Multiple phase variation in haemolytic, adhesive and antigenic properties of *Streptococcus gordonii*

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***Streptococcus gordonii* gave rise to β -haemolytic variants (Bhp⁺ for beta-haemolysin production) at frequencies of 10^{-4} – 10^{-3} on agar medium containing washed horse erythrocytes. Bhp⁺ variants reverted to the wild-type α -haemolytic phenotype (Bhp⁻) at the same frequencies. There was a significant probability (≥ 0.1) that phase variation in Bhp and phase variation in the previously described Spp (sucrose promoted phenotype) would occur concomitantly, but there was no correlation between these phenotypes. There was evidence also of independent phase variation in adhesion to saliva-coated hydroxyapatite (Asp for adhesion to salivary pellicles), in lactose-sensitive coaggregation (CIs for coaggregation, lactose-sensitive) and in the concentrations of particular cell surface antigens (Cap for cell antigen profile) in strains that had undergone phase changes in Spp and/or Bhp. Phase variation in all these phenotypes were transitions between high and low levels of activity and each appeared to occur as an independent event. Significant associations ($P \ll 0.0001$ by contingency table analysis) between particular phenotypes such as Bhp and Asp and between Asp, CIs and Cap phenotypes, however, were apparent. The results suggest that *S. gordonii* cells become predisposed to phase variation and that the resulting independent phenotypic changes may give rise to phenotypically diverse streptococcal populations able to accommodate rapid and transient environmental changes in the mouth.**

Keywords: phase variation, *Streptococcus gordonii*, adhesion, β -haemolysin, surface antigens

INTRODUCTION

Streptococcus gordonii is an early colonizer of the tooth surface (Kolenbrander & London, 1993), where it persists to become a common component of the supragingival dental plaque (Hamada *et al.*, 1980; Nyvad & Kilian, 1990). The species also may be found on the oral mucosae (Frandsen *et al.*, 1991) and is an agent of infectious endocarditis (Bayliss *et al.*, 1983a, b; Parker & Ball, 1976). The initial attachment of *S. gordonii* and other earlier colonizing streptococcal species (Frandsen *et al.*, 1991; Nyvad & Kilian, 1987) to the salivary pellicle on the tooth surface probably involves specific interactions between bacterial adhesins (Ganeskumar *et al.*, 1988; Rosan *et al.*, 1989) and adsorbed salivary components (Gibbons *et al.*, 1991; Morris & McBride, 1984). Following the saturation

of the tooth surface by bacteria, however, subsequent increases in bacterial numbers probably involve colonization mechanisms that are independent of initial attachment. One such mechanism may be accumulation in glucan polymers (Vickerman *et al.*, 1991a) synthesized from sucrose by the extracellular glucosyltransferase (GTF) enzyme. This mechanism of colonization is independent of initial attachment and compensates for reduced bacterial adhesiveness (Vickerman & Jones, 1995).

In *S. gordonii* strain Challis CH1, GTF activity undergoes reversible phase variation that significantly changes glucan production about fivefold (Tardiff *et al.*, 1989) and results in two distinct streptococcal colony phenotypes with very different abilities to accumulate in glucans on hydroxyapatite (HA) and saliva-coated hydroxyapatite (SHA) surfaces (Vickerman *et al.*, 1991a, b). Wild-type Spp⁺ (sucrose promoted phenotype, Tardiff *et al.*, 1989) cells grow and accumulate on HA and SHA surfaces when

Abbreviations: GTF, glucosyltransferase; HA, hydroxyapatite; SHA, saliva-coated hydroxyapatite.

sucrose is present, to reach population densities of $> 5 \times 10^{10}$ bacteria cm^{-3} (Vickerman *et al.*, 1991a), similar to bacterial densities of dental plaque (Gibbons & van Houte, 1975). Spp⁻ phase variants, in contrast, have about 20–30% of the GTF activity of Spp⁺ cells (Tardiff *et al.*, 1989) and do not accumulate in significant numbers in glucan polymers on HA or SHA surfaces (Vickerman *et al.*, 1991a). Earlier studies showed that Spp phase variants differed in the extent to which they attached to SHA surfaces in a sucrose-independent manner (Vickerman *et al.*, 1992) and that Spp⁻ phase variants may be more readily released from developing glucan polymer masses attached to HA substrata than Spp⁺ cells (Vickerman *et al.*, 1991b). These two observations suggested that growing Spp⁻ phase variants may be released from dental plaque *in vivo* and that released cells are able to colonize additional oral sites.

