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New Regulatory Gene That Contributes to Control of *Bacteroides thetaiotaomicron* Starch Utilization Genes

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***Bacteroides thetaiotaomicron* uses starch as a source of carbon and energy. Early steps in the pathway of starch utilization, such as starch binding and starch hydrolysis, are encoded by *sus* genes, which have been characterized previously. The *sus* structural genes are expressed only if cells are grown in medium containing maltose or higher oligomers of glucose. Regulation of the *sus* structural genes is mediated by SusR, an activator that is encoded by a gene located next to the *sus* structural genes. A strain with a disruption in *susR* cannot grow on starch but can still grow on maltose and maltotriose. A search for transposon-generated mutants that could not grow on maltose and maltotriose unexpectedly located a gene, designated *malR*, which regulates expression of an α-glucosidase not controlled by SusR. Although a disruption in *susR* did not affect expression of the *malR* controlled gene, a disruption in *malR* reduced expression of the *sus* structural genes. Thus, MalR appears to participate with SusR in regulation of the *sus* genes. Results of transcriptional fusion assays and reverse transcription-PCR experiments showed that *malR* is expressed constitutively. Moreover, multiple copies of *malR* provided on a plasmid (5 to 10 copies per cell) more than doubled the amount of α-glucosidase activity in cell extracts. Our results demonstrate that the starch utilization system of *B. thetaiotaomicron* is controlled on at least two levels by the regulatory proteins SusR and MalR.**

Bacteroides thetaiotaomicron and other human colonic *Bacteroides* species utilize a variety of polysaccharides as a source of carbon and energy (15). This trait may be important for their survival in the human colon because polysaccharides are the main form of carbohydrate available to colon bacteria. The starch utilization system of *B. thetaiotaomicron* is the best studied of the *Bacteroides* polysaccharide utilization systems. Previously, a cluster of starch utilization genes, designated *sus* genes, was identified and characterized (4, 5, 13, 14). This cluster contains seven structural genes (*susA* to *susG)*, most of which encode proteins that mediate the initial steps in starch utilization, such as starch binding and starch hydrolysis (13, 14). These genes are organized into two transcriptional units (5, 14), one containing *susA* and one containing *susB* to *susG.*

Expression of the structural genes is regulated at the transcriptional level by maltose and higher oligomers of starch. Regulation is mediated by SusR, a protein encoded by a gene that is located upstream of *susA.* Unlike the structural genes, *susR* is constitutively expressed (5). Since multiple copies of *susR in trans* increased *sus* gene expression and since a disruption in *susR* abolished expression of *susA-susG*, it appeared that SusR alone was responsible for controlling expression of the structural genes. In this report, we show that there is at least one other regulatory gene that participates in control of *sus* gene expression.

No other starch utilization genes were found in the region of the *sus* gene cluster, but it was clear that *B. thetaiotaomicron* must have other starch utilization genes. For one thing, disruption of *susB*, which encodes an α-glucosidase, did not eliminate all of the α-glucosidase activity in cell extracts. For another, the *susR* disruption mutant still grew as well as wild type on maltose and maltotriose even though it could not grow on higher oligomers. Thus, other maltose utilization genes must be located elsewhere on the chromosome. We report here that although a search for mutants unable to grow on maltose and maltotriose failed to locate the second α-glucosidase gene or any other genes encoding maltose utilization proteins, it did unexpectedly yield a second regulatory gene, *malR*, that controls expression of the *sus* genes, as well as expression of the second α-glucosidase.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. All *Escherichia coli* strains used in this study were grown in Luria-Bertani (LB) broth or on LB agar at 37°C. *B. thetaiotaomicron* 5482, transposon-generated derivatives, and some singly or doubly disrupted mutants used in this study have been described previously (1, 4, 14).

Bacteroides strains were grown initially in a prereduced Trypticase-yeast extract-glucose (TYG) medium. For the characterization of *Bacteroides* strains, cells were transferred to a defined minimal medium (9) containing glucose, maltose, maltotriose, amylopectin, or dextran (0.3% [wt/vol]), respectively, as a sole carbohydrate source. Antibiotic concentrations used in this study were as follows: ampicillin, 50 μg/ml; chloramphenicol at 20 μg/ml (*E. coli*) or at 15 μg/ml (*B. thetaiotaomicron*); erythromycin, 10 μg/ml; gentamicin, 200 μg/ml; and tetracycline at 10 μg/ml (*E. coli*), at 3 μg/ml for selection after conjugation and measurement of growth rates, and otherwise at 1 μg/ml (*B. thetaiotaomicron*), unless it is mentioned specifically.

