Molecular Ecology (1994) 3, 127–135

Epistatic effects of promoter and repressor functions of the Tn10 tetracycline-resistance operon on the fitness of *Escherichia coli*

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Abstract

We have been studying the effects of expression of plasmid-borne, Tn10-encoded, tetracycline resistance on the fitness of *Escherichia coli* K12. We previously demonstrated large reductions in fitness resulting from induced or constitutive expression of the resistance protein; however, any residual expression by the repressed operon was so slight that possession of an inducible resistance function imposed essentially no burden in the absence of antibiotic. Here, we demonstrate two distinct disadvantages for inducible genotypes relative to isogenic constitutive constructs. During the transition from antibiotic-free to antibiotic-containing media, the inducible genotype experiences a longer lag phase prior to growth. In the sustained presence of antibiotic, full induction of the resistance function in the inducible genotype is prevented by the continued action of its repressor. However, these disadvantages may be reduced by increasing the strength of the promoter for the resistance gene in the inducible genotype. Simultaneous consideration of the mode of gene regulation (i.e. constitutive or inducible) and the strength of the resistance-gene promoter (i.e. maximum level of expression) indicates an adaptive landscape with very strong epistasis and, perhaps, multiple fitness peaks.

Keywords: adaptive landscape, antibiotic resistance, epistasis, fitness, gene expression, gene regulation

Received 21 June 1993; accepted 13 September 1993

Introduction

Adaptive evolution may proceed by changes in regulatory as well as structural genes. The regulation of gene expression often plays a critical role in the timing and integration of developmental processes. In many other instances, gene regulation modulates the level of expression of one or more gene products in response to some ecological variable, such as the concentration of a resource. In this study, we are concerned with gene regulation in the latter context. Levins (1968) considered alternative strategies by which populations of organisms might cope with variable environments, including genetic polymorphisms as well as phenotypic plasticity. Levins assumed specific couplings between genotypes, phenotypes and fitness, but he did not consider the mechanistic bases of genetic control over phenotypic expression in any detail. Savageau (1976) formulated mechanistic models of gene expression and regulation, and he suggested several criteria for evaluating the functional effectiveness of regulatory systems. These criteria include the avoidance of wasteful protein synthesis and responsiveness to temporal changes in substrate concentration.

A rigorous analysis of the consequences of different levels of gene expression and alternative modes of gene regulation for fitness in constant and variable environments would be extremely difficult in most biological systems. However, bacteria such as *Escherichia coli* have several features that make them a powerful experimental system for testing hypotheses that involve such interactions of molecular and ecological phenomena. First, there exists a wealth of information about the environmental control of gene expression in bacteria. Secondly, the relative ease with which bacteria can be analysed genetically

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and propagated in the laboratory facilitates precise manipulations of both genotype and environment. Thirdly, their large populations, short generations and clonal reproduction make it feasible to estimate directly the relative fitness of two competing genotypes under specified environmental conditions by measuring the rate of change in their relative abundance (Dykhuizen & Hartl 1983; Hartl & Dykhuizen 1985; Dykhuizen 1990; Dykhuizen & Dean 1990; Lenski 1992).

We have sought to compare the relative fitness of several *Escherichia coli* genotypes that possess different tetracycline-resistance operons, under various environmental regimes. The operons are all derived from the transposon Tn10, but they differ in their promoter and repressor functions. All of the operons are encoded by isogenic plasmids transformed into isogenic host backgrounds. The 'wild-type' Tn10 *tet* operon encodes the resistance protein, designated TetA, and a repressor, designated TetR (Nguyen *et al.* 1983; Postle *et al.* 1984). The TetA protein causes an active efflux of tetracycline from the cell (McMurry *et al.* 1980). The TetR repressor inhibits transcription of *tetA* and *tetR* by binding to two operator sites that overlap *tetA* and *tetR* promoters. Tetracycline induces transcription from the *tetA* and *tetR* promoters by binding to the TetR repressor, which reduces its affinity for the operator sites (Hillen *et al.* 1984).

In an earlier paper (Nguyen *et al.* 1989), we examined the fitness effects of carriage and expression of several different Tn10 *tet* operons in the *absence* of antibiotic. Either induction or constitutive expression of the TetA resistance protein caused substantial reductions in fitness; however, carriage plus residual expression of the repressed operon imposed essentially no fitness burden. Thus, possession of the inducible system for regulation of the tetracycline resistance function is highly economical in the absence of antibiotic, consistent with Savageau's (1976) criterion of avoidance of unnecessary protein synthesis. Of course, one must also understand the relative efficacy of alternative modes of gene regulation in the *presence* of the relevant substrate. To that end, we now report the relative fitness of strains possessing constitutive and inducible tetracycline-resistance functions in environments that contain antibiotic some or all of the time.

