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Virulence of *Streptococcus mutans*: Restoration of Pathogenesis of a Glucosyltransferase-Defective Mutant (C4)

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Previous studies have shown that a mutant (designated C4) of *Streptococcus mutans* 6715 wild type (WT) is defective in glucosyltransferase (GTF)-synthesized insoluble glucan and is avirulent in gnotobiotic rats. This study investigated the factors which would render this mutant virulent in gnotobiotic rats. Microbial analysis of plaque from gnotobiotic rats (45 days old) infected with a mixture of C4 and virulent *S. mutans* PS-14 (approximately 15,000 C4 organisms to each *S. mutans* PS-14) yielded higher numbers of C4 organisms than *S. mutans* PS-14. These animals exhibited significantly lower caries scores than did gnotobiotic rats (age, 45 days) monoassociated with *S. mutans* PS-14. Similar mixed infection studies using C4 and an avirulent, aggregation-defective mutant of *S. mutans* 6715 WT (designated UAB 165) which exhibits GTF activity similar to that of the parent strain resulted in plaque consisting almost exclusively of UAB 165 and low caries activity. However, high levels of both C4 and UAB 165 in plaque and high caries activity were observed in gnotobiotic rats infected at weaning with C4 followed by UAB 165 3 days later. When dried *S. mutans* 6715 WT culture supernatant containing GTF activity was mixed with diet provided rats monoassociated with C4, significant caries activity was observed. Insoluble glucan supplemented in diet did not restore C4 to virulence; however, admixture of suboptimal GTF-rich supernatant with insoluble glucan and C4 resulted in high caries activity in gnotobiotic rats. These results suggest that in vivo restoration of pathogenesis of a GTF-defective mutant of *S. mutans* can be achieved either by complementation with a mutant defective in aggregation properties or by providing exogenous GTF and glucan from the parent *S. mutans* 6715 WT.

The most prevalent and costly bacterial disease afflicting humans is undoubedly dental caries (24). *Streptococcus mutans* is considered a prime cariogen in the induction of smooth surface decay in humans (17, 18) and in animal species (6, 16, 19). A special role for *S. mutans* in dental plaque formation has been related to the presence of the cell-associated and extracellular enzyme glucosyltransferase (GTF), which synthesizes water-soluble and -insoluble glucans from sucrose (9, 10). The highly branched, water-insoluble glucan is important for enhancement of attachment of the *S. mutans* cells to hard surfaces, including the tooth enamel (2, 8). The precise events which occur during this process are still not fully understood.

Some insight into the caries process has been provided by the isolation of mutants of *S. mutans* which either lack the ability to produce insoluble glucan (3, 20, 25) or excessively synthesize this product (20, 23). The former mutants are avirulent in gnotobiotic rats (20, 25), whereas the latter forms are hypercariogenic (19, 20). Although the properties of these mutants would suggest that insoluble glucan synthesis by GTF is of central importance for plaque formation and caries, more definitive proof could be provided by experiments which would allow restoration of mutants defective in insoluble glucan synthesis to full virulence in vivo. The present study used this approach, and the in vivo results suggested that an *S. mutans* mutant (C4) defective in production of insoluble glucan could: (i) effect a lower caries pattern when mixed at high proportions with a virulent strain, *S. mutans* PS-14, (ii) cooperate with another avirulent mutant, UAB 165, to produce carious lesions, and (iii) when supplemented with a cell-free, GTF-containing culture supernatant and glucan, be restored to full virulence.

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MATERIALS AND METHODS

Microorganisms and culture conditions. Two wild-type (WT) *S. mutans* strains, PS-14 (serotype *c*)

and 6715 (serotype *g*), and two mutant isolates derived from *S. mutans* 6715 WT were used. One mutant, designated C4, has been described in previous studies (12, 14, 20, 22). The second mutant, designated UAB 165, was isolated by using nitrous acid mutagenesis followed by four cycles of enrichment for cells that did not agglutinate when exposed to dextran T-2000 (Pharmacia Fine Chemicals, Piscataway, N.J.) under the conditions described by Gibbons and Fitzgerald (7). More detailed information on the isolation and properties of UAB 165 will be presented elsewhere (R. Curtiss, H. Harrison, and R. A. Hull, manuscript in preparation). A derivative of UAB 165 which was resistant to penicillin (100 μg/ml) was isolated and used in all studies described here. Each strain was maintained in brain heart infusion (BHI) agar stabs containing excess calcium carbonate. For infection of germfree weanling rats, cultures of each test strain were grown under anaerobic conditions in capped tubes containing BHI broth (with excess calcium carbonate). Sixteen- to 18-h cultures were used in all gnotobiotic infection studies. For in vitro assays, the test strains were grown in partially defined (PD) medium containing either 0.5% glucose (PD-glucose) or 0.5% sucrose (PD-sucrose) (13).

