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In Vitro and In Vivo Complementation of *Streptococcus mutans* Mutants Defective in Adherence

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Previous studies have shown that adherence-defective mutants of *Streptococcus mutans* PS14, serotype *c*, can be grouped into several different phenotypic groups. In this study a method was developed to test for complementation between pairs of nonadhering mutants which possess different genotypic defects. Mutant strains UAB95 and a spectinomycin-resistant derivative of UAB95 (UAB516) were found to exhibit increased levels of adherence when grown together with UAB230 in media containing sucrose as compared to the adherence of each strain grown separately. An increase in caries was also observed in gnotobiotic rats mixedly infected with the two mutants as compared to either strain alone. Tests revealed that UAB95 produced more water-insoluble glucan than its parent strain but had a defect in glucan binding. UAB230 was found to produce levels of a defective glucan that could not be bound by mutant or wild-type cells. Our results suggest that UAB95 produces a water-insoluble glucan which is bound by UAB230, thus allowing complementation for adherence and caries production.

Streptococcus mutans is indigenous to the oral cavity and is considered to be the principal causative bacterial agent of dental caries in humans (15) and experimental animals (9, 20). Various characteristics of *S. mutans* have been implicated in the virulence of this organism in inducing carious lesions in humans (14, 15) and experimental animals (6, 13, 18). These virulence characteristics of *S. mutans* include the ability of this microorganism to adhere to the tooth surface (3, 4, 25, 27, 28), produce insoluble glucans when sucrose serves as a substrate, which results in the subsequent formation of plaque (7–9, 21, 22), and produce acid (2, 25, 29, 30), which causes demineralization of the tooth and leads to the carious lesion. Evidence for the importance of these characteristics in the virulence of *S. mutans* is supported by the decrease in caries produced by mutants of *S. mutans* which are deficient in one or more of these characteristics (5, 9–11, 20). Since the numbers and types of genes involved in these processes are still unknown, we began to examine the ability of mutants to complement each other for specific defects. In this regard, we initially used avirulent *S. mutans* mutants of divergent phenotypes and showed that the mutants C4, defective in adherence (20), and UAB165, defective in aggregation (23), were able to complement in vivo (11).

We have previously isolated and characterized mutants of *S. mutans* for their inability to adhere or aggregate, and we were able to divide them into 13 different phenotypic groups (23). Since mutations in different genes might give rise to the same phenotype with regard to a given step or process that is necessary for cariogenicity, we reasoned that one might obtain complementation between two mutants of similar phenotype but with different genotypic defects. Traditionally, complementation requires a mechanism for gene transfer and the establishment of a partially diploid state. Although such classical systems of genetic analysis do not yet exist in most strains of *S. mutans*, including UAB62 and UAB66, most virulence attributes are expressed on the *S. mutans* cell surface; consequently, complementation could be evaluated by mixed cultivation of mutants of these strains.

In the present paper, we report an in vitro method which allowed testing of adherence-defective mutants of *S. mutans* for their ability to complement during mixed growth and result in adherence. One pair of nonadhering mutants found to complement for adherence in vitro was also found to complement for virulence in vivo by mixed infection of gnotobiotic rats. A possi-

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TABLE 1. Bacterial strains employed in this study

Strain	Description[a]
UAB62	Serotype *c*, strain PS14; Str^r Rif^r
UAB95	Adh^- mutant of UAB62; rough colony morphology on MS agar
UAB102	Adh^- Agg^- mutant of UAB62
UAB230	Adh^- mutant of UAB62; smooth colony morphology on MS agar
UAB516[b]	Spc^r mutant of UAB95
UAB418	Isolate from gnotobiotic rats monoassociated with UAB230 which retains UAB230 characteristics[c]
UAB534	Isolate from gnotobiotic rats monoassociated with UAB95 Spc^r which retains UAB95 Spc^r characteristics[c]
UAB545	Isolate from gnotobiotic rats diassociated with UAB230 and UAB95 Spc^r which retains UAB230 characteristics[c]
UAB547	Isolate from gnotobiotic rats diassociated with UAB230 and UAB95 Spc^r which retains UAB95 Spc^r characteristics[c]
UAB66	Serotype g, strain 6715; Str^r Spc^r
UAB108	Adh^- Dex^- mutant of UAB66
UAB122	Dex^- mutant of UAB66
UAB245	Adh^- Dex^- mutant of UAB66

[a] Str^r, streptomycin resistant; Rif^r, rifampin resistant; Spc^r, spectinomycin resistant; Adh^-, adherence defective; Agg^- aggregation defective; Dex^-, dextranase defective.

