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Evolution of a Second Gene for β-Galactosidase in *Escherichia coli*

(*lac* operon/gene mapping/enzyme characterization/lactose permease/enzyme evolution)

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ABSTRACT **Mutants of *E. coli* K12 with deletions of the β-galactosidase gene (*lacZ*) can reacquire the ability to hydrolyze β-galactosides during prolonged intense selection for growth on lactose. Full lactose competence is restored through a sequence of at least five mutations. Cell extracts of these derived strains hydrolyze *o*-nitrophenyl-β-D-galactoside, the standard substrate for assay of β-galactosidase. The enzyme responsible for this activity differs in its immunological, kinetic, and sedimentation characteristics from the *lacZ* β-galactosidase of wild-type *E. coli*. Its genetic determinant, designated *ebg*-5, maps at 59 min on the *E. coli* chromosome, whereas the *lac* operon maps at 10 min. We suggest that a gene not involved in lactose utilization has been progressively changed into a form capable of specifying a β-galactosidase and that this process is similar to that whereby genes with new functions are evolved by natural selection.**

Selection of bacterial mutants with new catabolic capacity has recently received considerable interest as an experimental model of gene evolution (for a current review see ref. 1). The usual procedure has been to select mutants able to utilize a novel carbon source not normally metabolized by the species. This constitutes selection for a new catabolic pathway, and characteristically gives complex results. For example, regulator genes and permeases may be altered, and pre-existing cryptic or latent genes may be activated. The catalytic properties of the catabolic enzymes themselves may or may not be modified. These complexities are of considerable evolutionary interest, but they decrease the attractiveness of selecting for a new catabolic *pathway* as a means of experimentally evolving a *single enzyme* activity.

In order to study the evolution of one enzyme, we have chosen instead to delete the gene for β-galactosidase (*lacZ*) in *Escherichia coli*, and then select for reacquisition of lactose competence. Our starting strain carries all the other genes involved in the uptake and metabolism of lactose, so that the only function selected for is lactose-hydrolyzing activity. Since *E. coli* has been adapted to grow in the presence of lactose and its metabolic intermediates, the evolution of a new lactose-splitting enzyme is not expected to be accompanied by other metabolic modifications. A further advantage of selecting for development of an alternate catalyst for a previously utilized reaction is that comparison can be drawn between a highly adapted enzyme and a newly evolved enzyme sharing a similar function.

Abbreviations: CRM, crossreacting material; Tet-Lac plates, tetrazolium–lactose indicator plates.

We report here our procedure for reverting *lacZ* deletion strains to forms able to metabolize lactose, and present biochemical and genetic characterization of the β-galactosidase of one of these revertants.

MATERIALS AND METHODS

Bacterial and Phage Strains. Transducing phage P1 was obtained from C. Yanofsky. The bacterial strains 3000 X44 (HfrH $lacZ^{CRM}_{X44}$), 3300 (HfrH *lacI*$^-$), 2300 (*lacI*$^-$), RV (*lacIZYAdel*-X74), and F05/RV (F'*lacIOZdel-05(694)*/*lacIZYAdel*-X74) were obtained from the collection of the Service de Biochemie Cellulaire et de Génétique Microbienne of the Institut Pasteur through the courtesy of J. Monod. The *lacI* mutation leads to constitutive production of the *lac* operon enzymes; $lacZ^{CRM}_{X44}$ is a point mutation that produces β-galactosidase crossreacting material (CRM) (2); and *lacIZYA-del*-X74 is a deletion of the entire *lac* operon (3, 4); the *lacIOZdel*-05(694) mutation deletes the *lacI* and *lacO* genes and the proximal one-third of the *lacZ* gene, but allows the *lacY* and *lacA* genes to be transcribed from a nearby, unidentified promoter (5, 6). Strain AB288 (*lacY*$^-$*his*$^-$*serA*$^-$*str*^{r}) was kindly supplied by P. J. Zamenhof and strains AT2685 (*his*$^-$*thyA*$^-$*argG*$^-$*str*^{r}) and AT2699 (*lacY*$^-$*his*$^-$*thyA*$^-$*metC*$^-$*argG*$^-$*str*^{r}) by A. L. Taylor. The $lacZ^{CRM}_{X44}$ locus was introduced into these three strains by conjugation with 3000 X44. The recombinants, with the $lacZ^{CRM}_{X44}$ locus of the donor and the auxotrophic and Str markers of the recipient, were designated LC1201, LC1215, and LC1225, respectively.

