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Evidence that the *Bacteroides thetaiotaomicron* Chondroitin Lyase II Gene Is Adjacent to the Chondro-4-Sulfatase Gene and May Be Part of the Same Operon

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The chondroitin lyase II gene from *Bacteroides thetaiotaomicron* has previously been cloned in *Escherichia coli* on a 7.8-kilobase (kb) fragment (pA818). In *E. coli*, the chondroitin lyase II gene appeared to be expressed from a promoter that was about 0.5 kb from the beginning of the gene. However, when a subcloned 5-kb fragment from pA818 which contained the chondroitin lyase II gene and the promoter from which the gene is expressed in *E. coli* was introduced into *B. thetaiotaomicron* on a multicopy plasmid (pEG800), the chondroitin lyase specific activity of *B. thetaiotaomicron* was not altered. Further evidence that the promoter that is recognized in *E. coli* may not be the promoter from which the chondroitin lyase II gene is transcribed in *B. thetaiotaomicron* was obtained by making an insertion in the *B. thetaiotaomicron* chromosome at a point which is 1 kb upstream from the chondroitin lyase II gene. This insertion stopped synthesis of the chondroitin lyase II gene product, as would be predicted if the gene was part of an operon and was transcribed in *B. thetaiotaomicron* from a promoter that was at least 1 kb upstream from the chondroitin lyase II gene. A region of pA818 which was adjacent to the chondroitin lyase II gene and which included the region used to make the insertional mutation was found to code for chondro-4-sulfatase, an enzyme that breaks down one of the products of the chondroitin lyase reaction. The upstream insertion mutant of *B. thetaiotaomicron* which stopped synthesis of chondroitin lyase II had no detectable chondro-4-sulfatase activity. This mutant was still able to grow on chondroitin sulfate, although the rate of growth was slower than that of the wild type.

Bacteroides thetaiotaomicron, an obligate anaerobe that is found in high concentrations in the human colon, can ferment a variety of complex polysaccharides including the mucopolysaccharide chondroitin sulfate (15). The steps in chondroitin sulfate breakdown by *B. thetaiotaomicron* are shown in Fig. 1. Chondroitin sulfate has two isomers, one which is sulfated at the C-6 position of the *N*-acetylgalactosamine residue (chondroitin 6-sulfate) and one which is sulfated at the C-4 position of this residue (chondroitin 4-sulfate). In both cases, the chondroitin sulfate molecule is first broken into sulfated disaccharides (ΔDi-4S for chondroitin 4-sulfate and ΔDi-6S for chondroitin 6-sulfate) by a β-eliminative cleavage of the bond next to the uronic acid residue (chondroitin lyase [EC 4.2.2.4]). The disaccharides are then desulfated (chondro-4 or 6-sulfatase [EC 3.1.6.4]) and finally hydrolyzed by a β-glucuronidase to produce *N*-acetylgalactosamine and Δ4,5-glucuronic acid.

B. thetaiotaomicron has two chondroitin lyases, chondroitin lyase I (108,000 daltons [Da]) and chondroitin lyase II (104,000 Da) (8). Both of these enzymes are soluble and cell associated and both can degrade chondroitin 4-sulfate, chondroitin 6-sulfate, and desulfated chondroitin sulfate (8). The sulfatase and glucuronidase activities are easily detectable in crude cell extracts from *B. thetaiotaomicron* (14), but none of these enzymes has been isolated and characterized. Thus, it is not known whether a single enzyme is responsible for the chondro-4-sulfatase and chondro-6-sulfatase activities or whether multiple enzymes are involved. Similar questions could be raised about the glucuronidase activity.

The enzyme activities shown in Fig. 1 are at least 30-fold higher when *B. thetaiotaomicron* is grown on chondroitin sulfate than when the bacteria are grown on monosaccharide components of chondroitin sulfate (13, 14). In addition to the degradative enzymes, there are outer membrane proteins that are associated with growth on chondroitin sulfate (6). The function of these outer membrane proteins is not known, but they could be involved in bringing chondroitin sulfate through the *Bacteroides* outer membrane. Since the genes that code for the degradative enzymes and the outer membrane proteins all appear to be coregulated, it seems reasonable to expect that they might be organized on the *Bacteroides* chromosome into one or more operons. However, owing to the lack of any method for genetic mapping of the *B. thetaiotaomicron* chromosome, no information has been obtained previously about the organization of these or any other *Bacteroides* genes. Recently, the gene for chondroitin lyase II was cloned (4). Results of γδ insertional mutagenesis of the cloned region demonstrated that the promoter which directs transcription of the *Bacteroides* chondroitin lyase II gene in *Escherichia coli* was located immediately adjacent to the gene. This finding indicated that there are no upstream genes in the same operon. Moreover, when insertional mutagenesis was used to interrupt the chondroitin lyase II gene in *B. thetaiotaomicron*, no evidence was found to suggest that this mutation affected the expression of any of the other chondroitin sulfate-associated genes in *B. thetaiotaomicron* (3). Since this insertional mutation should have been polar on downstream genes, it appears that none of the known chondroitin sulfate-associated genes are downstream of the chondroitin lyase II gene.

