APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1988, p. 1970–1976
0099-2240/88/081970-07$02.00/0

Vol. 54, No. 8

Importance of Mucopolysaccharides as Substrates for *Bacteroides thetaiotaomicron* Growing in Intestinal Tracts of Exgermfree Mice

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Received 23 February 1988/Accepted 6 May 1988

We used two approaches to determine whether the mucopolysaccharide chondroitin sulfate is an important source of carbon and energy for *Bacteroides thetaiotaomicron* in the intestinal tracts of germfree mice. First, we tested the ability of three mutants that grew poorly or not at all on chondroitin sulfate to colonize the intestinal tract of a germfree mouse and to compete with wild-type *B. thetaiotaomicron* in this model system. One mutant (CG10) was rapidly outcompeted by the wild type. However, since this mutant was unable to grow on chondroitin sulfate because it could not grow on *N*-acetyl-galactosamine, one of its monosaccharide components, this mutant might also be unable to utilize glycoprotein mucins. Two mutants (46-1 and 46-4) were isolated that grew poorly on chondroitin sulfate but normally on both component sugars. One of them was outcompeted by the wild type, but the percent wild type increased more slowly than with CG10. In one experiment, the percent wild type never reached 100%. The other (46-4) was not outcompeted by the wild type. These results indicate that, although chondroitin sulfate may be a carbon source in the animal, it is not of major importance. Our second approach was to determine by immunoblot analysis whether a 28-kilodalton outer membrane protein that is produced by *B. thetaiotaomicron* only when it is grown on chondroitin sulfate or hyaluronic acid was being produced at induced level by *B. thetaiotaomicron* growing in the ceca of exgermfree mice. There was no evidence for induction of this protein in vivo. Thus, the immunoblot results are consistent with results of the mutant competition experiments.

Some human colonic *Bacteroides* species such as *Bacteroides thetaiotaomicron* have evolved complex regulated systems for catabolism of mucopolysaccharides (7, 8, 11). Mucopolysaccharides such as chondroitin sulfate and hyaluronic acid are released into the lumen of the mammalian small intestine during sloughing of mucosal cells, but it is not known how much mucopolysaccharide actually enters the colon each day. A previous study of the composition of high-molecular-weight carbohydrate in human ileal contents showed that the concentration of uronic acid in this fraction was much lower than the concentrations of hexosamines, hexoses, and pentoses (16). Uronic acids are found primarily in mucopolysaccharides and pectins, whereas hexosamines are found both in mucopolysaccharides and glycoprotein mucins, which indicates that the amount of mucopolysaccharide entering the colon is low compared with other host polysaccharides, such as the glycoprotein mucins, and dietary polysaccharides, such as xylan and cellulose. However, the dietary polysaccharides that are thought to be present in highest concentrations, i.e., cellulose and xylan, are not fermented in *B. thetaiotaomicron* (12). Also, *B. thetaiotaomicron* does not ferment glycoproteins that are similar in structure to human intestinal mucin (12) and, for this reason, is generally assumed not to be a mucin degrader. Thus, polysaccharides such as the mucopolysaccharides that occur in relatively low concentrations cannot be ruled out as potentially important substrates for *B. thetaiotaomicron*.

Previously, we found that a mutant of *B. thetaiotaomicron* which no longer produced chondroitinase II (CSase II), an enzyme that accounts for 70% of the total cellular chondroitinase activity, was not outcompeted by the wild type (CSase II^+) strain in the intestinal tracts of germfree mice (10). One possible explanation of this finding is that the mutation was not deleterious in vivo because chondroitin sulfate is not an important source of carbohydrate in the animal. However, since the CSase II^- mutant grew nearly as well as the wild type on chondroitin sulfate in laboratory medium, we could not reach any definite conclusion about the importance of chondroitin sulfate as a source of carbohydrate for organisms growing in the animal.

To assess the importance of chondroitin sulfate as a source of carbohydrate for *B. thetaiotaomicron* growing in the intestine of a germfree mouse, we needed to test mutants that, unlike the CSase II^- mutant, grow poorly or not at all on chondroitin sulfate. In this paper, we describe the isolation and characteristics of three such mutants and report the results of experiments in which we tested their ability to colonize and compete with the wild type in the intestinal tracts of germfree mice. As a second approach to determining whether chondroitin sulfate is an important source of carbohydrate for *B. thetaiotaomicron* in the intestine, we have used immunoblotting to determine whether one of the outer membrane proteins that is associated with growth on mucopolysaccharides is produced at induced levels by bacteria growing in the ceca of exgermfree mice.

MATERIALS AND METHODS

Strains and growth conditions. *B. thetaiotaomicron* 5482 was obtained from the culture collection of the Virginia Polytechnic Institute Anaerobe Laboratory, Blacksburg. For most experiments, *B. thetaiotaomicron* was grown on tryptone-yeast extract-glucose (TYG; 4) broth or on TYG agar medium. For initial selection of transposon insertions, erythromycin (Em; 10 μg/ml) and gentamicin sulfate (200 μg/ml) were included in the medium. In some experiments, *B. thetaiotaomicron* was grown on a defined medium (11) that contained one of the following as the sole source of carbohydrate: chondroitin sulfate type II, hyaluronic acid, glucu-

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ronic acid, *N*-acetyl-glucosamine, *N*-acetylgalactosamine, or glucose. Chondroitin sulfate and hyaluronic acid were added to the medium before autoclaving. The rest were filter sterilized and added after autoclaving. All carbohydrates were obtained from Sigma Chemical Co., St. Louis, MO. *Escherichia coli* EM24 containing R751::Tn*4351* (Ω4; 13) was used as the donor in transposon mutagenesis experiments. *E. coli* EM24 was cultivated on Luria broth, Luria broth agar, or TYG agar.