[b] Isolated as a spontaneous resistant colony on BHI agar containing 1 mg of spectinomycin per ml.

[c] Isolates from each animal experiment were tested for antibiotic resistance markers, fermentation patterns, adherence, aggregation, and dextranase production as previously described (23), and representative isolates were stocked.

ble mechanism involved in complementation of these mutants is also presented.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Wild-type *S. mutans* PS14 (UAB62), 6715 (UAB66), and adherence-defective (Adh^-) mutants (23) used in this study are listed in Table 1. UAB95 and UAB230 were Adh^- mutants of UAB62. UAB516 was isolated from the Adh^- mutant UAB95 as a spontaneous spectinomycin-resistant isolate. We refer to UAB516 as UAB95 Spc^r throughout the text, however, to simplify and thus clarify the presentation and discussion of data. UAB418, UAB534, UAB545, and UAB547 were isolates from gnotobiotic rats infected with UAB230 or UAB95 Spc^r or with both mutant strains. All strains were maintained at −70°C in 1% peptone (Difco Laboratories, Detroit, Mich.) and 5% glycerol and at 4°C on brain heart infusion (BHI) agar (Difco) slants. Slants were transferred monthly.

Strains were cultured at 37°C in BHI broth (Difco) or partially defined medium (12) containing either 1% sucrose (PD-sucrose) or 1% glucose (PD-glucose). Colony morphology characteristics were examined after 48 h of anaerobic incubation (GasPak anaerobe system with an H_2-CO_2 atmosphere; BBL Microbiology Systems, Cockeysville, Md.) at 37°C on mitis salivarius (MS) agar (Difco) and BHI agar.

Adherence of growing cells. Microtiter plates (Costar; 96-well, flat-bottom plates) containing 300 μl of PD-sucrose medium per well were inoculated with 10 μl of overnight BHI broth cultures of each mutant individually and of all possible combinations of mutant pairs. The plates were incubated standing for 24 h at 37°C. The growth medium was then poured off, and the microtiter plates were washed with water to remove nonadhering cells (adherent cells of wild-type strains are not removed by this procedure). A crystal violet solution (1%) was added to stained cells which had adhered to the bottoms of the wells. The wells were washed with water, dried, and photographed with an Olympus OM10 35-mm camera to accurately record the differences in the amounts of adhered cells. The plates were then scored by assigning a value of + or − to each well based on the amount of stain retained.

Disposable borosilicate test tubes (13 by 100 mm) containing 3 ml of PD-sucrose medium were inoculated with 100 μl of overnight BHI broth cultures of selected mutants individually or in pairwise combinations. The tubes were incubated standing at 37°C for 24 h. Each culture was gently vortexed to remove nonadhering cells, the growth medium was then poured off (adherent cells of wild-type strains are not removed by this procedure), and tubes were stained with crystal violet, washed with ethanol, and dried. The tubes were then scored on a scale of 0 to 4+ for adherence (23).

Quantitation of cells in adherent mass. Disposable borosilicate test tubes containing 3 ml of PD-sucrose medium were inoculated with 100 μl of overnight BHI broth cultures of UAB230 and UAB95 Spc^r separately or together; UAB62 was inoculated separately as the wild-type adherence control. The tubes were incubated standing at 37°C for 24 h. After each culture was gently vortexed, the culture fluids were poured off, sonicated tor 30 s to disperse clumps of cells to short chains which averaged one to three cells in length (dispersal of clumps was confirmed by microscopic examination), diluted in BSG (buffered saline with gelatin; 23), and plated on MS agar. Three milliliters of BSG was added to the test tubes in which the cultures were grown, and the adherent mass was suspended with a glass pipet. After 30 s of sonication to break up clumps, the cell suspensions were diluted and plated on MS agar. After incubation, the plates were counted, and the numbers of UAB95 Spc^r (rough morphology) and UAB230 (smooth morphology) colonies in the culture fractions were determined.

