A Locus That Contributes to Colonization of the Intestinal Tract by *Bacteroides thetaiotaomicron* Contains a Single Regulatory Gene (*chuR*) That Links Two Polysaccharide Utilization Pathways

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Previously, we isolated two Tn4351-generated mutants of *Bacteroides thetaiotaomicron* (46-1 and CS3) that were unable to grow either on heparin or on chondroitin sulfate. This phenotype was unexpected, since the heparin and chondroitin sulfate utilization pathways had appeared from earlier studies to be independent of each other. Mutants 46-1 and CS3 were also of interest because both were unable to compete successfully with wild-type *B. thetaiotaomicron* in the intestinal tracts of germfree mice. Thus, both appeared to have a colonization defect. We have now cloned the chromosomal locus in which the transposon insertions in 46-1 and CS3 occurred. Southern blot analysis showed that the Tn4351 insertions in 46-1 and CS3 were about 100 bp apart. Using complementation and insertional mutagenesis, we localized the region affected by the 46-1 and CS3 insertions to within 2.5 kbp. This DNA segment was sequenced and found to contain a 401-codon open reading frame (ORF1) and the N-terminal segment of a second open reading frame (ORF2), which was downstream of ORF1 and transcribed in the same direction. The deduced amino acid sequence of ORF1 showed significant homology to that of a putative positive regulator of an arylsulfatase gene in *Klebsiella aerogenes*. ORF2 was at least 381 amino acids long and did not exhibit homology to any proteins in the data bases searched. Transposon insertions in both mutants 46-1 and CS3 disrupted ORF1. The results of insertional mutagenesis and complementation experiments indicated that ORF2 was not essential for growth on chondroitin sulfate or heparin. Thus, the chondroitin sulfate-negative and heparin-negative phenotypes of 46-1 and CS3 appear to be due to the interruption of a regulatory gene encoded by ORF1 and not to a polar effect of the insertions on a downstream gene(s). The gene encoding ORF1 has been designated *chuR*, for regulation of chondroitin sulfate and heparin utilization. Transcriptional fusion studies showed that the expression of *chuR* occurred at the same level under inducing and noninducing conditions, in contrast to the regulated expression of structural genes of the chondroitin sulfate utilization system. *chuR* was not autoregulated, nor was its expression affected by a mutation (46-4) that eliminated the expression of all chondroitin sulfate utilization genes but did not affect the utilization of heparin.

Bacteroides thetaiotaomicron, a gram-negative anaerobe found in large numbers in the human colon (18), can utilize a variety of dietary and host-produced polysaccharides. Dietary polysaccharides are clearly a major source of carbohydrate for colonic bacteria (24), but it was not clear whether host polysaccharides, such as mucins and mucopolysaccharides, were also important substrates for colonic bacteria. To test the importance of mucopolysaccharides as carbon and energy sources for *B. thetaiotaomicron* growing in the intestinal tract, we tested the ability of several Tn4351-generated mutants of *B. thetaiotaomicron*, which is unable to utilize the mucopolysaccharide chondroitin sulfate (CS), to compete with the wild type for the colonization of intestinal tracts of germfree mice. Two of these mutants (46-1 and CS3) exhibited a decreased ability to colonize the intestinal tracts of germfree mice (8a, 26). However, the colonization defect associated with these mutations could not have been due simply to the inability to utilize CS, because other mutants of *B. thetaiotaomicron* that were unable to utilize CS had no such colonization defect (26).

Mutants 46-1 and CS3 differed from the colonization-proficient CS-utilizing mutants in that they were also unable to utilize the mucopolysaccharide heparin (8a, 9). This phenotype was surprising, because although heparin and CS

have some structural similarities, heparin does not induce the expression of CS utilization genes. Because of this fact, it had been assumed that the CS and heparin utilization systems were independent of each other. However, the fact that the utilization of both polysaccharides was affected by a single transposon insertion suggested that there was some connection between the two systems. In addition to being unable to utilize heparin, both 46-1 and CS3 exhibited aberrant expression of several CS-associated proteins (8a, 9). In both mutants, chondro-6-sulfatase, an enzyme involved in CS breakdown, was no longer produced. Four CS-regulated cytoplasmic proteins of unknown function were also missing. One membrane protein, whose expression was regulated in the wild type, was now produced constitutively in 46-1 and CS3, and another membrane protein was overexpressed.

Since the expression of so many genes was affected by a single transposon insertion and since both 46-1 and CS3 had the same phenotypes, it was possible that the transposon insertions in 46-1 and CS3 had disrupted the same regulatory locus. If so, this locus controls only a subset of the genes that are induced during growth on CS and presumably controls some genes that are necessary for heparin utilization as well. Alternatively, the transposon insertions in 46-1 and CS3 might have interrupted an operon that contained most or all of the affected genes. In this paper, we report the cloning and sequencing of the region that was interrupted by

