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Location and Characterization of Genes Involved in Binding of Starch to the Surface of *Bacteroides thetaiotaomicron*

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Previous studies of starch utilization by the gram-negative anaerobe *Bacteroides thetaiotaomicron* have demonstrated that the starch-degrading enzymes are cell associated rather than extracellular, indicating that the first step in starch utilization is binding of the polysaccharide to the bacterial surface. Five transposon-generated mutants of *B. thetaiotaomicron* which were defective in starch binding (Ms-1 through Ms-5) had been isolated, but initial attempts to identify membrane proteins missing in these mutants were not successful. We report here the use of an immunological approach to identify four maltose-inducible membrane proteins, which were missing in one or more of the starch-binding mutants of *B. thetaiotaomicron*. Three of the maltose-inducible proteins were outer membrane proteins (115, 65, and 43 kDa), and one was a cytoplasmic membrane protein (80 kDa). The genes encoding these proteins were shown to be clustered in an 8.5-kbp segment of the *B. thetaiotaomicron* chromosome. Two other loci defined by transposon insertions, which appeared to contain regulatory genes, were located within 7 kbp of the cluster of membrane protein genes. The 115-kDa outer membrane protein was essential for utilization of maltoheptaose (G7), whereas loss of the other proteins affected growth on starch but not on G7. Not all of the proteins missing in the mutants were maltose regulated. We also detected two constitutively produced proteins (32 and 50 kDa) that were less prominent in all of the mutants than in the wild type. Both of these were outer membrane proteins.

Bacteroides thetaiotaomicron, a gram-negative anaerobe which is found in high numbers in the human colon, can ferment a variety of polysaccharides, including amylose, amylopectin, and pullulan (10). An unusual feature of the *B. thetaiotaomicron* starch utilization system is that the degradative enzymes are not extracellular but are located in the periplasm or cytoplasm (2, 14). Some membrane-bound activity was detected, but the enzymes appeared not to be exposed on the cell surface. These findings suggested that the first step in starch breakdown was binding of the polysaccharide to the bacterial surface, followed by translocation through the outer membrane into the periplasm, where the degradative enzymes were located.

Two types of evidence supported this model for starch breakdown. First, binding of labelled starch to the surface of *B. thetaiotaomicron* was demonstrated and was found to be protease sensitive, saturable, and specific for long stretches of α-1,4-linked glucose residues (2). Thus, it appeared that there might be a starch-specific receptor complex on the surface of the bacteria. Moreover, binding activity appeared to be regulated similarly to that of the degradative enzymes. That is, high-level binding activity was seen in bacteria grown on maltose but not in bacteria grown on glucose or other monosaccharides (2).

A second line of evidence supporting the hypothesis that binding of starch to the bacterial surface was an important step in starch utilization came from the analysis of transposon-generated mutants of *B. thetaiotaomicron* which were unable to grow on starch. Five mutants were obtained (Ms-1 through Ms-5) (1). All of these could grow on maltose (G2) and maltotriose (G3) but varied in their ability to grow on longer oligosaccharides such as maltoheptaose (G7). Three of the mutants (Ms-2, Ms-3, and Ms-4) produced normal or near-normal levels of the degradative enzymes when grown on maltose and appeared to be defective only in their ability to bind labeled starch (1). The fact that these mutants were unable to grow on starch showed that production of the degradative enzymes was not sufficient for starch utilization and indicated that starch binding was an essential step in starch utilization. The other two mutants, Ms-1 and Ms-5, were also deficient in starch binding but had other defects as well. Ms-1 did not produce any of the starch-degrading enzymes. Ms-5 produced fivefold-lower α-glucosidase activity and twofold-higher amylase and pullulanase activity than wild type. Thus, it appeared that both Ms-1 and Ms-5 might be regulatory mutants.

The availability of mutants which were defective in starch binding made it possible in theory to begin to identify the membrane proteins that mediated the binding of starch to the cell surface. However, comparisons of outer membrane protein profiles on one-dimensional sodium dodecyl sulfate-polyacrylamide gels did not reveal any proteins whose expression was maltose inducible, nor were there any apparent differences between the outer membrane protein profiles of the mutants and of the wild type (1). The outer membrane protein profile of *B. thetaiotaomicron* is quite complex (6, 7). Thus, if proteins involved in starch binding were not major membrane proteins, they might have been obscured on one-dimensional gels by comigrating outer membrane pro-

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teins. Accordingly, we took an immunological approach to detecting proteins that were missing in the mutants. In the present report, we describe the identification of four maltose-inducible and two constitutive proteins which were missing in one or more of the starch-binding mutants. Also, we show that the transposon insertions in Ms-1 through Ms-5 are clustered within an 18-kbp region of the *B. thetaiotaomicron* chromosome.

MATERIALS AND METHODS

Strains and growth conditions. All *Bacteroides* strains used in this study were derivatives of *B. thetaiotaomicron* 5482. The isolation and characterization of the Tn*4351*-generated mutants have been described previously (1). Construction of insertional disruptions in the *B. thetaiotaomicron* chromosome was done as described previously (13, 16), except that a newly constructed delivery plasmid, pBT-2, was used (see below). *Bacteroides* strains were grown on defined medium (6, 14) with glucose, maltose (G2), maltotriose (G3), maltoheptaose (G7), amylose, amylopectin, or pullulan as the sole carbohydrate source (final concentration of 0.5%). Amylose, amylopectin, and pullulan were autoclaved in the defined medium. The other carbohydrates were filter sterilized and added to the defined medium after being autoclaved. For cloning experiments, *Escherichia coli* DH5α MCR was used. *E. coli* was grown in Luria broth or on Luria broth agar supplemented either with tetracycline (10 μg/ml) or ampicillin (100 μg/ml).

