Altered adherence properties of a *Streptococcus gordonii* *hppA* (oligopeptide permease) mutant result from transcriptional effects on *cshA* adhesin gene expression

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Cell-surface polypeptide CshA (259 kDa) mediates multiple adherence interactions of *Streptococcus gordonii*. By generating a chromosomal *cshA* promoter (*p-cshA*)–*cat* gene fusion and measuring both CAT enzyme activity and *cat* mRNA levels, it was shown that *cshA* is expressed maximally in cells in the late exponential phase of growth in batch culture. The expression of CAT enzyme activity from the *p-cshA*–*cat* promoter fusion was 28% decreased in early stationary phase cell extracts of mutant strain OB528 in which the *hppA* (oligopeptide-binding lipoprotein) gene was insertionally inactivated. This effect was correlated with proportionally reduced cell-surface expression of CshA protein and with impaired adherence of *hppA* mutant cells to cells of an oral *Actinomyces naeslundii* strain. *cshA* promoter activity was enhanced in streptococcal cells that were incubated in conditioned culture medium as opposed to fresh medium, but this did not occur in an *hppA* genetic background. It is suggested that HppA is necessary for the response of cells to an extracellular factor that modulates *cshA* transcription, and hence affects cell-surface CshA expression and streptococcal cell adherence properties.

Keywords: streptococcal cell adhesion, cell-surface proteins, oligopeptide permease, promoter activity, *Streptococcus gordonii*

INTRODUCTION

The ability of oral bacteria to adhere to components in the oral environment such as deposited salivary proteins and glycoproteins, host epithelial cell surfaces and other micro-organisms is critical to their growth and survival within the host. A large number of potential adhesins in oral bacteria have now been identified and characterized (reviewed by Whittaker *et al.*, 1996) and the molecular complexities of these adhesion processes are becoming apparent. The adhesive and invasive properties of Gram-positive bacteria, which comprise a large proportion of

the human oral flora, are closely linked to the production of secreted proteins and activities of cell-wall-anchored polypeptides. The latter carry specialized sequences at their C-terminal regions that are necessary for polypeptide anchorage at the cell surface (Schneewind *et al.*, 1995), and typically they contain repeat blocks of amino acid residues comprising up to 65% of the entire polypeptide (see Kehoe, 1994; Jenkinson & Lamont, 1997). Both the amino acid residue repeat blocks and non-repetitive sequences within these proteins have been implicated in a wide range of colonization-associated reactions. These include, for example, the adhesion of streptococci and staphylococci to host tissue matrix components (Patti *et al.*, 1994), resistance of group A streptococci to phagocytosis (Podbielski *et al.*, 1996a), extracellular neuraminidase activity of pneumococci (Cámara *et al.*, 1994), cell-surface hydrophobicity (Mc Nab *et al.*, 1995) and adhesion of oral streptococci to salivary components and other oral bacterial cells (Jenkinson & Demuth, 1997), and invasion of epithelial cells by *Listeria monocytogenes* (Lebrun *et al.*, 1996).

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Abbreviation: CAT, chloramphenicol acetyltransferase.

The GenBank accession number for the *cshA* locus updated to include the upstream sequence is X65164.

Gram-positive bacteria also express a second group of cell-surface-anchored proteins that are N-terminally lipid-modified and thought to be linked to the outer face of the cytoplasmic membrane (Sutcliffe & Russell, 1995). These lipoproteins function in transport processes such as solute uptake (Tam & Saier, 1993) and in providing immunity to bacteriocins (de Vos *et al.*, 1995). The best characterized are the substrate-binding lipoproteins of ATP-binding cassette (ABC)-type transport systems (Higgins, 1992) necessary for the uptake of peptides and oligopeptides (Alloing *et al.*, 1994; Jenkinson *et al.*, 1996; Kunji *et al.*, 1996) and multiple sugars (Tao *et al.*, 1993). There is additional evidence that these binding proteins may be involved in the adhesion of oral streptococci to salivary components (Ganeshkumar *et al.*, 1991), fibrin (Burnette-Curley *et al.*, 1995) and other oral bacteria (Kolenbrander *et al.*, 1994).

Streptococcus gordonii is found at most sites within the human oral cavity (Frandsen *et al.*, 1991) and exhibits a wide range of adherence properties. *S. gordonii* produces two high-molecular-mass cell-wall-anchored polypeptides designated CshA (predicted molecular mass 259 kDa) and CshB (approximate molecular mass 245 kDa) that are involved in determining cell-surface hydrophobicity (McNab *et al.*, 1995), and that are essential for colonization of the oral cavity by *S. gordonii* (McNab *et al.*, 1994). The CshA polypeptide mediates binding of *S. gordonii* cells to immobilized human fibronectin, *Actinomyces naeslundii*, *Streptococcus oralis* and *Candida albicans* (Holmes *et al.*, 1996; McNab *et al.*, 1996), and *cshA* mutants are deficient in binding to these substrates. Mutation in *hppA* encoding a substrate (oligopeptide)-binding protein of a hexa-heptapeptide permease (Hpp) in *S. gordonii* (Jenkinson *et al.*, 1996) also affects adhesion of streptococcal cells to oral *A. naeslundii* cells and human serum components (Jenkinson & Easingwood, 1990).

