J. Gen. App!. Microbiol., 25, 71-93 (1979)

TECHNIQUE FOR FRACTIONATION OF BACTERIA IN RUMEN MICROBIAL ECOSYSTEM. III. ATTACHMENT OF BACTERIA ISOLATED FROM BOVINE RUMEN TO STARCH GRANULES
IN VITRO AND ELUTION OF BACTERIA ATTACHED THEREFROM

HAJIME MINATO AND TSUNEJI SUTO

National Institute of Animal Health, Yatabe-machi, Tsukuba-gun, Ibaraki-ken, 300-21 Japan

(Received August 3, 1978)

The attaching ability of a number of bacteria, isolated from bovine rumen and animal intestine, to starch granules was examined in vitro. Among bacteria examined in the present investigation, strains possessing the attaching ability to starch were those belonging to an untypable Bacteroides sp., Bacteroides amylophilus, Bacteroides oralis, Bacteroides ruminicola ss. ruminicola, an untypable Bifidobacterium sp., Bifidobac-terium thermophilum, Bifidobacterium pseudolongum, and Bifidobacterium longum.
The reaction temperature had a profound effect on the attachment of bacteria to starch. All of bacteria listed above attached well to starch at

38°. Strains of the untypable Bacteroides sp., B. oxalis, B. thermophilum, B. pseudolongum, and B. longum attached to starch even at the reaction temperature of 4°. On the other hand, those of B. amylophilus did not attach to starch at 4°.

The activity of several hydrolases of various kinds of bacteria was ex-amined. The amylase activity of cells of bacteria capable of attaching to starch was remarkably high, compared with that of bacteria incapable of attaching. Bacteria such as B. fibrisolvens and Streptococcus bovis, which were incapable of attaching to starch but possessed intense amy-lolytic activity, secreted extracellularly large parts of amylase produced by them. Only Peptostreptococcus productus among examined bacteria exhibited remarkably intense urease activity. There was no significant difference in the specific activity of the remaining three hydrolases be-tween bacteria capable and incapable of attaching to starch. The attach-ment of bacteria to starch was inhibited by various carbohydrates. Carbohydrates like dextrin, amylose, amylopectin, soluble starch, and

71

72 MINATO and SUTO VOL. 25

derivatives of starch except carboxymethyl-starch inhibited the attach-
ment of bacteria to starch. The degree of this inhibition by these carbohydrates depended on their concentrations. Other carbohydrates
examined, including derivatives of cellulose and dextran, did not inhibit significantly the attachment of bacteria to starch.
An attempt was made to detach or elute bacteria from starch by some means. Cells of B. amylophilus, once attached to starch at 38°, were detached by the cooling treatment at 4°. Although both cells of B. thermophilum and B. pseudolongum were not detached from starch by the cooling treatment at 4°, they were eluted from starch with a solution of
dextrin or partially-hydrolysed hydroxypropyl-starch. Cells of Bac-
teroides sp. 7-4, once attached to starch, were not detached either by the
cooling treatment at 4° or by the elution treatment with a solution of
above two carbohydrates.

To make it possible to progress the study on the constitution of a microflora
in a microbial ecosystem, it will be advisable to develop a new research technique as well as a further improvement of the usual culture method, as described in our previous report (1). In this respect, a method worthy of trial will be a fractionation of bacteria by utilizing the attaching ability of bacteria to solid substrates.
It has been found that the constitution of bacteria in solid digesta in the rumen is apparently different from that in liquid digesta (2, 3). Moreover, it has been shown that a determinant factor deciding the localization of bacteria in the rumen is their attaching ability to solid substrates (3).
We found that bacteria capable of attaching to starch granules among mixed rumen bacteria are different in morphology and physiological properties from those incapable of attaching, and that the attachment of rumen bacteria to starch granules is influenced profoundly by the reaction temperature (1).
The number and kinds of bacteria caught into test tubes so far by the anae-robic roll tube method are limited (4). Among bacteria isolated from bovine rumen, the only one bacterium whose attaching ability to starch granules has been noted is B. amylophilus (S). The attaching ability of other bacteria to starch granules has not been investigated. Therefore, it seemed of significance from the ecological standpoint to clarify the kinds of bacteria possessing the attaching ability to starch granules.
In this respect, we describe the results of our research study on the attaching ability of a number of bacteria isolated from bovine rumen to starch granules, on the influence of several factors on the attachment of bacteria to starch, on the comparison of several hydrolase activities of bacteria both capable and incapable of attaching to starch, and on the conditions necessary for the detachment or elu-tion of bacteria attached to starch.

