Theoretical and data-driven study of the influence of gene regulatory network on expression plasticity

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Abstract

Over the past two decades, a large body of theoretical and empirical work has been conducted with the aim of identifying the gene regulatory network topologies responsible for gene expression dynamics. Gene expression plasticity has been found to be preferentially associated with motifs such as feedforward loops, diamonds loops, or feedback loops. However findings were often equivocal for both theoretical and empirical work, as the same topologies have also been associated with expression robustness. Our objective is thus to finally decipher the regulatory causes of gene expression plasticity, using both theoretical predictions from a simulated network evolution model and the empirical data of *Escherichia coli* regulatory network. We investigated the link between network topology and gene expression at three levels: the number of regulators, the number and the proportion of loops (feedback loops, feedforward loops and diamond loops), and the proportion of unique motifs (here characterized by the position of up- or down-regulations within loops). Consistent results from our empirical and theoretical approaches revealed that plastic genes were regulated by more genes on average. Selection (as opposed to simulations under strict genetic drift) bias strongly the distribution of motifs, however this distribution was not different between plastic and non-plastic genes. Overall, this work illustrates that network topology as conceived today is insufficient to completely explain or predict gene expression dynamic.

Introduction

Understanding the mechanisms involved in the evolution of regulatory networks remains a major challenge in evolutionary and systems biology. It is widely acknowledged that biological gene regulatory networks are not randomly organized (Shen-Orr *et al.*, 2002; Burda *et al.*, 2011), but the interplay between natural selection, mutation bias, functional constraints, and genetic drift in the evolution of network topology is not clearly established. Some network topologies, such as feedforward loops (FFLs) and feedback loops (FBLs) have attracted considerable attention because of their abundance in biological systems (Mangan and Alon, 2003; Alon, 2007). This enrichment could be the consequence of selection indirectly promoting motifs through their effect on gene expression dynamics, including stability, plasticity, or oscillation.

Changes in gene expression is a molecular manifestation of phenotypic plasticity and it conditions the tempo and mode of the organism's response to environmental cues. At the gene level, plasticity is contingent on signal-sensitive regulation of transcription, and selection for plasticity might indirectly affect the underlying regulatory network. For instance, Vlková and Silander, 2022 demonstrated that *Escherichia coli* promoters involved in the metabolism of specific substrate are often selected to maximize plasticity. Yet, both theoretical and empirical work falls short of consensus when linking gene expression dynamics with specific network topology. From the theoretical side, feedforward loops (FFLs) (Shen-Orr *et al.*, 2002; Basu *et al.*, 2004; Ma *et al.*, 2009; de Ronde *et al.*, 2012; Xiong *et al.*, 2019; Wang *et al.*, 2020), diamond (DMD) loops (de Ronde *et al.*, 2012; Xiong *et al.*, 2019), and feedback loops (FBLs) (Hornung and Barkai, 2008; Panovska-Griffiths *et al.*, 2013) have been reported to be involved in plastic (environmentally-sensitive) gene expression. Counterintuitively, other theoretical findings associate those same loop categories with robustness-related functions (FFL: Osella *et al.*,

2011; Kadelka *et al.*, 2013; Roy *et al.*, 2017, DMD: Khammash, 2021, FBL: Kwon and Cho, 2007; Wang *et al.*, 2020). From the empirical side, many studies observed these motifs in both plastic and robust systems. Two studies on synthetic bacteria (Basu *et al.*, 2004) and *E. coli* (Sen *et al.*, 2014) brought experimental evidence of a link between feedforward loops and sensitivity to environmental signals. However these studies were restricted to a few genes, and the genome-wide picture remains unknown (Frei and Khammash, 2021; Weldemichael *et al.*, 2022). Overall, there is no clear indication whether specific network topologies are involved in the regulation of gene expression plasticity.

A considerable obstacle in the quest to identify biologically relevant topologies in networks is the lack of a clear theoretical framework indicating where information should be sought. Gene network topology can be described by multiple indicators that focus on different scales, including the entire network, the gene level (e.g., the number of incoming or outgoing connections), and local regulatory topologies, such as the nature of regulatory loops (feedback, feedforward, or diamond) and the loop motifs (the arrangement of up- and down-regulation within loops). Which of these levels, if any, is relevant for gene expression plasticity, and is it possible to explain the contradictory results from the literature? Our ambition here is to study the effect of local regulatory topologies in networks with a dual approach, coupling a theoretical model with a data-driven regulatory network analysis, in order to identify consistent predictions and observations. We derived theoretical predictions from evolutionary simulations, in which the regulatory interactions were free to evolve under a mutation-drift-selection process (need a reference here for this model?). Gene expression plasticity was then estimated as the response of individual genes to an environmental signal. In parallel, we collected lists of genes significantly responding to various environmental conditions according to 11 transcriptomic studies in *E. coli*. Despite that many regulatory pathways are known for this model species, the link between local regulatory network topology and gene expression plasticity is poorly investigated. In this study, we made use of one of the most up-to-date transcriptional regulation databases, EcoCyc (Keseler *et al.*, 2021), to retrieve regulatory interactions. Both simulated and empirical datasets were then analyzed within the same statistical framework, enabling direct comparison. We found that genes exhibiting expression plasticity were regulated by more genes on average. In addition, this study showed that selection biased the distribution of loop motifs, regardless of the level of gene expression plasticity.

Material and Methods

Empirical data: *E. coli*

We used *E. coli* K-12 substr. MG1655 annotation (RefSeq: GCF_000005845.2). *E. coli* K-12 known regulations have been obtained from Ecocyc v.28.5 (Keseler *et al.*, 2021, downloaded 29/01/2024). This release contained 9534 regulatory interactions.

To conduct our analyses, we established a list of "plastic" genes, to be contrasted to "non-plastic" genes. We defined plasticity as gene expression that respond to several environmental cues, with strong enough amplitude that it could be detected and statistically significant. Non-plasticity was attributed to genes never differentially expressed in any environmental cues considered. We selected 11 studies explicitly testing environmental conditions on gene expression in *E. coli*, and proposing lists of differentially-expressed genes. The environmental factors were pH (Tucker *et al.*, 2002; Maurer *et al.*, 2005), growth medium (urine, gluconate, and lysogeny broth) (Feugeas *et al.*, 2016), aerosolization and liquid suspension (Ng *et al.*, 2018), exposure to apple juice (Bergholz *et al.*, 2009), magnesium stress, sodium stress and carbon source (Caglar *et al.*, 2017), temperature (White-Ziegler *et al.*, 2007; Kim *et al.*, 2020), metabolic stress (low pH, low oxygen, low nutrients) (Bhatia *et al.*, 2022), oxidative stress (Wang *et al.*, 2009), and amino acid starvation (Durfee *et al.*, 2008). Genes were considered as plastic when reported as differentially expressed in at least two independent studies. By contrast genes were considered as "non-plastic" if they were never reported as differentially expressed in these studies.

Gene regulatory network simulations

We used a quantitative gene regulatory network model, inspired by A. Wagner's seminar work on gene expression robustness and plasticity (Wagner, 1994, 1996). This setting, coupling a traditional population genetics model with a gene network model as a genotype-to-phenotype map, was particularly

popular to explore the evolution of complex genetic architectures (Draghi and Wagner, 2008; Ng and Kinjo, 2023; Petit *et al.*, 2023).

