Transcriptional and Translational Regulatory Responses to Iron Limitation in the Globally Distributed Marine Bacterium \textit{Candidatus Pelagibacter Ubique}

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\textbf{Abstract}

Iron is recognized as an important micronutrient that limits microbial plankton productivity over vast regions of the oceans. We investigated the gene expression responses of \textit{Candidatus Pelagibacter} ubique cultures to iron limitation in natural seawater media supplemented with a siderophore to chelate iron. Microarray data indicated transcription of the periplasmic iron binding protein \textit{sfuC} increased by 16-fold, and iron transporter subunits, iron-sulfur center assembly genes, and the putative ferroxidase rubredoxin transcripts increased to a lesser extent. Quantitative peptide mass spectrometry revealed that \textit{sfuC} protein abundance increased 27-fold, despite an average decrease of 59% across the global proteome. Thus, we propose \textit{sfuC} as a marker gene for indicating iron limitation in marine metatranscriptomic and metaproteomic ecological surveys. The marked proteome reduction was not directly correlated to changes in the transcriptome, implicating post-transcriptional regulatory mechanisms as modulators of protein expression. Two RNA-binding proteins, CspE and CspL, correlated well with iron availability, suggesting that they may contribute to the observed differences between the transcriptome and proteome. We propose a model in which the RNA-binding activity of CspE and CspL selectively enables protein synthesis of the iron acquisition protein \textit{sfuC} during transient growth-limiting episodes of iron scarcity.

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\section*{Introduction}

The importance of iron as a nutrient in the oceans was first recognized by Martin [1] and later experiments verified that iron limits primary production over broad regions of the marine environment [2–4]. A variety of biological processes such as photosynthesis, \textit{N}_2 fixation, methanogenesis, respiration, oxygen transport, gene regulation, and DNA synthesis all depend on iron-containing proteins [5]. In pelagic surface waters, planktonic communities must cope with iron concentrations that average just 70 picomolar [6]. The inhibitory effect that this has on growth was most clearly illustrated by a series of iron fertilization experiments in which iron was added to large swaths of the ocean, resulting in a marked increase in nutrient utilization [2,3,7].

Bacteria commonly have specialized systems for responding to iron limitation. Genes for iron uptake and utilization are primarily regulated by the Fur protein [8,9]. When complexed with Fe(II) cations, Fur binds the “Fur box” recognition sequence, which is made of several GATAAT hexamers [10–14]. In some bacteria, this single transcription factor can directly repress or activate more than 100 genes in response to iron scarcity [9]. Irr is a similar transcription factor that couples intracellular heme levels to expression of many different iron-related pathways [15–21]. Small RNAs [22–25] and mRNA-binding proteins [26,27] can also regulate nonessential iron-utilizing proteins at the post-transcriptional level by selectively targeting their transcripts for degradation. To improve their chances of encountering Fe(III), many bacteria secrete siderophores [28–30]. These chelating agents help dissolve the poorly soluble particles and sequester them in a form that is unusable by competing microorganisms. Due to the spontaneous reactivity of iron ions, cells often encapsulate these atoms inside containers made of ferritin proteins to better modulate redox reactions [31,32].

\textit{Candidatus Pelagibacter} ubique was selected as an iron limitation model for two reasons. First, this alphaproteobacterium is regularly the most numerically abundant microorganism in surveys of marine microbial diversity. Second, its proteome of just 1,354 genes is possibly the simplest of any free-living heterotrophic organism [33]. \textit{Ca. Pelagibacter} ubique’s genome encodes Fur and Irr, but not ferritin or siderophore-related proteins, raising questions about how or if this bacterium can cope with iron stress. Investigating how this organism’s relatively small
genetic repertoire produces thriving populations in the variable ocean environment has been impeded by the lack of a genetic system able to create knockouts or other genetic modifications. Thus, observing how the entire transcriptome or proteome changes in response to growth conditions has become a primary approach for elucidating metabolic and regulatory schemes [34–36]. A comparison of cultures in exponential and stationary phase did not reveal a major remodeling of the proteome nor evidence of a global regulatory mechanism [34], suggesting that this organism may continue to benefit from temporary nutrient availability regardless of overall cellular activity. That study, along with a follow-up using a metaproteomics approach on environmental samples [35], found Ca. Pelagibacter ubique’s proteome to be consistently composed of an unusually high proportion of transport-related proteins.

