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Initial characterization of the *Streptococcus gordonii htpX* gene

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Examination of the *Streptococcus gordonii* chromosomal region, which lies immediately upstream of the glucosyltransferase positive regulatory determinant *rgg*, revealed two open reading frames. Based on nucleotide sequences, these genes were similar to the *Listeria monocytogenes lemA* gene, which is involved in antigen presentation, and the *Escherichia coli htpX* heat shock gene, which has an unknown function. Northern hybridization analysis indicated that *S. gordonii lemA* and *htpX* genes were associated with a *ca.* 1.7-kb polycistronic transcript. Although levels of the *lemA*/*htpX* transcript did not increase in response to heat to levels seen with *dnaK* controls, insertional inactivation of *htpX* resulted in changes in adhesiveness, cellular morphology and detergent-extractable surface antigens in cells grown at 41°C, implying that *htpX* may be involved in surface protein expression. Insertional inactivation of *lemA* and *htpX* indicated that, despite their proximity to *rgg* and the structural gene, *gtfG*, these upstream genes do not affect *S. gordonii* glucosyltransferase activity.

Key words: oral streptococci, glucans, bacterial adhesion, heat shock

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Streptococcus gordonii is an early colonizing bacterial component of dental plaque (7). When these bacteria enter the bloodstream, they can attach themelves to cardiac tissues, acting as pathogens in infective endocarditis (8). The accumulation of streptococcal cells on surfaces can be facilitated by extracellular glucosyltransferase (GTF) enzymes which synthesize glucan polymers from sucrose. *S. gordonii* has a single GTF enzyme encoded by the structural gene, *gtfG* (37). The genetic regulation of *gtfG* expression is complex and may involve multiple levels of control (28, 35). Transcription of *gtfG* is positively regulated by the upstream determinant, *rgg* (27). Although nucleotide sequence analysis of spontaneously occurring *S. gordonii* strain Challis variants has shown that decreased levels of GTF can result from mutations in either *rgg* or *gtfG*, data indicate that additional genes are also involved (35). The chromosomal DNA sequence of a spontaneous variant strain, with *ca.* 30% of the parental level of GTF protein, was determined upstream of the putative *rgg* promoter through the putative transcriptional terminator downstream of *gtfG*. This 5.95-kbp chromosomal region was identical to that of the parental strain, indicating that additional genes were involved in determining the level of GTF activity (35). The DNA region immediately downstream of the putative *rgg* promoter had a region of dyad symmetry suggestive of a regulatory factor binding site (28), which implied that *trans*-acting factor(s) may influence *rgg* expression.

Complementation and biochemical studies suggest that at least two unlinked genetic loci are responsible for the increased levels of GTF activity which are seen in mutants arising from chemical mutagenesis (12). In order to determine the potential roles that genes immediately upstream of *rgg* might play in influencing GTF activity in *S. gordonii*, this chromosomal region was examined. Previous studies indicate that a 1.7-kb transcript is associated with this region (27), and the present studies further characterized this upstream region. Based upon nucleotide sequence analysis, two genes were identified. The first was similar to the *lemA* gene involved in antigen presentation in *Listeria monocytogenes* (21), and the second was similar to *Escherichia coli htpX* (19). The latter is a heat shock gene of unknown function, which is induced by a temperature increase; the over-expression of a truncated form of the HtpX protein leads to an increase in the degradation of abnormal proteins. Initial characterization of the *S. gordonii lemA and htpX* genes was performed *via* an examination of *S. gordonii* mutant strains in which these genes were insertionally inactivated. The results suggest that, despite their proximity, the *S. gordonii lemA* and *htpX* genes immediately upstream of *rgg* do not influence the level of GTF activity.

Materials and methods

Bacterial strains, plasmids and culture conditions

All strains were stored at −80°C in 50% glycerol. *S. gordonii* strain Challis CH1 (30) and its derivatives were grown in a Todd Hewitt (TH; Difco Laboratories Inc., Detroit MI) or in defined, FMC medium (31) and incubated in an anaerobic chamber with a gas mixture of 85% N_2, 10% H_2 and 5% CO_2. *S. gordonii* strains examined for the effects of heat stress were each grown to early log stage (*ca.* 10^6 cells/ml) at 36°C, then transferred to a 42°C heat block, resulting in a culture temperature of 41.5°C, for various time periods until mid- to late-log stage. *S. gordonii* strains with integrated plasmids for insertional inactivation of genes were grown with 5 μg/ml erythromycin. *E. coli* DH5α strains were grown in a Luria-Bertani (LB) medium and incubated aerobically at 37°C. Strains carrying cloned inserts in pVA8912 (22) were grown with 300 μg/ml erythromycin.

DNA isolation and manipulations

Plasmid DNA was prepared using Qiagen purification columns (Qiagen Inc., Valencia, CA) according to the manufacturer's directions. In order to prepare *S. gordonii* chromosomal DNA, cells were grown overnight in broth, plus 0.5% glycine, and incubated with mutanolysin and lysozyme as previously described (28). Double-stranded polymerase chain reaction (PCR) products were obtained using Elongase enzyme (Invitrogen Life Technologies, Carlsbad, CA) in accordance with the manufacturer's directions. DNA fragments were eluted from agarose gels with a Qiaex bead kit (Qiagen Inc). For cloning, DNA subclones or polymerase chain reaction (PCR) fragments were digested with appropriate restriction enzymes, ligated with T4 DNA ligase into convenient restriction sites of vector DNA and transformed into *E. coli* DH5α $CaCl_2$-treated cells (3). *S. gordonii* cells were transformed according to Lawson and Gooder's method (20), using horse serum.

Southern blots

Chromosomal DNA was digested with restriction enzymes, electrophoresed in agarose gels and transferred to nylon membranes using standard procedures (3). Probe DNA was labelled with digoxigenin-dUTP, hybridized to the membrane under stringent conditions and detected by chemi-luminescence using the Genius System (Roche Molecular Biochemicals, Indianapolis, IN). The DNA probe for the *htpX* gene was a 392-bp PCR fragment, produced from the *S. gordonii* chromosomal template with primers 5′GTCCATGAATGGTGCT3′ and 5′AAAAGGCCAAGACCAC3′ (27). The probe used for the *lemA* gene was a 354-bp internal *Hin*dIII fragment (Fig. 1A).

