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Use of Suppressor Analysis To Find Genes Involved in the Colonization Deficiency of a *Bacteroides thetaiotaomicron* Mutant Unable To Grow on the Host-Derived Mucopolysaccharides Chondroitin Sulfate and Heparin

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***Bacteroides thetaiotaomicron*, one of the numerically predominant species of human colonic bacteria, can ferment two types of host-derived mucopolysaccharides, chondroitin sulfate (CS) and heparin (HP). Originally, the pathways for utilization of CS and HP appeared to be completely independent of each other, but we have recently identified a gene, *chuR*, that links the two utilization systems. *chuR* is probably a regulatory gene, but it controls only a small subset of genes involved in CS and HP utilization. Some of the genes controlled by *chuR* are important for survival of *B. thetaiotaomicron* in the colon because a mutant that no longer produced ChuR was unable to compete with the wild type for colonization of the intestinal tract of germfree mice. In an attempt to identify genes that either were controlled by ChuR or encoded proteins that interacted with ChuR, we used transposon mutagenesis to generate suppressor mutations that restored the ability of a *chuR* disruption mutant to grow on CS and HP. Two classes of suppressors were isolated. One class grew as well as the wild type on CS and HP and had recovered the ability to compete with the wild type for colonization of the germfree mouse intestinal tract. A second class grew more slowly on CS and HP and reached only a half-maximum level on CS. This mutant still had a colonization defect. Representatives of both classes of suppressor mutants have been characterized, and the results of this analysis suggest that the transposon insertions in the suppressor mutants probably affected regulatory genes whose products interact with ChuR.**

Bacteroides, a genus of gram-negative obligate anaerobes, accounts for 25 to 30% of the total bacterial population of the human colon (11). *Bacteroides* spp. require a fermentable carbohydrate for growth. In the colon, polysaccharides are probably the main source of carbon and energy because simple sugars are absorbed during passage through the small intestine and thus do not reach the colon in high concentrations. Some *Bacteroides* spp., such as *Bacteroides thetaiotaomicron*, are notable for their ability to ferment a variety of polysaccharides, including host-derived polysaccharides such as chondroitin sulfate (CS), hyaluronic acid (HA), and heparin (HP) (19). CS consists of repeating dimers of *N*-acetylgalactosamine-glucuronic acid, with the *N*-acetylgalactosamine residue sulfated either at the C-4 position or at the C-6 position. HA has a composition and structure similar to those of chondroitin sulfate, except that it has *N*-acetylglucosamine instead of *N*-acetylgalactosamine residues and contains no sulfate groups. CS is broken down into sulfated disaccharides by chondroitin lyases. Two chondroitin lyases have been characterized: chondroitin lyase I and chondroitin lyase II (5, 14). Sulfate groups are removed from the sulfated disaccharides by chondro-6-sulfatase (which removes sulfates from C-6-sulfated disaccharides) and chondro-4-sulfatase (which removes the sulfates from C-4-sulfated disaccharides). The unsulfated disaccharides are then hydrolyzed to monosaccharides by a β-glucuronidase. The chondroitin lyases and β-glucuronidase also act on HA and degrade it to monosaccharides. Expression of genes encoding the enzymes responsible for degrading CS and HA is tightly regulated, and the enzymes are produced only when cells are grown on CS, HA, or disaccharide components of these two polysaccharides. HP, another host-derived mucopolysaccharide, has a composition and structure different from those of CS and HA. HP is composed of glucuronic (or iduronic) acid and *N*-acetylglucosamine residues. It has linkages different from those of CS and HA and is much more highly sulfated, with sulfates on the uronic acid residues as well as on the hexosamine residues. HP is not degraded by the CS-utilizing enzymes, nor does it induce expression of genes encoding these enzymes (8).

CS, HA, and HP presumably enter the small intestine and colon because of sloughing of epithelial cells, which releases extracellular matrix components into the intestinal lumen. Although the concentration of these mucopolysaccharides in the colon is probably low, they might still be important substrates for *Bacteroides* spp. because they are constantly available, readily fermented, and not utilized by most other species of colonic bacteria. Moreover, mucopolysaccharides contain hexosamine residues, which could serve as a source of nitrogen as well as carbon. In fact, *Bacteroides* spp. can grow in defined medium that contains *N*-acetylglucosamine or CS as the sole nitrogen and carbon source as well as they grow in medium supplemented with ammonia (unpublished data). In previous studies to determine whether mucopolysaccharides were important substrates for *B. thetaiotaomicron* growing in the colon, we tested the ability of CS utilization mutants to compete with the wild type in the intestines of germfree mice (15). Results of these experiments were somewhat confusing. Some CS utilization mutants competed successfully with the wild type in the germfree mouse model, but two CS utilization mutants (46-1

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and CS3) were unable to compete with the wild type for colonization. One difference between CS3 and 46-1 on one hand and mutants that were still colonization proficient on the other was that CS3 and 46-1 were also unable to grow on HP, whereas the competition-proficient mutants could still grow on HP. This raised the possibility that the ability to utilize HP might be important for bacterial growth in the colon.

The mutations in CS3 and 46-1 inactivated the same gene, *chuR* (2). The finding that a transposon insertion in a single open reading frame (ORF) eliminated growth on both CS and HP was unexpected because the CS and HP utilization pathways had appeared to be completely independent of each other. Nonetheless, the phenotype of the *chuR* disruption mutant showed that there was a link of some sort between the two pathways and that this link had some connection to colonization proficiency. The deduced amino acid sequence of the *chuR* product suggested that ChuR was a regulatory protein, but if so, ChuR is not a general regulator of CS utilization genes. Only one of the enzymes involved in CS utilization, chondro-6-sulfatase, was missing in the *chuR* disruption mutant (2). Other enzymes involved in the breakdown of CS were produced at normal levels and were regulated normally. Thus, ChuR appears to control expression of only a subset of CS utilization genes. Presumably, ChuR also controls at least some genes essential for the breakdown of HP.

