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Invited Review: Adhesion Mechanisms of Rumen Cellulolytic Bacteria

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ABSTRACT

We divided the adhesion process of the predominant cellulolytic rumen bacteria *Fibrobacter succinogenes, Ruminococcus flavefaciens*, and *Ruminococcus albus* into four phases: 1) transport of the nonmotile bacteria to the substrate; 2) initial nonspecific adhesion of bacteria to unprotected sites of the substrate that is dominated by constitutive elements of bacterial glycocalyx; 3) specific adhesion via adhesins or ligands formation with the substrate, which can be dominated by several bacterial organelles including cellulosome complexes, fimbriae connections, glycosylated epitopes of cellulose-binding protein (CBP) or glycocalyx, and cellulose-binding domain (CBD) of enzymes; 4) proliferation of the attached bacteria on potentially digestible tissues of the substrate. Each of the phases and its significance in the adhesion process are described. Factors affecting bacterial adhesion are described including: 1) factors related to bacterial age, glycocalyx condition, and microbial competition; 2) factors related to the nature of substrate including, cuticle protection, surface area, hydration, and ionic charge; and 3) environmental factors including pH, temperature, and presence of cations and soluble carbohydrate. Based on the information available from the literature, it appears that each of the predominant rumen bacteria—*F. succinogenes, R. flavefaciens,* and *R. albus*—has a specific mechanism of adhesion to cellulose. In *F. succinogenes*, both the glycosidic residues of the outer membrane CBP and especially of the 180-kDa CBP, and the distinct CBD of EG2 EGF and Cl-stimulated cellobiosidase, may play a role in the adhesion to cellulose. No direct evidence, except scanning electron microscopy observations, yet supports the existence of either cellulosome complex or fimbriae structures involved in the adhesion mechanism of *F. succinogenes*. At least two mechanisms, cellulosome-like complexes and carbohydrate epitopes of the glycocalyx layer are involved in the specific adhesion of *R. flavefaciens* to cellulose. *Ruminococcus albus* possesses at least two mechanisms for specific adhesion to cellulose: a cellulosomal-like mechanism, and a CbpC (Pil)-protein mechanism that probably involves the production of fimbrial-like structures. Indirect and direct studies suggested that carbohydrate epitopes of CBPs and CBD epitope of cellulases may also be involved mostly in the nonspecific phase of adhesion of *R. albus*. (**Key words:** *Fibrobacter succinogenes, Ruminococcus albus, Ruminococcus flavefaciens*, specific and nonspecific adhesion)

Abbreviation key: CBD = cellulose-binding domain, **CBP** = cellulose-binding proteins, **CMC** = carboxymethylcellulose, **EG** = endoglucanase, **MC** = methylcellulose, **PAA** = phenylacetic acid, **PPA** = phenylpropanoic acid.

INTRODUCTION

The rumen microbial ecosystem comprises at least 30 predominant bacterial species at a total concentration of 10^{10} to 10^{11}/ml of rumen fluid, some 40 species of protozoa (10^5 to 10^7/ml), and five species of fungi (< 10^5/ml), (Orpin and Joblin, 1997; Stewart et al., 1997; Williams and Coleman, 1997). Bacterial species of the rumen are considered more important than protozoa and fungi in determining the extent and rate of feed degradation and utilization for the production of microbial protein and VFA (Stewart et al., 1997). Subsequently, the host ruminant animal absorbs VFA (mostly through the rumen wall) and digests proteins, lipids, and carbohydrate constituents of microbes and feed residues entering the small intestine to supply its maintenance needs and for the production of meat and milk.

Bacteria inhabiting the rumen have been classified into five groups dependent on their environmental existence: 1) free-living bacteria associated with rumen liquid phase; 2) bacteria loosely associated with feed particles; 3) bacteria firmly adhered to feed particles; 4) bacteria associated with rumen epithelium; and 5) bacteria attached to the surface of protozoa or fungal sporangia (Cheng and Costerton, 1980; McAllister et al., 1994). Under ordinary feeding conditions, bacterial populations associated with feed particles (groups 2 and 3) are numerically predominant and occupy up to 75%

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of the total microbial population and microbial ATP production in the rumen (Craig et al., 1987; Forsberg and Lam, 1977; Minato et al., 1993). Microbial populations associated with feed particles are estimated to be responsible for 88 to 91% of ruminal endoglucanase and xylanase activity, 70% of the amylase activity, and 75% of the protease activity in the rumen (Minato et al., 1993; Williams and Strachan, 1984). These percentages suggest that microbial populations associated with feed particles are pivotal for feed digestion in the rumen. Accordingly, microbes associated with the liquid phase (20 to 30% of the total microbes), including free-living bacteria and bacteria detached from solid substrate, have little direct involvement in the digestion of insoluble feed particles. The bacterial populations associated with rumen epithelium and those attached to the surface of protozoa and fungi (~1% of total rumen population) have a minor role in the process of feed digestion in the rumen.

The importance of adhesion on subsequent cell wall degradation was demonstrated in studies employing cellobiose or glucose grown mutant cells lacking the adhesion ability of *Fibrobacter succinogenes* S85, *Fibrobacter intestinalis* DR7, *Ruminococcus albus* SY3 and 8, and *Ruminococcus flavefaciens* 007. The mutant cells were characterized by smoother appearance of surface topology compared to the wild type cells, lacked adhesins or ligands formation with the substrate, and lost most of their cellulolytic capability (Gong and Forsberg, 1989; Miron et al., 1998; Miron and Forsberg, 1998 and 1999; Reddy and Morrison, 1998; Stewart et al., 1990). Additional support to the relationship between adhesion ability and subsequent cellulose degradation was given by Morris and Cole (1987), who demonstrated that isolates of *R. albus* strains that adhered to cellulose-degraded cellulose better than strains lacking adhesion ability. The necessity of adhesion for subsequent cellulose digestion by rumen bacteria was further demonstrated by the observations that a low concentration of methylcellulose, which mediated detachment of cellulolytic rumen bacteria to cellulose without affecting enzymes activity also blocked cellulose degradation (Kudo et al., 1987; Pell and Schofield, 1993). This review will focus mainly on the predominant rumen cellulolytic bacteria: *F. succinogenes, R. flavefaciens*, and *R. albus*, whose attachment mechanism to fibrous plant particles has been extensively studied over the last decade. These species are considered as firmly attached bacteria that can adhere to cellulose but are incapable of attaching to insoluble starch (McAllister et al., 1994; Minato et al., 1993; Miron et al., 1989; Pell and Schofield, 1993).

In this paper, we describe the understanding obtained so far on: phases of adhesion of rumen cellulolytic bacteria to plant tissues and their importance for subsequent fiber degradation; factors affecting bacterial adhesion to fiber; and mechanism of the adhesion process in each of the rumen cellulolytic species.

PHASES OF BACTERIAL ADHESION TO PLANT TISSUES

When the adhesion process is described, a distinction between specific and nonspecific adhesion should be emphasized (Pell and Schofield, 1993). Thus, the adhesion process occurring in the rumen can better be described by dividing the process into four phases: 1) transport of the bacteria to the fibrous substrate; 2) initial nonspecific adhesion of bacteria to proper sites of the substrate; 3) specific adhesion between the attached bacteria and the digestible tissue via the production of more extensive linkages and adhesins (Figure 1A, B, C, and F); and 4) proliferation of attached bacteria to form colonies on specific sites of the plant tissue (Figure 1C, D, and E). Each of these phases depends on the successful completion of the previous one. The multistep model summarized here is similar to the general mode for microbial adhesion to solid substrate that ultimately results in the formation of biofilm (Busscher and Weerkamp, 1987; Costerton et al., 1981; McAllister et al., 1994; Pell and Schofield, 1993).

