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Role of a Cell Surface-Associated Protein in Adherence and Dental Caries

W. H. BOWEN,[1]* K. SCHILLING,[1] E. GIERTSEN,[1]† S. PEARSON,[1] S. F. LEE,[2] A. BLEIWEIS,[2] AND D. BEEMAN[1]

University of Rochester, Rochester Cariology Center, Department of Dental Research, 601 Elmwood Avenue, Box 611, Rochester, New York 14642,[1] and University of Florida, Department of Oral Biology, J. H. Miller Health Science Center, Gainesville, Florida 32610[2]

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Insertional inactivation of the *Streptococcus mutans spaP* gene was used to construct an isogenic mutant (834) of strain NG8 (serotype c) which lacked the major cell surface-associated protein referred to as P1 (15). Results of several studies suggest that P1 is involved in the adherence of *S. mutans* to saliva-coated apatite surfaces. With an in vitro model system of hydroxyapatite (HA) beads coated with parotid saliva (PS) and additional HA surfaces coated with PS and in situ-formed glucan, it was observed that mutant 834 adhered poorly to the PS/HA surfaces. In contrast, both parent and mutant strains bound to the PS-glucan/HA surface. Groups of intact and desalivated rats were infected with each strain to determine relative capacities to induce dental caries. Rats were fed a highly cariogenic diet containing 56% sucrose for 3 to 5 weeks. Each strain colonized the rodent model and caused similar levels of smooth-surface caries under these dietary conditions. It was concluded that P1 influences the ability of organisms to adhere to saliva-coated surfaces and possibly affects primary colonization of the oral cavity in the absence of a glucan surface but has no effect on glucan-mediated adherence in vitro or in vivo.

The role played by specific cell surface-associated proteins of mutans streptococci in the colonization of tooth surfaces is the subject of intensive research by a number of investigators. Clarification of the molecular basis of adhesion could lead to the development of specific reagents that act to prevent colonization of tooth surfaces by potentially pathogenic organisms and thereby affect the accumulation of pathogenic plaque. Results from several studies suggest that a surface protein termed P1 (also known as antigen I/II, B, and PAc) is involved in the adherence of *Streptococcus mutans* to saliva-coated hydroxyapatite (sHA) (9, 15, 18). Furthermore, it is suggested that this protein could be an effective immunogen against dental caries (22). Indeed, it is reported that topical application of monoclonal antibodies specific for P1 can prevent colonization of tooth surfaces by *S. mutans* and thereby prevent dental caries (16).

Opinions differ as to what constitutes the primary mechanism of colonization of the tooth by *S. mutans*. Most attention is focused on the ability of organisms to adhere to the mammalian-derived constituents of salivary pellicles adsorbed to HA. Recent evidence demonstrates that bacterial glucosyltransferase is adsorbed to tooth surfaces and that it retains activity in the adsorbed state within the pellicle (24). As a result, glucan can be synthesized in situ from sucrose and thereby present a different type of surface for microorganisms to colonize.

The purpose of this study, therefore, was to determine whether an isogenic mutant of *S. mutans* (15), lacking the surface protein P1, could adhere to (i) parotid-saliva-coated HA (PS/HA) and (ii) glucan-coated HA and, finally, to compare the cariogenicity of the mutant and its parent in an animal model.

* Corresponding author.
† Present address: University of Oslo, Geitmyrsveien 71, N-0455 Oslo 4, Norway.

MATERIALS AND METHODS

An isogenic mutant (834) of *S. mutans* NG8 (Brathall serotype c) which lacked the major cell surface-associated P1 was constructed by insertional inactivation of the *spaP* gene and then by allelic exchange in the wild-type strain (15). Serotyping was reconfirmed frequently throughout the investigation by the use of highly specific antisera (25). A thorough characterization of the mutant indicated that it differed from the wild-type strain (NG8) only by its loss of the fibrillar surface protein, P1 (15).

The microorganisms used in this study were radiolabeled by being grown in a dialyzed medium (24) containing 10 mCi of [^{3}H]thymidine per ml. After being harvested in the exponential phase by centrifugation, the labeled organisms were dechained by brief sonication (Braun-Sonic 1510) by exposure to three or four 30-s pulses at 400 W. Bacterial concentrations were measured by optical density with a calibration curve for optical density at 540 nm in a Beckman DU 8 spectrophotometer versus bacterial numbers as quantitated in a Petroff-Hausser counting chamber. PS was collected from a single donor by means of a modified Lashley cup (13) by using sugar-free lemon-flavored candy as a stimulant for salivary flow.

The standard adherence assay was carried out as described previously (6), but modified to include incorporation of glucosyltransferase (23). Briefly, where in situ-formed glucan was required, after washing of PS/HA, glucosyltransferase was added. The preparation was again washed and exposed to sucrose. When pellicle-coated HA was required, the enzyme adsorption step was omitted. Bacteria were added at 1.0×10^9 cells per ml in either the presence or absence of 1 mg of 9,000-kDa dextran per ml (Sigma Chemical Co., St. Louis, Mo.). Adherent bacteria were quantitated after 1.0 h.

