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Characterization of Four Outer Membrane Proteins That Play a Role in Utilization of Starch by *Bacteroides thetaiotaomicron*

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Results of earlier work had suggested that utilization of polysaccharides by *Bacteroides* spp. did not proceed via breakdown by extracellular polysaccharide-degrading enzymes. Rather, it appeared that the polysaccharide was first bound to a putative outer membrane receptor complex and then translocated into the periplasm, where the degradative enzymes were located. In a recent article, we reported the cloning and sequencing of *susC*, a gene from *Bacteroides thetaiotaomicron* that encoded a 115-kDa outer membrane protein. SusC protein proved to be essential for utilization not only of starch but also of intermediate-sized maltooligosaccharides (maltose to maltoheptaose). In this paper, we report the sequencing of a 7-kbp region of the *B. thetaiotaomicron* chromosome that lies immediately downstream of *susC*. We found four genes in this region (*susD*, *susE*, *susF*, and *susG*). Transcription of these genes was maltose inducible, and the genes appeared to be part of the same operon as *susC*. Western blot (immunoblot) analysis using antisera raised against proteins encoded by each of the four genes showed that all four were outer membrane proteins. Protein database searches revealed that SusE had limited similarity to a glucanohydrolase from *Clostridium acetobutylicum* and SusG had high similarity to amylases from a variety of sources. SusD and SusF had no significant similarity to any proteins in the databases. Results of ^{14}C-starch binding assays suggested that SusD makes a major contribution to binding. SusE and SusF also appear to contribute to binding but not to the same extent as SusD. SusG is essential for growth on starch but appears to contribute little to starch binding. Our results demonstrate that the binding of starch to the *B. thetaiotaomicron* surface involves at least four outer membrane proteins (SusC, SusD, SusE, and SusF), which may form a surface receptor complex. The role of SusG in binding is still unclear.

Human colonic *Bacteroides* spp. can utilize a variety of polysaccharides as their sole source of carbon and energy. An unusual feature of *Bacteroides* polysaccharide utilization systems is that the degradative enzymes are cell associated rather than extracellular, and the binding of the polysaccharide to the cell surface appears to be an essential first step in the polysaccharide utilization process (2, 3, 28). We have used the starch utilization system of *Bacteroides thetaiotaomicron* as a model system for defining the various components involved in the polysaccharide utilization process, especially those that mediate early steps in starch uptake and digestion. *B. thetaiotaomicron* can utilize all forms of starch, including amylose, amylopectin, and pullulan, and their component maltooligosaccharides. Amylose consists of linear chains of α-1,4-linked glucose residues. Amylopectin consists of amylose chains linked to each other by α-1,6 branches. Pullulan is a linear chain of maltotriose residues connected by α-1,6 linkages.

To date, we have characterized three structural genes that are involved in the breakdown of starch by *B. thetaiotaomicron*. These genes have been designated *sus* genes, for starch utilization system. Two of these genes, *susA* and *susB*, encode degradative enzymes (6). *susA* encodes a neopullulanase, and *susB* encodes an α-glucosidase. The neopullulanase had been previously purified and characterized by Smith and Salyers (31). In contrast to amylases, which attack only the α-1,4 linkages of amylose and amylopectin, the neopullulanase hydrolyzes α-1,4 linkages in pullulan as well as those in amylose and amylopectin. The neopullulanase does not attack short oligomers of glucose. The oligomers produced by neopullulanase action are degraded by the α-glucosidase, which can hydrolyze either α-1,4 or α-1,6 bonds (31). Both the neopullulanase and the α-glucosidase are cell associated and loosely associated with the inner membrane (6). Downstream of *susB* is *susC*, a gene which encodes an outer membrane protein (OMP) that is essential not only for growth on starch but also for growth on maltooligosaccharides larger than maltotriose (25). *susA*, *susB*, and *susC* are all transcribed in the same direction (6). The neopullulanase gene *susA* is in its own transcriptional unit, whereas *susB* and *susC* are part of the same operon (Fig. 1). In this report, we describe four more starch utilization genes that appear to be in the same operon as *susB* and *susC* (i.e., *susD*, *susE*, *susF*, and *susG*) and show that these genes, like *susC*, all encode OMPs.

We had predicted the existence of starch-binding OMPs on the basis of some earlier findings. First, the fact that the neopullulanase and α-glucosidase were not exposed on the cell surface (2) suggested that starch must first transit the outer membrane before being degraded, presumably via some protein receptor or channel. Second, Anderson and Salyers (2) reported that ^{14}C-starch is bound by intact cells of *B. thetaiotaomicron* and that characteristics of the binding suggested that it was mediated by a protein receptor. That is, binding was specific for long-chain α-1,4-linked glucan segments, was saturable, and was susceptible to digestion by proteinase K. Finally, Tancula et al. (35) used polyclonal antisera generated against intact membranes of *B. thetaiotaomicron* to identify three maltose-inducible OMPs with molecular masses of 115, 43, and 65 kDa. Since these three proteins were missing in a transposon-generated mutant that was deficient in the ability to bind starch, *B. thetaiotaomicron* Ms-2, it seemed likely that one or more of them played a role in the binding process. Previously, we had identified the gene that encoded the 115-kDa protein, *susC*, and we showed that this gene was essential