The genetic basis of phase variation in the activity level of GTF is not fully understood (Vickerman *et al.*, 1995). The expression of the structural gene for GTF, *gtfG*, of *S. gordonii* strain CH1 is known to be positively regulated by a gene designated *rgg*, located 66 bp upstream of *gtfG* (Sulavik *et al.*, 1992). However, the involvement of at least two additional regulatory sites is apparent from the studies of Haisman & Jenkinson (1991). In addition to evidence implicating several loci in the regulation of *gtfG*, environmental factors also may have temporal effects on glucan production. Hudson & Curtiss (1990), for example, demonstrated increased transcription of a *Streptococcus mutans gtf* in the presence of sucrose when cells were attached to HA surfaces. Although the stability of both Spp phenotypes (Tardiff *et al.*, 1989) implies that changes in DNA structure may be involved in Spp phase variation, preliminary analysis has detected no DNA frame-shifts or rearrangements in the region of *S. gordonii* strain Challis *gtfG* (Sulavik *et al.*, 1992).

We have examined the parent strain CH1 of *S. gordonii* strain Challis and Spp⁺ and Spp⁻ phase variant strains for changes in other phenotypes under non-selective conditions. In particular we examined adhesion to salivary pellicles and interbacterial coaggregation reactions, since these reactions may dictate initial colonization of the tooth surface and subsequent increases in bacterial numbers following the saturation of initial sites of attachment. We have also included studies on a newly described β -haemolysin, since haemolytic activities may be associated with virulence in other oral streptococci (Woltjes *et al.*, 1982), and on streptococcal surface antigens, since the composition of these may reflect changes in other cell properties. The results suggest that significant changes in these phenotypes may occur concomitantly with Spp phase variation, but the particular phenotypic change does not correlate with either Spp phase variant, nor do all these phenotypes appear to be regulated by *rgg*.

METHODS

Bacterial cultures and transformation. The Spp⁺ parental strain CH1 of *S. gordonii* Challis and an Spp⁻ derivative strain, strain CH1C1, were used as the standard reference strains

(Tardiff *et al.*, 1989; Vickerman *et al.*, 1991a). Other Spp⁻ variant and Spp⁺ revertant strains have been described previously (Sulavik *et al.*, 1992; Tardiff *et al.*, 1989; Vickerman *et al.*, 1991a; Vickerman & Jones 1992), or were isolated during this study. β -Haemolytic variants of strain CH1 were isolated as part of this study (see below). The Spp phase of a culture was confirmed on 3% (w/v) sucrose agar plates incubated in air plus 5% (v/v) CO₂ for 20 h and by GTF activity gels (Tardiff *et al.*, 1989). The haemolytic phenotypes of cultures were established on plates containing washed horse erythrocytes (see below) incubated in an anaerobic chamber (Coy Industries). Frozen stocks of bacteria were maintained at -70°C in 50% (v/v) glycerol. Stock cultures were grown anaerobically on Brain Heart Infusion (BHI) agar plates (Difco) at 36°C . Unless otherwise stated, broth cultures were grown in Todd Hewitt (TH) broth (Difco) for 18 h under anaerobic conditions at 36°C .

Strain CH1 and the Spp⁻ strains CH1C1, CH1A8 and CH97, which exhibited different phenotype combinations (see Results), were transformed (Lawson & Gooder, 1970; Raina & Macrina, 1982) with plasmid pAMS57, which carries the *rgg* gene (Sulavik *et al.*, 1992). Transformants were selected on plates containing $5\text{ }\mu\text{g}$ erythromycin ml^{-1} . The presence of the plasmid clone was confirmed in DNA extracts (Clewett *et al.*, 1974) run on agarose gels. The functional activity of the *rgg* clone was demonstrated by the increased hardness of transformants on sucrose agar plates and by an increase in periodic acid/Schiff reagent positive band intensity detected on GTF activity gels (Tardiff *et al.*, 1989). Strains carrying pAMS57 were maintained and grown on media containing $5\text{ }\mu\text{g}$ erythromycin ml^{-1} .

Adhesion to SHA beads. Bacteria were grown in TH broth overnight, diluted to approximately 1×10^6 bacteria ml^{-1} in fresh broth containing $5\text{ }\mu\text{Ci}$ ($1.85 \times 10^4\text{ Bq}$) [^3H]thymidine ml^{-1} (Amersham) and incubated anaerobically for a further 20 h, when the culture entered late-exponential phase (Vickerman *et al.*, 1992). The bacteria were recovered by centrifugation at 1000 g for 15 min and the cells washed three times and resuspended in buffered KCl (Appelbaum *et al.*, 1979) to a concentration of 1×10^8 bacteria ml^{-1} (Vickerman *et al.*, 1992).