DNA methods. Isolation of plasmids was done by using a Wizard Plus DNA purification system (Promega Corp.). Dephosphorylation reactions and restriction digests were performed in accordance with the manufacturer's instructions (Bethesda Research Laboratories [Bethesda, Md.] or New England BioLabs [Beverly, Mass.]). Transformation of *E. coli* DH5αMCR was done by the method of Lederberg and Cohen (10). Conjugations, where constructs generated in *E. coli* were transferred to *Bacteroides* recipients, were performed as described by Shoemaker et al. (17). Insertional and replicative shuttle vectors were mobilized from *E. coli* donors to *Bacteroides* recipients by transfer genes of RP4 integrated in the chromosome of S17-1 (19). Southern blotting was done as described by Maniatis et al. (11) except that a Renaissance Detection Kit (DuPont-NEN) was used for detection of the bound DNA probe.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Description and/or reference
Strains		
E. coli		
DH5αMCR	RecA$^-$ Gn^s	8
S17-1	RecA$^-$ Gn^s	IncP RP4 inserted into the chromosome (19)
HB101	RecA$^-$ Gn^s Str^r	11
B. thetaiotaomicron		
BT5482	Wild type, Gn^r (G2–G7)$^+$ starch$^+$	Anaerobe Laboratory, Virginia Polytechnic Institute, Blacksburg, Va.
BT4009	Tc^r Em^r Gn^r (G2-G7)$^+$ starch$^+$	*B. thetaiotaomicron* mutant with CTn*7853* element carrying Tc^r (*tetQ*) and Em^r (*ermG*) genes; known as Tc^r Em^r 7853 (3)
BTMAL	Tc^r Em^r Gn^r G2$^+$ G3$^-$ starch$^-$	Tn*4351*-generated mutant of BTΩ*susR* (this study)
Ms-1	Em^r Gn^r G2$^+$ G3$^+$ starch$^-$	Tn*4351*-generated mutant of strain BT5482 (1); displays BTΩ*susR* phenotype since *susR* is disrupted
BTΩ*susR*	Tc^r Gn^r G2$^+$ G3$^+$ starch$^-$	*B. thetaiotaomicron* mutant with a *susR* disruption created by a suicide vector pBT-1 containing 185-bp *Cla*I-*Bst*YI internal *susR*; known as *susR*::pBT-1 (5)
BTΩ*malR*	Em^r Gn^r G2$^+$ G3$^+$ starch$^+$	*B. thetaiotaomicron* mutant with a *malR* disruption created by a suicide vector pGERM containing a PCR-generated 0.33-kbp internal fragment of *malR* (this study)
BTΩ*susR*Ω*malR*	Tc^r Em^r Gn^r G2$^+$ G3$^-$ starch$^-$	BTΩ*susR* with a *malR* disruption created by a suicide vector pGERM containing a PCR-generated 0.33-kbp internal fragment of *malR* (this study)
BTΩ*susB*	Tc^r Gn^r starch$^-$	*B. thetaiotaomicron* mutant with a *susB* disruption created by a suicide vector pBT-1 containing a PCR-generated 0.61-kbp internal fragment of *susB* (16)
BTΩ*susB1*	Em^r Gn^r starch$^-$	*B. thetaiotaomicron* mutant with a *susB* disruption created by suicide vector pNJR-6 containing a 0.57-kbp internal fragment of *susB*; known as *susB1*::pNJR6 (4)
BTΩ*susB*Ω*malR*	Tc^r Em^r Gn^r starch$^-$	BTΩ*susB* with a *malR* disruption created by a suicide vector pGERM containing a PCR-generated 0.33-kbp internal fragment of *malR* (this study)
BTΩ*malR*GUS	Em^r Gn^r G2$^+$ G3$^+$ starch$^+$	*B. thetaiotaomicron* mutant with a GUS fusion created by suicide vector pΩMALRGUS (this study)
Plasmids		
pBT-1	<u>Kn^r</u> Tc^r	RSF1010-based suicide vector used to make insertional disruptions in *Bacteroides* spp. (21)
pGERM	<u>Ap^r</u> Em^r	pUC19-based suicide vector (expressing *ermG*) used to make insertional disruptions in *Bacteroides* spp. (18)
pMALR	<u>Ap^r Tc^r</u> Tc^r	PCR-generated 1.2-kbp DNA fragment containing entire *malR* and its promoter region cloned into pT-COW (this study)
pMALRGUS	<u>Ap^r</u> Em	PCR-generated 0.82-kbp DNA fragment containing *malR* promoter region cloned into pMJF-2 (this study)
pΩMALRGUS	<u>Ap^r</u> Em^r	pCQW-1 containing a PCR-generated 0.33-kbp internal fragment of *malR* (this study)
pT-COW	<u>Ap^r Tc^r</u> Tc^r	pVAL-based shuttle vector with *tetQ* (7)
pMJF-2	<u>Ap^r</u> Em^r	pFD160-based shuttle vector containing a promoterless GUS gene (6)
pCQW-1	<u>Ap^r</u> Em^r	pUC19-based suicide vector containing a promoterless GUS gene (6)

[a] Abbreviations: G2, maltose; G3, maltotriose; G7, maltoheptaose; Tc, tetracycline; Em, erythromycin; Gn, gentamicin; Ap, ampicillin; Kn, kanamycin; Cm, chloramphenicol; Str, streptomycin. Underlined antibiotic resistances are expressed only in *E. coli*. Other resistances are expressed only in *Bacteroides* spp.

Chemicals. Glucose, maltose, maltotriose, amylopectin, dextran, phenylmethylsulfonyl fluoride, *p*-nitrophenyl-α-D-glucopyranoside, were purchased from Sigma Corp. 4-Nitrophenyl-α-D-maltoheptaoside-4,6-*O*-ethylidene was purchased from Boehringer Mannheim Biochemicals.

Isolation of a *B. thetaiotaomicron* mutant deficient in maltose and maltotriose utilization. To isolate a mutant of *B. thetaiotaomicron* that was deficient in maltose and maltotriose utilization, transposon mutagenesis was done by introducing the *Bacteroides* transposon Tn*4351* into two different hosts: *B. thetaiotaomicron* 5482 (wild type) and the *susR* disruption mutant *B. thetaiotaomicron* Ω*susR* (BTΩ*susR*). In the mutant strain, BTΩ*susR*, the *susR* gene had been disrupted by a single crossover insertion of the suicide vector, pBT-1, into which an internal segment of *susR* had been cloned (5). The selectable marker on pBT-1 was a tetracycline resistance gene, *tetQ*, so that the selectable marker on Tn*4351*, the erythromycin resistance gene *ermF*, could be used. Tn*4351* was introduced into the wild-type strain or the BTΩ*susR* mutant by conjugation, by using as a donor *E. coli* HB101 containing Tn*4351* on the self-transmissible IncP plasmid, R751 (2, 12). Transconjugants harboring Tn*4351* insertions were selected by growth on TYG agar plates containing erythromycin (10 μg/ml) and gentamicin (200 μg/ml). The gentamicin selection eliminated the *E. coli* donors. Tetracycline (3 μg/ml) was also included in the medium to ensure retention of the pBT-1 insertion in BTΩ*susR*. Transconjugants were screened for growth on maltose-defined medium agar plates.

