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Predicting epistasis: an experimental test of metabolic control theory with bacterial transcription and translation

R. C. MacLEAN

Department of Zoology, University of Oxford, Oxford OX1 3PS, UK

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Abstract

Epistatic interactions between mutations are thought to play a crucial role in a number of evolutionary processes, including adaptation and sex. Evidence for epistasis is abundant, but tests of general theoretical models that can predict epistasis are lacking. In this study, I test the ability of metabolic control theory to predict epistasis using a novel experimental approach that combines phenotypic and genetic perturbations of enzymes involved in gene expression and protein synthesis in the bacterium *Pseudomonas aeruginosa*. These experiments provide experimental support for two key predictions of metabolic control theory: (i) epistasis between genes involved in the same pathway is antagonistic; (ii) epistasis becomes increasingly antagonistic as mutational severity increases. Metabolic control theory is a general theory that applies to any set of genes that are involved in the same linear processing chain, not just metabolic pathways, and I argue that this theory is likely to have important implications for predicting epistasis between functionally coupled genes, such as those involved in antibiotic resistance. Finally, this study highlights the fact that phenotypic manipulations of gene activity provide a powerful method for studying epistasis that complements existing genetic methods.

Introduction

Epistatic interactions between mutations are thought to play a crucial role in a number of evolutionary processes, including adaptation (Weinreich *et al.*, 2005; Bell, 2008) and sex (Kondrashov, 1988; Kouyos *et al.*, 2007; de Visser & Elena, 2007). Experimental studies in a wide variety of species, including eukaryotes (Wloch *et al.*, 2001; Kelly, 2005; Malmberg *et al.*, 2005; Segre *et al.*, 2005; Jasnos & Korona, 2007; Dickinson, 2008), prokaryotes (Elena & Lenski, 1997, 2001; Remold & Lenski, 2004; Maisnier-Patin *et al.*, 2005; Weinreich *et al.*, 2006; Cooper *et al.*, 2008), and viruses (Bonhoeffer *et al.*, 2004; Sanjuan *et al.*, 2004, 2005) have found evidence for epistasis, but the average direction of epistasis (i.e. synergistic or antagonistic) varies within (Elena & Lenski, 1997; Segre *et al.*, 2005) and between (Sanjuan & Elena, 2006) species. The underlying causes of this variation are not yet understood and an important goal of current research in this area is to predict epistasis from first principles. Two approaches have been taken to overcome this problem. The first approach, derived from quantitative genetics, has been to predict epistasis from the distribution of fitness effects of single mutations using Fisher's geometric model of adaptation (Martin *et al.*, 2007). The second approach has been to predict epistasis from biochemistry, using metabolic network analysis (Segre *et al.*, 2005; Sanjuan & Nebot, 2008) and metabolic control theory (Szathmáry, 1993). This second approach is especially appealing, because it offers the potential to develop a mechanistic understanding of epistasis based on the biochemical properties of organisms, so that epistasis could potentially be predicted directly from genome sequences.

In a seminal paper, Szathmáry (1993) showed that metabolic control theory predicts that functional interactions between genes can generate epistasis. Metabolic

Correspondence: R. Craig MacLean, Department of Zoology, University of Oxford, Tinbergen Building, South Parks Road, Oxford OX1 3PS, UK. Tel.: +44 (0) 1865 271157; fax: +44(0) 1865 310447; e-mail: craig.maclean@zoo.ox.ac.uk

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control theory assumes a linear pathway made up of n unsaturated enzymes with flux through the pathway, J, equal to $a/(1/E_1 + 1/E_2 + \ldots 1/E_n + b)$, where a and b are constants and E_n is the activity of nth enzyme in the pathway (Fig. 1a). Provided that fitness is correlated with metabolic flux, as will be the case in a growing population, metabolic control theory predicts that interactions between mutations in different genes are always antagonistic (Szathmáry, 1993). A second, and more subtle prediction, is that the strength of epistasis increases with average mutational effect (Fig. 1b). These predictions can be explained mechanistically as follows. The control of flux through the pathway exerted by the ith enzyme can be described by a control coefficient, c_i, which describes how flux through the pathway changes varies as a result of the activity of enzyme, E_i, such that $c_i = dJ/dE_i$. A fundamental property of pathways is that the sum of all control coefficients is always equal to 1 (Klipp *et al.*, 2009), and this law of conservation of the control of flux drives antagonistic epistasis. Imagine a hypothetical pathway in which the control of flux is distributed evenly among all enzymes. As the activity of enzyme i is reduced, it exerts more and more control over flux through the pathway, hence flux becomes increasingly insensitive to perturbations of other enzymes in the pathway. For example, if enzyme i acquires a highly deleterious mutation so that it acts as a rate limiting step for the pathway (i.e. $c_i = 1$) then small effect mutations in other enzymes in the pathway will have no effect on flux.