Two-dimensional gel analysis of *B. thetaiotaomicron* membrane proteins. Membranes from *B. thetaiotaomicron* were prepared and analyzed on two-dimensional gels as described previously (17). An SE400 electrophoresis unit (Hoefer Scientific Instruments, San Francisco, Calif.) was used, and the conditions were those described in the appendix of the Hoefer Scientific Instruments catalog. Two-dimensional gels were stained with Coomassie blue. Wild-type *B. thetaiotaomicron* (grown on glucose or maltose) and the mutants Ms-2 and Ms-3 (grown on maltose) were analyzed in this way.

Polyclonal antiserum. Our approach to obtaining polyclonal antisera that detected maltose-inducible membrane proteins was based on the procedure described by King and Morrow (5) for preparing monoclonal antibodies against specific proteins in complex mixtures. However, since our method is a substantial modification of the procedure of King and Morrow, it is described in detail. *B. thetaiotaomicron* 5482 was grown on defined maltose medium to late exponential phase (optical density at 650 nm of 0.7 to 0.9). Membranes were obtained from sonically disrupted bacteria as described previously (14). Membranes (approximately 1 mg of protein total, in several different sites) were injected in a mixture with Freund's adjuvant into the back of a New Zealand White rabbit. A second injection of the same membrane preparation was given 2 weeks later. Approximately 1 to 2 weeks after a second injection, 10 to 15 ml of serum was obtained and cross-absorbed five times with membranes from bacteria grown on glucose-defined medium, as described previously (6), and the cross-absorbed antiserum was used to probe a Western blot which contained membranes from glucose-grown *B. thetaiotaomicron* (30 μg of protein) and membranes from maltose-grown *B. thetaiotaomicron* (30 μg of protein). At this stage, the antisera detected a number of constitutively expressed proteins but no maltose-inducible proteins.

Accordingly, membranes from maltose-grown bacteria were mixed 1:1 (vol/vol) with the undiluted cross-absorbed antiserum, and this mixture was used for a third and fourth injection into the same rabbit. Approximately 2 weeks after the fourth injection, serum was obtained and cross-absorbed as described before with membranes from glucose-grown *B. thetaiotaomicron*. This time, a Western blot of membranes from glucose-grown and maltose-grown bacteria revealed several maltose-specific proteins. It is not clear whether the approach described here worked because four rather than two injections were used or whether the antisera from the first bleed covered strong antigens and caused the immune system to respond to less immunogenic proteins. The procedure described above was used on two different rabbits to produce two different cross-absorbed antisera.

These cross-absorbed antisera were used to probe Western blots of membranes from wild type, transposon insertion mutants, and insertional mutants constructed with DNA segments from the region adjacent to Ms-2 (see below). Membranes were obtained from sonically disrupted cells by ultracentrifugation as described previously (14). Cells were grown in defined glucose or maltose medium. In some experiments, the soluble fractions (supernatants after ultracentrifugation) from the wild type and mutants were compared with membrane fractions on Western blots. On these blots, equal volumes of soluble fraction and resuspended membranes (resuspended in the original volume of 50 mM K-phosphate buffer [pH 7.0]) were loaded on the gel. To determine whether proteins detected by the antisera were located in the outer or cytoplasmic membrane of *B. thetaiotaomicron*, membranes from wild type (grown on maltose) were separated by the method described by Kotarski and Salyers (7), modified as described previously (4).

Cloning DNA adjacent to the transposon insertions in Ms-2 and Ms-3. Ms-2 and Ms-3 had been generated with the *Bacteroides* transposon Tn*4351* (1). Tn*4351* is introduced into *Bacteroides* species on the self-transmissible IncP plasmid R751, which cannot replicate in *Bacteroides* species (11). Tn*4351* sometimes cointegrates R751 (11), and such a cointegration event had occurred in both Ms-2 and Ms-3 (1). Preliminary mapping indicated that in both cases, R751 was flanked by one copy of Tn*4351* and one copy of IS*4351*. Within R751, there is a *Sal*I site that is close to one end of the Tn*4351* insertion in R751. Accordingly, we used *Sal*I to clone chromosomal DNA adjacent to Tn*4351*. Unless otherwise indicated, cloning and other DNA manipulations were done as described in Maniatis et al. (8). To distinguish the Tn*4351* end of the R751 insertion from the IS*4351* end of this insertion on Southern blots, we used ^{32}P-pBS9 as a probe. pBS9 contains an internal segment of Tn*4351* but does not cross-hybridize with IS*4351* (15). When Southern blots of *Sal*I-digested chromosomal DNA from Ms-2 were probed with ^{32}P-labeled pBS9, a single cross-hybridizing fragment with a size of about 16 kbp was detected. *Sal*I-digested chromosomal DNA from Ms-2 was then size fractionated on a sucrose gradient (8) to obtain fragments in the >10-kbp size range. This fraction was ligated into the *Sal*I site of pJRD215 (3). Tn*4351* carries a gene (*tetX*) that confers tetracycline resistance (Tc^r) on aerobically grown *E. coli* (15). Thus, pJRD215 clones containing Tn*4351* and linked chromosomal DNA could be obtained by selection of *E. coli* DH5α MCR transformants on Luria broth-tetracycline medium.

A clone with an insert of about 16 kbp was obtained. Tn*4351* accounted for about 5.5 kbp of this insert. From the restriction pattern of the non-Tn*4351* portion of the insert, it

was evident that the DNA adjacent to Tn*4351* was chromosomal DNA, not R751 DNA. There was an *Eco*RV site within the chromosomal DNA segment that was close to the end of the Tn*4351*. Another *Eco*RV site was found near the opposite end of the cloned chromosomal segment. This *Eco*RV fragment (7.7 kbp) was subcloned into the *Eco*RV site of pBR328 and used to generate a restriction map of the chromosomal DNA segment. The clone was designated pBK1-7. Using a similar strategy, we cloned a smaller segment of DNA (approximately 8 kbp in size) which contained Tn*4351* and about 3 kbp of chromosomal DNA adjacent to the Tn*4351* insertion in Ms-3.

