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Analysis of Proteins Associated with Growth of *Bacteroides ovatus* on the Branched Galactomannan Guar Gum

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***Bacteroides ovatus*, a gram-negative obligate anaerobe from the human colon, can ferment the branched galactomannan guar gum. Previously, three enzymes involved in guar gum breakdown were characterized. The expression of these enzymes appeared to be regulated; i.e., specific activities were higher in extracts from bacteria grown on guar gum than in extracts from bacteria grown on the monosaccharide constituents of guar gum, mannose and galactose. In the present study, we used two-dimensional gel analysis to determine the total number of *B. ovatus* proteins enhanced during growth on guar gum. Twelve soluble proteins and 20 membrane proteins were expressed at higher levels in guar gum-grown cells than in galactose-grown cells. An unexpected finding was that the expression of the two galactomannanases was induced by glucose as well as guar gum. Three other proteins, one membrane protein and two soluble proteins, had this same expression pattern. The remainder of the guar gum-associated proteins seen on two-dimensional gels and the guar gum-associated α-galactosidase were induced in cells grown on guar gum but not in cells grown on glucose. Two transposon-generated mutants (M-5 and M-7) that could not grow on guar gum were isolated. Both mutants still expressed the galactomannanases and the α-galactosidase. They also still expressed all of the guar gum-associated proteins that could be detected in two-dimensional gels of glucose-grown or galactose-grown cells. A second transposon insertion that suppressed the guar gum-negative phenotype of M-5 was isolated and characterized. The characteristics of this suppressor mutant indicated that the original transposon insertion was probably in a regulatory locus.**

The diets of humans and animals contain a variety of complex polysaccharides. These polysaccharides are not degraded in the stomach and small intestine but are fermented in the colon by the resident bacterial microflora. In ruminants, such polysaccharides may also be fermented by bacteria in the rumen. Although many of the polysaccharides found in foods are branched polysaccharides, most of the work done on polysaccharide utilization has focused on the simple linear polysaccharides, such as amylose, cellulose, chondroitin sulfate, and pectin (14). A good model polysaccharide for studying the utilization of branched polysaccharides is guar gum, a galactomannan consisting of a β-1,4-linked mannose backbone with α-1,6-linked galactose branches (11). Guar gum is one of the simplest and best-characterized branched polysaccharides. It is also relatively soluble and available commercially in pure form.

Bacteroides ovatus, a gram-negative obligate anaerobe from the human colon, ferments guar gum (15). Previously, Gherardini et al. (3–5) characterized two galactomannanases and one α-galactosidase that were involved in the breakdown of guar gum by *B. ovatus*. Since *B. ovatus* produces other α-galactosidases when grown on other substrates, the guar gum-associated enzyme was designated α-galactosidase I. The two galactomannanases cleaved the β-mannan backbone of guar gum into large pieces (>100 kDa; 4, 5). α-Galactosidase I removed galactose branches from these large galactomannan segments. The infrequent cutting of the galactomannan backbone by the galactomannanases was probably due to interference by galactose branches, because once galactose branches had been removed by α-galactosidase I, the galactomannanases could hydrolyze the backbone to single mannose residues (4, 5).

The three enzymes involved in galactomannan breakdown were found to be cell associated (3–5). One of the galactomannanases was located in the outer membrane. The other galactomannanase and α-galactosidase I were soluble and were located in either the periplasm or the cytoplasm. The results of previous studies had indicated that human *Bacteroides* species generally do not secrete polysaccharide-degrading enzymes into the extracellular fluid. Rather, the first step in polysaccharide breakdown appears to be binding of the polysaccharide to the outer membrane (2, 7). The polysaccharide is then internalized and degraded in the periplasm or cytoplasm (2, 13). In the case of guar gum, which not only is branched but also can have a molecular mass of over 1 MDa, this type of strategy seemed at first to be unlikely. In fact, one of the galactomannanases was found to be located in the outer membrane and could thus be exposed on the bacterial surface. Therefore, some breakdown of guar gum presumably occurs before uptake. However, the products of the outer membrane enzyme were quite large (100 kDa). Also, α-galactosidase I, which was needed in combination with the galactomannanases to degrade guar gum completely to monosaccharides, was intracellular. Thus, even though guar gum might be partially degraded at the cell surface, it appeared that the binding and uptake of large guar gum segments would still be necessary.