Isolation of mutants. Initially, we used ethyl methanesulfonate mutagenesis, coupled with penicillin selection, to obtain mutants unable to utilize chondroitin sulfate. The procedure was the same as that described by VanTassell and Wilkins (15) for isolation of amino acid auxotrophs.

Mutants of *B. thetaiotaomicron* 5482 that were impaired in the ability to utilize chondroitin sulfate were also obtained by transposon mutagenesis of *B. thetaiotaomicron* 5482 with Tn*4351*. Procedures for Tn*4351* mutagenesis were the same as those described previously for *Bacteroides uniformis* (13). In each mutagenesis experiment, approximately 3,000 Em^r transconjugants (Tn*4351* insertions) of *B. thetaiotaomicron* 5482 were picked onto chondroitin sulfate defined medium agar and glucose defined medium agar plates. Mutants that grew poorly or not at all on chondroitin sulfate defined medium agar plates were then tested for the ability to grow on defined medium containing glucuronic acid or *N*-acetylgalactosamine, the monosaccharide components of chondroitin sulfate. Their ability to grow on hyaluronic acid defined medium was also tested.

Tn*4351* is flanked by direct repeats (5). To eliminate the internal 4-kilobase region of Tn*4351* and thus eliminate as much extraneous DNA as possible, we screened approximately 1,500 colonies of each Em^r mutant of *B. thetaiotaomicron* for ability to grow on TYG but not on TYG plus Em (10 μg/ml). If the initial insertion was a simple Tn*4351* insertion, the resulting Em^s derivative should contain only a single copy of IS*4351* (5). Although Tn*4351* transposes at high frequency in *Bacteroides* spp. and is apparently random with respect to insertion site, it has one serious drawback as a mutagen. It has been observed that simple Tn*4351* insertions occur in only half of the Em^r *Bacteroides* colonies. In the other half, R751 was cointegrated along with Tn*4351* (13). Accordingly, mutants and Em^s derivatives were screened for R751 insertions by colony blot hybridization, with ^{32}P-labeled R751 as a probe. Colony hybridization and nick translation were done as described previously (2, 10).

Colonization and competition experiments in germfree mice. The source of the germfree mice and procedures used for their maintenance have been described in the accompanying paper (17). Each colonization or competition experiment was done with three mice, each in a separate cage, in the same isolator. Mutants or the wild type was grown in TYG broth to an optical density (600 nm) of 0.5 to 0.7 immediately before introduction into the isolator. Mixtures of mutant and wild type were made before introduction of the culture into the isolator.

The percent wild type in the original mixture and in fecal specimens taken at intervals from each colonized mouse was obtained by plating appropriate dilutions of each specimen onto TYG agar plates and then picking 80 isolated colonies onto chondroitin sulfate defined agar medium and glucose defined agar medium. Fresh fecal specimens were placed in 0.5 ml of sterile TYG broth and broken up with a sterile toothpick to obtain the bacterial suspensions from which the original dilutions were made. The lowest dilution of the fecal specimen was also plated onto TYG agar and incubated aerobically to check for contaminants. At least twice during each competition, colonies from the highest dilution of the fecal specimen were picked onto TYG agar, incubated anaerobically, and tested by colony hybridization with ^{32}P-labeled pUSH1, as described previously (10), to check for anaerobic contaminants.

In some experiments, mice colonized with wild-type *B. thetaiotaomicron* were switched from the conventional autoclavable chow diet (Wayne Lab-Blox; Allied Mills, Inc., Chicago, Ill.) to the defined diet described by Pleasants et al. (9). This is a fully absorbable liquid diet which consists of glucose, amino acids, and vitamins provided in liquid form. A lipid soluble vitamin mix is given separately. Shredded ashless Whatman paper (Whatman International Ltd., Maidstone, England) was used as bedding instead of the regular bedding (Wood Particles 1; American Excelsior Co., Arlington, Tex.). The cecal weights of these animals were 0.2 to 0.6 g, compared with nearly 2 g in the case of mice on the chow diet.

Immunoblot analysis of cecal contents. To determine whether *B. thetaiotaomicron* was using chondroitin sulfate as a major source of carbon and energy when it was growing in the germfree intestinal tract, we used immunoblot analysis to determine whether proteins regulated by chondroitin sulfate were being produced at induced levels in bacteria taken directly from the cecum of a colonized mouse.

To obtain a bacterial fraction from cecal contents, we diluted each homogenized cecum to 30 ml with potassium phosphate buffer (50 mM; pH 7) and centrifuged the mixture at 500 × *g* to pellet particulate material. The supernatant fluid was saved, and the pellet was suspended in 30 ml of phosphate buffer and recentrifuged. The two supernatant fractions were combined and centrifuged at 17,000 × *g* to pellet bacteria. The bacterial pellet was suspended in 30 ml of phosphate buffer. The mixture was centrifuged at 500 × *g*, the pellet was discarded, and the supernatant fluid was centrifuged at 1,700 × *g*. This pellet, which should contain all bacteria except those trapped by fibrous material in the cecum, was suspended in 5 ml of distilled water, and bacteria were disrupted by sonication.

