Dynamics of a starvation-to-surfeit shift: a transcriptomic and modelling analysis of the bacterial response to zinc reveals transient behaviour of the Fur and SoxS regulators

Alison I. Graham,1 Guido Sanguinetti,2 Neil Bramall,3 Cameron W. McLeod3 and Robert K. Poole1

1Department of Molecular Biology and Biotechnology, The University of Sheffield, Western Bank, Sheffield S10 2TN, UK
2School of Informatics, University of Edinburgh, Informatics Forum, 10 Crichton Street, Edinburgh EH8 9AB, UK
3Centre for Analytical Sciences, The University of Sheffield, Western Bank, Sheffield S3 7HF, UK

We describe a hybrid transcriptomic and modelling analysis of the dynamics of a bacterial response to stress, namely the addition of 200 μM Zn to Escherichia coli growing in severely Zn-depleted medium and of cells growing at different Zn concentrations at steady state. Genes that changed significantly in response to the transition were those reported previously to be associated with zinc deficiency (zinT, znuA, ykgM) or excess (basR, cpxP, cusF). Cellular Zn levels were confirmed by ICP-AES to be 14- to 28-fold greater after Zn addition but there was also 6- to 8-fold more cellular Fe 30 min after Zn addition. Statistical modelling of the transcriptomic data generated from the Zn shift focused on the role of ten key regulators; ArsR, BaeR, CpxR, CusR, Fur, OxyR, SoxS, ZntR, ZraR and Zur. The data and modelling reveal a transient change in the activity of the iron regulator Fur and of the oxidative stress regulator SoxS, neither of which is evident from the steady-state transcriptomic analyses. We hypothesize a competitive binding mechanism that combines these observations and existing data on the physiology of Zn and Fe uptake. Formalizing the mechanism in a differential equation model shows that it can reproduce qualitatively the behaviour seen in the data. This gives new insights into the interplay of these two fundamental metal ions in gene regulation and bacterial physiology, as well as highlighting the importance of dynamic studies to reverse-engineer systems behaviour.

INTRODUCTION

Zinc plays a structural, regulatory or catalytic role in proteins (Berg & Shi, 1996; Fraústo da Silva & Williams, 2001). However, despite its indispensable role, Zn is toxic if accumulated to excess (Hughes & Poole, 1989), and so tightly regulated import and export mechanisms are required for Zn homeostasis (Blencowe & Morby, 2003). The major inducible high-affinity Zn uptake system is the ABC transporter ZnuABC. Periplasmic ZnuA is thought to pass Zn to ZnuB for transport through the membrane (Patzer & Hantke, 1998; Berducci et al., 2004). Zn-bound Zur represses transcription of znuABC (Patzer & Hantke, 2000). Zur senses subfemtomolar concentrations of cytosolic Zn, implying that cellular Zn starvation commences at exceptionally low Zn concentrations (Outten & O’Halloran, 2001). Indeed, it is difficult to demonstrate experimentally Zn-limited growth of Escherichia coli (Graham et al., 2009). Recently, zinT was shown to be induced by Zur in response to Zn starvation. The encoded protein, a periplasmic Zn-binding protein, has a major role in surviving Zn limitation (Graham et al., 2009). The P-type ATPase, ZntA (Beard et al., 1997), and the cation diffusion facilitator protein, ZitB (Grass et al., 2001), export excess Zn ions from the inside of the cell and thus also contribute to metal homeostasis.