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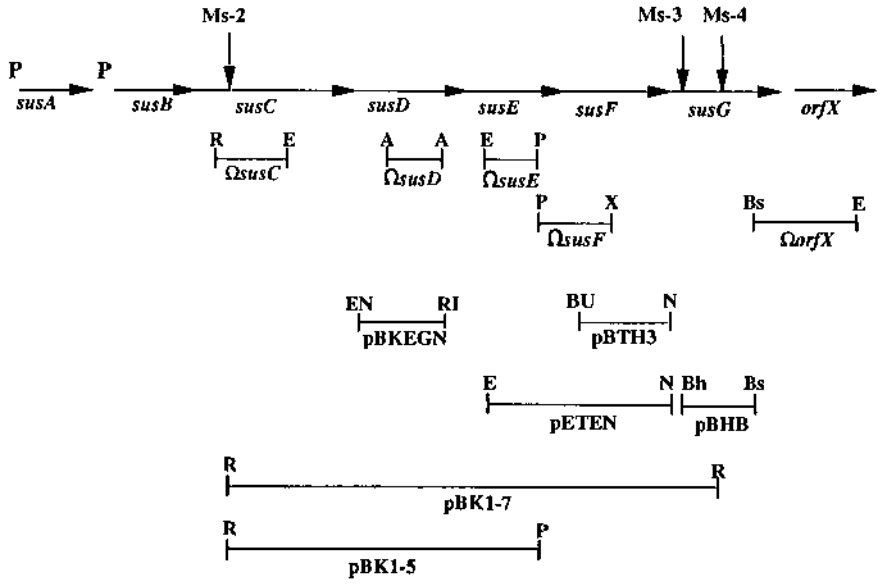

FIG. 1. Map showing the relative locations of the previously characterized *sus* structural genes (6, 25) and the locations of the four genes described in this paper. The positions of promoter regions are indicated by a P (6). The positions of the transposon insertions in mutants Ms-2, Ms-3, and Ms-4 are indicated by vertical arrows above the map. DNA segments used to make insertional disruptions are shown as horizontal lines under the map and marked with Ω. DNA segments cloned into plasmids used in this study are also shown as horizontal lines below the map. Abbreviations: Bh, *Bsp*HI; Bs, *Bst*I; Bu, *Bst*UI; E, *Eag*I; EN, *Eco*NI; P, *Pvu*II; R, *Eco*RV; RI, *Eco*RI; X, *Xmn*I.

for growth on starch (25). We also established that the transposon insertion in mutant Ms-2 had interrupted *susC*. Since the 43- and 65-kDa proteins were also missing in mutant Ms-2, it seemed reasonable to expect that genes in the region downstream of *susC* might encode either the 43- and 65-kDa OMPs or proteins that controlled their expression. In this paper, we show that the genes encoding these two OMPs, together with two genes that encode previously undetected OMPs, are in fact located downstream of *susC*. We further demonstrate that at least some of these proteins play a role in the binding of starch to the *Bacteroides* cell surface.

MATERIALS AND METHODS

Bacterial strains, plasmids, 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. For most experiments, *B. thetaiotaomicron* wild-type and mutant strains were grown in prereduced Trypticase (BBL Microbiology Systems, Cockeysville, Md.)-yeast extract-glucose liquid medium (31). Three transposon-derived mutants of *B. thetaiotaomicron* 5482 (Ms-2, Ms-3, and Ms-4) were described previously (2, 3). In experiments to determine whether genes were maltose inducible, *B. thetaiotaomicron* 5482 was transferred from the Trypticase-yeast extract-glucose liquid medium to a defined medium containing either glucose or maltose at a final concentration of 0.3% (31). In induction experiments to overexpress fusion proteins containing cloned *B. thetaiotaomicron* DNA in *E. coli*, IPTG (isopropyl-β-D-thiogalactopyranoside) added to the LB medium to a final concentration of 0.4 mM was used (33). Growth rates of *Bacteroides* strains were determined by first growing cells in the Trypticase-yeast extract-glucose liquid medium and then transferring them to defined medium containing either amylopectin or pullulan (0.5%) as the sole carbon source. Antibiotic concentrations used in this study were as follows: ampicillin, 200 μg/ml; chloramphenicol, 10 μg/ml; erythromycin, 10 μg/ml; gentamicin, 200 μg/ml; and tetracycline, 1 μg/ml.

DNA methods. Isolation of plasmids was done as described previously (25). Preparation of chromosomal DNA was performed by the method of Saito and Miura (27). Dephosphorylation reactions and ligations were performed as described in the manufacturer's instructions (Bethesda Research Laboratories, Inc., Gaithersburg, Md., or New England BioLabs, Inc., Beverly, Mass.). Transformation of *E. coli* DH5αMCR and BL21(DE3) was done by the method of Lederberg and Cohen (15). Conjugations, in which cloned *B. thetaiotaomicron* DNA was transferred from *E. coli* donors to *B. thetaiotaomicron* 5482 recipients, were done as described by Shoemaker et al. (30). Southern blotting followed the procedure described by Maniatis et al. (18).

Nucleotide sequence analysis. pBK1-7 was used as the source of DNA for sequencing the region immediately downstream of *susC*. pBK1-7 carries a 7.7-kb *Eco*RV fragment from the *B. thetaiotaomicron* chromosome (Fig. 1). The region containing *susG* was cloned from mutants Ms-3 and Ms-4, which have transposon insertions about 1 kbp apart. The transposon was used as a hybridization probe to clone adjacent chromosomal DNA, and the adjacent DNA was used as a hybridization probe to obtain a 2.2-kbp *Eco*RV fragment that was immediately downstream of the 3′ end of the DNA cloned in pBK1-7. The procedures used have been described previously (35). Portions of DNA from the two cloned regions were subcloned into the *Hin*cII site of pUC19. Overlapping deletion clones were generated and used to sequence the region. Where necessary, primers were constructed from an available sequence to close gaps. Sequencing was done by the method of Sanger et al. (29). Some of the DNA sequencing was done by the University of Illinois Automated Sequencing Facility (University of Illinois Biotechnology Center, Urbana). Derived amino acid sequences of the genes identified by sequence analysis were used to search for similar polypeptides in the GenBank, Swiss-Prot, and Prosite databases (1). Protein structure was analyzed by the method of Chou and Fasman (5). Direct comparisons of the amino acid sequences of two different proteins were done as described by Needleman and Wunsch (23). Clones carrying the transposon insertion in mutants Ms-3 and Ms-4 were sequenced in the region near the insertion site to determine where the transposon had inserted.