Phase I, Transport of Bacteria to the Substrate

Transport of rumen bacteria to the particulate substrate is problematic, because the predominant rumen cellulolytic bacteria lack any flagella or cilia and are therefore nonmotile; also, mixing in the rumen is poor (Weimer, 1996). Thus, the first contact between cellulolytic bacteria and substrate is dependent on the size of the free-suspended cellulolytic population, to bind new particles suspended in the rumen. Several studies (Gong and Forsberg, 1989; Miron et al., 1989, 1990, 1998; Miron and Forsberg, 1998; Morris and Cole, 1987) using various assays of adhesion measurement, showing that a state in which 100% of the cellulolytic rumen bacterial cells are attached to cellulose does not exist; there is always a free-suspended, unattached population of cellulolytic bacterial cells. Wells et al. (1995) presented evidence to demonstrate that *F. succinogenes* released cellodexterins during growth on glucose, cellobiose, or cellulose. Therefore, nonadherent cells and daughter cells of either *F. succinogenes* or ruminococci may have a source of nutrients for growth and survival in the rumen liquid phase (Russell, 1985) and possibly are poised for adhesion to new cell wall particles entering the rumen. Thus, incoming forages would be colonized primarily by nonadherent cells and by daughter cells released from cell division processes on the colo-

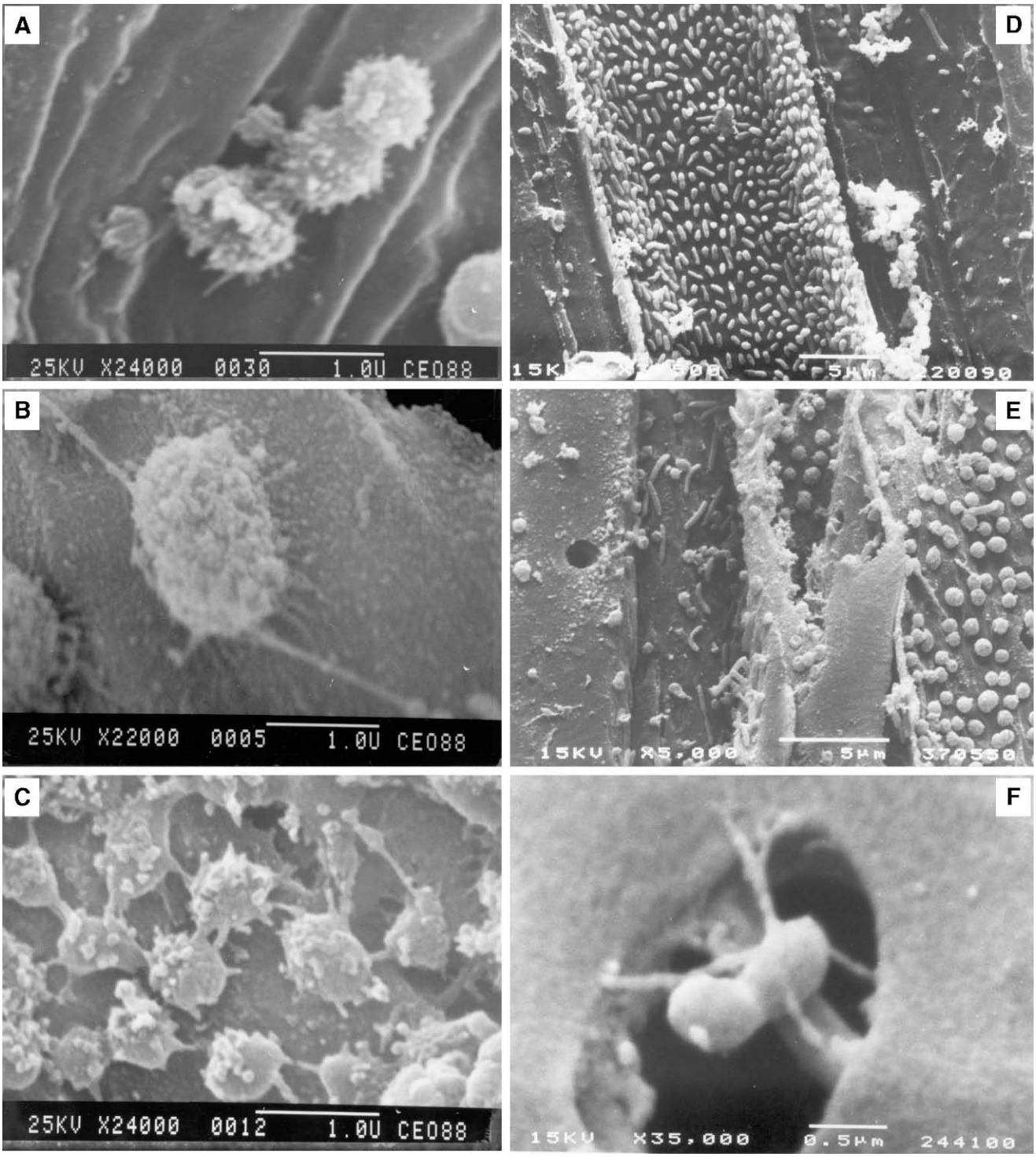

Figure 1. Scanning electron micrographs of cationized-ferritin-pretreated bacterial cells of the strains (A) *Ruminococcus albus* 7, (B) *Ruminococcus flavefaciens* FD1, (C) *Fibrobacter succinogenes* S85, (D) Co-culture of *F. succinogenes* BL2 + *Butyrivibrio fibrisolvens* D1, (E) Coculture of *F. succinogenes* S85 + *B. fibrisolvens* D1, and (F) *F. succinogenes* BL2. Bacterial strains were grown on cell walls of alfalfa (A, B and C) or ozone-treated cotton stalks (E and F) or SO_2-treated wheat straw (D) as the sole added carbohydrate substrate, and attached to cell wall particles. Note: 1. The presence of protuberant structures on bacterial surfaces and adhesins formation with the substrate (A, B, C, F); 2. The dense layer of firmly attached bacterial cells covering wrapped straw stem and broken edges of vascular cotton stalks tissue, compared to the absence of bacterial colonization on nearby unwrapped and protected tissues (D, E and F); 3. The mode of complimentary living of attached *F. succinogenes* cells with *B. fibrisolvens* (D and E). These micrographs were previously published in (Miron et al. 1989; Miron and Ben-Ghedalia, 1992, 1993a).

nized fibers or through direct physical contact between the incoming forage and the already colonized fibers (Weimer, 1996). Despite the poorer mixing in the rumen, this process apparently occurs with sufficient rapidity because of the relatively high volumetric concentration of both fluid-phase microorganisms and fiber (Weimer, 1996). Nonspecific initial adhesion in the rumen is dependent on various factors related to the nature of the bacteria, the substrate, and environmental conditions, as described later.

Phase II, Initial Nonspecific Adhesion

Nonspecific adhesion is initiated when the bacteria arrive within range (2 to 50 nm) of van der Waals forces—hydrophobic, ionic, and electrostatic interaction with the solid substrate (Busscher and Weerkamp, 1987; Pell and Schofield, 1993). Nonspecific adhesion is defined as a combination of reversible and irreversible processes without the involvement of specific adhesins or ligands between bacteria and substrate receptors (Pell and Schofield, 1993). In addition to the physiochemical forces, conformation to substrate shape and wedging into cavities of feed are also considered as nonspecific adhesion. The constitutive bacterial glycocalyx components appear to be involved in the initial binding process (Cheng et al., 1977, 1983; Cheng and Costerton, 1980). Glycocalyx was defined by Costerton et al. (1981) as those polysaccharide-, glycoprotein-, or protein-containing structures of bacterial origin lying outside the outer membrane of gram-negative cells (*F. succinogenes*) and the peptidoglycan of gram-positive cells (*R. flavefaciens* or *R. albus*). The components of bacterial glycocalyx involved in the nonspecific adhesion process may include constitutive carbohydrate epitopes (not induced by cellulosic substrate) of the glycocalyx layer and its proteins, cellulose binding proteins (**CBP**), and additional factors (Cheng et al., 1987; Cheng and Costerton, 1980; Gong et al., 1996; Latham et al., 1978a; Miron and Forsberg, 1998, 1999; Morrison and Miron, 2000; Ohara et al., 2000).

