

## Growth, Development, and Gene Expression in a Persistent *Streptococcus gordonii* Biofilm

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**A model for the protracted (30-day) colonization of smooth surfaces by *Streptococcus gordonii* that incorporates the nutrient flux that occurs in the oral cavity was developed. This model was used to characterize the biphasic expansion of the adherent bacterial population, which corresponded with the emergence of higher-order architectures characteristic of biofilms. Biofilm formation by *S. gordonii* was observed to be influenced by the presence of simple sugars including sucrose, glucose, and fructose. Real-time PCR was used to quantify changes in expression of *S. gordonii* genes known or thought to be involved in biofilm formation. Morphological changes were accompanied by a significant shift in gene expression patterns. The majority of *S. gordonii* genes examined were observed to be downregulated in the biofilm phase. Genes found to be upregulated in the biofilm state were observed to encode products related to environmental sensing and signaling.**

Tooth surfaces are persistently colonized by a complex but highly organized biota termed dental plaque, a microbial biofilm with the capacity to adapt to, and to endure, cyclic variation in nutrient availability as well as harsh mechanical and biological forces targeted at its containment and removal. *Streptococcus gordonii* is among the pioneering species to colonize a tooth surface (29, 30, 34, 39). Binding of these organisms to the tooth enamel creates a template for the subsequent attachment of other bacteria in establishment of the complex oral biofilm (20, 22). As succeeding layers of different bacterial species attach to the plaque, new binding templates and nutritional microenvironments are formed, which may ultimately favor the attachment and residence of periodontal pathogens (6). The net effect is the establishment of an ordered community of heterogeneous microbial species, with each member playing a role in maintaining the vitality and structure of plaque. A key element in the formation and stability of the plaque biofilm, therefore, is the persistent colonization of the smooth surface of the tooth at the base of this complex community.

Several studies have now demonstrated that cells existing in the biofilm state have phenotypic characteristics distinct from those of their planktonic counterparts, with significant changes in the patterns of gene expression (9, 45). This differential expression appears to be governed by communication between bacteria of the same or other species, in addition to cues emanating from the host and the environment (23). Specific intercellular communication mediated by *N*-(3-oxodecanoyl)-L-homoserine lactone has been shown to be central to the differentiation of the biofilm architecture by *Pseudomonas aeruginosa* (11).

Little is known of the physiologic changes that accompany persistent colonization of the tooth surface by pioneering spe-

cies in the formation of the dental plaque biofilm. It was therefore of interest to develop a model of protracted smooth-surface colonization with cyclic variation in nutrient availability as a first approximation of events that occur in the establishment of the dental plaque biofilm by pioneering bacterial species such as *S. gordonii*. Once the model was established, it was also of interest to determine whether this persistent colonization was accompanied by physiologic changes that may account for the long-term survival and durability of the plaque biofilm. We report the morphological characteristics of *S. gordonii* Challis DL1 exposed to cyclic variation in nutrient availability and persistently colonizing a smooth surface for as long as a month, and we identify transcriptional changes that accompany the formation of this persistent physiologic state.

### MATERIALS AND METHODS

**Bacterial strains and media.** To reduce the likelihood of contamination over the protracted cultivation period, a spontaneous streptomycin-resistant mutant of *S. gordonii* Challis DL1 (a gift from P.E. Kolenbrander, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, Md.) was selected by plating approximately  $10^{10}$  cells on tryptone-yeast extract medium (30 g of tryptone/liter–0.5 g of yeast extract/liter) (TY) supplemented with 10 mmol of sucrose, 200  $\mu$ g of streptomycin (Sigma-Aldrich Co.)/ml, and 1.5% (wt/vol) agar (2). *S. gordonii* appeared to preferentially partition into the biofilm phase.

**Culture conditions and generation of biofilms.** Streptomycin-resistant *S. gordonii* Challis DL1 biofilms were cultivated on 18- by 18-mm no. 1 glass coverslips (Fisher Scientific, Pittsburgh, Pa.) in 100-mm-diameter, 15-mm-deep polystyrene petri dishes at 37°C under anaerobic conditions. The biofilm was seeded with 15 ml of a 1:100-diluted overnight planktonic culture of *S. gordonii* and 200  $\mu$ g of streptomycin/ml. At 24-h intervals, the spent medium was aspirated from the dish, and the plates and coverslips were washed thoroughly twice with phosphate-buffered saline, pH 7.4 (PBS), to remove adventitiously associated cells. The biofilm was then further cultured in fresh, sterile, appropriately supplemented medium. This cycle of feeding and washing was repeated at 24-h intervals for as long as 30 days.