Arguably the most important characteristic of organisms is their ability to express the right proteins in the right amounts at the right times. The interplay between stimuli, sensors, and regulators precisely optimizes the combination of mRNA transcripts and protein products present in the cell. Several known and putative transcriptional regulators have been identified in the Ca. Pelagibacter ubique genome, as well as cis-acting riboswitches capable of modulating mRNA translation based on the concentration of particular metabolites. This method of decoupling production of mRNA from protein synthesis has been found on glycine metabolism genes in Ca. Pelagibacter ubique [37] and sequence motif searches [38] found additional candidate riboswitches for s-adenylmethionine [39,40] and thiamine pyrophosphate [40,41]. Meyer and colleagues also identified homologs to ribosomal proteins capable of regulating their own translation, as well as regions in the genome with riboswitch-like characteristics but lacking homologous annotated motifs. One of these putative structural RNA regions is located immediately upstream of the sufA–C operon, which encodes an iron-acquisition system. Post-transcriptional regulation schemes allow the cell to conserve amino acids while still rapidly providing ephemeral enzymes. The success of these characteristics are evidenced by direct observation; Ca. Pelagibacter ubique is the most abundant heterotroph in the oceans, accounting for one-third of surface water bacteria [42,43] and consuming up to half of some dissolved organic matter compounds [44].

Combining transcriptomic and proteomic data offers a perspective on cellular activity that cannot be obtained from either method individually. Numerous studies have shown that changes in the transcriptome poorly correlate with changes to the proteome, except for very highly expressed genes [45–48]. Although much of the disparity between these two types of datasets has been attributed to measurement inaccuracy [49] and differences in protein degradation rates [50], some studies have revealed systematic post-transcriptional regulatory schemes. For instance, in the eukaryotic protozoan Plasmodium falciparum, mRNAs were often upregulated an entire life phase before the one in which the encoded protein was needed [51]. Additionally, translation in Escherichia coli was found to be partially regulated by mRNA secondary structure [52]. Therefore, it is evident that the transcriptome does not necessarily represent the current state of the proteome, but is rather a mixture of transcripts being actively translated and others that are standing by, awaiting activation by post-transcriptional regulatory mechanisms. This study integrates both transcriptomic and proteomic analyses in order to attain a more complete understanding of the cellular response to iron limitation in Ca. Pelagibacter ubique. The results strongly suggest that transcription and translation are not always tightly coupled in this bacterium.

Results

Reaction to the Siderophore

Two iron-sequestering siderophores were tested on cultures of Ca. Pelagibacter ubique to determine their feasibility for creating iron-limiting conditions. Ferrichrome (Sigma #F8014) and deferoxamine mesylate salt (Sigma #D9533) were both found to arrest batch culture growth within 1/3 of a doubling – an inhibition which could be reversed by addition of iron (Figure 1A). Sufficient bioavailable iron was present in the natural seawater media collected from the Oregon coast to enable cultures to grow when supplemented with 10 nM siderophore, but not when supplemented with 100 nM siderophore.

Microarrays

Six 20 L carboys inoculated with Ca. Pelagibacter ubique were grown to near-maximum density, then randomly selected for treatment with either ferrichrome or ferrichrome plus excess iron (Figure 1B). To measure the amount of messenger RNA transcripts present in cells, mRNA from each carboy was hybridized to separate microarray chips containing probes for all Ca. Pelagibacter ubique genes. Microarray data was deposited in the NCBI GEO database under accession number GSE20962. Of the three time points where mRNA abundance was measured, the greatest difference in expression of known iron-related genes was observed 24 hours after the siderophore amendment. Table 1 lists the 23 transcripts that were expressed at least 50 percent higher in the iron-limited culture relative to the control. Two-thirds of the genes in this list come from two operons: the first containing iron-sulfur center assembly proteins including sufA–E and the second made up of iron uptake proteins such as sufA–C. The four other genes with a known function are: rubrerythrin, an iron-binding protein that is postulated to act as a ferroxidase for converting Fe(II) to Fe(III), hisU and hisV which together form a protease complex, and dimethylglycine dehydrogenase – an enzyme that is necessary for converting betaine to glycine.

