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Surface-Surface Associations in Microbial Communities Populating Epithelial Habitats in the Murine Gastrointestinal Ecosystem: Scanning Electron Microscopy

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Scanning electron microscopy has been used to visualize the residents of microbial communities populating habitats on epithelial surfaces in the gastrointestinal tracts of mice. In the stomach, bacteria form a dense layer on the stratified squamous epithelium of the nonsecreting area. Microbes of at least three morphological types can be seen in this layer, including short rods with round ends, rods in chains, and tapering filaments composed of repeating units of rod- or coccal-shaped elements varying in size from large at one end of the filament to small at the other end. These three forms all attach by one end to the epithelium. The latter two forms can be found only so attached; in both cases, the end is inserted into a hole or depression in the keratinized epithelium. In the small intestine, a microbe of morphology similar to that of the tapering filaments found in the stomach can be seen attached end-on to the epithelium. Again each filament has one end inserted into a hole in the epithelium. In this case, however, the repeating elements of each filament are all about the same size. In the cecum and colon, predominantly fusiform- and spiral-shaped microbes can be seen mixed together in layers on the epithelium. At least three types of fusiform-shaped microbes can be distinguished on the basis of surface texture, and one type of spiral-shaped microbe can be found. These microorganisms appear to be attached to each other and to the epithelium by weblike filaments. The numerous microbial types present in the various epithelial habitats associate intimately surface-to-surface with each other and with the epithelium. Such surface-surface association may be an important autogenic factor contributing to the stability of the murine gastrointestinal ecosystem.

The murine gastrointestinal ecosystem consists of numerous distinct microbial habitats (2, 13, 16). Each of these habitats is colonized normally by a characteristic community composed of populations of one or more types of autochthonous microorganisms (2, 4, 7, 8, 13, 14). Some of these autochthonous species are known to attach to or otherwise associate with gastric or intestinal epithelia in the habitat they colonize (1, 2, 11-14). The mechanisms of these microbe-epithelium associations are understood poorly (12). Moreover, the organization and structure of the microbial communities on the epithelial surfaces is only dimly appreciated. We have used scanning electron microscopy to examine these communities in their epithelial habitats in the gastrointestinal tracts of mice. Reported herein are our findings on the organization of the communities and the way various microbial types associate with the epithelial surface and with each other.

MATERIALS AND METHODS

Animals. Seven male CD-1 mice, 8 to 10 weeks of age, were obtained from the caesarean-originated, barrier-sustained colony of Charles River (Wilmington, Mass.). They were maintained in our animal room in plastic cages with paper covers (Isocage, Carworth-Bioquest, New City, N.Y.) and given acidified water (15) and commercial mouse food (Wayne, Allied Mills, Chicago, Ill.).

Preparation of specimens for scanning electron microscopy. Animals were sacrificed under ether anesthesia. Their gastrointestinal tracts were exposed quickly, but left in place. Cotton pads soaked in cold (4 C) fixative (2% glutaraldehyde in phosphate buffer, pH 7.3) were placed over the internal organs, including the gastrointestinal tract. Then, as quickly as possible, cold fixative was injected into the lumen of

each major segment of the tract. Immediately thereafter, from each mouse, four to five samples were taken from different areas of the mucosa of both the stomach and cecum. In addition, four to five short segments were taken from both the small and large intestines. The entire length of intestine was sampled in this manner. The samples were placed in vials containing cold fixative. After 1 h, they were placed in fresh fixative and then stored at 4 C for 24 h. They then were washed free of the glutaraldehyde fixative with four changes of cold phosphate buffer, pH 7.3, placed in cold 2% osmium tetroxide in phosphate buffer, pH 7.3, for 2 h, and then again washed, this time with three changes of the phosphate buffer. Thereafter, they were dehydrated with ethyl alcohol, infiltrated with amyl acetate, dried in a critical-point drying apparatus, fixed to aluminum stubs, and coated with carbon-gold-palladium.

Scanning electron microscopy. The coated specimens were examined in a Cambridge Mark II Stereoscan scanning electron microscope (Cambridge Instrument Co., Ltd., Cambridge, England). Photographs of the scanned fields were taken at 20 kV with Polaroid positive/negative film, type 55.

RESULTS

Stomach. In preparations from the nonsecreting portion of the stomach, bacteria in dense layers could be seen on the keratinized stratified squamous epithelium (Fig. 1A). Microbes of at least three morphological forms could be distinguished in the layers (Table 1). Most frequently seen were short rods with rounded ends (Fig. 1B). Also seen were rods in chains of two or more cells (Fig. 1C) and long tapering filaments composed of repeating units of rod- or coccal-shaped bodies varying in size from large at one end of the filament to small at the other end (Fig. 1D).

All three of these forms could be found attached by one end to the epithelium (Fig. 1B,E,F). The latter two forms were only found so attached (Fig. 1E,F). In both of these cases, one end of the chain of rod-shaped cells and the large end of the tapering filament were inserted into a hole or depression in the keratinized epithelium.

Small intestine. In preparations from the small intestine, a filamentous microbe also could be seen attached end-on to the epithelium (Fig. 2A, Table 1). Again, one of the ends of the organism was inserted into a hole or depression in the epithelium (Fig. 2B–D). Occasionally, two or more of the filaments were anchored in the same hole (Fig. 2D). The filaments of the microbe were composed of repeating rod- or coccal-shaped bodies similar to those making up the filaments of the microbe found in the stomach (Fig. 2E). In this case, however, the repeating elements did not appear to vary in size from large at one end to small at the other (Fig. 2F).