Inverse PCR

The DNA region upstream of the cloned DNA fragment in pAMS36 (28), containing *rgg* and the 5′ end of *gtfG*, was recovered from recircularized *Xba*I-digested *S. gordonii* chromosomal DNA. Southern blotting indicated that the internal *htpX* probe hybridized to a *ca.* 4.4-kb *Xba*1 fragment. Accordingly, *Xba*1-digested CH1 chromosomal DNA was electrophoresed on 0.7% agarose and fragments ranging in size from 4.0 to 5.0 kb were eluted, recircularized with T4 DNA ligase and used as a template for inverse PCR. The primers were 5′GCAGTTGAGCTCAATGGA3′, located *ca.* 575 bp upstream of the *Xba*I site within *gtfG* in pAMS36 (Fig. 1A), and 5′ATTCATGGACATGACTACCTCAG3′, located *ca.* 320-bp downstream from the 5′ *Hin*dIII site of pAMS36. The resulting single *ca.* 1.6-kb PCR product, which contained 752 base pairs upstream of the *S. gordonii* chromosomal region cloned in pAMS36, was electrophoresed, eluted from the agarose gel, and sequenced directly with custom oligonucleotide primers.

DNA sequencing and analysis

Both strands of DNA templates were sequenced via cycle sequencing with a PRISM-Ready Reaction Dye Deoxy Terminator Sequencing kit (Applied Biosystems Inc., Foster, CA) using custom oligonucleotide primers. Searches for homologs of the *S. gordonii* nucleotide and deduced amino acid sequences were carried out using National Center for Biotechnology Information databases (http://www.ncbi.nlm.nih.gov) in conjunction with the Basic Local Alignment Search Tool (BLAST) algorithm (1). Gene comparisons were done using MacVector (Oxford Molecular Group, Hunt Valley, MD) and the Genetics Computer Group Wisconsin Package, Version 10.0 Gap software programs (Madison, WI) for nucleotide (23) and protein (11) similarity. Computer analysis of protein domains was performed using the Pfam protein family database (4). Protein structural predictions were done using Signal P (24) and TMpred (14) programs to predict signal sequences and transmembrane orientations, respectively.

Construction of insertionally inactivated mutants

To construct strains in which the *lemA* and *htpX* genes were disrupted, internal fragments were cloned into the streptococcal integration vector pVA8912 in *E. coli* DH5α. To disrupt *lemA*, the 354-bp internal *Hin*dIII fragment (Fig. 1A) was cloned into the *Hin*dIII site of pVA8912. To disrupt *htpX,* oligonucleotide primers, 5′GGATCCATGAATGGTGCTCG3′ and 5′AAGCTTAAGGATTAGAACCCG3′, were designed with engineered restriction sites (underlined) and a codon change (G was replaced with an **A** to encode TAA in the chromosomally integrated fragment) in the downstream primer to introduce a translational stop to ensure a truncated gene product. These primers were used in PCR with pAMS36 template to amplify an internal region of *htpX* (Fig. 1B). The resulting 163-bp fragment was digested with restriction enzymes and directionally cloned into the *Bam*HI and *Hin*dIII sites of pVA8912. Plasmid nucleotide sequences were confirmed. The orientations of the *lemA* and *htpX* internal fragments in pVA8912 remained the same. The plasmids were transformed into the parental strain CH1, and erythromycin-resistant transformants, resulting from integration of the plasmid into the chromosome, were selected on TH agar containing 5 μg/ml of erythromycin. For each gene, the site of chromosomal integration was confirmed by Southern hybridization analyses using both pVA8912 and the internal gene fragments as probes. Constructs with predicted Southern hybridization patterns were confirmed by nucleotide sequence analyses of PCR products from reactions that used chromosomal templates of putative transformants and oligonucleotide primers designed to anneal to the DNA regions that flanked the duplicated DNA regions and the vector cloning sites. Two resulting strains, CH35, in which *lemA* was insertionally inactivated, and CH34, in which *htpX*