Generation of antibodies against the proteins encoded in the sequenced region. The T7 promoter-expression system (33, 34) was used to overexpress portions of the genes in *E. coli*. In the case of *susD*, a 1.3-kb *Eco*NI-*Eco*RI fragment from pBK1-5 was cloned into the *Bam*HI site of pET3A. In the case of *susE*, a 2.3-kb *Eag*I-*Nru*I fragment from pBK1-7 was cloned into the same site except in pET3B. In the case of *susF*, a 1.3-kb *Bst*UI-*Nru*I fragment from pARR10 was cloned into the *Bam*HI site of pET3A, and in the case of *susG*, a 1.65-kb *Bsp*HI-*Bst*BI fragment was cloned into the same site. BL21(DE3) served as the source of inducible T7 RNA polymerase. High-level expression of the fusion protein was accomplished by the procedure of Studier et al. (33, 34). Inclusion bodies were purified by the procedure of Marston et al. (19). The overexpressed proteins were excised from 4 to 5% agarose gels (FMC BioProducts, Rockland, Maine), and antigen preparation was done by the method of Litz (16), except that Metaphor agarose was used instead of ProSieve agarose. Immune ascites fluid was generated in mice and prepared as described previously (25).

Cellular localization of proteins. Western blot (immunoblot) analysis of membrane and soluble fractions from wild-type and mutant *B. thetaiotaomicron* strains was done as described previously (9). Protein concentrations of membrane and soluble fractions were determined by the modified method of Lowry et al. (17) with a final sodium dodecyl sulfate (SDS) concentration of 1% (19). Separation of inner and outer membranes was done as described by Kotarski and Salyers (14), except that the final gradient purification step was omitted. This

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)[a]	Description or source
E. coli		
BL21(DE3)	T7 RNA polymerase source	Studier et al. (34)
BL21(DE3) pLysS	T7 RNA polymerase source; $LysS^+$ Cm^r	Studier and Moffett (33)
B. thetaiotaomicron		
BT5482	Wild type; (Gn^r)	Virginia Polytechnic Institute Anaerobe Laboratory, Blacksburg, Va.
Ms-2	(Em^r) (Gn^r) $G2^+$ $G7^-$ starch$^-$ *susC susD susE susF susG*	Tn*4351*-generated mutant of strain 5482 (3)
Ms-3	(Em^r) (Gn^r) $G2^+$ $G7^+$ starch$^-$ *susF*$^+$ *susG*	Tn*4351*-generated mutant of strain 5482 (3)
Ms-4	(Em^r) (Gn^r) $G2^+$ $G7^+$ starch$^-$ *susF*$^+$ *susG*	Tn*4351*-generated mutant of strain 5482 (3)
Ω*susC*	(Tc^r) (Gn^r) $G2^+$ $G7^-$ starch$^-$ *susC susD susE susF susG*	*Bacteroides* suicide vector pBT-2 containing a 1.1-kbp *Eco*RV-*Eag*I fragment inserted in the *B. thetaiotaomicron* chromosome in the *susC* gene (35)
Ω*susD*	(Tc^r) (Gn^r) $G2^+$ $G7^+$ starch$^-$ *susC*$^+$ *susD susE susF susG*	pBT-2 containing a 0.6-kbp *Afl*III-*Afl*III fragment inserted in the *B. thetaiotaomicron* chromosome in the *susD* gene (35)
Ω*susE*	(Tc^r) (Gn^r) $G2^+$ $G7^+$ starch$^-$ *susC*$^+$ *susD*$^+$ *susE susF susG*	pBT-2 containing a 0.5-kbp *Eag*I-*Pvu*II fragment inserted in the *B. thetaiotaomicron* chromosome in the *susE* gene (35)
Ω*susF*	(Tc^r) (Gn^r) $G2^+$ $G7^+$ starch$^-$ *susC*$^+$ *susD*$^+$ *susE*$^+$ *susF susG*	pBT-2 containing a 1.0-kbp *Pvu*II-*Xmn*I fragment from the 3′ end of the *susE* gene to the downstream sequence inserted in the *B. thetaiotaomicron* chromosome (35)
Ω*orfX*	(Em^r) (Gn^r) $G2^+$ $G7^+$ starch$^+$ *susA*$^+$ to *-G*$^+$	Chromosomal insertion mutant in which a 1.1-kb *Bst*BI-*Eco*RV fragment that contained the 3′ end of *susG* and the 5′ end of *orfX* and used to generate a disruption; this study
ΩR1NS	(Tc^r) (Gn^r) $G2^+$ $G7^+$ starch$^+$ *susC*$^+$ *susD*$^+$ *susE*$^+$ *susF*$^\pm$ *susG*$^\pm$	25
ΩRIPB	(Tc^r) (Gn^r) $G2^+$ $G7^+$ starch$^+$ *susC*$^+$ *susD*$^+$ *susE*$^+$ *susF*$^\pm$ *susG*$^\pm$	*chuR* promoter cloned adjacent to a segment of DNA containing the 3′ end of *susE* and inserted in the chromosome downstream of the *susE* gene; this study
2ΩRIPB-1	(Tc^r) (Gn^r) (Cm^r) $G2^+$ $G7^+$ starch$^+$ *susC*$^+$ *susD*$^+$ *susE susF*$^\pm$ *susG*$^\pm$	Double insertion strain derived from ΩRIPB, in which pNLT-RP was used to generate an insertion in *susE* that was used to generate Ω*susE*; this study
2ΩRIPB-31	(Tc^r) (Gn^r) (Cm^r) $G2^+$ $G7^+$ starch$^+$ *susC*$^+$ *susD*$^+$ *susE*$^+$ *susF*$^\pm$ *susG*$^\pm$	Same as 2ΩRIPB-1, except that pNLY-RP crossed over into the OriT site of the plasmid inserted in ΩRIPB; this study
Plasmids		
pARR10	Ap^r	pUC19 containing as additional 2.3-kb *Eag*I-*Nru*I fragment from pBK1-7 (35) cloned into the *Hin*cII site; used for subcloning a portion of the *susF* gene; this study
pBT-2	Ap^r (Tc^r)	35
pBK1-7	Ap^r	See Fig. 1; 35
pBK1-5	Ap^r	pBK1-7 with a 0-kbp *Pvu*II-*Pvu*II fragment deleted; see Fig. 1; this study
pCQW-1	Ap^r (Em^r)	*Bacteroides* suicide vector containing the *E. coli* β-glucuronidase gene; used for reporter gene assays; 7
pCQW-DSG	Ap^r (Em^r)	pCQW-1 containing a 1.1-kb *Bst*BI-*Eco*RV fragment used to create an insertion in the *B. thetaiotaomicron* chromosome downstream of *susG*; this study
pET3A	Ap^r	33
pET3B	Ap^r	33
pETEN	Ap^r	pET3B containing a portion of the *susE* gene on a 2.3-kbp *Eag*I-*Nru*I fragment; this study
pBTH3	Ap^r	pET3A containing a 1.3-kb *Bst*UI-*Nru*I fragment from pARR10 cloned into the *Bam*HI site; this study
pBHB	Ap^r	pET3B containing a 1.65-kb *Bsp*HI-*Bst*BI fragment from pPE-15; this study
pBKEGN	Ap^r	pET3A containing a 1.3 kbp *Eco*NI-*Eco*RI fragment; this study
pCHURB	Ap^r (Tc^r)	*Bacteroides* suicide vector containing the *chuR* promoter cloned in the *Sph*I site of pLYL001B (25)
pRIPB	Ap^r (Tc^r)	pCHURB containing a 951-bp fragment beginning from within the *susE* gene into the downstream gene; this study
pNLY-2	Ap^r Cm^r (Cm^r)	pACYC-based suicide vector used to make insertions in the *B. thetaiotaomicron* chromosome; this study
pNLY-RP	Ap^r Cm^r (Cm^r)	p-NLY-2 containing a 1.084-kbp *Eco*RI-*Pvu*II fragment used to disrupt the *susE* gene; this study