Cecum and large intestine. The microbial communities on the epithelium appeared to be similar in composition in the preparations of mucosa from cecums and colons. Depressions in the epithelium marking openings to the crypts of Lieberkuhn could be seen in the preparations scanned at low magnifications (Fig. 3A). These depressions usually were filled with masses of microorganisms (Fig. 3B). When the preparations were scanned at higher magnification, the masses in the crypts proved to be composed mostly of fusiform- (Fig. 3C) and spiral-shaped (Fig. 3D) microorganisms. Masses of microbes were seen also in thick layers on the epithelium (Fig. 3E). These layers covered much of the epithelium, especially in preparations from colons. They were composed largely of fusiform- and spiral-shaped microorganisms (Fig. 3F).

When the preparations were scanned at higher magnifications, two types of fusiform-shaped microbes with quite different surface textures could be seen most often (Fig 4A). One of these organisms had a wrinkled surface; the other seemed to be generally smooth in texture with only an occasional wrinkle (Fig. 4B). Another type of fusiform-shaped microbe with a bumpy surface also could be seen infrequently in the preparations (Fig. 4C, Table 1).

Rod-shaped microbes other than fusiform-shaped organisms occasionally were found in the preparations. At high magnifications, these microbes could be seen to have rounded ends and a bumpy surface texture (Fig. 4D, Table 1).

Also seen only occasionally was a small curved microbe that seemed to have somewhat pointed ends (Fig. 4C, Table 1). This organism was seen in close association with much larger fusiform-shaped organisms. What appeared to us to be another small organism also could be seen in close association with the large rods with pointed ends (Fig. 4E, Table 1). In this case, however, the tiny microbe appeared to be a straight rod with pointed ends clamped onto the surface of the larger cell. The attachment of the smaller to the larger cell seemed to be mediated by filamentous appendages.

Scanning the preparations at high magnifications revealed only one morphological type of spiral-shaped microbe (Fig. 4D,F, Table 1).

Scanning the preparations at high magnifications revealed also that the fusiform-shaped microbes often appeared to be connected to

