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Agustino Martínez-Antonio

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Agustino Martínez-Antonio

Departamento de Ingeniería Genética. Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional. Unidad Irapuato. Km. 9.6 Libramiento Norte Carretera Irapuato-León. CP 36821. Irapuato, Guanajuato. México

*Correspondence

amartinez@ira.cinvestav.mx

Abstract

Bacterial populations transition between growing and non-growing phases, based on nutrient availability and stress conditions. The hallmark of a growing state is anabolism, including DNA replication and cell division. In contrast, bacteria in a growth-arrested state acquire a resistant physiology and diminished metabolism. However, there is little knowledge on how this transition occurs at the molecular level. Here, we provide new evidence that a multi-element genetic regulatory circuit might work to maintain genetic control among growth-phase transitions in *Escherichia coli*. This work contributes to the discovering of design principles behind the performance of biological functions, which could be of relevance on the new disciplines of biological engineering and synthetic biology.

Keywords

Growth phase, regulatory circuit, proteome, mRNA, bacteria

Abbreviations

NAPs, nucleoid-associated proteins

RNAP, RNA polymerase

TF; transcription factor

cAMP; cyclic adenosine monophosphate

CRP; catabolic repressor protein

Highlights

- The regulatory circuit is composed of five transcription factors, including three nucleoid-associated proteins, one transcription factor, and one sigma factor.
- Regulators in the circuit architecture are added in a consecutive order according the time they are maximally expressed and functional through the culture phases.
- This feedback biological loop is negative and designed to preserve homeostasis. Within this negative feedback loop are two small circuits, each with two elements that share a common, pivotal element: resulting in one negative and one positive circuit.
- The switch between these internal circuits is thought to be the checkpoint between active cell division and the arrested growth in stationary phase.
- Protein and mRNA abundance show that the abundance of these regulators peaks at times when they are supposedly operating maximally.

Introduction

It is well-known that bacteria multiply rapidly when nutrients are plentiful and arrest their growth when carbon sources are depleted or stress conditions occur. When bacteria are grown in batch culture, the population follows a well-defined curve with previously described growth phases (Monod 1949). These growth phases have been modeled mathematically (Zwietering 1990). One can assume that bacteria arrest their growth when nutrients are limiting and resume growth when conditions are favorable again (Kolter 1993). This simple supposition implies that the cellular machinery is designed to function in a continuous mode; designed to arrest and re-initiate function depending upon nutrient availability and/or stress conditions. Nevertheless, biochemical and genetics studies provide us with clues about the molecular processes that occur when bacteria transition between active and arrested growth. However, the molecular details of this mechanism are more complex than this simple conjecture implies. In fact, bacteria need to adapt their cellular machinery to changing conditions; this adaptation includes the altering of transcriptional expression profile. The phenotypic result of these molecular changes is transition of a bacterial population between growth phases.

Methods in molecular biology have enabled us to identify hundreds of genes and, in some cases, the regulators that control their expression. The most precise experiments that link the activity of regulators to their target genes are those that are investigated specifically and individually. These types of studies produce detailed results on the regulatory interactions between one regulator and one target gene. This information is gathered and curated on dedicated databases, such as RegulonDB (Salgado 2013). The activation and repression of gene transcription is a task executed by the regulatory machinery, which includes nucleoid-associated proteins, sigma factors, and transcription factors that operate in an intricate regulatory network (Martinez-Antonio 2011).

Previously, we described a multi-element genetic regulatory circuit that may be implicated in controlling the transition between growths phases in *Escherichia coli* (Martinez-Antonio 2012). In that

study, we described the components of the genetic regulatory circuit and offered rationale for this hypothesis. Additionally, we developed a mathematical model, consisting of differential equations based on power-law formalism, to determine how this circuit might be operating. Here, we searched transcriptome and proteome data that could lend further support to this hypothesis. We show that mRNAs and proteins corresponding to the regulators on the network are more abundantly expressed at times that corresponds to their peak activity within the growth phase circuit.

