JOURNAL OF BACTERIOLOGY, Dec. 1996, p. 7180–7186 Vol. 178, No. 24
0021-9193/96/$04.00+0

Effect of Regulatory Protein Levels on Utilization of Starch by *Bacteroides thetaiotaomicron*

JOHN N. D'ELIA AND ABIGAIL A. SALYERS*

Department of Microbiology, University of Illinois, Urbana, Illinois 61801

Received 8 May 1996/Accepted 10 October 1996

***Bacteroides thetaiotaomicron*, a gram-negative obligate anaerobe, appears to utilize starch by first binding the polymer to its surface and then translocating it into the periplasmic space. Several genes that encode enzymes or outer membrane proteins involved in starch utilization have been identified. These have been called *sus* genes, for starch utilization system. Previous studies have shown that *sus* structural genes are regulated at the transcriptional level and their expression is induced by maltose. We report here the identification and characterization of a gene, *susR*, which appears to be responsible for maltose-dependent regulation of the *sus* structural genes. The deduced amino acid sequence of SusR protein had a helix-turn-helix motif at its carboxy-terminal end, and this region had highest sequence similarity to the corresponding regions of known transcriptional activators. A disruption in *susR* eliminated the expression of all known *sus* structural genes, as expected if *susR* encoded an activator of *sus* gene expression. The expression of *susR* itself was not affected by the growth substrate and was not autoregulated, suggesting that binding of SusR to maltose might be the step that activates SusR. Three *susR*-controlled structural genes, *susA*, *susB*, and *susC*, are located immediately upstream of *susR*. These genes are organized into two transcriptional units, one containing *susA* and another containing *susB* and *susC*. *susA* was expressed at a lower level than *susBC*, and *susA* expression was more sensitive to the gene dosage of *susR* than was that of the *susBC* operon. An unexpected finding was that increasing the number of copies of *susR* in *B. thetaiotaomicron* increased the rate of growth on starch. This effect could be due to higher levels of *susA* expression. Whatever the explanation, the level of SusR in the cell appears to be a limiting factor for growth on starch.**

Bacteroides thetaiotaomicron is a gram-negative obligate anaerobe that is notable for its ability to utilize a variety of polysaccharides. A common feature of most *B. thetaiotaomicron* polysaccharide utilization genes is that their expression is tightly regulated (16). That is, polysaccharide-degrading enzymes and polysaccharide-associated membrane proteins are produced only if *B. thetaiotaomicron* is grown on that polysaccharide or on its subunits. *B. thetaiotaomicron* is one of the numerically predominant bacterial species in the human colon, and tight control of gene expression is presumably necessary to allow the bacteria to adapt efficiently to the constantly changing mixture of polysaccharides that passes through the colon. A considerable amount of information is now available about *Bacteroides* genes involved in the utilization of polysaccharides such as starch and chondroitin sulfate. Results of previous studies have suggested that expression of these polysaccharide utilization genes is regulated at the level of transcription (6, 7, 14, 16), but little is known about the regulatory genes that control the expression of polysaccharide utilization genes. We report here the first example of a *Bacteroides* central regulatory gene, *susR*, which controls the expression of a set of genes involved in the utilization of starch.

B. thetaiotaomicron utilizes three forms of starch, i.e., amylose, amylopectin, and pullulan. Amylose is a linear polymer of α-1,4-linked glucose residues. Amylopectin is a branched molecule, consisting of amylose chains linked to an amylose backbone by α-1,6 bonds. Pullulan consists of maltotriose residues linked by α-1,6 bonds. All of these starches are utilized via the same pathway, and all act as inducers of starch utilization gene expression. Maltose and higher oligomers of α-1,4-linked glucose residues also act as inducers of these genes. A number of starch utilization genes have been identified and characterized. These genes have been designated *sus* genes (for starch utilization system). *susA* encodes a neopullulanase, which attacks both amylose and pullulan (6). *susB* encodes an α-glucosidase, which hydrolyzes short oligomers of starch to produce glucose (6). *susC* encodes an outer membrane protein that is essential not only for utilization of starch but also for utilization of large α-1,4-linked oligomers, such as maltoheptaose (14). Gene fusion analysis has shown that all three of these genes are regulated at the transcriptional level and that their expression is stimulated by maltose (6, 14).

In our search for the gene(s) that controlled the expression of the *sus* structural genes, we focused our attention on a transposon-generated mutant of *B. thetaiotaomicron*, Ms-1, whose phenotype suggested that the transposon insertion might have inactivated a central regulatory gene (2). Ms-1 was able to grow on maltose and maltotriose but not on higher oligomers of glucose or on starch. When grown on maltose, Ms-1 did not produce any of the starch-associated degradative enzymes or outer membrane proteins (2). Since the transposon insertion was not in either of the enzyme-encoding genes, *susA* or *susB*, it was possible that it had occurred in a regulatory gene that controlled the expression of these and other *sus* structural genes. We report here that the transposon insertion in Ms-1 disrupted a gene, *susR*, which appears to encode an activator of *sus* structural genes. We also show that increasing the production of SusR protein not only increases the expression of the *sus* structural genes but also increases the growth rate of the cells on starch.