To investigate the regulation of expression of CshA we constructed a fusion of the *cshA* gene promoter with a chloramphenicol acetyltransferase (*cat*) gene reporter. In this article, we show, by measuring CAT enzyme activities and *cat* mRNA levels, that the *cshA* promoter is most active during the late exponential phase of growth. Furthermore, transcription of the *cshA* gene and expression of associated adherence properties are dependent upon production of HppA.

METHODS

Bacterial strains and media. The bacterial strains and plasmids utilized are listed in Table 1. Streptococci were grown at 37 °C on TSBY agar (Jenkinson *et al.*, 1993) in a GasPak System (BBL Microbiology Systems). Liquid cultures were grown without shaking in screw-cap tubes or bottles at 37 °C in BHY medium (Jenkinson *et al.*, 1993). *Escherichia coli* strains were grown aerobically at 37 °C in LB medium (Sambrook *et al.*, 1989). Concentrations of antibiotics used for selection were as follows: ampicillin, 100 µg ml⁻¹ (*E. coli*); chloramphenicol, 5 µg ml⁻¹ (*E. coli*); tetracycline, 15 µg ml⁻¹ (*E. coli*) or 10 µg ml⁻¹ (*S. gordonii*); erythromycin, 50 µg ml⁻¹ (*E. coli*) or 1 µg ml⁻¹ (*S. gordonii*).

DNA manipulations. Routine molecular biology techniques were performed as specified by Sambrook *et al.* (1989). Plasmid DNA was isolated from *E. coli* by using Wizard Minipreps (Promega), and PCR products were purified by using QIAquick (Qiagen). Chromosomal DNA was isolated from *S. gordonii* as described previously (Jenkinson, 1987). *S. gordonii* cells were transformed with DNA according to Haisman & Jenkinson (1991). Restriction and modifying enzymes (from New England BioLabs) were used under the conditions recommended by the manufacturer.

To obtain DNA sequence information 5' to the ATG start codon of *cshA*, inverse PCR was performed as outlined by McNab *et al.* (1994). Briefly, *S. gordonii* DL1 (Challis) genomic DNA was digested with *Pst*I, self-ligated at 5 ng µl⁻¹ and used as template in PCR (Expand High Fidelity System; Boehringer) with primers IPA (complementary to nucleotides 672–692, *cshA* locus; GenBank accession no. X65164), 5'-CGAATAGAAAACCTTACGCAGG-3', and IPB (nucleotides 926–946, *cshA* locus), 5'-ATACTGCACCTACAACTCAG-3' (30 cycles, 58 °C primer annealing temperature). The PCR product (2.04 kb) was purified, and DNA fragments were subcloned in pUC19. DNA sequencing was performed with M13 forward or reverse primers or custom-synthesized oligonucleotides (DNA Express; Colorado State University) in an Applied Biosystems model 373A automated DNA sequencer, or manually using Sequenase (United States Biochemical). Sequence data were analysed with the University of Wisconsin Genetics Computer Group package (Devereux *et al.*, 1984) and the BLAST algorithm (Altschul *et al.*, 1990) was utilized for database searches.

Construction of the *cshA* promoter-*cat* fusion strain. To facilitate PCR product cloning into pMH109 which contains a promoterless Gram-positive *cat* gene (Hudson & Stewart, 1986), restriction sites (underlined in the primer sequences below) were incorporated into the primers designed to amplify the *cshA* promoter region. The primer pair comprised SMAP1 (nucleotides 240–266, *cshA* locus), 5'-CTGCCCGGGATC-GTGACTATCTATTG-3', and SACP2 (complementary to nucleotides 619–645, *cshA* locus), 5'-AATGAGCTCCTAT-TTATTTCATTATATT-3'. By using Expand System, a PCR product of the predicted size was amplified with 30 cycles and a primer annealing temperature of 58 °C. The PCR-amplified product (405 bp) of *S. gordonii* DL1 chromosomal DNA containing the *cshA* promoter was digested with a combination of *Sma*I and *Sac*I and ligated with pMH109 likewise digested. The ligation mixture was transformed into *E. coli* HB101 with selection for resistance to tetracycline and chloramphenicol. The recombinant pMH109 was then digested with *Bam*HI to release a 1.3 kb *cshA* promoter (*p-cshA*)-*cat* fragment which was cloned into *Bam*HI-cut pSF143 (Tao *et al.*, 1992) to generate plasmid pSFCAT1. Plasmid pSFCAT1 was introduced onto the *S. gordonii* chromosome by transformation and selection for Tc^R to generate recombinant strain OB507. The insertion also conferred resistance of *S. gordonii* to 1 µg chloramphenicol ml⁻¹. To confirm that the plasmid had integrated with insertion duplication of the target sequence at the predicted chromosomal region, nylon blots of *Hind*III-digested *S. gordonii* genomic DNAs were probed each with the labelled promoter PCR product and, separately, the *Bam*HI fragment from pMH109 containing the *cat* gene.