1979 Fractionation of Rumen Bacteria 73

MATERIALS AND METHODS

Bacteria. Bacteria used in this investigation were a total of 122 strains listed
in Table 1. The majority of the strains examined were isolated from bovine rumen in our laboratory. Additional strains examined were supplied through the kindness of the researchers and described in our previous report (6).
Starch granules as an adsorbent for bacterial attachment. Corn (Zea mays) starch granules were mainly used as an adsorbent, as described previously (6). Test for attachment of bacteria to starch granules. The anaerobic technique used throughout this investigation for culturing bacteria was essentially that of HUNGATE (7) as modified by BRYANT and BURKEY (8).
The medium used for culturing the two strains of Veillonella spp. was RL medium, described in our previous report (6). The medium used for culturing other bacteria was RGMCS medium of the composition indicated in Table 2.

Table 1. List of bacteria examined.

74 MINATO and SUTO VOL. 25

Test organisms were grown in these media for 20 to 24 hr at 38°, unless stated otherwise. The attachment test of bacteria to starch granules was carried out according to the same procedures as described in our previous report (6), except that starch granules were used as the adsorbent in place of cellulose powder. Namely, 210 mg of starch granules was added to 3 ml of a culture. The percent-age of cells attached to starch was determined by measuring the decrease of optical density at 600 nm in the liquid phase after the addition of starch to the culture. Effect of reaction temperature, washings of cells with salt solution, and treat-ment of cells with Formalin on the attachment of bacteria to starch granules. For strains of bacteria which possessed the attaching ability to starch at 38°, their at-taching ability was examined at 4°. The test for the attachment of bacterial cells to starch at 4° was carried out according to the same procedures as described in our previous report (6), except that starch granules were used as an adsorbent in place of cellulose powder. After cells of B. amylophilus were washed aerobically in 0.85 % saline, the attaching ability of these cells to starch decreased remarkably. Therefore, the attachment test of cells of B. amylophilus to starch in the suspend-ing medium with or without sodium carbonate was carried out anaerobically. The suspending medium with sodium carbonate was the potassium phosphate buffer (0.05 M, pH 7), which contained 0.3 % sodium carbonate and 0.05 % cysteine-HC1 and was saturated with C02. The suspending medium without sodium carbonate was the potassium phosphate buffer (0.05 M, pH 7), which contained 0.05 % cysteine-HC1 and was saturated with N2. Bacterial cells were washed twice in each suspending medium and the washed cells were resuspended in the same suspending medium employed to wash them. The attaching ability of the washed cells to starch was examined, as described previously (6).

Table 2. Composition of RGMCS medium.

1979 Fractionation of Rumen Bacteria 75

Treatment of bacterial cells with Formalin and examination on the attaching ability of the treated cells to starch was carried out, as described previously (6). Carbohydrate used for inhibition test of bacterial attachment to starch granules and elution test of bacterial cells attached to them. The carbohydrates used in these examinations were glucose, maltose, cellobiose, dextrin, methylcellulose 400 cps, sodium carboxymethylcellulose (Wako Pure Chemical Industries, Ltd., Tokyo), amylose (Nagase Co., Osaka), amylopectin (Tokyo Kasei Kogyo Co., Tokyo), maltotriose (Hayashibara Biochemical Laboratories, Inc., Okayama), soluble starch (Merck & Co., Inc., U.S.A.), dextran T500 (Pharmacia Fine Chemi-cals, Uppsala). The following derivatives of starch used in these examination were supplied kindly by Mr. YosHLo HANNO (Matsutani Chemical Industry Co., Itami) : They were hydroxypropyl-starch (D.S. =0.1), partially-hydrolysed hy-droxypropyl-starch (D.S. ' ; 0.1), hydroxyethyl-amylose (Amylose HE-15U, D.S. 0.15), hydroxyethyl-starch (Avelex 1030, D.S. _ 0.05), carboxymethyl-starch (CMS-PPL, D.S. 0.3).
Each of these carbohydrates was dissolved in 0.85 % saline or the prereduced suspending medium with sodium carbonate at twice the concentration desired in the reaction mixture. The solutions of these carbohydrates were autoclaved at 121° for 10 min and preserved. The solutions of carbohydrates dissolved in 0.85 saline were used in the examination for Bacteroides sp. 7-4 and Bifidobacterium spp., and the solutions of carbohydrates dissolved in the prereduced suspending medium with sodium carbonate were used for B. amylophilus.
Inhibition of the attachment of bacteria to starch by various carbohydrates. Even though washed cells of B. amylophilus were suspended in the prereduced suspending medium with sodium carbonate, their attaching ability to starch de-creased gradually during storage at 4°. Therefore, in this examination, the washed cell suspensions of B. amylophilus were used on the day when they were prepared. Inhibition test of the attachment of bacteria to starch by each carbohydrate was carried out as described previously (6), except that starch granules were used as an adsorbent. The degree of inhibition of the attachment of bacteria to starch by each carbohydrate was calculated on the basis of the amount of cells attached to starch in both the presence and the absence of carbohydrate, by using the equa-tion described previously (6).
When the washed cell suspensions of B. pseudolongum Pa-17-124 and two strains of B. amylophilus were incubated at 38° in the presence of dextrin, soluble starch, or derivatives of starch, cell aggregates were formed and optical density of the suspensions increased approximately twice as much as that of the original. Therefore, it was impossible to determine the degree of inhibition of the attach-ment of these bacteria to starch by carbohydrate spectrophotometrically. Fortu-nately, the cell aggregates did not sediment after treatment by low-speed cen-trifugation at 377>< g for 1 min to sediment starch granules. Therefore, the number of cells in the cell suspensions before and after the treatment with starch

