Microbial Attachment and Feed Digestion in the Rumen[1,2]

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ABSTRACT: Direct microscopic examination of the rumen and its contents shows microbial populations largely attached to feed particles in the digesta. Most feeds contain a surface layer that is resistant to attachment and therefore to digestion. Infiltration of these recalcitrant epidermal layers through damage sites or through focused enzymatic attack is essential for initiation of the digestive process. Proliferation of primary colonizing cells produces glycocalyx-enclosed microcolonies. Secondary colonizers from the ruminal fluid associate with microcolonies, resulting in the formation of multispecies microbial biofilms. These metabolically related organisms associate with their preferred substrates and produce the myriad of enzymes necessary for the digestion of chemically and structurally complex plant tissues. Upon accessing the internal, enzyme-susceptible tissues, microbial "digestive consortia" attach to a variety of nutrients, including protein, cellulose, and starch and digest insoluble feed materials from the inside out. Substances that prevent microbial attachment or promote detachment (e.g., condensed tannins, methylcellulose) can completely inhibit cellulose digestion. As the microbial consortium matures and adapts to a particular type of feed, it becomes inherently stable and its participant microorganisms are notoriously difficult to manipulate due to the impenetrable nature of biofilms. Properties of feed that place constraints on microbial attachment and biofilm formation can have a profound effect on both the rate and extent of feed digestion in the rumen. Developments in feed processing (i.e., chemical and physical), plant breeding, and genetic engineering (both of ruminal microorganisms and plants) that overcome these constraints through the promotion of microbial attachment and biofilm formation could substantially benefit ruminant production.

Key Words: Rumen, Bacteria, Fungi, Protozoa, Bacterial Attachment, Feed Digestion

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Introduction

Microorganisms in aquatic ecosystems as diverse as alpine streams and the bovine rumen have a natural tendency to interact with and attach to surfaces (Matthysse, 1992; Pell and Schofield, 1993). In newborn ruminants, this natural phenomenon is observable 38 h after birth as bacteria from the ruminal fluid attach to and colonize the tissues of the digestive tract (Cheng et al., 1991a). Bacteria attached to the wall of the rumen sequester oxygen, hydrolyze urea and, in conjunction with bacteria in the fluid phase, modify the ruminal environment. Fungi and protozoa become established in the ruminal microbiota within 8 to 10 d and 12 to 20 d, respectively (Fonty et al., 1987, 1988; Stewart et al., 1988). This complex succession pattern culminates in the establishment of complex multispecies consortia that are often encased within biofilms and are adapted to specific microenvironments on the digestive tract tissue surface, in the ruminal fluid, and on the surfaces of feed particles. Attachment is pivotal in the development of surface-associated populations and is largely responsible for their inherently stable nature. The role of microbial populations attached to the tissues of the ruminant digestive tract has been addressed previously (Cheng et al., 1979; Cheng and Costerton, 1980; Cheng et al., 1981b); this paper will focus on updating the role of microbial association and attachment in the ruminal digestion of feed.

Association and Attachment of Microorganisms to Feed

Ruminal microorganisms rapidly associate with and attach to recently ingested feed particles (Cheng et al., 1983/84; Craig et al., 1987). Attachment to

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substrates is accomplished through specific mechanisms requiring adhesins and(or) receptors and nonspecific mechanisms that rely on physicochemical forces such as van der Waals forces (Pell and Schofield, 1993). Bacteria and protozoa often attach to plant tissues within 5 min after ingestion (Bonhomme, 1990). Microorganisms that interact with feed particles can be functionally described as three distinct subpopulations: 1) those associated with the ruminal fluid, 2) those loosely attached to feed particles, and 3) those firmly attached to feed particles (Czerkawski and Cheng, 1988). The subpopulation associated with the ruminal fluid is a mixture of microorganisms that have detached from feed particles as well as those that subsist on soluble feed components within ruminal fluid. Microorganisms contained within this subpopulation have little direct involvement in the digestion of insoluble feed particles (Latham, 1980). Despite its minor role in feed particle digestion, this subpopulation is an integral part of the ruminal ecosystem; the bacteria it contains attach to and initiate the digestion of newly ingested feed particles.

Bacteria that are loosely associated with feed particles are removed by gentle washing, whereas tightly associated bacteria remain attached. These two subpopulations account for 70 to 80% of the microbial matter in the rumen (Forsberg and Lam, 1977; Craig et al., 1987). It has been estimated that these two populations are responsible for 80% of the endoglucanase activity, 70% of the amylase activity (Minato et al., 1966), and 75% of the protease activity (Brock et al., 1982) in the rumen. Hemicellulase and cellulase activities are also notably higher in the particulate fraction of ruminal contents than in the fluid (Williams and Strachan, 1984), leaving no doubt that particle-associated microbial populations are responsible for the majority of feed digestion in the rumen.

Attachment of ruminal microorganisms to their nutrient substrata serves to appose digestive enzymes with their specific substrates and concentrate digestion within a small area (Chesson and Forsberg, 1988). In bacteria, digestive enzymes are often stabilized and protected within a fibrous polysaccharide glycocalyx on the cell surface (Cheng et al., 1981a; Lappin-Scott et al., 1992). Presumably, because these microorganisms are close to the digestion site, they receive a large proportion of the nutrients released from the digestion of feed particles. In contrast, microorganisms within the fluid phase must continuously seek out new sources of soluble substrates and the low activity of cell-free ruminal fluid indicates that the digestive enzymes produced by these organisms are quickly inactivated.

Depending on size, density, and susceptibility to digestion, feed particles are generally retained in the rumen two to three times longer than the fluid (Owens and Goetsch, 1986). Microorganisms that attach to feed particles with a slow rate of passage prolong their residence within the rumen. Retention with the particulate phase is reported to increase the amount of energy partitioned by microorganisms toward maintenance and to decrease the efficiency of microbial protein synthesis (Kennedy and Milligan, 1978; Russell and Wallace, 1988). However, prolonged residence is necessary for slowly growing organisms such as ruminal fungi and protozoa. With generation times of 5 to 14 h for ruminal protozoa (Williams and Coleman, 1988) and 24 to 30 h for ruminal fungi (Bauchop, 1981; Joblin, 1981), populations of these microorganisms are rapidly depleted if they are unable to attach to feed particles and delay passage from the rumen.