To further ensure that the correct fusion construct on the streptococcal chromosome had been generated, an oligonucleotide primer, CATPCR [5'-AACACTAATATCAATT-TCTGTGG-3'; complementary to nucleotides 1348–1370 of

Table 1. Bacterial strains and plasmids used

Strain/plasmid	Relevant characteristics	Reference/source
Strains		
<i>S. gordonii</i>		
DL1 Challis	Wild-type	Pakula & Walczak (1963)
OB235	<i>csbA3::ermAM</i>	McNab <i>et al.</i> (1994)
OB285	<i>hpaA1::ermAM</i>	Jenkinson <i>et al.</i> (1996)
OB423	<i>hpaH2::ermAM*</i>	This study
OB507	<i>p-csbA-cat</i> ; Tc ^R	This study
OB528	<i>p-csbA-cat hpaA1::ermAM</i> ; Tc ^R (OB285 DNA → OB507)†	This study
OB568	<i>p-csbA-cat hpaH2::ermAM</i> ; Tc ^R (OB423 DNA → OB507)†	This study
OB587	<i>p-csbA-cat csbA3::ermAM</i> ; Tc ^R (OB235 DNA → OB507)†	This study
<i>E. coli</i>		
HB101	F ⁻ Δ (<i>gpt-proA</i>)62 <i>leuB6 supE44 ara-14 galK2 lacY1 Δ(<i>mcrC-mrr</i>) rpsL20 (Str^R) xyl-5 mtl-1 recA13</i>	Boyer & Roulland-Dussoix (1969)
JM83	F ⁻ <i>ara Δ(lac-proAB) rpsL (Str^R)</i> [ϕ 80dlac Δ (<i>lacZ</i>)M15] <i>thi</i>	Yanisch-Perron <i>et al.</i> (1985)
Plasmids		
pUC19	2.68 kb; Ap ^R ColE1ori	Yanisch-Perron <i>et al.</i> (1985)
pMH109	7.4 kb; Tc ^R Km ^R ColE1ori pUB110ori, promoterless <i>cat</i> gene	Hudson & Stewart (1986)
pSF143	5.7 kb; Tc ^R pACYC184ori	Tao <i>et al.</i> (1992)

* OB423 is an Em^R equivalent of OB427 *hpaH1::tet* (Jenkinson *et al.*, 1996).

† Donor DNA → recipient strain by transformation.

the staphylococcal *cat* gene (GenBank accession number J01754)], was used for PCR with primer SMAP1 to amplify the promoter-*cat* fusion DNA region from OB507. The PCR product was sequenced directly using a *cat*-defined nested primer, CATSEQ (5'-CTAAAGTCGTTTGTGG-3'; complementary to nucleotides 1325–1342). For measurement of the effect of gene disruption on *csbA* promoter activity, mutations in *csbA*, *hpaA* or *hpaH* genes were constructed by transformation of the *csbA* promoter-*cat* fusion strain OB507 (Table 1).

RNA analyses. Total RNA was prepared from *S. gordonii* cells by a modification of the method of Lunsford (1995). Streptococcal cells were grown in BHY medium (20 ml) containing 10 µg tetracycline ml⁻¹ (BHY-Tc), harvested by centrifugation (4000 g, 10 min, 4 °C), washed once in diethyl pyrocarbonate (DEPC)-treated distilled H₂O (dH₂O, 10 ml) and suspended in spheroplasting buffer (0.2 ml; Jenkinson *et al.*, 1993) containing 0.1 mg spectinomycin ml⁻¹. Mutanolysin (Sigma; final concentration 500 U ml⁻¹) was added and the suspension was incubated at 37 °C for 30 min. Spheroplasts were collected by centrifugation (5000 g, 10 min, 4 °C), the supernatant was removed and discarded, and the spheroplasts were suspended in 1 ml TriPure Isolation Reagent (Boehringer). RNA was purified according to the manufacturer's instructions, suspended in DEPC-treated dH₂O, and the concentration was determined by measuring the A₂₆₀ (Shimadzu).

