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Use of Targeted Insertional Mutagenesis to Determine Whether Chondroitin Lyase II Is Essential for Chondroitin Sulfate Utilization by *Bacteroides thetaiotaomicron*

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Bacteroides thetaiotaomicron **produces two inducible chondroitin lyases (I and II) when it is grown on chondroitin sulfate. Both enzymes have very similar biochemical properties. To determine whether both enzymes are required for growth on chondroitin sulfate, we constructed a *Bacteroides* suicide vector, pE3-1, and used it to create an insertional mutation that interrupts the chondroitin lyase II gene of *Bacteroides thetaiotaomicron*. pE3-1 contains a 4.4-kilobase cryptic *B. eggerthii* plasmid (pB8-51), the *Escherichia coli* cloning vector pBR328, and the *Eco*RI D fragment from the conjugative *B. fragilis* plasmid pBF4. A 0.8-kilobase fragment from the center of the *B. thetaiotaomicron* chondroitin lyase II gene was inserted in pE3-1 to create pEG817. Although, pEG817 is stably maintained in *E. coli* and can be mobilized into *B. thetaiotaomicron* by the IncP plasmid R751, pEG817 is not maintained as a plasmid in *Bacteroides* spp. When pEG817 was mobilized into *B. thetaiotaomicron*, with selection for a drug marker on pEG817, transconjugants were obtained which had pEG817 inserted into the chondroitin lyase II gene. Western blot analysis was used to confirm that intact chondroitin lyase II is not produced in the mutant. The mutant was able to utilize chondroitin sulfate as a sole source of carbon, although no active chondroitin lyase II was produced. Thus chondroitin lyase I alone appears to be sufficient for growth on chondroitin sulfate. The mutant also had some minor changes in its outer membrane protein profile. However, there was no evidence that any of the major chondroitin sulfate-associated polypeptides in the outer membrane were affected by the insertion in the chondroitin lyase II gene.**

Approximately 30% of the bacteria that reside in the human colon are members of the genus *Bacteroides* (17). *Bacteroides* spp. are obligately anaerobic gram-negative rods that obtain carbon and energy by fermenting carbohydrates. Species such as *Bacteroides thetaiotaomicron* can ferment a variety of complex polysaccharides including the mucopolysaccharide chondroitin sulfate (21). Chondroitin sulfate and other mucopolysaccharides are components of epithelial tissue, which is constantly being sloughed into the intestinal lumen, and may be a source of carbon and energy for *B. thetaiotaomicron* and other colon bacteria which can utilize it.

The first step in the breakdown of chondroitin sulfate is the cleavage of the polysaccharide into unsaturated disaccharides by chondroitin lyase (20). Two chondroitin lyases, chondroitin lyase I and chondroitin lyase II, have been isolated from *B. thetaiotaomicron*. These chondroitin lyases are so similar with respect to physical characteristics that it is not clear why the organism needs both enzymes (12). Similar pairs of chondroitin lyases have also been isolated from the two other colonic *Bacteroides* species which can ferment chondroitin sulfate, *B. ovatus* and *Bacteroides* sp. strain 3452A (13). To determine whether both chondroitin lyases are required for the utilization of chondroitin sulfate by *B. thetaiotaomicron*, we wanted to obtain a mutant that did not produce one of the chondroitin lyases. Attempts to isolate such mutants by using chemical mutagenesis were unsuccessful (Salyers, unpublished results). Recently, we cloned the gene that codes for chondroitin lyase II (5). This made it possible to use insertional mutagenesis to interrupt the chondroitin lyase II gene. First, however, we had to develop a suicide vector which could replicate in *Escherichia coli* but was not maintained in *B. thetaiotaomicron* and which could be mobilized from *E. coli* to *B. thetaiotaomicron*. Such a vector could be used to introduce a fragment of the cloned chondroitin lyase II gene by homologous recombination into *B. thetaiotaomicron* so that the chondroitin lyase II gene could be interrupted. In this report we describe the construction of pE3-1, a suicide vector for *Bacteroides* species, and the use of this vector for insertional mutagenesis of the chondroitin lyase II gene of *B. thetaiotaomicron*. Also, we report the effect of this mutation on the ability of *B. thetaiotaomicron* to utilize chondroitin sulfate.

