Degradation of Blood Group Antigens in Human Colon Ecosystems

II. A GENE INTERACTION IN MAN THAT AFFECTS THE FECAL POPULATION DENSITY OF CERTAIN ENTERIC BACTERIA

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ABSTRACT The autosomal dominant ABH secretor gene together with the ABO blood type gene control the presence and specificity of A, B, and H blood group antigens in human gut mucin glycoproteins. Certain obligate anaerobes in feces produce extracellular antigen-specific glycosidases that degrade these ABH antigenic determinant glycoside structures. We estimated the populations of these bacteria in feces of 22 healthy subjects by determining the greatest dilution of feces that yielded A, B, or H blood group-degrading enzyme activity after 24 h incubation in anaerobic cultures. Comparatively small populations of fecal bacteria produce blood group-degrading enzymes; their estimated populations were 10^8 per g or less in 21 subjects. Fecal populations of B-degrading bacteria were stable over time, and their population density averaged 50,000-fold greater in blood group B secretors than in other subjects. We present evidence that the greater fecal populations of B-degrading bacteria in B secretors is due in part to a competitive nutritional advantage gained by their ability to enzymatically cleave the B antigenic determinant α-D-galactose from gut mucins of B secretors. Fecal populations of bacteria producing A and H antigen-degrading enzyme activities were comparable in all subjects to the fecal population of B-degrading bacteria in B secretors. The large populations of fecal anaerobes may be an additional source of A antigen substrate for A-degrading bacteria; thus, antigens cross-reacting with A antigen were detected on cell walls of anaerobic bacteria from 3 of 10 cultures inoculated with 10^{-10} g feces. Bacteria producing B-degrading activity likely represent a separate population from those producing A- or H-degrading activity since their fecal populations differed numerically in 14 subjects.

These findings suggest that adaptation of blood group-degrading enzymes to mucin structures in human colon ecosystems is chiefly by mutation-selection of comparatively small populations of constitutive enzyme-producing strains rather than by substrate induced enzyme synthesis in many strains.

INTRODUCTION

Populations of various human enteric bacteria may show large differences from one host to another that exceed the variation from day to day in the same host (1-3). The reasons for these differences are unclear. Although the age of the host (1) and alterations in diet (1, 4) are associated with changes in population counts, such changes are generally minor, even with abrupt, extreme changes in diet (4, 5) and do not account for the larger differences observed among healthy adults. In this paper we present evidence that the interaction of two inheritable traits, blood type B and ABH secretor capacity, affects the population density of a functionally distinct but incompletely characterized group of normal enteric bacteria: those that produce extracellular blood group B antigen-degrading enzyme activity. We investigated the basis for this difference and also estimated the population densities of fecal bacteria that produce A- and H-degrading activities. The findings are relevant to bacterial enzyme adaptation in human colon ecosystems.

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METHODS

Cultures of fresh feces were obtained from healthy biomedical colleagues of known ABO blood group and secretor status. The study group consisted of seven blood group B secretors, five blood group A secretors, five group O secretors, and five ABH nonsecretors. Two nonsecretors were blood group A, two were group O, and 1 was group B. Details of the materials, the preparation of prereduced anaerobic culture media, anaerobic techniques, the assay of A-, B-, and H-degrading enzyme activity in cell-free culture supernates, and the measurement of A, B, and H antigen titers by hemagglutination inhibition are described in the preceding paper (6). The fucose-inhibited lectin fraction from *Ulex europeus* (6) was used as anti-H.

Unless stated otherwise, the culture medium used was "anaerobic medium" (6) containing 2 mg/ml hog gastric mucin (HGM).[1] "Basal medium" is anaerobic medium lacking glucose and HGM. To compare the effect of various glycoproteins on the growth of the B-degrading enzyme-producing strain of *Ruminococcus* AB, we used basal medium alone or containing either 1 mg/ml D-galactose, HGM, or boiled, dialyzed and lyophilized A, B, or H saliva. To ensure that the media containing each of the crude glycoprotein preparations were comparable in glycoprotein content the L-fucose content of each glycoprotein preparation was determined (7) and the amount of each was adjusted to give a final glycoprotein L-fucose concentration of 120 μg/ml in the media. In three experiments where we compared the effect of glycoproteins versus glucose on proliferation of B-degrading enzyme-producing bacteria in 24-h fecal cultures (Fig. 5) we used basal medium containing 1 mg/ml glucose or 2 mg/ml HGM or boiled, dialyzed and lyophilized salivas. In exp. 3, 1 mg/ml glucose was included in the glycoprotein media and 3 mg/ml was present in the glucose medium.