* Corresponding author. Mailing address: Department of Microbiology, 407 South Goodwin Ave., University of Illinois, Urbana, IL 61801. Phone: (217) 333-7378. Fax: (217) 244-6697.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)[a]	Description or reference
E. coli		
DH5αMCR	RecA Gn^s	9
B. thetaiotaomicron		
5482	Wild type, Gn^r	Anaerobe Lab, Virginia Polytechnic Institute, Blacksburg
Ms-1	Em^r Gn^r Am^-	Tn*4351*-generated mutant of strain 5842 (1)
susR::pBT-1	Tc^r Gn^r Am^-	Insertional mutant of BT5482 that was generated with a 185-bp *Cla*I-*Bst*YI fragment (internal to *susR*), which had been cloned into suicide vector pBT-1 (this study) (Fig. 1)
susA1::*uidA*	Em^r Gn^r Am^+	Insertional mutant of BT5482 mutant that was generated with a 761-bp *Dra*I-*Dra*I fragment (internal to *susA*), which had been cloned upstream of the GUS gene in suicide vector pCQW-1 (this study) (Fig. 1)
susC::*uidA*	Em^r Gn^r Am^-	Insertional mutant of BT5482 that was generated with a 1.1-kbp *Eco*RV-*Xmn*I fragment (internal to *susC*), which had been cloned upstream of the GUS gene in suicide vector pCQW-1 (14).
susR::*uidA*	Em^r Gn^r Am^-	Insertional mutant of BT5482 that was generated with a 185-bp *Cla*I-*Bst*YI fragment (internal to *susR*), which had been cloned upstream of the GUS gene in suicide vector pCQW-1 (this study) (Fig. 1).
susA2::*uidA*	Em^r Gn^r Am^+	Insertional mutant of BT5482 that was generated with a 1.7-kbp *Aat*II-*Xba*I fragment (containing the amino-terminal part of *susA*), which had been cloned upstream of the GUS gene in suicide vector pCQW-1 (this study) (Fig. 1).
orf2::pLYL001B	Tc^r Gn^r Am^+	Insertional mutant of BT5482 that was generated with a 0.1-kbp *Eco*RI-*Sna*BI fragment (containing an internal disruption of the *orf2* gene), which had been cloned into pLYL001B (this study) (Fig. 1).
Plasmids		
pBT-1	$\underline{Kn}^r$ Tc^r	RSF1010-based suicide vector used to make insertional disruptions in BT5482 (18)
pLYL001B	$\underline{Ap}^r$ Tc^r	pUC19-based suicide vector (14).
pNJR-24	$\underline{Kn}^r$ $\underline{Sm}^r$ Cm^r	RSF1010-based vector (A. M. Stevens)
pCQW-1	$\underline{Ap}^r$ Em^r	pUC19-based vector containing a promoterless GUS gene (7)
pLYL02	$\underline{Ap}^r$ Em^r	pUC19-based vector containing a promoterless GUS gene (this study)
pMJF-2	$\underline{Ap}^r$ Em^r	pUC19-based vector containing a promoterless GUS gene (7)
pJND-81N	$\underline{Ap}^r$ Cm^r	1.7-kbp *Hin*dIII-*Pvu*II fragment (containing the entire *susR* gene) cloned into the *Hin*dIII-*Stu*I site of pNJR-24 (this study) (Fig. 1).
pJND-44R	$\underline{Ap}^r$ Em^r	3.2-kbp *Xba*I-*Xba*I fragment (containing the amino-terminal part of *susR*) cloned upstream of the GUS gene in pLYL02 (this study) (Fig. 1)
pJND-44A	$\underline{Ap}^r$ Em^r	Same as pJND-44R, except that the fragment was cloned in the other orientation, so that *susA* was now upstream of the GUS gene in pLYL02 (this study) (Fig. 1).
p580	$\underline{Ap}^r$ Em^r	1.8-kbp *Hae*III-*Nsi*I fragment (containing 580 bp upstream of the first start codon of *susB*) cloned upstream of the GUS gene in pLYL02 (this study) (Fig. 1).
p392	$\underline{Ap}^r$ Tc^r	392-bp *Dra*I-*Dra*I fragment (containing 398 bp upstream of the first start codon of *susA*) cloned upstream of the GUS gene in pMJF-3 (this study) (Fig. 1).

[a] Abbreviations: Am^+, grows on amylopectin; Am^-, no growth on amylopectin; Tc^r, tetracycline resistance; Em^r, erythromycin resistance; Gn^r, gentamicin resistance; Kn^r, kanamycin resistance; Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Sm, streptomycin resistance. Underlined antibiotic resistances are expressed only in *E. coli*; other resistances are expressed only in *Bacteroides* spp.

MATERIALS AND METHODS

Strain and growth condition. The bacterial strains and plasmids used in this study are listed in Table 1. *B. thetaiotaomicron* Ms-1, the Tn*4351*-generated mutant used in this study, has been described previously (2). All *Escherichia coli* strains were grown overnight in Luria-Bertani broth or on Luria-Bertani agar at 37°C. *Bacteroides* strains were grown in a prereduced Trypticase-yeast extract-glucose (TYG) liquid medium or agar (18). For growth and induction experiments, *Bacteroides* strains were transferred from TYG medium to defined medium (18) containing glucose, maltose, amylose, amylopectin, pullulan, or chondroitin sulfate as the sole source of carbon. In all cases, the concentration of carbohydrate in the medium was 0.3%. The concentrations of antibiotics used in cloning experiments and in the construction of disruption mutants were ampicillin, 200 μg/ml; erythromycin, 10 μg/ml; tetracycline, 3 to 10 μg/ml; gentamicin, 200 μg/ml; chloramphenicol, 15 to 20 μg/ml.