MATERIALS AND METHODS

Bacterial strains and media. Concentrations of antibiotics used were as follows: ampicillin, 50 to 100 μg/ml; tetracycline, 10 to 20 μg/ml; naladixic acid, 100 μg/ml; rifampin, 20 μg/ml; geneticin or G418, 400 μg/ml; erythromycin, 10 to 20 μg/ml; and clindamycin, 10 to 20 μg/ml.

B. thetaiotaomicron 5482 (ATCC 29148) was obtained from the culture collection of the Virginia Polytechnic Institute Anaerobe Laboratory, Blacksburg. *B. thetaiotaomicron* 5482 is Em^s Nal^r Gen^r. *E. coli* EM24N was used as a donor in the mating experiments. *E. coli* EM24N is a spontaneous Nal^r derivative of *E. coli* EM24. *E. coli* EM24N is Em^r Nal^r Gen^s and is also $RecA^-$. Geneticin, formerly designated G418, is an aminoglycoside that is effective against strains of *E. coli* which are resistant to streptomycin, kanamycin, or gentamicin. Also, geneticin is more effective than these other aminoglycosides against susceptible strains of *E. coli* that are growing under anaerobic conditions. For these reasons, geneticin was used to select against the *E. coli* donor in mating experiments.

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B. thetaiotaomicron 5482 was grown in prereduced Trypticase (BBL Microbiology Systems) yeast extract-glucose broth (TYG) (7) or in defined medium containing chondroitin sulfate type III (5 mg/ml) or glucose (5 mg/ml) as the sole carbon source (20). The atomosphere was 80% N_2–20% CO_2. *E. coli* EM24N was grown in Luria broth (LB; 16) or on LB agar plates. In most cases, erythromycin was added to medium that was used to grow the mutants because the insertional mutation made the mutants Em^r. However, in experiments to compare growth on chondroitin sulfate of *B. thetaiotaomicron* mutants and wild type, strains were inoculated into defined medium that contained chondroitin sulfate (5 mg/ml) but no erythromycin. Defined medium containing chondroitin sulfate or glucose, but no erythromycin, was also used to determine the frequency of revertants.

Plasmid construction, DNA isolation, and analysis. Plasmids were isolated by the Ish-Horowitz modification (16) of the method of Birnboim and Doly. Chromosomal DNA from *Bacteroides* species was isolated by the procedure of Saito and Miura (19). Standard procedures were followed in steps involving restriction endonucleases, calf intestinal phosphatase, linkers, and T4 DNA ligase (16). Restriction endonuclease digestions were loaded on a 1% agarose slab gels in 4× GGB (0.16 M Tris, 0.08 M sodium acetate, 8 mM EDTA [pH 8.3]) and electrophoresed for 10 to 15 h. Gels were stained with ethidium bromide and photographed. Preparation of competent *E. coli* cells and transformation of *E. coli* with plasmid DNA was performed as described by Lederberg and Cohen (11).

Bacterial matings. Donor (*E. coli* EM24N) and recipient (*B. thetaiotaomicron* 5482) strains were grown separately to early exponential phase (1×10^8 to 2×10^8 CFU/ml). Bacteria in 1 ml of broth were pelleted by centrifugation in an Eppendorf microfuge (1 min), and the bacterial pellet was suspended in 0.1 ml of TYG broth. Donor and recipient strains were cospotted on nitrocellulose filters placed on LB or TYG agar plates. The mating mixtures were incubated under aerobic conditions at 37°C for 16 h. Aerobic incubation increases the frequency of conjugal transfer from *E. coli* to *Bacteroides* species (23). After incubation, the cells were suspended in TYG by vortexing and were plated on TYG agar plates that contained geneticin and erythromycin. Gen^r Em^r transconjugants were tested for the inability to grow aerobically to confirm that they were Em^r *Bacteroides* species and not *E. coli*.

