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Infectivity of a glucan synthesis-defective mutant of *Streptococcus gordonii* (Challis) in a rat endocarditis model

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Abstract: Streptococcus gordonii, a member of the human indigenous oral microflora, colonizes smooth tooth surfaces and contributes to dental plaque formation. Although it is not recognized as being a cariogenic pathogen, it may cause endocarditis following invasion of the bloodstream. Using allelic exchange mutagenesis, we have constructed a mutant of *S. gordonii* (Challis) which is defective in its single functional glucosyltransferase gene and, hence, is unable to synthesize glucan exopolymers from sucrose. When examined in a rat endocarditis model, the sucrose-grown mutant did not differ significantly from *S. gordonii* wild-type, suggesting that glucan polymers did not contribute to infectivity. This result was in striking contrast to that previously observed with a polymer-defective *S. mutans* mutant.

Key words: Endocarditis; *Streptococcus gordonii*; Glucosyltransferase

Introduction

Streptococcus gordonii (formerly *Streptococcus sanguis* [1]) is a normal inhabitant of the human oropharyngeal mucosa and smooth tooth surfaces. In this environment it is considered indigenous and relatively avirulent. However, this organism may escape the oral cavity following routine dental or specialized procedures and enter the bloodstream [2,3]. The resulting bacteremia may cause infective endocarditis if underlying cardiac valvular disease exists in the host. Viridans streptococci including *S. mutans* and *S. gordonii* produce several extracellular enzymes which use sucrose to synthesize exopolymers. Glucosyltransferase (GTF) enzymes use the glucosyl moiety of sucrose to synthesize water-soluble or insoluble glucans. Fructosyltransferase (FTF) enzymes use the fructosyl moiety of sucrose to synthesize water soluble fructans. Based

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on both biochemical and genetic analysis of a limited number of isolates, *S. mutans* strains usually possess multiple GTF enzymes and a single FTF enzyme while *S. gordonii* appears to possess a single GTF enzyme [4–8]. Sucrose-derived polymers of *S. mutans* have been established as virulence factors in dental caries. Water-insoluble polymers are believed to facilitate tenacious adherence to the smooth tooth surface while the soluble polymers may contribute to virulence by acting as extracellular storage compounds, thus promoting long term organism survival [9–13].

Current evidence regarding pathogenesis of bacterial endocarditis suggests that a crucial step in the development of infection is the initial adherence of bacteria to endocardium. Morphological studies with experimental models of endocarditis have demonstrated that the initial colonization occurs in areas of endocardial trauma on fibrin-platelet deposits [14–17]. Synthesis of glucan polymer has been correlated with the ability of *S. sanguis* to adhere to damaged heart valve leaflets in vitro [18,19] and has been associated with increased infectivity in vivo [18]. Dextranase treated cells showed decreased adherence to fibrin-platelet matrix in vitro and decreased rate of infection in the animal model of endocarditis [18,19].

Recently, Munro and Macrina [20] have used a defined mutant of *S. mutans* to evaluate the contribution of sucrose-derived polymer formation to infectivity in a rat endocarditis model. This mutant, unable to synthesize either glucans or fructans from sucrose, was constructed by allelic exchange. Sucrose-grown cells were significantly less virulent than similarly-grown or glucose-grown wild-type cells. Using similar allelic exchange mutagenesis techniques, we have constructed a glucosyltransferase-deficient mutant of *S. gordonii*. As expected, this mutant was devoid of glucan polymer biosynthetic capability. When examined in a rat model for endocarditis, sucrose-grown mutant cells did not differ significantly from *S. gordonii* wild-type cells, suggesting that glucan polymers were not contributing to infectivity. This is in striking contrast to results seen with the polymer-defective *S. mutans* mutant.

Materials and Methods

Bacterial strains and media

The strain of *Streptococcus gordonii* used in this work, CH1, is a Challis strain which exhibits a characteristic hard colony phenotype designated Spp^+ (sucrose promoted phenotype positive; [4]) when grown on Todd Hewett media (Difco Labs, Detroit, MI) containing 3% sucrose. For use in the endocarditis experiments, the strains were grown overnight in Todd Hewett broth (Difco Laboratories) anaerobically at 37°C. Fresh Todd Hewett broth containing 3% sucrose was inoculated with 0.5 ml of the overnight culture and allowed to grow to late log phase prior to use as animal inocula. GTF assays were performed using broth culture supernatants of brain heart infusion (Difco Laboratories) grown cells. Transformation of *S. gordonii* was accomplished as previously described [21].