The B-degrading enzyme-producing bacterium used in the growth experiments was isolated from feces of a group B secretor and has been tentatively identified as a strain of a new species, *Ruminococcus* AB (8), by the staff of the Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg, Va. It is a gram-positive obligate anaerobe that constitutively produces extracellular B-degrading enzyme activity.[2]

Estimation of the numbers of blood group-degrading enzyme-producing bacteria. We estimated the populations of A-, B-, and H-degrading enzyme-producing bacteria in feces by making serial dilutions of 2 g feces in 20-ml tubes of anaerobic medium and determining the smallest fecal inoculum that yielded detectable blood group-degrading enzyme activity after anaerobic incubation for 24 h at 37°C. Except as noted below, six serial 100-fold dilutions were made. Different sterile pipettes were used for each dilution. After incubation a loopful of each culture was streaked onto blood agar and eosin-methylene blue agar plates which were incubated aerobically for 72 h to screen for facultative anaerobes. The cultures were then centrifuged for 30 min at 10,000 *g* and the supernatant fluid assayed for blood group-degrading enzyme activity. Our assumption that no decrease in antigen substrate titer after 4 h incubation with culture supernate indicated absence of enzyme activity appears valid since in 42 out of 43 instances cultures producing no decrease in substrate titer after 4 h also produced no decrease when incubated for an additional 20 h. The reciprocal of the smallest fecal inoculum that yielded detectable blood group-degrading enzyme activity was taken as the estimated population of enzyme-producing bacteria per gram of fecal sample.

In measurements of the weekly variation in counts in stools from three subjects (Results, Fig. 1) the procedure was similar except that serial 10-fold dilutions of feces were made.

In the studies observing the effect of mucin glycoproteins on the growth of B-degrading enzyme-producing bacteria in fecal cultures (Results, Fig. 5) we estimated the numbers of these bacteria in 24-h cultures by making serial 10-fold dilutions of 1 ml of each culture in tubes of anaerobic medium. These in turn were incubated 24 h anaerobically, and the smallest inoculum of each primary culture that produced detectable B-degrading enzyme activity in the serially diluted subcultures was determined.

Although populations of coliform bacteria in water samples have been estimated by fermentation reactions in serially diluted samples (9, 10), we have not found reports validating the present method of estimating populations of enzyme-producing bacteria in samples containing large numbers of other bacteria as in feces. We therefore performed studies to assess the validity of the method. These studies are summarized in the Appendix. They showed: (*a*) that the "most probable number" (M.P.N.) method of enumerating bacteria from their growth in cultures of serial dilutions of sample can be applied to enumerating enzyme-producing bacteria from their enzyme production in cultures from serially diluted feces, (*b*) that the lack of precision inherent in a population estimate derived from a single culture at each fecal dilution does not affect the coarse estimate obtained by using 100-fold serial dilutions of feces, and (*c*) that populations of enzyme-producing fecal bacteria estimated from cultures of serially diluted feces may underestimate by 100-fold the actual population density of such bacteria.

Detection of ABH antigens on the cell walls of fecal anaerobes. In order to screen for ABH antigens on cell walls of the large fecal anaerobe populations 1 ml of the 24-h culture that was originally inoculated with 2×10^{-10} g feces from each of 10 subjects was subinoculated into 300 ml anaerobic medium containing 5 mg/ml D-glucose and no HGM. After 24 h the cells from the subculture were harvested, washed twice with 0.1 M ammonium acetate, and lyophilized. Obligate anaerobes that grew in the subcultures were mainly gram-negative rods whose morphology differed from one culture to another. Preliminary trials with *Escherichia coli* 086:B7 revealed that the hemagglutination inhibition method of Springer et al. (11) adapted to microtiter plates with our procedure (6) gave clear and reproducible results. 10-mg/ml suspensions of each lyophilized bacteria preparation were prepared by stirring for 3 h in 0.15 M NaCl containing 0.02 M phosphate buffer, pH 7.0. In some instances to obtain homogeneous suspensions of bacteria, clumps were disrupted by a 3-5-s, 2.4-A burst of ultrasonication (6). Aliquots of each suspension were heated 2.5 h at 100°C to remove any masking effect of heat-extractable capsular antigens (11). This treatment increased the frequency of positive hemagglutination inhibition titers. Serial two-fold dilutions of the bacterial suspensions were then tested for hemagglutination inhibition. Positive inhibition by a suspension is defined (11) as that occurring at concentrations of 2.5 mg/ml or less.

Statistical methods. Statistical significance was evaluated by one-way analysis of variance and Tukey's honestly

[1] *Abbreviations used in this paper:* HGM, hog gastric mucin; M.P.N., most probable number.

[2] Hoskins, L. C., and M. Agustines. In preparation.

significant difference procedure for multiple comparisons at the 0.05 significance level (12).

RESULTS

Anaerobic cultures of serially diluted feces

In each of 26 serially diluted cultures of feces from 22 subjects obligate anaerobes grew in the culture with the smallest fecal inoculum, 2×10^{-10} g. Except for one subject, whose 2×10^{-10}-g fecal inoculum grew coliforms in two separate samples, the smallest fecal inoculum yielding facultative anaerobes ranged from 2×10^{-4} to 2×10^{-8} g, with a modal value of 2×10^{-6} g.