A modified radial coordinate visualization plot (Figure 2) of the microarray data shows four distinct clusters of genes: exponential growth, stationary phase, early iron stress, and late iron stress. As detailed in Supplementary Table S1, the early iron stress cluster is dominated by genes from three genomic loci: the sufA iron uptake operon, the suf iron-sulfur center assembly operon, and the functionally unclear loci SAR11_1157, SAR11_1158, SAR11_1163, and SAR11_1164. The late iron stress cluster also contains different genes that are located in or adjacent to the suf and suf operons, but is better characterized by lexA, recA, and mussA – three genes involved in the SOS response. The iron response regulators fur and irr cluster with stationary phase genes, indicating that the abundances of these two transcripts are more affected by stationary phase than by iron limitation.

Proteomics

Cellular protein fractions from each treatment were isolated and digested before being separated with liquid chromatography and injected into a tandem mass spectrometer. An Accurate Mass and Time Tag library, developed previously [34], was used to make quantitative comparisons of the abundance of individual peptides between samples. This dataset is available at http://omics.pnl.gov/. Of the 216 proteins detected with high certainty in this study, 18 were observed to be at least 50% more abundant in the iron-limited cultures: four on day 18, and 17 on day 28 (Table 2). The proteins SufC, CspL, and GroES were higher in the iron-limited cultures at both timepoints. The iron-binding SufC is unique in that it was the only one of these 18 proteins to increase
in both protein and mRNA abundance by at least 50%. CspL was originally annotated as a DNA-binding protein, however, similar proteins have been found to modulate the accessibility of mRNA binding sites by selectively melting secondary RNA structures [53]. The third protein, GroES, forms a complex with GroEL to mediate protein folding. Because the required GroEL subunit was much less abundant in iron-limited cultures, and since the three largest GroES peptide spectra (out of 9) were less pronounced in the iron-limited cultures, GroES may be a false positive. Mass spectrometry measurements did not reveal a significant change in Fur or Irr abundance between treatments or timepoints.

Iron-limitation had a marked impact on the overall proteome. Two days after addition of an iron-chelator, 181 of the 216 proteins were significantly (P ≤ 0.05) less abundant in the iron-limited cultures relative to the control cultures. Using the same criteria, only 32 of the 216 proteins were found to significantly decrease in the control cultures between days 18 and 28 as Ca. Pelagibacter ubique cells entered stationary phase due to an unknown, non-iron, limitation.

Comparing Changes in mRNA and ProteinAbundances

Aside from the highly expressed iron-binding protein SfuC, the abundances of individual proteins appeared to be independent of the amount of mRNA encoding them (Figure 3).

Discussion

We are studying keystone microbial plankton species such as Ca. Pelagibacter ubique in culture to provide a basis for interpreting data emerging from molecular ecology studies. In an era of rapid environmental change, metagenomics, and allied technologies such as metaproteomics and metatranscriptomics, are being used to monitor the structure and health of natural ecosystems and to identify ecological processes that impact biogeochemistry. Interpretations of these data depend on understanding how complex cellular systems respond to environmental factors. We focused on a microorganism, Ca. Pelagibacter ubique, that produces the largest signal in most environmental studies of marine macromolecules, and a process, iron limitation, that impacts marine ecology on very large geographical scales.

Upregulation of sfuC during iron limitation

The only gene to clearly increase in both mRNA and protein abundance during iron limitation was sfuC. This protein localizes to the periplasmic space and binds dissolved Fe(III) with high affinity. The SfuC-Fe complex associates with the ATPase (SfuA) and permease (SfuB) components of the tripartite ABC transporter complex to actively transport iron into the cell. The fact that sfuA and sfuB were not observed to increase in protein abundance is not wholly unexpected – SfuA-SfuB complexes only interact with iron-bound SfuC proteins, which are a very small fraction of the total SfuC pool in an iron-limited environment. Additionally, integral membrane proteins such as SfuB are particularly challenging to recover in proteomic studies because they are not readily soluble. This likely contributed to the complete absence of SfuB peptides in all mass spectrometry studies of Ca. Pelagibacter ubique to date.