Media and Buffers. Minimal and complete liquid and agar medium were as described by Pardee *et al.* (7); tetrazolium–lactose indicator (Tet–Lac) plates contained nutrient broth plus 10 mg/ml of lactose and 50 μg/ml of triphenyl tetrazolium chloride (8). Special supplements, as required, were added to the autoclaved media in the following concentrations: glucose, 10 mg/ml; lactose, 10 mg/ml; melibiose, 1 mg/ml; L-amino acids, 33–200 μg/ml; thymine, 50 μg/ml; streptomycin, 100 μg/ml; and isopropyl-β-D-thiogalactoside, 0.5 mM. KPM buffer consists of 0.5 M KCl–0.01 M $MgSO_4$–0.25 M KH_2PO_4 adjusted to pH 7.5 with KOH. TAM buffer contains 0.25 M Tris·HCl–0.3 M $(NH_4)_2SO_4$–0.01 M $MgSO_4$, and HCl to give pH 7.5. Phosphate buffer refers to solutions of KH_2PO_4 at the concentrations indicated brought to pH 7.5 with KOH.

Biochemical Methods. For preparation of cell extracts, cultures were grown overnight in glycerol broth, with or without isopropyl-β-D-thiogalactoside as inducer, and centrifuged.

TABLE 1. *Comparison of LacZ and Ebg β-galactosidase activities*

Strain designation	3300 (LacZ+)	LC110 (Ebg+)
Generation time on lactose minimal medium (min)	52	72
β-Galactosidase activity in:		
toluene-treated cells (units/10^9 cells)	1400	71
cell extracts (units/mg of protein)	1600	21
pH optimum	5.5–6.5	7.5
K_m for ONPG (M × 10^4)	3.5	14
K_i† for lactose (M × 10^4)	50	200
Estimated molecular weight (× 10^{-5})	5.4	10
Cell extract precipitates with anti-Z serum	Yes	No

ONPG, *o*-nitrophenyl-β-D-galactoside.
† Obtained by the graphic method of Dixon (18).

The cells were either ground with alumina and suspended in 0.1 M phosphate buffer or were suspended in 0.25 M phosphate and sonicated. Cell debris was removed by centrifugation. Protein concentration was determined by the method of Lowry *et al.* (9).

Quantitative assays and kinetic measurements of β-galactosidase activity were performed in cell extracts as we described earlier (10) and in toluene-treated cells as described by Miller (11) by monitoring the release of *o*-nitrophenol from *o*-nitrophenyl-β-D-galactoside, with a Zeiss PMQ II spectrophotometer. 1 Unit of β-galactosidase activity is defined as that which releases 1 μmol/min of *o*-nitrophenol from 0.5 ml of 0.23 mM *o*-nitrophenyl-β-D-galactoside in KPM buffer at 37°.

The presence of β-galactosidase activity was detected qualitatively by incubation of extracts of cells from 2 ml of culture in 0.5 ml of 0.25 M phosphate buffer containing 0.25 mM *o*-nitrophenyl-β-D-galactoside and 0.05 ml of toluene at 37°. The activity was recorded as + if a yellow color was detected in less than 10 min, ± if color appeared only after 2 hr, and − if no color was detected after 24 hr.

Immunological analysis was by double diffusion in agar plates (12), with a rabbit antiserum to purified wild-type β-galactosidase (anti-Z) generously provided by E. Sercarz.

Genetic Methods. Transducing lysates of P1 were prepared on plates and stored over $CHCl_3$ (13). Recipient cells were grown in broth to 1–5 × 10^8/ml. They were incubated with phage (multiplicity of infection = 1–3) for 20 min at 25° in broth containing 2.5 mM $CaCl_2$, and then plated on selective medium in soft agar containing 2% sodium citrate to prevent phage readsorption.

Uninterrupted Hfr matings were done for 90 min on Millipore filters (14) after mating pairs were allowed to form for 15 min at 37° in stationary liquid. Ebg+ recombinants were selected on lactose minimal plates containing isopropyl-β-D-galactoside, streptomycin, and appropriate supplements. They were purified on Tet–Lac plates containing isopropyl-β-D-thiogalactoside and streptomycin. Other recombinants were selected and purified on glucose minimal plates containing appropriate auxotrophic supplements and streptomycin. The Ebg+ character as an unselected marker was detected by placing a drop of toluene on a patch of bacterial growth on a broth plate, followed 30 min later by a drop of 1.33 mM *o*-nitrophenyl-β-D-galactoside in 0.25 M phosphate buffer (pH 7.0) with 1 mM $MgSO_4$ (15). The constitutive Lac+ strain 3300 gives intense yellow color in 2–3 sec, Ebg+ strains give yellow color in 10 min, and $lacZ^{CRM}_{X44}$ or Lac− deletion strains give no color after several hours.