In meal-fed ruminants and in ruminants surviving in nutritionally marginal environments, nutrient supply to microorganisms is characterized by short periods of abundance separated by longer periods of starvation. This effect is especially pronounced for microorganisms dependent on soluble substrates, because these are often at negligible concentrations during a portion of the feeding cycle (Johnson, 1976). In continuous culture, the viability of the soluble-substrate-dependent bacterium *Selenomonas ruminantium* is halved after 2.5 h of starvation (Mink and Hespell, 1981). In the rumen, however, this organism apparently survives prolonged periods of starvation. In Svalbard reindeer, resident ruminal bacterial species remain essentially unchanged between summer and winter, even though these animals are forced to subsist on low-quality feed and are frequently starved during the severe arctic winter (Orpin et al., 1985). One could surmise that the ability of these microorganisms to associate with feed particles (Latham, 1980) enables them to subsist during extended periods of nutrient deprivation.

Microbial Attachment and Penetration

Effect of Cuticle

Attachment to nutrient substrata is the initial step in the digestive process. However, for digestion to proceed microorganisms must often penetrate or skirt resistant barriers on the surface of feed particles to access their preferred substrates. The degree to which microorganisms attach to and penetrate these physical barriers is reflected in the fermentation lag time that characterizes the ruminal digestion of various feeds (Allen and Mertens, 1988). Forages and cereal grain tissues are protected by a cuticle that is almost completely resistant to attachment (Bauchop, 1980; Akin, 1989; McAllister et al., 1990c). In some forages, the cuticle contains 18 to 24% silica that enhances the rigidity of this outer layer and further impedes its digestion (Harbers et al., 1981). Usually, ruminal bacteria strategically circumvent this physical barrier

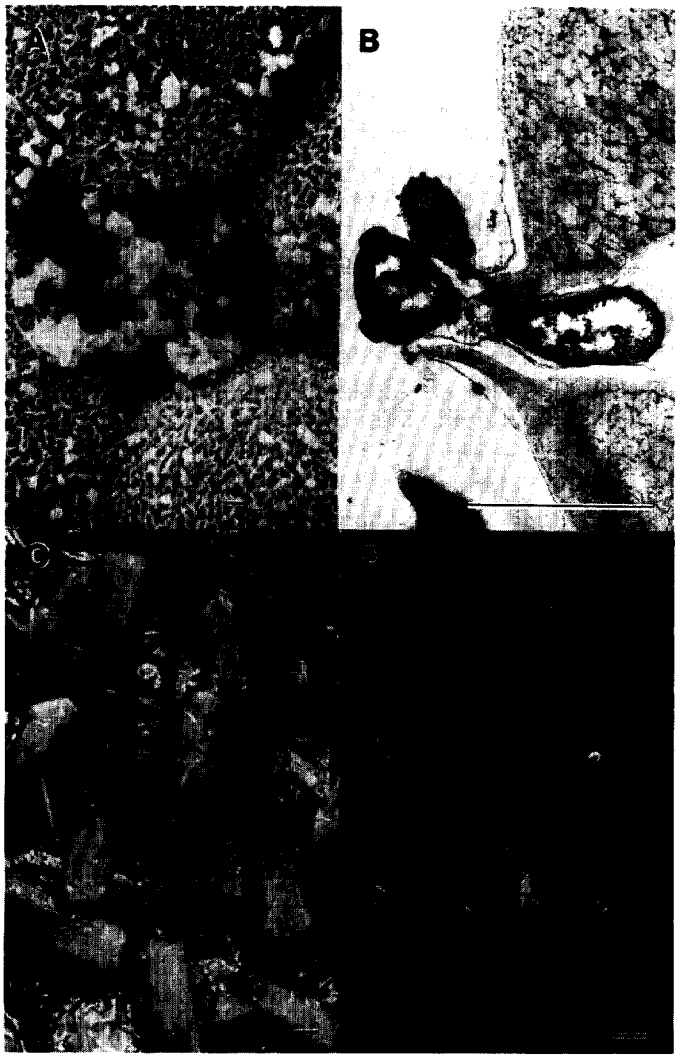

Figure 1. (A) Scanning electron micrograph of an alfalfa leaflet after 2 h of incubation with ruminal fluid. Note that bacteria have attached to and proliferated at the stomatal opening. (B) Transmission electron micrograph showing that bacteria have gained access to the interior of the leaf cell by penetrating the stomatal opening. (C) Photograph showing that digestion of alfalfa stems has preferentially occurred at the cut ends of an alfalfa stem. (D) Scanning electron micrograph showing that ruminal fungi have preferentially colonized the stomata of a corn leaf after 12 h in ruminal fluid. Bars in Figures A and B indicate 1 μm. Bars in Figures C and D indicate 1 mm and 50 μm, respectively. (Figures A and B from Cheng et al., 1980; Figure C, Cheng, unpublished data; Figure D courtesy of E. Gernet, INRA-Theix, France.)

and gain access to sheltered, readily digestible inner-tissues through stomata, lenticels, or damaged areas (Figure 1) and digestion essentially proceeds from the inside out (Chesson and Forsberg, 1988; Cheng et al., 1991a). Similarly, ruminal fungi focus their digestion on the vulnerable regions of plant tissues, but when confronted with recalcitrant tissues their hyphae possess the extraordinary capacity to directly penetrate the cuticle (Akin and Rigsby, 1987; Ho et al., 1988). This action substantially decreases the tensile strength of the tissue and may provide additional sites for bacteria to access and attach to protected plant tissues (Akin et al., 1983).

Effect of Internal Tissues and Phenolic Compounds

Additional uncolonizable barriers to digestion are commonly encountered internally by digestive microorganisms even after the protective cuticular layer of plant material is breached. In forages, the middle lamella, which lines the surface of lignified cell walls, presents such an obstruction and is in part responsible for the indigestible nature of vascular and sclerenchymal tissues (Engels, 1989). In contrast, tissues such as the mesophyll and phloem, which are easily penetrated and colonized by ruminal microorganisms, are rapidly and completely digested (Akin, 1979). Thus, the proportion of uncolonizable to colonizable tissues often dictates the extent to which a particular forage is digested in the rumen (Wilson, 1990).

Several workers have reported a negative correlation between digestibility and the concentration of phenolic compounds in forage cell walls (Chesson et al., 1982; Reeves, 1985; Jung, 1989). Phenolic monomers (5 m*M*) are toxic to ruminal bacteria, protozoa, and fungi in vitro (Chesson et al., 1982; Akin and Rigsby, 1987) and have been shown to interfere with the attachment of *Fibrobacter succinogenes* to cellulose (Varel and Jung, 1986). However, the concentration of free phenolics in ruminal fluid is low (.15 to 5.15 μM; Jung et al., 1983), and these compounds are readily metabolized by the ruminal microflora (Chesson et al., 1982). Thus, it is unlikely that free phenolics have similar effects in the rumen. Rather, phenolic acids seem to limit the digestion of plant cell walls by crosslinking cell wall polymers through ferulic acid and coumaric acid dimers (Hartley and Ford, 1989). As digestion of plant cell walls proceeds, polysaccharides are removed, and phenolics accumulate, forming a protective surface layer that shelters underlying tissues from further attack (Chesson and Forsberg, 1988). The effects of this protective layer on attachment have not been documented, but assuming that attachment is not markedly inhibited, microflora that possess the feruloyl and coumaroyl esterases necessary to cleave these phenolic dimers could have a major impact on the extent of plant cell wall digestion (Borneman et al., 1990; McDermid et al., 1990).