Saliva was obtained from healthy male donors, diluted 1:4 in buffered KCl, heated at 60°C for 30 min and then clarified by centrifugation (Clark *et al.*, 1978). HA beads (BDH) in 10 mg quantities were equilibrated in buffered KCl at 4°C overnight in polystyrene tubes ($17 \times 100\text{ mm}$ Falcon tubes) and then treated with 1 ml vols diluted saliva for 90 min at room temperature. After the adsorption of the saliva to the beads, the beads were washed in buffer and 1 ml radiolabelled bacterial suspension was added to each tube. Adhesion assays were incubated at 37°C for 90 min on a rotary drum turning at 10 r.p.m. The supernatants were then removed and the beads were washed three times in buffer to remove unattached bacteria. The beads and supernatants were counted using BioSafe II scintillation fluid. Radioactive counts were correlated with bacterial numbers and the results then expressed as the number of bacteria attached to 10 mg beads.

Coaggregation assays. *Streptococcus* sp. strain PK509 is a standard reference strain for lactose-sensitive (type 5) coaggregation (Kolenbrander *et al.*, 1990). Strain PK509 (a gift from P. Kolenbrander, NIDR, NIH, Bethesda, MD, USA) was grown anaerobically in pre-reduced TH broth for 24 h at 36°C . *S. gordonii* strains were grown in the same medium and under the same conditions. Cultures were centrifuged at 1000 g for 15 min and the bacteria suspended in 25 mM sodium phosphate buffer (pH 8.0) containing 25 mM NaCl (McIntire *et al.*, 1978) to give an OD₆₅₀ of 0.8–1.0 (1 cm light path, Pharmacia Ultraspec Plus

spectrophotometer). Equal volumes (0.5 ml) of strain PK509 and test *S. gordonii* strains were mixed together in 12 × 75 mm polystyrene tubes (Falcon). To one set of tubes, 0.25 ml phosphate buffer was added and to another set of control tubes, 0.25 ml buffer containing 0.8 M D-(+)-lactose (Fisher Scientific) was added to inhibit coaggregation. Tubes were mixed for 10 s on a vortex mixer (Fisher). After incubation at room temperature overnight, approximately 1 ml supernatant was removed carefully and the OD₆₅₀ read. Coaggregation was expressed as the difference between the optical density of supernatants of test mixtures without lactose (in which coaggregation occurred) and the optical density of the supernatants of control mixtures with lactose (in which no observable coaggregation occurred). The difference between test and control optical density (ΔOD_{650}) represented the increased sedimentation of the bacteria due to lactose-sensitive coaggregation. Tests were done in triplicate. Strain CH1 and strain CH1C1 were the reference strains to which the results of all other strains were compared. No homologous coaggregation was observed under these test conditions.

Haemolytic activity. β -Haemolytic activity of *S. gordonii* was detected on 4% (v/v) blood agar plates composed of 10 g LabLemco (Oxoid), 10 g type L37 peptone (Oxoid), 5 g NaCl and 15 g agar (agar number 3, Oxoid) (1 deionized water)⁻¹; the final pH was 7.3. To prepare washed horse-blood agar medium, defibrinated horse blood (Colorado Serum) was centrifuged and the erythrocytes washed three times in PBS (Oxoid). The erythrocytes were resuspended in PBS to their original packed cell volume of approximately 50%. Washed erythrocyte suspensions were added to the sterilized blood agar base medium at 50 °C to give a final concentration of 4% (v/v), and 15 ml volume plates were prepared immediately. Washed-blood agar plates were prepared from sheep, cow and human erythrocytes in the same way. Whole-blood agar plates were prepared with 4% (v/v) whole blood without further treatment.

To determine the frequency of variation in haemolytic activity, overnight TH broth cultures were diluted to give about 200 colonies per plate after anaerobic incubation for 24 h; the haemolytic activities of selected isolates were confirmed by streaking washed-horse-blood agar plates for isolated colonies and incubating the cultures under the same conditions.

Western blot analysis of surface antigens. Bacteria were grown anaerobically in TH broth overnight and pelleted by centrifugation. The bacteria were then incubated with gentle agitation in 1% (w/v) sodium lauroyl sarcosine, 10 mM Tris, 1 mM EDTA buffer (pH 7.5) at room temperature for 20 min to extract cell-surface antigens (Jenkinson, 1986). Bacteria were removed by centrifugation and the extracts were treated with boiling 5% (w/v) SDS, 0.2 M Tris buffer (pH 6.8). Gels (7.5%, w/v, polyacrylamide, Tris-glycine buffer, pH 8.6; Laemmli, 1970) were loaded with approximately equal amounts of protein per lane (20 µg ml⁻¹; Markwell *et al.*, 1978). After electrophoresis at 10 mA per gel for 16 h, components were electroblotted (Bio-Rad) onto polyvinylidene difluoride membranes (Millipore) using 10 mM CAPS buffer, 10% (v/v) methanol (pH 11.0). Antigens were detected with polyclonal rabbit serum made against whole live bacteria of strains CH1 and CH1C1, alkaline-phosphate-conjugated goat anti-rabbit sera (Gibco BRL) and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate (Sigma). Antisera were made by injecting rabbits intravenously with six incremental doses of bacteria in PBS (10⁷–10⁹ bacteria) at intervals of 3–4 d.