Materials and methods

Bacterial strains and plasmids

Escherichia coli K12 strain JA221 (F-*lacY1, leuB6, thi-1, tonA2, supE44, ΔtrpE5, recA1, hsr*: Moyed *et al.* 1983) is unable to utilize lactose (Lac⁻). We have isolated previously a spontaneous lactose utilizing (Lac⁺) revertant of JA221 (Nguyen *et al.* 1989). JA221 and this revertant are designated simply Lac⁻ and Lac⁺ in this paper. Lac⁻ and Lac⁺ form red and white colonies, respectively, on tetrazolium lactose indicator agar. The lactose marker is effectively neutral under the culture conditions used in this study (Nguyen *et al.* 1989; see also the Results section of this paper).

Table 1 Plasmids used in this study. All five of these plasmids confer resistance to ampicillin, kanamycin, and tetracycline

Designation	Description	Source
pBT1071S = pInd-Lo	Weak *tetA* promoter and functional repressor (inducible)	Moyed & Bertrand 1983
pBT1071 = pCon-Lo	Weak *tetA* promoter and non-functional repressor (constitutive)	Moyed & Bertrand 1983
pBT107–6A = pInd-Md	Intermediate *tetA* promoter and functional repressor (inducible)	Daniels & Bertrand 1985
pBT107–39L–6A = pCon-Md	Intermediate *tetA* promoter and non-functional repressor (constitutive)	This study
pBT107 = pInd-Hi	Strong *tetA* promoter and functional repressor (inducible)	Moyed *et al.* 1983

Five different plasmids were transformed into both the Lac⁻ and Lac⁺ backgrounds, following the basic procedures described in Maniatis *et al.* (1982). Table 1 summarizes the key properties of the plasmids; details of their construction can be found in the references cited therein. Plasmid pBT107-39L-6A was constructed in two steps. First, the 230-bp *Sna*BI–*Xba*I fragment from pBI501-39L (Isackson and Bertrand 1985) was ligated to the 7280-bp *Sna*BI–*Xba*I fragment from pBT7512 (Moyed & Bertrand 1983) to generate pBT107-39L-512, a derivative of pBT107 that contains a point mutation in *tetR* (*tetR39L*) and an insertion in *tetA* (*tetA512*::*IS1*). Secondly, the 1814-bp *Xba*I–*Pst*I fragment from pBT107-39L-512 was ligated to the 4901-bp *Xba*I–*Pst*I fragment from pBT107-6A to generate pBT107-39L-6A. All five plasmids are isogenic except for the *tet* operon: promoters are weak, intermediate or strong and the repressor is either functional or nonfunctional. None of the plasmids carry the IS10 insertion sequences derived from Tn10. None of the genetic manipulations impinge on the plasmids' origin of replication. Similar manipulations have little or no effect on plasmid copy number, which is about 20 copies per cell (Daniels & Bertrand 1985). All of the plasmids are nonconjugative. To minimize confusion, we have provided alternative designations for each of these plasmids, such that the first three letters indicate whether the Tet function is inducible (Ind)

or constitutive (Con) while the last two letters indicate whether the maximum level of TetA expression is low (Lo), medium (Md) or high (Hi).

All strains were stored at –80 °C in order to maintain isogenicity.

Culture conditions and measurements of relative fitness

Pure and mixed (competition) cultures were grown for 24 h in 10 mL of LB medium (Miller 1972) in 50-mL Erlenmeyer flasks at 37 °C and aerated by shaking at 120 r.p.m. LB was supplemented with 10 mg/L tetracycline as indicated in certain experiments. Tetrazolium lactose (TL) indicator agar was used to determine the ratio of competing strains (Nguyen *et al.* 1989). Agar plates were incubated overnight at 37 °C.

The fitness of one genotype relative to another was assayed by allowing them to compete directly in mixed culture. To ensure that the two genotypes had comparable physiological states at the start of a competition experiment, pure cultures of each genotype were separately grown overnight to saturation density in LB (supplemented as indicated). The two pure cultures were then mixed in a 1:1 volumetric ratio, diluted $1{:}10^6$ into fresh LB (supplemented as indicated), and immediately sampled on TL agar to obtain an initial estimate of the relative abundance of the two genotypes in the mixed culture. After 24 h, the saturated mixed culture was sampled again to obtain a second estimate of the relative abundance of the two genotypes, while an aliquot of the saturated culture was diluted $1{:}10^6$ into fresh medium, and so on for as many as 6 days. The exact duration of a competition experiment depended on the relative fitness of the competing genotypes; no further data were obtained when either of the genotypes had fallen below ~10% of the total population (< 30 colonies, with approximately 300 colonies total per plate).