In one experiment, sonicated cell extracts from *E. coli* DH5α MCR carrying pBK1-7 and from *E. coli* carrying pBR328 alone were analyzed on Western blots with the polyclonal antiserum that detected the starch-binding membrane proteins. Cell extracts of *E. coli* were prepared as described previously for cell extracts of *B. thetatiotaomicron*. These extracts were also assayed for amylase and pullulanase activity as described previously (14).

Relative locations of Tn*4351* insertions in mutants Ms-1 through Ms-5. The 7.7-kb *Eco*RV fragment adjacent to the Ms-2 Tn*4351* insertion was used to probe a Southern blot of *Eco*RI-digested chromosomal DNA from wild-type *B. thetaiotaomicron*. Two cross-hybridizing bands were detected (12 and 6.5 kbp). *Eco*RI cuts twice in Tn*4351*, about 26 bp from one end and about 1.2 kbp from the other end (11). Thus, if insertions in mutant Ms-1, Ms-3, Ms-4, or Ms-5 lay within the 18.5-kbp region spanned by the two *Eco*RI fragments, they would change the pattern of *Eco*RI bands which cross-hybridized with the 7.7-kbp *Eco*RV fragment. *Eco*RI digests of DNA from mutants Ms-1, Ms-3, Ms-4, and Ms-5 were probed on Southern blots with the ^{32}P-labeled 7.7-kbp *Eco*RV fragment, and the digest patterns were compared with that of wild type. When necessary, digests made with other enzymes were also probed with the 7.7-kbp *Eco*RV fragment to determine more precisely the location of insertions within the 18.5-kbp region around Ms-2.

Insertional mutations in the cloned region adjacent to the site of the Tn*4351* insertion in Ms-2. Insertional mutations in the wild-type *B. thetaiotaomicron* chromosome were created by subcloning segments of DNA from the 7.7-kbp *Eco*RV fragment into a suicide plasmid and introducing these subclones into *B. thetaiotaomicron*, with selection for single crossover insertions into the cloned region. Vectors constructed previously for this purpose were unsatisfactory. The mobilization frequency of pVAL7 (13) was so low that multiple attempts had to be made to obtain a single insertion mutant. A more recent construct which had a higher mobilization frequency (pNJR6 [16]) had the drawback that there was no simple screen for clones in this low-copy-number plasmid. Accordingly, we constructed a new suicide vector, pBT-2. pBT-2 (Fig. 1) was constructed by cloning the *Bacteroides* Tc^r gene on a 2.7-kbp *Sst*I fragment from pNFD13-2 (12, 16) into the *Sst*I site on pJRD215 (3), thereby inactivating the streptomycin resistance gene. This construct was designated pBT-1. pBT-1 was then digested with *Sal*I and *Hin*dIII to remove the multiple-cloning site of pJRD215. The ends of the digested vector were then blunted. Plasmid pUC18 was digested with *Afl*III and *Nde*I, and a 620-bp fragment carrying the *lacZ* α-complementing fragment and multiple-cloning site was extracted from a gel, blunted, and cloned into the blunted *Sal*I and *Hin*dIII sites. Restriction digests and other manipulations were done as described by Maniatis et al. (8).

pBT-2 was mobilized from *E. coli* to *Bacteroides* species by the IncP plasmid, R751. Conjugal transfers were performed as described previously (12, 13), with selection for Tc^r *B. thetaiotaomicron* transconjugants. Since pBT-2 contained no *Bacteroides* replication origin, Tc^r transconjugants were obtained only if the plasmid was integrated into the *B. thetaiotaomicron* chromosome via a single crossover in the DNA segment cloned into pBT-2. The location of each insertion made in the 7.7-kbp *Eco*RV region was checked by Southern blotting, with the 7.7-kbp *Eco*RV fragment or subclones of this fragment as the probe. Each insertion was scored for its ability to grow on G2, G3, G7, amylose, amylopectin, and pullulan. Also, membrane fractions from each insertional mutant were analyzed on Western blots for the proteins detected by the antiserum.

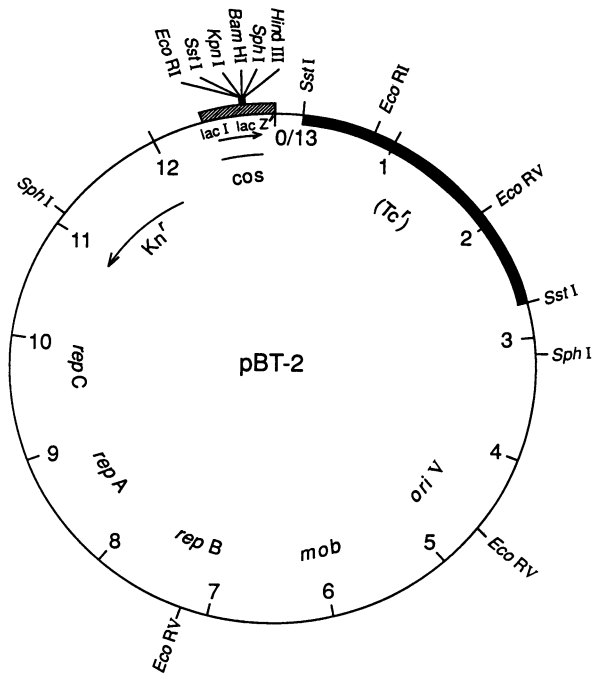

FIG. 1. Partial restriction map of pBT-2, the vector used to make gene disruptions in the *B. thetaiotaomicron* chromosome. The selectable marker in *E. coli* is kanamycin resistance (Kn^r). The selectable marker in *B. thetaiotaomicron* is tetracycline resistance (Tc^r). The DNA sequence of the Tc^r gene, *tetQ*, has been determined elsewhere (9). The α-complementing fragment of *lacZ* (*lacZ'*) is represented by the shaded bar. Locations of genes or regions on pJRD215 which are needed for replication (*repA*, *repB*, *repC*, and *oriV*), mobilization (*mob*), or cosmid packaging (*cos*) are also indicated. pBT-2 is mobilized by R751 from *E. coli* to *B. thetaiotaomicron*.

