JOURNAL OF BACTERIOLOGY, Dec. 1996, p. 7173–7179 Vol. 178, No. 24
0021-9193/96/$04.00+0

Contribution of a Neopullulanase, a Pullulanase, and an α-Glucosidase to Growth of *Bacteroides thetaiotaomicron* on Starch

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 colonic anaerobe, can utilize three forms of starch: amylose, amylopectin, and pullulan. Previously, a neopullulanase, a pullulanase, and an α-glucosidase from *B. thetaiotaomicron* had been purified and characterized biochemically. The neopullulanase and α-glucosidase appeared to be the main enzymes involved in the breakdown of starch, because they were responsible for most of the starch-degrading activity detected in *B. thetaiotaomicron* cell extracts. To determine the importance of these enzymes in the starch utilization pathway, we cloned the genes encoding the neopullulanase and α-glucosidase. The gene encoding the neopullulanase (*susA*) was located upstream of the gene encoding the α-glucosidase (*susB*). Both genes were closely linked to another starch utilization gene, *susC*, which encodes a 115-kDa outer membrane protein that is essential for growth on starch. The gene encoding the pullulanase, *pulI*, was not located in this region in the chromosome. Disruption of the neopullulanase gene, *susA*, reduced the rate of growth on starch by about 30%. Elimination of *susA* in this strain allowed us to detect a low residual level of enzyme activity, which was localized to the membrane fraction. Previously, we had shown that a disruption in the *pulI* gene did not affect the rate of growth on pullulan. We have now shown that a double mutant, with a disruption in *susA* and in the pullulanase gene, *pulI*, was also able to grow on pullulan. Thus, there is at least one other starch-degrading enzyme besides the neopullulanase and the pullulanase. Disruption of the α-glucosidase gene, *susB*, reduced the rate of growth on starch only slightly. No residual α-glucosidase activity was detectable in extracts from this strain. Since this strain could still grow on maltose, maltotriose, and starch, there must be at least one other enzyme capable of degrading the small oligomers produced by the starch-degrading enzymes. Our results show that the starch utilization system of *B. thetaiotaomicron* is quite complex and contains a number of apparently redundant degradative enzymes.**

Bacteroides thetaiotaomicron, a gram-negative obligate anaerobe, utilizes three forms of starch: amylose, amylopectin, and pullulan. Amylose is a linear polymer of α-1,4-linked glucose residues. Amylopectin is a branched polymer consisting of amylose chains attached to an amylose backbone by α-1,6 linkages. Pullulan is a linear polymer of maltotriose units (α-1,4-linked glucose trisaccharides) joined by α-1,6 bonds. *B. thetaiotaomicron* grows on all three of these starches nearly as rapidly as on glucose and maltose. Previously, Smith and Salyers (13, 14) reported the purification and characterization of two enzymes whose substrate spectrum suggested that they would be involved in the initial breakdown of starch. One of these enzymes was a neopullulanase, which attacked the α-1,4 linkages in all three forms of starch to produce disaccharides or trisaccharides (14). The second was a pullulanase, which attacked the α-1,6 bonds of pullulan and produced maltotriose (13). The pullulanase gene, *pulI*, was cloned by screening libraries of *B. thetaiotaomicron* DNA for starch-degrading activity in *Escherichia coli* (13), but no clones expressing neopullulanase activity were found by this approach. In their biochemical analysis of starch-degrading enzymes of *B. thetaiotaomicron*, Smith and Salyers (14) also identified and partially purified an α-glucosidase, which hydrolyzed the α-1,4 linkages of maltose and maltotriose and the α-1,4 and α-1,6 linkages of the trisaccharide products of the action of neopullulanase on pullulan. The α-glucosidase attacked only short oligomers and had no activity on full-length starch.

The neopullulanase appeared to be the major starch-degrading enzyme produced by *B. thetaiotaomicron*. The pullulanase accounted for less than 30% of the total pullulan-degrading activity and made no contribution to the breakdown of amylose or amylopectin (13). Moreover, since disruption of *pulI* in *B. thetaiotaomicron* had no effect on the ability of the mutant strain to grow on pullulan, this gene was clearly not essential for growth on pullulan (13). When the neopullulanase was purified, no other pullulan-degrading or amylose-degrading activities were noted during the purification process. However, since the final yield of enzyme after purification was low (14), it was possible that other starch-degrading enzymes had been present in cell extracts but had been lost during purification or were poorly detected by the assay conditions used in these studies. The α-glucosidase of *B. thetaiotaomicron* was only partially purified (14). The final α-glucosidase fraction contained three major proteins of 80, 57, and 50 kDa, and it was not clear which of these proteins was the α-glucosidase or whether there was more than α-glucosidase in this fraction. To determine whether the neopullulanase and α-glucosidase described by Smith and Salyers (14) played an important role in starch utilization by intact bacteria, it was necessary to clone the genes that encoded them and create mutants with disruptions in these genes.

We were able to clone these genes by taking advantage of a transposon insertion mutant we had obtained previously, Ms-5 (2). In our initial study of Ms-5, in which we tested growth on starch by using agar medium and an incubation time of 2 to 3

* 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.

days, Ms-5 had appeared to be completely unable to grow on starch. More recent studies of Ms-5 have revealed that Ms-5 can grow on amylose, amylopectin, and pullulan but only after a long lag period. Another characteristic of Ms-5 was that its α-glucosidase activity was much lower than that of the wild type and was produced almost constitutively (2). Normally, the production of α-glucosidase activity is tightly regulated, and high levels of this enzyme are seen only if the bacteria are grown in medium containing maltose or starch. The fact that the α-glucosidase activity in Ms-5 was low and was expressed constitutively suggested that the transposon either had interrupted a regulatory gene or had inserted in the promoter region of the α-glucosidase gene. In this paper, we report that the transposon insertion in Ms-5 occurred in the promoter region of the α-glucosidase gene and that this gene lies immediately downstream of the gene encoding the neopullulanase. We also report the effect of disrupting these two genes on the ability of *B. thetaiotaomicron* to utilize starch.

