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# A *Bacteroides thetaiotaomicron* Outer Membrane Protein That Is Essential for Utilization of Maltooligosaccharides and Starch

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**Previous studies suggested that the first step in utilization of starch by *Bacteroides thetaiotaomicron* was binding of the polysaccharide to the cell surface, followed by translocation of the polysaccharide across the outer membrane into the periplasm. In this study, we report the molecular characterization of a gene that encodes an outer membrane protein that is essential for utilization of both maltooligosaccharides and starch. The gene, *susC*, encoded a protein of 115.3 kDa. Antibodies were raised against SusC, and the outer membrane location of SusC could be confirmed by Western blot (immunoblot) analysis. SusC had a possible signal sequence of between 20 and 39 amino acids, depending on which N-terminal methionine initiates the start of the protein. It also had some features typical of well-characterized outer membrane proteins from members of the family *Enterobacteriaceae*, such as a terminal phenylalanine residue and a region in the amino portion of the protein thought to be involved in stabilizing the protein in the outer membrane. The amino acid sequence, together with results of gene disruption experiments, suggested that SusC was not an amylolytic enzyme. Transcriptional fusion experiments, using β-glucuronidase as a reporter group, showed that expression of *susC* was maltose regulated at the transcriptional level. This is the first molecular characterization of a *B. thetaiotaomicron* outer membrane protein involved in maltooligosaccharide and starch utilization.**

*Bacteroides thetaiotaomicron*, a gram-negative anaerobe found in high numbers in the human colon, utilizes a wide variety of plant polysaccharides, including the starches amylose, amylopectin, and pullulan (48). Amylose is a linear polymer of α-1,4-linked glucose units, which ranges in size from 100 to 400,000 glucose residues. Amylopectin is a branched polymer, consisting of amylose chains linked to an amylose backbone by α-1,6 bonds. Amylopectin ranges in size from 10,000 to 40,000,000 glucose units (13). Pullulan is a linear polymer consisting of maltotriose units linked together by α-1,6 bonds. Given the size and complexity of these polymers, it might seem likely that surface-exposed or extracellular enzymes would be involved in the initial breakdown of the polymer, because the polymers are too large to diffuse through porins. In fact, the pullulanase of *Klebsiella oxytoca* is exposed on the cell surface of the outer membrane during exponential phase and released into the medium during entry into stationary phase (36). Nonetheless, previous work on *Bacteroides* spp. has demonstrated that the starch-degrading enzymes are not extracellular but rather are located in the periplasm or cytoplasm (3). This finding suggested that the starch molecule might first be bound to a receptor on the *Bacteroides* outer membrane and then translocated through the outer membrane into the periplasm, where it could be degraded by the periplasmic starch-hydrolyzing enzymes. It is perhaps not surprising that the *Bacteroides* strategy for polysaccharide utilization is different from that of members of the family *Enterobacteriaceae* because these two groups of bacteria are so distant from each other phylogenetically (50).

Previously, Anderson and Salyers demonstrated that radiolabeled starch bound to the surface of *B. thetaiotaomicron* and found that this binding had the characteristics expected for a receptor-mediated event. That is, binding was saturable, protease sensitive, and inhibited by unlabeled amylose or amylopectin (3). Subsequently, Tancula et al. used an immunological approach to detect three outer membrane proteins (OMPs), which had molecular masses of approximately 115, 65, and 43 kDa (46). Production of these proteins was regulated in a way similar to that of the starch-degrading enzymes. Growth on maltose or higher oligomers of α-1,4-linked glucose was necessary to produce elevated levels of the degradative enzymes and the three OMPs. A transposon-generated mutant of *B. thetaiotaomicron*, Ms-2, was obtained in a screen for mutants unable to utilize starch. Ms-2 was able to grow on maltose (G2) and maltotriose (G3) but not on higher maltooligosaccharides such as maltopentaose (G5), maltohexaose (G6), and maltoheptaose (G7). Ms-2 was also unable to grow on amylose, amylopectin, or pullulan. The fact that Ms-2 lacked the three maltose-inducible OMPs suggested that the transposon insertion in Ms-2 might have occurred in an operon containing structural genes for the OMPs. However, it was also possible that the transposon had disrupted a gene essential for export of the OMPs or a gene involved in regulatory control of the OMP genes. In this study, we report the cloning and sequencing of the gene interrupted by the transposon insertion in Ms-2. We show that this gene encodes a 115-kDa OMP, which is essential both for utilization of G7 and for utilization of full-length starch.

## MATERIALS AND METHODS

**Strains and plasmids.** The bacterial strains and plasmids used in this study are listed in Table 1. The Tn*4351*-generated mutant used in this study, Ms-2, has been described previously (46). Ms-2 was unable to grow on the maltooligosaccharide G7 or on the starches amylose, amylopectin, and pullulan. It could, however, grow on maltose, which acts as an inducer of starch utilization genes.

**Growth conditions.** All *Escherichia 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 first grown in chopped meat broth (Carr-Scarborough Microbiologicals, Inc., Decatur, Ga.) at 37°C and subsequently transferred to prereduced Trypticase (BBL Microbiology Systems, Cockeysville, Md.)-yeast extract-glucose liquid medium or agar. In experiments in which differences in gene expression were measured, strains were transferred from Trypticase-yeast extract-glucose medium to a defined minimal medium containing one of the following as sole carbon source: glucose, 0.3%; maltose, 0.3%; G7, 0.3%; amylose, 0.5%; and amylopectin, 0.5%. All carbohydrates were obtained from