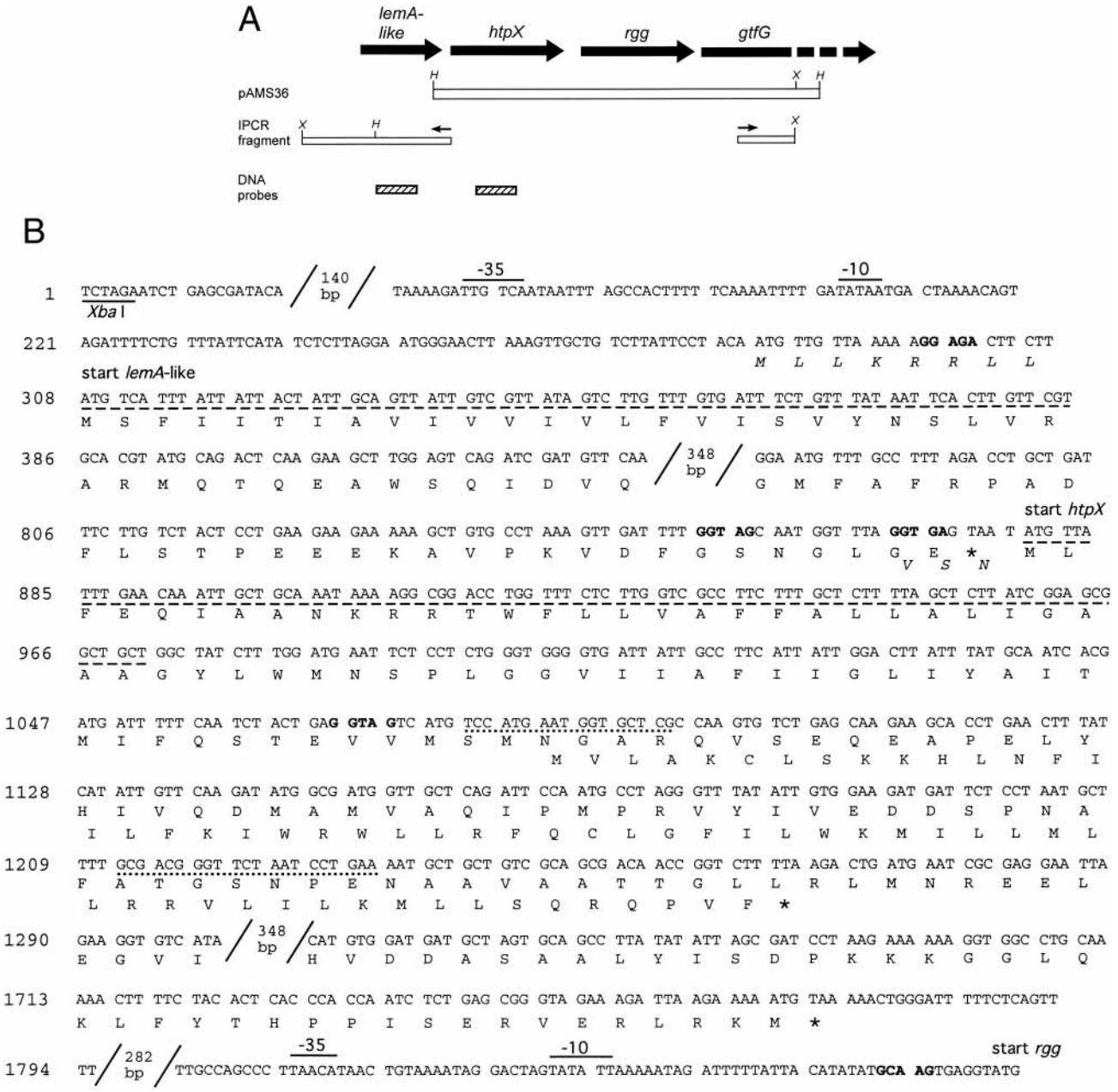

Fig. 1. A. Schematic diagram depicting *Streptococcus gordonii* chromosomal positions of *rgg* and the 5′ end of *gtfG* in plasmid pAMS36. The inverse PCR fragment was generated to examine the DNA region upstream of the *rgg* and *gtfG* genes. The large arrows above the restriction digest maps indicate the size and reading direction of potential open reading frames. For orientation, significant restriction sites are designated by H (*Hin*dIII) and X (*Xba*I). Position and direction of primers used for inverse PCR are shown by small arrows above the mapped inverse PCR (IPCR) product. Positions and sizes of *lemA* and *htpX* DNA probes used for hybridization analyses are shown by hatched bars. *B.* Partial nucleotide and amino acid sequences of the sequenced region upstream of *rgg*. Nucleotide numbers are indicated in the left-hand margin. Deduced amino acids for each open reading frame are shown by one letter codes beneath the first nucleotide of each codon. Putative −35 and −10 promoter sites are indicated with a single line. Putative ribosomal binding sites are emboldened. NH_2-terminal amino acids corresponding to alternative start sites of *lemA* and *htpX* are italicized. Computer-predicted signal sequences are underlined with a broken line. Nucleotides corresponding to the oligonucleotide primers used to insertionally inactivate *htpX* and the potential 60 amino acid internal protein, are underlined with a dotted line.

was insertionally inactivated, were chosen for further characterization.

Examination of GTF levels

Relative amounts of GTF were determined via activity gels as previously described (30). Briefly, strains were grown to the same mid- to late-log stage and equal volumes of cell-free supernatants were run on sodium dodecyl sulfate 8.75% polyacrylamide gel electrophoresis (SDS-PAGE). To determine GTF activity levels, gels were subjected to electrophoresis then incubated overnight in 3% sucrose, 0.5% Triton X-100 in 10 mM sodium phosphate, pH 6.8, at 37°C. The resulting glucan bands were

then treated with periodic acid and pararosanaline. The intensities of the stained bands reflect the relative amounts of active GTF protein (30,36). Relative GTF activity for each strain was determined via laser densitometry (LKB Ultrascan XL) for four independent gels. The sensitivity of this assay is comparable to GTF activities determined by measuring [^{14}Cglucose] sucrose incorporation into glucan polymers (30,36). To confirm that GTF activity correlated to amounts of GTF protein, immunoblots were done as previously described (32). Briefly, after electrophoresis proteins were transferred to PVDF membranes and incubated overnight with polyclonal antiserum directed against strain Challis GTF (a gift from H. F. Jenkinson), and antibody-reactive protein was detected with goat antirabbit IgG-alkaline phosphatase. The amounts of anti-GTF antibody reactive protein in each lane were determined densitometrically.

Determination of cell surface antigen profiles

In order to compare strains or growth temperatures, bacteria were grown to the same cell density in FMC and cell surface antigen profiles, were determined as previously described (17). Briefly, cells were extracted with 1% sodium lauryl sarcosinate (SLS) detergent in 1 mM NaEDTA, 10 mM Tris-HCl, pH 7.5 for 20 min at room temperature (16). Equal volumes of extracts (*ca.* 6 µg protein/lane) were run on SDS-PAGE. Proteins were transferred using an electroblot apparatus to PVDF membranes in 10 mM CAPS, 10% methanol, pH 11.0. Membranes were incubated overnight with polyclonal rabbit antiserum which had been raised against whole strain CH1 cells (17). Bound anti-CH1 antibody was detected with goat antirabbit IgG-alkaline phosphatase. Relative intensities of bands were determined via densitometric readings.

Northern blots

S. gordonii RNA was prepared from cultures grown with 0.5% glycine. Cells were collected by centrifugation at 4°C and the RNA extracted using the Purescript RNA isolation kit (Gentra Systems, Minneapolis MN) with the following modifications. Cell pellets were resuspended in 16.1 mg lysozyme ml^{-1} and 100 Units of mutanolysin in GET buffer(50 mM glucose, 10 mM EDTA, 25 mM Tris HCl, pH 8.0), and incubated at 37°C for 10 min. After the addition of 0.1 g of SDS, cells were incubated on ice for 5 min, then at 65°C for 30 s for lysis. RNA was isolated with the Purescript kit in accordance with the manufacturer's instructions, then further purified by two phenol/chloroform/isoamyl alcohol extractions and an ethanol precipitation. The final purified RNA was then resuspended in RNA hydration solution (Gentra). Equal amounts of RNA were electrophoresed on a 2% formaldehyde-1% agarose gel, transferred to a positively charged nylon membrane and probed under stringent conditions with digoxigenin-labelled DNA fragments (DIG System; Roche Biochemicals) according to the manufacturer's directions. Internal regions of *lemA* and *htpX* were used as probes (Fig. 1A). All solutions were prepared using sterilized dimethyl pyrocarbonate-treated water.