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics ^a	Description or source
Strains		
<i>E. coli</i>		
DH5 α	RecA Gn ^s	Hanahan (7)
DH5 α MCR	RecA Gn ^s	Hanahan (7)
S17-1	RecA (Tp ^r) (Sm ^r) Gn ^s ; RP4 inserted in chromosome	Simon et al. (30)
<i>B. thetaiotaomicron</i>		
5482	Wild type; Gn ^r	Anaerobe Laboratory, Virginia Polytechnic Institute, Blacksburg
BT4001	Rif ^r Gn ^r Em ^s	Spontaneous Rif ^r mutant of <i>B. thetaiotaomicron</i> 5482
Δ CSaseII	Rif ^r Gn ^r Em ^s CSaseII ⁻	Chromosomal deletion in the CSaseII gene in 5482 (22)
46-1	Rif ^r Gn ^r Em ^r CS ⁻ HA ⁺ HP ⁻	Tn4351 mutagenesis of 5482 (26)
46-4	Rif ^r Gn ^r Em ^r CS ⁻ HA ⁻ HP ⁺	Tn4351 mutagenesis of 5482 (26)
CS3	Rif ^r Gn ^r Em ^r CSaseII ⁻ CS ⁻ HA ⁺ HP ⁻	Tn4351 mutagenesis of Δ CSaseII; this study
Plasmids		
pMJF-3	(Ap ^r) Tc ^r	GUS fusion shuttle vector used to make multicopy GUS fusions (3)
pCQW-1	(Ap ^r) Em ^r	GUS fusion suicide vector used to make chromosomal GUS fusions (3)
pBT-1	(Kn ^r) Tc ^r	Suicide vector used to make gene disruptions (32)
pBT-2	(Kn ^r) Tc ^r	pBT-1 containing the α -complementing fragment of <i>lacZ</i> (32)
R751	(Tp ^r) (Tra ⁺)	IncP plasmid used to mobilize vectors from <i>E. coli</i> to <i>Bacteroides</i> recipients (29)
pJRD215	(Kn ^r Sm ^r)	RSF1010-based cosmid vector (2)
pNJR-12	(Kn ^r) Tc ^r	pJRD215-derived shuttle vector used for complementation experiments (31)
pCS3B	(Kn ^r Tc ^r)	pJRD215 containing the 3.4-kbp <i>tetX</i> portion of Tn4351 and a 1.7-kbp flanking chromosomal fragment from CS3; this study
pJH9-1	(Kn ^r)	pJRD215 containing an 11-kbp <i>Hind</i> III chromosomal fragment from wild-type <i>B. thetaiotaomicron</i> that spanned the transposon insertion site in CS3; this study
pNCS1	(Kn ^r) Tc ^r	pNJR-12 containing the 3.5-kbp <i>Eco</i> RI fragment from pJH9-1; this study
pNCS2	(Kn ^r) Tc ^r	pNJR-12 containing the 2.8-kbp complementing <i>Cla</i> I fragment from pJH9-1; this study
pSQW-6	(Ap ^r) Tc ^r	pMJF-3 containing the 1.6-kbp <i>Hind</i> III- <i>Nsi</i> I fragment from pJH9-1; this study
pSQW-7	(Ap ^r) Tc ^r	pMJF-3 containing the 1.2-kbp <i>Eco</i> RI- <i>Cla</i> I fragment from pJH9-1; this study
pCQW-14	(Ap ^r) Em ^r	pCQW-1 containing the same <i>Eco</i> RI- <i>Cla</i> I fragment in the same orientation as pSQW-7; this study

^a Abbreviations: Gn, gentamicin; Tp, trimethoprim; Sm, streptomycin; Rif, rifampin; Em, erythromycin; Ap, ampicillin; Tc, tetracycline; Kn, kanamycin; CSaseII, chondroitin lyase II; HP, heparin; HA, hyaluronic acid. Designations in parentheses are for genes expressed only in *E. coli*; others are for genes expressed in *B. thetaiotaomicron*.

the transposon insertions in 46-1 and CS3. We show that the gene affected by these insertions encodes a protein that shares amino acid homology with a regulatory protein from *Klebsiella aerogenes*.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. Three Tn4351-generated mutants were used in this study. Two of them, 46-1 and 46-4, have already been described (26). The third mutant, CS3, was obtained by mutagenizing *B. thetaiotaomicron* Δ CSaseII (22) as previously described (26, 28). Two separate mutagenesis experiments with strain Δ CSaseII yielded 7,000 to 8,000 Em^r transposon insertion mutants. Three of the Em^r mutants were unable to grow on CS but were still able to grow on the CS component sugars glucuronic acid and *N*-acetylgalactosamine. All three mutants (CS3, CS4, and CS11) grew as well as parent strain Δ CSaseII and the wild type on unrelated polysaccharides, such as polygalacturonic acid and arabinogalactan. CS3, like 46-1,

was unable to grow on heparin as well as on CS. CS4 and CS11 were able to grow on heparin. Since CS3 had a phenotype similar to that of 46-1, it was further analyzed by the same procedures as those used to analyze 46-1 (9, 26). These included an assay of CS utilization enzymes, a two-dimensional gel analysis of CS-associated proteins, and competition of CS3 with the wild type in germfree mice. The enzyme activities, two-dimensional protein profiles, and the results of the competition experiments were identical to those reported previously for 46-1, except that the chondroitinase specific activity was one-third that of the wild type (data not shown).

Growth conditions. *B. thetaiotaomicron* strains were grown anaerobically at 37°C. In most experiments, bacterial strains were grown on prerduced Trypticase-yeast extract-glucose (8) broth or agar. In some experiments, bacterial strains were grown in a defined medium that contained one of the following as the sole carbon source: CS A (5 mg/ml), hyaluronic acid (3 mg/ml), heparin (4 mg/ml), polygalacturonic acid (3 mg/ml), arabinogalactan (3 mg/ml), glucose (5

mg/ml), glucuronic acid (2 mg/ml), or *N*-acetylgalactosamine (2 mg/ml). All carbohydrates were obtained from Sigma Chemical Co. (St. Louis, Mo.). The gas phase of the liquid medium was 80% N₂-20% CO₂. The agar plates were incubated in GasPak jars (BBL Microbiology Systems, Cockeysville, Md.). *Escherichia coli* strains were grown in Luria broth or on Luria agar aerobically at 37°C. The concentrations of antibiotics used for selection were as follows: erythromycin, 10 µg/ml; gentamicin sulfate, 200 µg/ml; trimethoprim, 200 µg/ml; tetracycline, 10 µg/ml; ampicillin, 100 µg/ml; and kanamycin monosulfate, 100 µg/ml.