Fecal populations of bacteria that produce B antigen-degrading enzyme activity

With one exception, every subject's stools contained B-degrading enzyme-producing bacteria. The exception was a group O secretor whose cultures inoculated with 0.2 g feces contained these bacteria on two occasions in 1971 but not on five occasions over the ensuing 3 yr.

Fecal populations of B-degrading enzyme-producing bacteria appear to be stable over extended periods. This is shown in Fig. 1, which depicts the estimated populations in feces collected once weekly for 6 consecutive wk from three subjects. Also included is the estimated population in a single sample from each subject tested 18 mo previously. The average weekly variation in estimated populations in subjects 1, 2, and 3 was twofold, fourfold, and sevenfold, respectively. The estimated populations obtained in stools from subjects 1 and 2 corresponded to that obtained 18 mo previously in a single sample. In subject 3 there were slightly greater fluctuations in the weekly population densities and a greater disparity between them and the very low estimate obtained 18 mo previously. It should be noted that fluctuations over the 1,000-fold range of 10^3–10^6 bacteria per g seen in subject 3 could also occur in subjects 1 and 2

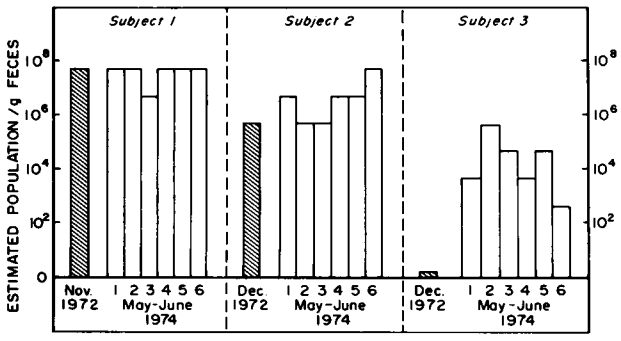

FIGURE 1 Comparative stability of fecal populations of bacteria producing B antigen-degrading enzyme activity in stools from three subjects collected once weekly for 6 wk. The fecal population in a single specimen from each subject collected 18 mo previously is included for comparison.

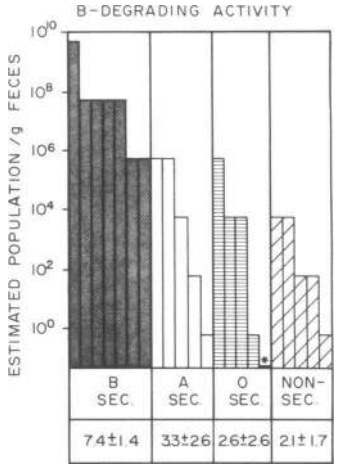

FIGURE 2 Estimated fecal populations of bacteria producing B antigen-degrading enzyme activity in 22 subjects. The mean±1 SD $\log_{10}$ count per gram feces for each group is shown below each panel. *One blood group O secretor had no B-degrading activity detected at any fecal dilution.

without affecting their estimated population densities which are in the range of 10^6–10^8 per g.

Feces from blood group B secretors contain larger populations of bacteria producing B-degrading enzyme activity than do feces from other subjects. This is shown in Fig. 2, where the estimated fecal population of these bacteria is shown for subjects grouped according to their blood type and secretor capacity. The mean±1 SD $\log_{10}$ estimated population per gram feces among the B secretors, 7.4±1.4, is statistically significantly greater than the mean $\log_{10}$ estimated population of 2.7±2.2 per g among the A secretors, O secretors, and nonsecretors combined. Their fecal populations of B-degrading bacteria did not differ significantly from each other. Thus, on the average, the B secretors had 50,000-fold greater numbers of B-degrading bacteria per gram feces than did the other subjects. Among subjects other than B secretors fecal populations of B-degrading bacteria varied downward from levels comparable to B secretors in three to none detected in any fecal dilution in one.

Basis for the greater fecal population of bacteria producing B-degrading enzyme activity in B secretors

An explanation for the greater numbers of B-degrading enzyme-producing bacteria in feces of B secretors is that their ability to produce B-degrading enzyme gives them a competitive nutritional advantage in B secretors compared to other subjects. B-degrading enzymes catalyze cleavage of the B antigenic determinant α-D-galactose from oligosaccharide side chains of B antigen glycoproteins (6, 13, 14), and the galactose thereby released could be utilized by the enzyme-producing bac-

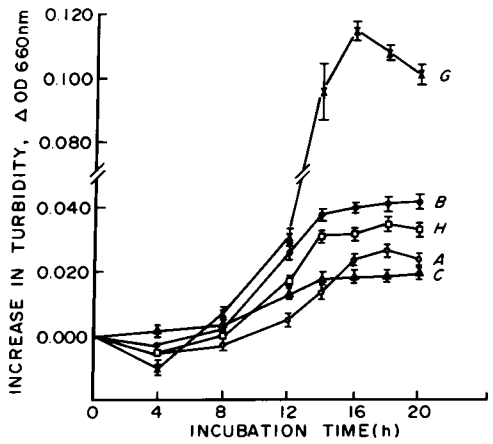

FIGURE 3 Growth of a B-degrading enzyme-producing strain of *Ruminococcus* AB in anaerobic media containing either D-galactose, a salivary glycoprotein, or neither of these. Each curve represents the mean of triplicate cultures. Vertical bars at each point = 1 SEM. Additives: G, D-galactose 1 mg/ml; A, A saliva 2.0 mg/ml; B, B saliva 1.8 mg/ml; H, O saliva 2.2 mg/ml; C, basal medium (no additives). Salivary glycoprotein concentration is equivalent to 120 μg/ml L-fucose.