To monitor transcription of *lemA*/*htpX* at elevated temperatures, transcription of *S. gordonii dnaK* was used as a positive control. A probe for the *S. gordonii dnaK* transcript was made by PCR with strain CH1 chromosomal template, using primers for an internal region of *dnaK* that was conserved among the *Streptococcus agalactiae, S. mutans*, and *Streptococcus pneumoniae dnaK* genes found in GenBank. The forward primer, 5′ TTAGGTACAACAAACTCAGCAGT3′ and reverse primer, CTGTTGAGAAGACTTGTGATTTAG3′ resulted in a *ca.* 1.2-kb DNA fragment that was sequenced and found to have 85% identity with *S. mutans*, 86% identity with *S. agalactiae*, and 92% identity with the *S. pneumoniae dnaK* genes (GCG Wisconsin Gap program). To measure potential heat-associated transcriptional effects, a single strain CH1 culture with *ca.* 5×10^5 cells/ml was dispersed into 2 ml aliquots and grown at 36°C. At the early to mid-log phase, tubes containing cultures to be heat-stressed were transferred to an environment where the temperature was 41.5°C. The heat-stressed cultures, as well as the control cultures that remained at 36°C, were then sampled for periods of 10 min for up to 3 h and total RNA was prepared immediately. Synchronicity of cultures was confirmed by OD_{520} readings.

Adhesion to surfaces

As an *in vitro* model of adhesion to tooth surfaces, the ability of bacterial cells to attach to hydroxyapatite and saliva-coated hydroxyapatite beads was measured as previously described (34). Briefly, [^{3}H]thymidine-labelled cells were washed and resuspended to a concentration of 10^8 cells/ml in buffered KCl, pH 6.8. After incubation for 3.5 h with 10 mg of hydroxyapatite beads that had been either buffer-equilibrated or coated with human saliva, supernatants containing unattached bacteria were removed and hydroxyapatite beads containing attached bacteria were counted in a scintillation counter. Radiolabel counts were correlated to bacterial numbers via viable counts, and adhesion was determined as the number of attached bacteria per 10 mg of beads. Percent recovery ([number of counts in the supernatant + number of counts on the beads ÷ the initial washed cell counts/ml] × 100) was ≥ 85%.

As an indicator of cell surface hydrophobicity, the attachment of *S. gordonii* cells, grown at control and elevated temperatures, to phenyl-Sepharose beads, was measured as previously described (33). Briefly, [^{3}H]thymidine-labelled bacteria were washed and resuspended in buffered KCl (*ca.* 10^9cells/ml) then mixed with an equal volume of 25% phenyl-Sepharose beads in buffer for 90 min at room temperature (26). The number of bacteria adsorbed to Sepharose beads was calculated from radiolabelled counts of unattached cells.

All tests were done in duplicate and repeated at least three times. Statistical comparisons between strains or growth conditions were done by a two-tailed Student *t*-test.

Scanning electron microscopy

To determine the potential effects that elevated growth temperature could have on the cellular morphology of mutant and parental *S. gordonii* strains, cells were washed in 0.1 M sodium phosphate buffer (pH 7.4) and fixed in 2.5% glutaraldehyde for 1 h. After graded dehydration to 100% ethanol, hexamethyldisialazane (HMDS) was used as the final dehydrating agent (6). Following the last HMDS rinse, small drops of the cell suspension were placed onto glass coverslips and allowed to dry. The coverslips were then glued to an aluminum stub and sputter-coated (approx. 15 nm) with gold, before being examined with an AMRAY 1000-B Scanning Electron Microscope (Bedford, MA, USA).

Nucleotide accession numbers

The *S. gordonii* chromosomal region upstream of *rgg,* which is partially shown in Fig. 1(B), has been deposited in GenBank with accession number AF017421.

Results

Identification and sequence analysis of the *lemA*-like and *htpX* genes upstream of *rgg*

Several potential open reading frames were present in the region immediately upstream of *rgg*. The first of these was a 570-bp open reading frame that shares 60.5% identity with the *L. monocytogenes lemA* gene that is involved in antigen presentation (21); the encoded proteins are 49.4% similar. Based upon the most likely potential ribosomal binding site (emboldened at nucleotide number 287, Fig. 1B), the *S. gordonii* encoded LemA protein has a molecular weight of 20.96 and a pI of 5.42. Like the *L. monocytogenes* LemA (21), the *S. gordonii* putative protein lacks basic residues before the hydrophobic stretch in the region of the predicted signal sequence, suggesting the possibility that the NH_2-terminus remains intact. However, based on computer analyses (14,24), the *S. gordonii* encoded protein has a predicted signal sequence cleavage site after amino acid 27 and two potential transmembrane helices with an NH_2- terminus outside orientation.

S. gordonii lemA also has a potential start site 24 nucleotides upstream (nucleotide number 284, Fig. 1B) which would increase the size of the protein to 197 amino acids; the additional NH_2-terminal amino acids are indicated by italics in Fig. 1(B). This larger protein would have a MW of 21,984 and a pI of 8.69. However, since there is not a strong potential ribosomal binding site for the alternative start codon for this protein, the 189 amino acid LemA protein (the coding sequence starting at nucleotide number 308, Fig. 1B), with an NH_2-terminal region that is more closely aligned with that of the expressed *L. monocytogenes* LemA protein, is more probable.

The *lemA*-like gene is immediately followed by an 891-bp open reading frame, beginning with an ATG start codon, that encodes a putative transmembrane protein with a calculated MW of 32,786 and pI of 6.71. The nucleotide sequence has 44.4% and 45.9% identity with *htpX* genes of *Escherichia coli* (19) and *Haenophilus influenzae*, respectively. The corresponding HtpX proteins are 53.19% and 55.56% similar. *S. gordonii htpX* also had 57% identity with the partial sequence of the *L. monocytogenes lemB* gene which is cotranscribed with *lemA* (21). Thus, it appears that the *S. gordonii* DNA region immediately upstream of *rgg* is similar to the *L. monocytogenes lemA*/*lemB* operon.

The *S. gordonii htpX* has two closely placed potential ribosomal binding sites and two potential translational start sites (Fig. 1B). If the protein starts with the upstream GTG codon and corresponding ribosomal binding site, then the resulting 900-bp open reading frame encodes a protein with a molecular weight of 33,086. Such closely placed possible translational start sites are also seen in *htpX* genes of *E. coli* and *H. influenza*, both of which have two adjacent methionine residues at the start of their open reading frames (GenBank accession numbers M58470 and U32755, respectively). Computer-assisted structural predictions (4) indicate that, like the *E. coli* HtpX, the encoded *S. gordonii* HtpX has a putative metallopeptidase motif, suggestive of a zinc-binding endopeptidase.