To test the predictions of metabolic control theory, I used a novel experimental approach involving phenotypic and genetic perturbations of a very well-characterized pathway, gene expression and protein synthesis, in the opportunistic human pathogen *Pseudomonas aeruginosa*. Although the motivation for the development of metabolic control theory was to understand the control of flux in metabolic pathways, the theory itself does not make any assumptions that are specific to any biological features of metabolism – in fact the theory ignores many features of metabolic biochemistry, such as feedback control. In other words, this is a theory about how linear processing chains respond to perturbation that is not specific to metabolism in any way. Because of this generality, metabolic control theory has been successfully used to model a number of processes in molecular biology that are completely unrelated to metabolism, such as signalling pathways and gene expression (Kholdenko *et al.*, 2000; Hofmeyr & Westerhoff, 2001, Bruggeman *et al.*, 2002; Klipp *et al.*, 2009). This study is motivated by the idea that gene expression and protein synthesis can be considered as a simple chain of reactions involving three substrates (DNA, RNA and protein) and two reactions (transcription and translation). Although this simplification neglects the many complexities of transcription and translation, the advantage of this simplification from an evolutionary perspective is that flux through this pathway is correlated with reproductive rate (i.e. fitness).

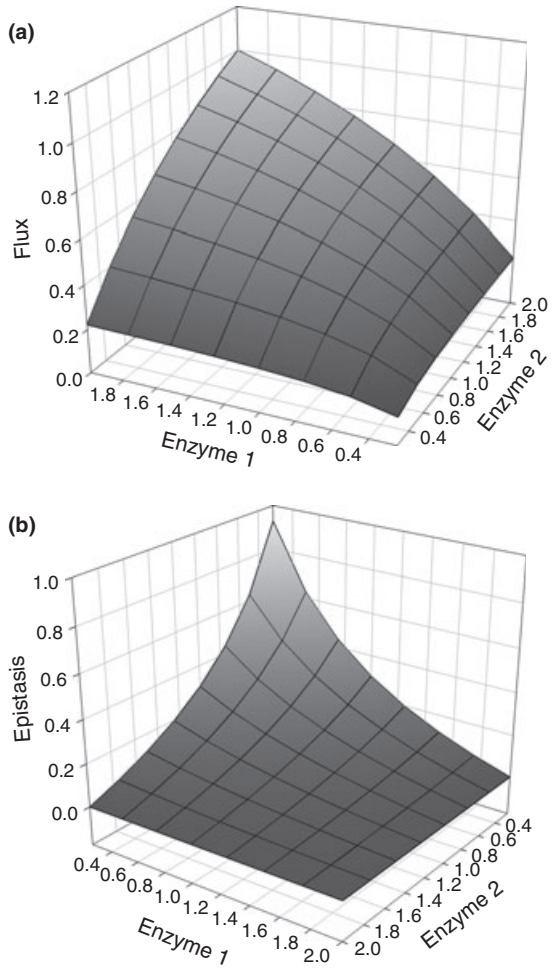

Fig. 1 A model of flux and epistasis in two enzyme pathway. This figure shows flux (a) and epistasis (b) in a model of a two-enzyme pathway with flux, J, equal to $1/(1/E_1 + 1/E_2)$, where E_i is the activity of ith enzyme. Epistasis is measured as $e = \ln(w_{x,y} - \ln(w_{x,2}{*}w_{2,y})$, where x and y are the activity of enzymes 1 and 2 respectively, when enzyme activity is below the wild type level (arbitrarily set at 2). A justification for the use of this measure of epistasis is given in the results section.

