EVOLUTION OF A REGULATED OPERON IN THE LABORATORY

BARRY G. HALL

Microbiology Section, U-44, University of Connecticut, Storrs, CT. 06268

Manuscript received November 6, 1981
Revised copy accepted April 12, 1982

ABSTRACT

The evolution of new metabolic functions is being studied in the laboratory using the EBG system of *E. coli* as a model system. It is demonstrated that the evolution of lactose utilization by *lacZ* deletion strains requires a series of structural and regulatory gene mutations. Two structural gene mutations act to increase the activity of ebg enzyme toward lactose, and to permit ebg enzyme to convert lactose into allolactose, an inducer of the *lac* operon. A regulatory mutation increases the sensitivity of the *ebg* repressor to lactose, and permits sufficient ebg enzyme activity for growth. The resulting fully evolved *ebg* operon regulates its own expression, and also regulates the synthesis of the lactose permease.

THE two basic questions in biology are "How do organisms function?" and "How did they get to be that way?" The great majority of modern biological research is devoted to answering the first question, but ever since DARWIN published *The Origin of Species* in 1859 curiosity about the second question has been one of the major unifying forces in biology. It is remarkable that these two basic questions have been approached in such fundamentally different ways.

The general approach to discovering "how organisms function" has been to isolate some system as much as possible, and then to deliberately perturb the system and observe its responses to those perturbations. Eventually hypotheses are constructed that predict responses to specific perturbations, and the accuracy of those predictions is taken as a measure of the validity of the hypothesis.

In contrast, the understanding of evolution, or "how things got to be this way," has depended upon fragmentary fossil evidence and upon comparisons of existing organisms with each other and with their extinct ancestors. Based upon an enormous number of observations at the anatomical and molecular levels, an assemblage of evolutionary hypotheses has been constructed. Remarkably absent from that set of observations are those based upon deliberate perturbation of the system. The time scale upon which evolution is perceived to act seems to prohibit deliberate perturbations, and to render futile any predictions of the consequences of specific perturbations. Evolutionists are in a position equivalent to that of a biochemist being shown the entire contents of a cell and being asked to deduce biochemical pathways. A good chemist could generate

plausible hypotheses, but could not verify their validity without perturbing the system in order to observe it in a dynamic, rather than a static, state.

To observe evolution in a dynamic state we can ask, "How do simple cells evolve new metabolic functions?" Because they can be cultured under well-defined conditions, and because of their short generation times, bacteria are excellent tools for this kind of study. Our detailed knowledge of how current cells function allows us to describe the results of historical evolution. A cell carries out a metabolic function by synthesizing a series of specific proteins, each of which catalyzes a step in a sequential pathway of reactions. In general, the synthesis of these proteins is controlled so that they are made only when there is a need for the pathway to function, and that need is recognized by specific regulatory proteins that respond to the concentration of small molecules in the cellular environment.

Evolution of new metabolic functions must therefore involve more than the acquisition of new enzymatic activities; it must also involve the organization of those activities so that just the right amount of each enzyme is made in direct response to an appropriate environmental signal. As organisms evolve enzymes to carry out new metabolic functions, they must also evolve regulatory systems that respond to environmental signals appropriate for new functions. How do organisms that are already well adapted to their environments evolve new enzyme systems that are well integrated into the organism's established metabolic system? It is a major point of this paper that evolution of regulatory organization may require several mutational steps. If evolution of a fully integrated new enzyme system requires several mutations, then some of those mutations may not be selectively advantageous in the genetic background in which they arise, but may confer a selective advantage only in the presence of a second mutation. Such mutations would be initially neutral, but would have adaptive potential. In this paper it is demonstrated that such mutations can and do arise in the laboratory.

I have been using the EBG system of *Escherichia coli* K12 as a model system to study the acquisitive evolution of new metabolic functions. Beginning with *E. coli* strain DS4680A, which carries a large internal deletion within the *lacZ* (β-galactosidase) gene, I have selected a large series of spontaneous mutants that have evolved the ability to grow on lactose and other β-galactoside sugars as sole sources of carbon and energy (HALL and HARTL 1974, 1975). The mutations that allow *lacZ* deletion strains of *E. coli* to utilize lactose occur in the genes of the *ebg* operon located at 66 min on the *E. coli* map (HALL and HARTL 1974, 1975; BACHMANN and LOW 1980). I have identified three genes in the *ebg* operon: *ebgA* is the structural gene for a β-galactosidase enzyme (HALL and HARTL 1975), *ebgB* is "downstream" from *ebgA*, and specifies a 79,000 mol. wt. polypeptide of unknown function (HALL and ZUZEL 1980b), and *ebgR* specifies a repressor protein that controls expression of *ebgA* (HALL and HARTL 1975). Mutations in both *ebgA* and *ebgR* are required for the evolution of lactose utilization (HALL and CLARKE 1977).