The caries studies were carried out with specific-pathogen-free Sprague-Dawley rats purchased from Charles River (Kingston, N.Y.). Litters (13 to 15 days old) were obtained

TABLE 1. Adherence of *S. mutans* NG8 and isogenic P1$^-$ mutant, *S. mutans* 834, to experimental pellicles

Species and strain	Phenotype	Bacteria bound to exptl pellicles (10^8)[a]				
		BSA/HA	PS/HA	PS/HA with dextran	PS-glucan/HA	PS-glucan/HA with dextran
S. mutans NG8	P1$^+$	0.14 (0.02)	1.36 (0.14)	1.53 (0.13)	1.21 (0.12)	0.64 (0.10)
S. mutans 834	P1$^-$	0.34 (0.04)	0.29 (0.03)	0.36 (0.05)	2.25 (0.04)	1.37 (0.19)

[a] The data shown are mean values (n = 3) from a single experiment and are representative of at least two experiments; standard deviations are indicated in parentheses.

with their dams and screened for the presence of indigenous mutans streptococci using our previously described techniques (3). The animals did not harbor *Streptococcus rattus*. The presence of *S. rattus* (*S. mutans* serotype b) in the indigenous population could interfere with the interpretation of data from our experiments. However, this species, even if present, does not possess the *spaP* gene responsible for the major surface protein (P1) under consideration as a putative virulence antigen in *S. mutans* (serotype c) (14). Thus, these two species should not compete for the same attachment sites on tooth surfaces. Two studies were carried out with rats. In one, salivary ducts from the parotid glands were ligated, and the submandibular and sublingual glands were removed by using previously described techniques (4). Results from the study would help to clarify the role of saliva in facilitating colonization of tooth surfaces by microorganisms. In the second study, animals with intact glands were used as described previously (5).

Rats were infected with either the parent strain, NG8, or the isogenic P1 mutant. An additional group of intact animals was infected with *Streptococcus sobrinus* 6715 as a positive control. Infection was confirmed in all animals by plating oral swabs on mitis salivarius agar plus streptomycin.

Rats were fed diet 2000 (12) containing 56% sucrose ad libitum. The investigation continued for 3 weeks with the desalivated rats and for 5 weeks with the intact animals. At the end of these periods, the animals were killed, and one-half of each lower jaw was aseptically removed and placed in 5 ml of saline. Following sonication, bacterial populations were estimated as previously described (17).

Caries was scored by means of the Keyes method (11), and data were analyzed by ANOVA.

RESULTS

The isogenic P1 mutant, 834, in contrast to the wild-type parent strain, NG8, adhered poorly to PS/HA and thereby confirmed the importance of this protein in facilitating the colonization of PS/HA surfaces (Table 1). When adherence to controls (bovine serum albumin [BSA]/HA) was considered, the mutant was totally nonadherent to PS/HA upon subtraction of background values. The addition of dextran to the incubation mixture did not affect the ability of the organisms to adhere to PS/HA. However, in contrast to the results observed with PS/HA, both parent and mutant strains adhered to glucan-coated HA (PS-glucan/HA). Inclusion of dextran in the incubation mixture reduced the ability of the parent and mutant to adhere to the glucan surface (Table 1), indicating the specific binding of α-1,6-glucan by both strains.

All of the animals survived the surgical procedures and ate and drank normally. Each strain readily colonized desalivated rats and intact animals. Differences were not detected in the total population or in recoveries of mutans streptococci in the desalivated groups (Table 2). However, compared with the parent strain, significantly higher numbers of the mutant strain were isolated from intact animals.

Smooth-surface-caries scores were essentially the same for animals infected with the parent strain and animals infected with the mutant strain (Table 2). In the intact group, animals infected with *S. sobrinus* developed significantly more smooth-surface caries than did the animals infected with either the parent or the mutant strain of *S. mutans*. However, intact animals infected with the mutant strain developed significantly more sulcal caries than did the other intact groups. Caries scores did not differ significantly among the desalivated groups, although the caries scores were substantially higher than those observed in the intact animals similarly infected.