DNA manipulation. Plasmids were isolated from *E. coli* and *Bacteroides* strains by the Ish-Horowitz modification of the method of Birnboim and Doly (17). Chromosomal DNA from *Bacteroides* strains was isolated by the procedure reported by Saito and Miura (21). Fractionation of chromosomal DNA fragments was performed by differential sucrose gradient centrifugation (17). Restriction endonuclease digestion and ligation with T4 DNA ligase were carried out in accordance with the manufacturer's instructions (Bethesda Research Laboratories, Inc., Gaithersburg, Md., or New England BioLabs, Inc., Beverly, Mass.).

Transformation and conjugation. Preparation of competent *E. coli* strains and transformation of *E. coli* with plasmid DNA were performed as described by Lederberg and Cohen (14). When suicide vector pCQW-1 (3), containing the *E. coli* β-glucuronidase (*GUS*) gene (*uidA*) as a reporter group, was used for cloning, *E. coli* S17-1 (30), which contains a copy of *Tra*⁺ plasmid RP4 in its chromosome, was transformed with the recombinant plasmids, and transformants were used as donors in matings with *B. thetaiotaomicron* recipients. RP4 can mobilize pCQW-1 derivatives because pCQW-1 contains *RK2 oriT* (5). Other plasmids and their derivatives were introduced into *E. coli* DH5α by transformation and were introduced from *E. coli* to *Bacteroides* strains by broad-host-range IncP plasmid R751 (29). Matings were performed as previously described (29).

Southern hybridization. Chromosomal DNA was isolated from *B. thetaiotaomicron* strains and digested with restriction endonucleases. The digested DNA was run on a 1% agarose gel and transferred to nitrocellulose paper by capillary blotting (17). Nick translation of probes with [³²P]dCTP and hybridization and washing of the filters were done as previously described (28). Colony hybridization (22) was used to screen for clones of wild-type *B. thetaiotaomicron* DNA that hybridized to DNA flanking the site of the transposon insertion in CS3.

Cloning of the wild-type chromosomal DNA segment that contained the transposon insertion sites in CS3 and 46-1. Southern hybridization experiments with plasmid R751 and Tn4351 as the hybridization probes indicated that CS3 contained a tandem duplication of Tn4351 and that R751 had not formed a cointegant (28) with Tn4351 (data not shown). The results of these experiments also indicated that the Tn4351 insertion in CS3 was close to the site of insertion in 46-1 (see Results). A two-step cloning procedure was used to clone the wild-type locus that was interrupted by the transposon insertions in CS3 and 46-1. In the first step, we took advantage of a *tetX* gene carried on Tn4351 expressed in *E. coli* (4). A unique *Hind*III site within Tn4351 cuts the transposon into two segments, the larger of which (3.4 kbp) carries the intact *tetX* gene (Fig. 1). Southern analysis of DNA from CS3 and 46-1, with *tetX* as the probe, showed that the *tetX*-containing *Hind*III fragment from CS3 was only about 5 kbp, whereas the *tetX*-containing *Hind*III fragment from 46-1 was larger than 12 kbp. The smaller *Hind*III

FIG. 1. Locations and orientations of the transposon insertions in mutants CS3 and 46-1. The position of the Tn4351 insertions in the cloned 11-kbp segment is indicated by the vertical arrow. The heavy horizontal arrows indicate the direct repeat insertion sequences (IS4351) that flank Tn4351. Tn4351 carries a clindamycin-erythromycin resistance gene (*ermF*), which is expressed only in *Bacteroides* strains, and a tetracycline resistance gene (*tetX*), which is expressed only in aerobically grown *E. coli* strains. Abbreviations: H, *Hind*III; C, *Cla*I; RI, *Eco*RI; RV, *Eco*RV.

fragment, from CS3, was chosen for cloning. DNA from CS3 was digested with *Hind*III and size fractionated as described by Maniatis et al. (17). The fraction containing the 5-kbp segments was ligated to the unique *Hind*III site in pJRD215 (2) and transformed into *E. coli* DH5αMCR with selection for Tc^r transformants. Five Tc^r transformants were obtained, all of which carried a *Hind*III insert of 5.1 kbp. Since the transposon segment of the clone was 3.4 kbp in size, the flanking chromosomal DNA was approximately 1.7 kbp in size. The Tc^r plasmid with the 5.1-kbp insert was designated pCS3B. To obtain the wild-type locus, we probed *Hind*III-digested chromosomal DNA from wild-type *B. thetaiotaomicron* on Southern blots with pCS3B. This analysis showed that an 11-kbp *Hind*III segment cross-hybridized with the probe. *Hind*III-digested DNA was then size fractionated on a sucrose gradient, and the fraction containing the 11-kbp segments was ligated to the *Hind*III site in pJRD215. The resulting plasmids were transformed into *E. coli* DH5αMCR with selection for Kn^r transformants. The 1.7-kbp *Hind*III-*Eco*RI segment of chromosomal DNA from pCS3B was used as a probe in colony hybridization experiments to screen the transformants. Six of the 800 colonies screened cross-reacted with the probe. Plasmids from these isolates were digested with *Hind*III and *Eco*RI, and a Southern blot was probed with a ³²P-labelled chromosomal junction segment. Four of the six had a 1.7-kbp *Hind*III-*Eco*RI fragment that hybridized to the probe. Three of the four shared the same restriction pattern but carried an extra *Hind*III fragment and were thus discarded. The final clone, designated pJH9-1, carried a single *Hind*III insert approximately 11 kbp in size.

Gene disruption and complementation experiments. To construct gene disruptions in the cloned wild-type region, we cloned small segments covering different parts of the 11-kbp *Hind*III fragment into suicide vector pCQW-1 (3). For some

experiments, more recently constructed suicide vector pBT-1 or pBT-2 (32) was used because it has a higher transfer frequency than pCQW-1. These constructs were introduced into wild-type *B. thetaiotaomicron* with selection for Em^r or Tc^r , depending on the vector used. The transconjugants were checked for the ability to grow on CS, heparin, hyaluronic acid, and glucose.