One approach to finding genes that either are controlled by ChuR or encode proteins that interact with ChuR is to isolate suppressor mutations that allow a *chuR* disruption mutant to grow on CS or HP. We decided to seek such suppressors by using transposon mutagenesis. The phenotype of the *chuR* disruption mutant, i.e., loss of ability to grow on CS and HP, suggested that ChuR either was an activator of gene expression or acted as an antirepressor. In the former case, a transposon insertion upstream of an essential gene controlled by ChuR might provide a new promoter that would allow constitutive expression of the gene and thus bypass the need for ChuR. In the latter case, a transposon insertion that interrupted the repressor gene should allow the strain to grow on CS. In this paper, we report the isolation and characterization of two classes of *chuR* suppressor mutations, which were generated by use of the *Bacteroides* transposon Tn*4351*. This is the first reported use of suppressor analysis to identify *Bacteroides* genes. The characteristics of the suppressor mutants suggest that the regulation of CS and HP utilization genes is much more complex than was previously thought to be the case.

MATERIALS AND METHODS

Growth conditions. *B. thetaiotaomicron* strains were grown anaerobically either on prereduced Trypticase-yeast extract-glucose medium or on a defined medium (6) that contained one of the following as the sole carbon source: CS type A (5 mg/ml, unless otherwise stated), HA (3 mg/ml), HP (4 mg/ml), and glucose (5 mg/ml). CS type A and HA contain some contaminants that allow mutants deficient in the ability to utilize either CS or HA to grow to an optical density at 650 nm (OD_{650}) of 0.1 to 0.2 (7). Whatever the identity of the contaminant, it does not act as an inducer of CS utilization genes and is probably unrelated to CS. Accordingly, OD_{650} values given in Results are the values obtained by subtracting this background OD_{650} value from the final OD_{650} value after the culture had reached maximal growth. *Escherichia coli* strains were grown aerobically on Luria-Bertani medium. The antibiotics and concentrations used for selection were erythromycin, 10 μg/ml; gentamicin sulfate, 200 μg/ml; trimethoprim, 200 μg/ml; tetracycline, 5 μg/ml; and ampicillin, 100 μg/ml.

Isolation of suppressor mutations. Suppressors were isolated by mutagenization of a *chuR* disruption strain with Tn*4351*. The isolation scheme is shown in Fig. 1. The tetracycline-resistant (Tc^r) *chuR* disruption strain ΩCRI1 was made by insertional disruption with an internal fragment of the *chuR* gene as described previously (2). Transposon mutagenesis of the *chuR* disruption strain was done as described previously, with plasmid R751 used to deliver the transposon by conjugation (17). Tn*4351* carries an erythromycin resistance (Em^r) gene that is used as the selectable marker. About 20,000 Tc^r Em^r transconjugants were isolated and replica plated onto defined agar medium in which CS was the sole carbon source. Twelve Tc^r Em^r transconjugants that could grow on CS were found.

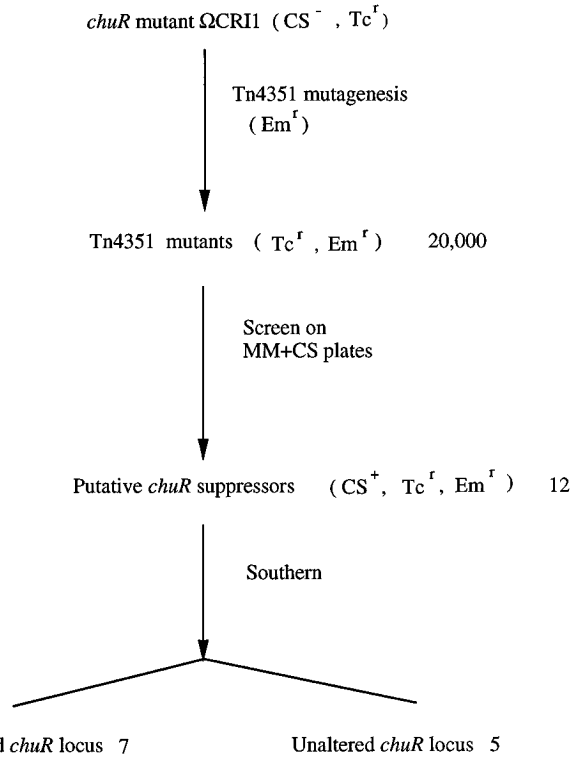

FIG. 1. Isolation of putative suppressors of the *chuR* mutant. The flow chart shows the major steps involved in isolation of putative suppressors of the *chuR* disruption mutant ΩCRI1. In parentheses are the relevant phenotypes of mutants or putative suppressors. The numbers on the side indicate how many of them were obtained. The last branch step indicates that the *chuR* loci of the 12 putative suppressors were examined by Southern hybridization (*Hin*dIII, *Cla*I, *Pst*I, and *Eco*RV digest blots). Seven of the twelve (S1, S2, S4, S5, S7, S8, and S10) exhibited altered patterns of the *chuR* locus compared with the ΩCRI1 strain. The other five of them (S3, S6, S9, S11, and S12) exhibited the same pattern of the *chuR* locus as did ΩCRI1. MM, defined (or minimal) medium.

Southern hybridization. Chromosomal DNA was isolated from *B. thetaiotaomicron* strains and digested with restriction endonucleases as described previously (17). Southern hybridization was done as described by Maniatis et al. (10).

Chondro-6-sulfatase assay. Sonicated cell extracts were made as previously described (14) from strains grown in defined medium containing either HA or glucose as the carbon source. The protein concentration in the extracts was measured by the method of Lowry et al. (9). A 30-μg sample of total cell protein was incubated with 50 μg of chondro-6-sulfate disaccharide (ΔDi-6S) at 37°C for 2 h as described previously (7), and then the mixture was boiled to stop the reaction. Portions of the reaction mixture were spotted on Whatman 3MM paper and resolved by descending paper chromatography in glacial acetic acid–*n*-butanol–1 M NH_4OH (3:2:1 [vol/vol/vol]) (14). ΔDi-6S substrate was purchased from ICN Biochemicals.