For each competition experiment, a selection rate constant, s, was obtained as the slope of the following log-linear model:

$$\log_e R(t) = \log_e R(0) + s\,t$$

in which R is the ratio of the abundances of the two competing genotypes, t is time in days, and s has units of day^{-1}. The selection rate constant was converted to a relative fitness, W, as follows:

$$W = 1 + s \,/\, \log_e d$$

in which d is the dilution factor (10^6 day^{-1}). This relative fitness is equivalent to the ratio of the two competing genotypes' Malthusian parameters (i.e. rates of population growth averaged over the entire growth cycle) when they are in competition with one another (Lenski *et al.* 1991). A relative fitness of one is expected if two genotypes have identical Malthusian parameters; a relative fitness of zero is expected if one genotype simply fails to replicate, while the other genotype increases by the dilution factor.

No statistical significance was attributed to any single estimate of relative fitness. Instead, we always ran multiple assays for each pair of genotypes, and we based our statistical inferences on the reproducibility of the estimates of their relative fitness (Lenski 1991). To ensure statistical independence of the replicate fitness estimates, the pure cultures used to initiate the competition experiments were always inoculated with different single colonies. To determine whether or not the fitness of one genotype relative to another (in a particular environment) was significantly different from one, 95% confidence intervals about the sample mean were constructed using the t-distribution with $N - 1$ degrees of freedom, where N is the number of replicate experiments.

Population growth trajectories and estimation of doubling time and duration of lag phase

Bacterial populations were grown to stationary phase in LB, with or without supplemental tetracycline as indicated, and then diluted 1:20 in fresh LB containing 10 mg/L tetracycline. Optical densities (*OD*) were obtained every ten minutes. From each population's trajectory, we estimated its doubling time during exponential

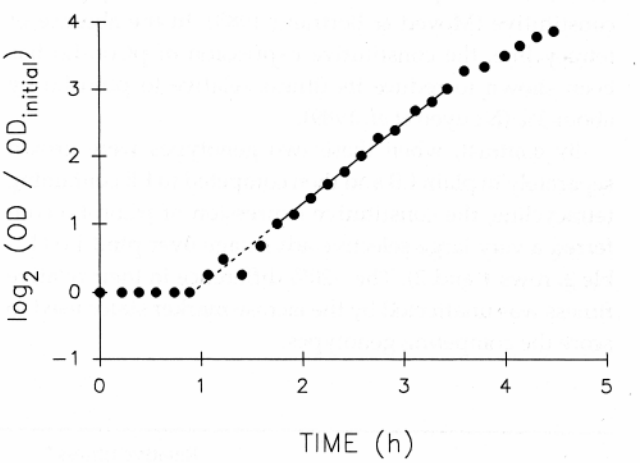

Fig. 1 Illustration of procedures used to estimate the duration of lag phase and the doubling time during exponential growth. A bacterial population was grown overnight to stationary phase and then diluted 1:20 into fresh medium. Optical densities (*OD*) were obtained at frequent intervals (indicated by circles). All *OD* were standardized by dividing by the initial *OD* (obtained immediately after dilution into fresh medium) and then $\log_2$-transformed. Linear regression was performed on the transformed *OD* that lay in the interval from 1 to 3 (shown as solid line); the estimated doubling time is given by the inverse of the slope of that regression. The regression was then extrapolated to a transformed *OD* equal to zero (shown as dotted line) to obtain the estimated duration of the lag phase.

phase growth, and the duration of its lag phase prior to the commencement of growth, as follows (see Fig. 1). Doubling time was estimated as the inverse of the slope obtained by regressing the $\log_2$-transformed *OD* against time for the period encompassing the second and third cell doublings (i.e. the period in which *OD* went from two to eight times its initial value). Duration of lag phase was estimated by extrapolating exponential growth back in time until that point where the extrapolated *OD* intersected the initial *OD* that was actually measured (Fig. 1). In effect, the duration of the lag phase was estimated by the difference between the time required for the first cell doubling and the average time required for the second and third doublings.

