JOURNAL OF BACTERIOLOGY, Oct. 1977, p. 219-223

Vol. 132, No. 1
Printed in U.S.A.

A Third β-Galactosidase in a Strain of *Klebsiella* That Possesses Two *lac* Genes

BARRY G. HALL†* AND ERIC C. R. REEVE[1]

Faculty of Medicine, Memorial University of Newfoundland, St. John's, Newfoundland, Canada, and Institute of Animal Genetics, University of Edinburgh, Edinburgh, Scotland EA9 3JN[1]

Received for publication 13 April 1977

Klebsiella strain RE1544 contains two *lac* operons, one on the chromosome and one on a *lac* plasmid. A mutant of RE1544, in which the *lacZ* genes of both operons produce no active enzyme, was found to synthesize a β-galactosidase that hydrolyzes *ortho*-nitrophenyl-β-D-galactopyranoside but not lactose. Synthesis of this β-galactosidase (BGase-III) is induced by lactose but not by isopropyl-1-thio-β-D-galactopyranoside or methyl-β-D-thiogalactopyranoside. In both the regulation of synthesis and substrate specificity, BGase-III strongly resembles the *ebg*^{0} enzyme of *Escherichia coli*. Nevertheless, by the criteria of immunological cross-reactivity and subunit molecular weight, BGase-III is not related to the *ebg*^{0} enzyme.

Many wild strains of *Klebsiella* species contain two *lac* operons, one plasmid-borne and one on the chromosome, each of which enables the host organism to grow on lactose as a sole carbon source (12). The plasmid operon is highly efficient and is very closely related to (and probably derived from) the *lac* operon of *Escherichia coli* (4, 13). The chromosomal *lac* operon is of lower efficiency and strains that have lost the *lac* plasmid are weakly positive in their phenotype on MacConkey lactose agar, contain about 10% as much β-galactosidase activity as plasmid-bearing cells (12), and grow more slowly than the latter on lactose as a sole carbon source (2; E. C. R. Reeve, unpublished observations).

We have examined the properties of a strongly lactose-negative mutant of a *Klebsiella* strain that does not express either the plasmid or chromosomal *lacZ* genes. This mutant synthesizes an enzyme that shares many properties with the *ebg*^{0} enzyme of *E. coli*. The *ebg*^{0} enzyme is the second β-galactosidase of *E. coli* (10), which is active on the synthetic galactoside *ortho*-nitrophenyl-β-D-galactopyranoside (ONPG), but virtually inactive on the natural β-galactoside lactose.

MATERIALS AND METHODS

Strains used. Strain RE1755 is a lactose-negative mutant of the *Klebsiella* strain RE1544, in which both the plasmid-borne and the chromosomal *lac* operons produce no active β-galactosidase (11). This strain is not designated specifically *K. pneumoniae* or *K. aerogenes* because of the lack of agreement among different authorities as to what should be included in these taxonomic groups. Strain 1B1 is an HfrC, *spc ebgR ebgA*^{0} *lacZ*Δ*w4680* strain of *Escherichia coli* K-12 (9). Strain 1B1 synthesizes *ebg*^{0} enzyme constitutively.

Media and culture conditions. The minimal medium for *E. coli* was described previously (8). The minimal medium for *Klebsiella* was identical except that it contained no citrate but did contain 0.05% NaCl. Carbon sources were, as indicated, 1% sodium succinate, 0.2% glycerol, or 0.5% melibiose. Cultures were maintained at 37°C with constant vigorous aeration. The buffers used for enzyme purification were potassium phosphate, pH 7.5, and have been described elsewhere (5). Antibody buffer was 0.025 M potassium phosphate (pH 7.5), 0.1 M NaCl, and 1% glycerol.

Cell extracts. Extracts were prepared by grinding the cells with alumina and precipitating the nucleic acids with streptomycin as previously described (5).