Stained Western blots were analysed with an Ambis image acquisition and software system. The backgrounds of lanes were

adjusted to the same degree and the concentrations of the antigen bands were determined as percentages of the total antigen present per lane.

Statistical analysis. The association of phenotypes was compared by contingency table analysis using Fisher's exact test (Instat, GraphPad Software). Comparisons of activity levels of phenotypic variants were made using one-tail Student's *t* test.

RESULTS

Phase variation in β -haemolytic properties of *S. gordonii* strain CH1

The parental strain CH1 (Spp⁺) and the Spp⁻ phase variant, strain CH1C1 (Tardiff *et al.*, 1989), were selected for use in preliminary experiments since they are phenotypically distinct in terms of GTF activity (Tardiff *et al.*, 1989), sucrose-associated accumulation (Vickerman *et al.*, 1991a) and adhesive properties (Vickerman *et al.*, 1992). Colonies of both strains grown anaerobically on sheep and cow whole- and washed-blood agar were α -haemolytic. However, strains CH1 and CH1C1 caused β -haemolysis of human and horse erythrocytes when grown on whole-blood agar plates, whereas only strain CH1C1 was β -haemolytic on washed-human- and washed-horse-blood agar plates (data not shown). These results indicated that the β -haemolytic activity detected on washed-horse-blood agar plates may vary with the Spp phase and that β -haemolytic activity, therefore, may be subject to phase variation. The β -haemolytic colony type of strain CH1C1

Table 1. Changes in the Bhp phenotype of Spp phase variants

(a) Strain CH1 is the parental strain from which the lineage of strains CH1A8, CH1B1, CH1C1 and CH1D2 were sequentially derived (Tardiff *et al.*, 1989); frequencies were about 1×10^{-4} in all cases. (b) Strains CH1C1 through CH1C11 are Spp⁻ variants that were obtained from strain CH1B1 at the same time (Tardiff *et al.*, 1989); the frequency was 8.5×10^{-4} .

Strain	Spp phenotype	Origin	Bhp phenotype*
(a) Sequentially derived strains			
CH1	Spp ⁺	Parental	Bhp ⁻
CH1A8	Spp ⁻	CH1	Bhp ⁺
CH1B1	Spp ⁺	CH1A8	Bhp ⁺
CH1C1	Spp ⁻	CH1B1	Bhp ⁺
CH1D2	Spp ⁺	CH1C1	Bhp ⁻
(b) Concomitantly derived strains			
CH1C1	Spp ⁻	CH1B1	Bhp ⁺
CH1C2	Spp ⁻	CH1B1	Bhp ⁺
CH1C3	Spp ⁻	CH1B1	Bhp ⁻
CH1C9	Spp ⁻	CH1B1	Bhp ⁺
CH1C11	Spp ⁻	CH1B1	Bhp ⁻

* Bhp⁺/Bhp⁻, β -haemolytic/not β -haemolytic on washed-horse-blood agar plates.

Table 2. Frequency of isolation of β -haemolytic variants (Bhp⁺) from strain CH1 and the frequency of their reversion to the Bhp⁻ phenotype

TH broth cultures were diluted and spread on agar plates containing washed horse erythrocytes to give about 200 colonies after 24 h anaerobic incubation. (a) Results of five independent experiments to determine the frequency of Bhp⁻ → Bhp⁺ phase variation for strain CH1. (b) Reversion of Bhp⁺ variants of strain CH1 to the Bhp⁻ state in eight independently isolated Bhp⁺ strains.

Strain	Experiment	No. of haemolytic variants/no. examined*	Frequency†
(a) Phase variation (Bhp⁻ → Bhp⁺)			
CH1	1	1/9396	1.1×10^{-4}
	2	2/12936	1.5×10^{-4}
	3	7/7058	9.9×10^{-4}
	4	8/9568	8.4×10^{-4}
	5	3/7749	3.9×10^{-4}
(b) Reversion (Bhp⁺ → Bhp⁻)			
HL2.1	1	2/9040	2.2×10^{-4}
HL3.1	2	3/10832	2.8×10^{-4}
HL3.2	3	1/4200	2.4×10^{-4}
HL3.4	4	4/19802	2.0×10^{-4}
HL3.7	5	5/22202	2.3×10^{-4}
HL3.8	6	12/15650	7.7×10^{-4}
HL4.2	7	6/8549	7.0×10^{-4}
HL5.2	8	3/2827	1.1×10^{-3}

* Total number showing a change in phenotype/number of colonies examined.