Two-dimensional gel electrophoresis. Proteins were radiolabeled first by growth of the bacterial strains in defined medium to an OD_{650} of 0.6 and then by addition of 50 μCi of ^{35}S-labeled methionine-cysteine (Tran^{35}S-label; ICN Biochemicals) per ml and incubation for 30 min at 37°C. Cells were pelleted by centrifugation, and the resuspended cell pellet was boiled in sodium dodecyl sulfate (SDS) lysis buffer. Incorporation of label was assessed by precipitation of solubilized protein with 10% trichloroacetic acid on filters and scintillation counts of the filters. A total of 5×10^6 cpm from each sample was loaded on the first-dimensional isoelectric focusing gel with a pH range from 4 to 8. The equilibrated isoelectric focusing tube gel was then laid on top of an SDS–11.5% polyacrylamide gel electrophoresis (PAGE) slab gel and resolved at constant current of 30 mA per gel on the second dimension (12). The labeling and two-dimensional gel analysis of each sample were repeated at least twice. The protein profile seen in different experiments involving the same mutant was reproducible.

Cloning chromosomal DNA flanking the transposon insertion site in the suppressors. When Tn*4351* was inserted by itself, without cointegration of the

R751 delivery vector, chromosomal DNA flanking both sides of the transposon insertion site could be cloned by taking advantage of a Tc^r gene (*tetX*) which is carried on Tn*4351* and is expressed in *E. coli* (4). Chromosomal DNAs prepared from suppressors were first digested with *Eco*RV and run on a 0.8% low-melting-point agarose gel. The region containing the band that cross-hybridized with the Tn*4351* probe (a 3.8-kbp internal *Eco*RI fragment of Tn*4351*) was cut out from the gel. DNA in this fraction was extracted and ligated with *Hin*cII-digested pUC19. *E. coli* DH5α transformants were selected on Luria-Bertani plates containing ampicillin and tetracycline. Since *Eco*RV cuts outside Tn*4351*, the Ap^r Tc^r transformants should carry the inserts of the entire Tn*4351* with flanking chromosomal DNA on both sides.

DNA sequence analysis. DNA sequencing was done by the M13 dideoxynucleotide chain termination method (16). Sequencing reactions were performed with the Sequenase 2.0 kit purchased from United States Biochemicals, Inc. (Cleveland, Ohio).

Competition assay. The source of the germfree mice and the procedures used for their maintenance have been described previously (20). The competition assay was performed in the same way as previously described (15). Five mice were used to test each suppressor mutant and were kept in a separate isolator, with each mouse in its own cage. Two mice were used for the ΩCRI1 control and were kept in separate cages in a different isolator. Mice were fed a mixture of the mutant and the wild type, in which the wild type accounted for about 10 to 15% of the mixture. Fecal samples from each mouse were taken at 3- or 4-day intervals.

Construction of suppressor derivatives with a wild-type *chuR* locus. To determine whether the phenotype associated with the transposon insertion depended on the presence of a disrupted *chuR* locus, we isolated derivatives of the suppressor mutants that had lost the insertion and now had a wild-type *chuR* locus. Since the *chuR* disruption was made with an single crossover, the *chuR* disruption could revert to the wild type by homologous recombination. We took advantage of a competition defect associated with the *chuR* disruption to isolate derivatives of suppressor strains that had lost the *chuR* disruption by homologous recombination. Suppressor mutants were used to colonize the intestinal tract of a germfree mouse. After 1 to 2 weeks, feces from the colonized animals were diluted and plated to obtain isolated colonies, and colonies were screened to identify Em^r Tc^s derivatives of the Em^r Tc^r suppressors. The Em^r Tc^s derivatives were then checked by Southern hybridization, with the ^{32}P-labeled *chuR* locus as a probe, to ascertain that the *chuR* locus had been restored to its wild-type form.

RESULTS AND DISCUSSION

Isolation of two classes of *chuR* suppressors. The original *chuR* disruption mutant was unable to grow either on CS or on HP. In a screen of over 20,000 transposon insertion mutants for the ability to grow on CS, 12 mutants that were able to utilize CS were obtained. These were designated S1 to S12. All of these mutants also grew on HP. Mutants that had regained the ability to grow on CS could be the desired suppressor mutants, or they could have sustained alterations in the *chuR* locus that restored wild-type *chuR* function (pseudorevertants). To determine whether the original *chuR* mutation remained unaltered in the mutants, the *chuR* locus was examined by Southern hybridization, with a 2.9-kbp *Cla*I fragment containing the entire *chuR* gene as the probe (Fig. 2A). *Hin*dIII digestion of DNA from the original *chuR* insertional mutant (ΩCRI1) produced two cross-hybridizing fragments (10.3 and 15.3 kbp). DNAs from 7 of the 12 putative suppressor mutants had the same hybridization pattern (S3, S4, S6, S8, S9, S11, and S12), indicating that the *chuR* locus containing the disruption was unaltered. Five of the twelve mutants (S1, S2, S5, S7, and S10) had an altered *chuR* locus. Since all five of the mutants with an altered *chuR* locus were in the class that exhibited wild-type growth on CS and HP (see below), it is likely that some sort of genetic rearrangement that restored a wild-type *chuR* locus occurred in these mutants. These pseudorevertants were excluded from further investigation. Subsequent experiments, in which DNA from the suppressor strain was digested with *Cla*I, *Eco*RV, or *Pst*I and probed with the *chuR* locus probe (data not shown), revealed that S3 was the only suppressor exhibiting wild-type growth that had an unaltered *chuR* locus. Suppressor mutants S6, S9, S11, and S12 (mutants that did not grow on CS as well as did the wild type), however, were all found to have an unaltered *chuR* locus.