Two conditions must be met in order for *E. coli* cells to use lactose: lactose must be transported to the inside of the cell, and once inside it must be hydrolyzed to glucose and galactose at a sufficient rate to permit growth (that minimum rate is about 5 nmol hydrolyzed per minute per milligram of soluble cell protein (HALL and CLARKE 1977). In this paper I show how a series of mutations in the *ebg* operon act in concert to permit synthesis of all proteins for lactose transport and hydrolysis in response to the presence of lactose in the environment.

The wild-type allele is designated *ebgA*^{o} [the "o" standing for original (HALL and CLARKE 1977)]; and its product, ebg^{o} enzyme, hydrolyzes lactose very ineffectively [$V_{max} = 620$ nmol min^{-1} mg^{-1}, $K_m = 150$ mM lactose (HALL 1981)]. That represents about 0.05% of the efficiency of *lacZ* β-galactosidase under physiological conditions (HALL and CLARKE 1977; HUBER, KURZ and WALLENFELLS 1976), and even constitutive strains that synthesize 5% of their soluble protein as ebg^{o} enzyme cannot grow on lactose (HALL and CLARKE 1977; HALL 1976).

There are two sites, about 1 kilobase apart, within *ebgA* where spontaneous mutations can alter ebg enzyme so that it hydrolyzes lactose effectively (HALL and ZUZEL 1980a). A single mutation in the Class I site alters the enzyme so that it hydrolyzes lactose effectively but not lactulose (4-O-β-D-galactopyranosyl-D-fructose) or galactosyl-arabinose (3-O-β-D-galactopyranosyl-D-arabinose).

A single mutation in the Class II site within *ebgA* also alters the enzyme so that it hydrolyzes lactose effectively, but the properties of Class II enzymes are quite distinct from those of Class I enzymes. In addition to lactose, Class II enzymes hydrolyze lactulose effectively (HALL 1978a, 1981; HALL and ZUZEL 1980a).

When *ebgA* carries mutations in both the Class I and the Class II sites simultaneously the resulting double mutant enzyme, designated Class IV, acquires a new function: it hydrolyzes galactosyl-arabinose effectively. The properties of Class IV enzymes are quite distinct from those of either Class I or Class II enzymes, the most dramatic difference being that the K_ms for all three β-galactosides are five to ten fold lower than those of Class I or Class II enzymes (HALL 1981).

The mutant alleles specifying Class I, Class II, or Class IV ebg enzymes are all designated *ebgA*$^{+}$, and are distinguished by allele numbers. *EbgA*$^{+}$ mutants cannot necessarily utilize lactose. Their ability to do so depends entirely upon the state of the *ebgR* gene.

The wild-type allele, *ebgR*$^{+}$, specifies an extremely effective repressor that permits the synthesis of only three to five molecules of ebg enzyme per cell (HALL and CLARKE 1977; HALL 1978b). The repressor does not respond to isopropyl-thio-β-D-galactoside (IPTG) or to thio-β-methyl-galactoside (TMG), although both are powerful gratuitous inducers of the *lac* operon. When grown in the presence of lactose, synthesis of ebg enzyme is induced 100-fold (HALL and CLARKE 1977). Other β-galactoside sugars, such as lactulose and galactosyl-arabinose, are not recognized as inducers by the wild-type repressor. Even the

most efficient ebg enzyme, when synthesized at only 100-fold above the basal level, does not permit growth on lactose (HALL and CLARKE 1977). A mutation in the *ebgR* gene is required to increase the level of expression of the *ebgA* gene, and thus to synthesize enough activity for growth on lactose. One class of such mutations is *ebgR*$^-$, which results in defective repressor and leads to constitutive ebg enzyme synthesis about 2000-fold above the basal level (HALL and CLARKE 1977). Constitutive expression of the *ebg* operon uses about 3% of the total protein synthesis capacity of the cell (HALL and ZUZEL 1980b), an enormous burden under conditions where the ebg proteins are not required. Constitutive synthesis, therefore, does not represent an appropriate response of the cell to its environment.

