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Enzyme Activity and Fitness: Evolution in Solution

Daniel E. Dykhuizen and Antony M. Dean

Natural selection should be studied as an end in itself, and this requires rigorous experimental tests of theoretical models linking molecular phenotypes to differences in fitness. We describe the experimental verification of one such model and thereby demonstrate that the causal relations between genotype and fitness need not be as hopelessly complex as many have assumed. The model uses metabolic control theory to link enzyme activity to metabolic flux and then assumes that fitness is proportional to flux. The model was tested using the pathway for the uptake and metabolism of growth-rate-limiting concentrations of lactose in E. coli inhabiting chemostats. Many of the properties expected of natural selection are manifest in this system.

Only two questions can be asked about natural selection: what are its causes and what are its effects? The laws of population and quantitative genetics describe the effects of natural selection and are usually invoked when demonstrating the action of natural selection in nature[1]. These laws are called 'consequence laws', to distinguish them from the 'source laws' that determine the causes of natural selection[2]. Attempts to delineate these source laws all too often rely on consistency arguments – the 'just-so' stories[3]. Hence, our understanding of evolution remains incomplete. Can the source laws, the 'hows and whys' of natural selection, be understood?

Some have assumed that the source laws will be very complicated[4–6]. The same genotype in different environments can give rise to different phenotypes (plasticity), while different genotypes in the same or different environments can yield the same phenotypes (canalization). Clearly, there are potentially many ways to map genotypes onto phenotypes and vice versa. Likewise, the interaction between phenotype and environment may also generate complex mappings of phenotype onto fitness. Important environmental properties that interact with the genotype to produce phenotype (the epigenetic environment) may often be different from those that actually cause selection (the selective environment). Unfortunately, the components in the epigenetic and selective environments are generally unknown, so the problem of understanding the source laws seems hopeless.

These potential complexities have not deterred others from searching for source laws. Starting with the seminal paper of Bates[7], where he provided the first independent evidence of natural selection, studies of mimicry and visual predation have continued to provide much-needed data on the mechanisms of natural selection[8,9]. Studies of 'life history strategies', particularly mating and reproduction, have given many additional insights[10,11]. Although viability, mating and reproduction are obviously closely related to fitness, the full dynamics of a selective process and its various constraints cannot be fully understood unless a causal chain, from genetic differences to fitness differences, is delineated.

During this century, the development of biochemistry and molecular genetics led to the conclusion that natural selection is ultimately a consequence of variation at the molecular level. The discovery of widespread natural variation in enzymes, as visualized by protein electrophoresis, allowed the possibility of investigating the source laws of natural selection using

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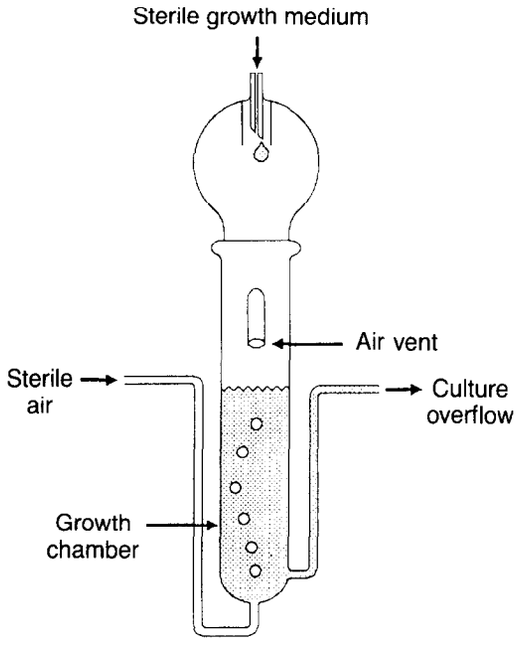

Fig. 1. A simple chemostat. A peristaltic pump feeds fresh sterile medium into the growth chamber. An overflow siphon removes the spent medium and cells so that a constant volume is maintained. Sterile air is forced through the culture to ensure an adequate supply of oxygen and thorough mixing in the growth chamber.

molecular phenotypes rather than morphological or behavioral phenotypes. The many-to-many mappings of genotype to phenotype are often reduced to a simple one-to-one mapping of gene to enzyme function. However, the problem of relating enzyme activity to fitness remained.