76 MINATO and SuTo VOL. 25

granules was counted. Namely, the cell suspensions were mixed with an equal volume of saline containing 10 % Formalin and the cell aggregates were dispersed. The number of cells in the suspensions treated with Formalin was determined by direct microscopic counting method (1).
Detachment or elution of bacteria from starch granules by various treatments. Detachment and elution tests of bacterial cells from starch granules were carried out as described previously (6). These examinations for the two strains of Bifido-bacterium spp. and Bacteroides sp. 7-4 were performed aerobically, while examina-tions for B. amylophilus 7-9 were performed anaerobically. The amount of cells detached and eluted from starch granules was determined by measuring spectro-photometrically both strains of Bifidobacterium spp. and Bacteroides sp. 7-4, and by direct microscopic counting method for B. amylophilus 7-9 as mentioned above. The degree of detached or eluted cells from starch granules was calculated by using the equation described previously (6).
Determination of hydrolase activities of washed cells and culture filtrates. After the growth of bacteria entered the stationary phase, autolysis of bacterial cells proceeds and intracellular amylase of the cells may be released gradually into the medium. When the relationship between culture period and rate of amylase released into the medium to the total amount of amylase produced by bacteria were examined for Bacteroides sp. 7-4, the rates of amylase released into the medium were 12-24 % at the culture period of 24 hr and 38-50 % at the culture period of 72 hr. Therefore, the culture period of 24 hr for obtaining each culture of bacteria was employed in this examination. Activities of amylase, urease, j3-glucosidase, acid and alkaline phosphatases of washed cells, and of amylase of culture filtrates were assayed according to the methods described previously (1). Scanning electron microscopic observation of bacteria attached to starch gran-ules. Bacteroides sp. 7-4 used in this examination for the observation by a scan-ning electron microscope was cultured at 38° for 20 hr in RMS medium. By using the cultures of Bacteroides sp. 7-4, the following three samples were prepared :
(1) The mixture, in which starch granules (7 %, w/v) were added to the culture, was centrifuged at 250x g for 30 sec. The supernatant was decanted and the sediment, consisting of starch granules and attached cells, was fixed at room tem-perature for 1 hr in 2.5 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.2). (2) Cells in the culture were harvested by centrifugation, washed twice in the phos-phate buffer, and fixed at 4° for 48 hr in 2 % Formalin in the phosphate buffer. Starch granules (7 %, w/v) were added to the treated cell suspension. (3) Cells in the culture were harvested by centrifugation, washed twice in the phosphate buffer, and fixed at 4° for 2 hr in 2.5 % glutaraldehyde in the phosphate buffer. Then the cells were washed four times in the phosphate buffer, fixed at 4° for 2 hr in 2 osmium tetroxide in the phosphate buffer, again washed four times in the phos-phate buffer, and suspended in the phosphate buffer. Thereafter, starch granules (7 %, w/v) were added to the cell suspension. These mixtures were used as samples.

1979 Fractionation of Rumen Bacteria 77

These three samples were dehydrated through a graded series of 50, 70, 90, and 100% ethanol diluted with distilled water for 10 min each, and infiltrated with amyl acetate for 10 min. The amyl acetate-treated samples were dropped on cover glasses (18 x 18 mm) and dried by the usual critical point method. These cover glasses with the dried specimens were mounted on metal stubs and coated with a thin layer of carbon-gold in the rotating vacuum evaporator. These gold-coated specimens were observed with a scanning electron microscope of Nihon Denshi Co., JEOL Model JSM-35, at an accelerating voltage of 20 kV. Micro-graphs of the scanned fields were taken with Fuji Neopan SS film.