Since labeled guar gum is not commercially available, it was not possible to measure the binding and uptake of guar gum or guar gum fragments directly. However, it was possible to identify proteins that might be involved in early steps in guar gum utilization by assuming that these proteins would be regulated similarly to the enzymes and using two-dimensional gel analysis to determine how many proteins, especially membrane proteins, exhibited the same

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type of regulation. In previous studies, elevated levels of α-galactosidase I and the two galactomannanases (I and II) were seen in bacteria grown on guar gum but not in bacteria grown on galactose or mannose (3–5). Thus, our initial goal was to use two-dimensional gel analysis of *B. ovatus* protein profiles to determine how many proteins were induced during growth on guar gum but not during growth on its monosaccharide constituents.

Although some investigations of selected polysaccharide-induced membrane proteins have been done with another *Bacteroides* species, *Bacteroides thetaiotaomicron* (2, 7, 8), no systematic study of polysaccharide-inducible *Bacteroides* proteins has been attempted. We report here that over 30 membrane and soluble proteins were induced during growth on guar gum. We also made the surprising observation that a subset of the proteins induced by guar gum were also induced by glucose. Finally, we characterized two transposon-generated mutants of *B. ovatus* that had lost the ability to utilize galactomannan, and we provide evidence suggesting that at least one of the transposon insertions might have affected a regulatory locus.

MATERIALS AND METHODS

Strains and growth conditions. *B. ovatus* 0038 was obtained from the culture collection of the Virginia Polytechnic Institute Anaerobe Laboratory, Blacksburg. Bacteria were grown on defined medium containing glucose, galactose, or guar gum as the sole carbohydrate source (3). Erythromycin was added to the medium used to grow strains carrying Tn*4351*.

Tn*4351* was used to make transposon-generated mutants of *B. ovatus*. The procedure for introducing Tn*4351* into *B. ovatus* was the same as that described previously for *B. thetaiotaomicron* (2). The frequency of Tn*4351* insertions in *B. ovatus* was 10-fold lower than that reported for *B. thetaiotaomicron*. Nonetheless, we obtained a total of over 10,000 erythromycin-resistant *B. ovatus* transconjugants in several matings and screened them for growth on defined guar gum agar. Three mutants unable to grow on guar gum, M-4, M-5, and M-7, were found. Since the Tn*4351* insertions in M-4 and M-7 appeared to be in the same region (16), only M-7 was used in the analyses reported here.

To isolate second transposon insertions that enabled either M-5 or M-7 to grown on guar gum, we incubated M-5 and M-7 in defined guar gum broth at 37°C until growth was visible (about 5 days). Erythromycin (10 μg/ml) was included to prevent loss of the transposon. Cultures that grew on guar gum were reinoculated into defined guar gum broth and allowed to reach an optical density at 650 nm of about 1. The cultures were then streaked on defined guar gum agar to obtain single colonies, and DNA from these isolates was analyzed. Because of the difficulty of the analysis, which relied on two-dimensional gel comparisons, only one suppressor of each mutant was analyzed. We considered this sufficient to answer the question of what protein(s) had been missing in the original mutant.

The guar gum-positive phenotype could arise either from the loss of the original transposon insertion or from a second insertion at another site. Since the desired suppressor mutations would be ones that still had the original transposon insertion, we checked putative suppressor mutants for the number and location of Tn*4351* insertions by Southern hybridization. Chromosomal DNA was isolated and cut with *Eco*RI, an enzyme that cuts twice in Tn*4351*, and electrophoresed and blotted as described by Maniatis et al. (9). Blots were probed with ^{32}P-labeled pVOH 2C, a plasmid that contains one of the insertion sequence elements that flank Tn*4351* cloned into pBR328 (6). The procedures for nick translation and hybridization were those described by Maniatis et al. (9).

Enzyme assays. Extracts of *B. ovatus* grown on defined medium containing glucose, galactose, or guar gum were made by sonicating washed, resuspended bacteria as described previously (3–5). Galactomannanase activity was assayed by measuring the increase in the reducing sugar concentration when an extract was incubated with guar gum (5). *B. ovatus* produces two galactomannanases, one soluble and one membrane bound (4, 5). Although the specific activities reported in the Results are for total galactomannanase activity (galactomannanase I plus galactomannanase II), we also checked the soluble and membrane-bound activities as described previously (4, 5) to ascertain that the relative proportions of the two enzymes were the same as in the wild type.