[a] Abbreviations: G2, maltose; G7, maltoheptaose; Ap, ampicillin; Tc, tetracycline; Em, erythromycin; Cm, chloramphenicol; Gn, gentamicin; Kn, kanamycin. Antibiotic resistances in parentheses are expressed only in *B. thetaiotaomicron*. Antibiotic resistances not in parentheses are expressed only in *E. coli*. Superscript ± indicates that production of protein encoded by this gene was at a level 8- to 10-fold lower than that by the wild type.

step has proved to be unnecessary for preparation of an enriched outer membrane fraction (36).

Gene disruption experiments. The strategy for determining whether *susE* was essential for growth on starch was similar to the one used previously to determine whether *susC* was essential for starch utilization (25). That is, a heterologous, constitutively expressed *Bacteroides* promoter, the *chuR* promoter (4), was used to drive expression of the genes downstream of *susE*, and then *susE* was disrupted. A 951-bp *Pvu*II-*Bam*HI fragment contained in pΩR1SS, which began at the 3′ end of *susE* and extended into the region downstream of *susE*, was cloned into suicide vector pCHURB, which had been digested with *Hin*cII and *Bam*HI. The cloned fragment contained an additional 15 bp from the multiple-cloning site of pΩR1SS as well as *B. thetaiotaomicron* DNA. The resulting clone was mobilized into *B. thetaiotaomicron* 5482 to generate ΩRIPB, a single crossover insertion in the *B. thetaiotaomicron* chromosome that placed the *chuR* promoter upstream of *susF*. A second suicide plasmid containing a 1,084-bp *Eco*RI-*Pvu*II fragment from pBK1-5 was cloned into the *Eco*RV site in suicide vector pNLY-2, producing strain pNLY-RP, and then mobilized into the ΩRIPB background, with selection for Tc^r and Cm^r transconjugants. The strain containing both insertions was called 2ΩRIPB-1. Transconjugants were tested for the ability to grow on amylopectin as the sole carbon source in defined liquid medium or defined medium agar plates. The growth rate on amylopectin defined medium of strain 2ΩRIPB-1 was determined and compared with the growth rate of ΩRIPB and a strain that contained pNLY-RP inserted in the OriT site (*B. thetaiotaomicron* 2ΩRIPB-31), which was common to both suicide vectors. This strain, 2ΩRIPB-31, acted as a control for the effects on growth rate of the two antibiotics needed to maintain the insertions in the strain.

To determine whether there were any genes essential for starch utilization downstream of *susG*, a 1.0-kb *Bst*BI-*Eco*RV fragment, which contained the 3′ end of *susG* and a small amount of downstream DNA, was cloned into the *Sma*I site of pCQW-1. The insertion mutant generated with this construct, *B. thetaiotaomicron* ΩDSG, was tested for the ability to grow on amylose or amylopectin as the sole carbohydrate source.