RESULTS

Two-dimensional gel analysis of maltose-inducible proteins. Since our previous failure to detect maltose-inducible membrane proteins using one-dimensional gels could have been due to comigration of these proteins with more abundant constitutively expressed proteins, we attempted to detect maltose-inducible proteins using two-dimensional gel analysis. A comparison of the protein profiles of membranes from glucose-grown and maltose-grown *B. thetaiotaomicron* on two-dimensional gels revealed many proteins that were expressed at a higher level in cells grown on glucose than in

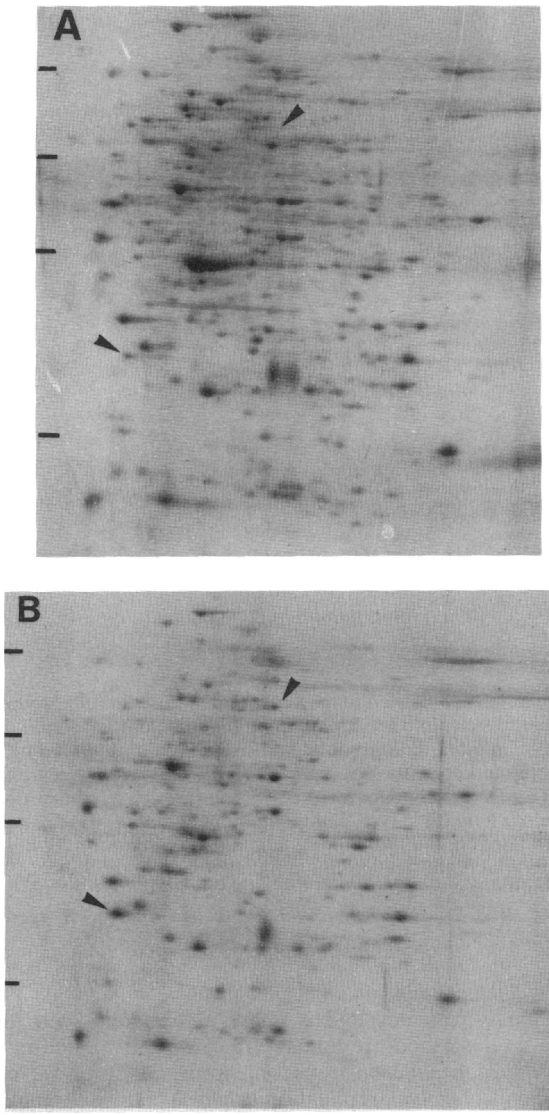

FIG. 2. Two-dimensional gels of membrane proteins from *B. thetaiotaomicron*. Gels were stained with Coomassie blue. (A) Membrane proteins from bacteria grown on glucose; (B) membrane proteins from bacteria grown on maltose. Arrowheads indicate the positions of proteins which were expressed at higher levels in bacteria grown on maltose than in bacteria grown on glucose. The top arrowhead indicates a protein doublet. Hatch marks at the left of the gels indicate migration distances of the following molecular mass markers (from top to bottom): 110, 71, 44, and 29 kDa.

cells grown on maltose. However, we detected only three proteins that were expressed at higher levels in maltose-grown cells than in glucose-grown cells (Fig. 2A and B). None of these was missing in the two mutants that were deficient only in starch binding, Ms-2 or Ms-3 (data not shown). Thus, either the proteins affected by transposon insertions in Ms-2 and Ms-3 were not membrane proteins or they were expressed at too low a level to be detectable on Coomassie-stained gels. Accordingly, we tried an immunological approach to detecting maltose-inducible membrane proteins.

Identification and localization of four maltose-inducible membrane proteins. Two polyclonal antisera raised against

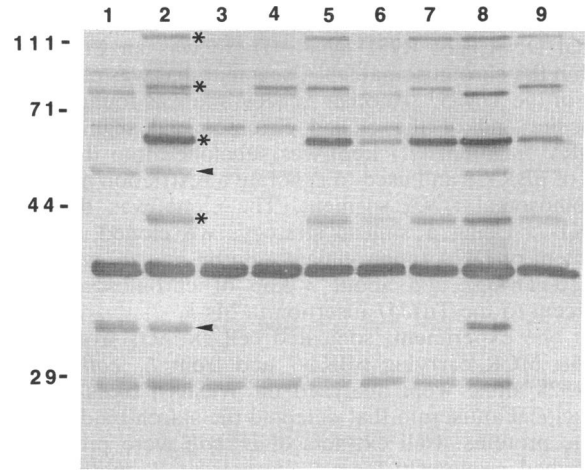

FIG. 3. Western blot probed with antiserum which detected four maltose-induced membrane proteins. Migration distances of molecular mass standards (in kilodaltons) are shown at the left of the blot. Lanes: 1, membranes from wild type grown on glucose; 2, membranes from wild type grown on maltose. Asterisks indicate the maltose-inducible proteins. Arrowheads indicate two constitutively produced proteins which were missing in the starch-minus mutants. Lanes 3 to 7, membranes from the following starch-minus mutants: Ms-1 (lane 3), Ms-2 (lane 4), Ms-3 (lane 5), Ms-4 (lane 6), and Ms-5 (lane 7). All mutants were grown on maltose; lane 8, outer membranes from wild type grown on maltose; lane 9, inner membranes from wild type grown on maltose. In all lanes, approximately 100 μg of protein was loaded.

membranes of maltose-grown *B. thetaiotaomicron* and cross-absorbed as described in Materials and Methods detected four maltose-inducible membrane proteins on Western blots (Fig. 3, lanes 1 and 2). Both antisera also detected several constitutively expressed proteins which served as internal standards to confirm that the same amount of protein was loaded in each lane.