Enzyme assays. The assay for ONPG hydrolysis was described elsewhere (9), as was the assay for lactose hydrolysis (5). For both assays, 1 U equals the hydrolysis of 1 nm of substrate per min in 0.125 M potassium phosphate buffer, pH 7.5, containing 5 mM Mg^{2+}, at 37°C. Protein concentrations were determined from the absorbance at 255 nm as previously described (9).

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS) was carried out as previously described (5).

Preparation of anti-*ebg*^{0}-IgG. Adult male New Zealand White rabbits were injected intramuscularly with 1.0 mg of pure *ebg*^{0} enzyme (purified as in [5]) emulsified in Freund complete adjuvant (Difco). A second identical injection was administered 35 days later, and the rabbits were bled on day 63. The clotted blood was centrifuged at 15,000 × *g* for 20

† Present address: Biological Sciences Group, Microbiology Section, U-44, University of Connecticut, Storrs, CT 06268.

min after standing overnight at 6°C, and the supernatant was retained. The supernatant was adjusted to 50% saturation with saturated, neutralized ammonium sulfate, stirred for 20 min in ice, and centrifuged at 30,000 × *g* for 20 min at 4°C. The pellet was dissolved in the original volume of antibody buffer and adjusted to 40% saturation with ammonium sulfate. The resulting precipitate was centrifuged, and the pellet was suspended in one-half the original volume of antibody buffer and dialyzed overnight against 100 volumes of antibody buffer. The protein concentration was adjusted to 8 mg/ml, and the anti-*ebg*^{0}-immunoglobulin G (IgG) was stored frozen in small portions.

Immunoprecipitation of enzyme activity. Fifty microliters of an extract (which had been diluted to an appropriate enzyme concentration with antibody buffer) was mixed with the indicated volumes (see Table 2) of anti-ebg^0-IgG and allowed to stand for 8 h at 6°C. The indicated volume of commercial goat anti-rabbit-immunoglobulin antiserum (Calbiochem) was added; the volume was adjusted to a total of 0.4 ml, and the preparation was allowed to stand overnight at 6°C. On the next day, the mixture was centrifuged for 2 min in a Beckman Microfuge in the cold. The supernatant was withdrawn and assayed for enzyme activity. The pellet was washed twice in cold antibody buffer and suspended in 0.2 ml of antibody buffer, and the suspension was assayed for enzyme activity.

RESULTS

Strain RE1755 arose spontaneously as a completely lactose-negative mutant of *Klebsiella* strain RE1544, which produces both a plasmid-specified β-galactosidase (BGase-I) and a chromosomally specified β-galactosidase (BGase-II). RE1755 does not synthesize either BGase-I or BGase-II (at least in an active form), cannot grow on lactose, and has not reverted to *lac*$^+$ in large-scale selection experiments (11; unpublished data). This mutant is thought to have arisen through recombination between the chromosomal and plasmid-borne *lac* operons of RE1544, with loss or inactivation of both *lac* genes, and behaves like *lacZ* deletion strains of *E. coli*.

Studies of *lacZ* deletion strains of *E. coli* have shown that *E. coli* synthesizes a second β-galactosidase, which is active on the synthetic galactoside ONPG, but virtually inactive on lactose (10). This second β-galactosidase is the *ebg*^{0} enzyme, and it is the product of the *ebgA*^{0} gene located at 66 min on the recalibrated map of *E. coli* (1, 9). The *ebg*^{0} enzyme has physical and kinetic properties very different from the *lacZ* β-galactosidase (5), but the most striking difference between *ebgA* and *lacZ* lies in the regulation of their expression. Synthesis of *ebg*^{0} enzyme is induced by growth in the presence of lactose (10), but it is not induced by common powerful inducers of the *lacZ* gene such as isopropyl-1-thio-β-D-galactoside (IPTG), methyl-β-D-thiogalactoside (TMG), glycerolgalactoside, or melibiose (7). Because it is not an effective substrate of *ebg*^{0} enzyme, lactose may be considered a gratuitous inducer of the *ebgA*^{0} gene.