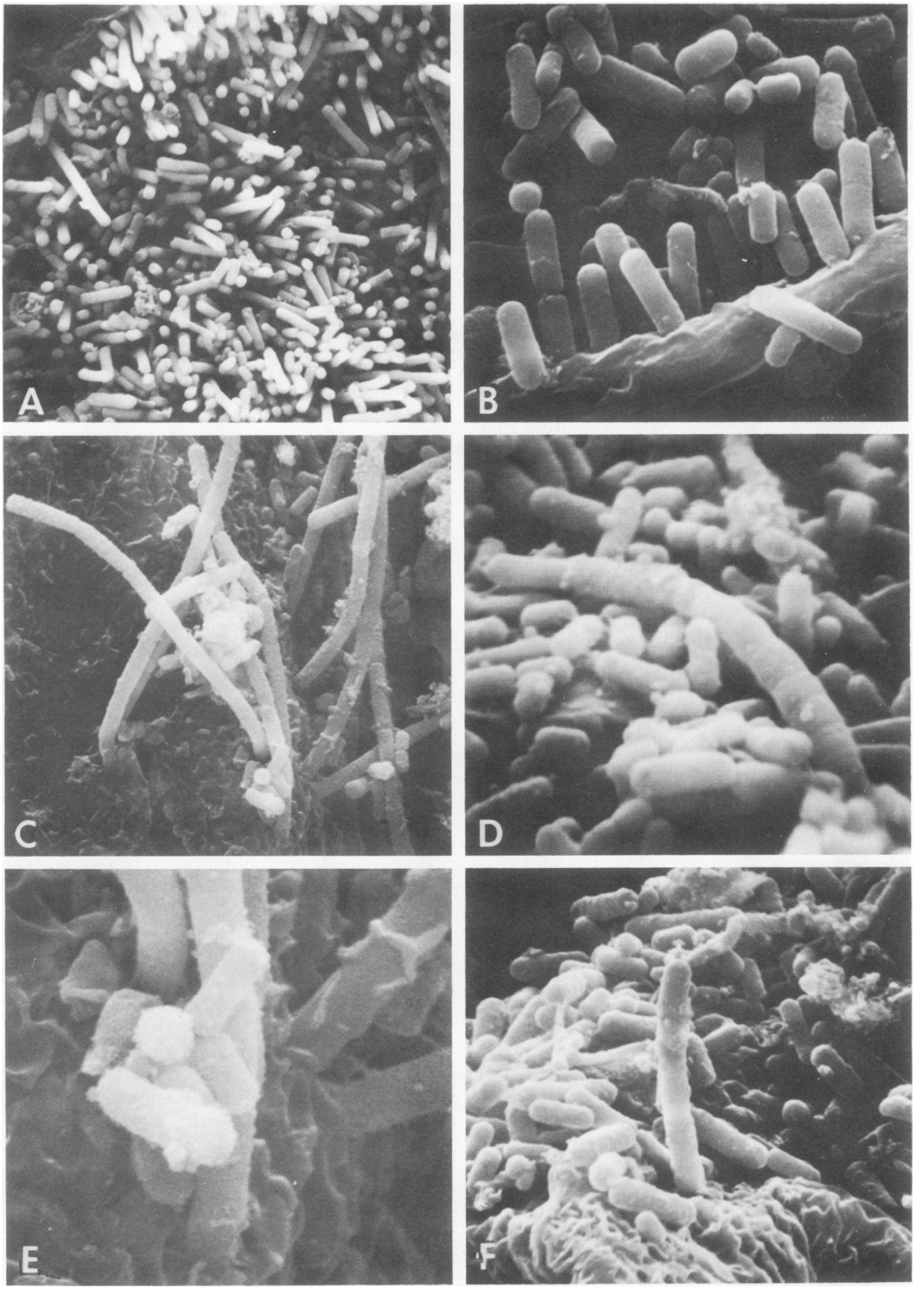

FIG. 1. *Microbial community as viewed with the scanning electron microscope on the surface of the keratinized stratified squamous epithelium of the stomachs of adult CD-1 mice. (A) Overview of the community at low magnification showing the population density, heterogeneity, and end-on attachment to the epithelium of the microorganisms. ×1,785. (B) End-on attachment to the epithelium of the most numerous microbial type in the community, short, rod-shaped bacteria that are probably Lactobacillus. ×4,930. (C) End-on attachment to the epithelium of filaments composed of several rod-shaped bacteria of uniform length. ×2,100. (D) Long microbial filament composed of repeating units of rod- or coccal-shaped elements large at one end of the chain tapering to small at the other end. ×6,120. (E) Higher magnification of the site of attachment illustrated in (C). The ends of the filaments are inserted into holes in the keratinized epithelium. ×8,500. (F) End-on attachment to the epithelium of a microbial filament similar in structure to the one shown in (D). ×4,420.*

TABLE 1. *Frequency of observation in preparations viewed in the scanning electron microscope of various indigenous microbial types on the epithelial habitats of the gastrointestinal ecosystem of male CD-1 mice*

Epithelial habitat	Microbial type	Frequency[a]
Gastric nonsecreting	Short rods, round ends	7/7
	Short rods in chains	7/7
	Rod- or coccal-shaped forms in tapering filaments	2/7
Small intestinal	Rod- or coccal-shaped forms in filaments	7/7
Large intestinal	Fusiform-shaped, smooth surface	7/7
	Fusiform-shaped, wrinkled surface	7/7
	Fusiform-shaped, bumpy surface	7/7
	Spiral-shaped	6/7
	Short rods, round ends, bumpy surface.	7/7[b]
	Tiny curved rod with pointed ends associated with fusiform-shaped bacteria	7/7
	Tiny straight rod with pointed ends attached by filaments to fusiform-shaped bacterium	7/7

[a] Number of mice in which microbe was seen/number examined.

[b] Seen in every animal examined, but only in a few fields.

each other (Fig. 5A) and to the epithelium (Fig. 5B) by tangled, weblike filaments. Likewise, when fusiform- and spiral-shaped microbes were associated intimately, the organisms seemed to be connected to each other and to the epithelium by filaments (Fig. 5C). The filaments often were associated closely with globules of some material present both on the surface of the bacteria (Fig. 5B,D) and on the epithelial surface (Fig. 5D).

DISCUSSION

The murine stomach is known to harbor populations of autochthonous lactic acid bacteria. One or more species of *Lactobacillus* (2, 13, 16) and often one or more species of group N streptococci (2, 16) can be cultured from the keratinized stratified squamous epithelium of the nonsecreting portion of the mouse stomach. Likewise, layers of gram-positive bacteria, principally rod-shaped, can be seen on the keratinized epithelium in frozen sections of murine stomach viewed in a light microscope (2, 12–14). Such gram-positive layers also can be seen in the stomachs of ex-germfree mice monoassociated with a particular *Lactobacillus* strain isolated from a conventional mouse stomach (19). Therefore, at least some of the microbes seen with the scanning electron microscope on preparations of the squamous epithelium of the mouse stomach are undoubtedly lactobacilli.

Particular candidates for this identification are the short rods with round ends. These organisms outnumbered all others in the preparations. In fact, their population levels appeared to us to be far higher than those of the rods in chains and the tapered filaments. Therefore, the populations of these individual rods are probably composed of one or more species of *Lactobacillus*.

No identity can be suggested with as much confidence for the rods in chains found on the gastric epithelium. These microbes are similar morphologically to lactobacilli and, because they are found in the gastric habitat, could be tentatively identified on ecological grounds as lactobacilli. However, an identification made on such grounds cannot be taken too seriously. Therefore, further investigation is required before these particular organisms can be identified.

Likewise, we cannot name the tapered filaments. To the best of our knowledge such organisms have never been reported to be present in the murine stomach. Moreover, we know of no reports that microbes of similar morphology have ever been cultured from the murine gastrointestinal ecosystem.

Organisms of similar morphology have been seen, however, attached to epithelial cells in preparations of rat and mouse ilea viewed by light (11) and scanning and transmission electron microscopy. These microbes were identified tentatively as members of the order *Caryophanales* (C. P. Davis, S. L. Erlandsen, and D. C. Savage. Abstr. Annu. Meet. Amer. Soc. Microbiol., 1973, p. 57). They differ morphologically, in some respects (Fig. 2A) from the ones we saw in the stomach (Fig. 1D), but may differ only because they are growing in different habitats. Such a decision cannot be made with any certainty until one or more of the organisms is cultured in vitro in recognizable morphological form.