Materials and Methods

Regulatory Interactions Data

The pairwise transcriptional regulatory interactions between genes and regulators were obtained from RegulonDB v8.0 (Salgado 2013). To reduce the network, nodes corresponding to non-regulatory genes were eliminated; however, the primary network of regulatory genes was kept intact. From this last subset of nodes and interactions, the regulators forming the circuit were extracted, as shown in Fig. 1.

Transcriptome Data

Data on the mRNA levels of genes on the circuit were searched at the NCBI GEO database (Barrett 2011). Care was taken to ensure that included information was not generated by experiments using gene deletion, gene over-expression, environmental stressors, or any other condition that could mask or influence the presence of transcripts beyond that of the normal transition of bacteria between growth phases. Useful data corresponding to the genes in the circuit were extracted manually.

Proteome Data

Due the scarcity this data type on dedicated databases, proteome data were mined from the original literature on PubMed (http://www.ncbi.nlm.nih.gov/pubmed/). The key words "proteome data" and "*E. coli*" were used for these searches. Using the same inclusion criteria as above for mRNA data, useable data corresponding to the proteins in the circuit were extracted manuals from primary and supplementary figures within the articles.

Results and Discussion

The genetic regulatory circuit controlling growth phase in E. coli

When all experimentally validated, pairwise, regulatory interactions are combined, a number of multi-element regulatory circuits begin to emerge (Martínez-Antonio 2008). One of these circuits (Fig. 1) involves global regulators at the core of the entire genetic regulatory network in *E. coli*. One multi-element genetic regulatory circuit, comprised a set of genes and regulators that activate and repress expression in a way that form a closed path, is shown in Fig. 1; a description of the components in the circuit is given in Table 1. Overall, this circuit is a negative feedback loop, designated by a negative sign; the products of the signs of its edges obtained this result. This result means that the circuit displays homeostatic control and a periodic behavior.

Embedded within this genetic regulatory circuit are two additional regulatory circuits, one negative (HNS-GadX) and one positive (GadX-RpoS). Both embedded circuits have GadX as the common element. GadX has been proposed as the master switch for the activity of this circuit because inactive GadX protein maintains activity of the HNS-GadX circuit, while active GadX shifts the activity of the circuit to RpoS and IHF (Martinez-Antonio, 2012). Dynamic studies on gene regulatory circuits reveal that circuits like the one described here could have multiple functions and complex behaviors if positive and negative circuits are embedded within them (Thomas 1995). In other words, this kind of circuit can produce different steady states of gene expression patterns under different

physiological conditions (Kaufman 2007). In the case of this circuit, the biological implication of such a regulator switch is that the activity of these regulatory components and their functions may be linked to the various growth phases of this bacterium. In subsequent sections, we provide some evidence that this circuit is regulating gene expression in a growth phase-dependent manner in *E. coli*.

The regulatory factors of the circuit

Three of regulators in the circuit on Fig. 1 are nucleoid-associated proteins or NAPs (FIS, HNS and IHF) (Dillon 2010). These proteins bend and bridge DNA in different conformations. The Ishihama laboratory studied the abundance of NAPs in *E. coli*, primarily by western blot analysis. They reported that the NAPs present in this circuit were maximally expressed in a growth phases-dependent manner. First, FIS expression peaks when cells start to divide before the exponential phase. Next, HNS expression is maximal during exponential growth. Finally, IHF is expressed mostly in stationary phase (Azam 1999). The circuit also contains the acid-stress resistance regulator, GadX. GadX belongs to a group of transcriptional regulators that respond to low pH, mainly due to intracellular acidification by the accumulation of organic acids resulting from fermentative metabolism (Tramonti 2002, Ma 2002). The circuit is completed with a general stress response sigma factor, RpoS, which replaces the activity of the housekeeping sigma factor RpoD during stress conditions. Transcription of the anti-sigma factor RSD, inactivates RpoD. RpoS is the master sigma factor that directs RNAP to the transcription of genes, including the promoters of IHF subunits, whose products respond to multiple stress types (Lange 1991).

How might this circuit operate?