Results

Relative fitness of inducible and constitutive genotypes with identical weak promoters

Plasmids pInd-Lo and pCon-Lo have identical promoters, which permit maximum levels of *tetA* synthesis that are only ~0.5% of the level found in pInd-Hi (Daniels & Bertrand 1985). They differ in that pInd-Lo has a functional repressor, and hence is inducible, whereas pCon-Lo has a mutation in *tetR* that renders the TetR repressor nonfunctional, so that expression of the TetA resistance protein is constitutive (Moyed & Bertrand 1983). In the absence of tetracycline, the constitutive expression of pCon-Lo has been shown to reduce its fitness relative to pInd-Lo by about 3% (Nguyen *et al.* 1989).

By contrast, when these two genotypes were grown separately in plain LB and then competed in LB containing tetracycline, the constitutive expression of pCon-Lo conferred a very large selective advantage over pInd-Lo (Table 2, rows 1 and 2). The ~20% difference in their relative fitness was unaffected by the lactose marker states used to score the competing genotypes.

Effect on fitness of growth in tetracycline prior to competition

The poor fitness of the inducible genotype relative to its constitutive counterpart, in medium containing tetracycline, could reflect either (or both) of two distinct phenomena. First, the inducible genotype may experience a delay in growth after the sudden transition from antibiotic-free medium to medium that contains tetracycline, as the TetA protein becomes induced. Second, continued expression of the TetR repressor by the inducible genotype may prevent maximal induction of the resistance function, such that the rate of synthesis of the TetA resistance protein is lower for the inducible genotype than for the constitutive genotype even though they have identical promoters.

If an induction delay accounts for the entire 20% difference in fitness between the inducible and constitutive genotypes, then this difference should be eliminated when the two competing strains have been grown in medium containing tetracycline *before* they are mixed. On the other hand, if this entire difference results from submaximal expression of the resistance function caused by the action of the repressor, then it should not matter whether the two competing strains have been grown previously in plain LB or in LB supplemented with tetracycline.

When the two competing strains were separately grown (as well as competed) in LB supplemented with tetracycline, the advantage associated with the constitutive expression was reduced from ~20% to ~15% (Table 2, rows 3 and 4). Again, the effect is independent of the lactose marker used to distinguish the strains carrying pCon-Lo and pInd-Lo. A two-way fixed-effect analysis of variance using the data summarized in Table 2 (rows 1–4) indicates that the effect of the antibiotic treatment prior to competition was highly significant ($F_{1,32} = 11.3$, $P < 0.01$), whereas neither the effects of the marker nor the interaction between marker and antibiotic treatment were significant ($P > 0.5$ for both). Therefore, we infer that both an induction delay and submaximal expression of the TetA resist-

Pretreatment and competing strains	Relative fitness*		
	Mean	SD	95% CI
Pretreatment – Tc:			
Lac⁻/pInd-Lo vs. Lac⁺/pCon-Lo	0.799	0.052	0.760, 0.839
Lac⁺/pInd-Lo vs. Lac⁻/pCon-Lo	0.806	0.046	0.771, 0.842
Pretreatment + Tc:			
Lac⁻/pInd-Lo vs. Lac⁺/pCon-Lo	0.848	0.044	0.814, 0.881
Lac⁺/pInd-Lo vs. Lac⁻/pCon-Lo	0.855	0.027	0.834, 0.875

Table 2 Relative fitness of inducible and constitutive genotypes with weak *tetA* promoters (pInd-Lo and pCon-Lo, respectively) in LB supplemented with 10 mg/L tetracycline. Prior to each assay, the two competing strains were grown separately in medium with (+ Tc) or without (– Tc) supplemental tetracycline, as indicated

*Fitness of the first strain relative to the second; a value of 1 indicates equal fitness. Each mean is based on $N = 9$ replicate assays. SD = sample standard deviation. 95% CI = confidence interval calculated using the *t*-distribution with $N - 1$ degrees of freedom and $P = 0.05$.

ance protein contribute to the reduced fitness of the inducible genotype relative to the constitutive genotype when challenged with antibiotic.

Direct estimation of doubling time and duration of lag phase

From the results in the preceding section, it appears that pCon-Lo has a shorter doubling time (i.e. higher maximal growth rate) than pInd-Lo in medium supplemented with tetracycline *and* a shorter lag prior to the commencement of growth upon transfer from antibiotic-free medium into medium containing tetracycline. To check these inferences, we estimated doubling times during exponential growth and lag times prior to the commencement of exponential growth in LB supplemented with 10 mg/L tetracycline. The growth trajectories of four replicate populations of bacteria were monitored for each of the following four treatments: (a) bacteria carrying pCon-Lo and previously grown to stationary phase in medium with supplemental tetracycline; (b) bacteria carrying pCon-Lo but grown to stationary phase in medium without tetracycline; (c) bacteria carrying pInd-Lo and grown to stationary phase in medium with tetracycline; (d) bacteria carrying pInd-Lo and grown to stationary phase in medium without tetracycline.