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TABLE 1. Bacterial strains and plasmids[a]

| Strain or plasmid | Relevant characteristic(s) | Description, reference, or source |
|---|---|---|
| Strains | | |
| *E. coli* | | |
| DH5αMCR | RecA $Gn^s$ | Hanahan (16) |
| BL21(DE3) | T7 RNA polymerase source | Studier and Moffett (44) |
| MCR106 | *lamB106* | Emr et al. (9) |
| *B. thetaiotaomicron* | | |
| BT5482 | Wild type, $Gn^r$ | Anaerobe Laboratory, Virginia Polytechnic Institute, Blacksburg |
| Ms-2 | $Em^r$ $Gn^r$ $G2^+$ $G7^-$ $Am^-$ | Tn*4351*-generated mutant of strain 5482 (46) |
| ΩMA-1 | $Em^r$ $Gn^r$ $G2^+$ $G7^-$ $Am^-$ | Chromosomal insertion mutant of BT5482 containing GUS inserted downstream of a 1.45-kbp *Eco*RV-*Xmn*I fragment within *susC* (this study, Fig. 6) |
| ΩMB-1 | $Em^r$ $Gn^r$ $G2^+$ $G7^-$ $Am^-$ | Same as ΩMA-1, except with the GUS gene inserted in the opposite orientation (this study, Fig. 6) |
| ΩJND-42R | $Em^r$ $Gn^r$ $G2^+$ $G7^-$ $Am^-$ | BT5482 containing GUS inserted downstream of a 0.6-kbp *Ssp*I-*Hin*cII fragment located within *susC* (this study, Fig. 3) |
| Ω3 | $Tc^r$ $Gn^r$ $G2^+$ $G7^+$ $Am^-$ | Disruption mutant of BT5482 made with a 1.0-kbp *Xmn*I-*Xmn*I fragment downstream of *susC* (46) |
| ΩR1NS | $Tc^r$ $Gn^r$ $G2^+$ $G7^+$ $Am^+$ | Mutant of BT5482 that has the *chuR* promoter inserted via a 3.8-kbp *Nsi*I-*Sca*I fragment at the 3′-terminal end of *susC* (this study, Fig. 7) |
| 2ΩRLN-42 | $Tc^r$ $Cm^r$ $Gn^r$ $G2^+$ $G7^-$ $Am^-$ | Same as ΩR1NS, except that it contains an internal disruption of *susC* (this study, Fig. 7) |
| Plasmids | | |
| pCQW-1 | $\underline{Ap^r}$ $Em^r$ | GUS-containing suicide vector used to make insertions in the BT5482 chromosome (10) |
| pET3B | $\underline{Ap^r}$ | T7 overexpression vector used to overproduce a portion of *susC* in *E. coli* (45) |
| pDRST-42 | $\underline{Ap^r}$ | Same as pET3B but containing a 2.5-kbp *Dra*III-*Stu*I fragment from *susC* cloned into the *Bam*HI site (this study) |
| pNLY-2 | $\underline{Cm^r}$ $\underline{Tc^r}$ $Cm^r$ | pACYC-based suicide vector used to make insertions in the BT5482 chromosome |
| pNLY-RE | $\underline{Cm^r}$ $\underline{Tc^r}$ $Cm^r$ | pNLY-2 containing a 1.1-kbp *Eco*RV-*Eag*I fragment cloned into the *Eco*RV site (this study) |
| pLYL001B | $\underline{Ap^r}$ $Tc^r$ | *Bacteroides* suicide vector used to make chromosomal insertions |
| pCHURB | $\underline{Ap^r}$ $Tc^r$ | *Bacteroides* suicide vector containing *chuR* promoter cloned into the *Sph*I site of pLYL001B (this study, Fig. 7) |
| pR1NS | $Ap^r$e $Tc^r$ | *Bacteroides* suicide vector pCHURB containing a 3.8-kbp *Nsi*I-*Sca*I fragment cloned into the *Hin*cII site. Used to make C-terminal insertion in *susC* (this study) |
| RP4 | $\underline{Ap^r}$ $\underline{Kn^r}$ $\underline{Tc^r}$ | IncP plasmid used to mobilize *E. coli* plasmids containing the RK2 OriT (15) |

[a] Abbreviations: G2, maltose; Am, amylose; Ap, ampicillin; Gn, gentamicin; Kn, kanamycin; Tc, tetracycline; Em, erythromycin; Cm, chloramphenicol. Underlined antibiotic resistances are expressed only in *E. coli*. The antibiotic resistances that are not underlined are expressed in *B. thetaiotaomicron*.

Sigma Chemical Co. (St. Louis, Mo.). The concentrations of antibiotics used in the selection of cloned fragments were as follows: erythromycin, 10 μg/ml; gentamicin, 200 μg/ml; ampicillin, 200 μg/ml; kanamycin, 50 μg/ml; chloramphenicol, 10 μg/ml; and tetracycline, 1 μg/ml.

**DNA manipulation.** Plasmid DNA was isolated from *E. coli* and *Bacteroides* strains by the method of Ish-Horowitz as originally described by Birnboim and Doly (28). Total cellular DNA from *Bacteroides* strains was isolated by the method of Saito and Miura (39). All restriction digests, blunting reactions, and ligations were performed in accordance with the manufacturer's instructions (Bethesda Research Laboratories, Inc., Gaithersburg, Md., or New England BioLabs, Inc., Beverly, Mass.). Transformation of *E. coli* DH5αMCR followed the method of Lederberg and Cohen (25). Chromosomal insertions in *Bacteroides* genes were performed as described previously (41). Southern hybridizations were carried out as described in the work of Maniatis et al. (28).

**Transcriptional fusion experiments.** To determine whether the gene was regulated at the transcriptional level, chromosomal transcriptional fusions were constructed. A 1.45-kbp *Eco*RV-*Xmn*I fragment located within the region thought to contain the 115-kDa protein gene was subcloned in both orientations in the *Sma*I site of suicide vector pCQW-1 (10). The reporter group in pCQW-1 is the *E. coli* gene *uidA*, which encodes β-glucuronidase (GUS). GUS activity was measured as described by Feldhaus et al. (10). Protein concentrations were determined by the modified method of Lowry et al. (27).