Clark's postulates[12] outline a procedure for studying allozyme polymorphisms in natural populations: show how the allozymes are biochemically different; postulate a selective agent; test it by experimentally manipulating the environment; and re-examine natural populations, seeking a comprehensive explanation for observed patterns. Many of the studies attempting to carry out this program have been reviewed[13,14] and criticized[15]. This program has proved to be enormously difficult[14], and no more than a handful of studies have demonstrated clearly that natural selection discriminates between allelic variants. Uncertainties arise at three levels: the possibility that the selection seen is due to linked loci; the conversion from phenotypic variation at the molecular level to variation in fitness; and the nature of the selective environment. These problems make it very difficult to draw rigorous conclusions, even when the proposed causes of natural selection are highly plausible. Thus, the source laws remain elusive.

Simplification of the problem

The problem is considerably simplified by accepting that the study of natural selection need not be confined to natural variation, natural populations or natural environments, and can be profitably investigated in the laboratory using controlled experiments. In contrast to artificial selection, where the investigator directly establishes relative fitness by differentially removing phenotypes from the population, natural selection is investigated whenever the fitnesses of the phenotypes are established by the environment, even if that environment is created by the investigator. Moreover, an ability to manipulate the laboratory environment enables the source of selection to be unambiguously identified.

One objection to laboratory studies of natural selection is that the environment may not be sufficiently complex to be realistic. But the very strength of laboratory experiments is the deliberate reduction of complexity to a point where an understanding of the process of natural selection is possible. Once the basic principles are understood, they can be combined in various ways to explain more complex situations. Such simplifications are a recognized method of science. Mendel studied the heredity of discrete traits in a single simplified system – garden peas – and yet his simple laws provide quantitative genetics with a basis for understanding the inheritance of continuous traits. Similarly, we expect results from molecular studies, such as the one reviewed here, to provide the necessary framework to explain many other adaptations that evolutionary biologists are interested in, such as peacocks' tails and pretty snails.

The experimental system

A simple experimental system for investigating the source laws is the bacterium *Escherichia coli* inhabiting a chemostat. Here, we give a brief account of the salient features of this system; more extended descriptions are available[16,17].

The single, genetically haploid cells of *E. coli* reproduce clonally by binary fission to produce nearly identical daughter cells. Relative fitness can be determined by growing two strains of *E. coli* in competition. These strains are genetically identical except for those alleles that have been deliberately introduced into the common genetic background by the investigator. The isogenicity is confirmed by control experiments[18], and the absence of differences in the genetic backgrounds ensures that any selection seen is caused by the introduced alleles.

Figure 1 illustrates a simple chemostat, a device that enables a microbial culture to be maintained in permanent exponential growth in a constant and homogeneous environment. The growth medium is a chemically defined salt solution supplemented with a source of carbon and energy. Concentrations of the components of the fresh medium entering the growth chamber are such that only one is exhausted by the culture. This one (the limiting nutrient) is usually a sugar, such as lactose or glucose, and all the other nutrients present are non-substitutable[19]. When substitutable resources are used (for example both lactose and glucose), they are both considered to be the same limiting nutrient, and the environment is more complex.

The demography of bacteria in chemostats is also simple. Under the conditions used, bacterial cells do not die and their removal from the growth chamber by outflow is random with respect to natural selection. Hence, only scramble competition between strains for the limiting nutrient can cause a difference in growth rate. From this difference in growth rate, natural selection is estimated.

Chemostat experiments are sufficiently reproducible that differences in growth rates as small as 0.5% per generation are readily detected in replicate experiments (see Fig. 2). Thus the selection coefficients can be estimated in chemostat experiments far more accurately than is usually possible with more complex organisms in natural environments. This accuracy makes possible quantitative tests of theories of natural selection.

The metabolic pathway

Any attempt to describe fitness in terms of the entire chemistry of a single living bacterial cell in its immediate environment would present a problem of such staggering

complexity that it would be doomed to failure. However, metabolism can often be considered as a collection of quasi-independent subsystems. This property is implicitly recognized when analysing many simple mendelian traits. The trick, then, is to find a relatively simple subsystem whose behavior is, nevertheless, directly related to fitness.