**Confocal microscopy.** For microscopic imaging of biofilms, coverslips were washed in PBS to remove cells nonspecifically associated with the biofilm and were stained with 100  $\mu$ l of freshly prepared 10-mg/ml aqueous acridine orange, which was applied directly to the coverslip, followed immediately by three sequential PBS rinses. Confocal microscopy was conducted at a magnification of

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TABLE 1. Genes whose expression in biofilm and planktonic cultures was compared

Gene	Description	Source or reference	Primer sequence	
			Forward	Reverse
<i>scaR</i>	Manganese-dependent repressor of <i>scaCBA</i>	16	TTCTTGCCAAGGAACCTGAACAT	GGTCACATGAGGCAGTGGAA
<i>sgg</i>	GTP-binding protein	19	TCGGTAAAGGCGGATCCA	CCCCAGCATAAGCTCAATAT
<i>ropA</i>	Chaperone-trigger factor (prolyl isomerase)	This study	CCTTCAGCTGCTGGACCTTC	TGCACGTATCGAACGCGA
<i>dltA</i>	D-Alanine-D-Alanyl carrier protein ligase	5	TTGCTGCTTATCATACTGGAGAT	GAAATCCATTGACCACCGTAA
<i>scaA</i>	Coaggregation-mediating adhesin	21	TGTCTGGGCTTGAGGAGTT	CTCTGCGGTTTCGCAATACAAC
<i>hhpH</i>	Oligopeptide-binding lipoprotein (hexa- or heptapeptide)	17	ACCCGACAAAATGGTGGATTATT	AGAGCTGACACAACATCTTGGTTT
<i>arcB</i>	Omithine carbamoyltransferase	3	CGTACGCGTGCAGCCTT	TCATTAGACCAAGATATTCTGG ATGC
<i>abpA</i>	Amylase binding protein	33	AGTGAAGGTGGAAGCCACAAT	ACGTACAGCGTTGAAAGCGTT
<i>hugX</i>	Putative heat shock protein	41	GGTCGCTTCTTTGCTCTTTT	CACCCAGAGGAGAATTCATCCA
LDH	Lactate dehydrogenase	This study	CATGATGTAGGCGTGCAGTGA	AGCTCGTTTCCGTCGAAGCA
tRNA-arg	tRNA for Arg	14	GGTCCCATAGCTCAGCTGGATA	TCCCAGCAAGATTCGAACITG
<i>hsa</i>	Streptococcal hemagglutinin	38	CGTGGGACCCTTCAGGAAAT	TTGACCGTAAAGGACAAATCTTT CTAG
<i>rpoC</i>	DNA-directed RNA polymerase beta subunit	24	TGTCTGGGCTTGAGGAGTT	CTCTGCGGTTTCGCAATACAAC
int/CoA	Intragenetic coaggregation-relevant adhesin	46	TCCAGCAGTCTGTGGCAGATA	TTAGCGACACGGTGGTGGT
<i>scaA</i> orf	Hydrophobic membrane protein	21	TGATTACAGCCATTGTGATTGGA	TGCCCCGAGAATGATAAACT
<i>ddl</i>	D-Alanine: D-alanine ligase	13	CCCGTCTTACCAAACCTTCTA	CGCAGTCTTCTCTGATTATCAGAT
<i>soda</i>	Manganese-dependent superoxide dismutase	32	GCTGACATTGATGCTACTTTTGGT	CAAACGAGTTGTTGCTGCAG
<i>flpA</i>	Fibronectin-binding protein-like protein A	27	TCTGCTTCTCCGTTGCTAGG	CCGCCGAGTAGAAAATGAGTTG
<i>xdhA</i>	Extracellular glyceraldehyde-3-phosphate dehydrogenase	28	TCGGTCGTATCGGTCGTCTT	AGGTGCTGTATGCGAGTAACCTC
<i>tdkF</i>	Thymidine kinase	26	ATGACCAGCGCAGTTGACAC	GCCTGGCGTTTCATACCAATT
<i>cysK</i>	Cysteine kinase	A. J. M. Vriesema, unpublished data	TTTTCAATGGTAAAGTTCAGTTA AGCA	GCTATGGCTATCATAGCTTTTTTT TATATC
<i>comYA</i>	ABC transporter subunit	24	TCCGGACTTGCTCATCATTG	AAACTGTAGCTCCCGTCAAGCT
<i>sspA</i>	Surface adhesin A	12	AAGAGTTGGCTGAGTATCCGA CTAA	AGTCTACAAGTGCCGCCTTAATT
<i>spaA</i>	<i>spaA</i> homolog adhesin protein I/II V-region	4	ACAACCTCAATACCAAGAAC ACCA	TGGTGTGTCAGGTTTGTCAAG
<i>msrA</i>	Methionine sulfoxide reductase	44	TTGCCGGTGACAAGTTTGAG	CTCTGGCAATAGGCCGACTAA
orf pH	Neutral pH-inducible promoter region and unknown gene	43	GGCCTTCAGATTTTTTCAGAGA TTAA	CGTGTCCGAAATACAGGTCATTT
<i>gtfG</i>	Glucosyltransferase	42	AGAGCGTTTGCCAGAACCA	CAAACACATCGTCATCATGCT
<i>rggD</i>	<i>gtfG</i> regulator (positive)	37	TCGTAAAGTCGTCGGGAAAAA	CGAGACAGCTGGGCAACAG
<i>pbpG</i>	Phospho-beta-glucosidase	44	CGGCGGCAAATCAATATGA	AGACAGGCCTTTTCCATTAGAT
<i>comD</i>	Competence pheromone receptor (histidine kinase)	14	AAATGCACATCTTAATAGCTTTGC TAGT	CATATTGTTACAGAGCAGACT TCAG
<i>comE</i>	Competence pheromone regulator	14	TTGAGTCAGACGAGGTAATCA ACTT	GCATAGGGATTATGTTGGCG TATA

×63 under PBS immersion by using a model TCS NT confocal microscope (Leica Lasertechnik GmbH, Heidelberg, Germany) equipped with an argon-krypton laser.

