Stress induces biphasic-rewiring and modularization patterns in the metabolomic networks of *Escherichia coli*

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Abstract— **Metabolomic networks describe correlated change in metabolite levels that crucially link the transcriptome and proteome with the complex matter and energy dynamics of small molecule metabolism. These networks are atypical. They do not directly portray regulatory and pathway information, yet they embed both. Here we study how stress rewires the metabolomic networks of *Escherichia coli*. Networks with vertices describing metabolites and edges representing correlated changes in metabolite concentrations were used to study time resolved bacterial responses to four non-lethal stress perturbations, cold, heat, lactose diauxie, and oxidative stress. We find notable patterns that are common to all stress responses examined: (1) networks are random rather than scale-free, i.e. metabolite connectivity is dictated by large network components rather than 'hubs'; (2) networks rewire quickly even in the absence of stress and are therefore highly dynamic; (3) rewiring occurs minutes after exposure to the stressor and results in significant decreases in network connectivity, and (4) at longer time frames connectivity is regained. The common biphasic-rewiring pattern revealed in our time-resolved exploration of metabolite connectivity also uncovers unique structural and functional features. We find that stress-induced decreases in connectivity were always counterbalanced by increases in network modularity. Remarkably, rewiring begins with energetics and carbon metabolism that is needed for growth and then focuses on lipids, hubs and metabolic centrality needed for membrane restructuring. While these patterns may simply represent the need of the cell to stop growing and to prepare for uncertainty, the biphasic modularization of the network is an unanticipated result that links the effects of environmental perturbations and the generation of modules in biology.**

Keywords- Dynamic behavior; environmental perturbation; metabolism; metabolite; metabolomics; module; network connectivity; random networks.

I. INTRODUCTION

Organisms interact with their environment and respond to environmental perturbations by implementing changes in their physiology at both cellular and molecular levels. Some environmental perturbations can be damaging and can compromise cellular persistence. Responses to stresses of these kinds require fast adjustments of physiology, which can be specific or general. Specific stress responses minimize deleterious effects and repair damage, including catalase–mediated responses during oxidative damage or chaperone-mediated repair of proteins subjected to temperature stress. Examples include specific thermosensory systems that exist in bacteria, such as the heat shock response (HSR) that involves molecular chaperones and proteases [1]. General stress responses down regulate genes controlling central cellular processes, including translation, and usually result in growth reduction. For example, stress responses in *E. coli* are mediated by general response regulators such as Sigma S (RpoS) [2], which controls the expression of hundreds of genes involved in catalysis, protein processing, transport, and transcriptional regulation [3], and (p)ppGpp, the global regulator responsible for the 'stringent response' that tunes metabolism [4], typically during depletion of amino acids and carbon starvation [5].

Metabolic responses to environmental stimuli are fundamental since they modulate the energetic status of the cell and control metabolic balance, biosynthesis of cofactors, and complex biological processes involving transcription factors, translational and RNA regulators, chromatin structure, and ion channels [6]. Metabolic networks operate in concert with the transcriptome and proteome [7,8]. Representative metabolites behave as reporters that bridge the worlds of small molecules and macromolecules. Molecules such as ATP, NAD(H) and S-adenosylmethionine (SAM) are not only metabolite hubs of interconnectivity but they are also general regulators that relay for example energy shortages to kinase signaling pathways, which ultimately halt cellular growth and prevent cellular collapse (e.g., the rapamycin [TOR] kinase and AMP-activated protein kinase signaling; [9,10]). The NAD(H) balance signals broad physiological changes and impacts a multiplicity of regulatory processes, including histone deacetylation and poly-ADP-ribosylation-dependent methylation important for post-translation chromatin modifications (reviewed in [6]). Calcium signaling, transcriptional regulation and the immune response are also linked to this global metabolite regulator, which reports the overall metabolic state of the cell.

Metabolic networks can be subdivided into modules [11,12]. These modules are generally metabolite subsets that are highly interconnected and have similar functional roles [13,14]. They represent structural and functional units that are generally robust to perturbations [15,16]. Metabolic modules are also connected by regulatory processes and adapt within a large space of possible phenotypes [6]. They likely arise as a consequence of periodic environmental change [17,18]. Reporter metabolites that belong to these modules respond to environmental perturbations that are

usually transient, reconfiguring the network structure of metabolic fluxes within the confines of varying concentrations and kinetics. These dynamic responses can be captured by global correlations between metabolite concentrations and graph theoretical approaches [19,20]. These metabolomic networks describe correlated change in metabolite levels that crucially link the transcriptome and proteome with the complex matter and energy dynamics of small molecule metabolism [21]. While complex, these correlations are the consequence of the underlying network of enzymatic reactions and metabolite balance linked to the physiology of the cell. Here we study how non-lethal stress rewires the metabolomic networks of *E. coli*. Using time resolved bacterial responses to different stress perturbations we reveal that stress elicits biphasic-rewiring patterns in the metabolite networks that are very dynamic. We argue these patterns are the natural consequence of metabolic modularity.