The metabolism of lactose by *E. coli* is such a subsystem. This pathway (Fig. 3) has three steps: diffusion of lactose through the porin pores of the cell wall, active uptake of lactose by the lactose permease and, finally, irreversible hydrolysis by β-galactosidase. The permease and β-galactosidase are encoded by the lactose operon, which has served as a paradigm for the regulation of gene expression[20]. The genes encoding the porin pores are not part of the operon.

The prediction

In a bacterial chemostat, any advantage in capturing or processing the limiting nutrient will be reflected in an increased growth rate. This process is described by the steady-state model of lactose metabolism presented in Box 1. The model relates the *in vivo* rate of lactose hydrolysis (the flux) to the activities at the three steps. We assume that mutations not affecting enzyme activity are selectively neutral.

The metabolic model requires that each step in the pathway is unregulated and unsaturated. The use of deregulated operons ensures that the enzyme activities remain constant among all experiments, while the intense competition for growth-rate-limiting concentrations of lactose in the chemostat ensures that each enzyme is unsaturated. The rest of cell metabolism is unable to affect the lactose flux (Box 1), and can be viewed as a sink that soaks up the output from this pathway. Hence, these three steps – diffusion, active uptake and hydrolysis – should constitute an isolated subsystem amenable to experimental analysis.

If central metabolism behaves as a sink, then the lactose flux should be directly proportional to the growth rate. If relative fitness is nothing more than the ratio of the growth rates of the two competing strains, then

$$w = J_t/J_c$$

where w is the relative fitness, J is the lactose flux (defined by Eqn 4 in Box 1), and the subscripts t and c refer, respectively, to the test and control strains.

The test

The simplifying assumptions required to construct the above equation may not be justified, and the relations between fitness and enzyme function could be more complicated than has been assumed. Thus, an experimental verification of the proposed relationship was absolutely necessary. The model was tested[21–23] by determining the activities and fitnesses conferred by various alleles of the β-galactosidase and the permease, from both natural polymorphisms and laboratory mutants. The diffusion constant associated with the porin pores of the cell wall and the apparent equilibrium constant for lactose uptake were also determined[23]. This allowed the relative flux to be calculated using the enzyme activity measurements and Eqn 4 of Box 1.

As predicted, fitness is directly proportional to flux (Fig. 4). From Eqn 4 in Box 1, the relationship between fitness and the activity of a single enzyme, with all other activities held constant, is predicted to be a rectangular hyperbola. This is confirmed for the permease and β-galactosidase (Fig. 5). Thus the fitness of a new mutant can be predicted solely from a knowledge of its kinetic properties.

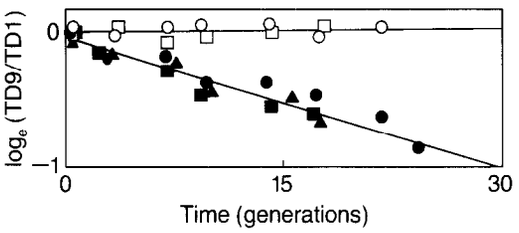

Fig. 2. An example of natural selection. In this plot, the $\log_e$ of the ratio of the frequencies of the two competing *E. coli* strains (TD9 and TD1) is plotted against time (data from Ref. 23). The gradient of the slope is the selection coefficient (s). Filled symbols: three replicate experiments with lactose as the limiting nutrient. Open symbols: two replicate experiments using the same strains with glucose as the limiting nutrient. Since the replicate experiments within each environment were statistically homogeneous, the selection coefficients were pooled. On glucose, the pooled $s = 0.0045 \pm 0.0078$ and is not significantly different from zero. On lactose, the pooled $s = -0.0334 \pm 0.0023$, which is highly significantly different from zero; this selection is caused by differences encoded by the lactose operon. The y-axis intercepts were normalized so that the pooled regression lines pass through the origin. Fitness, $w = 1 + s$.