The sonicated suspension was centrifuged at 12,000 rpm for 5 min to remove unbroken cells, and the supernatant fluid was centrifuged at 200,000 × *g* for 3 h to pellet membranes. The membrane fraction was suspended in 0.3 ml of distilled water. The supernatant fluid was lyophilized to dryness and then dissolved in 0.3 ml of distilled water. The membrane and supernatant fractions were loaded onto a 10% polyacrylamide gel, electrophoresed, and then blotted onto Zeta-probe paper (Bio-Rad Laboratories, Richmond, Calif.). Composition of the gel, electrophoresis conditions, and immunoblotting procedure were the same as described previously, except that the conditions described in the Zeta-probe booklet were used (2, 6).

To ascertain that we were recovering *B. thetaiotaomicron* in the bacterial fraction and, if so, to determine whether we could detect the chondroitin sulfate-regulated proteins on immunoblots if bacteria in the cecum were growing on chondroitin sulfate, we added 2×10^{10} wild-type bacteria, which had been grown in chondroitin sulfate defined medium, to a homogenized cecum from a germfree mouse that had not been colonized with bacteria. The number of bacteria added approximates the number detected in ceca from mice colonized with wild-type *B. thetaiotaomicron* (10). The mixture was allowed to stand for 20 min at room temperature to allow cecal proteases to act on the bacteria and was then processed as described above.

To detect chondroitin sulfate-associated proteins on the immunoblot, we first tested an antiserum that detects CSase II for the ability to detect this enzyme in the final supernatant solution from the sonicated extracts. We also tested an antiserum that detects a chondroitin sulfate-associated outer membrane protein for the ability to detect this protein in the membrane fraction obtained from the sonicated bacterial fraction as described above. Both antisera have been described previously (3, 6).

RESULTS

Characteristics CG10. Several mutants unable to utilize chondroitin sulfate (CS) were obtained by ethyl methanesulfonate mutagenesis. All of these mutants had the same phenotype; i.e., they were CS^- because they were unable to grow on *N*-acetylgalactosamine ($NAGal^-$), one of the component sugars of chondroitin sulfate. All grew normally on glucuronic acid, the other component sugar of chondroitin sulfate. All reverted to CS^+ at a frequency of 10^{-8}, and CS^+ revertants were $NAGal^+$. Since all of the mutants had the same properties, one of them, CG10, was chosen for use in the colonization studies. When CG10 was mixed with wild type and the mixture was transferred six times in glucose defined medium (about 50 generations), the final mixture was not significantly different from that in the inoculum. Thus, the mutation in CG10 did not have a pleiotropic effect on growth.

Colonization and competition experiments with CG10. CG10 colonized germfree mice at levels comparable to wild-type *B. thetaiotaomicron*, i.e., 1×10^{10} to 3×10^{10} per g of cecal wet weight. When the mice were killed 7 days after colonization to obtain cecal contents, 1 to 8% of the bacteria in the cecal contents (from all five mice tested) were CS^+ $NAGal^+$ revertants. This indicated that there was a selection for reversion in vivo. When three mice were colonized with a mixture of CG10 (70%) and wild type (30%), 100% of the bacteria recovered from fecal samples taken on day 3 after colonization had the wild-type phenotype. In a second experiment, also involving three mice, the mixture contained only 3% wild type and fecal specimens were taken 2 days after colonization. In this case, 98 to 100% of the bacteria recovered from all three mice had the wild-type phenotype. Thus, there was clearly a strong selection in the germfree intestinal tract against a strain of *B. thetaiotaomicron* carrying the CG10 type of mutation.

These results seem to suggest that the ability to utilize chondroitin sulfate is important for survival in vivo. But there is a serious drawback to using CG10 to assess the importance of mucopolysaccharides as carbon sources in the intestinal tract. Since CG10 cannot utilize *N*-acetyl-hexosamines, it would be unable to utilize glycoprotein mucins as well as mucopolysaccharides. Although a previous survey of polysaccharide utilization by *B. thetaiotaomicron* had shown that this species does not ferment porcine gastric mucin or bovine submaxillary mucin (12), possible utilization of intestinal mucin by *B. thetaiotaomicron* could not be ruled out because the structure of intestinal mucin is not identical to that of the commercially available mucins. Thus, to test the importance of chondroitin sulfate in vivo, we needed a mutant unable to utilize chondroitin sulfate but able to grow normally on both of its constituent monosaccharides. Since three attempts with ethyl methanesulfonate mutagenesis had not yielded any such mutants, even after several attempts, we decided to try mutagenizing *B. thetaiotaomicron* 5482 with the recently characterized *Bacteroides* transposon, Tn*4351* (13).