terium. B antigenic determinant structures are secreted in gut mucin glycoproteins of B secretors but not in mucin glycoproteins of A secretors, O secretors, and nonsecretors (13, 15). Thus, the presence of B antigenic structures in the gut mucins of B secretors should sustain a larger population of B-degrading bacteria in B secretors than in other subjects. We tested this hypothesis by comparing proliferation of B-degrading enzyme-producing bacteria in media containing B antigen with their proliferation in other media.

Growth in pure cultures. In the first study we compared the growth of a pure culture of a strain of *Ruminococcus* AB in medium containing B saliva with its growth in media containing A or O saliva, D-galactose, or none of these. This strain produces B-degrading enzyme activity but no A- or H-degrading activity. Hence, it should cleave B antigenic determinant α-D-galactose from B saliva but not cleave the A- and H-antigenic determinant monosaccharides from A or O saliva. Growth of *Ruminococcus* AB in each of these media is shown in Fig. 3. This strain readily utilizes D-galactose, the cleavage product of B-degrading enzyme, as shown by its 5–6-fold greater growth in medium containing galactose than in basal medium. In addition, its growth in B saliva medium was twice that in basal medium. At each time from 12 to 20 h growth of this microorganism in B saliva was statistically significantly greater than in A or O saliva except at 18 h, when growth in B saliva was no different than in O saliva.

In the growth study shown in Fig. 3 the ABH antigen titers were measured in the saliva media before and 20 h after inoculation with *Ruminococcus* AB (Table I). B antigen in the B saliva medium was almost completely degraded, and the H titer in this medium increased 16-fold. This increase in H titer is the expected result if the B-degrading enzyme were an α-D-galactosidase since cleavage of the B antigenic determinant α-D-galactose results in unmasking of the underlying H antigenic determinant structure (6, 13, 14). Lacking both A-degrading and H-degrading activity, this organism effected no decrease in either the A antigen titer in the A saliva medium or the H antigen titer in the O saliva medium. This immunological evidence indicates that the greater growth of this bacterium in B saliva is related to selective cleavage of the B antigenic determinant α-D-galactose from B saliva without further degradation of the underlying oligosaccharide side chains.

If production of B degrading enzyme permits greater growth of *Ruminococcus* AB in media containing B antigen, then the extent of growth should vary with the concentration of B antigen. This is seen in Fig. 4, which shows the extent of growth in tubes of basal media containing different concentrations of either B saliva or HGM. Growth was clearly related to the concentration of B saliva. In contrast, growth increased only slightly with the concentration of HGM, which has A and H but no B antigenic structures.

Studies similar to that shown in Fig. 3 were performed with a pure culture of a fecal strain of *E. coli* that does not produce A-, B-, or H-degrading enzymes. It grew well in medium containing galactose (2.5-fold greater growth than in basal medium). But its growth in B saliva was no different from its growth in H saliva, its growth in these media was only slightly (15%) greater than its growth in basal media, and it effected no change in the B and H antigen titers of the saliva media. Thus, although *E. coli* can utilize the B antigenic determinant D-galactose *after* its cleavage from oligosaccharide chains, growth of *E. coli* in B saliva was no greater than in O saliva since it lacked the enzymatic capability to perform this cleavage.

TABLE I

Reciprocal Titers of A, B, or H Antigen in Culture Media before and 20 h after Inoculation with a B-Degrading Enzyme-Producing Strain of Ruminococcus AB

Culture medium containing:	Reciprocal antigen titers					
	Before			After		
	A	B	H	A	B	H
B saliva		512	16		2	256
A saliva	1,024			1,024		
O saliva			64			128

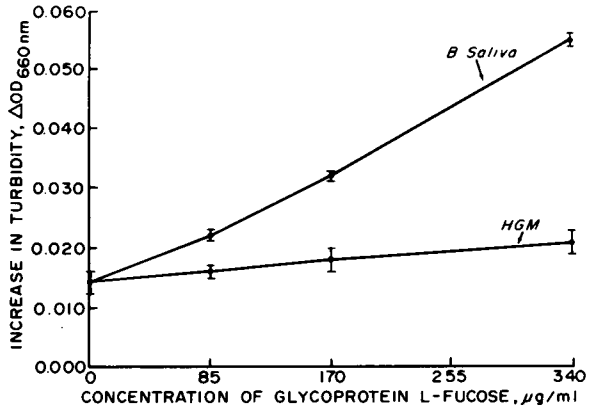

FIGURE 4 Growth of *Ruminococcus* AB as a function of the concentration of B saliva or HGM in anaerobic media. Growth was measured by increase in turbidity after 24 h incubation. Each point represents the mean±SEM of triplicate cultures. The glycoprotein L-fucose concentration of 340 μg/ml is equivalent to 5.0 mg/ml B saliva and 5.5 mg/ml HGM.