Smith and Salyers (14) reported that most of the neopullulanase and α-glucosidase activity in sonicated cell extracts partitioned to the membrane fraction but could be eluted from the membranes by washing them with 0.5 M NaCl (14). Thus, the enzymes appeared to be membrane associated but not to be integral membrane proteins. The pullulanase, by contrast, was located in the soluble fraction. No further localization of the membrane-associated neopullulanase and α-glucosidase activities to the inner or outer membrane was done. Anderson and Salyers (1), working with spheroplasted cells, found that some of the neopullulanase activity was released by the spheroplasting process, suggesting that the enzyme might be located on the periplasmic side of the inner membrane whereas the α-glucosidase activity was released only when the spheroplasts were disrupted. These results suggested that the starch-degrading enzymes were in the periplasm or cytoplasm, not on the bacterial surface. However, Anderson and Salyers (1) also found a small but significant fraction of the neopullulanase and α-glucosidase activities in extracellular fluid from centrifuged cells. This could have been due to cell lysis, because similar amounts of known cytoplasmic proteins were released under the same conditions, but it raised the possibility that there are some excreted enzymes in addition to the cell-associated ones. In this report, we provide evidence that the enzyme activities detected in extracellular fluid from centrifuged cells were not true extracellular enzymes.

MATERIALS AND METHODS

Strain and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. The construction of various mutants used in this study is also described in Table 1. Ms-5, the Tn*4351*-generated mutant, has been described previously (2). All *E. coli* strains used in this study were grown overnight in Luria-Bertani broth or on Luria-Bertani agar at 37°C. All *Bacteroides* strains were grown in a prereduced Trypticase-yeast extract-glucose (TYG) liquid medium or agar (14). For growth rate or gene expression experiments, strains were transferred to defined medium (14) containing 0.3% glucose, maltose, amylose, amylopectin, or pullulan as the sole carbon source. The concentrations of antibiotics used for selection were ampicillin, 200 μg/ml; erythromycin, 10 μg/ml; tetracycline, 3 to 10 μg/ml; and gentamicin, 200 μg/ml.

DNA manipulation. Plasmid DNA was isolated from *E. coli* and *Bacteroides* strains by the method of Ish-Horowitz as described by Maniatis et al. (10). Total cellular DNA from *Bacteroides* strains was isolated by the method of Saito and Muira (12). All restriction digests, blunting reactions, and ligations 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 (9). Chromosomal insertions in *Bacteroides* genes were done as previously described (15). Southern hybridization was done as described by Maniatis et al. (10). To disrupt the *susA* gene, an internal 768-bp region of the gene was amplified by PCR and cloned into pLYL001B, a plasmid that does not replicate in *Bacteroides* spp. (Table 1). The primers were GCG GAT CCG CCC CGA CCT TTT GAT GGG CAG G and GCG GAT CCT TCG TAA TCG GAA GCA TAC GGG. The left primer was designed to introduce a stop codon in the correct reading frame near the amino terminus of the gene, in hopes that this would cause early termination of translation. The disruption mutant constructed with the cloned PCR fragment was designated *susA*::pLYL001B.

Two strains with disruptions of *susB* were constructed. One was generated with a 905-bp *Xba*I-*Hin*cII fragment cloned into the suicide vector pBT-1. The cloned insert extended from 100 bp upstream of the first possible translational start site and extended into the open reading frame (*susB1*::pBT-1 [see Fig. 1]). A smaller, 507-bp *Bst*UI fragment, which was completely internal to the open reading frame, was cloned into suicide vector pNJR6 and used to create a disruption of *susB* (*susB2*::pNJR6 [see Fig. 1]). Different suicide vectors, with little sequence similarity to each other, were used to facilitate the later construction of double mutants. A disruption that separated *susB* from the downstream gene, *susC*, but left both open reading frames intact and provided a heterologous promoter to run the downstream genes was constructed by using a 953-bp *Bst*UI-*Hin*cII fragment that extended from the interior of *susB* into the interior of *susC*. This fragment was cloned downstream of the promoter region from the constitutively expressed *B. thetaiotaomicron* gene *chuR* (3) in suicide vector pCHURB and used to construct the mutant *susBC*::pCHURB (Table 1). A *Pvu*I-*Hin*cII internal fragment of the *pulI* gene was cloned into suicide vector pCQW-1. This construct was used to generate the *pulI* disruption strain, *pulI*::*uidA* (Table 1). Double mutants were constructed with combinations of these same suicide plasmids. All disruption mutants were checked by Southern blot analysis to confirm that the insertions had occurred in the correct location.

Sequence analysis. Previously, Tancula et al. (18) reported the cloning of a 10-kbp *Eco*RI-*Eco*RV fragment that spanned the region in the *B. thetaiotaomicron* chromosome where the transposon insertion in Ms-5 had occurred. An internal 3.4-kbp *Xba*I fragment and an adjacent 3.1-kbp *Xba*I-*Eco*RV fragment from this large clone were each subcloned into pUC19 in both orientations. Southern blot analysis indicated that the transposon insertion in Ms-5 lay near the end of the 3.1-kbp segment that was nearest one of the *Xba*I sites of the 3.4-kbp clone. We also sequenced a 4.1-kbp *Hin*dIII segment containing the cloned *pulI* gene, which had been cloned into pMJF-2 (pKS30-1). Both strands of these clones were sequenced with the DNA Sequence 2.0 kit (United States Biochemicals, Inc., Cleveland, Ohio) or in an automated sequencing facility at the Biotechnology Center, University of Illinois, Urbana. M13 universal and reverse sequencing primers were used for sequencing unless there was a gap, in which case primers were designed based on known sequences in the region. The GenBank, Swiss-Prot, and Prosite databases were searched for proteins related to the proteins encoded by open reading frames contained within the cloned region. Sequence alignments were prepared with the Genetics Computer Group (GCG) software (4). The phylogenetic tree of the pullulanase genes was constructed by using the Jukes-Cantor distance tree program (GCG).