Most genome-wide microarray studies of the effects of metal stresses to date have been carried out in batch culture, but continuous culture offers major benefits for such studies (Pullan et al., 2008; Bull, 2010). The ability to control growth conditions, especially growth rate, eliminates the masking effects of secondary stresses and growth-rate changes (Hayes...
et al., 2002; Hoskisson & Hobbs, 2005), and the reproducibility of transcriptomic analyses is greater when chemostat, rather than batch, cultures are used (Piper et al., 2002). Therefore, we used E. coli chemostat cultures to assay the transcriptional response to Zn excess (Lee et al., 2005). These transcriptome profiling experiments comparing E. coli grown under control conditions and cells grown with a toxic, sublethal ZnSO₄ concentration (200 μM) revealed 64 genes to be significantly upregulated (including basR, cpxP, cusF and zntA) whilst 58 genes were significantly downregulated by Zn stress. The protein encoded by the basR gene is part of a two-component regulator system that has been implicated in Fe responses (Hagiwara et al., 2004). The cpxP and cusF genes encode a periplasmic chaperone (Danese & Silhavy, 1998; Fleischer et al., 2007) and a periplasmic metal binding protein (Franke et al., 2003), respectively.

Only a few attempts have been made to study the global consequences of metal deficiency in bacteria and most have employed chelators of poor specificity (Sigdel et al., 2006). Therefore, we previously grew E. coli in continuous culture in which severe Zn depletion was achieved without recourse to chelating agents by thorough extraction of medium and scrupulous attention to metal contamination (Graham et al., 2009). Microarray analysis identified only nine genes that respond significantly to Zn starvation, including znuA, ykgM and zinT. The znuA and zinT genes are clearly implicated in Zn homeostasis as described above; ykgM is predicted to encode a non-Zn ribbon-containing paralogue of the ribosomal protein L31 (Panina et al., 2003), which normally binds Zn.

Most transcriptomic studies compare transcript abundances between a control state and a perturbed system that has achieved steady state (Hayes et al., 2002; Tai et al., 2007). While this can give useful information on physiology in different states, it necessarily falls short of providing an explanation of the kinetics and mechanisms of adaptation to different conditions. Therefore, in this study, we present a dynamic transcriptional profile of the transition from severe Zn starvation to excess Zn (200 μM) and its analysis using a probabilistic model (Sanguinetti et al., 2006) to infer the activity profile of key transcription factors (TFs) involved in gene regulation. While most TFs exhibited a monotonically changing pattern in both the dynamic and steady-state data, the inferred profile of the Fe-responsive regulator Fur exhibited a sharp transient peak in activity in the dynamic data that could not be discovered by considering the steady-state measurements alone. The inferred activity profile of SoxS was also discontinuous, falling before rising again. Guided by these observations, we have formulated an ordinary differential equations model that formalizes a plausible mechanism by which Zn and Fe interact following changes in Zn availability. The model robustly reproduces the qualitative behaviour of the observations, and relies on a minimal set of parameters and assumptions that are well established in the literature on metal ion physiology.

METHODS

Zn-depleted cultures and exposure to excess Zn. For preliminary RT-PCR experiments, a saturated culture of E. coli strain MG1655 was grown in LB medium. Cells were collected from approximately 0.5 ml culture by centrifugation and the pellet was resuspended in 10 ml Zn-depleted GGM (Graham et al., 2009) containing <60 nM Zn in a polypropylene tube and cultured for 24 h at 37 °C with shaking. A 3 ml aliquot was used to inoculate 60 ml Zn-depleted GGM in a 250 ml Zn-depleted flask. These cultures were incubated at 37 °C with shaking until an OD₆₀₀ of 0.6 was reached. At this point, ZnSO₄·7H₂O was added to a final concentration of 50 μM or 200 μM in two different flasks. A third flask to which nothing was added acted as a control. A 10 ml sample of culture was taken using a polypropylene pipette tip immediately prior to Zn addition and 5, 15 and 60 min after addition. The culture was pipetted into RNAProtect (Qiagen). RNA was extracted and RT-PCR carried out as before (Lee et al., 2005). Genes analysed were ansS (control), basR, cpxP, cusF, holB (control), ykgM, zinT and znuA. The mRNA levels of ansS and holB were unchanged as determined previously by microarrays (Graham et al., 2009; Lee et al., 2005) and were thus used as internal controls. The normalized Ci value for each gene and Zn concentration was divided by its corresponding 0 time point value. Then, 50 μM and 200 μM time points were divided by the corresponding ‘no added Zn’ time point. Values were calculated from a single biological experiment.