RESULTS

Selection of lactose competent mutants from a lac-deletion strain

Strain F05/RV (F'*lacIOZdel/lacIZYAdel*) was streaked onto several Tet–Lac plates. The plates were incubated for 48 hr at 37° and maintained thereafter at room temperature (25°). During the initial incubation period deep red colonies appeared, indicative of growth supported by the broth and not by the lactose in the agar. The colonies spread and became flattened during the subsequent 8 weeks as the plates slowly dehydrated. Probably a low rate of growth was sustained by remaining traces of broth or by turnover as cells died. After 25–40 days, white papillae began to appear, growing out of the red colonies. The length of time before the first papilla appeared varied from plate to plate depending on the rate at which the plates dried out. The first plates to show papillae were the first to dessicate. After the first papilla appeared on a plate, others became visible on that plate at a constant rate of several per day for about 2 weeks. Subsequently, no new papillae emerged although those present continued to enlarge.

The papillae initially appeared as minute smooth white spheres. After several days they increased in diameter to 1–2 mm and their surfaces appeared like minute raspberries, presumably due to secondary protrusion of faster-growing clones of cells. 8 Weeks after the plates had been streaked, the papillae had expanded into flat irregular masses. One papilla was picked on the thirtieth day and streaked on lactose minimal agar. After incubation for 7 days at 37°, colonies of various sizes appeared. The lactose competence of the largest was designated Ebg-1, standing for evolved β-galactosidase activity§.

The Ebg-1 cells had a low but detectable (±) *o*-nitrophenyl-β-D-galactoside hydrolase activity. They produced uniform small colonies on lactose minimal agar after 5 days at 37°. When the plates were incubated for 5 additional days, faster growing mutant papillae grew out of several of the colonies. The largest papilla, whose Lac+ character was designated Ebg-2, was restreaked on lactose minimal agar. Two additional rounds of selection gave rise to the character Ebg-4. This strain grew poorly on lactose minimal agar at 42° until selection for growth at this temperature was done. The resulting character was designated Ebg-5.

Ebg-5 cells form colonies on lactose minimal agar as rapidly as does wild-type *E. coli*. They produce pink colonies on Tet-Lac plates, distinct from the white colonies formed by Lac+ strains and the dark-red colonies formed by the parent strain FO5/RV. Extracts of Ebg-5 cells hydrolyze *o*-nitrophenyl-β-

§ Ebg± derivatives have been observed from various LacZ− deletion strains. Strain RV generates Ebg± derivatives more readily than some other strains, although the reason for this is obscure. The presence of absence of the F' *lacZ*$^-$*Y*$^+$ episome has no noticeable effect on the kinetics of appearance of Ebg± papillae on our selection plates, probably because strain RV readily gives rise to an alternative lactose permease.

D-galactoside more rapidly than do extracts of Ebg-1 cells, but more slowly than do extracts of LacZ$^+$ cells. The Ebg-5 cells retain the parental F'*lac* factor with the original 05(694) deletion, as shown by their pattern of recombination when mated with a series of female strains carrying known, mapped *lacZ* point mutations (10). When cured of F'*lac* factor with acridine (16), the Ebg-5 cells form dark-red colonies on Tet–Lac plates, can no longer grow on lactose or melibiose minimal agar [a test for the LacY gene (17)], and do not transfer either the Ebg$^+$ or the LacY character. However, extracts of the cured strain still hydrolyze *o*-nitrophenyl-β-D-galactoside, and the cured cells can be restored to lactose competence by reinfecting with the original F'*lac* Z^-Y^+ episome. These results indicate that the Ebg-5 character is chromosomal and that the only function of the episome in promoting growth on lactose is to supply the lactose permease gene.

Further selection was applied to develop a new lactose permease. The cured Ebg-5 strain was streaked on a Tet–Lac plate and incubated for 24 hr at 37° and thereafter at 25°. By the sixth day, several hundred white papillae had arisen. Cells from four papillae were checked for ability to grow on lactose–minimal agar at various temperatures. Two isolates formed colonies at 25°, but not at 37°; one formed colonies at 25° and 37°, but not at 42°; and the fourth formed colonies as rapidly as wild type at 25°, 37°, and 42°. This last isolate was assigned the strain number LC110, and its acquired β-galactoside permease function is referred to as Ebp-1.