RESULTS

Bacteria possessing the attaching ability to starch granules
The attaching ability of cells of many kinds of bacteria, listed in Table 1, to starch granules was examined in vitro. Strains which belong to both B. pseudo-

Table 3. Bacteria possessing the attaching ability to starch granules.

78 MINATO and SUTo VOL. 25

longum and an untypable Bacteroides sp. possessed the ability to attach firmly to starch. Those belonging to B. amylophilus, B. oxalis, B. thermophilum, B. longum, and an untypable Bifidobacterium sp. possessed a distinct ability to attach to starch. Those belonging to B. ruminicola ss. ruminicola possessed a weaker ability than those listed above (Table 3).
Two strains of an untypable Bifidobacterium sp., three strains of B. thermo-philum, and one strain of B. pseudolongum possessed the attaching ability to starch, and all of them were isolated from bovine rumen. Therefore, strains of Bifidobacterium spp. isolated from other animal intestine were examined for their attaching ability to starch. Both B, pseudolongum PNC-2-9G isolated from swine intestine and B. longum R101-8 isolated from a rat intestine possessed the attaching ability to starch. Two strains of B. thermophilum, however, which were isolated from swine intestine and avian intestine, respectively, did not attach to starch at all.

Only B. longum R101-8 among bacteria possessing the ability to attach to starch did not have any amylolytic properties. All other bacteria capable of attaching to starch had amylolytic properties.
Succinimonas sp., Butyrivibrio sp., Selenomonas sp., Borrelia sp., and Suc-cinivibrio sp. did not attach to starch, and all of them were motile.
Strains belonging to an untypable Bacteroides sp., B. amylophilus, and an untypable Bifidobacterium sp. showed gradually decreasing attaching ability to starch during repeated transfers on a maintenance agar medium. Therefore, to carry out further examination of the attachment of bacteria to starch, the strain which possessed the most powerful ability to attach to starch among the strains reisolated from the cultures of these bacteria was used. On the other hand, two strains of B. ruminicola ss. ruminicola abruptly and entirely lost their attaching ability to starch during repeated transfers and their attaching ability did not
recover.

Comparison of the amount of bacteria attached to starch granules from djfferent origins
By using both Bacteroides sp. 7-4 and B, amylophilus 7-9, an attempt was made to compare the amount of cells attached to starch granules from different origins. Rice starch, wheat starch, corn starch, and sweat potato starch were better as an adsorbent than potato starch or tapioca starch (Table 4). The results obtained here are in agreement with our previous results obtained by using mixed rumen bacteria (1). To carry out further examination on the attachment of bacteria to starch, corn starch was employed as an adsorbent for its superiority to others in easiness of handling as well as in the amount of cells attached per unit weight of starch.
By using the washed cell suspension of Bacteroides sp. 7-4, prepared in 0.85 saline to give cell concentration of McFarland No. 10, an attempt was made to

1979

Table

Fractionation of Rumen Bacteria

4. Difference in the amount of cells attached to starch granules
from different origins.

79

Fig. 1. Attaching ability of bacterial cells to starch at various stages of growth. RGMCS medium (80 ml) was inoculated with 5 ml of the culture of bacteria grown
in the same medium at 38° for 20 hr, and incubated at 38°. Both growth and attaching ability of cells to starch were determined by using 8-ml samples of the culture, which were removed aseptically under a stream of CO2 at various period of incubation. The amount of cells attached to starch was determined as described in MATERIALS AND METHODS.

• growth curve of bacteria; o amount of bacteria attached to starch.

80 MINATO and SUTO VoL. 25

examine the relationship between the quantity of starch granules added and the amount of cells attached to them. When starch granules added to the cell sus-pension amounted to 7 % (w/v), the amount of attached cells became saturated. Therefore, the number of cells attached to starch at this saturation point was estimated by direct microscopic counting method. As a result, it was found that the number of cells attached to 1 g of starch was 1.03>< 1010

The attaching ability of bacteria to starch at various stages of growth
By selecting both Bacteroides sp. 7-4 and B, amylophilus 7-9, the attaching ability of these bacteria to starch was examined at various stages of growth. The attaching ability of these bacteria was maximum at the stages of late logarithmic and early stationary growth (Fig. 1). After early stationary phase elapsed, de-terioration of bacterial cells proceeded and their attaching ability to starch de-creased gradually. Therefore, to perform further examination on the attachment

Table 5. Comparison of the attaching ability of cells grown on different carbon source, to starch granules.