A more economical solution to the problem of synthesizing enough ebg enzyme is represented by *ebgR*$^+$*L* mutations. Repressor specified by the *ebgR-105*$^+$*L* allele allows the same low basal level of synthesis as does wild-type repressor, but it is induced 460-fold by lactulose, 800-fold by galactosyl-arabinose, and 1000-fold by lactose (HALL 1978b and see Table 2). *EbgR*$^+$*L ebgA*$^+$ (Class II) strains can utilize both lactose and lactulose as sole carbon and energy sources (HALL 1978b).

Lactose utilization by such *ebgA*$^+$ strains is subject to an additional pair of constraints: (a) the *lacZ* deletion strain must possess a functional lactose permease (*lacY* gene product); and (b) unless the *lac* operon is itself expressed constitutively an artificial inducer of the *lac* operon, such as IPTG, must be included in the medium. This is because lactose itself is not an inducer of the *lac* operon (JOBE and BOURGEOIS 1972; BARKELY *et al.* 1975), but is converted to allolactose (6-O-β-galactopyranosyl-D-glucose), the natural inducer of the *lac* operon (JOBE and BOURGEOIS 1972), by the transgalactosylase activity of *lacZ* β-galactosidase. Ebg$^+$ enzymes do not convert lactose into allolactose (HALL and HARTL 1974; ROLSETH, FRIED and HALL 1980). Our "evolved" strains are therefore capable of utilizing lactose only under very artificial conditions: either inclusion of IPTG in the medium, or constitutive expression of the *lac* operon. Those strains do not meet the criterion of making all the proteins necessary for lactose utilization in response to the presence of lactose in the environment.

We recently described a mutant ebg$^+$ enzyme that does convert lactose into an inducer of the *lac* operon (ROLSETH, FRIED and HALL 1980), and I have since identified that inducer as allolactose (HALL 1982). Strains that synthesize that mutant enzyme constitutively grow on lactose without the presence of IPTG in the medium. The strains carrying the allele *ebgA205*$^+$, which specifies the enzyme capable of converting lactose into allolactose, were positive for galactosyl-arabinose utilization (ROLSETH, FRIED and HALL 1980), a property unique to strains expressing Class IV *ebgA* alleles (HALL 1981; HALL and ZUZEL 1980a). When ebg enzyme from one of those strains (SJ60) was purified and characterized it was indistinguishable from other Class IV enzymes based upon kinetic analysis utilizing ten substrates (HALL 1981). These observations suggested that all Class IV ebg enzymes might convert lactose into inducer.

Using MACCONKEY-lactose indicator plates, I screened seven Class I, twenty-two Class II, and twelve Class IV strains for IPTG independent lactose utilization. All of the Class IV strains were lactose positive without IPTG, while no Class I and no Class II strains were lactose positive without IPTG. *In vitro* studies with purified ebg enzymes confirmed that only Class IV ebg enzymes synthesize allolactose at a significant rate, a rate that is about 100-fold greater than that of Class I or Class II enzymes (HALL 1982). Taken together with the earlier study (ROLSETH, FRIED and HALL 1980), and with studies demonstrating that the properties of ebg enzymes are very homogeneous within a class (HALL 1981), it seems likely that synthesis of a Class IV ebg enzyme is both a necessary and a sufficient condition for *in vivo* synthesis of allolactose by a *lacZ* (deletion) Y^+ strain of *E. coli*.

Because they synthesized ebg enzyme constitutively, these strains also failed to meet the criteria for having fully evolved ebg enzyme systems.

Strain 5A1 synthesizes the Class II enzyme designated "C" (HALL 1976, 1981) under control of the wild-type *ebg* repressor, and is therefore lactose negative even in the presence of IPTG (HALL and CLARKE 1977, HALL 1978b). Its derivative, strain 5A103, synthesizes the same enzyme under control of the evolved *ebgR*$^+$*L* repressor, and is therefore lactose positive in the presence of IPTG (HALL 1978b and see Table 1).