The main properties of regulators in the circuit and the functional classes their regulons are shown in Table 2 and Fig. 2, with brief descriptions of each elements of the circuit (an additional,

detailed description was presented in [Martínez-Antonio 2012]). FIS should be a very important player at the beginning of bacterial growth because it activates the transcription of important cellular elements dealing with the process of cell division, such as tRNAs, rRNAs, and stable RNAs, as well as ribosomal RNAs and genes for translation (Finkel SE 1992). Some of these same genes are also regulated by HNS (Free A 1995), which is the regulator that follows FIS in the circuit. Interestingly, *hns* is activated by FIS. GadX regulates primary the genes for pH homeostasis; most of these genes are co-regulated by HNS. *gadX* activates *hns* and is repressed by *hns*. This mutual regulation with opposed signs constitute a negative circuit of regulation. During ideal growth conditions, the inactive form of GadX should stop the main activity of the whole circuit at this point in the pathway.

GadX can be allosterically activated by organic acids, such as acetate and formate (Shin S 2001). Usually, the presence of such acids is indicative of acidic stress conditions, such as those produced by cells entering fermentative metabolism. Organic acids activate GadX, which increases the transcription of *rpoS*. Because the *gadX* gene has a promoter for RpoS, a robust positive circuit forms. RpoS transcribes many genes that prepare the cell to acquire a resistant physiology, including those that induce a smaller, rounded morphology, such as the regulator BolA (Aldea M 1989). RpoS also transcribes two of the IHF subunits *(ihfA* and *ihfB*) and IHF activates the transcription of *dps*, which encodes a small protein in late stationary phase that forms crystals with DNA to protect it (Altuvia S 1994). IHF regulates many genes, but notable for this discussion are those for anaerobic respiration. In addition, IHF activates *fis*, and with this interaction, the circuit is closed. At the DNA origin of replication in the *E. coli*, there is a DNA-binding site for IHF, which suggests that this regulator may be involved in this process. IHF may function by bending the DNA and preventing or facilitating the access of the replication machinery to the origin of DNA replication (Goosen N 1995).

The overall activity of this circuit was modeled (Martinez-Antonio 2012) and revealed that GadX might serve as a checkpoint of the circuit by maintaining the negative circuit while inactive and

activating the positive circuit in response to organic acids. It is proposed that this circuit should contribute to the robust population-wide decision to continue or arrest growth. By activating the second part of the circuit, starting from GadX and RpoS, the regulatory machinery ensures that bacteria change the pattern of gene expression upon growth arrest. One can expect that an analogous checkpoint should exist to facilitate the transition from arrested growth to an active growing state. At this point in the circuit architecture, no such analogous switch has been found. Certainly, this transition could not be explained solely by the activation of *fis* by IHF, however, this transition might also depend on the control of CRP over *fis*. CRP is the most global regulator in *E. coli* and its activity depends allosterically on the presence of cAMP (Harman 2001). It means that CRP could sense the overall energetic status of the cell, including information on the carbon sources availability, and might have the capability to activate or repress *fis*, thus controlling the decision for growth is when conditions are suitable.

mRNA levels of the regulatory genes in the circuit support the circuit model

We searched the mRNA levels of genes on the circuit in the NCBI GEO database (Barrett 2011). Ideally, the data used in this analysis should not be obtained from experiments that involve gene deletions, gene over-expression, environmental stress, or any other condition that could mask or influence the presence of transcripts beyond those that result from the natural transition of bacteria through growth phases. One such exceptionally useful study was published by Sangurdekar et al., (Sangurdekar DP 2006). In this work, the authors measured mRNA abundance of a culture of *E. coli* MG1655 grown in the minimal medium Bonner-Vogel at 0.5 DO and compared the results to those obtained from the same strain grown in LB medium at multiple time points that covered all the growth phases. From this study, we could recover information about the mRNA abundance for five of the six regulatory genes of the regulatory circuit (since IHF is constituted by two genes: *ihfA* and *ihfB*).