Comparison between the Ebg and LacZ β-galactosidases

Growth rates on lactose and β-galactosidase activities of the strain LC110 (Ebg$^+$Ebp$^+$) and 3300 (constitutive Lac$^+$) are compared in Table 1. Strain LC110 grows almost as rapidly on lactose as strain 3300, yet has only 1–5% the *o*-nitrophenyl-β-D-galactose hydrolyzing activity of strain 3300. A possible explanation is that optimum conditions for assay of the Ebg activity may not have been achieved, since the conditions under which the two enzymes are active are quite different. Compared to the LacZ enzyme, Ebg activity is extremely sensitive to inhibition by ammonium ions, displays a much sharper pH optimum (data not shown), and is relatively more active when assayed in toluenized cells than in cell extracts. The K_m of the Ebg enzyme for *o*-nitrophenyl-β-D-galactoside is 1.4×10^{-3} M, which is only 4-fold higher than the K_m of wild-type β-galactosidase. Lactose acts as competitive inhibitor of both enzymes, with inhibition constants 5-fold higher for the Ebg enzyme than for the *lacZ* enzyme.

The sedimentation behavior of Ebg activity in a sucrose gradient is compared with that of LacZ β-galactosidase in Fig. 1. The enzymes were centrifuged individually and as a mixture. The difference in degree of activity of the Ebg enzyme in TAM and KPM buffer allowed us to identify the positions of the two enzymes in the gradient from the mixture. The Ebg enzyme migrated as a single major band with a sedimentation coefficient of 24.4 S, by comparison with the LacZ enzyme as a 16S standard (19). We calculate (20) the molecular weight of the Ebg enzyme to be 1.0×10^6 using 5.4×10^5 as the molecular weight of wild-type β-galactosidase (19).

In order to determine whether the LacZ and Ebg enzymes are antigenically related, extracts of strains 3300, RV, LC110, and 3000 X44 were tested by immunodiffusion for precipitation by anti-Z antiserum. A dense precipitin band was obtained only with the constitutive Lac$^+$ strain 3300 and induced LacZ^{CRM} strain 3000 X44. Two faint bands, representing traces of antibodies to two contaminating proteins in the immunizing antigen, were formed by extracts of all strains tested. Upon 10-fold dilution, the serum still gave a strong reaction against extracts of strain 3300, but no visible reaction with extracts of the Lac deletion strain RV, the Ebg$^+$ strain LC110, or the uninduced strain 3000 X44.

Genetic analysis of Ebg-5

Although lactose competence arose through a series of at least five independent mutations, the *ebg*-5 genetic determinant behaves as a discrete locus during transduction from strain LC110 into strain 3000 X44 (HfrH LacI$^+$O$^+$Z^{CRM}Y$^+$). Transductants were selected on lactose minimal plates contain-