Based upon detailed biochemical studies of the various evolved ebg enzymes (HALL 1981, 1982), and upon studies of the evolved repressors (HALL, 1978b), it was now possible to predict the evolutionary consequences of a specific perturbation of the environment. I predicted that when galactosyl-arabinose is the sole available carbon source in the environment of strain 5A103, galactosyl-arabinose utilizing mutants that arose would possess a fully integrated regulatory system that controlled expression of both the *ebg* β-galactosidase and the *lac* permease in response to the presence of lactose in the environment.

MATERIALS AND METHODS

Bacterial strains and culture conditions: All strains are *Escherichia coli* K12, and carry *lacZ* deletion W4680. Genotypes are given in Table 1. Minimal medium was previously described (HALL and HARTL 1974). Cultures were grown at 37° with constant aeration.

Enzyme assays: Ebg enzyme activity was assayed in crude extracts using o-nitrophenyl-β-D-galactoside (ONPG) as a substrate as previously described (HALL and HARTL 1974). Units are nanomoles of ONPG hydrolyzed per minute. *Lac* permease activity was determined from the

TABLE 1

Genotypes of E. coli *K-12 strains used*

Strain	Genotype						
DS4680A	HfrC	*spc*	*ebgA*^{o}		*ebgR*+		
5A1	HfrC	*spc*	*ebgA51*		*ebgR*+		
5A103	HfrC	*spc*	*ebgA51*		*ebgR105*+*L*		
5A1032	HfrC	*spc*	*ebgA109*		*ebgR105*+*L*		
SJ7	F-	*rpsL*	*tolC*	*argG*	*metC*	*ebgA*^{o}	*ebgR*+

rate of ^{14}C-thiomethyl-galactoside (TMG) uptake as previously described (ROLSETH, FRIED and HALL 1980). Units are micromoles of TMG accumulated per minute at 15°.

Growth rate determinations: Cultures were grown overnight in 0.03% sodium succinate minimal medium plus any additions indicated under "pregrowth conditions." That concentration of succinate limits cell density to about 10^8 per ml, and cells enter exponential growth within a few minutes of the addition of a metabolizable carbon source. Cells were washed and resuspended in minimal medium containing the indicated concentration of lactose, and the turbidity of the culture growing at 37° was monitored at 600 nm in a Gilford Model 250 spectrophotometer. Growth rates are given as the first order growth rate constant in reciprocal hours ± the 95% confidence limits as determined from a minimum of four independent cultures.

RESULTS

Selection of the fully evolved strain: A spontaneous galactosyl-arabinose utilizing mutant was selected by plating strain 5A103 onto galactosyl-arabinose minimal medium containing 0.2 mM IPTG. Colonies that arose were purified by restreaking them onto MACCONKEY galactosyl-arabinose indicator plates containing 0.2 mM IPTG. One of these galactosyl-arabinose positive isolates was designated strain 5A1032. Strain 5A1032 was positive on MACCONKEY indicator plates containing either lactose+IPTG or lactulose+IPTG. As expected, strain 5A1032 was positive on MACCONKEY-lactose plates without IPTG, indicating that it was converting lactose into an inducer of the lac operon.

Characterization of the fully evolved strain: Strain 5A1032 was mated with the *tolC rpsL ebgA*^{o} *ebgR*$^+$ strain SJ7, and *rpsL tolC*$^+$ recombinants were selected by plating the mating mixture onto MACCONKEY-lactose-IPTG medium containing 300 mg per liter streptomycin sulfate (MACCONKEY medium kills *tolC* cells). Of 512 colonies 349 were *ebgA*$^+$ (phenotypically lactose positive), giving 32% recombination between *ebgA* and *tolC*. This value is in good agreement with previous studies (HALL and HARTL 1975; HALL and ZUZEL 1980a). Of 100 *ebgA*$^+$ colonies tested, all were lactose positive in the absence of IPTG. The IPTG independent lactose positive phenotype of strain 5A1032 is thus the consequence of a mutation in *ebgA*, and the allele present in strain 5A1032 is designated *ebgA*$^+$*109*.

To confirm that strain 5A1032 synthesized a Class IV ebg enzyme, I partially purified its ebg enzyme, and determined the K_m for O-nitrophenyl-β-D-galactoside (ONPG) and for O-nitrophenyl-β-D-fucoside (ONPF). The K_m for ONPG was 0.045 ± 0.0015mM, and for ONPF was 3.1 ± 0.16 mM. Only Class IV ebg enzymes have K_m values in those ranges (HALL 1981). Because the enzyme from 5A1032 was not pure the V_{max} values obtained are only relative, not absolute. However, the ratio of the observed V_{max} (ONPG) to V_{max} (ONPF) was 2.8, compared with a ratio of 2.4 for purified Class IV enzymes (HALL 1981). By these criteria 5A1032 synthesized a typical Class IV ebg enzyme.