Short- and long-term incubation studies demonstrated that bacteria randomly transported to sites of potential adhesion start their initial adhesion mainly on cut or macerated surfaces of forage particles as demonstrated in Figure 1D and E and reported in several studies (Dinsdale et al., 1978; Latham et al., 1978a, 1978b; Miron and Ben-Ghedalia, 1992, 1993a, 1993b, 1993c). Forage tissues protected by cutin are resistant to adhesion (Akin, 1989; Bauchop, 1980). Some forage tissues contain 18 to 24% silica, which impedes its digestion (McAllister et al., 1994). Physical or chemical pretreatments of fibrous substrate before feeding may create more adhesion sites compared with untreated substrates (Ben-Ghedalia et al., 1993; Miron and Ben-Ghedalia, 1992, 1993a; and Figure 1D and E). The chewing that occurs during eating and rumination by the host animal is necessary to disrupt the protective cutin layer to expose more digestible portions of the plant and to increase the surface area of the substrate and its hydration. This process increases the probability that cellulolytic bacteria will initiate nonspecific binding to fibrous sites. Several studies have shown that although the rate of adhesion of different bacterial species to cellulose varies, this generally occurs shortly after contact with solid substrates. Adhesion of ruminococci species to damaged plant tissues or cellulose occurs within 1 to 5 min after their addition into the medium (Latham et al., 1978a, 1978b; Morris, 1988; Morris and Cole, 1987; Roger et al., 1990). However, maximum adhesion of *F. succinogenes* cells is not attained until 15 to 30 min after contact with cellulose (Gong and Forsberg, 1989; Roger et al., 1990). The initial nonspecific adhesion is a prerequisite for the third phase, in which bacterial-substrate linkages and adhesins are created.

Phase III: Specific Adhesion

Specific adhesion is defined as a process in which ligands or adhesins on the bacterial cell surface recognize receptors on the substrate tissue (Pell and Schofield, 1993). The surface topology of *F. succinogenes, R. albus*, and *R. flavefaciens* cells grown on and attached to plant cell walls were characterized by extensive aggregation of protuberances on the glycocalyx layer and formation of adhesins connections with the plant cell walls sites, that could be seen only after cationized-ferritin prestaining (Figure 1A, B, C, and F). These protuberance organelles created after several hours of incubation have been suggested to be cellulosome complexes and, more recently, to have fimbriae structure (Kim et al., 1999; Miron et al., 1989, 1990; Morrison and Miron, 2000; Pegden et al., 1998). We have suggested (Miron et al., 1989, 1990) that attached ruminal bacteria receive stimulating signals during initial cell wall polysaccharide digestion for the subsequent manufacture of inducible linkages and adhesins between the outside glycocalyx layer of the bacteria and the digestible tissue and for the manufacture of cellulolytic enzymes as shown in Figure 1A, B, C, and F. This suggestion has been supported by previous studies (Bera et al., 1999; Doerner et al., 1992; Flint et al., 1999; Gong et al., 1996; Karita et al., 1997; Malburg et al., 1997; McGavin and Forsberg, 1989; Mitsumori and Minato, 1995, 1997; White et al., 1997) based on incubation for several hours, showing that the presence of cellulose or xylan substrates induced the production of some bac-

terial cellulolytic and xylanolytic enzymes and also possibly of some cellulose binding proteins.

The importance of adhesin formation in plant cell wall digestion has been demonstrated in several electron microscopy observations (Akin, 1979; Kudo et al., 1987; Latham 1980; Miron et al., 1989, 1990; Morris and Cole, 1987), and adhesins always formed later in the adhesion process. Structures that have been frequently proposed as adhesins in cellulolytic bacteria include polycellulosome complexes, fimbriae or pili, glycocalyx capsule, cellulosic fibrils, cellulose binding proteins, and enzyme binding domains (Morrison and Miron, 2000; Pell and Schofield, 1993).

Fibrobacter succinogenes, R. flavefaciens, and *R. albus* cells preadapted to grow on plant cell walls produce protuberant-like organelles on their cell surface, adhere to cellulose better via adhesins formation, and degrade plant cell wall faster than bacteria preadapted to grow on cellobiose (Kim et al., 1999; Miron et al., 1989, 1990; Miron and Ben-Ghedalia, 1993a). Based on these data, we suggest that a high concentration in the rumen of viable daughter bacterial cells containing the induced glycocalyx organelles needed for specific adhesion and adhesins formation is responsible for reducing the lag time of cell wall degradation in the rumen.

The strategy of specific and strong adhesion to the cellulosic substrate provides several advantages for the bacteria. First, the cellulolytic enzymes are concentrated on their substrate, and other microbes are excluded from the site of hydrolysis, which allows the attached bacteria to have first access to the products of cellulose hydrolysis (Minato et al., 1993; Pell and Schofield, 1993; Figure 1D and E). Moreover, strong adhesion protects the attached bacteria from grazing by ruminal protozoa and protects their cellulolytic enzymes themselves from ruminal proteases (Pell and Schofield, 1993; Weimer, 1996). Finally, bacteria attached to food particles have a retention time in the rumen as much as three times longer than these free in the liquid phase, thus having better opportunity to digest plant cell walls polysaccharides (Minato et al., 1993; Weimer, 1996).

The intimate and specific linkages between the attached bacteria and the potentially digestible substrate results in a proliferation of new generations of induced bacteria for the production of colonies, as described in phase IV. If a bacterial cell is attached to an undigestible site of plant substrate, it would not proliferate to produce a colony, as demonstrated in unwrapped plant tissues (Figure 1D, E, and F).

Phase IV: Proliferation and Colonization on Plant Tissues

During this phase, the adhered bacteria proliferate to create colonies on potentially digestible sites of forage particles (Figure 1C, D, and E). Latham et al. (1978a, 1978b) observed that the epidermis of perennial ryegrass is colonized by *R. flavefaciens* within 30 min of incubation in rumen fluid. *R. flavefaciens* predominates only on uncut surfaces of epidermis, phloem and sclerenchyma cell walls. In contrast, *F. succinogenes* colonizes more slowly on cut edges of most plant cells, except those of xylem, and also colonizes uncut surfaces of mesophyll, epidermis, and phloem cell walls. Dinsdale et al. (1978) found that cell walls of grass leaves are digested primarily by ruminococci while fibrous cell walls and cotton fibers were colonized by *F. succinogenes*. Thus, bacterial species demonstrate different specificity for binding and colonization, which serves to reduce competition. Additional documentation of adhesion and colonization specificity was provided by Bhat et al. (1990), who found that *R. flavefaciens* and *F. succinogenes* have separate specific adhesion sites on barley straw. After adhesion and initial colonization, a film of bacterial layer were evident after several hours of incubation on the interior of the tissues between plant parenchyma and phloem cells and subsequently begins the digestion of the plant cell walls (Cheng et al., 1983; Latham et al., 1978a, 1978b; Figure 1C, D, and E). The cell wall rich tissue is degraded by slowly diffused nonmotile bacteria and their enzymes from the "inside-out" namely: starting from cell lumen of broken cells, toward S3, S2, and S1 layers of the secondary cell wall, and terminates in the lignified middle lamella (Cheng et al., 1983; McAllister et al., 1994; Weimer, 1996). The mode of degradation of plant cell walls by either *F. succinogenes* or *R. flavefaciens* is essentially through the production of well-defined pits in the colonized tissue. Cells of pure *R. albus* cultures are identified as loosely adherent, always being at a distance from the plant cell walls and surrounded by extensive condensed glycocalyx (Cheng et al., 1983). These observations suggest that each of the cellulolytic bacterial species have different modes of adhesion to plant cell walls, as will be discussed later. Akin (1989) suggested the following general pattern of ease and extent of grass tissue digestion by rumen bacteria: mesophyll, phloem > epidermis, parenchymal boundle sheath > sclerenchyma > lignified vascular xylem.

FACTORS AFFECTING BACTERIAL ADHESION

Several factors that affect both the nonspecific and the specific phases of the adhesion process include: 1) factors related to bacterial age, bacterial glycocalyx condition and microbial competition; 2) factors related to the substrate including, cuticle protection, surface area, hydration, ionic charge and cation exchange capacity; and 3) environmental factors including pH, tempera-

ture, and the presence of O_2, cations (Na^+, Ca^{+2}, and Mg^{+2}) and soluble carbohydrate. Much of the work on the effects of bacteria, substrate, and environmental conditions on the adhesion process, as described below, has been done in short-term incubations before de novo glycocalyx synthesis. Therefore, the implications of these studies are more relevant to the initial nonspecific adhesion phase than to the specific phase.