Although these findings seem to indicate that the chondroitin lyase II gene is not part of an operon, they do not rule out this possibility. In particular, the promoter from which the chondroitin lyase II gene is expressed in *E. coli*

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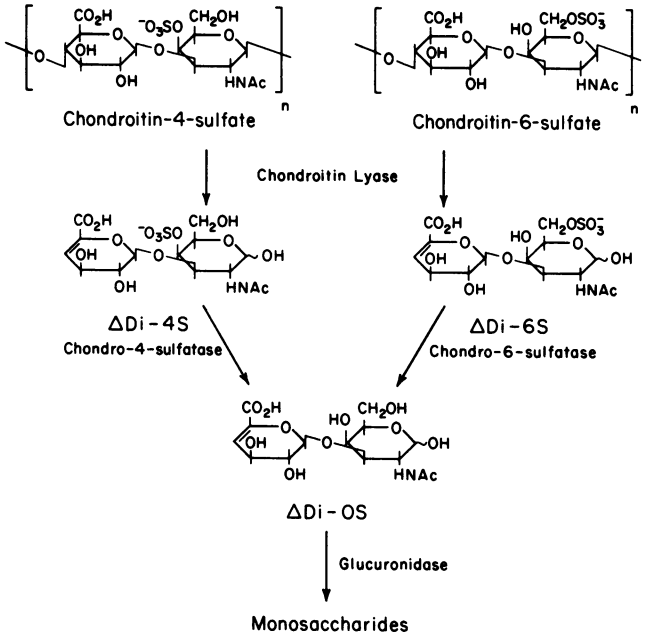

FIG. 1. Enzymes involved in bacterial breakdown of the two isomers of chondroitin sulfate to monosaccharides. HNAc, *N*-Acetyl.

might not be the promoter from which the gene is expressed in *B. thetaiotaomicron*. In this report, we present evidence that the promoter from which the chondroitin lyase II gene is expressed in *B. thetaiotaomicron* may not be adjacent to the gene and that the gene which codes for chondro-4-sulfatase is adjacent to the chondroitin lyase II gene on the *Bacteroides* chromosome and may be part of the same operon.

MATERIALS AND METHODS

Bacterial strains and media. Bacterial strains and plasmids used are listed in Table 1. *B. thetaiotaomicron* 5482 was grown in prereduced Trypticase (BBL Microbiology Systems, Cockeysville, Md.)-yeast extract-glucose broth (TYG) (5) or in defined medium containing chondroitin sulfate type III (5 mg/ml) or glucose (5 mg/ml) as the carbon source (14). When chondroitin sulfate A and chondroitin sulfate C were used in growth experiments for mutant analysis, they were filter sterilized and added to the media after autoclaving. The atmosphere used was 80% N_2–20% CO_2. *E. coli* EM24 or HB101 (11) was grown in Luria broth or on Luria plates (11). Concentrations for the antibiotics used for selection were as follows: ampicillin, 50 μg/ml; erythromycin or clindamycin, 10 to 20 μg/ml; geneticin, also known as G418, 400 μg/ml; nalidixic acid, 100 μg/ml; tetracycline, 10 to 20 μg/ml.

Plasmid isolation, analysis, and construction. Plasmids were isolated from *E. coli* and *B. thetaiotaomicron* strains by the Ish-Horowitz modification of the method of Birnboim and Doly (11). Standard procedures were followed in steps involving restriction endonuclease, calf intestinal phospha-

TABLE 1. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Relevant characteristics[a]	Construction or source
Plasmids		
pA818	Ap^r Tc^r; contains the structural gene for chondroitin lyase II	7.8-kb fragment of *B. thetaiotaomicron* DNA cloned into pBR328 (4)
pE5-2	Em^r Tc^r Su^r; maintained as a multicopy plasmid in *E. coli* and in *Bacteroides* spp.	17
pEG800	Em^r Tc^r; contains the structural gene for chondroitin lyase II; maintained as a multicopy plasmid in *E. coli* and in *Bacteroides* spp.	5-kb *Pst*I fragment from pA818 cloned into pE5-2 (Fig. 2); this study
R751	IncPβ, Tp^r Tra$^+$; mobilizes pE5-2 and pE3-1 from *E. coli* to *Bacteroides* spp.	3, 17
pE3-1	Em^r Tc^r Ap^r; suicide vector that is maintained as a multicopy plasmid in *E. coli* but not in *Bacteroides* spp.	3
pEG815	Em^r Tc^r; suicide vector used for insertional mutagenesis	1.6-kb *Pst*I fragment from pA818 which lies upstream of the chondroitin lyase II gene cloned into pE3-1 (Fig. 3) (this study)
pEG817	Em^r Tc^r; suicide vector used for insertional mutagenesis	0.6-kb *Pst*I fragment from pA818 which lies within the chondroitin lyase II gene (Fig. 5) (4)
Bacterial strains		
E. coli EM24	RecA$^-$ Str^r Gn^s	J. Cronan; RecA$^-$ Str^r derivative of LE392 (13)
B. thetaiotaomicron 5482	Em^s Gn^r	4
E. coli EM24(pEG800)	RecA$^-$ Str^r Gn^s Tc^r	Transformant of *E. coli* EM24 which contained pEG800 (this study)
E. coli EM24(R751-pEG800)	RecA$^-$ Str^r Gn^s Tp^r Tc^r	Transconjugant of mating which introduced R751 into *E. coli* EM24(pEG800) (this study)
B. thetaiotaomicron 5482(pEG800)	Em^r Gn^r	Transconjugant of mating with *E. coli* EM24 (R751-pEG800) (this study)
B. thetaiotaomicron 5482 (Ω817)	Em^r Gn^r	Insertion of pEG817 into the chondroitin lyase II gene (4)
B. thetaiotaomicron 5482 (Ω815)	Em^r Gn^r	Insertion of pEG815 upstream of the chondroitin lyase II gene (this study)

[a] Abbreviations used for antibiotic resistances or sensitivities: ampicillin, Ap; erythromycin, Em; geneticin (also known as G418), Gn; sulfonamide, Su; tetracycline, Tc; trimethoprim, Tp; and streptomycin, Str. Concentrations used to select for antibiotic resistances are given in Materials and Methods. Other abbreviations used: Tra, ability to self-transfer; Inc, plasmid incompatibility group; Rec, *E. coli* recombination mutation.

tase, and T4 DNA ligase (11). Restriction endonuclease digestions were loaded on a 1% agarose slab gel 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* and transformation of *E. coli* with plasmid DNA were performed as described by Lederberg and Cohen (7).