Growth in mixed cultures. To determine if the presence of B antigen offered a growth advantage to B-degrading bacteria in the presence of other fecal bacteria, we estimated their populations in fecal cultures after 24 h anaerobic incubation in media containing glucose or glycoproteins with A, B, or H antigen specificity. The results of three experiments (Fig. 5) show that 24 h after inoculation the population of B-degrading bacteria was at least 10-fold greater in the medium containing B saliva than in media containing other glycoproteins. Little or no proliferation of these bacteria occurred in media supplemented only with glucose, which is a carbon source readily utilized by enteric bacteria. Thus, in glucose medium the ability to produce B-degrading enzyme should not confer an advantage, and bacteria producing B-degrading enzyme activity could be easily overgrown by other bacteria.

In exps. 2 and 3 greater proliferation of B-degrading bacteria occurred in media containing glycoproteins with A, H, or A + H specificities than in media containing only glucose. The basis for the greater growth of B-degrading bacteria in media containing glycoproteins lacking B antigen is unclear, for the production of B-degrading enzyme activity should not confer a competitive advantage in such media.

An alternative explanation for the smaller populations of fecal B-degrading bacteria in subjects other than B secretors is that their feces either contain growth inhibitors that select against B-degrading bacteria or contain inhibitors of B-degrading enzyme activity. This explanation is unlikely, for a Millipore filter-sterilized, particle-free fecal extract from the group O secretor with no detectable B-degrading bacteria inhibited neither B-degrading enzyme activity nor growth of B-degrading bacteria in anaerobic medium.

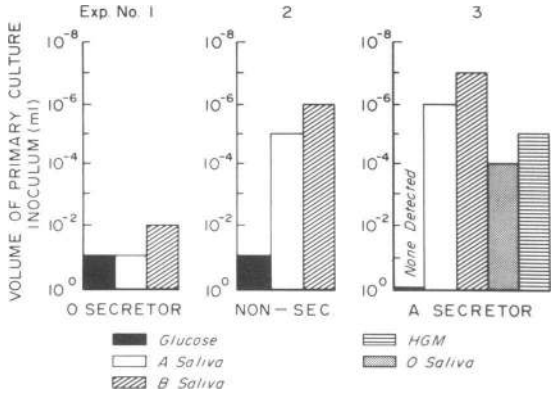

FIGURE 5 Estimation of the population of B-degrading bacteria in media containing glucose or various mucin glycoproteins 24 h after inoculation with feces. The height of each bar represents the smallest inoculum of fecal culture that produced B-degrading enzyme activity in serially diluted subcultures. Results of three separate experiments using fecal inocula from an O secretor, a nonsecretor, and an A secretor. HGM, hog gastric mucin (blood group A + H specificity).

Fecal populations of bacteria producing A and H antigen-degrading enzyme activities

Stools from every subject contained A- and H-degrading enzyme-producing bacteria. In contrast to bacteria that produce B-degrading enzyme activity, the fecal populations of bacteria that produce A- and H-degrading enzyme activities appeared to be unrelated to the blood group and secretor status of the host. This is shown in Fig. 6, where the estimated fecal populations of these bacteria are plotted for each subject. There was comparatively little variation in populations among the individual subjects and no statistically significant differences when

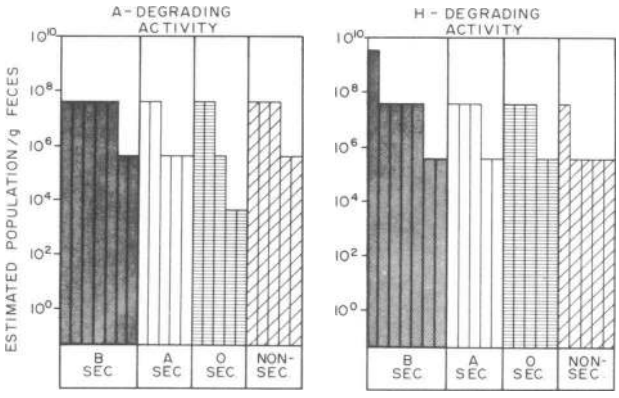

FIGURE 6 Estimated fecal populations of bacteria producing A or H antigen-degrading enzyme activity in 22 subjects.

comparisons were made with subjects grouped according to blood group and secretor status. The mean±SD $\log_{10}$ counts per gram feces for A-degrading and H-degrading bacteria among the 22 subjects were 6.6±1.3 and 6.9± 1.2, respectively. These are nearly comparable to the population of B-degrading bacteria in B secretors. In order to determine if the cell walls of the large anaerobe populations in the colon provided an additional source of A and H antigenic substrates, we tested for cell wall ABH antigen cross-reactivity in cell suspensions of obligate anaerobes subcultured from the 2×10^{-10} g fecal cultures from each of 10 subjects. Cross-reactivity with A antigen was detected in 3 of the 10 bacterial subcultures. None showed B or H antigen activity.