Some plants harbor phenolic compounds that are not associated with the plant cell wall. For example, condensed tannins are high molecular weight polyphenolic compounds that are deposited within vacu-

oles in the cells of some legumes such as sainfoin and birdsfoot trefoil. These compounds readily form hydrophobic and hydrogen bonds with protein, a tendency that is likely responsible for the bloat-safe properties of forages that contain condensed tannins (Waghorn, 1990). Condensed tannin-protein complexes resist microbial digestion, and considerable interest has been shown in using condensed tannins as a means of increasing the ruminal escape protein value of forages (Mangan, 1988; Waghorn, 1990). In pure culture studies, condensed tannins readily form complexes with microbial enzymes, interfere with microbial attachment (Figure 2), and consequently inhibit the digestion of fiber by cellulolytic bacteria (Bae et al., 1993). Although these compounds inhibit virtually all ruminal cellulolytic bacteria (Bae, McAllister, and Cheng, unpublished data) their effect on proteolytic bacteria is variable (Figure 3; Jones et al., 1993). Condensed tannins at concentrations of 200 μg/mL completely inhibited the growth of *Butyrivibrio fibrisolvens*, and at 100 μg/mL flocculation and elongation of these cells was observed (Figure 3B). In contrast, *Prevotella ruminicola* and *Streptococcus bovis* grew in the presence of 600 μg/mL of condensed tannin. At this concentration, condensed tannins had no apparent effect on the morphology of *P. ruminicola* (Figure 3D) but caused extensive chain formation and incomplete division in cells of *S. bovis* (Figure 3F).

The formation of condensed tannin-protein complexes depends on a variety of physicochemical factors (Mangan, 1988) and currently it is difficult to determine whether condensed tannins exert effects on bacteria in the rumen similar to those observed in vitro. It is known that condensed tannins often decrease the ruminal digestibility of both carbohydrates and protein (Leinmuller et al., 1991). Further characterization of the effects of condensed tannins on microbial attachment and penetration of plant tissues may help define the overall effect that these compounds have on ruminal fermentation.

Even in cereal grains, which are generally viewed as readily fermentable feeds, internal barriers to digestion are encountered. Endosperm cells are surrounded by a β-glucan-rich cell wall and contain starch granules encased within a protein matrix. After the cuticle is cracked, the protein matrix and endosperm cell wall determine the rate at which ruminal microorganisms attach to and penetrate starch granules (Kotarski et al., 1992; McAllister et al., 1993b). In wheat and barley, these structures are easily penetrated, and consequently starch in these cereal grains is rapidly fermented in the rumen (Orskov, 1986). In contrast, the protein matrix in the horny endosperm of sorghum and corn is extremely resistant to attachment and penetration. To date, the only microorganisms observed to have penetrated this structure are the ruminal fungi (McAllister et al., 1993a). Undoubtedly, the resistant nature of the protein matrix in corn and sorghum contributes to the

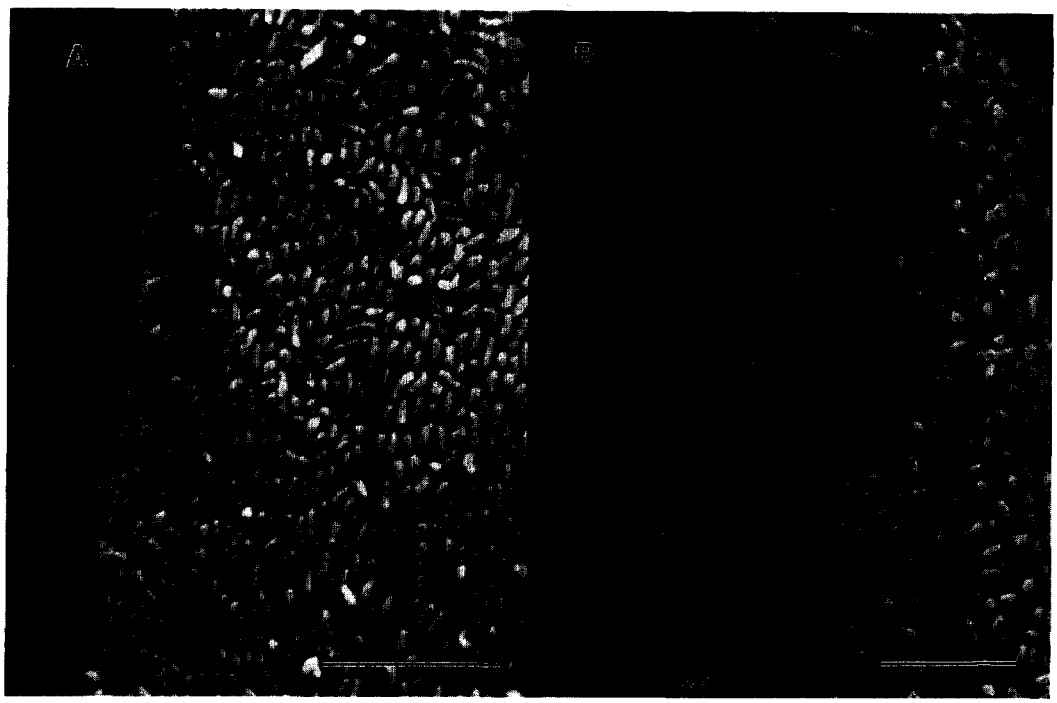

Figure 2. Effect of condensed tannins on the attachment of *Fibrobacter succinogenes* S85 to filter paper. (A) alignment and attachment of cells in the absence of condensed tannin. (B) Pitted and partly digested surface of filter paper from which most cells have been detached by exposure to 400 μg/mL of Birdsfoot trefoil condensed tannin for 30 min. Bars in scanning electron micrographs indicate 5 μm (from Cheng et al., 1993).