For complementation experiments, different fragments that spanned the transposon insertion region on the 11-kbp *Hind*III fragment were subcloned on low-copy-number shuttle vector pNJR-12 and introduced into CS3 and 46-1 with selection for Tc^r . The transconjugants were tested for the ability to complement the phenotypes of mutant CS3 or 46-1.

Sequence analysis. The DNA sequence was determined by the M13 dideoxynucleotide chain termination method (27). Sequencing reactions were performed with a Sequenase 2.0 kit purchased from United States Biochemicals, Inc. (Cleveland, Ohio). The sequencing reaction products were resolved on 6% polyacrylamide-8 M urea denaturing gels. Gels were run at 40 mA (50 to 55°C), fixed, dried, and exposed to X-ray films. The deduced amino acid sequence was used to search for homologous polypeptides in the GenBank data base (20).

Insertional disruption of *chuR* and construction of a *chuR* 46-4 double mutant. A 600-bp internal fragment of *chuR* was amplified by the polymerase chain reaction (PCR) with a standard PCR amplification protocol (11). The amplified fragment was subsequently cloned into a unique blunt *Stu*I site of suicide vector pBT-1. The resulting construct was introduced into wild-type *B. thetaiotaomicron* or 46-4 with selection for Tc^r . Transconjugants were checked for the ability to grow on CS, heparin, hyaluronic acid, and glucose.

Construction of GUS fusions. Two multicopy GUS fusions, pSQW-6 and pSQW-7, were constructed in this study (see Fig. 6) by cloning of DNA segments into multiple cloning sites upstream of the GUS gene on pMJF-3. pMJF-3 (3) contains a promoterless GUS gene (12) and replicates in *B. thetaiotaomicron*. pSQW-6 contained a 1.6-kbp *Hind*III-*Nsi*I fragment from pJH9-1, cloned into the *Hind*III and *Pst*I sites of pMJF-3 so that the *Nsi*I end of the insert was immediately upstream of the GUS gene. pSQW-7 contained a 1.2-kbp blunted *Eco*RI-*Cla*I fragment from pJH9-1, cloned into the blunted *Bam*HI and *Acc*I sites of pMJF-3 so that the *Eco*RI end of the insert was immediately upstream of the GUS gene. The clones were introduced into *B. thetaiotaomicron* as described above. Chromosomal GUS fusion Ω CQW-14 (see Fig. 6) was constructed by integration of plasmid pCQW-14 into the *B. thetaiotaomicron* chromosome. pCQW-14 was cloned by use of vector pCQW-1, which contains a promoterless GUS gene but does not replicate in *B. thetaiotaomicron*. pCQW-1 was digested with *Pst*I and *Sst*I to remove the GUS gene, and then a 3-kbp *Pst*I-*Sst*I fragment from pSQW-7 that contained the *B. thetaiotaomicron* DNA segment plus the GUS gene was cloned into pCQW-1. Cloning in this way ensured that the GUS gene was placed in the right orientation with respect to *chuR* and that the junction region remained the same as it was in pSQW-7. To test for titration of GUS expression from the Ω CQW-14 fusion by multiple copies of the promoter-operator region of *chuR*, we introduced a 3.5-kbp *Eco*RI fragment cloned in pNJR-12 (pNCS1) into the Ω CQW-14 fusion strain. To determine whether *chuR* was autoregulated, we also introduced a 2.8-kbp *Cla*I fragment containing the wild-type *chuR* gene cloned in pNJR-12 (pNCS2) into the Ω CQW-14 fusion strain. Cell extracts from transconjugants were assayed for GUS activity.

Preparation of cell extracts. *B. thetaiotaomicron* strains were grown in a defined medium containing glucose, CS, hyaluronic acid, or heparin as the sole carbohydrate source. Cell extracts were prepared as described previously (25). Extracts of *E. coli* cells were prepared in the same way. The protein concentration was determined by the method of Lowry et al. (16).

Assay of enzyme activities. Cell extracts were assayed for GUS, chondroitin lyase, chondro-4-sulfatase, chondro-6-sulfatase, CS-associated β -glucuronidase, and arylsulfatase activities. GUS activity was determined by measuring the increase in the A_{415} when the extracts were incubated with *p*-nitrophenyl glucuronide (Sigma) at 37°C (3). Chondroitin lyase (I plus II; 15) activity was assayed by incubating the extracts with CS A (2 mg/ml) and measuring the increase in the A_{235} due to the production of unsaturated disaccharides (25). Chondro-4-sulfatase, chondro-6-sulfatase, and CS-associated β -glucuronidase activities were detected by paper chromatography as described previously (25). The respective substrates were Δ Di-4S, Δ Di-6S, and Δ Di-0S. They were purchased from ICN Biomedicals, Inc. (Irvine, Calif.). Arylsulfatase activity was determined as described by Adachi et al. (1).

Nucleotide sequence accession number. The nucleotide sequence reported in this paper has been deposited in GenBank under accession number L00678.

RESULTS

Cloning of the wild-type chromosomal locus interrupted in mutants 46-1 and CS3. An 11-kbp *Hind*III fragment containing the region of the wild-type chromosome in which the Tn4351 insertion in CS3 had occurred was cloned as described in Materials and Methods (pJH9-1), and a restriction map of the region was constructed (Fig. 1). A comparison of the restriction maps of pJH9-1 and pCS3B, showing the cloned junction region in which Tn4351 had been inserted in CS3, showed that the Tn4351 insertion in CS3 had occurred about 400 bp from the *Eco*RI site (Fig. 1). To determine whether the Tn4351 insertion in 46-1 was close to the Tn4351 insertion in CS3, we digested chromosomal DNA from 46-1 with *Hind*III and probed it with the wild-type 1.2-kbp *Eco*RI-*Cla*I fragment, which contained the CS3 transposon insertion site. There were two cross-hybridizing bands, one extending from the left *Hind*III site in Fig. 1 to the *Hind*III site in the transposon insertion in 46-1 (3.6 kbp) and one extending from the *Hind*III site in the transposon to the right *Hind*III site in Fig. 1 (12 kbp). Since the distance between the *Hind*III site in the transposon to its left end in the 46-1 insertion was 1.6 kbp, the distance between the left chromosomal *Hind*III site and the left end of the 46-1 insertion was 2.0 kbp, i.e., the insertion in 46-1 had occurred about 300 bp from the *Eco*RI site (which was 1.7 kbp from the left *Hind*III site) and about 100 bp from the insertion in CS3.