DISCUSSION

The use of an isogenic mutant may allow the role of a potential virulence factor(s) to be clearly defined, provided

TABLE 2. Ability of *S. mutans* NG8 (parent) and 834 (mutant) and *S. sobrinus* to colonize and induce caries in intact and desalivated rats[a]

Animal status and infecting organism	Smooth-surface caries	Sulcal caries	Total cultivable count (10^7)	% *S. mutans* or *S. sobrinus* of total cultivable count
Intact rats				
S. sobrinus	12.3 (10.4)[b]	22.4 (13.0)	1.0 (7.0)	24.7
NG8 (parent)(P1$^+$)	3.5 (6.6)	21.8 (5.2)	1.8 (1.0)	6.1
834 (mutant)(P1$^-$)	3.5 (4.6)	31.1 (6.4)	2.8 (1.5)	15.7[c]
Desalivated rats				
NG8 (parent)(P1$^+$)	80.0 (17.2)	44.0 (9.2)	4.1 (1.7)	20.2
834 (mutant)(P1$^-$)	81.5 (25.4)	48.0 (5.6)	3.9 (1.3)	15.4

[a] There were 12 animals in each group; standard deviations are given in parentheses.
[b] Significantly higher than P1$^+$ or P1$^-$.
[c] Significantly higher than P1$^+$ ($P < 0.05$).

the gene deleted is not part of an operon which could have secondary effects on metabolism. We have no evidence that such a situation prevails here. Allelic-exchange mutagenesis also clearly has advantages over ill-defined nondirected chemical- or radiation-induced mutagenesis. Results from several investigations show that the major surface protein of *S. mutans* (known variously as P1, I/II, B, or PAc) confers surface hydrophobicity to this oral species and mediates binding of this microorganism to PS/HA (9, 15, 18, 19, 21).

The data presented here support the concept that P1 is essential for the attachment of *S. mutans* to host-derived-binding sites in the acquired pellicle. In addition, results reported by Russell (21) show that purified I/II (P1) binds effectively to PS/HA surfaces and that it also binds to salivary proteins immobilized on nitrocellulose. These observations suggest that P1 may interact directly with salivary pellicle. Lee et al. (15) isolated high-molecular-weight salivary agglutinins that react directly with this bacterial surface adhesin. Demuth et al. (8) suggest that this interaction may involve lectinlike domains on the P1 molecule and on similar surface proteins derived from *Streptococcus sanguis*.

There is also some indirect evidence which indicates that P1 may be involved in sucrose-mediated binding. Antibodies raised against P1 inhibit adherence of *S. mutans* to PS/HA and also inhibit sucrose-induced accumulation of *S. mutans* on glass surfaces (9). Antibodies against SpaA, a closely related surface protein produced by *S. sobrinus*, are reported by Curtiss et al. (7) to inhibit sucrose-induced aggregation of that species. Curtiss et al. further suggest that SpaA and P1 are adhesins involved in binding mutans streptococci to saliva- and glucan-coated surfaces.

Our results, however, indicated that the ability of the P1 mutant to adhere in vitro to saliva-coated surfaces differed significantly from that of the parent strain, confirming observations made previously (15). Of no less importance is the observation that the mutant and parent strains adhered to a glucan-coated surface, thereby providing clear evidence that *S. mutans* possessed at least two mechanisms to allow it to colonize animal hosts in vivo. Such an observation was hardly surprising, considering the extraordinary range of environments which may prevail in the mouth.

Understandably, much attention is focused on the colonization of salivary pellicle on the tooth surface (10). However, it is clear that salivary pellicle in vivo contains glucosyltransferase as well as fructosyltransferase activity (20). Given the universality of sucrose (1), a glucan surface may frequently be the primary surface of colonization for many oral microorganisms.

The latter hypothesis is supported in part by the observations reported here, namely that mutant and parent strains alike readily colonized our animals. Furthermore, removal of the salivary glands, preventing formation of salivary pellicles, was without apparent influence over the ability of either the parent or mutant strain to colonize the animals. Available evidence indicates that *S. sobrinus* and *S. mutans* colonize poorly in the absence of diets administered in a conventional manner. Animals receiving their entire diet by gastric gavage are either not colonized or poorly colonized by *S. sobrinus* or *S. mutans* (2). It appears that sucrose in the diet may help provide a surface on the tooth to facilitate colonization by oral microorganisms. The singular importance of antigen P1 as a virulence factor is placed in doubt by the observation that both parent and mutant strains had equal ability to induce smooth-surface caries in intact and desalivated rats, although the reason for the mutant inducing significantly more caries on sulcal surfaces in the intact animals is unclear.

Superficially, our results may appear at variance with reports that vaccination with antigen P1 (I/II or B) confers protection against caries (22). However, it is conceivable that antibodies attached to this antigen may sterically block other surface structures associated with virulence. Alternatively, P1 may be required for binding to saliva-coated, nondental tissues, thereby allowing some retention of *S. mutans* in the oral cavity until the glucan pellicle is formed on enamel surfaces upon introduction of sucrose into the diet.

This study emphasized the importance of conducting studies in vivo as well as in vitro when exploring the virulence of microorganisms. It was also apparent, on the basis of the high scores with desalivated animals, that the expression of virulence was influenced considerably by host factors, the host factor in this instance being the absence of saliva. In any event, it was concluded that *S. mutans* possessed multiple modes of adhering to host tissues and that effective antimicrobial reagents may have to interfere with each of these adhesins.

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