α-Galactosidase activity was measured with *p*-nitrophenyl-α-D-galactoside as the substrate (3). Two α-galactosidases are detectable under the growth conditions used in the studies reported here. One is induced specifically by guar gum, and the other is induced either by α-galactosides (melibiose, raffinose) or partially by galactose (3). These α-galactosidases can be differentiated on the basis of their migration on electrofocusing gels (3). The α-galactosidases reported here were identified as described previously (3). Protein concentrations were determined by the method of Markwell et al. (10).

Two-dimensional gel electrophoresis. Extracts from 1-liter cultures of *B. ovatus* grown on defined medium and disrupted by sonication were centrifuged to pellet membranes as described previously (4, 5), except that RNase A (20-μg/ml final concentration), DNase I (50-μg/ml final concentration), and freshly made phenylmethylsulfonyl fluoride (14-μg/ml final concentration) were added to the resuspended cells prior to sonication. The supernatant fluid was designated the soluble fraction. Membranes were washed once and resuspended in 50 mM potassium phosphate buffer to a concentration of approximately 10 mg of protein per ml.

Two-dimensional gels were run on an SE400 electrophoresis unit (Hoefer Scientific Instruments, San Francisco, Calif.) as described in the appendix of the Hoefer Scientific Instruments catalog. The method was based on that described originally by O'Farrell (12). The amount of protein loaded on each gel was 80 μg. Membrane proteins were solubilized prior to first-dimensional electrofocusing as described by Ames and Nikaido (1). After electrophoresis in the second dimension, gels were stained with Fast Stain (Zoion Research, Allston, Mass.) as described by the manufacturer.

RESULTS

Glucose induction of galactomannanase expression. Previously, Gherardini et al. (3–5) measured galactomannanase and α-galactosidase I specific activities in extracts from *B. ovatus* grown on galactose, mannose, and guar gum, but they did not test glucose-grown cells. We made the surprising observation that extracts from *B. ovatus* grown on glucose had galactomannanase specific activities as high as those in extracts from *B. ovatus* grown on guar gum (Table 1). In contrast, α-galactosidase specific activity in extracts from glucose-grown cells was even lower than that in extracts from galactose-grown cells. The higher activity in the

TABLE 1. Effect of the substrate on α-galactosidase and galactomannanase specific activities in extracts from *B. ovatus* strains[a]

Enzyme	*B. ovatus* strain	Sp act (U/mg) in bacteria grown on:		
		Glucose	Galactose	Guar gum
α-Galactosidase	Wild type	0.02	0.10	0.45
	M-5	0.02	0.09	ND[b]
	M-7	0.02	0.10	ND
Galactomannanase	Wild type	0.65	<0.05	0.55
	M-5	0.59	<0.05	ND
	M-7	0.60	<0.05	ND

[a] The values reported are the arithmetic means from at least two separate experiments done in duplicate.
[b] ND, not determined. The mutants were unable to grow on guar gum-defined medium.

galactose-grown cells was due to an increase in the level of a second α-galactosidase (II), not α-galactosidase I. In guar gum-grown cells, the higher α-galactosidase activity was due entirely to the induction of α-galactosidase I, and α-galactosidase II was barely detectable. Since there appeared to be two different induction patterns for guar gum-associated proteins (induction by glucose and guar gum, induction by guar gum but not by glucose), we analyzed two-dimensional protein profiles of *B. ovatus* grown on glucose as well as galactose and guar gum.

Two-dimensional gel analysis of guar gum-associated proteins. A comparison of two-dimensional gels of soluble polypeptides in extracts from *B. ovatus* grown on galactose or guar gum revealed 12 soluble polypeptides that were consistently expressed at higher levels in bacteria grown on guar gum than in bacteria grown on galactose (Fig. 1 and Table 2). A comparison of these profiles with the two-dimensional gel profile of soluble polypeptides in extracts from *B. ovatus* grown on glucose revealed only two polypeptides (designated d and g) that were also present in elevated levels in bacteria grown on glucose (Table 2). The molecular masses of soluble polypeptides d and g were both different from the molecular mass reported for the soluble galactomannanase (61 kDa; 5), so neither of these polypeptides was the soluble galactomannanase. The soluble polypeptide designated b was presumably α-galactosidase I because it had the same expression pattern and the same molecular mass (85 kDa) as α-galactosidase I (3).

