Colonization of a Portion of the Bovine Tongue by Unusual Filamentous Bacteria

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Tongue samples from cattle on varied diets and ranging in age from 2 months to adult were studied by transmission and scanning electron microscopy to observe the in situ distribution and adhesion patterns of two readily identifiable genera of filamentous bacteria. The two, both members of the *Simonsiellaceae*, adhere to the epithelium by means of fibers which are produced on one side of the bacterial filaments and subsequently display a sidedness in their manner of adhesion to epithelial surfaces. Other bacterial populations found on the tongue were normally members of chains and seldom present as single cells. This suggests that filamentous or chain-forming bacteria may have a selective advantage over single bacteria in their ability to colonize and remain attached to the epithelium of the tongue.

Unusual filamentous bacteria have been reported in the oral cavities of a variety of warm-blooded hosts, including humans (2, 3, 5), chickens (11), dogs (10), rabbits, and sheep (12). Based on their habitat and their unusual morphological characteristics, these aerobic gram-negative filamentous bacteria can be recognized as members of the *Simonsiellaceae* and can easily be classified to genus as either *Simonsiella* or *Alysiella*. Thus, morphological identification has played a key role in their classification (1, 2, 3, 11). Although more precise diagnostic tests are now being developed, especially for *Simonsiella* (7) the tests essentially serve only as a means of differentiating species or strains, with morphological characteristics still being the essential first step in classifying the filamentous bacteria to genera.

Simonsiella filaments exhibit a dorsal-ventral convex-concave differentiation with extensive extracellular fibrous material produced predominantly on the ventral surface (11). Investigators have suggested that this fibrous material may function in gliding and also in facilitating adhesion of the bacteria to epithelial surfaces (11). Although these bacteria are often found attached to desquamated epithelial cells when observed in saliva or oral smears (2, 3, 10, 11), these distinctive filamentous bacteria have not previously been observed in situ on the tissue.

We have made use of the distinctive morphological characteristics of these filamentous bacteria to study their distribution and adhesive characteristics in a natural system. The bacteria were observed by scanning electron microscopy

(SEM) and transmission electron microscopy (TEM) of tissue taken from the tongues of cattle of different ages and on different diets.

MATERIALS AND METHODS

Animals. Three Hereford bulls and one 6-month-old Holstein calf were maintained on a high-plane ration consisting of 60% barley, 10% oats, 10% beet pulp, and 20% chopped alfalfa. One other male Holstein (2 months) was maintained on a milk diet.

Preparation for electron microscopy. The animals were sacrificed, and at least three spatially separated tissue blocks (2 by 2 cm) were removed from the dorsal surface of the tongue. Two of the tissue blocks were prepared for SEM, and the third was reduced in size and prepared for TEM by techniques described in detail by McCowan et al. (9).

Axenic cultures were prepared for SEM as follows. Agar colonies were fixed in their plates in 0.5% glutaraldehyde in 0.067 M cacodylate (pH 7.2) for 2 h followed by a 2-h fixation in 5% glutaraldehyde in cacodylate. Cubes of agar with colonies attached were then removed and carried through the preparative procedure for SEM previously described (9).

Sections of ruthenium red-stained tissue were examined by using an AEI 801 electron microscope at an accelerating voltage of 60 kV. Samples prepared for SEM were examined on a Cambridge Stereoscan 180 or a Hitachi 450 scanning electron microscope at an accelerating voltage of 20 kV.

Analysis of populations. A minimum of four random sites (each 1 by 1 mm) per tissue sample were examined by SEM. Each site consisted of a suitably oriented papilla or epithelial surface and was examined at a magnification of $\times 1,000$ to $\times 2,000$. The distribution patterns and composition of the bacterial populations were noted. At these magnifications, relatively large areas of tissue could be seen and bacteria could

be distinguished from the epithelium. After this, the bacterial-epithelial interactions and the structures mediating the interactions were studied at higher magnifications from $\times 5,000$ to $\times 10,000$. TEM was used to provide details of the bacterial interactions with the tissue and yielded no information about the distribution of the colonizing populations.