I have usually characterized the evolved strains in terms of their growth rates in minimal medium containing 0.1% (w/v) β-galactoside sugar and 0.2 mM IPTG. As expected the parental strain 5A103 grew on 0.1% lactose only if pregrown in the presence of IPTG (Table 2). Growth was monitored for only one generation. Significant dilution of the lactose permease requires two generations of growth in the absence of IPTG (HALL and HARTL 1974). Since 5A-

TABLE 2

Growth rates under various conditions

Pre-growth condition	Growth condition	Growth rates, hr^{-1} 5A103	5A1032
No addition	0.1% lactose	<0.01	<0.01
0.1% lactose + 0.2 mM IPTG	0.1% lactose	0.124 ± 0.001	0.176 ± 0.001
0.8% lactose	0.8% lactose	<0.01	0.168 ± 0.004
0.8% lactose	0.1% lactose	not done	0.072 ± 0.011

Cultures were grown overnight in 0.03% sodium succinate minimal medium plus any additions indicated under "pregrowth conditions."

1032 colonies were clearly positive on MacConkey lactose medium without IPTG, I was surprised to find that strain 5A1032 also failed to grow on 0.1% lactose when pregrown in the presence of 0.1% lactose without IPTG. Since MacConkey medium contains 1.0% lactose, I measured the growth of strain 5A1032 at various lactose concentrations in the absence of IPTG, and found that the threshold concentration for growth was 0.2%, with the maximal rate obtained at 0.8% lactose (data not shown).

Table 2 shows that when induced with 0.8% lactose, strain 5A1032 grew well in 0.8% lactose, and grew much more slowly in 0.1% lactose. By contrast, the parental strain, 5A103, failed to grow on any concentration of lactose unless induced with IPTG. If induced with 0.8% lactose, or shifted from 0.8% lactose, strain 5A1032 grew slowly in 0.1% lactose; however if innoculated directly from succinate minimal into 0.1% lactose minimal medium strain 5A1032 failed to grow. To understand this behavior, and to understand the difference between strains 5A103 and 5A1032, I measured the levels of ebg enzyme and of lactose permease in cells grown under various conditions. In strain 5A103, ebg enzyme synthesis was induced about 100-fold by 0.1% lactose, about 600-fold by 0.8% lactose, and in agreement with earlier results (Hall 1978b) about 1000-fold by 0.1% lactose + IPTG (Table 3). The induction levels are similar for strain 5A1032 except that it is induced only 350-fold by 0.8% lactose. The lower levels of activity in extracts of strain 5A1032 simply reflect the fact that

TABLE 3

Egb enzyme and Lac Permease activities

Growth medium	ebg enzyme activity (Units mg^{-1} cell protein)		Lac permease activity (Units g^{-1} dry weight)	
	5A103	5A1032	5A103	5A1032
Succinate	0.232 ± 0.062	0.083 ± 0.003	<0.3	<0.3
Succinate + 0.1% lactose	32.2 ± 1.2	7.5 ± 0.25	<0.3	0.4
Succinate + 0.8% lactose	139 ± 11	29 ± 1.3	<0.3	2.7
Succinate + 0.1% lactose + 0.2M IPTG	234 ± 27	74 ± 13	8.3	8.5
0.8% lactose	ND*	31 ± 0.8	ND	2.3
Succinate + 0.2M IPTG	0.26 ± 0.054	0.08 ± 0.004	7.9	9.0

Cultures were harvested in late exponential phase.
* ND = not determined.

Class IV enzymes are typically about one third as active toward 5 mM ONPG as are Class II enzymes (HALL 1981). The failure of strain 5A103 to grow on 0.8% lactose can not, then, be attributed to a failure to synthesize sufficient ebg enzyme for growth.

Table 3 shows that lactose failed to induce synthesis of the lactose permease in strain 5A103, but that 0.8% lactose yielded about 30% of the fully induced level of lac permease in strain 5A1032. The failure of strain 5A1032 to grow when shifted from succinate to 0.1% lactose minimal medium is explained by the low levels of both ebg enzyme and lactose permease induced by 0.1% lactose.

