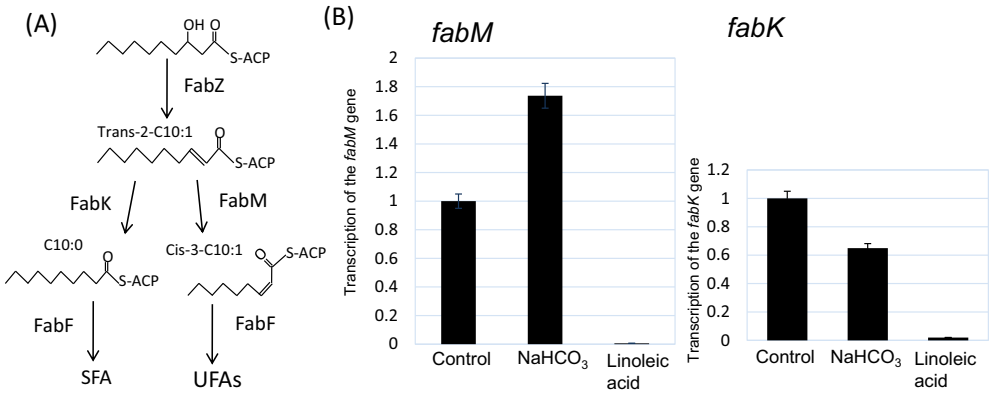

FIG. 5. HCO_3^- affects fatty acid synthesis pathway. (A) Putative UFA synthesis pathway in *S. anginosus*. (B) Transcriptions of the *fabK* and *fabM* genes of *S. anginosus* were determined by RT-qPCR under atmospheric conditions with addition of 10 mM $NaHCO_3$ or 10 μM linoleic acid, or without any addition (control). The expression level of the control sample was defined as 1. The data are shown as averages with standard deviations from three independent assays.

inaccurate especially under CO_2 poor condition though the standardization seems not to be affected by sonication conditions. According to our results, CP production standardized with bacterial amount shows similar tendency to that standardized with protein concentration though the difference is less in the former than that in the latter (Figs. 1B and S1B–D). Probably, this seems to be due to the difference of the extent of cell disruption by sonication between well-encapsulated and poorly-encapsulated *S. anginosus*. In fact, the values of protein concentration / OD_{600} seem to decrease as CP amount increases (Fig. S2). However, in any case, it would be certain that CP amount of *S. anginosus* increases in response to CO_2 or UFAs. The results of RT-qPCR analysis also support this conclusion. Direct observation by electron microscope will be very helpful.

A previous report suggests that the positive influence of CO_2 on CP production in *S. pneumoniae* may be due to a decrease in oxygen availability accompanied by an increase in CO_2 concentration (13). Our results indicate that CP production in *S. anginosus* seems to be affected by CO_2 availability, and not by O_2 availability. We confirmed that *S. anginosus* does not show clear enhancement of growth and CP production under 100% N_2 condition, in our preliminary experiment (Fig. S3). While oxygen-mediated regulation of CP production seems to occur through protein phosphorylation of CpsD in *S. pneumoniae*, and not through gene regulation (13), CP production in *S. anginosus* was found to be regulated by CO_2 at transcriptional level. The previous study reported that there are two types of *S. pneumoniae* strains showing low O_2 -related increase in CP production (O type) and not showing any enhancement (T type) (13). The authors of the previous study speculated that the difference between O and T strains may be due to *cps* expression. However, the authors were not able to clarify the reason behind the *cps* gene expression being affected. In the present study, we showed that the presence of CO_2, HCO_3^- and UFAs can stimulate transcription of the *cps* gene in *S. anginosus*. These factors may also play some roles in expression of the *cps* genes in different *S. pneumoniae* strains.

In this study, we found that expression of the *cps* operon requires the presence of the *cpsA* gene. This is consistent with the previous study (12). Furthermore, *cpsB–D* genes seem to enhance the *cpsA* mediated transcription. Thus, *cpsB–D* genes might also possess functions enhancing the transcription of the *cps* operon. As far as we know, there is no report showing relationship between these genes and transcription of the *cps* operons. These genes may regulate CpsA function by tyrosine phosphorylation or direct interaction. As CpsA is a membrane bound transcription factor for the *cps* gene cluster, the protein may sense the presence of UFA or HCO_3^- and use them to express the *cps* genes. The membrane protein may recognize UFAs, although it is still unclear whether the protein directly binds UFAs or indirectly senses them through compositional changes in membrane fatty acids. To further clarify this, a binding experiment using ligands such as radioisotope-labeled UFAs will be required.