^{14}C-starch binding experiments. ^{14}C-starch binding experiments were performed on maltose- or glucose-grown *B. thetaiotaomicron* strains, which had been harvested in the exponential phase (optical density at 600 nm, 0.6 to 0.8). Cells (10 ml) were pelleted by centrifugation at room temperature, washed twice, and resuspended in phosphate-buffered saline (pH 7.4, 0.1 M) to a final optical density at 600 nm of 0.7. This cell suspension was used to measure ^{14}C-starch binding by the procedure described by Anderson and Salyers (2). The values in counts per minute per microgram of cell protein presented in Results are the experimentally determined values minus background binding. Background binding was determined with strain *B. thetaiotaomicron* Ω*susC*, which did not produce any of the Sus OMPs. Background was 167 cpm/μg of cell protein or 2% of the wild-type values. Variation between replicate measurements of binding by the same strain was 10 to 15%. The percent inhibition of ^{14}C-starch binding caused by incubation of cells with unlabeled starch (as amylopectin or pullulan) or maltoheptaose, before the addition of radiolabeled starch, was calculated with the equation [1 − (cpm in the presence of inhibitor)/(cpm in the absence of inhibitor)] × 100%, where cpm is counts per minute. The final concentration of the unlabeled carbohydrate in the inhibition experiments was 0.5 mg/ml.

Membrane treatments. Detergent extraction of proteins from the outer membrane fraction was done with 200 μg of membrane protein resuspended in 0.15 M KCl–0.1 M KPO_4 buffer to a final concentration of 5 mg/ml. The nonionic detergents octyl-β-D-glucoside and Triton X-100 were tested for their ability to extract the membrane proteins at final concentrations ranging from 0 to 1.5% in 0.15 M KCl–0.1 M KPO_4 buffer. Extractions were performed at 4°C with gentle shaking for 1.5 h (11). The suspension was then centrifuged to pellet membranes in an Airfuge (Beckman, Palo Alto, Calif.) set at 28 lb/in^2 for 1.0 h. Proteins from the membrane and soluble fractions were separated on SDS–10% polyacrylamide gels and electrotransferred to nylon membranes, and antisera directed against the proteins were used to detect the proteins on Western blots. In a separate set of experiments, outer membranes were washed with 0.5 or 1.0 M NaCl or with carbonate buffer (pH 10.5) at 10 or 50 mM as described previously (25).

Nucleotide sequence accession numbers. The sequences of all four genes have been deposited with GenBank under the accession numbers L77614 (*susD*), L77615 (*susE*), L77733 (*susF*), and L77732 (*susG*).

RESULTS

Sequence analysis of the region downstream of *susC*. The nucleotide sequence of this region revealed four complete and one incomplete open reading frames, all of which were transcribed in the same direction as that of *susC* (Fig. 1). We have designated these open reading frames as *susD*, *susE*, *susF*, and *susG*. *susD* encoded a polypeptide with a predicted molecular mass of 62.8 kDa and a predicted isoelectric point of 5.2. The amino acid sequence of the SusD protein had no significant similarity to any other proteins in the protein databases. *susE* encoded a polypeptide with a predicted molecular mass of 42.7 kDa and a predicted isoelectric point of 4.2. In database searches, SusE showed highest similarity to an α-1,4-glucanohydrolase from *Clostridium acetobutylicum*. This enzyme attacks α-1,4 linkages of maltooligosaccharides and starch (8). We consider the similarity between these two proteins not to be significant, however, since the amino acid sequence of SusE had only 41% similarity and 17% identity to that of the glucanohydrolase.

The *susF* gene encoded a protein with a predicted molecular mass of 52.1 kDa and a predicted pI of 4.2. SusF had highest similarity to AmyH, a glucoamylase from *Saccharomyces diastaticus*. The overall similarity between the two proteins was 33%, and the identity was 17%. This similarity may not be significant, however, because regions found in many amylases that are thought to be important for substrate binding or catalysis (20, 21) were absent from the SusF sequence (data not shown). The SusF sequence contained a possible lipoprotein processing site at the N terminus. The sequence was Phe-Ser-Ala-Cys, which is very similar to lipoprotein processing sites recognized by signal peptidase II in *E. coli* (Leu-Ser-Ala-Cys) (26). *susG* was predicted to encode a protein of 76 kDa with a pI of 4.9. SusG had high sequence similarity to a number of amylases. The closest match was to an α-amylase (AmyC) from *Dictyoglomus thermophilum* (12), with a percent identity of 37% and a percent similarity of 57%. In contrast to SusF, SusG contained the four regions thought to be involved in starch binding or starch hydrolysis. These regions have been found to be conserved in amylases from a variety of organisms, such as *Bacillus* spp. (37), hog (13), mouse (10), and human (22).

Previously, Anderson and Salyers (2, 3) had identified five classes of transposon insertion mutants that lacked the ability to grow on starch. These were designated Ms-1 to Ms-5. Mapping of the transposon insertion sites in mutants Ms-3 and Ms-4 by Southern hybridiation (data not shown) demonstrated that these insertions occurred approximately 1-kb apart and were both within *susG* (Fig. 1). Since neither Ms-3 nor Ms-4 was able to grow on starch, either SusG is essential for growth on starch or the transposon insertions in Ms-3 and Ms-4 were having a polar effect on a gene downstream of *susG*. There was an open reading frame (*orfX* [Fig. 1]) which was located downstream of *susG*. We made an insertional disruption at the 3′ end of *susG*, which left *susG* intact but would have disrupted expression of *orfX* if it was in the same operon as *susG* (*B. thetaiotaomicron* Ω*orfX* [Fig. 1]). This insertional disruption had no effect on the ability of *B. thetaiotaomicron* to grow on amylose or amylopectin. Thus, there are no essential genes downstream of *susG*, and *susG* is essential for growth on starch.