Bacterial matings. Donors (*E. coli*) and recipients (*B. thetaiotaomicron*) were grown separately to the 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 microcentrifuge (1 min), and the bacterial pellet was suspended in 0.1 ml of TYG broth. Donors and recipients were cospotted on nitrocellulose filters placed on TYG agar plates. The mating mixtures were incubated under aerobic conditions at 37°C for 16 h (16). After incubation, the cells were suspended in TYG by vortexing and were plated on TYG agar plates that contained various drugs.

Southern blot analysis. Chromosomal DNA was isolated from *B. thetaiotaomicron* as described previously (3) and digested with restriction enzymes by standard procedures (11). The DNA from agarose gels was transferred to nitrocellulose paper by capillary blotting (11). DNA probes were labeled with ^{32}P by nick translation (12) and hybridized to the DNA on the nitrocellulose paper for 48 h at 42°C in a hybridization solution which contained 50% formamide (11). After hybridization, the filter-paper blots were washed twice for 30 min each time with 0.2% sodium dodecyl sulfate in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and twice with 0.2% sodium dodecyl sulfate in 0.5× SSC. The filters were then autoradiographed.

Western blot analysis. Crude cell extracts and crude membrane preparations of *B. thetaiotaomicron* were prepared, and proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described previously (3). The proteins were then transferred onto nitrocellulose sheets by the procedure of Erickson et al. (1). Blocking of the nitrocellulose sheets and detection of the bound protein with antibody were done as described previously (3). Antiserum against chondroitin lyase II was obtained as described previously (4). The immunoglobulin G fraction was prepared by the procedure of Lopatin and Voss (9).

Chondroitin lyase assay. *B. thetaiotaomicron* strains were grown in 100 ml of defined medium containing 5 mg of chondroitin sulfate III (or glucose) per ml as the carbon source. Medium used for erythromycin-resistant (Em^r) insertion mutants of *B. thetaiotaomicron* also contained 10 μg of erythromycin per ml. Crude extracts from disrupted bacteria were assayed for chondroitin lyase activity by measuring the increase in A_{235} due to the production of unsaturated disaccharides that absorb at this wavelength (13, 16). Protein concentration was determined by the method of Lowry et al. (10).

Sulfatase and glucuronidase assays. To determine whether pA818 carries a gene that codes for glucuronidase, chondro-4-sulfatase, or chondro-6-sulfatase and, if so, to localize the gene within the cloned region, *E. coli* HB101 (carrying pA818 or pA818 with various γδ insertions) was grown overnight in L broth with 10 μg of tetracycline per ml. Bacteria were harvested by centrifugation (17,000 × *g*, 10 min, 4°C), washed once and resuspended in 5 ml of 50 mM potassium phosphate buffer (pH 7.2), and disrupted by sonication (6 min, 50% pulse). Disrupted bacteria were centrifuged for 10 min at 17,000 × *g* (4°C) to remove cell debris, and the supernatant fluid (crude cell extract) was assayed for enzyme activity.

Crude cell extract (60 to 80 μg of protein) was mixed with 30 μg of one of the following dissacharides: ΔDi-0S (glucuronidase assay), ΔDi-4S (chondro-4-sulfatase assay), or ΔDi-6S (chondro-6-sulfatase assay), all in 50 mM potassium phosphate buffer (pH 7.2). The mixture was incubated at 37°C for 2 h and then placed in boiling water for 2 min to stop the reaction and finally centrifuged in a microcentrifuge for 3 min to pellet insoluble material. For comparison, an identical mixture was boiled immediately after mixing without being incubated at 37°C.

The supernatant fluid (20 μl) was spotted on Whatman no. 1 paper, and the component sugars were separated by descending paper chromatography (14) in acetic acid–*n*-butanol–1 M NH_3OH (3:2:1, vol/vol/vol). Chromatograms were air dried, sprayed with 0.1 M *p*-anisidine–0.1 M phthalic acid in 95% ethanol, and heated at 100°C to visualize disaccharides and monosaccharide (14). Disaccharides and *N*-acetylgalactosamine in the incubation mixtures were identified by comigration with authentic standards and by the color of the spot (brown for ΔDi-4S and *N*-acetylgalactosamine, yellow for ΔDi-0S and ΔDi-6S).

Chondro-4-sulfatase activity in *B. thetaiotaomicron* 5482, *B. thetaiotaomicron* 5482 (Ω817), and *B. thetaiotaomicron* 5482 (Ω815) was measured as described above for *E. coli*, except that the bacteria were grown in 100 ml of defined medium containing chondroitin sulfate. The amount of cell protein in the reaction mixture was 20 μg, and incubations lasted 20, 40, 60, and 120 min.