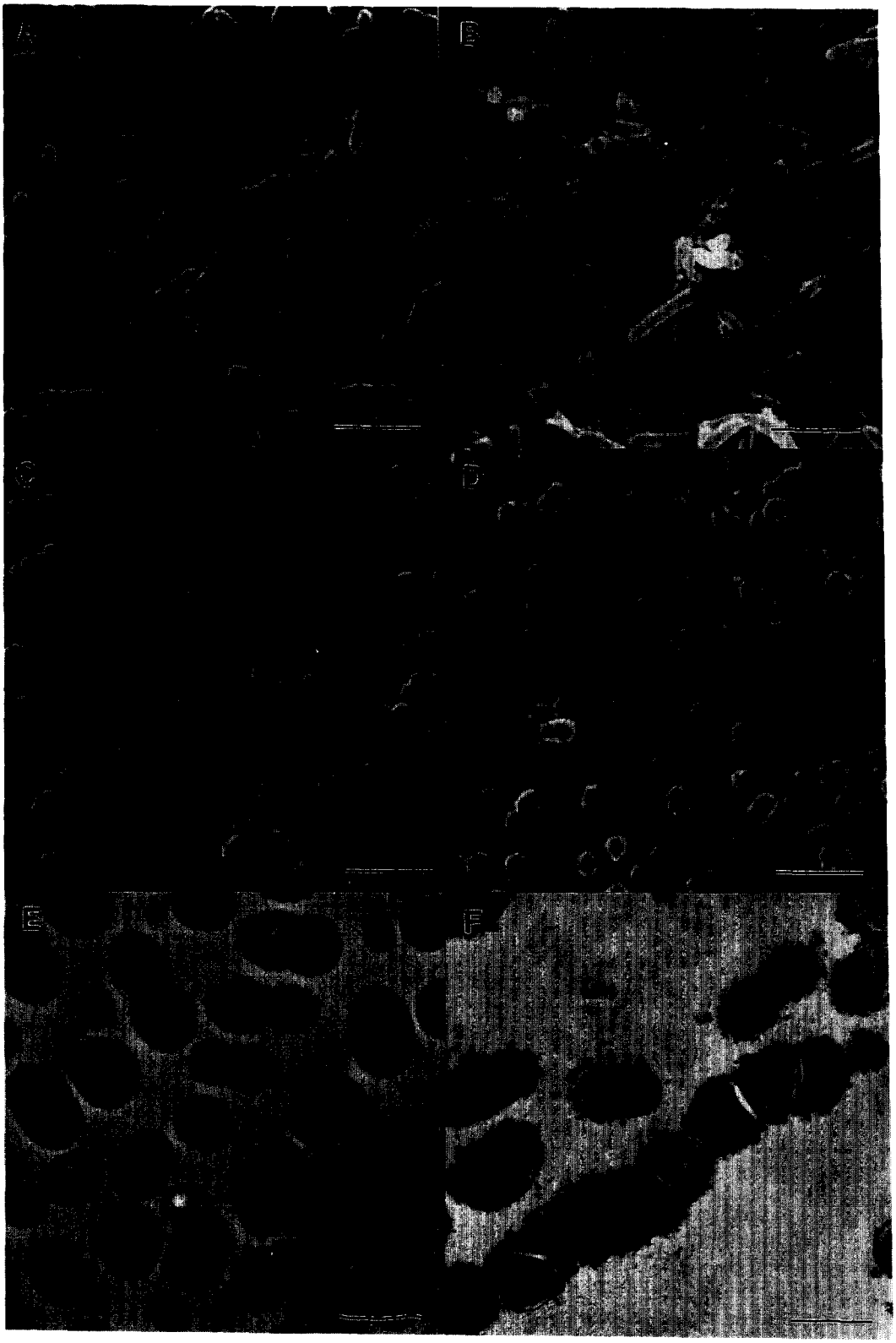

Figure 3. Effect of condensed tannins on the cell morphology of *Butyrivibrio fibrisolvens*, *Prevotella ruminicola*, and *Streptococcus bovis*. (A) *B. fibrisolvens* without condensed tannins, (B) *B. fibrisolvens* with 100 μg/mL of condensed tannin. Note flocculation and elongation of cells. (C) *P. ruminicola* without condensed tannins, (D) *P. ruminicola* with 500 μg/mL of condensed tannin. Note that although cell morphology is apparently normal, condensed extracellular material is associated with the cells. (E) *S. bovis* without condensed tannins, F) *S. bovis* with 500 μg/mL of condensed tannins. Note that cell division is incomplete and extensive chain formation has occurred. Bars in scanning electron micrographs (Figures A, B, C, and D) indicate 2 μm, whereas bars in transmission electron micrographs (Figures E and F) indicate 1 μm. (Jones, unpublished data).

greater passage of starch to the small intestine of cattle fed these grains as compared with those fed barley (Spicer et al., 1986). Thus, subtle changes in the structural integrity of plant tissues can have a profound effect on the microbial processes of attachment and penetration and, ultimately, the efficiency with which ruminants digest a particular feedstuff.

Role of Mastication and Feed Processing

The pentration of cuticular layers by ruminal fungi is by itself inadequate for the efficient digestion of intact plant tissues. Grazing ruminants rely almost entirely on mastication to disrupt plant tissues that create physical barriers to digestion (Pond et al., 1987), whereas feed for confined ruminants is often mechanically processed (i.e., rolled, chopped, ground) to augment mastication damage. The extent of mechanical processing required to facilitate microbial attack often depends on the degree to which the feedstuff is damaged by ingestive and ruminative mastication. For example, damage to corn kernels during eating is sufficient to expose the endosperm so that both the rate and extent of in sacco DM digestion of masticated corn is greater than that for halved or quartered kernels. In contrast, unprocessed barley and wheat kernels are not extensively damaged by mastication during eating. Much of the endosperm remains sheltered, and the rate and extent of in sacco DM digestion is lower for masticated kernels than for halved or quartered kernels (Figure 4; Beauchemin et al., 1994). Thus, unprocessed corn is effectively digested by ruminants and the feed:gain ratios in cattle fed whole corn are similar to those fed rolled corn (Chester-Jones and Ziegler, 1991). Predictably, the feed:gain ratio of cattle fed whole barley is consistently lower than that of cattle fed rolled barley (Mathison et al., 1991; Hironaka et al., 1992), and both barley and wheat must be processed for efficient ruminal digestion.

In addition to disrupting physical barriers, mastication and mechanical processing reduce the size of feed particles and increase the surface area available for microbial attachment and enzymatic attack (Bowman and Firkins, 1993). Salivation during mastication serves to wet the ingested feed, a process that is required for the association of microorganisms with feed particles and the initiation of the attachment process. Saliva also buffers the ruminal fluid and helps prevent the development of a low ruminal pH. This is especially important for cellulolytic bacteria (i.e., *Ruminococcus albus*, *F. succinogenes*, *Ruminococcus flavefaciens*), which are particularly sensitive to low pH (Russell and Dombrowski, 1980) even though it does not inhibit their attachment (Morris, 1988; Roger et al., 1990).

