

# Response of Fatty Acid Synthesis Genes to the Binding of Human Salivary Amylase by *Streptococcus gordonii*

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***Streptococcus gordonii*, an important primary colonizer of dental plaque biofilm, specifically binds to salivary amylase via the surface-associated amylase-binding protein A (AbpA). We hypothesized that a function of amylase binding to *S. gordonii* may be to modulate the expression of chromosomal genes, which could influence bacterial survival and persistence in the oral cavity. Gene expression profiling by microarray analysis was performed to detect genes in *S. gordonii* strain CH1 that were differentially expressed in response to the binding of purified human salivary amylase versus exposure to purified heat-denatured amylase. Selected genes found to be differentially expressed were validated by quantitative reverse transcription-PCR (qRT-PCR). Five genes from the fatty acid synthesis (FAS) cluster were highly (10- to 35-fold) upregulated in *S. gordonii* CH1 cells treated with native amylase relative to those treated with denatured amylase. An *abpA*-deficient strain of *S. gordonii* exposed to amylase failed to show a response in FAS gene expression similar to that observed in the parental strain. Predicted phenotypic effects of amylase binding to *S. gordonii* strain CH1 (associated with increased expression of FAS genes, leading to changes in fatty acid synthesis) were noted; these included increased bacterial growth, survival at low pH, and resistance to triclosan. These changes were not observed in the amylase-exposed *abpA*-deficient strain, suggesting a role for AbpA in the amylase-induced phenotype. These results provide evidence that the binding of salivary amylase elicits a differential gene response in *S. gordonii*, resulting in a phenotypic adjustment that is potentially advantageous for bacterial survival in the oral environment.**

It is well established that tooth-borne bacterial biofilms (also known as dental plaques) are etiologic for the two most common oral diseases, dental caries and periodontal disease (29). Essential to oral biofilm development is the initial colonization of the oral cavity by the commensal (also known as “normal”) microflora. Throughout evolutionary history, humans and their commensal microbial partners have evolved together with mutual advantage. While most commensal organisms benefit human health, some can transition to serve as pathogens when ecologic factors are perturbed, as, for example, following the administration of broad-spectrum antibiotics that select for antibiotic-resistant forms, resulting in imbalances of the microflora that foster opportunistic infections. The presence of abundant commensal bacteria may prevent pathogens from increasing in numbers and thus prevent shifts in the ecological balance toward a pathogenic state. It is thus imperative to learn details of the biology of commensal bacteria, such as oral streptococci, in order to understand the ecologic factors that support a healthy flora and prevent the transition to a pathogenic flora (1).

Dental plaque formation is a complex process that involves the participation of a variety of salivary components (43). One such interaction is that between amylase and several predominant oral commensal streptococcal species (7, 10, 11, 14, 26, 36, 37, 40–42, 44, 46, 49). Amylase binds to a number of oral streptococcal species, collectively referred to as the amylase-binding streptococci (ABS) (13, 14, 22, 40). The binding of amylase to *Streptococcus gordonii* is saturable, irreversible, calcium independent, and inhibited by starch and maltotriose, but not by maltose, suggesting the involvement of a specific receptor on the bacterial surface (13, 15, 40). Amylase bound to streptococcal cells retains enzymatic activity to mediate the hydrolysis of starch to fermentable oligosaccharides *in vitro* (13, 15, 37, 41). Thus, streptococcus-bound salivary amylase hydrolyzes dietary starch that can be further metabolized for streptococcal nutrition. ABS are abundant in supra-

gingival plaque immediately after tooth cleaning (43), highlighting their role in the initiation of plaque development. Notably, only animals with measurable salivary amylase activity have ABS in their mouths (43), suggesting that streptococcal amylase binding has evolved with the ability of mammalian hosts to produce salivary amylase. These findings offer compelling evidence that the binding of amylase to these bacteria influences their colonization of the oral cavity. Furthermore, the ABS survive and persist in this host niche for prolonged periods and facilitate colonization by other bacterial species, including commensals and pathogens, to form multispecies biofilms (23, 39).

Previous *in vitro* studies found that amylase-binding proteins (ABPs) play a role in bacterial adhesion and biofilm formation (30, 37). Amylase binding is mediated by a specific protein, amylase-binding protein A (AbpA), found on the surfaces of *S. gordonii* cells (36, 42). Interestingly, AbpA-deficient *S. gordonii* mutants were able to colonize rat mouths as well as the parental strains, suggesting that bacterial factors in addition to AbpA are involved in colonization and survival *in vivo* (46). In light of these findings, we have considered other, potentially novel functions for this interaction. Thus, the goals of this study were (i) to determine the transcriptional profile of *S. gordonii* in response to salivary  $\alpha$ -amylase, (ii) to determine the role of AbpA in the amylase-

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induced gene response, and (iii) to examine the phenotypic changes that occur in *S. gordonii* in response to amylase binding.

## MATERIALS AND METHODS

**Bacterial strains and culture conditions.** The fully sequenced *S. gordonii* strain Challis CH1 (GenBank accession no. CP000725) was the primary strain for study. A kanamycin-resistant derivative of *S. gordonii* strain CH1 that carries a chromosomal *aphIII* gene replacing a phage integrase gene (SGO\_2076) was constructed previously for use in animal studies (J. M. Tanzer et al., submitted for publication) and was designated strain KS1. The analysis of growth curves of *S. gordonii* strains showed no decline in KS1 vitality. An *abpA*-deficient derivative of KS1 was constructed by inserting the *tet(M)* gene, encoding tetracycline resistance (EMBL accession no. X56353), into the *SnaBI* site of the chromosomal *abpA* gene of KS1, as previously described (Tanzer et al., submitted). Briefly, a 1.5-kb fragment containing *abpA* was PCR amplified from the CH1 chromosomal template and was cloned into pGEM-T (Promega). A 2.3-kb fragment containing the *tet(M)* gene and its promoter was PCR amplified from pAM620 (50) using primers with engineered 5' *HincII* sites and was cloned into pGEM-T. The *tet(M)* fragment was released from the plasmid by digestion with *HincII* and was cloned into the internal *SnaBI* site of *abpA* in pGEM-T. Cloned fragments were checked for fidelity by nucleic acid sequencing. After digestion of the plasmid with *BamHI*, the cloned 3.8-kb *abpA::tet(M)* double-stranded DNA (dsDNA) fragment was used to transform competent strain KS1. Putative transformants were selected on agar medium with 10  $\mu$ g tetracycline/ml, and the expected chromosomal sequence was confirmed by nucleic acid sequencing. Growth curves of strains CH1 and KS1 and of the KS1 *abpA* mutant were conducted in chemically defined medium. All three strains grew essentially identically to each other, as shown in Fig. S3 in the supplemental material. Statistical analysis using a one-way analysis-of-variance (ANOVA) test found no significant difference between the growth curves of these three strains (with 3 growth curve experiments conducted on separate days for each strain).

All *S. gordonii* strains were routinely cultured in the chemically defined medium (CDM) (20, 30). Bacteria from frozen stocks were streaked onto Todd-Hewitt (TH) (Difco, Detroit, MI) agar plates and were cultured overnight at 37°C under microaerophilic conditions in a candle jar. Tetracycline supplementation (10  $\mu$ g/ml) was used only for initial culture of the KS1 *abpA* mutant from freezer stock; no antibiotics were added to subsequent subcultures used in experiments, and the disruption of the *abpA* gene was confirmed by an amylase ligand dot blot assay.

**Purification of human salivary  $\alpha$ -amylase.** Human parotid saliva from several healthy donors was duct collected, as described previously (6, 33). This saliva was extensively dialyzed against distilled water, lyophilized, resuspended in chromatography buffer (40), and subjected to Bio-Gel P60 gel filtration chromatography (6, 40). The peaks corresponding to nonglycosylated amylase were analyzed for purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting; selected peaks were pooled, dialyzed, lyophilized, and stored at -20°C. For the experiments in this study, nonglycosylated amylase was resuspended in simulated salivary buffer (0.021 M sodium phosphate buffer, 0.036 M NaCl, 0.96 mM CaCl<sub>2</sub>) (2, 6) and was used at 0.4 mg/ml, corresponding to its salivary concentration.

To ensure that the amylase fractions were not contaminated with salivary IgA, the chromatography peaks from saliva purifications were tested by Western blotting (8) with an anti-amylase or anti-IgA antibody. Briefly, 20  $\mu$ l from the apex fraction of each peak (see Fig. S1, p6 and p7, in the supplemental material) and from the pooled peaks (see Fig. S1, A/g and A/n) was resolved on a 12% SDS-polyacrylamide gel, transferred to an Immobilon-P polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA), blocked with 5% milk in Tris-buffered saline containing 0.1% Tween 20 (TBST), washed in TBST, and overlaid with a rabbit anti-amylase antibody (1:1,000) (Calbiochem-Merck, Rockland, MA) in 1% milk-TBST for 30 min (10). After washing, the blot was incubated with a

goat anti-rabbit IgG antibody conjugated with alkaline phosphatase (1:7,500) (Bio-Rad, Hercules, CA) in 1% milk-TBST for 30 min and was developed with SigmaFAST 5-bromo-4-chloro-3-indolylphosphate (BCIP)-nitroblue tetrazolium (NBT) (Sigma). Alternatively, the blot was directly overlaid with a goat anti-human IgA antibody conjugated with alkaline phosphatase (Sigma) and was developed with SigmaFAST BCIP-NBT.