II. Materials and Methods

Metabolomic data retrieved using gas chromatography-mass spectrometry (GC-MS) was obtained directly from Jozefczuk et al. [22]. The data set comprised metabolite concentrations for 188 metabolites and 545 observations, from which 95 metabolites could be positively identified, 58 were chemically classified, and 40 were unknown. For the purpose of this study we used 95 metabolites that were measured in triplicates as technical replicates within three independent biological replicates at each time point over the course of 90 min. Correlations among the metabolites and within each time point were performed using the CORR procedure of SAS (version 9.2; SAS Institute Inc., Cary, NC). We used replicates as independent biological samples, instead of taking the mean or median for the replicates, in order to avoid having more variables than observations. We applied significance thresholds of $p \leq 0.05$ and removed weak correlations ($-0.6 < r < 0.6$). Such stringency guarantees a conservative network structure, with related metabolites being controlled by the same enzyme system. Metabolite correlation networks were visualized using PAJEK [23]. To assess how metabolites are related to pathways, we used the subnetwork classification scheme provided by KEGG [24], which includes nine major pathway maps: carbohydrate metabolism, energy metabolism, lipid metabolism, nucleotide metabolism, amino acid metabolism, glycan biosynthesis and metabolism, metabolism of cofactors and vitamins, biosynthesis of secondary metabolites and biodegradation of xenobiotics. Network modularity was assessed using measurements of community structure [25,26]. The modularity of metabolite graphs with respect to some division of vertices measures how good is the division or how separated are vertex types from each other. Networks were also shrunk to combine all vertices of the same functional class. Vertex and edge size were made proportional to the extent of connectivity to allow a coarse grained view of the networks. Supplementary information (SI) and high-resolution images of networks can be found at: http://www.manet.illinois.edu/reference.php.

III. Results and Discussion

A. Metabolomic networks are constrained by stress responses

Stress responses have been widely studied in *E. coli*, notably because laboratory strains are easy to manipulate [27,28]. However, most studies have focused on microarray-based transcript profiling. Stress responses at metabolomic level have not been adequately explored. We have taken advantage of an integrated metabolic and transcript profiling analysis of stress responses that was recently published [22]. In this study, a gas GC-MS platform was used to measure metabolite changes in response to: (1) cold treatment (16° C), (2) heat treatment (45° C), (3) glucose-lactose diauxic shift, and (4) oxidative stress induced by hydrogen peroxide. An unperturbed bacterial culture was used as control. Changes along a time resolved series that started in mid-log phase of growth and lasted until advanced stationary phase were used to explore the structural and functional make up of the metabolic network of *E. coli*. Experimental conditions were repeated 3 times and 3 technical samplings were taken from biological end points along the time series, totaling >550 samples. Metabolite concentrations were sampled in 2-3 time points prior to perturbation and 9-10 time points (10-260 min) after perturbation. A total of 95 metabolites (out of 188) could be positively identified, and these formed the basis for co-clustering and canonical correlation analysis on combined metabolite and transcript data in the original study.

Here we re-examine stress-induced patterns in metabolomic data by visualizing changes in metabolite concentration using metabolomic correlation networks [19-21]. In these graph representations, metabolites are defined as vertices and any two metabolites showing strong correlation in their concentration levels are represented by vertices joined by an edge in the graph. This approach is superior to clustering or principal component approaches. Correlations are made explicit and the dynamic aspects of stress-induced change can be quantified by studying connectivity and modularity parameters in the network structure. We selected time points 1 (prior to perturbation), 2 (at perturbation), 6 (40 min after perturbation) and 8 (90 min after perturbation) as representative biological end points (SI Table I), and produced metabolomic networks for each of the four stresses and the control (SI Fig. 1). Figure 1A illustrates with the glucose-lactose diauxic shift the timeline of correlation networks and corresponding 'reduced graphs', in which components linked by related biochemistries are shrank into a vertex. Remarkably, these networks show significant changes in metabolite connectivity and a clear biphasic pattern in the network make up, with a decrease in metabolite vertex connectivity occurring at time points 2 and 6 and an increase at time point 8. These 'hourglass' patterns were similarly observed in all stress responses, but were absent in the control, which showed only a slight increasing trend overall (Fig. 1B). For example, at time point 1 of the diauxic shift we see a cluster of highly connected modules that apportion 'carbohydrate' (blue), 'energy' (green), 'amino acid' (purple), and 'hub' (yellow) metabolites and

