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# Virulence of *Streptococcus mutans*: Biochemical and Pathogenic Characteristics of Mutant Isolates[1] (39064)

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Recent studies have clearly shown that dental caries is a multifactorial infectious disease in which a caries-promoting diet, a cariogenic microorganism and a susceptible host contribute major roles (1). The importance of a sugar substrate in caries promotion has been clearly demonstrated for oral cariogens, especially members of the viridans streptococci, most notably *Streptococcus mutans* (2–5).

The pathogenesis of *S. mutans* infection has been ascribed to the microorganism's ability to adhere to the tooth surfaces (6, 7) and to produce large amounts of lactic acid which results in demineralization of the tooth with subsequent decay (8, 9). These two characteristics are associated with the ability of *S. mutans* to utilize dietary sucrose (10–12). Furthermore, the ability of *S. mutans* to adhere has been correlated with the presence of the cell associated enzyme, dextran–sucrase, which synthesizes insoluble glucans which in turn, form an adherent mass on tooth enamel, thus contributing to plaque formation (11–15). Studies employing mutants of *S. mutans* which cannot form adherent masses on hard surfaces *in vitro* have been found to be avirulent in mono-infected gnotobiotic rats (16, 17).

This study was undertaken to determine the relationship between dextran–sucrase activity and adherence of *S. mutans* mutants with virulence of these isolates in gnotobiotic rats. A direct correlation between levels of these biochemical characteristics with *in vivo* virulence would allow more detailed investigations on the precise mechanism(s) of *S. mutans* pathogenesis.

*Materials and methods. Microorganisms.* Two strains of *S. mutans* were employed in this study, PS14 and 6715 which correspond to Bratthall's serologic groups c and d, respectively (18). Each strain was resistant to streptomycin (10 mg/ml), fermented mannitol and sorbitol and appeared as rough spheroid shaped colonies on Mitis-salivarius (MS) agar. Cell suspensions of each strain were cultured in the presence of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine at concentrations of 2, 5 and 10 g/ml medium (19). Several mutants from PS14 and 6715 were isolated which were identical to the parental strain with respect to streptomycin resistance (10 mg/ml), fermentation of mannitol and sorbitol and serological group specificity. Two mutants of *S. mutans* 6715, designated C211 and C229, resembled the parental strain morphologically following growth on MS agar, whereas *S. mutans* 6715 mutant C4 and *S. mutans* PS14 mutants B414 and B421 appeared as smooth lens shaped colonies on MS agar.

*Quantitation of dextran–sucrase activity and adherence to glass.* Dextran–sucrase activity was determined for each parental and mutant isolate by incubating the supernatant fluid of 18 hr glucose grown cultures (0.5–1.6 mg protein) with 675 μmoles sucrose, $2.8 \times 10^6$ cpm of [U-$^{14}$C] glucose labeled sucrose and 67.5 μmoles of potassium phosphate buffer, pH 6.8 (final volume, 4.5 ml). Following incubation of the reaction mixture for 6 hr at 37°, 2.2 ml was removed and placed in a boiling water bath for 5 min. The insoluble glucans in the heated reaction mixture were collected on Millipore filters (0.45 μm) and washed with four portions of water. The filters with the collected polysaccharides were assayed for radioactivity using Bray's scintillation fluid (20) in a

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Packard Scintillation Spectrophotometer (Model 3375).

The ability of each *S. mutans* isolate to adhere to glass was quantitated following growth in 10% sucrose medium. Cultures were incubated at 37° in preweighed test tubes for 24 hr. The growth medium and nonadherent material were carefully decanted and fresh sterile medium added to each tube followed by incubation at 37° for 24 hr.

After the fourth cultivation, the supernatant fluid was discarded and each tube filled gently with ethanol and washed three times. The adherent masses were dried *in vacuo* at 60–70° and the tubes weighed. The adherent mass represents the alcohol insoluble material which was determined by difference.

*Evaluation of bacterial cariogenicity in vivo.* The methods employed for *in vivo* determination of bacterial cariogenicity have been described previously (21–23). Briefly, 19 day old gnotobiotic rats were weaned, transferred to a new Trexler plastic isolator (24), orally swabbed with a log phase culture of the test *S. mutans* and fed a sterile caries-promoting diet (No. 305) containing 5% sucrose until day of sacrifice (21–23).