Western blot analysis. Crude cell extracts of *B. thetaiotaomicron* were prepared by growing 100 ml of bacteria to an optical density at 650 nm of 0.6 to 0.8. The bacteria were harvested by centrifugation at 7,000 × *g* for 15 min at 4°C. The pelleted bacteria were washed twice and suspended in 7 ml of 0.02 M potassium phosphate buffer (pH 7.6). The suspended bacteria were disrupted by three 2-min periods of sonication. Undisrupted cells were removed by centrifugation at 12,000 × *g* for 20 min at 4°C. To obtain a crude membrane preparation, the disrupted cell suspension was centrifuged at 175,000 × *g* for 2.5 h at 4°C. The pellet was washed once and resuspended in 0.02 M potassium phosphate buffer (pH 7.6).

Antibodies against chondroitin lyase II and purified outer membranes from *B. thetaiotaomicron* 5482 were obtained as described previously (5, 9). The immunoglobulin G fraction was obtained as described by Lopatin and Voss (15).

Proteins in crude cell extracts or in crude membrane preparations were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis as described previously (9, 10) and then transferred onto nitrocellulose sheets by the procedure of Erickson et al. (2). The nitrocellulose filters were blocked with bovine lactotransfer technique optimizer (8) and treated with the primary antibody. The antibody-protein conjugate was detected on the nitrocellulose sheets by using the immunodetection system of Bethesda Research Laboratories, Gaithersburg, Md. Molecular weight standards were β-galactosidase (116,000), phosphorylase *b* (97,000), bovine serum albumin (66,000), catalase (57,000), ovalbumin (45,000), carbonic anhydrase (29,000), and myoglobin (18,000). Molecular weight standards were visualized on the nitrocellulose sheets with Indian ink staining (6).

Southern blot analysis. Chromosomal DNA was digested, electrophoresed in an agarose gel, stained, and blotted as described previously (1, 5). pA818, which contains the cloned chondroitin lyase II gene, was labeled with ^{32}P by nick translation (18) and hybridized with the DNA on the nitrocellulose paper for 48 h at 42°C in a hybridization solution that contained 50% formamide (16). After hybridization, the blots were washed twice for 30 min each with 0.2% SDS in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and twice with 0.2% SDS in 0.5× SSC at 58°C. The filters were then autoradiographed.

Chondroitin lyase assay. Strains of *B. thetaiotaomicron* were grown in 100 ml of defined media with 5 mg of chondroitin sulfate III per ml and 10 μg of erythromycin per ml. Crude extracts from disrupted bacteria were assayed for chondroitin lyase activity by measurement of the increase in A_{235} as a result of the production of unsaturated disaccharides that absorb at this wavelength (20). Protein concentration was determined by the method of Lowry et al. (15).

RESULTS

Construction of pE3-1. A method for the transformation of *B. fragilis* has been reported (24), but this method does not work for *B. thetaiotaomicron*. We therefore had to introduce the suicide vector into *B. thetaiotaomicron* by conjugation. Construction of a mobilizable suicide vector, pE3-1, is shown in Fig. 1. pB8-51, a cryptic plasmid which was originally isolated from *B. eggerthii* B8-51 (23), is cotransferred in conjugative crosses by R751 (22). pB8-51 is stably maintained in several *Bacteroides* species, but the *Mbo*I site that was used to insert pB8-51 into pBR328 interrupts a region on the plasmid that is critical for stability in *Bacteroides* species (see below). The *E. coli* cloning vector pBR328 (25) is maintained in *E. coli* but not in *Bacteroides* species. In addition, none of the markers carried on pBR328 express in *Bacteroides* species (4). The *Eco*RI D fragment from pBF4 was included in pE3-1 to furnish a drug resistance marker (Em^r) that is expressed in *Bacteroides* species (23). The *Eco*RI D fragment also contains a tetracycline resistance gene which is not expressed in *Bacteroides* species and is expressed only in aerobically grown *E. coli* strains (3). This tetracycline resistance is designated $*Tc^r$ to distinguish it from other Tc^r. R751 mobilizes pE3-1 from *E. coli* to *E. coli* at a frequency of 10^{-5} (22). We did not detect transfer of pE3-1 to *B. thetaiotaomicron* ($<10^{-9}$), as expected if the *Mbo*I site interrupts a region of pB8-51 which is required for the stability of the plasmid in *Bacteroides* species.