Isolation and characterization of transposon-derived mutants. Although it has been shown that Tn*4351* can transpose in *Bacteroides* spp. (13), this transposon has not been used previously as a mutagen in *B. thetaiotaomicron*. From 3,000 Tn*4351* insertions, we obtained five mutants that grew poorly or not at all on chondroitin sulfate defined medium agar. Three of the mutants were unable to grow on chondroitin sulfate because they could not use *N*-acetyl-hexosamines; i.e., they had the same phenotype as CG10. These mutants were not characterized further. Two mutants grew poorly on chondroitin sulfate but grew normally on its component sugars, glucuronic acid and *N*-acetyl-galactosamine. In broth medium containing 0.5% chondroitin sulfate, both mutants grew to an optical density of 0.2 to 0.3, compared with an optical density of >1.2 for the wild type. These mutants were not identical because one (46-1) grew on hyaluronic acid and the other one (46-4) did not. Both mutants grew normally on polygalacturonic acid, another negatively charged polysaccharide, and on arabinogalactan. Thus, the effect of the mutation was specific to chondroitin sulfate or closely related polysaccharides.

Both 46-1 and 46-4 were quite stable. When 0.1 ml of culture (ca. 10^8 bacteria) was inoculated into tubes of chondroitin sulfate defined medium, no growth above an optical density of 0.3 was detected even after 1 week of incubation. Reversion rates could not be determined on agar plates because of background growth by the mutant. Although both mutants exhibited some growth on chondroitin sulfate, they were easily distinguishable from the wild type when picked onto agar medium containing chondroitin sulfate as the sole source of carbohydrate.

Previous work on Tn*4351* insertions in the *Bacteroides* chromosome had shown that, in about half of the insertions, Tn*4351* cointegrated R751 (13). Colony hybridization of the 46-1 mutant, with ^{32}P-labeled R751 as a probe, demonstrated that this mutant contained a simple insertion of Tn*4351* and had not cointegrated R751. An Em^s derivative of this strain, which presumably contained only IS*4351*, was used in all colonization and competition experiments. When a *Hin*dIII digest of chromosomal DNA from this strain was probed on Southern blots with a labeled probe that contained IS*4351*, only one cross-hybridizing band was detected (data not shown). Since *Hin*dIII does not cut within IS*4351*, this is the pattern expected from a single insertion of IS*4351*.

Colony hybridization analysis showed that the other mutant, 46-4, contained R751 as well as Tn*4351*. Since R751 insertions are flanked by copies of Tn*4351* or IS*4351*, it was possible that some Em^s derivatives might have lost R751 along with Em^s. Several Em^s derivatives were obtained and tested but all contained R751; i.e., recombinatory excision of the Em^r gene occurred between the two IS*4351* copies at one end of the insertion. Despite the presence of R751 in this mutant, we decided to use it in colonization experiments because no other mutants with the desired phenotype were available. When a *Hin*dIII digest of chromosomal DNA from this strain was probed on a Southern blot with a labeled probe containing IS*4351*, two cross-hybridizing bands were detected (data not shown). This is the pattern expected if the mutation was a single insertion.

Both mutants appeared to have the same growth rate as wild type when cultures were grown in parallel in glucose defined medium. To confirm that the growth rates of the mutants were identical to that of wild type in this medium, i.e., that there was no general growth deficit resulting from the mutations, we mixed mutant and wild type and then determined the mutant/wild-type ratio after 50 generations in

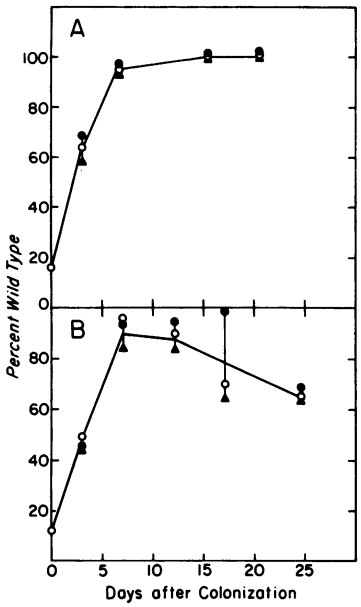

FIG. 1. Results of a competition experiment between wild-type *B. thetaiotaomicron* and mutant 46-1, which grows poorly on chondroitin sulfate but normally on hyaluronic acid and on the monosaccharide components of chondroitin sulfate. Panels A and B show the results of two separate experiments, each involving three mice. Fecal specimens were taken from each mouse at intervals after colonization, diluted, and plated to obtain isolated colonies. The percent wild type was determined by screening 80 colonies for the ability to grow on chondroitin sulfate defined medium agar plates. Each mouse is indicated by a different symbol. A solid line connects the means of the percent wild type values from the three mice at each time point.

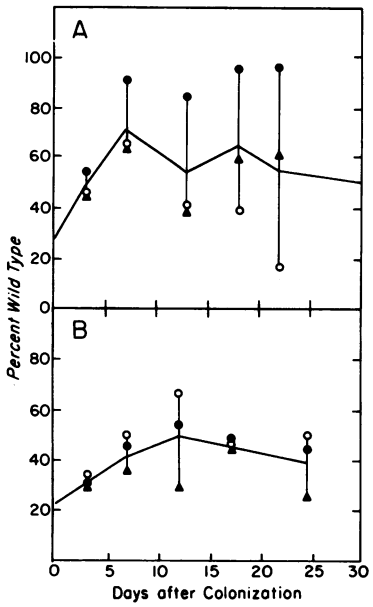

FIG. 2. Results of a competition experiment between wild-type *B. thetaiotaomicron* and mutant 46-4, which grows poorly on chondroitin sulfate and hyaluronic acid but normally on the monosaccharide components of these mucopolysaccharides. Panels A and B show the results of two separate mucopolysaccharides. Panels A and B show the results of two separate experiments, each involving three mice. Each mouse is indicated by a different symbol. A solid line connects the means of the values from the three mice at each time point.

glucose defined medium. In both cases, the percent wild type after 50 generations was the same as that in the initial mixture.