Enzyme assays. β-Glucuronidase (GUS) assays were done as described by Feldhaus et al. (5). α-Glucosidase and neopullulanase assays were done with *p*-nitrophenyl-α-D-glucopyranoside and *p*-nitrophenyl-α-D-maltopentaoside, respectively, as substrates. Conditions were the same as those described previously by Anderson and Salyers (1). Previous biochemical experiments had suggested that the *p*-nitrophenyl-α-D-maltopentaoside substrate detects the neopullulanase described by Smith and Salyers (14) but not the pullulanase, which attacks only α-1,6 linkages, or the α-glucosidase. This was confirmed by testing crude cell extracts from strains with disruptions in *susA* (neopullulanase gene) or in *susB* (α-glucosidase gene). An extract from the *susA* disruption strain (*susA*::pLYL001B) had no activity on this substrate, indicating that the enzymes encoded by *susB* or *pulI*, which were still produced in this strain, could not attack this substrate. Similarly extracts from the *susB* disruption strain (*susB1*::pNJR6) had no activity on *p*-nitrophenyl-α-D-glucopyranoside, indicating that neither the neopullulanase nor the pullulanase had activity against this substrate. In our experience, levels of enzyme activity detected with the *p*-nitrophenyl-D-maltopentaoside substrate reflect accurately the levels of neopullulanase activity measured by a reducing-sugar assay with amylopectin or pullulan as the substrate, except that the *p*-nitrophenyl-D-maltopentaoside assay is somewhat more sensitive. Neither of these assays would detect a glycosidase that cleaves phosphorylated sugars. Efforts to detect such an enzyme in extracts from *B. thetaiotaomicron* have so far yielded negative results (data not shown).

Polyclonal antisera that recognized the *susA* and *susB* gene products. Portions of *susA* and *susB* (see Fig. 1) were subcloned in the T7 expression vector, pET3B (16), to produce pSUSA and pETSB. Overexpression of both of these proteins in *E. coli* produced inclusion bodies. The inclusion bodies were collected by centrifugation and solubilized as described previously (11). The overproduced proteins were extracted from sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and used to immunize rabbits. The resulting antisera were used to determine the actual size and cellular location of SusA and SusB in *B. thetaiotaomicron.*

Cellular localization of SusA and SusB. Preparation of the various cellular fractions and Western blot analysis of these fractions were done as described previously by Reeves et al. (11). Both the soluble fraction and the extracellular fraction were concentrated 50-fold in a Centricon to make them comparable in concentration to the membrane fraction, which was resuspended in 1/50 the initial volume of phosphate buffer. In the concentrated fractions, 8 to 12% of the neopullulanase and α-glucosidase activities were found in the extracellular fraction, 48 to 50% were found in the membrane fraction, and 39 to 41% were found

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	6
BL21(DE3)	T7 RNA polymerase source	15
B. thetaiotaomicron		
5482	Wild type, Gn^r	Anaerobe Lab, Virginia Polytechnic Institute, Blacksburg
Ms-5	Em^r Gn^r $G7^+$ Am^+	Tn*4351*-generated mutant of strain 5482 (1)
susA::pLYL001B	Tc^r Gn^r $G7^+$ Am^+	*B. thetaiotaomicron* mutant with a disruption created by a 768-bp PCR product internal to *susA*, which was cloned into a suicide vector, pLYL001B (this study) (Fig. 1).
susB1::pNJR6	Em^r Gn^r $G7^-$ Am^-	*B. thetaiotaomicron* mutant with a disruption created by a 507-bp *Bst*BI-*Bst*BI fragment, which was cloned into a suicide vector, pNJR-6 (this study) (Fig. 1).
susC::*uidA*	Em^r Gn^r $G7^-$ Am^-	*B. thetaiotaomicron* GUS fusion mutant created by a 1.1-kbp *Eco*RV-*Xmn*I fragment of *susC*, which was cloned upstream of the β-glucuronidase gene in pCQW-1 (10)
susB2::pBT-1	Tc^r Gn^r $G7^-$ Am^-	*B. thetaiotaomicron* mutant with a disruption created by the 905-bp *Xba*I-*Hin*cII fragment which was cloned into a suicide vector, pBT-1 (this study) (Fig. 1).
susB2::pBT-1/*susC*::*uidA*	Tc^r Em^r Gn^r $G7^-$ Am^-	*B. thetaiotaomicron* mutant, *susC*::*uidA* with an additional disruption created with a 905-bp *Xba*I-*Hin*cII fragment, which was cloned into suicide vector pBT-1 (this study)
susBC::pCHURB	Tc^r Gn^r $G7^+$ Am^+	*B. thetaiotaomicron* mutant with a disruption created by a 953-bp *Bst*UI-*Hin*cII fragment, containing the 3′ end of the *susB* gene and the 5′ end of the *susC* gene, which was cloned into suicide vector, pCHURB, downstream of the *chu*R promoter (this study)
susB1::pNJR6/*susBC*::pCHURB	Tc^r Em^r Gn^r $G7^+$ Am^+	*B. thetaiotaomicron susBC*::pCHURB with a second disruption created with a vector used to generate *susB1*::pNJR6 (this study)
susA::pLYL001B/*pulI*::pVAL7	Tc^r Em^r Gn^r $G7^+$ Am^+	*B. thetaiotaomicron susA*::pLYL001B with a second disruption created with a vector used to generate *pulI*::pVAL7 (13) (this study)
Plasmids		
pLYL001B	$\underline{Ap}^r$ Tc^r	pUC19-based suicide vector used to make insertional mutations *B. thetaiotaomicron* 5482 (10)
pNJR-6	$\underline{Kn}^r$ $\underline{Sm}^r$ Em^r	RSF1010-based suicide vector used to make insertional mutations of *B. thetaiotaomicron* 5842 (14)
pBT-1	$\underline{Kn}^r$ Tc^r	RSF1010-based suicide vector used to make insertional mutations of *B. thetaiotaomicron* 5842 (18)
pCHURB	$\underline{Ap}^r$ Tc^r	pUC19-based suicide vector used to provide the *chuR* promoter (10)
pET-3	$\underline{Ap}^r$	T7 overexpression vector used to overproduce portions of *susA* and *susB* in *E. coli* (14)
pSUSA	$\underline{Ap}^r$	Same as pET-3 but containing a 1.7-kbp *Aat*I-*Xba*I fragment from *susA* cloned into the *Bam*HI site (this study)
pETSB	$\underline{Ap}^r$	Same as pET-3 but containing a 2.4-kbp *Nla*IV-*Nla*IV fragment from *susB* cloned into the *Bam*HI site (this study)