196 exceptLacking information was for one of the subunits of the IHF protein (*ihfB*). This analysis revealed
197 that the quantity of mRNA varies for each gene over the growth phases (Fig. 3). In the case of *fis* and
198 *hns*, their transcripts are more abundant before mid-exponential phase. In contrast, the transcripts for
199 *gadX*, *ihfA*, and *rpoS* are more abundant after mid-exponential phase. For comparison, we decided to
200 look for the mRNA quantity *dps* because it is expected to be abundant in the late stationary phase. We
201 found that transcripts level of *dps* were most abundant in stationary phase, further supporting the
202 hypothesis that this circuit modulates the transition into stationary phase.
203 To validate the accuracy of the data used for this analysis, we examined the expression pattern
204 of several control genes with known expression changes over the entire growth curve. The repressor
205 LacI is not required in these conditions (Semsey S 2013), and we observed no major changes in *lacI*
206 mRNA. Topoisomerase 1 *(topA)* is supposed to be active during DNA replication to separate the DNA
207 strands (Valjavec-Gratian M 2005). We noted more abundant mRNA of this gene from the beginning
208 of growth through the first half of the exponential phase. Lastly, the global regulator CRP is subject to
209 dual regulation, including self-regulation, was slightly more abundant in exponential phase (Fig. 3).
210 Thus, at the mRNA level, these regulators in the circuit are differentially transcribed, likely because
211 they are required at different bacterial growth phases.
212
213 *Protein levels of the regulators in the circuit*
214 Next, we examined expression of the regulator are the protein level. Despite exhaustive
215 searching, we found only one proteomic study in minimal medium were the authors applied stable
216 isotope labeling to amino acids in cell culture (SILAC) and performed a quantitative analysis of
217 proteome dynamics in *E. coli* BW25113 during five distinct phases of growth (Soares NC 2013). Our
218 analysis is summarized in Fig 4. Soares *et al*. took as reference the quantity of proteins of a culture just
219 entering the stationary phase ("point 4" on Fig. 4) and compared the relative abundance of proteins in

the samples from other growth phases. With these data in hand, we looked for the relative protein abundance of the regulators in the circuit. We obtained information for four of the five regulators of the circuit (FIS, HNS, RpoS, IHF). Data for GadX were not available; thus, we used data from GadE, which is directly activated by GadX and is involved on the acid-stress response. Abundance of GadE may provide indirect information about the abundance of GadX. The profiles of relative protein abundance are shown in Fig. 4. FIS protein levels are more abundant when cells start to divide and enter into exponential phase; and levels of FIS fall as the bacteria decelerate their rate of growth. The sigma factor RpoS and the two subunits of IHF (IHFA and IHFB) augment their quantities as the culture enters stationary phase. The protein levels of HNS and CRP seem to be slightly more abundant at some points in exponential phase; however, changes in the abundance of these proteins are less robust. Dps protein was more abundant in late stationary phase. Finally, the protein levels of TopA and LacI remain almost constant across all growth phases. Similar to the results seen with the mRNA analysis, the protein profiles of the elements of the circuit support the notion that these regulators should be more abundant when their activity peaks.

Closing remarks

In this study, we describe a multi-element genetic regulatory circuit whose components function to provide control, fitness, and robustness to the process of population growth of *E. coli*. The elements and architecture of the circuit are organized such that they regulate each other in periodic fashion that makes biological sense. This circuit is arranged into two smaller parts, operating in either active growth or growth-arrested conditions. Given what is known about the architecture and biological roles of each regulator of the circuit, it makes biological sense they control population growth. Here, we provide proteomic and transcriptomic data that support this hypothesis of growth regulation. The presence and abundance of mRNA and proteins of these components peaked when they are more active.

It is rare to find proteomic and transcriptomic data at several phases of growth for the same culture; fortunately we found data that, although generated for other purposes, served nicely for this analysis. With these data, we confirmed the notion that the maximal abundance of these elements occurs when these regulators should be required, offering a form of temporal support for this hypothesis. The architecture and proposal activity of the genetic regulatory circuit could explain how it operates to start and arrest bacterial growth.