Localization of the region on the cloned 11-kbp fragment that was essential for CS and heparin utilization. The complex phenotypes of mutants CS3 and 46-1 suggested that the single transposon insertion in 46-1 or CS3 either disrupted an operon that contained the affected genes or disrupted a regulatory locus. To determine which possibility had occurred, we first made gene disruptions in the regions adjacent to the CS3 insertion in wild-type *B. thetaiotaomicron* as described in Materials and Methods and then tested whether the gene disruptions abolished growth on CS or heparin. The DNA segments used to make the disruptions and the growth phenotypes of the resulting mutants are shown in Fig. 2. The

FIG. 2. Locations and growth phenotypes of the insertion mutations generated by homologous recombination of a suicide vector carrying the indicated fragment into the *B. thetaiotaomicron* chromosome. The PCR fragment was 600 bp in size and spanned the *NsiI* and *EcoRI* sites. Abbreviations: H, *HindIII*; Hc, *HincII*; C, *ClaI*; N, *NsiI*; RI, *EcoRI*; S, *SspI*; RV, *EcoRV*; P, *PstI*; Glu, glucose; HA, hyaluronic acid; HP, heparin.

fact that all but one of the disruption mutants were still able to grow on CS and heparin suggested that only a small region on the 11-kbp *HindIII* fragment, and possibly only a single gene, was essential for CS and heparin utilization. The only disruption that yielded the 46-1 and CS3 mutant phenotypes was generated by a 600-bp PCR fragment containing the *NsiI* and *EcoRI* sites (see below). The fact that neither the 1.2-kbp *EcoRI-ClaI* disruption nor the 1.2-kbp *HincII-NsiI* disruption yielded this phenotype indicated that the essential region was located within the 2.5-kbp *HincII-ClaI* segment.

Complementation was also used to locate the region containing the gene(s) affected by the CS3 and 46-1 insertions (Fig. 3). The smallest complementing clone was the one that contained the 2.8-kbp *ClaI* fragment. CS3 or 46-1 containing this fragment in *trans* could grow on both CS and heparin, and wild-type levels of chondro-6-sulfatase activity were detectable in cell extracts. The fact that neither the 3.5-kbp *EcoRI* fragment nor the 3.3-kbp *NsiI* fragment complemented the 46-1 and CS3 mutations indicated that the affected gene spanned the *EcoRI* and *NsiI* sites in the 2.8-kbp *ClaI* fragment.

Nucleotide sequence of the fragment complementing the mutations in 46-1 and CS3. The complete nucleotide sequence of the 2,894-bp *HindIII-ClaI* segment containing the complementing *ClaI* fragment was determined. Two open reading frames (ORFs) (ORF1 and ORF2) were found (Fig. 3). The two ORFs were clearly separated by a number of stop codons in all three reading frames. The nucleotide sequence and the predicted amino acid sequence of ORF1 and the first part of ORF2 are shown in Fig. 4. ORF1 contained 401 amino acids, with the first potential ATG initiation codon 365 bp from the upstream *ClaI* site and a TGA termination codon at 1,568 bp. The first potential ATG initiation codon in ORF2 was at 1,751 bp. No termination

FIG. 3. DNA segments tested for complementation of the 46-1 and CS3 mutations. +, mutant 46-1 or CS3 containing the indicated fragment in *trans* could grow on both CS and heparin and had a wild-type level of chondro-6-sulfatase activity; -, the indicated fragment in *trans* did not rescue any of the 46-1 or CS3 mutant phenotypes. The locations of two ORFs within the 2.9-kbp *HindIII-ClaI* fragment, deduced from the DNA sequence, are shown at the bottom of the figure. The horizontal arrows indicate the direction of transcription of the ORFs. The vertical arrows indicate the positions of the transposon insertions in 46-1 and CS3. The positions of the first potential start codons of ORF1 and ORF2, the stop codon of ORF1, and the *Tn4351* insertion in CS3 are given in base pairs (relative to the right-hand *ClaI* site). The stop codon of ORF2 did not lie within the sequenced region. Abbreviations: H, *HindIII*; C, *ClaI*; N, *NsiI*; RI, *EcoRI*; RV, *EcoRV*.

codon for ORF2 was found on the 2,894-bp *HindIII-ClaI* fragment. By sequencing the junction between IS4351 and chromosomal DNA in pCS3B, we found that the transposon in CS3 had been inserted 925 bp from the *ClaI* site and hence disrupted ORF1. The insertion in 46-1 also interrupted ORF1. A gene disruption made with a 600-bp internal PCR fragment of ORF1 reproduced the original transposon insertion phenotypes of 46-1 and CS3. A gene disruption within ORF1 might have had a polar effect on ORF2 if both were in the same operon. The complementation results indicated that ORF2 was not required for complementation. Consistent with this conclusion, a gene disruption made with a 350-bp internal *HincII* fragment of ORF2 had no effect on growth on CS or heparin (Fig. 2). This result confirmed that a disruption of ORF1, rather than a polar effect on ORF2, was responsible for the 46-1 and CS3 mutant phenotypes.

ORF1 encoded a protein with a deduced molecular mass of 46,401 Da. The EMBL and SWISS data bases were searched for proteins with homology to the ORF1-encoded protein. The latter showed 36% overall amino acid identity and 60% amino acid similarity (conservative amino acid changes included) to a regulatory protein encoded by the *atsB* gene of *Klebsiella aerogenes* (Fig. 5). *AtsB* is thought to be a positive regulator of the expression of an arylsulfatase.