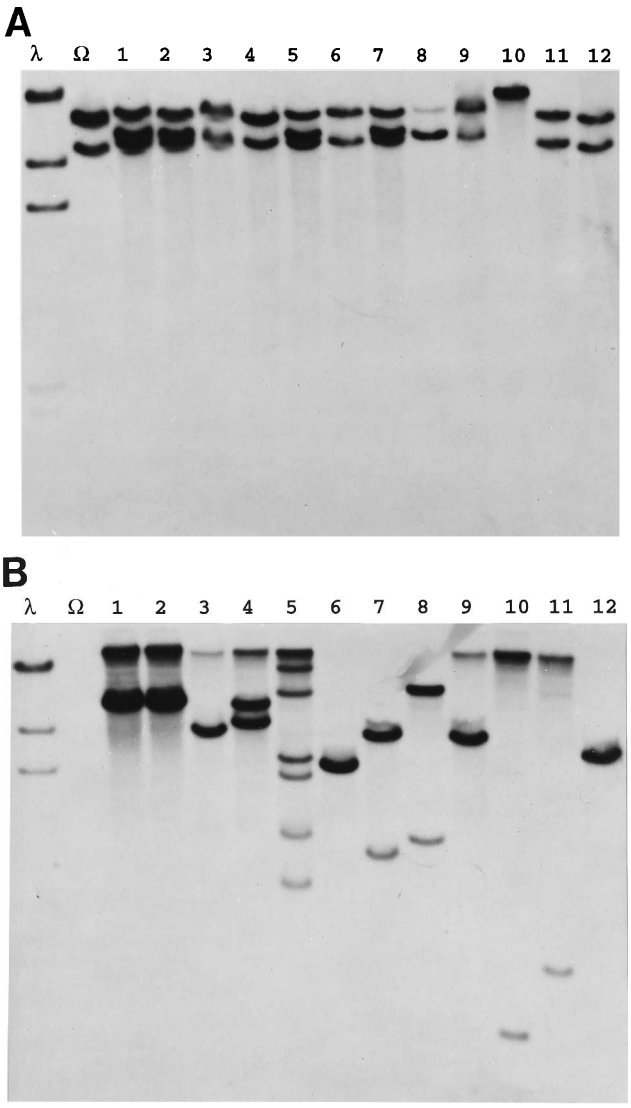

FIG. 2. Southern hybridization of the *chuR* insertional mutant ΩCRI1 (lane Ω) and the 12 isolated putative *chuR* suppressors (lanes 1 to 12). (A) Chromosomal DNAs of ΩCRI1 and the 12 suppressor strains were digested with *Hin*dIII and were hybridized with a ^{32}P-labeled *chuR* locus probe (a 2.9-kbp *Cla*I fragment containing the entire *chuR* locus). (B) Chromosomal DNAs of ΩCRI1 and the 12 suppressor strains were digested with *Eco*RV and were hybridized with a ^{32}P-labeled Tn*4351* probe (a 3.8-kbp *Eco*RI fragment containing most of the Tn*4351*). ^{32}P-labeled *Hin*dIII-digested λ DNA was used as the size standard shown in lanes λ for both blots.

Tn*4351* not only integrates itself into the *Bacteroides* chromosome but can also cointegrate R751, the plasmid used to introduce Tn*4351* into the strain (17). Moreover, some recipients of Tn*4351* can contain multiple insertions of Tn*4351* or insertions of Tn*4351* plus separate insertions of IS*4351*. To determine whether there was a single Tn*4351* insertion in each of the suppressor mutants, a 3.8-kbp *Eco*RI fragment containing most of Tn*4351* was used as the probe on Southern blots containing *Eco*RV digests of the original *chuR* insertional mutant ΩCRI1 and the 12 suppressor mutants (Fig. 2B). Since *Eco*RV does not cut inside Tn*4351*, a single insertion of Tn*4351* should give only one cross-hybridizing band with a molecular size larger than that of Tn*4351*. Two of the seven mutants with an unaltered *chuR* locus, S6 and S12, had a single

TABLE 1. Phenotypes of two classes of *chuR* suppressors

Type	Strain	Growth (final OD_{650}) on[a]:		Chondro-6-sulfatase activity[b]
		CS	HP	
Wild type	BT4001	+ (1.0)	+ (0.6)	+, R
chuR	ΩCRI1	− (0)	− (0)	−
Class A[c]	S3	+ (1.0)	+ (0.5)	+, R
	S4	+ (1.0)	+ (0.5)	+, R
	S8	+ (1.0)	+ (0.4)	+, R
Class B	S6	± (0.5)	± (0.5)	−
	S9	± (0.5)	± (0.4)	−
	S11	± (0.5)	± (0.4)	−
	S12	± (0.5)	± (0.4)	−

[a] + indicates that the growth rate was the same as that of the wild type; ± indicates that the growth rate was slower than that of the wild type; − indicates no growth.

[b] +, R indicates that the chondro-6-sulfatase activity was detected at the wild-type level and expression was regulated; − indicates that no chondro-6-sulfatase activity was detected.

[c] Grouping of suppressor mutations into two classes (class A and class B) was based on the growth phenotype and the presence or absence of chondro-6-sulfatase activity.