Gene network The gene network model was dynamic with discrete time-steps. Genotype consisted in $n = 36$ genes encoding a gene network represented by a $n \times n$ gene regulatory matrix $\mathbf{W}$. Genes were expressed quantitatively between $P = 0$ (no expression) and $P = 1$ (maximum expression). Initial gene expressions were set to their basal level (constitutive expression) $\kappa = 0.15$, representing the gene expression in absence of regulation, and updated as a function of the concentration of the other genes in the network during 24 time steps:

$$\mathbf{P}_{t+1} = F(\mathbf{W}\mathbf{P}_t), \tag{1}$$

where $\mathbf{P}$ was a vector of n quantitative gene expressions scaled between 0 and 1 by a multivariate sigmoid function $F(x_1, \ldots, x_n) = (f(x_1), \ldots, f(x_n))$, with $f(x) = [1 + (1/\kappa - 1)\exp(-x/\kappa(1-\kappa))]^{-1}$ as in Rünneburger and Le Rouzic, 2016.

Individuals were characterized by their phenotype, consisting in the average expression of their n genes during the four last time-steps $\bar{\mathbf{P}} = [\bar{P}_1, \ldots, \bar{P}_i, \ldots, \bar{P}_n]$, with $\bar{P}_i = (1/4)\sum_{t=21}^{24} P_{it}$.

Reproduction, mutation, and selection Populations consisted in 20,000 haploid, clonally-reproducing individuals, parameterized to match approximately *E. coli*'s life cycle. Simulations were carried on for 4,000 non-overlapping generations.

The genotype $\mathbf{W}$ was affected by mutations of the regulation strength W_{ij}, at a rate of $\mu = 0.15$ per individual. Each gene had the same probability $\mu/n = 0.004$ to be affected by a mutation. A mutated gene i had a random regulation W_{ij} shifted by a Gaussian random number of standard deviation 0.05. This setting (many mutations of small effect) ensured a substantial amount of polymorphism in the population, so that evolution was not limited by mutations.

Successive generations were produced by drawing clones with a probability proportional to their relative fitness. The individual fitness w was the product of two components: $w = w_e \times w_s$. The first term w_e was determined by a stabilizing bell-shaped fitness function and decreased with the Euclidean distance to the gene expression optima $\boldsymbol{\theta}$ (Lande, 1979): $w_e = \exp(-\sum_{i=1}^{n} s_i(\overline{\mathrm{P}_i} - \theta_i)^2)$. Unless specified otherwise, the selection coefficient was $s_i = 0$ for "unselected" genes, and $s_i = 10$ for "selected" genes. The second term w_s corresponds to the selection for network stability, calculated as $w_s = \exp(-s' \sum_i^n V_i)$, where s' quantifies the selection against unstable gene expression, and $V_i = (1/4)\sum_{t=21}^{24}(\mathrm{P}_{it} - \overline{\mathrm{P}_i})^2$ was the variance in the expression of gene i during the last 4 steps of the network dynamics. We set $s' = 46,000$ (as in Rünneburger and Le Rouzic, 2016; Petit *et al.*, 2023), which was a large penalty, reflecting the popular conjecture that unstable networks are not viable (Siegal and Bergman, 2002).

Fluctuating selection and plasticity In our simulations, the environment was modeled as a numerical value E_g changing randomly every generation g, drawn from an uniform distribution $U(0.15, 0.85)$. The state of the environment was transmitted to the network via a sensor gene, as in Draghi and Whitlock, 2012; Vadée-Le-Brun *et al.*, 2015. The model implementation follows Burban *et al.*, 2022, the expression of the sensor gene was constant $P_s = E_g$ during all the network dynamics. The sensor gene was free to evolve regulations toward any genes of the network, but it was not genetically regulated, as its expression depends solely of the environment.

The other 35 network genes were divided in 4 categories, see Table 1. 10 genes were "Transcription factors" not subjected to direct selection; 10 "Plastic Target Genes" were directly selected to have their expression correlated with the environment; 10 "Non-plastic Target Genes" were directly selected to have a constant expression; and 5 unselected "neutral genes" were let free to evolve.

Selection on plastic genes was modelled by changing the gene expression optimum every generation g as a function of the environment E_g, as $\theta_i = a_i E_g + b_i$. The intercept b_i and the slope a_i were randomly drawn for each gene i at the beginning of each simulation under the constraint that optimal gene expression had to remain between 0 and 1 for any $E_g \in (0.15, 0.85)$, and stayed constant over generations. "Non-plastic" genes had their constant optimum drawn from a uniform distribution $U(0.15, 0.85)$.

The reaction norm is the function describing the trait response to an environmental variation. We measured the expression plasticity of a gene i as the slope β_i of the linear regression between a set

of environments and the corresponding gene expression $\bar{P}_i$. Simulated genes were classified into the category "plastic genes" and "non-plastic genes" according to their reaction norm slope. Genes with $|\beta_i| \geq 0.5$ were considered as plastic and genes with $|\beta_i| \leq 0.025$ as non-plastic. Genes outside of these categories were not kept during the analysis.

	Sensor gene	Transcription factor (TF)	Plastic Target gene	Constant Target gene	Neutral gene
Number	1	10	10	10	5
Selection s_i	0	0	10	10	0
Optimum θ_i			$a_i E_g + b_i$	b_i	
Evolution	Environment only	Free	Towards plasticity	Towards robustness	Drift
Regulating	All genes	All genes but sensor	TF	TF	None
Regulated by	None	All genes but Neutral	TF and Sensor	TF and Sensor	TF and Sensor

Table 1: Description of the five gene categories implemented in the gene regulatory network model.

Network Analysis

We used *E. coli* Ecocyc regulation data to construct a discrete 4638-gene network as a 4638×4638 square regulatory matrix **Ec**. A cell $\mathrm{Ec}_{ij} \in \{-1, 0, 1\}$ represents the regulation of gene j on i. Regulations that were both activating and inhibiting their target in the Ecocyc dataset were considered as null.

To study the regulatory motifs responsible for plasticity in the simulated networks with the same pipeline used for *E. coli*, we discretized the regulation matrix **W** into a matrix **Et**, based on the concept of "essentiality" (Burda *et al.*, 2011). We tested one by one every element of the **W** matrix by changing their value to 0. If it changed the reaction norm slope or the intercept by more than 0.005, then the regulation was considered as "essential": $\mathrm{Et}_{ij} = \mathrm{sign}(\mathrm{W}_{ij})$, and $\mathrm{Et}_{ij} = 0$ otherwise.

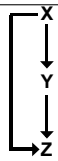
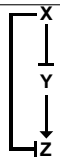
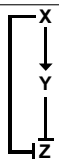
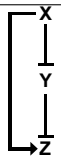
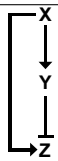
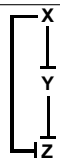
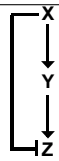
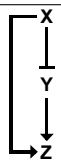
Coherent				
Nomenclature Abbreviation	Coherent 1 C1	Coherent 2 C2	Coherent 3 C3	Coherent 4 C4
Incoherent				
Nomenclature Abbreviation	Incoherent 1 I1	Incoherent 2 I2	Incoherent 3 I3	Incoherent 4 I4

Table 2: Three-gene feedforward loop nomenclature.