For microarray experiments, a 0.25 ml aliquot of a saturated culture of strain MG1655 grown in LB medium was centrifuged and the pellet used to inoculate 5 ml of Zn-depleted GGM that was incubated overnight at 37 °C with shaking. A 2.4 ml aliquot was then used to inoculate the Zn-depleted custom-built chemostats described before (Graham et al., 2009). Chemostats were grown for 50 h to allow 5 culture volumes through the vessel to reach an apparent (pseudo-) steady state. At this point, ZnSO₄·7H₂O in water was added to a final concentration of 200 μM in the chemostat. A 10 ml sample was taken using a polypropylene pipette tip immediately prior to Zn addition and 2.5, 7, 10 and 30 min after addition. The culture was pipetted into RNAProtect. RNA was extracted and microarray analysis carried out as described by Graham et al. (2009). In a control experiment, water was added instead of Zn. The chemostat culture was maintained at 37 °C and stirred at 440 r.p.m. Samples were taken throughout to check pH, OD₆₀₀ and glycerol content, and for contaminants. Steady-state values for pH and OD₆₀₀ were 6.9 and 0.6, respectively. Glycerol assays (Garland & Randle, 1962) showed cultures to be glycerol-limited (more than 99.5 % consumed during growth). Control (water added) and experiment (ZnSO₄ added) chemostats were grown separately twice. Slides were analysed as described previously (Graham et al., 2009) so that time points ‘with Zn’ were compared to the same time point ‘without Zn’ (e.g. 30 min after Zn was added was compared to 30 min after water was added). Twenty slides were analysed in total, namely control and dye swap for the five time points in two biological replicates.

ICP-AES and calculation of dry cell weight. Metal quantification by ICP-AES and calculation of dry cell weight were carried out exactly as described before (Graham et al., 2009).

Probabilistic modelling of the transcriptome. The dynamics of the transcriptomic response in the Zn shift were modelled using a simple Bayesian statistical model of transcription (Sanguinetti et al., 2006; Partridge et al., 2007). This models log expression ratios as linear combinations of TF concentration:

\[ y_i(t) = \sum_{m} X_{nm} b_{nm} e_{nm}(t) + e_i \]  

Here \( X_{nm} = 1 \) if TF \( m \) binds gene \( n \), zero otherwise. The term \( b_{nm} \) quantifies the effect of TF \( m \) on gene \( n \), and is given a zero mean
A Gaussian prior, allowing equal likelihood of repression and activation. The term $c_m(t)$ represents the activity of TF $m$ at time $t$ and is given a state-space model prior which enforces smooth changes. Having observed transcriptomic time-course data $y_c(t)$, the model returns a posterior distribution over the TF activity and the regulatory effects on each target gene. A schematic of the model is shown in Fig. 1.

To clarify the results obtained with the model we provide here a brief intuitive explanation of how the model works. Equation (1) implies that log-changes in mRNA expression of a gene are obtained as a weighted linear combination of the changes in activity of the TFs binding the gene. While this is undoubtedly a simplistic model of the complex process of transcription, it does capture the first-order (i.e. more evident) effects and its simplicity makes it amenable to a statistical treatment, leading to data-driven predictions of TF activities. In the simplest case, when the genes in a regulon have a single regulator (i.e. all genes in the regulon are controlled by a specific TF and no others), the model essentially infers suitable weights so that all gene expression profiles become comparable, thus extracting the predicted regulator profile as the consensus profile. The outcome is more complex when regulons overlap. In that case, the model statistically disentangles the signal from the different regulators in order to fit the observed data. An illustration of this process is provided in Fig. 2.