Doubling times during exponential-phase growth were unaffected by the pretreatment for either pCon-Lo ($P > 0.4$) or pInd-Lo ($P > 0.2$), indicating that any effect of exposure to tetracycline during the previous day had worn off by the time that cells were in exponential growth. However, pCon-Lo had a significantly shorter doubling time (i.e. faster growth) during exponential phase than did pInd-Lo (Table 3, rows 1 and 2), in agreement with the competition experiments.

Prior growth in medium containing tetracycline significantly reduced the duration of lag phase for bacteria carrying the inducible resistance plasmid, pInd-Lo (Table 3, rows 3 and 4). This pretreatment also reduced the duration of lag phase for bacteria carrying pCon-Lo, although to a lesser degree (Table 3, rows 5 and 6). The latter result implies that even bacteria possessing a constitutive resistance function are not completely insensitive to a sudden transition to medium containing antibiotic. A two-way fixed-effect analysis of variance using the data summarized in Table 3 (rows 3–6) indicates no main effect of the plasmid ($F_{1,12} = 0.214$, $P > 0.5$), a highly significant main effect of antibiotic treatment prior to competition ($F_{1,12} = 27.7$, $P < 0.001$), and a marginally significant interaction between plasmid and antibiotic treatment ($F_{1,12} = 3.95$, $0.05 < P < 0.1$). Given that the direction of the interaction was predicted in advance, a one-tailed *t*-test corresponding to the interaction effect is significant ($t_s = 1.99$, $P < 0.05$).

Table 3 Doubling time and duration of lag phase for inducible (pInd-Lo) and constitutive (pCon-Lo) genotypes in LB supplemented with 10-mg/L tetracycline. Prior to determination of population growth trajectories, strains were grown to stationary phase in medium with (+ Tc) or without (– Tc) supplemental tetracycline, as indicated

	Estimated parameters *	
Pretreatment and strain	Mean ± SD	Significance
Doubling time (h) ($N = 8$)		
pCon-Lo (+ Tc and – Tc)	0.523 ± 0.091	$P < 0.05$
pInd-Lo (+ Tc and – Tc)	0.649 ± 0.140	
Duration of lag phase (h) ($N = 4$)		
pInd-Lo (+ Tc)	0.419 ± 0.124	$P < 0.01$
pInd-Lo (- Tc)	0.889 ± 0.182	
pCon-Lo (+ Tc)	0.518 ± 0.049	$P < 0.05$
pCon-Lo (- Tc)	0.730 ± 0.128	

*Doubling time and duration of lag phase were estimated from population growth trajectories, as illustrated in Fig. 1. Each mean is based on *N* trajectories. SD = sample standard deviation. Significance level based on a one-tailed *t*-test.

Relative fitness of inducible and constitutive genotypes with promoters of different strength

We have demonstrated that an inducible genotype with a weak promoter is much less fit than a constitutive genotype with the same weak promoter, even when both strains are separately grown (as well as competed) in medium containing tetracycline (Table 2, rows 3 and 4). This difference in fitness presumably occurs because the equilibrium level of expression of the TetA resistance protein is lower for the inducible genotype than for the constitutive genotype. Therefore, it is of interest to determine the fitness of an inducible genotype with a strong *tetA* promoter, such as pInd-Hi, relative to a constitutive genotype with a weak *tetA* promoter, such as pCon-Lo, in medium containing antibiotic. In particular, we expect that an inducible genotype with a stronger *tetA* promoter will have a higher equilibrium concentration of the TetA resistance protein than an inducible genotype with a weaker *tetA* promoter. The higher concentration of the TetA resistance protein should thereby reduce the equilibrium intracellular concentration of tetracycline. All else equal, we predict that the disadvantage experienced by an inducible genotype, when competed against a constitutive genotype in medium containing tetracycline, should be reduced or eliminated by having the stronger *tetA* promoter.

Two specific caveats can be issued with respect to the 'all else equal' qualification. First, promoters for the *tetA* and *tetR* genes are physically adjacent and functionally not independent. However, the 200-fold difference between

the maximum level of mRNA synthesis for the *tetA* promoters in pInd-Hi and pInd-Lo is opposed by only a 3.3-fold difference in the maximum level of mRNA synthesis for their *tetR* promoters (Daniels & Bertrand 1985). Secondly, the improved fitness that would result from a reduction in the intracellular concentration of tetracycline might be offset to some extent by the adverse side-effects caused by increased expression of the TetA resistance protein (Moyed *et al.* 1983; Eckert & Beck 1989; Nguyen *et al.* 1989).