Mutagenesis

The *S. gordonii* mutant AMS12 was constructed by allelic exchange in order to inactivate the GTF gene, *gtf*G (see Fig. 1). Disruption of the *gtf*G gene in strain AMS12 was verified by Southern blot analysis using four different DNA hybridization probes: pTV32(Ts), and the 4-, 1.5-, and 3.3-kb *Hind*III fragments which contained portions of the *gtf*G gene (see Fig. 1). The *Xba*I 3.2-kb chromosomal component identified in the wild type strain was missing in strain AMS12 and was replaced by a 6.4-kb fragment (see *Xba*I sites in Fig. 1). This fragment is larger than the 3.2-kb fragment by the difference in size (approx. 3.2 kb) between the *lacZ-erm* DNA cassette (4.7 kb) and the 1.5-kb *Hind*III fragment. Thus, this analysis confirmed the replacement of the 1.5-kb fragment of the *gtf*G gene by the *lacZ-erm* cassette as illustrated in Fig. 1.

Assay of glucosyltransferase activity

The production of extracellular GTF in culture supernatants of both CH1 and AMS12 was assayed using acetone precipitates of brain heart infusion-grown cells cultivated at 37°C to late log phase. Glucan assays monitored ^{14}C-glucose incorporation into methanol-insoluble material using radioisotopic sucrose as a substrate [10].

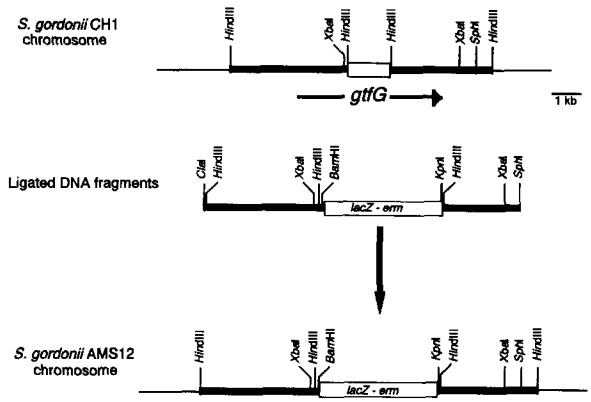

Fig. 1. Construction of the *gtf*G mutant of *S. gordonii*. The *gtf*G gene spans three *Hind*III fragments as indicated by the top line labelled '*S. gordonii* CH1 chromosome' in Fig. 1 [5]. The 5′ end of the chromosomal *gtf*G gene begins within a 4-kb *Hind*III fragment, extends through a 1.5-kb *Hind*III fragment (open rectangle), and terminates within a 3.3-kb *Hind*III fragment as indicated by the arrow. The two flanking *Hind*III fragments were isolated and ligated to the *lacZ-erm* cassette as seen in Fig. 1 (second line marked 'Ligated DNA fragments'). They are: i) a 4.7-kb *lacZ-erm* DNA cassette containing *Kpn*I and *Bam*HI cohesive ends isolated from pTV32(Ts) [26]; ii) the 4-kb *Hind*III fragment flanked by DNA containing *Cla*I and *Bam*HI cohesive ends; and, iii) a 2.5-kb fragment containing *Sph*I and *Kpn*I cohesive ends. The ligated fragments were introduced into *S. gordonii* CH1 by transformation. Erythromycin-resistant colonies were obtained, one of which was designated strain AMS12. Two reciprocal recombination events, between the target cell's chromosome and DNA flanking the *lacZ-erm* determinants, resulted in the replacement of the 1.5-kb chromosomal *Hind*III fragment with the *lacZ-erm* DNA giving rise to the structure seen at the bottom of Fig. 1 ('*S. gordonii* AMS12 chromosome').

Endocarditis testing

The technique used to produce endocarditis in male Sprague-Dawley rats was adapted from Santoro and Levison [22] and our use of it has been previously described [20].

Results

Construction and analysis of glucosyltransferase defective mutant

A glucosyltransferase-deficient mutant of *S. sanguis* CH1 was prepared by allelic exchange as described in Materials and Methods and illustrated in Fig. 1. This mutant displayed a stable erythromycin resistance phenotype as expected from the loss of an internal segment of the *gtf*G gene with a concomitant insertion of a drug resistance cassette. Extracellular protein preparations of the wild-type CH1 and AMS12 mutant strains were prepared and assayed for GTF activity as measured by incorporation of ^{14}C-glucose (from ^{14}C-glucose-labeled sucrose) into methanol insoluble material. Activities were standardized by expressing incorporated cpm per microgram of protein in the assayed material. The wild type strain displayed activity defined in this manner at a level of 218 ± 41 (total of 9 repititions using 2 independently-prepared protein extracts). Similar analysis using extracellular protein preparations from the AMS12 mutant strains failed to reveal GTF activity. Enzyme levels could not be distinguished from negative control baseline (3.8 ± 7.7). These results corroborated the predicted genotype of the mutant and confirmed the presence of a single active GTF gene in the wild type CH1 strain. A genetic switching mechanism which affects GTF activity in *S. gordonii* CH1 has been reported [4,5]. Switching involved differences in GTF levels that range from 4- to 10-fold, with low-level producing variants still displaying detectable GTF activity. This is in contrast to the *gtf*G mutant reported here. Enzyme activity is reduced approximately 60-fold in the AMS12 mutant, to a level which is statistically consistent with its absence.