Insertional mutagenesis. A 0.8-kilobase (kb) *Hin*cII fragment from the center of the chondroitin lyase II gene was inserted into the *Pst*I site of pE3-1 with *Pst*I linkers (Fig. 2). The resulting plasmid, pEG817, was mobilized by R751 into *B. thetaiotaomicron* 5482, and Em^r Gen^r transconjugants were selected. Em^r Gen^r transconjugants should be mutants that have pEG817 inserted in the chondroitin lyase II gene

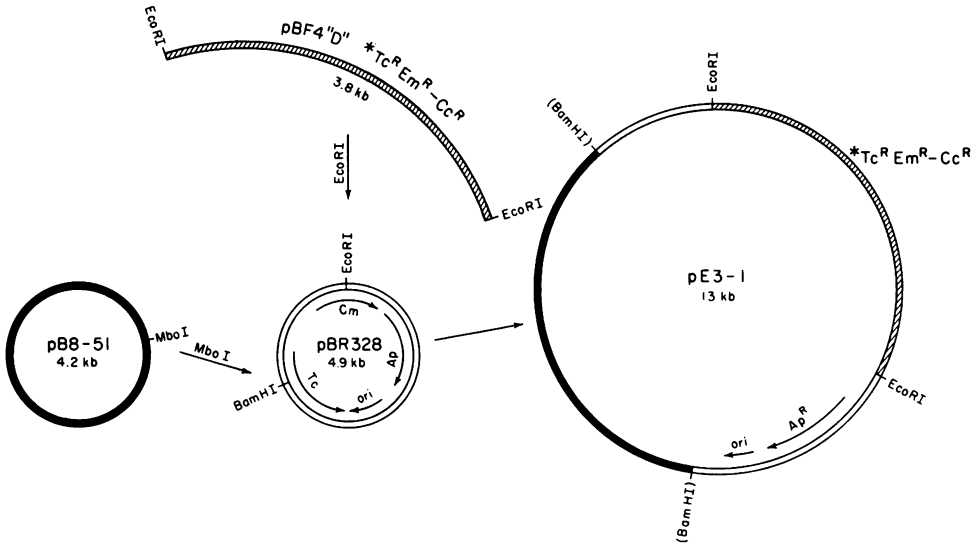

FIG. 1. Construction of pE3-1. The cryptic *Bacteroides* plasmid pB8-51 (represented by the solid region) was cloned into the *Bam*HI site, and the 3.8-kb *Eco*RI D fragment of pBF4 (represented by the hatch-marked region) was cloned into the *Eco*RI site of pBR328. The resultant 13-kb chimeric plasmid (pE3-1) has Ap^r and *Tc^r determinants that are expressed in *E. coli* and an Em^r-Cc^r determinant that is expressed in *Bacteroides* species. It can be mobilized from *E. coli* to *Bacteroides* species, but is not maintained in *Bacteroides* species.

by homologous recombination. Twenty-nine Em^r transconjugants were obtained from the same mating, and four were chosen for characterization.

Characterization of Em^r isolates. To confirm that the Em^r phenotype in the *B. thetaiotaomicron* transconjugants was not due to maintenance of pEG817 as a plasmid, four transconjugants were checked for plasmids. The only plasmid that was visible in any of the strains was a 40-kb cryptic plasmid, p5482, that is also found in wild-type *B. thetaiotaomicron* 5482 (data not shown). To confirm that pEG817 had inserted in the chromosome, chromosomal DNA was isolated from three of the four transconjugants, digested with *Eco*RI or *Bam*HI, and hybridized on a Southern blot with ^{32}P-labeled pA818, a plasmid which contains the entire chondroitin lyase II gene (5) (Fig. 2). The pattern seen on the Southern blot was consistent with the pattern that would be expected if pEG817 was inserted into the chondroitin lyase II gene (Fig. 3). *Bam*HI does not cut pEG817 or the segment cloned in pA818. Thus the lanes containing DNA from the transconjugants have a single band which cross-reacts with pA818 and which is 13.2 kb larger than the *Bam*HI fragment in the wild type owing to insertion of pEG817 into the chondroitin lyase II gene. *Eco*RI cuts twice within pEG817, but does not cut within the segment cloned in pA818 (Fig. 2). Thus there should be three *Eco*RI fragments in the mutant but only one in the wild type. Only two of the three *Eco*RI