These caveats not withstanding, the prediction was confirmed that a stronger *tetA* promoter would reduce the disadvantage associated with inducible expression in medium containing tetracycline. When strains were conditioned and competed in LB supplemented with 10 mg/L tetracycline, the inducible genotype with a strong promoter, pInd-Hi, was only ~6% less fit than the constitutive genotype with a weak promoter, pCon-Lo (Table 4, rows 1 and 2). By contrast, the fitness difference was ~15% when inducible and constitutive strains had identical weak promoters (Table 2, rows 3 and 4).

Although the fitness disadvantage associated with inducible expression was substantially reduced by virtue of the very strong *tetA* promoter, it was not eliminated entirely. The remaining disadvantage could occur either because (i) pInd-Hi still does not produce as much TetA protein as pCon-Lo, so that pInd-Hi suffers more from the toxic effect of tetracycline; or (ii) pInd-Hi produces much more TetA protein than pCon-Lo, so that pInd-Hi suffers from the adverse side-effects caused by over-expression of this protein (Moyed *et al.* 1983; Eckert & Beck 1989; Nguyen *et al.* 1989).

To distinguish these possibilities, we also competed an inducible genotype expressing a *tetA* promoter of intermediate strength, pInd-Md, against pCon-Lo. This intermediate promoter is approximately 50 times stronger than the weak promoter, but only about one-quarter as strong as the strongest promoter (Daniels & Bertrand 1985). The inducible genotype with an intermediate strength promoter, pInd-Md, is also disadvantaged relative to pCon-Lo (Table 4, rows 3 and 4), but to a lesser degree than the inducible genotype with the very strong promoter (Table 4, rows 1 and 2), consistent with explanation (ii) above. Evidently, the *optimal* promoter strength for an inducible genotype in this medium lies somewhere between Lo and Hi. (However, we cannot discern from these data whether the optimum lies between Lo and Md or between Md and Hi.)

We can also ask whether a constitutive genotype might do better with a stronger promoter in medium containing antibiotic. To that end, we competed bacteria carrying pCon-Md with those carrying pCon-Lo. The intermediate strength promoter is also favoured when resistance is expressed constitutively, although by only ~4% (Table 4, rows 5 and 6). We cannot perform competition experiments with pCon-Hi, however, as constitutive expression of TetA from the strongest promoter is effectively lethal (Moyed & Bertrand 1983; Moyed *et al.* 1983; Nguyen *et al.* 1989).

Discussion

We have avoided labelling any of the genotypes used in this study as the 'wild-type', even though pBT107 (= pInd-Hi) does indeed encode a *tet* operon that has the same DNA sequence as the Tn10-derived *tet* operon. Such a label could be misleading since the relative fitnesses of the various genotypes have been examined under artificial conditions, with respect to both the culture medium in which the genotypes were competed (a rich broth) and the genetic context in which the *tet* operons were inserted (a high-copy-number plasmid). We have not sought to test the hypothesis that the 'wild-type' operon is adaptively superior to any alternative configuration, nor do we believe that this hypothesis could be properly tested. Rather, we have used well-characterized genotypes and defined environmental conditions as a model experimental system to address the following general questions: What selective

Table 4 Relative fitness of genotypes with weak (Lo), intermediate (Md), and strong (Hi) *tetA* promoters in LB supplemented with 10 mg/L tetracycline

Competing strains	Relative fitness*		
	N	Mean ± SD	95% CI
Lac⁻/pInd-Hi vs. Lac⁺/pCon-Lo	10	0.927 ± 0.023	0.911, 0.943
Lac⁺/pInd-Hi vs. Lac⁻/pCon-Lo	10	0.947 ± 0.023	0.931, 0.964
Lac⁻/pInd-Md vs. Lac⁺/pCon-Lo	5	0.969 ± 0.005	0.962, 0.975
Lac⁺/pInd-Md vs. Lac⁻/pCon-Lo	5	0.968 ± 0.010	0.955, 0.981
Lac⁻/pCon-Md vs. Lac⁺/pCon-Lo	10	1.038 ± 0.010	1.031, 1.045
Lac⁺/pCon-Md vs. Lac⁻/pCon-Lo	10	1.039 ± 0.011	1.031, 1.047

*Fitness of the first strain relative to the second; a value of 1 indicates equal fitness. Each mean is based on *N* replicate assays. SD = sample standard deviation. 95% CI = confidence interval calculated using the *t*-distribution with $N - 1$ degrees of freedom and $P = 0.05$.

constraints determine the optimum level of gene expression in a constant environment? How does the responsiveness of gene expression affect fitness in a changing environment? Of what evolutionary importance are interactions between the genetic components that govern the level and the responsiveness of gene expression?