[a] Abbreviations: $G7^+$, grows on maltoheptose; Am^+, grows on amylopectin; Tc^r, tetracycline resistance; Em^r, erythromycin resistance; Gn^r, gentamicin resistance; Kn^r, kanamycin resistance; Ap^r, ampicillin resistance; Sm^r, spectomycin resistance. Underlined antibiotic resistances are expressed only in *E. coli*. Other resistances are expressed only in *Bacteroides* spp.

in the soluble fraction. The inner and outer membranes were separated by the procedure of Kotarski and Salyers (8). This procedure produces inner and outer membrane fractions that contain less than 10% contamination by the other type of membrane (8). Portions of the extracellular, soluble, and membrane fractions were electrophoresed on an SDS-PAGE gel, transferred to a membrane, and probed with antibodies to SusA or SusB. The antibodies were visualized by an alkaline phosphatase-labeled secondary antibody.

Nucleotide sequence accession numbers. The accession number for the *susA* and *susB* sequence is BankIt63729 U66897. The accession number for the *pulI* sequence is BankIt 63855 U67061.

RESULTS

Sequence of the region containing the Ms-5 transposon insertion. Previously, we had found that the transposon insertion in Ms-5 lay about 3 kbp upstream of *susC*, a gene which encodes a 115-kDa maltose-inducible outer membrane protein (11). *susC* has been shown to be essential for growth on maltoheptaose (G7) and starch. We sequenced the region of DNA that started with the amino-terminal end of *susC* and extended about 4 kbp upstream. This 4-kbp region contained two complete open reading frames, which were designated *susA* and *susB* (Fig. 1). Both of these genes were transcribed in the same direction as *susC*. Upstream of *susA* was the beginning of an open reading frame that was transcribed in the opposite direction (results not shown). A clone that contained the junction between the end of Tn*4351* in Ms-5 and the adjacent chromosomal DNA was also sequenced to locate the precise site at which the transposon had inserted. The point of insertion was 13 bp upstream of the first possible start codon of the *susB* coding region (Fig. 1). The location of Tn*4351* suggested the possibility that the transposon insertion had provided a new promoter for *susB* rather than inactivating a gene.

***susA* encodes the neopullulanase.** The first open reading frame, *susA*, encoded a protein with an estimated molecular mass of 65 kDa and a predicted pI of 5.4. A search of the databases for similar proteins revealed that the *susA* gene product shared amino acid sequence similarity with a number

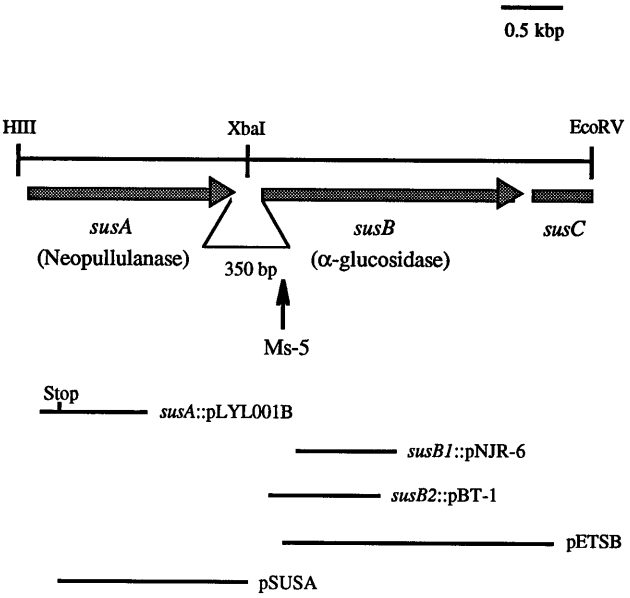

FIG. 1. The location and direction of transcription of *susA*, *susB*, and *susC*, as well as the size of the intergenic region between *susA* and *susB*, are shown at the top. A vertical arrow underneath the intergenic segment shows the location of the transposon insertion in mutant Ms-5. The regions used to construct various gene disruptions and the regions cloned into the pET-3 to overexpress portions of SusA and SusB are shown below the map.