† Frequency = number of colonies showing a change in phenotype/total number of colonies examined.

was termed Bhp⁺ for beta-haemolysis production, and the α -haemolytic phenotype of strain CH1 was called Bhp⁻.

To determine if changes in the haemolytic properties were reproducibly co-ordinated with Spp phase variation, the Spp⁺ parent strain CH1 and a collection of independently derived Spp⁻ phase variants and Spp⁺ revertants were examined for β -haemolytic activity on washed-horse-blood agar plates. Changes in the Bhp phenotype of the parent strain CH1 from Bhp⁻ to Bhp⁺ were apparent in one third of the Spp isolates examined (8 of 24 strains). However, the occurrence of the Bhp⁺ trait was independent of the Spp phase ($P > 0.4$ by contingency table analysis using Fisher's exact test) in that approximately equal numbers of Spp⁻ and Spp⁺ strains were Bhp⁺; i.e. 5 of 13 Spp⁻ and 3 of 11 Spp⁺ strains were Bhp⁺. The independence of the Bhp and the Spp phenotypes in strains screened for Spp phase variation also was clearly evident (Table 1a) in a lineage of Spp⁺ and Spp⁻ strains derived sequentially from strain CH1. Moreover, the high rates of change in Bhp during Spp phase variation were apparent in five Spp⁻ strains obtained from a single

Fig. 1. Adhesion (hatched bars) and coaggregation (solid bars) of representative strains CH1 and CH1C1. Adhesion is expressed as bacteria ($\times 10^6$) per 10 mg SHA beads and coaggregation in units of ΔOD_{650} ; the error bars represent the SEM for four to seven assays. Asp⁻ and Cls⁻ strains had mean adhesion and coaggregation values within 2 SD of the values for CH1 for that assay; Asp⁺ and Cls⁺ strains had mean values statistically indistinguishable from the mean value for strain CH1C1.

screening of the Spp⁺ Bhp⁺ strain, strain CH1B1 (Table 1b), when two Spp⁻ isolates were found to have reverted to Bhp⁻ phenotype.

These observations suggested that the expression of the Bhp phenotype was subject to phase variation. Evidence of phase variation in haemolytic properties of strain CH1 was sought by plating cultures of strain CH1 onto 4% (v/v) washed-horse-blood agar plates to give approximately 200 colonies per plate after 24 h anaerobic incubation at 36 °C. Under these conditions, strain CH1 produces α -haemolytic or γ -haemolytic colonies typical of the Bhp⁻ phenotype. However, at rates of between 1×10^{-4} and 1×10^{-3} , Bhp⁺ colonies surrounded by clear zones of β -haemolysis were detected (Table 2). Bhp⁺ variants gave rise to stable Bhp⁻ revertants at similar frequencies (Table 2). The examination of the Bhp⁺ phase variants and the Bhp⁻ revertants confirmed that Spp and Bhp were not correlated. Of the 10 Bhp⁺ isolates selected for further study, only one had undergone phase variation to the soft colony phenotype of Spp⁻ strains, and all Bhp⁻ revertant strains remained Spp⁺.

Changed adhesion to SHA and lactose-sensitive coaggregation of Spp and Bhp phase variants

Strains CH1C1 and CH1 exhibited consistent differences in both adhesion to SHA and in coaggregation and were included in all experiments as controls. Approximately fivefold more cells of strain CH1C1 attached to SHA compared to strain CH1 ($P < 0.0001$, Student's *t* test) and strain CH1C1 coaggregated significantly better with the substratum strain PK509 than did strain CH1 ($P \leq 0.001$) (Fig. 1). Other strains were assigned to the low adhesion

Table 3. Association of high (Asp⁺) and low (Asp⁻) adhesion to SHA with the Spp or Bhp phenotypes in strains that have undergone Spp phase variation

Twenty-four Spp variant strains were classified as Spp⁺ or Spp⁻, and as either Bhp⁺ or Bhp⁻. Each strain was then assigned to the high (Asp⁺) or to the low (Asp⁻) adhesion groups according to its Spp or its Bhp phenotype. The parental Spp⁺ strain CH1 and the Spp⁻ strain CH1C1 were used as standard reference strains for low (Asp⁻) and high (Asp⁺) adhesion groups, respectively. Strains within 2 SD of the mean adhesion value of strain CH1 were assigned to the Asp⁻ group. The adhesion of strains assigned to the Asp⁺ group was similar to the adhesion of strain CH1C1 ($P > 0.03$, by Student's *t* test) and significantly higher than the adhesion of Asp⁻ strains ($P < 0.0001$). The probability was calculated by Fisher's exact test for the association of Spp or Bhp traits with Asp⁺ or Asp⁻ phenotypes.