**Amylase ligand dot blot.** Dot blot analysis was modified from the method of Konto-Ghiorghi et al. (24). Briefly, whole bacteria at an optical density at 600 nm (OD<sub>600</sub>) of 0.3 were pelleted by centrifugation, washed in phosphate-buffered saline (PBS), and resuspended in PBS to obtain a similar OD<sub>600</sub> of ~2.9. A 10- $\mu$ l bacterial suspension was spotted onto a nitrocellulose membrane, dried for 20 min at room temperature, blocked in 5% milk-PBS for 30 min, incubated with amylase (0.1 mg/ml) in 1% milk-PBS for 30 min, washed in PBS, and then overlaid with a rabbit anti-amylase antibody (1:1,000) (Calbiochem) in 1% milk-PBS for 30 min. After washing, the blot was incubated with goat anti-rabbit IgG, conjugated with alkaline phosphatase (1:7,500) (Bio-Rad), in 1% milk-PBS for 30 min and was developed with SigmaFAST BCIP-NBT (Sigma).

**Isolation of RNA from *S. gordonii*.** In order to identify amylase-regulated genes, *S. gordonii* CH1 was cultured statically in 40 ml CDM at 37°C in a candle jar to mid-log phase, corresponding to an optical density at 600 nm of 0.5 to 0.6. The mid-log-phase bacterial culture was then divided into two aliquots of equal volume. Bacterial cells from all aliquots were pelleted by centrifugation at 6,000  $\times$  g in a Sorvall RC6 centrifuge at 20°C and were washed once with simulated salivary buffer preconditioned to 37°C. Simulated salivary buffer containing 0.4 mg/ml purified, nonglycosylated salivary amylase (native amylase) and warmed to 37°C was added to the cells of the first aliquot. As a negative control, simulated salivary buffer containing 0.4 mg/ml of the same salivary amylase that had first been denatured by heating to 100°C for 15 min (18) and cooled to 37°C was added to the cells of the second aliquot. Each aliquot (the amylase-treated aliquot and the control aliquot) was incubated statically for 15 min at 37°C in a candle jar.

Total RNA was immediately isolated by the hot acid-phenol method as described previously (48), followed by treatment with Turbo DNase (Applied Biosystems/Ambion, Austin, TX) according to the manufacturer's protocol. The remaining contaminants were removed using the RNeasy minikit column (Qiagen, Valencia, CA) with the cleanup protocol. Total RNA was quantified using the NanoDrop 2000 spectrophotometer, and RNA integrity was determined by agarose gel electrophoresis. Total RNA was used immediately for cDNA synthesis.

**Microarray probe preparation.** *S. gordonii* total RNA was reverse transcribed to aminoallyl-cDNA and was indirectly labeled with Cy dyes according to the standard operating protocol of the Pathogen Functional Genomics Resource Center (PFGR) at the J. Craig Venter Institute (<http://pfgr.jcvi.org/index.php/microarray.html>). Briefly, 4  $\mu$ g of total RNA was reverse transcribed using SuperScript II or III (Invitrogen, Carlsbad, CA) reverse transcriptase in a deoxynucleotide mixture containing aminoallyl-dUTP (Ambion). After alkaline hydrolysis to remove the RNA template, aminoallyl-cDNA was purified with QIAquick PCR columns (Qiagen), and was dried in a SpeedVac instrument. The cDNA was labeled with the appropriate Cy3 or Cy5 *N*-hydroxysuccinimide (NHS) dye (GE Healthcare Bio-Sciences Corp., Piscataway, NJ), and unbound Cy dyes were removed from the probe using the QIAquick PCR column (Qiagen). The quality of the probes and their specific activity were confirmed by a spectrophotometric scan from 200 to 700 nm on a NanoDrop 2000 spectrophotometer. Only labeled cDNAs with >200 pmol of dye incorporation per sample and a ratio of <50 nucleotides/dye molecule were used for hybridizations. The Cy dye-labeled cDNA from the native-amylase-treated aliquot of the culture was mixed with Cy dye-labeled cDNA from the denatured-amylase-treated control aliquot, and this mixture was used to probe the *S. gordonii* microarray slides. Initially, cDNA from cells exposed to native amylase was labeled with Cy5, and cDNA from cells exposed to denatured amylase was labeled with Cy3. Each amylase exposure experiment was repeated four times. To confirm microarray results, the

cDNA from each strain was labeled with the opposite Cy dye and was hybridized to similar arrays for the flip-dye comparison.

**Microarray hybridization.** A collection of 2,151 70-mer oligonucleotides (Invitrogen, Carlsbad, CA) representing all loci in the *S. gordonii* Challis CH1 genome (48) were spotted onto Nexterion Slide E microarray glass slides (Schott North America Inc., Louisville, KY) in replicates of four for each *S. gordonii* microarray slide, along with the DNA microarray controls described below. The oligonucleotides were spotted onto a QArray2 printer (Genetix USA Inc., Boston, MA) located in the genomics core at Roswell Park Cancer Institute in Buffalo, NY. This number of replicates compensates for spot-to-spot variation within each slide and allows for greater overall precision of the method. Quality control experiments were rerun on each new batch of printed microarray slides. These consisted of self-self hybridizations and determination of total spot saturation using SYBR dyes (Molecular Probes, Eugene, OR) that intercalate between DNA strands to check spotting consistency.

Cy dye-labeled cDNA probes were hybridized to *S. gordonii* microarray slides according to the Nexterion Slide E DNA application protocol (Schott). Briefly, slides were prewashed sequentially in a 0.1% Triton X-100 solution, a 1 mM HCl solution, and a 100 mM KCl solution before blocking with Nexterion blocking solution (Schott). The Cy dye-labeled cDNAs from the two samples were mixed, dried, and resuspended in Nexterion Hyb solution. The probe mixture was heated to 95°C and was pipetted onto a dried microarray slide. A coverslip (Lifter Slip; Erie Scientific Co., Portsmouth, NH) was placed over the slide, which was then sealed in a hybridization chamber (Corning Inc., Lowell, MA) and incubated in a 42°C water bath for 16 h. After hybridization, the slides were washed sequentially with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.2% SDS, 2× SSC, and 0.2× SSC solutions, dried briefly by centrifugation, and kept in a desiccator in a light-tight container until scanning.

**Microarray analysis.** The fluorescent spots on the microarray were scanned using the Axon 4000A laser scanner (Axon Instruments, Foster City, CA), and Genepix software, version 6.0, was used to extract the raw data, which were analyzed using the TM4 Microarray software suite ([www.jcvi.org/cms/research/software](http://www.jcvi.org/cms/research/software)). The Spotfinder program was used to analyze the quality and fluorescence of individual spots. Raw Spotfinder data were then normalized in MIDAS by applying the LOWESS algorithm (block mode; smooth parameter, 0.33). The MeV program was used for comparison of the results of the normalized data, providing numerical values corresponding to each specific gene expressed, and for visualization of the data. Statistical analysis was performed in MeV using the *t* test (alpha parameter, 0.1) and the statistical analysis of microarray (SAM) test; a change of ±1.7-fold was considered significant.

**qRT-PCR.** To confirm the transcription of selected differentially expressed genes identified by microarray analysis, *S. gordonii* strain CH1 cells were exposed to native or denatured amylase for 15 min at 37°C in a candle jar in the same manner as for microarray experiments. Total RNA was extracted as described previously and was reverse transcribed to cDNA with SuperScript II (Invitrogen, Carlsbad, CA) reverse transcriptase using the manufacturer's protocol. Prior to quantitative reverse transcription-PCR (qRT-PCR), the cDNA in each sample was normalized to the same concentration (2 ng/ml) using a NanoDrop 2000 spectrophotometer. The gene-specific primers designed using Primer BLAST (National Center for Biotechnology Information [[www.ncbi.nlm.nih.gov/](http://www.ncbi.nlm.nih.gov/)]) and synthesized by Invitrogen are listed in Table 1. To ensure that the efficiency of the assay was between 90 and 100%, qRT-PCR was performed using serial dilutions of the cDNA template with *gyrA* primers. Serial 10-fold dilutions of primers were tested with cDNA as a template to optimize each reaction. Each reaction mixture (total volume, 25 µl) contained 5 µl of the template, 3.5 µl of diethyl pyrocarbonate (DEPC)-treated water, 12.5 µl of Power SYBR green PCR master mixture (Applied Biosystems, Foster City, CA), and 2 µl each of the forward and reverse primers (100 nM). Relative qRT-PCR was performed on the ABI 7500 thermal cycler (Applied Biosystems), using uniform cycling conditions