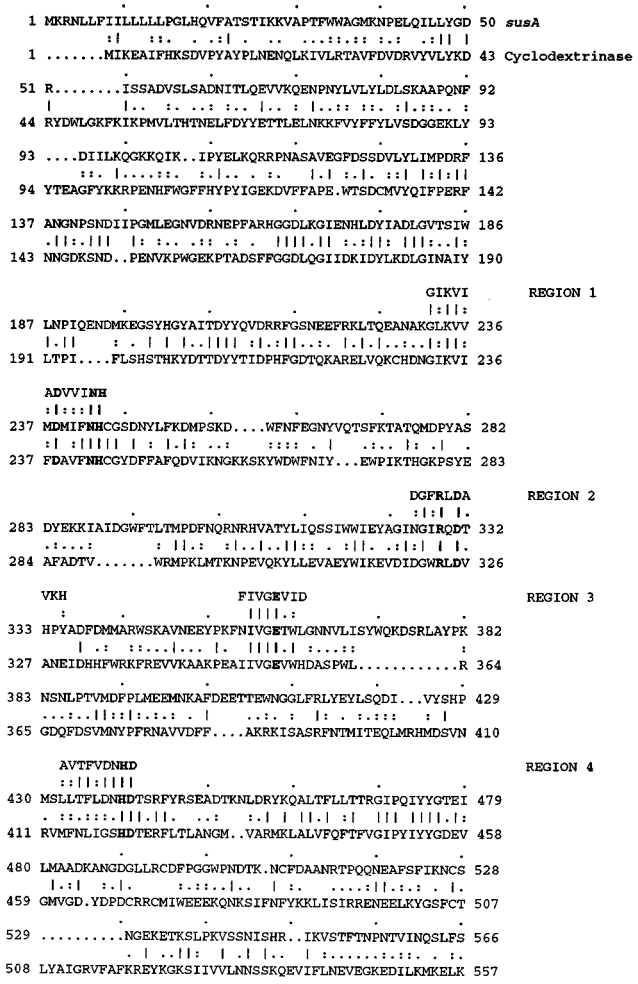

FIG. 2. Alignment of the deduced amino acid sequence of *susA* with that of the closest match, a cyclodextrinase from *C. thermohydrosulfuricum*. Consensus regions found in members of the amylase family are shown by letters above the aligned sequences, and conserved amino acid residues are indicated by boldface type (7, 17). Gaps are indicated by dots between amino acid residues. Vertical bars indicate identical amino acids; : and . indicate conservative differences.

of α-amylases. The closest match was to a *Clostridium thermohydrosulfuricum* cyclodextrinase (47% similarity, 25% identity [Fig. 2]). The neopullulanase, SusA, also has cyclodextrinase activity (14). Janse et al. (7) and Svensson (17) have defined four consensus regions that are normally found in the active sites of amylases. Similar sequences were found in the deduced amino acid sequence of SusA and were present in the expected order (Fig. 2). Moreover, the derived amino acid sequence of SusA had the Asp-331, Glu-360, and Asp-440 residues, which have been shown to be essential for the catalytic activity of α-amylases and other starch-degrading enzymes. α-Amylases differ from neopullulanases in that they attack only the α-1,4 linkages in amylose and amylopectin, not the α-1,4 linkages in pullulan. This is presumably because the α-amylases require a longer stretch of α-1,4-linked glucose residues than the maltotriose units of pullulan. Only a few neopullulanases have been characterized in detail, so it is not known whether the residues that are essential for functioning of the active site of α-amylases are also required for functioning of the active site of neopullulanases.

The predicted size and pI of the SusA protein were virtually identical to those measured previously for the purified *B. thetaiotaomicron* neopullulanase (molecular mass, 70 kDa; pI, 5.4 [14]). Antiserum generated against a segment of SusA reacted with several proteins, but only one of these, a 68-kDa protein, exhibited maltose-regulated expression (Fig. 3). This protein was distributed about equally between the membrane fraction and the soluble fraction of sonicated cells, with a small amount visible in concentrated supernatant fluid (Fig. 3). We used the anti-SusA antiserum to probe inner and outer membrane fractions from wild-type, maltose-induced *B. thetaiotaomicron*. The 68-kDa protein was seen primarily in the inner membrane fraction, with only a trace amount detectable in the outer membrane fraction (Fig. 3).

To determine if a disruption of *susA* eliminated all the neopullulanase activity in cell extracts, we constructed *B. thetaiotaomicron susA*::pLYL001B (Table 1) and tested extracts from this mutant for neopullulanase activity. Extracts from this mutant had less than 8% of the activity detectable in extracts from wild-type *B. thetaiotaomicron* (Table 2). Western blot analysis of these extracts with the anti-SusA antiserum confirmed that 68 kDa was missing in the *susA* disruption mutant. Taken together, these results demonstrate that *susA* encodes the neopullulanase purified previously by Smith and Salyers (14).

Although an extract from the *susA*::pLYL001B mutant contained no detectable SusA protein, as detected by the antiserum, some residual starch-degrading activity could still be detected in cell extracts by using the *p*-nitrophenyl-D-maltopentaoside substrate. This remaining activity was not due to the previously described pullulanase, PulI, because the *p*-nitrophenyl-α-D-maltopentaoside substrate does not detect the pullulanase. Thus, there must be at least one other enzyme in *B. thetaiotaomicron* with activity against the *p*-nitrophenyl-D-maltopentaoside substrate. The residual starch-degrading activity in the *susA* disruption mutant partitioned exclusively with the membrane fraction, and none was detectable in the soluble or extracellular fraction (data not shown).

The *susA* disruption mutant had the same generation time

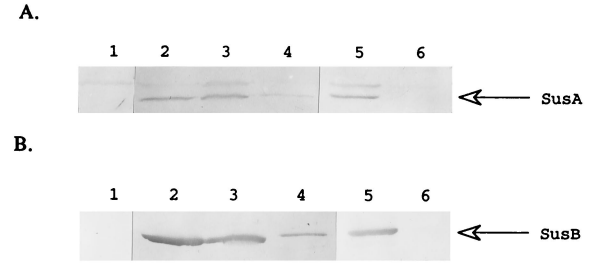

FIG. 3. Western blot showing the distribution of SusA and SusB in different cellular fractions. Only the relevant portions of the blots are shown. (A) Fractions probed with anti-SusA antiserum. This antiserum detected two bands, the lower of which is SusA. Lanes: 1, cell extract from *susA*::pLYL001B; 2, membrane fraction from the wild type; 3, soluble fraction from the wild type; 4, extracellular fraction from the wild type; lanes 5, inner membrane fraction; 6, outer membrane fraction. (B) Fractions probed with anti-SusB antiserum. Lane 1, cell extract from *susB2*::pNJR6. Other lanes were loaded as described for panel A.

on glucose as did the wild type, but when grown on amylopectin or pullulan, it had a generation time that was about 30% longer than that of the wild type (Table 2). Thus, the neopullulanase SusA makes some contribution to growth on amylopectin and pullulan but is not essential for growth on these substrates.