Motifs We investigated three loop categories: 3-gene feedforward loops (corresponding to 8 unique motifs, Table 2), four-gene diamonds loops (10 unique motifs, Table 3), and 2 to 5-gene feedbacks loops. The nomenclature of the FFLs was based on (Mangan and Alon, 2003), the genes under consideration hold the position Z (regulated gene) in the motifs. FFLs were divided into two groups, coherent and incoherent. The coherence of a motif was defined by the product of all regulations of the motif: coherent if the product was positive, incoherent otherwise (Table 2). The nomenclature of diamonds

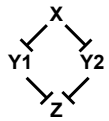
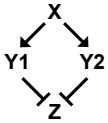
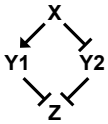
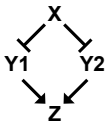
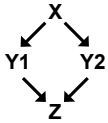
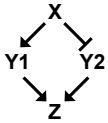
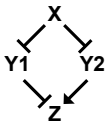
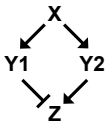
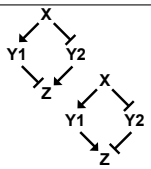
$X \rightarrow Y_n$ Sign	Negative	Positive	Mixed
Negative $Y_n \rightarrow Z$			
Nomenclature Abbreviation	Negative negative NN	Positive negative PN	Mixed negative MN
Positive $Y_n \rightarrow Z$			
Nomenclature Abbreviation	Negative positive NP	Positive positive PP	Mixed positive MP
Mixed $Y_n \rightarrow Z$			
Nomenclature Abbreviation	Negative mixed NM	Positive mixed PM	Mixed mixed 1 and 2 MM1 ; MM2

Table 3: Diamond motifs nomenclature.

was defined by the the signs of the $X \rightarrow Yn$ regulations (positive, negative, or mixed) for the first part of the name and the sign of $Yn \rightarrow Z$ for the second part (Table 3). Feedback loops were divided into subcategories by size, from 2 to 5 genes.

Control datasets We considered two types of control: drifting simulation and randomized networks. In drifting simulations, populations of 200 individuals (i.e., smaller than in the main simulations) evolved for 20000 generations with a mutation rate of $\mu = 0.4$ (larger than in the main simulations). "Shuffled" controls were obtained by randomly shuffling the values of non-null gene regulations. The number of regulations and loops toward genes were thus kept, but not the motifs.

Availability and reproducibility

The simulation program was coded in C++, and is available at https://github.com/lerouzic/simevolv. The program was compiled and executed on the IFB-core cluster (https://www.france-bioinformatique.fr/en/ifb-core-cluster/). Simulation pipelines (in R and Bash) and analysis scripts (in R) are available at https://github.com/apetit8/Topoplast.

Results

The *E. coli* regulatory network featured 3084 genes, including 326 regulators (graphic representation of the network in Suppl. Fig. 1). Out of the 4638 genes annotated in the reference annotation, 2144 (46%) genes were never reported as differentially expressed in our 11 reference studies, and were thus considered as "non-plastic". In contrast, 1074 (23%) genes were differentially expressed in at least two studies, and therefore considered as "plastic" (Suppl. Table 1 and Suppl. Fig. 2). The 1420 (31%) genes reported only once as differentially expressed were excluded from the analysis.

Computer simulations were performed by coupling a dynamic gene network model and a classical population genetics framework. At the end of the simulations, genes were classified as plastic and non-plastic based on thresholds on the slope of the reaction norm; Because the sample size with simulation was virtually unlimited, we were more stringent and rejected 55% of the genes (mild plasticity). 73% of the remaining genes were clearly plastic, and 27% were clearly non-plastic.

Regulation of plastic *vs.* non-plastic genes

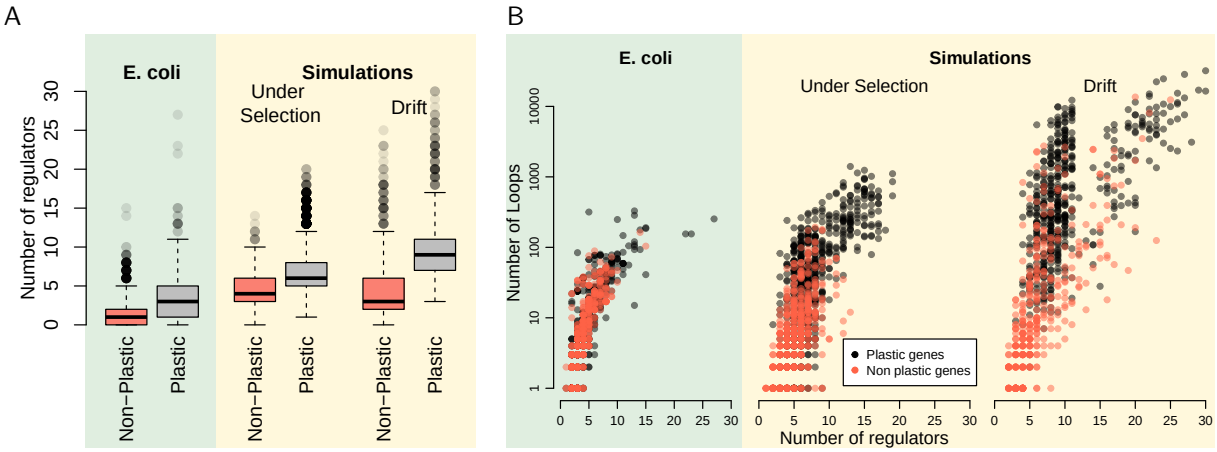


Figure 1: Regulation of plastic and non-plastic genes. A) Distribution of the number of regulations toward the genes. B) Number of feedforward, diamond, and feedback loops by which genes were regulated (only genes regulated by at least one loop are displayed). The discontinuity in the "drift" panel separates the transcription factors (which can get feedback from all the network genes, i.e. $>$ 10 regulators) and the rest of the genes which can get regulated by 10 transcription factors at most. Simulation results have been downsampled to show the same number of data points in all panels.

We first considered the number of regulators of plastic and non-plastic genes, see Fig. 1A. Plastic genes were more regulated than non-plastic genes, both in *E. coli* and in simulations. In *E. coli*, plastic genes were regulated by 3.2 genes on average (7.2 in simulations), while non-plastic genes were regulated by 1.4 genes on average (4.2 in simulations, to be compared to the strict minimum of 1 regulator necessary to deviate from the constitutive expression). The mean number of regulators of *E. coli* plastic and non-plastic genes were found significantly different by a t-test (one-sample t-test, $t = -11.3$; $df = 1480.3$, $p = 2 \cdot 10^{-16}$; statistical tests were conducted on *E. coli* data only, any difference being statistically supported in highly-replicated simulations). In absence of selection (drift simulations), genes that happened to evolve a plastic response were also characterized by a larger number of regulators, suggesting that plasticity tends to be passively induced by a larger number of regulators. In Fig. 1B, we looked at the number of feedforward, diamond, and feedback loops targeting genes. The number of loops targeting plastic genes was again larger than for non-plastic genes in both *E. coli* and in simulations, including in control simulations where mutations could accumulate freely with genetic drift. In drift simulations, there were more regulatory loops than in regular simulations for the same number of regulators, suggesting that stabilizing selection on gene expression tends to limit the number of regulatory loops.