TABLE 1 Primers used in qRT-PCR

Primer name	Primer sequence
SGO_0091 IMP-F	TCAGTCGTGGCTGGGGTGT
SGO_0091 IMP-R	TCCCTGCACTAGAGGCCAGC
SGO_1834-F	AGTTGCTCAAGCAGCTCACGAA
SGO_1834-R	AGAAGCCACCGTCAACAGCC
SGO_0671 <i>murD</i> -F	GCAGGTGGCTTGACCAGG
SGO_0671 <i>murD</i> -R	GCTGGCGTTGGCAGGACTGA
SGO_1541 <i>atpC</i> -F	AGCTGAGTCCGCCACAATCG
SGO_1541 <i>atpC</i> -R	AGGTCAAGGTACGCCGGGTC
SGO_1546 <i>atpF</i> -F	CTGCCACATCGCCCTTGATTGA
SGO_1546 <i>atpF</i> -R	GAAGCACTGGCAGGAAGCCG
SGO_1599 <i>sodA</i> -F	TGGTGGACACCTTAACCACGCT
SGO_1599 <i>sodA</i> -R	ACCAAGCCCAGCCTGATCCA
SGO_1890 PTS-F	TCAACCGCTTTGTCGCCAG
SGO_1890 PTS-R	CGTGCAAATGGTGTGACGGA
SGO_1833 <i>lip</i> -F	AGCAGGCATTATTCTAGCGTGT
SGO_1833 <i>lip</i> -R	CTGCCCCCTAGAGTAGCGCAA
SGO_1697 <i>acpP</i> -F	GAGTCAACTTTCGATGATCTTGAAGCA
SGO_1697 <i>acpP</i> -R	ACCTTCCTCAGTTTCGATTTGGATGT
SGO_1688 <i>accD</i> -F	TGGAGGGGCACGTATGCAGG
SGO_1688 <i>accD</i> -R	ACTCGACGACCAGCGAATCCA
SGO_1686-F	TGCCTTGGGGGACTGTAAACCT
SGO_1686-R	AGCCAACTCCCAGTCCCA
SGO_0832-F	CACITTCAGCCGCTGTGTCCG
SGO_0832-R	GCCTGCTTCATAGCATCACGTCC
SGO_1693 <i>fabG</i> -F	TACGAGCCGCAACCTCACGA
SGO_1693 <i>fabG</i> -R	TGACTAAGGCGCGTCAGGGAG
SGO_1698 <i>fabH</i> -F	ATGCCATCAACAGCGGCTCG
SGO_1698 <i>fabH</i> -R	AGACCGCAGTGCCTCGATCA
SGO_1495-F	AGCATGCCGGGAAACCTGGA
SGO_1495-R	TTGACTCGCTTTCAGCAGCGG
SGO_0107-F	AGGCTGAGGCTCGTCCAAGAC
SGO_0107-R	AAGGGCGAGCCTGATCCAA
SGO_1273 <i>pol-rpoD</i> -F	GCACGGGTAATGGCCTGACG
SGO_1273 <i>pol-rpoD</i> -R	GAGCAGGGCGATACTGAGGCT
<i>GyrA</i> -F	GCGGATTGTTGTAACCGAGT
<i>GyrA</i> -R	ACGGACACCCTCACGATTAG

(95°C for 10 min, followed by 40 cycles of 30 s at 95°C and 1 min at 56°C) for Power SYBR green (Applied Biosystems). Melt (dissociation) curve analysis was done at the conclusion of the initial run of each set of primers to verify the amplification of a single product. Assay controls for each run included (i) amplification with a primer set for gyrase A (*gyrA*) as the endogenous control and (ii) amplification with a reaction mixture without a template. Results for samples exposed to native amylase were normalized to those for samples exposed to denatured amylase. All reactions were run in triplicate and were repeated with four independent biological replicates. Statistical analysis of qRT-PCR data was performed on the relative quantity (RQ) values or log<sub>10</sub> RQ values by using the Student paired *t* test to compare the expression of each specific gene in native-amylase-treated cells with the expression of the same gene in denatured-amylase-treated cells. Intrareplica comparisons between the microarray and qRT-PCR data for the selected upregulated and downregulated genes showed Pearson correlation coefficients (*r*) of 0.99 for SGO\_1697, 0.93 for SGO\_1693, 0.92 for SGO\_1698, 0.97 for SGO\_1686, 0.8 for SGO\_1688, 0.88 for SGO\_1890, and 0.83 for SGO\_0091. For the scope of genes, the correlation resulted in a Pearson coefficient of 0.89.

**Phenotypic characterization of *S. gordonii* following amylase binding. (i) Effect of salivary amylase on bacterial growth.** *S. gordonii* strains were cultured statically at 37°C in a candle jar to mid-log phase and were then exposed to native or denatured salivary amylase. The growth of the bacterial cultures was monitored by measuring the OD<sub>600</sub> of the vortexed

culture at every hour for an additional 6 h after the addition of amylase. The difference between the OD of native-amylase-treated cells and the OD of denatured-amylase-treated cells at each time point was calculated for *S. gordonii* strain CH1. In the same manner, the difference between the ODs of cells treated with native versus denatured amylase at each time point was calculated for the KS1 AbpA<sup>-</sup> mutant. Similarly, CFU were determined after incubation for 3 and 4 h at 37°C in a candle jar by plating serial dilutions on TH plates, and the CFU counts were used to compare native-amylase-treated with denatured-amylase-treated parts of the same *S. gordonii* strain CH1 or KS1 *abpA* mutant culture at the 3- and 4-h time points. Statistical analysis was performed using Student's *t* test, where the means of the differences for strain CH1 were compared to the means of the differences for the mutant. In addition, to rule out effects of amylase binding on chain length, mean bacterial chain lengths were determined for each condition microscopically by counting 10 random fields per slide and 10 random chains per field.

(ii) **pH sensitivity.** Wild-type (WT) *S. gordonii* CH1 was tested for resistance to low pH. Bacteria from frozen stocks were streaked onto a TH plate and were cultured overnight at 37°C in a candle jar. The KS1 *abpA* mutant was cultured under the same conditions on a TH plate supplemented with tetracycline (10 µg/ml). Several colonies from strain CH1 were inoculated into 10 ml of CDM and were cultured statically to late-log phase (OD<sub>600</sub>, 0.7 to 0.8). The culture was then divided equally (5 ml each) and was pelleted at 6,000 × *g* for 5 min. To the bacterial pellet, 0.5 ml of salivary buffer containing either native or denatured salivary amylase (0.4 mg/ml in each case) was added, resuspended, and incubated for 15 min at 37°C. After incubation, bacteria were vortexed, and 10 µl was transferred into 1 ml of simulated salivary buffer previously adjusted with 2 N HCl to a pH of either 3, 5, 7, or 9 and was incubated for 1 h at room temperature. Following this, serial dilutions were plated on TH plates and were cultured for 24 h at 37°C in a candle jar in order to enumerate the remaining CFU. In parallel, the same experiment was conducted with the AbpA<sup>-</sup> mutant. The numbers of CFU were compared using paired two-tailed Student *t* tests, where the amylase-treated cells were paired with cells derived from the same culture exposed to denatured amylase.

(iii) **Triclosan resistance.** The bacteria were tested for sensitivity to triclosan, an antibacterial agent known to affect fatty acid synthesis (FAS) (32). Wild-type *S. gordonii* CH1 and the KS1 *abpA* mutant were cultured to mid-log phase and were exposed to native or heat-treated amylase as described above. After 15 min of incubation with salivary amylase, triclosan (Irgasan; Sigma, St. Louis, MO) was added to a final concentration of 0.01%. The OD<sub>600</sub> was measured every 30 min for 5 h.

(iv) **Resistance to detergent (bile salts).** *S. gordonii* strain CH1 and the KS1 *abpA* mutant were tested for resistance to deoxycholate, a bile salt that emulsifies lipids and disrupts bacterial lipid membranes, causing cell lysis. Bacteria were cultured to mid-log phase as described above. After exposure to either native or denatured salivary amylase, sodium deoxycholate (Calbiochem) was added to a final concentration of 100 µg/ml, and the OD<sub>600</sub> was measured every hour for 3 h (31).

**Microarray data accession number.** Microarray data were deposited in NCBI's Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/geo/>) and are accessible through GEO Series accession no. GSE31830.

## RESULTS

**Transcriptome profiling of *S. gordonii* in response to amylase binding.** In order to determine the potential role of host salivary amylase as an environmental signal to *S. gordonii*, we used microarray analysis to test the effect of *S. gordonii* exposure to salivary amylase on its gene expression. CDM was used to culture the bacteria in all experiments, in order to eliminate the possible variable effects of complex carbohydrates or proteins found in a compound medium. As a control for protein exposure, we exposed the bacteria to heat-denatured salivary amylase. It was found previously that treating amylase with heat eliminates its ability to bind

to *S. gordonii* and abolishes its enzymatic activity (4). Bacterial cultures were cultured to mid-log phase, when AbpA is maximally present on the surfaces of bacteria (13). The culture was divided into two aliquots; one was treated with purified native salivary amylase and the other with denatured salivary amylase. Nonglycosylated amylase was used in order to avoid possible interactions of the bacteria with glycosyl moieties of amylase. This amylase fraction was tested by Western blotting (see Fig. S1 in the supplemental material) and was found to be free of IgA, which could possibly bind to the bacteria to elicit a response. Based on preliminary experiments, *S. gordonii* gene expression was found to be optimal after a 15-min exposure to salivary amylase.

Microarray analysis identified 17 genes upregulated 2-fold or more in response to a 15-min amylase exposure, while 16 genes were downregulated (Table 2). Several genes belonging to the fatty acid synthesis (FAS) locus (Fig. 1A and B) were upregulated by as much as 4-fold in response to the binding of native amylase compared to exposure to denatured amylase. These genes, which are involved in the initiation of fatty acid synthesis and product elongation, include *acpP*, encoding acyl carrier protein (ACP) (SGO\_1697); *accD*, encoding acetyl coenzyme A (acetyl-CoA) carboxylase subunit beta (SGO\_1688); *fabG*, encoding 3-ketoacyl-(acyl-carrier-protein) reductase (SGO\_1693); *fabH*, encoding 3-oxoacyl-(acyl carrier protein) synthase III (SGO\_1698); and one of the genes from the same chromosomal location, SGO\_1686, whose product has been designated a hypothetical protein. These genes were selected for confirmation by qRT-PCR.