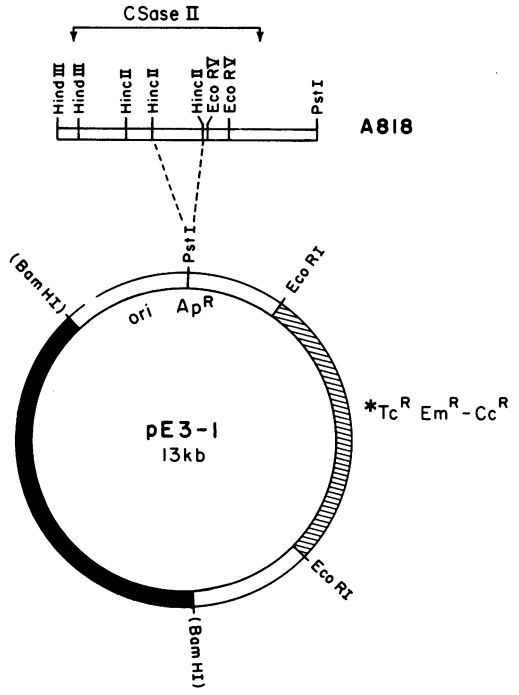

FIG. 2. Construction of pEG817. A 0.8-kb *Hinc*II fragment from the center of the chondroitin lyase II gene (CSase II) contained on a portion of pA818 was inserted into the *Pst*I site of pE3-1 by using *Pst*I linkers. The shading of pE3-1 is the same as that described in the legend to Fig. 1.

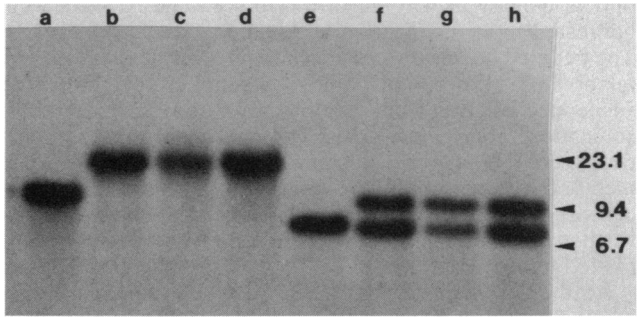

FIG. 3. Autoradiogram of a Southern blot of *Bam*HI- (lanes a through d) and *Eco*RI- (lanes e through h) digested chromosomal DNA from *B. thetaiotaomicron* wild type (lanes a and e) and three different transconjugants, 2 (lanes b and f), 3 (lanes c and g), and 4 (lanes d and h). ^{32}P-labeled pA818 was used as the hybridization probe. Arrows to the right of the autoradiogram indicate the migration distances of *Hin*dIII-digested lambda DNA in kilobases.