GTF production and assay. Standard GTF preparations were obtained from *S. mutans* 6715 WT and assayed as described previously (12, 13). For in vivo supplementation experiments, the culture supernatant was sterilized by filtration (0.45 μm) and lyophilized. By aseptic techniques, portions of sterile, dried supernatant were added to sterile screw-capped tubes and entered into the isolator. Just before the animals were fed, a preweighed amount of diet 305 (19) was mixed with the dried supernatant portion with the aid of large mortar and pestle in the isolator. Diet was subsequently provided to the animals ad libitum.

Insoluble glucan production. With GTF preparations similar to those described above, a large amount of insoluble glucan was produced as follows. To flasks containing 10 ml of enzyme supernatant (1 mg of protein/ml) was added 50 ml of sucrose (0.5 M) and 10 ml of sodium acetate buffer (0.25 M, pH 5.5). After overnight incubation at 37°C, insoluble glucan was removed by centrifugation (10,000 × *g*, 20 min) and washed five times with water. These preparations were lyophilized, and preweighed amounts were added to diet 305 (as described below).

In vitro aggregation assay. A modification of the method originally described by Gibbons and Fitzgerald (7) was used in these studies. *S. mutans* cells grown in PD-glucose medium were washed three times in saline and suspended to an optical density of 1.0 (540 nm) in 0.2 M glycine-NaOH buffer, pH 8.5. To 0.2 ml of cells was added 0.1 ml of sucrose (1 mg/ml) or dextran T-2000 (100 μg/ml), and the reaction mixture was incubated for 18 h at 37°C. The degree of aggregation was recorded as described previously (7).

In vitro plaque assay. The ability of each test *S. mutans* strain to form plaque was assessed by determining the amount of adherent material on glass surfaces after overnight growth in PD-sucrose medium at 37°C in an atmosphere of 95% N_2 and 5% CO_2. The preweighed culture tubes (10 by 100 mm) were washed twice with distilled water and once with alcohol (100%) to remove nonadherent material and then dried, and the dry weight of the adherent material was determined.

A modification of the above plaque assay was used for the in vitro reconstruction studies. Overnight cultures of either *S. mutans* 6715 WT or C4 grown in BHI broth were used to inoculate (0.2 ml) preweighed glass screw-cap minivials (4 ml) containing PD-sucrose medium (3.7 ml) supplemented with either ^{14}C-labeled insoluble glucan (approximate activity, 1,500 cpm/100 μg), *S. mutans* 6715 WT culture supernatant (described above), or both. After incubation at 37°C for 20 h in an atmosphere of 95% N_2 and 5% CO_2, the culture vials were gently washed three times with distilled water to remove nonadherent material and dried by overnight incubation at 37°C. The dried vials were either filled with Bray scintillation fluid (1) and counted in a Packard Tri-Carb scintillation counter to determine the amount (percentage) of added insoluble glucan adhering to the glass surfaces or weighed to determine the amount of adherent material.

In vivo gnotobiotic rat studies. In all studies reported here, the young gnotobiotic rat model was used as described previously (19). After infection of weanling rats (age, 20 days) with either a single strain or a designated mixture (see Results), a sample of each culture was tested for numbers of colony-forming units (CFU). Two days after infection, colonization was assessed by culturing oral swab samples collected on mitis-salivarius agar. The colonial morphology on mitis-salivarius agar of UAB 165 resembled that of the parent strain, *S. mutans* 6715 WT (rough texture), and was easily distinguished from the smooth textured colonies of C4 (12). Experiments were terminated when rats reached age 45 days, and the proportions of bacteria in plaque were assessed as described previously (21). Caries activity was determined on mandibular molars of individual animals as described previously (19, 20).