The target of natural selection

In the lactose-limited chemostat, an increased lactose flux is selectively advantageous (Fig. 4). If the cells evolve in a chemostat, which steps will natural selection target to further increase this flux? Figure 5 illustrates the responses in fitness caused by modulating the activities at each step while holding the other two activities constant at the values of the control strain. Dramatic increases in β-galactosidase activity will hardly affect fitness. In contrast, increases in the activities of either the permease or the porin pores will

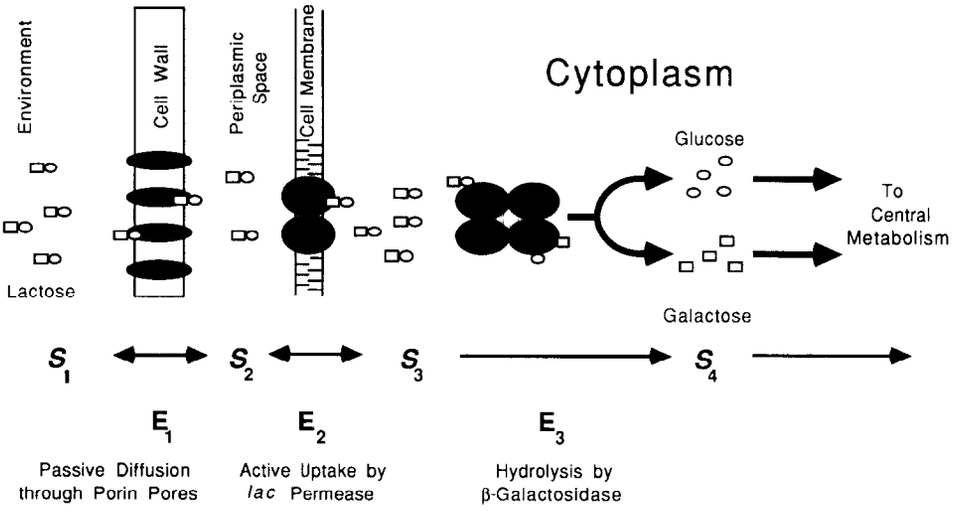

Fig. 3. The lactose pathway in *E. coli*. Lactose in the medium (S_1), passively diffuses through the porin pores (E_1) of the cell wall into the periplasmic space (S_2). The lactose permease (E_2) actively transports this periplasmic lactose across the cell membrane into the cytoplasm (S_3), where it is irreversibly hydrolysed by β-galactosidase (E_3). The pathway products, glucose and galactose (S_4), then enter central metabolism.

Box 1. A simple flux: the steady-state model of lactose metabolism

The relations between enzyme activity and metabolism are described by the two closely related theories of metabolic control[29] and biochemical systems analysis[30]. These sophisticated theories have been developed to the extent that virtually any complex pathway, with branches, cycles, cascades, enzyme–enzyme interactions and various regulatory systems at both the genetic and metabolic levels, can be described. Here, we need only consider a simple linear metabolic pathway at steady state, with each enzyme catalysing a reversible monomolecular reaction. The velocity of the ith reaction (v_i), catalysed by enzyme i, is described by the Briggs–Haldane relation

$$v_i = \frac{(V^f_{max,i}/K^f_{m,i})(S_i - S_{i+1}/K_{eq,i,i+1})}{1 + S_i/K^f_{m,i} + S_{i+1}/K^r_{m,i}} \quad (1)$$

where $V^f_{max,i}$ is the maximum rate of the forward reaction, $K^f_{m,i}$ and $K^r_{m,i}$ are the half-saturation constants of the forward and reverse reactions, and S_i and S_{i+1} are the concentrations of the substrate and product of the enzyme. The thermodynamic equilibrium constant, $K_{eq,i,i+1}$, is a function of the change in Gibbs free energy of the reaction, the gas constant and the temperature.

An enzyme is unsaturated if the concentrations of substrate and product are low in relation to their respective half-saturation constants. Under these conditions, the denominator is approximately equal to one and Eqn 1 reduces to

$$v_i = E_i(S_i - S_{i+1}/K_{eq,i,i+1}) \quad (2)$$

where $E_i = V^f_{max,i}/K^f_{m,i}$ is called enzyme activity.