TABLE 2. Summary of proteins expressed differently in relevant strains as identified by two-dimensional SDS-PAGE analysis

Protein spot	Estimated mol mass (kDa)	Presence of protein in indicated strain on indicated medium[a]							
		BT4001		ΩCRI1		S3		S6	
		Glu	HA	Glu	HA	Glu	HA	Glu	HA
1	16–17	+	−	+	+	+	−	+	±
2	43–44	+	+	−	−	−	−	−	−
3	>100	+	+	−	−	+	++	+	+

[a] BT4001, wild-type *B. thetaiotaomicron* strain; ΩCRI1, *chuR* insertional mutant strain; S3, class A *chuR* suppressor S3; S6, class B *chuR* suppressor S6. Extracts were made from cells grown in defined medium containing either glucose (Glu) or HA as the sole carbon source. + indicates that the protein was present; − indicates that the protein was not detectable; ++ indicates an increased level of expression; ± indicates a decreased level of expression.

band that cross-hybridized with the transposon probe. S3, S8, S9, and S11 had two cross-hybridizing bands. S5 had more than two bands on the Southern blot. The bands in some lanes that are smaller than Tn*4351* (<5 kbp) probably arise from IS*4351* insertions. In cases in which a single insertion occurred but the transposon cointegrated R751, there were two cross-hybridizing bands because R751 contains a number of *Eco*RV sites. To check strains with two cross-hybridizing bands for cointegration of R751, R751 was used to probe the same Southern blot. The results of this analysis showed that S3, S4, S8, S9, and S11 contained R751 cointegrated with the transposon (data not shown). Therefore, S3, S6, S8, S9, S11, and S12 all contained only a single insertion, even though some of them had two *Eco*RV bands that cross-hybridized with the Tn*4351* probe.

Characteristics of the suppressor mutants. The suppressor mutants exhibited two types of growth phenotype (Table 1). Three of them (S3, S4, and S8) grew as well as the wild type on both CS and HP. The others (S6, S9, S11, and S12) grew more slowly on CS than did the wild type and reached a final OD_{650} of 0.5, which is about half the maximum OD attained by wild-type *B. thetaiotaomicron* under the same conditions. They also grew more slowly on HP, but they reached the same final OD as did the wild type. Normally, the concentration of CS used in the growth medium was 0.5%. To determine whether the inability of mutants S6, S9, S11, and S12 to grow to the full wild-type level on CS was due to accumulation of an inhibitory intermediate or to utilization of only part of the CS molecule, we also tested the growth of these mutants in medium containing twice the normal level of CS (1.0%). The mutants reached a final OD_{650} of about 0.7 in the medium containing 1% CS, whereas the wild type attained an OD_{650} of about 1.3. This result suggests that the mutants were growth limited because they were able to utilize only part of the CS molecule and not because a toxic intermediate was accumulating, because accumulation of a toxic intermediate should have stopped their growth at an OD_{650} of 0.5.

Further support for the hypothesis that mutants S6, S9, S11, and S12 were able to utilize only part of the CS molecule came from assays of chondro-6-sulfatase activity in extracts from putative suppressor mutants grown on HA. Extracts from the original *chuR* disruption strain, ΩCRI1, had no detectable chondro-6-sulfatase activity. Extracts from the suppressor mutants S6, S9, S11, and S12 lacked chondro-6-sulfatase activity (minus signs in Table 1), whereas mutants S3, S4, and S8 had chondro-6-sulfatase activities at the wild-type level (plus signs). Failure to produce chondro-6-sulfatase would limit the mutant to using only 4-sulfated disaccharides, which account for about half of the disaccharide residues in the form of CS used in these experiments, and this could explain why mutants S6, S9, S11, and S12 grew only to about half-maximal levels on CS. The fact that they were able to grow on CS, whereas the original *chuR* disruption strain could not, indicates that the suppressor mutants may have lost some control circuit that shuts off growth when sulfated disaccharides begin to accumulate intracellularly.

The suppressor mutants S3, S4, and S8 could have regained chondro-6-sulfatase activity because some regulatory barrier to expression of this enzyme had been removed or because the transposon insertion had provided the chondro-6-sulfatase gene with a new promoter that was now independent of CS regulation. We found that chondro-6-sulfatase activity was still normally regulated in the suppressor mutants. That is, chondro-6-sulfatase activity was detectable only when the mutants were grown on HA, and no activity was detected when the mutants were grown on glucose (Table 1). This finding suggests that the effect of the Tn*4351* insertion in S3, S4, and S8 was to eliminate a regulatory gene that prevented expression of chondro-6-sulfatase in the *chuR* disruption strain, ΩCRI1, because if the Tn*4351* insertion had provided a new promoter for the chondro-6-sulfatase gene, expression of chondro-6-sulfatase activity should have become constitutive.

Protein profiles of suppressor mutants. To determine whether proteins other than the chondro-6-sulfatase were affected by the disruption in *chuR*, two-dimensional gel analysis was used to compare the *chuR* disruption mutant and the wild type. One representative from each class of suppressor mutant was also analyzed by two-dimensional gel electrophoresis to determine the effects of the suppressor mutation on proteins affected by the loss of *chuR*. Comparison of the protein profiles of the wild type and the *chuR* disruption mutant, grown on HA and glucose, identified three proteins that were expressed differently in the wild type and the *chuR* disruption mutant, ΩCRI1 (Table 2). The behavior of these proteins in the two suppressor mutants S3 and S6 was also examined. In the wild type, a small protein of 16 to 17 kDa was expressed when cells were grown on glucose but not when cells were grown on HA. In the *chuR* disruption mutant, ΩCRI1, this protein was expressed constitutively. In the suppressor mutants, regulated expression was regained (S3) or partially regained (S6). A

second difference involved a 43- to 44-kDa protein, which was expressed constitutively in the wild type and was missing in ΩCRI1. This protein, which was also missing in the suppressor mutants S3 and S6, was probably ChuR because previous work has shown that ChuR is constitutively expressed and has the same size and predicted pI as the 43- to 44-kDa protein (2). The fact that ChuR was not detectable in the original disruption strain demonstrates that this mutant was a true null mutant, not a mutant that produces a truncated form of ChuR. The fact that ChuR was not detectable in S3 or S6 confirmed that these mutants were not pseudorevertants. The third protein that showed aberrant expression was a large protein (>100 kDa). It was expressed constitutively in the wild type but was missing from extracts of ΩCRI1 grown on glucose or HA. In both suppressors, this protein was expressed constitutively, although in the case of S6, expression was somewhat higher in cells grown on HA than in cells grown on glucose.