Sequence of the pullulanase gene, *pulI*, and contribution of this gene to growth on pullulan. The *B. thetaiotaomicron* pullulanase gene, *pulI*, has been cloned previously but was not sequenced. To determine how this gene was related to other pullulanase genes in the databases, we obtained the sequence of this gene. *pulI* could encode a protein of 75 kDa, a molecular mass similar to that obtained from biochemical studies of the purified PulI protein, 72 kDa (13). A comparison of the deduced amino acid sequence of PulI with the sequences of other pullulanases in the databases, using a tree-building program, showed that PulI was more closely related to a pullulanase from *Bacillus stearothermophilus* than to a pullulanase from *Klebsiella pneumoniae* (Fig. 4).

pulI has been shown previously not to be essential, because a disruption in the *pulI* gene, *pulI*::pVAL7, did not affect the growth of *B. thetaiotaomicron* on pullulan (13). It was possible, however, that the pullulanase encoded by *pulI* was making some contribution to growth on pullulan that was not easily detectable in a strain that was still producing the neopullulanase, SusA. To test this, we created a double mutant that had a disruption in *susA* and a disruption in *pulI* (*susA*::pLYL001B/*pulI*::pVAL7). The double mutant still grew on pullulan as well as the *susA*::pLYL001B strain did (Table 2).

***susB* encodes the α-glucosidase.** *susB* encoded a protein with an estimated molecular mass of 84 kDa and a pI of 6.0. The molecular mass was close to the molecular mass of an 80-kDa protein in the partially purified α-glucosidase fraction described by Smith and Salyers (14). The predicted pI was also similar to the pI of 5.7 measured for the partially purified α-glucosidase activity (14). A search of the databases revealed no significant similarity between this protein and any known α-glucosidases. This is not surprising, because there are only a few prokaryotic α-glucosidase sequences in the databases and they have little sequence similarity to each other.

Antiserum raised against a fragment of SusB reacted with a single protein whose molecular mass was approximately 84 kDa. The protein was evenly distributed between the soluble and membrane fractions of sonicated cells (Fig. 3). A small amount of SusB was detectable in concentrated extracellular fluid. Western blot analysis of inner and outer membrane fractions revealed that the membrane-associated SusB, like SusA, fractionated almost exclusively with inner membranes rather than with outer membranes (Fig. 3).

A 507-bp internal fragment of *susB* was used to make a single-crossover disruption in *susB*. The protein detected by the anti-SusB antiserum was missing in this mutant (Fig. 3), and cell extracts had lost all detectable α-glucosidase activity (Table 2). Since *susB* was located immediately upstream of *susC*, another gene involved in starch utilization, and since integration of pNJR6 is known to have a polar effect on downstream genes, it was possible that the loss of α-glucosidase activity was not due to disruption of *susB* but, rather, to nonexpression of some downstream gene. This was not the case, however, because a transposon insertion in *susC* (in mutant Ms-2) did not affect the α-glucosidase activity in cell extracts (2, 11). Taken together, our results demonstrate that *susB* encodes the α-glucosidase described by Smith and Salyers (14).

***susA* is in a different transcriptional unit than *susB* and *susC*.** Since *susA*, *susB*, and *susC* were all transcribed in the same direction, the question arose whether these genes were all in the same operon. This was important mainly because if they were, then a *susA* disruption would have a polar effect on *susB* and *susC* and a *susB* disruption would have a polar effect on *susC*, thus complicating the interpretation of experiments to determine the importance of SusA and SusB in intact cells. The *susA* disruption in mutant *susA*::pLYL001B was clearly not polar on *susB*, because extracts from mutant *susA*::pLYL001B had high levels of α-glucosidase activity (Table 2) and production of the enzyme was regulated normally (data not shown). The fact that the α-glucosidase activity of this

TABLE 2. α-Glucosidase, neopullulanase, and GUS activities when the strains where grown on maltose

B. thetaiotaomicron strain	Generation time on amylopectin (hs)[a]	Neopullulanase activity (μmol/min/mg)[b]	α-Glucosidase activity (μmol/min/mg)[b]	GUS activity (U/mg of protein)[c]
Wild type	1.9–2.1	122	250	<0.6
susA::pLYL001B	2.6–2.9	7	111	<0.6
susB1::pNJR-6	—[d]	234	1.7	<0.6
susB2::pBT-1	—	212	1.2	<0.6
susC::*uidA*	—	119	325	63
susB2::pBT-1/*susC*::*uidA*	—	190	2.0	<0.6
susA::pLYL001B/*pulI*::pVAL7	2.7–2.9	6.5	160	<0.6

[a] Generation times on pullulan were essential the same as generation times on amylopectin.
[b] Cells grown on maltose. The specific activity in extracts from cells grown on glucose was <1.5 μmol/min/mg of protein. Variation between replicate values was less than 10%.
[c] Cells grown on maltose. The specific activity in extracts from cells grown on glucose was <0.3 U/mg of protein. Variation between replicate values was less than 10%.
[d] —, not applicable because strains did not grow on starch.

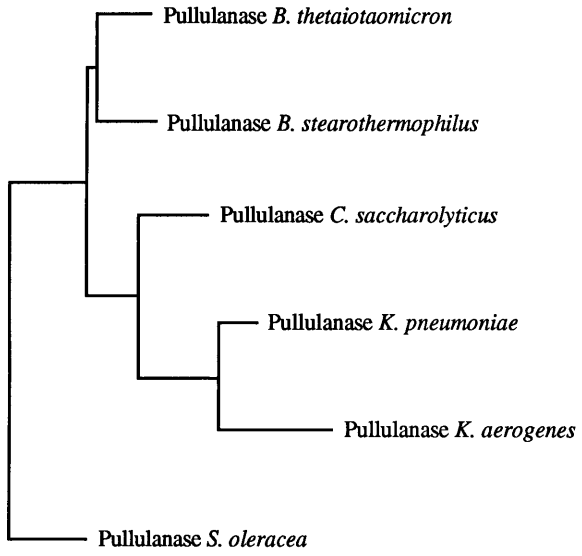

FIG. 4. Phylogenetic tree constructed from the deduced amino acid sequences of *pulI* and pullulanases in the databases, using the Jukes-Kantor distance method (GCG).

mutant was nearly twofold lower than that of the wild type could be because there is some readthrough from the *susA* promoter. Nonetheless, the *susA* disruption in mutant *susA*::pLYL001B did not abolish production of SusB. The *susB* promoter must be located within the 350-bp intergenic region between the end of *susA* and the beginning of *susB*. A disruption mutant, *susB2*::pBT1, which was made with a DNA segment that contained 100 bp upstream of the first possible start site for *susB*, had no α-glucosidase activity (Table 2). Thus, the *susB* promoter lies more than 100 bp upstream of the start site of the gene.