In vivo gnotobiotic rat studies. The virulence of *S. mutans* strains reported here was determined in the young gnotobiotic rat model (18). Weanling germfree Fischer CD F (344) GN rats (age, 20 days) were transferred to experimental Trexler plastic isolators and challenged with an 18-h culture of each strain (UAB62, UAB230, or UAB95 Spc^r) or a mixture of UAB230 and UAB95 Spc^r consisting of an equal number of CFU. Rats were provided sterile diet 305 (18) and water ad libitum throughout the experiment. Two days after infection, colonization was assessed by culturing oral swab samples collected on MS agar. Experiments were terminated when rats reached 45 days of age, and the proportions of bacteria in plaque were assessed (19). UAB230 and UAB95 Spc^r were easily distinguished on MS agar due to colony morphology differences and could also be distinguished by comparing titers on BHI agar (UAB230 plus UAB95 Spc^r) and on BHI agar containing 500 μg of spectinomycin per ml (UAB95 Spc^r alone). Random isolates from rats were cultured and tested for original mutant characteristics as described previously (23). Caries activity was determined on mandibular molars of individual animals (18, 20).

Determination of cell-associated and extracellular water-insoluble glucan production. One liter of PD-glucose medium was inoculated and incubated at 37°C for 20 h. Cultures were centrifuged for 30 min at 8,000 rpm in a Sorvall refrigerated centrifuge with a GSA rotor. The pelleted cells were washed three times with water and suspended in 0.04 M potassium phosphate buffer (pH 6.8) to an absorbance of 1.0 at 540 nm. This suspension was centrifuged and suspended in 10% of the original volume in the same buffer. The culture supernatant fluids were precipitated with ammonium sulfate (70% of saturation). The precipitate was dialyzed against 0.05 M potassium phosphate buffer (pH 6.8), lyophilized, and suspended in 2 ml of 0.05 M potassium phosphate buffer (pH 6.8). These samples were used as the cell-associated and extracellular glucosyltransferase (GTF) preparations, respectively. The total amount of protein contained in each sample was measured by the method of Lowry (16), with bovine serum albumin as the standard.

Cell-associated GTF activity or extracellular GTF activity was determined by incubating either the washed *S. mutans* cells or the GTF preparation in 0.05 M sodium acetate buffer (pH 5.5)–0.1 μCi of glucose-[*U*-^{14}C]sucrose–0.01 M sucrose in a total volume of 0.5 ml for 1 h at 37°C. The activities for water-insoluble and water-soluble glucan production by cell-associated and extracellular GTF preparations were determined as previously described (12, 24). One unit of GTF activity is that amount of enzyme which catalyzes the incorporation of 1 μmol of glucose moiety of sucrose into water-insoluble glucan per h (24).

Adherence due to glucan binding. The adherence assay used was a modification of the procedure described by Schachtele et al. (27). Cells were grown in 25 ml of PD-glucose medium containing 2.5 μCi of L-[4,5-^{3}H]leucine per ml at 37°C for 20 h. The cells were harvested by centrifugation and washed three times with distilled water. The cells were treated at 100°C for 30 min and washed two times with 0.05 M potassium phosphate buffer (pH 6.8), and the absorbance of the cell suspension was adjusted to 1.0 at 660 nm. The assay mixture, containing 0.2 ml of labeled cells (UAB62, UAB230, or UAB95 Spc^r), 0.2 mg of sodium Merthiolate, 10 mg of sucrose, 1.0 U of water-insoluble glucan-synthesizing activity of the extracellular GTF preparation (UAB62, UAB230, or UAB95 Spc^r), and 0.01 M potassium phosphate buffer (pH 6.8) to give a total volume of 0.5 ml, was incubated for 18 h at 37°C at a 25° incline in 4-ml-volume screw-cap glass vials (Fisher Scientific Co., Pittsburgh, Pa.). The nonadhering cells were removed by aspiration, and the vials were washed three times with 0.05 M potassium phosphate buffer (pH 6.8) and dried by overnight incubation at 37°C. The vials were counted in a Packard Tri-Carb scintillation spectrometer, with Bray scintillation fluid (1) to determine the amount of cells adhering to the glass surfaces.