Sequence of the region containing the S6 insertion. A 6.3-kbp *Eco*RV fragment containing Tn*4351* and the chromosomal DNA flanking the Tn*4351* insertion was cloned from S6 (Fig. 3). Since Tn*4351* is 5.0 kbp in size, the 6.3-kbp fragment should contain 1.3 kbp of adjacent chromosomal DNA. Detailed restriction mapping of this 6.3-kbp *Eco*RV fragment showed that it contained 0.6 kbp of chromosomal DNA on one side and 0.7 kbp on the other side. An *Ava*I site in IS*4351* was 0.7 kbp from the end of the transposon. The 1.4-kbp *Ava*I-*Eco*RV fragment (0.7 kbp of chromosomal DNA plus 0.7 kbp of IS*4351*) from one side of the cloned *Eco*RV fragment and the 1.1-kbp *Ava*I-*Eco*RV fragment (0.6 kbp of chromosomal DNA plus 0.5 kbp of IS*4351*) from the other side of the cloned fragment were subcloned into pCQW1 to generate pCQW24 and pCQW25 (Fig. 3). The chromosomal portions in pCQW24 and pCQW25 were sequenced. pCQW24 contained a 656-bp stretch of the right junction chromosomal DNA. pCQW25 contained a 613-bp stretch of the chromosomal DNA adjacent to the left junction of the insertion. Double-stranded sequencing of both sides of the junction region revealed that Tn*4351* had been inserted with no duplication of target site DNA.

A DNA sequence of a total of 1,269 bp of the wild-type locus containing the Tn*4351* insertion site in S6 was obtained when the 613-bp left junction sequence and the 656-bp right junction sequence were combined. Two ORFs were found on the 1,269-bp DNA (Fig. 3). The upstream ORF (ORF1) was in frame 2 and was capable of encoding at least 146 amino acids. The downstream ORF (ORF2) was in frame 3 and was capable of encoding more than 229 amino acids. The first possible start codon of ORF2 was at bp 583, and no stop codon was found within the sequenced region. Tn*4351* had been inserted in ORF2, between bp 613 and 614. Although the 229-amino-acid sequence deduced from the N-terminal DNA sequence of ORF2 showed no overall similarity to any proteins listed in GenBank, it contained two regions that had similarity to two blocks of sequences in the Block search database (BLOCKS version 7.01). One region was a stretch of 31 amino acids (143 to 173) that had similarity to a heat shock factor type of DNA-binding domain (18). Another region was a stretch of 55 amino acids (101 to 155) that had similarity to HP-binding growth factors I and II (1). The two partially overlapping regions are shown as boxed regions in Fig. 3. This suggests that the ORF2 protein might have the ability to bind DNA and HP-related substrates.

The finding that Tn*4351* was inserted between 613 and 614 bp from the left *Eco*RV site, just 30 bp downstream of the first potential start codon (bp 583) for ORF2, raised the question of whether the Tn*4351* insertion in S6 disrupted the function of ORF2 or still allowed ORF2 to be expressed. Earlier work showed that IS*4351* can activate promoterless genes and provides a fairly strong promoter (3, 13). To determine whether the S6 suppressor phenotype was due to the loss of function of ORF2 or to the gain of constitutive ORF2 expression, the suicide construct pCQW24 (which contained 0.7 kbp of the end of IS*4351* and a 0.7-kbp internal fragment of ORF2) was integrated into the ΩCRI1 chromosome. This integration event would produce a disruption of ORF2. If loss of ORF2 function were responsible for restoring growth on CS, this strain should have been able to grow on CS. The strain was still unable to grow on CS and HP, i.e., the disruption of ORF2 did not suppress the ΩCRI1 phenotype. This result suggests that the Tn*4351* insertion in S6 provided a promoter for ORF2. The pCQW24 insertion was generated in such a way that the wild-type promoter of ORF2 was fused to a β-glucuronidase reporter gene when the plasmid integrated (3). No β-glucuronidase activity was detected, indicating that expression of the