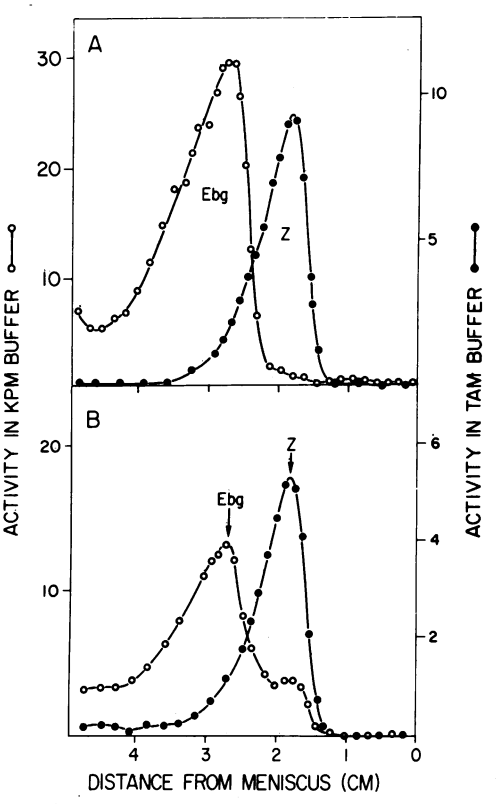

FIG. 1. Sedimentation of Ebg and LacZ β-galactosidases on a sucrose gradient. (*A*) Cell extracts of strains 3300 (LacZ$^+$) and LC 110 (Ebg$^+$) were treated with 25 μg/ml of streptomycin sulfate to remove nucleic acids. Proteins were precipitated by addition of ammonia sulfate to 40% saturation, resuspended in and dialyzed against 0.1 M potassium phosphate (pH 7.5), and diluted appropriately. Aliquots of 100 μl were layered onto individual linear 5–20% sucrose gradients in 0.1 M phosphate buffer, and centrifuged 6 hr at 37,000 rpm and 4° in a Spinco SW40 rotor. Three-drop fractions were collected. LacZ activity was assayed in 0.33 mM *o*-nitrophenyl-β-D-galactoside in TAM buffer (O——O) and Ebg activity in 0.33 mM *o*-nitrophenyl-β-D-galactoside in KPM buffer (O——O). Activity is expressed as ΔA_{420}/min per μl of gradient fraction $\times 10^{-4}$. The two profiles are superimposed with different ordinate scales. (*B*) As above except that 50 μl of each diluted extract were mixed together and centrifuged in a single gradient, simultaneously with the two gradients in (*A*). Gradient fractions were assayed in TAM and in KPM buffers. The Ebg enzyme has significant activity only in the KPM buffer, while the LacZ enzyme is active in both buffers.

TABLE 2. *Frequency of recombinants from the cross: LC172 (HfrH $lacZ^{CRM}_{X44}$ebg-5 x LC1215 ($lacZ^{CRM}_{X44}$his$^-$ thyA$^-$ argG$^-$ str^r)*

	Recombination frequency (%)	No. analyzed	% of recombinants that score as: His$^+$	ThyA$^+$	Ebg$^+$	ArgG$^+$
A. Selected recombinants:						
ThyA$^+$Str^r	0.022	112	20	—	23	11
Ebg$^+$Str^r	0.017	188	20	46	—	47
ArgG$^+$Str^r	0.016	112	20	33	54	—
B. Three-factor analysis of above recombinants:						
Ebg$^+$ThyA$^+$Str^r		113		—	—	42
Ebg$^+$ArgG$^+$Str^r		148		43	—	—
ArgG$^+$ThyA$^+$Str^r		49		—	71	—
ArgG$^+$ThyA$^-$Str^r		75		—	44	—

ing isopropyl-β-D-thiogalactoside, and were obtained at a frequency of 6×10^{-6}.

23 Ebg$^+$ transductants were purified, and all were shown to have the following characteristics: (*i*) They produce LacZ-CRM in the presence but not in the absence of isopropyl-β-D-thiogalactoside, which is an inducer of the *lac* operon. (*ii*) They produce Ebg activity in the presence and in the absence of the thiogalactoside. (*iii*) They grow on lactose minimal medium only if thiogalactoside is present. (*iv*) On Tet–Lac indicator plates they form pink colonies (i.e., metabolize lactose) if thiogalactoside is present, but form red colonies if it is absent. From these findings we deduce the following: (*i*) The Ebg$^+$ transductants retain and express the LacI$^+$, O$^+$, and Z^{CRM} alleles of the recipient. (*ii*) The LacI repressor does not affect the expression of the *ebg* gene. (*iii*) Metabolism of lactose requires the expression of one of the Lac operon genes, obviously the *lacY* permease gene. (*iv*) Lactose does not induce the *lac* operon in Ebg$^+$,Z$^-$ cells. Ordinarily the presence of lactose induces the *lac* operon because the LacZ enzyme converts some of the lactose into an inducer compound by transgalactosidation to a small acceptor molecule (21). Lactose itself is not an inducer, whereas isopropyl-β-D-thiogalactoside is. The Ebg enzyme is either incapable of carrying out this transfer reaction (e.g., it acts only as a hydrolase) or has an activity too low to induce the *lac* operon. (*v*) Since the Ebg$^+$Ebp$^+$ donor strain metabolizes lactose in the absence of thiogalactoside, the Ebg$^+$ transductants did not also receive the Ebp character. When 5×10^7 transducing phage-infected cells were plated on lactose minimal agar without isopropyl-β-D-thiogalactoside, no transductants appeared, indicating that *ebp* is cotransduced with *ebg* at a frequency less than 0.3%. We therefore conclude that *lac*, *ebg*, and *ebp* are completely independent genetic determinants. One of the transductants, designated strain LC172, thus has the genotype HfrH $lacZ^{CRM}_{X44}$*ebg*-5.