DNA manipulation. Various plasmids and chromosomal insertions used in this study are described in Table 1 and Fig. 1. Plasmid DNA was purified as described by Maniatis et al. (12). Total cellular DNA was isolated by the method of Saito and Miura (15). All restriction digestions and blunting and ligation reactions were performed as specified by the manufacturer (Bethesda Research Laboratories, Inc., Gaithersburg, Md., or New England BioLabs, Inc., Beverly, Mass.). Transformation of *E. coli* DH5αMCR was done by the method of Lederberg and Cohen (10). Chromosomal insertions in *Bacteroides* genes were constructed as previously described (4, 14). Southern blot analysis was done as described by Maniatis et al. (12).

Sequence analysis. A 1.5-kbp *Eco*RI-*Xba*I fragment and an adjacent 0.9-kbp *Xba*I-*Hin*dIII fragment from pJRD-L1 (18), a plasmid that contained *susR*, *susA*, *susB*, and part of *susC* (Fig. 1), were cloned separately in pUC19. Overlapping deletion clones were generated (Erase-a-base kit; Promega, Madison, Wis.). DNA sequencing was done with the Sequenase 2.0 kit (United States Biochemical, Inc., Cleveland, Ohio) or at the DNA Sequencing Center, University of Illinois, Urbana, Ill.). Both strands were sequenced. The position of the transposon insertion in Ms-1 was determined by Southern blot analysis. The GenBank, Prosite, and Swiss-Prot databases were searched to identify similar proteins of known function. Sequence alignments were prepared with the Genetics Computer Group software (13).

Enzyme assays. β-Glucuronidase (GUS) assays were done as described by Feldhaus et al. (7). α-Glucosidase activity was measured as described by Anderson and Salyers (1). Neopullulanase activity was assayed with *p*-nitrophenyl-α-maltoside as the substrate, in the same reaction buffer used for the α-glucosidase assays (6). Although the *p*-nitrophenyl-α-maltoside substrate is generally considered an amylase substrate, it can also be used to measure neopullulanase activity, the main starch-degrading activity in *B. thetaiotaomicron* extracts, because neopullulanases attack α-1,4 linkages in pullulan and amylose (6). The neopullulanase did not hydrolyze the α-glucosidase substrate, *p*-nitrophenyl-α-glucopyranoside, and the α-glucosidase did not hydrolyze the neopullulanase substrate, *p*-nitrophenyl-α-maltoside (6).

^{14}C-starch binding assays. The starch-binding assay was based on the assay originally described by Anderson and Salyers (1). Cultures were grown in mal-

