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A Mutant Ebg Enzyme That Converts Lactose into an Inducer of the *lac* Operon

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Lactose is not itself an inducer of the *lac* operon, nor is it converted to an inducer by *ebg*$^+$ β-galactosidase of *Escherichia coli*. We report here the isolation of a mutant Ebg β-galactosidase which is capable of converting lactose into an inducer of the *lac* operon.

Ebg enzyme, the second β-galactosidase synthesized by *Escherichia coli* K-12, is the product of the structural gene *ebgA*°. Even when synthesized constitutively (*ebgR* mutants), Ebg° enzyme does not hydrolyze lactose rapidly enough to permit lactose-dependent growth (5, 7). Point mutations within the *ebgA*° gene can alter Ebg enzyme so that it hydrolyzes lactose effectively, permitting *lacZ* deletion strains to utilize lactose as a sole carbon and energy source (4). Lactose utilization by such *ebgA*$^+$ strains is subject to two constraints: (i) the *lacZ* deletion strain must possess a functional lactose permease (*lacY*$^+$); and (ii) unless the *lacY*$^+$ gene is expressed constitutively, an inducer of the *lac* operon such as isopropyl-thio-β-galactoside (IPTG) must be included in the medium (6). This is because lactose itself is not an inducer of the *lac* operon (2, 10). In wild-type strains *lacZ* β-galactosidase converts lactose to allolactose, the natural inducer of the *lac* operon (10). Ebg enzyme, however, is not capable of this reaction (6). As a result *ebgA*$^+$ strains are white (Lac-negative phenotype) on MacConkey lactose agar, but red (Lac-positive phenotype) on MacConkey lactose agar containing 2×10^{-4} M IPTG (6).

Strain 5A2 is an *ebgA*$^+$ strain which synthesizes Ebg enzyme constitutively (see Table 1 for genotypes). After long-term (300 generations) selection for rapid growth on both lactose (4-*O*-β-D-galactopyranosyl-D-glucose) and lactulose (4-*O*-β-D-galactopyranosyl-D-fructose), an unstable mutant population arose which segregated clones that were red on MacConkey lactose agar without IPTG. One such stable segregant, strain 4R1, was the subject of this study. Strain 4R1 was distinguishable from its parent, strain 5A2, by three characteristics: (i) 4R1 colonies were red on MacConkey lactose agar without IPTG, whereas 5A2 colonies were white; (ii) 4R1 colonies required 2 days to become red on MacConkey lactulose agar containing IPTG, whereas 5A2 colonies were deep red within 1 day; (iii) 4R1 colonies were red on MacConkey galactosyl-arabinose (3-*O*-β-D-galactopyranosyl-D-arabinose) agar containing IPTG, whereas 5A2 colonies were white.

The simplest explanation of the IPTG-independent lactose fermentation would be constitutive expression of the *lacY* gene function, although this would not explain the fermentation of galactosyl-arabinose by strain 4R1. Expression of the *lac* permease was measured directly as the rate of thio-[^{14}C]methyl-β-galactoside ([^{14}C]TMG) transport in strains 5A2 and 4R1 grown in glycerol, glycerol + 2×10^{-4} M IPTG, or glycerol + 0.1% lactose minimal medium (Table 2). Minimal medium was previously described (6). Strain 5A2, as expected, transported TMG only when synthesis of the *lac* permease was induced with IPTG; however, strain 4R1 transported TMG when induced with either IPTG or lactose, but not when uninduced. We also measured the rate of [^{14}C]IPTG transport, since IPTG is not known to enter by other permeases, whereas TMG is known to be transported by at least two permeases in addition to the *lac* permease in *E. coli* (11, 13) (Table 2). These data clearly showed that the *lac* permease was not expressed constitutively in strain 4R1, although the basal level of permease was significantly higher than in strain 5A2.

There were several possible explanations for the expression of a TMG-IPTG permease in lactose-induced 4R1 cells.

(i) Strain 4R1 might be a *lac i*$^+$*Z*$^+$*Y*$^+$ contaminant. This seemed unlikely, since (1) strain 4R1 retains the parental HfrC, and (2) β-galactosidase activity assays showed that *ebg* β-galactosidase activity was synthesized constitutively in strain 4R1, and that the specific activity of crude extracts was unaffected by the addition of IPTG to the growth medium (data not shown). Furthermore, wild-type (*lac i*$^+$*Z*$^+$*Y*$^+$) *E. coli* colonies

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TABLE 1. *Genotypes of E. coli K-12 strains employed*