Colonization of the test *S. mutans* was confirmed the day after infection and monitored at weekly intervals until sacrifice (35 or 45 days of age) by culturing oral swabs of individual rats on MS agar. The *S. mutans* which was isolated at each sampling time always demonstrated morphologic and metabolic characteristics identical with the original test strain. Furthermore, no other bacteria or fungi could be detected in any of the experiments reported here.

In the first series of experiments, rats were monoinfected with either *S. mutans* 6715 wild-type (WT), mutant C4, C211 or C229. Two groups of rats were monoinfected with either *S. mutans* 6715 WT or mutant C4 and sacrificed at 45 days of age. Three additional groups of rats were moninfected with either *S. mutans* 6715 WT, mutants C211 or C229 and sacrificed at 35 days of age. At the termination of each experiment, rats were sacrificed with a guillotine and the mandibles dissected, cleaned and stained with 0.4% murexide in 70% alcohol. Following staining, the buccal and proximal surfaces of molars were scored for caries by the Keyes procedure (25). Differences among caries scores were evaluated by analysis of variance and multiple mean comparisons using the Duncan test (26).

In the second series of experiments, gnotobiotic rats were monoinfected with either *S. mutans* PS 14 wild type, mutant B414 or mutant B421. All rats were sacrificed at 45 days of age by cardiac exsanguination and the mandibles dissected. The right mandible was stained with safranin and the level of plaque on the molars determined by recording the stained regions with the aid of a dissecting microscope. The number of viable *S. mutans* was estimated from plaque collected from the left mandible into cold phosphate buffered-saline, pH 7.2. Each suspension was sonically-treated for 15 sec, diluted and plated on MS and blood agar. All plates were incubated at 37° for 24 hr under anaerobic conditions. Both mandibles were then stained with murexide and scored for caries as described above.

*Results. Dextran–sucrase activities, adherence and in vivo cariogenicity of S. mutans 6715 wild type and mutants.* As shown in Table I, *S. mutans* 6715 mutant C4 exhibited approximately 87% less dextran–sucrase activity and 90% less adherence than the parental strain. *In vivo* this mutant was less virulent than the wild type with 24% and 56% fewer enamel lesions and 44% and 71% fewer dentinal lesions on buccal and proximal smooth surfaces, respectively. These results suggest that a mutant of *S. mutans* which demonstrated less enzyme activity and adherence caused fewer smooth surface carious lesions than WT in young gnotobiotic rats fed a caries-promoting diet.

On the other hand, 1.2× and 1.1× higher dextran–sucrase activity and 1.6× and 2.2× more adherence were exhibited by mutants C211 and C229, respectively, when compared with WT (Table I). Furthermore, 35 day old gnotobiotic rats monoinfected with either mutant C211 or C229 had significantly more smooth surface lesions than animals infected with the parental strain. These results indicate that mutants of *S.*

TABLE I. BIOCHEMICAL CHARACTERISTICS OF *Streptococcus mutans* 6715 WILD TYPE AND MUTANTS C4, C211 AND C229 AND SMOOTH SURFACE LESIONS IN 35 OR 45 DAY OLD, MONOINFECTED GNOTOBIOTIC RATS.

| *S. mutans* strain | Age (days) | Percentage of wild type: Dextran-sucrase activity[a] | Adherence[b] | Smooth surface lesions[c]: Buccal Enamel | Buccal Dentin | Proximal Enamel | Proximal Dentin |
|---|---|---|---|---|---|---|---|
| 6715 Wild type | 45 | 100 (3.43) | 100 (15.8) | 100 (23.4 ± 0.6) | 100 (18.0 ± 0.8) | 100 (9.2 ± 0.5) | 100 (7.7 ± 0.5) |
| 6715 mutant C4 | 45 | 13.4 | 10.1 | 76.1 (17.8 ± 1.7) | 55.6 (10.0 ± 1.2) | 43.5 (4.0 ± 0.3) | 28.6 (2.2 ± 0.6) |
| 6715 Wild type | 35 | 100 (3.43) | 100 (15.8) | 100 (15.3 ± 0.7) | 100 (10.3 ± 0.9) | 100 (3.5 ± 0.4) | 100 (2.9 ± 0.5) |
| 6715 mutant C211 | 35 | 121 | 160 | 134 (20.5 ± 0.9) | 172 (17.7 ± 1.0) | 194 (6.8 ± 0.5) | 234 (6.8 ± 0.5) |
| 6715 mutant C229 | 35 | 114 | 222 | 120 (18.3 ± 2.2) | 112 (11.5 ± 1.9) | 171 (6.0 ± 0.7) | 207 (6.0 ± 0.7) |