Different types of assays have been used to measure adhesion: 1) a turbidimetric assay that measure adhesion based on change in optical density after cellulose has been added to a culture and allowed to sediment (Gong and Forsberg, 1989; Minato et al., 1993; Roger et al., 1990); 2) an assay using bacteria radiolabeled with ^{14}C or ^{3}H (Morris, 1988; Mosoni et al., 1997; Pell and Schofield, 1993; Rasmussen et al., 1989); and 3) an assay based on protein determination of bacteria adhered to cellulosic substrate (Bhat et al., 1990; Weimer and Schmidt, 1989). The assay used may greatly influence the outcome of these studies (McAllister et al., 1994; Minato et al., 1993; Pell and Schofield, 1993). However, the results summarized below are necessary to better understand the adhesion mechanism and can serve to identify areas for further research aimed at improving the adhesion process in the rumen.

Factors Related to Bacteria

Age. Maximal adhesion of *F. succinogenes* and *R. flavefaciens* was obtained when bacterial cultures were in their midexponential stage of growth (Bhat et al., 1990; Weimer and Schmidt, 1989).

Bacterial envelope condition. Several studies demonstrated the effects of various treatments of bacterial cultures on their adhesion ability. In *F. succinogenes*, treatments of bacterial cells with several enzymes (including trypsin, pronase, proteinase K, thermolysin, protease, and lipase), carbohydrate removal by periodate treatment, and protein fixation with glutaraldhyde (0.1%) significantly decreased bacterial adhesion to cellulose. Killing the bacterium by sodium azide or formalin without protein denaturation had no effect on adhesion, whereas killing the bacteria by heat treatment that also cause complete protein denaturation, strongly inhibited the adhesion (Gong and Fors berg, 1989; Minato et al., 1993; Pell and Schofield, 1993; Roger et al., 1990). Similarly, in *R. albus* protease treatments with trypsin and pronase, and carbohydrate removal by dextranase or periodate oxidation, decreased bacterial adhesion to cellulose (Pell and Schofield, 1993). In *R. flavefaciens*, fixation of bacterial proteins with formaldhyde strongly inhibited adhesion, whereas killing the bacterium with sodium arsenate or heat treatment reduced adhesion only by 15% (Rasmussen et al., 1989; Roger et al., 1990). These studies suggest that both proteins and carbohydrate components of these bacteria were involved in their adhesion to cellulose, and adhesion also occurred by dead bacteria if their protein structures were not modified or denaturated. Features of these protein and carbohydrate structures involved in adhesion mechanism are discussed below.

Microbial competition. In vitro studies have demonstrated that adhesion of *F. succinogenes* is inhibited to a limited quantity of cellulose when the ruminococci species are added simultaneously (Bhat et al., 1990; Mosoni et al., 1997). This competition was explained by the faster adhesion of Ruminococci species (within 5 min) compared with the slower adhesion of *F. succinogenes* (Mosoni et al., 1997). When *R. flavefaciens* and *F. succinogenes* are already adherent, *R. albus* 20 adhesion occurred without inhibition but involved *R. flave faciens* detachment (Mosoni et al., 1997). These data suggest that the two *Ruminococcus* species have the same adhesion site or physically hinder each other during their adhesion. However, the *Ruminococcus* species and *F. succinogenes* probably have different adhesion sites (Mosoni et al., 1997). The rumen xylanolytic bacteria *Butyrivibrio fibrisolvens* and *Prevotella ruminicola* do not interfere with adhesion to and digestion of plant cell walls by ruminococci and *F. succinogenes* and act with the cellulolytic species in a complementary manner (Miron and Ben-Ghedalia, 1992, 1993a, 1993b, 1993c; Miron et al., 1994; Figure 1D and E). Some evidence suggests that rumen fungi can inhibit bacterial fibrolytic activity (Joblin, 1997); however, interaction for adhesion to cellulose between protozoa or fungi and the rumen cellulolytic bacteria have not been identified.

Factors Related to the Substrate

We have already noted the need to remove parts of the cutin wrapping of the plant tissue before consequent rumen bacterial adhesion.

Using a variety of cellulosic substrates, Weimer and Schmidt (1989) demonstrated that *F. succinogenes* cells adhered better to cationic cellulose ethers than to neutral crystalline cellulose, whereas anionic cellulose-ether substrate reduced adhesion of this bacterium. Increasing the surface area of the cellulosic substrate by fine grinding resulted in increasing adhesion of *F. succinogenes*.

Environmental Factors

Temperature. The adhesion to cellulose of the three cellulolytic species was completely inhibited at temperatures below 4°C, and in *R. albus* and *F. succinogenes*

adhesion also decreased in temperature above 50°C and achieved maximal values at 30 to 38°C (Gong and Forsberg, 1989; Minato et al., 1993; Morris and Cole, 1987; Pell and Schofield, 1993; Roger et al., 1990). Heat denaturation (100°C) completely reduced *F. succinogenes* adhesion, but was less effective on *R. flavefaciens* (Roger et al., 1990). Some differences between studies may be ascribed to variations in the technique used for measuring adhesion.

pH. The effect of pH on adhesion of cellulolytic bacteria to cellulose varied according to bacteria. Roger et al. (1990) showed that the adhesion of *F. succinogenes* to cellulose increased as pH was increased from 4.5 to 6, remained stable between pH 6 and 7, and fell abruptly above pH 7.5. Notwithstanding, Gong and Forsberg (1989) reported that the adhesion of this bacterium did not change over a pH range of 5.3 to 6.8. Roger et al. (1990) also showed that the adhesion of *R. flavefaciens* to cellulose was stable at pH values between 3.3 and 7.5, and decreased at pH 8, whereas Rasmussen et al. (1989) reported that the adhesion of the bacterium was not affected by changes in pH between 6 and 8. The adhesion of *R. albus* was not affected by changes in pH between 5.5 and 8 (Morris, 1988). These differences between studies may be ascribed to variations in the technique and bacterial strains used for measuring adhesion.

Presence of cations. Gong and Forsberg (1989) reported that the adhesion of *F. succinogenes* to cellulose was enhanced by the presence of either Ca^{+2} or Mg^{+2} and Na^{+}, whereas Roger et al. (1990) reported that the adhesion of this bacterium was insensitive to the presence of divalent cations, although Na^{+} was essential for adhesion. The adhesion of *R. flavefaciens* was not affected by deprivation of either Ca^{+2}, Mg^{+2}, or Na^{+}, but was significantly reduced by the deprivation of both divalent cations. Therefore, Roger et al. (1990) suggested that interaction between the bacterial cell and the divalent cations Ca^{+2} and Mg^{+2} is the main mechanism involved in *R. flavefaciens* adhesion, although hydrophobic interactions and enzymes may also be involved.

Soluble carbohydrates. The effect of soluble carbohydrate on adhesion of cellulolytic rumen bacteria to cellulose-solid substrate has been studied in several works. The adhesion of *F. succinogenes, R. albus*, and *R. flavefaciens* to cellulose is not inhibited by glucose, mannose, xylose, maltose, cellodextrins, and soluble starch added at concentration of 1% (Minato et al., 1993). However, the adhesion of *F. succinogenes* to cellulose is strongly inhibited by 1% cellobiose, whereas that of ruminococci cells is only slightly affected by cellobiose (Minato et al., 1993; Morris, 1988; Rasmussen et al., 1989). Cellulose-binding factors of *F. succinogenes* specifically therefore recognize a cellobiose moiety site of cellulose and therefore addition of excess cellobiose blocks the bacterial binding factors (Minato et al., 1993).

The adhesion of the three rumen cellulolytic species to cellulose is strongly inhibited by soluble derivatives of cellulose including sodium-carboxymethylcellulose (**CMC**) and methylcellulose (**MC**) added at concentrations of 0.1% (Bhat et al., 1990; Kudo et al., 1987; Minato et al., 1993; Morris, 1988; Rasmussen et al., 1989). However, these results are in conflict with the data of Roger et al. (1990), reporting that the adhesion of *F. succinogenes* and *R. flavefaciens* is not inhibited by the addition of CMC. These findings suggest that the recognition site of cellulose binding factors of *R. albus* and *R. flavefaciens* is larger than a cellobiose unit or repeating cellobiose moiety, and therefore adhesion of these bacteria to cellulose is blocked when the bacterial cells are coated with high molecular weight MC or CMC.