place more in the periphery 'lipid' (red) and other functions. We note that the highly connected 'hub' metabolites participate in numerous metabolic subnetworks and appear central. At time point 2 the cluster splits in two and at time point 6 in three, in both cases forming less connected (diffuse) arrangements of metabolites and harboring 'lipid' functions that were before peripheral in one of the subclusters. At time point 8 the trend reverts and now the three clusters join in a single highly connected and dense cluster that is again highly populated with 'hub' and other metabolites but is now markedly rewired. Similar trends were observed in other stress responses (SI Fig. 1).

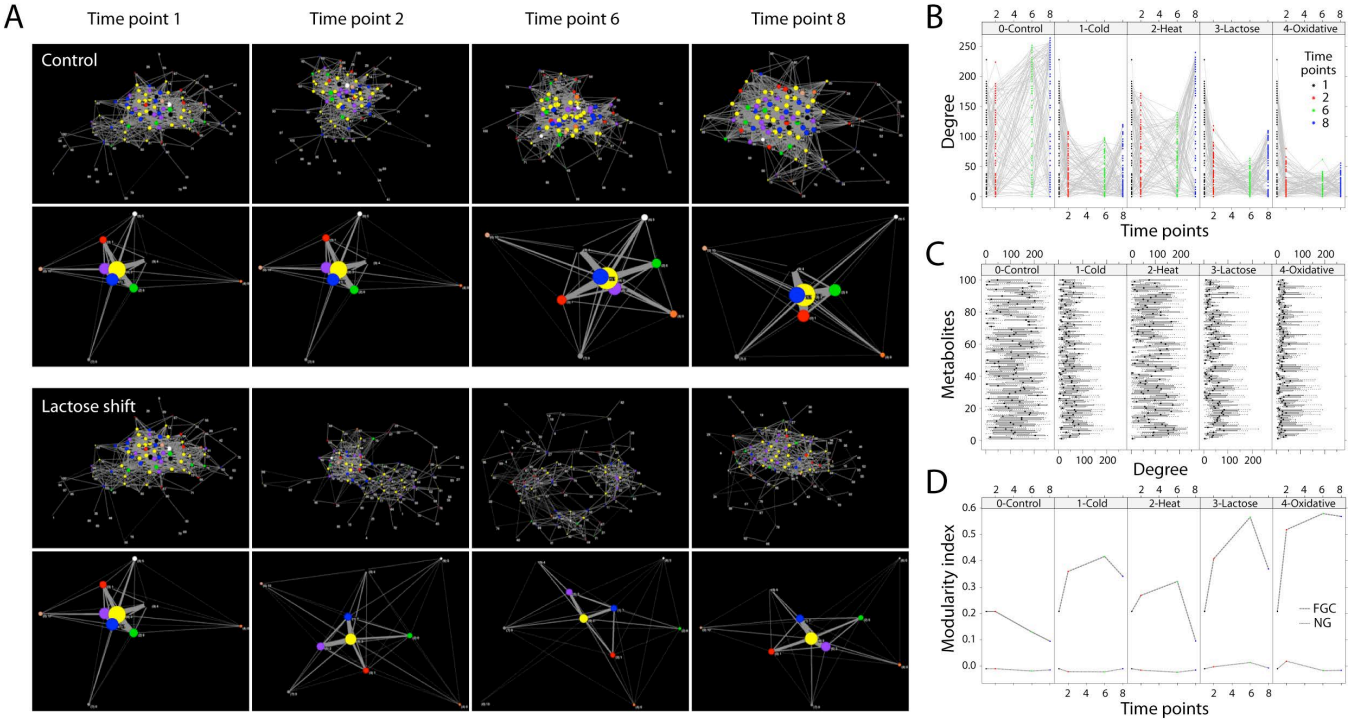

Figure 1. Metabolomic networks. A. Example timelines of metabolomic networks (top) and reduced derivatives (bottom) showing biphasic-rewiring patterns in response to glucose-lactose diauxic shift. The Fruchterman-Reingold force-directed algorithm places nodes that are more connected with shorter paths in the center of the reduced graphs and pushes lightly connected vertices towards the periphery. Nodes are colored according to pathway maps in KEGG: yellow, hubs; blue, carbohydrate; green, energy; red, lipid; orange, nucleotide; purple, amino acid; brown, glycan; white, cofactors/vitamins; grey, secondary metabolites and xenobiotics; and black, miscellaneous. The group named 'hubs' unifies metabolites associated with more than one pathway and are considered central to metabolism. Vertex size and edge width are proportional. B. Metabolite connectivity measured as vertex degree for each time point in time resolved bacterial responses. C. Boxplot of metabolite connectivities. D. Analysis of network modularity using the FGC and the NG algorithms.

Reduced graphs show again remarkable changes in metabolite connectivity. We find metabolites that are pooled into 'carbohydrate' (blue) and 'energy' (green) nodes are initially located around the center of the reduced graph but after stress they migrate to the periphery. This observation supports stress-induced trends towards energy conservation, such as rapid decrease of central carbon metabolism intermediates belonging to the TCA cycle, glycolysis and the pentose phosphate pathway that coincides with down regulation of genes related to cell growth [22]. With an exception in temperature perturbations, a similar trend is observed with 'amino acid' (purple) metabolites, which start at the center and are pushed out to the periphery. However, these nodes regain their connections and are pulled back to the center at time point 8, markedly in diauxic shift and weakly in oxidative stress. Remarkably, temperature perturbations keep the 'amino acid' nodes in central positions tightly linked to 'hubs'. These patterns are consistent with the