Phenotype of adhesion to SHA	No. strains to have undergone Spp phase variation characterized according to their Spp or Bhp phenotypes			
	Spp		Bhp	
	Spp ⁺	Spp ⁻	Bhp ⁺	Bhp ⁻
Asp ⁺	3	5	7	1
Asp ⁻	8	8	1	15
	$P \geq 0.04$		$P < 0.0002$	

or low coaggregation group if the mean value of adhesion or coaggregation fell within 2 standard deviations (2 SD) of the mean value of strain CH1 for that experiment. Other strains were assigned to the high adhesion or coaggregation groups represented by strain CH1C1. Such strains had adhesion and coaggregation levels similar to that of strain CH1C1 ($P > 0.03$ by Student's *t* test) and significantly greater than strain CH1 ($P < 0.001$). Strains assigned to the high adhesion group were termed Asp⁺ (for adhesion to salivary pellicles), and strains with low adhesion levels as Asp⁻. Likewise, the higher level of coaggregation was termed Cls⁺ for coaggregation, lactose-sensitive, and lower coaggregating activity as Cls⁻.

Changes in the Asp⁻ phenotype of strain CH1 were apparent in 8 of 24 Spp phase variant strains examined, although no statistical associations between Spp and Asp characters were apparent ($P \geq 0.4$ by Fisher's exact test, Table 3). In contrast, the Asp⁺ phenotype of these strains was significantly associated with the Bhp⁺ phenotype ($P = 0.0002$ by Fisher's exact test) (Table 3). Eight of the ten Bhp⁺ phase variant strains isolated from strain CH1 (see above) also showed the increased adhesion to SHA of the Asp⁺ phenotype (data not shown).

Changes in the Cls phenotypes of strains screened for Spp and Bhp phase variation were also found. However, there were no statistical associations between the Cls⁺ phenotype and either Spp phase (i.e. 5 of 13 Spp⁻ and 1 of 11 Spp⁺ were Cls⁺; $P \geq 0.7$), nor between Cls⁺ and either

Table 4. Association of high (Cls⁺) and low (Cls⁻) lactose-sensitive coaggregation activity with the Asp⁺ and Asp⁻ phenotype in Spp and Bhp phase variant isolates

Independently isolated Spp phase variants and independently isolated Bhp phase variants were classified as Asp⁺ or Asp⁻, as described in Table 3. All strains were examined for coaggregation and assigned to either the Cls⁺ or Cls⁻ groups as follows. Strain CH1 was the control for the Cls⁻ group; strains with ΔOD_{650} within 2 SD of the mean ΔOD_{650} of strain CH1 were assigned to the Cls⁻ group. Strain CH1C1 was the standard reference strain for the Cls⁺ group. Differences between the controls and the two groups were consistent and significant ($P < 0.001$ by Student's *t* test). The probability of association between Cls and Asp phenotypes in isolates selected because of changes in Spp or Bhp was calculated by Fisher's exact test.

Coaggregation phenotype	Spp isolates		Bhp isolates	
	Asp ⁺	Asp ⁻	Asp ⁺	Asp ⁻
Cls ⁺	6	2	1	0
Cls ⁻	0	16	7	2
	$P = 0.0002$		$P = 1.0$	

Bhp phenotype ($P \geq 0.4$, data not shown) in these Spp isolates. In contrast, the Cls⁺ phenotype was significantly associated with the Asp⁺ phenotype in these Spp isolates (Table 4; $P \leq 0.0002$). No similar association between Cls and Asp phenotypes was apparent for the 10 Bhp⁺ phase variant isolates (Table 4).