Whatever their identity, the microbes attached to the epithelium in the stomachs and small intestines must be important inhabitants of the ecosystem. In particular, their ability to attach to epithelia must give them survival

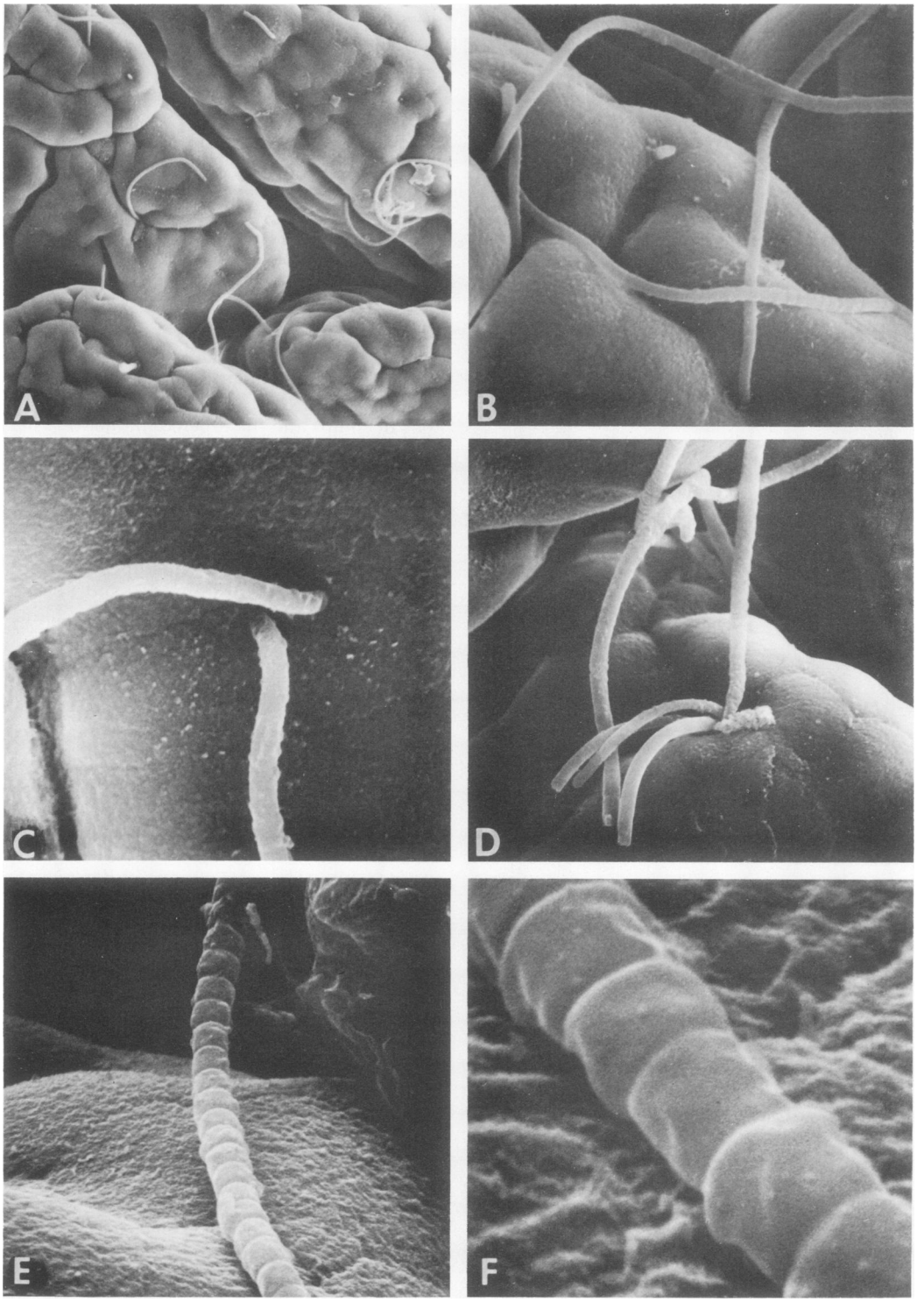

FIG. 2. *Microbial community as viewed with the scanning electron microscope on the surface of the columnar epithelium covering the villi in the ilea of adult CD-1 mice. (A) Overview at low magnification. The tips of four villi appear as grayish masses covering the entire area of the photograph. Long filamentous microorganisms lie on the surface of the villous epithelium. One end of each filament is inserted into a hole in the epithelium. ×425. (B) View more highly magnified than (A) showing filaments inserted into holes in the villous epithelium. ×1,700. (C) View more highly magnified than (B) showing holes in epithelium into which microbial filaments are inserted. ×4,250. (D) View showing that more than one microbial filament may appear to be inserted into the same hole. ×1,998. (E) Microbial filament lying on the epithelium. The filament is composed of repeating units of rod- or coccal-shaped elements of uniform size and shape. ×5,225. (F) View more highly magnified than (E) of microbial filament lying on the epithelium. ×18,700.*