DISCUSSION

We began with a *lacZ* deletion strain, DS4680A, that could not grow on lactose because (a) it lacked an enzyme that could hydrolyze lactose effectively, (b) it had an *ebg* repressor that was insufficiently sensitive to lactose as an inducer, and (c) synthesis of the lactose permease was not inducible by lactose (Figure 1).

The first evolutionary step was a mutation in the *ebgA* gene that allowed ebg enzyme to hydrolyze lactose effectively. The resulting strain, 5A1, was isolated by chance during selection for lactose utilization. It was isolated from a papilla on the surface of a colony of strain DS4680A growing on a lactose-tetrazolium-IPTG plate as first described by CAMPBELL, LENGYEL and LANGRIDGE (1973).

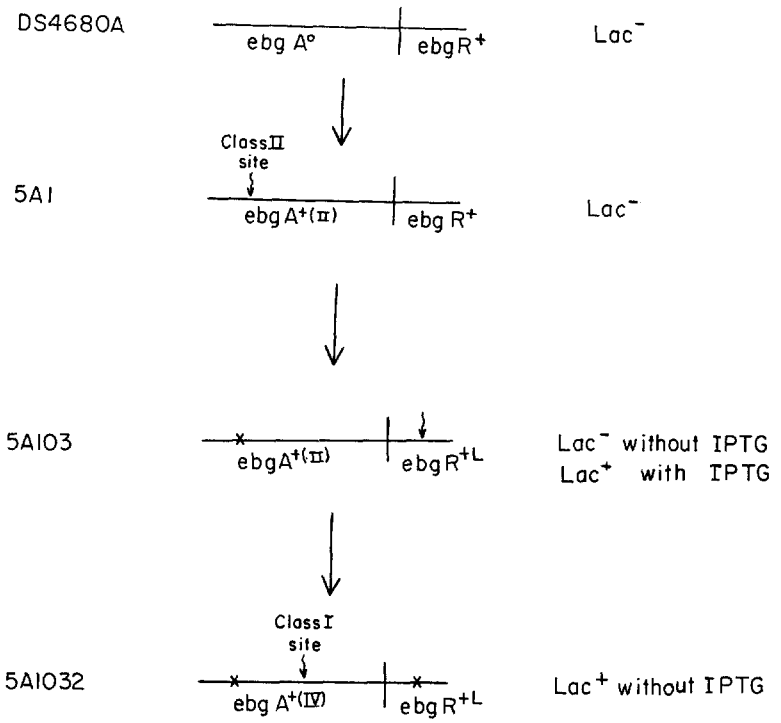

FIGURE 1.—Evolutionary pathway.

Although strain 5A1 is lactose negative it formed faintly pink colonies on the sensitive fermentation indicator MacConkey-Lactose-IPTG agar. The presence of 5A1 cells in the papilla suggests that the Class II *ebgA* mutation may have conferred a slight selective advantage within the colony as the other nutrients in the medium were depleted. On the other hand, "false" papillae—those which yield no cells with a detectable change in the lactose phenotype—often arise on the surface of such colonies. Those papillae presumably result from adaptations to elements other than lactose utilization. Strain 5A1 may simply have been a neutral mutation within such a false papilla.

A second spontaneous mutation, in *ebgR*, made the *ebg* repressor hypersensitive to lactose as an inducer. The resulting strain, 5A103, could use lactose, but only if synthesis of the lactose permease was artificially induced by IPTG. The second mutation may also increase the fitness of the strain in the absence of IPTG. While unable to grow without IPTG, strain 5A103 survives longer than does its parent, strain 5A1, in lactose minimal medium without IPTG.

The third evolutionary step was the result of a second mutation in *ebgA* that altered ebg enzyme so that it could convert lactose into an inducer of the lactose permease. The resulting strain, 5A1032, is capable of growth on lactose without artificial inducers. The *ebg* operon in strain 5A1032 is very nearly perfectly evolved for lactose utilization: in the absence of lactose neither ebg enzyme nor the lactose permease is synthesized above its normal basal level. In the presence of lactose, synthesis of ebg enzyme is induced and that enzyme in turn converts lactose into a *lac* operon inducer and lactose permease is synthesized.