Among the downregulated genes, a large effect was noted for a gene encoding an integral membrane protein (IMP), SGO\_0091. This gene was analyzed using STRING 8.3 software (<http://string-db.org>) and was found to be a predicted functional partner of SGO\_0090, encoding a transcriptional regulator of the TetR family (21, 48). Other genes significantly downregulated in response to salivary amylase included a gene encoding phosphotransferase transport system (PTS) fructose (mannose)-specific subunit IID (SGO\_1890); *murD*, encoding UDP-*N*-acetylmuramoyl-L-alanyl-D-glutamate synthetase (SGO\_0671); two genes involved in oxidation phosphorylation, *atpF*, encoding ATP synthase subunit B (SGO\_1546), and *atpC*, encoding ATP synthase F<sub>1</sub>, epsilon subunit (SGO\_1541); and a gene encoding a putative lipoprotein (SGO\_1833) (Table 2).

**qRT-PCR analysis.** Genes encoding proteins of the FAS pathway, including *accD* (encoding acetyl-CoA carboxylase subunit beta), *acpP* (encoding acyl carrier protein), *fabG* [encoding 3-ketoacyl-(acyl-carrier-protein) reductase], and *fabH* [encoding 3-oxoacyl-(acyl carrier protein) synthase III], were confirmed by qRT-PCR to be significantly (*P* < 0.001) upregulated (Table 3) in native-amylase-treated bacteria compared to bacteria treated with denatured amylase. Two proteins of unknown function—hypothetical proteins SGO\_0832 and SGO\_1686—were also significantly upregulated in amylase-treated bacteria (Table 3). Six genes confirmed by qRT-PCR to be downregulated following exposure to salivary amylase compared to exposure to denatured amylase included genes encoding an integral membrane protein (SGO\_0091), PTS fructose (mannose)-specific IID, ATP synthase subunit B (*atpF*), a putative lipoprotein (SGO\_1833), a conserved hypothetical protein (SGO\_1834), and manganese-dependent superoxide dismutase (*sodA*) (SGO\_1599) (Table 3). Among the downregulated genes, *atpC*, encoding ATP synthase F<sub>1</sub>, epsilon subunit (SGO\_1541), and

TABLE 2 *S. gordonii* CH1 genes upregulated or downregulated upon exposure to amylase as determined by microarray analysis

Protein encoded	NCBI locus tag	Data analysis			
		High stringency		Low stringency	
		Log 2 (ratio)	Fold	Log 2 (ratio)	Fold
<b>Upregulated genes</b>					
Acyl carrier protein	SGO_1697	1.57	2.98	2.09	4.28
Acetyl-CoA carboxylase, carboxyl transferase, beta subunit	SGO_1688	2.12	4.34	2.13	4.39
Hypothetical protein	SGO_0832	1.92	3.79	1.69	3.22
Imidazoleglycerol-phosphate dehydratase	SGO_1407	1.22	2.34	1.18	2.27
Hypothetical protein	SGO_1686	1.89	3.71	2.11	4.32
Phosphoribosyl-AMP cyclohydrolase	SGO_1403	1.05	2.07	0.92	1.89
Putative transcriptional regulator	SGO_1495	1.08	2.11	1.29	2.44
3-Oxoacyl-(acyl-carrier-protein) reductase	SGO_1693	1.01	2.01	1.10	2.14
Conserved domain protein	SGO_2052	1.31	2.49	0.94	1.91
LPXTG cell wall surface protein	SGO_0107	0.92	1.89	1.10	2.15
3-Oxoacyl-[acyl-carrier-protein] synthase III (beta-ketoacyl-ACP synthase III) (KAS III)	SGO_1698	0.87	1.83	1.01	2.02
4-Hydroxybenzoate octaprenyltransferase-related protein	SGO_0939	1.00	2.00	0.80	1.74
Trehalose PTS enzyme II	SGO_1653	0.72	1.65	0.77	1.71
Transcriptional regulator	SGO_1792	0.78	1.71	0.65	1.57
ATP synthase (F/14-kDa) subunit	SGO_0133	0.86	1.82	0.82	1.77
RNA polymerase sigma factor ( <i>rpoD</i> )	SGO_1273	0.78	1.72	0.83	1.78
Conserved domain protein	SGO_0018	0.63	1.55	0.98	1.97
<b>Downregulated genes</b>					
Integral membrane protein	SGO_0091	-1.59	3	-1.69	3.33
Conserved hypothetical protein	SGO_1834	-1.76	3.4	-1.73	3.33
PTS system, fructose (mannose)-specific IID	SGO_1890	-1.65	3.2	-1.83	3.57
UDP-N-acetylmuramoylalanine-D-glutamate ligase	SGO_0671	-1.34	2.5	-1.38	2.6
Ribosomal protein L7A family	SGO_0545	-1.89	3.8	-1.86	3.7
ABC transporter, ATP-binding protein SP1553	SGO_1118	-1.23	2.3	-1.27	2.4
ATP synthase F <sub>o</sub> , B subunit	SGO_1546	-1.01	2.0	-0.77	1.7
Pyruvate oxidase	SGO_0292	-1.04	2.0	-0.63	1.56
Manganese-dependent superoxide dismutase	SGO_1599	-0.86	1.8	-0.82	1.78
Lipoprotein, putative	SGO_1833	-0.96	1.9	-0.86	1.84
Glycosyl hydrolase, family 38	SGO_1768	-0.89	1.8	-0.98	2.00
Voltage-gated chloride channel family protein	SGO_1166	-0.80	1.7	-0.89	1.75
ATP synthase F <sub>1</sub> , epsilon subunit	SGO_1541	-0.81	1.78	-0.88	1.85
Phosphate import ATP-binding protein PstB 1 (ABC phosphate transporter 1)	SGO_1059	-0.81	1.78	-0.90	1.88
SUF system FeS assembly protein, NifU family	SGO_1719	-0.88	1.85	-0.87	1.84
Conserved hypothetical protein	SGO_0010	-0.79	1.75	-0.81	1.78

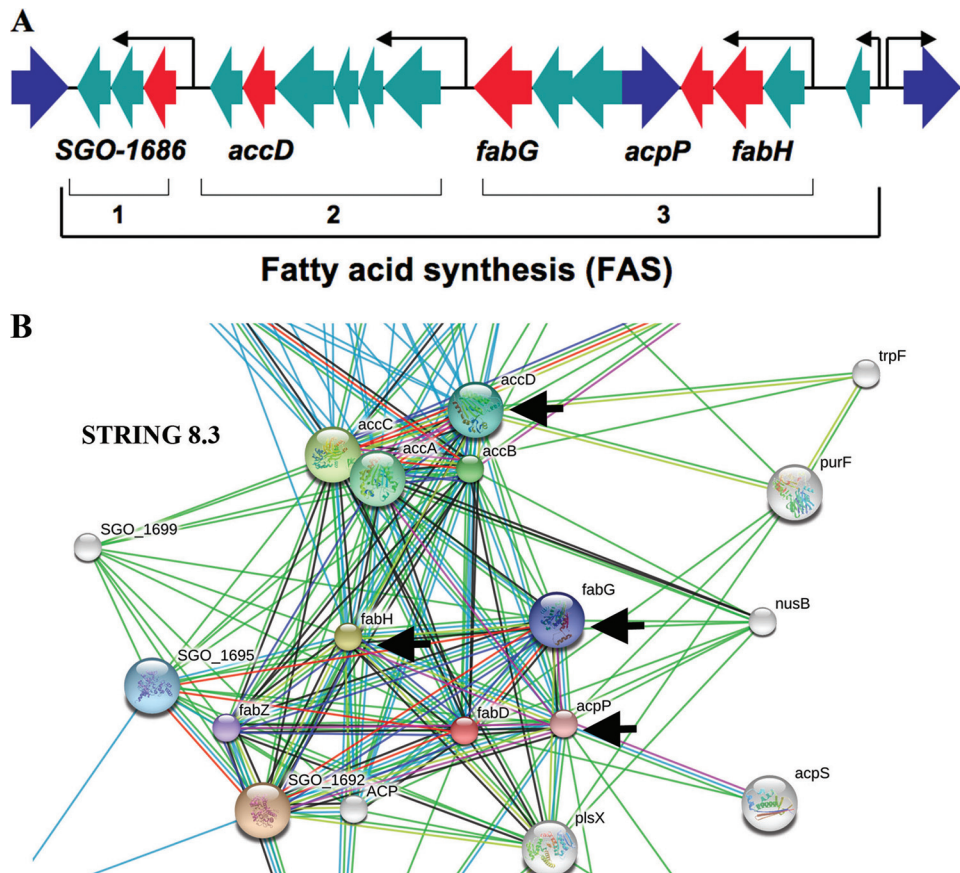
*murD*, encoding UDP-N-acetylmuramoyl-L-alanyl-D-glutamate synthetase (SGO\_0671), showed statistically significant ( $P < 0.05$ ) 1.54- and 1.55-fold downregulation, respectively. These data are consistent with the microarray results.

Two transcription regulators, SGO\_1273 and SGO\_1495, and the “LPXTG cell wall surface protein” (SGO\_0107), which were upregulated by microarray analysis, were also tested by qRT-PCR. The expression of these three genes was not significantly changed by the addition of amylase in the qRT-PCR experiments, which used the same RNA as that used in the microarray experiments. Overall, the qRT-PCR results verified the microarray data for 12 genes showing significant changes in expression in *S. gordonii* in response to a 15-min exposure to purified human parotid non-glycosylated amylase.