**Sequence analysis.** Restriction fragments from a 3.5-kbp region containing the putative 115-kDa protein gene were subcloned into pUC19 in both orientations. Exonuclease III digestion was performed on all subclones with an Erase-a-Base kit (Promega, Madison, Wis.) to generate overlapping deletion clones. DNA sequencing was done with a Sequenase 2.0 kit purchased from United States Biochemicals, Inc. (Cleveland, Ohio). Both strands were sequenced (40). In all cases, the universal and reverse primers of M13 were used, except when gaps between deletion clones needed to be filled, in which case sequence-specific primers were obtained from the University of Illinois Bioengineering Center (Urbana, Ill.). The deduced amino acid sequence was used to search for homologous polypeptides in the GenBank, Swiss-Prot, and Prosite databases (1). Hydropathy and amphiphilicity predictions of the derived amino acid sequence of open reading frames (ORFs) were based on the Kyte-Doolittle (23) and Chou-Fasman (6) algorithms, respectively, by using the DNA Strider program. Sequence alignments were prepared with the Genetics Computer Group package (35).

**Generating antibodies against the protein encoded by the cloned region.** The T7 promoter expression system (44) was used to overexpress a fusion protein that contained a 2.5-kbp *Dra*III-*Stu*I fragment from within the putative *orf115* gene cloned into the unique *Bam*HI site on plasmid pET3B. BL21-DE3 served as the source of inducible T7 RNA polymerase (44). A final IPTG (isopropyl-β-D-thiogalactopyranoside) concentration of 0.4 mM was used to initiate high-level expression of the protein in strain pDST-42. Inclusion bodies were purified according to the procedure described by Marston et al. (29). The overexpressed protein was run and excised from an agarose gel (Metaphor; FMC Bioproducts, Rockland, Maine) according to the procedure of Litz (26). Approximately 25 to 50 μg of the purified protein was mixed with an equal volume of Freund's complete adjuvant and injected into the peritoneal cavities of four BALB/c mice. Booster injections were given at 3-week intervals with approximately the same amount of protein, but instead with equal volumes of Freund's incomplete adjuvant. Ascites fluid production was initiated by injecting 25 to 50 μg of protein into the peritoneal cavities and then 3 days later injecting the tumorigenic cell line SP210. Ascites fluid was collected, delipified, and stored in saturated ammonium sulfate until needed.

**Localization of the ORF115 gene product.** Membrane and soluble fractions from *B. thetaiotaomicron* wild-type and recombinant strains grown in defined glucose or maltose medium were prepared as described previously (47). Membrane and soluble protein (50 μg) were electrophoresed on sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis gels. Proteins were electrotransferred to Zeta probe nylon membranes (Bio-Rad Laboratories, Hercules, Calif.),

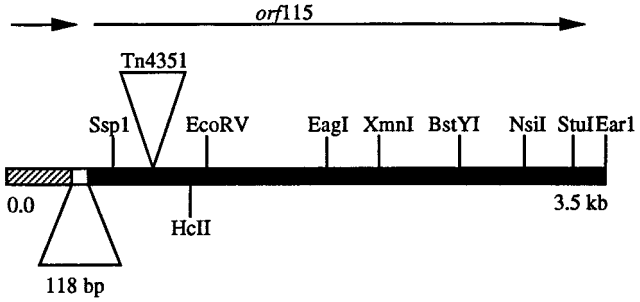


FIG. 1. Physical map and organization of the 3.5-kbp region disrupted in Ms-2. Restriction sites and ORFs are shown. The black rectangle represents ORF115. The hatched rectangle represents a partial ORF upstream of ORF115. An intergenic region between the ORFs is indicated by a square with the length in base pairs given below the triangle. Tn*4351* is the transposon insertion site in Ms-2. The horizontal arrows above the line indicate the direction of transcription of the ORFs.

and antibodies bound to the protein were detected with biotinylated secondary antibodies followed by treatment with streptavidin β-galactosidase reagent (Bethesda Research Laboratories, Bethesda, Md. [12]). Protein concentration was determined by the modified method of Lowry et al. (27). Separation of inner and outer membranes was done as described previously by Kotarski and Salyers (21), except that the final gradient purification step was omitted.

**Determining whether the ORF115 gene product is essential for growth on starch.** To determine if the protein encoded by ORF115 is essential for growth on starch, two separate single-crossover insertions were made in the *B. thetaiotaomicron* chromosome, one to disrupt the gene and one to provide a promoter to restore expression of downstream genes in the operon. One suicide vector was constructed by cloning the heterologous promoter *chuR* (4), contained on a 0.443-kbp *Sph*I fragment, into the unique *Sph*I site of the *Bacteroides* suicide vector pLYL001B. This construct was called pCHURB. Then, a 3.8-kbp restriction fragment extending from the 3′-terminal *Nsi*I site of ORF115 to a downstream *Sca*I site was cloned into the unique *Hinc*II site of pCHURB in an orientation that would place the *chuR* promoter in front of the downstream genes when integrated into the chromosome. This suicide construct was mobilized into the wild-type *B. thetaiotaomicron* background, with selection for tetracycline resistance. The strain containing the insert was called ΩR1NS. Strain ΩR1NS was then tested to make sure it could still grow on G7 and starch. The second suicide vector was made by cloning a 1.1-kbp *Eco*RV-*Eag*I fragment internal to ORF115 into suicide vector pNLY-2 and then conjugating into *B. thetaiotaomicron* ΩR1NS, to obtain the double-insertion strain 2ΩRLN-42. This strain was tested for growth in defined minimal medium containing chloramphenicol and tetracycline, to maintain the inserted plasmids, and either G7 or amylopectin as the carbohydrate source.

**Testing whether expression of the 115-kDa protein in *E. coli* increases the binding of radiolabeled starch to the *E. coli* surface.** A 3.2-kbp *Ear*I-*Ear*I fragment that contained the entire *susC* gene was cloned downstream of the pTac promoter for overexpression of SusC protein. Expression of SusC was done at a low level, by omitting IPTG, or at a slightly higher level by the addition of only 0.1 mM IPTG to induce expression of the cloned gene. Addition of greater amounts of IPTG was toxic to the cells. The same strain, containing the *Ear*I-*Ear*I fragment cloned in the opposite orientation, was used as a control. *E. coli* inner and outer membranes were obtained by the method of Kotarski and Salyers (21). We have found that this separation method works as well for *E. coli* as for *Bacteroides* spp. Antiserum generated against SusC protein was used to detect the 115-kDa protein in membrane and soluble fractions, as described in an earlier section about detecting the 115-kDa protein in *B. thetaiotaomicron*. Binding of [$^{14}$C]starch to *B. thetaiotaomicron* and *E. coli* and testing for inhibition of binding by amylose, amylopectin, dextran, and G7 were done as described previously by Anderson and Salyers (3). All values obtained in the [$^{14}$C]starch binding experiments were the result of at least three trials.