Statistics. The caries scores from each group of rats were statistically reduced by computing means and standard errors. Differences among means were evaluated by an analysis of variance and by multiple mean comparisons using the Duncan test (4). The results on the number of *S. mutans* CFU per one mandible per rat per group of rats were expressed as the mean ± standard error. The significance of difference between means was determined by Student's *t* test.

RESULTS

Attempts to establish C4 in plaque of gnotobiotic rats by using a virulent strain of *S. mutans*. The initial experiments attempted to establish a plaque of C4 with a matrix provided by *S. mutans* PS-14. *S. mutans* PS-14 was selected since C4 reverts to WT-like organisms in vivo (12); therefore, if revertants occurred in the experimental animals, all three types could be distinguished morphologically on mitis-salivarius agar plates (C4 is smooth and raised and 6715 WT is rough, hard, and pulvinate, whereas PS-14, although similar to 6715

WT, is more moist and smooth). Regardless of the ratio of C4 to *S. mutans* PS-14 used, the latter always predominated in plaque (Table 1). However, it was of interest that at the highest ratio of C4 to *S. mutans* PS-14 (14,921:1), C4 did reach significant numbers in plaque. Furthermore, this mixture ratio appeared much less virulent in gnotobiotic rats than the other mixtures studied or with *S. mutans* PS-14 alone. The results of this study indicated that the virulent strain of *S. mutans* quickly reached predominance in plaque even when the ratio of *S. mutans* PS-14 to C4 (1:14,921) significantly favored the colonization of the latter. However, the diminution of the cariogenic potential of the plaque microflora with increasing proportions of C4 suggested that this avirulent mutant of serotype *g S. mutans* was unable to use extracellular virulence factors provided by the serotype *c S. mutans* PS-14 strain in an in vivo situation.

Mixed infection studies using two avirulent mutants with complementary properties. From the initial experiments in this investigation and from previous observations (12), it was obvious that virulent, WT strains of *S. mutans* exhibit preferential colonization of smooth surfaces over C4. Therefore, a simplified model for reconstruction of virulence in vivo was investigated by using a second avirulent mutant of *S. mutans* which exhibited complementary properties to C4 (Table 2). This mutant of *S. mutans* 6715 WT, designated UAB 165, exhibits GTF and plaque-forming activities similar to those of the WT strain. However, this mutant, unlike the WT or the C4 strain, does not aggregate with dextran T-2000 or with sucrose. In this series of experiments, C4 was admixed with UAB 165 in increasing ratios favoring C4 and was used to infect weanling germfree rats (age, 20 days). After infection, rats (age, 45 days) were assessed for their plaque microflora and caries activity (Table 3). As in the studies with *S. mutans* PS-14, UAB 165 predominated in plaque of gnotobiotic rats regardless of the ratio of C4 to UAB 165 used. Furthermore, as with the WT strains of *S. mutans*, UAB 165 predominated in plaque 1 week after infection (data not shown). The results of this series of experiments clearly indicated that UAB 165 exhibited characteristics which were more favorable than those of C4 in the early stages of colonization and plaque formation.

In a second series of experiments, C4 was allowed to establish an infection in weanling gnotobiotic rats, and after 3 days UAB 165 was superimposed by infecting these same animals. Although UAB 165 ultimately gained predominance in the plaque microflora, C4 was also

TABLE 1. *Potential of S. mutans PS-14 to support colonization of an avirulent mutant (C4) of S. mutans 6715 (serotype g) in gnotobiotic rats*[a]

Mixture ratio, C4:PS-14[b]	No. of rats/group	Mean CFU/mandible[c]			Mean caries score[d]					
					Buccal		Sulcal		Proximal	
		C4 ($\times 10^6$)	PS-14 ($\times 10^6$)	C4/PS-14 ratio	Enamel	Dentinal (slight)	Dentinal (slight)	Dentinal (extensive)	Enamel	Dentinal (extensive)
2:0 (94.8:1)	24	1.2 ± 0.2	8.8 ± 1.2	0.01	19.1 ± 0.3	10.2 ± 0.3	16.3 ± 0.2	9.0 ± 0.3	7.0 ± 0.2	3.2 ± 0.2
3:0 (768:1)	17	3.3 ± 1.1	11.7 ± 1.7	0.03	16.8 ± 0.8	9.4 ± 0.5	14.3 ± 0.3	8.3 ± 0.7	4.5 ± 0.5	2.4 ± 0.3
4:0 (14,921:1)	12	81.6 ± 4.8	9.6 ± 0.5	0.85	12.0 ± 0.7	6.6 ± 0.3	14.2 ± 0.5	8.0 ± 0.9	4.1 ± 0.4	2.4 ± 0.2
C4 alone	18	33.3 ± 3.9			11.0 ± 0.5	6.5 ± 0.3	10.8 ± 0.6	6.3 ± 0.9	2.6 ± 0.4	0.0
PS-14 alone	14		10.7 ± 0.8		19.2 ± 0.6	10.7 ± 0.3	16.4 ± 0.5	7.1 ± 0.5	7.2 ± 0.4	3.3 ± 0.2