1979 Fractionation of Rumen Bacteria 81

of bacteria to starch, the culture periods of 16 hr for B. amylophilus and 20 hr for the remaining bacteria were employed.

Influence of various carbon sources for growth on the attaching ability of bacteria to starch

The attaching ability of cells grown on several different carbon sources was compared. Since all of bacteria possessing the attaching ability to starch were incapable of utilizing cellobiose as an energy source, mainly glucose, maltose, and soluble starch were used in this examination. The attaching ability of bacteria to starch was not influenced significantly by the kind of carbon sources for growth (Table 5).

Effect of environmental factors and Formalin treatment of cells on their attaching ability to starch
The reaction temperature had a very profound effect on the attachment of bacteria to starch (Table 6). Cells of seven strains of B. amylophilus attached well to starch at the reaction temperature of 38°, but not at 4°. On the other hand, cells of Bacteroides sp. 7-4, three strains of B. thermophilum, two strains of B, pseudolongum, and one strain of B. longum attached abundantly to starch at both reaction temperatures of 38° and 4°. Cells of two strains of B. ruminicola ss. ruminicola attached to starch at 38°, even though weakly. Since these two strains lost their attaching ability to starch in the early stage of repeated transfers, regretfully their attaching ability at 4° was not examined.
An attempt was made to examine how the attachment of bacteria to starch is influenced by the presence or absence of sodium carbonate in the suspending medium. Cells of four strains of B. amylophilus attached more abundantly to starch in the suspending medium with sodium carbonate than in that without sodium carbonate. The attaching ability of other bacteria to starch, however, was independent of the presence or absence of sodium carbonate in the suspending medium (Table 6). These results suggest that the presence of sodium carbonate in the suspending medium did not determine the kind of bacteria capable of at-taching to starch, but the amount of cells attached to starch.
After cells of B. amylophilus were treated with 2 % Formalin, the cells did not attach to starch. As to B. ruminicola ss. ruminicola, the attaching ability of cells after the treatment with Formalin was not examined. Cells of other bacteria

tested retained their attaching ability to starch, even after treatment with 2 Formalin (Table 6).

Observation of cells attached to starch granules by a scanning electron microscope By using Bacteroides sp. 7-4, an attempt was made to observe the cells at-tached to starch granules by a scanning electron microscope. Scanning electron micrographs of cells attached to starch granules revealed that extracellular coat material mediates the binding between cells and starch granules (Fig. 2a, b and c).

82 MINATO and SUTO VOL. 25

Even after the cells were washed with phosphate buffer and fixed with Formalin, these treated cells still possessed the attaching ability to starch. Scanning elec-tron micrographs of these treated cells attached to starch have shown that extra-cellular coat material remained on the cell surface (Fig. 2d, e and f). After the cells were washed with phosphate buffer and fixed with glutaraldehyde and osmium tetroxide, these treated cells markedly lost the attaching ability to starch. There-

Table 6. Effect of washing of cells and reaction temperatures on their attachment to starch granules.

1979 Fractionation of Rumen Bacteria 83

fore, it was impossible to take scanning electron micrographs of these treated cells attached to starch.

Activity of several hydrolases of bacteria
It was found that the most characteristic properties of bacteria attached to starch among mixed rumen bacteria were their remarkably high intracellular amylase activity and nil or extremely low urease activity (1). In order to sub-stantiate the above findings from the properties of bacteria isolated from bovine

Fig. 2. Scanning electron micrographs of cells
starch granules : (a, b and c) cells in the culture; (d,
maim.

of Bacteroides sp. 7-4 attached to
e and f) cells treated with 2 % For-

84

Table

MINATO and SUTO

7. Several hydrolase activities of bacteria isolated from rumen content.

VOL. 25

1979 Fractionation of Rumen Bacteria 85

rumen, the activity of hydrolases of many kinds of bacteria was examined. Broadly speaking, the amylase activity of cells of bacteria capable of attaching
to starch was remarkably high, compared with that of bacteria incapable of at-taching. However, both B. oxalis ATCC 15930 and two strains of B, ruminicola ss. ruminicola, among bacteria possessing the attaching ability to starch, exhibited weak intracellular amylase activity. Furthermore, strains belonging to both B. pseudolongum and B. thermophilum exhibited either weak or intense intracellular amylase activity. On the other hand, strains of both B. fibrisolvens and S. bovis, which are incapable of attaching to starch and possess intense amylolytic property, exhibited extremely weak intracellular amylase activity.
All of the strains which were isolated from bovine rumen and used in this

examination exhibited nil or extremely weak intracellular urease activity. On the other hand, remarkably intense urease activity was demonstrated for the cells of P. productus 0191 (Table 7). Strains of P. productus have been isolated from human intestine or bovine rumen, etc. Now, the strain used in this examination was isolated from human feces.