Although mRNA was reported in relative units; however, to appreciate their small quantity, studies on the total mRNA have determined a median value of less than 10 mRNA copies per gene per cell in a single-cell study on *E. coli* (Taniguchi 2010). This observation may be explained by the fact that mRNA is quickly degraded, often within minutes. In contrast, many proteins have half-lives greater than the *E. coli* cell cycle. In the case of proteins, one recent study by (Wiśniewskia and Rakusb 2014) stated that *E. coli* (ATCC 25922 grew at 37ºC, 250 rpm, 15 hrs in LB medium) has 75 fg of proteins in late stationary phase. This number corresponds to approximately 1.3×10^6 proteins/cell. The specific values on late stationary phase for the proteins referred to here are in molecules/cell: 2534 for IHFA, 1582 for IFB; 2980 for CRP; 206 for FIS; 6059 for HNS; 55 for GadC (there are not data for GadX); 1.7 for RpoS (101 for RpoD) ; 9 for LacI; 125 for TopA and 4339 for Dps.

Isogenic mutants for all the regulators in the circuit are available in the Keio collection (Baba T 2006). The mutants for FIS and RpoS are the most sensitive for growth in our hands; a strain with a *fis* deletion is unable to grow in minimal medium without additional supplements, including carbon and nitrogen sources (e.g., casamino acids). The RpoS mutant was lost from the collection with three successive freezings to -80ºC (personal observation). With new methodologies at hand, now it is possible to determine the importance of intracellular macro-components to physiological requirement, such as ribosomes, mRNA, proteins, etc., depending on the quality of nutrients (Klumpp 2009). It is possible that regulators of this circuit, although not essential for bacterial growth in normal conditions,

are evolutionarily important to the fitness of *E. coli*. Our description of this kind of circuit reveals a potential mechanism to explain observed phenotypes and could guide the engineering of certain biological processes in synthetic biology.

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Competing Interest

The author declares no competing interests.

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415 **Table 1.** Validated regulatory interactions between elements of the circuit.

Gene	Promoter	Transcription Factor	Mode of regulation	Evidence	Reference
fis	*dusBp*	CRP-cAMP	Activation	Microarrays	(Zheng 2004)
fis	*dusBp*	CRP-cAMP	Dual	DNase I footprinting	(W. S. Nasser 2001)
fis	*dusBp*	FIS	Repression	DNase I footprinting	(Ball 1992) (Hengen 1997)
fis	*dusBp*	IHF	Activation	Site mutation, reporter assays	(W. R. Nasser 2002) (Pratt 1997)
hns	*hnsp*	FIS	Activation	DNase I footprinting	(M. B. Falconi 1996) (M. G. Giangrossi 2001)
hns	*hnsp*	Gadx	Activation	Microarrays, TF overexpression	(F. Hommais 2004)
hns	*hnsp*	HNS	Repression	DNase I footprinting	(M. B. Falconi 1996) (M. H. Falconi 1993) (M. G. Giangrossi 2001) (Ueguchi 1993)
gadX	gadXp	GadX	Activation	Microarrays, RT-PCR	(Z. R. Ma 2002) (F. Hommais 2004) (A. D. Tramonti 2008)
gadX	gadXp	HNS	Repression	DNase I footprinting	(M. Z. Giangrossi 2005) (F. K.-W. Hommais 2001)
rpoS	rpoSp	GadX	Activator	Microarrays	(F. Hommais 2004)
ihfA	*ihfAp4*	IHF	Repression	DNase I footprinting	(Aviv 1994) (Bykowski 1998)
ihfB	*ihfBp*	IHF	Repression	DNase I footprinting	(Aviv 1994) (Bykowski 1998)
dps	*dpsp*	FIS	Repression	DNase I footprinting, Electrophoretic mobility shift, reporter assays	(Grainger 2008) (Yamamoto 2011)
dps	*dpsp*	FIS	Repression	DNase I footprinting, Electrophoretic mobility shift, reporter assays	(Grainger 2008) (Yamamoto 2011)
dps	*dpsp*	FIS	Activation	Computational evidence	(Altuvia S 1994)
Genes transcribed by the sigma RpoS, in addition to RpoD					
Gene	Promoter	Sigma	Mode	Evidence	Reference
gadX	gadXp	RpoS	Transcription	Electrophoretic mobility shift assay and DNase I footprinting	(A. V. Tramonti 2002)
ihfA	*ihfAp4*	RpoS	transcription	Transcription initiation mapping	(Aviv 1994) (Mechulam 1987)
ihfB	*ihfBp*	RpoS	transcription	Transcription initiation mapping	(A. V. Tramonti 2002) (Węgleńska 1996)

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418 **Table 2.** The regulatory genes of the circuit and the functional classes of their regulated genes.