Cloning of DNA adjacent to the Tn*4351* insertion in *B. thetaiotaomicron* MAL (BTMAL). The strategy used to clone DNA adjacent to the Tn*4351* insertion is shown in Fig. 1. Chromosomal DNA was isolated from the mutant and digested with *Eco*RI. *Eco*RI cuts Tn*4351* near one end of each of the directly repeated insertion sequence (IS) elements that flank Tn*4351* but nowhere else in the transposon. The *Eco*RI fragments were ligated into the *Eco*RI site of pUC19. *E. coli* DH5α MCR transformants were plated onto Luria agar containing ampicillin (100 μg/ml) and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; 40 μg/ml). White colonies on X-Gal plates were screened for the Tn*4351* junction fragment by colony hybridization, by using a probe that contained IS

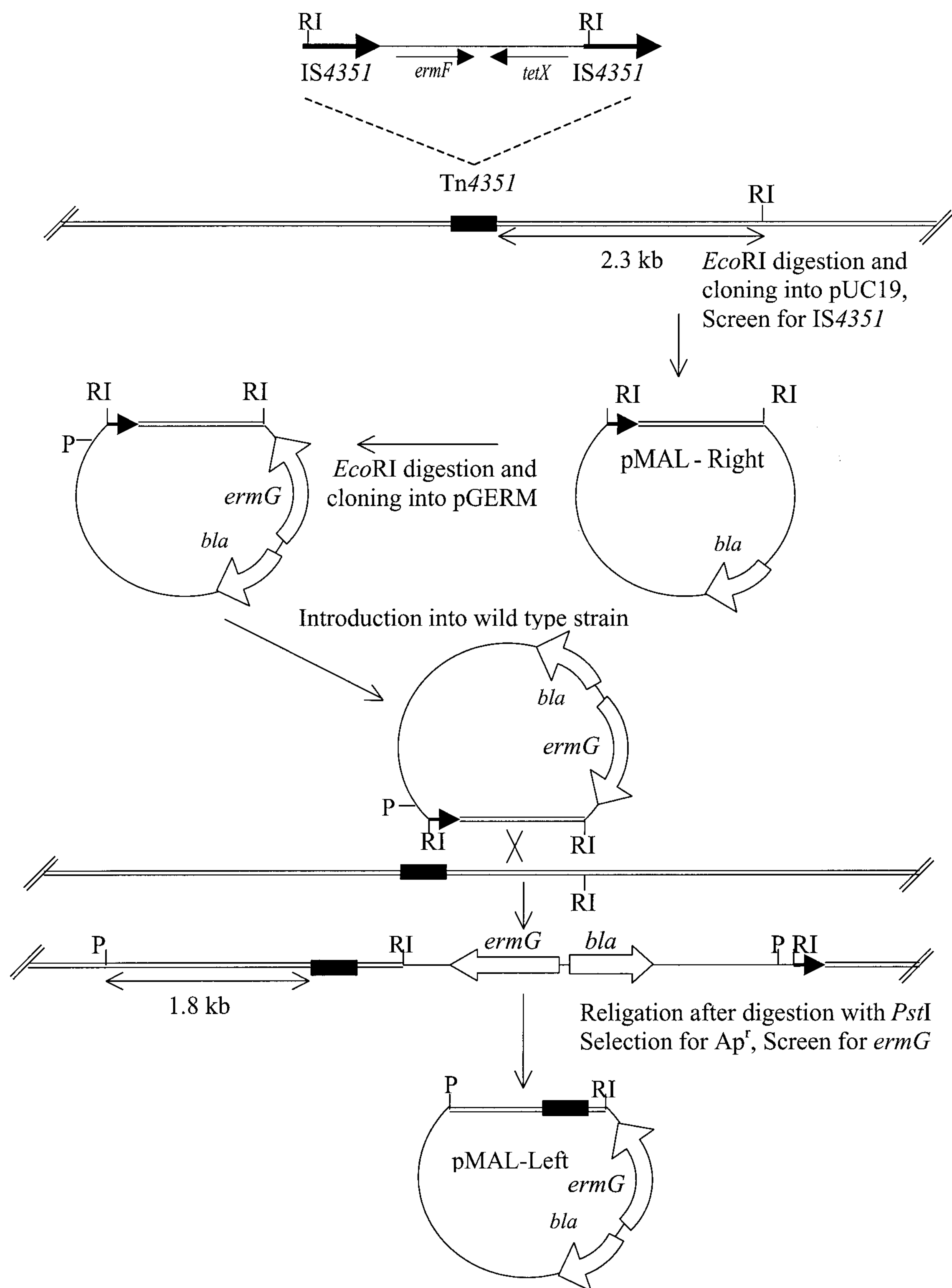

FIG. 1. Cloning of pMAL-Right and pMAL-Left to sequence the region where Tn*4351* is inserted in BTMAL. The position of the transposon insertion in BTMAL is indicated by the black rectangle. The heavy horizontal arrows in Tn*4351* indicate the direct repeat insertion sequence (IS*4351*). Tn*4351* carries an 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. pGERM, a suicide vector in *Bacteroides*, has a different erythromycin resistance gene (*ermG*). The double lines (=) indicate *Bacteroides* chromosomal DNA. Abbreviations: RI, *Eco*RI; P, *Pst*I