An important experimental design decision is the selection of the regulators to be included in the statistical modelling. While there is no hard-and-fast rule, this is generally done by preliminary exploration of the data, e.g. by selecting the regulators of genes that exhibit a significant change in expression, and/or by invoking domain knowledge, e.g. in this case by selecting metal-responsive regulators. It should be remarked that it is possible that the model will infer no change of activity for certain regulators, even if some of their target genes change in expression, if these changes are not consistent across the regulon. Conversely, it is plausible (and desirable) that modest changes which are consistent across a regulon will lead to a very confident prediction of regulatory activity, thus providing a more meaningful criterion than the arbitrary thresholds in fold change often employed. The probabilistic modelling shown here is designed primarily to analyse TF activity so the small regulatory RNAs are not included.

**RESULTS**

### Zn addition to Zn-starved batch cultures of *E. coli* strain MG1655

Having reported the response of chemostat-cultured *E. coli* to Zn excess (Lee et al., 2005) and Zn starvation (Graham et al., 2009), we dissected the response of cells growing under severe Zn depletion to (excess) Zn addition. The exhaustive procedures used to deplete Zn and prevent Zn contamination were described before (Graham et al., 2009). Due to the irreversible nature of the experiment (that is, once Zn is added back to a Zn-depleted chemostat vessel, the vessel can no longer be used without arduous and prolonged leaching of the added metal ion), we established the overall dynamics of the transition by analysing expression of several genes by...
RT-PCR in cells grown in batch cultures in simple shake flasks subjected to the same washing protocol (Graham et al., 2009). Zn-depleted cells were grown to mid-exponential phase (OD$_{600}$ 0.6) and either 50 μM or 200 μM ZnSO$_4$ was added. RNA was harvested immediately prior to Zn addition and then at 5, 15 and 60 min after addition. RT-PCR was carried out on six genes known to be upregulated under Zn excess (Lee et al., 2005) and Zn starvation (Graham et al., 2009).

The genes known to be upregulated under Zn excess, namely basR, cpxP and cusF, encode respectively part of a two-component regulator system (Hagiwara et al., 2004), an auxiliary protein for a two-component system (Zhou et al., 2011) and a periplasmic metal chaperone (Bagai et al., 2008). Expression of these increased (Fig. 3), although basR was only slightly upregulated at 50 μM Zn (Fig. 3a) and the fold increase was lower than in our previous chemostat experiments (Lee et al., 2005). Genes ykgM, znuA and zntT are known to be upregulated under Zn starvation. The gene ykgM encodes a non-Zn-containing paralogue of a ribosomal protein (Panina et al., 2003). ZnuA forms the soluble periplasmic metallochaperone that captures and delivers Zn to ZnuBC, the membrane-bound component of the high-affinity Zn importer (Patzer & Hantke, 1998; Patzer & Hantke, 2000). ZinT has recently been suggested to be an additional component of this system (Graham et al., 2009; Gabbianelli et al., 2011). Expression of all these genes decreased in this experiment (Fig. 3), but znuA was not significantly downregulated at 200 μM Zn (Fig. 3b). Again, the difference is probably attributable to the use of batch cultures for these trial experiments.