A comparison of two-dimensional gel patterns of membrane proteins from *B. ovatus* grown on galactose and guar gum revealed 20 membrane polypeptides that were present in elevated levels in bacteria grown on guar gum compared with bacteria grown on galactose (Fig. 2 and Table 3). Only one polypeptide (designated o) was induced both by guar gum and glucose (Fig. 2 and Table 3). The molecular mass of this polypeptide (62 kDa) was different from the molecular masses of the two polypeptides in the partially purified membrane galactomannanase preparation obtained by Gherardini and Salyers (49 and 94.5 kDa; 4). Thus, this polypeptide was probably not the membrane galactomannanase. The remaining 19 guar gum-associated membrane polypeptides had the same expression pattern as α-galactosidase I, i.e., enhanced expression in guar gum-grown cells but not in glucose-grown cells (Table 1).

Analysis of mutants M-5 and M-7. Of the 10,000 Tn*4351* insertions in the *B. ovatus* chromosome that were screened,

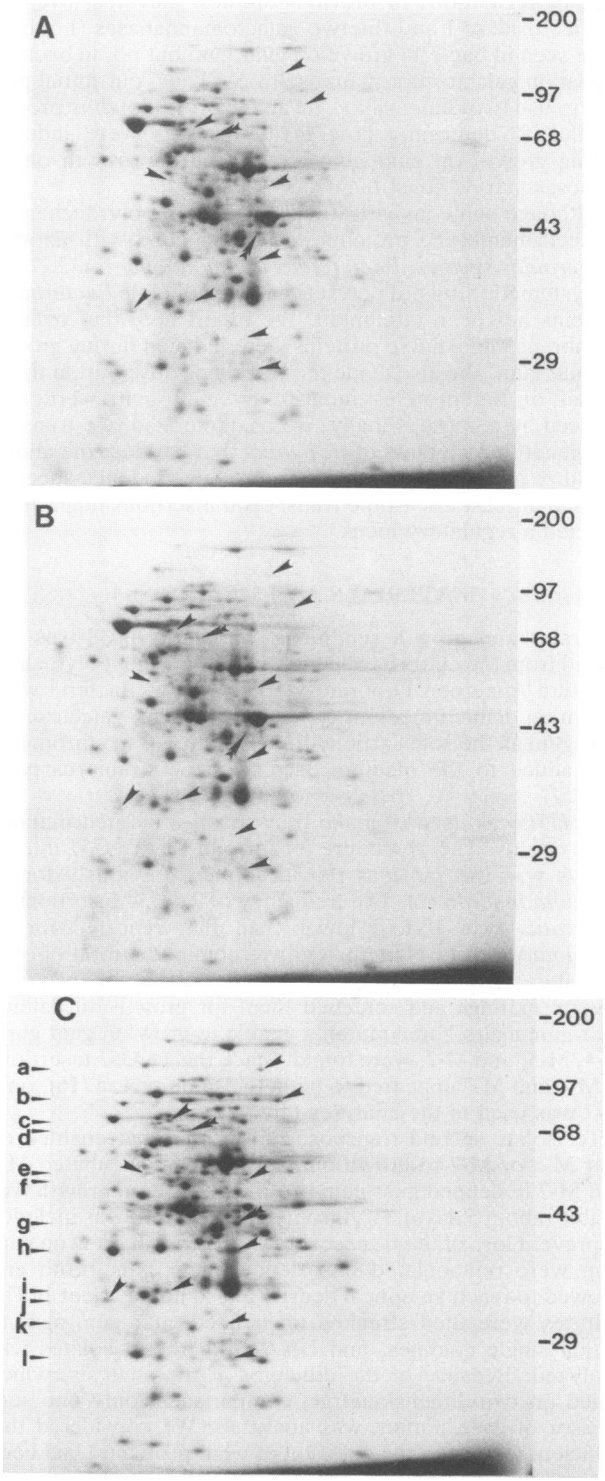

FIG. 1. Two-dimensional profiles of soluble polypeptides in extracts from wild-type *B. ovatus* grown on glucose (A), galactose (B), and guar gum (C). The isoelectric focusing dimension (pH 3 to 10) is horizontal, with the acidic proteins migrating on the left side of the gels. The sodium dodecyl sulfate dimension is vertical. Migration distances of molecular mass standards (in kilodaltons) are shown to the right of the gels. Arrowheads indicate the positions of guar gum-inducible polypeptides. Letter designations are shown to the left of the gel in panel C. Polypeptides that were induced by glucose as well as by guar gum are indicated by double arrowheads.