Chemicals. Unless otherwise indicated, chondroitin sulfate III (Sigma Chemical Co., St. Louis, Mo.) was used as the carbohydrate source in all growth media. This grade of chondroitin sulfate contains approximately equal amounts of chondroitin 4-sulfate and chondroitin 6-sulfate (13). Chondroitin sulfate A (Sigma) was used as a substrate for chondroitin lyase assays and as a carbohydrate source in one growth experiment. This preparation of chondroitin sulfate contains approximately 75% chondroitin 4-sulfate and 25% chondroitin 6-sulfate (13). In one growth experiment, chondroitin sulfate C (Sigma) was used as the carbohydrate source. This preparation contains approximately 90% chondroitin 6-sulfate and 10% chondroitin 4-sulfate (8). The disaccharides ΔDi-0S [3-*O*-(β-D-Δ4,5-glucopyranosyl)-*N*-acetylgalactosamine], ΔDi-4S [3-*O*-(β-D-Δ4,5-glucopyranosyl)-*N*-acetylgalactosamine 4-sulfate], and ΔDi-6S [3-*O*-(β-D-Δ4,5-glucopyranosyl)-*N*-acetylgalactosamine 6-sulfate], which were used as substrates for the sulfatase and glucuronidase assays and as authentic standards on the chromatograms, were obtained from ICN Immunobiochemicals, Lisle, Ill.

RESULTS

Construction of pEG800. Previously, the *B. thetaiotaomicron* chondroitin lyase II gene had been localized to a 3-kilobase (kb) region of a 7.8-kb cloned DNA segment (pA818; Fig. 2). From the results of subcloning experiments and γδ insertion mutagenesis of the 7.8-kb region, it was clear that the promoter that was being recognized in *E. coli* was within the cloned fragment and adjacent to the chondroitin lyase II gene (4). To determine whether the promoter that was being recognized in *E. coli* was the same as the regulated promoter from which the chondroitin lyase II gene is expressed in *B. thetaiotaomicron*, we cloned a 5-kb *Pst*I fragment from pA818 which contained the chondroitin lyase II gene plus about 0.5 kb of DNA on either side into the *E. coli-Bacteroides* shuttle vector pE5-2 (Fig.

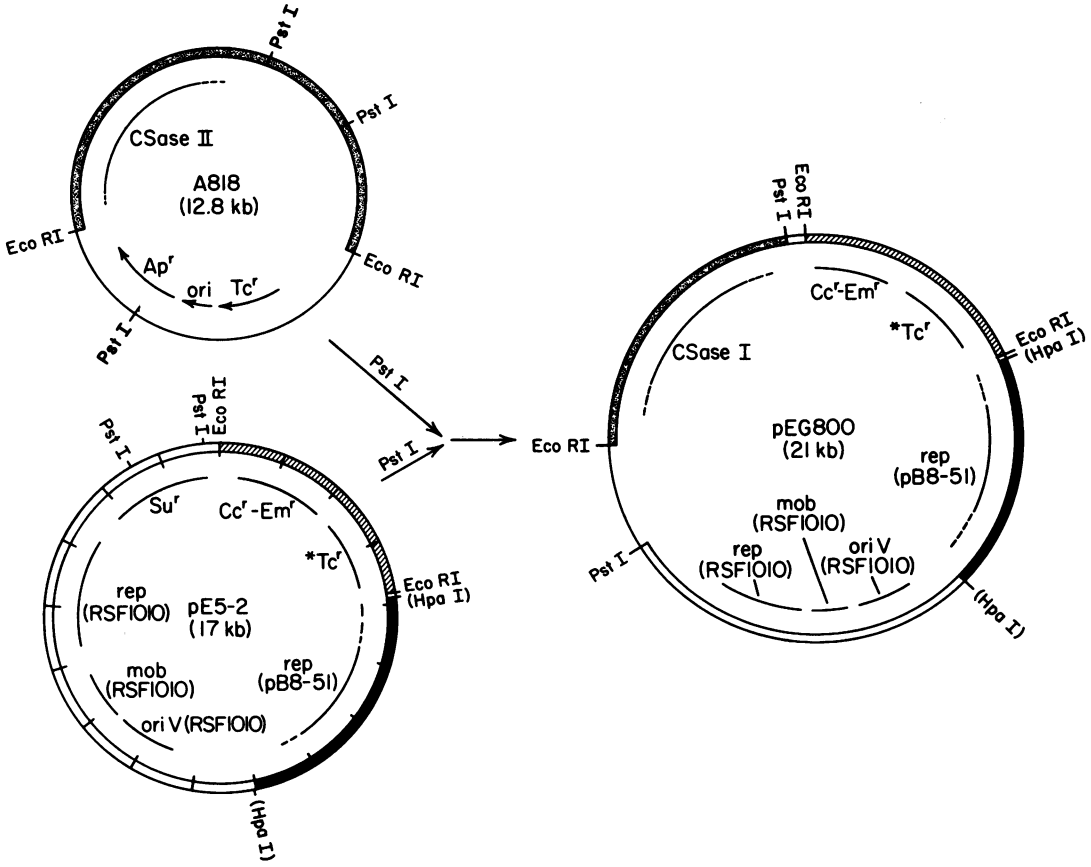

FIG. 2. Construction of pEG800. A 5-kb *Pst*I fragment from pA818 (denoted by the bold-face *Pst*I sites) which carried the cloned chondroitin lyase II gene (CSase II) was inserted into the two *Pst*I sites of pE5-2. The resultant 21-kb plasmid carries a Tc^r determinant that confers resistance to tetracycline on *E. coli* and a Cc^r Em^r determinant that confers resistance to clindamycin and erythromycin on *Bacteroides* spp. pEG800 is stably maintained as a plasmid in both *E. coli* and *Bacteroides* spp. and can be mobilized from *E. coli* to *Bacteroides* spp. by the broad-host-range IncP plasmid R751.