**Enumeration of adherent cells.** To enumerate cells in the biofilm phase, the biofilms were dislodged from the substratum by scraping and were transferred to a 2-ml Bead Beater tube (BioSpec Products, Bartlesville, Okla.) containing 0.5 ml of 1-mm-diameter glass beads (BioSpec Products) in 1 ml of PBS. The tubes were subsequently transferred to a Bead Beater (BioSpec Products) and horizontally shaken for 1 min to disaggregate the cells from the biofilm matrix, a condition determined experimentally to yield the maximum number of CFU with no detectable loss of viability. Disaggregated biofilm phase cells and planktonic phase cells were then enumerated by quantitative track dilution plating as described previously (18).

**RNA extraction.** RNA was rapidly purified from planktonic cells or 10-day-old biofilm cells as previously described (35, 36), with minor modifications. Cells from the biofilms were disaggregated by scraping the biofilm into a 2-ml microcentrifuge tube (BioSpec Products) containing 0.5 ml of 1-mm-diameter glass beads and 1.5 ml of PBS. The tube was placed in the Mini Beadbeater (BioSpec Products) and shaken twice at 5,000 rpm for 60 s. The tube was immediately transferred to ice, and the beads were allowed to settle out. The suspended cells were removed from the beads and transferred to a 17- by 100-mm snap-cap tube (VWR International, Westchester, Pa.). To recover additional cells interspersed

within the bead bed, the beads were washed with an additional 1.5 ml of PBS, and fractions were combined. Disaggregated biofilm or planktonic phase cells were pelleted by centrifugation at  $2,500 \times g$  for 4 min at 4°C and then resuspended in 1.5 ml of Tri-Reagent (Sigma-Aldrich). The suspension was immediately transferred to a 2-ml microcentrifuge tube containing approximately 0.5 ml of packed 100- $\mu$ m-diameter zirconia-silica beads, which was placed in a high-speed reciprocating shaker (BioSpec Products) and horizontally shaken at 5,000 rpm for 1 min to lyse the cells. RNA was recovered from the cell lysate, treated with RQ1 RNase-free DNase (Promega, Madison, Wis.) as described previously (39, 40), and stored in diethyl pyrocarbonate (DEPC)-treated water. The integrity of the RNA was assessed by electrophoresis of 2  $\mu$ l of each sample through a 1.2% agarose-0.66 M formaldehyde gel in MOPS running buffer (20 mM morpholinopropanesulfonic acid [pH 7.0] [MOPS], 8 mM sodium acetate, 1 mM EDTA [pH 8.0]) at 3 to 4 V/cm (40). The RNA concentration was determined spectrophotometrically by measuring the  $A_{260}/A_{280}$  ratio of a 1:50 dilution in DEPC-treated water.

**Real-time quantitative PCR.** Genes for which expression in biofilm and planktonic cultures was compared included those known or previously suggested to be involved in biofilm formation or in *S. gordonii* adhesion or coaggregation and are listed in Table 1. Amplification, detection, and analysis were performed using the ABI Prism 7700 Sequence Detection system (Applied Biosystems, Foster City,

Calif.) as described previously (36). Briefly, primers were developed by using the algorithms provided in Primer Express (version 1; Applied Biosystems) for uniformity in size (approximately 100 bp) and melting temperature. Primer sequence data are provided in Table 1.

RNA concentrations were normalized by using amplification of the 23S rRNA gene of *S. gordonii* as an internal standard. For each experimental reaction, cDNA synthesis and PCR amplification were performed in a two-step reaction. Reverse transcriptase reactions were performed using the Taqman reverse transcription (RT) reagent kit (Applied Biosystems). Each 50- $\mu$ l reaction mixture contained the following in a 1 $\times$  RT buffer: 5.5 mM MgCl<sub>2</sub>, 500  $\mu$ M each deoxynucleoside triphosphate, 0.4 U of RNase inhibitor/ $\mu$ l, 1.25 U of reverse transcriptase/ $\mu$ l, 2.5  $\mu$ M reverse primer, and 10 ng of total RNA. The RT reaction was incubated at 48°C for 30 min and then at 95°C for 5 min.

Real-time PCR amplifications were performed in 50- $\mu$ l reaction mixtures that contained 1 $\times$  SYBR Green I PCR master mix (Applied Biosystems), 300 nM forward primer, 50 nM additional reverse primer, and 5  $\mu$ l of cDNA template. PCR conditions included an initial denaturation at 95°C for 10 min, followed by a 40-cycle amplification consisting of denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min. All primer pairs were checked for primer-dimer formation by using the two-step protocol described above without the addition of RNA template. As an additional control for each primer pair and each RNA sample, the cDNA synthesis reaction was carried out without reverse transcriptase in order to identify contamination of RNA samples by residual genomic DNA.