Population differences of bacteria producing A-, B-, and H-degrading enzyme activities in single fecal samples

The population densities of bacteria that produced A-, B-, and H-degrading activity were compared in the same stool specimen from each of the 22 subjects. Estimated populations of bacteria producing B-degrading activity differed from those producing either A- or H-degrading activity by more than 100-fold in 11 subjects and by 100-fold in 3. This strongly suggests that B-degrading activity is produced by a different population of bacteria in these subjects than is A- or H-degrading activity. It is possible that A- and H-degrading enzymes are each produced by separate populations of bacteria but the evidence is less clear: the estimated population of A-degrading bacteria differed from that producing H-degrading activity in feces from nine subjects but in only two was the difference more than 100-fold.

DISCUSSION

Enteric bacteria extensively degrade and utilize the carbohydrate moities of gut mucins (16). Bacterial degradation of the oligosaccharide side chains of mucin glycoproteins requires their production of glycosidases with the requisite specificity for each of the various glycoside linkages. Among these glycosidases are the A, B, and H blood group antigen-degrading enzymes which are produced by normal members of the enteric microflora (6, 17) in adaptation to the blood group antigenic determinant structures in the host's gut mucins (18). A, B, and H antigen specificity is conferred by the nonreducing terminal glycosides on oligosaccharide side chains of mucin glycoproteins (6, 13). Although not yet fully characterized, A-, B-, and H-degrading enzymes from fecal bacteria appear to be exoglycosidases that cleave these antigenic determinant glycosides from the oligosaccharide side chains (6). They would be required for the initial cleavage in the stepwise degradation of oligosaccharide chains possessing these structures. Since production of glycosidases by members of the enteric flora is an important step for obtaining nutrition from gut mucin saccharides, it is noteworthy that the ability to produce extracellular A, B, and H blood group-degrading enzymes is not a property common to most fecal bacteria but is limited to comparatively small populations of obligate anaerobes numbering on the order of 10^6–10^8 per g feces. Since our method of estimating fecal populations of enzyme-producing bacteria may underestimate their true population by 100-fold (see Appendix), the true population densities of these bacteria are probably higher, but they would still represent a small fraction of the anaerobes in feces, which number on the order of 10^{11} per g (19). A comparable situation exists in rumen ecosystems. Hungate (20) observed that cellulose-degrading organisms comprise only 1-5% of the total rumen microflora even though cellulose is a substantial portion of the total fermentable substrate. Furthermore, our evidence that bacterial populations producing B-degrading enzymes differ from those producing A- or H-degrading enzymes indicates further specialization with respect to production of glycosidases active against closely related substrates. This is also illustrated by the strain of *Ruminococcus* AB isolated from feces of a B secretor that produces B-degrading but no A- or H-degrading enzyme activity.

Our findings permit two conclusions about bacterial enzyme adaptation to environmental substrates in human colon ecosystems. First, extracellular enzymes important for the adaptation are produced by comparatively small populations of bacteria with specialized enzyme producing capacity. It is significant from the standpoint of ecosystem nutrition that blood group-degrading enzymes produced by such strains are extracellular (6) and are readily recovered in the particle-free supernatant fraction of fecal suspensions (18, 21), for the monosaccharides released by these enzymes into the microenvironment can be utilized by other bacteria.

Second, bacterial enzyme adaptation to environmental substrates includes proliferation of bacteria that produce the enzyme. This is clearly shown by the bacteria producing B-degrading enzyme activity whose populations in B secretors averaged 50,000-fold greater than in other subjects and by their greater proliferation in fecal cultures when grown in the presence of B antigen. One basis for the increase appears to be the competitive nutrient advantage conferred by their ability to produce B-degrading enzyme activity in environments containing B antigen. But there are also indications that other mechanisms may be operating. Thus, B-degrading bacteria multiply more in media containing HGM or A or O salivary glycoproteins than in media containing glucose alone (Fig. 5). B-degrading bacteria might have a competitive advantage in these glycoprotein me-

dia if they were able to degrade oligosaccharide chains other than those with ABH antigen structures. Thus, growth of the B-degrading fecal strain of *Ruminococcus* AB was slightly but significantly greater in medium containing H saliva than in basal medium (Fig. 3). On the other hand, its growth was unaffected by increasing concentrations of HGM (Fig. 4). An alternative possibility is that certain oligosaccharides in mucin glycoproteins are growth factors for these bacteria providing that they are exposed by prior cleavage of the terminal glycosides. Thus, 4-*O*-β-D-galactosyl-*N*-acetyl-D-glucosamine ("bifidus factor"), a disaccharide underlying the antigenic determinant glycosides of type 2 chains in human blood group substances (13) and a constituent of several other glycoproteins, including HGM (22, 23), is a growth factor for *Lactobacillus bifidus var. Penn* isolated from breast-fed infants' stools (24).