**Nucleotide sequence accession number.** The nucleotide sequence of *susC* has been deposited with GenBank (accession number L49338).

## RESULTS

**Nucleotide sequence of the region spanning the Ms-2 transposon insertion.** A 3.5-kbp region spanning the transposon insertion site in Ms-2 was sequenced and was found to contain a single ORF of 3,118 bp (Fig. 1). The derived amino acid sequence of the ORF is shown in Fig. 2. The polypeptide had a predicted molecular mass of 115,327 Da, which is consistent

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1 MIREITINFKDKNMKKGNFMFKVLLMLIAGIFLSIDAFAQQITVKGIVKD 50
  TTGEPVIGANVVVKGTTTGTITDFDGNFQLSAKQGDIIVVSFIGYQPQEL 100
  PVAAQMNVILKDDTEILDEVVVIGYGQVKKNDMTGSVMAIKPDELSKGIT 150
  TNAQDMLSGKIAGVSVISNDGTPGGGAQIRIRGGSSLNASNDPLIVIDGL 200
  AIDNEGIKGMANGLSMVNPADIETLTVLKDASATAIYGSRASNGVIIITT 250
  KKGKNGQAPSVTYNGSVSFSKTQKRYDVLSGDEYRAYANQLWGDKLPADL 300
  GTANTDWQDQIFRTAVSTDHHVSINGGFKNLPYRVSLGYTDDNGIVKTSN 350
  FRRFTASVNLAPSFFEDHLKFNINAKFMNGKNRYADSRCRYWRALAIDPT 400
  RPVYSNEDPYQFTGGYWQNINSTTGFSNPDWKYTSNPNSPQNPLAALELK 450
  NDKGNSNDFVGNVDVDYKFHFLPDLRHASIGGEYAEGTQTTIVSPYSFGN 500
  NYYGWNGDVTQYKYNLSYNIYVQYIKSLGANDFDIMVGGEEQHFHRNGFE 550
  EGQGWDSYTQEPHDAKLREQTAYATRNTLVSYFGRLNYSLLNRYLFTFTM 600
  RWDGSSRFSKDNRWGTFPSLALGWKIKEENFLKDVNVLSDLKLRLGWGIT 650
  GQQNIGDDFAYLPLYVVNNEYAQYPFGDTYYSTSRPKAFNENLKWEKTTT 700
  WNAGLDFGFLNGRITGGIDGYFRKTMTCVTALRSPMNILQCPDDTEYRFT 750
  GKLRYGFSINAKPIVTKDFTWDLSYNITWNHNEITKLTGGDDSDYYVEAG 800
  DKISRGNNTKVQAHKVGYAANSFYVSRGNNTKVQAHKVGYAANSFYVYQQ 850
  VYDENGKPIENMFVDRNGNGTIDSGDKYIYKKPAGDVLMGLTSKMQYKNF 900
  DFSFSLRASLNNYVYYDFLSNKANVSTSGLFSNNAYSNTSAEAVALGFSG 950
  QGDYYMSDYFIHNASFLRCDNITLGYSFQNLWKTQTYKGVGGRVYATVQN 1000
  PFIISKYKGLDPEVKSGIDANPYPRAMTFLLGLSLQF 1038
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FIG. 2. Derived amino acid sequence of ORF115. The first methionine residue in the coding region of ORF115 is shown at position 1. Additional possible start methionines at positions 14 and 20 are underlined. A protein beginning at the second methionine residue would have a standard hydrophobic signal peptide, on the basis of a plot of hydropathy of ORF115 (window size 11 [data not shown]), whereas a protein beginning at the first methionine residue would have a hydrophilic amino-terminal sequence. The predicted signal sequence cleavage site is indicated by a vertical arrow. Residues in the boxed region spanning amino acids 194 to 253 indicate a region that may be involved in stabilizing the protein in the outer membrane. The last 10 residues, in boldface type, are similar to the C termini of known transmembrane OMPs (18, 43).

with the hypothesis that the ORF encoded the 115-kDa OMP that was one of three proteins missing in Ms-2 (46). The first possible start codon is marked at position 1 in Fig. 2. Translation initiation at this methionine would produce a hydrophilic amino acid sequence preceding the hydrophobic region, which has the features of a signal sequence. A second possible start site is located 13 amino acids downstream from the first (underlined in Fig. 2). Results of a hydropathy analysis suggested that, if translation started at the second methionine residue, a standard signal peptide would result, consisting of a charged amino terminus, a hydrophobic core, and a polar cleavage site. A third possible translation start site is located six residues down from the second methionine (underlined, Fig. 2). In either case, the signal peptide cleavage site is predicted to be after an alanine residue (arrow, Fig. 2 [49]). Cleavage at this site would give a mature protein with a predicted molecular mass of 111,311 Da. The predicted isoelectric point of the mature protein was 7.62. By sequencing the junction between the end of Tn*4351* in Ms-2 and the adjacent chromosomal DNA, we found that the transposon had inserted 437 bp downstream of the first putative start site (Fig. 1).