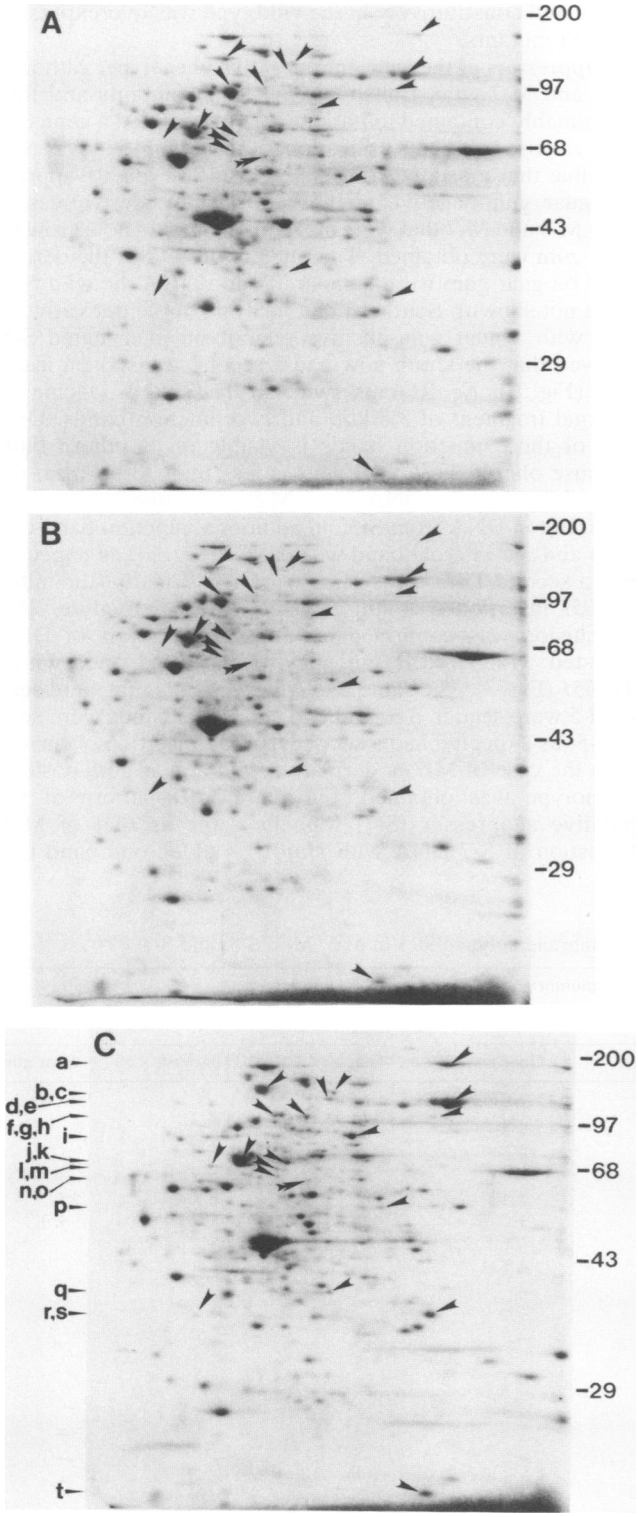

FIG. 2. Two-dimensional profiles of membrane polypeptides in extracts from wild-type *B. ovatus* grown on glucose (A), galactose (B), and guar gum (C). The isoelectric focusing dimension (pH 3 to 10) is horizontal, with the acidic proteins migrating on the left side of the gels. The sodium dodecyl sulfate dimension is vertical. Migration distances of molecular mass standards (in kilodaltons) are shown to the right of the gels. Arrowheads indicate the positions of guar gum-inducible polypeptides. Letter designations are shown to the left of the gel in panel C. In some cases, when polypeptides had similar molecular masses, the letter designations were assigned from left to right. A polypeptide that was induced by glucose as well as by guar gum is indicated by double arrowheads.

TABLE 2. Molecular masses and patterns of expression of guar gum-regulated soluble polypeptides in wild-type *B. ovatus*

Designation[a]	Molecular mass (kDa)	Level of expression in bacteria grown on[b]:		
		Glucose	Galactose	Guar gum
a	108	−	−	+
b	86	−	−	+
c	72	−	−	+
d	67	+	±	+
e	55	−	−	±
f	52	−	−	+
g	42	+	±	+
h	38.5	−	−	++
i	34	±	±	+
j	33	±	±	+
k	30.5	−	−	±
l	27.5	±	±	+

[a] See Fig. 1C.
[b] ++, +, ±, and − refer to the relative size and darkness of the spot on stained two-dimensional gels.

only 3 did not permit growth on guar gum. The mutants were designated M-4, M-5, and M-7. Southern analysis of DNA from these mutants showed that the transposon insertions in M-4 and M-7 were in the same locus on the *B. ovatus* chromosome (16). Thus, only M-5 and M-7 were analyzed in the present study. M-5 and M-7 appeared to be affected specifically in the utilization of guar gum because they grew normally on galactose, mannose, and unrelated polysaccharides, such as chondroitin sulfate and arabinogalactan (data not shown). Guar gum is the only known inducer of guar gum utilization genes which are not induced by glucose. This fact made M-5 and M-7 difficult to analyze because only extracts from M-5 or M-7 grown on glucose and galactose could be tested.