Unlike the *E. coli* and *H. influenzae htpX* genes, however, the *S. gordonii htpX* has a 180-bp internal open reading frame with a potential ribosomal binding site (beginning at nucleotide number 1084, Fig. 1B). This putative cytoplasmic protein, with a molecular weight of 7,414 and a pI of 13.08, is 53.45% similar to the 69 carboxyl-terminal amino acids of HlyB, an ATP-dependent *E. coli* hemolysin transport protein involved in the translocation of substrates across membranes (18).

Insertional inactivation of *lemA* or *htpX* does not affect GTF activity

To determine the effects of insertional activation of *lemA* or *htpX* on GTF activity, comparisons were made of strains CH35 and CH34, respectively, to the parental strain CH1 (Fig. 2A). Densitometric scanning of GTF activity gels showed that the product band intensities differed by less than 10% among all three strains for four independent experiments, indicating that there were no detectable differences in GTF activity within the limits of this assay. Because of the possibility that *S. gordonii htpX* is involved in a heat stress response, activity was also examined in cultures shifted to the potential heat-shock temperature, 41.5°C. Again, no differences were observed in levels of GTF activity among strains CH1, CH34, and CH35. Immunoblots using antiserum directed against *S. gordonii* GTF confirmed that the levels of activity corresponded to levels of GTF protein (Fig. 2B). As seen when activity was measured, GTF antibody-reactive protein bands for all three strains differed in intensity by less than 10%. Similarly, colony morphologies on TH 3% sucrose agar plates, which are influenced by the presence of glucans synthesized by GTF (30,36), were indistinguishable for mutant and parent strains grown at 36°C or 42°C. There were also no differences among colony morphologies of strains CH1, CH34 and CH35 when grown on TH agar without sucrose at either temperature and incubated either anaerobically or aerobically in 5% CO_2. The growth curves of strains CH1, CH34 and CH35 in FMC medium demonstrated similar doubling times (data not shown).

Temperature and insertional inactivation of *htpX* affects cell surface antigen profiles

Because of its similarity to the *E. coli* heat shock gene, *htpX*, the effects of insertional inactivation of *htpX* on *S. gordonii* cells grown at normal or elevated temperatures were further examined. To examine cell surface antigens, detergent extracts of bacterial cells were immunoblotted with antiserum directed against whole parental cells (Fig. 3). Although the predicted size of HtpX is *ca.* 33 kDa, no loss of antigen band in this size range was seen in strain CH34, which suggests that, either the putative transmembrane HtpX was not removed by the detergent extraction methods used, or that HtpX was not detected by the whole cell antiserum used in these studies. As expected, some differences in levels of cell surface antigens were seen in parental cells grown at 36°C and 41.5°C, reflecting expected responses to temperature stress. Differences were also observed between strains CH1 and CH34. The change in detergent-extractable antigens was also seen in two independently constructed *htpX*-insertionally inactivated strains (data not shown). Moreover, these differences were most noticeable in cells grown at elevated temperatures. Most noticeably, SLS extracts of strain CH34 cells grown at 41.5°C showed a consistent reduc-

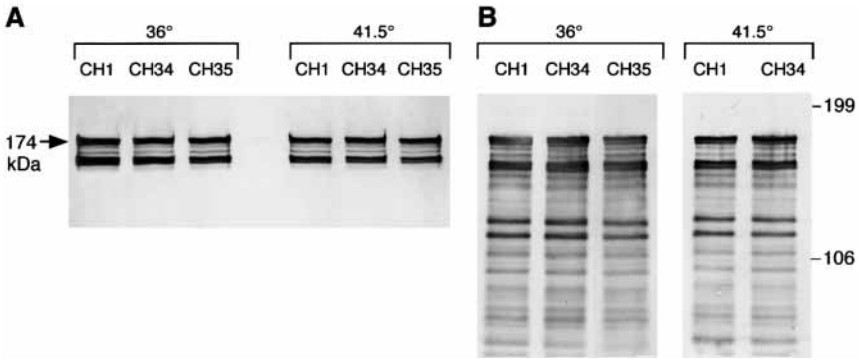

Fig. 2. Examination of GTF levels. Cells were grown at 36°C to early log stage and then either left at 36°C or shifted to a temperature of 41.5°C and grown to the same mid- to late-log stage as determined by OD_{520} readings. Equal volumes of cell-free culture supernatants were run on SDS-PAGE. *A.* To determine GTF activity, gels were incubated in sucrose and Triton X-100, treated with periodic acid and the resulting glucan bands stained with pararosanaline. Band intensity is proportional to GTF activity. The position of the *ca.* 174 kDa native GTF protein is shown by an arrow. Beneath the native protein band are lower molecular weight bands with various amounts of GTF activity. *B.* To confirm that the levels of GTF activity were related to levels of GTF protein, gels were immunoblotted, a process described in Materials and methods, and GTF antibody-reactive protein was detected with antiserum directed against strain Challis GTF. These lower molecular weight forms of GTF are thought to be due to the degradation of the native enzyme by endogenous proteases, and occur even in the presence of protease inhibitors, as previously described (10,32). Results shown are representative of a minimum of four independent experiments. Protein size markers ran as indicated. Similar GTF activities and protein levels for all three strains were also seen when cells were grown in TH medium (data not shown).

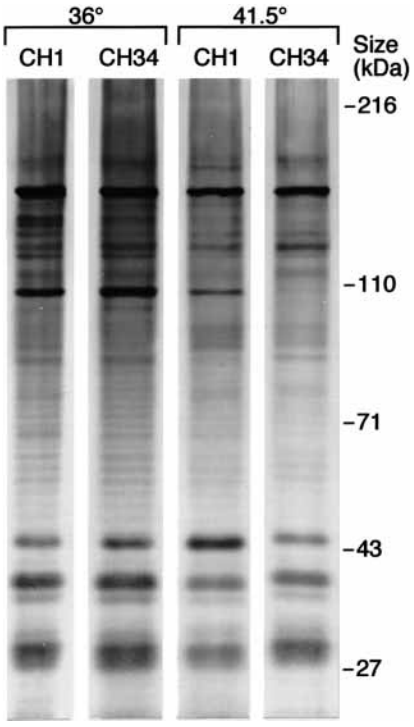

Fig. 3. Effects of increased temperature on *Strepococcus gordonii* cell surface antigens. FMC cultures of strains CH1 and CH34, either incubated at 36°C or shifted to 41.5°C. Pairs of strains to be compared were grown to the same OD_{520} and cell pellets were extracted with 1% SLS. Equal volumes of cell extracts were run on SDS-PAGE and immunoblotted with polyclonal rabbit antiserum directed against *S. gordonii* strain CH1 whole cells. Protein size markers ran as indicated. Results shown are representative of four independent immunoblots.