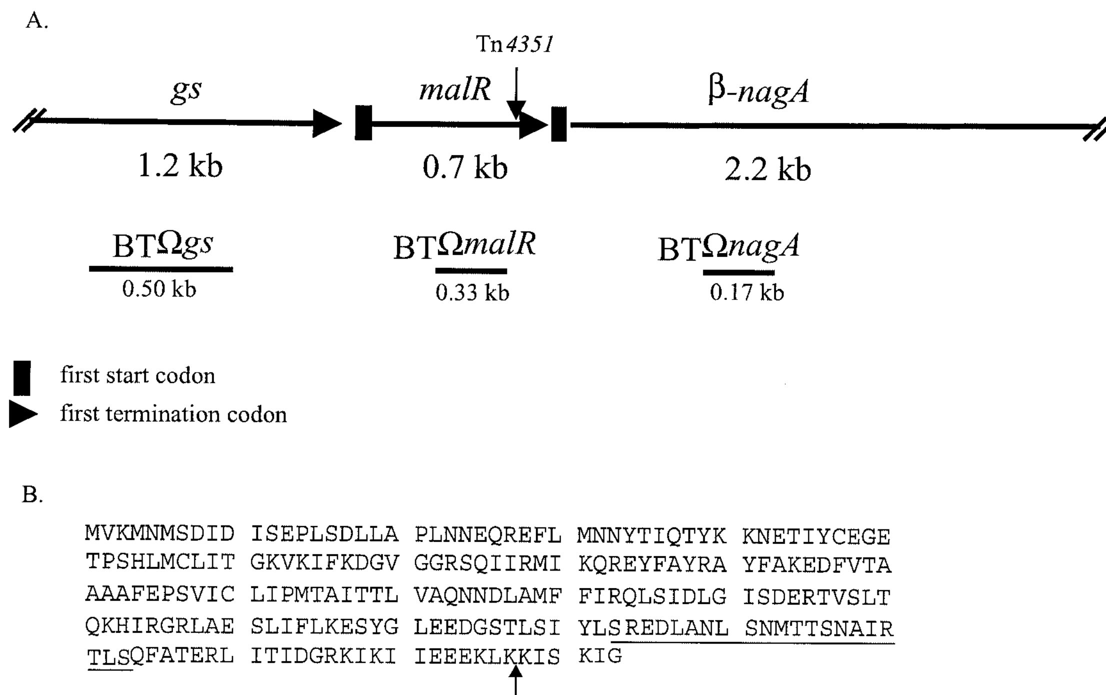

FIG. 2. (A) Map showing the relative locations of ORFs in the *malR* gene area. The position of the transposon insertion in mutant BTMAL is indicated by the vertical arrow above the map. DNA segments used to make insertional disruptions are shown as horizontal lines under the map and marked with an "Ω." The sizes of the DNA fragments used to make the disruptions are also indicated under the lines. *gs*, putative glutamine synthetase gene; *malR*, putative regulatory protein gene; β-*nagA*, putative β-*N*-acetylglucosaminidase gene. (B) Deduced amino acid sequence of *malR*. A possible carboxy-terminal helix-turn-helix motif is underlined. The vertical arrow indicates the transposon insertion site in the transposon-generated mutant, BTMAL.

element DNA. To clone the other chromosomal junction, we first recloned the *Eco*RI fragment containing IS DNA into pGERM. This clone was transferred to wild-type *B. thetaiotaomicron* to create a single crossover insertion in the cloned region. Chromosomal DNA from the resulting strain was digested with *Pst*I, religated, and then transformed into *E. coli* with selection for ampicillin. *Pst*I cuts only once in pGERM and not at all in the IS element. Thus, ampicillin-resistant transformants should contain chromosomal DNA adjacent to the insertion site on both sides of the transposon insertion.

The clones were sequenced by the University of Illinois Biotechnology Automated Sequencing Facility (University of Illinois Biotechnology Center, Urbana). The BLAST network service was used to search for proteins in the databases that have homology with the open reading frames (ORFs) of the sequenced DNA.

Enzyme assays. α-Glucosidase activity and amylase activity in sonically disrupted cell extracts were measured by determining the rate of hydrolysis of *p*-nitrophenyl-α-D-glucopyranoside and 4-nitrophenyl-α-D-maltoheptaoside-4,6-*O*-ethylidene, respectively. α-Glucosidase activity was measured as described by Smith and Salyers (20). Amylase activity was measured with 2 mM 4-nitrophenyl-α-D-maltoheptaoside-4,6-*O*-ethylidene in potassium phosphate buffer (20 mM, pH 6.5) at 37°C (Boehringer Mannheim Biochemicals). The protein concentration in each extract was measured by using the Bio-Rad DC protein kit. The K_m values of the α-glucosidases were calculated from a Lineweaver-Burke plot. BTΩ*susB* was used to determine the K_m of the second α-glucosidase, and BTΩ*malR* was used to determine the K_m of the *susB*-encoded α-glucosidase. β-Glucuronidase (GUS) assays, used to monitor expression of *uidA* (GUS) fusions, were done as described by Feldhaus et al. (6).

malR gene expression. To provide *malR in trans* on a multicopy plasmid, *malR* was amplified by PCR with primers TCAAAGTACTGGATCCCGAAATGA CC, which lies ca. 300 bp upstream of the first start codon in the ORF, and TATATTGACAGGATCCATGTACTTGT, which lies ca. 150 bp downstream of the first stop codon in the ORF. This product (ca. 1.2 kb) was cloned into pT-COW, which has a copy number in *Bacteroides* of 5 to 10 (22). The resulting vector was called pMALR.

To create a *malR-uidA* (GUS) fusion for studies of *malR* expression, a DNA segment including the promoter region of *malR* was first amplified by PCR with primers TCAAAGTACTGGATCCCGAAATGACC and AATCAGCGATGG ATCCAGACGTCCAC, a sequence which lies ca. 210 bp upstream of the first stop codon in the ORF, and then cloned into pMJF-2, a shuttle vector which has the GUS gene clomed downstream of a multiple cloning site. The resulting vector was called pMALRGUS.