The identification of sfuC expression as a readily quantifiable iron limitation marker is particularly useful for ecological surveys.
As its name suggests, *Ca.* Pelagibacter ubique’s genome, transcriptome, and proteome regularly dominate bacterial surveys throughout the pelagic environment. Future oceanographic studies seeking evidence of iron availability limiting bacterioplankton growth may use metatranscriptomic or metaproteomic analyses to assess the expression of iron-related genes such as *sufA–E* 

*Iron Limitation in *P.* ubique*

Transcriptome distinct from proteome

Protein abundance was generally uncorrelated with changes in mRNA abundance, suggesting that post-transcriptional mechanisms might be acting at the RNA level to suppress translation. As reviewed in the introduction, previous studies have shown that disparities between a cell’s transcriptome and proteome are the norm rather than the exception. However, the observation that iron-related genes such as *sufA–E* increased in mRNA but not protein indicates that expression of these proteins are controlled at both the level of transcription and at the level of translation.

Cold-shock proteins correlated with iron stress

*CspL* was significantly more abundant in iron-limited cultures compared to the control cultures, 24 hours after addition of an iron-chelator. This is the first report describing the general suppression of translation across the entire transcriptome of the cell. In this case, the apparent adaptive significance of protein synthesis suppression is related to urgent cellular requirements to acquire an essential nutrient. The model we propose to explain this phenomenon incorporates activity previously observed in cold-shock proteins, however, the essence of our model assigns cold-shock proteins a new systemic role in *Ca.* Pelagibacter ubique cells with the apparent result of focusing protein synthesis on transporters that target a missing essential nutrient. The validation of this model is needed to prevent spurious stem loop structures from interfering with transcription and translation [54–59]. Despite their homology to CspA, many CSP variants are not cold-inducible, but rather are involved in regulating cellular processes [54,60–62] and can even target their activity to specific RNA sequences [63]. A growing body of literature has described mRNAs which modulate their own expression via temperature- (RNA thermometer) or ligand-sensitive (riboswitch) secondary structures [64,65]. Due to the episodic nature of iron deposition into ocean surface waters [66] and the resulting selective pressure favoring rapid response systems for this limiting nutrient [67], we speculate that *Ca.* Pelagibacter ubique CspE and/or CspL affects a reversible inhibition of translation by facilitating an mRNA secondary structure unfavorable for ribosome processing, thereby maintaining the transcriptome in a state of cell growth readiness during times of stress such as iron limitation.

**Table 1.** All 23 *Ca.* Pelagibacter ubique mRNA transcripts that were at least 50 percent more abundant in the iron-limited cultures compared to the control cultures, 24 hours after addition of an iron-chelator.

| Locus ID | Gene   | Description                                    | Ratio | P Value | Cluster |
|----------|--------|------------------------------------------------|-------|---------|---------|
| SAR11_0144 | hslV   | ATP-dependent protease: peptidase               | 1.54  | 0.001   | Early   |
| SAR11_0333 | hslU   | ATP-dependent protease: ATP-binding            | 1.63  | 0.000   | Stat.   |
| SAR11_0334 | rbr    | Rubrythrin, hyp. ferroxidase (Fe$^{3+}$→Fe$^{2+}$) | 1.57  | 0.025   |         |
| SAR11_0399 | sufA   | Transcriptional regulator                      | 1.76  | 0.062   | Stat.   |
| SAR11_0738 | sufB   | Cysteine desulfurase activator complex         | 2.00  | 0.009   | Early   |
| SAR11_0740 | sufC   | FeS assembly ATPase                            | 1.80  | 0.017   | Early   |
| SAR11_0741 | sufD   | FeS assembly protein                           | 2.29  | 0.019   | Early   |
| SAR11_0742 | csdB   | Selenocysteine lyase chain A                    | 2.44  | 0.046   | Early   |
| SAR11_0743 | sufE   | Putative NifU-like protein                     | 2.24  | 0.004   | Late    |
| SAR11_0744 | paaD   | Phenylacetic acid degradation protein          | 2.00  | 0.001   | Early   |
| SAR11_0745 | hesB   | HesB protein                                   | 1.95  | 0.000   | Early   |
| SAR11_0785 | CspE   | Conserved hypothetical protein (DUF952)        | 1.52  | 0.051   | Late    |
| SAR11_1233 | a2IC   | A2IC protein                                   | 2.14  | 0.016   | Early   |
| SAR11_1236 | sufA   | Iron(III) ABC transporter: ATP-binding         | 4.99  | 0.000   | Early   |
| SAR11_1237 | sufB   | Iron(III) ABC transporter: permease            | 10.36 | 0.000   | Early   |
| SAR11_1238 | sufC   | Iron(III) ABC transporter: periplasmic          | 16.00 | 0.003   | Early   |
| SAR11_1239 | aceA   | Isocitrate lyase                               | 2.47  | 0.006   | Late    |
| SAR11_1240 | dmdgdh | Dimethylglycine dehydrogenase                  | 1.54  | 0.054   | Late    |
| SAR11_1279 | unknown membrane protein                       | 1.56  | 0.056   |         |