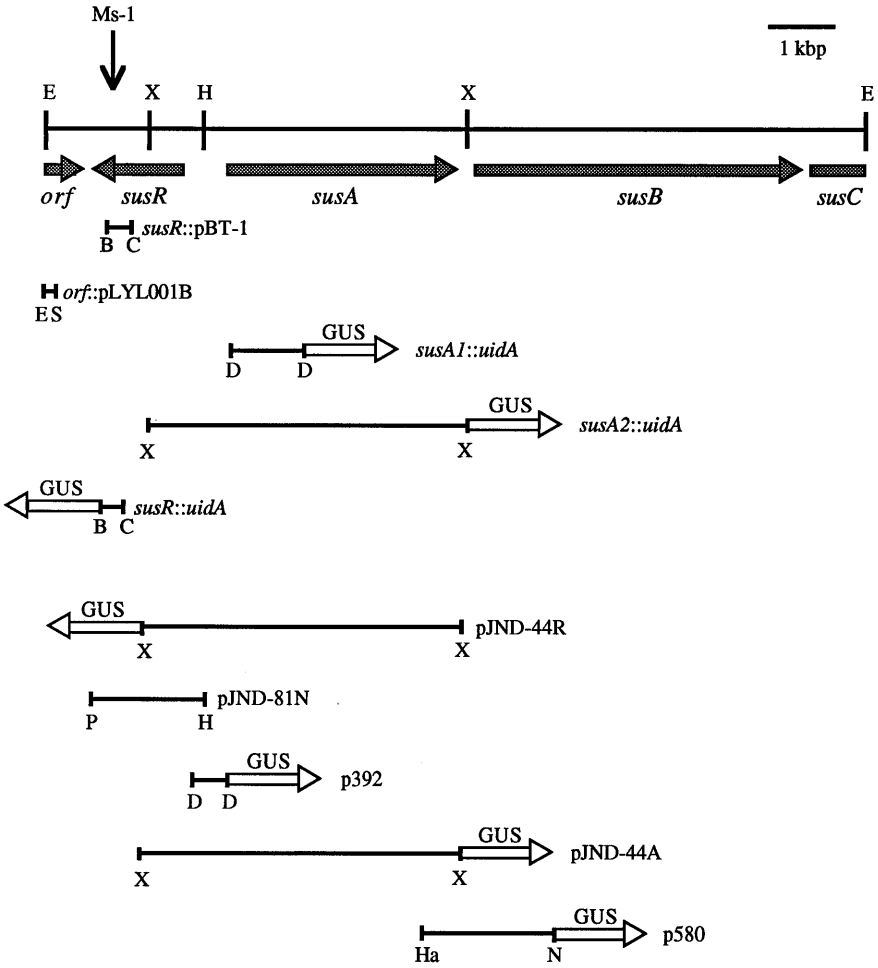

FIG. 1. A map of the region containing *susR*, *susA*, *susB*, and *susC* is shown at the top. A vertical arrow indicates the location of the Tn*4351* insertion in mutant Ms-1. Below the map are shown the segments used to make various *B. thetaiotaomicron* insertion mutants or plasmids used in this study. The ends of each fragment are labeled with the appropriate restriction site. Abbreviations: B, *Bst*YI; C, *Cla*I; D, *Dra*I; E, *Eco*RI; H, *Hin*dIII; Ha, *Hae*III; N, *Nsi*I; P, *Pvu*II; S, *Sna*BI; X, *Xba*I. Fusions to *uidA* (GUS) are indicated by large open arrows. The beginning of the arrow shows where the 5′ end of the promoterless *uidA* gene was fused to the cloned segment, and the arrowhead indicates the direction of transcription of *uidA*. Details of these constructions are given in Table 1. A construct not shown in Fig. 1 is ΩMB, in which GUS was fused to the interior of the *susC* gene (14).

tose defined medium to an optical density at 600 nm of 0.6. Cells were pelleted by centrifugation, washed once, and resuspended in 8 ml of 0.05 M phosphate buffer (pH 7.2). A portion of the resuspended cells (0.5 ml) was incubated at 20°C for 5 min and 0.5 μCi of ^{14}C-starch was added. After 1 min, the cells were pelleted in a microcentrifuge at 10,000 × *g* for 3 min. The supernatant fluid was removed, and the cell pellet was resuspended in 100 μl of phosphate buffer. The entire 100 μl was counted in scintillation fluid. The protein concentration was determined by the method of Lowry et al. (11).

RESULTS

The transposon insertion in Ms-1 disrupted a single open reading frame. Previously, we had cloned a 13-kbp *Eco*RI fragment (pJRD-L1) that proved to contain three starch utilization structural genes, *susA*, *susB*, and *susC* (Fig. 1) (18). Southern blot analysis of DNA from the Ms-1 mutant, with segments of the cloned 13-kbp fragment as probes, revealed that the transposon insertion had occurred about 1 kbp from left end of the 13-kbp cloned segment, as depicted in Fig. 1, in a region that had not been sequenced (data not shown). We sequenced the 2.5-kbp segment that extended from the amino terminus of *susA* to the left end of the DNA cloned in pJRD-L1 (Fig. 1). Analysis of the DNA sequence revealed only one complete open reading frame, *susR*, which began immediately upstream of *susA* and was transcribed in the opposite direction. There was also an incomplete open reading frame, *orf*, which was located downstream of *susR* (Fig. 1). This *orf* was transcribed in the opposite direction from *susR*.

susR could encode a protein of 433 amino acids, with an estimated molecular mass of 50 kDa and an estimated pI of 9.0. Tfasta and Blast searches of the sequence databases revealed no proteins with a statistically significant degree of alignment with SusR, but a Blocks search showed that the SusR protein contained a helix-turn-helix motif near its carboxy terminus (Fig. 2). In this region, SusR was most similar to the prokaryotic transcriptional activators LuxR, MalT, and GerE. An insertional disruption in *susR*, using a large internal segment of the gene, produced a copy of *susR* that lacked the sequence encoding the carboxy-terminal 47 amino acids of SusR (Fig. 2). This disruption mutation, *susR*::pBT-1, caused the same phenotype as Ms-1. That is, the strain was unable to grow on starch and did not produce any of the starch-associated enzymes and membrane proteins when grown on maltose. Thus, the carboxy-terminal region appeared to be important for the activity of the SusR protein.

A.

```
1    MEILPQLNRP DLEYEIVINR ATVMGVMGMY IEAMEQLEKI DPKKLNEWTL
51   LSYYQTYRAC YGWLADYTTN KTEKEKYLKK TDLYRDSIIA AMPPEENKTI
101  VMAERCIVTG KADTAIGMLN DALKDMEDER QKVYIYYTLS EAYSMKKDVE
151  KEVYYLILTA IADLESSVRE YASLQKLAHL MYELGDIDRA YKYLSCSMED
201  AVACNARLRF MEVTEFFPII DKAYKLKEER ERAVSRAMLI SVSLLSLFLL
251  IAIFYLYRWM KKISVMRRNL SLANKQMSAV NKELEQTGKI KEVYIARYLD
301  RCVNYLDKLE TYRRSLAKLA MSSRIDDLFK AIKSEQFIRD ERNEFYNEFD
351  KSFLNCSHTL LLLSITAGRR SKSLSKIRRT ADNRTPDLCL IRLGVVDSNK
401  IAHFLGYSLA TIYNYRSRIA IKLPEIKIGS NRM
```

B.

```
                1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
SusR            S N K I A H F L G Y  S  L  A  T  I  Y  N  Y  R  S
LuxR            S W D I S K I L G C  S  E  R  T  V  T  F  H  L  T
MalT            N E Q I A G E L E V  A  A  T  T  I  K  T  H  I  R
GerE            T K E I A S E L F I  S  E  K  T  V  R  N  H  I  S
```