For estimation of relative mRNA concentrations, total cell RNA samples (50 µg) were denatured by heating (15 min,

65 °C) in the presence of formaldehyde (7.4%, w/v) and 1 × SSC (0.15 M NaCl, 0.015 M trisodium citrate; pH 7.0). The solutions were diluted to 0.1 µg RNA µl⁻¹ in 10 × SSC and diluted serially twofold in 10 × SSC. Duplicate samples in the range 10–1.25 µg RNA were applied to nylon membrane held in a well-manifold, RNA was fixed to the membrane by UV treatment (10 min), and hybridization of blots with ³²P-labelled *cat* gene fragment was performed as described by Church & Gilbert (1984), with solutions containing 8% (w/v) dextran sulphate. Blots were washed three times each for 20 min with 2 × SSC containing 0.1% SDS at 65 °C. Relative RNA concentrations were quantified from laser densitometer scans of autoradiographs exposed for 48 h and expressed as mean peak height (µg RNA)⁻¹ relative to the peak height (µg RNA)⁻¹ for an *S. gordonii* OB507 16 h stationary phase sample.

Primer extension analysis. Purified RNA (60 µg) was incubated for 15 min at 20 °C with DNase I (2 U; amplification grade, Life Technologies), annealed with 1.6 pmol ³²P-5'-end-labelled CATSEQ oligonucleotide in a final volume of 10 µl dH₂O by heating for 2 min at 80 °C and allowing to cool slowly to room temperature. Primer extension was performed in the presence of RNasin (10 U; Life Technologies) using Superscript II RNase H⁻ reverse transcriptase (200 U; Life Technologies) (50 min, 42 °C). Reactions were heat-inactivated (15 min, 70 °C), cooled on ice and incubated for 20 min at 37 °C with RNase H (2.1 U; Life Technologies). Extension products were ethanol-precipitated following phenol/chloroform extraction, and analysed on an 8% poly-

acrylamide sequencing gel adjacent to a sequence reaction for the relevant promoter-*cat* PCR product primed with the CATSEQ oligonucleotide.

Preparation of cell-free extracts and CAT assay. Streptococcal cells in BHY-Tc medium (10 ml) were harvested by centrifugation (4000 g, 10 min, 4 °C) and washed once in TPE buffer (100 mM Tris/HCl, pH 7.8, containing 1 mM PMSF and 1 mM EDTA). Bacteria were suspended in spheroplasting buffer containing mutanolysin (see above) and incubated for 30 min at 37 °C. Cells were then disrupted by adding 0.4 ml ice-cold TPE buffer and 0.1 ml glass beads (0.10 mm diameter) and vortex mixing for two periods of 30 s. Suspensions were centrifuged (12000 g, 20 min, 4 °C) to remove glass beads and cell debris, and supernatants were removed for enzyme assays. Protein concentrations were determined by using a Bio-Rad protein assay kit with bovine γ -globulin as the standard. CAT enzyme activity was determined by the spectrophotometric method of Shaw (1975), utilizing a Shimadzu UV-240 recording spectrophotometer with temperature-controlled cuvette chamber. The reaction rate was determined from the linear portion of the graph and corrected for background change in A_{412} , and divided by 0.0136 to yield CAT activity expressed as nmol chloramphenicol acetylated min^{-1} at 37 °C.

Antibodies and ELISA. CshA-specific antibodies were raised to the N-terminal non-repetitive amino acid sequence region of CshA (N-CshA; McNab *et al.*, 1996) and used to determine the immunoreactivity of CshA present on intact streptococcal cells by ELISA (Holmes *et al.*, 1995) utilizing an EL340 plate reader (Bio-Tek Instruments).

Bacterial adherence assays. Streptococci were radioactively labelled by growth in medium containing [*methyl*- ^3H]-thymidine (0.22 MBq ml^{-1} , 3 TBq mmol^{-1} ; Amersham). Adherence of streptococci to immobilized actinomyces cells was measured as described previously (Jenkinson *et al.*, 1993).

Statistical analysis. Student's *t*-test was used for all data and *P* values < 0.05 were considered significant.

RESULTS

Sequence of the *cshA* gene promoter region

The initial aim of this work was to identify factors regulating the expression of the *cshA* adhesin gene in *S. gordonii*, by utilizing a *cshA* promoter (*p-cshA*)-reporter gene fusion. Since sequence information was available for only seven nucleotides 5' to the *cshA* start codon (McNab *et al.*, 1994), upstream DNA was obtained by inverse PCR (see Methods). Nucleotide sequence data were obtained from a 2.04 kb amplicon that overlapped with previously reported *cshA* sequence (GenBank accession number X65164 updated to include upstream sequence). Computer analysis of the nucleotide sequence extending 645 bp upstream of the *cshA* start codon predicted an open reading frame (nucleotides 1–313) encoding the C-terminal 104 aa residues of a polypeptide that exhibited 66% identity with α -acetolactate decarboxylase (AldB) from *Lactococcus lactis* (GenBank accession number X82620) and 38% identity with the homologous enzyme (AldC) from *Bacillus subtilis* (SWISS-PROT accession number Q04777). The *aldB-cshA* intergenic region in *S. gordonii* DL1 comprised 332 nucleotides (see Fig. 1). Between nucleotides 339 and 372 was a putative rho-independent transcriptional terminator predicted to form a stem-loop structure. A potential RBS was identified six nucleotides upstream from the *cshA* translational start site (see Fig. 2). Two possible –10 promoter sequences (each TATAAT) were identified at 20 and 35 nucleotides 5' to the *cshA* start codon, and a potential –35 sequence was located 18 nucleotides 5' to the RBS distal –10 sequence (see Fig. 2).