Seventy-eight percent of these genes are found in Figure 2’s early and late iron stress clusters. Bullet points in the first column indicate contiguous loci.

ªAverage fluorescence of three replicates, (iron limited culture/iron replete culture).

ßResult of a two-tailed Student’s t-test comparing the three biological replicates for each treatment.

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beyond the scope of this study. Future work may more precisely identify interactions between cold-shock proteins with specific RNA motifs.

Summary
Census information has left little doubt that Ca. Pelagibacter ubique plays its role in biological oceanography on a vast scale. To understand this role, we turned inward, investigating the mechanisms used by these cells to respond to a common form of nutritional stress. One motivation for this study can be described with a term borrowed from satellite remote sensing: the term “ground truth” was coined to describe the validation, by direct measurements, of remotely sensed observations. Metatranscriptomic and metaproteomic measurements are being widely adopted by microbial ecologists anticipating that these approaches will reveal the metabolic status of cells in microbial communities, providing information that can be extrapolated to interpret broader levels of ecosystem function. Essential to this vision is an understanding of how cells respond to environmental variables. Our findings indicate that the periplasmic iron binding protein sfuC is uniquely suitable for assessing the iron limitation status of Ca. Pelagibacter ubique cells. We anticipate that ecologists will use this data for interpreting the nutritional status of Ca. Pelagibacter ubique cells in nature.

This study, one of the few to simultaneously examine both transcriptional and translational responses in a bacteria cell, uncovered evidence suggesting that cpl might play a role in the cellular response to iron limitation. We offer the model that this protein controls translation in response to environmental conditions for a specific subset of genes present in the transcriptome. We hypothesize that this activity might serve an emergency function, limiting the synthesis of proteins to those that are critical for survival. This finding is consistent with previous reports of post-transcriptional regulation of the iron stress response in which a protein was found to facilitate the degradation of specific mRNAs which encoded nonessential iron-consuming pathways [26,27].

Not only is Ca. Pelagibacter ubique one of the most successful cells known, it is also one of the simplest, giving it value as a model for understanding bacterial cell responses. Indeed, numerous new structural RNAs, some widely distributed among bacteria, have been discovered and described in Ca. Pelagibacter ubique [37,38]. It is perhaps hubris to imagine that the concept of systems biology might one day be extended from the machinery of cells to the machinery of microbial ecosystems at work on the scale of oceans. But, if that vision has a chance, it will be by combining studies that cross scales and disciplines to understand the keystone species of the oceans.

Materials and Methods

Growth Media and Harvesting
Seawater was collected on 6/14/08 at the Newport Hydroline station NH5 (44°39.1”N, 124°10.6’W) from a depth of 10 m. The water was then filtered through a 0.2 μm filter, autoclaved, and sparged with CO2 for 24 hours followed by air for 24–48 hours as previously described [68,69]. Immediately prior to inoculation with Ca. Pelagibacter ubique HTCC1062, the media was amended with 50 μM pyruvate, 50 μM glucose, 10 μM nitrogen, 1 μM methionine, 1 μM glycine, 1 μM phosphate, and vitamins. Cells were grown at 20°C (flasks) or 16°C (carboys) with intermittent light and sparging with air. On day 16, three 20 L control cultures were amended with 100 nM ferrichrome and

Figure 2. Genes transcribed during iron limitation were different from stationary phase genes. The four clusters indicate up-regulation of similar condition-specific mRNA. Symbols for each microarray sample (open circles) were manually positioned on a circle according to each sample’s iron availability and growth rate. Genes were “attracted” to the samples in which they were most abundant. Larger points indicate genes with larger condition-to-condition variation; a key for the 10 largest points in each cluster is provided. The complete list of gene locations for this graph can be found in Supplementary Table S1.