Nonetheless, the fact that all three guar gum-induced enzymes and many of the guar gum-induced proteins seen on two-dimensional gels were detectable in extracts from cells grown on glucose or galactose allowed us to ascertain whether any of these proteins was missing in either M-5 or M-7 (Tables 1 to 3). The three enzymes had not been inactivated by the transposon insertions because the galactomannanase and α-galactosidase specific activities were the same in extracts from M-5 and M-7 as in extracts from the wild type (Table 1). In two-dimensional gels of soluble polypeptides, all of the guar gum-associated proteins detectable in glucose-grown or galactose-grown cells were detectable (data not shown). Thus, at least 5 of the 12 soluble polypeptides were still present in M-5 and M-7. The expression of soluble polypeptides d and g was still induced by glucose in M-5 and M-7, as was the expression of the galactomannanases.

In two-dimensional gels of membrane polypeptides in extracts from M-5 and M-7, all of the guar gum-associated polypeptides visible in glucose-grown or galactose-grown cells were still present (Table 4). Thus, at least half of the guar gum-associated membrane polypeptides were still present in M-5 and M-7. Membrane polypeptide o was still

TABLE 3. Molecular masses and patterns of expression of guar gum-regulated membrane polypeptides in wild-type *B. ovatus*

Designation[a]	Molecular mass (kDa)	Level of expression in bacteria grown on[b]:		
		Glucose	Galactose	Guar gum
a	155	±	−	++
b	124	+	+	++
c	118	±	±	+
d	114	−	−	+
e	110	++	++	++++
f	99	−	−	+
g	98	±	±	+
h	98	−	−	±
i	83	−	−	++
j	70	++	++	+++
k	68	−	−	+
l	67	−	−	+
m	66	−	−	+
n	63	−	−	+
o	62	+	−	+
p	55	±	±	+
q	38	−	−	+
r	36	−	−	±
s	36	±	±	++
t	20	+	+	++

[a] See Fig. 2C.
[b] ++++, +++, ++, +, ±, and − refer to the relative size and darkness of the spot on stained two-dimensional gels.

induced by glucose in M-5 and M-7. A marked difference between the mutants and the wild type was that membrane polypeptide q, which had been regulated in the wild type, was now produced constitutively in both mutants (Table 4). Also, a 107-kDa membrane polypeptide which had been expressed constitutively in the wild type was overexpressed in both mutants.

Suppressors of the guar gum-negative phenotype. Although M-5 and M-7 were transposon insertion mutants and thus presumably contained mutations that interrupted a gene that was required for the utilization of guar gum, it was still possible that a second transposon insertion might suppress the guar gum-negative phenotype. In fact, derivatives of both M-5 and M-7 that were still Em^r and could now grow on guar gum were obtained. The rates of growth of the derivatives on guar gum were comparable to that of the wild type (data not shown). Southern blot analysis of the derivative of M-5 with a guar gum-positive phenotype (designated S-5) showed that the strain now had a second transposon insertion (Fig. 3). *Eco*RI cuts twice in Tn*4351*, producing an internal fragment of 3.8 kbp and two junction bands. Only one of these junction bands is visible on Southern blots because one of the *Eco*RI sites is less than 30 bp from the end of Tn*4351*. In DNA from M-5, only two bands were seen, but in DNA from S-5, an additional junction band was seen and the internal band was darker (Fig. 3), as expected had a second Tn*4351* insertion occurred. Clearly, the initial Tn*4351* insertion was still in place in this derivative. This conclusion was confirmed by Southern analysis of DNA digested with *Hin*dIII, an enzyme that cuts once within Tn*4351* (Fig. 3). The same two *Hin*dIII bands that were seen in M-5 were seen in S-5, and two additional bands were seen in S-5, as expected had a second Tn*4351* insertion occurred.