These data show the effect of several factors on the nonspecific adhesion phase, since adhesion measurements are for short incubation periods (~1 h). However, some environmental factors that affect the viability of bacteria (e.g., pH, Na^{+} depletion, and temperature) or inhibition of bacterial growth due to bacteriocin secretion by coculture bacterial species (Chan and Dehority, 1999) may also inhibit the specific adhesion phase that occurred after several hours of fermentation.

MECHANISMS OF BACTERIAL ADHESION TO CELLULOSE

Investigators have focused on four structures believed to be important in specific adhesion to cellulose of the rumen cellulolytic bacteria: 1) large multicomponent complexes called cellulosomes (Flint et al., 1999; Karita et al., 1997; Lamed et al., 1987; Miron et al., 1989, 1990; Morrison and Miron, 2000; Ohara et al., 2000); 2) fimbriae or pili adhesins (Morrison and Miron, 2000; Pegden et al., 1998); 3) carbohydrate epitopes of bacterial glycocalyx layer (Cheng and Costerton, 1980; Gong et al., 1996; Miron and Forsberg, 1998, 1999; Pell and Schofield, 1993); and 4) enzyme binding domains (Gong et al., 1996; Karita et al., 1997; McGavin and Forsberg, 1989; Mitsumori and Minato 1995, 1997). Evidence for the occurrence of these structures in each of the rumen bacterial species will be discussed below.

Adhesion via Cellulosome-like Complexes

Cellulosomes are large, stable, multienzyme complexes specialized in the adhesion to and degradation of cellulose that reside within protuberances visible on the cell surface (Bayer et al., 1998, 1999; Beguin et

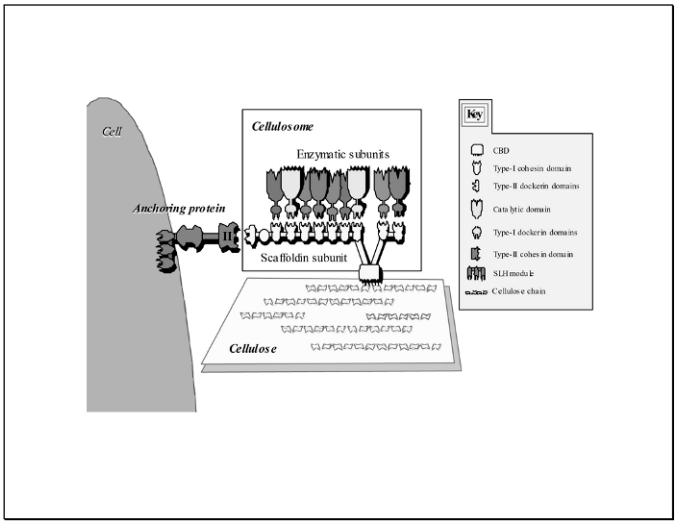

Figure 2. A schematic model of the *Clostridium thermocellum* cellulosome. Provided by Ed Bayer, Dept. of Biological Chemistry, The Weizmann Institute of Science, Rehovot, Israel.

al., 1996; Lamed et al., 1983). Electron microscopic, biochemical, and immunochemical methods were used to establish the existence of "cellulosomes" in a phylogenetically diverse range of anaerobic bacteria, including rumen cellulolytic species (Lamed et al., 1983, 1987; Miron et al., 1989, 1990). Much of our knowledge of cellulosomes has been derived from the study of *Clostridium* spp., and a model of the *Clostridium thermocellum* cellulosome is reproduced in Figure 2. An integral feature of the cellulosome is the "scaffoldin" or "cellulosome-integrating protein." It is a large, glycosylated protein that possesses a series of functional domains shown to facilitate either enzyme adhesion (via type I cohesin domains), cellulose binding or, in the case of the *C. thermocellum* scaffoldin, anchoring to the bacterial cell surface (via a type II dockerin domain). The catalytic components of the cellulosome include enzymes involved with the hydrolysis of plant cellulose and heteroxylan. These proteins are also characterized by their modular architecture, with discrete domains coordinating either cleavage of polysaccharide bonds, cellulose binding or enzyme activity, and adhesion to the scaffoldin (via a type I dockerin-type I cohesin interaction) (Bayer et al., 1998, 1999). A recent study of two *Clostridium* species indicated that the type I dockerin of the catalytic proteins interact selectively and in a species-specific manner with the type I cohesins of the scaffoldin (Pages et al., 1997). The anchoring of the *C. thermocellum* cellulosome to the bacterial surface also appears to be mediated by a cohesin-dockerin type of interaction. The type II dockerin of the *C. thermocellum* scaffoldin forms a stable interaction with a type II cohesin domain, which is present in a surface protein incorporated into the bacterium's S-layer (Bayer et al., 1998 and 1999).

Adhesion of bacterial cellulosome to cellulose is mediated by cellulose-binding domain (**CBD**) of the scaffoldin plus CBD of enzymes connected to the scaffoldin. Although six distinct families of CBD have been identified in cellulolytic microorganisms, the CBD of *C. thermocellum* provides a good example of the structure and function of scaffoldin's CBD. Scaffoldin's CBD is composed of nine-stranded β-sandwich of jellyroll topology, that form two antiparallel β sheets. The planar face of the CBD molecule interacts with three successive chains on the cellulose surface, by harboring a planar strip of aromatic residues of amino acids aligned precisely along one of the cellulose chains. The stacking interactions performed between the planar strip residues and the glucose rings along the cellulose chain are considered the major cause of the specificity and strong binding of the CBD to crystalline cellulose substrate (Bayer et al., 1998). Some CBD also appear to catalyze the disruption of the hydrogen bonds interactions between the chains of the crystalline cellulose (Din et al., 1991).

Our knowledge of "cellulase" enzyme structure and function has progressed quite rapidly, and molecular details describing the structure and function of various scaffoldins are also now beginning to emerge (Bayer et al., 1999). The discovery and characterization of the cellulosomes from a number of bacterial species has helped to explain why many anaerobic cellulolytic bacteria adhere tightly to plant surfaces, and why their "cellulase" activity is principally located on the bacterial cell surface.

Based on electron microscopy observations, Miron et al. (1989, 1990) suggested that the predominant rumen cellulolytic bacteria contain cellulosome-like complexes. Recently, genetic evidence was provided to this suggestion, showing that some of the enzymes of *R. flavefaciens* and *R. albus* are integrated to form cellulosome-like complexes (Flint et al., 1999; Karita et al., 1997; Kirby et al., 1997; Morrison and Miron, 2000; Ohara et al., 2000; Reddy and Morrison, 1998).

Adhesion via Fimbriae or Pili

Fimbriae or pili, which have been implicated in bacterial adhesion, are surface appendages that are 5 to 7 nm in width and 100 to 200 nm in length in gram-negative bacteria (Pell and Schofield, 1993). Initially, fimbriae were identified on gram-negative bacteria, but they also are involved in adhesion of gram-positive bacteria (Fives-Taylor and Thompson, 1985). The fimbriae of gram-positive bacteria are aggregated proteins

rather than the highly ordered structures observed in gram-negative cells (Doyle and Sonnenfeld, 1989). As more has been learned about the role of fimbriae in adhesion, it has become clear that structural subunits of fimbriae are the actual adhesins. Some subunits in the gram-positive bacteria *Actinomyces viscosus* (Yeung and Cisar, 1990) and *S. sanguis* (Fenno et al., 1989) associated with the fimbriae have been identified as adhesins. In *E. coli*, the carbohydrate-binding sites of three types of fimbriae are in small (28 to 35 kDa) repeated subunits, most of which are in the tips of the fimbriae with a few additional sites along their length (Lindberg et al., 1987).