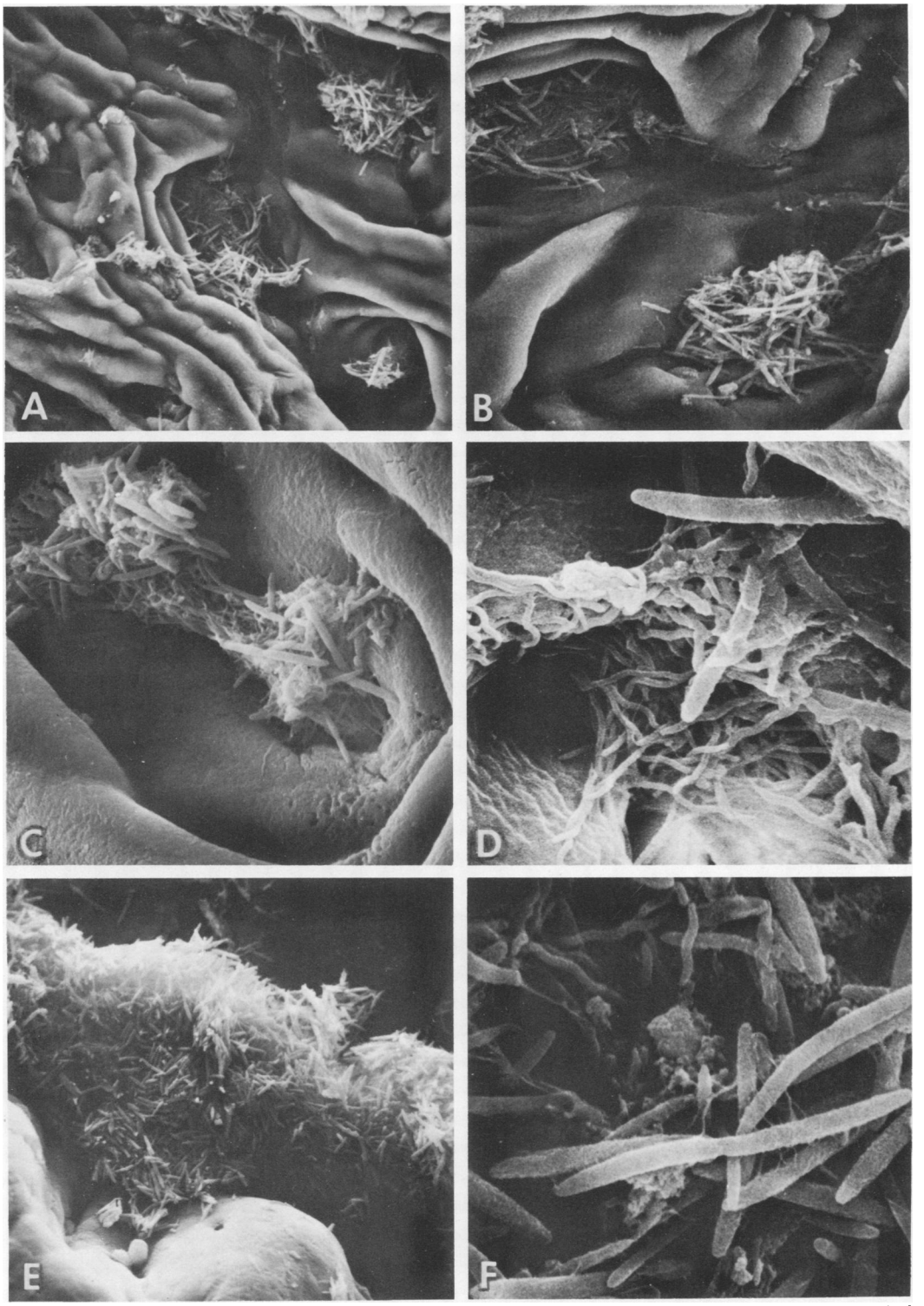

FIG. 3. *Microbial community as viewed with the scanning electron microscope on the surface of the columnar epithelium of the colons of adult CD-1 mice. (A) Overview at low magnification of the surface of the colonic epithelium. Each hole in the wrinkled surface is the entrance to a crypt of Lieberkuhn. The openings to most of the crypts are filled with masses of microorganisms. ×476. (B) Surface of the colonic epithelium viewed at higher magnification than in (A). Openings to two crypts of Lieberkuhn are filled with masses of microorganisms. ×956. (C) View of colonic epithelium more highly magnified than (B) showing that the openings to the crypts are filled with masses of fusiform- and spiral-shaped microorganisms. ×1,700. (D) View of opening to crypt in colonic epithelium showing that fusiform- and spiral-shaped microbes associate intimately with each other and the epithelium around the mouth of the crypt. ×4,760. (E) Overview at low magnification of the colonic epithelium (lower left of photograph) showing a thick layer of microbes on the surface of the epithelium. ×553. (F) View of mass of microbes on surface of colonic epithelium more highly magnified than in (E). The mass contains fusiform- and spiral-shaped microbes. ×4,760.*