In the case of M-7, a derivative with a guar gum-positive phenotype was obtained, but the *Eco*RI pattern of the putative suppressor (S-7) was the same as that of M-7. Digestion of S-7 DNA with *Hin*dIII yielded one band that

TABLE 4. Patterns of expression of guar gum-regulated membrane polypeptides in M-5, M-7, S-5, and S-7

Designation	Level of expression in the following mutant grown on the indicated substrate[a]:							
	M-5 and M-7		S-5			S-7		
	Glucose	Galactose	Glucose	Galactose	Guar gum	Glucose	Galactose	Guar gum
a			+++[b]	++[b]	+++[b]			
b			++[b]	+	++			
c			±	±	±[c]			
d			−	−	−[c]	−	−	+++[b]
e			++	++	++[c]	+[c]	+[c]	+[c]
f			±[b]	±[b]	+	+[b]	+[b]	++[b]
g								
h			+[b]	+[b]	+[b]			
i								
j								
k			−	−	−[c]			
l								
m								
n								
o						−[c]	−	+
p			−[c]	−[c]	−[c]	−[c]	−[c]	−[c]
q	+[b]	+[b]	+[b]	+[b]	++[b]	+[b]	+[b]	+
r								
s			±	±	±[c]	±	±	±[c]
t						+	+	+[c]
107 kDa[d]	+++	+++	+	+	+	+++	+++	+++

[a] Only levels of expression that differed from those in the wild type are shown. Blanks indicate that the suppressor phenotype was the same as the wild-type phenotype. See Table 3 for wild-type expression levels and explanation of symbols.
[b] Expression was higher than that in the wild type grown under the same conditions.
[c] Expression was lower than that in the wild type grown under the same conditions.
[d] This polypeptide was expressed constitutively in the wild type but was overexpressed in M-5, M-7, and S-7.

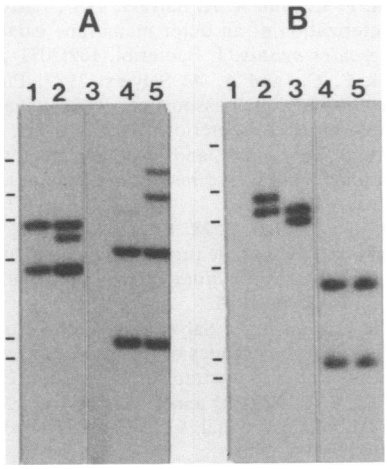

FIG. 3. (A) Southern blot of chromosomal DNA from M-5 digested with *Eco*RI (lane 1), S-5 digested with *Eco*RI (lane 2), the wild type digested with *Hin*dIII (lane 3), M-5 digested with *Hin*dIII (lane 4), and S-5 digested with *Hin*dIII (lane 5). (B) Southern blot of chromosomal DNA from the wild type digested with *Hin*dIII (lane 1), S-7 digested with *Hin*dIII (lane 2), M-7 digested with *Hin*dIII (lane 3), S-7 digested with *Eco*RI (lane 4), and M-7 digested with *Eco*RI (lane 5). The positions of the molecular size standards are indicated by lines to the left of the Southern blots. The sizes of the standards (in kilobase pairs) were as follows: 23.1, 9.4, 6.7, 4.4, 2.3, and 2.0. The *Eco*RI segment internal to Tn*4351* was 3.8 kbp in size.

was the same size as a band in the *Hin*dIII digest of M-7 and one band that was larger than either band in M-7. Thus, the phenotype of S-7 appeared not to have resulted from a second Tn*4531* insertion but rather from some DNA rearrangement or mutation in the vicinity of the M-7 insertion that altered the size of one of the *Hin*dIII junction fragments. Nonetheless, since it appeared from the *Eco*RI digest that the original Tn*4351* insertion was still present in S-7, we included this suppressor in the analysis.

Since we were interested primarily in the guar gum-associated membrane proteins, we used two-dimensional gel analysis to evaluate the polypeptides in membranes from S-5 and S-7 grown on glucose, galactose, and guar gum (Table 4). Only the polypeptides with changed expression patterns are shown, for emphasis. In S-5, three guar gum-associated polypeptides were missing (d, k, and p), and some polypeptides that had formerly shown regulated expression were now expressed in galactose-grown cells at the same level or nearly the same level as in glucose-grown or guar gum-grown cells (a, b, f, h, and q). Some polypeptides still present in extracts from S-5 grown on guar gum were expressed at lower levels than in the wild type grown on guar gum (b, c, e, and s; Tables 3 and 4). In S-7 extracts from cells grown on guar gum, only one polypeptide was missing (p), five were expressed constitutively (e, f, q, r, and s), and one was overexpressed (d). One polypeptide (o) was still inducible by guar gum but was no longer inducible by glucose.