**Localization of the gene product to the outer membrane.** To determine whether the ORF encoded a membrane protein, and if so whether it was located in the outer or inner mem-

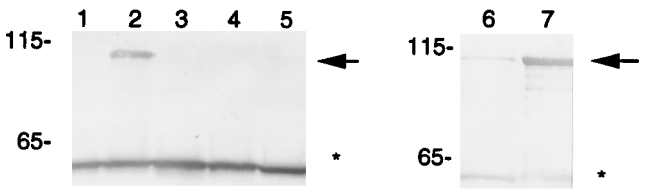


FIG. 3. Localization of SusC by immunoblotting. The same amount of total protein (50 μg) was loaded in each lane. Lanes 1 and 2, total membranes from wild-type *B. thetaiotaomicron* grown on defined glucose or defined maltose medium, respectively. Lane 3, soluble fraction from wild-type *B. thetaiotaomicron* grown on defined maltose media. Lanes 4 and 5, total membranes from *B. thetaiotaomicron* Ms-2 and ΩJND-42R, respectively, grown on defined maltose media. Lane 6, inner membrane fraction. Lane 7, outer membrane fraction from *B. thetaiotaomicron* grown on defined maltose medium. The arrow at the right indicates the location of SusC. The asterisk at right marks a *B. thetaiotaomicron* streptavidin-binding protein that cross-reacts with the streptavidin β-galactosidase detection reagent. Numbers at left indicate molecular masses of standards in kilodaltons.

brane, we first overproduced a portion of the protein from a 2.5-kbp *Dra*III-*Stu*I fragment by cloning the fragment into the *Bam*HI site of T7 overexpression vector pET3B. The resulting overproduced polypeptide was then used to generate antiserum for use in Western blot (immunoblot) analysis. A protein of approximately 115 kDa was detected by the antiserum (Fig. 3). It was seen only in membrane fractions from wild-type cells grown on maltose (Fig. 3, lane 2) and not in the soluble fraction (Fig. 3, lane 3). This protein was also missing from membranes of Ms-2 (Fig. 3, lane 4), *B. thetaiotaomicron* ΩJND42R (Fig. 3, lane 5), or wild type grown on glucose (Fig. 3, lane 1). When *B. thetaiotaomicron* membranes were separated into inner and outer membranes (Fig. 3, lanes 6 and 7, respectively), the 115-kDa protein partitioned to the outer membrane. These results demonstrate that the protein encoded by the ORF is an OMP, which is regulated in a way similar to that of the starch-degrading enzymes. We now have some preliminary data suggesting that the ORF encoding the 115-kDa protein is not the first gene in the operon and that there are at least two ORFs upstream of the ORF encoding the 115-kDa protein. Accordingly, we have named the ORF that encodes the 115-kDa protein *susC* for starch utilization system gene C.

To determine whether SusC was tightly associated with the outer membrane, we subjected outer membranes from *B. thetaiotaomicron* to various treatments known to remove at least some peripheral membrane proteins (30, 52). We treated outer membranes from *B. thetaiotaomicron* with each of the following: 0.5 M NaCl, 1.0 M NaCl, 10 mM carbonate buffer (pH 10.5), or 50 mM carbonate buffer (pH 10.5). We then pelleted the membranes by centrifugation and tested the membrane and soluble fractions by Western blotting to determine whether SusC was released by any of these treatments. We found that SusC partitioned solely with the membrane fraction in all cases (data not shown). Under these conditions, SusC remained tightly associated with the outer membrane and is thus probably an integral OMP.

**Similarity to other OMPs in the protein databases.** The EMBL and Swiss-Prot databases were searched for proteins with similarity to SusC (1). The region of SusC that showed the highest percent similarity to proteins in the databases was located 194 amino acids downstream from the first methionine residue (Fig. 2, boxed region). There was a high degree of similarity over a region of approximately 60 amino acids between SusC and various OMPs known to be involved in TonB-dependent iron or vitamin uptake by members of the *Enterobacteriaceae*. This region is thought to be involved in stabilizing certain OMPs in the outer membrane (7, 34). A multiple sequence alignment comparing SusC with several TonB-dependent receptors as well as another *B. thetaiotaomicron* OMP (CsuF) is shown in Fig. 4. The pairwise percent similarity between SusC and the other OMPs in this region was as follows: Csuf, 82%, an OMP essential for chondroitin sulfate utilization in *B. thetaiotaomicron* (5); IrgA, 60%, the iron-regulated OMP from *Vibrio cholerae* (14); BtuB, 66%, the vitamin $B_{12}$ receptor of *E. coli* (17); FepA, 62%, the enterochelin receptor of *E. coli* (38); CirA, 59%, the colicin I receptor of *E. coli* (34); IutA, 62%, the ferric-aerobactin receptor of *E. coli* (22); FhuA, 61%, the ferrichrome receptor of *E. coli* (7); and PfeA, 63%, the ferric enterobactin receptor of *Pseudomonas aeruginosa* (8). This is similar to the pairwise percent similarities among the *Enterobacteriaceae* sequences. This finding was somewhat surprising since *Bacteroides* spp. are as distant phylogenetically from the *Enterobacteriaceae* as are the gram positives (50). Apparently, signal sequences and regions involved in the stable association of certain proteins in the outer membrane are conserved in different phylogenetic groups. By contrast, the promoter sequences of *Bacteroides* spp. do not function in *E. coli* and do not have the standard *E. coli* promoter structure (42).

Another protein to which SusC showed significant similarity was a recently identified *B. thetaiotaomicron* OMP encoded by the *csuF* gene (Fig. 5) (5). CsuF is essential for growth on the charged mucopolysaccharide chondroitin sulfate and is the only other *Bacteroides* OMP sequence currently in the databases. SusC showed considerable sequence similarity to CsuF, especially in the amino-terminal 245 residues (Fig. 5) (66% similarity, 50% identity). In particular, there was significant amino acid sequence similarity between the two proteins in the