Comparison of outer membrane and cytoplasmic membrane fractions from *B. thetaiotaomicron* grown on maltose (Fig. 3, lanes 8 and 9) suggested that three of the maltose-induced proteins (115, 65, and 43 kDa) were outer membrane proteins, whereas the other maltose-induced protein (80 kDa) was located in the cytoplasmic membrane. As can be seen with some of the bands in Fig. 3 (lanes 8 and 9), there was some cross-contamination between the outer and cytoplasmic membrane fractions. Such cross-contamination is not uncommon in separations of *Bacteroides* membranes and results from the fact that these two membranes differ less in density than *E. coli* cytoplasmic and outer membranes (6, 7). Nonetheless, the concentration of each of the four maltose-inducible proteins was clearly higher in one membrane fraction than the other.

Comparison of soluble and membrane fractions from the wild type grown on glucose and maltose (data not shown) revealed that in all but one case, the proteins shown in Fig. 3 fractionated with the membranes and not with the soluble fraction. The exception was the 80-kDa protein. Western blot analysis revealed that there was nearly as much of this protein in the soluble fraction as in the membrane fraction.

Analysis of starch utilization mutants Ms-1 through Ms-5. The maltose-inducible proteins identified by the polyclonal antisera were missing in one or more of the starch utilization mutants (Fig. 3, lanes 3 to 8; Table 1). Ms-2 was missing all three maltose-inducible outer membrane proteins. Ms-4 was

TABLE 1. Effect of different starch-minus mutations on expression of four maltose-inducible membrane proteins

Strain[a]	Presence of maltose-inducible membrane proteins[b]				Growth on[c]:	
	OM115	OM43	OM65	IM80	G2	G7
Wild type	+	+	+	+	+	+
Ms-1	−	−	−	−	+	−
Ms-5	+	+	+	+	+	+
Ms-2	−	−	−	+	+	−
Ω1	−	−	−	+	+	−
Ω2	−	−	−	+	+	−
Ω3	+	+	−	+	+	+
Ω4	+	−	−	+	+	+
Ω5	+	−	−	+	+	+
Ω6	+	−	−	+	+	+
Ω7	+	+	−	+	+	+
Ms-3	+	+	+	+	+	+
Ms-4	+/−	+/−	+/−	−	+	+

[a] All strains were grown on maltose. See Fig. 4 and 5 for locations of transposon and single-crossover insertions.

[b] OM115, 115-kDa outer membrane protein; OM43, 43-kDa outer membrane protein; OM65, 65-kDa outer membrane protein; IM80, 80-kDa protein associated with the inner membrane.

[c] G2, maltose; G7, maltoheptaose.

missing the cytoplasmic membrane protein. However, Ms-4 also exhibited lower levels of the three outer membrane proteins (Fig. 3, lane 6). Ms-1 did not produce any of the maltose-inducible proteins, whereas Ms-3 and Ms-5 still produced all four of them.

Only one of the proteins detected by the antiserum, the 80-kDa inner membrane protein, was similar in size to the maltose-inducible proteins seen on the Coomassie-stained two-dimensional gels (Fig. 2). A Western blot of two-dimensional gels of membrane proteins from glucose-grown and maltose-grown wild type showed that the antiserum reacted with a pair of maltose-inducible proteins, which migrated at the position of the pair of proteins marked by the uppermost arrow in Fig. 2 (data not shown). Like the 80-kDa protein identified by the antiserum, this pair of proteins was present in Ms-2 and Ms-3. Thus, the 80-kDa protein identified by the antiserum is probably the same as the maltose-inducible protein doublet seen in Coomassie blue-stained two-dimensional gels.

Constitutively produced proteins detected by the antisera. Although both of the antisera detected the same four maltose-inducible membrane proteins, the antisera differed somewhat in the constitutively produced proteins they detected. Moreover, although the pattern of absence or presence of the maltose-inducible proteins in extracts from the different mutants remained constant over a number of different Western blots, this was not true of two constitutively produced proteins (indicated by arrows in Fig. 3). Initially, proteins migrating at the positions of these two proteins appeared to be present in all of the mutants. However, as the antisera were reused and presumably became further cross-absorbed by the blotting procedure, the concentrations of these two proteins (32 and 50 kDa) appeared to be lower in the mutants than in the wild type. The Western blot shown in Fig. 3 is one in which the difference between the wild type and the mutants was particularly pronounced. However, a substantially decreased level of these two proteins in all of the mutants was observed in several different Western blots, made with different extracts. The 32- and 50-kDa proteins fractionated with the outer membrane (Fig. 3, lanes 8 and 9).

Proximity of the Tn*4351* insertions in Ms-1, Ms-2, Ms-3, Ms-4, and Ms-5. We wanted to clone DNA adjacent to Ms-2 and Ms-3 because these two mutants appeared to be deficient only in starch binding. Thus, the transposon insertions might have interrupted genes essential for binding. DNA segments adjacent to both Ms-2 and Ms-3 were obtained. When restriction maps of the two chromosomal segments were compared, it became evident that the map of the DNA segment adjacent to the Ms-3 insertion overlapped with one end of the map of the segment adjacent to the Ms-2 insertion (data not shown). Thus, it appeared that the Tn*4351* insertion site in Ms-3 was approximately 7 kbp from the Tn*4351* insertion site in Ms-2. This raised the question of whether Tn*4351* insertion sites in the other starch-minus mutants were located in the same region of the *B. thetaiotaomicron* chromosome. To determine whether this was so, the 7.7-kbp *Eco*RV fragment of chromosomal DNA adjacent to the Ms-2 insertion was used to probe Southern blots containing DNA from Ms-1, Ms-3, Ms-4, and Ms-5. The results of Southern analysis revealed that all of these insertions were within a region of approximately 18 kbp (Fig. 4A).