FIG. 4. Nucleotide sequence and predicted amino acid sequence of ORF1 and part of ORF2.

tase gene (*atsA* [19]). These results suggested that the defects in 46-1 and CS3 were due to a loss of function of a single regulatory gene encoded by ORF1. This gene has been designated *chuR*, for regulation of CS and heparin utilization.

ORF2 encoded more than 381 amino acids, since it extended past the end of the cloned *Hind*III fragment. In *K. aerogenes*, the structural gene for arylsulfatase (*atsA*) is downstream of its regulatory gene (*atsB*), and the two genes are organized in an operon (19). This fact raised the question of whether ORF2 might encode a previously undiscovered *B. thetaiotaomicron* arylsulfatase gene. However, the partial N-terminal 381-amino-acid sequence deduced from ORF2 did not exhibit significant amino acid homology to *Klebsiella* arylsulfatase (*AtsA*) or any other proteins in the data base, and no arylsulfatase activity was detected in extracts of *B. thetaiotaomicron* grown on CS. Moreover, as mentioned above, a gene disruption made with an internal *Hinc*II segment of ORF2 (Fig. 2) did not affect growth on CS or heparin. Nor were the activities of any CS-associated enzymes affected (data not shown). Thus, we concluded that ORF2 was not an arylsulfatase gene and was not essential for CS or heparin utilization.

Expression of *chuR*. To confirm that *chuR* was expressed in *B. thetaiotaomicron* and to check the direction of *chuR* transcription, we constructed and tested GUS fusions

pSQW-6 and pSQW-7 (Fig. 6 and Table 2). The fact that the strain carrying pSQW-7 was GUS positive, whereas the strain carrying pSQW-6 was GUS negative, showed that *chuR* was expressed in *B. thetaiotaomicron* and that the direction of *chuR* transcription was that predicted from the DNA sequence. The GUS specific activity associated with pSQW-7 was the same in extracts from bacteria grown on glucose as in extracts from cells grown on CS, hyaluronic acid, and heparin (Table 2). Thus, the expression of *chuR* appeared not to be regulated at the transcriptional level. The vector used to construct pSQW-7 has a copy number of 10 to 20 in *Bacteroides* spp. To eliminate the possibility that the apparently unregulated transcription of *chuR* was due to the titration of a repressor by multiple copies of the promoter region, we constructed a chromosomal GUS fusion (Ω CW-14). The chromosomal fusion results confirmed that the expression of the *chuR* gene was not regulated at the transcriptional level (Table 2).

An unexpected result was that the chromosomal fusion exhibited the same level of GUS specific activity as the multicopy fusions (Table 2). A possible explanation for this observation is that *chuR* expression depends on an activator that is not present in a sufficient quantity to activate more than one copy of *chuR*. If so, multiple copies of the 3.5-kbp *Eco*RI fragment containing the upstream region of *chuR* should reduce the expression from chromosomal GUS fu-

1441 GACAGCGGACGGCGAACCCGGTCTGAACATATCTGTGCAAGGATATTACCAATACTTTCA 1500
 CTGTGCGCTGCGCTTGGGCGAGACTTGTATAGACAGCTTCTCTATAATGTTATGAAGT
 T A D G E P G L N Y L C K G Y Y Q Y F Q -
 1501 GCATGTAGCTCCCTATATGGATTTTCATGAAAAAGAAATTAATGAATCAACAGGCTCCTGC
 CGTACATCGAGGATATACCTAAAGTACTTTTCTTAATTAATCTAGTTGTGCGAGGACG
 H V A P Y M D F M K K E L M N Q Q A P A -
 1561 AACATCATGAAAGCACTAAAGACGGAAGTTAAAAATAGAATATTAACATCATAATTTA
 TTGTAGTACTTTCTGATTTTCTGCTTCAAATTTTATCTTATAATTTAGTATTAAAT
 T S *
 1621 TAATTGTTATCATAAAGTAATGAACAGACTAAGACTTTTATTATTAGCGCTGACAGCGCT
 ATTAACAATAGTATTTCATTACTTGTCTGATTCTGAAATAAATAATCGGACTGTGCGGA
 1681 GCTGGTGTACGTAAAAGCGGACGAAGGTATGTGGTTATTGCAACTAATGCAACAGCAA
 CGACCATACATGCAATTTTGCCTGCTTCCATACACCAATAAGCTTGATTACGTTGTCGTT
 1741 CATTTCATAGTATGATGAAGAAAAAGGATTAAAACTGGAAGCCAGGATTATATATCC
 GTAAGTATCATACTTCTTTTCTTCTAAATTTTACCTTCGGGCTCTAAATATTATAGG
 M M K K K G L K L E A Q D L Y N -

FIG. 4—Continued.

sion Ω CW-14 by titrating the activator. To test this idea, we introduced pNCS1 (containing the *EcoRI* fragment shown in Fig. 3) into the Ω CW-14 fusion strain. However, the GUS activity of this strain was the same as that of the strain not carrying multiple copies of the *chuR* upstream region (Table 2).