Because of the formal analogy between *Klebsiella* strain RE1755 and *lacZ* deletion strains of *E. coli*, we examined RE1755 to determine whether it synthesized an *ebg*^{0}-like enzyme.

Synthesis of BGase-III. Table 1 shows the β-galactosidase levels of strain RE1755 grown in succinate minimal medium with various galactosides present. Lactose alone induces synthesis of this β-galactosidase. Thiogalactosides, which are powerful inducers of both BGase-I and BGase-II in *Klebsiella* (13), fail to induce synthesis of the β-galactosidase that we shall refer to as BGase-III. Even fully induced cultures of RE1755 are incapable of growing on lactose as a sole carbon source. Thus, strain RE1755 closely resembles *lacZ* deletion strains of *E. coli* in that (i) it synthesizes a β-galactosidase (BGase-III), which is quite active toward ONPG, but which does not hydrolyze lactose effectively; and (ii) synthesis of BGase-III is induced by lactose, but not by IPTG or TMG. From Table 1 it is evident that RE1755 can accumulate lactose, so that it must possess some permease capable of transporting lactose. The origin of that permease is not yet known.

The regulation of BGase-III is clearly distinct from that of either the plasmid or chromosomal *lac* operons of *Klebsiella*. IPTG (10^{-4} M) induces the chromosomal operon about 30-fold, and the plasmid operon about 60-fold, over basal level (13), whereas 10^{-4} M IPTG fails to induce BGase-III at all. To be sure that the BGase-III repressor does not respond to thiogalactosides, we assayed RE1755 grown in glycerol plus 10^{-3} M thiogalactosides. Table 1 shows the results of this experiment. No significant induction was

TABLE 1. *BGase-III activity in extracts of strain RE1755*

Carbon source	Addition	U/mg[a]
Succinate	None	12.4 ± 1.4
	2 × 10^{-4} M IPTG	14.4 ± 3.4
	10^{-4} M TMG	21.9 ± 9.7
	0.5% Lactose	2,150 ± 410
Glycerol	None	10.8
	10^{-3} M IPTG	28.0
	10^{-3} M TMG	22.4
Melibiose	None	24.3 ± 3.3

[a] Values ± 95% confidence intervals.

observed even at these very high thiogalactoside concentrations.

An important distinction between the repressors of the chromosomal and plasmid *lac* operons of *Klebsiella* is that the plasmid *lac* operon is strongly induced by melibiose, but the chromosomal *lac* operon is not induced by melibiose; indeed, it is repressed (13). Table 1 shows that BGase-III synthesis is not significantly induced by growth on melibiose. In this respect, the BGase-III repressor is like the *ebg* repressor (*ebgR* gene product) of *E. coli*, which is also insensitive to melibiose as an inducer (7).

Immunological comparison of BGase-III with *ebg*^{0}. The similarities between the *ebg*^{0} enzyme of *E. coli* and BGase-III, both in terms of activity and of regulation, led us to experiments designed to explore the question of their molecular similarity. Antibody was prepared against *ebg*^{0} enzyme, which had already been purified (5). Double-diffusion tests of extracts of induced RE1755 versus anti-*ebg*^{0} enzyme IgG failed to reveal any precipitin lines, although precipitin lines were observed on the same plates close to wells containing extracts of an induced *lacZ* deletion strain of *E. coli* and wells containing pure *ebg*^{0} enzyme (controls showed no precipitin lines when the anti-*ebg*^{0}-IgG was tested against an uninduced extract, or an extract of a *ebgA* deletion strain).