Although mastication and mechanical processing promote microbial feed digestion, care must be taken to ensure that feeds are not overprocessed. Over-

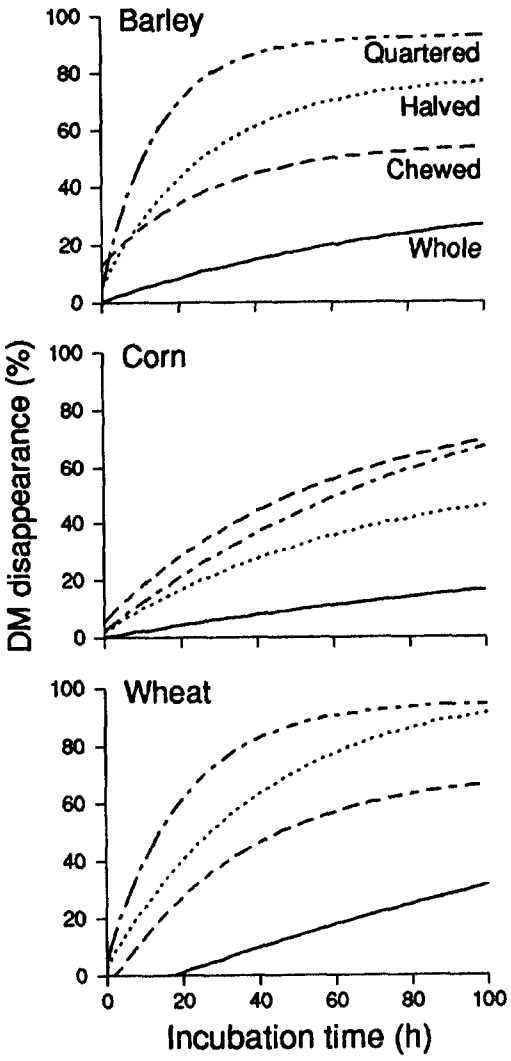

Figure 4. *In sacco* disappearance of DM from whole, chewed, halved, and quartered kernels of barley, corn, and wheat (from Beauchemin et al., 1994).

processing of refractory feeds such as grasses often causes feed particles to pass from the rumen before the microbial processes of attachment, penetration, and digestion are completed. As a result, the apparent digestibility of OM may be as much as 15 percentage units lower for pelleted or ground grass hay than for long hay (Thomson and Beever, 1980). However, in most instances the negative effects of processing on the digestibility of these feeds is offset by an increase in DMI (Galyean and Owens, 1991). In contrast, overprocessing of readily fermentable feeds such as barley and fresh legume forage dramatically increases the rate of microbial fermentation (Orskov, 1979; Robles et al., 1980) and often results in digestive disturbances such as lactic acidosis and bloat (Johnson, 1991; Wikse, 1991). Consequently, fermentable feeds should be mechanically processed only to the extent that primary barriers to microbial attachment

and penetration are disrupted. Selection of an optimal degree of mechanical processing requires not only consideration of the properties of the feed, but also an appreciation for the extent of mastication and the fundamental microbial processes involved in feed digestion.

Mechanisms of Microbial Attachment

Knowledge of the cellular mechanisms of attachment is rudimentary for ruminal bacteria and virtually nonexistent for fungi and protozoa. The series of papers by Minato and Suto remains the most comprehensive examination of the attachment of ruminal bacteria to cellulose and starch (Minato and Suto, 1976, 1978, 1979, 1981).

Attachment to Fiber

To focus of most studies on adhesion has been on the capsular carbohydrate coat or glycocalyx, a structure that is instrumental in the initial attachment of the three principal cellulolytic bacteria, *F. succinogenes*, *R. flavefaciens*, and *R. albus*, to cellulose (Patterson et al., 1975; Latham et al., 1978a; Stack and Hungate, 1984). Addition of phenylpropanoic acid to growth medium enhances the formation of the glycocalyx by *R. albus* but does not affect the development of this structure by *R. flavefaciens* or *F. succinogenes* (Stack and Hungate, 1984; Stack and Cotta, 1986; Morrison et al., 1990). Disruption of the glycocalyx prevents bacterial attachment (Latham, 1980). The glycocalyces of *R. flavefaciens* and *R. albus* are notably thicker than that of *F. succinogenes* (Costerton et al., 1974; Latham, 1980), and the factors that mediate attachment in these organisms have been shown to differ (Cheng et al., 1983/84; Morris, 1988; Roger et al., 1990).

F. succinogenes S85 associates closely with cellulose and its attachment is sensitive to low and high temperatures, low and high pH, lack of Na^+, inhibitors of membrane ATPases and ionophores, but is insensitive to electron transport inhibitors or the absence of divalent cations (Roger et al., 1990). Treatment with a variety of proteases reduces the attachment of this bacterium to cellulose, strongly implying that proteins on the cell surface participate in the attachment process (Gong and Forsberg, 1989; Mitsumori and Minato, 1993). In fact, recent work suggests that there may be multiple proteins as well as lipids consummating the attachment of *F. succinogenes* to cellulose (Mitsumori and Minato, 1993). The suppressive effects of ionophores, O_2, or inhibitors of membrane ATPases suggests that energetic processes may play a role in the attachment of *F. succinogenes*, but this hypothesis remains to be tested.

In contrast, *R. flavefaciens* associates less closely with the cellulose surface and its attachment is unaffected by metabolic inhibitors, lack of Na^+, pH, O_2, and temperature but is sensitive to formaldehyde (Rasmussen et al., 1989) and the removal of the divalent cations Ca^{++} and Mg^{++} (Roger et al., 1990). Attachment of *R. albus* is insensitive to pH and O_2 (Morris and Cole, 1987; Morris, 1988). When grown in mixed culture, *R. flavefaciens* and *F. succinogenes* attach noncompetitively to specific sites on complex substrates such as ryegrass and barley straw (Latham et al., 1978b; Bhat et al., 1990). It remains to be determined whether this specificity in attachment sites stems from differences in the mechanisms used by these cellulolytic species to attach to fibrous substrates.