The experimental approach used to investigate epistasis in this study relies on the idea that phenotypic and genetic perturbations provide complementary methods for understanding the response of biological systems to changes in their component parts, an idea that is central to systems biology (Segre *et al.*, 2005; Yeh *et al.*, 2006; Lehar *et al.*, 2007; Yeh & Kishony, 2007); for example, if deleterious mutations in different enzymes in a metabolic pathway antagonize each other's effects, it follows that inhibitors of these enzymes should also antagonize each other. The main advantage of using drug–drug interactions to measure epistasis as opposed to conventional

mutation–mutation interactions is that drug–drug interactions allow precise and quantitative manipulation of gene activity using dose-response curves over the entire range of enzyme activity (Lehar *et al.*, 2007; Yeh & Kishony, 2007).

Methods and materials

Strains and culture conditions

Strains used in this study were *P. aeruginosa* PAO1 or derivatives of PAO1 carrying a single nonsynonymous mutation in *rpoB*, named according to the mutation in each strain (A455T, A455G, A1592G, A1562G and C1550T). Further details of RNAP mutants can be found in (MacLean & Buckling, 2009). Cultures were propagated in M9KB broth (10 g L^{-1} glycerol, 20 g L^{-1} proteose peptone #3, 12.8 g L^{-1} $Na_2HPO_4.7H_2O$, 3 g L^{-1} KH_2PO_4, 0.5 g L^{-1} NaCl, 1 g L^{-1} NH_4Cl, 2 mM $MgSO_4$) incubated at a constant temperature of 37 °C without shaking.

Measuring fitness

To assay fitness, I measured the growth rate of cultures of *Pseudomonas* in exponential growth phase by assaying optical density at 600 nM using an automated microplate reader (Synergy HT Powerwave XS; Biotek, VT, USA). Under the assay conditions used, optical density is correlated with the log of cell density, so that the linear rate of increase of optical density in exponential growth phase provides an estimate of the growth rate, *r*, of each culture, such that $N_t = N_o e^{rt}$. Fitness estimates were standardized by diving each growth rate by the mean growth rate of PAO1 in antibiotic-free culture medium. Preliminary experiments involving direct competition between PAO1 and RNAP mutants demonstrated that this method provides fitness estimates that are highly correlated ($r > 0.95$) with those obtained in traditional pairwise competition experiments using genetic markers (R.C. MacLean, unpublished).

Co-inhibittion with rifampicin and streptomycin

Overnight cultures of PAO1 were diluted 100 fold into fresh M9KB supplemented with variable concentrations of rifampicin (0, 2, 4, 8 or 16 mg L^{-1}) and streptomycin (0, 15, 30 or 60 mg L^{-1}) and fitness was assayed over a 20–24 h period. I measured the fitness of four replicate cultures of PAO1 in each pairwise combination of antibiotics.

Streptomycin inhibition assay of PAO1 and RNAP mutants

Overnight cultures of PAO1 and RNAP mutants were diluted 100 fold into M9KB supplemented with streptomycin at the following concentrations (0 mg L^{-1}, 7.5 mg L^{-1}, 15 mg L^{-1}, 30 mg L^{-1}, 60 mg L^{-1} and 120 mg L^{-1}) and fitness was measured over a 24 h period. I assayed the fitness of four replicate cultures of each strain at each antibiotic concentration. Resistance was calculated as IC_{50}, the concentration of streptomycin necessary to cause a 50% reduction in fitness, using the following regression model: $y = y_{min} + y_{max} - y_{max}/(1 + 10^{(\log IC_{50} - x)*H})$, where y is fitness, x is the concentration of streptomycin and H is parameter that estimates the rate of decay in fitness with increasing streptomycin concentration. Relative fitness was measured as y_{max}, which provides an estimate of fitness in the absence of antibiotics.