The value used for quantitation and comparison among the samples was the threshold cycle ( $C_T$ ), or the number of cycles required to cross the midpoint of the detectable amplification curve, which was normalized to a passive reference dye (carboxy-x-rhodamine [ROX]) included in each reaction. Real-time PCR analysis was performed on three independent RNA preparations from three separate planktonic and biofilm cultures. The  $C_T$  values for RNA obtained from biofilm cultures were compared to the  $C_T$  values of the same products amplified from planktonic-culture RNA in order to determine the fold difference. Fold difference was calculated by dividing the larger of the mean  $C_T$  values for a particular gene (biofilm or planktonic) by the smaller. If the larger mean (which indicates more cycles of amplification required to cross the midpoint of the visible range of detection, and hence a smaller starting RNA concentration) derived from the planktonic culture, a plus sign was affixed to the fold difference to indicate an increase in RNA abundance in the biofilm sample. If the reverse was true, a minus sign was affixed to indicate a decrease in the abundance of that specific RNA in the biofilm-derived sample. Student's *t* test was used to calculate the significance of the difference between the mean expression of a given gene in a 10-day biofilm and its mean expression in planktonic culture. A *P* value of <0.05 was considered significant.

## RESULTS

**Microscopic characterization of the persistent biofilm model.** To simulate the cyclic exposure to nutrients experienced by *S. gordonii* in the oral cavity over protracted periods, a batch cycling and rinsing model was developed, where *S. gordonii* Challis DL1 was cultured in sucrose-supplemented 0.5 $\times$  TY medium on glass coverslips in petri dishes as the substratum. A visible film of adherent bacteria was observable 24 h after inoculation in PBS-rinsed dishes. Initially, the adherent cells took the form of a smooth, uniform coating of the support substratum. However, by 8 days, a visibly rough, coherent, sheet-like structure developed, which was maintained and continued to evolve over the duration of the study. There was no observable difference between the biofilm that formed on the glass coverslips and that on the polystyrene surfaces of the petri dishes that contained them.

To characterize the developing *S. gordonii* Challis DL1 biofilm in greater detail, its evolution was monitored throughout development by confocal microscopy, with concurrent enumeration of the adherent population. At regular intervals over a period of approximately 1 month, biofilms developing on the glass coverslips were stained and examined (Fig. 1). Initially and through the first 4 days of cyclic feeding and washing,

adherent cells formed a thin, confluent layer of uniform thickness, with no observable differentiation of higher-order architectures, confirming observations made visually. However, following 8 to 9 days of cyclic feeding and washing, adherent cells began to differentiate into discrete microcolonies. By 10 days, a stippled mat of *S. gordonii* Challis DL1 covered the surface, with obvious differentiation into microcolony and channel architectures (Fig. 1), similar to those reported during maturation of *P. aeruginosa* biofilms (7, 8). These complex structures continued to develop throughout continued cycles of feeding and washing (Fig. 1), achieving a thickness of 100  $\mu$ m by day 30 (Fig. 2).

**Growth kinetics of *S. gordonii* Challis DL1 biofilms.** Because of the observable evolution of higher-order architecture in the growing biofilm over its monthlong development, it was of interest to determine the relationship between biofilm structure and the population dynamics of the cultivable bacteria comprising the biofilm. Therefore, adherent cells were harvested in triplicate, disaggregated with glass beads, and enumerated by track dilution quantitation (18) (Fig. 3).

Bacterial growth was found to be biphasic. Over the first 2 days, the bacterial population increased in number; after that point, the population of cultivable cells declined until day 8. This change in population appears to parallel the log, stationary, and decline phases characteristically observed for planktonic organisms. From day 8 through day 10, however, there was a resurgence in the adherent cell population, which reached a second plateau by day 11. Cell numbers remained remarkably constant over the remainder of the 30-day study period. Interestingly, the period corresponding to the initial decline in population (days 4 to 8) followed by resurgence (days 8 to 10) (Fig. 3) corresponded precisely with the emergence of highly organized channel and microcolony architectures (Fig. 1).

Because the nutrient content of the medium has been found to regulate the development of biofilms by other organisms (31, 47), we tested global nutrient components for their influence on biofilm formation by *S. gordonii* DL1. The availability of simple carbohydrates and nitrogenous nutrients was varied as follows: (i) unsupplemented TY, (ii) 0.5 $\times$  unsupplemented TY, (iii) TY plus 10 mmol of sucrose, (iv) 0.5 $\times$  TY plus 10 mmol of sucrose, (v) 0.5 $\times$  TY plus 10 mmol of glucose, and (vi) 0.5 $\times$  TY plus 10 mmol of fructose. The extent of partitioning between adherent populations and planktonic cells was determined after 4 days of cyclic feeding and washing, a time found in preliminary experiments to yield substantial populations of cells in both phases. As shown in Fig. 4, when the medium was not supplemented with simple carbohydrates (i.e., sucrose, glucose, or fructose), similar numbers of cells were found in the planktonic and biofilm phases (culture in TY or 0.5 $\times$  TY). The addition of sucrose to TY resulted in numbers of adherent-population cells comparable to, or nominally higher than, those observed with unsupplemented TY but, unexpectedly, even higher numbers of cells in the planktonic phase at this relatively early point in biofilm development. On the other hand, when the concentration of nitrogenous medium components was reduced by 50% (i.e., 0.5 $\times$  TY) and the medium was supplemented with sucrose, glucose, or fructose, the cells appeared to preferentially partition into the biofilm phase, indicating that nitrogen limitation in the presence of