Virulence in the rat model of endocarditis

The ability of the GTF deficient strain (AMS12) to cause endocarditis was compared with that of the wild-type (CH1) in the rat model. Seventy-two hours following injection of a 10^7 inoculum of either the sucrose-grown wild-type (CH1) or the mutant (AMS12) cells the animals were sacrificed and their hearts removed and visually inspected for proper catheter placement across the aortic valve. In every case where catheter placement was correct, vegetations were observed. The number of animals that developed infective endocarditis as determined by positive vegetation cultures was similar in both the wild-type and mutant inoculated animals, 53.2% and

Table 1

Endocarditis cases in rats inoculated with *S. gordonii* CH1 or AMS12 [a]

Strain	Cases [b]	Total [c]	Percent infected
CH1	16	30	53.3
AMS12	18	32	56.2

[a] Animals were inoculated with 10^7 cells; [b] Number of animals with streptococci recovered from vegetation; [c] Surviving animals with proper catheter placement at autopsy.

56.2% respectively (Table 1). These data were analyzed using both the Test of Proportions and the Chi Square Test. Both statistical analyses indicated the differences between the CH1 and the AMS12 infection frequencies were not significant ($P > 0.01$ for the Test of Proportions and $P > 0.05$ for the Chi Square Test). Animals in both groups were inoculated either 24 or 48 h following catheterization. In order to determine if any difference existed within these groups, we compared the proportion of endocarditis cases among animals injected after 24 h to the proportion of endocarditis cases among those injected at 48 h. No significant difference was found (data not shown).

Discussion

Streptococci of the viridans group, including *S. gordonii*, are the leading cause of native valve endocarditis accounting for 45–65% of cases [23,24]. Earlier reports have suggested that glucan formation by viridans streptococci may enhance adherence to the damaged cardiac valvular endothelium in vitro and have a greater etiological association with endocarditis in vivo as compared to strains of streptococci that do not produce glucan [18,19]. Our application of allelic exchange mutagenesis has allowed the direct comparision of the contribution of sucrose-derived polymers in endocarditis infectivity using *S. gordonii* isogenic strains. Recently, Munro and Macrina [20] have reported that a non-polymer synthesizing, multiple mutant of *S. mutans* (devoid of all glucosyltransferase and fructosyltransferase activity) is significantly less infective in the rat endocarditis model as compared to wild-type, sucrose grown cells. In their study, the mutant constructed involved the inactivation of all four polymer synthesizing determinants (three GTFs and a single fructosyltransferase gene). These results prompted us to look in a similar manner at *S. gordonii* to evaluate specifically the role in virulence of sucrose-derived polymers in this species. Our rat endocarditis data clearly indicate no significant difference in virulence between the glucan negative mutant and the glucan positive wild-type *S. gordonii* strain (Table 1).

We conclude that generalizations about the role of sucrose-derived polymers in endocarditis infectivity are not possible as regards the viridans streptococci. Many of the oral viridans streptococci are able to synthesize glucans and, in some cases, fructans from sucrose. Our results taken together with those of Munro and Macrina [20], indicate that exopolymers vary in their contribution to endocarditis infectivity when comparing *S. mutans* and *S. gordonii*. Not surprisingly, this suggests a varied, multifactoral array of virulence factors are operative in *mutans*-initiated vs. *gordonii*-initiated lesions. Furthermore, it is important to note that single strain, isogenic mutant comparisons must be viewed with caution due to the heterogeneity of the virdans streptococci in general [25]. It is possible that the examination of several independent isolates must be performed in order to gain a more generalized picture of the factors important to infectivity. It is also important to note the previous *S. mutans* analyses involved a mutant defective in 4 different enzymes. It can be argued that the array of glucans and fructans made by wild-type *S. mutans* collectively contributes to infectivity in a way which is significantly different from the glucan produced by the single GTF of *S. gordonii*. The results reported here are important in that they provide new, comparative information, using a precisely-defined isogenic strain pair representing another oral species of *Streptococcus*. Data with *S. mutans* suggested that the sucrose-derived polymers contributed to endocarditis infectivity in two, non-mutually exclusive ways: promoting adherence to fibrin and interfering with phagocytic killing [20]. We infer from our infectivity studies

with *S. gordonii* CH1 and its glucan-defective mutant that fibrin adherence mechanisms and anti-phagocytosis strategies important in endocarditis are independent of sucrose-derived exopolymer formation in this strain.

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