FIG. 2. (A) Deduced amino acid sequence of *susR*. A carboxy-terminal helix-turn-helix motif is underlined. The vertical arrow indicates the last amino acid (P) of the truncated SusR produced in the *susR*::pBT-1 strain. (B) A Block search showed that SusR had a helix-turn-helix homology, which is similar to other transcriptional activators: GerE, LuxR, and MalT. Residues 8 to 10 are almost buried, and they should be hydrophobic or weakly polar amino acids. Residue 9 is most often a glycine. Residues 4 and 15 are completely buried within the protein; therefore, they must be hydrophobic or weakly polar amino acids. Residue 5 should not be branched, because it is between two helices, and a branch side chain would throw off the alignment of the helices.

The sequence similarity between the carboxy-terminal portion of the SusR protein and the carboxy termini of known transcriptional activators, together with the fact that disruption of *susR* abolished the production of a number of starch utilization proteins, suggested that *susR* encoded a regulatory protein that controlled *sus* gene expression at the transcriptional level. Since previous studies had used the starch-degrading enzyme activities as indicators of the effect of the insertion in *susR*, however, it was also possible that SusR actually exerted its effects at the posttranscriptional level. To determine whether the effect of the *susR* disruption occurred at the transcriptional level, we introduced GUS transcriptional fusions into the *susA* gene or the *susB* gene of the *susR* disruption mutant *susR*::pBT-1 to yield *susR*::pBT-1/*susA*::*uid*A1 and *susR*::pBT-1/*susC*::*uidA*, respectively (Table 2). For comparison, the same fusions were made in a wild-type background (intact *susR*). No GUS activity was detectable in the strains with a disrupted *susR* (*susR*::pBT-1/*susA*::*uidA* or *susR*::pBT-1/*susBC*::*uidA*), whereas GUS activity was easily detectable in the fusion strains with an intact *susR* gene (*susA-uidA* or *susC-uidA*) (Table 2). These results demonstrate that SusR exerts its effect at the transcriptional level.

TABLE 2. Evidence that SusR controls *susA* and *susBC* at the transcriptional level

B. thetaiotaomicron strain	*susR*	GUS sp act (U/mg of protein)[a] on growth substrate:	
		Glucose	Maltose
Wild type[b]	+	<0.3	<0.6
susA1::*uidA*	+	<0.3	2
susC::*uidA*	+	0.4	63
susR::pBT-1/*susA1*::*uidA*	−	<0.3	<0.6
susR::pBT-1/*susC*::*uidA*	−	<0.3	<0.6

[a] The variation in values shown was less than 10%.
[b] No *uidA* fusion in the strain.

TABLE 3. Expression of *susR* in cells grown on different carbohydrates

B. thetaiotaomicron strain	GUS sp act (U/mg of protein)[a] on growth substrate:		
	Glucose	Maltose	Chondroitin sulfate
Wild type[b]	<0.3	<0.6	<0.6
susR::*uidA*	<0.3	<0.6	<0.6
pJND-44R[c]	1.3	1.7	1.6
pJND-44R pJND-81N[c]	1.2	1.6	1.6

[a] The variation in values shown was less than 10%.
[b] No *uidA* fusion in the strain.
[c] pJND-44R carries the *susR*::*uidA* fusion; pJND-81N carries *susR*.

The *orf* downstream of *susR* was incomplete and was transcribed in the opposite direction from *susR*; therefore, it was clearly not in an operon with *susR*. Since this *orf* was in an area that contained many *sus* genes, we considered the possibility that this gene was involved in starch utilization. We constructed a mutant with a disruption in the *orf*, *orf*::pLYL001B (Fig. 1). This mutant grew normally on starch. Moreover, production of the starch-degrading enzymes, SusA and SusB, occurred at wild-type levels, and their expression was still regulated by maltose (data not shown). Thus, the *orf* is not essential for growth on starch and has no regulatory function. A search of the databases for homologs of the protein that could have been encoded by this *orf* revealed that the most similar protein was a *Pseudomonas cepacia* transposase subunit (3).

SusR is expressed constitutively. We had shown previously that transcription of *susA* and *susB* was stimulated by maltose (6). To determine whether the expression of *susR* was also controlled by maltose, as might be the case if SusR mediates a secondary or tertiary step in a regulatory cascade, we created a GUS fusion in the chromosomal copy of *susR* (strain *susR*::*uidA*) and measured the GUS activity in extracts from this mutant grown on glucose, maltose, or an unrelated carbohydrate, chondroitin sulfate. No GUS activity higher than background was detectable under any of these conditions (Table 3). Thus, it appeared that the level of *susR* expression was too low to be detected with a GUS fusion. Accordingly, we constructed a fusion of the *susR* promoter region with *uidA* in a plasmid, pJND-44R, which has a copy number of about 10 per cell (8). The GUS activity in extracts prepared from this strain, which was grown on glucose, maltose, or chondroitin sulfate, was determined. GUS activity was detectable in all the extracts and was the same in cells grown on all three substrates (1 U/mg of protein). Although this activity is low and is close to background GUS activity (0.3 U/mg of protein), it was reproducibly higher than background in all experiments performed.