Transcription factor	Description	Functional classes of target genes (numbers), take from RegulonDB
FIS (Factor for Inversion Stimulation)	A 22 kDa homo-dimeric protein. FIS bends the DNA between 50º and 90º ().	tRNAs (53), anaerobic respiration (34), membrane (34), translation (29), ribosome (27), aerobic respiration (23), rRNA and stable RNAs (22), carbon compounds (20), electron donors (20), transcription related (18).
HNS (Histone-like Nucleoid Structuring protein)	A 15.4 kDa protein that forms bridges between adjacent DNA duplexes.	Transcription related (24), carbon compounds (24), membrane (23), activators (20), translation (17), ribosomes (17), rRNA and stable RNAs (16), uncharacterized proteins (14), pH homeostasis (13).
GadX (Regulator of Glutamic Acid Decarboxylase)	Contributes to pH homeostasis by consuming intracellular H+ and producing gamma-amino butyric acid	pH homeostasis (8), Porters (5), membrane (5), transcription related (4), activators (3), amino acids (2).
RpoS (Sigma S or sigma38)	A sigma subunit of RNAP for general stresses and stationary phase transcription	Diverse stress-responses (60)
IHF (Integration Host Factor)	A protein composed of α (himA) and β (himB) subunits. It bends the DNA and compact the chromosome length by about 30%	Anaerobic respiration (42), membrane (41), carbon compounds (21), transcription related (19), aerobic respiration (16), electron donor (15), activators (14), porters (13), oxide-reduction transporters (13)

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Figure legends

Figure 1. The regulatory circuit controlling growth-phases in *E. coli*. This cartoon represents the growth phases and the regulators of the circuit, illustrated which is more active in each case: Green = represent activation; red = repression; blue = dual regulation (activation and repression).

Figure 2. Genes controlled by each regulator of the genetic circuit. Some of these regulated genes are also regulated by other transcription factors of *E. coli*, but for clarity, only the regulation exerted by regulators of this circuit are included here.

Figure 3. mRNA profiles of regulators on the circuit. Relative quantities of mRNA are shown (left y-axis) over time (x-axis). The bacterial growth curve (red line, right Y-axis) is shown to illustrate growth phases. The strain used was K12 MG1655 grown in LB medium (Sangurdekar DP 2006).

Figure 4. Relative protein levels of regulators in the circuit. Relative quantities of proteins are shown (left y-axis) over time. The bacterial growth curve (red line, right y-axis) is shown to illustrate growth phases. The strain used was W3110 grown in M9 minimal medium (Soares et al, 2013).