[a] Based on μmoles of glucosyl moieties of sucrose incorporated into insoluble glucan per mg protein. Actual value for wild type shown in parenthesis.

[b] Based on mg of mass adhering to glass surface. Actual value for wild type shown in parenthesis.

[c] Evaluated by the Keyes procedure (25). Means for 8–16 rats per group used for each test strain. All mutant values were significantly different from the wild type at $P \leq 0.01$. Actual value ± standard error shown in parentheses.

TABLE II. BIOCHEMICAL CHARACTERISTICS OF *Streptococcus mutans* PS14 WILD TYPE AND MUTANTS B414 AND B421 AND LEVELS OF PLAQUE, VIABLE *S. mutans* IN PLAQUE AND SMOOTH SURFACE LESIONS IN 45 DAY OLD, MONOINFECTED GNOTOBIOTIC RATS.

| *S. mutans* strain | Percentage of wild type: Dextran-sucrase activity[a] | Adherence[b] | Plaque | Smooth surface lesions[c]: Buccal Enamel | Buccal Dentin | Proximal Enamel | Proximal Dentin | Total viable *S. mutans* |
|---|---|---|---|---|---|---|---|---|
| PS14 wild type | 100 (6.34) | 100 (35.2) | 100 (60.7) | 100 (24.6 ± 0.6) | 100 (22.7 ± 0.6) | 100 (8.0 ± 0.0) | 100 (8.0 ± 0.0) | $9.56 \times 10^7$ |
| PS14 mutant B414 | 13.9 | 22.4 | 55.2 | 65.9 (16.2 ± 0.9) | 59.5 (13.5 ± 1.2) | 38.8 (3.1 ± 0.3) | 32.5 (2.6 ± 0.3) | $4.42 \times 10^6$ |
| PS14 mutant B421 | 43.8 | 14.5 | 47.4 | 30.9 (7.6 ± 0.6) | 14.1 (3.2 ± 0.6) | 18.8 (1.5 ± 0.3) | 12.5 (1.0 ± 0.3) | $4.30 \times 10^6$ |

[a] Based on μmoles of glucosyl moieties of sucrose incorporated into insoluble glucan per mg protein. Actual value for wild type shown in parenthesis.

[b] Based on mg of mass adhering to glass surface. Actual value for wild type shown in parenthesis.

[c] Evaluated by the Keyes procedure (25). Means for 8–16 rats per group used for each test strain. All mutant values were significantly different from the wild type at $P \leq 0.01$. Actual value ± standard error shown in parentheses.

*mutans* exhibiting increased adherence, which could be attributed to increased insoluble glucan synthesis, are more virulent in young gnotobiotic rats than the parental strain. In addition, these findings suggest that adherence, including dextran–sucrase directed insoluble glucan formation, is directly correlated with *in vivo* virulence as suggested by the results using mutants C211 and C229, since both mutants exhibit greater activity and increased virulence than the parental strain, whereas, mutant C4 exhibits less activity and is lowly virulent.

