Aggregative Behavior of Bacteria Isolated from Canine Dental Plaque

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Interbacterial adhesion of bacteria isolated from canine dental plaque was assessed by performing a visual coaggregation assay. Using conditions mimicking those likely to be encountered in vivo, the entire cultivable plaque microbiota from a single dog was assessed, and eight (6.7%) unique coaggregation interactions were detected for 120 crosses. Transmission electron microscopy was used to visualize several of the bacteria in isolation and as coaggregates, which revealed surface structures that may be involved in adhesion and coaggregation. The results of this study indicate that the prevalence of coaggregating pairs of dental plaque bacteria in dogs is similar to the prevalence of coaggregating pairs of dental plaque bacteria reported in humans. In addition, genera found in both hosts generally exhibited similar coaggregation reactions; however, autoaggregation was found to be more common among oral bacteria isolated from dogs.

Coaggregation is the term used to describe adhesion of genetically distinct microbial cells to each other in a suspension (12), and autoaggregation is the term used to describe the same phenomenon for genetically identical cells. These processes, in conjunction with adhesion to a substratum, are important factors in the development of bacterial biofilms (14). Coaggregation may have evolved in concert with structural and metabolic codependencies which in turn facilitate the development of complex biofilm communities, such as dental plaque. This possibility is supported by the findings of Bradshaw et al. (1), who showed that certain bacterial aggregates could allow obligate anaerobes to persist in aerated cultures.

Gibbons and Nygaard (5) were the first workers to realize the potential importance of cell-to-cell adhesion in the development of dental plaque and to develop a method for measuring interbacterial aggregation, which is now more commonly called coaggregation. They used this method to assess the coaggregation of 23 strains of numerically dominant culturable bacteria and found that 18 of these strains participated in coaggregation reactions; a total of 23 interactions were observed for the 253 pairs tested. This work has been continued by many workers, who often assess coaggregation by using the convenient visual assay described by Cisar et al. (2), which uses a system of scores from 0 to 4. Kolenbrander and Andersen (10) extended this work further by using radioactively labeled cells in coaggregation assays, which allowed them to address more elaborate questions, such as the stability of coaggregation bonds and the trapping of noncoaggregating cells by coaggregation networks.

Coaggregation research has focused primarily on bacteria isolated from human dental plaque; however, coaggregation has recently been shown to occur between bacteria from water systems, leading to the suggestion that it may be a universal

phenomenon among biofilm-forming bacteria (14). Coaggregation studies using bacteria from the canine oral microbiota should allow comparisons with the large volume of data related to the human oral microbiota and may help identify interactions important for the development of canine dental plaque.

In this study, coaggregation assays were performed using conditions described previously for use with bacteria isolated from the human oral microbiota. The conditions were adjusted to more accurately replicate the canine in vivo conditions. Finally, the whole cultivable bacterial community from a single plaque sample was assayed in duplicate using the canine-adapted assay conditions.

MATERIALS AND METHODS

Organisms. All bacteria used in this study were isolated from the dental plaque of dogs undergoing routine dental treatment either at the Waltham Centre for Pet Nutrition (WCPN), Waltham-on-the-Wolds, United Kingdom, or at a United Kingdom veterinary dental referral clinic. The dogs at WCPN were housed in environmentally enriched conditions under simulated pet conditions. They were fed a nutritionally complete, commercial dry dog food twice daily (Pedigree Complete; Masterfoods, United Kingdom). The dogs whose plaque was sampled were four Miniature Schnauzers (female neutered), three Cocker Spaniels (male; one entire and two neutered), one Cairn Terrier (female), and one Collie cross (female neutered) that were between 2 and 13 years old. All animals were in good health and had not been given antibiotics within the preceding 3 months. Plaque samples were taken from the buccal surfaces of upper second or third premolars using a dental probe. Samples were taken from the gingival margin, except where a periodontal pocket was present, in which case the sample was taken from the base of the pocket. In addition, a single supragingival plaque sample was taken from an upper second premolar, and a distal/mesial pooled sample was taken from the periodontal pocket on an upper second incisor; both of these extra samples were taken from one of the nine dogs mentioned above. Samples were diluted, plated on blood-containing agar media, and incubated aerobically (Columbia agar base [Oxoid, Basingstoke, United Kingdom]) and anaerobically (anaerobe agar [Bioconnections, Leeds, United Kingdom]) to isolate bacteria. Isolates were identified by comparative 16S rRNA gene sequencing.