2). The resulting plasmid, pEG800, was introduced into *E. coli* EM24 by transformation. The crude cell extract from the resulting transformant, *E. coli* EM24(pEG800), was positive for chondroitin lyase activity (Table 2). Thus, the cloned *Pst*I fragment contained the entire chondroitin lyase II gene plus the promoter from which the gene was transcribed by *E. coli*. pEG800 was mobilized from *E. coli* EM24 to *B. thetaiotaomicron* 5482 by the conjugative plasmid R751. Em^r *B. thetaiotaomicron* transconjugants contained a multicopy plasmid that had the same molecular weight and restriction pattern as pEG800.

TABLE 2. Effect of pEG800 on the chondroitin lyase specific activity of *B. thetaiotaomicron*

Species and strain	Plasmid	Sp act (nmol/min/mg of protein)[a]	
		Uninduced	Induced
B. thetaiotaomicron 5482	None	25	2,000
B. thetaiotaomicron 5482	pE5-2	12	1,700
B. thetaiotaomicron 5482	pEG800	14	1,900
E. coli EM24	None	<3	NA[b]
E. coli EM24	pEG800	22	NA

[a] Uninduced, Grown on glucose; induced, grown on chondroitin sulfate.
[b] NA, Not applicable. *E. coli* EM24 does not utilize chondroitin sulfate.

When *B. thetaiotaomicron* 5482(pEG800) was grown on chondroitin sulfate, the chondroitin lyase specific activity in crude cell extracts was the same as the specific activity in crude cell extracts from *B. thetaiotaomicron* 5482 which did not contain pEG800 (Table 2). When the bacteria were grown on glucose instead of chondroitin sulfate, chondroitin lyase specific activity in extracts from *B. thetaiotaomicron* 5482(pEG800) was somewhat lower than the specific activity in extracts from *B. thetaiotaomicron* 5482 with no plasmid. This difference appeared to be an effect of the vector rather than of the cloned chondroitin lyase II gene because specific activity in extracts from *B. thetaiotaomicron* 5482(pE5-2) was lower than in extracts from *B. thetaiotaomicron* 5482 with no plasmid (Table 2). Since pEG800 did not affect the chondroitin lyase specific activity of *B. thetaiotaomicron* it appeared that the chondroitin lyase II gene is not being expressed in *B. thetaiotaomicron* from pEG800.

Insertional mutagenesis of the region upstream of the chondroitin lyase II gene. If the chondroitin lyase II gene was not expressed in *B. thetaiotaomicron* 5482 from a promoter that is next to the gene but rather was part of an operon in which it was not the first gene, an insertion which was upstream from the chondroitin lyase II gene in the same operon should not only stop synthesis of the upstream gene but should also interrupt the transcript and thus stop synthesis of the chondroitin lyase II gene. To test this, we

cloned a 1.6-kb *Pst*I fragment which was located 1 kb from the end of the chondroitin lyase II gene on pA818 into the suicide vector pE3-1 to obtain pEG815 (Fig. 3). pE3-1 is maintained in *E. coli* and can be mobilized from *E. coli* to *Bacteroides* spp. by R751, but it is not maintained as a plasmid in *Bacteroides* spp. (3). pEG815 (erthromycin resistant [Em^r]) was mobilized into *B. thetaiotaomicron* 5482 by R751, with selection for Em^r. Em^r transconjugants should have pEG815 inserted in the *B. thetaiotaomicron* 5482 chromosome by homologous recombination involving the cloned region.

To confirm that pEG815 had been inserted into the chromosome in the region corresponding to the cloned *Pst*I fragment in pEG815, we did a Southern blot analysis of chromosomal DNA from six Em^r transconjugants. When the chromosomal DNA was digested with *Eco*RI (which cuts at either end of the 7.8-kb region in pA818) or with *Sal*I (which does not cut within this 7.8-kb region) and then blotted and probed with ^{32}P-labeled pA818, the sizes of the restriction fragments that hybridized with the probe were those expected if insertion of pEG815 had occurred within the cloned *Pst*I fragment (data not shown).

Effect of Ω815 insertion. To determine whether the insertion of pEG815 into the *B. thetaiotaomicron* chromosome

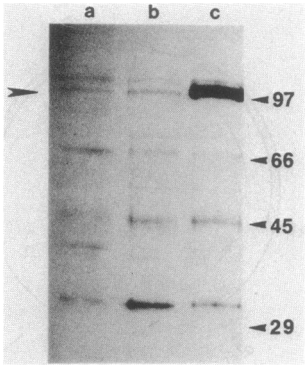

FIG. 4. Western blot of electrophoretically separated cell extracts from *B. thetaiotaomicron*. After electrophoresis and transfer of the proteins, the nitrocellulose paper was incubated with antiserum raised against chondroitin lyase II. Crude cell extracts (100 μg of protein) from *B. thetaiotaomicron* wild type (lane c), a mutant of *B. thetaiotaomicron* created by insertion of pEG815 into the chromosome (lane b), and a mutant of *B. thetaiotaomicron* created by insertion of pEG817 into the chromosome (lane a) were loaded on the gel. The migration distance of chondroitin lyase II is marked by an arrowhead on the left side of the figure. Arrowheads on the right side of the figure indicate migration distances of molecular size standards in kilodaltons.