[a] Germfree rats were infected at 20 days of age and sacrificed at 45 days of age.
[b] Expressed as the $\log_{10}$ CFU per milliliter. Values given in parentheses represent the actual number of CFU per milliliter in the mixture after microbial analysis.
[c] Expressed as mean ± standard error of the number of microorganisms present in plaque on one mandible per rat.
[d] Expressed as mean ± standard error.

TABLE 2. *Biochemical characteristics of S. mutans 6715 WT (serotype g) and its mutants C4 and UAB 165*

S. mutans 6715 strain	GTF activity (μmol/mg of protein per h)[a]		Plaque formation (mg, dry wt)[a]	Aggregation[b]	
	Water-insoluble glucan	Water-soluble glucan		Dextran, 100 μg	Sucrose, 1 mg
C4	0.0	48.3 ± 3.0	0.2 ± 0.1	4+	3+
UAB 165	54.4 ± 2.2	6.6 ± 0.3	9.2 ± 0.6	0	0
WT	47.9 ± 1.7	0.0	9.2 ± 0.2	4+	3+

[a] Expressed as mean ± standard error.
[b] Values represent the degree of aggregation observed (7) on a scale of 0 to 4+.

found in significant numbers in plaque of these animals (Table 3). Perhaps of greater interest was the observation that these animals exhibited significantly higher caries activity than occurred in rats monoassociated with either strain. Although UAB 165 was present in greater numbers than C4 in plaque, it was not possible from this study to determine which strain contributed most to the caries activity.

Cell-free culture supernatants containing GTF and glucan restore C4 to virulence. The principal defect in C4 is the inability of its GTF enzyme to synthesize insoluble glucan when sucrose is provided as substrate. Supernatant containing GTF provided C4 with plaque-forming potential (Table 4). However, the addition of insoluble glucan alone was insufficient to allow this microorganism to adhere. The results of this in vitro study suggested that the addition of an insoluble glucan-synthesizing GTF preparation to C4 cells in the presence of sucrose allowed this microorganism to exhibit plaque-forming characteristics very similar to those of *S. mutans* 6715 WT.

In the first series of animal experiments, partially purified, dried culture supernatant from *S. mutans* 6715 WT containing GTF activity was supplemented in diet 305 (19) and provided to rats monoassociated with C4. Monoassociated rats ingesting 20 μg of dried supernatant per g of diet exhibited more smooth surface lesions than did rats provided diet supplemented with a similar amount of heat-treated, dried supernatant (Table 5). When diet containing 10-fold greater amounts of the dried supernatant was provided to C4 monoassociated rats, quite significant levels of caries were observed (Table 5). Although a lower caries activity was observed in these animals when compared with levels in *S. mutans* 6715 WT monoassociated rats, it was clear that the virulence potential of C4 had been significantly ($P \leq 0.01$) increased. The factor(s) responsible for enhancement of C4 virulence was heat labile, since provision of dried supernatant previously autoclaved (15 min) did not facilitate caries induction in C4 monoassociated animals.

In the second series of experiments, we assessed the contribution of insoluble glucan to the virulence potential of C4. In these studies a level of GTF-containing dried supernatant (20 μg/g of diet) was chosen which partially enhanced the pathogenic potential of C4 (Table 5). Supplementation of diet with insoluble glucan alone did not affect the virulence pattern of C4 (Table 6). However, when a low level of dried supernatant containing GTF was mixed with 100 μg of glucan per g of diet and given to animals monoinfected with C4, significant caries activity occurred which was similar to that observed in C4 monoassociated rats provided diet supplemented with the higher amount of dried supernatant containing GTF (200 μg/g of diet) (Table 5). The results of these investigations indicate that cell-free culture supernatants derived from virulent *S. mutans* 6715 WT enhanced the pathogenic potential of the avirulent C4 in vivo. Furthermore, an amount of supernatant enzyme which did not restore C4 to virulence was sufficient to allow the GTF-defective C4 to form a virulent plaque when a small amount of insoluble glucan was also provided.