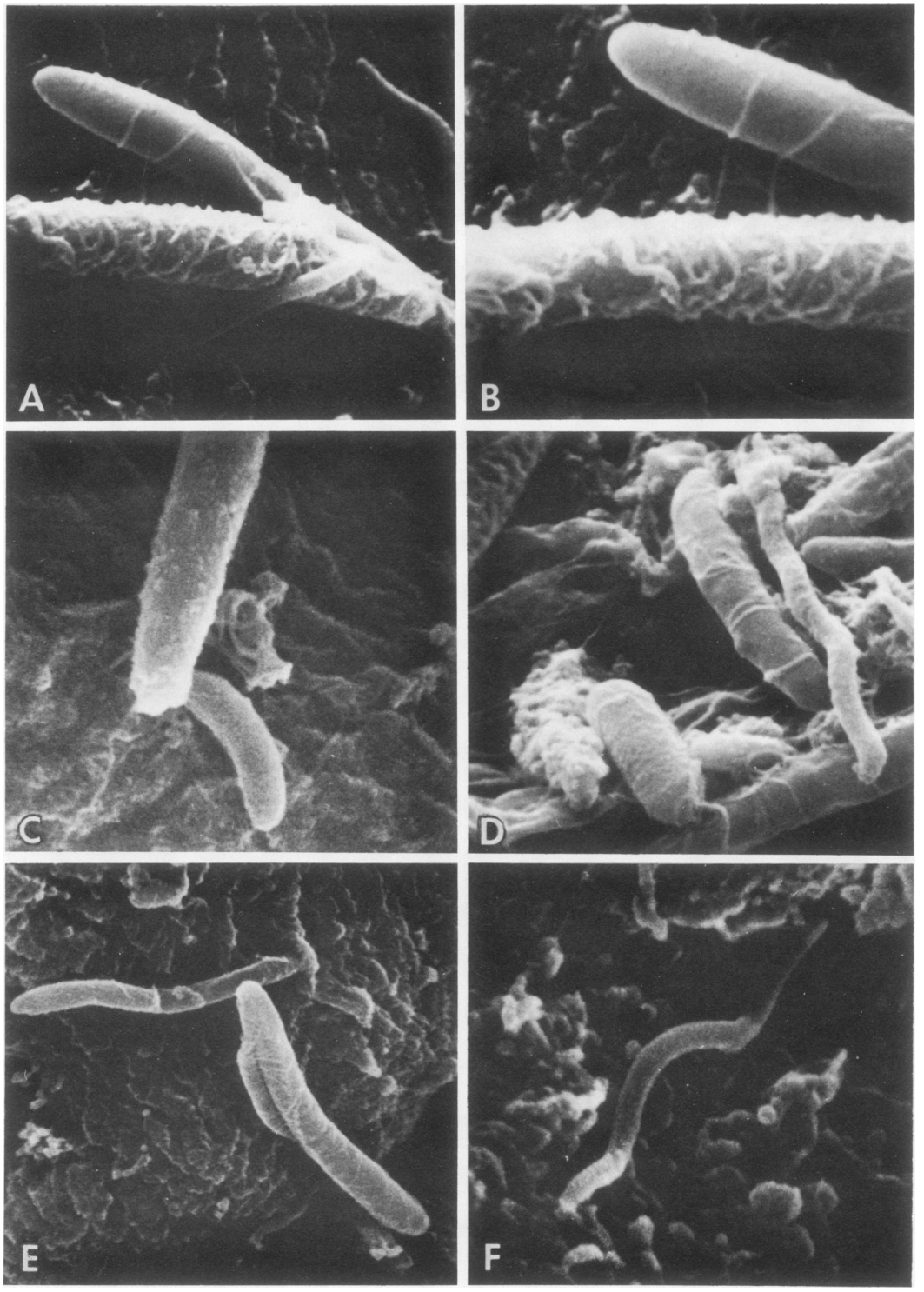

FIG. 4. *Highly magnified views obtained with the scanning electron microscope of individual microbial types resident in the communities populating the surface of the epithelia of the cecums and colons of adult CD-1 mice. (A) Fusiform- and spiral-shaped microorganisms in intimate association with each other and the epithelial surface. ×14,365. (B) Highly magnified view of fusiform-shaped bacteria shown in (A). The textures of the surfaces of the two microbes differ markedly, suggesting that the two are different microbial types. ×22,100. (C) Tip of a large fusiform-shaped bacterium partially covering a small curved rod-shaped microbe. The latter microbial type was seen frequently in the microbial masses, but always near a large fusiform-shaped*

advantage in the habitats they occupy, i.e., the higher reaches of the gastrointestinal tract. Microbes able to attach to epithelia would not be propelled down the tract by normal gastrointestinal motility. Thus, they would have a selective advantage over microbes unable so to attach for growing in a habitat high in the tract.

Such attachment need not necessarily be end-on, and yet all of the organisms seen on the epithelia of the stomach or small intestine could be found so attached. Some of the forms could be found *only* so attached. Therefore, end-on attachment must have some significance in the ecosystem. Unfortunately, that significance is difficult to assess. Such organization would allow more bacterial cells per unit area to contact the epithelium. But the survival advantage to the bacteria of such contact is unknown. Likewise, it would allow the bacterial cells to contact each other over a greater surface area, and possibly thus facilitate transfer of metabolites from one bacterium to another. Again, though, the need for intimate cell-to-cell contact in such transfer is not known. Thus, at this time the ecological significance of end-on attachment to epithelia of rod-shaped bacteria in the mouse stomach and small intestine is simply unknown.

Likewise, not a great deal is known about the mechanisms by which the microbes attach end-on or otherwise to the epithelia in the murine gastrointestinal tract. The lactobacilli may attach to the keratinized epithelium via acid mucopolysaccharides on their surfaces (A.Takeuchi and D. C. Savage. Abstr. Annu. Meet. Amer. Soc. Microbiol., 1973, p. 115). In contrast, certain other microbial types may adhere to teeth and other surfaces via trypsin-sensitive macromolecules (9). Thus, microbial adherence to surfaces of animal cells may be mediated by macromolecules of several types. Generally, however, the specific substances involved in adherence of microbes in the murine gastrointestinal tract are not known.