To monitor *malR* expression in the chromosome, a DNA segment containing an internal portion of the *malR* gene was amplified by PCR, by using the forward primer CCTCTCATTTGAGGATCCTCATTACC and the reverse primer AAT CAGCGATGGATCCAGACGTCCAC, and then cloned into pCQW-1, a GUS suicide vector (6). This vector was called pΩMALRGUS. The plasmids were transferred to *Bacteroides* strains by conjugation.

Reverse transcription-PCR (RT-PCR) was also used to examine the expression of *malR. B. thetaiotaomicron* was grown on the defined medium containing 0.3% (wt/vol) glucose or maltose as a sole carbon source. A portion (5 ml) of the culture was harvested at an optical density at 650 nm of 0.3. A Qiagen RNeasy kit (Qiagen, Chatsworth, Calif.) was used for the isolation of total RNA from cells. To prevent DNA contamination, the RNA was treated with Recombinant RNasin RNase Inhibitor and RQ1 RNase-free DNase (Promega, Madison, Wis.). Superscript II RNase$^-$ reverse transcriptase (Gibco-BRL) was used for the synthesis of cDNA from the isolated RNA.

Nucleotide sequence accession number. The nucleotide sequence of *malR* region (Fig. 2A) has been deposited in GenBank under accession number AF391102.

RESULTS

Isolation of a mutant with reduced ability to grow on maltose and maltotriose. A screen of thousands of transposon-generated mutants of wild-type *B. thetaiotaomicron* was done to find mutants that had lost the ability to utilize maltose or maltotriose. No such mutants were found. A possible explanation for the failure to find such mutants was that the *sus* genes were contributing to the utilization of maltose and maltotriose,

and this redundancy with the other maltose or maltotriose utilization genes made it impossible for a single transposon insertion to abolish maltose utilization. Accordingly, a mutant with a disruption in *susR* (BTΩ*susR*) was used as the background for transposon mutagenesis. This mutant did not produce any of the Sus structural proteins. Over 15,000 transposon-generated mutants were screened for maltose and maltotriose utilization. One mutant was found that was deficient in the ability to grow on maltose and maltotriose. The mutant was named BTMAL. The growth rate of BTMAL on glucose (0.45 h^{-1}) was the same as that of the parent strain, BTΩ*susR,* but BTMAL had one-fourth the growth rate of the parent strain on maltose (0.11 h^{-1}) and did not grow at all on maltotriose.

The DNA sequence of a 4.1-kb chromosomal DNA segment, in which the transposon had inserted, was analyzed. There were three possible ORFs in this segment. The transposon had disrupted a small *orf* in the middle of the segment (Fig. 2A). This small *orf*, which was 699 bp in size, was designated *malR*. The deduced amino acid sequence of the MalR protein had a low amino acid sequence homology (21 to 26% identity, 45 to 47% similarity) to transcriptional regulators of the Crp/Fnr family from *Bacillus subtilis*, *Pseudomonas* spp., and *Aquifex aeolicus*. A possible helix-turn-helix DNA-binding motif was found in the carboxy terminus (Fig. 2B). Tn*4351* inserted 18 bp upstream of the 3′ end of *malR*.

The amino acid sequences of the ORFs upstream and downstream of *malR* had significant sequence homology to a glutamine synthetase from *Bacteroides fragilis* and a putative β-*N*-acetylhexosaminidase from *Porphyromonas gingivalis*, respectively. Thus, they seemed unlikely to be involved in maltose utilization. Nonetheless, single crossover disruptions were constructed in each of the three ORFs in BTΩ*susR*. The only disruption that had the same phenotype as the mutant was the disruption in *malR* (BTΩ*susR*Ω*malR*; Fig. 3). Hence, *malR* alone among these ORFs was responsible for the reduced maltose utilization phenotype (Fig. 3). A disruption of *malR* in the wild-type background (BTΩ*malR*) reduced somewhat the rate of growth on both maltose and maltotriose, but the bacteria were still able to grow on these substrates (Fig. 3). This result supports the hypothesis that maltotriose and maltose are utilized via the *sus* system, as well as by the *mal* system.

The *malR* gene controls expression of the second α-glucosidase. α-Glucosidase and amylase activities in the various mutant strains were measured. Amylase activity was used as an indicator of the *sus* gene expression because the only amylases produced by *B. thetaiotaomicron* are encoded by *susA* and *susG* (16). Thus, the activity of these proteins is a good indicator of the activity of proteins encoded in this region. As expected, BTΩ*susR* had no detectable amylase activity and had lower α-glucosidase activity than the wild type (Table 2). The α-glucosidase activity remaining in extracts from the BTΩ*susR* mutant did not come from SusB; when we disrupted *susB*, the level of α-glucosidase activity was similar to that of BTΩ*susR*. When *malR* in BTΩ*susR* was disrupted to create BTΩ*susR*Ω*malR*, virtually all of the α-glucosidase activity disappeared. This was also true of BTΩ*susB*Ω*malR*. Hence, *malR* controls expression of the second α-glucosidase.