As for the remaining three hydrolase activities of bacteria, there was no definite difference in the specific activities between bacteria capable and incapable of attaching to starch. The patterns of these three hydrolase activities varied with species or strains among the same species.
The results obtained here support the findings that there was a difference in the characteristics of hydrolase activity between bacteria capable and incapable of attaching to starch among mixed rumen bacteria.

Influence of various carbon sources for growth on amylase activity of bacteria
It is well known that the production of amylase by bacteria is affected re-markably by a kind of carbohydrate added to the medium. Therefore, an at-tempt was made to compare the production of amylase by bacteria grown on glucose, maltose, or soluble starch as a carbon source. The production of amy-lase by both S. bovis A-30 and B, thermophilum A-6 was remarkably strengthened by soluble starch as the carbon source for growth (Table 8). As to other bacteria tested, both maltose and soluble starch were more effective as the carbon source for the production of amylase by them, compared with glucose. Furthermore, production of amylase by both B. fibrisolvens and B. ruminicola was significantly repressed by glucose as the carbon source for growth.
In the case of both S. bovis A-30 and two strains of B. fibrisolvens, most of amylase produced by them was secreted extracellularly into the medium. As to bacteria capable of attaching to starch, generally the amount of amylase associated with bacterial cells was more abundant than that secreted extracellularly into the medium. However, strains A-6 of B. thermophilum among bacteria capable of attaching to starch secreted extracellularly a large part of amylase produced by the strain into the medium.

86 MINATO and SUTO VOL. 25

Inhibition of the attachment of bacteria to starch granules by various carbohydrates It was assumed that surface substances of bacterial cells would be involved

in the attachment of cells to starch. Therefore, an attempt was made to examine whether the attachment of cells of bacteria to starch is specifically inhibited by a specific carbohydrate or not.

Table 8. Amylase activity of cultures of bacteria grown on different carbon sources.

1979 Fractionation of Rumen Bacteria 87

The attachment of cells of bacteria to starch was remarkably inhibited by dextrin, amylose, and amylopectin (Table 9). The attachment of cells of bacteria to starch was also definitely inhibited by both soluble starch and derivatives of

Table 9. Inhibition of the attachment of cells to starch granules by
various carbohydrates.

88 MINATO and SUTO VOL. 25

starch, which included hydroxyethyl-starch,
hydrolysed hydroxypropyl-starch (Table 10).

hydroxyethylamylose, and partially-
Hydroxypropyl-starch had an in-

Table 10. Inhibition of the attachment of cells to starch granules by soluble starch and derivatives of starch.

1979 Fractionation of Rumen Bacteria 89

hibitory effect on the attachment of cells of both Bacteroides sp. 7-4 and B. thermo-philum A-6 to starch, but not on the attachment of other three strains tested. The degree of inhibitory effect of the carbohydrates on the attachment of cells to starch increased as the concentration of the carbohydrates in the reaction mixtures became higher.
On the other hand, the attachment of cells of bacteria to starch was not sig-nificantly inhibited by glucose, maltose, cellobiose, maltotriose, dextran, sodium carboxymethylcellulose, and methylcellulose. Strange to say, the attachment of five strains of tested bacteria to starch was not at all inhibited by carboxymethyl-starch, which is a derivative of starch.
The results obtained here suggest that there are specific attaching sites on the cell surface of bacteria and that these sites can be blocked largely by dextrin, soluble starch, and partially-hydrolysed hydroxypropyl-starch.

Detachment and elution of bacteria from starch granules
An attempt was made to detach or elute cells of bacteria from starch granules
by various treatments. In B. amylophilus, the cells once attached to starch at 38° were detached by the cooling treatment at 4°. This fact was true of other two strains of B, amylophilus, which are not shown in the table. In Bacteroides sp. 7-4, B. pseudolongum Pa-17-124, and B, thermophilum A-6, the cells once attached to starch were not detached by the cooling treatment at 4° (Table 11).
An attempt was made to elute bacterial cells once attached to starch at 38° with a solution of various carbohydrates. With B. pseudolongum Pa-17-124 and B. thermophilum A-6, the cells once attached to starch were eluted with a solution of dextrin or partially-hydrolysed hydroxypropyl-starch, which have a great in-hibitory effect on the attachment of bacteria to starch. The amount of cells eluted with a solution of carbohydrates from starch increased as the concentration of carbohydrates in the solutions became higher. Though not shown in the table, a solution of hydroxyethyl-starch, hydroxyethylamylose, carboxymethyl-starch, amylose, or amylopectin was ineffectual for the elution of all of tested bacteria from starch. As for Bacteroides sp. 7-4 and B. amylophilus 7-9, the cells once attached to starch at 38° were not significantly eluted with a solution of soluble starch, dextrin, or partially-hydrolysed hydroxypropyl-starch (Table 11).