Effect of insertional mutations adjacent to the Ms-2 insertion site. Ms-2 lacked all but one of the maltose-inducible membrane proteins (Table 1; Fig. 3). A possible explanation was that the Tn*4351* insertion in Ms-2 had occurred in an operon and had a polar effect on expression of downstream genes. Alternatively, Ms-2 might be a regulatory mutant which affected expression of the genes encoding these three proteins. If the genes encoding the three membrane proteins are part of an operon, the size of the operon should be at least 7 kbp. The insertions closest to the Ms-2 insertion were Ms-5 and Ms-3, both of which expressed all three of the maltose-inducible outer membrane proteins. Since Ms-5 was within 3 kbp of Ms-2 on one side, it seemed likely that the operon would extend from Ms-2 toward the Ms-3 insertion rather than toward the Ms-5 insertion, because the Ms-3 insertion was far enough away for the intervening DNA to accommodate an operon of the expected size.

To determine whether the three outer membrane proteins were clustered between Ms-2 and Ms-3, segments of the 7.7-kbp *Eco*RV fragment were subcloned into pBT2 (Fig. 1) and introduced into *B. thetaiotaomicron* by conjugation, with selection for Tc^r. The DNA segments used to make the insertions are diagrammed in Fig. 4B, and the phenotype of each insertion is listed in Table 1. All of the insertion mutants were unable to grow on starch, but only Ω1 and Ω2 were unable to grow on G7 (i.e., they had the Ms-2 growth phenotype). Since inability to grow on G7 was associated with loss of the 115-kDa outer membrane protein, this protein presumably has some role in utilization of short oligomers of glucose.

With one exception, the phenotypes of the insertional mutants suggested that the genes for the three outer membrane proteins were in an operon and that the gene for the 115-kDa protein had been interrupted by the Ms-2 insertion (Fig. 4A). The exception was insertion Ω3. This insertion mutant produced the 43-kDa proteins, whereas insertion mutants Ω4, Ω5, and Ω6 did not. We confirmed by Southern blotting that the Ω3 insertion was actually in the location indicated in Fig. 4B and was not incorrectly located on the restriction map. This was done by digesting DNA from mutant Ω3 with enzymes that cut outside the 1-kbp *Xma*III fragment used to make the Ω3 insertion and probing the digests with the 1-kbp *Xma*III fragment (data not shown).

If the genes encoding the three outer membrane proteins were arranged as depicted in Fig. 4A, only a portion of the

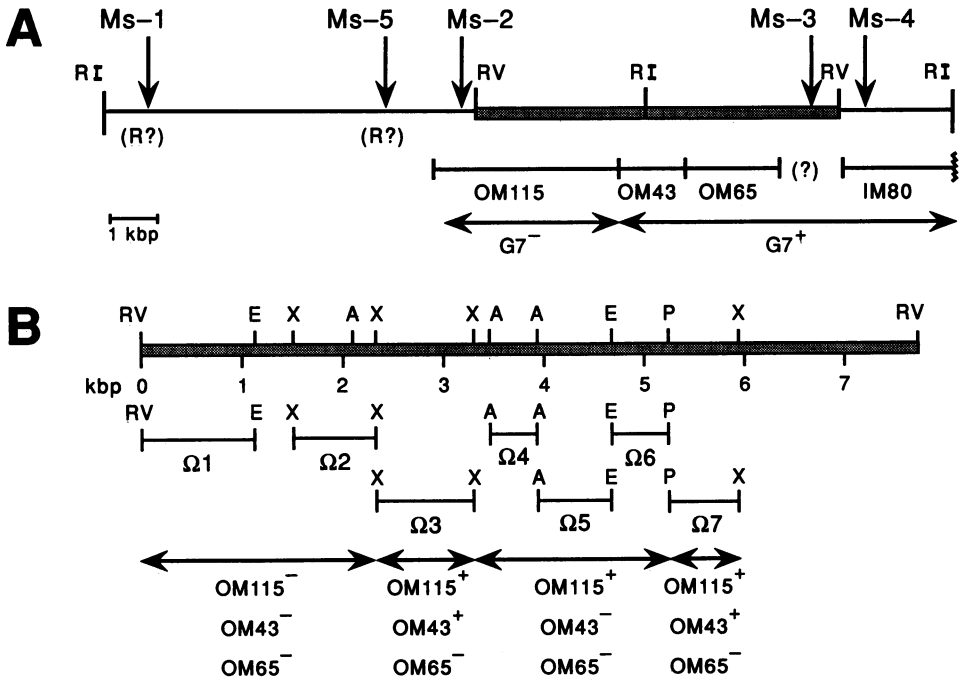

FIG. 4. (A) Location of Tn*4351* (or Tn*4351*-R751) insertions (vertical arrows) in different starch-minus mutants. Possible locations of genes encoding the inner membrane protein (IM80) and the outer membrane proteins (OM115, OM43, and OM65) are shown under the line representing the chromosomal segment. These locations are based on phenotypes of the Tn*4351* mutants (Table 1) and of the insertional disruption mutants (Table 1 and panel B). (R?) indicates that insertions in Ms-1 and Ms-5 probably interrupt regulatory genes. (?) indicates that no protein phenotype has yet been found for the gene interrupted by the Tn*4351* insertion in Ms-3. (B) Insertional disruptions made in the *Eco*RV segment that lies between the Ms-2 and Ms-3 insertion sites (indicated by the thick shaded line in panels A and B). Segments used to make the disruptions are shown below the thick shaded line. Each segment was cloned into pBT-2 and introduced into the *B. thetaiotaomicron* chromosome by selection for the Tc^r gene on pBT-2. The strains containing the insertional mutations were designated Ω1 through Ω7. All mutants still grew on G2 and G3 but were unable to grow on amylose, amylopectin, and pullulan. Insertion mutants Ω1 and Ω2 were unable to grow on G7, whereas all of the other insertion mutants grew on G7. The presence (+) or absence (−) of OM115, OM43, and OM65 in the insertion mutants (Table 1) is indicated at the bottom of the figure. RI, *Eco*RI; RV, *Eco*RV; X, *Xmn*I; A, *Afl*III; P, *Pvu*II; E, *Eag*I.