This solution to the evolutionary problem of acquiring a new function (lactose utilization) is particularly interesting in that the *ebg* operon in strain 5A1032 controls not only its own expression, but the expression of another operon required for the lactose utilization pathway to function. These results are an experimental verification of Wilson's hypothesis that most significant evolutionary changes arise from regulatory mutations that result in the co-ordinate control of sets of genes that had previously been regulated separately (Wilson 1977).

The mutations described above have been deliberately selected in the laboratory as a model for the way biochemical pathways might evolve so that they are appropriately organized with respect to both the cell and its environment. It is reasonable to ask whether this model might have any relationship to the real world outside the laboratory. If it is assumed that the selection is strictly for lactose utilization, then a growth advantage exists only when all three mutations are present simultaneously. Any one of the mutations alone could well be neutral (it is unlikely that any would be disadvantageous); but neutral mutations do enter populations by random chance events, and are fixed by a chance process termed genetic drift. In the background of a neutral *ebgA* or *ebgR* mutation, a second mutation in the alternative gene increases the fitness slightly by increasing the survival of the double mutant in the presence of lactose. Selection could thus increase the frequency of double mutants in the population. The third mutation, occurring in the background of the double mutant, is clearly strongly advantageous.

An alternative evolutionary route could involve intragenic recombination. We have previously shown that Class IV *ebgA* alleles can arise via recombination between Class I and Class II alleles (HALL and ZUZEL 1980a). A cross between two neutral mutants could result in a slightly advantageous double mutant; similarly a cross between a neutral single mutant and a double mutant could result in the strongly advantageous triple mutant.

We can fairly conclude that evolution of a well organized, integrated biochemical pathway can involve several mutations, not all of which must be individually advantageous. Likewise, this study provides a concrete example of a way in which neutral mutations can contribute not only to the genetic diversity of populations, but can also contribute to the adaptive potential of organisms.

Supported by N.I.H. Grant AI14766. The author is a recipient of Research Career Development Award 1 KO4 AI 00366 from the N.I.A.I.D.

LITERATURE CITED

BACHMANN, B. J. and K. B. Low, 1980 Linkage map of *Escherichia coli* K12, edition 6. Microbiol. Rev. **44**: 1–56.

BARKLEY, M. D., A. D. RIGGS, A. JOBE and S. BOURGEOIS, 1975 Interaction of effecting ligands with lac repressor and repressor-operator complex. Biochemistry **14**: 1700–1712.

CAMPBELL, J. H., J. A. LENGYEL and J. LANGRIDGE, 1973 Evolution of a second gene for β-galactosidase in *Escherichia coli*. Proc. Nat. Acad. Sci. U.S.A. **70**: 1841–1845.

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. ——, 1978a Experimental evolution of a new enzymatic function. II. Evolution of multiple functions for EBG enzyme in *E. coli*. Genetics **89**: 453–465. ——, 1978b Regulation of newly evolved enzymes. IV. Directed evolution of the ebg repressor. Genetics **90**: 673–691. ——, 1981 Changes in the substrate specificities of an enzyme during directed evolution of new functions. Biochemistry **20**: 4042–4049. ——, 1982 Transgalactosylation activity of EBG β-galactosidase synthesizes allolactose from lactose. J. Bacteriol. **150**: 132–140.

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.

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. ——, 1975 Regulation of newly evolved enzymes. II. The ebg repressor. Genetics **81**: 427–435.

HALL, B. G. and T. ZUZEL, 1980a Evolution of a new enzymatic function by recombination within a gene. Proc. Nat. Acad. Sci. U.S.A. **77**: 3529–3533. ——, 1980b The ebg operon consists of at least two genes. J. Bacteriol. **144**: 1208–1211.

HUBER, R. E., G. KURZ and K. WALLENFELS, 1976 A quantitation of the factors which affect the hydrolase and transgalactosylase activities of β-galactosidase (*E. coli*) on lactose. Biochemistry **15**: 1994–2001.

JOBE, A. and S. BOURGEOIS, 1972 Lac repressor-operator interaction. VI. The natural inducer of the lac operon. J. Mol. Biol. **69**: 397–408.

ROLSETH, S. J., V. A. FRIED and B. G. HALL, 1980 A mutant ebg enzyme which converts lactose into an inducer of the lac operon. J. Bacteriol. **142**: 1036–1039.

WILSON, A., 1977 Gene regulation in evolution, pp. 225–234. In: *Molecular Evolution*. Edited by F. AYALA. Sinaeur Assoc.

Corresponding editor: D. L. HARTL