**Zn addition to Zn-starved continuous cultures of E. coli strain MG1655**

The experiments in batch culture and previous work (Lee et al. (2005) showed that 200 μM ZnSO$_4$ yielded the largest transcriptional changes for most genes without killing the cells, so it was decided to use this concentration for microarray analysis. These experiments also showed that many of the changes in mRNA abundance happened soon after the addition of Zn, so we concentrated on a smaller time frame with time points clustered immediately after Zn addition. E. coli strain MG1655 was grown in continuous culture in a Zn-depleted chemostat. Under these conditions, at a dilution rate of 0.1 h$^{-1}$, the doubling time is 6.9 h. Once a (pseudo-)steady state had been reached (after 50 h), ZnSO$_4$·7H$_2$O was added to a final concentration of 200 μM. Samples were taken for RNA extraction immediately prior to Zn addition and 2.5, 7, 10 and 30 min after addition. These time points were also used in a control experiment in which water, not Zn, was added to a Zn-starved chemostat. Two biological repeats from four successful chemostat runs were analysed. Supplementary Table S1, available with the online version of this paper, shows the genes with a significant change in mRNA level in response to ZnSO$_4$ addition at the five different time points. The entire dataset has been deposited with GEO (accession number GSE26187) (Edgar et al., 2002). Fig. 4 shows the fold changes obtained using microarray analysis of selected genes; the changes exhibit the same pattern as in Fig. 3 but cover a wider dynamic range, perhaps due to the use of batch cultures for RT-PCR analysis but chemostats for microarrays. Expression of the gene encoding ZntA, a Zn efflux pump, was shown using microarrays to increase on the addition of Zn, as expected (Fig. 4). This gene was not included in the RT-PCR analysis shown in Fig. 3.

**Zn addition leads to increased cellular Zn, Fe and Cu**

Chemostats were harvested immediately after the last time point was taken and analysed for cellular metal as before (Graham et al., 2009). Here, ‘cellular metal’ is defined as metal that cannot be removed by three successive washes with 0.5 % nitric acid. Cellular Zn levels were confirmed by ICP-AES to be 14- to 28-fold greater after Zn addition (Table 1). Interestingly, there was also 6- to 8-fold more cellular Fe 30 min after Zn addition than after water addition (Table 1) and 7- to 8-fold more cellular Cu (Table...
1). Future studies will characterize this iron and the mechanism(s) underlying its accumulation.

Statistical modelling of the transcriptome

Data were analysed using the TFInfer statistical modelling tool, which integrates the architecture of the regulatory network with gene expression data to infer the activity profile of TFs (Sanguinetti et al., 2006; Asif et al., 2010) (see Methods for the mathematical details and further comments on the model). The analysis of the transcriptomic data generated from the Zn shift focused on the role of ten key regulators: ArsR, BaeR, CpxR, CusR, Fur, OxyR, SoxS, ZntR, ZraR and Zur. A list of genes populating the (overlapping) regulons of these TFs, together with information on the scale of the regulation, where available, was obtained from regulonDB (http://regulondb.ccg.unam.mx/). Details are shown in Supplementary Table S2, but briefly this consisted of 184 genes that could be divided into the following categories: repressed by Fur, 63; activated by Fur, 15; repressed by CpxR, 21; activated by CpxR, 37; repressed by SoxS, 1; activated by SoxS, 23; repressed by OxyR, 2; activated by OxyR, 16; activated by CusR, 6; activated by BaeR, 9; repressed by Zur, 4; repressed by ArsR, 3; activated by ZraR, 3; activated by ZntR, 1. Note that some genes are controlled by more than one regulator. With the sole exception of OxyR (see below), the model predicted with high confidence significant changes in the activity of all the regulators (Fig. 5).

No response of OxyR was detected. A closer analysis of the regulons reveals that there are 11 genes whose sole regulator is OxyR; their changes across the transition are modest (fold change less than 2) and uncorrelated, which implies that the log-linear model is unable to detect statistically significant changes in OxyR activity. It is interesting to compare this situation with the situation for BaeR: here, there is only one gene whose sole regulator is BaeR, and its fold change is also modest. Nevertheless, the profiles of all the genes across the regulon are very strongly correlated (mean Pearson correlation coefficient 0.8), leading to a very confident prediction of a change in activity for BaeR. Obviously, the model performed its analysis automatically, obtaining a statistically optimal solution without the need for time-consuming data exploration. There are also three regulators (ArsR, ZntR and ZraR) that do not share any targets with any other regulators in the chosen set, making the inference task trivial. The remaining six regulators have densely overlapping regulons.