gene for the 115-kDa protein would be on the *Eco*RV clone. However, the genes for the 43- and 65-kDa proteins should have been intact on this clone. Previous experience has shown that even though *Bacteroides* promoters generally do not express in *E. coli*, *Bacteroides* DNA contains many fortuitous *E. coli* promoter sequences (see, for example, references 9 and 13). Thus, it might be possible to detect expression in *E. coli* of some of the proteins encoded on the 7.7-kbp *Eco*RV fragment. We used one of the polyclonal antisera to probe Western blots of extracts from *E. coli* DH5α MCR carrying the cloned 7.7-kbp *Eco*RV fragment (pBK1-17) and extracts from *E. coli* DH5α MCR carrying only the vector (pBR328). A protein which comigrated with the 43-kDa protein was detected by the antiserum in extracts from cells carrying the clone but not in extracts from cells carrying the vector (data not shown). Thus, the structural gene for the 43-kDa protein, at least, appeared to be encoded on the 7.7-kDa *Eco*RV fragment. There was also a faint doublet which migrated slightly above the 50-kDa constitutively expressed protein and which was detected only in extracts from cells carrying the cloned 7.7-kbp *Eco*RV fragment. Previously, we had found that a cloned *B. thetaiotaomicron* pullulanase was expressed as a doublet in *E. coli* maxicells (13), possibly because the initiation or processing of the protein in *E. coli* was not the same as in *B. thetaiotaomicron*. Thus, this doublet could be the 65-kDa protein, aberrantly expressed in *E. coli*.

DISCUSSION

Analysis of two-dimensional gels of *B. thetaiotaomicron* membrane proteins identified three maltose-inducible proteins. An immunological approach identified four maltose-inducible proteins on one-dimensional gels. Two of the maltose-inducible proteins seen on the Coomassie blue-stained two-dimensional gels migrated close together and had molecular masses of approximately 80 kDa. Since they reacted with the antiserum and were present in membranes from both Ms-2 and Ms-3, it is likely that the protein doublet seen on the two-dimensional gels is the same as the 80-kDa maltose-inducible protein that was identified by the antiserum and was missing in Ms-4. It is not clear whether the protein doublet seen in the two-dimensional gels is two forms of the same protein or two different proteins that happen to comigrate on one-dimensional gels. The other maltose-inducible protein seen on the Coomassie blue-stained two-dimensional gels had a molecular mass (approximately 35 kDa) different from those of the maltose-inducible proteins identified by the antiserum. Also, the 35-kDa protein seen in the two-dimensional gels was present in Ms-2, whereas 43-, 65-, and 115-kDa maltose-inducible proteins detected by the antisera were missing in this mutant. Thus, we have identified a total of at least five maltose-inducible membrane proteins. None of these was a major membrane protein.

The 43-, 65-, and 115-kDa maltose-inducible proteins were clearly outer membrane proteins, because little or no protein with these molecular masses was seen in the cytoplasmic membrane or soluble fraction. The location of the 80-kDa protein is less certain. It is not an outer membrane protein because it was not detected in an outer membrane fraction. The absence of the 80-kDa protein from this fraction also indicates that it is not a protein that binds nonspecifically to membranes. The 80-kDa protein fractionated with the cytoplasmic membrane fraction in the inner-outer membrane separation experiments, but comparisons of membrane and soluble fractions showed that nearly equal amounts of the protein were seen in the soluble and membrane fractions. *Bacteroides* cytoplasmic membranes are difficult to pellet quantitatively, but at least 90% of the cytoplasmic membrane should have been pelleted under the conditions used (6). Thus, the 80-kDa protein could be a peripheral membrane protein rather than an integral membrane protein. Alternatively, if the 80-kDa band seen on the Western blots really represents two comigrating proteins, it is possible that one of these proteins is soluble and that the other is a cytoplasmic membrane protein.

Determining the functions of the four maltose-inducible proteins seen on Western blots was complicated by the surprising observation that two constitutively expressed proteins in all of the mutants were produced at levels much lower than those in the wild type. The starch-minus phenotype of Ms-3, a mutant which produced wild-type levels of all of the degradative enzymes and all of the maltose-inducible proteins detected on two-dimensional gels or by the antisera, could have been due to lack of these proteins. Alternatively, lack of some maltose-inducible protein not detected by any of the methods we employed might have been responsible for the starch-minus phenotype. If the constitutively produced 32- and 50-kDa proteins are required for starch utilization, we cannot conclude from our results whether any of the maltose-inducible membrane proteins are essential for starch utilization, because mutants that lacked one or more of the maltose-inducible proteins also exhibited decreased synthesis of the 32- and 50-kDa proteins. We can, however, conclude that the 115-kDa protein is important for utilization of G7, because the inability to grow on G7 was associated specifically with insertions that prevented synthesis of the 115-kDa protein (Ω1 and Ω2 [Fig. 4B; Table 1]).

We can also conclude that the maltose-inducible 80-kDa protein (or proteins), which may be associated with the cytoplasmic membrane, has no role in transport of maltose or maltotriose, because both mutants that lacked this protein (Ms-1 and Ms-4) could still grow on maltose and maltotriose. It is not clear whether the Tn*4351* insertion in Ms-4 interrupted the gene encoding the 80-kDa protein or a gene that encodes a regulatory protein. The latter possibility is suggested by our observation that Ms-4 also exhibited reduced levels of the three maltose-inducible outer membrane proteins. Two alternative possibilities are that the 80-kDa protein itself regulates expression of the three outer membrane protein genes or that loss of the 80-kDa protein disrupts a complex and leads to increased turnover of the outer membrane proteins. Cloning and further characterization of the Ms-4 locus will be necessary to resolve these questions.