***susD*, *susE*, *susG*, and *susG* all encode maltose-inducible outer membrane proteins.** The sizes of the predicted SusD and SusE gene products suggested at first that they might encode the 65- and 43-kDa proteins, respectively, that were seen previously by Tancula et al. (35). However, a disruption in what we now know is *susE* eliminated production of both the 43- and 65-kDa proteins. Thus, the 65-kDa protein identified by Tancula et al. (35) was not encoded by *susD*. However, neither SusF nor SusG had a predicted size of 65 kDa. To determine the actual molecular mass and the cellular location of SusD, SusE, SusF, and SusG, we overexpressed portions of all four proteins in *E. coli* and raised antibodies against each of the resulting polypeptides. Results of Western blot analysis using the anti-SusD antiserum are shown in Fig. 2. The membrane fraction from cells grown on maltose (lane 2) contained a band that reacted with the anti-SusD antiserum. This protein was close to the size predicted from the sequence data. SusD was

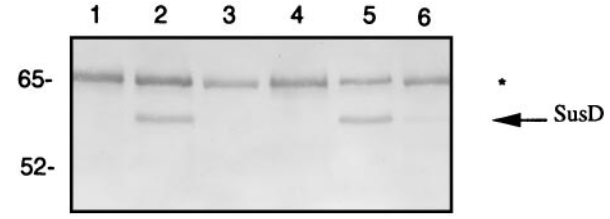

FIG. 2. Localization of the SusD protein by immunoblotting. Fifty micrograms of protein from membranes or soluble fraction was loaded per lane. All membrane and soluble fractions were obtained from cells grown on maltose except in the case of lane 1, which was obtained from cells grown on glucose. Lanes: 1, membrane fraction from *B. thetaiotaomicron* 5482 (glucose); 2, membrane fraction from *B. thetaiotaomicron* 5482 (maltose); 3, soluble fraction from *B. thetaiotaomicron* 5482; 4, membrane fraction from *B. thetaiotaomicron* Ms-2; 5, outer membrane fraction from *B. thetaiotaomicron* 5482; 6, inner membrane fraction from *B. thetaiotaomicron* 5482. The arrow identifies SusD. The asterisk identifies a streptavidin-binding protein that is present in *B. thetaiotaomicron* extracts and is detected by the Western blot detection reagents. Molecular masses in kilodaltons are shown on the left.

TABLE 2. ^{14}C-starch binding and inhibition of starch binding seen with whole cells of *B. thetaiotaomicron*

Strain	Phenotype[a]	^{14}C-starch binding (% of wild type)[b]	% Inhibition[c]		
			AP	G7	Pull
5482	C^+ D^+ E^+ F^+ G^+	100	95	90	71
Ω*susC*	C^- D^- E^- F^- G^-	2	NA[d]	NA	NA
Ω*susD*	C^+ D^- E^- F^- G^-	7	NA	NA	NA
Ω*susE*	C^+ D^+ E^- F^- G^-	68	94	83	52
Ω*susF*	C^+ D^+ E^+ F^- G^-	24	76	91	14
Ms-3 or -4	C^+ D^+ E^+ F^+ G^-	100	95	70	72
Ω*orfX*	C^+ D^+ E^+ F^+ G^+	100	97	91	73

[a] Production of proteins encoded by *susC* (C) and the downstream genes in the operon *susD* (D), *susE* (E), *susF* (F), and *susG* (G).

[b] Wild-type binding was 7,608 (±558) cpm/μg of cell protein (mean ± standard deviation) and arbitrarily set at 100%. The difference between replicate values was always within 10 to 15%.

[c] Calculated by the equation 1 − [(cpm/μg of cell protein in the presence of inhibitor)/(cpm/μg of cell protein in the absence of inhibitor)]. Abbreviations: AP, amylopectin; G7, maltoheptaose; Pull, pullulan.

[d] NA, not appropriate (indicates that the binding level was too low).

not detected in the membrane fraction from cells grown on glucose (lane 1). SusD was detected only in the outer membrane fraction (Fig. 2, lane 5) and not in the inner membrane or soluble fractions. The fact that the membrane fraction from Ms-2 (Fig. 2, lane 4) did not contain SusD is consistent with the hypothesis that *susD* and *susC* are in the same transcriptional unit and that the Ms-2 insertion in *susC* was having a polar effect on *susD* expression. Similar analyses using anti-SusE antiserum and anti-SusG antiserum demonstrated that SusE and SusG, like the SusD protein, were OMPs whose expression was induced by maltose (data not shown). The sizes of the proteins detected on Western blots were the sizes expected from the sequence data. SusE was missing in Ms-2, a finding consistent with the hypothesis that *susE* is in the same operon as *susC* and *susD*.

SusF also proved to be a maltose-inducible OMP, but the size of the protein detected on Western blots by the anti-SusF antiserum (65 kDa) was larger than that predicted from the DNA sequence (52 kDa) (Fig. 3, lanes 2 and 3). SusF could be the 65-kDa protein seen by Tancula et al. (35). We eliminated the possibility that the difference in size resulted from a sequencing error by careful reanalysis of the carboxy-terminal sequence of *susF*. The difference between the actual and expected molecular masses, together with the observation that the deduced amino acid sequence contained a possible lipoylation site, suggested the possibility that SusF might have been posttranscriptionally modified. When we expressed SusF in *E. coli*(pBK1-7), we found that SusF had a different molecular mass than when expressed in *B. thetaiotaomicron*. The apparent molecular mass of the protein produced in *E. coli* was close

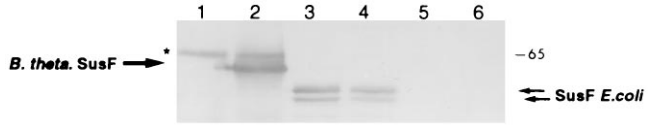

FIG. 3. Difference between apparent molecular masses of SusF expressed in *E. coli* and that expressed in *B. thetaiotaomicron*. Approximately 50 μg of protein was loaded in each lane. The outer membrane fraction from *E. coli* was obtained by the same method as that used for *B. thetaiotaomicron* (11). Lanes: 1, membranes from *B. thetaiotaomicron* grown on glucose; 2, membranes from *B. thetaiotaomicron* grown on maltose; 3, outer membrane fraction from *E. coli* carrying pBK1-7; 4, inner membrane fraction from *E. coli* carrying pBK1-7; 5, membrane fraction from *E. coli* carrying pBK1-5 (pBK1-7 with the *susF* gene deleted); 6, membrane fraction from *E. coli* carrying pBR328.