Regulatory loops

In *E. coli*, 64% of plastic genes (Fig. 2A), and 30% of non-plastic genes (Fig. 2B) were regulated by at least one of the loop categories considered (*i.e.* feedforward, feedback, and diamonds). The most common observed pattern for *E. coli* genes (plastic or not) was the regulation by both FFLs and DMDs, with a slight excess of FFLs compared to DMDs. Feedback loops (FBLs) involving 2 to 5 genes were very rare in *E. coli* (68 genes regulated by FBLs out of 3214). Overall, the proportions of genes regulated by FFLs, DMDs, FBLs, and any combination thereof were strikingly similar between plastic and non-plastic genes, and none of these loop categories appeared to be preferentially associated with plastic gene expression.

In simulated gene networks, plastic genes were also more frequently regulated by feedforward, feedback, or diamond loops than non-plastic genes. Most genes were targeted by several categories of loops, and the proportion of FFLs *vs.* DMDs was even (Fig. 2C and D). Overall, there was no clear difference in the relative proportion of FFL and DMD involved in the regulation of plastic *vs.* non-plastic genes. FBLs were common in simulated networks (although less frequent than DMD and

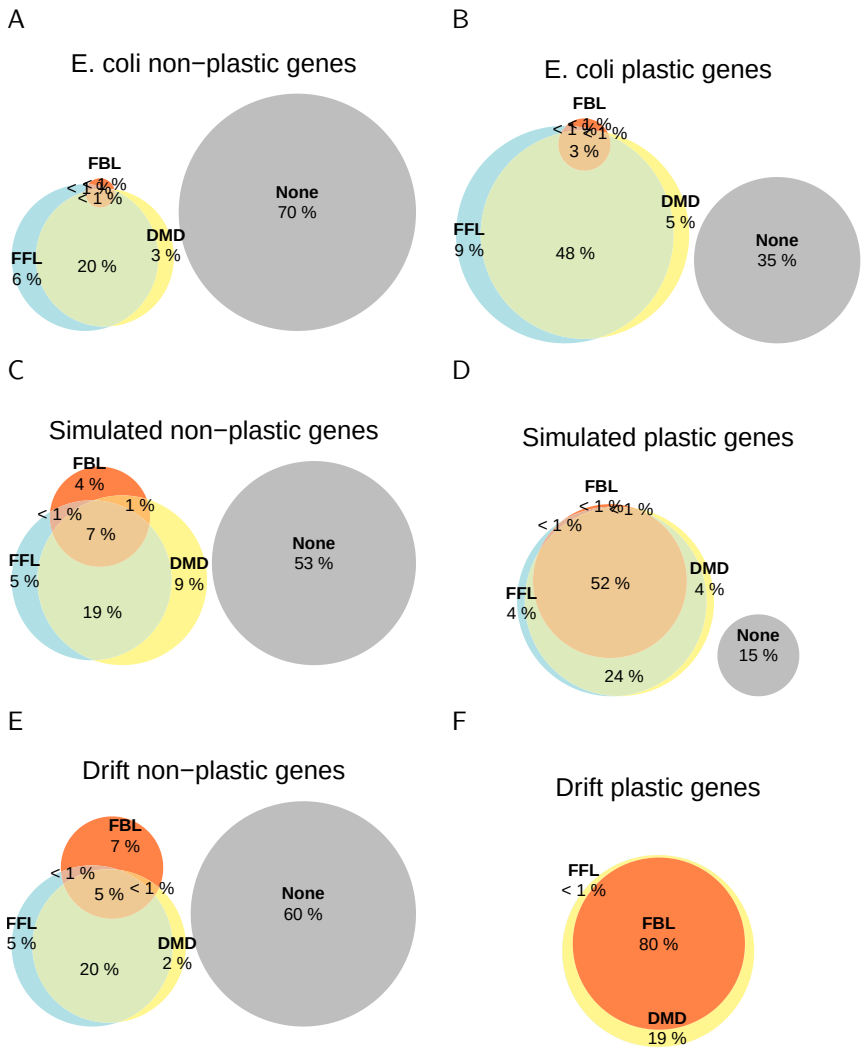


Figure 2: Genes classified by incoming regulation loops. A,C,E) Non-plastic genes in *E. coli*, simulations and drift. B,D,F) Plastic genes in *E. coli*, simulations, and drifting simulations. FFL: Feedforward loops, FBL: Feedback loops, DMD: Diamond loops. "None" stands for genes which regulators were unknown, or which were regulated by other motifs (no loops, or loops involving more or less genes than investigated here).

FBLs), and were often associated with FFL and DMD. For non-plastic genes, the regulatory loops were similar between selection and drift simulation. However, this was not the case for plastic genes, which regulation involved more FBLs and virtually no FFLs when plasticity arose by drift. This points out a substantial effect of selection on the regulation of plastic genes; stabilizing selection on gene expression tends to homogenize the regulatory network topologies around plastic and non-plastic genes.

Loop motifs occurrence

Loop categories remains a coarse-grained motif classification, as the precise regulatory function necessarily depends on the sign and the coherence of interactions. We have thus determined the motif percentage within FFL and DMD loops (8 motifs for FFLs, Table 2, and 10 motifs for DMD, Table 3), for both the *E. coli* dataset and for simulated networks. Feedback loops were too rare in *E. coli* networks (only 68), and thus not included in Fig. 3A and B.

In both *E. coli* and simulated gene networks, the most frequent feedforward motif was C1 (coherent, two genes up-regulating), followed by I1 (incoherent, one gene up-regulating and the other down-