Strain	Genotype	Source
5A2	HfrC Δ *lacZ(W4680) ebgA52 ebgR52*	(7)
4R1	HfrC Δ *lacZ(W4680) ebgA205 ebgR52*	This study
SJ7	F⁻ *argG tolC metC rpsL* Δ *lacZ(W4680) ebgA° ebgR⁺*	(7)
SJ12	F⁻ *argG metC rpsL* Δ *lacZ(W4680) ebgA52 ebgR52*	(7)
SJ60	F⁻ *argG rpsL* Δ *lacZ(W4680) ebgA205 ebgR52*	This study
SJ61	F⁻ *argG metC rpsL* Δ *lacZ(W4680) ebgA205 ebgR52*	This study
CSH26	F⁻ Δ(*lac-pro*) *thyA serA ara thi*	(12)

are deep red within 18 h on MacConkey lactulose agar, whereas 4R1 colonies require 2 days to turn red. Genetic evidence (below) confirmed the idea that strain 4R1 did not carry a *lacZ*⁺ gene, and is thus not a wild-type contaminant.

(ii) Lactose, TMG, and IPTG might enter 4R1 cells via an "evolved" permease, previously unknown, which was inducible by lactose. Hobson (8) has described such a new lactose permease which arose as a consequence of mutation(s) in a gene located between 43 and 53 min on the *E. coli* map. Were this the case, the *lac* region of the chromosome would not be required for lactose utilization. To test this hypothesis, strain 4R1 was mated for 2 h with the *lac-pro* deletion strain CSH26, and the *thyA*⁺ gene (distal to both the *lac* operon at 7 min and the *ebg* genes at 66 min) was selected. The recombinants were scored for the presence of the *lacY*⁺ gene by their ability to ferment melibiose at 42°C (12); for *pro*⁺; for the ability to ferment lactose in both the presence and absence of IPTG; and for the ability to ferment galactosyl-arabinose in the presence of IPTG. Of 283 *thyA*⁺ *rpsL* recombinants tested, 149 were *lacY*⁺ *pro*⁺. Of these, 69 were Lac⁻, ruling out the possibility that 4R1 was a *lacZ*⁺ contaminant. The remainder were Lac⁺ in the presence and in the absence of IPTG and were also galactosyl-arabinose positive. Of the 134 *lacY pro* isolates, none was Lac⁺. This demonstrates that the *lacY*⁺ gene product was required for lactose fermentation, and argues against the hypothesis that some other permease was responsible for lactose transport.

(iii) A mutation in the *lacI* gene might have resulted in the *lac* repressor becoming sensitive to lactose as an inducer, or (iv) a mutation in the *ebgA* gene might have led to Ebg enzyme capable of converting lactose to an inducer of the *lac* operon. To distinguish between these alternatives, the *ebgA* gene from strain 4R1 was transduced by cotransduction with *tolC* by bacteriophage P1 into strain SJ7. *tolC*⁺ transductants were selected on MacConkey lactose agar (the dyes present in MacConkey agar select against *tolC* mutants) containing IPTG. *ebgA*⁺ cotransductants were scored as red colonies. The cotransduction frequency between *tolC* and *ebgA* was 30% (43 *ebgA*⁺ among 144 total colo-

TABLE 2. *Thiogalactoside transport rates*[a]

Strain	Addition	Transport rate (μmol min⁻¹ g⁻¹ [dry wt])	
		TMG	IPTG
5A2	None	<0.3	<0.3
	IPTG	5.1	4.2
	Lactose	<0.3	<0.3
4R1	None	0.7	0.5
	IPTG	11.2	4.9
	Lactose	13.6	6.8
SJ7	None	<0.3	<0.3
	IPTG	4.7	2.4
	Lactose	<0.3	<0.3
SJ12	None	<0.3	<0.3
	IPTG	5.2	1.8
	Lactose	<0.3	<0.3
SJ60	None	<0.3	<0.3
	IPTG	4.5	2.4
	Lactose	5.9	2.6
SJ61	None	<0.3	<0.3
	IPTG	4.6	5.0
	Lactose	5.6	4.2

[a] Cells were grown in glycerol minimal medium with no addition, with 2×10^{-4} M IPTG, or with 0.1% lactose. Cells were harvested in mid-exponential phase, washed twice with minimal salts buffer, and resuspended in minimal salts buffer at about 5×10^9 cells per ml; the suspension was maintained at 15°C until used. Uptake was initiated by the addition of 1 μCi of [^{14}C]TMG per μmol (to give a concentration of 0.25 mM TMG), or 1 μCi of [^{14}C]IPTG per μmol (to give a concentration of 0.42 mM IPTG). The cell suspension was maintained at 15°C without shaking throughout the assay. At 0.5-min intervals, 200-μl samples were pipetted onto Millipore type HA filters and washed twice with 5 ml of 15°C minimal salts buffer. Filters were dried and counted in a liquid scintillation counter. Blank values, to correct for nonspecific binding, were obtained by killing a portion of cell suspension with 5% formaldehyde 10 min before the addition of [^{14}C]TMG. The rates were calculated from a least-squares fit of counts incorporated versus time for 3 min of incorporation. In all cases where the rate exceeded 0.3 μmol min⁻¹ g⁻¹ (dry weight), the correlation coefficient was greater than 0.98. The dry weight was estimated from the turbidity at 600 nm, where a value of 1.0 is equal to 10^9 cells, or 0.2 mg (dry weight).