Recently, the research team of Morrison has identified in *R. albus 8* a novel form of cellulose-binding protein (cbpC, 17.7 kDa) that belongs to the Pil protein family, being most similar to the type 4 fimbrial proteins of gram-negative, pathogenic bacteria (Larson et al., 1999; Pegden et al., 1998). Thus, *R. albus* appears to produce a fimbriae mechanism involved in its adhesion to cellulose, which is consistent with the morphology of the cell glycocalyx observed by electron micro scopy (Costerton et al., 1981; Morrison and Miron, 2000; Patterson et al., 1975; Stack and Hungate, 1984).

Adhesion via Carbohydrate Epitopes of Bacterial Glycocalyx

Most of the evidence that implicated polysaccharides in adhesion is from electron microscopy observations (Cheng and Costerton, 1980; Cheng et al., 1983; Dinsdale et al., 1978; Latham et al., 1978a, 1978b). Several studies reported that the slime layer surrounding *R. albus* and *R. flavefaciens* was composed of glycoproteins that their carbohydrate residues were involved in adhesion of the bacteria (Cheng and Costerton, 1980; Latham et al., 1978a). Treatment with protease (trypsin and pronase) and dextranase and removal of glycocalyx carbohydrate by periodate oxidation significantly decreased adhesion of *R. albus* and *F. succinogenes* to cellulose (Pell and Schofield, 1993). Using specific lectins, Baintner et al. (1993) demonstrated that *R. flavefaciens, R. albus*, and *F. succinogenes* react with lectins that can specifically bind to glucose or mannose of bacterial envelope, and *F. succinogenes* also reacts with lectins specific to galactose. These indirect studies suggest that both protein and carbohydrate are involved in adhesion mechanism of cellulolytic bacteria.

More direct evidence for the role of carbohydrate in adhesion was given recently in *Fibrobacter* species (Gong et al., 1996; Miron and Forsberg, 1998, 1999) and in *R. albus* SY3 (Miron, 2001, unpublished data), as described later. However, additional biochemical and genetic evidence is needed to explore the role of glycocalyx carbohydrate in the adhesion process of the rumen cellulolytic bacteria.

Adhesion via Cellulose-Binding Domains of Cellulolytic Enzymes

Examination of cellulase structure in some organisms has revealed two functional domains: the active catalytic domain that is responsible for the hydrolytic cleavage of the glycosidic bonds, and the binding domain that binds the bacterial enzymes to its substrate. In many of these cases, the CBD is linked to the catalytic core by linkers rich in hydroxy amino acids. Most of the CBD identified in nonruminal bacteria share considerable homology in the presence of four conserved tryptophan and additional two cysteine residues, and the CBD do not participate in catalysis (Tomme et al., 1995). Some CBD may participate in disruption of hydrogen bonds between cellulosic chains (Din et al., 1991). Because of the conserved aromatic residues, it is thought that CBD attached to cellulose either by hydrogen bonding or hydrophobic interactions (Tomme et al., 1995). Previous experiments have identified CBD and shown that bacteria lacking these domains were less adherent, and in some cases, less able to digest crystalline cellulose (McGavin and Forsberg, 1989; Tomme et al., 1995).

Distinct binding domains have been identified in *F. succinogenes*, including the CBD of endoglucanases 2 (EG2) and EGF, which are induced by cellulose, and the chloride stimulated cellobiosidase (Clcbsase) (Fors berg et al., 1993; Gong et al., 1996; Huang et al., 1988; Malburg et al., 1997; McGavin and Forsberg, 1989; Mitsumori and Minato, 1995, 1997).

Recently, Karita et al. (1997) cloned a gene *egVI* encoding a family 9 cellulase from *R. albus* F-40 and found that the enzyme contained a distinct CBD.

No genetic evidence is available to demonstrate whether *R. flavefaciens* contains a distinct CBD in its enzymes; however, a noncatalytic 30-kDa CBP has been identified in this bacterium (Mitsumori and Minato, 1997).

ADHESION MECHANISMS IN *FIBROBACTER SUCCINOGENES*

Fibrobacter succinogenes binds tightly to the surface of plant materials via adhesins leading to extensive plant cell wall degradation (Forsberg et al., 1993; Miron et al., 1989; Miron and Ben-Ghedalia, 1993a, 1993b). At least nine different glucanase genes and one cellodextrinase gene plus four xylanase genes have been cloned from *F. succinogenes* S85 (Bera et al., 1999; Forsberg et al., 1993; Iyo and Forsberg, 1996; Malburg

and Forsberg, 1993; Malburg et al., 1997). Cellulolytic enzymes of *F. succinogenes* S85 that have been purified, cloned, and characterized in these studies include endoglucanases (**EG**) EG1, EG2, EG3, EGB, EGD, EGE, EGF and EGG, a chloride stimulated cellobiosidase (Clcbsase); a cellodextrinase (CEDA); and the endoxylanases EX1, EX2, and XynC. In addition, a number of heteroxylan-debranching enzymes, including acetyl-xylan esterase, arabinofuranidase, α-glucoronidase, and lichenase were also identified in this bacterium. Parts of those enzymes were cell associated with the outer layers of the bacterium, and production of almost all enzymes was quantitatively induced by cellulosic substrate (Bera et al., 1999). However, only three of the *F. succinogenes* enzymes including the endoglucanases EG2 and EGF and the chloride-stimulated cellobiosidase (Clcbsase) probably contained a distinct CBD. Thus, at least those enzymes of *F. succinogenes* that contain a CBD may be involved in bacterial adhesion to cellulose (Forsberg et al., 1993; Huang et al., 1988; McGavin and Forsberg, 1989).

In addition, evidence suggests that seven CBP of 40, 45, 50, 120, 180, 220, and 240 kDa from outer membrane of *F. succinogenes*, which possess a common carbohydrate epitope, are involved in the adhesion mechanism. Immunogold labeling of the 180-kDa CBP demonstrated its importance in adhesion to cellulose via the common glycosidic epitope (Gong et al., 1996). Periodate oxidation of carbohydrate and protease treatments of bacterial cells prior to adhesion demonstrate the possible importance of these glycoproteins in mediating the adhesion to cellulose of *F. succinogenes* (Pell and Schofield, 1993).

In a recent study employing another member of the *Fibrobacter* family (the lower gut bacterium *F. intestinalis* DR7), the role of carbohydrate in *Fibrobacter* adhesion was further supported. Using immunochemical methods, we have demonstrated that the carbohydrate components of glycosylated CBP isolated from the outer membrane and periplasm of *Fibrobacter intestinalis* DR7 (lower gut bacteria) play a significant role in the adhesion of this bacterium to cellulose. These isolated CBP included residues of glucosamine, galactosamine, glucuronic acid, and galacturonic acid that blocked adhesion to cellulose when premixed as neutral monosaccharides with cellulose in the growth medium before bacterial addition (Miron and Forsberg, 1998, 1999). However, additional biochemical and genetic evidence is needed to explore the role of glycocalyx carbohydrate in the adhesion process *F. succinogenes*.

Thus, both the glycosidic residues of the outer membrane CBP and especially of the 180-kDa CBP, and the distinct CBD of EG2, EGF, and Cl-stimulated cellobiosidase may play a role in the adhesion of *F. succinogenes* to cellulose. The only evidence to support the existence of either cellulosome complex or fimbriae structures involved in the adhesion mechanism of this bacterium is scanning electron microscopy observations (Gong and Forsberg, 1989; Miron et al., 1989; Miron and Ben-Ghedalia, 1992, 1993a, 1993b, 1993c; Figure 1 C to F). However, in bacterial cells prestained with cationized ferritin, the presence of ultrastructural protuberances is sometimes connected to growth rate rather than to induction of cellulolytic systems (Blair and Anderson, 1998).