RESULTS

Screening of mutants capable of complementation for adherence. To determine whether wild-type virulence phenotypes were restored when nonadhering mutants (23) were grown pairwise in vitro, a rapid screening method using microtiter plates was developed to select possible complementing mutant pairs. Several different brands of microtiter plates were tested for efficacy of adherence and ease of scoring. We found that Costar plates allowed adherence similar to that obtained with glass test tubes. Nonadhering mutants previously obtained by us (23) were tested for complementation in all possible combinations. As seen in Fig. 1, complementation for adherence was observed between mutant pairs UAB95 and UAB102, UAB95 and UAB230, and UAB230 and UAB102, but the amount of adherence was not as great as that observed for control strains, wild-type UAB62 and UAB66, and mutant UAB122. In addition, some inhibition of the wild-type level of adherence was observed when UAB62, UAB66, or UAB122 was grown together with UAB108 or UAB245. This phenomenon will be the subject of a future publication (T. Shiota et al., manuscript in preparation). Mutant pairs which showed increases in adherence were also tested for complementation in test tubes to rule out artifactual increases in adherence by using the microtiter plates and to also give a better indication of the relative amounts of adherence expressed by complementing pairs. As shown for UAB95 and UAB230 in Fig. 2, a pattern of complementation was observed which was similar to that observed in the microtiter plate assay.

Quantitative complementation analysis. To test for complementation of UAB95 and UAB230 quantitatively, it was necessary to add an additional antibiotic resistance marker to one strain to aid in reisolation of each strain after mixed cultivation. UAB516 was isolated as a spontaneous spectinomycin-resistant derivative of UAB95 which retained all other characteristics

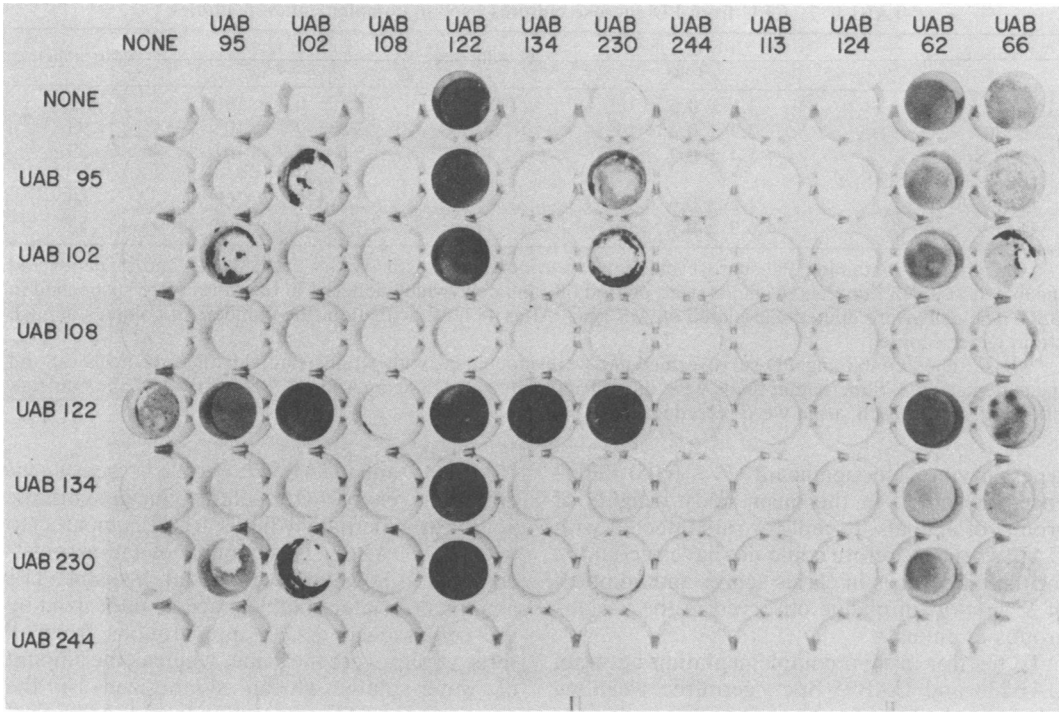

FIG. 1. Microtiter wells containing 300 μl of PD-sucrose medium were inoculated with 10 μl of overnight BHI broth cultures individually and in pairs, incubated overnight at 37°C, washed to remove nonadhering cells, and stained with a 1% crystal violet solution.

of UAB95 (23). Quantitative measures for the level and type of complementation which occurred were made (Table 2). As expected, control cultures indicated that both UAB230 and UAB95 Spc^r were much less able to adhere than wild-type UAB62. When UAB95 Spc^r and UAB230 were grown together, however, the percentage of UAB230 cells which adhered to the glass surface increased from 7 to 40%, whereas the percentage of UAB95 Spc^r cells which adhered remained nearly the same. The increase in the total percentage of cells found in the adherent mass was less than that of UAB62 but sufficient to suggest complementation of UAB230 by UAB95 Spc^r in growing cultures.