Culture preparation. Bacterial cultures were grown in 20 ml static brain heart infusion broth (Oxoid) for 18 h or 40 h. When bacteria grew poorly in broth or if a specific nutrient requirement was known, supplements were added as appropriate. The supplements used were 5 $\mu\text{g ml}^{-1}$ hemin (Sigma) (stock dissolved in 1 M NaOH), 0.5 $\mu\text{g ml}^{-1}$ menadione (Sigma) (stock dissolved in 70% ethanol), 750 ng ml^{-1} cysteine hydrochloride (BDH), and 5 $\mu\text{g ml}^{-1}$ yeast extract (Oxoid). Hemin and menadione were filter sterilized and added to sterile broth,

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TABLE 1. Coaggregation interactions corrected for autoaggregation for bacteria from several dogs when standard conditions were used

Organism	Coaggregation or autoaggregation score ^a																														
	<i>Granulicatella</i> sp. (abio1)	<i>Actinomyces canis</i> (actino1)	<i>Actinomyces canis</i> (actino2)	<i>Actinomyces bowdenii</i> (actino3)	<i>Actinomyces</i> sp. (actino4)	<i>Actinomyces hordeovulnens</i> (actino8)	<i>Bergeyella zoohelcum</i> (berg1)	<i>Porphyromonas</i> sp. (bpp10)	<i>Porphyromonas macacae</i> (bpp11)	<i>Prevotella</i> sp. (bpp4)	<i>Porphyromonas gulae</i> (bpp6)	<i>Porphyromonas canons</i> (bpp7)	<i>Porphyromonas cangingivalis</i> (bpp8)	<i>Porphyromonas cansulci</i> (bpp9)	<i>Corynebacterium</i> sp. (cory2)	<i>Corynebacterium</i> sp. (cory3)	<i>Fusobacterium</i> sp. (fuso3)	<i>Haemophilus</i> -like sp. (haem1)	<i>Streptococcus</i> sp. (lac1)	<i>Filifactor villosus</i> (fuso4)	<i>Neisseria</i> sp. (nei1)	<i>Neisseria canis</i> (nei3)	<i>Neisseria canis</i> (nei4)	<i>Neisseria weaveri</i> (nei5)	<i>Peptostreptococcus anaerobius</i> (pep1)	<i>Propionibacterium</i> sp. (prop2)	<i>Staphylococcus epidermidis</i> (staph1)	<i>Streptococcus minor</i> (strep2)			
abio1	0	0	0	1	1	1			0	0	1	0	0	0					0	0	0	1	0	0	0	0	0	0	1		
actino1		0		0	0	0			0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
actino2			0	0	0	0	0		0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
actino3				0	0	0		0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
actino4					0	0		0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	
actino8					0	0	0		0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
berg1						0	0	1	0	0	0			0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
bpp10									0	0	1		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
bpp11								0	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
bpp4									0	0	0		1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
bpp6										0	0			0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
bpp7											0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	
bpp8													0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
bpp9														0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
cory2															0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
cory3																0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
fuso3																	1	0	0	0	0	0	0	0	0	0	0	0	0	0	
haem1																		4	1	1	0	0	0	0	0	0	0	0	0	0	
lac1										1	1								1	0	0	0	0	0	0	0	0	0	0	0	
fuso4																				1	0	0	0	0	0	0	1	1	0	0	
nei1																					0	0	0	0	0	0	1	1	0	0	
nei3																						0	0	0	0	0	1	1	0	0	
nei4																						0	0	0	0	0	1	1	0	0	
nei5																									0	0	1	1	0	0	
pep1																										0	1	1	0	0	
prop2																											1	1	0	0	
staph1																														0	
strep2																														0	1

^a Blanks indicate crosses which were not tested. To correct for autoaggregation, the highest autoaggregation score for a pair was subtracted from the coaggregation score. Raw autoaggregation data are on the diagonal.