The failure to detect a reaction between BGase-III and anti-*ebg*^{0}-IgG might have resulted from low concentrations of enzyme (in spite of its high activity) in the extracts. We therefore compared the ability of anti-*ebg*^{0}-IgG to precipitate BGase-III activity with its ability to precipitate *ebg*^{0} activity in the test tube. Since our procedure employed a second antibody, goat-anti-rabbit-IgG, to precipitate enzyme-antibody complexes, the relative concentrations of antigen and antibody were not critical. Table 2 clearly shows that anti-*ebg*^{0}-IgG fails to bind BGase-III enzyme. The failure to detect any cross-reaction between *ebg*^{0} enzyme and BGase-III was surprising in light of the similarities discussed; we therefore felt it necessary to purify and further characterize BGase-III enzyme.

Purification of BGase-III enzyme. Thirty liters of RE1755 culture grown in succinate minimal medium plus lactose was harvested near the end of the exponential growth phase. All further operations were carried out at 6°C. An extract was prepared and brought to 40% saturation with $(NH_4)_2So_4$ by the dropwise addition of neutralized saturated ammonium sulfate while stirring at 6°C. The solution was allowed to stand for 20 min in ice, and was then centrifuged for 20 min at 35,000 × *g* at 4°C. The pellet was gently dissolved in buffer A-125 (see Materials and Methods), and the solution was applied to a column (5 by 60 cm) of Bio-Gel A1.5M (an agarose molecular sieve with a fractionation range of 60,000 to 1,500,000 daltons). The column was eluted by the upward-flow technique with buffer A-125 at a flow rate of 2 ml/min, and 300-drop fractions (about 18 ml) were collected. The fractions with the highest specific activity were pooled. The pool was concentrated by precipitation with 60% $(NH_4)_2SO_4$, and the pellet formed after centrifugation was suspended in and dialyzed overnight against buffer A-5 (see Materials and Methods). The dialyzed solution was applied to a column (2.5 by 20 cm) of hydroxylapatite type III (Sigma Chemical Co.). The column was washed with 200 ml of buffer A-5, and 300-drop fractions were collected. Activity was present in fractions 5 and 6. (The column was further eluted with a 5 to 50 mM phosphate gradient, but no further activity was eluted.) The specific activities of each step in the purification are given in Table 3.

Portions of fractions 5 and 6 were applied to

TABLE 2. *Immunoprecipitation of β-galactosidase activity with anti-ebg*^{0}*-IgG*

Extract	Enzyme	IgG (μl)	GARGG[a] (μl)	Supernatant (U)	Pellet (U)	% of activity in supernatant
1B1	*ebg*^{0}	0	0	750	N[b]	100
		0	160	720	N	95
		5	40	400	170	53
		10	80	290	250	38
		20	160	60	590	8
RE1755	BGase-III	0	0	410	N	100
		0	160	400	N	98
		5	40	400	0	99
		10	80	410	0	100
		20	160	410	0	100

[a] GARGG, Goat anti-rabbit immunoglobulin antiserum.
[b] N, No pellet present.

TABLE 3. *Purification of BGase-III*

Step	Activity (U/ml)	Protein (mg/ml)	Sp act (U/mg)	Enrichment (-fold)	Total U	Recovery (%)
Crude extract	59,200	32	1,860		3,060,000	100
First $(NH_4)_2SO_4$ precipitation	175,000	25	7,030	3.8	3,170,000	103
Bio-Gel pool	15,400	1.4	10,800	5.8	1,360,000	45
Second $(NH_4)_2SO_4$ precipitation	136,000	9.5	14,300	7.7	1,330,000	43
Hydroxylapatite						
Fraction 5	30,000	0.23	132,000	71	528,000	17
Fraction 6	19,800	0.17	115,000	62	346,000	11

SDS-polyacrylamide gels to determine the purities of these fractions (Fig. 1). Fraction five is seen to be pure BGase-III, and fraction 6 is seen to be 87.8% pure, as calculated from the areas under the curves. This value is in good agreement with the ratio of specific activities of fractions six and five (6/5 = 0.871).

Characterization of BGase-III enzyme. We have estimated the molecular weight of the BGase-III subunit from mobility on SDS-polyacrylamide gels. From the mobility of BGase-III, and the mobility of several proteins of known subunit molecular weight, we conclude that BGase-III has a subunit molecular weight of about 68,000.