Fig. 1. Construction of the chromosomal *cshA* promoter-*cat* fusion. (a) Schematic diagram of the chromosomal region containing the *cshA* promoter. Numbers indicate base pairs from the start of the nucleotide sequence (in GenBank). The coding sequences for *cshA* and *aldB* are indicated by open arrows, and the associated numbers delineate the coding sequences. Oligonucleotide primers (arrowed) were utilized in PCR to obtain a promoter-containing DNA fragment (dark hatching) that was cloned downstream of a promoterless *cat* gene in plasmid pSF143 to generate pSFCAT1 (see Methods). (b) Diagram depicting the introduction of the promoter-*cat* fusion onto the streptococcal chromosome by insertion duplication following recombination between the cloned promoter sequence (dark hatching) and the homologous chromosomal sequence (light hatching). The thick line represents *S. gordonii* chromosomal DNA, and the thin line represents pSF143 DNA.

Fig. 2. Comparison of the sequence containing the *cshA* promoter region with the corresponding sequence generated by chromosomal promoter-*cat* fusion. SACP2 is the complementary strand oligonucleotide primer used for amplification of *p-cshA* sequence. Lower-case letters within the primer sequence indicate changes made from the chromosomal sequence that introduced a *SacI* recognition site (given in italics) to facilitate cloning of the PCR product. Potential -10 (underscored) and -35 (overscored) promoter sequences are indicated, and RBS sequences are in bold type. The experimentally defined transcriptional start site for *p-cshA-cat* is indicated by an arrow.

Construction of the *cshA* promoter-*cat* fusion

A segment of *S. gordonii* chromosomal DNA (405 bp) containing *p-cshA* was obtained following PCR amplification (Fig. 1) and cloned 5' to a promoterless *cat* gene present in plasmid pMH109 (Table 1). Since pMH109 contained an origin of replication that functioned in *S. gordonii*, the *p-cshA-cat*-containing fragment (1.3 kb) was excised and cloned into pSF143, which does not replicate in *S. gordonii* (Tao *et al.*, 1992), to generate pSFCAT1 (Fig. 1). Plasmid pSFCAT1 was transformed onto the *S. gordonii* DL1 chromosome with selection for tetracycline resistance (Tc^R) to generate strain OB507 containing an insertion duplication of the *cshA* promoters expressing *cat* (see Fig. 1b).

To check that the predicted promoter fusion had been obtained, PCR was used to amplify the corresponding chromosomal regions from *S. gordonii* OB507. The promoter fusion construct sequence obtained is shown in Fig. 2 and is aligned with the wild-type sequence. The RBS sequence for *cshA* (GGAGA) was replaced during construction of the promoter-*cat* fusion with the *cat* RBS sequence (GGAGG), and the RBS-ATG spacing was increased from five nucleotides to eight nucleotides. The *cshA* promoter region contained at least two potential -10 sequences (see Fig. 2). Therefore, primer extension was used to map the transcriptional start site of the gene by utilizing a *cat*-defined oligonucleotide (see Methods). A single primer extension product was obtained (Fig. 3). The *p-cshA-cat* mRNA initiated at nucleotide position -43 relative to the start codon of *cat*, equivalent to -27 nucleotides from the ATG start codon of *cshA* (Fig. 2).

cshA promoter activity in cells at different stages of growth in batch culture

To determine *cshA* promoter activity at different stages of growth of *S. gordonii* cells in batch culture, strain OB507 (*p-cshA-cat*) was cultured in BHY-Tc medium and samples were taken at intervals for measurement of CAT activity in cell-free extracts. CAT enzyme activity within OB507 cultures increased throughout growth, lagging behind the growth curve, and reached a maximum at early stationary phase (Fig. 4).

Fig. 3. Primer extension analysis of the transcriptional start point of *cshA*. The extension reaction product is shown in lane P. The predicted transcriptional initiation site is indicated by an arrow in the expanded sequence on the left, which is the reverse complement of that shown in Fig. 2. Sequences (lanes T, G, C and A) were derived from the same CATSEQ primer utilized for the primer extension reaction.