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All samples were kept at 4°C until analysis. All 17 Ca. Pelagibacter ubique proteins that were at least 50 percent more abundant in the iron-limited cultures compared to the iron replete cultures, two and 12 days after addition of an iron chelator.

| Day | Locus ID | Gene | Description | Ratio | P Value |
|-----|----------|------|-------------|-------|---------|
| 18  | SAR11_1238 | sfuC | Iron(III) ABC transporter: periplasmic | 11.41 | 0.000 |
| 18  | SAR11_1274 | cspL | DNA-binding cold shock protein | 6.20 | 0.000 |
| 18  | SAR11_0161 | groES | Protein-folding chaperonin | 2.01 | 0.028 |
| 18  | SAR11_1062 | dapA | Dihydriodipicolinate synthase | 1.53 | 0.849 |
| 28  | SAR11_1238 | sfuC | Iron(III) ABC transporter: periplasmic | 26.96 | 0.000 |
| 28  | SAR11_1161 | sbC | ATPase involved in DNA repair | 4.59 | 0.011 |
| 28  | SAR11_0161 | groES | Protein-folding chaperonin | 3.59 | 0.008 |
| 28  | SAR11_0601 | ftsH | Metalloprotease | 3.28 | 0.001 |
| 28  | SAR11_1124 | rplL | 50S ribosomal protein L31 | 3.16 | 0.006 |
| 28  | SAR11_0430 | aceF | Dihydriolipoamide S-acetyltransferase | 3.02 | 0.094 |
| 28  | SAR11_0171 | rhodH | Rhodanese-related sulfurtransferase | 2.74 | 0.002 |
| 28  | SAR11_0791 | cspL | Ring-cleaving dioxygenase | 2.42 | 0.336 |
| 28  | SAR11_1274 | cspL | DNA-binding cold shock protein | 2.27 | 0.437 |
| 28  | SAR11_0235 | pdhD | Dihydriolipoyl dehydrogenase | 2.26 | 0.035 |
| 28  | SAR11_0401 | pilA | Conserved hypothetical protein | 2.21 | 0.003 |
| 28  | SAR11_0054 | pilA | Pin protein | 2.16 | 0.020 |
| 28  | SAR11_0727 | accB | Acetyl-CoA carboxylase | 2.03 | 0.301 |
| 28  | SAR11_0987 | ppiB | Peptidylprolyl isomerase | 1.99 | 0.291 |
| 28  | SAR11_0793 | unknown | Unknown protein | 1.74 | 0.128 |
| 28  | SAR11_0599 | groES | Protein-folding chaperonin | 1.70 | 0.623 |
| 28  | SAR11_0708 | acpP | Acyl carrier protein | 1.55 | 0.198 |

**Table 2. All 17 Ca. Pelagibacter ubique proteins that were at least 50 percent more abundant in the iron-limited cultures compared to the iron replete cultures, two and 12 days after addition of an iron chelator.**

**Genes in bold were more abundant in the iron-limited cultures at both timepoints.**

- **a**Average spectra height of at least three peptides, (iron limited culture/iron replete culture).
- **b**Combined one-tailed Student’s t-test comparing the three technical replicates for each treatment.

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Microarray Clustering

A modified radial coordinate visualization plot was used for illustrating mRNA expression in a manner that accentuated condition-specific preferential transcription. In Figure 2, dimensional anchors (DA) representing each of the six microarray samples were positioned manually around the circumference of a circle such that iron-limited samples are on the left, samples with excess iron are on the right, and the vertical placement corresponds to the culture’s transition from exponential growth (bottom) to stationary phase (top). Each gene is represented by a single point, positioned according to the relative abundances between every sample pair, and sized according to the largest observed change in expression level. \( PT_g \) is the point for gene \( g \), with attributes \( x, y \), and \( s \) describing its x-axis position, y-axis position, and size, respectively. \( S_{max} \) is the log base-10 average fluorescence for gene \( g \) in sample \( i \). \( DA_i \) is sample \( i \)'s dimensional anchor positioned at \( \langle DA_{1i}, DA_{2i}, DA_{3i} \rangle \) on the graph.