We also examined the kinetic properties of BGase-III enzyme on the two substrates ONPG and lactose. Table 4, which shows the results of these measurements, also gives the properties of *ebg*^{0} enzyme for comparison (5). It is seen that BGase-III and *ebg*^{0} enzyme are qualitatively similar in that both are very active on ONPG, but have little activity toward lactose; however, the detailed kinetic parameters are very different. We have tested the activity of purified BGase-III toward ONP-α-galactopyranoside and toward ONP-β-glucoside. The enzyme is completely inactive toward these substrates, a property that it has in common with *ebg*^{0} enzyme (B. G. Hall, unpublished observations).

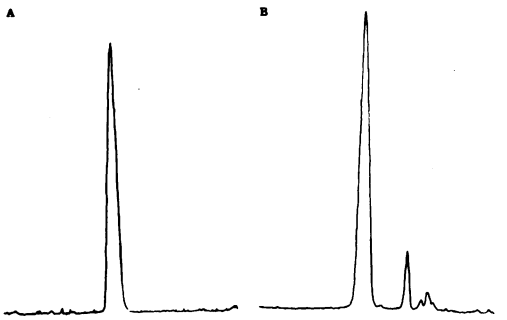

FIG. 1. *Densitometer traces of SDS-7.5% polyacrylamide gels at 600 nm. In each panel, the top of the gel is at the left. (A) Thirty micrograms of fraction 5; (B) 30 μg of fraction 6. Coomassie brilliant blue stained.*

TABLE 4. *Comparison of properties of β-galactosidase*

Property	Enzyme		
	BGase-III enzyme	*ebg*^{0} enzyme	β-Galactosidase (*lacZ* gene product)
K_m ONPG (mM)	34	160	0.16
K_m lactose (mM)	29	1,200	2
V_{max} ONPG	990,000	340,000	134,000
V_{max} lactose	420	1,900	9,000
Subunit molecular weight	68,000	120,000	135,000
Cross-reaction with anti-*ebg*^{0} antibody	None	Strong	None

DISCUSSION

Klebsiella strain RE1755 appears to be very similar to *lacZ* deletion strains of *E. coli* in that it has completely lost the ability to utilize lactose as a carbon source, yet it synthesizes a β-galactosidase that is induced by lactose and is specific for ONPG as a substrate. Because *Klebsiella* and *E. coli* are closely related (14), one would expect that the two enzymes BGase-III and *ebg*^{0} enzyme would be closely related homologs. The similarity of the regulation of the two enzymes served to reenforce this view. Comparison of the BGase-III enzyme with the *ebg*^{0} enzyme of *E. coli*, however, has clearly shown that they are unrelated.

First, the kinetic properties of BGase-III of *Klebsiella* are different from those of *ebg*^{0} enzyme of *E. coli*, as summarized in Table 4. Although both BGase-III and *ebg*^{0} enzyme cannot hydrolyze lactose efficiently, the efficiencies of hydrolysis of ONPG compared with those of lactose are different from each other and from BGase-I (*lacZ* gene product), as indicated by

the ratio of V_{max}(ONPG)/V_{max}(lactose). This ratio is 2,400 for BGase-III, compared with 180 for *ebg*^{0} enzyme and 14.9 for β-galactosidase (15). Likewise, the K_m values for lactose and ONPG of BGase-III are substantially different from those of *ebg*^{0} enzyme and β-galactosidase (15) (Table 4).

A second major difference between BGase-III and *ebg*^{0} enzyme is the molecular weight of their subunits. BGase-III has a subunit molecular weight of 68,000, wherease *ebg*^{0} enzyme has a subunit molecular weight of 120,000 (5). Likewise, BGase-III is very different from β-galactosidase enzyme of *E. coli* (and BGase-I of *Klebsiella*), which have subunit molecular weights of 135,000 (3).