To confirm that CAT enzyme activity was a direct estimate of promoter activity in *S. gordonii*, *cat*-specific mRNA present in extracts of *S. gordonii* OB507 cells

Fig. 4. Total culture CAT activity ($\square$) and relative amount of *cat* mRNA ($\bullet$) in cells of *S. gordonii* OB507 *p-cshA-cat* at points during growth in batch culture ($\circ$; culture density at OD_{600}). For each sample taken, a portion was utilized for CAT activity determination, while total RNA was prepared from the remainder for *cat*-specific mRNA determination by hybridization (see Methods). Results presented are from a representative experiment of four. Error bars are standard deviations of the mean of triplicate samples.

was quantified by blot hybridization analysis. RNA samples were reacted with a probe comprising the *Bam*HI fragment from pMH109 encoding the promoterless *cat* gene. *cshA* promoter activity as quantified by *cat* mRNA levels was found to be maximal at late exponential phase (Fig. 4). The continued increase in culture CAT activity that was associated with a decrease in *cat*-specific mRNA level in early stationary phase cell extracts (Fig. 4) indicated that *cshA* gene expression continued well into stationary phase. The *cshA* promoter region contained a strong transcriptional terminator sequence upstream (data not shown), sug-

gesting that transcriptional read-through of *cshA* from upstream was unlikely.

The growth-phase-related expression of CshA was examined in more detail for cells harvested at mid exponential phase (T1; Table 2) or at early stationary phase (T2; Table 2) of growth. The increased CAT specific activity of early stationary phase cell extracts compared with mid exponential phase cell extracts (50% increase) correlated with an increased cell-surface expression of CshA (33% increase) as determined by ELISA, and with statistically significant ($P < 0.005$) improved adherence of cells to immobilized *A. naeslundii* WVU627 cells (Table 2).

Effects of inactivating *hppA* on *cshA* gene expression

It was demonstrated previously (see Introduction) that insertional inactivation of the *hppA* gene affected binding of *S. gordonii* cells to *A. naeslundii* (Jenkinson & Easingwood, 1990), which is now known to be mediated, at least in part, by the CshA polypeptide (McNab *et al.*, 1996). To determine if inactivation of *hppA* affected *cshA* transcription, we measured specific CAT activity in cell extracts of strain OB528 (*p-cshA-cat*) in which *hppA* was insertionally inactivated (see Table 1). The CAT specific activity of mid exponential phase cell extracts of OB528 (*hppA*) was reduced somewhat compared with extracts of cells of OB507 (wild-type) taken at the same time-point (Table 2). However, this was not associated with a significant difference in levels of adhesion of the two strains to *A. naeslundii* cells (Table 2). By early stationary phase of growth (T2 in Table 2), the expression of CAT enzyme activity from the *p-cshA-cat* promoter fusion in extracts of strain OB528 (*hppA*) was 28% less than that in OB507 (wild-type *HppA*⁺) extracts. This statistically significant ($P < 0.0001$) reduced CAT activity was associated with reduced cell-surface expression of CshA ($P < 0.0001$) and with impaired adhesion ($P < 0.005$) of cells to *A. naeslundii* WVU627 (Table 2). As a control

Table 2. Effect of inactivating *cshA*, *hppA* or *hppH* genes on *cshA* gene expression and adhesion of *S. gordonii*

Cells were harvested for analysis at mid exponential (T1) or early stationary (T2) phase of growth. Values are the means $\pm$ SD of quadruplicate determinations from a representative experiment repeated three times. ND, Not determined.

Strain	CAT specific activity [nmol min ⁻¹ (mg protein) ⁻¹]		Amount of cell-surface CshA antigen by ELISA*		Input cells† attached to <i>A. naeslundii</i> WVU627 (%)	
	T1	T2	T1	T2	T1	T2
OB507	40.22 $\pm$ 0.59	60.38 $\pm$ 1.52	0.199 $\pm$ 0.008	0.265 $\pm$ 0.007	18.54 $\pm$ 0.98	28.46 $\pm$ 3.04
OB528 <i>hppA</i>	35.49 $\pm$ 1.30‡	43.64 $\pm$ 0.57‡	0.191 $\pm$ 0.008	0.205 $\pm$ 0.001‡	16.16 $\pm$ 1.76	21.38 $\pm$ 1.58‡
OB587 <i>cshA</i>	41.61 $\pm$ 0.99	62.30 $\pm$ 0.80	0.008 $\pm$ 0.002‡	0.004 $\pm$ 0.001‡	12.24 $\pm$ 0.42‡	14.90 $\pm$ 1.74‡
OB568 <i>hppH</i>	ND	61.07 $\pm$ 0.78	ND	0.274 $\pm$ 0.006	ND	ND

* N-CshA antibodies were diluted 1:2000 and ELISA values (A_{492}) were corrected for values obtained with pre-immune serum (diluted 1:2000).

† Input of 2×10^7 streptococcal cells.