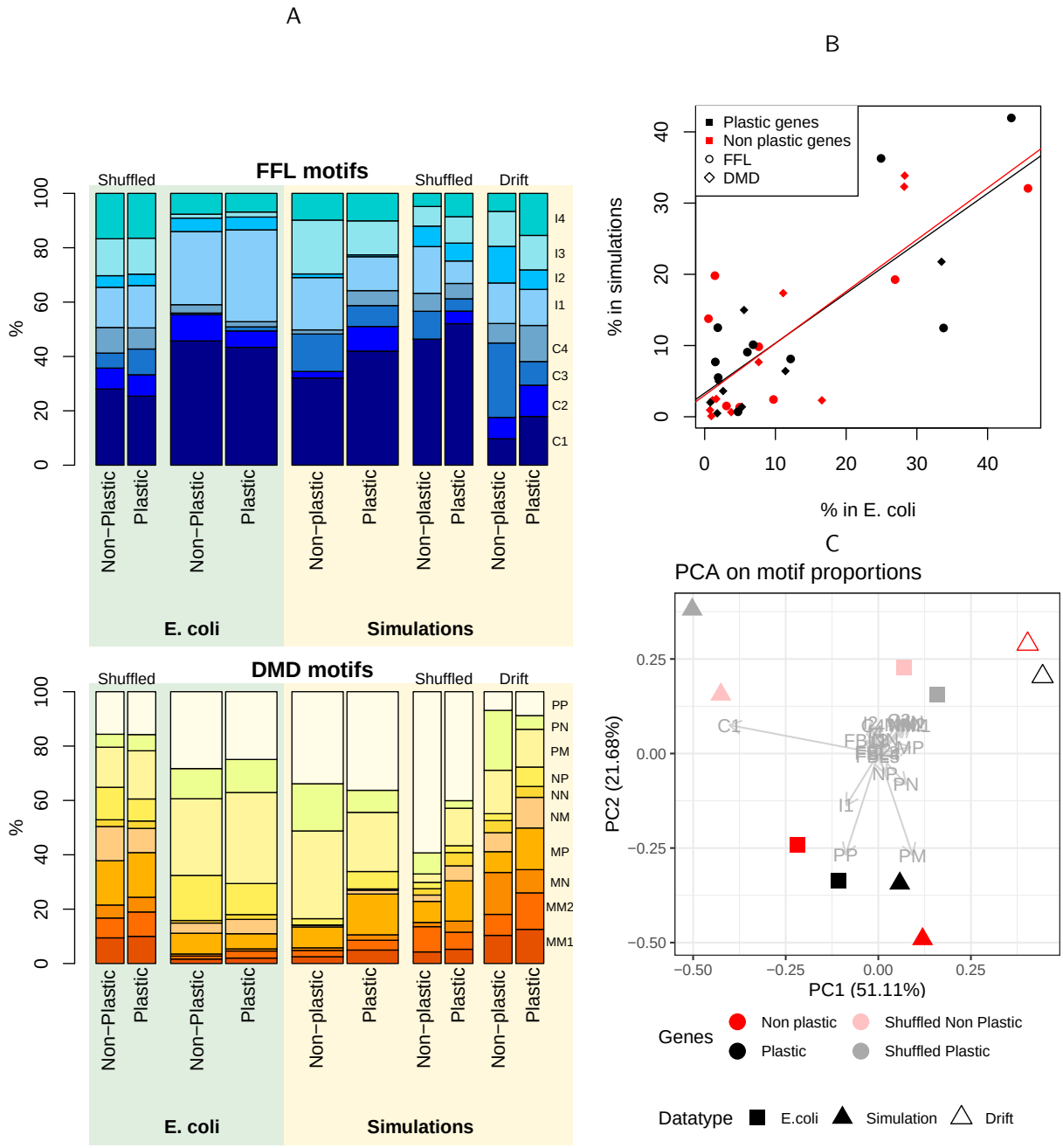


Figure 3: Loop motifs regulating plastic and non-plastic genes. A) Percentage of loop motifs within feedforward (top) and diamond (bottom) loop categories. Motif nomenclature corresponds to Tables 2 and 3. Motif percentages were normalized per gene (i.e., all genes have the same weight in the figure, regardless of the number of regulatory motifs that target them). Shuffled controls were obtained by randomly permuting all non-null interactions. Simulation runs in the absence of selection are indicated as "drift". B) Correlation plot of DMD and FFL motif frequencies (shown in panel A) between simulation and *E. coli*. C) Principal component analysis on the percentage of loop motifs (FFL, DMD and FBL).

regulating) (Fig. 3A). Other motifs were found in similar proportions, with the exception of I3, frequent in simulations but almost absent in *E. coli*. For diamond loops, the most frequent motifs in both *E. coli* and simulations were PP (up-regulated by both genes) and PM (up-regulated by one gene, down-regulated by the other one). The motifs frequency was similar among all the studies used to identify plastic genes (Suppl. Fig. 3). Motifs involving negative regulations (NN, NM, and NP) were more frequent in *E. coli* than in simulated networks; but overall, the motif distribution remained quite similar and the frequency of motifs found in *E. coli* and simulations was highly correlated, see Fig. 3B (plastic genes: $r = 0.80$; p-value $= 5.9 \cdot 10^{-5}$; non-plastic genes: $r = 0.79$; p-value $= 1.0 \cdot 10^{-4}$).

In contrast, the motif frequency distribution resulting from an evolutionary process occurring both in simulations and in *E. coli*, was substantially different from the randomly shuffled networks. In particular, the most frequent motifs (C1 and I3 for FFLs, PP and PM in DMDs) were enriched in evolved networks compared to the shuffled and drift controls. This enrichment was a consequence of selection for an optimal gene expression in simulations, and it is reasonable to postulate that the same explanation holds for *E. coli*.

While there was a clear contrast between control and selected networks, differences in motif composition between plastic and non-plastic genes for *E. coli* were non-significant. We performed Two-Proportion Z-tests for each motifs to determine which loop motifs differed between plastic and non-plastic genes. None of loop motifs were found to be significantly different in proportion between plastic and non-plastic genes after correction for multiple testing (Suppl. Table 2).

In a Principal Component Analysis on the relative proportion of each motifs of the three loop categories (Fig. 3C), PC1 (50% of the variance) was discriminating *E. coli* vs. simulations, while PC2 (22% of the variance) was associated with the contrast between selected and random networks. Plastic and non-plastic genes from the same conditions were found closer to each other than any other points, reinforcing that the contribution of plasticity to the distribution of motif frequency was minor. Moreover, *E. coli* and simulations were closer to each other than to their respective shuffled networks or drift.

Discussion

Our objective was to identify the gene regulatory topologies involved in gene expression plasticity, from both empirical and evolutionary simulated regulatory networks. We found that plastic genes indeed had specific regulatory characteristics: genes which expression depends on the environment had more regulators, and were the target of more regulatory loops. The analysis of the frequency of the different regulatory loops and regulatory motifs showed that both real and simulated networks were not random, as a consequence of selection on gene expression. The difference between plastic and non-plastic genes seems to be related to the number of regulators, but we found no evidence of an association between plasticity and regulatory motif.

Plastic genes have more regulators We identified some regulatory differences between plastic genes and non-plastic genes in both real and simulated data. On average, plastic genes were regulated by more genes, and were the target of more regulatory loops (feedforward loops, diamond loops, and feedback loops). These results reinforce previous observations, which associated the number of regulators with the complexity of the gene expression dynamics (Promislow, 2005; Mochizuki, 2008; Bei *et al.*, 2024). Nevertheless, the distributions of the number of regulators and the number of regulatory loops largely overlap between plastic and non-plastic genes, both in simulated and in empirical networks. As a consequence, the number of regulators remains a poor predictor of gene expression plasticity.

An obvious advantage from simulations to complement the analysis of empirical network lies in the possibility to go beyond association statistics. We showed for instance that plasticity was statistically associated with a higher number of regulators, even in the drift simulations. We suspect here that increasing the number of regulators increases the probability to connect the gene to a sub-network influenced by the environmental factor, thus enriching the proportion of plastic genes among highly-connected genes. We propose the following scenario for the changes in the regulatory networks associated with the evolution of plasticity: selection acting on plastic gene expression directly promotes the recruitment of *cis*-regulators, which indirectly increases the number of regulatory loops. Thus, the reported association between some regulatory loops like FFL with plasticity may only be a consequence of the higher number of regulation towards plastic genes.