To determine whether *ChuR* represses its own transcription, we assayed the GUS activity of chromosomal *chuR* fusion Ω CW14 in the presence or absence of multiple copies of the wild-type *chuR* gene. The Ω CW14 strain containing pNCS2, a multicopy plasmid containing the wild-

ChuR	1	MKATTYAPFAKPLVVMKPVGAVCNLACEYCYYLEKANL	39
AtsB	1	MLNIAALRQQQIPLAAEPRSPVFFHLMKPIGACNLACRYCYYPQ....	46
ChuR	40	YKENPKHVMSELEKFIIDEYINSQ...TMPQVLFTWHGGETLMRPLSFYK	87
AtsB	47	.DETVPVNMKDARLEQFIRRYIAAQFAGAREINFVWQGEPLLAGLSFYK	95
ChuR	88	KAMELQKKYAR.GRTIDNCIQNTGTLTDEWCEFFRENNWLGVGSIDGPQ	136
AtsB	96	KALALQARYAPDGVTTISNSLQNTGTLINDAWCLFREHGFITGLSLBEGNE	145
ChuR	137	EFHDEYRKNNKMGKPSFVKVMQGINLLKKHGVENAMAVNDFNAEYPLDF	186
AtsB	146	ALQDYHRPDKRGRSTWSAALRGIDLLHQHGVDFNLLVVVHNEMAAHAAAI	195
ChuR	187	YNFFKEIDCHYIQFAPIVERIVSHQDGRHLASLAEKGEALADFGVSPEQ	236
AtsB	196	YDLVSLGARYLQFQPLMS.....EGAAALREG.....YQLSADN	229
ChuR	237	WGNFLCTIDFEWVKE.DVGKFFIQIFDSTLANWMEQPGVCTMAKHCGHA	285
AtsB	230	WGRFMVGIWRQWRKRCRGRVFINIEQAWAQYFTHTSGSCVHSARCGSN	279
ChuR	286	GVMEFNGDVYSCDHFVPEYKLGNIYSQTLVEMHSEHQHNGFTMKYQSL	335
AtsB	280	LVMEPDGQLYACDHLINAEHRLGRLEQTLAAAVDASVQLPPGQK...SL	327
ChuR	336	PTQCKECDFLACNGECPKNRFSRTADGEPGLNYLCKGYYQYFQHVAPYM	385
AtsB	328	RRECQTCVKNMVCQGGCPAHL.....NAGNNRLCGGYYRFFSDILAPL	371
ChuR	386	DFMKELMNNQAPATS*.....	401
AtsB	372	RPFSRDLNGLKAWRAAFVGTAAVCVAPYDDIPL*	405

FIG. 5. Comparison of amino acid sequences of *ChuR* from *B. thetaiotaomicron* and *AtsB* from *K. aerogenes*. Identical amino acids are indicated by a vertical line. Amino acids whose comparison value (scoring based on a simplification scheme for amino acid differences involving a University of Wisconsin Genetics Computer Group computer analysis program) was greater than or equal to 0.50 are indicated by a colon. Amino acids whose comparison value was greater than or equal to 0.10 are indicated by a period. The amino acid identity between the two sequences was 36%, and the amino acid similarity between the two sequences (a comparison value of greater than or equal to 0.50) was 60%.

FIG. 6. Transcriptional GUS fusions constructed in this study. The heavy arrows indicate the position and direction of transcription of the GUS gene in each construct, and the lighter lines show the region of DNA cloned upstream of the GUS gene. The GUS specific activities in extracts from cells grown on different substrates are shown in Table 2. The horizontal arrows above the restriction map at the top of the figure show the direction of transcription and length of *chuR* and ORF2, as deduced from the DNA sequence. Abbreviations: H, *HindIII*; C, *ClaI*; N, *NsiI*; RI, *EcoRI*.

type *chuR* gene, exhibited the same level of GUS expression as the Ω CW14 strain itself (Table 2). The chromosomal fusion strain (Ω CW14) contains one copy of the wild-type *chuR* gene and one copy of the fusion. To determine whether *ChuR* activates its own transcription, we introduced the multicopy *chuR-uidA* fusion (pSQW-7) into mutant 46-1 (*chuR*). The pSQW-7 fusion exhibited the same level of GUS expression in 46-1 as in the wild-type strain (Table 2). Thus, the transcription of *chuR* appears not to be autoregulated.

46-4 is a Tn4351-generated mutant of *B. thetaiotaomicron* that cannot grow on CS, hyaluronic acid, or the disaccharide subunit of CS. None of the CS-associated enzymes was detectable in extracts from 46-4 grown on glucose or heparin (9). This and other phenotypes of 46-4 had suggested either that a central regulatory locus controlling CS utilization genes had been disrupted or that 46-4 was unable to take up or process the inducer of CS utilization genes (9). The

TABLE 2. GUS specific activities associated with the fusions shown in Fig. 6

<i>B. thetaiotaomicron</i> strain (fusion) ^a	GUS specific activity ^b (U/mg of cell protein) with the following carbohydrate source ^c :			
	Glu	CS	HA	HP
5482 (pSQW-6)	<0.1	<0.1	<0.1	<0.1
5482 (pSQW7)	20.1	20.6	23.4	20.3
5482 (Ω CW14)	18.3	22.3	21.5	20.1
5482 (Ω CW14/pNCS1)	20.2	20.4	ND	19.3
5482 (Ω CW14/pNCS2)	14.1	15.1	ND	16.7
46-1 (pSQW7)	23.3	NG	25.4	NG
46-4 (pSQW7)	24.8	NG	NG	24.0

^a 5482 is a wild-type *B. thetaiotaomicron* strain. Mutants 46-1 and 46-4 have been described (9, 26). Ω CW14 is a chromosomal GUS fusion. The other fusions are on multicopy plasmids.

^b One unit of GUS activity is defined as an increase in the A_{415} of 0.01 per min at 37°C. Each value is the mean of at least triplicate determinations from independent experiments, with standard deviations of less than 20%. ND, not determined; NG, no growth.

^c In the defined medium used to grow cells. Glu, glucose; HA, hyaluronic acid; HP, heparin.

expression of GUS from pSQW-7 was the same in 46-4 as in the wild-type strain (Table 2). Thus, even if the 46-4 insertion affected a central regulatory locus, this mutation had no effect on the transcription of the *chuR* gene.