‡ Significantly different from OB507 (wild-type) value within column, $P < 0.05$.

we also measured specific CAT activity of *S. gordonii* OB587 (*cshA3*) cells in which the chromosomal *cshA* gene was insertionally inactivated. As expected, these cells did not express CshA protein on their surfaces as determined by ELISA (Table 2), and they were approximately 50% reduced in adhesion (Table 2). However, specific CAT activities were similar to those present in OB507 (wild-type) cells at both time-points (Table 2); therefore it appears that expression of *cshA* does not normally modulate its own transcription. The 'residual' adhesion of *S. gordonii* OB587 (*cshA3*) cells to *A. naeslundii*, which is mediated primarily by SspA and SspB adhesins (see Jenkinson & Demuth, 1997), increased slightly by early stationary phase, suggesting that expression of these adhesins may also be enhanced at this time. This may account, at least in part, for the increased adherence of *S. gordonii* OB528 (*hppA*) stationary phase cells compared with mid exponential phase cells that was observed in the absence of a significant increase in CshA production (see Table 2).

To determine if the observed effects of inactivating the *hppA* gene were specific, we measured *cshA* expression in *S. gordonii* OB568 (*p-cshA-cat*) in which the *hppH* gene was inactivated. Expression of the *hppH* gene is necessary for oligopeptide uptake via the Hpp transport system (Jenkinson *et al.*, 1996), but *hppH* mutants, unlike *hppA* mutants, are unaffected in their adherence properties (R. A. Baker & H. F. Jenkinson, unpublished data). The amounts of cell-surface CshA and specific CAT activity associated with early stationary phase OB568 (*hppH*) cells were found to be identical to those in OB507 (wild-type) (Table 2). This suggested that the previously observed effects of *hppA* gene inactivation on *cshA* transcription were specific to *hppA* and not simply due to inhibition of general oligopeptide uptake. Lastly, we determined that CAT enzyme activities were similarly stable in cell extracts from all the different strains, thus excluding the possibility that reduced specific CAT activities in OB528 (*hppA*) extracts resulted from an increased rate of protein degradation.

***cshA* expression is influenced by pre-conditioned culture medium**

The observation that *cshA* was maximally expressed in late exponential growth phase raised the possibility that an environmental cue and/or cell-density-related phenomenon might influence *cshA* promoter activity. In further support of this idea were the data showing the requirement of HppA for *cshA* expression, since *hppA* is essential for the natural development of competence (Jenkinson *et al.*, 1996). This is a cell-density-associated event mediated in part by a sensor-regulator relay that is responsive to an extracellular peptide pheromone (Håvarstein *et al.*, 1996). Therefore, to determine if *cshA* expression might be influenced by a quorum-associated factor, cells of *S. gordonii* OB507 (wild-type) in mid exponential phase of growth were harvested and suspended in either fresh medium or the medium from which they had been harvested (conditioned medium). After 2 h incubation there was 27% reduced ($P < 0.001$)

Fig. 5. Effect of pre-conditioned medium on *p-cshA-cat* activity. Mid exponential growth phase cultures of *S. gordonii* OB507 *p-cshA-cat* (a) or *S. gordonii* OB528 *p-cshA-cat hppA1::ermAM* (b) in BHY-Tc medium were divided into four equal portions and cells were harvested by centrifugation (6000 g, 20 min). One portion of cells was prepared for determination of CAT activity. Bacteria in the remaining portions were suspended at the original culture density in either conditioned medium from which they had been harvested (designated *own* in Figure), fresh BHY-Tc (designated *fresh*) or in the conditioned cell-free culture medium from the opposing strain (designated *swap*). After incubating the cultures for 2 h at 37 °C, bacteria were harvested, cell-free extracts were prepared and specific CAT activities were determined. Results shown are from a representative experiment of four. Error bars are standard deviations of the mean of triplicate samples.

specific CAT activity associated with cells growing in fresh medium as compared with conditioned medium (Fig. 5). Conversely, in *hppA* mutant OB528 cells, *cshA* transcription, as measured by specific CAT activity, was similar in both fresh or conditioned media (Fig. 5). When cells of each strain were suspended in the opposing conditioned medium, identical results were obtained (Fig. 5). These observations are consistent with the idea that cells require HppA to respond to an extracellular factor that influences *cshA* gene expression. This putative factor was nonetheless still produced by OB528 (*hppA*) cells in which the *hppA* gene was inactivated (Fig. 5).