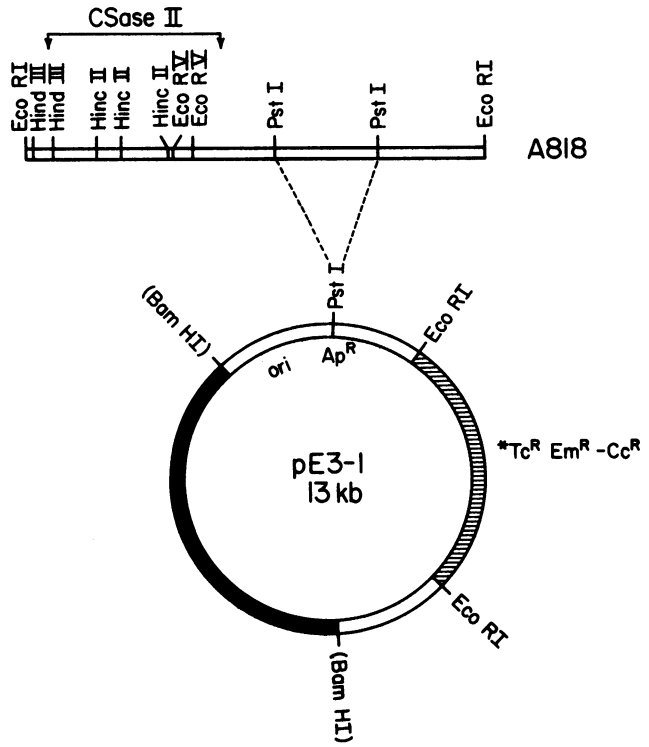

FIG. 3. Construction of pEG815. A 1.6-kb *Pst*I fragment from a region approximately 1 kb away from the chondroitin lyase II gene (CSase II) on pA818 was inserted into the *Pst*I site of pE3-1. The resultant 14.6-kb plasmid has a Tc^r determinant that is expressed in *E. coli* and an Em^r Cc^r determinant that is expressed in *Bacteroides* spp. The solid region on pE3-1 indicates the region which was derived from the cryptic *Bacteroides* plasmid pB8-51 (3). The hatchmarked region is a 3.8-kb *Eco*RI fragment from the *Bacteroides fragilis* plasmid pBF4 which carries the Tc^r and Cc^r Em^r genes. The remaining open area on the pE3-1 map indicates regions of the plasmid which are derived from pBR328. pEG815 is maintained as a plasmid in *E. coli* but not in *Bacteroides* spp. (3).

(designated Ω815) had interrupted a gene that was important for growth on chondroitin sulfate, we compared growth on chondroitin sulfate of the pEG815 insertion mutant [*B. thetaiotaomicron* 5482 (Ω815)] with that of the wild type (*B. thetaiotaomicron* 5482) and of a mutant containing an insertion in the chondroitin lyase II gene [*B. thetaiotaomicron* 5482 (Ω817)]. The doubling time of the Ω815 insertion mutant (5 h) was slower than that of the wild type or the Ω817 insertion mutant (1.5 to 2 h). The lag time was also much longer for Ω815 (14 h) than for the wild type or Ω817 (4 h). All three strains reached the same final optical density (650 nm) of 1.0. The effect on growth of the Ω815 insertion was specific to chondroitin sulfate. When glucose was the substrate, growth of the Ω815 insertion mutant was identical to that of the wild type and the Ω817 insertion mutant. The Ω815 insertion mutant also grew normally on *N*-acetylgalactosamine and glucuronic acid, the two monosaccharide components of chondroitin sulfate. Thus, the Ω815 insertion appeared to interrupt a gene that was important for growth on chondroitin sulfate but was not essential.

To determine whether the Ω815 insertion stopped the synthesis of the chondroitin lyase II gene, we compared the chondroitin lyase specific activity in crude cell extracts from *B. thetaiotaomicron* 5482 (Ω815) with that in extracts from the wild type and from *B. thetaiotaomicron* 5482 (Ω817). The chondroitin lyase specific activity of the Ω815 mutant was the same as that found with the Ω817 mutant (insertion interrupting the chondroitin lyase II gene), i.e., 20 to 30% of that found with the wild type.

To confirm that the lower chondroitin lyase specific activity in Ω815 was due to loss of chondroitin lyase II activity, we grew the Ω815 insertion mutant, the Ω817 insertion mutant, and the wild type in medium containing chondroitin sulfate to induce synthesis of chondroitin lyase. Then Western blots of crude extracts from these cultures were incubated with polyclonal antiserum that detects chondroitin lyase II. Chondroitin lyase II was clearly visible in the wild type, but no chondroitin lyase II was detected in the Ω815