nies scored), in good agreement with previously published results (7). When tested on MacConkey lactose agar without IPTG, all *ebgA*$^+$ transductants were lactose positive; furthermore, all were positive on MacConkey galactosyl-arabinose agar. The transductants, however, required 2 days to turn red on MacConkey lactose agar without IPTG, whereas the donor strain, 4R1, became red within 1 day. Two of these transductants, strains SJ60 and SJ61, were assayed for TMG and IPTG transport (Table 2). As did 4R1, the transductants transported TMG and IPTG when induced by either IPTG or lactose. Strains SJ7 and SJ12 (Table 1), on the other hand, transported TMG and IPTG only when induced by IPTG. These data clearly rule out the hypothesis that a mutant *lac* repressor gene in strain 4R1 is responsible for the induction of *lac* permease synthesis by lactose itself, since *lacI* does not cotransduce with *tolC*, and they strongly support the hypothesis that a mutation in the *ebgA* gene itself (*ebgA205*) has altered the Ebg enzyme so that it can convert lactose to an inducer of the *lac* operon.

To provide supporting evidence that the *lac* operon was being expressed as a consequence of lactose induction, we measured the level of thiogalactoside transacetylase, the *lacA* gene product, in uninduced cells and in cells induced with either IPTG or lactose (Table 3). Thiogalactoside transacetylase synthesis is inducible by lactose in *lacZ* deletion strains if they carry the *ebgA205* allele (strains 4R1, SJ60, and SJ61). This rules out the possibility that the *lac* operon itself was not induced, and shows that other permeases (such as the *araE* gene product, which can transport lactose [11]) are not responsible for the TMG and IPTG transport observed in strains SJ60 and SJ61.

The results of this study show that Ebg enzyme specified by the *ebgA205* allele is capable of converting lactose into an inducer of the *lac* operon. The nature of that inducer is not yet known. Allolactose, the natural inducer of the *lac* operon, is a direct transgalactosylation product catalyzed by *lacZ* β-galactosidase; i.e., a molecule of lactose is rearranged to form allolactose, rather than the galactose moiety being transferred to a free glucose acceptor molecule (9, 10). Transfer of galactose to free sugars can occur, however, and glycerol galactoside so formed is an inducer of the *lac* operon (2). It may well be that the mutant Ebg enzyme is synthesizing allolactose; however, it is also possible that it is synthesizing other inducers of the *lac* operon. Direct analysis of the transgalactosylation products formed will be required to determine the nature of the *lac* operon inducer in strain 4R1.

TABLE 3. *Thiogalactoside transacetylase activity*[a]

Strain	Addition	Acetylase activity (A_{350})
SJ7	None	<0.02
	IPTG	1.70 ± 0.03
	Lactose	<0.02
4R1	None	<0.02
	IPTG	3.00 ± 0.02
	Lactose	2.43 ± 0.02
SJ60	None	<0.02
	IPTG	3.00 ± 0.05
	Lactose	2.26 ± 0.09
SJ61	None	<0.02
	IPTG	2.71 ± 0.05
	Lactose	1.88 ± 0.02

[a] Cultures were grown at 32°C in M63 minimal salts buffer (12) containing 0.2% glycerol and 100 μg of methionine, 100 μg of arginine, and 0.5 μg of thiamine per ml. As indicated, 0.5 mM IPTG or 0.1% lactose was added to this medium. Cultures were harvested in late exponential phase by centrifugation, washed once, and suspended to a turbidity of approximately 10 measured at 350 nm (1 unit of absorbancy at 350 nm [A_{350}] = 3×10^8 cells per ml). The suspensions were lysed by a freeze-thaw lysozome procedure and assayed for thiogalactoside transacetylase activity by using a modification of the procedure described by Alpers et al. (1) (Fried, manuscript in preparation). Assays were performed at 28°C at initial substrate concentrations of 2×10^{-4} M acetyl-coenzyme A and 0.1 M IPTG. One unit of activity is equivalent to 1 nmol of coenzyme A produced per min. All assays were performed in triplicate. Values shown are the mean ± the standard error.

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