With the exceptions of Fur (Fig. 5d) and SoxS (Fig. 5e), the regulators exhibit a monotonic change from a low level of activity to a high level of activity or vice versa, which is compatible with their physiological role (e.g. the activity of Zur is predicted to increase as Zn concentration increases). The predicted activity profile of Fur exhibits a transient behaviour, with a rapid increase in activity levels followed by a slow decrease to return it close to the initial state after 30 min (Fig. 5d). The converse is true for SoxS, with a rapid decrease in activity followed by a slow increase back to approximately the initial state (Fig. 5e).

Some of the genes considered in our analysis also have other regulators (e.g. FNR, ArcA), and secondary effects may lead to a change in activity of some other regulators. To check against this, and to assess the robustness of our predictions, we repeated our analysis including three further regulators, FNR, ArcA and IscR. The results on the previous set of regulators were unchanged, with transient activity of Fur and SoxS still predicted (Supplementary Fig. S1). No significant activity of IscR and FNR was predicted. Moderate changes in activity for ArcA were predicted, perhaps indicative of membrane effects. However, since our focus is the transient behaviour of Fur and SoxS, we will not investigate ArcA activity any further.

Table 1. Change in cellular metal concentration after addition of ZnSO$_4$ to a Zn-limited chemostat culture of E. coli strain MG1655

(a) and (b) denote sequential runs in the same chemostat vessel.

<table>
<thead>
<tr>
<th>Metal</th>
<th>$10^4 \times$Metal content [mg metal (mg cells)$^{-1}$]</th>
<th>Fold change</th>
<th>Control (30 min after</th>
<th>30 min after addition of 200 μM ZnSO$_4$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>after addition of</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ZnSO$_4$</td>
<td></td>
</tr>
<tr>
<td>Zn</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a)</td>
<td>0.155</td>
<td>4.38</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>(b)</td>
<td>0.105</td>
<td>1.47</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Fe</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a)</td>
<td>1.40</td>
<td>11.6</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>(b)</td>
<td>1.50</td>
<td>8.4</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Cu</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a)</td>
<td>0.06</td>
<td>0.433</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>(b)</td>
<td>0.055</td>
<td>0.433</td>
<td>8</td>
<td></td>
</tr>
</tbody>
</table>
Dynamic effects underlie the observed transients in Fur and SoxS activity

The above observations can be explained in two different ways: first, Fur activity could be maximal at intermediate Zn concentrations, such as might be experienced as cells perceive the increased Zn concentration; or second, the transient profile of Fur activation could be a dynamic effect resulting from the shock of the addition of Zn to a Zn-depleted culture. Similarly, for SoxS, activity may be minimal at intermediate Zn concentrations or the transient profile of SoxS repression may be a dynamic effect. To test the first hypothesis, we generated a dataset containing microarray data from E. coli growing at steady state in Zn starvation and E. coli growing at steady state in Zn excess, linked via a culture grown in 'adequate Zn' to form an artificial time-course with three time points. The time-course was constructed using existing microarray data, i.e. those (a) of Lee et al. (2005) comparing E. coli grown with high Zn (200 μM) to cells grown with adequate Zn (6.14 μM) and (b) of Graham et al. (2009) comparing E. coli grown with low Zn (<60 nM) to cells grown with adequate Zn (6.14 μM). In both cases, the cultures grown in high or low Zn were compared to a culture grown with ‘adequate Zn’ of 6.14 μM. However, it is worth noting that, due to the differing methods of medium preparation, the ‘adequate Zn’ culture used (Lee et al., 2005) is likely to contain more Zn introduced as contamination from other chemicals, glassware, etc. The results of this analysis are shown in Fig. 6. While the profiles of the ‘monotonic’ regulators BaeR, CpxR, Zur and CusR are compatible with the predicted profile from the dynamic time-course dataset, neither Fur nor SoxS exhibits the transient behaviour predicted from the dynamic data. This analysis does not support the hypothesis that the activities of Fur and SoxS depend solely on Zn concentration, lending support to the hypothesis that their transient behaviour may be a result of the dynamics of Fe concentration during the transition.