to that predicted by the sequence of the gene (Fig. 3, compare lane 2, *B. thetaiotaomicron* membrane fraction, with lanes 3 and 4, *E. coli* outer and inner membrane fractions, respectively). Cross-reacting bands that migrated at approximately 54 kDa (assumed to be unprocessed SusF) and 51 kDa (processed SusF) could be seen in both the outer and inner membrane fractions of *E. coli*(pBK1-7), although the amount contained in the outer membrane fraction appeared to be present at a three- to fourfold-higher level than the amount in the inner membrane fraction. To make sure that the anti-SusF antisera was cross-reacting with a protein encoded by the cloned *susF* gene, we made a 4.0-kb deletion in pBK1-7, so that the entire *susF* gene was deleted [*E. coli*(pBK1-5)]. Both cross-reacting bands disappeared in this strain (Fig. 3, lane 5) and in *E. coli* carrying only the vector pBR328 (Fig. 3, lane 6).

To determine whether these OMPs were integral membrane proteins or proteins associated peripherally with the outer membrane, we subjected *B. thetaiotaomicron* 5482 membranes to various treatments, including NaCl washes (0.5 or 1.0 M) or exposure to high pH (10 or 50 mM carbonate buffer [pH 10.5]). None of these treatments released SusD, SusE, or SusG from the membrane (data not shown). Small amounts of SusF were released by the highest salt concentration and by exposure to high pH, but most of the SusF remained in the membrane fraction. The KCl-phosphate buffer used in the detergent extractions also released some of the SusF, but addition of Triton X-100 did not further increase the solubilization. Octylglucoside at a final concentration equal to or greater than 0.75% (detergent/protein ratio of 3 or greater) completely solubilized all four proteins. Although Triton X-100 (1.5%) did not solubilize SusD, SusE, or SusF, a lower concentration (0.5%) was sufficient to solubilize SusG. These results suggest that SusF and SusG may be less tightly embedded in the outer membrane than SusC, SusD, and SusE.

Starch binding experiments. To determine whether SusD, SusE, SusF, or SusG was involved in starch binding, we tested the ability of mutants with insertions in each of these genes to bind ^{14}C-starch. The results are summarized in Table 2. A mutant with a disruption in the *susC* gene (Ω*susC*) had 2% of the binding activity of the wild type. When *susC* expression was restored but the downstream genes were not expressed due to polarity (Ω*susD*), ^{14}C-starch binding increased slightly, to approximately 7% of the wild-type levels. When *susC* and *susD* were both expressed (Ω*susE*), binding increased to 68% of

wild-type levels. A mutant with a disruption in *susF*, Ω*susF*, had a binding capacity that was only 24% of wild-type levels. Mutant Ms-3, which expressed all of the *sus* genes except *susG*, bound starch at the same level as did the wild type under the conditions used in our assay system. Some evidence suggests that there may be a difference between the *susG* disruption strain and the wild type at higher starch concentrations (3), but SusG clearly has less of an effect on binding than the other starch-associated OMPs.

Table 2 also shows the results of ^{14}C-starch binding experiments when *B. thetaiotaomicron* wild-type and mutant strains were preincubated with unlabeled amylopectin, pullulan, or maltoheptaose (final concentration, 0.5 mg/ml) prior to the addition of ^{14}C-starch. In the case of wild-type *B. thetaiotaomicron*, unlabeled amylopectin inhibited starch binding better than maltoheptaose, and maltoheptaose inhibited starch binding better than pullulan (95 versus 90 versus 71%). Dextran, a linear polymer of α-1,6-linked glucose, had no inhibitory effect (data not shown). In the Ω*susE* mutant, in which a significant level of starch binding was present, the same trend and the same relative degree of inhibition were seen when cells were preincubated with amylopectin and maltoheptaose. However, inhibition of starch binding by pullulan decreased to 52%. The reduction of inhibition by pullulan was even more dramatic in the mutant Ω*susF*, in which pullulan inhibited only 14% of starch binding, whereas amylopectin and maltoheptaose still inhibited starch binding like the wild type and mutant Ω*susE*.