The results of insertional mutagenesis of DNA adjacent to the Ms-2 insertion suggested that genes encoding the three maltose-inducible outer membrane proteins are all located in this region of the *B. thetaiotaomicron* chromosome. The phenotype of the one insertion mutant that does not fit this hypothesis, Ω3, could be explained by assuming that the Ω3 insertion created a fortuitous promoter that allowed expression of a downstream gene. However, another possible explanation is that this region did not contain the genes encoding the membrane proteins but rather genes encoding regulatory proteins which controlled expression of the membrane protein genes. The fact that the cloned *Eco*RV segment which contained DNA adjacent to the Ms-2 insertion expressed (in *E. coli*) a protein that comigrated with the 43-kDa outer membrane protein indicates that the gene encoding this protein is in the region between the Ms-2 and the Ms-3 insertions. However, we cannot be sure that the genes for the other two outer membrane proteins are in this region. Further genetic analysis, including DNA sequence analysis and transcriptional fusion experiments, will be needed to ascertain whether there is an operon encoding three outer membrane proteins arranged as drawn in Fig. 4.

All four of the maltose-inducible proteins were missing in Ms-1, the mutant which also did not produce any of the degradative enzymes. This finding, together with the fact that the insertion in Ms-1 is not located in the same region of the chromosome that appears to contain the genes for the maltose-inducible membrane proteins (Fig. 4A), supports the hypothesis that the transposon insertion in Ms-1 interrupts a regulatory locus that controls expression of many starch utilization genes. Ms-5 had also appeared to be a regulatory mutant because of its aberrant expression of starch-degrading enzymes (2). If the Tn*4351* insertion in Ms-5 has in fact disrupted a regulatory locus, this locus does not control expression of the four maltose-inducible proteins detected on the Western blots.

Our results indicate that starch binding and uptake by *B. thetaiotaomicron* may involve a number of proteins. In this study, we focused on membrane-associated proteins because we expected that at least some of the proteins required for binding and uptake would be outer membrane proteins. However, periplasmic proteins could also be part of the complex that mediates binding and translocation. The results of previous studies had indicated that the amylase and pullulanase activities were either located in the periplasm or associated with membranes (1, 14). These enzymes might be part of a complex which also includes the outer membrane proteins. Our finding that all of the previously isolated starch-minus Tn*4351* insertions are clustered in the same region of the *B. thetaiotaomicron* chromosome raises the possibility that a number of starch utilization genes may be located in this region. Cloning and further characterization of the regions linked to the insertions in Ms-1, Ms-4, and Ms-5 may reveal genes encoding other proteins involved in starch binding.

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REFERENCES

1. **Anderson, K. L., 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.
2. **Anderson, K. L., 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.
3. **Davidson, J., M. Heusterspeute, N. Chevalier, V. Ha-Thi, and F. Brunel.** 1987. Vectors with restriction site banks. V. pJRD215, a wide host range cosmid vector with multiple cloning sites.

Gene **51:**275–280.

4. **Gherardini, F. C., and A. A. Salyers.** 1987. Partial purification and characterization of an outer membrane galactomannanase from *Bacteroides ovatus*. J. Bacteriol. **169:**2038–2043.
5. **King, S. W., and K. J. Morrow.** 1988. Monoclonal antibodies produced against antigenic determinants present in complex mixtures of proteins. BioTechniques **6:**856–861.
6. **Kotarski, S. F., J. Linz, D. M. Braun, and A. A. Salyers.** 1985. Analysis of outer membrane polypeptides that are associated with growth of *Bacteroides thetatiotaomicron* on chondroitin sulfate. J. Bacteriol. **163:**1080–1086.
7. **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.
8. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
9. **Nikolich, M. P., N. B. Shoemaker, and A. A. Salyers.** 1992. A *Bacteroides* tetracycline resistance gene represents a new class of ribosome protection tetracycline resistance. Antimicrob. Agents Chemother. **36:**1005–1012.
10. **Salyers, A. A., J. R. Vercellotti, S. E. H. West, and T. D. Wilkins.** 1977. Fermentation of mucin and plant polysaccharides by strains of *Bacteroides* from the human colon. Appl. Environ. Microbiol. **33:**319–322.
11. **Shoemaker, N. B., C. Getty, J. F. Gardner, and A. A. Salyers.** 1986. Tn*4351* transposes in *Bacteroides* spp. and mediates the integration of plasmid R751 into the *Bacteroides* chromosome. J. Bacteriol. **165:**929–936.
12. **Shoemaker, N. B., R. D. Barber, and A. A. Salyers.** 1989. Cloning and characterization of a *Bacteroides* conjugal tetracycline-erythromycin resistance element by using a shuttle cosmid vector. J. Bacteriol. **171:**1294–1302.
13. **Smith, K. A., and A. A. Salyers.** 1989. Cell-associated pullulanase from *Bacteroides thetaiotaomicron*: cloning, characterization, and insertional mutagenesis to determine role in pullulan utilization. J. Bacteriol. **171:**2116–2123.
14. **Smith, K. A., and A. A. Salyers.** 1991. Characterization of a neopullulanase and an α-glucosidase from *Bacteroides thetaiotaomicron* 95-1. J. Bacteriol. **173:**2962–2968.
15. **Speer, B. S., and A. A. Salyers.** 1989. Novel aerobic tetracycline resistance gene that chemically modifies tetracycline. J. Bacteriol. **171:**148–153.
16. **Stevens, A. M., N. B. Shoemaker, and A. A. Salyers.** 1990. The region of a *Bacteroides* conjugal tetracycline resistance element which is responsible for production of plasmid-like forms from unlinked chromosomal DNA might also be involved in transfer of the element. J. Bacteriol. **172:**4271–4279.
17. **Valentine, P. J., and A. A. Salyers.** 1992. Analysis of proteins associated with growth of *Bacteroides ovatus* on the branched galactomannan guar gum. Appl. Environ. Microbiol. **58:**1534–1540.