DISCUSSION

Streptococcal cells express multiple surface adhesins and some of these, such as CshA in *S. gordonii*, have multiple binding functions (see Jenkinson & Lamont, 1997). CshA is involved in the determination of cell-surface hydrophobicity and adherence properties (McNab *et al.*, 1994, 1995) and mediates, in part, the binding of *S. gordonii* cells to fibronectin (McNab *et al.*, 1996). To identify factors regulating expression of this important colonization factor we generated a *cshA* promoter-reporter gene fusion on the streptococcal chromosome. The *cshA* promoter was most active in late exponential phase cells as deduced by quantification of *cat* mRNA levels and measurement of CAT enzyme activity. Transcriptional mapping of the *cshA* promoter

indicated that the RBS distal –10 sequence was likely to be active and this positioned a potential –35 sequence (TAGCCA) 18 nucleotides 5' to the active –10 sequence. The *csbA* promoter sequences thus strongly resembled –35 and –10 consensus promoter sequences of *E. coli* (Hawley & McClure, 1983) and proven promoter sequences in *Streptococcus pneumoniae* (Sabelnikov *et al.*, 1995). They also matched, but not closely, the *sspB* adhesin gene promoter sequences in *S. gordonii* DL1 (Demuth *et al.*, 1997). Neither the *csbA* nor *sspB* promoters appear to have extended –10 sequences, which are more frequently found in streptococcal gene promoters than in *E. coli* promoters (Sabelnikov *et al.*, 1995).

The Hpp system in *S. gordonii* is involved in the uptake of larger oligopeptides (up to heptapeptides) and has three associated substrate-binding proteins designated HppA, HppG and HppH. Two of these three lipoproteins, HppA and HppH, are essential for oligopeptide uptake (Jenkinson *et al.*, 1996). Therefore, the observation that inactivation of *hppA*, but not inactivation of *hppH*, affected *csbA* expression suggests that the transcriptional effect was not due to inhibition of general peptide uptake. It is likely that HppA has some unique specificity for either substrate binding or other physiological response. Indeed, it has been shown that HppA, but not HppG or HppH, is necessary for the development of competence for transformation in *S. gordonii* (Jenkinson *et al.*, 1996). The data presented in this article suggest that HppA might be involved in sensing extracellular signals that not only modulate competence development in *S. gordonii* but also expression of the *csbA* adhesin gene. Evidence for an extracellular signal is provided by the experiments in which *csbA* expression was shown to be repressed in exponential phase cells suspended in fresh culture medium compared with cells suspended in conditioned medium. The *csbA* promoter in *hppA* mutant cells failed to respond to conditioned medium, although an *hppA* mutant was still capable of conditioning the medium that subsequently effected a *p-csbA* response in an HppA⁺ background. In light of the evidence that the transport preference of the Hpp system is for oligopeptides of 5–7 aa residues (Jenkinson *et al.*, 1996), it would seem most likely that the putative extracellular factor is an oligopeptide. In this respect, it is significant that the competence-stimulating factor recognized by Spo0K (oligopeptide permease) in *B. subtilis* is a pentapeptide (Solomon *et al.*, 1996), conjugative transfer of plasmid pCF10 by *Enterococcus faecalis* donor cells occurs as a result of uptake of a heptapeptide pheromone secreted by recipient cells (Leonard *et al.*, 1996), and an octapeptide pheromone modulates late exponential phase expression of *Staphylococcus aureus* virulence-associated genes (Ji *et al.*, 1995). By utilizing the promoter fusion constructs described in this article we hope to identify the extracellular factor that stimulates *csbA* promoter activity in *S. gordonii*. It is worth emphasizing that these data demonstrate that only relatively small differences in *csbA* promoter activity

appear sufficient to cause significant effects on cell adhesion properties. The growth-phase-related modulatory effects on CsbA production are clearly distinct from other induction systems that may lead to anywhere between severalfold to thousandfold increases in transcript levels. The modulation of *csbA* promoter activity described is reminiscent of the approximately 50% increase in expression of *gtfBC* genes, encoding glucosyltransferases B and C synthesizing extracellular glucans, that was associated with adherent as opposed to non-adherent cells of *Streptococcus mutans* (Hudson & Curtiss, 1990).

Oligopeptide transport systems in Gram-positive bacteria are essential for the uptake of peptides as sources of nutrients, but evidence has accumulated suggesting they are involved in determining other critical cellular functions and properties. These include the development of competence (Pearce *et al.*, 1994; Alloing *et al.*, 1996; Jenkinson *et al.*, 1996), and the expression of virulence and adhesion properties of streptococci; for example, inactivating the oligopeptide permease of *Streptococcus pyogenes* affects production of secreted proteins and reduces expression of extracellular cysteine protease, a major virulence factor (Podbielski *et al.*, 1996b). In particular though, the activities of peptide permeases have been linked consistently with expression of streptococcal adherence properties: thus, inactivation of *hppA* affects adherence and aggregation properties of *S. gordonii* (Jenkinson & Easingwood, 1990), while mutations in *ami* genes reduce the ability of pneumococcal cells to adhere to human epithelial and endothelial cells (Cundell *et al.*, 1995). These studies raised the possibility that oligopeptide permease proteins had dual functions in substrate binding and adherence. In light of the results presented in this article, it is likely that the reduced adherence properties demonstrated by *hppA* mutants of *S. gordonii* are due to, at least in part, reduction in transcriptional activity of the *csbA* promoter and thus reduced cell-surface expression of a major adhesin.

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