Comparison of properties of the two α-glucosidases. Cell extracts from BTΩ*malR* and BTΩ*susB* were used to assay SusB and the second α-glucosidase, respectively. The K_m values of the two α-glucosidases were similar: 132 μM for SusB and 103 μM for the second α-glucosidase. Also, the cellular location of these two α-glucosidase were the same. SusB partitioned to the inner membrane fraction but could be eluted by washing the

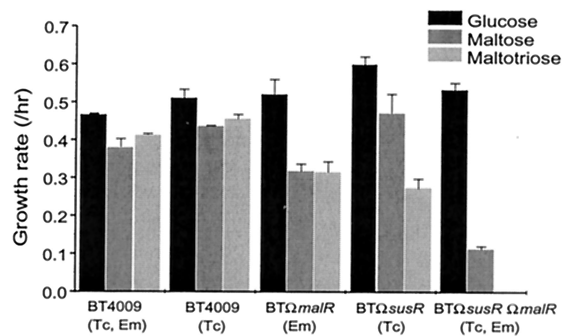

FIG. 3. Growth rates of various mutants on glucose, maltose, and maltotriose. The medium used was defined medium that contained glucose, maltose, and maltotriose, and the results are indicated by the three shaded bars in each set from left to right, respectively. The concentrations of antibiotics used in this experiment were 3 μg/ml for tetracycline (Tc) and 10 μg/ml for erythromycin (Em). These measurements were done in triplicate; the range of values is indicated by the error bars. *B. thetaiotaomicron* BT4009 was used as the wild-type control because it contains a single copy of *tetQ* and *ermF.* Thus, either tetracycline or erythromycin or both (Tc, Em) can be added to the medium used to grow both the control and the mutant strains. This eliminates the slight differences in growth rate that can sometimes occur due to the presence of antibiotics in the medium (note the difference between "Tc" columns versus the "Tc, Em" columns). The selectable marker used to create the BTΩ*malR* strain was *ermF*, and the marker used to create the BTΩ*susR* strain was *tetQ*. The double mutant BTΩ*susR*Ω*malR* contained both resistance genes.

TABLE 2. α-Glucosidase and amylase activities of whole-cell extracts of strains[a]

Strain	Growth substrate (carbon source)	Activity[b] (U/g of cell protein)	
		α-Glucosidase	Amylase
BT5482	Glucose	2.2[c]	<0.6
	Maltose	60.3	49.1
BTΩ*susR*	Glucose	<0.6	<0.6
	Maltose	7.4	<0.6
BTΩ*susB*	Glucose	ND	ND
	Maltose	6.1	84.9
BTΩ*susR*Ω*malR*	Glucose	<0.6	<0.6
	Maltose	1.5	<0.6
BTΩ*malR*	Glucose	<0.6	<0.6
	Maltose	14.0	5.7
BTΩ*susB*Ω*malR*	Glucose	ND	ND
	Maltose	<0.6	4.5

[a] For this assay, strains were grown on minimal medium containing glucose or maltose (0.3%).

[b] The substrates used to detect α-glucosidase and amylase activities are *p*-nitrophenyl-α-D-glucopyranoside and 4-nitrophenyl-α-D-maltoheptaoside-4,6-*O*-ethylidene, respectively. One unit of activity was defined as one micromole of *p*-nitrophenol liberated per minute. This experiment was conducted in duplicate, and the variation between replicates was <10%. ND, not determined.

[c] There was some α-glucosidase activity from wild type on glucose, so there was a basal level expression of α-glucosidase on glucose.

TABLE 3. α-Glucosidase and amylase activities of whole-cell extracts of strains[a]

Strain	Genotype	Growth substrate (carbon source)	Activity[b] (U/g of cell protein)	
			α-Glucosidase	Amylase
BT5482	One copy of *malR* in the chromosome	Glucose	2.2	<0.6
		Maltose	60.3	49.1
BT5482(pMALR)	One chromosomal *malR* and *malR* in *trans*	Glucose	1.8	<0.6
		Maltose	148.5	88.1
BTΩ*malR*(pMALR)	Disrupted chromosomal *malR* and *malR* in *trans*	Glucose	1.8	<0.6
		Maltose	126.4	78.1
Ms-1(pMALR)	Disrupted chromosomal *susR* and *malR* in *trans*	Glucose	ND	ND
		Maltose	6.3	<0.6
BTΩ*susB1*(pMALR)	Disrupted chromosomal *susB* and *malR* in *trans*	Glucose	ND	ND
		Maltose	3.7	80.0

[a] For this assay, strains were grown on minimal medium containing glucose or maltose (0.3%).
[b] The substrates used to detect α-glucosidase and amylase activities are *p*-nitrophenyl-α-D-glucopyranoside and 4-nitrophenyl-α-D-maltoheptaoside-4,6-*O*-ethylidene, respectively. One unit of activity was defined as one micromole of *p*-nitrophenol liberated per minute. This experiment was conducted in duplicate, and the variation between replicates was <10%. ND, not determined.

membrane with 0.5 M NaCl (4). This was also the case for the second α-glucosidase; about two-thirds of the α-glucosidase activity partitioned with the membrane fraction. There was, however, a difference in the stability of the two enzymes in cell extracts. The second α-glucosidase was not stable even at 4°C. The enzyme activity that was detectable in a cell extract from BTΩ*susB* after 14 h at 4°C was just 33% of the initial activity. In contrast, 87% of the original activity was detectable in an extract from BTΩ*malR* that had been stored for 14 h at 4°C.

***malR* regulates expression of *sus* genes.** If *malR* regulates only the expression of the second α-glucosidase, BTΩ*malR* should still have full SusB and amylase (SusA and SusG) activity. That is, the α-glucosidase activity of the *malR* disruption strain (BTΩ*malR*) should be ca. 53 U/g of cell protein, the value of the activity in BT5482 extracts minus the activity in BTΩ*susR* extracts, and the amylase activity should be 49 to 50 U/g of cell protein. However, the α-glucosidase activity in BTΩ*malR* extracts was only 14 U/g cell protein, one-fourth of the expected value (Table 2). Moreover, the amylase activity in BTΩ*malR* extracts was much lower than in extracts from wild type. Thus, *malR* seems to be necessary for full expression of the *sus* genes. To make sure that the α-glucosidase of BTΩ*malR* was due to SusB, we disrupted *susB* in BTΩ*malR* to create BTΩ*susB*Ω*malR*. BTΩ*susB*Ω*malR* has no detectable α-glucosidase activity. Thus, the α-glucosidase activity in extracts from BTΩ*malR* came from SusB. The α-glucosidase specific activity in BTΩ*susR* extracts was almost the same as in BTΩ*susB* extracts, so SusR does not control the expression of the α-glucosidase controlled by MalR.