stress-induced accumulation of amino acids in these experiments [22], probably as a result of protein denaturation and degradation [29,30]. In contrast, we find that 'lipid' metabolites migrate towards the network periphery and remain selectively associated with 'hub' and 'carbohydrate' metabolites in diauxic shift, 'hub', 'energy' and 'amino acid' metabolites in oxidative stress, and 'hub', 'carbohydrate' and 'amino acid' in temperature stress. 'Lipid' metabolites maintain centrality and high connectivity after heat treatment. These trends may be related to stress-related changes in membrane composition and fluidity. One example is the 'homeoviscous adaptation' of membrane lipid composition in bacteria exposed to heat [31] that maintains adequate physical state of the membrane by incorporation of more saturated fatty acids (e.g. palmitic acid) [32].

B. Metabolomic networks are highly dynamic

Time-resolved analysis of metabolite connectivity can explicitly measure how dynamic are metabolite correlations. Boxplot graphs describing connectivity variation, measured as degrees of vertices (number of projecting connections). show significant and unanticipated change in metabolite

correlations (Fig. 1C). Variation was minimal in diauxic shift and cold stress, and maximal in the control. These variations may be more than stochastic events but rather adaptive physiological changes of the bacterial population to local nutrient availability, gradual regulatory changes, and small external perturbations. Remarkably, variations are largely constrained when external perturbations are present, being minimal in heat responses and maximal in nutrient shifts. Comparative analysis of connectivity variation between stresses and control for each individual time point again reveals the biphasic pattern, with spread of values and outliers increasing with time (SI Fig. 2). However, these trends never exceed the variation observed in controls without stress perturbations (Fig. 1C). Clear criss-cross patterns of differential connectivity make variation explicit (SI Fig. 3). Results showcase how stress constrains dynamic behavior in metabolomic networks.

C. *Metabolomic networks are random and not scale-free*

Network connectivity can be characterized by probability $P(k)$ that a vertex has k edges. For random networks of the Erdös and Rényi type [33], $P(k)$ peaks at $k = \langle k \rangle$ and decays exponentially at large k, usually following $P(k) \approx e^{-k}$, *for* $k >> \langle k \rangle$. In the case of scale-free networks, most nodes have only a few links and very few have many (hubs). For these networks, $P(k)$ has a not well defined peak and for large k it decays as a power law, following $P(k) \approx k^{-\gamma}$, *for* $k >> \langle k \rangle$. A log-log plot will show a straight line with slope $-\gamma$.