No population differences attributable to host blood type and secretor capacity were found for A- or H-degrading enzyme-producing bacteria. Their populations were comparable to that of B-degrading bacteria in B secretors. If their population levels depend upon the presence of A or H antigen substrates, then sources of these antigens unrelated to the host's blood type should be present. Dietary sources are improbable (25). A more likely source is the capsular and cell-wall heterosaccharides of the resident enteric flora itself, since Springer et al. (11) showed that a number of strains of facultative anaerobes have cell-wall heterosaccharides that cross-react with ABH antigens. But these bacteria comprise a comparatively small segment of the total microflora. Obligate anaerobes comprise 99% of the total population, but they were not tested for ABH cross-reactivity by Springer et al. Our finding cross-reactivity with A antigen on the cell walls of anaerobes from 3 of 10 cultures inoculated with 10^{-10} g feces indicates that strains of the dominant obligate anaerobe populations of fecal flora are an additional source of A-like antigen which may be quantitatively sufficient to sustain populations of A-degrading bacteria. Levels of H antigen adequate to sustain fecal populations of H-degrading bacteria could be present in A, B, and AB secretors as well as in H secretors since the H antigenic determinant α-(1,2)-L-fucoside is also present on mucin oligosaccharides of A, B, and AB secretors.

Findings reported here provide an explanation for two observations made in the preceding paper (6): (*a*) that production of a blood group-degrading enzyme is enhanced in media containing mucin glycoproteins that lack the enzyme's specific antigen substrate, and (*b*) that fecal cultures of B secretors yield higher specific activities of B-degrading than A- or H-degrading enzymes in media containing A and H but no B antigen (HGM). These can be explained on the basis of (*a*) enhanced population growth of enzyme-producing bacteria in mucin glycoprotein media compared to glucose medium (Fig. 5) and (*b*) the greater population of B-degrading enzyme-producing bacteria in feces of B secretors. As noted previously (6), both observations are inconsistent with substrate-induced enzyme synthesis. Instead, they are more compatible with proliferation of enzyme-producing bacteria whose synthesis of ABH blood group-degrading enzymes is constitutive, i.e., occurs independently of the presence or absence of substrate. Isolation of the fecal strain of *Ruminococcus* AB that produces B-degrading enzyme constitutively provides support for this argument. The findings reported in this and the preceding paper suggest that adaptation of blood group-degrading enzymes in human colon ecosystems is primarily by mutation-selection of constitutive enzyme-producing strains rather than by substrate-induced enzyme synthesis in many strains. An analogous situation has been shown to occur with *E. coli* during prolonged continuous culture in a chemostat (26). Starting with a strain that produced β-galactosidase by induced synthesis, Horiuchi et al. found that during prolonged incubation at low lactose concentrations this strain was replaced by constitutive and ultimately by "hyper" constitutive mutants that produced high levels of β-galactosidase.

There are important implications if future research shows that this mode of bacterial enzyme adaptation includes other hydrolases active in degradation of complex organic molecules. Induced enzyme synthesis responds within minutes to changes in the level of environmental substrate. On the other hand, the development of an enzyme-producing mutant population is a much slower process that depends upon the spontaneous mutation rate and the generation time of the mutant. In both continuous (27) and batch (28) culture systems this may take days. We have observed that blood group-degrading enzyme activity may disappear from feces and fecal cultures for periods of at least 10 days for unknown reasons. This suggests that the dominant enzyme-producing strain has disappeared and considerable time is required before a new one becomes established. During this period the host's colon may be exposed to antigenic structures that normally are degraded. Such antigens may include the heterosaccharide antigens on the cell walls of the enteric flora, for we have recently shown (29) that the B-like antigen on *E. coli* 086 is degraded by bacterial glycosidases in fecal extracts and fecal cultures. In view of the possibility that immune responses to cell-wall heterosaccharides of the enteric flora play a role in idiopathic ulcerative colitis (30), such apparently minor population changes in the colon ecosystem may have a significant role in the pathogenesis and unexplained exacerbations of this disease.

APPENDIX

Estimating the population density of enzyme-producing bacteria in feces

We performed studies to evaluate the validity, precision, and accuracy of population estimates of enzyme-producing bacteria in feces derived from enzyme production in cultures of serially diluted feces. Because the findings should also be applicable to fecal bacteria producing enzymes other than A-, B-, and H-degrading enzymes they are summarized here.