Changes in the relative concentrations of surface antigens of Spp and Bhp phase variants

Western blot analyses revealed changes in the relative concentrations of cell antigens. Three antigens [designated antigens A (146 kDa), B (102 kDa) and C (53 kDa)] in particular showed distinct and consistent differences in extracts of 9 of the 38 independently isolated Spp and Bhp phase variant strains. On the basis of these antigenic changes, strains were placed in one of two groups designated CapA (for cell antigen pattern) or CapB. Stained blots of the two antigenic patterns are shown in Fig. 2 and the quantitative analyses of the three antigens of six independently isolated CapA and six independently isolated CapB strains are shown in Fig. 3. Antigen A was significantly higher in extracts of CapA strains ($P < 0.0001$), whereas antigens B and C were significantly elevated in CapB strains ($P < 0.0001$). Each of the three bands appeared to be a single antigen in that no additional bands were apparent when gels of different concentrations were run under different conditions for different periods of time (data not shown). Neither Cap phenotype was associated with either Spp phenotype, nor with either Bhp phenotype ($P < 0.06-0.03$). The CapB phenotype ($P < 0.0001$), however, was significantly associated with the more adhesive/coaggregative Asp⁺ and Cls⁺ phenotypes, but no exact correspon-

Fig. 2. Western blots of antigens extracted from strain CH1C1 (CapB) and from strain CH97 (CapA). Sodium lauroyl sarcosine extracts of cell-surface proteins were electrophoresed on 7.5% (w/v) polyacrylamide gels, blotted to polyvinylidene difluoride membranes and treated with polyvalent rabbit antiserum to whole *S. gordonii* cells and alkaline-phosphatase-conjugated goat anti-rabbit antiserum. The positions of antigens A, B and C are indicated on the right and molecular mass standards on the left. Variations between strains in the concentrations of other proteins appeared to be strain specific.

dence between the calculated molecular masses of antigens A, B or C and streptococcal cell surface components identified as being involved in various adhesive and coaggregating activities (Demuth *et al.*, 1988; Erickson & Herzberg, 1990; Fenno *et al.*, 1989; Jenkinson, 1986, 1992; Jenkinson *et al.*, 1993; Kolenbrander *et al.*, 1994;

Fig. 3. Mean concentrations ($\pm$ SEM, $n = 6$) of antigens A (146 kDa), B (102 kDa) and C (53 kDa) of six independently isolated CapB (solid bars) and six CapA (hatched bars) strains. Western blots were analysed with Ambis image acquisition equipment and software and the relative concentrations of antigens A, B and C were expressed as percentages of the total components present. The significance of the differences in the concentrations of antigens A, B and C for CapB and CapA strains was $P < 0.0001$ in all three instances.

Lamont *et al.*, 1988, 1994; Scannapieco *et al.*, 1992; Sommer *et al.*, 1992; Vacca-Smith *et al.*, 1994) was apparent.

The responsiveness of phenotypes to the *rgg* regulatory gene of GTF synthesis

Four strains were used to determine the influence of *rgg* on the Bhp, Asp, Cls and Cap phenotypes, since these phenotypes may change during Spp phase variation. These were strains CH1 ($Spp^+ Bhp^- Asp^- Cls^- CapA$), strains CH1C1 and CH1A8 ($Spp^- Bhp^+ Asp^+ Cls^+ CapB$) and strain CH97 ($Spp^- Bhp^+ Asp^- Cls^- CapA$); the phenotypes of these strains are representative of phase variants identified in this study. The transformation of these strains with plasmid pAMS57 increased the colony hardness of all four strains and increased GTF activity on activity gels (data not shown). In contrast, the presence of *rgg* on pAMS57 had no detectable influence on the Bhp, Asp or Cls phenotypes of any of the four test strains (data not shown). The presence of the *rgg* clone, however, resulted in an approximately threefold increase in the concentration of antigen C in CapA strains CH1 and CH97, to levels similar to those found in CapB strains (data not shown). In contrast, *rgg* did not elevate antigen C in CapB isolates, nor did *rgg* cause observable changes in the concentration of antigens A or B in either phenotype.

DISCUSSION

Based on the observations that only Spp^+ bacteria accumulate significantly in glucan polymers (Vickerman *et al.*, 1991a) and that Spp^- cells tend to be excluded from

polymers produced by Spp⁺ cells (Vickerman *et al.*, 1991a, b), it was proposed that phase variation in GTF activity may be ecologically relevant. Specifically, it was suggested that Spp⁻ strains are released from the bacterial populations of dental plaque and are then more able to attach to cells on other oral surfaces (Vickerman *et al.*, 1991a, b). The latter was indicated by the increased attachment of some phase variants to salivary pellicles (Vickerman *et al.*, 1992). The present results extend support for these conclusions and show that *S. gordonii* Challis strain CH1 exhibits phenotypic phase variations in addition to changes in the activity of GTF, and that these changes in phenotype may also be relevant to colonization.