| Protein | Organism | | Sequence | |
|---|---|---|---|---|
| SusC | *B.theta.* | 194 | LIVIDGLAID N.....E | GIKGMANGLS MVPPAD.IET LTVLKDASAT |
| CsuF | *B.theta.* | 186 | LFVIDGFPIE DS..... | S.....AAS TLNPSD.IES LDFLKDASAT |
| IrgA | *V.chol.* | 101 | LILVDGK... .RQTSRQTRP | NSDGPGIEQG WLPPLQAIER AEVIRGP.MS |
| BtuB | *E.coli* | 97 | LVLIDG.... | ....VRLNLA GVSGSADLSQ F..PIALVQR VEVIRGP.RS |
| FepA | *E.coli* | 105 | LILIDGKPVS | SRNSVRQGWR GERDTRGDTS WVPP.EMIER IEVLRGP.AR |
| CirA | *E.coli* | 100 | LILVDGKRVN | SRNAV..... .FRHNDFDLN WI.PVDSIER IEVVRGP.MS |
| IutA | *E.coli* | 100 | VVLVDGVRLN | S..... .SRTDSRQLD SIDPFNM.HH IEVIFGA.TS |
| FhuA | *E.coli* | 123 | LIIRGFAAEG | QSQNNVLNGL KLQGNFYNDA VIDPY.MLER AEIMRGP.VS |
| PfeA | *P.aerug.* | 110 | LILVDGKPVS | SRNSVRYGWR GERDSRGDTN WV.PADQ.ER IEVIRGP.AA |
| | | | LILIDG---- | ---------- ---------- ---P---IER -EV-RGP--S |
| SusC | *B.theta.* | | AIYGSRASNG | VIIITTKKGK 253 |
| CsuF | *B.theta.* | | AIYGSRASNG | VIIITTKKGK 240 |
| IrgA | *V.chol.* | | TLYGSDAIGG | VINITTKDQ 166 |
| BtuB | *E.coli* | | AVYGSDAIGG | VVNITTRDEP 156 |
| FepA | *E.coli* | | ARYGNGAAGG | VVNITTKKGS 173 |
| CirA | *E.coli* | | SOYGSRCARR | CSEYHHQKNR 163 |
| IutA | *E.coli* | | .LYGGGSTGG | LINIVKKGQP 157 |
| FhuA | *E.coli* | | VLYGKSSPGG | LLNMVSKRPT 191 |
| PfeA | *P.aerug.* | | ARYGNGAAGG | VVNITTKQAG 177 |
| | | | A-YG--A-GG | V-NI-TKK-- |

FIG. 4. Multiple sequence alignment representation of the putative N-terminal outer membrane stabilization region in SusC, CsuF (another *B. thetaiotaomicron* OMP), and various OMPs of members of the *Enterobacteriaceae*. The alignment was generated with the PILEUP program of the Genetics Computer Group package (35). Underlined letters indicate highly conserved amino acids (present in at least five sequences), and these are given as the consensus at that position (bottom line). Several amino acids are conserved in all the sequences: a Pro (P) residue as the sixth underlined residue in the consensus line; a Tyr (Y) residue as the 17th underlined residue in the consensus line; and a Gly (G) residue as the 18th underlined residue in the consensus line. The sizes of the proteins and the location of the domain sequences are as follows: SusC, 1,038 amino acids with the domain starting at residue 194; CsuF, 1,065 amino acids with the domain starting at residue 186; IrgA, 652 amino acids with the domain starting at residue 101; BtuB, 614 amino acids with the domain starting at residue 97; FepA, 745 amino acids with the domain starting at residue 105; CirA, 663 amino acids with the domain starting at residue 100; IutA, 723 amino acids with the domain starting at residue 100; FhuA, 747 amino acids with the domain starting at residue 123; PfeA, 746 amino acids with the domain starting at residue 110.

FIG. 5. Amino acid sequence alignment of SusC (top) with CsuF, a *B. thetaiotaomicron* OMP essential for utilization of the negatively charged polysaccharide chondroitin sulfate (bottom). Bars represent identical amino acids; a colon represents a comparison value of 0.50 or greater based on the simplification scheme for amino acid differences from the Wisconsin Genetics Computer Group computer analysis program; a period represents a comparison value between the amino acids of 0.10 or greater. The overall similarity was 53%, and the overall identity was 32%. The underlined region spanning residues 194 to 253 in SusC and 186 to 240 in CsuF is the putative outer membrane stabilization region shown in Fig. 4. In this region, SusC and CsuF shared 65% identity and 82% similarity. The SusC amino acid sequence was begun at the second possible start methionine (position 14 in Fig. 2 but treated as position 1 in Fig. 5) in order to optimize the alignment.

region thought to be involved in the stable association of the protein in the outer membrane (82% similarity and 65% identity [Fig. 5, underlined region]). Since the putative N-terminal OMP stabilization region is located at the same relative position in the two amino acid sequences (residues 194 to 253 in SusC; residues 186 to 240 in CsuF [Fig. 5]), the region may serve a similar function in the two proteins. In the other portions of the two proteins, there was little sequence similarity, except for short regions in the middle of the proteins and in the C-terminal region (Fig. 5). Regions of dissimilarity between the two proteins could be involved in substrate binding, because starch and chondroitin sulfate differ considerably in composition. Starch is a neutral polymer of glucose residues, whereas chondroitin sulfate is a negatively charged polymer of sulfated *N*-acetyl-galactosamine and uronic acid residues. CsuF has a predicted pI of 9.86, which would make it positively charged at the estimated pH of the colon (pH 6 to 7) and thus compatible with the binding of the highly negatively charged substrate chondroitin sulfate. SusC has a predicted pI of 7.62, which would make it slightly positively charged at pH 6 to 7 and thus compatible with the binding of starch, a neutral molecule.

The C-terminal 10 amino acids of SusC also exhibited amino acid sequence similarity to the TonB-dependent receptors and many other OMPs from members of the *Enterobacteriaceae* (18, 43). An almost absolute requirement for a C-terminal phenylalanine residue in the TonB-dependent receptors and many porins, as well as hydrophobic amino acids at positions −3, −5, −7, and −9 from the C terminus (taking the terminal phenylalanine residue as −1), has been shown to be important for OMP assembly and is also thought to be involved in OMP sorting (43). Two other residues besides the terminal phenylalanine residue are highly conserved in the C termini of the TonB-dependent receptors (20). These are an arginine at the −11 position and a proline, glycine, or glutamate at the −12 position. In SusC, as well as CsuF, these residues were at −13 and −14, respectively (Fig. 5).