To determine whether a *susB* disruption would have a polar effect on expression of *susC*, we created a *susB* disruption in a mutant that had *uidA* (GUS) fused to *susC* (mutant *B. thetaiotaomicron susC*::*uidA*, previously called ΩMB [11]) to produce mutant *susB1*::pNJR6/*susC*::*uidA*. The *susB* disruption completely abolished the GUS activity of this mutant (Table 2). Thus, the *susB* disruption had a polar effect on the expression of *susC*.

***susB* contributes to starch utilization but is not essential for growth on starch.** As shown in the previous section, the *susB* disruption in mutant *susB1*::pNJR6 eliminates the expression of *susC* by polarity (11). Since *susC* is essential for growth on starch, a mutant with a disruption in *susB* should be unable to grow on starch, and this was in fact the case. Thus, to determine whether SusB made an important contribution to growth on starch, it was necessary to provide a heterologous promoter to ensure the expression of *susC*. To this end, the promoter region of *chuR*, a *B. thetaiotaomicron* gene that is involved in the utilization of chondroitin sulfate and heparin (3), was used to construct a mutant that retained one intact copy of *susB* but now had the *chuR* promoter upstream of *susC* (Fig. 5). This strain was designated *susBC*::pCHURB. We used the *chuR* promoter because it has been localized to a relatively small region (<500 bp) and is expressed constitutively. The small size of this promoter region helps to reduce the likelihood that the suicide vector carrying the *chuR* promoter region will insert in the *chuR* locus rather than in the desired location upstream of *susB*. A drawback to the *chuR* promoter is that it is about eightfold weaker than the normal promoter of *susB* (3, 11). This is probably the reason why *susBC*::pCHURB, the strain with the *chuR* promoter running *susC*, had a doubling time that was more than twice as long as that of the wild type (Fig. 5).

A second insertion was then made in *susB* to create mutant *susB1*::pNJR6/*susBC*::pCHURB (Fig. 5). The double mutant had no detectable α-glucosidase activity, but it grew nearly as rapidly on starch as did the parent strain, *susBC*::pCHURB (Fig. 5). Thus, although the *susB* gene product may make a small contribution to growth on starch, it is not essential. The neopullulanase activity of the double mutant was twofold higher than that of the wild type. We noticed a similar effect

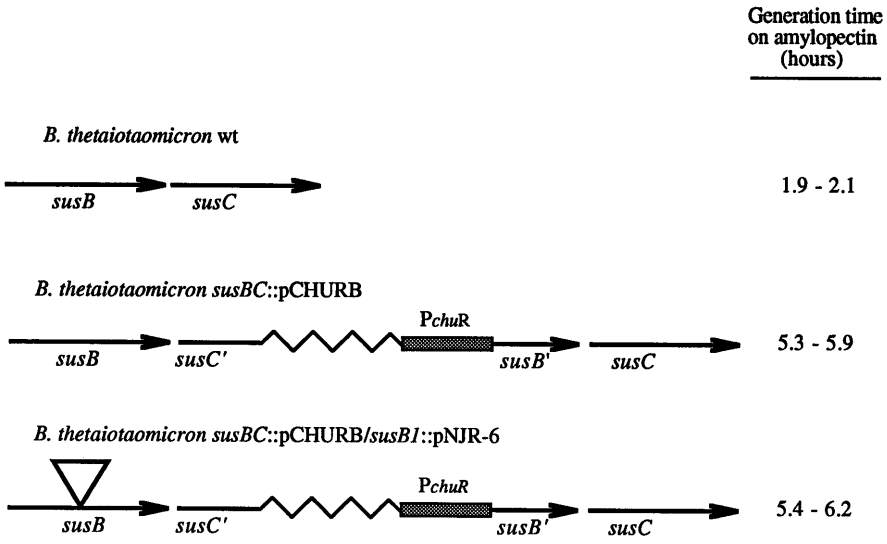

FIG. 5. Mutants used to test the importance of *susB* for starch utilization. Horizontal arrows indicate the extent and direction of transcription of *susB* and *susC*. The wild type (wt) is shown at the top of the figure for comparison. For the mutants, wavy lines indicate where a suicide vector carrying the *chuR* promoter (P*chuR*) was inserted into the chromosome. P*chuR* is shown as a gray rectangle. A triangle represents the *susB* disruption, which was made with the same suicide vector used to make *susB*::pNJR6. The generation times of the mutants grown on amylopectin are given in minutes to the right of the figure. These values indicate the range of values seen in several independent experiments. Generation times of these mutants on pullulan (not shown) were the same as seen with amylopectin.

with the transposon insertion in Ms-5, which occurred immediately upstream of *susB* but outside the coding region of *susA*. We do not know why insertions in *susB* affect the level of SusA activity.