Forsberg (1986) hypothesized that the cell surface of cellulolytic bacteria may have specific "attachment factors" that facilitate their binding to cellulose. Some cellulases possess cellulose binding domains (**CBD**) (McGavin and Forsberg, 1989; Gilbert et al., 1990), but the specific role of CBD in cellulose digestion remains to be determined (Pell and Schofield, 1993). *Clostridium* spp. produce a protrudent cellulase enzyme complex termed a cellulosome (Lamed et al., 1987; Lamed and Bayer, 1988). Goldstein et al. (1993) recently characterized the CBD of the cellulose-binding protein (CbpA) within the cellulase enzyme complex of *Clostridium cellulovorans*. These researchers hypothesized that CbpA directs the enzyme complex to the surface of crystalline cellulose. Cellulosome-like organelles have been observed on the cell surfaces of *F. succinogenes*, *R. albus*, and *R. flavefaciens* grown on alfalfa cell walls (Miron et al., 1989), but it is not known whether these structures contain cellulose binding proteins. An analogous structure involved in the digestion of xylan, termed a xylanosome, has been described in *B. fibrisolvens* (Lin and Thomson, 1991). The involvement of this structure in mediating attachment is questionable, however, because *B. fibrisolvens* seems to be capable of partial degradation of plant cells without obvious attachment (Akin and Rigsby, 1985). Further support for the importance of attachment in cellulose digestion is evident in work with methylcellulose, which prevents the attachment of cellulolytic ruminal bacteria to cellulose fibers and inhibits cellulose digestion (Minato and Suto, 1978; Kudo et al., 1987a). Kudo et al. (1987a) demonstrated that treatment of *R. flavefaciens*, *R. albus*, and *F. succinogenes* with .1% methylcellulose inhibited filter paper digestion completely but inhibited endoglucanase activity by less that 20%, a result that supports the involvement of noncatalytic binding proteins in the attachment process. However, more recently methylcellulose (< 3 m*M*) was also shown to completely inhibit the exo-β-1,4-glucanase A of *R. flavefaciens* (White et al., 1988), and Roger et al. (1990) found that methylcellulose did not reduce the attachment of *F. succinogenes* to Avicel as it did to filter paper (Kudo et al., 1987a). Thus, it seems that

the attachment and cellulolytic processes may be intertwined to a greater degree than was initially suspected.

At least one of the cellulases in *F. succinogenes* may have a direct role in attachment because it was found to contain a CBD (McGavin and Forsberg, 1989). The possibility that attachment factors (i.e., nonspecific binding proteins and[or] cellulases) may be transitory in nature (Roger et al., 1990) and differ not only among strains of the same species (Morris and Cole, 1987), but also with the source of substrate (Wood et al., 1982), further emphasizes the potential complexity of the process of attachment in cellulolytic bacteria.

Ruminal fungi and protozoa, like ruminal bacteria, readily attach to plant tissues within the rumen (Williams, 1986; Fonty and Joblin, 1991). Soluble components diffuse from masticated plant tissues and act as chemoattractants, allowing fungal zoospores (Orpin and Bountiff, 1978) and protozoa (Orpin, 1979) to locate and attach to feed particles. Holotrich ciliates such as *Isotricha* spp. attach to feed particles by means of specialized regions on their cell surfaces (Orpin and Letcher, 1978), whereas Ophryoscolecid ciliates such as *Eudiplodinium maggi* and *Epidinium ecaudatum* use their oral cavity to attach to damaged fibers (Bauchop, 1980). Although the mechanisms remain obscure, the ability of protozoa to freely attach and dissociate from feed particles suggests that their attachment is a purely physical phenomenon. Positioning within the particulate digesta not only places protozoa in juxtaposition to available sources of carbohydrate, but also aids in their retention and survival in the rumen (Williams, 1986).

As with cellulolytic bacteria, methylcellulose prevents the attachment of fungi to cellulose (Cheng et al., 1991b) and suggests a commonality in the mechanisms of attachment between cellulolytic ruminal fungi and bacteria. In coculture, *R. flavefaciens* and *R. albus* inhibit the cellulolytic activity of *Neocallimastix frontalis* and *Piromyces communis* (Fonty and Joblin, 1991). Two research groups have recently isolated extracellular protein factors from *R. flavefaciens* (Stewart et al., 1992; Bernalier et al., 1993) that inhibit the attachment of *N. frontalis* to cellulose. It is tempting to speculate that the attachment factors of cellulolytic ruminococci are sufficiently similar to those of ruminal fungi that they block a common attachment site on cellulose fibers. Wilson and Wood (1992a) isolated a cellulosome-type enzyme fraction from *N. frontalis*, but unlike cellulase complexes isolated from ruminal bacteria, the cell-free cellulase complex of *N. frontalis* degrades crystalline cellulose (Wood et al., 1986). However, the cellulase complex of *N. frontalis* is inactivated by chitinase (Wilson and Wood, 1992b), which suggests that even these "cell free" complexes must be associated with the fungal cell wall to actively digest crystalline cellulose.

Attachment to Starch and Protein

Several species of ruminal bacteria attach avidly to starch granules (Minato and Suto, 1979; McAllister et al., 1990c) even though cell-free preparations of ruminal bacteria have been shown to be capable of digesting starch (McWethy and Hartman, 1977; Cotta, 1988). In general, those ruminal bacterial species that attach to starch granules exhibit amylase activities that are significantly higher than those exhibited by non-attaching amylolytic species (Minato and Suto, 1976, 1979). As with attachment to cellulose, attachment to starch in some species of ruminal bacteria is sensitive to temperature and formaldehyde.

Pure culture studies with *S. bovis*, *Ruminobacter amylophilus*, and *B. fibrisolvens* have shown that these bacteria differ in their sites of attachment to cereal grains (McAllister et al., 1990b). In *S. bovis*, attachment is not site-specific and cells randomly attach to both starch granules and the protein matrix, whereas *R. amylophilus* preferentially attaches to the surface of starch granules. *B. fibrisolvens* readily digests isolated starch granules (Cotta, 1988), yet when grown on cereal grains, this organism colonizes the cell wall of endosperm cells. These data suggest that the affinity of amylolytic bacteria for starch granules may differ substantially among bacterial species.

Detailed experiments on the mechanisms of attachment to starch have been completed for *Bacteroides thetaiotaomicron* (Anderson and Salyers, 1989a,b) and *Lactobacillus amylovorus* (Imam and Harry-O'kuru, 1991). In these nonruminant species, attachment seems to be mediated through surface proteins. In *B. thetaiotaomicron* there are two distinct attachment sites on the cell surface for large starch oligomer and maltodextrins (Anderson and Salyers, 1989b). In this organism, attachment seems to be a prerequisite for starch digestion, because non-attaching mutants are incapable of digesting amylose or amylopectin. The affinity of surface receptors for starch and the adhesion of *L. amylovorus* to starch are reduced when starch is treated with mixtures of HCl and alcohol (Imam and Harry-O'kuru, 1991). Assuming that comparable attachment mechanisms are employed by amylolytic ruminal bacteria, similar chemical treatments of cereal grains could prove to be beneficial in controlling the rate and extent of starch digestion in the rumen.