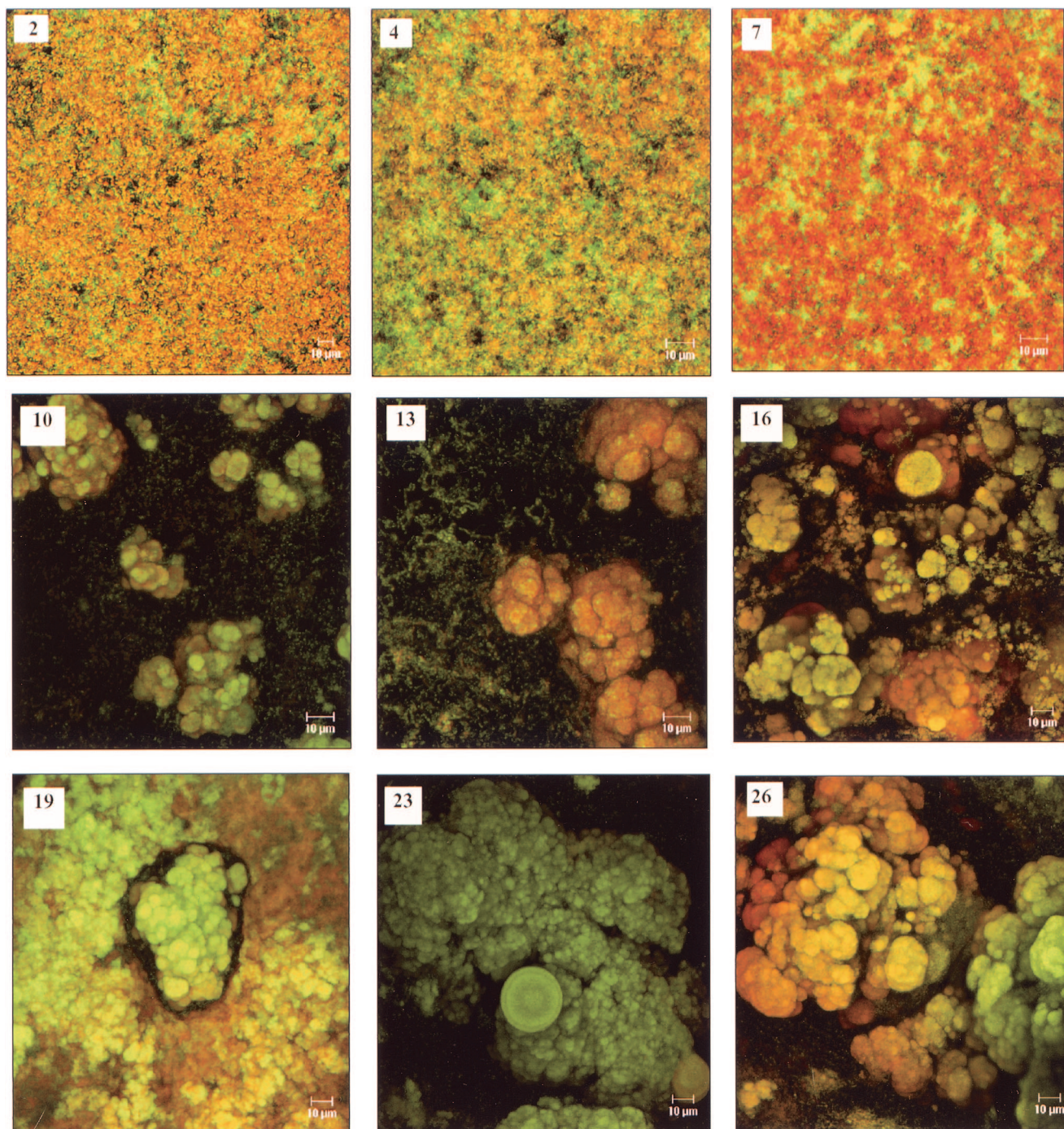


FIG. 1. Confocal scanning laser micrographs of the development of *S. gordonii* Challis DL1 biofilms over 26 days. The biofilms were generated on glass coverslips in 0.5× TY medium supplemented with 10 mM sucrose and were stained immediately prior to microscopy with acridine orange. Numbers indicate the age of the biofilm in days. Magnification, ×63.

excess carbohydrate promotes biofilm formation by this species.

**Differential gene expression.** To assess the expression of genes known or thought to be involved in biofilm formation by *S. gordonii*, real-time PCR was used to quantify gene expression in planktonic cells for comparison to that in 10-day-old biofilms. In general, most genes tested were observed to be

downregulated in the biofilm (Table 2). The greatest reduction in gene expression in the biofilm phase was observed for *scaR*, which codes for a metalloregulator of the manganese uptake system in *S. gordonii* (16). Levels of mRNA encoding ScaR were observed to be reduced approximately 45-fold in biofilm-derived cells. This reduction in expression was accompanied by an eightfold reduction in the abundance of mRNA encoding