Consider a simple linear metabolic pathway of n steps each catalysed by reactions with the properties described by Eqn 2. At steady state, the rate of each reaction will be the same and equal to the flux, J, of metabolites through the pathway ($v_i = J$, for $i = 1,2,3 \ldots n$). Thus we have a set of simultaneous equations which, after much tedious algebra, can be solved to yield

$$J = \frac{S_1 - S_{n+1}/K_{eq.1,n+1}}{\sum_{i=1}^{n} 1/(E_i K_{eq.1,i})} \quad (3)$$

where $K_{eq.1,i}$, the equilibrium constant from the 1st to the ith substrate, is equal to the product of the intervening equilibrium constants.

The flux through the lactose pathway (Fig. 3) can be found from Eqn 3. The hydrolysis of lactose is irreversible and hence its equilibrium constant, $K_{eq.3,4}$, is very large. The activities of enzymes downstream from the β-galactosidase step must be multiplied by $K_{eq.1,i}$ values that contain the factor $K_{eq.3,4}$. Hence the value of each $1/E_iK_{eq.1,i}$ downstream from the hydrolysis step is zero. Thus

$$J = \frac{S_1}{\frac{1}{E_1} + \frac{1}{E_2 K_{eq.1,2}} + \frac{1}{E_3 K_{eq.1,3}}} \quad (4)$$

where S_1 is the concentration of lactose in the external medium and E_1, E_2 and E_3 are the activities of the porin pores, the lactose permease and the β-galactosidase respectively. Note that no step is rate limiting because the flux is a function of the activities at all three steps. Notice, too, that the irreversible hydrolysis of lactose helped define this subsystem of metabolism.

Equation 4 is an approximation for unsaturated pathways. If the same derivation is made allowing for saturation[23], and recognizing that the Michaelis constant, K_m, for the exit of lactose from the cell is very large, then

$$J = \frac{S_1}{\frac{1}{D} + \frac{K_{m.2}+S_2}{V_{max.2}K_{eq.1,2}} + \frac{K_{m.3}+S_3}{V_{max.3}K_{eq.1,3}}} \quad (5)$$

where E_1 is now represented by D, the unsaturatable diffusion constant of the porin pores.

cause marked increases in fitness. Natural selection will target these two steps as *E. coli* adapts to this lactose-limited environment.

Although the evolutionary action appears to be concentrated at the cell wall and membrane, the β-galactosidase is of significant interest, because it provides an example of how metabolic architecture can place a constraint on the evolution of enzyme activity. Naturally occurring allozymes, even ones that display significant kinetic variation, may be selectively equivalent – even when the phenotype to which the enzyme contributes is the major determinant of fitness.

The conditional nature of fitness

Epistasis is a natural consequence of the metabolic architecture of even the simplest pathway[22]. Suppose the activity of the porin pores for the entire population is increased to 300% of that of the original control strain. Then, what is the selective coefficient for a mutant that increases the activity of one of the three steps by, say, 10%? Such values can be calculated from Eqn 4 in Box 1 using the parameters as estimated from the data. For this example, the increase in activity of the porin pores would cause the selective coefficient of a permease mutant to increase from 1.51% to 3.25% and of a β-galactosidase mutant to increase from 0.04% to 0.08%, while the selective advantage of a porin pore mutant would decrease from 8.45% to 6.23%. Hence, selection for increased activity at one step may increasingly expose kinetic variation at other steps to natural selection.

Gene-by-environment interaction can be described by incorporating the effects of an environmental variable, such as pH or temperature, on each kinetic parameter. Saturating concentrations of environmental lactose may also cause gene-by-environment interactions in the absence of changes in the kinetic parameters. This is modeled using Eqn 5 in Box 1. Figure 6 illustrates the predicted effect of changes in lactose concentration on the selective advantage of three mutants, each with a 10% increase in maximum velocity at one of the steps. Increasing the concentration of lactose results in smaller selection coefficients at the porin pores and the β-galactosidase, and larger ones at the permease. This is caused by the permease becoming saturated and virtually rate limiting at high lactose concentrations. Thus, one should neither assume that the intensity of natural selection is diminished when resources are abundant, nor assume that it is maximized when resources are scarce.