We also obtained interesting contrasts between selected and drift simulations, since selection tends to (i) decrease the number of loops for a given number of regulators, and (ii) homogenize the distribution of loops and loop motifs between plastic and non-plastic genes. Selection for gene expression control thus implies the elimination of supernumerary and/or inadequate loops and loop motifs, and the enrichment in motifs such as C1, I1, PP, and PM. C1 and I1 have for instance been predicted to be efficient at buffering expression noise (Ghosh *et al.*, 2005; Osella *et al.*, 2011; Reeves, 2019; Chakravarty and Csikász-Nagy, 2021). Strikingly, this selection-related enrichment makes plastic and non-plastic gene regulation more similar than what could be observed without selection. Therefore, the regulation of plastic vs. non-plastic genes could theoretically be different (as we observed in the drift simulations for instance), but they happen to be similar because the regulatory structure was overwhelmed by the necessity to control for gene expression (for a fixed or an environmentally-driven optimal value, indifferently).

The *E. coli* motif distribution was consistent with previous work (Mangan *et al.*, 2006; Cooper *et al.*, 2008), who also identified C1 and I1 as the most represented motifs. Up-regulating motifs (including C1 for feed-forward loops or PP for diamond loops) were over-represented in *E. coli* compared to randomly-permuted networks, suggesting some specific preference for such motifs. However the high frequency of up-regulating motifs (including C1 for feed-forward loops or PP for diamond loops) could also partially be a statistical consequence of the uneven ratio between up- and down-regulation within the network. "Pure" up-regulating motifs C1 and PP were indeed rare in simulations evolving by drift only, featured by an equal number of up- and down-regulation interactions. Yet, arguments related to up/down-regulation ratios are fragile, as it is difficult to discard that the uneven balance could be a consequence, rather than a cause, of an evolutionary bias towards specific motifs. In our simulations, the gene network dynamics was deterministic, so that it was impossible to disentangle the control of the equilibrium expression from the control of random perturbations (noise). Alternative theoretical settings as proposed in Draghi and Whitlock, 2015; Schmutzer and Wagner, 2020 could be considered in the future to address this interesting issue.

Theoretical and empirical results are converging Systems biology is rather convincing at describing general patterns in biological networks, based on measurement tools derived from graph theory (Mason and Verwoerd, 2007; Pavlopoulos *et al.*, 2011). For instance, it is often admitted that biological networks tend to be scale-free (Babu *et al.*, 2004), relatively scarce (Espinosa-Soto, 2018), and modular (Wagner *et al.*, 2007). Yet, there is a surprising lack of theory from which such patterns could be interpreted. Typically, the extent to which network evolution is driven by mutation bias, genetic drift, adaptation, or indirect selection remains widely unknown (Tsuda and Kawata, 2010; Halfon, 2017; Petit *et al.*, 2023). Here, we show how a simplified gene network evolutionary model, generally exploited to address genetic architecture-related questions, can nourish the field of systems biology and predict empirical patterns. Our simulations could retrieve, both qualitatively and quantitatively, the larger number of regulatory genes for plastic genes, motif enrichment and depletion in selected *vs.* randomized networks, the most abundant motifs, and the relationship between the number of regulation and number of motifs. This validates the capacity of such simple models in which gene regulations are encoded in an interaction matrix (Wagner, 1994; Fierst, 2011), to contribute to the theoretical corpus on gene network evolution, in complement to the more cumbersome physiologically-realistic models (Xiong *et al.*, 2019).

Nevertheless, we noticed some differences between empirical and simulations results, that might be worthwhile to explore. First, the most obvious qualitative difference between both approaches was the $\sim$10-fold decrease in the frequency of feedback loops in *E. coli* regulation network compared to simulations. Although feedback regulation exists in *E. coli* and is known to be involved into noise reduction or bistability (Smits *et al.*, 2006), feedback loops happen to be outnumbered by feedforward loops in the Ecocyc transcriptional dataset and in pathway databases in general (Karp, 2001; Kumar *et al.*, 2018). A possible explanation for this discrepancy lies in the fact that most feedback mechanisms in prokaryotes rely on autoregulation (in *E. coli*, 50% of TFs self-regulate, Rosenfeld *et al.*, 2002), while the minimum size for a motif in our analysis was set to 2 genes. Secondly, a more subtle discrepancy between theoretical and empirical networks was that simulations predicted a small but systematic difference between motif frequencies in plastic vs. non-plastic genes, which was not statistically supported in *E. coli*. Insufficient statistical power could partly explain this observation, but more generally, the classification of *E. coli* genes into plastic and non-plastic categories remained coarse-grained, and may

not match the same definition of plasticity; simulations define plastic genes based on the slope of the reaction norm to a single environmental factor, while in *E. coli* we defined plastic genes based on their differential expression to different environmental treatments, which might not reflect exactly the same mechanisms. The observed convergence between theoretical and empirical observations in spite of these different measurements of plasticity strengthens our conclusions.

In our model, the ratio of up- vs. down-regulation interactions was not a parameter, but rather the outcome of the evolutionary process. As a result, this rate could not be easily controlled and ended up to differ from the observed network database (78% of up-regulation in simulations vs. 61% in *E. coli*). This difference limits the robustness of quantitative conclusions, as it conditions the expected proportions of the loop motifs (differences in the "shuffled" control in simulations vs. *E. coli*). We are therefore confident that the striking correlation between motif frequencies in simulations vs. *E. coli* reflects the capacity of the model to simulate realistic evolutionary trajectories, but we cannot be certain that this convergence shoud be attributed to analogous selection pressures since motif frequencies had to evolve from a different baseline.

Conclusion If not the network topology, which regulatory features could reveal whether the expression of a specific gene has been selected for robustness, stability, cyclic expression, or sensitivity to an environmental or physiological input? To what extent is it possible to predict gene expression profiles from pathway databases (Fang *et al.*, 2017; Mercatelli *et al.*, 2020; Escorcia-Rodríguez *et al.*, 2023)? Three major hypotheses can be proposed: (i) the information about network topologies involved in plasticity is present in existing databases, but they were not caught by known descriptors of network topologies and properties (loops, motifs, or graph metrics). For instance, it might depend on a more general regulatory context (Lipshtat *et al.*, 2008; Jiang and Hao, 2021), or on complex combinations of several intertwined network topologies (Chakravarty and Csikász-Nagy, 2021). Alternatively, (ii) regulation databases remain the correct approach, but existing databases are not precise nor exhaustive enough. Typically, presence/absence data for regulatory interactions might be insufficient, and quantitative estimation of interaction strengths (weighted edges in the network graph (Freyre-González *et al.*, 2022)) could be necessary as suggested by others (Ingram *et al.*, 2006; Panovska-Griffiths *et al.*, 2013; Kim and Wysocka, 2023). The last hypothesis (iii) presumes that regulation might be too complex to be abstracted in regulatory networks, which omit important epigenetic and cellular mechanisms, including the spatial organization of the genome, methylation, phosphorylation, dimerization (Klumpe *et al.*, 2023), protein conformation, or "missing regulation" from intergenic sequences (Connally *et al.*, 2022). The next challenge for the systems biology community may therefore not be to generate more data, but to identify which data is relevant to understand the evolutionary properties of biological systems, a task for which theoretical approaches might bring invaluable insights.