Colonization and competition experiments with 46-1 and 46-4. Both the Em^s 46-1 and the Em^s 46-4 mutants colonized three germfree mice at levels of 1×10^{10} to 3×10^{10} per g of cecal contents. That is, they colonized the cecum at levels comparable to the wild type. No evidence of revertants was seen in either case when 80 colonies were picked onto chondroitin sulfate defined medium agar plates from fecal specimens taken 3 weeks after colonization.

Two separate competition experiments, each involving three mice, were done with each mutant. The initial mixture used to colonize the animals contained 15 to 25% wild type. The wild type outcompeted the 46-1 mutant within 10 days in one competition experiment (Fig. 1A). In the second experiment involving 46-1, the percent wild type rose to about 90% in all three mice during the first 7 to 10 days, but then dropped to about 60% over the next 20-day period (Fig. 1B).

The 46-4 mutant was not outcompeted by the wild type, although the percent wild type increased during the competition period in both experiments (Fig. 2). The leveling off of the percent wild type that followed the initial rise in some animals could be explained if loss of R751 by homologous recombination had occurred and if this loss had made the mutant more competitive. To test this, we isolated 36 colonies having the mutant phenotype from fecal specimens obtained from each of the mice in Fig. 2. Samples from the mice in Fig. 2A were taken after 32 days, and samples from the mice in Fig. 2B were taken after 16 days. Colony blots were hybridized with ^{32}P-labeled R751. All of the colonies that had the mutant phenotype contained DNA that cross-hybridized strongly with this probe, whereas no hybridization was detectable with wild-type colonies.

Effect of defined diet. If the effect of the various mutations described above was to reduce the ability to utilize chondroitin sulfate, rather than some pleiotropic effect on cell surface properties or growth rate in vivo, the effect ought to be more severe if mice were fed a diet that contained no utilizable polysaccharides. In this case, the bacteria would be forced to rely entirely on host products such as mucopolysaccharides or mucins for survival. To test this, we did some experiments with the defined liquid diet described by Pleasants et al. (9). In our hands, the mice were healthy for the first 7 days on this diet, but often did not thrive if continued longer on this diet. For this reason, we tried to design experiments that did not exceed 7 days.

CG10 was outcompeted so rapidly by the wild type in mice eating the normal diet that we decided to test the effect of the defined diet on survival of this mutation by measuring the percent revertants after 7 days in mice colonized with a pure culture of CG10 rather than by doing a competition experiment. The results (Table 1) show that revertants of CG10 predominated after 7 days in mice on the defined diet, whereas revertants were only beginning to appear at appreciable levels in mice eating the chow diet. This indicates that the selection for revertants in mice eating the defined diet was much stronger than in mice eating the chow diet.

46-1 was outcompeted rapidly enough in mice eating the chow diet so that we were not sure that we could detect a significant difference in the rate of increase of the percent wild type by doing a competition experiment similar to the ones shown in Fig. 1. We had found, however, that if we

TABLE 1. Effect of diet on reversion rate of CG10 in ceca of germfree mice colonized with CG10 and then sacrificed after 7 days

Type of diet	Mouse	Cecal wt (g)	Bacteria/g of cecal wet wt, 10^{10}	% Revertants
Chow	1	2.1	1.5	1
	2	1.8	2.1	1
	3	1.0	1.9	7
Defined	4	0.2	1.6	100
	5	0.2	2.4	100
	6	0.2	1.4	100

placed a Rif^r wild-type strain in competition with Rif^s 46-1, the wild type did not outcompete the mutant. Apparently, the slower growth rate associated with the Rif^r mutation was sufficient to cancel the growth advantage of the wild type over Rif^s 46-1. Accordingly, we placed a Rif^r derivative of the wild type in competition with Rif^s 46-1 in mice eating the chow diet and in mice eating the defined liquid diet. The results (Fig. 3) show that the advantage of the wild type over the mutant was increased significantly if mice were fed the defined diet rather than the chow diet. We did not test 46-4 in mice fed the defined diet because of the presence of R751 in this strain.

Immunoblot analysis of chondroitin sulfate-regulated proteins in cecal contents. We were unable to detect CSase II in the soluble portion of cecal contents from mice colonized with wild-type bacteria (data not shown). This appeared to be because the level of CSase II in the bacterial extract from a single mouse cecum was too low to detect with the antiserum even after concentrating the extract by lyophilization. We could not detect CSase II in our control sample, which consisted of a germfree cecum to which wild-type bacteria, grown on chondroitin sulfate, had been added to a final concentration comparable to that found in colonized ceca.