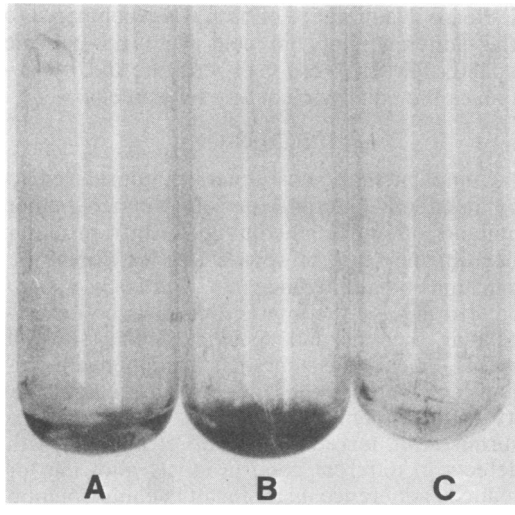

FIG. 2. Adherence of (a) UAB95, (b) UAB95 and UAB230, and (c) UAB230 grown in glass tubes containing PD-sucrose medium (see text).

In vivo complementation analysis. Gnotobiotic rats monoinfected with either UAB95, UAB230, or UAB95 Spc^r exhibited significantly ($P \leq 0.01$) fewer carious lesions on buccal and sulcal surfaces than observed in rats infected with UAB62 (Table 3). Essentially no caries activity was observed on proximal molar surfaces of rats infected with UAB62, UAB95, or UAB230, whereas a low level of caries activity was noted in rats infected with UAB95 Spc^r. Rats infected with the mutant strains had lower numbers of recoverable *S. mutans* in mandibular molar plaque than obtained from rats infected with UAB62. Colonies from each experimental group of animals were isolated and characterized (23). No differences were noted in the biochemical characteristics of animal isolates from those of the infecting microorganism; therefore, the strains appeared to stably maintain their pheno-

TABLE 2. CFU from PD-sucrose cultures used in complementation studies

Strain	CFU of adhered cells[a]	% Adhering	CFU of nonadhered cells[b]	% Nonadhering
UAB62	$3.1 \pm 0.6 \times 10^8$	88	$4.1 \pm 0.4 \times 10^7$	12
UAB230	$2.9 \pm 0.2 \times 10^7$	7	$3.8 \pm 0.2 \times 10^8$	93
UAB95 Spc^r	$5.4 \pm 0.9 \times 10^7$	13	$3.6 \pm 0.7 \times 10^8$	87
UAB230 + UAB95 Spc^r				
UAB230	$1.5 \pm 0.6 \times 10^8$	40	$2.2 \pm 0.6 \times 10^8$	60
UAB95 Spc^r	$6.9 \pm 0.7 \times 10^7$	17	$3.4 \pm 0.9 \times 10^8$	83

[a] Glass tubes containing PD-sucrose medium were inoculated with strains (individually and in pairs) and incubated at 37°C. Supernatant fluids were poured off, and cells which adhered to the tubes were suspended in BSG. The cells were diluted and plated on MS agar. After 48 h of incubation, the numbers of colonies of each strain were recorded.

[b] Glass tubes containing PD-sucrose medium were inoculated with strains (individually and in pairs) and incubated at 37°C. Supernatant fluids were diluted and plated on MS agar. After 48 h of incubation, the numbers of colonies from each strain were recorded.

types in vivo. No significant ($P \leq 0.05$) difference was noted in the mean body weights of groups of animals, except for rats infected with UAB95, and therefore could not have accounted for the differences in caries scores and numbers of *S. mutans* in plaque observed in the various groups of animals.