Interestingly, the amylase activity of BTΩ*susB* was higher than that of wild type. We measured the amylase activities of several mutants with disruptions in genes downstream of *susB* such as *susC*, *susE*, or *susG* and amylase activities of those strains were almost the same as that of wild type (data not shown). Thus, only the disruption in *susB* increased amylase activity from SusA. This increased amylase activity could be due to the fact that maltose, the inducer of *sus* gene expression, was not broken down as rapidly in the cell. Higher levels of maltose could make SusR and/or MalR better able to activate gene expression.

The *malR* disruption in BTΩ*susB* abolished virtually all of the α-glucosidase in the cell extract, the double-disruption strain, BTΩ*susB*Ω*malR*, yet the mutant still grew slowly on maltose (0.11 h^{-1}). Thus, there might be the third α-glucosidase in BT5482 that is not detected by our enzyme assay.

The growth rate of BTΩ*malR* on the starch amylopectin was measured. Even though the *malR* gene disruption lowered the α-glucosidase and amylase activities, the growth rate of BTΩ*malR* (0.39 h^{-1}) on amylopectin was only slightly lower than that of the wild type (0.49 h^{-1}).

Effect of providing *malR* in *trans*. The data from disruption mutants indicated that *malR* had its own promoter, because disruptions in the adjacent ORFs had no effect on maltose utilization. Accordingly, *malR* plus ca. 300 bp of upstream DNA was cloned to produce pMALR and was introduced into wild type and BTΩ*malR*. The plasmid in which *malR* was cloned has a copy number of ca. 5 to 10 copies per cell (22). When pMALR was present in BTΩ*malR,* α-glucosidase and amylase activities were twofold higher than those of BTΩ*malR* (Table 3)*,* so *malR* in *trans* complemented the disrupted chromosomal *malR* and increased expression of starch utilization genes. This same effect was seen when pMalR was introduced into the wild-type strain (Table 3). These results showed that MalR is not an α-glucosidase but is a regulatory protein that regulates positively genes encoding α-glucosidase, amylase genes (*susA* and *susG*) and presumably *susC* to *susF* as well. The growth rate of BT5482(pMALR) on amylopectin was 0.61 h^{-1}, a value slightly higher than that of a control strain, BT5482(pT-COW), which contained only the vector into which *malR* was cloned (0.52 h^{-1}).

To determine whether *malR* exerted its effect on expression of the *sus* genes in the absence of SusR, pMALR was transferred into Ms-1, a mutant strain of *B. thetaiotaomicron* strain that has a transposon insertion in *susR*. In this background multiple copies of *malR* in *trans* did not restore expression of the *sus* genes (Table 3).

pMALR was transferred into BTΩ*susB*, a *susB* disruption strain, to determine whether multiple copies of *malR* in *trans* increased the activity of the second α-glucosidase. The α-glucosidase activity in BTΩ*susB*(pMALR) did not increase com-

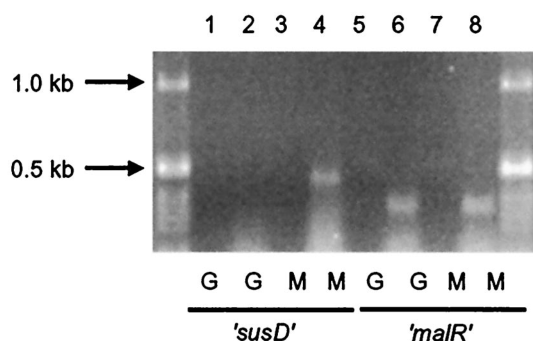

FIG. 4. Detection of *malR* expression on glucose and maltose by using RT-PCR. Odd-numbered lanes contain negative controls in which the reaction was done without the RT step. The expression of the maltose-regulated *susD* gene was used as a control to assess whether we could detect regulated gene expression (lane 2, glucose [G]-grown cells; lane 4, maltose [M]-grown cells). The region amplified from *susD* was 0.5 kb in size. To check *malR* expression (lanes 6 and 8), a 0.4-kbp region of the mRNA was amplified. The 1-kb ladder (Gibco-BRL) is seen in the two outside lanes.

pared to BTΩ*susB*. Thus, excess MalR does not have the same effect on the expression of the second α-glucosidase as it does on *sus* gene expression. The amylase activity of BTΩ*susB* (pMALR) was almost the same as that of BTΩ*susB*. Thus, multiple copies of *malR* in BTΩ*susB*(pMALR) did not increase amylase activity further.

malR is expressed constitutively, and its expression is not autoregulated. RT-PCR was used to determine whether *malR* is expressed constitutively or is induced by maltose. Expression of *susD* was used as a control. RT-PCR detected *susD* mRNA only when cells were grown on maltose (Fig. 4). In contrast, *malR* mRNA was detected in cells grown on glucose as well as in cells grown on maltose (Fig. 4). Thus, *malR* is expressed constitutively.

To confirm the RT-PCR data and to determine whether the expression of *malR* is autoregulated, a GUS fusion shuttle vector was used to monitor *malR* expression. A DNA segment that contained the *malR* promoter region and the 5′ end of *malR* cloned into pMJF-2 (pMALGUS) produced similar levels of GUS activity on glucose and maltose (Table 4). pMALR was transferred into BT5482(pMALRGUS) to create BT5482 (pMALRGUS, pMALR). The two plasmids are compatible and have approximately the same copy number (5 to 10). Therefore, this arrangement should provide a similar level of MalR relative to the *malR* promoter, as is present in the wild type, but a higher level of MalR relative to the GUS fusion than in the strain that contained only the GUS fusion plasmid and one copy of *malR* in the chromosome. The GUS activity of this strain was almost the same as that of BT5482 (pMALRGUS) (Table 4). The fact that expression from the *malR* promoter was not affected by different amounts of MalR in the cell suggests that the expression of *malR* is not autoregulated.