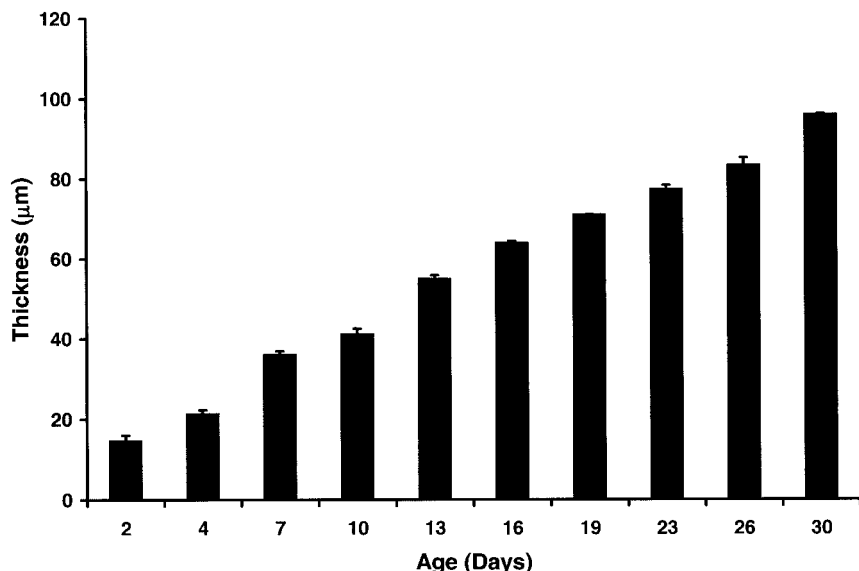


FIG. 2. Change in thickness of biofilms with time, as measured by confocal microscopy. The depth of the biofilm represents the mean of five randomly chosen sites within each biofilm.

ScaA, one component of the putative manganese transport system. The second greatest reduction in mRNA abundance in the biofilm phase was observed for that encoding Sgg, a GTP-binding protein shown to be a member of the G protein su-

perfamily in streptococci (19). The third greatest reduction in biofilm gene expression occurred in a homolog of *ropA* in group A streptococci, encoding a putative peptidyl-prolyl isomerase and chaperone (25). Genes observed to be expressed at in-

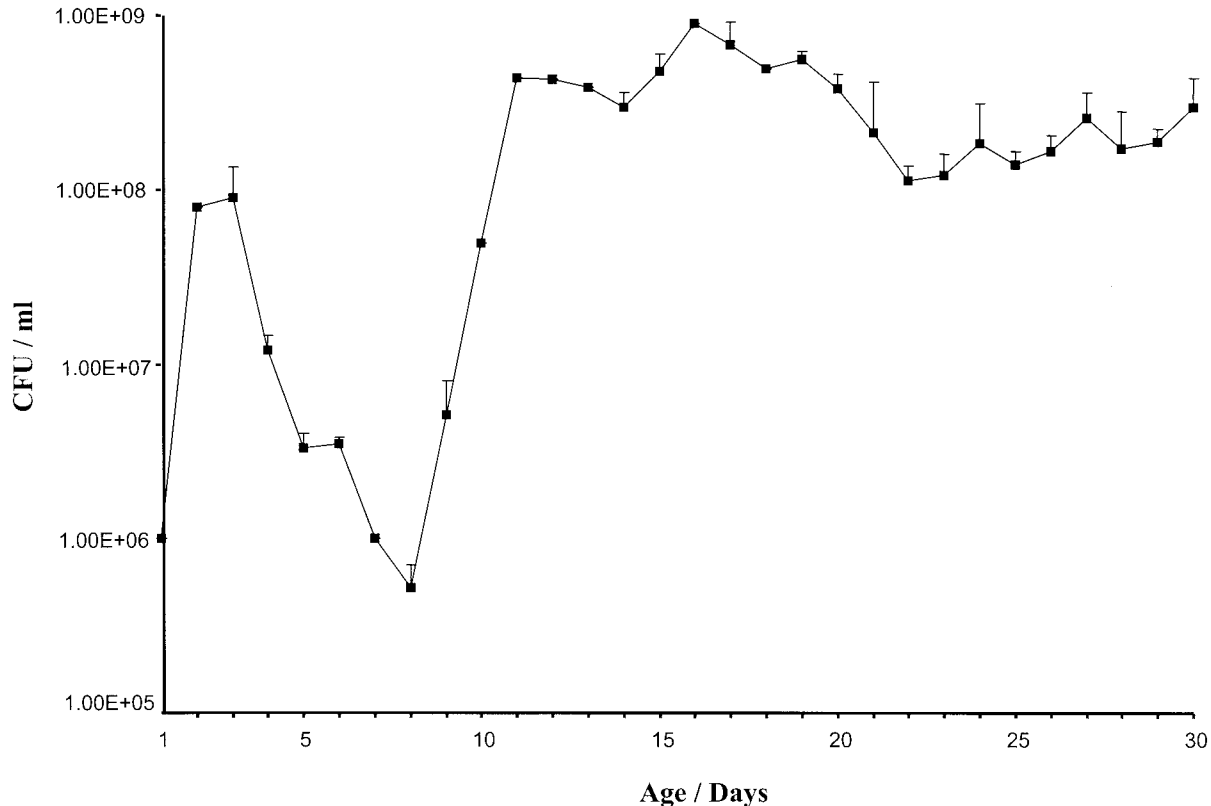


FIG. 3. Change in CFU within *S. gordonii* Challis DL1 biofilms cultured in 0.5× TY medium supplemented with sucrose, which was replaced at 24-h intervals. Cells were enumerated over 30 days of cultivation. Numbers of CFU for biofilms harvested at each time point are expressed as means of triplicate determinations. Error bars represent the standard errors of the means and are not detectable where the error is smaller than the symbol.