The Tn10 *tet* operon is subject to strong, opposing selection pressures. Tetracycline inhibits protein synthesis, and hence growth, by binding to bacterial ribosomes. The TetA resistance protein reduces the intracellular concentration of tetracycline by causing an active efflux of this antibiotic (McMurry *et al.* 1980), but the TetA protein itself has a detrimental effect on bacterial fitness (Moyed *et al.* 1983; Eckert & Beck 1989; Nguyen *et al.* 1989). Because there is strong selection against either under-expression or over-expression of the TetA resistance protein, the optimum clearly depends upon the concentration of tetracycline that is present in the environment.

In the absence of tetracycline, repression of the *tetA* gene is so tight that there is no discernible cost (< 0.3%) associated with carriage and residual expression of the TetA protein, even when resistance is expressed from the strongest (Hi) *tetA* promoter (Nguyen *et al.* 1989). Constitutive expression of the TetA protein from the weak (Lo) promoter reduces fitness by ~3%, whereas constitutive (or fully induced) expression from the strongest *tetA* promoter is effectively lethal (Moyed & Bertrand 1983; Moyed *et al.* 1983; Nguyen *et al.* 1989).

In medium containing 10-mg/L tetracycline, the inducible genotype with the weak *tetA* promoter is ~15% less fit than the constitutive genotype with the same weak *tetA* promoter, if the competitors were separately grown in medium supplemented with antibiotic prior to their competition (Table 2, rows 3 and 4). If these same competitors were grown in medium without antibiotic prior to their competition in medium containing tetracycline, then the inducible genotype has an even larger competitive disadvantage (Table 2, rows 1 and 2). This additional handicap for the inducible genotype can be attributed to the delay associated with induction of a formerly repressed operon, which results in a disproportionately longer lag phase prior to the commencement of growth (Table 3, rows 3–6).

The disadvantage of inducible relative to constitutive expression in medium containing tetracycline is manifest as a reduced maximal growth rate even after the effect of pretreatment (with or without antibiotic) has worn off (Table 3, rows 1 and 2). Induction is not an all-or-nothing phenomenon, and continued repression of the synthesis of the TetA protein evidently causes the resistance function to be expressed at a sub-optimal level. Dykhuizen & Davies (1980) observed a similar growth-inhibiting effect of the *lacI*-encoded repressor when inducible and constitutive genotypes were competed in medium in which lactose was the limiting resource.

Consistent with this explanation, the fitness disadvantage of the inducible genotype in medium containing tetracycline was reduced from ~15% to ~3% (relative to the same competitor) by substantially increasing the strength of the TetA promoter (Table 2, rows 3 and 4, versus Table 4, rows 3 and 4). A comparable increase in promoter strength also improves the fitness of a constitutive genotype in medium containing tetracycline, but by only ~4% (Table 4, rows 5 and 6). If the promoter strength is increased still further, however, then both inducible (Table 4, rows 1 and 2) and constitutive (see Moyed & Bertrand 1983) genotypes suffer from the deleterious side-effects of over-expression of the TetA protein. Thus, in medium containing tetracycline, a *tetA* promoter of intermediate strength is optimal for both inducible and constitutive genotypes (Fig. 2). However, an inducible genotype evidently suffers more strongly from a promoter that is too weak, which leaves it vulnerable to the effects of tetracycline; whereas a constitutive genotype suffers more from having a promoter that is too strong, which causes the cell to be poisoned by the toxic side-effects of the resistance protein itself.

These experiments also demonstrate highly epistatic (i.e. nonadditive) interactions between the loci that make up the tetracycline operon in determining fitness. That is, the selective value of any particular gene substitution depends upon its genetic context, as well as the environment. Therefore, in order to decide whether constitutive or inducible expression is optimal in a particular environment, it is also necessary to specify the strength of the *tetA* promoter. For example, in LB containing 10-mg/L tetracycline, a constitutive genotype is favoured over its inducible counterpart if the genotypes both possess the same weak or intermediate promoter (Fig. 2). However, the inducible genotype is favoured relative to the constitutive genotype if both possess a very strong promoter.