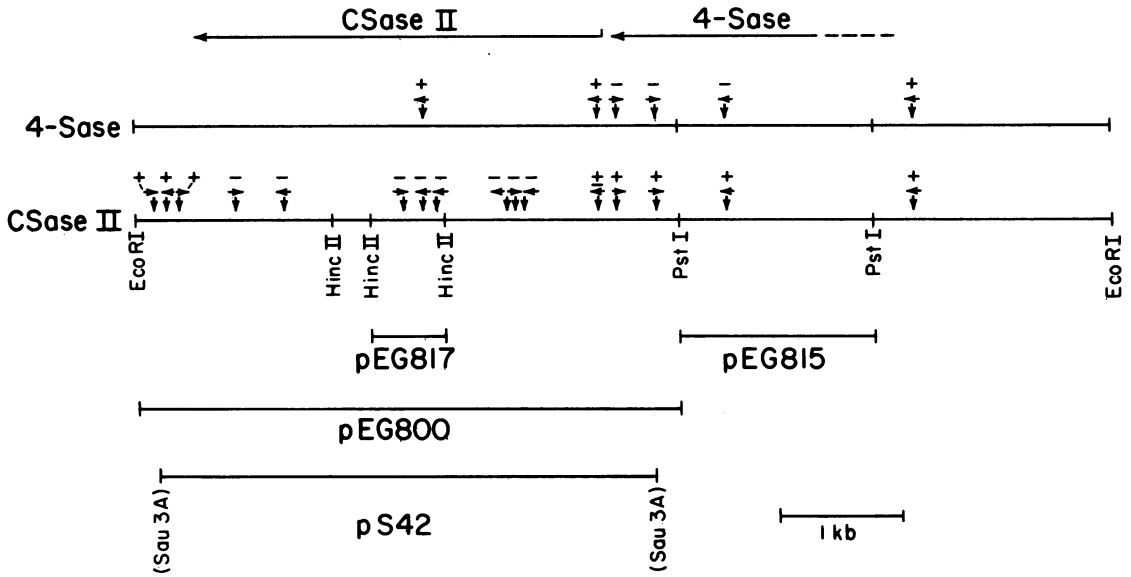

FIG. 5. Location and effect of γδ insertions in the 7.8-kb cloned region from pA818. The top line indicates the regions required for the production of active chondroitin lyase II (CSase II) and for the production of active chondro-4-sulfatase (4-Sase). The second line shows the effect of different γδ insertions on the production of chondro-4-sulfatase. For comparison, the effect of these insertions on production chondroitin lyase activity, taken from a previous study (4), is also shown. Both pS42 and pEG800 express chondroitin lyase II activity in *E. coli*. The horizontal arrows indicate the orientation of the insertion, and the vertical arrows indicate the site of the insertion. The + or − symbols above the arrows indicate whether or not there was any enzyme activity detected from these insertions. The lower lines indicate the fragments of pA818 which have been cloned to create the different plasmids used in this study. A more complete map of the cloned region is also given in Guthrie et al. (4).

mutant (Fig. 4). A lightly staining band that comigrated with chondroitin lyase in the lane containing crude cell extract from the Ω815 mutant was not chondroitin lyase II because this same band appeared in the lane containing crude extract from the Ω817 mutant in which the chondroitin lyase II gene is interrupted.

Characterization of the upstream gene. Slow growth and a long lag period in medium containing chondroitin sulfate but normal growth in medium containing the monosaccharide components of chondroitin sulfate was the sort of effect that would be expected if the Ω815 insertion interrupted a gene that coded for one of the enzymes that degrade the disaccharide products of the chondroitin lyases, i.e., chondro-4-sulfatase, chondro-6-sulfatase, or glucuronidase. To determine whether one of these genes was upstream from the chondroitin lyase II gene, we first tested *E. coli* EM24(pA818) for the ability to alter ΔDi-0S (glucuronidase activity) or one of the sulfated disaccharides, ΔDi-4S and ΔDi-6S (sulfatase activity). A crude cell extract from *E. coli* EM24 had no effect on any of these disaccharides. A crude cell extract from *E. coli* EM24(pA818) had no effect on ΔDi-0S or on ΔDi-6S but did convert ΔDi-4S to ΔDi-0S. Thus, pA818 carries a gene from *B. thetaiotaomicron* which codes for chondro-4-sulfatase and which is expressed in *E. coli*.

To confirm that the chondro-4-sulfatase gene on pA818 was located next to the chondroitin lyase II gene, we tested various γδ insertions in pA818 for chondro-4-sulfatase activity. The results of this experiment, summarized in Fig. 5, demonstrated that the chondro-4-sulfatase gene lies immediately adjacent to the chondroitin lyase II gene and in the same region as the *Pst*I fragment that was used to make the Ω815 insertion mutation.

The effect of the Ω815 insertion on sulfatase and glucuronidase activities in *B. thetaiotaomicron* was determined by comparing these activities in a crude cell extract from the Ω815 insertion mutant with sulfatase and glucuronidase activities in crude cell extracts from the wild type and the Ω817 insertion mutant. Extracts from the wild type and the Ω817 insertion mutant degraded ΔD-6S, ΔDi-4S, and ΔDi-0S to monosaccharides within 20 min. The extract from the Ω815 insertion mutant degraded ΔDi-6S and ΔDi-0S to monosaccharides within 20 min but had no effect on ΔDi-4S, even when the incubation time was as long as 2 h. Thus, the Δ815 insertion abolished chondro-4-sulfatase activity in *B. thetaiotaomicron* 5482 but had no effect on chondro-6-sulfatase and glucuronidase activity.

Since the Ω815 insertion mutant appeared to be unable to break down ΔDi-4S, it should be able to grow on chondroitin 6-sulfate but unable to grow on chondroitin 4-sulfate. The fact that the final optical density of the Ω815 mutant when grown on chondroitin sulfate III was about the same as that of the wild type could have been due to the fact that although chondroitin sulfate III is only half chondroitin 6-sulfate, 2.5 mg of chondroitin 6-sulfate per ml could have been enough to allow maximum growth yield. If the Ω815 mutant has no chondro-4-sulfatase activity, a difference in growth yield should be seen if the Ω815 mutant was grown on lower amounts (2 mg/ml) of chondroitin sulfate A (25% chondroitin 6-sulfate) and of chondroitin sulfate C (75% chondroitin 6-sulfate). When Ω815 was grown on defined medium containing 2 mg of chondroitin sulfate A or chondroitin sulfate C per ml, Ω815 grew slower on chondroitin sulfate A than on chondroitin sulfate C (about 12 h longer lag period), but the final growth yields on both chondroitin sulfate A and chondroitin sulfate C were identical (optical density at 650 nm of 0.4). From the final optical density, it is clear that chondroitin sulfate was limiting in this experiment and thus that the Ω815 mutant must be able to utilize ΔDi-4S from chondroitin 4-sulfate breakdown.