DISCUSSION

The number of guar gum-associated proteins identified by two-dimensional gel analysis was unexpectedly large: 20 membrane polypeptides and 12 soluble polypeptides. This number is probably an underestimate, because neither of the galactomannanases was visible on the Coomassie-stained gels. Other guar gum-associated proteins may also have been too light to be visible on these gels. The large number of guar gum-associated membrane proteins indicates that the breakdown of guar gum is a complex process which involves many proteins other than the degradative enzymes.

All of the proteins identified as being guar gum associated were expressed at higher levels in bacteria grown on guar gum than in bacteria grown on galactose. However, within this group of guar gum-associated proteins, there were two different patterns of expression. One was low expression in galactose-grown cells but high expression in glucose-grown cells and in guar gum-grown cells. Galactomannanases I and II, membrane polypeptide o, and soluble polypeptides d and g had this expression pattern. The fact that these proteins appeared to be induced by glucose as well as guar gum was unexpected, since the primary monosaccharide components of guar gum, galactose and mannose, did not act as inducers. There are two possible explanations for this unusual expression pattern. One is that the bacteria are adapted to respond to plant cell wall complexes which contain a mixture of polysaccharides. A glucose-containing linkage may be linked to galactomannans in plant cell walls. Alternatively, commercial preparations of glucose may contain very low levels of contaminating disaccharides. If so, the concentrations must be very low, because we have been unable to detect any contaminants in our glucose stocks on paper chromatograms.

Most of the guar gum-associated polypeptides exhibited the same expression pattern as α-galactosidase I; i.e., they were induced during growth on guar gum but not during growth on glucose or galactose. This result indicated that the regulation of the guar gum utilization genes might be complex. Further evidence for this conclusion was obtained from analysis of mutants with a guar gum-negative phenotype (M-5 and M-7) and derivatives that were suppressors of this phenotype (S-5 and S-7).

M-5 and its suppressor S-5 had features that suggested that the original mutation in M-5 might have been a regulatory mutation. First, several proteins that were regulated in wild-type cells were expressed at high levels when M-5 or S-5 was grown on glucose, galactose, or guar gum (a, b, f, h, and q). Second, had the Tn*4351* insertion in M-5 removed an essential structural gene, it is unlikely that the effect of this insertion could have been suppressed by a second Tn*4351* insertion. The fact that the M-5 phenotype was suppressed by a second Tn*4351* insertion suggests that the gene products of the locus interrupted by the insertion in M-5 and the locus interrupted by the second insertion in S-5 interact in some way.

In the case of S-7, there was no apparent second Tn*4351* insertion. Thus, a small deletion in the region of the Tn*4351* insertion or a mutation elsewhere on the chromosome was presumably responsible for the suppression of the guar gum-negative phenotype of M-7. Whatever the mechanism of suppression, the pattern of expression of membrane polypeptides in S-7 was not the same as that in S-5, although there was some overlap. With S-7, as with S-5, some proteins that were formerly regulated were now expressed constitutively or were overexpressed. Thus, M-7 may also contain a regulatory mutation. If mutations in both M-5 and M-7 define regulatory loci, these two loci together with the gene(s) responsible for glucose induction of a subset of the guar gum-associated polypeptides identify three different levels of regulation of the guar gum utilization system. Thus, the regulation of this system is clearly complex.

Given the number of guar gum-associated proteins seen in the two-dimensional gel analysis, it is remarkable that a

screen of 10,000 transposon insertions from more than one mating yielded only 3 insertions causing a guar gum-negative phenotype, and these defined only two separate loci. Since Tn*4351* appears to be integrated randomly, the small number of mutants with a guar gum-negative phenotype that we obtained may reflect the fact that many of the guar gum-associated proteins are not essential for growth on guar gum. Since three membrane polypeptides (d, k, and p) were missing in S-5 and S-7, these polypeptides are clearly not essential for growth on guar gum. Similarly, a number of the membrane proteins were expressed at lower levels in S-5 and S-7 than in the wild type and thus are probably not rate limiting for growth.

Frequently, in studies of polysaccharide utilization systems, attention is focused exclusively on the enzymes involved in the breakdown of the polysaccharide. It is clear from our two-dimensional gel analysis that the enzymes account for only a small portion of the guar gum-associated proteins. Moreover, the two mutants with a guar gum-negative phenotype that we obtained still expressed the degradative enzymes. Thus, other proteins besides the enzymes clearly play an essential role in guar gum utilization.

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