We plotted $P(k)$ versus k (SI Fig. 4) and log $(P(k))$ versus log k (data not shown) for all constructed networks. In all cases the maximum likelihood scaling-exponent or alpha-values (ranging 0.937-1.233) indicate wide departure from scale-free behavior (SI Fig. 4), and in contrast, Poisson plots showed connectivity patterns matching random graphs. Log-log plots also showed poor correlation fit to linear power-law behavior (γ ranging 0.06074-0.4965) (data not shown). The fact that metabolomic networks are not scale-free is significant. The large-scale organization of metabolic networks has been represented as graphs with metabolites (vertices) connected by chemical reactions (edges). These networks are scale-free and in different organisms exhibit the same scaling properties [34] that are present in complex non-biological systems [35]. Moreover, they have been shown to be both robust and error-tolerant [36]. The fact that metabolite correlation networks are not scale-free and do not exhibit power law behavior despite of being hardwired to the networks of metabolic reactions signify that structure and function are loosely linked in metabolism. This does not mean that there is not a backbone structure behind dynamic interactions in these networks. Dissection of a 'stable network component' from metabolomics networks [20] of the same four stress conditions examined here [22], in which only metabolites correlations that are homogeneous along all stresses are considered, uncovered networks with scale-free topologies and small-world characteristics. This suggests that behind the stochastic nature and the wide fluctuations of metabolomic networks there is a hardwired power-law structure that follows the 'rich-get-richer' principle of scale-free organization.

D. *Network modularity paraphrases biphasic rewiring*

Here we define modularity as the decomposition of a system, in our case a network, into nearly independent subsystems. Metabolomic network modularity was assessed using the Newman-Girvan (NG) [25] and the fast-greedy Clauset-Newman-Moore (FGC) [26] community scores that measure the community structure of the networks. Analysis of modularity with the FGC algorithm reveals that stress-induced connectivity decreases were always counterbalanced by increases in network modularity (Fig. 1D). In these analyses, high indices indicate that interaction among vertices belonging to modules is higher than those between modules. In turn, low indices indicate high community structure. Modularity slightly decreased in the control as bacterial cultures progressed towards stationary phase. However, it increased markedly upon stress application, decreasing back to normal levels in heat stress or showing slight decreases in the other stress responses at time point 8. In contrast, analyses with the NG algorithm that constrains modules to physiologies of KEGG mesonetworks did not reveal significant changes in modularity. This suggests that stress-induced modularity occurs at physiological levels that are distinct to the module definitions of KEGG.

These results are remarkable. Recent studies [17,18] have shown by simulation that networks generate modular structure when goals (environments) change over time, such that new goals share a same set of network constraints with previous goals as environments change. This feature of systems has the property of speeding up their evolution [18]. The fact that different environmental perturbations induce modularity as networks rewire and that modularization patterns are biphasic is important. These biphasic patterns exist widely in nature, in chemistries, molecular repertoires, and even development, and are probably linked to evolution of systems and the rise of biological modules [37].

IV. CONCLUSIONS

Our study reveals that metabolomic networks are not scale-free, and consequently, fail to be dominated by a group of 'hub' metabolites. Instead, networks have relatively large components of highly connected metabolites. More importantly, metabolomic networks appear to be stochastic and highly dynamic. Remarkably, we uncover a common biphasic-rewiring pattern in our time-resolved exploration of metabolite connectivity. In all stress responses, one densely connected network cluster becomes diffuse upon application of the stressor and later on regains connectivity. In the process, overall rewiring patterns change, moving from a focus on energetics and carbon metabolism that fosters growth to a focus on lipid metabolism, metabolic hubs and modules that foster membrane restructuring and metabolic centrality. This pattern is consistently recovered regardless of the stress that is applied and may simply represent the need of the cell to stop growing and to prepare for uncertainty.

While identification of 'stable components' in metabolomic networks informs on 'backbones' of common physiology elicited by sets of environmental perturbations [20], the dynamic and transient nature of metabolite

concentrations may be a necessity in light of environmental noise. Kashtan and Alon [17] proposed that systems must respond constantly to varying external stimuli in order to evolve modular network structures. This may well be the case with metabolic rewiring, since we find an association of rewiring and modularity in these networks. While responsive modules are different from KEGG metabolic modules, we find that stress-induced biphasic patterns of modularization explain changes in network connectivity and physiology.

In summary, networks of metabolite correlations provide useful information about metabolic connectivity and modularity, which generally gets overlooked when focusing on macroscopic changes in metabolite profiles. We posit that in order to understand metabolic physiology, these metabolic complexities must be carefully dissected and must be integrated with proteome and transcriptome information.

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