The "most probable number" (M.P.N.) method (31, 32). This method, as used in enumerating bacteria from the presence or absence of growth in liquid cultures of serially diluted sample, requires making several replicate cultures in liquid medium from each serial dilution and observing the proportion of fertile cultures at each dilution. It has been shown that at low bacterial population densities the probability, P, of obtaining a sterile culture follows the Poisson distribution: $P = e^{-vd}$, where d is the mean bacterial density and v is the volume of inoculum. Hence, the probability of obtaining a fertile culture is $1 - e^{-vd}$. From this relationship, "the most probable number," or the value for d most likely to give the observed proportion of fertile cultures at each of several serial dilutions, is obtained from an equation (32) or from published tables derived from it (31). In order to use the M.P.N. method for enumerating enzyme-producing bacteria in mixed populations it is necessary to show that: (*a*) the end-point measurement, presence or absence of enzyme in each culture, can be readily distinguished and (*b*) the frequency of enzyme-positive cultures agrees with the expected probability of enzyme-positive cultures predicted from the Poisson distribution (31). As noted in Methods the 4-h assay for blood group-degrading enzyme activity distinguished between enzyme-positive and enzyme-negative cultures. To determine whether the frequency of enzyme-positive cultures in a dilution series of feces follows a Poisson distribution, we made fourfold serial dilutions of feces from a B secretor in culture medium and inoculated five tubes of medium with 1 ml from each dilution. After 24 h incubation the number of enzyme-positive cultures was determined at each dilution. The results (Table II) show that the observed numbers of positive cultures agreed closely ($P < 0.90$) with the number expected if the probability of obtaining enzyme-positive cultures followed a Poisson distribution.

TABLE II

Number of Enzyme-Positive Cultures among Five Made from Each Fourfold Serial Dilution of Feces

Fecal concn	Observed no.	Expected no.*
g/ml		
2.5×10^{-8}	5	4.89
6.25×10^{-9}	3	3.06
1.56×10^{-9}	1	1.05
3.9×10^{-10}	0	0.25

* Expected no. $= nP = n(1 - e^{-vd})$, where n = no. of cultures per dilution = 5; P = expected probability of enzyme-positive cultures; d = the most probable number of B-degrading bacteria in 2.5×10^{-8} g feces = 3.78; v = volume of inoculum from the 2.5×10^{-8}-g/ml sample. Chi square = 0.3810; df = 3; $P > 0.90$.

TABLE III

Comparison of the Fecal Population of B-Degrading Bacteria Estimated from a Single Culture at Each Fecal Dilution with That Estimated by the M.P.N. Method on the Same Sample

Subject	Estimated populations of B-degrading bacteria per gram feces: Single culture at each dilution	M.P.N. method: M.P.N.	M.P.N. method: 95% confidence limits*
1	13×10^7	15×10^7	$(5–50) \times 10^7$
2	5×10^7	26×10^7	$(8–86) \times 10^7$
3	50×10^6	29×10^6	$(6–63) \times 10^6$
4	5×10^2	80×10^2	$(24–263) \times 10^2$

* Calculated from the factor provided by Cochran (32).

Comparison of the population estimates of enzyme-producing fecal bacteria obtained by using a single culture at each fecal dilution with those obtained by using the M.P.N. method. Because of the inherent sampling error at low population densities of enzyme-producing bacteria, the precision of a population estimate is less when a single culture is made at each serial dilution, as in the present study, than when multiple cultures are made at each dilution as in the M.P.N. method. In order to evaluate the sampling errors attendant to the use of single cultures at each dilution, we compared the populations of B-degrading enzyme-producing bacteria estimated from single cultures at each serial 10-fold dilution with estimates obtained simultaneously by the M.P.N. method by using five replicate cultures inoculated with 1 ml from each serial dilution. The results from four fecal samples (Table III) showed that two of the population estimates obtained by using a single culture at each dilution were outside of the 95% confidence limits obtained by the M.P.N. method on the same sample. In these cases the estimated populations by the M.P.N. method were, respectively, 5- and 16-fold greater than by the single culture method. Differences of this magnitude are less than the 100-fold serial fecal dilutions used in the present study. Thus, the sampling error that arises from using a single culture at each dilution is unlikely to affect population estimates obtained with 100-fold serial dilutions as used in the present study.

Accuracy of population estimates of enzyme-producing fecal bacteria derived from fecal cultures. In order to assess the accuracy of the serial dilution culture method for enumerating fecal enzyme-producing bacteria we compared the M.P.N. obtained for a pure culture of the B-degrading enzyme-producing strain of *Ruminococcus* AB with the M.P.N. obtained for the same culture in the presence of fecal bacteria. 1 ml of the pure culture alone and 1 ml together with 1 g fresh feces from a group O secretor whose feces had no B-degrading bacteria were each suspended in 10 ml anaerobic medium and serial 10-fold dilutions were made. Five replicate subcultures were made from each dilution and the M.P.N. of B-degrading bacteria per milliliter of the original culture was determined from the frequency of enzyme-positive subcultures at each dilution after 24 h incubation. The M.P.N. obtained by using serial dilu-

tions of *Ruminococcus* alone was 24×10^7/ml whereas the M.P.N. obtained for *Ruminococcus* serially diluted with fecal bacteria was 24×10^5/ml. Thus, the serial dilution culture method of estimating populations of enzyme-producing bacteria in the presence of large numbers of other fecal bacteria may underestimate by 100-fold the actual population density of such bacteria.

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