A *lac* of Dr Pangloss

Why has evolution molded this pathway so that the cell wall exerts a marked influence at low concentrations of lactose and the permease becomes rate limiting at high concentrations? We don't know, but can hazard a guess – our contribution to the glut of 'just-so' stories. Porin pores of greater diameter would allow lactose to diffuse faster through the cell wall, but would also allow bile salts, present in the mammalian gut where *E. coli* lives, to penetrate into the periplasmic space and solubilize the lipid bilayer of the cell membrane[24]. The

actose-killing effect – a process whereby starving cells expressing high amounts of lactose permease die if suddenly presented with excess lactose – may be sufficient to prevent further increases in permease activity[22,25]. These two phenomena, together with other unknown effects, may serve to prevent further increases in activity at these two steps. Thus, investigations of alternative environments may contribute insights into the selective constraints that have molded this metabolic pathway.

Prospectus

Any investigation of the source laws of natural selection requires both that the environmental cause of selection be identified, and an ability to relate the phenotype of molecules to fitness. The work described here achieves this goal for a simple metabolic system in a simple prokaryote in a simple environment. Although the model merely describes the mechanistic relationships between simple kinetic parameters and fitness, it is surprisingly accurate, explaining over 95% of the variance in fitness. The model illustrates how a metabolic architecture may result in phenomena such as epistasis and gene-by-environment interactions. In addition, predictions regarding the order in which metabolic steps will be targeted by natural selection and speculations on the selective constraints imposed in natural environments are possible.

One might choose to dismiss this as a special case; an oversimplified analysis, applicable only to *E. coli* in the artificial environment of the laboratory, which does not solve the problem of how to analyse natural selection in higher organisms in nature. However, the causes of heredity were equally intractable during the 19th century, and at the turn of the century the results of Mendel were rejected as just a special case[26].

We accept that the study of natural selection is difficult and that we do not, according to a reviewer of an earlier draft of this article, 'solve the problem of analysing natural selection where most people want to study it'. Yet there is no reason to suppose that the principles of natural selection studied in the laboratory are fundamentally different from those in natural populations, where they may frequently be obscured by other important phenomena. An experimental approach demands the ability to manipulate causes, and to observe effects – in other words, the environmental causes of natural selection must be identified and subjected to direct manipulation by the investigator so that rigorous experimental control can be used to confirm or reject hypotheses.

The concept of phenotype has a complex and hierarchical nature. There is a conversion from gene to enzyme, often one gene to one enzyme. There is a conversion from enzyme to local metabolic behavior, which in turn is combined with other local pieces into a higher level of phenotype, that of general metabolism. The process of nesting subsystems continues until the phenotype upon which selection acts is reached. This is usually the phenotype of the whole organism. By limiting the genetic variation to that within a subsystem, and keeping the rest of the genome isogenic, the phenotype on which selection acts appears much lower in the hierarchy. This simplification permits the study of what is otherwise an intractable problem.

Are we forever condemned to the analysis of simple cases? No! As more information is gained, the kinetic, metabolic and fitness studies can be expanded to several or more subsystems and to several environments, so that various interactions can be investigated. This experimental approach gradually adds complexity in manageable chunks, until the intricacy of the complete system can be fully comprehended. For example, the effects of regulation of the lactose operon might be included. The concentration of inducer or repressor inside the cell becomes part of the epigenetic environment because it affects the concentration of enzymes and hence their activities.

Exact molecular models of fitness for many important traits in complex higher eukaryotes await quantitative theories for the conversion of metabolism to development and morphology. Yet some studies, for example of leucine aminopeptidase in the blue mussel[27] and of lactate dehydrogenase in the common killifish[28], have shown that the probable causes of selection can be identified by determining the relations between enzyme activity, various metabolic parameters and the various components of fitness. The laboratory experiments show that natural selection is not hopelessly complex, suggesting that natural systems are also simpler than might be expected. However,