*Dextran-sucrase activities, adherence and in vivo levels of plaque and viable S. mutans in plaque and cariogenicity of S. mutans PS*

*14 wild type and mutants.* In the course of this study, the direct correlation of biochemical characteristics with *in vivo* virulence suggested that differences may exist in the amount of plaque and the number of viable *S. mutans* present in plaque on the molars of rats monoinfected with different mutants of *S. mutans*. As shown in Table II, *S. mutans* PS14 mutant B414 exhibited approximately 80% less dextran-sucrase activity and adherence than the parental strain. Mutant B421 demonstrated 56% less dextran–sucrase activity and 85% less adherence than the parental strain. Rats monoinfected with either mutant B414 or B421 had approximately 50% less plaque, greater than 10-fold less viable *S. mutans* in plaque, and significantly fewer smooth surface lesions than rats monoinfected with the parental strain. Furthermore, mutant B421 exhibited significantly less adherence to glass and *in vivo* virulence than mutant B414. These results suggest that the level of plaque and the number of viable *S. mutans* present in plaque are directly related to degree of carious lesions observed in monoinfected gnotobiotic rats.

*Discussion.* Although previous reports support the hypothesis that two important factors in virulence of *S. mutans* are the ability of this microorganism to produce insoluble glucans (11, 12, 16, 17) and to adhere to hard surfaces (2, 13–17), a correlation has not been clearly documented between quantitative differences in dextran-sucrase activity and adherence of *S. mutans* isolates with *in vivo* cariogenicity. This study reports the isolation of several mutants of *S. mutans* 6715 and PS14 which were identical to WT in antibiotic resistance, sugar fermentation and serological characteristics. However, these mutants differed from the parental strain in dextran-sucrase activity, adherence and virulence in gnotobiotic rats.

Our *in vivo* method for determining virulence of *S. mutans* employs young gnotobiotic rats fed a purified caries-promoting diet (No. 305) which contains only 5% sucrose. This model has been shown to be very sensitive and reproducible in determining the cariogenicity of various *S. mutans* strains (21–23) and has allowed differentiation of *S. mutans* isolates which exhibit higher or lower virulence. When young gnotobiotic rats were monoinfected with *S. mutans* 6715 mutant C4, which exhibited decreased activity for insoluble glucan synthesis and subsequently less adherence, significantly fewer smooth surface lesions were observed. This finding was in agreement with the reports of others (16, 17) which demonstrated that mutants of *S. mutans* that could not form adherent masses on hard surfaces *in vitro* were avirulent in monoinfected gnotobiotic rats. This would suggest that isolates of *S. mutans* with less dextran–sucrase activity and adherence than WT are less virulent.

On the other hand, *S. mutans* 6715 mutants C211 and C229 exhibited more dextran-sucrase activity and were more adherent than the parental strain and caused more smooth surface carious lesions in gnotobiotic rats. These findings lend experimental support to the suggestions of others (2, 11–17) that two important factors in *S. mutans* virulence are the ability of this microorganism to synthesize insoluble glucans and to adhere to hard surfaces. This study would suggest a direct correlation between *S. mutans* pathogenesis in gnotobiotic rats and the ability of the organism to synthesize insoluble glucan and to adhere.

When gnotobiotic rats were monoinfected with either *S. mutans* PS14 mutant B414 or B421, significantly less plaque and viable *S. mutans* were observed when compared with the parental strain. These mutants also exhibited less adherence and caused significantly fewer carious lesions in monoinfected gnotobiotic rats when compared with wild type. However, it should be stressed that other, yet unknown, factors probably contribute to *S. mutans* pathogenesis.

These findings have significance in that; (a) mutants of *S. mutans* have been isolated which are either more or less virulent than parental strains, (b) a correlation has been demonstrated between *S. mutans* pathogenesis in gnotobiotic rats, adherence and insoluble glucan synthesis and (c) these mutants, with varying caries potential, will allow further investigation in more

detail of the precise mechanism(s) in *S. mutans* pathogenesis.

*Summary.* The *in vitro* dextran-sucrase activities and adherence to glass of *S. mutans* 6715 and PS14 wild types and mutants were quantitated and compared with their *in vivo* cariogenicity in young, gnotobiotic rats. In general, *S. mutans* PS14 mutants B414 and B421 and 6715 mutant C4 demonstrated less dextran-sucrase activity and adherence than parental strains and caused fewer carious lesions in gnotobiotic rats. Rats monoinfected with either PS14 mutants B414 or B421 had less plaque and viable *S. mutans* in plaque than rats infected with parental strain. Both *S. mutans* 6715 mutants C211 and C229, demonstrated greater enzyme activity and adherence than the parental strain and produced more carious lesions.

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