FIG. 1. Summary of autoaggregation and coaggregation interactions between bacteria from a single canine plaque sample, using canine-adapted conditions. The numbers in parentheses are autoaggregation scores, and the numbers next to lines are coaggregation scores. Organisms for which no coaggregation reactions were detected are indicated at the bottom left.

and cysteine hydrochloride and yeast extract were added to the broth before autoclaving. Cultures were grown long enough to produce a dense suspension in order to obtain a sufficient concentration of cells. The cells were harvested by centrifugation (4,000 relative centrifugal force for 10 min at 4°C) and suspended in 5 ml coaggregation buffer (see below) three times. The optical density at 600 nm of each suspension was then adjusted to 1.0 using sterile buffer.

Coaggregation buffers. The buffer for the initial experiments was prepared by using the method described by Cisar et al. (2), and it contained 0.15 M NaCl, 0.001 M Tris, 0.0001 M CaCl₂, 0.0001 M MgCl₂, and 0.02% NaN₃. To adapt the standard coaggregation buffer for bacteria isolated from dogs, the pH was lowered to 7.5, and the salts composition was adjusted to match measurements made at WCPN (Neil Culham, Waltham Centre for Pet Nutrition, personal communication). In addition, the pH buffer was changed to HEPES, which is more biologically compatible than Tris (6), and the buffer strength was increased to 0.01 M because the pH of the standard buffer was observed to be unstable. The canine-adapted buffer contained 0.04 M NaCl, 0.01 M HEPES, 0.001 M CaCl₂, 0.02 M KCl, and 0.02% NaN₃.

Coaggregation assay. The coaggregation assay was performed by using the standard method described by Cisar et al. (2). Briefly, this assay involves mixing 0.2-ml portions of suspensions and visually scoring the formation of flocs from 0 (no visible aggregates) to 4 (large aggregates which settle immediately).

All coaggregation crosses were performed twice, and autoaggregation crosses were also duplicated. Assays were performed blind with isolate numbers identifying the crosses; at the end of each scoring session the grid of results was examined for any discrepancies between the duplicate crosses. When a discrepancy was found, the pair was rechecked and rescored; invariably, the discrepancy was due to a difference in score interpretation rather than an actual difference in coaggregation, as shown by a comparison of suspensions after scoring. The effect of autoaggregation was corrected for by subtracting the highest autoaggregation score of the two partners from the coaggregation score, as described by Rickard et al. (14).

Transmission electron microscopy of coaggregates. Several bacterial isolates were examined by transmission electron microscopy (TEM), using methylamine tungstate as a negative stain to visualize bacterial cell surfaces and coaggregates. Cells were prepared for TEM by removing 1 ml of the coaggregation assay cell suspension and centrifuging it at 7,200 relative centrifugal force for 2 min. The supernatant was removed and replaced with sterile distilled water, and then the cells were resuspended and pelleted again in the same manner. Finally, cell preparations were suspended in 200 µl sterile distilled water, which concentrated the cells fivefold and removed the coaggregation buffer, which was found to be incompatible with TEM. Negative staining of cells was performed on plasma discharged Formvar/carbon-coated 400-mesh copper grids (Agar Scientific, Essex, United Kingdom). The following protocol was performed quickly to prevent the grids from drying out, based on the method of Handley et al. (7): (i) float 20 µl of culture 1 on top of a grid for 1 min; (ii) draw off the culture with damp filter

paper; (iii) float 20 µl of culture 2 on top of the grid for 1 min (if required) and draw off the culture with damp filter paper; and (iv) float 20 µl of 1% (wt/vol) methylamine tungstate on top of the grid for 1 min and draw off the solution with damp filter paper.

Nucleotide sequence accession numbers. The sequences used for identification have been deposited in the GenBank database under accession numbers AY827856 to AY827945.

RESULTS

Standard coaggregation assay. In the initial experiments we used the standard coaggregation conditions described by Cisar et al. (2) to make 224 different test crosses with 28 strains obtained from the oral microbiota of several dogs. Repeats of 10% of the crosses produced identical results after correction for autoaggregation in 95% of the cases. These experiments revealed a total of 35 different coaggregation interactions between 15 bacterial strains isolated from the canine oral microbiota, as shown in Table 1.

Canine-adapted coaggregation assay. Duplicate coaggregation experiments performed with the cultivable oral microbiota from a single dog using canine-adapted conditions were in agreement in 74% of the cases. All possible crosses of 15 strains were performed (120 crosses), which revealed a total of eight different coaggregation reactions involving eight different isolates (Fig. 1).