In a similar vein, little is understood of the mechanisms of the various microbial associations we saw on the epithelia of the cecums and colons of mice. Fusiform- and spiral-shaped bacteria (12–14) have been known for some time to colonize the epithelium of the cecal and colonic mucosa of both mice (12) and rats (1). In histological sections, especially of proximal colon, the microbes frequently can be seen in dense layers on the epithelial surface (12–14). In preparations of colonic epithelium viewed in the scanning electron microscope, we also saw such thick dense layers on the epithelium (Fig. 3D). The layers covered much of the epithelium in most preparations. They cannot be seen, however, in Fig. 3A–C. These figures illustrate the appearance in the scanning electron microscope of the mucosal epithelium. Such views of the epithelium were difficult to find microscopically when the mucosa had been prepared so that the thick microbial layers were preserved intact. We believe that the thick layers are washed away relatively easily, and now exercise great care in preparing the specimens so as to preserve them. Nevertheless, even when the layer cannot be seen, the crypts of Lieberkuhn in both cecal and colonic mucosal are packed full of spiral- and fusiform-shaped microbes. The crypts may serve as reservoirs of these microbes when the surface layers are removed in the living animal.

Fusiform- and spiral-shaped microbes also have been cultured from both the cecums and colons of mice (4, 7, 8, 14). Fusiform-shaped bacteria of three genera, *Clostridium*, *Fusobacterium*, and *Eubacterium*, have been cultured from mouse cecums (4). Spiral-shaped microbes cultured from that source have yet to be identified (4, 14). At least three morphological forms of these latter organisms can be seen by transmission electron microscopy in negatively stained preparations of sediments washed from cecal epithelia from mice of various strains (14). In mice of any one strain, though, only two spiral-shaped forms could be found. Thus, the epithelial habitats could be populated by fusiform-shaped bacteria of at least three genera and spiral-shaped microorganisms of at least two morphological forms.

Those findings were confirmed in part and extended by our observations with the scanning electron microscope of preparations of cecal and colonic mucosa. In agreement with the earlier findings, fusiform-shaped bacteria of at least three types could be distinguished on the basis of surface texture. But in contrast to the earlier findings, only one morphological type of spiral-shaped microbe was seen in the scanned prepa-

microbe of the type shown. This particular fusiform-shaped bacterium had a lumpy surface that differed from the surfaces of both of the types shown in (A) and (B). ×19,040. (D) Fusiform- and spiral-shaped microbes near a rod-shaped bacterium with rounded ends. The latter microbial type was seen occasionally in the masses of microbes on the epithelial surface. ×16,065. (E) Fusiform-shaped bacterium to which a small rod-shaped microbe is attached intimately by some fine filaments. This latter small microbe was only seen attached in such a manner to larger fusiform-shaped bacteria. ×9,563. (F) Spiral-shaped microbe on the surface of the epithelium. ×20,655.