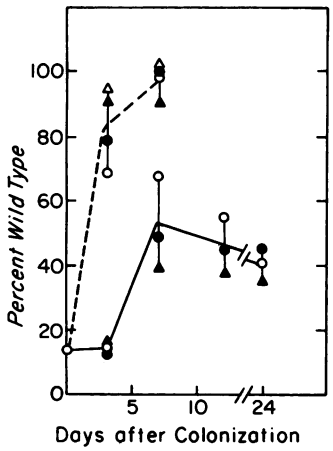

FIG. 3. Effect of a totally absorbable diet on results of a competition experiment between mutant 46-1 (Rif^s) and a Rif^r derivative of the wild type. The Rif^r derivative was used to make the competition between mutant and wild type equal on the chow diet so that differences due to the effect of the defined diet would be more apparent. The solid line shows the outcome of the competition in three mice fed the chow diet. The dashed line shows the outcome of a competition in four mice fed the totally absorbable diet described by Pleasants et al. (9).

We were, however, able to detect *B. thetaiotaomicron* outer membrane proteins in cecal contents by using a cross-absorbed antiserum that was raised against cells grown on chondroitin sulfate and cross-absorbed with cells grown on glucose. This antiserum detects several constitutive proteins as well as proteins regulated by chondroitin sulfate. Although more than one chondroitin sulfate-associated protein was visible on some blots, especially if the load was heavy, only one of them, a 28-kilodalton (kDa) protein, gave a strong and consistent signal. This protein has been shown previously to be specific for chondroitin sulfate utilization and is not seen in extracts from *B. thetaiotaomicron* grown on other polysaccharides (6). Accordingly, we decided that this protein was a suitable indicator of chondroitin sulfate utilization. That is, the relative darkness of this band on immunoblots, compared with the darkness of the constitutive protein band immediately below it in the blot, can be used to determine whether the cells are growing on chondroitin sulfate rather than on some other carbohydrate. The constitutive band thus serves as an internal standard to standardize the amount of extract loaded on the gel. This is particularly important in the case of bacterial extracts from cecal samples because standard protein determinations are unreliable.

When a culture of *B. thetaiotaomicron* that had been grown on chondroitin sulfate minimal medium was added to germfree cecal contents and then taken through the differential centrifugation procedure to obtain a bacterial membrane fraction, the immunoblot pattern was virtually identical to that of a membrane fraction from bacteria grown in chondroitin sulfate defined medium (Fig. 4). The immunoblot patterns of bacterial membrane fractions from monoassociated mice is not the same as that of membranes from *B. thetaiotaomicron* grown on chondroitin sulfate as the sole source of carbohydrate. As can be seen from the darkness of the band below the 28-kDa band, the amount of membrane protein loaded in lanes 1 to 3 is lower than that loaded in lanes 4 to 7. A light band comigrating with the 28-kDa protein can be seen in some of the cecal samples, but the ratio of this band to that below it is clearly less than expected if the bacteria had been growing on chondroitin sulfate as the sole carbohydrate source. The 28-kDa band is generally not seen even in heavy loads of glucose-grown cells (data not shown), so the small amount of this protein seen in some cecal samples could indicate that a partial induction by chondroitin sulfate had occurred. This is the result expected if small amounts of chondroitin sulfate were being used in the mouse cecum, but chondroitin sulfate was not the major carbohydrate source.

A 60-kDa protein is missing in the bacteria added to cecal contents. This protein is also missing in the lanes containing bacteria obtained from colonized exgermfree mice. The absence of this protein could have been due to selective action of proteases in cecal contents. Bacterial membrane fractions from the ceca of monoassociated animals colonized with *B. thetaiotaomicron* had at least one protein that was not present in the three control lanes (Fig. 4). These polypeptides could be proteolytic digestion products of larger proteins or they could be proteins that are induced as bacteria adapt to the animal.

In one experiment, we obtained the bacterial membrane fraction from cecal contents of two animals that had been receiving the defined liquid diet for 7 days. The immunoblot pattern of these samples was essentially the same as that shown in Fig. 4, lanes 5 to 8 (data not shown). Thus, even in