tion($24 \pm 12\%$ of the parental level) in the level of a *ca.* 102 kDa antigen in comparison to the levels observed in the parental strain, which was grown to the same cell density. Differential expression of this antigen has previously been noted in *S. gordonii* phase variant strains and is not related to the integrated vector; rather, decreased levels of this antigen have been associated with changes in hemolytic zones surrounding *S. gordonii* colonies grown on Oxoid blood agar plates (17). However, differences in hemolysis were not seen in strains CH1 and CH34. Overall, the data indicated that inactivation of *htpX* affected detergent-extractable cell surface antigens. However, it was beyond the scope of the present studies to identify the specific protein changes that occur.

Inactivation of *htpX* affects cell surface morphology

To determine if growth temperature, *htpX* disruption and any resulting changes in cell surface antigens affected cell surface morphology, cells of strains CH1 and CH34 were examined by scanning electron microscopy. Results from two independent preparations indicated that cells of both strains had similar smooth surfaces when grown at 36°C (Fig. 4, panels A and B). The parental strain looked similarly smooth when grown at 41.5°C (Fig. 4, Panel C). However, the cell surfaces of strain CH34, grown at 41.5°C, were distinctly rough (Fig. 4, Panel D).

Growth temperature affects *S. gordonii* adhesive properties

Strains CH1 and CH34 grown at 36°C did not differ in their abilities to attach to hydroxyapatite or saliva-coated hydroxyapatite beads ($P > 0.6$; Table 1). Growth at 41.5°C significantly increased adhesion of both strains to buffer-equilibrated hydroxyapatite ($P < 0.0002$ for both CH1 and CH34 compared to 36°C-grown strain CH1 cells; Table 1). This increased adhesion may be related to increased surface hydrophobicity of cells grown at elevated temperature (see below). Adhesion of both strain CH1 and CH34 cells grown at 41.5°C to saliva-coated hydroxyapatite was lower in comparson to adhesion of cells grown at 36°C, but this difference was not statistically significant ($P > 0.05$).

When grown at 36°C, strain CH34 cells were slightly less able to attach to phenyl-Sepharose beads than the parental cells ($P \leq 0.02$), suggesting that disruption of *htpX* resulted in a less adhesive phenotype, which was possibly due to decreased surface hydrophobicity. Growth at 41.5°C significantly increased the numbers of strain CH1 and CH34 cells that attached to phenyl-Sepharose beads ($P \leq 0.0001$ compared to cells grown at 36°C), suggesting that growth at higher temperature increased the hydrophobicity of both strains. Differences between the parent and mutant cells grown at 41.5°C in their adhesiveness to phenyl-Sepharose were not significant ($P \geq 0.4$), suggesting that the increased adhesiveness in response to elevated temperature overcomes the decreased adhesiveness of strain CH34 cells in comparison to that displayed by the parental strain at 36°C. Examination by phase contrast microscopy indicated that streptococcal chain lengths of

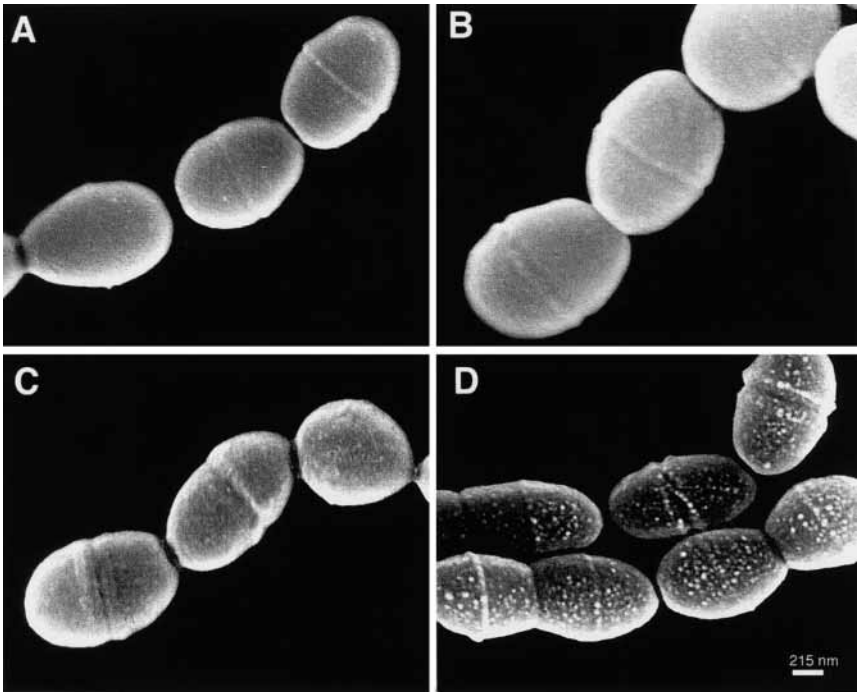

Fig. 4. Scanning electron micrographs of strain CH1 and strain CH34 cells grown in FMC medium at 36°C and 41.5°C. To examine cells for any morphological changes bacteria were grown to early log stage (OD_{520} *ca.* 1.0) and then either kept at 36°C or transferred to 41.5°C for approximately three hours until they reached the same cell density (OD_{520} *ca.* 1.6). Cells were then pelleted and prepared for scanning electron microscopy as described in Materials and methods. *A.* Parental strain CH1 grown at 36°C. *B.* Insertional *htpX* mutant strain CH34 grown at 36°C. *C.* Strain CH1 transferred to 41.5°C. *D.* Strain CH34 transferred to 41.5°C. The surface of the *htpX* insertionally inactivated strain grown at 41.5°C is distinctly rougher than the surface of the parental cells grown at 41.5°C. These cellular morphologies were noted in two independent cell preparations.

strains CH1 and CH34 grown at both 36°C and 41.5°C were similar.