A limitation of this experiment was that the *susR* promoter was present in multiple copies (10 to 20 copies per cell [8]) whereas the intact *susR* gene was present in only one copy. If SusR regulated its own expression, this difference in gene dosage could produce misleading results. To test the effect of increasing the level of SusR relative to the plasmid-borne *susR*::*uidA* fusion, we introduced pJND-81N (intact *susR*) into

TABLE 4. Effects of increasing the gene dosage of *susR* on the expression of *susA* and *susBC*

B. thetaiotaomicron strain	GUS sp act (U/mg of protein)[a] on growth substrate:	
	Glucose	Maltose
Wild type[b]	<0.3	<0.6
p392[c]	<0.3	8
p580[d]	<0.3	54
susA1::*uidA*	<0.3	2
susA1::*uidA*/pJND-81N[e]	<0.3	20
susC::*uidA*	0.4	63
susC::*uidA*/pJND-81N[e]	1	200

[a] The variation in values shown was less than 10%.
[b] No *uidA* fusion in strain.
[c] p392 carries the *susA* promoter region.
[d] p580 carries the *susB* promoter region.
[e] pJND-81N carries *susR*.

TABLE 5. Effect of multiple copies of the *susA* and *susBC* promoter regions on neopullulanase (*susA*) and α-glucosidase (*susB*) specific activity

B. thetaiotaomicron strain	Sp act (μmol/min/mg)[a] of:	
	Neopullulanase	α-Glucosidase
Wild type	122	240
p392[b]	48	184
p580[c]	37	131
pJND-81N[d]	944	842
p392 pJND-81N	189	698
p580 pJND-81N	54	466

[a] Values shown here are for cells grown on maltose. The variation in values shown was less than 10%.
[b] p392 carries the *susA* promoter region.
[c] p580 carries the *susB* promoter region.
[d] pJND-81N carries *susR*.

the same strain as pJND-44R (*susR*::*uidA* fusion) and measured the GUS activity of this strain when grown in glucose, maltose, or chondroitin sulfate. The two plasmids are compatible and have approximately the same copy number (8); therefore, this arrangement should provide the same level of SusR relative to the *susR* promoter as is present in the wild type. The GUS activity of this strain was the same as the GUS activity of the strain containing only the *susR-uidA* fusion plasmid (Table 3). We also tested the GUS activity associated with pJND-44R (*susR-uidA1*) in mutant Ms-1 (transposon insertion in the chromosomal copy of *susR*). The GUS activity in extracts from this construct was the same as that detected in extracts from wild-type cells carrying pJND-44R (Table 3). This result shows that the *susR*::*uidA* fusion is expressed at the same level regardless of whether an intact copy of *susR* is present in the strain. Taken together, our results suggest that *susR* is expressed constitutively and is not autoregulated.

Different response levels of the promoters of *susA* and *susBC*. We had found previously that a gene disruption in *susA* was not polar on *susB* (6). Thus, the two genes appeared to be in different transcriptional units. To determine whether *susA* and *susB* had separate promoters, we localized the promoter regions of these genes by GUS fusion analysis. When a 392-bp *Dra*I fragment upstream of *susA* was cloned upstream of *uidA* (p392; Fig. 1), extracts from the strain carrying this plasmid had easily detectable maltose-regulated GUS activity (Table 4). Thus, the *susA* promoter is presumably located within this region. The intergenic region between *susA* and *susB* is 350 bp. Plasmid p580, which contained 580 bp of DNA upstream of the first possible start codon of *susB* and thus extended only a short distance into the carboxy-terminal end of *susA*, conferred high-level, maltose-inducible GUS activity when fused to *uidA* (Table 4). Thus, there is a separate promoter upstream of *susB*, a promoter that is more than 30-fold stronger than the promoter upstream of *susA*. In the intergenic region between *susA* and *susB*, there was no apparent stem-loop structure characteristic of rho-dependent terminators. This raised the question whether the *susA* transcript terminated in this intergenic region or continued into the *susB* operon. To determine whether readthrough was occurring, we compared the GUS activity of a strain in which *uidA* had been fused to a point 249 bp past the stop codon of *susA* (*susA2*::*uidA*) with that of *susA1*::*uidA*, which had a GUS fusion inside the *susA* open reading frame. Extracts prepared from both mutants had the same GUS activity (2 U/mg of protein). This result suggests that there is some readthrough from the *susA* promoter into the *susB* region. If so, readthrough makes only a minor contribution to *susBC* expression, because the promoter of the *susBC* operon is so much stronger than that of the *susA* gene.