DISCUSSION

The strains which possessed the attaching ability to starch among the strains
of bacteria tested in the present investigation were those belonging to B. amy-lophilus, B. oralis, B. ruminicola ss. ruminicola, an untypable Bacteroides sp., B. thermophilum, B. pseudolongum, B. longum, and an untypable Bifidobacterium sp. On the other hand, the strains of bacteria which exhibited no attaching ability to starch were those belonging to B. succinogenes, R, flavefaciens, R. albus, E. cel-lulosolvens, M. elsdenii, V. alcalescens, V. parvula, S. amylolytica, P. productus,

90 MINATO and SUTO VOL. 25

E. ruminantium, an untypable Eubacterium sp., B. fibrisolvens, L. multiparus, Borrelia sp., S. ruminantium, Succinivibrio sp., P. aches, and S. bovis. Further-more, we have to point out that all of bacteria exhibiting the attaching ability to starch in the present investigation were incapable of attaching to cellulose as described previously (6).
The kinds of the species of bacteria capable of attaching to starch were limited within the range of the present examination. Therefore, it was assumed that the attaching ability of bacteria to starch could be used as one of items of test for the identification of bacteria. In this case, it should be pointed out that this ability has to be tested using fresh isolates from the rumen and other microbial ecosys-tems, because the attaching ability of nine strains of B. amylophilus, Bacteroides sp. 7-4, two strains of an untypable Bifidobacterium sp., and three strains of B. thermophilum to starch decreased gradually during repeated transfers on a main-tenance medium. However, several strains reisolated from these old cultures had

Table 11. Detachment of cells attached to starch granules by various treatments.

1979 Fractionation of Rumen Bacteria 91

a powerful attaching ability to starch.
Although five strains of B. thermophilum were examined for their attaching ability to starch, only three strains isolated from bovine rumen possessed the attaching ability to starch. Other two strains, each of which was isolated from swine and avian intestines, were not able to attach to starch. Our colleague, MIYAGAWA, examined the attaching ability of a number of strains of B. oxalis, which were isolated from various kinds of domestic animals. The results he

obtained showed that only strains of B. oralis isolated from cattle possessed the attaching ability to starch. On the basis of this finding and our above finding, it seems possible that the examination of the attaching ability of bacteria to starch becomes a useful means to determine ecovar of strains belonging to the same species.
Two strains of B. ruminicola ss. ruminicola lost their attaching ability to starch at an early stage of repeated transfers on a medium. B. ruminicola ss. ruminicola ATCC 19189, which was received from the American Type Culture Collection, did not attach to starch. However, we could not decide whether this strain was not able to attach to starch at the primary isolation or lost their at-taching ability during repeated transfers for a long time. These facts show that the property of the attachment of bacteria to starch is not a long-lasting property. This was true in the case of bacteria possessing the attaching ability to cellulose as described previously (6). Therefore, it will be interesting to clarify whether such a property of bacteria is regulated by episomes as a genetic substance.
It was found that the specific amylase activity of bacteria capable of attaching
to starch among mixed rumen bacteria was remarkably higher than that of incap-able bacteria (1). After this finding, we compared the specific amylase activity of bacteria attaching to starch with that of non-attaching bacteria, by using the rumen contents collected from cattle slaughtered in a slaughter-house. The results obtained showed that the specific amylase activity of bacteria capable of attaching to starch was two to five times higher than that of incapable bacteria. In the present investigation on the amylase activity of pure cultures of bac-teria, we can explain part of these phenomena. Namely, the intracellular amylase activity of most of bacteria capable of attaching to starch was remarkably higher than that of incapable bacteria. However, this fact is not common for all of bacteria capable of attaching to starch. The intracellular amylase activity of B. oralis ATCC 15930, isolated from other than bovine rumen, was particularly lower than that of other bacteria, and B. longum R101-8 capable of attaching to starch did not possess the ability for digestion of starch.
A large amount of starch and cellulose are degraded by the activities of many kinds of bacteria and protozoa in the rumen. As mentioned above, bacteria capable of attaching to starch among those isolated from bovine rumen had a powerful intracellular amylase activity, while strains of both B. fibrisolvens and S. bovis, which had a powerful capacity for the digestion of starch but were incapable