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Conflict of interest

The authors declare no conflict of interest.

References

Alon U. 2007. Network motifs: theory and experimental approaches. Nature Reviews Genetics. 8:450.

Babu MM, Luscombe NM, Aravind L, Gerstein M, Teichmann SA. 2004. Structure and evolution of transcriptional regulatory networks. Current opinion in structural biology. 14:283–291.

Basu S, Mehreja R, Thiberge S, Chen MT, Weiss R. 2004. Spatiotemporal control of gene expression with pulse-generating networks. Proceedings of the National Academy of Sciences. 101:6355–6360.

Bei C, Zhu J, Culviner PH, Gan M, Rubin EJ, Fortune SM, Gao Q, Liu Q. 2024. Genetically encoded transcriptional plasticity underlies stress adaptation in Mycobacterium tuberculosis. Nature Communications. 15:3088.

Bergholz TM, Vanaja SK, Whittam TS. 2009. Gene expression induced in *Escherichia coli* O157:H7 upon exposure to model apple juice. Applied and Environmental Microbiology. 75:3542–3553.

Bhatia RP, Kirit HA, Predeus AV, Bollback JP. 2022. Transcriptomic profiling of *Escherichia coli* K-12 in response to a compendium of stressors. Scientific Reports. 12:1–14.

Burban E, Tenaillon MI, Le Rouzic A. 2022. Gene network simulations provide testable predictions for the molecular domestication syndrome. Genetics. 220:iyab214.

Burda Z, Krzywicki A, Martin OC, Zagorski M. 2011. Motifs emerge from function in model gene regulatory networks. Proceedings of the National Academy of Sciences of the United States of America. 108:17263–17268.

Caglar MU, Houser JR, Barnhart CS, Boutz DR, Carroll SM, Dasgupta A, Lenoir WF, Smith BL, Sridhara V, Sydykova DK *et al*. 2017. The *E. coli* molecular phenotype under different growth conditions. Scientific Reports. 7:1–15.

Chakravarty S, Csikász-Nagy A. 2021. Systematic analysis of noise reduction properties of coupled and isolated feedforward loops. PLoS Computational Biology. 17:1–15.

Connally NJ, Nazeen S, Lee D, Shi H, Stamatoyannopoulos J, Chun S, Cotsapas C, Cassa CA, Sunyaev SR. 2022. The missing link between genetic association and regulatory function. eLife. 11:1–35.

Cooper MB, Loose M, Brookfield JF. 2008. Evolutionary modelling of feed forward loops in gene regulatory networks. BioSystems. 91:231–244.

de Ronde WH, Tostevin F, ten Wolde PR. 2012. Feed-forward loops and diamond motifs lead to tunable transmission of information in the frequency domain. Physical Review E. 86:021913.

Draghi J, Wagner GP. 2008. Evolution of evolvability in a developmental model. Evolution: International Journal of Organic Evolution. 62:301–315.

Draghi J, Whitlock M. 2015. Robustness to noise in gene expression evolves despite epistatic constraints in a model of gene networks. Evolution. 69:2345–2358.

Draghi JA, Whitlock MC. 2012. Phenotypic plasticity facilitates mutational variance, genetic variance, and evolvability along the major axis of environmental variation. Evolution: International Journal of Organic Evolution. 66:2891–2902.

Durfee T, Hansen AM, Zhi H, Blattner FR, Ding JJ. 2008. Transcription profiling of the stringent response in *Escherichia coli*. Journal of Bacteriology. 190:1084–1096.

Escorcia-Rodríguez JM, Gaytan-Nuñez E, Hernandez-Benitez EM, Zorro-Aranda A, Tello-Palencia MA, Freyre-González JA. 2023. Improving gene regulatory network inference and assessment: The importance of using network structure. Frontiers in Genetics. 14:1–14.

Espinosa-Soto C. 2018. On the role of sparseness in the evolution of modularity in gene regulatory networks. PLoS computational biology. 14:e1006172.

Fang X, Sastry A, Mih N, Kim D, Tan J, Yurkovich JT, Lloyd CJ, Gao Y, Yang L, Palsson BO. 2017. Global transcriptional regulatory network for *Escherichia coli* robustly connects gene expression to transcription factor activities. Proceedings of the National Academy of Sciences of the United States of America. 114:10286–10291.

Feugeas JP, Tourret J, Launay A, Bouvet O, Hoede C, Denamur E, Tenaillon O. 2016. Links between Transcription, Environmental Adaptation and Gene Variability in *Escherichia coli*: Correlations between Gene Expression and Gene Variability Reflect Growth Efficiencies. Molecular Biology and Evolution. 33:2515–2529.

Fierst JL. 2011. A history of phenotypic plasticity accelerates adaptation to a new environment. Journal of evolutionary biology. 24:1992–2001.

Frei T, Khammash M. 2021. Adaptive circuits in synthetic biology. Current Opinion in Systems Biology. 28:100399.

Freyre-González JA, Escorcia-Rodríguez JM, Gutiérrez-Mondragón LF, Martí-Vértiz J, Torres-Franco CN, Zorro-Aranda A. 2022. System Principles Governing the Organization, Architecture, Dynamics, and Evolution of Gene Regulatory Networks. Frontiers in Bioengineering and Biotechnology. 10.

Ghosh B, Karmakar R, Bose I. 2005. Noise characteristics of feed forward loops. Physical Biology. 2:36–45.

Halfon MS. 2017. Perspectives on Gene Regulatory Network Evolution. Trends in Genetics. 33:436–447.

Hornung G, Barkai N. 2008. Noise Propagation and Signaling Sensitivity in Biological Networks: A Role for Positive Feedback. PLoS Computational Biology. 4:e8.

Ingram PJ, Stumpf MP, Stark J. 2006. Network motifs: structure does not determine function. BMC Genomics. 7:108.

Jiang Y, Hao N. 2021. Memorizing environmental signals through feedback and feedforward loops. Current Opinion in Cell Biology. 69:96–102.

Kadelka C, Murrugarra D, Laubenbacher R. 2013. Stabilizing gene regulatory networks through feed-forward loops. Chaos: An Interdisciplinary Journal of Nonlinear Science. 23:1–19.

Karp PD. 2001. Pathway databases: a case study in computational symbolic theories. Science. 293:2040–2044.

Keseler IM, Gama-Castro S, Mackie A, Billington R, Bonavides-Martínez C, Caspi R, Kothari A, Krummenacker M, Midford PE, Muñiz-Rascado L *et al*. 2021. The ecocyc database in 2021. Frontiers in Microbiology. 12.

Khammash MH. 2021. Perfect adaptation in biology. Cell Systems. 12:509–521.

Kim S, Kim Y, Suh DH, Lee CH, Yoo SM, Lee SY, Yoon SH. 2020. Heat-responsive and time-resolved transcriptome and metabolome analyses of *Escherichia coli* uncover thermo-tolerant mechanisms. Scientific Reports. 10:1–13.

Kim S, Wysocka J. 2023. Deciphering the multi-scale, quantitative cis-regulatory code. Molecular Cell. 83:373–392.