We had noted that when the *susA* promoter fused to *uidA* was provided on a plasmid (p392 [Table 4]), the GUS activity in extracts from this strain was fourfold higher than the GUS activity in extracts from a strain with a chromosomal fusion of *susA* to *uidA* (*susA1*::*uidA* [Table 4]). By contrast, multiple copies of a *susB-uidA* fusion (p580 [Table 4]) did not increase the GUS activity at all compared to a single chromosomal copy of a *susBC*::*uidA* fusion (*susC*::*uidA* [Table 4]). These results suggested that SusR was limiting in the cell and that the promoter of *susBC* had a higher affinity for SusR than did the promoter of *susA*. To test the hypothesis that SusR was limiting the expression of *susA* and *susBC*, we increased the production of SusR by introducing the *susR*-containing plasmid, pJND-81N, into strains carrying a chromosomal *susA1-uidA* or *susC-uidA* fusion. The GUS activity in extracts from the strain carrying the *susA1-uidA* chromosomal fusion and multiple copies of *susR* (*susA1*::*uidA*/pJND-81N [Table 4]) was 10-fold higher than the GUS activity of a strain with only a single chromosomal copy of *susR* (*susA1*::*uidA* [Table 4]). The GUS activity of the strain carrying the *susC-uidA* chromosomal fusion and multiple copies of *susR* (*susC*::*uidA*/pJND81N [Table 4]) was increased by threefold compared to that of the strain carrying only a single chromosomal copy of *susR* (*susC*::*uidA1* [Table 4]). Thus, the low level of SusR in wild-type *B. thetaiotaomicron* is limiting the expression of both the *susA* gene and the *susBC* operon.

To test the hypothesis that the promoter of *susA* had a lower affinity for SusR than did the promoter of *susBC*, we determined the effect of multiple copies of the *susA* promoter region (p392) or the *susB* promoter region (p580) on the neopullulanase (SusA) and α-glucosidase (SusB) activity of the strain and did a similar experiment with the promoter region of *susA*. Multiple copies of the *susB* promoter caused a somewhat larger decrease in neopullulanase (SusA) and α-glucosidase (SusB) activity than did multiple copies of the *susA* promoter, although this difference was barely significant (Table 5). The difference was more evident in a strain that contained multiple copies of *susR* (Table 5). Also, multiple copies of both the *susA* and *susB* promoters had a greater effect on SusA levels than on SusB levels. These results are consistent with the hypothesis that the promoter of *susA* is not as strong as that of *susBC*.

A somewhat unexpected result was that increasing the amount of SusR in the cell did not suppress the titration effect exerted by the promoter regions. In fact, multiple copies of the

susB promoter reduced the level of SusA to be almost as low in the strain with multiple copies of *susR* as in the wild-type background. A possible explanation of this effect is that the cloned forms of the promoters did not have as high an affinity for SusR as did the chromosomal promoter regions. Thus, an increased level of SusR allowed the cloned promoter regions to bind SusR more efficiently than they did in the strain with a single copy of *susR*.

Effect of SusR levels on the ability of *B. thetaiotaomicron* to bind starch and grow on starch. The fact that increasing the gene dosage of *susR* increased the expression of the *susBC* operon, which has been shown to encode proteins involved in the binding of starch to the bacterial surface (2, 14), raised the question whether multiple copies of *susR* would increase the ability of the cell to bind radioactive starch. To test this, we compared the binding of radiolabeled starch to a strain containing pJND-81N (intact *susR* gene) with binding of labeled starch by the wild type. The strain containing multiple copies of *susR* bound only 112% as much starch as did the wild type. This increase is close to the limit of significance, since variation in this assay can be as high as 10 to 15%. This difference was seen consistently in different experiments, however, and might thus represent a real increase in binding. If so, the increase in binding was not proportional to the increase in levels of binding proteins in the cell, as indicated by the more than threefold increase in the expression of the *susBC* operon in the strain with multiple copies of *susR* (Table 4). Surprisingly, however, the presence of multiple copies of *susR* did increase the growth rate on starch. *B. thetaiotaomicron* pJND-81N had a doubling time of 2.2 (±0.1) h on amylopectin and 2.1 (±0.1) h on pullulan, which was significantly shorter than the doubling time of 2.8 (±0.1) h of *B. thetaiotaomicron* pNJR-24 (vector but no *susR* gene) on both substrates.

DISCUSSION

Although it has been shown that many of the *Bacteroides* polysaccharide utilization systems are regulated, there had been little previous information about the nature of the regulatory apparatus. There has been one report of a gene, *chuR*, which appears to control a subset of genes in the chondroitin sulfate utilization system but is not the central controller of chondroitin sulfate-induced expression of chondroitin sulfate utilization genes (4). Also, none of the genes controlled by *chuR* has been cloned and characterized. *susR* is the first gene to be identified that is a central controller of a set of known *Bacteroides* polysaccharide utilization genes. One other central regulatory gene from *Bacteroides* spp. has been described: *rteB*, a gene that controls transfer genes of conjugative transposons (17). Since *rteB* is on a broad-host-range transmissible element, however, it is not clear whether *rteB* is truly a *Bacteroides* regulatory gene or one that was acquired from some other organism.

susR appears to encode a transcriptional activator, because disruption of the gene abolished the expression of *sus* structural genes and increasing the gene dosage of *susR* increased *sus* gene expression. Nonetheless, we cannot be completely certain that SusR itself binds directly to the promoters of *susA* and the *susBC* operon. It is still possible that SusR protein controls the expression of another regulatory protein, which acts directly on these promoters, or that SusR interacts with another protein to form the activation complex. We attempted to demonstrate binding of SusR, which had been overexpressed in *E. coli*, to the promoter of *susBC* in vitro but did not succeed in demonstrating a band shift of the promoter fragment (5). Thus, direct proof that SusR binds the *susA* or *susB* promoter is still lacking. It is clear, however, that SusR plays an important regulatory role in expression of *sus* genes. The fact that *susR* was expressed constitutively suggests that the stimulatory effect of maltose on the expression of *susA* and the *susBC* operon may be mediated by the binding of maltose to SusR. Alternatively, maltose may stimulate the expression of another protein that interacts with SusR to form the activation complex.