DISCUSSION

The simplest interpretation of the finding that an insertion in the chondro-4-sulfatase gene was polar on the chondroitinase II gene which is located immediately adjacent to it on the *Bacteroides* chromosome is that both genes are part of an operon which is transcribed from a promoter located somewhere upstream of the chondro-4-sulfatase gene. However, this evidence is not conclusive. Further work is needed to locate the putative *Bacteroides* promoter and to demonstrate that a transcript of the appropriate size is made in *Bacteroides* species. Since the two genes can be transcribed independently in *E. coli* (Fig. 5), it is clear that regions of the DNA adjacent to the genes can function as promoters, at least in *E. coli*. These promoters are very weak, as can be seen by the fact that the activity of the chondroitinase II produced in *E. coli* from genes cloned on multicopy plasmid pA818 or pEG800 (20 nmol/min per mg of protein) is approximately equal to the uninduced activity owing to one copy of the gene in the *Bacteroides* chromosome. Although the sulfatase activity was not quantitated, the sulfatase activity expressed from the multicopy plasmid pA818 in *E. coli* also appeared to be comparable to the uninduced level owing to the single copy of the gene in *Bacteroides* species. Nonetheless, these promoter regions could have some function in *Bacteroides* species even if they are not the promoters responsible for inducible expression of these genes.

Another possible explanation of the finding that the insertion in the chondro-4-sulfatase gene eliminates production of detectable amounts of chondroitinase II gene product in *Bacteroides* species is that the chondro-4-sulfatase forms a complex with the chondroitinase II that protects the chondroitinase II from degradation. This possibility cannot be completely ruled out. However, there is no evidence for such a complex in *Bacteroides* species. In fact, the chondroitinase II can be separated by standard chromatographic procedures from the sulfatase activity (8) and appears to be located in a different cellular compartment (14).

The effect of the insertion in the chondro-4-sulfatase gene on cellular sulfatase activity and on growth of *B. thetaiotaomicron* on chondroitin sulfate provides new information about the number and role of the sulfatases. First, the sulfatase cloned on pA818 clearly has an absolute specificity for the 4-sulfated disaccharide and has no activity on the 6-sulfated disaccharide or the unsulfated disaccharide. Second, the results of γδ insertional mutagenesis taken together with the size of the cloned region used to make the Ω815 insertion indicate that the monomeric molecular size of the chondro-4-sulfatase is at least 50 kDa. Previously, we had tried to detect the gene products encoded by pA818 in extracts from maxicells that contained pA818 and in extracts from an *E. coli* transcription translation system in which pA818 and pS42, a subclone of pA818, were used as templates. A 57-kDa polypeptide was observed in both the maxicell extracts and in the S-30 extracts when pA818 was the template, but not in the S-30 extract obtained with pS42 as a template (3; E. P. Guthrie, unpublished data). We concluded that this polypeptide could not be the chondroitin lyase II since pS42 coded for chondroitin lyase II activity and since the protein was the wrong size. It is possible that the 57-kDa polypeptide is the chondro-4-sulfatase. If the 57-kDa polypeptide is the *B. thetaiotaomicron* chondro-4-sulfatase, the end of the *Pst*I fragment in pEG815 which is farthest upstream from the chondroitin lyase II gene must lie very close to one end of the gene (Fig. 5).

Third, loss of chondro-4-sulfatase activity has a profound effect on the ability of *B. thetaiotaomicron* to grow on chondroitin sulfate. However, loss of this activity does not completely abolish growth on this substrate. The fact that no chondro-4-sulfatase activity was detectable in crude cell extracts from the Ω815 mutant seems to indicate that there is only one chondro-4-sulfatase. However, the ability of the Ω815 mutant to attain a high turbidity on a limiting concentration of chondroitin sulfate A, which is predominantly chondroitin 4-sulfate, supports the hypothesis that there is a second form of the enzyme. If there is a second chondro-4-sulfatase in *B. thetaiotaomicron*, its activity is not detectable by using the assay conditions that detect the enzyme which is encoded by the gene on pA818. Also, it is not as efficient at degrading ΔDi-4S in the intact cell. Alternatively, *B. thetaiotaomicron* may have some other pathway for utilizing ΔDi-4S from the breakdown of chondroitin 4-sulfate.

Although chondroitin lyase II can degrade both chondroitin 4-sulfate and chondroitin 6-sulfate, it has a slightly higher activity and a lower K_m on chondroitin 4-sulfate than on chondroitin 6-sulfate (8). Chondroitin lyase I, by contrast, has a higher activity on chondroitin 6-sulfate than on chondroitin 4-sulfate (8). Thus, the operon which contains the chondroitin lyase II and the chondro-4-sulfatase seems to be adapted for preferential use of the 4-sulfate rather than the 6-sulfate form of chondroitin sulfate. The chondroitin lyase I and chondro-6-sulfatase genes have not yet been located in *B. thetaiotaomicron*. If they are in a separate operon, this could give *B. thetaiotaomicron* the ability to induce one or the other preferentially in response to a predominance of 4-sulfate or 6-sulfate isomers. In nature, chondroitin sulfate molecules generally contain both 4-sulfate and 6-sulfate regions (2), but one isomer usually predominates.

To date, no Hfr strains or transducing phages of *Bacteroides* species which could be used for genetic mapping of the *Bacteroides* chromosome have been isolated. However, the approach used in this study can yield information about chromosomal organization of genes if one or more of the genes has been cloned.

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