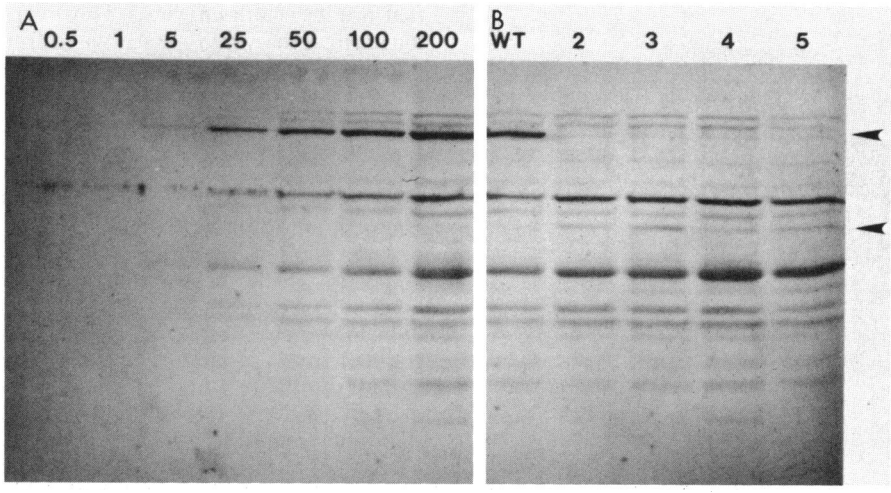

FIG. 4. Western blot of crude cell lysates from *B. thetaiotaomicron* loaded on a 10% SDS-polyacrylamide gel. After electrophoresis and transfer of the proteins, the nitrocellulose paper was incubated with antiserum raised against chondroitin lyase II. (A) Sensitivity of the procedure. Crude cell lysate (0.5 to 200 μg; the amount of protein in micrograms is shown at the top of the photograph) from wild-type *B. thetaiotaomicron* was loaded on the SDS-polyacrylamide gel. Chondroitin lyase II, indicated by the top arrow on the right, could be detected in as little as 5 μg of crude lysate protein. (B) Crude lysate (100 μg) from *B. thetaiotaomicron* wild type (lane WT) and four transconjugants, 2 (lane 2), 3 (lane 3), 4 (lane 4), and 5 (lane 5) were loaded on the SDS-polyacrylamide gel. No chondroitin lyase II was detected (top arrow) in any of the transconjugants. However, one cross-reacting band (lower arrow), possibly a truncated chondroitin lyase II, was observed in the transconjugants but was not present in the wild type.

fragments in the mutants cross-hybridize with pA818 on the Southern blot, because the 3.8-kb *Eco*RI fragment which lies within pE3-1 (Fig. 1) and which carries the Em^r and $*Tc^r$ genes has no homology with pA818. Two cross-hybridizing *Eco*RI fragments of the predicted size were seen in lanes containing *Eco*RI digests of DNA from the mutant (Fig. 3).

Since it appeared that pEG817 had inserted into the chondroitin lyase II gene, the four transconjugants were tested for the ability to grow on defined medium with chondroitin sulfate as the sole carbon source. All four were able to grow on chondroitin sulfate. The final optical density of these cultures was the same as that of the wild type.

To confirm that intact chondroitin lyase II was not being produced in the mutant, mutant and wild-type strains were grown in medium containing chondroitin sulfate to induce synthesis of chondroitin lyase II. Then Western blots of crude extracts from these cultures were incubated with polyclonal antiserum that detects chondroitin lyase II. Chondroitin lyase II was visible in the wild type, but no chondroitin lyase II was detected in any of the four Em^r transconjugants (Fig. 4). In Fig. 4B, 100 μg of protein was loaded on each well for SDS-polyacrylamide gel electrophoresis. We found that the immunoblot procedure was able to detect chondroitin lyase II in wild-type cell extracts if as little as 5 μg of protein was loaded on the gel (Fig. 4A). Therefore, production of chondroitin lyase II in the mutant was at least 20-fold lower than in wild-type *B. thetaiotaomicron* cells. In addition, chondroitin lyase specific activity in cell extracts from the mutant was 20 to 40% of the chondroitin lyase specific activity in cell extracts from the wild type.

Growth rates. The growth rate of the mutant in defined medium containing chondroitin sulfate but no erythromycin was similar to the growth rate of the wild type in this medium (data not shown). However, if the mutant was first grown in glucose defined medium and then inoculated into chondroitin sulfate defined medium, it took 2 to 4 h longer for it to reach the same optical density as that of the wild type (Fig. 5).