A surprising finding was that increasing the level of SusR actually enabled the bacteria to grow more rapidly on starch. Thus, the *B. thetaiotaomicron* starch utilization system has not evolved to achieve the highest possible growth rate. This makes sense since the generation times of bacteria in the colon are probably much longer than those achievable in broth culture, so that maximizing growth rates beyond those sufficient for survival in the colon would not confer any advantage. It is also interesting that although increasing the level of SusR in the cell made growth on starch more rapid, it caused only a 12% increase in the binding of radiolabeled starch to the bacterial surface. This small increase seems unlikely to be responsible for the increase in growth rate. Also of interest is that there was little if any increase in starch binding although SusC, one of the proteins involved in the binding process (14), was being produced at a threefold-higher level. This suggests that higher production of binding-complex components does not necessarily result in larger amounts of functional binding complex.

The increased rate of growth conferred by multiple copies of *susR* could be due to increased production of SusA. In an accompanying paper, we have shown that although *susA* is not essential for growth, loss of this gene decreases the growth rate of the mutant on starch by about 30% (6). We report here that the expression of *susA* was over 30-fold lower than the expression of the *susBC* operon and that *susA* expression increased more in a strain carrying multiple copies of *susR* than did *susBC* expression. The sizeable increase in neopullulanase activity could have made the starch utilization process more efficient. We cannot rule out the possibility, however, that other, as yet uncharacterized, *sus* genes might be responsible for the growth rate enhancement. Results of previous studies have shown that binding of starch to the cell surface is an important, and possibly rate-limiting, step in the utilization process (2, 14). Our finding of an increased growth rate without a significant increase in the binding of starch to the cell surface suggests that starch binding is not the only rate-limiting step in the utilization process.

ACKNOWLEDGMENTS

This work was supported by grant number AI 17876 from the U.S. National Institutes of Health.

We thank Lhing-Yew Li for providing pLYL001B.

REFERENCES

1. **Anderson, K., and A. A. Salyers.** 1989. Biochemical evidence that starch breakdown by *Bacteroides thetaiotaomicron* involves outer membrane starch-binding sites and periplasmic starch-degrading enzymes. J. Bacteriol. **171:** 3192–3198.
2. **Anderson, K., and A. A. Salyers.** 1989. Genetic evidence that outer membrane binding is required for starch utilization by *Bacteroides thetaiotaomicron*. J. Bacteriol. **171:**3199–3204.
3. **Bryne, A. M., and A. Lessie.** 1994. Characteristics of IS401, a new member of IS3 family implicated in plasmid rearrangment in *Pseudomonas cepacia*. Plasmid **31:**138–147.
4. **Cheng, Q., V. Hwa, and A. A. Salyers.** 1992. 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. J. Bacteriol. **174:**7185–7193.
5. **D'Elia, J. N.** Unpublished results.
6. **D'Elia, J. N., and A. A. Salyers.** 1996. Contribution of a neopullulanase, a pullulanase, and an α-glucosidase to growth of *Bacteroides thetaiotaomicron*

on starch. J. Bacteriol. **178:**7173–7179.
7. **Feldhaus, M. J., Q. Cheng, V. Hwa, and A. A. Salyers.** 1991. Use of *Escherichia coli* β-glucuronidase (GUS) gene as a reporter gene for investigation of *Bacteroides* promoters. J. Bacteriol. **173:**4540–4543.
8. **Guthrie, E. P.** Unpublished results.
9. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557–580.
10. **Lederberg, E. M., and S. M. Cohen.** 1974. Transformation of *Salmonella typhimurium* by plasmid deoxyribonucleic acid. J. Bacteriol. **119:**1072–1074.
11. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
12. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
13. **Needleman, S. B., and C. D. Wunsch.** 1970. A general method applicable to the search for similarities in the amino acid sequence of two proteins. J. Mol. Biol. **48:**443–453.
14. **Reeves, A. R., J. N. D'Elia, J. Frias, and A. A. Salyers.** 1996. A *Bacteroides thetaiotaomicron* outer membrane protein that is essential for utilization of maltooligosaccharides and starch. J. Bacteriol. **178:**823–830.
15. **Saito, H., and K. I. Miura.** 1963. Preparation of transforming deoxyribonucleic acid by phenol treatment. Biochim. Biophys. Acta **72:**619–629.
16. **Salyers, A. A.** 1992. Molecular biology of *Bacteroides* polysaccharide utilization systems, p. 121–129. *In* B. I. Duerden et al. (ed.), Medical and environmental aspects of anaerobes. Wrightson Biomedical Publishing Ltd., London.
17. **Salyers, A. A., N. B. Shoemaker, and Y. Li.** 1995. In the driver's seat: the *Bacteroides* conjugative Transposons and the elements they mobilize. J. Bacteriol. **177:**5727–5731.
18. **Tancula, E., M. J. Feldhaus, L. A. Bedzyk, and A. A. Salyers.** 1992. Location and characterization of genes involved in binding starch to the surface of *Bacteroides thetaiotaomicron*. J. Bacteriol. **174:**5609–5616.