To test for in vivo complementation between UAB230 and UAB95 Spc^r, germfree weanling rats were challenged with an inoculating suspension containing approximately equal numbers of CFU of each strain. Rats diassociated with the two mutants exhibited significantly ($P \leq 0.01$) higher caries activity on buccal and proximal molar surfaces (but not on sulcal surfaces) than observed in rats monoassociated with either mutant strain (Table 3). Of interest was the finding that rats diassociated with UAB230 and UAB95 Spc^r had total caries activity which was similar to that seen in rats monoassociated with wild-type UAB62 (although the caries scores were the same, lower, and higher in the mixed infections compared to the wild-type infections for the buccal, sulcal, and proximal surfaces, respectively). Furthermore, UAB230 cells were found in higher number than UAB95 Spc^r cells on mandibular molars of rats diassociated with the two mutants, although the total number of organisms recovered was lower than that observed in rats monoinfected with UAB230, UAB95 Spc^r, or UAB62. These results indicate that these nonadhering mutants complemented each other in vivo and are more cariogenic than the wild-type organism.

GTF activity and glucan binding. To determine the mechanism of complementation for adherence by UAB230 and UAB95 Spc^r, a comparison of water-insoluble glucan synthesis by UAB230 and UAB95 Spc^r was made. The results shown in Table 4 indicate that the water-insoluble glucan synthesis abilities of UAB230, UAB418, and UAB545 were lower than those of wild-type UAB62. However, UAB95 Spc^r, UAB534, and UAB547 showed greatly increased levels of water-soluble glucan synthesis ability in supernatant fluids when compared to wild-type UAB62. The amounts of cell-associated activity, however, were about the same. The amounts of water-soluble glucan synthesized by the cell-associated GTF preparations from all three strains were the same, whereas the amount of water-soluble glucan synthesized by the extracellular GTF preparation from UAB95 Spc^r was about 25% of the amount synthesized by the UAB62 and UAB230 preparations (data not shown).

Based on the assumption that adherence of cells requires both the appropriate cell surface receptor to bind glucan and production of a glucan which can bind to the cell or glass surface, an adherence assay of heat-killed cells to glass surfaces was performed (Table 5). When UAB62 or UAB230 cells were incubated with the UAB95 Spc^r GTF enzyme, there was an increase in adherence of both when compared to the adherence of the cells with no enzyme added. The UAB230 GTF enzyme did not increase the adherence of any tested cells.

DISCUSSION

Complementation is generally considered to be an effective method for determining whether mutants of similar phenotype arise from mutations in the same or separate genes. Since the mechanism of adherence in *S. mutans* is considered to involve the interaction of several extracellular proteins and specific binding sites, it seems logical to assume that a number of mutations in different genes could result in an inability of the organism to adhere. One might also surmise that mixed cultivation of mutants with defects in different gene products allowing for reduced adherence might result in complementation for adherence in vitro and in vivo.

The results presented show that mutants which complement each other for adherence

during mixed cultivation can be identified and the process by which complementation occurs can be explained biochemically. When UAB230 was grown in a sucrose-containing medium, the percentage of adherent UAB230 cells increased from 7 to 40% when UAB95 Spc^r cells were added to the culture, whereas the level of UAB95 Spc^r remained the same (Table 2). In addition, when gnotobiotic rats were diassociated with the complementing pair, UAB230 was found in higher numbers than UAB95 Spc^r in saliva and fecal samples (data not shown), as well as on mandibular surfaces, and wild-type caries activity was restored (Table 3). Thus, in all cases of mixed cultivation, UAB95 Spc^r provided a more favorable environment for the adherence of UAB230.

As stated above, UAB95 Spc^r was isolated from UAB95 and was shown to be identical to UAB95 for all in vitro tests except for its resistance to spectinomycin. However, in vivo, the proximal caries scores were significantly higher for UAB95 Spc^r than for either UAB95 or wild-type UAB62. Furthermore, an additional increase in proximal lesions was seen in mixed cultivation of the complementing pair (Table 3). Unfortunately, there is not as yet any quantitative method for analyzing the number of bacteria adhered to proximal surfaces alone, and it was unlikely that sonication of the entire mandible would result in removal of all adhered bacteria between individual molars. Thus, the values for numbers of bacteria per mandible in Table 3 most likely represent an overview of the ratio of UAB230 to UAB95 Spc^r which adhered during mixed infection. If the trend toward higher numbers of UAB230 adhered relative to numbers of UAB95 Spc^r adhered were maintained on the proximal surface, then the additional increase in proximal caries seen in mixed infection was probably the result of increased adherence of UAB230 relative to UAB95 Spc^r proximally, as indicated from our in vitro studies, rather than of an additional increase in numbers of UAB95 Spc^r on those surfaces.