Mutagenesis assay

Overnight cultures of PAO1 and RNAP mutants were diluted down and spread on M9KB plates. These plates then immediately received a moderate mutagenic dose of UV (15 s of exposure to a germicidal UV light) or a mock no-UV control. Plates were then incubated overnight at 37 °C to allow colonies derived from single mutagenized cells to form. The following day, cells from the 16 or 32 colonies closest to a randomly chosen point on the agar plate were transferred to a microtiter plate containing M9KB for a fitness assay. I measure the fitness of one replicate culture from each colony. UV-induced mortality rates were the same in all strains tested.

Results

To investigate the fitness consequences of phenotypic perturbations of bacterial gene expression and protein synthesis, I assayed the fitness of *P. aeruginosa* PAO1 across a gradient of rifampicin, which inhibits RNA polymerase (Trinh *et al.*, 2006), and streptomycin, which inhibits the 30S subunit of the ribosome (Poehlsgaard & Douthwaite, 2005). Gene interactions are often quantified as $e_{1.2} = \ln w_{1.2} - \ln (w_1 * w_2)$, where $w_{1.2}$ is relative fitness in the presence of both mutations 1 and 2, and w_1 and w_2 are relative fitness in the presence of either mutation 1 or mutation 2 (Martin *et al.*, 2007; Gros *et al.*, 2009). With this definition epistasis is a relative deviation from single-mutant effects and is therefore not directly connected to the amplitude of the effect of single mutations on fitness. If interactions are additive, $e_{1.2}$ is equal to 0; antagonistic epistasis occurs when $e_{1.2} > 0$ and synergistic epistasis occurs when $e_{1.2} < 0$. If enzyme inhibitors are used to compromise enzyme activity instead of mutations, the same mathematical approach can be used to quantify epistasis, except that w_1 and w_2 are now fitness in the presence of either inhibitor 1 or 2 and $w_{1.2}$ is fitness in the presence of both inhibitor 1 and inhibitor 2.

Systematic co-inhibition experiments reveal that the interaction between rifampicin and streptomycin is, on average, antagonistic (Fig. 2a, mean $e_{Rif.Strep} = 0.072$,

(a)

[Streptomycin] mg L^{-1} \ [Rifampicin] mg L^{-1}	16	8	4	2	0	
60			0.44	0.41	0.34	Relative fitness
30		0.27	0.67	0.7	0.73	
15		0.33	0.84	0.84	0.96	
0	0.128	0.33	0.9	0.98	1	
60			0.353	0.202		$e_{Rif.Strep}$
30		0.142	0.023	–0.032		
15		0.058	–0.046	–0.126		
0						

(b)

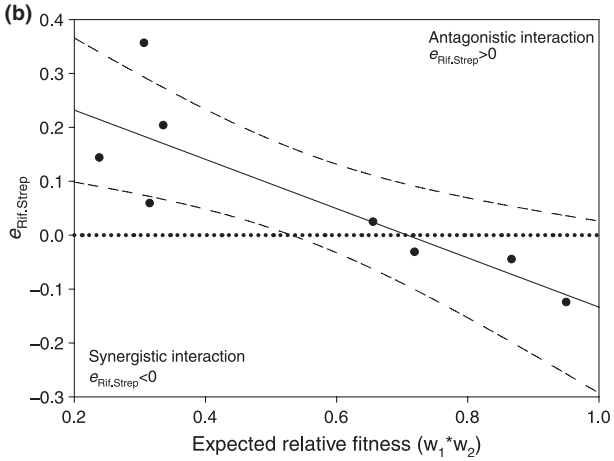

Fig. 2 Co-inhibition with streptomycin and rifampicin. Panel a shows the mean relative fitness (n = 4) and $e_{Rif.Strep}$ for cultures of *Pseudomonas aeruginosa* PAO1 grown across a range of concentrations of rifampicin and streptomycin. Panel b shows shows $e_{Rif.Strep}$ plotted against expected fitness. The solid line is a linear regression of y on x, the dashed lines give the 95% confidence interval about this regression and the dotted line shows $e_{Rif.Strep}$ = 0.