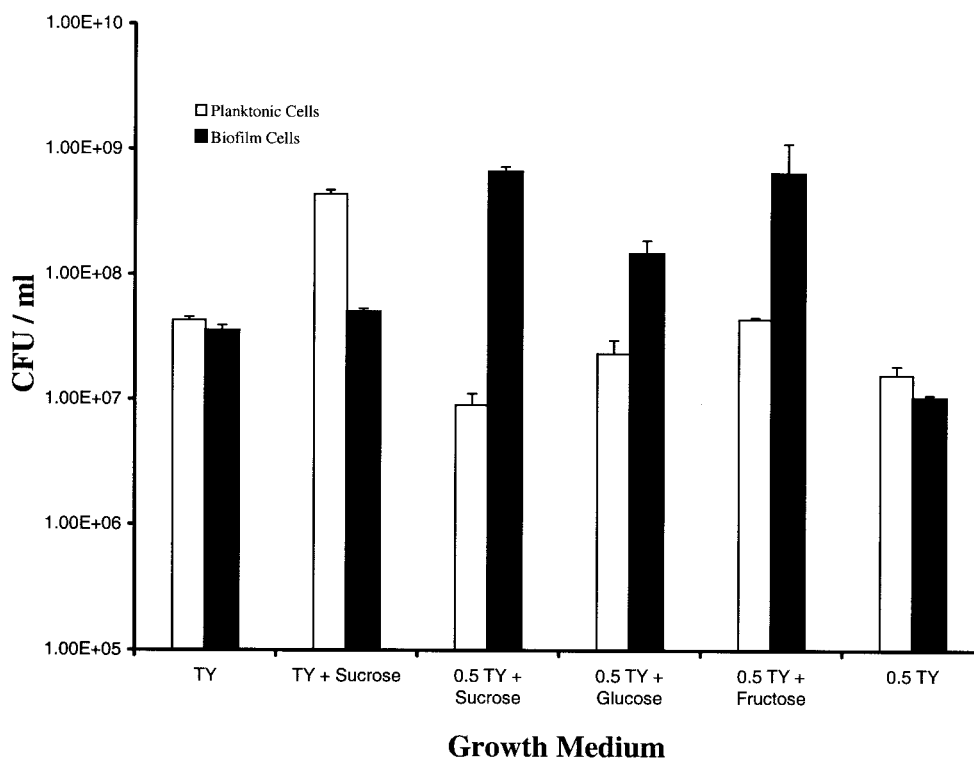


FIG. 4. Effects of medium composition on partitioning of *S. gordonii* DL1 into biofilm and planktonic phases after 4 days of cultivation. Reduction in the yeast extract and tryptone contents of the medium resulted in increased numbers of CFU occurring in the biofilm phase in all cases where the medium was supplemented with 10 mmol of a simple sugar (sucrose, glucose, or fructose). Lack of carbohydrate supplementation of either TY or 0.5× TY resulted in comparable numbers in the biofilm and planktonic phases, and supplementation of TY with sucrose resulted in an unexpected increase in the number of planktonic cells, with no net change in numbers in the biofilm phase.

creased levels in the biofilm phase were largely limited to *comD* and *comE*, which were observed to occur at four- and ninefold-increased abundances, respectively. The *comD* and *comE* genes encode the histidine kinase and response regulator for the *S. gordonii* competence pathway for DNA uptake (14).

## DISCUSSION

Using a cyclic feeding and washing model of persistent colonization, we have shown that *S. gordonii* Challis DL1 forms highly differentiated biofilms capable of supporting stable populations of organisms over a protracted period. The differentiated architectures that developed over the 30-day experimental period were reminiscent of the microcolony and channel structures seen in biofilms of *Pseudomonas fluorescens* and *Streptococcus mutans* (1, 31). An interesting bimodality of growth was observed during *S. gordonii* biofilm development. Confocal microscopic observation, accompanied by quantitative determination of population size, revealed an initial cycle of growth, spanning days 0 to 8, which appears to represent the conventional log, stationary, and decline phases of growth commonly observed for bacteria grown in planktonic culture. This early period was characterized by adherence to glass coverslips in a fine, confluent, and evenly distributed pattern, with little evidence of community organization. The second phase initiated with a resurgence in bacterial numbers after day 8 and achieved population stability by day 11, which corresponded in

time with the appearance of organization within the adherent community. Characteristic mushroom and pillar formations emerged, and channels appeared to be cleared in place of the uniformly distributed individual cells that initially attached to the substratum. In many ways, the emergence of organization, involving growth, remodeling, and potentially specialization within the community, from an initial mass of uniform cells resembles early stages in the tissue differentiation of higher organisms.