Isolation of filamentous bacteria. Tissue blocks (2.5 by 2.5 cm) were excised from the tongue at sites adjacent to where samples for SEM and TEM were removed. Some tissue blocks were then impressed and subsequently spread on tryptose agar or nutrient agar plates. Other blocks were washed in phosphate-buffered saline (pH 7.4), placed in fresh phosphate-buffered saline, and homogenized in a Waring blender, and the resultant suspension was then spread on blood agar and nutrient agar plates. After 24 h of incubation at 37°C, colonies were examined for the presence of filaments.

The blood agar consisted of tryptose blood agar (Difco Laboratories) with 10% sheep blood. The nutrient agar consisted of 2.5% nutrient agar (Difco), 0.01% sodium acetate, and 0.5% yeast extract (Difco).

RESULTS

Simonsiella and *Alysiella* both consist of flat multicellular filaments. *Simonsiella* filaments are segmented into repeating packets of eight cells, with the terminal cells of each filament being rounded and smaller (Fig. 1). The broad sides of the filaments display a dorsal-ventral differentiation. As expected from previously described axenic cultures (11), we observed that the ventral, concave side of the filament produced most of the fibrous material, and it was that side which always faced the epithelial cells

to which the filaments adhered. On the tissue, the fibrous material was only visible on those bacterial filaments which were not completely adhering to the epithelial surface (Fig. 2). The ventral concave side of the *Simonsiella* filaments appeared to be strongly adherent, because this side of the filament always remained tightly opposed to the surface and closely followed the contours of the epithelium (Fig. 1).

Alysiella filaments are readily distinguished from those of *Simonsiella* (11, 13). The filaments consist of continuous pairs of cells rather than repeating packets of eight cells, and the terminal cells are not rounded but resemble the rest of the cells in the filament (Fig. 3, 6).

Another unusual feature which separates *Alysiella* from *Simonsiella* can best be seen in axenic culture (Fig. 4). *Alysiella*, like *Simonsiella*, produces an extracellular fibrous material asymmetrically, but instead of producing this material from the flat (ventral concave) side of the filament (11) the fibers are produced along the edge of the filament. This is the first time that this phenomenon has been reported in *Alysiella*. Again, the fibers appear to serve as a means of anchoring the filaments to the epithelial surface, but instead of holding the filaments flat against the epithelial cells, as with *Simonsiella*, the filaments form a palisade structure, with the result that far less of the bacterial filament is in actual contact with the host (Fig. 3, 6).

When examined in thin section (Fig. 5), the bacterial filaments appear to adhere to squa-

FIG. 1. Scanning micrograph of *Simonsiella* filaments adhering to the epithelial surface. The filaments are composed of repeating packets of eight cells, with the terminal cells rounded and smaller. The filaments lie flush against the epithelium and tightly follow the epithelial contours. The bar in this and subsequent scanning micrographs represents 5 μ m.

FIG. 2. One *Simonsiella* filament in this scanning micrograph is not fully attached to the epithelium (arrow), and the ventral fibrous material is therefore visible.

FIG. 3. Scanning micrograph of *Alysiella* filaments forming a palisade structure on the surface epithelium. The filaments consist of repeating bacterial pairs, with the terminal cells neither rounded nor smaller. The fibrous material along the edge of the filament (arrow) appears to anchor the filament to the epithelium.

FIG. 4. Scanning micrograph of an axenic culture of *Alysiella*. The filaments consist of repeating bacterial pairs which produce fibrous material only along one edge of the filament (arrows).

FIG. 5. The filaments in this sectioned material appear to produce ruthenium red-stained fibers from one side of the filaments only (large arrows). These filaments appear to bind to the epithelium and to each other by means of the fibrils. A thin ruthenium red-staining coat surrounds the lobulate gram-negative cell wall (small arrows). The bar in this and the other sectioned sample represents 0.5 μ m.

FIG. 6. Scanning micrograph of a mixed population on the epithelium. Note that most of the bacteria are present as filaments or as members of chains. The palisade-forming filaments, consisting of repeating bacterial pairs with the terminal cells identical to the rest, are those of *Alysiella*.

FIG. 7. Scanning electron micrograph of a mixed population on the epithelium. Almost all the bacteria are either filamentous or chain forming, and many follow the contours of the epithelial cells, often appearing wedged between them (arrows).