Proximal surfaces (those between the teeth) account for only a small percentage of the total caries seen, and the level of activity was still low when compared with that induced by a highly virulent strain of *S. mutans* (18). Nevertheless, these surfaces probably provide a unique environment with regard to the availability of nutrients and oxygen; thus, these scores might reflect some unique characteristic of UAB95 Spc^r for colonization of this tooth surface. Ultimately, we hope to resolve these questions by examining optimum growth conditions for UAB95 Spc^r and by developing a method of accurately determining relative numbers of bacteria adhered to proximal surfaces.

TABLE 3. Virulence of *S. mutans* PS14 and selected mutant isolates in gnotobiotic rats[a]

S. mutans PS14 strain	No. of rats per group	Mean caries scores[b]						Mean body wt (g)	Mean no. of *S. mutans* CFU per mandible[c] ($\times 10^6$)
		Buccal		Sulcal		Proximal			
		Enamel	Dentinal slight	Dentinal slight	Dentinal extensive	Enamel	Dentinal slight		
UAB62	18	17.8 ± 1.3	12.6 ± 0.9	14.5 ± 0.2	4.8 ± 0.4	0.2 ± 0.2	0.0	102 ± 3.5	4.5
UAB95	14	13.7 ± 0.6	8.1 ± 0.6	10.4 ± 0.4	1.0 ± 0.3	0.1 ± 0.1	0.0	68.7 ± 10.5	2.3
UAB95 Spc^r	16	11.6 ± 0.6	8.0 ± 0.5	11.7 ± 0.2	0.5 ± 0.2	1.3 ± 0.5	0.1 ± 0.1	89.5 ± 3.7	2.9
UAB230	22	9.1 ± 0.4	8.1 ± 0.5	12.1 ± 0.2	1.9 ± 0.7	0.0	0.0	107.6 ± 8.7	1.4
UAB95 Spc^r + UAB230	25	17.3 ± 1.4	12.8 ± 0.8	12.6 ± 0.2	2.2 ± 0.4	2.8 ± 0.4	1.0 ± 0.3	93.6 ± 3.3	0.4 (UAB95 Spc^r) 0.8 (UAB230)

[a] Weanling germfree rats (age, 20 days) were infected with an overnight log-phase culture of the test microorganisms (s); for mixed infection, approximately equal numbers of CFU were combined: UAB95 Spc^r = 15.9×10^8 CFU + UAB230 = 15.8×10^8 CFU in the inoculating suspension. Rats were sacrificed at 45 days of age for assessment of complementation and virulence.

[b] Values are the means ± standard error of the mean as determined by the method of Keyes, as previously described (17, 18).

[c] Determined on MS and MS + spectinomycin agar. Values are the mean number of *S. mutans* CFU per mandible per rat per group.

TABLE 4. Water-insoluble GTF activity[a]

Strain	Extracellular GTF[b] (μmol/mg of protein per h)	Cell-associated GTF[c] (μmol/mg of protein per h)
UAB62	7.4 ± 0.8	9.4 ± 0.9
UAB230	1.7 ± 0.9	4.4 ± 0.2
UAB418	2.4 ± 0.4	5.4 ± 0.5
UAB545	2.4 ± 0.1	5.6 ± 0.4
UAB95 Spc^r	112.5 ± 6	10.1 ± 1.5
UAB534	129 ± 1	10.4 ± 1.5
UAB547	132 ± 9	9.4 ± 1.1

[a] Assays were performed twice with duplicate samples for each experiment.
[b] Synthesis of water-insoluble glucan by concentrated culture supernatant fluids (see text).
[c] Synthesis of water-insoluble glucan by washed cells grown in media containing glucose (see text).

The increase in caries on buccal and proximal surfaces in rats infected with both mutant strains over that observed in animals monoinfected with either mutant strain clearly shows that these mutants complemented each other for increased virulence in vivo. Furthermore, the twofold higher number of UAB230 over UAB95 Spc^r that was observed was in agreement with the observation made in vitro.