DISCUSSION

Recent findings by van Houte and his associates (27–29) have indicated that colonization of the tooth surface by *S. mutans* involves at least two separate stages. The first stage is sucrose independent and involves the initial attachment of *S. mutans* to the tooth surface (27), whereas further colonization and finally disease is dependent on the presence of sucrose (5, 11, 15, 26). The results obtained in the present investigation suggest that several sucrose-dependent stages may be involved in the establishment of a virulent *S. mutans* plaque microflora. A mutant of *S. mutans* (C4) which is unable to synthesize insoluble glucan was rapidly supplanted in plaque by WT strains of *S. mutans* which exhibit normal metabolic behavior. Therefore, sucrose-dependent insoluble glucan synthesis may be one of the most important steps in the formation of an *S. mutans* plaque (2, 5, 7–10, 25, 26) since, in addition to *S. mutans* WT strains, the aggregation-negative *S. mutans* 6715 mutant

TABLE 3. *Microbial plaque and caries analysis of gnotobiotic rats (age, 45 days) infected with two avirulent mutants with complementary biochemical characteristics*

Mixture ratio, C4:UAB165[a]	No. of rats/group	Mean CFU/mandible[b]			Mean caries score[c]				
					Buccal		Sulcal	Proximal	
		C4 ($\times 10^9$)	UAB 165 ($\times 10^6$)	C4/UAB 165 ratio	Enamel	Dentinal (slight)	Dentinal (slight)	Enamel	Dentinal (extensive)
0:0 (2.91:1)	12	12.6 ± 5.5	3.7 ± 0.7	0.004	12.0 ± 0.6	7.5 ± 0.5	13.7 ± 0.6	4.0 ± 0.6	3.0[d] ± 0.4
2:0 (163:1)	11	0.6 ± 0.1	3.8 ± 1.6	0.0001	12.7 ± 1.1	6.9 ± 0.3	14.2 ± 0.4	3.2 ± 0.7	2.0 ± 0.4
4:0 (24,645:1)	10	5.8 ± 2.1	2.9 ± 0.5	0.002	14.0 ± 1.3	6.8 ± 0.2	15.3[d] ± 0.3	4.2 ± 0.4	3.0[d] ± 0.2
C4, then UAB 165[e]	14	900.0 ± 400.0	6.4 ± 1.7	0.14	18.9[d] ± 0.7	8.2[d] ± 0.2	17.5[d] ± 0.2	10.1[d] ± 0.5	6.9[d] ± 0.4 4.8[d] ± 0.1
C4 alone	18	3,019 ± 388			11.0 ± 0.5	6.5 ± 0.3	10.8 ± 0.6	6.3 ± 0.9	2.6 ± 0.4 0.0
UAB 165 alone	20		1.9 ± 0.3		10.4 ± 0.4	5.1 ± 0.4	11.2 ± 0.5	3.8 ± 0.5	2.1 ± 0.3 0.9 ± 0.3

[a] Expressed as the $\log_{10}$ CFU per milliliter. Values given in parentheses represent the actual number of CFU per milliliter in the mixture after microbial analysis.
[b] Expressed as mean ± standard error of the number of microorganisms present in plaque on one mandible per rat.
[c] Expressed as mean ± standard error.
[d] Values significantly higher than those obtained with either C4 or UAB 165 alone; $P \leq 0.01$.
[e] Rats (age, 20 days) infected with C4 followed by infection with UAB 165 (age, 23 days).