Figure 1

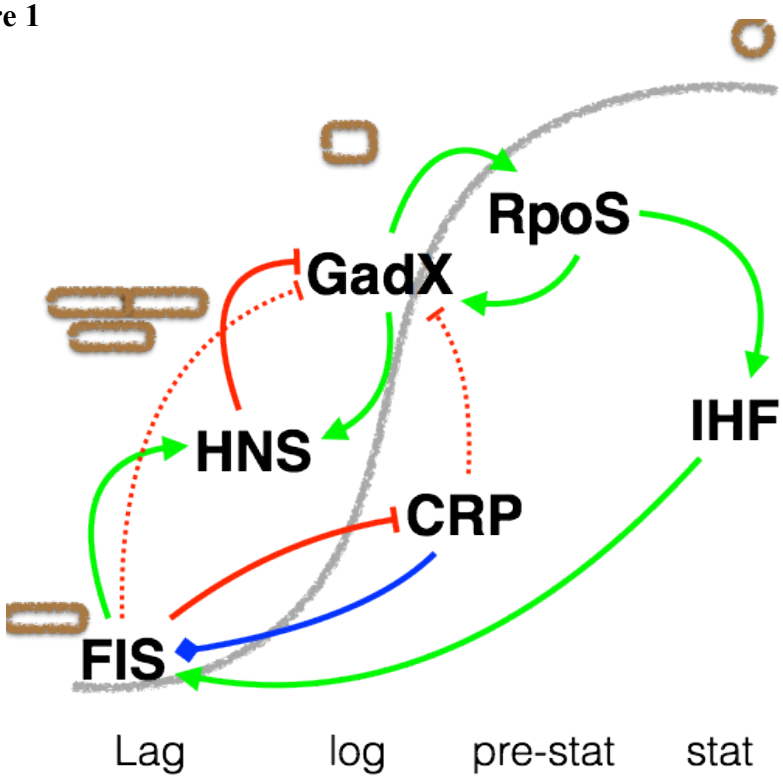
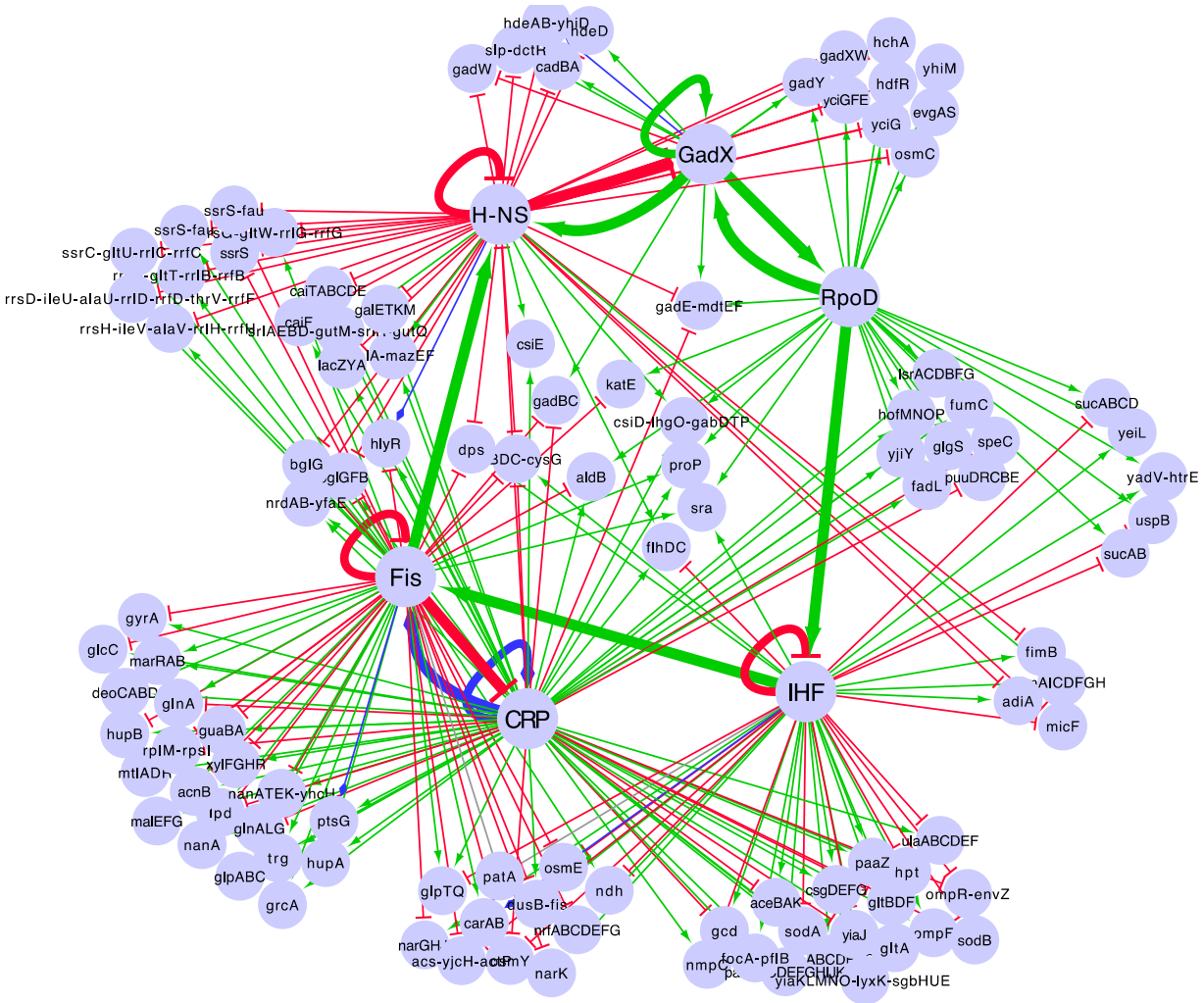