We also constructed similar promoter reporter vectors derived from the *cps* gene cluster of other streptococci, *S. agalactiae* (7) and studied the expression in *S. anginosus*. *S. agalactiae* does not require CO_2 for sufficient growth. These constructs did not show any expression regardless of the presence of CO_2, HCO_3^- or UFAs (Fig. S4). This result suggested that the regulatory system sensing these environmental factors is specific for *S. anginosus*. It is interesting to clarify which factors including CpsA, promoter sequence or other transcription factors are important for the sensing.

With respect to the relationship between CO_2 and UFAs, there is a speculation that CO_2 or HCO_3^- may positively regulate the biosynthetic pathway of UFAs. It is also possible that HCO_3^- acts as a substrate for the biosynthesis of UFAs. Burghout et al. described that an HCO_3^--mediated pathway for the synthesis of SFA and UFA should be common in *S. pneumoniae*, and the dependency of strains lacking carbonic anhydrase (*Δpca*) on supplied UFAs may be due to the depletion of UFAs in growth media because UFAs are more readily depleted (4). However, other study suggested that there is a specific branched pathway for UFA biosynthesis in *S. pneumoniae* (20). Our results demonstrated that CO_2 or HCO_3^- might stimulate the UFA synthesis branch by regulating expression of synthetic enzymes (FabM and FabK) existing at the branch point. *S. anginosus* usually exists in oral CO_2-rich environment. In this condition, the bacteria may be able to sense CO_2 or converted HCO_3^- to stimulate UFA synthesis. Then, UFAs might enhance CP production to protect bacterial cells from host immune system. Although other functions of UFAs such as influences on physical characteristics of cell membrane may be also important, further study will be required to clarify this. From these points of view, UFAs may be regarded as signal transduction molecules rather than environmental factors. Synthesized UFAs would accumulate in cell membrane and be able to affect CpsA. Alternatively, a part of the UFAs may be secreted to outside of bacterial cells and influence other cells as environmental factors.

In the present study, we first reported that CP production of streptococci is regulated by environmental factors at transcription level. Many bacteria producing CPs are known to have similar *cps* gene clusters and these operons always have *cpsA-D* type genes in the first part. Therefore, these bacteria may use *cpsA-D* genes for sensing some environmental factors to produce CPs. CPs of many pathogenic bacteria are thought to be good materials for the

development of sugar pharmaceuticals because these often confer resistance to complement-mediated opsonophagocytosis and mimic host diverse polysaccharides to avoid the specific immune response of hosts (21,22). CO_2, HCO_3^- and UFAs may be able to synergistically enhance productivity of CPs in several bacteria.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbiosc.2019.04.010.

ACKNOWLEDGMENTS

This study was supported by the Nagase Science Technology Foundation. The authors declare no conflict of interest.