TABLE 4. *In vitro reconstruction of S. mutans 6715 mutant C4 virulence with insoluble glucan and with culture supernatant containing GTF*

S. mutans 6715 strain	Added *S. mutans* 6715 WT GTF (U)[a]	Added ^{14}C-labeled insoluble glucan[b]	Plaque formation	
			Dry wt (mg)[c]	Insoluble glucan (%)[d]
C4	—[e]	—	0.2 ± 0.1	—
C4	0.001	—	2.6 ± 0.1	—
C4	0.01	—	13.4 ± 1.1	—
C4	0.1	—	14.7 ± 0.5	—
WT	0.01	—	13.6 ± 0.5	—
WT	—	+	14.3 ± 0.4	78.62
C4	—	+	0.6 ± 0.1	0.64
C4	0.001	+	0.7 ± 0.2	1.18
C4	0.01	+	13.9 ± 0.9	66.42
C4	0.1	+	14.7 ± 0.4	74.55

[a] One unit of GTF represents the amount of enzyme that will incorporate 1 μmol of the glucosyl moiety of sucrose into water-insoluble glucan.
[b] Approximate activity, 1,500 cpm/100 μg of insoluble glucan per reaction mixture.
[c] Expressed as mean ± standard error.
[d] Expressed as the amount (percentage) of added ^{14}C-labeled insoluble glucan adhering to the glass surfaces. Similar results were obtained with ^{14}C-labeled autoclaved (15 min) insoluble glucan.
[e] —, None added.

UAB 165, which exhibits normal GTF activity, also supplants the GTF-deficient C4 in plaque. Nevertheless, UAB 165 alone was unable to form a virulent plaque in vivo. One could speculate that this mutant can undergo the initial sucrose-dependent colonization steps involving insoluble glucan synthesis by GTF. However, the aggregation defect prohibits a cell-to-cell attachment which may be required for the development of a virulent plaque. Studies are currently underway to investigate this possibility.

Although attempts to reconstruct virulence of a deficient mutant such as C4 with other *S. mutans* strains would seem feasible, our studies indicate that formation of a virulent plaque of predominantly C4 organisms is technically difficult. It was interesting that ratios favoring establishment of C4 in plaque with *S. mutans* PS-14 or UAB 165 always resulted in a microflora dominated by the latter. One contributing factor obviously involved the total amount of inoculum used. In our previous studies, we used inocula derived from approximately 10^8 CFU per ml of broth for infectivity (19, 20). Therefore, establishing a microflora of predominantly C4 with lesser numbers of other *S. mutans* types (such as UAB 165) will likely require ratios of organisms similar to those reported here; however, much lower inocula should be used (e.g., 10^4 to 10^6 total bacteria). Such experiments are presently underway.

TABLE 5. *Reconstruction of virulence in S. mutans 6715 mutant C4 monoassociated gnotobiotic rats provided diet supplemented with culture supernatant containing GTF*

Dietary *S. mutans* 6715 WT culture supernatant, (μg, dry wt)	No. of rats/ group	Mean CFU/mandible ($\times 10^6$)[a]	Mean caries score[b]					
			Buccal		Sulcal		Proximal	
			Enamel	Dentinal (slight)	Dentinal (slight)	Dentinal (extensive)	Enamel	Dentinal (extensive)
20 (0.001 U)[c]	11	4.6 ± 0.9	15.7[d] ± 0.6	6.5 ± 0.3	13.3 ± 0.6	5.9 ± 0.4	4.1 ± 0.4	1.8 ± 0.6
20 (heat inactivated)	11	4.1 ± 0.9	12.5 ± 0.5	6.5 ± 0.2	11.6 ± 0.4	4.9 ± 0.6	3.7 ± 0.3	1.4 ± 0.2
200 (0.01 U)	12	4.4 ± 0.7	19.4[d] ± 0.7	9.9[d] ± 0.2	16.8[d] ± 0.3	9.3 ± 0.4	5.5[d] ± 0.2	1.9[d] ± 0.1
200 (heat inactivated)	12	2.5 ± 0.5	12.9 ± 0.6	6.2 ± 0.3	11.2 ± 0.4	3.6 ± 0.4	3.8 ± 0.4	0.9 ± 0.2
None, mutant C4 alone	18	3.3 ± 0.4	11.0 ± 0.5	6.5 ± 0.3	10.8 ± 0.6	6.3 ± 0.9	2.6 ± 0.4	0.0
None, WT alone[e]	14	6.5 ± 1.4	22.4 ± 0.6	14.4 ± 0.8	21.4 ± 0.5	14.6 ± 0.5	7.7 ± 0.2	4.0 ± 0.0

[a] Expressed as mean ± standard error of the number of microorganisms present in plaque on one mandible per rat.
[b] Expressed as mean ± standard error.
[c] One unit of GTF represents the amount of enzyme that will incorporate 1 μmol of the glucosyl moiety of sucrose into water-insoluble glucan.
[d] Values significantly higher than those obtained with C4 alone; $P \leq 0.01$.
[e] Rats monoinfected with *S. mutans* 6715 WT alone.