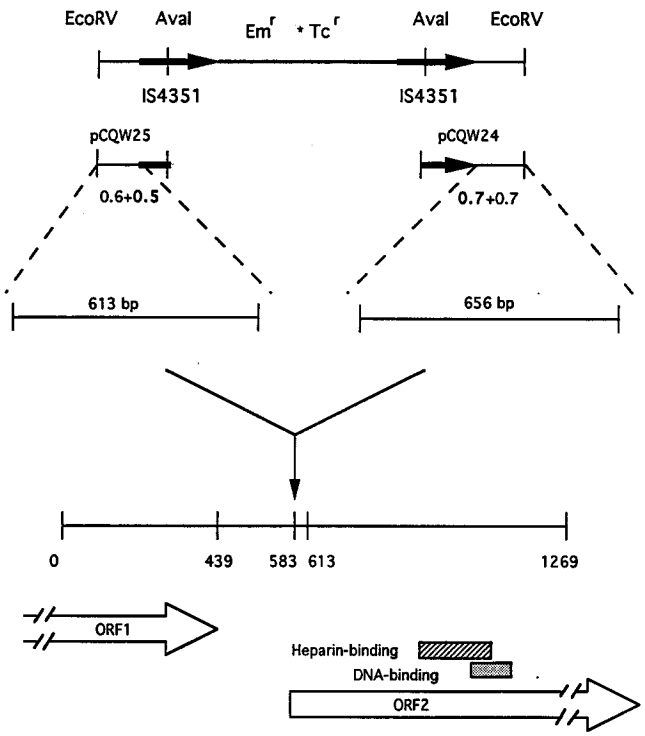

FIG. 3. Chromosomal DNA adjacent to the Tn*4351* insertion from S6. Tn*4351* is indicated by a thick line (top of figure). The heavy horizontal arrows indicate the direct repeat insertion sequences (IS*4351*) that flank Tn*4351*. Tn*4351* carries a clindamycin-erythromycin resistance (Em^r) gene, which is expressed only in *Bacteroides* strains, and a tetracycline resistance ($*Tc^r$) gene, which is expressed only in aerobically grown *E. coli* strains. The 1.4-kbp *Ava*I-*Eco*RV fragment cloned into pCQW24 contained 0.7 kbp of IS*4351* (thick line) and 0.7 kbp of the right junction chromosomal DNA (thin line). The 1.1-kbp *Ava*I-*Eco*RV fragment cloned into pCQW25 contained 0.5 kbp of IS*4351* (thick line) and 0.6 kbp of the left junction chromosomal DNA (thin line). The locations of two ORFs (upstream ORF1 in frame 2 and downstream ORF2 in frame 3) within the 1,269 bp of the wild-type sequence reconstituted from the adjacent chromosomal DNA sequence from both sides of the insertion are shown at the bottom of the figure. The open horizontal arrows indicate the directions of transcription of the ORFs. The positions of the Tn*4351* insertion (613), the potential start codon of ORF2 (583), and the stop codon of ORF1 (439) are given in base pairs (relative to the left *Eco*RV site). The stop codon of ORF2 did not lie within the sequenced region. The Tn*4351* insertion was within ORF2. The stippled box indicates the putative DNA-binding domain (amino acid residues 143 to 173) of ORF2. The hatched box indicates the putative HP-binding domain (amino acid residues 101 to 155) of ORF2. The two regions partially overlap each other.

gene is normally quite low. Attempts to clone DNA adjacent to the Tn*4351* insertion in S3 were not successful.

The Tn*4351* insertion in S6 by itself did not affect growth on CS or regulation. The reduced growth of S6 on CS could have been due to a partial suppression of the *chuR* disruption mutation, but it could also have been a direct effect of the Tn*4351* insertion itself on CS utilization. To determine the effect of the S6 Tn*4351* insertion in a wild-type background, we obtained revertants that had lost the *chuR* disruption and now had a wild-type *chuR* locus. Such revertants were readily obtained by taking advantage of the fact that the *chuR* disruption was deleterious in germfree mice. Three Em^r Tc^s derivatives of S6 were obtained, one from a day 4 sample and two from a day 8 sample of the S6-wild type competition experiment. These strains were designated C6-1, C6-2, and C6-3. The *chuR* locus and the transposon insertion in these Em^r Tc^s derivatives were checked by Southern hybridization (data not shown). All three C6 strains had a wild-type *chuR* locus and retained the same transposon insertion as S6. C6-1, C6-2, and C6-3 exhibited wild-type growth on both CS and HA, in contrast to the partial growth of S6 on CS. Chondro-6-sulfatase activity was assayed in extracts from C6 mutants grown on glucose or CS. Chondro-6-sulfatase activity was detected in extracts from C6 mutants grown on CS, but not in extracts from mutants grown on glucose. Thus, the effect of the second site mutation in S6 was seen only when this mutation was present in combination with the *chuR* disruption mutation.

Suppression of the colonization defect. When germfree mice were fed a mixture of ΩCRI1 and the wild type, in which the wild type accounted for 20% of the inoculum, the percentage of wild type rose to over 97% in less than 2 weeks (Fig. 4A). This is the same result obtained previously (15). In mice colonized with a mixture of S3 and the wild type (Fig. 4B), the wild type accounted for 17.5% of isolates at day 0 and remained below 50% throughout the 3-week experimental period. The ability of S3 to coexist with the wild type in the murine intestinal tract suggested that the colonization defect conferred by the *chuR* mutation was overcome by the second site mutation in suppressor S3. In the case of mice colonized with a mixture of S6 and the wild type (Fig. 4C), the colonization defect was still apparent, although the percentage of wild type rose only to 80% rather than nearly 100%. Given the animal-to-animal variation seen in these experiments, however, we cannot be sure that the difference in competitiveness between S6 and ΩCRI1 was significant. At best, the colonization defect in the original *chuR* disruption strain, ΩCRI1, was only slightly suppressed in S6.

Conclusions. The simplest explanation for the effects of the S3 suppressor mutation is that the transposon insertion in this strain disrupted a gene encoding a repressor that interacts with or counters the effect of ChuR. Disruption of *chuR* would have left the putative repressor intact and would thus eliminate the ability of the cells to express genes controlled by this repressor. Elimination of the repressor would restore expression of proteins controlled by ChuR and the repressor. Such a restoration was seen not only in the case of chondro-6-sulfatase activity but also in the case of the 16- to 17-kDa and >100-kDa proteins identified on two-dimensional gels. The only protein not restored in the suppressor mutant S3 was ChuR itself, as expected since the suppressor mutation occurred in a strain with a disruption of ChuR. The fact that the suppressor mutant S3 was now able to compete with the wild type in the intestinal tracts of germfree mice indicates that the colonization defect in the *chuR* disruption mutant was not due directly to loss of ChuR itself but rather to effects of this mutation on genes controlled by ChuR. Since the proteins controlled by ChuR