DISCUSSION

Our results suggest that the genes controlled by *susR* and *malR* encode most or all of the proteins responsible for utilization of maltotriose. Disrupting both of these genes abolished growth on maltotriose and severely reduced growth on maltose. Proteins encoded by the *sus* genes aid in maltotriose utilization but do not contribute significantly to utilization of maltose because disruption of *susR* decreases growth on maltotriose but not growth on maltose. In contrast, disruption of *malR* decreases the rate of growth on both maltose and maltotriose. The *malR* gene was not linked genetically to the gene encoding the second α-glucosidase or to any other structural genes that may be under MalR control, so there is as yet little information about the genes MalR controls other than the *sus* genes. An attempt to purify the second α-glucosidase failed due to the instability of the enzyme (data not shown), so it was not possible to use N-terminal sequencing as a basis for designing a probe to locate the gene encoding it. The K_m for this enzyme was virtually identical to that of SusB. In fact, the only difference between the two enzymes was stability in cell extracts. This raises the question of why there are two α-gluco-

TABLE 4. GUS specific activities of whole-cell extracts of strains[a]

Strain	Genotype	Growth substrate (carbon source)	GUS activity (U/mg of cell protein)[b]
BT5482	One copy of *malR* in the chromosome	Glucose	<1.0
		Maltose	<1.0
BT5482(pMALRGUS)	One copy of *malR* in the chromosome and GUS gene downstream of the promoter of *malR* in *trans*	Glucose	33.6
		Maltose	40.4
		Amylopectin	41.9
BT5482(pMALRGUS, pMALR)	One chromosomal *malR*, *malR* in *trans*, and GUS gene downstream of the promoter of *malR* in *trans*	Glucose	39.5
		Maltose	31.2
BTΩ*malR*GUS	GUS gene downstream of the promoter of the chromosomal *malR*	Glucose	ND
		Maltose	<1.0
BTΩ*malR*GUS(pMALR)	GUS gene downstream of the promoter of chromosomal *malR* and *malR* in *trans*	Glucose	ND
		Maltose	<1.0

[a] For this assay, strains were grown on the defined medium plus glucose or maltose (0.3%).
[b] Units are expressed in micromoles per minute. The substrate used to detect GUS activity is *p*-nitrophenyl glucuronide. This experiment was conducted in duplicate, and the variation between replicates was <10% except for BT5482(pMALRGUS, pMALR). BT5482(pMALRGUS, pMALR) produced a lot of capsule so the cell mass was much less than in other strains (ca. 10 to 20% of the wild-type level). The variation between replicates of this strain was 20 to 25%. ND, not determined.

sidases, which appear to have redundant characteristics, and why one is associated with the *sus* system and one is not.

MalR appears to be a regulatory protein. Not only does it have amino acid similarity to known regulatory proteins, but its loss affects the activities of more than one protein. This latter observation supports the hypothesis that MalR controls the transcription of the genes encoding these proteins and probably other genes as well. The fact that the transcription of *susB* is affected by a *malR* disruption indicates that transcription of the other genes in the *susB-susG* operon would also be affected. An unexpected role of *malR* is its effect on *sus* gene expression. The *sus* genes were thought to be controlled only by *susR*, but results reported here show that disruption of *malR* reduced expression of the *sus* structural genes by 5- to 10-fold. Loss of SusR, however, did not seem to affect the expression of the α-glucosidase controlled by MalR. Since both *susR* and *malR* are expressed constitutively, it is likely that SusR and Mal R proteins interact with each other rather than one controlling the other's expression.

An alternative possibility is that the second α-glucosidase or one of the other proteins encoded by MalR-controlled genes is not involved in catabolism of maltose but rather converts maltose to a derivative that is a more effective inducer than maltose itself. In this case, the drop in the expression of *susA* and *susB* in the *malR* disruption strain would be due to the fact that maltose is now the only inducer available to interact with SusR. This would explain why MalR seems to influence expression of the *sus* genes but SusR does not seem to influence expression of the MalR-controlled α-glucosidase. If SusR and MalR proteins formed a complex that increases transcription of the *sus* genes, one would expect this same complex to be responsible for expression of the MalR-controlled genes. The hypothesis that a MalR-controlled gene encodes an enzyme that makes a better inducer would also explain why expression of the *sus* genes is decreased in the *malR* disruption strain but does not fall to zero.

The results reported here suggest that the MalR regulon and the SusR regulon together account for all of the genes needed to grow on maltotriose and starch. The very low rate of growth of the BTΩ*susB*Ω*malR* strain on maltose could be due to uptake and breakdown of maltose by transporters and enzymes that normally handle a closely related substrate such as melibiose or lactose. Given that the *mal* and *sus* systems account for most or all of the utilization of small glucosides, the finding that the BTΩ*malR* strain still grew on starch confirms that the *susA-susG* gene products are solely responsible for the processing of starch. The fact that the *malR* disruption strain was able to grow on starch nearly as well as wild type was surprising since expression of the *sus* genes was decreased five- to ninefold in that strain. This observation can be explained by noting that *B. thetaiotaomicron* probably never encounters in nature concentrations of starch as high as those it encounters in laboratory medium. The bacteria may be optimized to operate with enzyme levels lower than those seen in bacteria growing on high concentrations of maltose. Whatever the explanation for the effect of the disruption in *malR* on *sus* gene expression, it is clear that the starch utilization pathway and the pathway controlled by MalR are linked at the metabolic and/or regulatory level.

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