Figure 2



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465 **Figure 3**

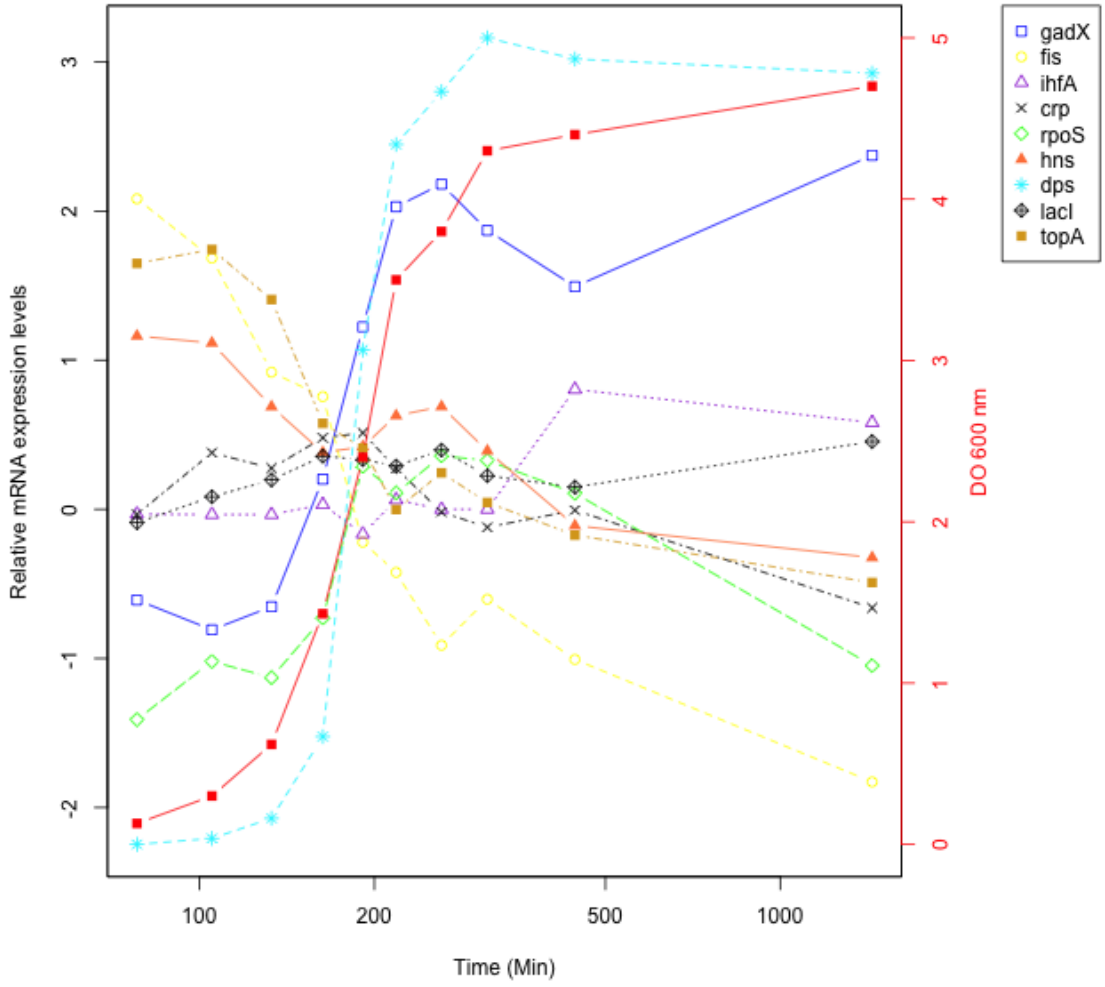

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Figure 4