**Role of AbpA in amylase-induced gene response.** The KS1 *abpA* mutant was tested to determine whether mutation of the *abpA* gene affects the response of bacteria to amylase exposure. Amylase ligand dot blot analysis of whole cells demonstrated that

inactivation of *abpA* eliminated amylase binding to the surface of the *S. gordonii* KS1 *abpA* mutant (Fig. 2). We tested the expression of selected genes shown to be upregulated in the *S. gordonii* strains CH1 and KS1 after amylase exposure compared to the KS1 *AbpA*<sup>-</sup> mutant. As expected, these genes were upregulated in both CH1 and KS1. In contrast, qRT-PCR analysis of the FAS genes in the *AbpA*<sup>-</sup> mutant did not show significant differences between cells treated with native versus denatured amylase (Fig. 3). Thus, mutation of *abpA* eliminated the amylase-dependent gene response, suggesting that the gene response observed following amylase binding involves *AbpA*.

**Effect of amylase on bacterial growth.** One of the genes of the *fab* cluster, *fabG*, which was highly upregulated in response to salivary amylase in *S. gordonii* strain CH1, has been reported previously to be an essential gene for bacterial growth in *Escherichia coli* (51). The fact that *fabG* was induced by amylase exposure led us to test the effect of amylase exposure on bacterial growth. After incubation with salivary amylase, *S. gordonii* strain CH1 increased



**FIG 1** Fatty acid synthesis (FAS) genes. (A) FAS gene loci (green). Genes that are upregulated in amylase-treated bacteria compared to denatured-amylase-treated bacteria (red) positioned in the three distinct polycistronic regions (1, 2, and 3) and clustered in the chromosome among other genes transcribed in the opposite direction (blue). (B) Predicted functional relationship of FAS genes plotted by STRING 8.3. Black arrows indicate genes upregulated upon exposure to native amylase.

in optical density faster, by 12% on average, than did cells from the same culture exposed to denatured amylase (Fig. 4A). In contrast, no difference in growth was noted between native-amylase-treated and denatured-amylase-treated *AbpA*<sup>-</sup> mutant cells (Fig. 4A). A change in bacterial growth was also verified by plating the amylase-treated culture at 3 and 4 h and comparing the CFU of native-amylase-treated bacteria with those of denatured-amylase-treated bacteria (Fig. 4B). These data suggest that the growth of *S. gordonii* may be enhanced upon exposure to salivary amylase. The fact that there was no increase in growth in the *AbpA*<sup>-</sup> mutant exposed to native amylase compared to denatured amylase further suggests that *AbpA* plays a role in the amylase response by *S. gordonii*. No significant change in bacterial chain length between treated and control bacteria was observed (data not shown), suggesting that the observed increase in CFU following exposure to salivary amylase is not due to a reduction in chain length.

**Amylase-induced resistance to low pH.** We hypothesized that the observed increased expression of genes responsible for the initiation of fatty acid synthesis may have an effect on membrane-dependent pH resistance. The presence of host amylase in the bacterial milieu could be indicative of the availability of additional utilizable carbohydrates, thus priming the bacterium for carbohydrate uptake. Hydrolysis of starch by amylase would supply maltose, which, when metabolized by bacteria, would lower the culture

pH. Exposure of *S. gordonii* to salivary amylase could increase its resistance to low pH. Indeed, the survival of *S. gordonii* CH1 was significantly increased in a buffer at pH 3 and pH 5 after amylase treatment. As expected, there was no difference in survival between native-amylase-treated and denatured-amylase-treated bacteria at pH 7 or pH 9 (Fig. 5). The survival of the *AbpA*<sup>-</sup> mutant under acidic or basic conditions was not affected by amylase treatment, again suggesting a role for *AbpA* in survival in an acidic environment after amylase exposure.

**Amylase exposure increases resistance to triclosan.** To further support the finding that amylase binding affects the expression of fatty acid synthesis genes, resulting in a change in phenotype, we compared the triclosan resistance of *S. gordonii* cells exposed to native or denatured amylase. Triclosan is a bacteriostatic antiseptic that inhibits enoyl-ACP reductases encoded by the *fab* gene cluster (9). *S. gordonii* cells were found to be more resistant to triclosan following amylase binding than control cells exposed to denatured salivary amylase (Fig. 6). The effect on the *AbpA*<sup>-</sup> mutant was reversed, i.e., cells exposed to denatured salivary amylase were more resistant to triclosan than cells exposed to native salivary amylase (Fig. 6). The amylase-induced expression of FAS genes allows *S. gordonii* strain CH1 to overcome the growth suppression caused by triclosan, while the *AbpA*<sup>-</sup> mutant abolishes this effect, and growth is suppressed. These results suggest

TABLE 3 S. gordonii CH1 genes upregulated or downregulated upon exposure to amylase as determined by qRT-PCR

NCBI locus tag	Gene description by NCBI and STRING 8.3	Mean fold difference <sup>a</sup> ± SE	P
<b>Upregulated genes</b>			
SGO_0107 <sup>b</sup>	LPXTG cell wall surface protein	0.72 ± 0.04	<0.001
SGO_0832	Hypothetical protein	33.95 ± 14.33	<0.05
SGO_1495 <sup>b</sup>	Putative transcriptional regulator	1.33 ± 0.17	<0.001
SGO_1686	Hypothetical protein	10.42 ± 0.71	<0.001
SGO_1273 <sup>b</sup>	RNA polymerase sigma factor ( <i>rpoD</i> )	0.99 ± 0.08	0.4655
SGO_1688	Acetyl-CoA carboxylase subunit beta ( <i>accD</i> )	18.28 ± 2.42	<0.001
SGO_1697	Acyl carrier protein ( <i>acpP</i> )	20.89 ± 2.71	<0.001
SGO_1693	3-Ketoacyl-(acyl-carrier-protein) reductase ( <i>fabG</i> )	35.51 ± 3.41	<0.001
SGO_1698	3-Oxoacyl-(acyl carrier protein) synthase III ( <i>fabH</i> )	18.38 ± 1.90	<0.001
<b>Downregulated genes</b>			
SGO_0091	Integral membrane protein	201.56 ± 73.22	<0.05
SGO_1890	PTS system fructose (mannose)-specific IID	39.58 ± 25.02	<0.1
SGO_1546	ATP synthase subunit B ( <i>atpF</i> )	2.49 ± 0.58	<0.05
SGO_1833	Lipoprotein, putative	2.84 ± 0.66	<0.05
SGO_1834	Conserved hypothetical protein	2.81 ± 0.99	<0.1
SGO_1599	Manganese-dependent superoxide dismutase ( <i>sodA</i> )	2.09 ± 0.22	<0.01
SGO_1541	ATP synthase F <sub>1</sub> , epsilon subunit ( <i>atpC</i> )	1.54 ± 0.10	<0.01
SGO_0671	UDP-N-acetylmuramoyl-L-alanyl-D-glutamate synthetase ( <i>murD</i> )	1.55 ± 0.18	<0.05
SGO_1786	Glycosyl hydrolase, family 38	1.46 ± 0.72	0.2724

<sup>a</sup> From four independent assays.

<sup>b</sup> Gene upregulated by microarray analysis that did not prove to be upregulated by qRT-PCR.

that the expression of FAS enzymes is increased following exposure to salivary amylase and that this increase is sufficient to overcome the suppressing effect of triclosan.

**Resistance to detergent (bile salts).** We considered the possibility that transcriptional regulation of *fab* cluster genes could alter the fatty acid composition of the membrane and increase its sensitivity to lysis by the detergent deoxycholate, as described for *Streptococcus pneumoniae* (31). After exposure of native-amylase-treated or denatured-amylase-treated *S. gordonii* CH1 to deoxycholate, no change in sensitivity to bile salts was observed. Similarly, there was no difference in lysis between KS1 AbpA<sup>-</sup> mutant cells treated with native versus denatured amylase following exposure to deoxycholate. These results suggest that increased expression of fatty acid synthesis genes in amylase-treated *S. gordonii* strain CH1 likely does not cause a change in the composition of

the membranes; however, increased expression of FAS genes may modulate overall bacterial growth and membrane turnover.

## DISCUSSION

*S. gordonii* expresses endogenous intracellular amylase, which is not secreted, and is thus unable to metabolize starch as a primary nutritional source unless it binds host salivary amylase (13, 17, 37, 41, 45).  $\alpha$ -Amylase hydrolyzes starch at  $\alpha$ -1,4 glucosidic linkages to form various maltooligosaccharides and eventually maltose. Once bound to the streptococcal surface, host amylase retains its enzymatic activity, allowing the organism to metabolize dietary starch efficiently (13, 37, 41). The presence of both starch and amylase in the oral cavity likely allows *S. gordonii* to persist and proliferate. Previous studies identified genes in *S. gordonii* that were differentially expressed in response to exposure to human

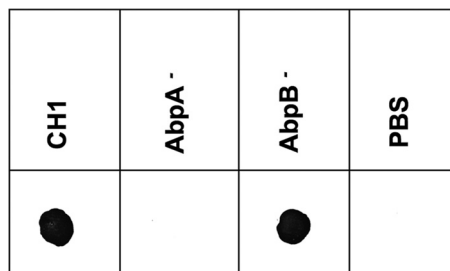


FIG 2 A dot blot amylase overlay assay was used to demonstrate amylase binding to bacterial cells. Whole bacterial cells were pelleted from broth culture, washed, and standardized to an OD<sub>600</sub> of 2.9 in PBS. Each sample (10  $\mu$ l) was spotted onto a nitrocellulose membrane, blocked with skim milk, overlaid with salivary amylase (0.1 mg/ml) followed by a rabbit anti-amylase antibody, and developed with alkaline phosphatase-conjugated goat anti-rabbit IgG and SigmaFAST BCIP-NBT (Sigma). CH1, *S. gordonii* Challis CH1; AbpA<sup>-</sup>, the KS1 amylase-binding protein A mutant; AbpB<sup>-</sup>, the KS1 amylase-binding protein B mutant.