Transmission electron microscopy of coaggregates. Several isolates from the canine-adapted coaggregation assay were examined by TEM using a negative stain to highlight cell surface detail. In each case, single-species suspensions were also viewed. Cell clumping appeared to be less frequent in monocultures than in the mixed populations; however, the differences were not quantified.

The strong autoaggregator haem1 (*Actinobacillus*-like) was observed to be a highly fimbriated bacterium (Fig. 2). Large aggregates were present, but some of the bacteria occurred singly.

lep1 (*Leptotrichia* sp.) cells appeared to be predominantly

FIG. 2. Negatively stained TEM micrograph of haem1 (*Actinobacillus*-like). Scale bar = 1 μ m.

isolated slender rods which were poorly stained, while actino3 (*Actinomyces naeslundii*-like) cells were strongly stained, isolated or clumped, short, thick rods (Fig. 3). This staining distinction facilitated discrimination of cells in the mixed population by TEM, and coaggregates of the two cell types were clearly observed (Fig. 3), despite the fact that they were not detected by the assay. actino3 cells frequently had irregularities on the cell surface, which may have resulted from adhesion of debris from the medium.

fuso3 (*Fusobacterium* sp.) cells were large rods which stained variably, and cory2 (*Corynebacterium felinum*-like) cells were shorter rods which stained more darkly and had a thick coat which stained variably (Fig. 4). In mixed populations cory2 cells were darker and shorter cells that coaggregated with the lighter fuso3 cells (Fig. 4).

DISCUSSION

Using standard coaggregation conditions, 35 unique interactions were detected for 224 crosses (15.6%) of bacteria from several dogs. Using canine-adapted conditions, eight unique interactions were detected for 120 crosses (6.7%) of bacteria isolated from a single dog. These results indicate that the prevalence of coaggregation of plaque bacteria in dogs is similar to the prevalence of coaggregation of plaque bacteria in humans; Gibbons and Nygaard (5) reported coaggregation in 9% of their crosses with human plaque bacteria. Although the frequency of coaggregation of bacteria in dogs was similar to the frequency of coaggregation in humans observed in previous studies, autoaggregation was more common in dogs. In studies of oral bacteria from humans, autoaggregation often is not

FIG. 3. Negatively stained TEM micrographs of lep1 (*Leptotrichia* sp.) (top left), actino3 (*Actinomyces bowdenii*) (top right), and mixed coaggregate (bottom). Scale bars = 1 μ m.

detected or is not mentioned. This means that there is no need to correct for autoaggregation in human studies, and thus, coaggregation may be detected more readily. In the present study, eight isolates (29%) were found to autoaggregate under standard conditions (Table 1), but in a similar study using 23 isolates of human origin autoaggregation was not detected (5), although in a recent study workers detected autoaggregation using a spectrophotometric method with bacteria from root caries lesions (17). Although the difference may be an artifact

of different methods used by different groups, the apparently higher level of autoaggregation in canine dental plaque may indicate an ecological difference compared to human dental plaque. It seems possible that dog teeth experience greater hydrodynamic shear forces and are thus less able to sustain thick biofilms. In such a situation there may be greater competition for direct adhesion to the substratum, and autoaggregation may be an adaptation for this purpose. This speculation is supported by the in vitro work of Rickard et al. (15), which

FIG. 4. Negatively stained TEM micrographs of fus03 (*Fusobacterium* sp.) (top left), cory2 (*Corynebacterium* sp.) (top right), and mixed coaggregate (bottom). Scale bars = 1 μ m.

showed that high hydrodynamic shear forces were associated with a high frequency of autoaggregation in freshwater biofilms and that coaggregation was more frequent under lower-shear-force conditions. In the absence of coaggregation partners, autoaggregation may assist the exit of bacteria from the bulk fluid and subsequently stabilize the biofilm structure in the same way as coaggregation; however, the opportunity for metabolic cooperation would be reduced.