SusE is not essential for growth on starch. The fact that the mutant with an insertion in *susE* (Ω*susE*) was still able to bind starch at 68% of the wild-type level suggested that SusC and SusD were playing a major role in starch binding. However, when *susE* expression was restored in a background that expresses SusC and SusD (in mutant *B. thetaiotaomicron* Ω*susF*), the level of starch binding was reduced by more than half compared with that of strain Ω*susE*. This was perplexing because it suggested that SusE might actually have a negative effect on formation of the starch binding complex. To determine whether SusE was essential for starch utilization, we first inserted a heterologous promoter (P*chuR*) downstream of the *susE* coding region. This was necessary because there is at least one essential gene downstream of *susE*. We found that the resulting strain, ΩRIPB, was able to grow on amylopectin with a doubling time of 7 h, compared with a doubling time of 2.5 h for the wild-type strain. The decrease in growth rate was probably due to the fact that the heterologous promoter, *PchuR*, is about five- to eightfold weaker than the promoter normally running the genes in this region. We then made a disruption in *susE* in the ΩRIPB strain and tested for growth on starch. This strain, 2ΩRIPB-1, was still able to grow in medium containing amylopectin as the sole carbohydrate source. Thus, SusE is not essential for growth on starch. Initial growth rate experiments indicated that the *susE* knockout mutant 2ΩRIPB-1 grew more slowly than did the parent strain, with a generation time of 14 h. A problem with this comparison was that we had to include both chloramphenicol and tetracycline in the medium used to grow the double mutant 2ΩRIPB-1 to maintain the two insertions in the chromosome. We knew from previous experience that even low levels of chloramphenicol reduced the growth rate of strains with the *cat* gene integrated in the chromosome, even though the strain became resistant to chloramphenicol. To control for the chloramphenicol effect, we compared the growth rate of 2ΩRIPB-1 (SusD$^+$ SusE$^-$) with that of a strain in which pNLY-RP had inserted in the *oriT* site common to the two suicide vectors rather than in *susE* (2ΩRIPB-31 [SusD$^+$ SusE$^+$]). This insertion had no effect on *susE* expression, as confirmed by Western blot analysis, but made the cells resistant to chloramphenicol. The strain 2ΩRIPB-1, which lacked SusE, grew somewhat more slowly (14-h generation time) than strain 2ΩRIPB-31, which produced SusE protein (11-h generation time). Thus, SusE protein makes some contribution to growth on starch even though it is not essential.

DISCUSSION

We have now identified five *B. thetaiotaomicron* genes that encode maltose-inducible OMPs, namely, *susC* (25) and the four genes described in this paper. We had shown previously that *susC* is essential for starch utilization (25). In this paper, we reported that *susE* is not essential, although loss of it makes the cells grow more slowly on starch. We have also shown that *susG* is essential. We were not able to determine whether *susD* or *susF* is essential, but SusD clearly makes a major contribution to starch binding, because a mutant producing only SusC and SusD bound labeled starch almost as well as the wild type whereas loss of SusD reduced binding to background levels. Whether the major contributor to starch binding is SusD alone or a combination of SusC and SusD remains to be established.

The fact that all of the OMP genes appear to be in the same operon raises the possibility that the gene products act together in some fashion, possibly as an outer membrane receptor complex that binds starch molecules at the cell surface. Although we do not have direct evidence that these OMPs do in fact form a complex, our results suggest that at least some of these proteins are interacting. The evidence is clearest in the case of SusE. Although a mutant with a disruption in *susE* (*susC*$^+$ *susD*$^+$) bound ^{14}C-starch nearly as well as the wild type did, a disruption in the next gene, *susF*, reduced the level of starch binding to 24% of that of the wild type. Binding was back to wild-type levels in the *susG* disruption mutant, where SusF production was restored. The simplest explanation for this surprising observation is that SusE normally interacts with SusF, but when SusF is not present, SusE can interact with either SusC or SusD or both and interfere with their ability to bind starch. It is interesting that the disruption in *susE* was associated with a preferential reduction in the ability of pullulan to inhibit starch binding. One explanation for this result is that pullulan binds the complex less avidly than amylopectin or maltoheptaose because pullulan lacks long regions of α-1,4-linked glucose residues. Thus, a slight decrease in the binding affinity of the complex might have a much greater effect on binding of pullulan than amylose or amylopectin. If so, our results suggest that SusE has some effect on the strength of starch binding by SusD and perhaps SusC.

In the case of the pullulanase system of *Klebsiella* spp., most of the genes in the pullulanase operon are involved in the export of the pullulanase itself (24). This appears not to be the case for *susD*, *susE*, *susF*, or *susG*. If one or more of the genes encoded export system proteins, disruptions in them should prevent other Sus proteins from reaching the outer membrane. Immunoblot analysis of outer membrane fractions showed that none of the mutants tested exhibited altered localization of the proteins that were still being produced (data not shown). This observation, together with the results of the binding studies, suggests that most, if not all, of the proteins encoded in this region of the chromosome play a structural role in binding rather than an export role.

We noted in an earlier paper that the only two *Bacteroides* OMPs for which sequence data were available at that time, SusC and CsuF (a protein involved in utilization of chondroitin sulfate), had considerable sequence similarity in their amino-terminal regions and both had a terminal phenylalanine resi-

due (25). Many OMPs of members of the family *Enterobacteriaceae*, including the porins and the TonB-dependent receptors, have a terminal phenylalanine residue (32). This residue is thought to be important for the final positioning of the OMP in the outer membrane. SusD, SusE, SusF, and SusG all lacked a terminal phenylalanine residue, and none had amino acid sequence similarity with either SusC or CsuF. However, SusD and SusE appear to be tightly associated with the outer membrane. Thus, the amino-terminal conserved sequence in SusC and CsuF and the terminal phenylalanine residue are evidently not obligatory features of *Bacteroides* OMPs.

The fact that some of the mutants we tested could bind starch at wild-type or nearly wild-type levels but still could not grow on starch demonstrates that binding of starch to the bacterial cell surface is not sufficient for internalization. If it was, these mutants should have been able to grow on starch because the periplasmic neopullanase, which is present in all of these mutants, would have had access to the starch molecule. Also, the fact that these mutants did not grow on starch at all, not even slowly, supports previous biochemical evidence suggesting that the neopullulanase, which was tentatively localized to the periplasm of *B. thetaiotaomicron* (2), is in fact not exposed on the cell surface. If it had been, the strain should have been able to grow on starch because the enzymes could have degraded the bound starch into oligomers small enough to diffuse through porins or be taken up by SusC, which appears to form a specific channel for α-1,4-linked maltooligosaccharides (25). The sequence analysis suggests that SusG might be a starch-degrading enzyme. If so, its activity is very low because mutants Ms-3 and Ms-4 had the same level of starch-degrading activity as wild type (3). Further work is needed to determine whether SusG or any of the other OMPs has starch-degrading activity and whether they are exposed on the cell surface.

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