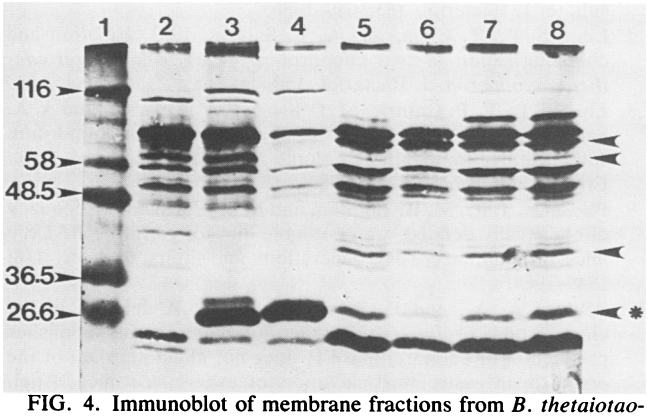

FIG. 4. Immunoblot of membrane fractions from *B. thetaiotaomicron* grown in pure culture or obtained directly from the ceca of colonized mice by differential centrifugation. Lane 1 contains prestained molecular weight standards. Molecular sizes (10^3) are indicated at the left. Lanes 2 and 3 contain membrane fractions (ca. 30 μg of protein) of *B. thetaiotaomicron* grown in defined medium plus glucuronic acid (lane 2) or defined medium plus chondroitin sulfate (lane 3). Lane 4 contains a mixture consisting of bacteria grown on chondroitin sulfate defined medium and added to a homogenized cecum taken from a mouse that had not been colonized. The mixture was processed as described for the homogenized ceca of mice colonized with *B. thetaiotaomicron*. Lanes 5 to 8 contain membrane fractions from *B. thetaiotaomicron* obtained directly from cecal contents by differential centrifugation. The blot was probed with antiserum raised against membranes of bacteria grown on chondroitin sulfate and cross-absorbed with membranes of bacteria grown on glucose (6). The 28-kDa chondroitin sulfate-specific protein detected by this antiserum is indicated by an arrow and asterisk on the right. The faint band above the 28-kDa protein is also associated with growth on chondroitin sulfate but is not always well resolved. Arrows on the left indicate a 60-kDa protein that is not seen in lanes 5 to 8 and some proteins in the 40-kDa range that are seen only in samples from the ceca of colonized animals. Mice represented in lanes 5 to 8 were fed regular chow diet. Bacterial fractions from ceca of mice fed the defined liquid diet described by Pleasants et al. (9) exhibited the same pattern (data not shown).

these animals, the chondroitin sulfate-associated 28-kDa protein was not being produced at induced levels.

DISCUSSION

Our findings that 46-1, a mutant that appears to be defective specifically in the ability to utilize chondroitin sulfate, was outcompeted by the wild type and that the competitiveness of 46-1 was decreased even further in mice fed a liquid diet that contained no dietary polysaccharides support the hypothesis that mucopolysaccharides are being used at some level in the germfree intestinal tract. However, another mutant (46-4) with a similar phenotype was able to compete successfully with the wild type. This is surprising because 46-4 appeared to be more deficient in use of mucopolysaccharides that 46-1. 46-4 could not use either hyaluronic acid or chondroitin sulfate, whereas 46-1 could utilize hyaluronic acid. Moreover, 46-4, unlike 46-1, carried 53 kilobases of foreign (R751) DNA. Since both mutants seem to be affected specifically in mucopolysaccharide utilization, as indicated by the fact that they compete equally with wild type in glucose defined medium and grow well on all other polysaccharides fermented by the wild type, this result indicates that inability to utilize mucopolysaccharides is not by itself enough to put the organism at a disadvantage.

Preliminary evidence indicates that the 46-1 mutant induces both chondroitinases when exposed to chondroitin sulfate, whereas the 46-4 mutant does not (V. Hwa, unpublished data). It is possible that production of proteins that cannot be used productively is the basis for the lower competitiveness of the 46-1 mutant in vivo. If this is the case, the finding that 46-1 was outcompeted whereas 46-4 was not would support the hypothesis that low levels of chondroitin sulfate are being utilized in the mouse.

The results of our immunoblot analysis also support the hypothesis that chondroitin sulfate is not a major source of carbohydrate for *B. thetaiotaomicron* in the germfree intestine. That is, a membrane protein that is prominent when *B. thetaiotaomicron* is grown on chondroitin sulfate or hyaluronic acid was not seen at induced levels in a bacterial fraction from mouse cecal contents. All previous studies of regulation of this and other chondroitin sulfate-associated outer membrane proteins have been done with bacteria grown in high concentrations of substrate. Thus, we do not know what is the smallest amount of polysaccharide that will produce high levels of this protein, especially under conditions encountered by bacteria in the cecum. Also, the proteins detected by the antiserum are outer membrane proteins, and it is possible that proteins that are exposed on the outside of the cell are degraded by cecal proteases. Since the 28-kDa chondroitin sulfate-associated protein did not disappear in cells added to germfree cecal contents and incubated for 20 min at room temperature, it appears not to be very accessible to protease action. Nonetheless, we cannot rule out the possibility that the 28-kDa protein is being selectively degraded by proteases in the colonized animal. Despite these problems, the immunoblot approach appears to be a promising one for probing the activities of specific groups of organisms in vivo. To our knowledge, this is the first attempt to apply this method to an ecological problem.

Although the results of our experiments seem to point to a rather minimal role for mucopolysaccharides as carbon sources in vivo, it should be kept in mind that in the experiments reported here *B. thetaiotaomicron* is not being forced to compete with other polysaccharide-utilizing species. It is possible that competition from other bacteria in the colonic microflora of conventional animals would cause *B. thetaiotaomicron* to rely more heavily on mucopolysaccharides than is indicated by the results of experiments involving mice associated with a single species.

That CG10, a mutant that cannot utilize either chondroitin sulfate or *N*-acetylhexosamines, is at a greater disadvantage in competition with the wild type than is 46-1, which can utilize *N*-acetylhexosamines, could indicate that *B. thetaiotaomicron* growing in the intestine relies heavily on the hexosamine-containing glycoprotein mucins. Indirect support for this conclusion also comes from the fact that concentrations of *B. thetaiotaomicron* were so high (ca. 10^{10} per g of cecal wet weight) in the ceca of mice eating the defined liquid diet. It is unlikely that enough mucopolysaccharide is being generated from sloughing mucosal cells to support this population level. Thus, the assumption that *B. thetaiotaomicron* cannot ferment intestinal mucin, an assumption based on the previous finding that *B. thetaiotaomicron* could not ferment two commercially available glycoprotein mucins, hog gastric and bovine submaxillary (12) mucins, needs to be reevaluated. *B. thetaiotaomicron* can ferment mucin components such as fucose and hexosamines (12), and it may be that this organism can ferment some types of glycoprotein mucin.