Klumpe HE, Garcia-Ojalvo J, Elowitz MB, Antebi YE. 2023. The computational capabilities of many-to-many protein interaction networks. Cell Systems. 14:430–446.

Kumar S, Mahajan S, Jain S. 2018. Feedbacks from the metabolic network to the genetic network reveal regulatory modules in E. coli and B. subtilis. PLOS ONE. 13:e0203311.

Kwon YK, Cho KH. 2007. Analysis of feedback loops and robustness in network evolution based on Boolean models. BMC Bioinformatics. 8:430.

Lande R. 1979. Quantitative genetic analysis of multivariate evolution, applied to brain: body size allometry. Evolution. 33:402–416.

Lipshtat A, Purushothaman SP, Iyengar R, Ma'ayan A. 2008. Functions of bifans in context of multiple regulatory motifs in signaling networks. Biophysical Journal. 94:2566–2579.

Ma W, Trusina A, El-Samad H, Lim WA, Tang C. 2009. Defining Network Topologies that Can Achieve Biochemical Adaptation. Cell. 138:760–773.

Mangan S, Alon U. 2003. Structure and function of the feed-forward loop network motif. Proceedings of the National Academy of Sciences of the United States of America. 100:11980–11985.

Mangan S, Itzkovitz S, Zaslaver A, Alon U. 2006. The incoherent feed-forward loop accelerates the response-time of the gal system of *Escherichia coli*. Journal of Molecular Biology. 356:1073–1081.

Mason O, Verwoerd M. 2007. Graph theory and networks in biology. IET systems biology. 1:89–119.

Maurer LM, Yohannes E, Bondurant SS, Radmacher M, Slonczewski JL. 2005. pH regulates genes for flagellar motility, catabolism, and oxidative stress in *Escherichia coli* K-12. Journal of Bacteriology. 187:304–319.

Mercatelli D, Scalambra L, Triboli L, Ray F, Giorgi FM. 2020. Gene regulatory network inference resources: A practical overview. Biochimica et Biophysica Acta - Gene Regulatory Mechanisms. 1863:194430.

Mochizuki A. 2008. Structure of regulatory networks and diversity of gene expression patterns. Journal of Theoretical Biology. 250:307–321.

Ng ETH, Kinjo AR. 2023. Plasticity-led evolution as an intrinsic property of developmental gene regulatory networks. Scientific Reports. 13:1–19.

Ng TW, Ip M, Chao CY, Tang JW, Lai KP, Fu SC, Leung WT, Lai KM. 2018. Differential gene expression in *Escherichia coli* during aerosolization from liquid suspension. Applied Microbiology and Biotechnology. 102:6257–6267.

Osella M, Bosia C, Corá D, Caselle M. 2011. The Role of Incoherent MicroRNA-Mediated Feedforward Loops in Noise Buffering. PLoS Computational Biology. 7:e1001101.

Panovska-Griffiths J, Page KM, Briscoe J. 2013. A gene regulatory motif that generates oscillatory or multiway switch outputs. Journal of the Royal Society Interface. 10.

Pavlopoulos GA, Secrier M, Moschopoulos CN, Soldatos TG, Kossida S, Aerts J, Schneider R, Bagos PG. 2011. Using graph theory to analyze biological networks. BioData mining. 4:1–27.

Petit AJR, Guez J, Le Rouzic A. 2023. Correlated stabilizing selection shapes the topology of gene regulatory networks. Genetics. 224. iyad065.

Promislow D. 2005. A regulatory network analysis of phenotypic plasticity in yeast. The American Naturalist. 165:515–523.

Reeves GT. 2019. The engineering principles of combining a transcriptional incoherent feedforward loop with negative feedback. Journal of Biological Engineering. 13:62.

Rosenfeld N, Elowitz MB, Alon U. 2002. Negative autoregulation speeds the response times of transcription networks. Journal of molecular biology. 323:785–793.

Roy S, Raj M, Ghosh P, Das SK. 2017. Role of motifs in topological robustness of gene regulatory networks. IEEE International Conference on Communications. 1.

Rünneburger E, Le Rouzic A. 2016. Why and how genetic canalization evolves in gene regulatory networks. BMC evolutionary biology. 16:239.

Schmutzer M, Wagner A. 2020. Gene expression noise can promote the fixation of beneficial mutations in fluctuating environments. PLoS computational biology. 16:e1007727.

Sen S, Kim J, Murray RM. 2014. Designing robustness to temperature in a feedforward loop circuit. In: . volume 2015-Febru. pp. 4629–4634. IEEE.

Shen-Orr SS, Milo R, Mangan S, Alon U. 2002. Network motifs in the transcriptional regulation network of *Escherichia coli*. Nature Genetics. 31:64–68.

Siegal ML, Bergman A. 2002. Waddington's canalization revisited: developmental stability and evolution. Proceedings of the National Academy of Sciences. 99:10528–10532.

Smits WK, Kuipers OP, Veening JW. 2006. Phenotypic variation in bacteria: the role of feedback regulation. Nature Reviews Microbiology. 4:259–271.

Tsuda ME, Kawata M. 2010. Evolution of gene regulatory networks by fluctuating selection and intrinsic constraints. PLoS computational biology. 6:e1000873.

Tucker DL, Tucker N, Conway T. 2002. Gene expression profiling of the pH response in *Escherichia coli*. Journal of Bacteriology. 184:6551–6558.

Vadée-Le-Brun Y, Rouzaud-Cornabas J, Beslon G. 2015. Epigenetic inheritance speeds up evolution of artificial organisms. In: . pp. 439–446. MIT Press.

Vlková M, Silander OK. 2022. Gene regulation in Escherichia coli is commonly selected for both high plasticity and low noise. Nature Ecology & Evolution. 6:1165–1179.

Wagner A. 1994. Evolution of gene networks by gene duplications: a mathematical model and its implications on genome organization. Proceedings of the National Academy of Sciences. 91:4387–4391.

Wagner A. 1996. Genetic redundancy caused by gene duplications and its evolution in networks of transcriptional regulators. Biological cybernetics. 74:557–567.

Wagner GP, Pavlicev M, Cheverud JM. 2007. The road to modularity. Nature Reviews Genetics. 8:921–931.

Wang J, Belta C, Isaacson SA. 2020. How Retroactivity Affects the Behavior of Incoherent Feedforward Loops. iScience. 23:101779.

Wang S, Deng K, Zaremba S, Deng X, Lin C, Wang Q, Tortorello ML, Zhang W. 2009. Transcriptomic response of *Escherichia coli* O157:H7 to oxidative stress. Applied and Environmental Microbiology. 75:6110–6123.

Weldemichael T, Asemoloye MD, Marchisio MA. 2022. Feedforward Loops: Evolutionary Conserved Network Motifs Redesigned for Synthetic Biology Applications. Applied Sciences. 12:8292.

White-Ziegler CA, Malhowski AJ, Young S. 2007. Human body temperature (37°C) increases the expression of iron, carbohydrate, and amino acid utilization genes in *Escherichia coli* K-12. Journal of Bacteriology. 189:5429–5440.

Xiong K, Lancaster AK, Siegal ML, Masel J. 2019. Feed-forward regulation adaptively evolves via dynamics rather than topology when there is intrinsic noise. Nature Communications. 10:2418.