SEM = 0.032, n = 32, t_{31} = 2.21, P = 0.017). However, the strength of antagonism between rifampicin and streptomycin varies substantially depending on the concentration of each inhibitor (Fig. 2a). Much of this variation can be explained by the fact that the interaction between rifampicin and streptomycin becomes increasingly antagonistic as expected fitness (i.e. w_1*w_2) declines (Fig. 2b, slope = −0.46, SE = 0.094, t = 4.86, $P \leq 0.001$, r^2 = 0.44). In other words, the overall tendency towards antagonistic epistasis is driven by the fact that the interaction between rifampicin and streptomycin becomes highly antagonistic when large doses of either or both drugs are administered. When expected fitness is close to 1, e is approximately equal to 0, and in some cases our estimate of e is less than 0. However, none of these negative estimates are e are significantly different from zero after correcting for multiple comparisons (P > 0.05).

If the antagonistic interaction between rifampicin and streptomycin reflects a genuine functional antagonism between the ribosome and RNA polymerase, genotypes carrying deleterious mutations in RNA polymerase should show reduced sensitivity to ribosomal inhibitors and *vice versa*. To test this idea, I measured the streptomycin inhibition kinetics of strains carrying mutations in RNA polymerase and a wild-type control strain (Fig. S1). Tolerance to streptomycin increases linearly with the fitness cost of RNAP mutations (Fig. 3; r^2 = 0.987, t = 17.11, P < 0.001), demonstrating that deleterious mutations in RNAP increase robustness to ribosomal inhibition. Moreover, this antagonism becomes more pronounced as flux through the pathway decreases, as we would expect from theory (Fig. 1b) co-inhibition experiments with streptomycin and rifampicin (Fig. 2b).

One possible alternative explanation for the antagonism between perturbations of the ribosome and RNAP is that a compromised RNAP increases robustness to deleterious mutations at a genomic scale. If this were the case, then it could be argued that the ribosomal-RNAP antagonism demonstrated above was simply a by-product of increased robustness that did not arise from the functional interaction that occurs between RNAP and the ribosome in gene expression. To test this possibility, I measured the fitness cost of UV-induced spontaneous mutations in strains carrying deleterious mutations in RNAP and a wild-type control strain (Fig. S2). Across all strains, UV mutagenesis decreased mean fitness (average relative fitness = 0.94, SEM = 0.017, n = 72, t_{71} = 3.19, P = 0.002). However, I failed to detect differences in the fitness cost of UV mutagenesis between genotypes carrying deleterious RNAP mutations and the wild-type control (Dunnett's test; P > 0.05 for all comparisons), demonstrating that RNAP mutations do not change robustness to random deleterious mutations at a genomic scale.

Discussion

This study reports two findings that are in agreement with the predictions of metabolic control theory. First, this work shows that there is a tendency for antagonistic epistasis between perturbations of the ribosome and RNA polymerase. In support of this conclusion, a recently published study found that epistasis between deleterious mutations in RNA polymerase and the ribosome tends to be antagonistic (Trindade *et al.*, 2009). Second, the strength of epistasis correlates with the average magnitude of perturbation; epistasis is weak to nonexistent for mild perturbations and epistasis is highly antagonistic for harsh perturbations. Interestingly, the same correlation between mutational severity and epistasis emerges from theoretical models of epistasis using either a discrete model of fitness based on RNA secondary structure (Wilke & Adami, 2001) or a continuous model based on stabilizing selection on a pair of phenotypic traits (Gros *et al.*, 2009). However, it is difficult to relate the results of this study to these models because there is nothing in common between the underlying biological assumptions of these models.

In summary, I argue that metabolic control theory can successfully predict epistasis between genes involved in the same pathway, or biological process. It is possible that

this source of epistasis makes only a very minor contribution to epistasis at the scale of the entire genome. For example, in an organism with N pathways of equal length, the probability that two random mutations occur at random in the same pathway is $(1/N)^2$. Instead, I think that metabolic control theory could emerge as an important tool for predicting epistasis in functionally linked modules of genes, such as metabolic pathways and regulatory networks. As I have emphasized throughout this article, metabolic control theory need not only apply to metabolic pathways; this is a general theory that applies to any set of genes that act in a linear and sequential manner. For example, most of the major classes of antibiotics that are currently in clinical use target a pathway that links gene expression (rifamycins) with protein synthesis (amino-glycosides) and DNA replication (fluoroquinolones). Metabolic control theory predicts that targeting different components of this pathway is a poor strategy for antimicrobial chemotherapy, because carrying a costly resistance mutation in one enzyme involved in this pathway will both increase phenotypic resistance to antibiotics that target other enzymes in the pathway (Fig. 3), and reduce the genetic cost associated with acquiring costly resistance mutations in other enzymes in this pathway (Fig. 2, Trindade *et al.*, 2009).