It is interesting to note that inserted R751 in mutant 46-4 did not interfere with the ability of this mutant to compete

with the wild type. Moreover, the R751 insertion was stable in vivo, even after periods as long as 32 days. Since inserted R751 is flanked by homologous regions (IS*4351*), it could easily have been lost by homologous recombination if its presence in the strain had been deleterious. We had found previously that R751 cannot mobilize itself out of *Bacteroides* spp. and that the trimethoprim resistance gene carried on it appears not to be expressed in *Bacteroides* spp. (14). The stability of R751 insertions in vivo could be because none of the genes on this large plasmid are being expressed in *Bacteroides* spp.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI 17876 from the National Institute of Allergy and Infectious Diseases.

We thank D. C. Savage for helpful advice and suggestions and for the use of his germfree facility, which is supported by Public Health Service grant AI 11858 from the National Institute of Allergy and Infectious Disease.

LITERATURE CITED

1. **Davis, R. W., D. Botstein, and J. R. Roth.** 1980. Advanced bacterial genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
2. **Guthrie, E. P., and A. A. Salyers.** 1986. Use of targeted insertional mutagenesis to determine whether chondroitin lyase II is essential for chondroitin sulfate utilization by *Bacteroides thetaiotaomicron*. J. Bacteriol. **166**:966–971.
3. **Guthrie, E. P., N. B. Shoemaker, and A. A. Salyers.** 1985. Cloning and expression in *Escherichia coli* of a gene coding for chondroitin lyase from *Bacteroides thetaiotaomicron*. J. Bacteriol. **164**:510–515.
4. **Holdeman, L. V., E. P. Cato, and W. E. C. Moore.** 1977. Anaerobe laboratory manual, 4th ed. Virginia Polytechnic Institute and State University, Blacksburg.
5. **Hwa, V., N. B. Shoemaker, and A. A. Salyers.** 1988. The direct repeats flanking Tn*4351* are insertion sequences. J. Bacteriol. **170**:449–451.
6. **Kotarski, S. F., J. Linz, D. M. Braun, and A. A. Salyers.** 1985. Analysis of outer membrane polypeptides that are associated with the growth of *Bacteroides thetaiotaomicron* on chondroitin sulfate. J. Bacteriol. **163**:1080–1086.
7. **Linn, S. P., T. Chan, and A. A. Salyers.** 1983. Isolation and characterization of two chondroitin lyases from *Bacteroides thetaiotaomicron*. J. Bacteriol. **156**:859–866.
8. **Lipeski, L., E. P. Guthrie, M. O'Brien, S. F. Kotarski, and A. A. Salyers.** 1986. Comparison of proteins involved in chondroitin sulfate utilization by three colonic *Bacteroides* species. Appl. Environ. Microbiol. **51**:978–984.
9. **Pleasants, J. R., M. H. Johnson, and B. S. Wostmann.** Adequacy of chemically defined, water-soluble diet for germfree BALB/c mice through successive generations and litters. J. Nutr. **116**: 1949–1964.
10. **Salyers, A. A., and E. P. Guthrie.** 1988. A deletion in the chromosome of *Bacteroides thetaiotaomicron* that abolishes production of chondroitinase II does not affect survival of the organism in gastrointestinal tracts of exgermfree mice. Appl. Environ. Microbiol. **54**:1964–1969.
11. **Salyers, A. A., and M. O'Brien.** 1980. Cellular location of enzymes involved in the breakdown of chondroitin sulfate. J. Bacteriol. **143**:772–780.
12. **Salyers, A. A., J. R. Vercellotti, S. E. H. West, and T. D. Wilkins.** 1977. Fermentation of mucin and plant polysaccharides by strains of *Bacteroides* from the human colon. Appl. Environ. Microbiol. **33**:319–323.
13. **Shoemaker, N. B., C. Getty, J. F. Gardner, and A. A. Salyers.** 1986. Tn*4351* transposes in *Bacteroides* spp. and mediates the integration of plasmid R751 into the *Bacteroides* chromosome. J. Bacteriol. **165**:929–936.
14. **Shoemaker, N. B., and A. A. Salyers.** 1987. Facilitated transfer of IncP R751 derivatives from the chromosome of *Bacteroides uniformis* to *Escherichia coli* recipients by a conjugative *Bacteroides* tetracycline resistance element. J. Bacteriol. **169**:3160–3167.
15. **VanTassell, R. L., and T. D. Wilkins.** 1978. Isolation of auxotrophs of *Bacteroides fragilis*. Can. J. Microbiol. **24**:1619–1621.
16. **Vercellotti, J. R., A. A. Salyers, W. S. Bullard, and T. D. Wilkins.** 1977. Breakdown of mucin and plant polysaccharides in the human colon. Can. J. Biochem. **55**:1190–1196.
17. **Whitt, D. B., and D. C. Savage.** 1980. Kinetics of changes induced by indigenous microbiota in the activity levels of alkaline phosphatase and disaccharidases in small intestinal enterocytes in mice. Infect. Immun. **29**:144–151.