One possible explanation of the function of ChuR arises from the observation that *B. thetaiotaomicron* grows more slowly on heparin than on CS and thus may have evolved a system for utilizing CS first if a mixture of CS and heparin is encountered. Such a scheme might occur if the 46-4 locus positively regulates CS utilization genes but negatively regulates heparin utilization genes. In such a scheme, ChuR may serve to counteract the effects of the 46-4 product under certain conditions and allow the induction of heparin utilization genes; i.e., 46-1 is heparin negative because ChuR no longer counteracts 46-4. If so, a *chuR* 46-4 double mutant should be able to grow on heparin. This was not the case. Double mutant *chuR* 46-4 did not grow on any of the mucopolysaccharides tested (CS, hyaluronic acid, and heparin); i.e., the 46-4 mutation did not suppress the heparin-negative phenotype of 46-1.

DISCUSSION

Our earlier finding (9) that a single transposon insertion eliminated growth on both CS and heparin, despite the fact that the CS and heparin utilization pathways had appeared to be completely independent of each other, could be explained in one of three possible ways. The insertion could have occurred early in an operon and could thus have eliminated the expression of multiple genes by polarity. Alternatively, the insertion could have disrupted a regulatory locus that controlled the expression of essential CS and heparin utilization genes. It was also possible that a promoter provided by the end of the transposon caused constitutive expression of a gene that prevented the expression of key CS and heparin utilization genes under all growth conditions. We have now shown that the second of these explanations is correct. That is, the phenotype of 46-1 was due to the disruption of a single gene that appears to have a regulatory function.

Two lines of evidence suggest that *chuR* is a regulatory gene. First, both the CS-negative and heparin-negative phenotypes of 46-1 and CS3 resulted from the disruption of a single gene, *chuR*, and were complemented by the fragment carrying only the intact *chuR* gene. Although the disruption of *chuR* may have had a polar effect on a downstream gene(s), the inability of 46-1 and CS3 to utilize CS and heparin was not due to the possible polar effect because a mutant with an insertional disruption of downstream ORF2 was able to grow on heparin and CS and had a wild-type level of chondro-6-sulfatase activity. However, the aberrant expression of some proteins of unknown function in protein profiles of 46-1 and CS3 may have been due to a polar effect on a downstream gene(s) as well as the disruption of *chuR* itself.

A second line of evidence is that ChuR shares homology with a protein (AtsB) that activates the transcription of the arylsulfatase gene (*atsA*) of *K. aerogenes*. In contrast to AtsB, ChuR appears not to regulate a *Bacteroides* arylsulfatase gene, since no arylsulfatase activity was detectable in *B. thetaiotaomicron* extracts. However, ChuR is required for the expression of a gene encoding another type of sulfatase, chondro-6-sulfatase. Interestingly, ChuR is not required for the expression of the gene encoding chondro-4-sulfatase, the other sulfatase needed for the breakdown of CS. ChuR also differs from AtsB in that ChuR appears to

regulate the expression of a number of unlinked genes rather than a single closely linked gene.

Transcriptional fusion analysis of *chuR* expression indicated that its expression was not regulated at the transcriptional level. All of the CS-degrading enzymes and a number of CS-associated membrane proteins are produced at 30- to 100-fold-higher levels when cells are grown on CS or hyaluronic acid (a nonsulfated inducer of the CS utilization system) than when they are grown on other carbohydrates (13, 23). Previous analysis of fusions of the GUS gene to the promoter-operator region of an operon (6) containing two genes encoding CS-degrading enzymes demonstrated that the expression of this operon is regulated at the level of transcription (3). Thus, it is likely that other structural genes involved in CS utilization are also regulated at the transcriptional level. The fact that *chuR* seems to control the expression of a regulated gene encoding chondro-6-sulfatase, despite the fact that its own expression is not elevated under inducing conditions, raises the possibility either that *chuR* expression is regulated at the translational level or that ChuR may have to interact with an inducer or with a CS-regulated protein to exert its regulatory activities.

The CS utilization system is quite complex and involves regulations at different levels (10). A disruption of *chuR* affected only a small subset of the CS-regulated genes. The rest were expressed at wild-type levels and were regulated normally when cells were grown on hyaluronic acid. Thus, there must be at least one other regulatory locus involved in the control of the CS utilization system. A previous characterization of another CS-negative, Tn4351-generated mutant of *B. thetaiotaomicron* (46-4) had suggested that the transposon insertion in 46-4 disrupted either a central regulatory locus or a gene required for the uptake or modification of the inducing disaccharides. If the 46-4 locus encodes a regulatory gene, the product of this gene does not affect the transcription of *chuR* because GUS fusion experiments showed that *chuR* was expressed normally in 46-4. Nor was the 46-4 mutation a suppressor of the heparin-negative phenotype of 46-1. Thus, the transcription of *chuR* appears to be independent of the locus affected in 46-4.

An unexplained feature of *chuR* expression was that the level of GUS expression from the chromosomal GUS fusion (Ω CQW-14) was the same as that from plasmid fusion pSQW-7, which had a copy number of at least 10 per cell. The expression of *chuR* appeared not to be autoregulated. Moreover, the *chuR* promoter-operator region in *trans* did not titrate the expression of a chromosomal *chuR*-*uidA* fusion, a finding that suggests that the unexpectedly low expression from the multicopy fusion was not caused by a limiting amount of some activator required for *chuR* expression. Thus, it is still not clear why the multicopy fusion did not increase GUS expression from the *chuR* promoter-operator region.

Our finding that a regulatory protein connects the CS utilization and heparin utilization systems raises the question of whether other mucopolysaccharide utilization systems are also controlled in part by ChuR. Two other types of mucopolysaccharides, dermatan sulfate and keratan sulfate, have not been tested as carbon sources for *B. thetaiotaomicron* because these compounds are so expensive. These two mucopolysaccharides, in addition to CS and heparin, are components of the mammalian extracellular matrix and could be released into the lumen of the gastrointestinal tract during sloughing of mucosal cells. It is possible that the colonization defect associated with the loss of ChuR is due to an inability to utilize multiple mucopolysaccharides. Fur-

ther work is needed to determine whether ChuR also controls other mucopolysaccharide utilization systems besides those for CS and heparin.

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