A number of experimental methods have been developed for quantifying epistasis using meiotic recombination or direct genetic manipulation (Kouyos *et al.*, 2007). The drawback of these methods is that they are all labour intensive, and until recently large-scale genome-wide scale studies of epistasis were only possible in viruses

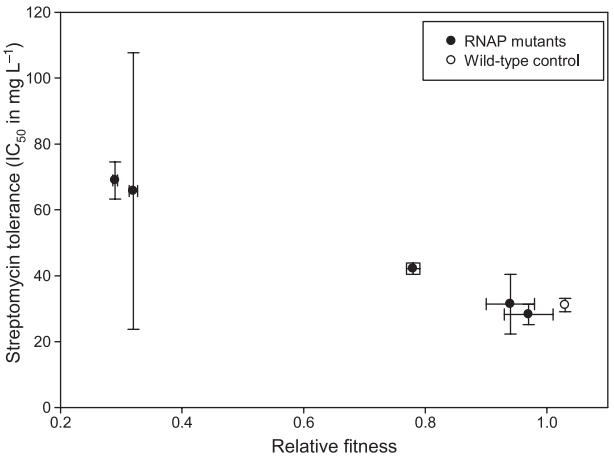

Fig. 3 Sensitivity of RNAP mutants to streptomycin inhibition. Plotted points show the relative fitness (± SE) in antibiotic-free medium and streptomycin tolerance (± SE) of RNAP mutants (filled symbols) and a wild-type control (open symbol). Full inhibition curves are given in Fig. S1 and details of the regression used to estimate both relative fitness and tolerance are given in the methods. One of the RNAP mutants has a high standard error associated with its measurement of streptomycin resistance because it displays unusual inhibition kinetics. Excluding this mutant does not alter the conclusions of the linear regression analysis.

(Sanjuan *et al.*, 2004). Recent work in this area has focused on measuring epistasis by creating pairwise combinations of knock-out mutations (Elena & Lenski, 1997; Jasnos & Korona, 2007). Although this approach has led to a substantial increase in the power of experimental studies in this area, it is associated with two important drawbacks. First, transformation and recombination are both mutagenic processes, and it is difficult to control for the potential creation of undesired second site mutations when constructing strains carrying combinations of mutations. Second, the approach is somewhat artificial, in that most spontaneous deleterious mutations probably lead to only partial loss of gene activity (Eyre-Walker & Keightley, 2007) and this study suggests that using knock-out mutations is likely to bias these studies in favour of detecting antagonistic epistasis (Fig. 2).

In this study, I have used an alternative method for studying epistasis using pairwise combinations of gene inhibitors, in this case antibiotics. Of course this approach suffers from its own limitations, such as the potential for off-target effects, chemical interactions between inhibitors, and the limited number of genes that can currently be targeted using specific inhibitors. In this case, these potential difficulties do not seem to be important: I have shown that phenotypic and genetic manipulations of RNAP inhibition mimic each others' effects and a recently published study (Trindade *et al.*, 2009) provides direct evidence of antagonistic epistasis between deleterious mutations in RNAP and the 30S subunit of the ribosome, providing a good validation of this method. What is exciting about this approach is that technological progress in the development of synthetic small molecule inhibitors and siRNA have the potential to dramatically increase the power of this method, so that it might be possible in the future to use inhibitor–inhibitor interactions to quantify epistasis at a genome-wide scale.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Plotted points show the mean relative fitness (± SEM, $n = 4$) of cultures of RNAP mutants (panels B to F) and the wild-type strain (panel A) across a concentration gradient of streptomycin.

Figure S2 This figure shows the average relative fitness (± SEM) of colonies derived from single cells that were exposed to 15 s of UV mutagenesis in strains carrying deleterious mutations in RNAP (A455G, A455T and C1550T) and a wild-type control (PAO1).

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