Normal growth after a lag period could be due to reversion of the mutant in the absence of erythromycin selection. To test the stability of the insertional mutation in the absence of erythromycin selection, the mutant was inoculated into medium containing glucose or chondroitin sulfate and allowed to grow to stationary phase. It was then reinoculated into the same type of broth medium, grown to mid-exponential phase, and plated on plates made with the same medium. A total of 2,000 colonies picked from the plates which contained glucose and 2,000 colonies picked from the plates that contained chondroitin sulfate were tested for resistance to erythromycin. None of them were Em^s. Thus,

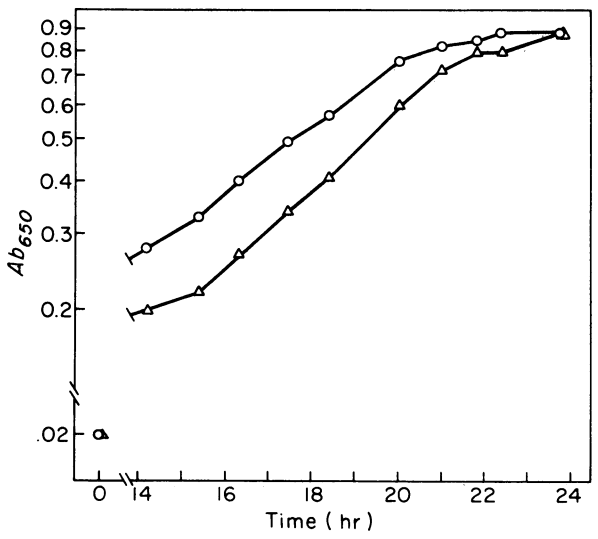

FIG. 5. Growth of *B. thetaiotaomicron* wild-type (○) and mutant (△) strains on chondroitin sulfate defined medium after being grown on glucose defined medium. The mutant was consistently slower to reach the same level of absorbance than was the wild type in repeated experiments. The curve shown for the mutant is an average of several different experiments.

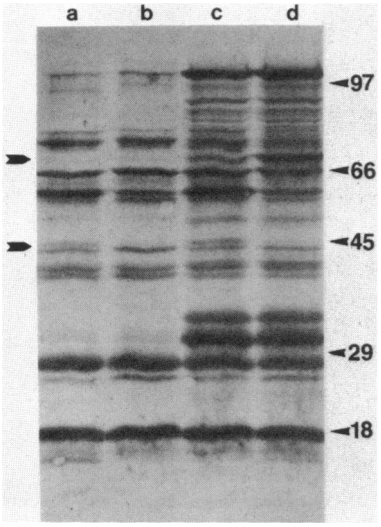

FIG. 6. Western blot of crude membranes isolated from *B. thetaiotaomicron* wild-type (lanes a and c) and mutant (lanes b and d) strains grown on glucose (lanes a and b) or chondroitin sulfate (lanes c and d) defined media. Approximately 60 μg of protein was loaded in each lane of an 8 to 15% gradient SDS-polyacrylamide gel. The antiserum was raised against purified outer membrane from *B. thetaiotaomicron* chondroitin sulfate-grown cells and was cross-absorbed with outer membranes from glucose grown cells. Arrows on the right indicate the migration distance of molecular weight standards in kilodaltons. Arrows on the left indicate differences consistently observed in the peptide patterns.

the organisms that grew on chondroitin sulfate were not revertants.

Effect of the insertion on synthesis of outer membrane polypeptides. If the chondroitin lyase II gene is part of an operon, expression of downstream genes should be affected by an insertional mutation. Many components of the chondroitin sulfate-degrading system have not yet been characterized, but it is known that there are outer membrane polypeptides that are coregulated with the chondroitin lyases (9). To determine whether levels of any of these outer membrane polypeptides were affected in the mutant, membrane polypeptides from the mutant and from the wild type were compared on a Western blot by using a cross-absorbed antiserum that detects most of the chondroitin sulfate-associated outer membrane polypeptides (9). This cross-absorbed antiserum also detects some constitutively produced polypeptides, as seen in the lanes which contain membranes from bacteria grown on glucose (Fig. 6, lanes a and b). In the mutant, a 45-kilodalton (kDa) polypeptide was missing and a 70-kDa polypeptide was enhanced (Fig. 6). These differences were seen in repeated Western blots. The 70-kDa polypeptide, but not the 45-kDa polypeptide, is associated with growth on chondroitin sulfate. None of the other major chondroitin sulfate-associated polypeptides were affected.