On the basis of the above sequence analysis, along with information obtained from a hydropathy analysis of SusC (6, 23), database searches (1), and topological mapping of domains from other OMPs (32), we have defined four putative functional domains in SusC. Domain 1 consists of residues 194 to 253 of the preprocessed protein and could represent a region involved in the stable association of the protein in the outer membrane (7); domains 2 and 3 consist of residues 476 to 501 and 857 to 888, respectively, and share some amino acid similarity with a repeat sequence motif known to be involved in carbohydrate binding in several gram-positive bacteria (51); and domain 4 consists of residues 1015 to 1025, shares amino acid sequence similarity with the C-terminal 10 amino acids of the TonB-dependent receptors and many other polytopic OMPs from members of the *Enterobacteriaceae*, and has been suggested to form the terminal β-strand in those proteins (42).

**SusC is maltose regulated at the transcriptional level.** SusC was seen only in cells that had been grown on maltose or starches. To determine whether *susC* was regulated at the

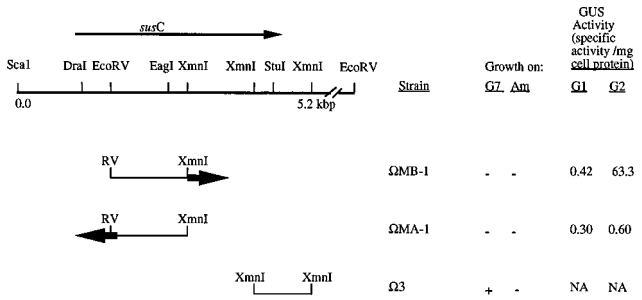


FIG. 6. GUS transcriptional fusions and insertion mutations used in this study. Thick black arrows indicate the amino-terminal end and direction of transcription of the GUS gene inserted in the *B. thetaiotaomicron* chromosome. The fragment attached to the thick black area is the cloned fragment used to make the insertion. The direction of transcription of *susC* is indicated by the black arrow above the restriction map. The plus and minus signs indicate whether the insertion mutant grew or did not grow on G7 or amylose. GUS specific activities (units per milligram of cell protein) were determined for strains grown on either defined glucose (G1) or defined maltose (G2) medium.

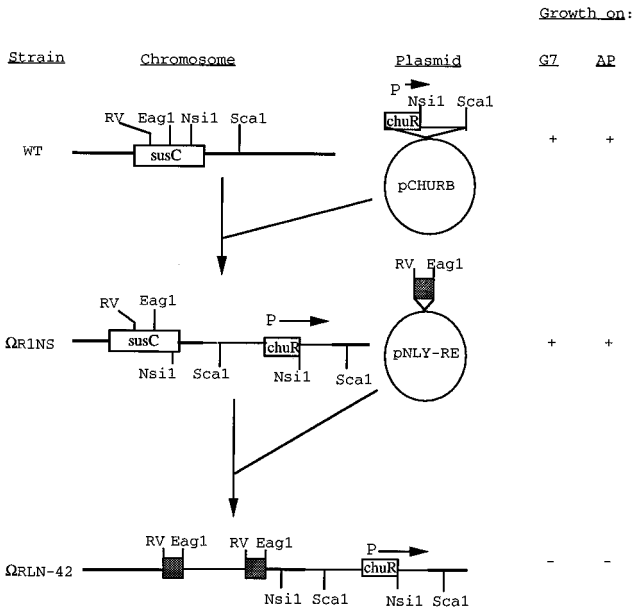


FIG. 7. Strategy used to determine whether SusC is essential for growth on starch. Suicide plasmid pCHURB, which contains the heterologous promoter, *chuR*, was cloned upstream of a 3.8-kbp *Nsi*I-*Sca*I fragment. The resulting construct was mobilized into wild-type *B. thetaiotaomicron* and integrated into the chromosome to produce strain ΩR1NS (*susC*$^+$ G7$^+$ and starch positive). pNLY-RE, containing a 1.1-kbp *Eco*RV-*Eag*I fragment from within the *susC* gene, was then used to disrupt *susC* in ΩR1NS to yield the final construct, 2ΩRLN-42. The ability of wild-type and recombinant strains to grow in defined G7 and defined amylopectin (Ap) starch media is indicated at the right. Thick black lines refer to chromosomal DNA, whereas thin black lines refer to plasmid DNA.

transcriptional level, a 1.45-kbp *Eco*RV-*Xmn*I fragment internal to the *susC* gene was subcloned in both orientations into the GUS suicide vector pCQW-1 and introduced into *B. thetaiotaomicron* to produce strains ΩMA-1 and ΩMB-1, respectively. The results of GUS assays from whole-cell extracts of strains ΩMA-1 and ΩMB-1 are shown in Fig. 6. Only one orientation produced detectable GUS activity, and only extracts from cells grown on maltose were positive. Thus, *susC* expression is regulated at the transcriptional level by maltose.

**SusC is essential for growth on starch.** *B. thetaiotaomicron* Ω3, a mutant that had a disruption immediately downstream of *susC*, was able to grow on G7 (46). Thus, the fact that insertions in *susC* abolished growth on G7 suggested that SusC was essential for growth on G7. However, it was not clear whether SusC was also essential for growth on starch because the disruption in mutant Ω3 rendered the strain unable to grow on starch, a finding which suggested that there were more genes downstream of *susC* that were essential for growth on starch. Thus, the transposon insertion in Ms-2 might have abolished growth on starch simply by exerting a polar effect on these downstream genes.

To ascertain whether SusC was essential for growth on starch, we first introduced a heterologous promoter downstream of the 3′ end of the *susC* gene in such a way as to retain an intact copy of *susC* and at the same time allow the downstream genes to be transcribed from the heterologous promoter (Fig. 7). This strain was able to grow on starch, although it grew somewhat more slowly than wild type, with a doubling time of approximately 5 h compared with about 2 h in the wild-type strain, and had a longer lag period in minimal media of about 9 h compared with 2 to 3 h in the wild-type strain. This slower growth phenotype was not surprising since GUS fusion analysis suggested that the *chuR* promoter is about eightfold weaker than the one controlling expression of *susC* (4). A second insertion was then made to disrupt the *susC* gene, in a region separate from the first insertion, but internal to the gene, in strain ΩR1NS (Fig. 7). The double insertion strain was tested for growth on starch and G7. Strain 2ΩRLN-42 did not grow on starch or G7 (Fig. 7). This result indicates that *susC* is essential for growth on starch as well as for growth on G7.