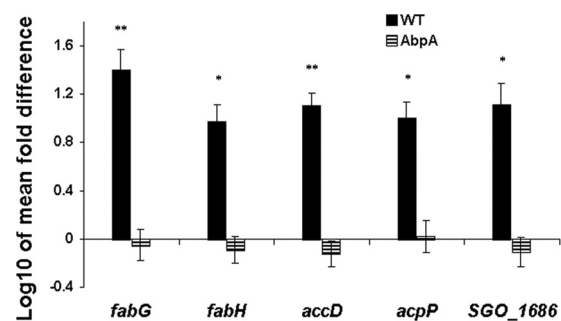
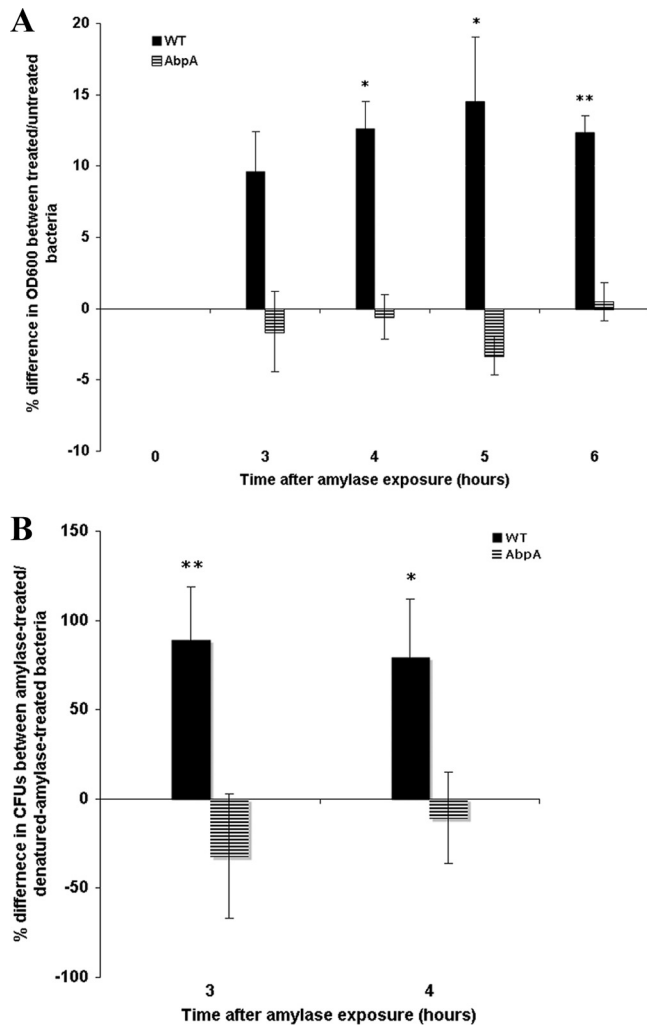


FIG 3 Expression of fatty acid synthesis genes after a 15-min exposure of bacteria to salivary amylase compared to the control culture exposed to denatured salivary amylase, as determined by qRT-PCR. Filled bars, WT *S. gordonii*; striped bars, KS1 AbpA<sup>-</sup> mutant. *gyrA* was used as the endogenous control. Each bar represents the log<sub>10</sub> of the mean fold difference in expression (native amylase – denatured amylase) ± the standard deviation from four independent experiments. Significance levels: \*,  $P < 0.01$ ; \*\*,  $P < 0.001$ . Results similar to those seen with CH1 were obtained with KS1.



**FIG 4** (A) Comparison of culture growth between bacteria treated with native amylase and those treated with denatured amylase. Filled bars, WT *S. gordonii*; striped bars, KS1 *AbpA*<sup>-</sup> mutant. Bacteria were cultured in chemically defined medium to mid-log phase, when salivary amylase was added to a final concentration of 0.4 mg/ml (0 h). (A) The OD<sub>600</sub> was measured after 3 h and was monitored every hour thereafter for the next 3 h. (B) The culture was plated so as to determine CFU. Each bar represents the mean difference in culture growth between native-amylase-treated and denatured-amylase-treated bacteria of the same culture ( $n = 3$ ). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

whole saliva (16). Considering that amylase is one of the most abundant components of human saliva, this interaction could mediate differential gene expression and could serve as an important factor for bacterial host adaptation.

The results of this study provide evidence that the binding of amylase to the surface of *S. gordonii* elicits differential gene expression in the bacterial cell. This appears to result in an alteration of the bacterial phenotype that may be advantageous for bacterial survival in the oral environment. Here we show evidence that after exposure to salivary amylase, *S. gordonii* is better able to tolerate an acidic environment. The bacterium also appears to grow better in the presence of bound amylase. Salivary amylase upregulates genes involved in the synthesis of fatty acids, which are key components of bacterial membranes. Considering the fact that in all the experiments we performed, the presence of complex carbohy-

drate as a source of nutrition in the medium was avoided through the use of a chemically defined medium, it is clear that *S. gordonii* not only binds host amylase but also responds to its binding by differential regulation of gene expression and phenotype adjustment.

The amylase-induced upregulation of FAS genes could be a mechanism for the “fine tuning” of bacterial metabolism to redirect acetyl-CoA from the citric acid cycle to fatty acid synthesis for membrane building and bacterial proliferation. In the first step of fatty acid synthesis, acetyl-CoA from the citric acid cycle receives a CO<sub>2</sub> group and forms malonyl-CoA; this involves the action of acetyl-CoA carboxylase (*accD*) (9). Next, 3-oxoacyl-(acyl carrier protein) synthase III (*fabH*) initiates the condensation of acetyl-CoA with malonyl-CoA and attaches it to acyl carrier protein (*acpP*), creating acetoacetyl-ACP (9). Then 3-ketoacyl-(acyl-carrier-protein) reductase (*fabG*) reduces the 3-carbon ketone of acetoacetyl-ACP to hydroxyl and converts it to hydroxybutanoyl-ACP. These steps are repeated to elongate the fatty acid chain. The acyl carrier protein is essential for the cycle, since it carries the synthesizing fatty acid chain. In fact, *acpP* is an essential gene in *E. coli* that possesses several promoters, ensuring that mutation of one of the promoters does not prevent the transcription of fully functional protein (51). Another essential gene, *accD*, previously reported in *E. coli* temperature-sensitive mutations, blocked fatty acid synthesis (9, 27). KAS III-3-oxoacyl-(acyl carrier protein) synthase III, encoded by *fabH*, produces only C<sub>4</sub> through C<sub>6</sub> compounds and cannot make long-chain fatty acids (47), suggesting that this enzyme initiates fatty acid synthesis (9, 19, 35). *fabG*, encoding 3-ketoacyl-(acyl-carrier-protein) reductase, is also an essential gene; its transcriptional termination in *Salmonella enterica* serovar Typhimurium blocked cell growth (51). Considering the fact that several amylase-induced genes affect bacterial growth and proliferation, the findings of the present study suggest that upregulated fatty acid synthesis genes in amylase-exposed *S. gordonii* modulate the initiation of fatty acid synthesis and stimulate proliferation. Thus, the effect of upregulation of FAS genes may be to increase the overall amount of fatty acids, but it does not change the proportion of fatty acids in the membrane (see Table S1 in the supplemental material).

We established that the cell wall-attached protein AbpA plays a role in the amylase-induced gene response. Insertional inactivation of *abpA* drastically reduced the binding of salivary amylase to the bacterial surface and eliminated amylase-induced gene expression and phenotypic response.

Considering the fact that AbpA is a cell wall-associated protein localized at the cell surface (41), the question of its ability to convey a signal upon binding to salivary amylase arises. AbpA is maximally expressed during the mid-log phase of *S. gordonii* growth (13). Recent sequence analysis indicates that AbpA shares homology with several AbpA-like proteins produced by other amylase-binding oral streptococci. To date, no conserved domains have been identified (see Fig. S2 in the supplemental material). The AbpA protein is translated with a leader peptide directing its secretion outside the bacterial cell, where it attaches to the cell surface to serve as the receptor for amylase (H. Wu, personal communication). It has been suggested recently (28) that sortase B (*SrtB*), encoded by *srtB* and located immediately downstream of *abpA*, plays a role in the attachment of AbpA to the cell wall. AbpA does not have a classic sortase B recognition domain, such as NXZTN, as described for *Listeria monocytogenes*, or NPQTN, for