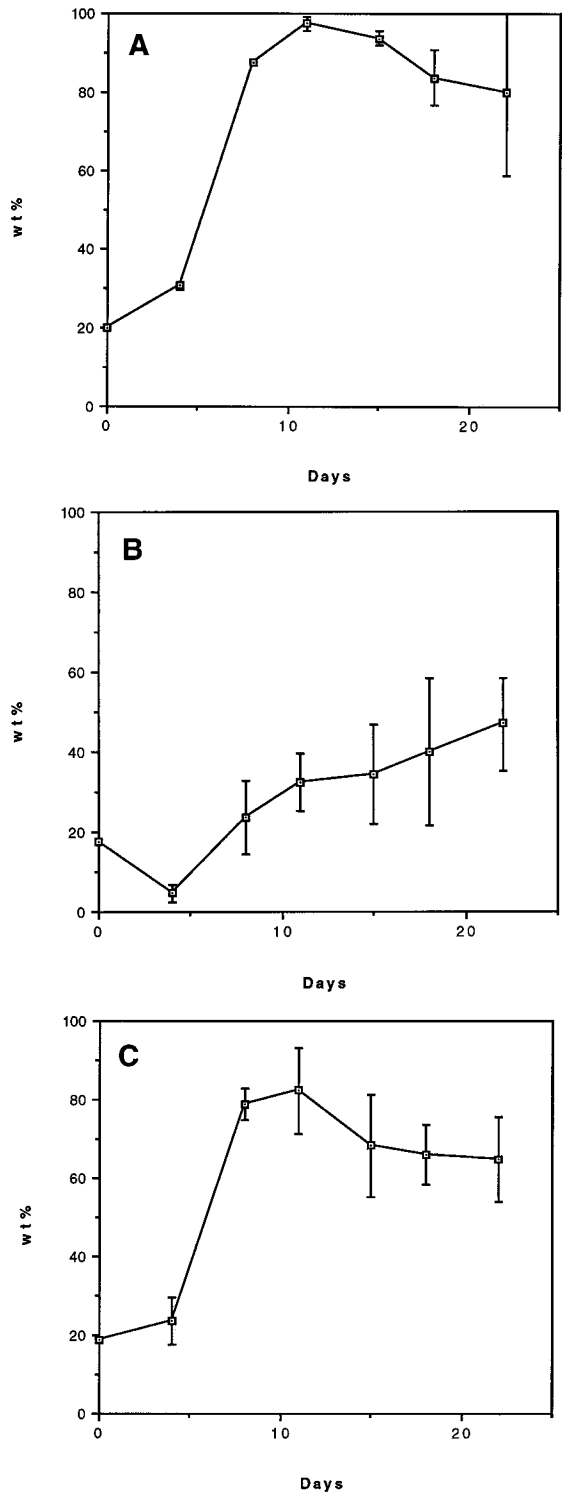

FIG. 4. Competition of mutants with wild-type *B. thetaiotaomicron* for colonization of intestinal tracts of germfree mice. (A) Competition of the *chuR* insertional mutant ΩCRI1 with the wild type (Ω/wt); (B) competition of the class A *chuR* suppressor S3 with the wild type (S3/wt); (C) competition of the class B *chuR* suppressor S6 with the wild type (S6/wt). The *x* axes indicate days after the mice were orally inoculated with a mixture of a mutant and the wild type. The *y* axes indicate the percentage of wild-type bacteria in each sample isolate. The small squares connected by the line mark the average values of data obtained from a group of five mice colonized with S3/wt, a group of five mice colonized with S6/wt, and a group of two mice colonized with Ω/wt. The error bars indicate the standard deviations of the data for every sample.

and the putative repressor were still inducible by CS and HA in S3, the proteins controlled by ChuR are probably also controlled by the same central regulatory locus that controls expression of other CS utilization proteins, and this central regulatory locus acts independently of ChuR.

The effects of the suppressor mutation in S6 are harder to explain. Previous analysis of the original *chuR* disruption mutant, 46-1 (7), had suggested that disruption of the *chuR* locus might have affected a regulatory circuit that prevented growth when sulfated disaccharides began to accumulate inside the cell. 46-1 did not grow at all on CS, whereas the remaining chondro-4-sulfatase activity should have allowed it to grow on the 4-sulfated residues of CS. Also, in the absence of chondro-6-sulfatase activity, a sulfated monosaccharide accumulated in the extracts. The sulfated monosaccharide proved to be the result of β-glucuronidase cleavage of the 6-sulfated disaccharide. There was no evidence that the accumulation of sulfated monosaccharides was toxic. That is, the cells were not killed by exposure to CS. Rather, the mutant seemed to respond to CS by failing to induce CS utilization genes. These results suggested that accumulation even of low levels of sulfated sugars, which would occur because of basal expression of CS utilization genes, was interpreted by the cell as a signal to shut down CS gene expression completely. Mutant S6 acts as if this response to unused sulfated sugars has been counteracted. That is, the mutant could now grow partially on CS, as expected if it could induce CS utilization genes and grow on the 4-sulfated CS residues. We used paper chromatography to test medium in which S6 had grown to determine whether 6-sulfated disaccharides were being released from the cells. No evidence for accumulation of sulfated disaccharides in the medium was obtained (data not shown). This does not rule out the possibility that slow breakdown of the 6-sulfated disaccharide by the β-glucuronidase occurs in the cytoplasm and that cells can somehow utilize or rid themselves of the sulfated galactosamine by converting it to a form that was not detectable on paper chromatograms.

It is interesting to note that the protein affected by the transposon insertion in S6 (ORF2) contained a DNA-binding motif and an HP-binding motif. A protein that responded to increased levels of sulfated disaccharides by binding to DNA would be expected to have these features. The transposon insertion in S6 appears not to be disrupting ORF2 expression since a disruption of ORF2 constructed by a single crossover insertion did not suppress the effect of the *chuR* disruption, but we cannot be sure whether the ability of S6 to grow partially on CS is due to overexpression of ORF2 or to the fact that ORF2 is now expressed independently of ChuR. Whatever the effects of the suppressor mutation in S6, this mutation was not sufficient to restore the ability of the mutant to compete with the wild type in the intestines of germfree mice. Previously, we had suggested that the colonization defect in the *chuR* disruption mutant might be explained by its inability to grow on HP. The fact that S6 was able to grow on HP but still had the colonization defect indicates that this simple explanation is not correct.

It is important to note that we cannot rule out the possibility that the suppressor mutations in S3 and S6 were actually caused by some point mutation or rearrangement rather than by the transposon insertion. The ease with which we isolated pseudorevertants of the *chuR* disruption raises the possibility that other types of genetic rearrangements might be taking place. If the actual suppressor mutation in S3 or S6 was caused by some event other than the transposon insertion, this would not change any of our conclusions except one, the conclusion that the ORF identified as ORF2 encodes a protein that interacts somehow with ChuR. The conclusion that both types of suppressor mutations probably affected regulatory proteins that interact with ChuR would remain unaffected, since this conclusion was based on analysis of the phenotypes of the mutants.

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