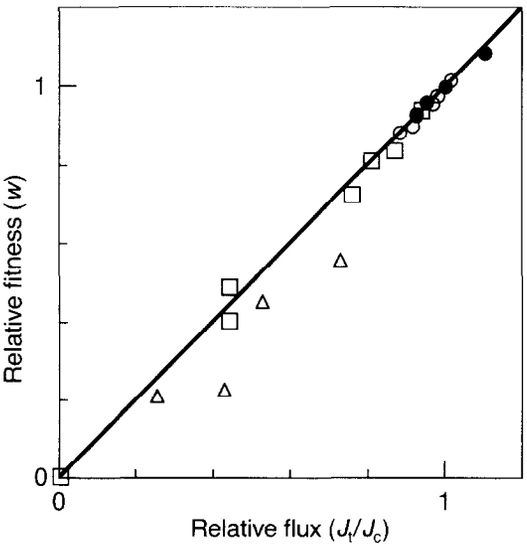

Fig. 4. The relation between fitness and the rate of lactose metabolism in *E. coli* when lactose is the growth-limiting nutrient. The straight line is the theoretical relation, $w = J_t/J_c$. The control strain carries a lactose operon from the K12 isolate of *E. coli* and is positioned at (1,1). Filled circles: lactose operons from natural isolates[23]. Open circles: laboratory mutants of β-galactosidase[21]. Open triangles: four mutants of *ebg*A (this gene encodes an enzyme that is normally incapable of hydrolysing lactose, but that can mutate to yield a second, rather inefficient lactase)[31]. Open squares: data from a constitutive K12 operon in competition with an inducible counterpart whose expression is controlled by using various concentrations of the gratuitous inducer, IPTG[22,32].

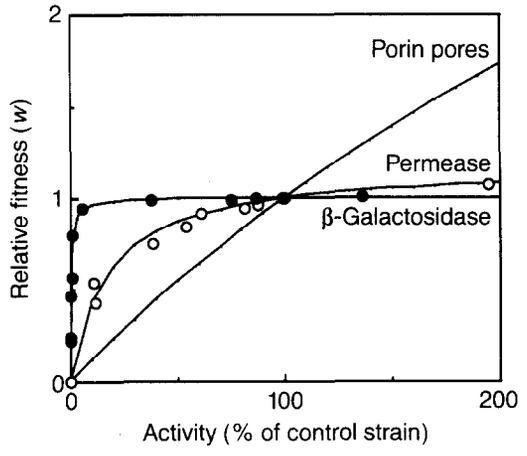

Fig. 5. The relationship between fitness and activity at each step in the lactose pathway. Each curve is a rectangular hyperbola, drawn from the exact model[23] when one activity at a time is changed. Filled circles: alleles of β-galactosidase[21,23,31]. Open circles: alleles of the permease[22,23]. Such concave fitness functions, an important feature in some models of molecular evolution[33], account for the recessive nature of inborn errors in metabolism[34] and the viability of small deletions in *Drosophila melanogaster*[35].

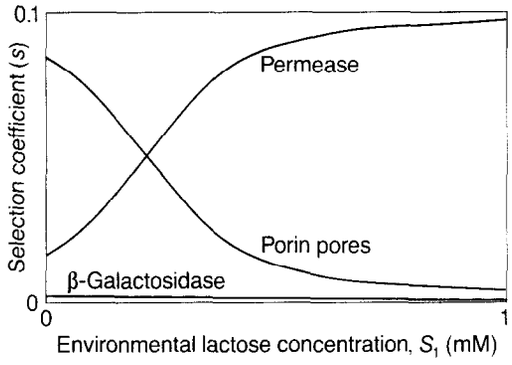

Fig. 6. The predicted effects of the concentration of environmental lactose on the selection coefficients at each step in the lactose pathway. The model uses differences of 10% in maximum velocity at each step while holding the other steps constant, and assumes that fitness remains proportional to flux. Note that as the abundance of resource increases, the intensity of selection on the permease increases.

where possible the studies on natural systems should be extended using a set of mutants covering a broad spectrum of activities, rather than concentrating on the most common alleles which may have only minor kinetic differences. This allows the relations between enzyme activity and fitness to be empirically determined and any last lingering doubts about hitchhiking effects dispelled. Also, by extending these studies to other loci within the metabolic subsystem, it may be possible to demonstrate rigorously that a particular aspect of metabolism (rather than some correlated effect) is important to fitness. Thus, a sequence of cause and effect from the molecular level to the population level might still be demonstrated rigorously, even when exact models and isogenicity are impractical.

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