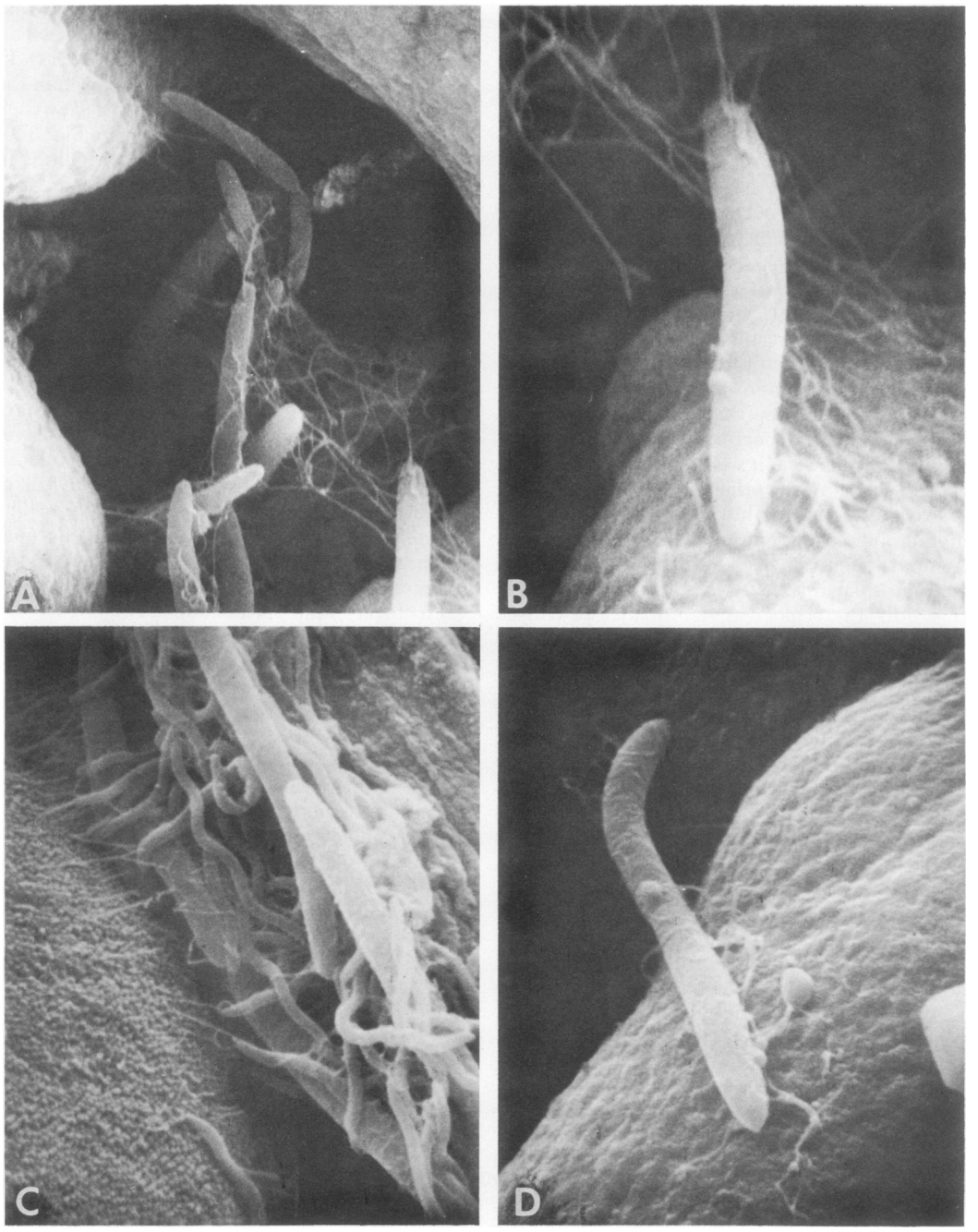

FIG. 5. *Views obtained with the scanning electron microscope of microbial communities resident on the surface of the epithelia of the cecums and colons of adult CD-1 mice. (A) Fusiform-shaped bacteria at the opening of a crypt of Leiberkuhn. The bacteria are connected one to the other and to the epithelium by a web of fine filaments. ×5,865. (B) Fusiform-shaped microbe connected to the epithelium by weblike filaments. ×11,900. (C) Fusiform- and spiral-shaped microbes intimately associated one with the other and with the epithelium. As in (A), these organisms are connected to each other and to the epithelium by weblike filaments. ×9,350. (D) Fusiform-shaped microbe on the epithelium with associated filaments and globules. ×9,818.*

rations. Mice used in this study were obtained from one of the suppliers (Charles River) from which animals were purchased for one of the earlier studies (14). We have no explanation of why two types of spiral-shaped microbes were found in the animals in the previous study, and only one type in this study. Quite possibly, scanning electron microscopy is not useful for discriminating morphologies of spiral-shaped microbes of certain types. We are still studying this problem.

The earlier findings were extended by our observations on the cecal and colonic epithelia of some rods with round ends, some small curved rods with pointed ends found closely associated with fusiform-shaped bacteria, and a tiny rod-shaped organism with pointed ends found attached to the surface of fusiform-shaped bacteria. The latter two are of especial interest because of their apparent close association with the much larger fusiform-shaped microbes. At present, however, their identity is not known.

The microbial community populating the epithelial habitat in the cecum and colon is composed of fusiform- and spiral-shaped microbes and a few microbes with other morphological forms. These organisms are mixed together in complex masses that do not seem to have any particular organization. They associate closely one with another, however, and also with the epithelial surface upon which they live. Some of them seem to be bound to each other and to the epithelium by weblike filaments.

Using the scanning electron microscope, we have so far been unable to resolve the identity of these tangled filaments. At least some of them may be known bacterial appendages such as flagella or pili. However, some may be strands of mucus or mucus-like material. They are often associated with globules of material on the surface of the bacteria and the epithelium. Whatever their identity, they may be holding the microorganisms in close association with each other and with the epithelium.

We believe that this close association is an important factor in the structure and stability of these microbial communities. As noted earlier, microorganisms of dissimilar types attached to an epithelial surface may find nutritional value in being closely associated with each other. Metabolic products of one microbial type are known to be used as nutrients by other types in natural environments (6). Any such matter produced by bacteria on a mammalian epithelial surface could be absorbed quickly by that epithelium and thus not be available as nutrients for other microbes unless the organisms were intimately associated one with the other. Similarly, one microbial type could feed another by excreting enzymes that hydrolyze mucins (5) or other substances near the epithelium. Again, though, the products of this hydrolysis might be absorbed quickly by the epithelium. So any microbial recipient of this largesse, itself unable to produce the hydrolases, would have to be in close contact with the producer. The cell-to-cell contact we see in these microbial communities would be the most efficient circumstance for such sharing.

The close association with epithelial surfaces of the various microbial types in the alimentary tract has already been discussed in part. As noted, microbes able to attach to epithelia would have a survival advantage in that habitat over organisms not able to so attach. The gastrointestinal tract can be viewed as a flowing stream. Any microbes able to attach to the bed of the stream could remain in the habitat; any unable to attach would be washed down stream.

Indigenous microorganisms of several types are known to attach to or otherwise associate intimately with epithelial surfaces in the gastrointestinal tracts of rats (1, 11, 12), mice (2, 12-14), chickens (3), swine (2, 18), monkeys (17), and humans (10). Therefore, microbial attachment to epithelial surfaces must be an important autogenic factor in maintaining stability in these ecosystems.

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