To our knowledge, there are no reports on the role of attachment in the ruminal digestion of feed protein. Observations that the proteolytic enzymes of ruminal bacteria are predominantly cell-associated (Wallace and Brammall, 1985), that cell-free ruminal fluid is virtually free of proteolytic activity (Blackburn and Hobson, 1960), and that 75% of proteolytic activity in the rumen is associated with the solid portion of ruminal contents (Brock et al., 1982) all imply that

attachment is central in the ruminal digestion of feed protein. The direct observation of attachment to insoluble proteins by electron microscopy has been hampered by the fact that plant proteins rarely exist in a "pure" form and are often encased in or complexed with carbohydrate. We have observed ruminal bacteria adhered to protein-rich substrates such as canola meal and the protein matrix of cereal grains (McAllister et al., 1990b,c). Protein-rich substrates that are not readily colonized, such as the germ in corn, are also resistant to microbial digestion (McAllister et al., 1990c).

Adsorption of soluble proteins to the cell surface is the first step in proteolysis (Nugent and Mangan, 1981; Wallace, 1985) and, as with cellulases, much of the proteolytic activity of ruminal bacteria is associated with the extracellular glycocalyx (Kopecny and Wallace, 1982). *Prevotella ruminicola*, a principal proteolytic species in the rumen, produces protuberant structures on its cell surface that are similar to those produced by cellulolytic bacteria (Wallace and Brammall, 1985). Given the apparent importance of attachment in protein digestion, treatments that protect feed proteins from ruminal digestion (e.g., heat, formaldehyde) may partially owe their effectiveness to their ability to alter the structure of feed proteins and to inhibit the attachment of proteolytic bacteria.

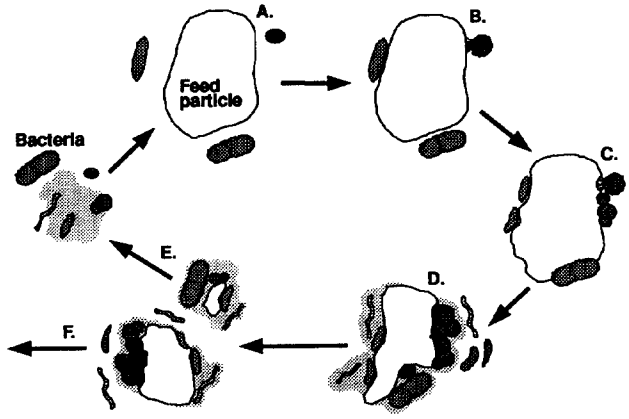

Figure 5. Diagrammatic representation of the formation of microbial consortia and biofilms on the surface of feed particles. (A) Bacteria associate with feed either randomly or through chemoattractants. (B) Bacteria adhere to feed particle by bacterial glycocalyx and accompanying binding proteins. (C) Bacteria divide to produce sister cells and digestive pits become visible on the feed particle surface. (D) Additional bacteria combined with primary colonizers to form rich biofilms and particle digestion is accelerated. (E) Portions of the biofilm dissociate from feed particles. (F) Other portions remain attached to feed particles and pass from the rumen to the lower digestive tract.

Role of Attachment in Adaptation and Biofilm Development

Direct microscopic examination clearly shows that both forage and grain particles from the rumen are colonized by morphologically diverse microbial populations (Dinsdale et al., 1978; McAllister et al., 1990c). A single microbial species is incapable of producing the myriad of enzymes required to digest chemically and structurally complex plant tissues. Digestive processes are instead accomplished by physiologically complementary bacterial species that combine to form complex microbial consortia on the surface of plant tissues (Cheng et al., 1991a). Establishment of microbial consortia is a sequential process, and attachment by primary digestive microorganisms is pivotal for the formation of the climax digestive population (Figure 5). Initially, primary digestive bacteria (e.g., *F. succinogenes, R. albus, R. flavefaciens, R. amylophilus*) from the fluid milieu interact and attach to the surface of feed particles via their glycocalyces and associated binding proteins. Primary colonizers divide to produce sister cells and their digestive enzymes attack the insoluble substrate, form digestive pits and release soluble nutrients. The resultant microcolony produces a surface environment that attracts secondary colonizers from the ruminal fluid to the site of digestion and these newly associated bacteria subsist on the soluble substrates released by primary colonizers (Kudo et al., 1987b; Wolin and Miller, 1988).

Some of the secondary colonizers attach to the glycocalyces of primary colonizers. As this process continues, a rich and varied microbial biofilm is formed, which often completely covers the surface of feed particles (Figure 6). Within the biofilm, nutrients are concentrated, substrates (e.g., succinate, isobutyrate, H_2) are exchanged among microbial members (Wolin and Miller, 1988; Fonty and Joblin, 1991), and end products (e.g., VFA) are released. The gel-like nature of the biofilm matrix limits the access of anti-bacterial agents such as bacteriophage (Costerton et al., 1987), and one can easily envision it sheltering component bacteria from predatory protozoa (Newbold et al., 1989).

Eventually, some of the plant cell wall polymers are completely digested. Previously attached species are released into the ruminal fluid and are free to associate with and colonize freshly ingested feed particles (Bowman and Firkins, 1993). Many of the more recalcitrant feed particles pass from the rumen before they are completely digested. The biofilms that they harbor are a rich source of both carbohydrates and AA and make an important contribution to the nutrition of the host animal (Czerkawski, 1976; Merry and McAllan, 1983).

Development of microbial biofilms on the surface of feed particles is a dynamic process. Whereas the

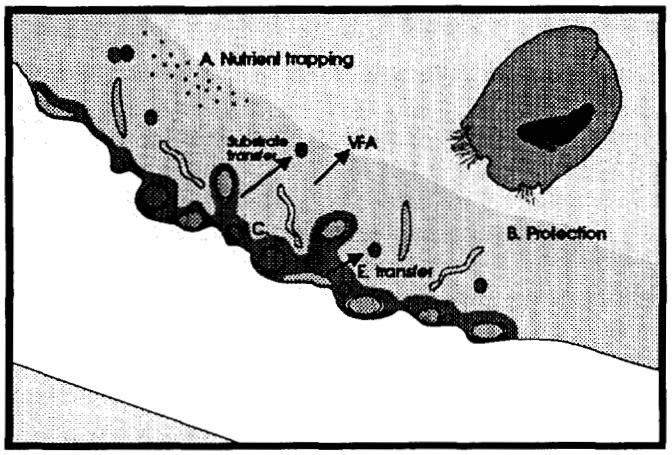

Figure 6. Functions of the biofilm on the surface of feed particles include (A) nutrient trapping, (B) protection from antibacterial substances (e.g., bacteriophages and protozoa), and (C) establishment of an environment for substrate and electron transfer and removal of end products (e.g., VFA).