FIG. 8. Electron micrograph of sectioned epithelial surface. The extent of bacterial penetration is clearly visible. The terminal end of one filament (arrow) appears wedged between 2 epithelial cells and held in place by fibers. A single bacterial cell, identical in ultrastructure to the filaments, has penetrated below the epithelial surface and has become wedged between 3 epithelial cells.

FIGS. 1-4.

FIGS. 5-8.

mous epithelial cells and to each other by means of ruthenium red-staining fibrils which emanate from one side of the filament. Also, the bacteria have a thin, intensely ruthenium red-positive coat which reflects the lobulate contours of their gram-negative cell walls. The ruthenium red character of these external components suggests that they are composed, at least partially, of acid polysaccharide (8).

In terms of the total adherent bacterial populations on the tongue, several trends were observed. Very few bacteria were present on the papillae covering the tongue. The majority were found either between the papillae or at their base and were usually in micro regions which provided the bacteria with some protection from mechanical abrasion.

The majority of bacteria remaining after washing were present either as filaments or as members of chains (Fig. 6). The bacteria both in chains and in filaments often followed the contours of the epithelial surface in such a way that they appeared wedged between adjacent epithelial cells (Fig. 7). The bacteria may penetrate some distance under the superficial squamous cells (Fig. 8). Terminal members of one filament sit between folds in the surface, whereas another bacterial cell of identical ultrastructure has apparently become wedged under one superficial epithelial cell. Ruthenium red-stained fibers can be seen linking the bacteria to the epithelial surface.

The filamentous bacteria displayed a contagious distribution on the tongue. That is to say that, at any site examined, if any of the filamentous bacteria were present they were usually in high numbers. *Alysiella* and *Simonsiella* were seldom found to occupy the same microsite together. At no time did we find that they colonized a site to the exclusion of other bacteria.

DISCUSSION

The dorsal surface of the tongue is populated predominantly by chain-forming, and to a lesser degree, by filamentous bacteria (Fig. 6). The high frequency of chain-forming and filamentous bacteria on the surface suggests that there may be some selective advantage given to the adherent bacteria when in this form. The bacteria often follow the contours of the epithelial cells and appear to be wedged between the cells (Fig. 7, 8). This wedging may function as a means of holding the cells on the surface and could aid other adhesion factors such as extracellular polysaccharides in securely fixing the bacteria to the surface (Fig. 8). The high rate of sloughing of squamous cells from the epithelium may give an advantage to chain-forming and filamentous

bacteria which are able to cover more than one epithelial cell. As the epithelial cells, with their colonizing bacteria, slough off the surface, they will expose new surfaces for colonization and will leave behind segments of chains or filaments on neighboring epithelial cells which are now advantageously positioned to colonize the virgin surfaces. The only other route available to colonizing bacteria would be attachment to the epithelium from the saliva, which may not be as favorable a method. A bacterial chain in which each individual cell adheres to the epithelial surface may be less susceptible to being mechanically removed than are single cells.

The importance of extracellular fibers to bacterial adhesion is indicated in our observations of two bacterial strains which produce this material asymmetrically. Both *Alysiella* and *Simonsiella* filaments were anchored to the host epithelium by that side of the filament which carried the fibrous coat. That this fibrous coat at least partially consists of anionic polysaccharide is suggested by its ruthenium red-staining characteristics in sectioned material (Fig. 5, 8) (8).

The relatedness of *Alysiella* to *Simonsiella* can be seen in that they both form flat filaments, are normal oral residents in most warm-blooded animals, and show a sidedness reflected in their production of extracellular fibers. However, while *Simonsiella* produces this material on the concave ventral side of the filaments (11) (Fig. 2), *Alysiella* extrudes the fibers along the filament edge (Fig. 4). This difference is reflected in the manner with which the filaments adhere to the epithelium. The filaments of *Simonsiella* lie flush against the epithelial cells, ventral side down (Fig. 1, 2), whereas the filaments of *Alysiella* are anchored to the epithelium along their edge (Fig. 3, 6), giving them a distinct palisade structure when viewed on the tissue.

Because of their distinct morphology, both genera of *Simonsiellaceae* can be readily identified and studied in situ by electron microscopy. That these bacteria are normal residents of the bovine oral cavity is strongly suggested, since they were observed on the dorsal surface of the tongue in all the animals studied which ranged in age from 2 months to full adults and in diet which ranged from milk only to a high-plane diet.

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