ADHESION MECHANISMS IN *RUMINOCOCCUS FLAVEFACIENS*

The rumen cellulolytic bacterium *Ruminococcus flavefaciens* adhere immediately and firmly to fibrous plant particles and degrade grass and straw cell-wall polysaccharides faster than the other ruminal cellulolytic species (Latham et al., 1978a, 1978b; Miron et al., 1994). For example, *R. flavefaciens* FD-1 has a maximum dilution rate on crystalline cellulose (0.1/h) that is higher than that of other ruminal bacteria (Shi and Weimer, 1992). Ultrastructural observations of several strains of *R. flavefaciens* cells grown on and attached to plant cell walls demonstrated that the bacterium contained protuberances on its surface and forms adhesins with the cellulosic substrate (Latham et al., 1978a; Miron et al., 1989, 1990; Stewart et al. 1990). White et al. (1997) reported that the endoglucanases of *R. flavefaciens* FD1 exist in two forms: a large enzyme complex of molecular mass greater than 3000 kDa termed as complex A and a smaller fraction of enzyme activity (89 kDa) designated as complex B. They suggested that complex A contains at least 13 different endoglucanase activities, whereas complex B has five unique endoglucanase activities. Furthermore, some of the polypeptides in these complexes were glycosylated.

Gene sequence analysis of the three endoglucanases and one cellodextrinase identified in *R. flavefaciens* FD1, and of the three xylanases (Xyn B, C, and D), endoglucanase (endA), and esterase (EstA) identified in *R. flavefaciens* 17, demonstrate that these enzymes lack any distinct CBD (Doerner et al., 1992; Flint et al., 1999; White et al., 1997). However, some of *R. flavefaciens* 17 enzymes including Xyn B, Xyn D, EndA, and EstA contain a dockerin-like domain, suggesting that they are integrated to form a cellulosome-like complex that may be involved in adhesion mechanism (Flint et al., 1999; Kirby et al., 1997). Recently, in a collaborative study between the research groups of Flint (Scotland) and the Israeli group of Lamed and Bayer, the scaffoldin protein of the cellulosome complex of *R. flavefaciens* 17 has been identified and sequenced, and

several cohesins connecting the scaffoldin with type 1 dockerin of the catalytic enzymes have been identified. However, it is not clear yet whether *R. flavefaciens* scaffoldin contains a distinct CBD or not (Lamed and Bayer, 2000, personal communication).

Mitsumori and Minato (1995 and 1997) identified a firmly attached CBP of 30 kDa in some *R. flavefaciens* strains; however, its role in the adhesion process is not clear yet.

The early study of Latham et al. (1978a) regarding the possible role of glycocalyx glycoproteins in mediating adhesion of *R. flavefaciens* cells to cellulose, was further supported by a study with lectins responding with bacterial glycocalyx (Baitner et al., 1993). The finding that polypeptides of the two complexes identified in *R. flavefaciens* FD1 are glycosylated (White et al., 1997) supports the possible importance of carbohydrate epitopes in adhesion of the bacterium. The adhesion of *R. flavefaciens* to cellulose was inhibited by MC or CMC added to medium (0.1%) but not by the addition of cellobiose (1%), suggesting that the recognition site of cellulose binding factors of this bacterium is larger than a repeating cellobiose moiety (Bhat et al., 1990; Minato et al., 1993; Rasmussen et al., 1989).

Thus, at least two mechanisms, cellulosome-like complexes and carbohydrate epitopes of the glycocalyx layer, are involved in the adhesion of *R. flavefaciens* to cellulose.

ADHESION MECHANISMS IN *RUMINOCOCCUS ALBUS*

The Cellulosome Paradigm

Indirect evidence for the presence of cellulosome-complex organelles in *R. albus* was obtained by a combination of electron microscopic and immunohistochemical methods (Kim et al., 1999; Lamed et al., 1987; Miron et al., 1989, 1990, 1998). Wood et al. (1982) reported that the cellulases activity of cellobiose grown *R. albus* SY3 was cell associated and of high molecular mass unstable complex (1.5 MDa) and could be disrupted by dissociating agents into proteins of low molecular mass. Later it was found that rumen fluid factors identified as phenylpropanoic acid (**PPA**) or phenylacetic acid (**PAA**), stabilized *R. albus* 8 cellulases and prevent dissociation of its surface organelles (Pegden et al., 1998; Stack and Hungate, 1984). In contrast to the findings of Wood et al. (1982), we have isolated and separated the glycocalyx capsule, inner membranes, and peptidoglycan cell walls of cellobiose grown *R. albus* SY3, from the extracellular fluid and the cytoplasm, by using a combination of different buffers extraction, centrifugation, ultracentrifugation, and enzymatic solubilization. We found that most of the cellulases, xylanases, and cellulose-degrading activities of *R. albus* SY3 were indeed associated with outer bacterial layers (capsule and cell walls) and not secreted into the extracellular medium. However, only small portion of the cellulolytic and xylanolytic activities were associated with a cellulosome-like enzymatic complexes of molecular mass >400 kDa (Miron, 2000, unpublished data). Our findings are consistent with electron microscopy observations that the adherent *R. albus* cells are embedded in a glycocalyx capsule and their cell membranes do not directly contact cellulose surface (Cheng et al., 1983; Dinsdale et al., 1978). Genetic support for our quantitative activity data is provided by sequence analysis of endoglucanases celA and celB of *R. albus* SY3 and endoglucanases I, II, III, and IV of *R. albus* F-40, and several xylanases, showing that these enzymes lack any epitope of either a CBD or a dockerin-like domains, suggesting that they are not integrated as part of a cellulosome complex (Karita et al., 1997; Nagamine et al., 1997; Ohara et al., 2000; Poole et al., 1990; White et al., 1997). Even though most of the *R. albus* SY3 endoglucanases are not integrated into cellulosome-like organelles, we have isolated high molecular weight complexes that contain mainly xylanases and some endoglucanase activity, and most of the activity was attached to cellulose. Thus it is suggested that the cellulosome complex of *R. albus* may contain a CBD or enzymes employing CBD (Miron, 2001, unpublished data).

In parallel, a cellulosome complex was isolated from the culture supernatant of *R. albus* F-40 grown on cellulose, and its components were identified as three previously sequenced endoglucanase (egV, egVI, and egVII) plus additional unidentified five endoglucanases, three xylanases, and four nonenzymatic proteins (Karita et al., 1997; Ohara et al., 2000). Genetic evidence for the presence of cellulosome-like organelles in *R. albus* 8 were also provided by Reddy and Morrison (1998), who isolated mutants incapable of adhesion and found that mutant strains lacked a 115- (CbpD) and 95-kDa (CbpE) CBP. Several independent clones that hybridized to the CpbD probe have been isolated and partially sequenced. Sequences highly similar to the type I dockerins of various *Clostridium* endoglucanases and xylanases, as well as the dockerin domain recently reported by Karita et al. (1997), were identified in these clones (Morrison and Miron, 2000; Reddy and Morrison, 1998).

The questions whether the cellulosome-like complex of *R. albus* contains a CBD on its scaffoldin skeleton is still open, and the structure and sequence of the scaffoldin, dockerins, cohesions, and CBD elements are still unknown.

The Fimbriae Paradigm

Microscopic examinations of several strains of *R. albus* revealed structures other than cellulosome-like protuberances that appear to mediate adhesion to cellulose. Patterson et al. (1975) described the presence of "extensive amounts of fibrillar, extracellular material," which projected as much as 600 nm from the cell surface, and were believed to be primarily responsible for adhesion to cellulose. Stack and Hungate (1984) later described the presence of "fimbrial-like structures" when *R. albus* 8 was provided with PAA or PPA, and it was shown that *R. albus* adhesion to cellulose is increased by the inclusion of PAA orPPA in the growth medium (Pegden et al., 1998). Electron microscopic examination of *R. albus* strain F-40 also illustrated similar structures mediating adhesion to cellulose (Kim et al., 1999). With this background, Morrison and coworkers decided to take a functional proteomics approach to identify and isolate cellulose-binding proteins (Larson and Morrison, 1999; Larson et al., 1999; Pegden et al., 1998; Reddy and Morrison, 1998). Several proteins of relatively small molecular mass (16 to 25 kDa) were identified, and initial characterization of one of these proteins, hereafter referred to as cellulose-binding protein type C (*cpb*C) was completed (Larson and Morrison, 1999; Larson et al., 1999; Pegden et al., 1998). The cbpC gene was isolated by a combination of reverse genetics and genomic walking procedures, and shown to encode a protein of 169 amino acids with a calculated molecular mass of 17,655 Da. Although the CbpC protein possesses no sequence similarity with existing CBD families, motifs characteristic of other, relatively well-characterized proteins are present. Notably, the amino-terminal third of the CbpC protein possesses a leader peptide, cleavage site, and motif characteristic of the Pil-family of proteins, especially the type 4 fimbrial subunit proteins from the gram-negative species: *Dichelobacter nodosus, Moraxella bovis, Neisseria gonorrhoeae, and Pseudomonas aeruginosa*. The type 4 fimbrial subunits are relatively low molecular mass polypeptides (20 to 25 kDa) and the resulting fimbriae are located at the polar ends of the bacterial cell (Hobbs and Mattick, 1993). The CbpC and the fimbrial proteins all possess a fairly long stretch of relatively hydrophilic residues at their carboxy-terminus, while other members of the Pil-family do not (Pegden et al., 1998). The similarities among CbpC and type 4 fimbrial proteins suggest that cbpC binding to cellulose is most likely mediated via the carboxy two-thirds of the protein. Significant sequence identity exists between this part of the CbpC protein and 72- and 75-amino acid motifs that are tandemly repeated 13 times within the 190-kDa surface antigen protein of *Rickettsia* spp. (Anacker et al., 1985). These motifs are thought to have an important role in the recognition and adhesion of *Rickettsia* spp. to cell surfaces. These similarities in structure and function add credibility to the proposed role for CbpC in adhesion to plant surfaces.