DISCUSSION

We have shown that a mutant of *B. thetaiotaomicron* which does not produce active chondroitin lyase II can still grow on chondroitin sulfate. Clearly, chondroitin lyase II is not essential for the utilization of chondroitin sulfate. The fact that the mutant did not revert at high frequency when grown on chondroitin sulfate without erythromycin selection indicates that there was not a strong selection for revertants that produce both enzymes. However, the mutant did take longer than the wild type to switch from utilizing glucose to utilizing chondroitin sulfate, even though the growth rate of the mutant appeared to be approximately the same as that of the wild type once they started growing. Although the contribution of chondroitin lyase II to the growth of *B. thetaiotaomicron* appears to be small under the conditions used in these experiments, i.e., substrate excess and no competition, the contribution of chondroitin lyase II could be more important if the organisms were growing in the colon, in which substrate concentration is probably quite low and competition for a limited nutrient pool is present.

In an earlier report on the purification of chondroitin lyase I and II from *B. thetaiotaomicron*, the total activity in the chondroitin lyase I fractions was about one-half that in the chondroitin lyase II fractions (12). From this, it appeared that only one-third of the chondroitin lyase activity in extracts of *B. thetaiotaomicron* was due to chondroitin lyase I. However, it was possible that chondroitin lyase I was more sensitive than chondroitin lyase II to some purification step. The results of the present study confirm that chondroitin lyase I accounts for only 20 to 40% of the total chondroitin lyase activity of *B. thetaiotaomicron*. Apparently, the loss of over half of the total chondroitin lyase activity of the organism does not significantly affect growth on chondroitin sulfate.

The insertion of the suicide vector into the chondroitin lyase II gene should be polar on any genes which are downstream from chondroitin lyase II part of the same operon. Aside from a 70-kDa outer membrane polypeptide, which was enhanced in the mutant, none of the chondroitin sulfate-associated outer membrane polypeptides was affected. Thus, we can conclude tentatively that none of the genes that code for these proteins are downstream of chondroitin lyase II in the same transcriptional unit. In addition to the two chondroitin lyases and the 8 to 10 outer membrane proteins which are coregulated with the chondroitin lyases, several inner membrane proteins as well as one or more sulfatases and glucuronidases are induced during growth on chondroitin sulfate (20). None of these proteins has been characterized in enough detail to permit us to determine whether they are absent in the mutant. Since the mutant was able to grow on chondroitin sulfate, enough sulfatase and glucuronidase activity was being produced to degrade the disaccharide products of chondroitin lyase I. However, there could be multiple forms of these enzymes. If so, the loss of one might not be easily detectable.

The results of the experiments reported here illustrate the utility of pE3-1. Our results show that pE3-1 is mobilized into *B. thetaiotaomicron* at sufficiently high frequencies to be useful for making insertional mutations in *Bacteroides* species. The approach we used is the only feasible one for creating the type of mutations reported here, which do not have an easily detectable phenotype or for which the phenotype is unknown. The fact that such mutations can now be made in *Bacteroides* species makes any cloned gene accessible to mutation. Moreover, although at present there is no method for genetic mapping in *Bacteroides* species, our results show that local mapping based on insertional mutations could be done. pE3-1 could also be used to locate and clone *Bacteroides* genes that are not expressed in *E. coli* Small fragments of *Bacteroides* DNA could be cloned into pE3-1, followed by mobilization of the resulting plasmids into *Bacteroides* species. This would generate a variety of insertions. The desired insertion could be located by screening *Bacteroides* transconjugants. Since pE3-1 contains a

replication origin which is recognized in *E. coli*, regions of the DNA adjacent to the insertion could be retrieved by self-cloning and used as hybridization probes to locate the intact gene in a clone bank.

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