Direct comparison of coaggregating pairs with examples from humans is difficult because the species found in canine dental plaque are not usually found in human dental plaque. Although there are many shared genera, streptococci and fusobacteria are rare in dogs but are among the most prolific coaggregators in human dental plaque, along with *Actinomyces* species (9). Several *Actinomyces* species were isolated from canine samples, and these species were found to coaggregate with *Leptotrichia*, *Neisseria*, *Porphyromonas*, and *Streptococcus* species, consistent with their promiscuous coaggregation interactions in human dental plaque. The *Fusobacterium* isolate used in the assay of a single dog's oral microbiota did not

appear to have the nearly universal coaggregation behavior that is often reported for *Fusobacterium nucleatum* from humans; however, autoaggregation may have masked this property. Any coaggregation reactions of the *Actinobacillus*-like isolate haem1 could also have been masked by the strong autoaggregation of this organism.

Porphyromonas species have been recognized as important bridging organisms in human dental plaque; by having multiple coaggregation partners, some species facilitate the association of other species which do not coaggregate directly with each other (11). The results of this study suggest that *Porphyromonas* species may also do this in canine dental plaque; for example, *Porphyromonas gulae* (bpp6) was found to coaggregate with *Granulicatella* sp., *Streptococcus* sp., *Neisseria* sp., *Staphylococcus epidermidis*, *Actinomyces bowdenii*, and *Fusobacterium* sp.

The greater number of coaggregation interactions that were detected when the standard conditions were used than when the canine-adapted conditions were used may have been due to the fact that not all possible crosses were tested in the former experiment. The visual assay rather than a spectrophotometer

was used to assess coaggregation because it is easier to perform, and Cisar et al. (2) found that comparable results were obtained with both methods; however, Shen et al. (17) recently reported that a spectrophotometric method is more sensitive. Correction for autoaggregation had the undesirable effect of masking some interactions, but it was important to avoid the possibility of declaring false-positive interactions. It should also be noted that experimenting with a selection of growth media and conditions may have revealed additional or different interactions.

TEM revealed dense fimbriae on haem1, which may be related to the strong autoaggregation observed with this bacterium. Although the exact identity of this isolate was not established, based on its 16S rRNA gene sequence and its morphology, it seems likely to be a member of the genus *Actinobacillus* or *Haemophilus*. One such organism, *Actinobacillus actinomycetemcomitans*, is recognized as an opportunistic periodontal pathogen of humans and is known to be a highly adhesive organism (8). Nonfimbriated smooth-colony variants of *A. actinomycetemcomitans* have a reduced ability to adhere to hydroxyapatite and saliva-coated hydroxyapatite (16); therefore, the presence of fimbriae on this isolate may enable it to adhere to the tooth surface as a primary colonizer.

Coaggregates of lep1 with actino3 and coaggregates of fuso3 with cory2 were also clearly observed using TEM. The coat surrounding cory2 cells appears to be similar to the cell wall layering observed for corynebacteria by Puech et al. (13). This effect is due to the unusual structure of the cell envelope of corynebacteria, which contains an outer polysaccharide barrier layer whose function is similar to the function of the outer membrane of gram-negative bacteria. *C. felinum* was first isolated from a necrotic mouth lesion in a wild cat (3), and later the *C. felinum*-like organism (cory2) used here was isolated from the dental plaque of three dogs. Coaggregation of cory2 with a *Fusobacterium*-like species isolated from canine plaque was detected by TEM but not by the coaggregation assay, further highlighting the problem of autoaggregation masking coaggregation reactions. The genera *Leptotrichia* and *Fusobacterium* are closely related (4) but were found to have different coaggregation partners. The coaggregation differences may be related to the structural differences observed by TEM; lep1 has a very plain and uniform cell surface, but fuso3 has a complex undulating appearance.

In conclusion, our experiments showed that coaggregation occurs among the bacteria of the canine oral microbiota in a manner similar to the manner observed in many studies of bacteria isolated from human dental plaque. Although the species in these communities differ, genera common to both communities seem to exhibit similar coaggregation behavior. In particular, there is evidence that *Porphyromonas* species may perform a bridging function and that *Actinomyces* species also coaggregate prolifically in canine dental plaque. No uni-

versal coaggregators which could play a role similar to the role of *F. nucleatum* in human systems were detected, but this may have been due to masking by autoaggregation. Autoaggregation was found to be more common in bacteria of canine origin than in bacteria from humans, as reported in the literature, and this may indicate that primary colonizers rather than secondary colonizers have a selective advantage in canine dental plaque.

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