DISCUSSION

Dynamic analysis of the changes in activity of TFs during a shift from Zn starvation to excess Zn reveals a transient behaviour for both Fur and SoxS. Neither behaviour is revealed by the steady-state analyses shown in Fig. 6, leading us to conclude that these unexpected patterns are transients caused directly by the starvation-to-surfeit shift imposed upon the cultures. The origins of these patterns are likely to be complex. For Fur, which responds primarily to intracellular iron status, we note that there is a substantial increase in cellular Fe within the 30 min of the experiment (Table 1). It was not possible to measure cellular metal levels during the time-course due to the amount of biomass needed for ICP-AES measurements and the small scale of the chemostats, and so the timing of the rise in iron levels is uncertain. Nevertheless, when Fe is plentiful, regulatory sites in Fur will be occupied, and Fur binds to DNA and functions as a repressor of genes...
involved in Fe uptake. Since the activities calculated here are a proxy for the inferred concentration of protein in the DNA-binding form, this phase is reflected in an increase in Fur activity in Fig. 5(d) over the first 10 min of the transit. When Fe is scarce, Fur loses its DNA-binding activity, the Fur regulon is de-repressed and Fe is taken up by the cell. The declining activity in Fig. 5(d) is therefore presumably a reflection of homeostatic mechanisms operating. A further explanation for the activation of Fur is that the sudden increase in intracellular Zn is sensed as a surfeit of iron. Indeed, in vitro, several divalent cations can activate Fur and cause it to bind DNA; in the case of the E. coli protein, Fe(II), Fe(III), Co(II), Mn(II) and Zn(II) all activate Fur (Mills & Marletta, 2005). However, the nature of the ‘regulatory’ and ‘structural’ metal-binding sites in proteins of the Fur family remains controversial (Sheikh & Taylor, 2009; Dian et al., 2011).

These considerations can be given a more formal expression with the aid of a simple mathematical model. A simple coarse-grained model of Fe homeostasis can be formulated as:

\[
\frac{d[Fe]}{dt} = -\theta[Exp][Fe] + \gamma[Imp]
\]

\[
[Fur] = \frac{k[Fe]}{k + [Fe]}
\]

\[
\frac{d[Imp]}{dt} = \frac{\alpha}{\beta + [Fur]} - \lambda[Imp]
\]