References

1. **Cisar, J. O., Kolenbrander, P. E., and McIntire, F. C.:** Specificity of coaggregation reactions between human oral streptococci and strains of *Actinomyces viscosus* or *Actinomyces naeslundii*, Infect. Immun., **24**, 742–752 (1979).
2. **Flanagan, P. G. and Paull, A.:** Carbon dioxide requirements of *Streptococcus pneumoniae* and *Haemophilus influenzae*, J. Antimicrob. Chemother., **42**, 669–670 (1998).
3. **Kempner, W. and Schlayer, C.:** Effect of CO_2 on the growth rate of the pneumococcus, J. Bacteriol., **43**, 387–396 (1942).
4. **Burghout, P., Cron, L. E., Gradstedt, H., Quintero, B., Simonetti, E., Bijlsma, J. J. E., Bootsma, H. J., and Hermans, P. W. M.:** Carbonic anhydrase is essential for *Streptococcus pneumoniae* growth in environmental ambient air, J. Bacteriol., **192**, 4054–4062 (2010).
5. **Burghout, P., Zomer, A., van der Gaast-de Jongh, C. E., Janssen-Megens, E. M., Francoijs, K. J., Stunnenberg, H. G., and Hermans, P. W. M.:** *Streptococcus pneumoniae* folate biosynthesis responds to environmental CO_2 levels, J. Bacteriol., **195**, 1573–1582 (2013).
6. **Supuran, C. T.:** Bacterial carbonic anhydrases as drug targets: toward novel antibiotics? Front. Pharmacol., **2**, 34 (2011).
7. **Yamamoto, S., Miyake, K., Koike, Y., Watanabe, M., Machida, Y., Ohta, M., and Iijima, S.:** Molecular characterization of type-specific capsular polysaccharide biosynthesis genes of *Streptococcus agalactiae* type Ia, J. Bacteriol., **181**, 5176–5184 (1999).
8. **Kolkman, M. A. B., Wakarchuk, W., Nuijten, P. J. M., and van der Zeijst, B. A. M.:** Capsular polysaccharide synthesis in *Streptococcus pneumoniae* serotype 14: molecular analysis of the complete *cps* locus and identification of genes encoding glycosyltransferases required for the biosynthesis of the tetrasaccharide subunit, Mol. Microbiol., **26**, 197–208 (1997).
9. **Glucksmann, M. A., Reuber, T. L., and Walker, G. C.:** Family of glycosyl transferases needed for the synthesis of succinoglycan by *Rhizobium meliloti*, J. Bacteriol., **175**, 7033–7044 (1993).
10. **Tsunashima, H., Miyake, K., Motono, M., and Iijima, S.:** Organization of the capsule biosynthesis gene locus of the oral streptococcus *anginosus*, J. Biosci. Bioeng., **113**, 271–278 (2012).
11. **Yoshida, Y., Yang, J., Peaker, P. E., Kato, H., Bush, C. A., and Cisar, J. O.:** Molecular and antigenic characterization of a *Streptococcus oralis* coaggregation receptor polysaccharide by carbohydrate engineering in *Streptococcus gordonii*, J. Biol. Chem., **283**, 12654–12664 (2008).
12. **Hanson, B. R., Lowe, B. A., and Neely, M. N.:** Membrane topology and DNA-binding ability of the streptococcal CpsA protein, J. Bacteriol., **193**, 411–420 (2011).
13. **Weiser, J. N., Bae, D., Epino, H., Gordon, S. B., Kapoor, M., Zenewicz, L. A., and Shchepetov, M.:** Changes in availability of oxygen accentuate differences in capsular polysaccharide expression by phenotypic variants and clinical isolates of *Streptococcus pneumoniae*, Infect. Immun., **69**, 5430–5439 (2001).
14. **Willenborg, J., Fulde, M., de Greeff, A., Rohde, M., Smith, H. E., Valentin-Weigand, P., and Goethe, R.:** Role of glucose and CcpA in capsule expression and virulence of *Streptococcus suis*, Microbiology, **157**, 1823–1833 (2011).
15. **Diaz, E. and Garcia, J. L.:** Construction of a broad-host-range pneumococcal promoter-probe plasmid, Gene, **90**, 163–167 (1990).
16. **Dunny, G. M., Lee, L. N., and LeBlanc, D. J.:** Improved electroporation and cloning vector system for gram-positive bacteria, Appl. Environ. Microbiol., **57**, 1194–1201 (1991).
17. **Livak, K. J. and Schmittgen, T. D.:** Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}{}_T$ method, Methods, **25**, 402–408 (2001).
18. **Hardie, J. M.:** Oral streptococci, p. 1054, in: Sneath, P. H. A., Mair, N. S., Sharpe, M. E., and Holt, J. G. (Eds.), Bergey's manual of systematic bacteriology, vol. 2. The Williams & Wilkins Co., Baltimore, MD (1986).
19. **Biswas, I., Jha, J. K., and Fromm, N.:** Shuttle expression plasmids for genetic studies in *Streptococcus mutans*, Microbiology, **154**, 2275–2282 (2008).
20. **Marrakchi, H., Choi, K. H., and Rock, C. O.:** A new mechanism for anaerobic unsaturated fatty acid formation in *Streptococcus pneumoniae*, J. Biol. Chem., **277**, 44809–44816 (2002).
21. **Moxon, E. R. and Kroll, J. S.:** The role of bacterial polysaccharide capsules as virulence factors, Curr. Top. Microbiol. Immunol., **150**, 65–85 (1990).
22. **Cross, A. S.:** The biologic significance of bacterial encapsulation, Curr. Top. Microbiol. Immunol., **150**, 87–95 (1990).