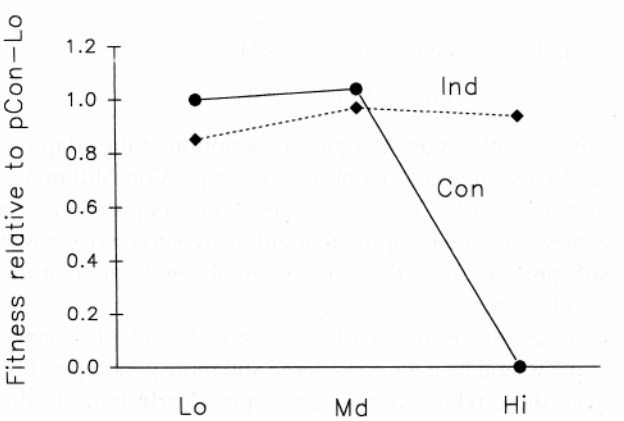

Fig. 2 Summary of the relative fitness of inducible (◆) and constitutive (●) genotypes with weak (Lo), intermediate (Md) and strong (Hi) *tetA* promoters, in LB supplemented with 10 mg/L tetracycline.

When there is such strong epistasis, then there may not be a globally stable fitness maximum (even in a constant environment) that can be found by progressively changing one locus at a time, such that each intermediate genotype has higher fitness than its predecessor. Instead, the selective surface, or 'adaptive landscape', may contain multiple local maxima, or 'peaks', separated by maladapted intermediate states (Wright 1931, 1977, 1982, 1988). If there exist multiple fitness peaks in the landscape, then the equilibrium genetic composition of an evolving population may be constrained by its initial state.

The potential for this type of constraint is clearly suggested by the fitness levels of the four genotypes with the weakest and strongest promoters:

Con	1.00 ←	0.00
	↑	↓
Ind	0.85 →	0.94
	Lo	Hi

Imagine introducing the inducible genotype with the strong *tetA* promoter into an environment in which tetracycline is present. A constitutive genotype with the weak *tetA* promoter would be more favourable, but substitution of either of the two mutations necessary to achieve this genotype, by itself, would reduce fitness. Hence, the population could be effectively stuck with the suboptimal Ind-Hi genotype.

However, it is difficult to make a strong inference about the global or even local optimality of a particular genotype from an incomplete picture of the adaptive landscape (Lewontin 1974; Barton & Charlesworth 1984; Barton & Rouhani 1987; Lenski 1988). If we now add the two genotypes with the intermediate-strength promoter to the picture, the fitness landscape in medium containing antibiotic becomes:

Con	1.00 →	1.04 ←	0.00
	↑	↑	↓
Ind	0.85 →	0.97 ←	0.94
	Lo	Md	Hi

This (slightly) more complete description of the adaptive landscape suggests an optimal genotype (Con-Md) that is globally stable. That is, an evolving population can achieve this quasi-optimal solution by successive single substitutions, regardless of the initial genetic state of the population.

It remains to be seen how rugged this adaptive landscape would be if we considered still more genotypes. But even these relatively few genotypes clearly indicate the strong epistasis and complex evolutionary dynamics that may arise in the context of a very simple and common mechanism for regulating gene expression.

Coda

The experiments reported here and in an earlier paper (Nguyen *et al.* 1989) were designed to address basic questions about structure–function relationships at the interface of molecular biology, ecology, genetics and evolution. However, similar questions are relevant to the proliferation of antibiotic-resistant bacteria, which has become a serious problem for public health (Levy 1986; O'Brien *et al.* 1986; Cohen 1992; Johnson & Adams 1992; Neu 1992). For example: What levels of expression and modes of regulation of antibiotic-resistance genes are optimal (from the bacterium's perspective)? How do these optimality criteria depend on the concentration of antibiotic as well as its variability over time and space? Does the optimal configuration depend on an antibiotic's mode of action (e.g. bacteriostatic versus bacteriocidal) or the mechanism of resistance (e.g. efflux versus detoxification)? What constraints are there on the evolution of bacteria, and can these be exploited to control the spread of antibiotic-resistant bacteria?

Acknowledgements

We thank Larry Mueller, Michael Rose and two anonymous referees for comments on this manuscript, Toai Nguyen and Mike Travisano for helpful discussions, and Colleen Hough and Matthew Powell for constructing pBT107–39L–6A. This work was supported by grants from the Whitaker Foundation (to R.E.L), the National Science Foundation (to R.E.L.) and the National Institutes of Health (to K.P.B.)

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