92 MINATO and SUTO VoL. 25

of attaching to starch, secreted extracellularly most of amylase produced by them. On the basis of these facts, it should be concluded that there exist mainly two types of bacteria different in the mode of participation to starch degradation in the rumen. Bacteria of the first type were those that attach to starch and digest starch by the action of amylase associated with cells. Bacteria of the second type were those that do not attach to starch and digest starch by the action of amylase extracellularly secreted by them.
It was found that the specific urease activity of bacteria capable of attaching
to starch among mixed rumen bacteria was nil or extremely lower than that of incapable bacteria (1). Therefore, the intracellular urease activity of many strains of bacteria maintained in our laboratory was examined. As a result, only one strain of P. productus among examined bacteria exhibited an intense activity of intracellular urease. Other strains of tested bacteria, including those both capable and incapable of attaching to starch, did not show any significant activity of in-tracellular urease (Table 7). The contradiction of these results with the previous finding that cells of mixed rumen bacteria exhibited an intense activity of urease, suggest a possibility that bacteria playing an important role in the ureolytic ac-tivity in the actual rumen have not yet been caught into test tubes.
Scanning electron micrographs of cells of Bacteroides sp. 7-4 attaching to starch showed that an extracellular coat material was involved in the association

of cells with starch (Fig. 2). Moreover, it was found that the attachment of bacterial cells to starch was remarkably inhibited by dextrin, soluble starch, and derivatives of starch except carboxymethyl-starch, but not inhibited by monosac-charides, disaccharides, dextran, and derivatives of cellulose (Tables 9 and 10). However, the degree of inhibition by derivatives of starch on the attachment of bacteria to starch was smaller than that by derivatives of cellulose on the attach-ment of bacteria to cellulose powder (6). It will be an interesting problem from the standpoint of microbial physiology to clarify what kind of cell surface sub-stances are involved in the attachment of bacteria to starch.

For developing a technique for fractionation of bacteria in the rumen micro-bial ecosystem by utilizing the attaching ability of bacteria to starch, the environ-mental factors necessary for the attachment of bacteria to starch were investigated in detail in the previous investigation and some interesting phenomena were found (1). One of the interesting phenomena was the fact that the reaction tem-perature affected profoundly the attachment of bacteria to starch. Especially, the results obtained suggested that there exist bacteria in the rumen capable of attaching to starch even at the low reaction temperature of 4°. Therefore, a trial was made to substantiate this assumption by examining the attaching ability of isolated bacteria to starch at the reaction temperatures of both 4° and 38°. As a result, it was found that there were three types of bacteria in their ability to attach to starch. The first type of bacteria were those capable of attaching to starch at the reaction temperatures of both 4° and 38 Those capable of attaching to

1979 Fractionation of Rumen Bacteria 93

starch at 38°, but incapable of attaching at 4° belonged to the second type. The third type were those incapable of attaching at either 4° or 38° (Table 6). We have to point out here that the attachment of bacteria to cellulose was also affected profoundly by the reaction temperature, as described previously (6).
Another interesting phenomenon was the fact that the concentration of sodium carbonate in the suspending medium greatly affected the attachment of mixed rumen bacteria to starch (1). The results obtained in the present investiga-tion by using isolated bacteria showed that the concentration of sodium carbonate in the suspending medium did not determine the kind of bacteria attached to starch, but the amount of attached bacteria (Table 6).
If bacteria once attached to starch granules can be detached or eluted by some means, it will become a promising technique for collecting selectively a given group of bacteria in the rumen and other microbial ecosystems. The results obtained in the present investigation suggested that bacteria capable of attaching to starch in the rumen microbial ecosystem can be further fractionated into the following three groups of bacteria on the basis of conditions necessary for their detachment and elution : The first group of bacteria are those that can be detached by the cooling treatment at 4°; those that cannot be detached by the cooling treatment at 4°, but be eluted with a solution of dextrin or partially-hydrolysed hydroxypropyl-starch are the second group; and those of the third group are those that cannot be detached by the cooling treatment at 4° and also not eluted with a solution of dextrin or partially-hydrolysed hydroxypropyl-starch. Our study will require further investigation as to whether bacteria capable of attaching to starch in the rumen microbial ecosystem can be subfractionated into the three groups of bacteria by means of the treatments mentioned above.

The authors wish to thank Dr. Y. HASEGAWA and K. OGURA of JEOL, Ltd. for taking the scanning electron micrographs.

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