where [Fe] denotes the intracellular Fe concentration, [Exp] and [Imp] are collective variables denoting Fe exporter(s) and importer(s) concentrations, and [Fur] denotes the concentration of metallated Fur, which is assumed to depend on [Fe] through a simple Michaelis–Menten equation. The model assumes a reservoir of extracellular Fe, so that Fe import is proportional to [Imp] (first equation). The importers are downregulated by metallated Fur via a Michaelis–Menten dependence. The proposed hypothesis of metal dynamics relies on the assumption that, in low Zn, cellular Zn importers will be highly expressed, while Zn exporters will be absent. A sudden influx of extracellular Zn will result in a marked increase of intracellular Zn (as the Zn importers are highly expressed at that point) without a corresponding mechanism for excess Zn removal (since the Zn exporters are repressed). Excess Zn may then be binding competitively to Fe exporters, thus reducing the effectiveness of the Fe export mechanism described in the first equation in (2). Support for this conjecture is found in the fact that both ZupT and FieF (YiiP) can transport both Zn and Fe (Grass et al., 2005a, b). Simulation of the transition using equation (2) consistently shows a transient activation of Fur and an elevated final concentration of Fe, which is robust to changes in the parameters across an order of magnitude. Full parameterization and validation of the model in equation (2) would require substantial further experimental evidence (such as Fe concentrations across the shift), so the mechanism we suggest has to be considered solely as a hypothesis. Nevertheless, the model shows the consistency of the competitive mechanism with the transient behaviour of Fur implied by the data.
In this analysis, most Fur-regulated genes are repressed by Fur and so their expression will decrease initially then increase, the converse of the pattern for Fur activity (Fig. 5d). For example, expression of tonB and exbD/exbB, which are repressed by Fur and encode proteins involved in Fe uptake and enterochelin uptake respectively, decrease up to sixfold between 0 and 10 min but, after 30 min, return to the same level as immediately prior to Zn addition. For the purpose of this analysis, Fur was classed as an activator of zinT, since one study found that a mutation in fur drastically attenuated the induction of zinT by cadmium (Puškárová et al., 2002). Expression of the zinT gene shows a massive decay towards the end of the time-course, which is consistent with the inferred profile of Fur. Recently in *Salmonella enterica*, it has been found that ZnuA is involved in the first response to Zn deficiency, and ZinT appears when the Zn deficiency becomes more severe; however, both rely on ZnuBC for transport of Zn into the cell (Petrarca et al., 2010). Our results show that the expression of the znuABC genes and zinT decreases at a similar rate when Zn is added to a severely Zn-depleted culture, but that, ultimately, expression of zinT is reduced to almost zero whereas znuABC continue to be expressed (albeit at a lower rate) in keeping with the findings of Petrarca et al. (2010).

SoxS exhibits a mirror-image pattern to Fur, with a rapid decrease followed by a gradual increase (Fig. 5e). However, SoxS primarily functions as an activator whereas Fur mainly acts to repress genes under its control (Supplementary Table S2). Because activity is a proxy for concentration of DNA-binding protein, increasingly positive values for SoxS (Fig. 5e) imply more activation, which may result from changes in conformation of existing proteins, and/or changes in total concentration of the protein (due to transcriptional responses). Recent work has shown that SoxR is activated when reductant agents oxidize its Fe–S cluster, leading to an activation of SoxS (Gu & Imlay, 2011). It was thought that activation of the SoxRS regulon formed a defence against superoxide. However, it appears now that the critical function of SoxRS is in the protection against reductant agents. Within the first 10 min of Zn addition, it appears that Fur senses extra iron (from unknown sources), reflected in an increase in DNA-binding activity (Fig. 5d). On the other hand, during this period, SoxS activity declines (Fig. 5e), so it seems unlikely that the function of this change is to counteract oxidative stress generated by iron overload (Halliwell & Gutteridge, 2007).

It is unknown how an increase in the cellular Zn levels leads to an increase in Cu, but what is certain from this and many other studies is that the interplay between Cu, Fe and Zn homeostasis is complex and delicately balanced. It is known that Cu can displace Fe from Fe–S clusters and that it may also inhibit the Isc Fe–S cluster assembly apparatus (Macomber & Imlay, 2009). Consequently, it is possible that elevated Fe levels seen in cells subject to a transition from very low Zn to high Zn may be mediated indirectly by Cu. It has been shown for yeast and mammalian cells that elevated levels of Zn result in an accumulation of Fe (Niles et al., 2008; Pagani et al., 2007).

We stress that the main insights into Fe regulation reported here are possible only because of the analysis of a dynamic dataset. No transient behaviour of Fur was inferred from the analysis of steady states (Fig. 6d). This highlights the importance of time-course data in the study of perturbations to biological systems, and should, in our opinion, guide experimental design in similar studies. However, this is, of course, context-dependent, and steady-state analyses are also valuable depending on the question being addressed.

**ACKNOWLEDGEMENTS**

This work was funded by the Biotechnology and Biological Sciences Research Council UK through the SysMO initiative (http://www.sysmo.net/) and project grants BB/F003463/1 and BB/C509831/1. G. S. is funded by the Scottish Government through the SICSA initiative.

**REFERENCES**