Third, anti-*ebg*^{0} antibody does not cross-react with BGase-III.

The function of *ebg*^{0} enzyme in *E. coli* is unknown, but the similarity of subunit sizes has led to the speculation that it may be ancestrally related to the *lacZ* enzyme (5). *ebg*^{0} enzyme can be altered by a single mutation to a form that hydrolyzes lactose effectively enough to permit lactose utilization (6). Likewise, the function of BGase-III enzyme in *Klebsiella* is unknown, and it remains to be determined whether or not it can be mutated to hydrolyze lactose effectively.

ACKNOWLEDGMENTS

We thank Norma Churchill for her expert technical assistance.

E. C. R. Reeve is a member of the Agricultural Research Council's Unit of Animal Genetics. This work was supported by MRC (Canada) grant MA-5391 to B. Hall and by grants to E. C. R. Reeve.

LITERATURE CITED

1. **Bachmann, B. J., K. B. Low, and A. L. Taylor.** 1976. Recalibrated linkage map of *Escherichia coli* K-12. Bacteriol. Rev. 40:116–167.
2. **Brenchley, J. E., and B. Magasanik.** 1972. *Klebsiella aerogenes* strain carrying drug-resistance determinants and a *lac* plasmid. J. Bacteriol. 112:200–205.
3. **Craven, G. R., E. Steers, Jr., and C. B. Anfinson.** 1965. Purification, composition, and molecular weight of the β-galactosidase of *Escherichia coli* K-12. J. Biol. Chem. 240:2468–2477.
4. **Guiso, N., and A. Ullmann.** 1976. Expression and regulation of lactose genes carried by plasmids. J. Bacteriol. 127:691–697.
5. **Hall, B. G.** 1976. Experimental evolution of a new enzymatic function. Kinetic analysis of the ancestral (ebg^0) and evolved (ebg$^+$) enzymes. J. Mol. Biol. 107:71–84.
6. **Hall, B. G.** 1977. Number of mutations required to evolve a new lactase function in *Escherichia coli*. J. Bacteriol. 129:540–543.
7. **Hall, B. G., and N. D. Clarke.** 1977. Regulation of newly evolved enzymes. III. Evolution of the ebg repressor during selection for enhanced lactase activity. Genetics 85:193–201.
8. **Hall, B. G., and D. L. Hartl.** 1974. Regulation of newly evolved enzymes. I. Selection of a novel lactase regulated by lactose in *Escherichia coli*. Genetics 76:391–400.
9. **Hall, B. G., and D. L. Hartl.** 1975. Regulation of newly evolved enzymes. II. The ebg repressor. Genetics 81:427–435.
10. **Hartl, D. L., and B. G. Hall.** 1974. Second naturally occuring β-galactosidase in *E. coli*. Nature 248:152–153.
11. **Reeve, E. C. R.** 1976. The lactose system of *Klebsiella aerogenes* V9A. 5. Lac-permease defective mutants of two *Klebsiella* lac plasmids and their apparent reversion to wild type. Genet. Res. 28:61–74.
12. **Reeve, E. C. R., and J. A. Braithwaite.** 1973. Lac$^+$ plasmids are responsible for the strong lactose-positive phenotype found in many strains of *Klebsiella* species. Genet. Res. 22:329–333.
13. **Reeve, E. C. R., and J. A. Braithwaite.** 1974. The lactose system of *Klebsiella aerogenes* V9A. 4. A comparison of the lac operons of *Klebsiella* and *Escherichia coli*. Genet. Res. 24:323–331.
14. **Sanderson, K. E.** 1976. Genetic relatedness in the family Enterobactericeae. Annu. Rev. Microbiol. 30:327–349.
15. **Wallenfels, K., and O. P. Malhotra.** 1961. Galactosidases. Adv. Carbohydr. Chem. 16:239–298.
16. **Weber, K., and M. Osborn.** 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. 244:4406–4412.