Reversible phase variation occurred in the haemolytic properties of *S. gordonii* strain CH1. This phase variation gave rise to a β -haemolytic (Bhp⁺) phenotype, which occurred at high frequency in independently isolated Spp phase variants. Statistical analyses suggested, moreover, that concomitant changes in cell-surface properties that modify the attachment of the bacteria to salivary pellicles (Asp phenotype) and alter their participation in lactose-sensitive coaggregation (Cls phenotype), as well as changes in surface antigen patterns (Cap phenotype), also occurred during phase variation in Spp and/or Bhp. In all these cases, phase variation consisted of the alternation of cells between high and low expression of a phenotype. These events appear to be linked in some instances in that there is a significant statistical association between the Bhp⁺ and Asp⁺ phenotypes and between the Asp⁺ and Cls⁺ phenotypes in strains that had undergone phase variation Spp and Bhp, respectively. Moreover, the CapB phenotype is very significantly associated with the adhesive phenotypes Asp⁺ and Cls⁺, although changes in the Cap phenotype probably reflect more extensive changes in surface properties. These changes may be due to alterations in specific adhesive activities, but may also reflect changes in the stability of cell surface components and/or the ease with which they may be extracted. The differing effects of *rgg* on the concentrations of antigens A, B and C, however, suggest that antigens B and C are not the breakdown products of antigen A. In other instances, variations in the phenotypes appeared to occur independently (e.g. Spp⁻ strains were not consistently Bhp⁺ and Bhp⁺ strains were not predominantly Cls⁺ etc.). Notwithstanding the absence of strict correlations between phenotypes, there was sufficient evidence to suggest that switching between phenotypes is regulated by a common process. First, Bhp phase variation occurred at a frequency very similar to that of Spp phase variation (i.e. 10^{-4} – 10^{-3} ; Tardiff *et al.*, 1989). Second, the magnitude of the differences in the level of the activities of both the Asp and Cls phenotypes was similar to the magnitude of the change in GTF activity following Spp phase transition (Tardiff *et al.*, 1989). Third, there was a 10–30% chance that cultures undergoing phase variation in Spp or Bhp would also show changes in one or more of the other phenotypes examined. For example, the greatly increased chance of detecting Bhp⁺ $\leftrightarrow$ Bhp⁻ transitions following Spp variation is about 1000 times greater than the measured frequency of reversible Bhp phase variation of

1×10^{-4} . One explanation for these results is that there is an increased chance of change in all phenotypes during some common event that predisposes the bacteria to phase transitions. Whether or not the expression of a particular phenotype increases or decreases may be only a function of the pre-existing level of expression.

The mechanism of GTF phase variation that gives rise to Spp phase variants is unknown, although it is unlikely that it involves mechanisms of gene conversion similar to those proposed for pilus variation in *Neisseria gonorrhoeae* (Seifert *et al.*, 1988; Swanson *et al.*, 1990) because only a single copy of *gtfG* is present on the *S. gordonii* chromosome (Sulavik, 1992; Sulavik *et al.*, 1992). Moreover, Spp phase variation does not entail major rearrangement(s) of the chromosome at sites within or immediately flanking *gtfG* or *rgg* (Sulavik *et al.*, 1992) and preliminary studies indicate that there are no DNA frame-shift mutations, such as are involved in phase variation in other bacteria (Murphy *et al.*, 1989; Stibitz *et al.*, 1989). *rgg* regulates *gtfG* expression, possibly at the level of transcription (Sulavik, 1992; Vickerman *et al.*, 1995), but does not appear to be involved in the differential expression of Bhp, Asp or Cls. Cells transformed with *rgg* clones, however, did exhibit increased concentrations of antigen C in both Spp⁺ and Spp⁻ variants.

The alternation of cells between high and low expression of several potentially important phenotypes would give rise to populations of oral streptococci with high degrees of phenotypic diversity. Interbacterial coaggregation, adhesion to salivary pellicle components and the synthesis of glucan *in vitro* are thought to be involved in colonization of the oral cavity. In contrast, there have been relatively few studies on the roles that the haemolysins of oral streptococci may play in the oral cavity (Wolff & Liljemark, 1978; Woltjes *et al.*, 1981, 1982; Woltjes & de Graff, 1983), although β -haemolysin production is more prevalent among mutants streptococci from pathological oral conditions (Woltjes *et al.*, 1982). Whether or not the β -haemolysin of *S. gordonii* is similar to the β -haemolysin(s) of the mutants streptococci, and whether or not the *S. gordonii* haemolysin is cytotoxic, remains to be determined. It would seem, however, that compared to co-ordinate regulation of gene expression (Miller *et al.*, 1989) in response to specific environmental factors, the apparent random phenotypic changes described here may be advantageous to oral bacteria. Environmental changes in the mouth can be rapid, variable and of short duration. Phenotypically diverse streptococcal populations, therefore, may be more able to accommodate and take advantage of such environmental changes and would be better able to colonize a wider spectrum of oral sites.

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