The possible mechanism by which UAB95 Spc^r complemented UAB230 for adherence was more clearly elucidated by examining the levels of extracellular and cell-associated GTF that synthesizes insoluble glucan for each strain. Assays for extracellular and cell-associated GTF activities showed that UAB95 Spc^r produced GTF that synthesized extremely high levels of water-insoluble glucan and that most of this enzyme activity was in the extracellular fraction (Table 4). UAB230, however, produced less GTF that synthesized water-insoluble glucan than wild-type UAB62, and most of the activity was cell associated. These data suggested that UAB95 Spc^r might have produced a glucan which allowed for the increased adherence of UAB230 and that UAB95 Spc^r was unable to efficiently bind the glucan formed, since the level of cell-associated GTF in UAB95 Spc^r was similar to that for UAB62.

Additional data from glucan-binding experiments (Table 5) supported these hypotheses. If glucan is bound to the cell surface by the GTF enzyme itself or by some other cell surface receptor which specifically binds insoluble glucan, then a mutation which affects the ability of either to bind to the glucan produced might also result in an inability of UAB95 Spc^r to efficiently adhere to smooth surfaces. This otherwise normal glucan, then, should still be able to bind to normal GTF or glucan-binding proteins present on other cell surfaces and ultimately lead to adherence. As seen in Table 5, GTF from UAB95 Spc^r produced an insoluble glucan which allowed for the adherence of both UAB230 and UAB62 heat-killed cells. The results obtained for UAB230, on the other hand, suggested that this mutant could adhere only in the presence of glucan produced by either UAB62 or UAB95 Spc^r. Its own glucan could not initiate the adherence of any strain, suggesting a defect in the glucan itself. Thus, whereas UAB95 Spc^r probably produced a normal glucan which could be bound by cells of UAB230, allowing for adherence of UAB230, neither the defective glucan produced by the UAB230 GTF preparation nor normal glucan produced by the UAB62 GTF preparations could complement for adherence of UAB95 Spc^r.

Hamada and Slade suggest with their model for adherence of *S. mutans* cells that GTF binds to cellular serotype-specific polysaccharides and cell-bound glucans and, in the presence of sucrose, synthesizes water-insoluble glucans which facilitate adherence (8). Additionally, there is evidence that a cell surface receptor which is a glucan-binding protein is present in *S. mutans* strains, and this protein may be involved in adherence (17, 26). Based on the GTF assays, glucan-binding experiments, and these models,

TABLE 5. Adherence of cells due to binding of in vitro-synthesized glucan

Source of GTF added	Adhering ^{3}H-labeled, heat-killed cells[a]					
	UAB62		UAB230		UAB95 Spc^r	
	dpm	Relative adherence[b]	dpm	Relative adherence	dpm	Relative adherence
None	7,846 ± 975	1.0	6,366 ± 940	1.0	8,908 ± 1,235	1.0
UAB62	20,471 ± 1,075[c]	2.6	43,358 ± 1,975	6.8	10,959 ± 2,485	1.1
UAB230	4,919 ± 800	0.6	2,390 ± 325	0.4	5,293 ± 1,025	0.6
UAB95 Spc^r	18,423 ± 3,835	2.3	26,092 ± 2,945	4.1	10,525 ± 2,375	1.2

[a] 100,000 dpm of [^{3}H]leucine-labeled cells was added to each tube (see text). One unit of water-insoluble glucan-synthesizing activity of GTF was used in each assay (see text).
[b] Adherence of cells due to glucan binding compared to adherence without glucan.
[c] Experiments were performed twice with duplicate samples each time.

we believe that (i) UAB230 has a defect in the synthesis of water-insoluble glucans by GTF and therefore cannot adhere; (ii) UAB95 Spc^r produces increased amounts of water-insoluble glucan which can initiate adherence but has a defect in either GTF or a glucan-binding protein which results in an inability of this strain to bind this glucan (or other glucans), and therefore a defect in adherence is observed; and (iii) when UAB230 and UAB95 Spc^r are grown together in sucrose-containing medium, the glucan produced by UAB95 Spc^r binds to UAB230 cells, and thus adherence is observed.

Our work has shown that complementation analysis between mutants with a similar phenotype can be used as an additional means, in the absence of classical methods of genetic analysis, to indicate the existence of difference biochemical lesions that yield the same phenotype with regard to avirulence. We plan to continue complementation analysis of nonadhering and nonaggregating mutants.

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