A series of preliminary conjugation experiments with strain LC172 mapped the *ebg*-5 determinant in the vicinity of *serA* [56.0 min on the *E. coli* linkage map (22)]. Strain LC172 was then mated with strain LC1215 (F$^-$LacZ^{CRM}His$^-$ThyA$^-$ ArgG$^-$). The markers *thyA* and *argG* are located at 54.3 and 61.0 min, respectively. The frequencies of selected Thy$^+$, Ebg$^+$, and Arg$^+$ recombinants shown in Table 2 suggest the gene order *thyA-ebg-5-argG*. This order is confirmed by the frequency of unselected markers among these recombinants and by three factor analysis of the three markers Thy$^+$, Ebg$^+$, and Arg$^+$. When the third of these was scored among recipients recombinant for the other two, it was found that 70% of the Arg$^+$Thy$^+$ recombinants were also Ebg$^+$, while only 40% of the Ebg$^+$Thy$^+$ and Ebg$^+$Arg$^+$ recombinants carried the third donor marker.

The Ebg-5 determinant was mapped more precisely by transduction from LC172 into strains LC1225 (MetC$^-$ LacZ^{CRM}Ebg$^-$) and LC1201 (SerA$^-$LacZ^{CRM}Ebg$^-$). Of 191 Met$^+$LC1225 transductants tested, 11 (5%) were Ebg$^+$. This frequency of cotransduction indicates that the *ebg*-5 locus is about 1.2 min from *metC* at 57.7 min (23). None of 91 Ebg$^+$ transductants of LC1201 tested as also SerA$^+$. Since *serA* lies at 56.0 min, the *ebg*-5 locus must lie on the opposite side of *metC* at about 59 min.

DISCUSSION

An enzyme capable of catalyzing hydrolysis of β-galactosides can be generated in *lac* deletion strains of *E. coli* K12 by a series of mutations. The new enzyme permits rapid growth on lactose minimal medium and has a high affinity for lactose and *o*-nitrophenyl-β-D-galactoside. It differs in its kinetic properties from the LacZ protein, does not detectably crossreact with anti-Z antiserum, and has almost twice the molecular weight of the LacZ protein. The *ebg* genetic determinant occupies a discrete locus that cotransduces at a frequency of 5% with *metC*. This places it at about 59 min on the *E. coli* map, almost exactly opposite from the *lac* operon (10 min out of a total map length of 90 min).

It is not known which gene of the original *lac* deletion strain has been developed into the new β-galactosidase gene. Only one other gene has been mapped near 59 min, *tolC*, which determines tolerance to colicin E_1 and which is probably involved in membrane structure (24).

The antigenic, enzymatic, and size differences between the Ebg and LacZ enzymes, in addition to the independence in map position and expression of the *ebg* and *lacZ* genes, suggest that the ancestral *ebg* gene is not closely related to the *lacZ* gene. Since such a function would have been found during the intensive study of lactose utilization by *E. coli* over the past three decades, it would be surprising if the function of the ancestral *ebg* gene product were to metabolize β-galactosides. No evidence has been found for multiple genes for β-galactosides. No evidence has been found for multiple genes for β-galactosidase in *E. coli*, although β-galactosidase isozymes do occur in higher organisms (25).

An alternative lactose permease gene (*ebp*) can also be developed by mutation in strain RV, independently of the

generation of the Ebg character. A new lactose permease is less unexpected than a new β-galactosidase because bacterial permeases are rather broad and often overlapping in their specificity. Wild-type *E. coli* has only one permease capable of accumulating lactose, but has three other permeases capable of taking up galactose or methyl β-galactosides (26). The Ebp permease may correspond to a modified form of one of these other permeases. The modification probably involves the structure of the permease since the Ebp^+ phenotype is temperature sensitive in some isolates.

The Ebg character is developed by the progressive modification of a precursor gene through mutation and selection, which increases the fitness of the organism in its environment. We therefore consider its genesis to be a process of evolution and suggest that this process is similar to the way other bacterial genes have evolved in nature.

NOTE ADDED IN PROOF

Lactose-utilizing mutants have been isolated from *E. coli lac* deletion strains by another selection method [Warren R. A. J. (1972) *Can. J. Microbiol.* **18**, 1439–1444]. Although not extensively characterized, their properties are similar to those of the Ebg^+ strain LC110.

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