**Binding of radiolabeled starch by an *E. coli* strain expressing *susC*.** To determine whether SusC had starch-binding activity, we determined the effect of moderate production of SusC on the ability of *E. coli* MCR106 (*lamB106*) to bind $^{14}$C-labeled starch. *E. coli* MCR106 carrying the vector alone or the *susC* clone in the wrong orientation bound 1,500 to 2,500 cpm of labeled starch. *E. coli* MCR106 carrying *susC* cloned in the right orientation bound 6,000 to 6,500 cpm of starch if no IPTG was added (low-level expression of *susC*) and 9,000 to 10,000 cpm of labeled starch if 0.1 mM IPTG was added (moderate level of *susC* expression). This level of binding is about eightfold lower than that seen when wild-type *B. thetaiotaomicron* was tested but was consistently higher than the level seen with *E. coli* that was not producing SusC protein. Moreover, addition of unlabeled amylose or amylopectin to the incubation mixture containing the expressed SusC protein reduced binding by 30 to 50%, whereas addition of dextran (a glucan that is not used by the starch utilization system of *B. thetaiotaomicron*) had no effect on binding.

## DISCUSSION

We have identified a 115-kDa protein that is essential not only for growth on higher maltooligosaccharides such as G7 but also for growth on starch. This protein is probably an integral OMP. Two regions that might interact with the polysaccharide are the regions that have some sequence similarity to carbohydrate-binding proteins (51). It seems unlikely that SusC is a starch-degrading enzyme. First, Ms-2, which has a transposon insertion that eliminates production of SusC, had wild-type levels of amylase and pullulanase activities. Moreover, a number of amylase sequences are available in the databases and four amino acid motifs that are thought to be essential for enzyme activity have been identified (33). These motifs were not found in SusC. Two lines of evidence suggest that SusC might contribute to the binding of starch to the bacterial surface. First, SusC had greatest amino acid similarity to receptor proteins. Second, we found that *E. coli* producing

moderate levels of SusC could bind more radiolabeled starch than *E. coli* not producing this protein, although the amount of binding seen with the *E. coli* strain was appreciably lower than that seen in *B. thetaiotaomicron*. The lower level of binding is not surprising given the different membrane lipid composition of *B. thetaiotaomicron* outer membranes compared with those of *E. coli*. We have some preliminary evidence to suggest that there are other OMP genes downstream of *susC* that appear to encode the 65- and 43-kDa OMPs, which were also missing in Ms-2. This raises the possibility that SusC is part of a complex of OMPs. If so, this could explain why binding of starch by the *E. coli* strain producing SusC bound starch less effectively than wild-type *B. thetaiotaomicron.*

It is unlikely that SusC is a diffusion-type porin, because SusC was essential for growth on amylopectin as well as for growth on G7. Amylopectin is a large, branched molecule with a Stokes radius far too large to allow it to diffuse through a porin. SusC is probably not a protein involved in the export of starch-binding proteins, because such proteins are usually located in the cytoplasm or the cytoplasmic membrane, not in the outer membrane (36). Future cloning and identification of other OMPs would allow us to test this hypothesis more conclusively by providing antibodies that could be used to detect other membrane proteins in a mutant that has a disrupted *susC* gene. If SusC is part of the export apparatus, other starch-associated OMPs should not be localized to the outer membrane in a *susC* disruption mutant.

It is interesting that all of the receptors to which SusC had sequence similarity are known to interact with TonB. The *Bacteroides* equivalent of *tonB* has not been identified conclusively. We used PCR to amplify and clone a region of the *B. thetaiotaomicron* chromosome that had some sequence similarity to *tonB*, and we used this cloned fragment to make a gene disruption in *B. thetaiotaomicron* (37). This disruption did not affect growth on starch or any other polysaccharide that we tested. However, we cannot be sure that we amplified and disrupted the real *B. thetaiotaomicron tonB* because we have so far failed to identify any phenotype associated with the mutation. Thus, the region we amplified could be a pseudogene.

The fact that SusC was essential both for growth on G7 and for growth on starch indicates that the receptor complex involved in utilization of higher maltooligosaccharides is the same as that involved in starch utilization. Moreover, the fact that the *susC* disruption mutant Ms-2 grew normally on maltose and maltotriose, but poorly on maltotetraose and not at all on higher oligomers, indicates that there is a maltose uptake system that is distinct from the G7-starch uptake system. At present, we cannot rule out the possibility that the G7-starch uptake system can admit maltose and maltotriose, but if so, it is completely dispensable for growth on these substrates. It is also important to note that, although Ms-2 could not grow on starches, it could still grow on other polysaccharides, including the closely related polysaccharide dextran. Dextran is a linear polymer of glucose residues that are linked with α-1,6 bonds rather than α-1,4 bonds. Thus, the G7-starch uptake system is specific for polysaccharides that contain α-1,4-linked glucose residues.

A considerable amount of effort has gone into the characterization of the cellulose utilization complex (cellulosomes) of *Clostridium* spp. (24). Cellulosomes are a complex of proteins, which include scaffolding proteins as well as enzymes (31). It is thought that the cellulosome complex might be anchored to the cell surface via the ORF3 protein (11). The *Bacteroides* starch utilization system could prove to be a gram-negative equivalent of the clostridial cellulosomes. An important difference between the *Bacteroides* system and the clostridial system is that genetic tools available for use in *Bacteroides* spp. allow mutational analysis of gene function. Thus, the importance of *Bacteroides* starch utilization genes can be assessed by disrupting them and determining the effect of the disruption on the ability of the bacteria to utilize polysaccharides. So far, this type of genetic analysis has not been done with any of the cloned clostridial cellulosome genes.

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