TABLE 6. *Reconstruction of virulence in S. mutans 6715 mutant C4 monoassociated gnotobiotic rats provided diet supplemented with insoluble glucan and with culture supernatants containing GTF*

Dietary supplements (μg, dry wt) in *S. mutans* 6715 WT culture supernatant	Glucan	No. of rats/ group	Mean CFU/ mandible ($\times 10^6$)[a]	Mean caries scores[b]					
				Buccal		Sulcal		Proximal	
				Enamel	Dentinal (slight)	Dentinal (slight)	Dentinal (extensive)	Enamel	Dentinal (extensive)
None	1 mg	12	2.7 ± 0.6	10.2 ± 0.4	4.9 ± 0.7	11.5 ± 0.5	5.0 ± 0.3	1.6 ± 0.2	0.6 ± 0.2
20 (0.001 U)[c]	10 μg	10	3.4 ± 1.0	9.6 ± 0.2	5.4 ± 0.9	11.4 ± 0.4	4.5 ± 0.5	1.9 ± 0.3	0.4 ± 0.2
20 (0.001 U)	100 μg	13	6.5 ± 1.0	17.6[d] ± 1.4	8.6[d] ± 0.4	14.3[d] ± 0.4	8.2[d] ± 1.0	6.2[d] ± 0.5	1.5[d] ± 0.2
20 (0.001 U)	None	13	6.2 ± 1.0	14.5[d] ± 1.2	6.7 ± 0.4	12.5 ± 0.3	5.8 ± 0.9	3.8 ± 0.6	1.2 ± 0.2
None, C4 alone	None	18	3.3 ± 0.4	11.0 ± 0.5	6.5 ± 0.3	10.8 ± 0.6	6.3 ± 0.9	2.6 ± 0.4	0.0
None, WT alone[e]	None	14	6.5 ± 1.4	22.4 ± 0.6	14.4 ± 0.8	21.4 ± 0.5	14.6 ± 0.5	7.7 ± 0.2	4.0 ± 0.0

[a-e] See Table 5.

The model of mixed infection reported here is suitable for other oral ecology studies, e.g., the possible contribution of bacteria other than *S. mutans* which employ GTF enzyme systems to generate insoluble glucan. Thus, association of these strains with C4 in gnotobiotic rats could be assessed to determine their overall contribution to the caries process. Without question, many such collaborative events occur in the plaque microflora. However, to date most studies of this type have been hampered by our inability to determine which of the two infecting organisms was of most importance in the induction of dental caries. The study involving colonization of C4 followed by UAB 165 was a good example of this point. From the data presented in Table 3, it was clear that when both C4 and UAB 165 were present in appreciable quantities in plaque, caries ensued. Although these microorganisms were acting synergistically (neither organism was virulent alone), the precise contribution of each strain to the caries process was not clear. Several studies using mutants of *S. mutans* in animal models have correlated various deficiencies (3, 20, 25) or increased GTF activity (20, 23) with caries activity. Although such results are suggestive, they do not prove that a particular characteristic is essential for virulence. The studies reported here for the first time demonstrate that a mutant defective in insoluble glucan synthesis by GTF can be restored to virulence with cell-free products. This was first accomplished by adding supernatants derived from the virulent *S. mutans* parent strain which contained the heat-labile enzyme GTF. Although insoluble glucan alone could not restore C4 to virulence, it could enhance caries activity when mixed with suboptimal levels of

supernatant containing extracellular enzyme.

The approach of supplanting missing components in vivo enables one to correlate certain gene products produced by the bacterium *S. mutans* with its virulence. This has allowed us to confirm the observations of others (7–9, 20, 23, 25) that GTF and insoluble glucans are important virulence characteristics of this bacterium. Finally, when we have firmly established all the traits of *S. mutans* which contribute to virulence, we can logically begin to consider therapeutic approaches which will interupt these events in the oral cavity.

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