DISCUSSION

We have cloned and sequenced the genes encoding a neopullulanase, a pullulanase, and an α-glucosidase from *B. thetaiotaomicron*. Previously, Smith and Salyers (13) showed that disruption of the pullulanase gene did not affect growth on pullulan. We have now shown that the neopullulanase and α-glucosidase are also not essential for growth on amylopectin or pullulan. In fact, a mutant lacking both the pullulanase and the neopullulanase was still able to grow almost as well as the wild type on pullulan. We had assumed that the neopullulanase and the α-glucosidase would be important enzymes because they were responsible for most of the starch-degrading activity detected in our assay system. Clearly, there are other starch-degrading enzymes that remain to be characterized. Smith and Salyers (14) reported a low level of starch-degrading activity that fractionated with membranes even after repeated washes with 0.5 M NaCl. A portion of this activity had a different pI from that of SusA protein, but we could not be sure whether it was a different enzyme or just a different form of SusA, such as unprocessed SusA. The fact that the mutant with a disruption in *susA* still had some neopullulanase activity that partitioned with membranes suggests that there is a membrane-associated enzyme distinct from SusA.

The phenotype of the transposon-generated mutant, Ms-5, initially caused us to think that the transposon had disrupted a regulatory gene, because the α-glucosidase activity of this mutant was lower than that of the wild type and was no longer regulated by maltose to the same extent as in the wild type. This phenotype could also occur if the transposon inserted upstream of the α-glucosidase gene and provided it with a new promoter. Our results suggests that this is in fact what happened in Ms-5. The very slow growth of Ms-5 on starch is presumably due to the lowered expression of *susC*, which is essential for growth on starch (11).

Although most of the neopullulanase and α-glucosidase activity of *B. thetaiotaomicron* is cell associated, about 10% of the activity can be found in extracellular fluid after centrifugation of the cells (1). This observation raised the question whether the extracellular enzyme activity was due to a separate set of enzymes, distinct from the enzymes responsible for the cell-associated activity, and whether this extracellular activity played a significant role in starch digestion by intact bacteria. Our results strongly support the hypothesis that the neopullulanase and α-glucosidase are entirely cell associated, not extracellular. First, the extracellular neopullulanase activity was due to SusA and not to some other enzyme, because extracellular fluid contained a protein that reacted with anti-SusA antibodies and there was no detectable extracellular neopullulanase activity when the *susA* disruption mutant was tested. Similarly, the α-glucosidase activity in the extracellular fluid was due to SusB. The release of small amounts of SusA and SusB into the extracellular fluid was not due to release of surface-bound enzymes, because the membrane-associated SusA and SusB fractionated preferentially with inner membranes. Release of inner membrane-associated enzymes suggests that these enzymes were released due to lysis of the bacteria.

It could be argued that the release of enzymes by lysis of some cells in a growing culture may be necessary for the initial breakdown of the starch molecule into segments that are easier for the remaining bacteria in the culture to take up. If so, the *susA* disruption strain should have been unable to grow on starch. Yet this strain grew nearly as well as the wild type on amylopectin and pullulan. Further evidence for the hypothesis that the extracellular SusA activity released by lysing cells makes no contribution to starch utilization by growing cells is that a mutant with a disruption in *susC* (mutant Ms-2) cannot grow on starch even though it grows normally on maltose and maltotriose (11). If some SusA were released during normal growth, this mutant should be able to grow on starch, because the extracellular SusA would break down starch in the medium into di- and trisaccharides, which can be utilized by Ms-2. Although our results rule out participation of extracellular SusA and SusB in growth on starch, they do not rule out the possible involvement of some other extracellular or surface-exposed enzyme. Our results show clearly that our assay is not detecting, or is detecting very poorly, at least one starch-degrading enzyme and at least one maltooligosaccharide-degrading enzyme. One or more of these enzymes could still prove to be surface exposed or extracellular.

ACKNOWLEDGMENT

This work was supported by grant AI17876 from the National Institutes of Health.

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 of starch is required for starch utilization by *Bacteroides thetaiotaomicron*. J. Bacteriol. **171:**3199–3204.
3. **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.
4. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
5. **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.
6. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557–580.
7. **Janse, B. J. H., A. J. C. Steyne, and I. S. Pretorius.** 1993. Regional sequence homologies in starch-degrading enzymes. Curr. Genet. **24:**400–407.
8. **Kotarski, S. F., and A. A. Salyers.** 1984. Isolation and characterization of outer membranes from *Bacteroides thetaiotaomicron* grown on different carbohydrates. J. Bacteriol. **158:**102–109.
9. **Lederberg, E. M., and S. M. Cohen.** 1974. Transformation of *Salmonella typhimurium* by plasmid deoxyribonucleic acid. J. Bacteriol. **119:**1072–1074.
10. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
11. **Reeves, A., J. 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.
12. **Saito, H., and K. I. Miura.** 1963. Preparation of transforming deoxyribonucleic acid by phenol treatment. Biochim. Biophys. Acta **72:**619–629.
13. **Smith, K., and A. A. Salyers.** 1989. Characterization of a cell-associated *Bacteroides* pullulanase and determination of its contribution to pullulan utilization. J. Bacteriol. **171:**2116–2123.
14. **Smith, K., and A. A. Salyers.** 1991. Purification and characterization of enzymes involved in starch utilization by *Bacteroides thetaiotaomicron*. J. Bacteriol. **173:**2962–2968.
15. **Stevens, A. M., N. B. Shoemaker, and A. A. Salyers.** 1990. The region of *Bacteroides* conjugal chromosomal tetracycline resistance elements which is responsible for production of plasmidlike form from unlinked chromosomal DNA might also be involved in transfer of the element. J. Bacteriol. **172:** 4271–4279.
16. **Studier, F. W., and B. A. Moffet.** 1986. Use of T7 RNA polymerase to direct selective high-level expression of cloned genes. J. Mol. Biol. **189:**113–130.
17. **Svensson, B.** 1994. Protein engineering in the α-amylase family: catalytic mechanism, substrate specificity and stability. Plant Mol. Biol. **25:**141–157.
18. **Tancula, E., M. J. Feldhaus, L. A. Bedzyk, and A. A. Salyers.** 1992. Location and characterization of genes involved in binding of starch to the surface of *Bacteroides thetaiotaomicron*. J. Bacteriol. **174:**5609–5610.