Following the emergence of organization over days 8 to 11 postinoculation, the population remained remarkably stable through the end of the 30-day experimental period. However, the biofilm did not remain static. Biofilm thickness was observed to increase steadily to a maximum of 100  $\mu\text{m}$ . The increase in biofilm thickness without an observable increase in culturable CFU may represent the continuing deposition of the extracellular polysaccharide matrix, the embedding of dead cells and their replacement by live progeny, or perhaps other physiological changes which render some members of the community less capable of reversion to a mode of growth that permits colony formation on an agar surface (i.e., viable but not culturable).

The physiology of biofilm formation has become a topic of considerable interest and investigation. Biofilms involving several different species have been studied, perhaps none more intensively than those of *P. aeruginosa* (7, 8, 9, 45). The pattern of biofilm development in *P. aeruginosa* has been dissected into



TABLE 2. mRNA abundance in biofilm relative to the planktonic culture

Gene	Fold difference <sup>a</sup>
Reduced expression in biofilm	
<i>scaR</i> .....	-45.68
<i>sgg</i> .....	-33.21
<i>ropA</i> .....	-11.50
<i>dltA</i> .....	-8.08
<i>scaA</i> .....	-8.01
<i>hhpH</i> .....	-5.89
<i>arcB</i> .....	-5.79
<i>abpA</i> .....	-5.50
<i>higX</i> .....	-5.02
2856 LDH.....	-4.57
<i>tRNA-arg</i> .....	-3.93
<i>hsa</i> .....	-3.76
<i>rpoC</i> .....	-3.68
<i>int/CoA</i> .....	-3.05
<i>scaA orf</i> .....	-2.75
<i>ddl</i> .....	-2.60
<i>sodA</i> .....	-2.45
<i>fpA</i> .....	-2.40
<i>xdhA</i> .....	-2.30
<i>tdkF</i> .....	-1.97
<i>cysK</i> .....	-1.82
No significant difference	
<i>comYA</i> .....	-1.52
<i>sspA</i> .....	-1.44
<i>spaA</i> .....	-1.23
<i>msrA</i> .....	-1.14
<i>orf pH</i> .....	1.26
<i>gtfG</i> .....	1.58
Increased expression in biofilm	
<i>rggD</i> .....	1.95
<i>pbg</i> .....	2.29
<i>comD</i> .....	4.23
<i>comE</i> .....	9.40

<sup>a</sup> Except for genes in the group labeled "No significant difference," comparisons of means were statistically significantly different ( $P < 0.05$ ).

distinct phases: initial attachment to a solid surface and microcolony formation, followed by maturation of microcolonies into exopolysaccharide-encased biofilms (7, 8). Cook et al. (6) recently analyzed biofilm formation by *S. gordonii* Challis DL1 on saliva-coated glass coverslips in a flow cell system similar to that used successfully for analysis of *P. aeruginosa* biofilm development. Biofilm development by *S. gordonii* Challis DL1, as determined by the formation of microcolonies around adherent bacteria, was assessed after 4 h. It was noted that under these conditions, *S. gordonii* Challis DL1 failed to establish a definable biofilm in this brief monoculture but did form a template for attachment of the periodontal pathogen *Porphyromonas gingivalis*.

Differences in the ability of *S. gordonii* Challis DL1 to form a biofilm are clearly model dependent. The results of the present study show that the culture medium exerts an important influence on the partitioning of cells between planktonic and biofilm phases. It is not known whether this partitioning is the simple result of a shift in physiology resulting from quorum-sensing cues, which have been shown to be important for *P. aeruginosa* biofilm development (11), or whether there is an underlying genetic switch, such as that seen with phase variation by some species facilitating adaptation to new ecological conditions (15) (a phenomenon known to occur in *S. gordonii* and to affect glucan production by cell surface glucosyltrans-

ferases [40]), with the subsequent selection and outgrowth of adherent-phase variants in a time- and population size-dependent manner.

Direct comparison of the gene expression profiles of cells in biofilm and planktonic phases consistently demonstrates that biofilm formation is accompanied by significant shifts in cell physiology. Others have shown that in *P. aeruginosa*, *algC* and *algD*, required for the synthesis of the exopolysaccharide matrix, alginate, are upregulated upon adhesion to an inanimate surface (10). More recently, it was shown by use of a microarray that in comparatively shorter term biofilms, approximately 0.5% of *P. aeruginosa* genes were upregulated and 0.5% were downregulated at significant levels (45). Because of the sensitivity of real-time PCR and its amenability to the performance of replicates for individual statistical determination, we were able to measure changes that achieved statistical significance for a larger fraction of the genes tested, although our results are in good agreement with the finding that changes of large magnitude occurred only in a minority of the genes examined. Also, in the absence of a completed genome, our experiments focused only on factors previously known or suspected of being involved in biofilm formation and adhesion, which may be a selected group more likely to be environmentally responsive.

In conclusion, a simple system for the analysis of biofilm formation by *S. gordonii* Challis DL1 was developed that permits study of the persistent colonization of smooth surfaces by this organism. Biofilm formation by this organism was influenced by the availability of several simple carbohydrates, importantly including two other than sucrose, demonstrating the importance of bacterial traits, potentially in addition to surface glucosyltransferases, in biofilm establishment. More-global differential gene expression studies to characterize *S. gordonii* biofilm formation will be greatly facilitated by the availability of genome sequence information.

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