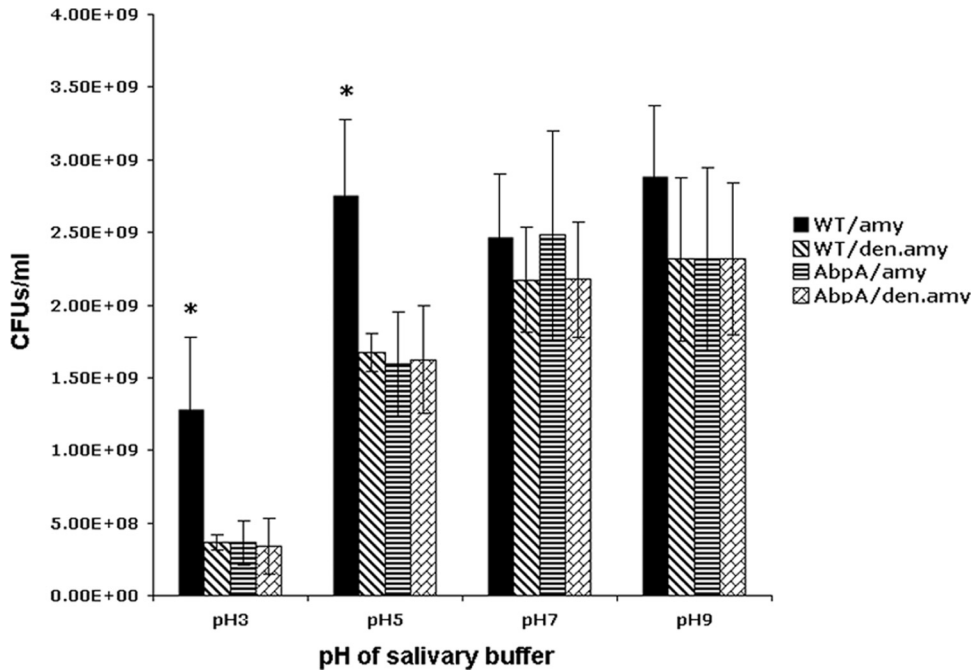


FIG 5 Effects of salivary amylase on *S. gordonii* survival under various pH conditions. WT *S. gordonii* was first cultured in chemically defined medium to mid-log phase, then exposed either to native salivary amylase (WT/amy) or to salivary amylase denatured by boiling (WT/den.amy), and finally incubated further in a salivary buffer adjusted to various pHs. The KS1 AbpA<sup>-</sup> mutant was also similarly exposed to native (AbpA/amy) or denatured (AbpA/den.amy) amylase and was further incubated in a salivary buffer adjusted to various pHs. \*,  $P < 0.05$ .

*Staphylococcus aureus* (3, 5). It apparently does possess a novel, yet to be identified sortase recognition motif at the C terminus (28), which is likely responsible for the covalent binding of AbpA to the cell wall.

AbpA is present on the surface of the nascent cell wall in the

annular and polar zones but is later shed into the environment (42). Its function, other than mediating the binding of amylase to streptococci, remains unknown. The fact that AbpA is present only at cell division sites and is later shed into the environment

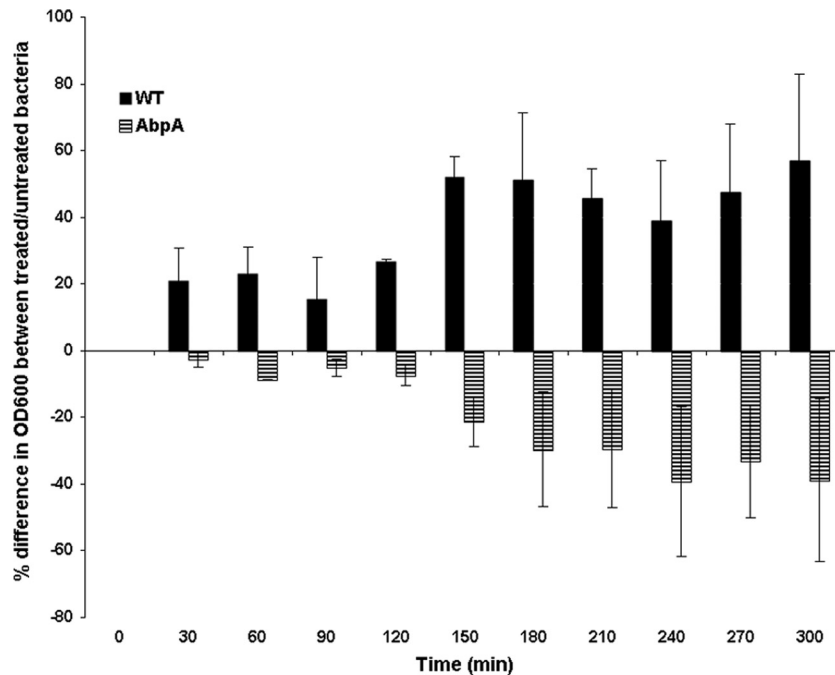


FIG 6 Resistance of *S. gordonii* to triclosan after exposure to salivary amylase. Filled bars, WT *S. gordonii*; striped bars, KS1 AbpA<sup>-</sup> mutant. Each bar represents the mean difference in growth (as indicated by the OD<sub>600</sub>) between bacteria treated with native versus denatured amylase after the addition of triclosan to a final concentration of 0.1 mg/ml at 0 min and upon monitoring every 30 min. Data represent three independent experiments ( $P < 0.001$ ).

leads us to postulate that AbpA may play a role in conveying information about the environment to new daughter cells during cell division. Although AbpA is an extracellular cell wall-attached protein and lacks a membrane-spanning domain, it could potentially function as a transient coreceptor or signal transducer. Consequently, it is possible that AbpA is part of a putative signal transduction system. Analysis of the AbpA protein sequence suggests several sites for protein-protein interaction, phosphorylation, and ATP binding.

AbpA may interact with another component(s) of a putative transduction system, such as an integral membrane protein or a transport system. The latter, activated by AbpA, could carry the signal to the transcription machinery. In fact, our microarray analysis revealed two candidates for such an interaction. Two genes downregulated upon exposure to salivary amylase encoded an IMP (SGO\_0091) and a PTS (SGO\_1890). One or both could be involved in transmitting the signal. The IMP is a predicted functional partner of a TetR repressor family regulator (SGO\_0090) and could be a member of the putative two-component signaling system (TCS) (34). Interestingly, the downregulation of this predicted TetR repressor system could be linked to upregulation of the FAS operon through the release of the suppression effect (31, 51). However, the function of the IMP as part of an amylase-triggered transduction system, as well as its role in the regulation of the FAS operon, has yet to be determined experimentally. The second candidate for such a possible regulatory role is a PTS (SGO\_1890), which was downregulated upon exposure to salivary amylase. Multiple phosphotransferase systems responsible for carbohydrate transport have been shown previously to be involved in signal transduction in Gram-positive bacteria (25) and *E. coli* (12, 38). Thus, it is possible that AbpA could interact with the PTS on the cell surface level and initiate signaling through this carbohydrate transport system. The actual roles of these candidates in amylase-induced signal transduction have yet to be determined.

While wild-type *S. gordonii* showed increased resistance to triclosan after exposure to native versus denatured amylase, peculiarly, the AbpA mutant, when exposed to amylase, showed less resistance to triclosan than did the control. Since triclosan binds to and inhibits the enzymes of fatty acid synthesis, it is logical that increased expression of FAS genes in the WT after amylase exposure would cause increased levels of FAS enzymes that overcome the effect of triclosan. We can only speculate on the possible explanations for why the AbpA mutant showed the opposite effect. Perhaps the absence of AbpA protein on the surface of the bacterium results in an as yet unknown perturbation that affects the bacterial response to the presence of unbound amylase in the environment. AbpA may not be the only protein that is involved in signaling following the binding of amylase. Perhaps the deletion of AbpA from the signaling system creates a countersignal in the presence of amylase that actually reverses triclosan resistance. Studies to identify other components that may be involved in amylase-dependent signaling are in progress. We anticipate that information on the regulatory pathway will be valuable in the interpretation of this phenomenon.

Identification of the mechanisms of amylase-induced signaling in differential gene expression has implications for understanding and controlling bacterium-host interactions and also for the detection of new paradigms for the regulation of bacterial fatty acid

synthesis that may stimulate the development of novel antimicrobial agents.

Now that we have demonstrated that amylase may serve as an environmental signal for the modification by *S. gordonii* of its gene expression and phenotype in order to adapt to a changing host environment, other questions arise. Does amylase binding provide an advantage for *S. gordonii* in competition with other bacterial species within the niche? Does it aid in colonization by other species? We observed that exposure to salivary amylase stimulates *S. gordonii* growth, as well as its survival under acidic conditions, which are often found in the oral environment. Does the interaction of *S. gordonii* with amylase decrease colonization by pathogenic bacteria, such as *Streptococcus mutans*, or does it benefit *S. mutans* by delivering an available source of utilizable carbohydrates? These results suggest further avenues for research to determine the fundamental role of *S. gordonii* in bacterium-host interaction and oral colonization.