Examination of *lemA/htpX* transcription

Northern blots of strain CH1, probed with internal fragments of the *lemA* and *htpX* open reading frames (Fig. 1A), indicated that both probes hybridized to the same *ca.* 1.7-kb fragment (data not shown). This result was consistent with the size of the polycistronic transcript suggested by the nucleotide sequence data (Fig. 1B). This also confirmed previous results indicating that the region upstream of *S. gordonii rgg* was associated with a 1.7-kb transcript that was not cotranscribed with *rgg and gtfG* (27). Northern blots suggested that the *lemA/htpX* transcript was expressed at a higher level during the early to mid-log stage of growth and decreased thereafter (Fig. 5A). There is also a 600-bp transcript that hybridized to the *htpX* probe. It is not known if this was due to the cross-reaction of the *htpX* probe with another transcript or due to mRNA processing.

To examine potential responses of *S. gordonii htpX* to elevated temperature, Northern blots were carried out to examine the transcription of *htpX* at the heat shock temperature of 41.5°C. The well-characterized *dnaK* heat shock gene was used as a positive control. The *S. gordonii dnaK* probe hybridized primarily to *ca.* 2.5-kb transcript (Fig. 5B). Although the *dnaK* chromosomal region of *S. gordonii* has not been studied and its genomic organization is not known, studies in the closely related *S. mutans* species are consistent with the idea that multiple *dnaK* transcripts may be due to mRNA processing (15). The transcript sizes and timing of the *dnaK* response to temperature increase were similar to those seen in the characterized response of *Lactococcus lactis* (2). The results (Fig. 5B) showed that the *dnaK* transcript clearly increased in response to a temperature shift to 41.5°C. The *S. gordonii dnaK* response was evident after 10 min at 41.5°C. Response was maximum at 20 min and decreased by 60 min. However, elevated temperatures did not elicit a similar response from *htpX* (Fig. 5A). No detectable changes in *htpX* transcription were seen when temperature stresses of up to 43°C were used (data not shown).

Discussion

Glucan production is an important colonization determinant of oral streptococci. Genetic data indicate that chromosomal region(s) in addition to

Table 1. Adhesive properties of cells grown at 36°C and 41.5°C

Strain	Growth temperature[a]	substratum		
		hydroxyapatite [b]	saliva-coated hydroxyapatite [b]	phenyl-Sepharose[c]
CH1	36°	11.29 ± 0.78	4.03 ± 0.46	41.1 ± 1.8
CH34	36°	11.50 ± 0.84	4.13 ± 0.27	36.7 ± 2.1
CH1	41.5°	21.25 ± 1.84	3.46 ± 0.72	69.9 ± 2.3
CH34	41.5°	19.75 ± 1.73	3.65 ± 0.13	71.1 ± 2.2

[a]Growing cells remained at 36°C or were shifted to 41.5°C; growth continued to the same OD_{520} reading before centrifugation and resuspension in buffered KCl.

[b]Number of buffer-washed radiolabelled bacteria × 10^6 (± S. D) attached to 10 mg of hydroxyapatite and saliva-coated hydroxyapatite beads after 3.5 h of rotation in tubes at room temperature.

[c]Number of radiolabelled bacteria × 10^7 (± S. D) attached to phenyl-Sepharose beads after 1.5 h of rotating incubation at room temperature. Tests were done in duplicate and repeated at least 3 times.