Further studies with the *R. albus* 8 wild type and mutant strains have further established CbpC role in the adhesion process. Morrison's group showed (Larson and Morrison, 1999; Larson et al., 1999; Pegden et al., 1998) that *R. albus* adhesion in cellulose-binding assays and cbpC transcript abundance were significantly increased by the inclusion of either ruminal fluid or micromolar concentrations of both PAA or PPA in the growth medium, that probably induce the production and stabilization of fimbriae structures (Morrison and Miron, 2000). A series of cellulose-binding experiments have also been conducted with preparations of the CbpC protein in the presence or absence of CMC (Pegden et al., 1998). Results suggest that CMC affects the association constant (Ka) of cbpC binding to cellulose, but not its maximal binding (Vmax), and, therefore, CMC serves as a competitive inhibitor of cbpC binding to cellulose. Southern blot analysis has confirmed that a number of other *R. albus* strains possess cbpC gene homologue(s). Additionally, Western immunoblot analysis identified a protein of V25-kDa present in *R. albus* SY3 that was cross-reactive with anti-CbpC antibodies, and in subsequent experiments the same protein was shown to be a cellulose-binding protein.

Based on these findings, it seems reasonable to propose that the CbpC protein that is the repeated building block for fimbriae creation, is a newly identified strategy for the adhesion of gram-positive bacteria to cellulose (Morrison and Miron, 2000; Pegden et al., 1998).

CBD of Enzymes

Recently, Karita et al. (1997) cloned a gene *egVI* encoding a family 9 cellulase from *R. albus* F-40. The sequence analysis revealed that this enzyme consists of three domains: a family 9 catalytic domain, a family IIIb CBD, and a dockerin-like reiterated sequence, similar to clostridial type dockerin. This is the first genetic evidence available for the presence of a cellulase CBD among the seven endoglucanases and three xylanase that have been cloned and sequenced from *R. albus* F-40 (Karita et al., 1997; Nagamine et al., 1997; Ohara et al., 2000). Although egVI contained dockerin type 1 element and thus is a part of the cellulosome complex of *R. albus* F-40, the CBD of the enzyme provides additional mechanism for bacterial adhesion to cellulose, apart from the CBD of the scaffoldin (whose existence has not yet been proven).

Table 1. Summary of adhesion mechanisms in rumen cellulolytic bacteria.[1]

Adhesion mechanism	*Fibrobacter succinogenes*	*Ruminococcus flavefaciens*	*Ruminococcus albus*
Surface protuberances (electron microscopy)	+ (1)	+ (1)	+ (1,2,3)
Adhesins connections (electron microscopy)	+ (1,4)	+ (1,15) Dockerin, (5)	+ (1,3)
Cellulosome organelles (genetic evidence)	?	Scaffoldin, (6) Cohesins (6)	Dockerin, (7,8,11)
Fimbriae or pili (genetic evidence)	?	?	CbpC (9)
CBD of enzymes (biochemical evidence)	EG2, EGF CI-Cbsase (10,16)	?	EGVI (11)
Carbohydrate epitope of identified CBP	+ (12)	?	+ (17)
Exopolysaccharides of glycocalyx layer	+ (12,13)	+ (15)	+ (2,13,14)

[1]References: 1 = Miron et al. (1989), 2 = Lamed et al. (1987); 3 = Kim et al. (1999); 4 = Miron et al. (1993a); 5 = Flint et al. (1999); 6 = Lamed and Bayer (2000, unpublished); 7 = Ohara et al. (2000); 8 = Reddy and Morrison (1998); 9 = Pegden et al. (1998); 10 = Forsberg et al. (1993); 11 = Karita et al. (1997); 12 = Gong et al. (1996); 13 = Pell and Schofield (1995); 14 = Cheng et al. (1977); 15 = Latham et al. (1978a); 16 = Malburg et al. (1997); 17 = Miron. (2001, unpublished data). CBD = cellulose-binding domain, CBP = cellulose-binding proteins, EG = endoglucanase.

Glycocalyx Exopolysaccharides

Cheng et al. (1977) reported that the slime layer surrounding *R. albus* was composed of glycoproteins and formed a layer of approximately 100 nm at the cell surface. They suggested that the slime layer involved in adhesion of these bacteria. Treatment with protease (trypsin and pronase) and dextranase, and removal of glycocalyx carbohydrate by periodate oxidation, significantly decreased adhesion of *R. albus* to cellulose (Pell and Schofield, 1993). *R. albus* contained several monosaccharide residues on its glycocalyx capsule, including glucose or mannose and galactose that reacted with several specific lectins (Baitner et al., 1993) and in particular with a lectin that recognized specific glycosylated epitope of *C. thermocellum* cellulosome (Lamed et al., 1987). These indirect studies suggest that carbohydrates are also involved in adhesion mechanism of *R. albus*, although their role has not yet been clarified.

We have recently demonstrated that part of the CBP of *R. albus* SY3 contain glycosidic epitopes that are specifically immunoreactive with antibodies specific to adhesion, suggesting their possible involvement in the adhesion process. These organelles were found in cells grown either on cellulose or on cellobiose + glucose as the sole carbohydrate substrate, suggesting constitutive nature, which is not induced by the substrate (Miron, 2001, unpublished data).

Based on these results, we propose that *R. albus* possesses at least two mechanisms for specific adhesion to cellulose: a cellulosomal-like mechanism, and a CbpC (Pil)-protein mechanism probably involving the production of fimbrial-like structures, whose interaction with cellulose can be competitively inhibited by CMC. Carbohydrate epitopes of some CBP and distinct CBD of cellulases are probably involved mostly in the nonspecific phase of the adhesion process.

CONCLUSIONS

Based on the literature, it appears that each of the predominant rumen bacteria *F. succinogenes, R. flavefaciens* and *R. albus* has a specific mechanism of adhesion to cellulose as summarized in Table 1.

In *F. succinogenes*, both the glycosidic residues of the outer membrane CBP and especially of the 180-kDa CBP, and the distinct CBD of EG2 EGF and Cl-stimulated cellobiosidase, may play a role in the adhesion to cellulose. There is no direct evidence yet, except scanning electron microscopy observations, to support the existence of either cellulosome complex or fimbriae structures involved in the adhesion mechanism of *F. succinogenes*.

At least two mechanisms, cellulosome-like complexes and carbohydrate epitopes of the glycocalyx layer are involved in the specific adhesion of *R. flavefaciens* to cellulose.

Ruminococcus albus possesses at least two mechanisms for specific adhesion to cellulose: a cellulosomal-like mechanism, and a cbpC (Pil)-protein mechanism probably involving the production of fimbrial-like structures. Indirect and direct studies suggested that carbohydrate epitopes of CBP and CBD epitope of cellulases

may also be involved mostly in the nonspecific phase of adhesion of *R. albus*.

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