sequence of events that leads to biofilm formation remains essentially the same, the participant microorganisms may change as new insoluble substrates are introduced into the rumen. In medical microbiology, it is well known that genes controlling attachment are regulated in response to changes in the environment (Matthysse, 1992; Stromberg et al., 1992). For example, *Escherichia coli* cells only produce fimbriae within the digestive tract and cease to produce these structures when they encounter the colder temperatures outside their animal host. Addition of soluble carbohydrates to pure cultures of fibrolytic ruminal bacteria decreases cellulolytic activity, but not the production of cellulase enzymes (Hiltner and Dehority, 1983). We have observed that cellulolytic bacteria transferred weekly have a slow rate of cellulose digestion, but if transferred every 2 d, they develop a rapid rate of cellulose digestion and utilize the same amount of substrate within 2 d as they previously did in a week (Kudo and Cheng, unpublished data). Roger et al. (1990) observed that repetitive transfers (10×) of *F. succinogenes* cultures with 4% cellobiose in the medium caused a large proportion (80%) of the cells to lose their ability to attach to cellulose. Given these data and the central role of attachment in cellulose digestion, it seems probable that attachment mechanisms in cellulolytic bacteria are also subject to regulation and adaptation.

Adaptive mechanisms in microbial attachment within the rumen are yet to be demonstrated, but it is known that when ruminants are switched from a low- to high-soluble carbohydrate diet some bacteria augment the production of their extracellular glycocalyx (Cheng et al., 1977). Undoubtedly, an abundance of readily available energy promotes glycocalyx formation, but this thickened structure may also alter the attachment of these bacteria and provide them with resistance to the low pH and high osmolarity that are often associated with finely processed concentrate diets.

In the rumen, adaptation is often perceived as a change in the component species of the biofilm, a phenomenon that is readily apparent as ruminants are gradually transferred from a forage to a concentrate diet (Mackie et al., 1978; Mackie and Gilchrist, 1979). After this transition is complete, however, adapted biofilms are inherently stable and it seems that the major bacterial species are remarkably similar in ruminants on forage and concentrate diets (Mackie and Gilchrist, 1979; Leedle et al., 1982; McAllister et al., 1993b). Thus, after adapted to a particular diet, with respect to component species, adaptive responses by individual bacteria within biofilms may be sufficient to cope with the subtle environmental changes that occur within the rumen on a daily basis.

Strategies to Manipulate Attachment

Essentially, digestion in the rumen involves a sequential attack of ruminal microorganisms on feed (Cheng et al., 1991a). To optimize feed digestion, we must expedite this attack on low-quality feeds (e.g., straw) and slow it with highly digestible feeds (e.g., cereal grains) to prevent digestive disturbances. Microbial attachment initiates the sequential microbial attack of feed and is a logical point at which to attempt to manipulate feed digestion.

For decades, physical and chemical processes have been used to manipulate feed digestion. Many of these techniques were developed before an appreciation for the importance of microbial attachment in feed digestion. When these processes are examined in detail, it is evident that much of their effectiveness arises from their ability to alter the number of available attachment sites on the surface of feed. Grinding and alkali treatment increase the number of microbial attachment sites on the surface of forages (Latham et al., 1979; Kolankaya et al., 1985; Bowman and Firkins, 1993) and enhance the digestion of these feeds in the rumen. Biological delignification using white rot basidiomycetes has been shown to increase the in vitro digestibility of oat straw, presumably by breaking down microbial barriers to penetration and attachment (Jung et al., 1992). In contrast, fat and formaldehyde reduce the number of attachment sites and limit the microbial digestion of feeds (Stewart, 1977; McAllister et al., 1990a). Many of the significant advances possible in physical and chemical processing of feed have likely already been made and future strategies to enhance the sequential microbial

attack on plant tissues are likely to arise from technologies in plant breeding and genetic engineering.

In the past, plant breeding programs have focused largely on the improvement of agronomic traits of forages, and comparatively few studies have been directed to improving their nutritional utilization (Wheeler and Corbett, 1989). The brown midrib mutants of maize, sorghum, and pearl millet contain substantially lower lignin (25 to 50%) than normal genotypes and have higher digestibilities in vitro (33%) and in dairy cows and sheep (Porter et al., 1978; Wedig et al., 1988; Cherney, 1990). Recently, Watson (1990) has advocated using antisense RNA mutagenesis or catalytic RNA molecules as a means of reducing the expression of lignin genes and perturbing the biosynthesis of lignin in alfalfa. The improved ruminal digestibility of these selected or genetically altered forages is most likely due to a reduction in the barriers to microbial attachment and penetration. Further characterization of natural plant compounds (e.g., condensed tannins) that alter these microbial processes could provide additional selection criteria for plant breeding programs or potential targets for genetic manipulation.

Our present lack of knowledge of the basic molecular mechanisms of attachment is a major impediment to the development of molecular strategies to manipulate microbial attachment. Current evidence indicates that both binding proteins in the cellulosome as well as binding regions in cellulases may be important for cellulose digestion (Beguin et al., 1992). Construction of a hybrid endoglucanase composed of the 40.5-kDa catalytic domain of *P. ruminicola* and the cellulose binding domain of a *Thermomonospora fusca* E2 cellulase produced an enzyme with activities on carboxymethylcellulose, amorphous cellulose, and ball-milled cellulose that were, respectively, 1.5, 10, and 8 times greater than the original 40.5-kDa *P. ruminicola* enzyme (Maglione et al., 1992). These studies support the conclusion that the binding of cellulases is important for cellulose digestion but provide little information on the mechanisms by which microbial cells attach to feed.

It is apparent that transfer of the genes coding for fibrolytic enzymes alone is unlikely to provide sufficient genetic information to allow recipient organisms to attach to and digest their solid substrates (Forsberg and Cheng, 1992). The development of nonadherent mutants through transposon mutagenesis is one approach that may identify the specific genes responsible for attachment (Mackie and White, 1990). After these genes are identified and characterized, the true magnitude of the task of transferring cellulolytic capacity to selected species of ruminal bacteria can be adequately assessed.

Implications

Microbial attachment is absolutely essential for the development of the complex microbial populations required for feed digestion in the rumen. Alterations in the properties of the feed (e.g., chemical or physical processing, plant breeding) that promote or deter attachment can be used to control the rate and extent of microbial feed digestion. Further characterization of cellular attachment mechanisms may enable genetic engineering to play a more prominent role in the manipulation of feed digestion through regulation of the genes responsible for the attachment of microorganisms to feed.

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