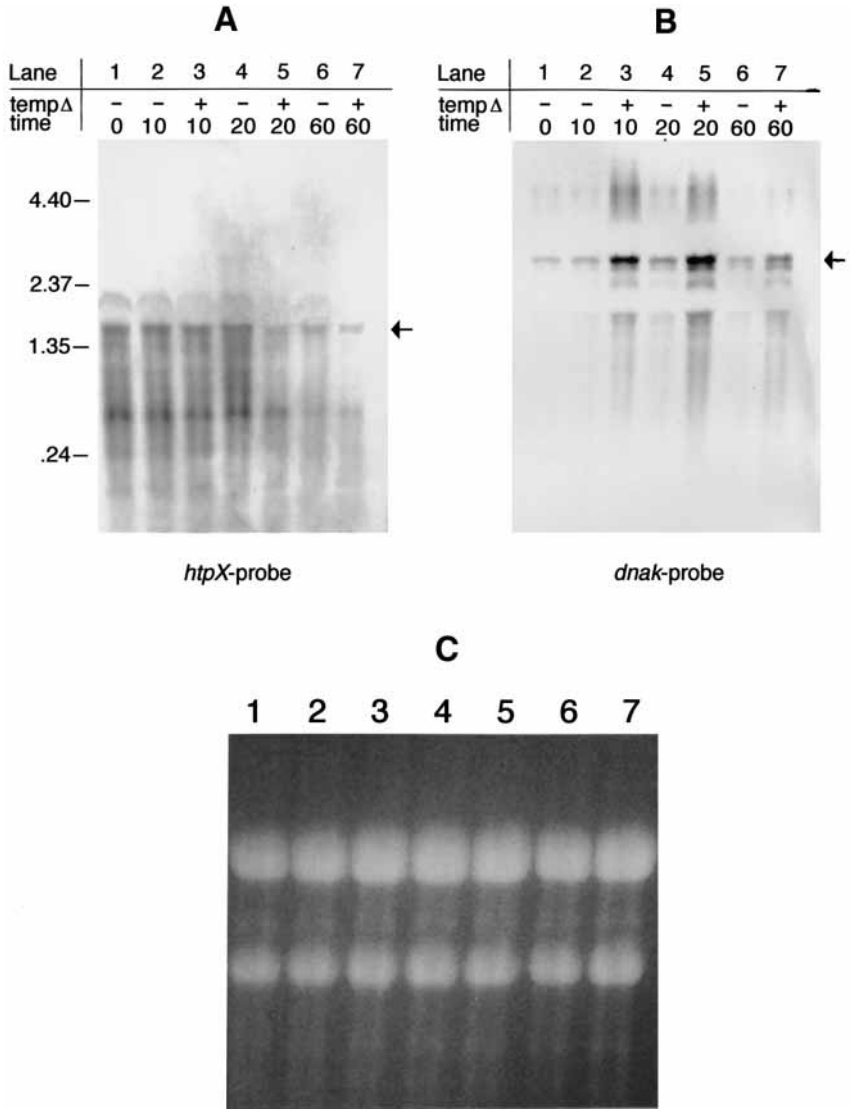

Fig. 5. Northern analysis of *Streptococcus gordonii htpX* transcription after a shift to 41.5°C, a potential heat shock temperature. Total RNA from strain CH1 was prepared after remaining at 36°C (temperature change -) or a shift to growth at 41.5°C (temperature change +) for various periods of time. Equal amounts of RNA were electrophoresed on 1% agarose-2% formaldehyde gels (Panel *C*). Samples were loaded in the following order: Lane 1, OD_{520} = 1.00, starting time for transcript analysis experiment (T_0); Lane 2: cultures remaining at 36°C for 10 min (T_{10}); Lane 3, cultures shifted to 41.5°C for 10 min (T_{10}); Lane 4, cultures remaining at 36°C for 20 min (T_{20}); Lane 5, cultures shifted to 41.5°C for 20 min (T_{20}); Lane 6, cultures remaining at 36°C for 60 min(T_{60}); Lane 7, cultures shifted to 41.5°C for 60 min (T_{60}). RNA was blotted to Hybond and probed with an internal fragment of *htpX* (Panel *A*). As a control, transcription of the well-described heat shock gene, *dnaK,* was monitored using an internal fragment of *S. gordonii dnaK* as a probe (Panel *B*). RNA size markers (Invitrogen Life Technologies) ran as indicated. Blots shown are representative of three independent experiments. Results shown are from cells grown in FMC medium. Similar results were seen when cells were grown in TH medium (data not shown).

the structural gene, *gtfG,* and the regulatory gene, *rgg*, are involved in determining the level of GTF activity in *S. gordonii* (35). Previous studies have demonstrated that the region upstream of *rgg* is associated with a *ca.* 1.7-kb transcript, which is transcribed independently of *rgg and gtfG.* Expression of the 1.7-kb transcript is not affected by chromosomal disruptions of *rgg* or by additional copies of *rgg* provided in *trans* (27). These results did not preclude the possibility that the genes of this region either affect *rgg*/*gtfG* expression, or are involved in post-transcriptional modifications of *rgg* or *gtfG,* thereby influencing the level of GTF expression. Because bacterial chromosomes are often organized with genes of similar functions in proximity to one another, the nucleotide sequence of the 2,156-bp region upstream of *rgg* was determined. Initial characterization of the identified *lemA* and *htpX* genes was performed, with the primary goal of determining potential effects on GTF activity.

Northern hybridization analysis indicated that the *S. gordonii lemA* and *htpX* genes are cotranscribed. This is similar to the polycistronic transcription of *L. monocytogenes lemA* and *lemB* (21) which share sequence similarity with the *S. gordonii* genes. In both organisms, there are multiple potential polypeptides that can be translated from the polycistronic transcript. There are up to five potential open reading frames greater than 15 amino acids in the *lemA*/*lemB* region of *L. monocytogenes*; there are four potential open reading frames in the *S. gordonii lemA*/*htpX* region. The *L. monocytogenes* LemA polypeptide has been reported to elicit specific immune responses to this intracellular pathogen, but the function of the *S. gordonii* LemA protein has not been determined.

In contrast to the *S. gordonii* gene, *E. coli htpX* is not flanked by a *lemA*-like determinant (5) and its monocistronic transcript is expressed from a σ^{32}-dependent promoter that responds to elevated temperatures (19). No sequences corresponding to heat shock consensus −35 and −10 promoter sequences (25) or *cis*-acting inverted repeat elements (13) were noted near the *S. gordonii lemA*/*htpX* putative promoter region. Nevertheless, because the sequence of the *S. gordonii htpX* gene is similar to the *E. coli* heat shock gene, transcription was examined at elevated temperatures. Under the conditions of the present studies, no increase in *S. gordonii lemA*/*htpX* transcription was observed at increased temperature in comparison to the increased transcription of the *dnaK* positive control. Thus, the Northern analyses do not support a role for *S. gordonii htpX* as a heat shock gene.

However, some heat shock genes do not respond as distinctly to elevated temperature as *dnaK* does (2). Limitations in the effectiveness of Northern

analyses in monitoring the transcription of heat shock genes in oral streptococci has been noted (15). Because heat shock responses and regulatory regions are less well-described in oral streptococci and bacteria other than *E. coli* (15,25), these results do not preclude the possibility that the *S. gordonii lemA/htpX* promoter responds to temperatures or environmental stresses under conditions other than those of the present studies. There are multiple conditions that induce stress responses in oral streptococcal species, which often overlap heat shock responses (15,29).

No macroscopic changes were observed in colonies of *S. gordonii* in which *htpX* was disrupted when grown at either control or elevated temperatures. This finding agrees with the report that disruption of *E. coli htpX* did not result in any detectable phenotype (19). However, in the present studies, *S. gordonii htpX*-disruption resulted in changes to the adhesive properties of the cell surface and altered the levels of some detergent-extractable antigens in comparison to parental cells. Furthermore, scanning electron micrographs indicated morphological changes in *htpX*-inactivated cells grown at 41.5°C; disruption of *htpX* appeared to affect the ability of cells grown at elevated temperature to maintain the parental cell morphology during growth and/or processing conditions for electron microscopy. Since computer-assisted protein motif analysis indicated that the *E. coli* and *S. gordonii htpX* genes share a metalloprotease motif (4), it is possible that inactivation of *htpX* affects protease-dependent processing of *S. gordonii* proteins, with a variety of functions. Such processing or chaperone functions are well-recognized roles for stress proteins (9). Thus, the differences in adhesive properties, surface antigens and cellular morphology observed in strain CH34 may be directly or indirectly due to loss of functional HtpX. Some of these differences were more evident in cells grown at elevated temperatures. Interestingly, growth at 41.5°C increased the adhesion of both parental and *htpX*-inactivated *S. gordonii* cells to hydroxyapatite and phenyl-Sepharose beads, whereas adhesion to saliva-coated hydroxyapatite was not increased. Such temperature-related cell surface changes could affect the ability of *S. gordonii* cells to attach or detach from different oral surfaces and play important ecological roles under stressful environmental conditions *in vivo*. Despite the putative role of HtpX as a protease involved in processing streptococcal proteins, the present studies do not support a role for *htpX* in influencing the level of GTF protein. Neither disruption of *htpX* nor the *lemA* gene immediately upstream, affected the level of *S. gordonii* GTF activity or the sucrose-promoted colony phenotype associated with glucan synthesis.

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