## ACKNOWLEDGMENTS

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## REFERENCES

- Avila M, Ojcius DM, Yilmaz O. 2009. The oral microbiota: living with a permanent guest. *DNA Cell Biol.* 28:405–411.
- Bennick A, Cannon M. 1978. Quantitative study of the interaction of salivary acidic proline-rich proteins with hydroxyapatite. *Caries Res.* 12:159–169.
- Bentley ML, Gaweska H, Kielec JM, McCafferty DG. 2007. Engineering the substrate specificity of *Staphylococcus aureus* sortase A. The  $\beta 6/\beta 7$  loop from SrtB confers NPQTN recognition to SrtA. *J. Biol. Chem.* 282:6571–6581.
- Bergmann JE, Gulzow HJ. 1995. Detection of binding of denatured salivary  $\alpha$ -amylase to *Streptococcus sanguis*. *Arch. Oral Biol.* 40:973–974.
- Bierne H, et al. 2004. Sortase B, a new class of sortase in *Listeria monocytogenes*. *J. Bacteriol.* 186:1972–1982.
- Bradway SD, Bergey EJ, Jones PC, Levine MJ. 1989. Oral mucosal pellicle. Adsorption and transpeptidation of salivary components to buccal epithelial cells. *Biochem. J.* 261:887–896.
- Brown AE, Rogers JD, Haase EM, Zelasko PM, Scannapieco FA. 1999. Prevalence of the amylase-binding protein A gene (*abpA*) in oral streptococci. *J. Clin. Microbiol.* 37:4081–4085.
- Burnette WN. 1981. “Western blotting”: electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.* 112:195–203.
- Campbell JW, and Cronan JE, Jr. 2001. Bacterial fatty acid biosynthesis: targets for antibacterial drug discovery. *Annu. Rev. Microbiol.* 55:305–332.
- Chaudhuri B, et al. 2008. Amylase-binding protein B of *Streptococcus gordonii* is an extracellular dipeptidyl-peptidase. *Infect. Immun.* 76:4530–4537.
- Chaudhuri B, Rojek J, Vickerman MM, Tanzer JM, Scannapieco FA. 2007. Interaction of salivary  $\alpha$ -amylase and amylase-binding-protein A (AbpA) of *Streptococcus gordonii* with glucosyltransferase of *S. gordonii* and *Streptococcus mutans*. *BMC Microbiol.* 7:60.
- De Reuse H, Danchin A. 1991. Positive regulation of the *pts* operon of *Escherichia coli*: genetic evidence for a signal transduction mechanism. *J. Bacteriol.* 173:727–733.
- Douglas CW. 1990. Characterization of the  $\alpha$ -amylase receptor of *Streptococcus gordonii* NCTC 7868. *J. Dent. Res.* 69:1746–1752.
- Douglas CW. 1983. The binding of human salivary  $\alpha$ -amylase by oral strains of streptococcal bacteria. *Arch. Oral Biol.* 28:567–573.
- Douglas CW, Heath J, Gwynn JP. 1992. Enzymic activity of salivary

- amylase when bound to the surface of oral streptococci. *FEMS Microbiol. Lett.* 71:193–197.
16. Du LD, Kolenbrander PE. 2000. Identification of saliva-regulated genes of *Streptococcus gordonii* DL1 by differential display using random arbitrarily primed PCR. *Infect. Immun.* 68:4834–4837.
  17. Eglund PG, Palmer RJ, Jr, Kolenbrander PE. 2004. Interspecies communication in *Streptococcus gordonii*-*Veillonella atypica* biofilms: signaling in flow conditions requires juxtaposition. *Proc. Natl. Acad. Sci. U. S. A.* 101:16917–16922.
  18. Heinen W, Lauwers AM. 1976. Amylase activity and stability at high and low temperature depending on calcium and other divalent cations. *Experientia Suppl.* 26:77–89.
  19. Jackowski S, Murphy CM, Cronan JE, Jr, Rock CO. 1989. Acetoacetyl-acyl carrier protein synthase. A target for the antibiotic thiolactomycin. *J. Biol. Chem.* 264:7624–7629.
  20. Jenkinson HF. 1986. Cell-surface proteins of *Streptococcus sanguis* associated with cell hydrophobicity and coaggregation properties. *J. Gen. Microbiol.* 132:1575–1589.
  21. Jensen LJ, et al. 2009. STRING 8—a global view on proteins and their functional interactions in 630 organisms. *Nucleic Acids Res.* 37:D412–D416.
  22. Kilian M, Nyvad B. 1990. Ability to bind salivary  $\alpha$ -amylase discriminates certain viridans group streptococcal species. *J. Clin. Microbiol.* 28:2576–2577.
  23. Kolenbrander PE. 2000. Oral microbial communities: biofilms, interactions, and genetic systems. *Annu. Rev. Microbiol.* 54:413–437.
  24. Konto-Ghiorgi Y, et al. 2009. Dual role for pilus in adherence to epithelial cells and biofilm formation in *Streptococcus agalactiae*. *PLoS Pathog.* 5:e1000422.
  25. Lengeler JW, Jahreis K. 2009. Bacterial PEP-dependent carbohydrate: phosphotransferase systems couple sensing and global control mechanisms. *Contrib. Microbiol.* 16:65–87.
  26. Li L, Tanzer JM, Scannapieco FA. 2002. Identification and analysis of the amylase-binding protein B (AbpB) and gene (*abpB*) from *Streptococcus gordonii*. *FEMS Microbiol. Lett.* 212:151–157.
  27. Li SJ, Rock CO, Cronan JE, Jr. 1992. The *dedB* (*usg*) open reading frame of *Escherichia coli* encodes a subunit of acetyl-coenzyme A carboxylase. *J. Bacteriol.* 174:5755–5757.
  28. Liang X, Chen YY, Wu H. 2011. Sortase B assembles amylase-binding protein A to streptococcal cell surface, abstr 20. 89th IADR, San Diego, CA. <http://iadr.confex.com/iadr/2011sandiego/webprogramcd/Paper150380.html>.
  29. Loesche W. 2007. Dental caries and periodontitis: contrasting two infections that have medical implications. *Infect. Dis. Clin. North Am.* 21:471–502.
  30. Loo CY, Corliss DA, Ganeshkumar N. 2000. *Streptococcus gordonii* biofilm formation: identification of genes that code for biofilm phenotypes. *J. Bacteriol.* 182:1374–1382.
  31. Lu YJ, Rock CO. 2006. Transcriptional regulation of fatty acid biosynthesis in *Streptococcus pneumoniae*. *Mol. Microbiol.* 59:551–566.
  32. McMurry LM, Oethinger M, Levy SB. 1998. Triclosan targets lipid synthesis. *Nature* 394:531–532.
  33. Miller JL. 1960. Method of pure parotid saliva collection without cannulization. *J. Dent. Res.* 39:1075.
  34. Ramos JL, et al. 2005. The TetR family of transcriptional repressors. *Microbiol. Mol. Biol. Rev.* 69:326–356.
  35. Rock CO, Cronan JE. 1996. *Escherichia coli* as a model for the regulation of dissociable (type II) fatty acid biosynthesis. *Biochim. Biophys. Acta* 1302:1–16.
  36. Rogers JD, et al. 1998. Identification and analysis of a gene (*abpA*) encoding a major amylase-binding protein in *Streptococcus gordonii*. *Microbiology* 144(Pt 5):1223–1233.
  37. Rogers JD, Palmer RJ, Jr, Kolenbrander PE, Scannapieco FA. 2001. Role of *Streptococcus gordonii* amylase-binding protein A in adhesion to hydroxyapatite, starch metabolism, and biofilm formation. *Infect. Immun.* 69:7046–7056.
  38. Rohwer JM, Postma PW, Kholodenko BN, Westerhoff HV. 1998. Implications of macromolecular crowding for signal transduction and metabolite channeling. *Proc. Natl. Acad. Sci. U. S. A.* 95:10547–10552.
  39. Rosan B, Lamont RJ. 2000. Dental plaque formation. *Microbes Infect.* 2:1599–1607.
  40. Scannapieco FA, Bergey EJ, Reddy MS, Levine MJ. 1989. Characterization of salivary  $\alpha$ -amylase binding to *Streptococcus sanguis*. *Infect. Immun.* 57:2853–2863.
  41. Scannapieco FA, Bhandary K, Ramasubbu N, Levine MJ. 1990. Structural relationship between the enzymatic and streptococcal binding sites of human salivary  $\alpha$ -amylase. *Biochem. Biophys. Res. Commun.* 173:1109–1115.
  42. Scannapieco FA, Haraszthy GG, Cho MI, Levine MJ. 1992. Characterization of an amylase-binding component of *Streptococcus gordonii* G9B. *Infect. Immun.* 60:4726–4733.
  43. Scannapieco FA, Solomon L, Wadenya RO. 1994. Emergence in human dental plaque and host distribution of amylase-binding streptococci. *J. Dent. Res.* 73:1627–1635.
  44. Scannapieco FA, Torres G, Levine MJ. 1993. Salivary  $\alpha$ -amylase: role in dental plaque and caries formation. *Crit. Rev. Oral Biol. Med.* 4:301–307.
  45. Shiroza T, Kuramitsu HK. 1993. Construction of a model secretion system for oral streptococci. *Infect. Immun.* 61:3745–3755.
  46. Tanzer JM, et al. 2003. Amylase-binding proteins A (AbpA) and B (AbpB) differentially affect colonization of rats' teeth by *Streptococcus gordonii*. *Microbiology* 149:2653–2660.
  47. Tsay JT, Oh W, Larson TJ, Jackowski S, Rock CO. 1992. Isolation and characterization of the beta-ketoacyl-acyl carrier protein synthase III gene (*fabH*) from *Escherichia coli* K-12. *J. Biol. Chem.* 267:6807–6814.
  48. Vickerman MM, Iobst S, Jesionowski AM, Gill SR. 2007. Genome-wide transcriptional changes in *Streptococcus gordonii* in response to competence signaling peptide. *J. Bacteriol.* 189:7799–7807.
  49. Vorrasi J, Chaudhuri B, Haase EM, Scannapieco FA. 2010. Identification and characterization of amylase-binding protein C from *Streptococcus mitis* NS51. *Mol. Oral Microbiol.* 25:150–156.
  50. Yamamoto M, Jones JM, Senghas E, Gawron-Burke C, Clewell DB. 1987. Generation of Tn5 insertions in streptococcal conjugative transposon Tn916. *Appl. Environ. Microbiol.* 53:1069–1072.
  51. Zhang Y, Cronan JE, Jr. 1998. Transcriptional analysis of essential genes of the *Escherichia coli* fatty acid biosynthesis gene cluster by functional replacement with the analogous *Salmonella typhimurium* gene cluster. *J. Bacteriol.* 180:3295–3303.