\[
PT_{g,s} = \max(S_{1,s,g}, S_{2,s,g}, S_{3,s,g}) - \min(S_{1,s,g}, S_{2,s,g}, S_{3,s,g})
\]

\[
PT_{g,s} = \sum_{1 \leq i < j \leq 6} \left[ \frac{(DA_{i,s} - DA_{j,s}) \times (S_{i,s,g} - S_{j,s,g})}{PT_{g,s}} \right]
\]
This type of graph is ideal for revealing if a given gene’s transcript abundance is changing as a result of iron limitation or as a result of the stationary phase transcriptome remodeling induced by iron limitation.

**Global TFE Protein Preparation**

Four samples were prepared using the TFE (2,2,2-Trifluoroethanol) digestion method. The cell pellets were reconstituted in 100 mM NH₄HCO₃, pH 8.4 buffer and transferred to a siliconized 0.6 mL microcentrifuge tube. 0.1 mm Zirconia/Silica Beads were added to the top of the tube and bead beat at maximum speed for 3 minutes and immediately placed on ice. A hole was poked in the base of the 0.6 mL siliconized eppendorf tube and placed in a 1.5 mL siliconized eppendorf tube. The sample was then centrifuged for 5 minutes at 14,000 rpm at 4°C. The cell lysis was mixed to a homogenized state and the volume was determined using a pipette. The sample concentration was determined with a Coomassie protein assay and read on a microplate reader. TFE was added to a concentration of 50%. The sample was then homogenized by sonication for one minute.
in an ice bath followed by incubation at 60°C for two hours with gentle shaking (300 rpm). Proteins were reduced by adding DTT to a final concentration of 2 mM, sonicated for one minute in an ice bath and incubated at 37°C for one hour with gentle shaking. Samples were then diluted 5-fold with 100 mM NH₄HCO₃ to reduce the salt concentration, and CaCl₂ was added to a final concentration of 1 mM. The sample was digested for 3 hours with Trypsin (Promega, Madison WI) at 37°C at a concentration of 1 unit trypsin/50 units protein. After trypsin incubation, a BCA protein assay was performed on the sample to determine the final concentration and vialed for mass spectrometer analysis.

**Capillary LC-MS Analysis**

The custom HPLC system was configured using 65-mL Isco Model 65D syringe pumps (Isco, Inc., Lincoln, NE), 2-position Valco valves (Valco Instruments Co., Houston, TX), and a PAL autosampler (Leap Technologies, Carrboro, NC), allowing for fully automated sample analysis across four separate HPLC columns. Reversed-phase capillary HPLC columns were manufactured in-house by slurry packing 3-μm Jupiter C18 stationary phase (Phenomenex, Torrance, CA) into a 70-cm length of 360 μm o.d.×75 μm i.d. fused silica capillary tubing (Polymicro Technologies Inc., Phoenix, AZ) that incorporated a 0.5-μm retaining screen in a 1/16” custom laser-bored 75 μm i.d. union (screen and union – Valco Instruments Co., Houston, TX; laser bore – Lenox Laser, Glen Arm, MD). Mobile phases consisted of 0.2% acetic acid and 0.05% TFA in water (A) and 0.1% TFA in 90% acetonitrile/10% water (B). The mobile phase flowed through an in-line Degassex DG4400 degasser (Phenomenex, Torrance, CA). The HPLC system was equilibrated at 10 k psi with 100% mobile phase A. Fifty minutes after sample injection the mobile phase was switched to 100% B, which created a near-exponential gradient from sample 1 was divided by the peptide average from sample 2.

To represent the likelihood that a protein was equally abundant in both samples, the multiple peptide measurements were combined into a single chi-square statistic using Fisher’s method:

\[ \chi^2 = -2 \sum \ln(P_i) \]

**Supporting Information**

**Table S1** Coordinates of All Genes Plotted in Figure 2. Found at: doi:10.1371/journal.pone.0010487.s001 (0.25 MB XLS)

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Author Contributions
Conceived and designed the experiments: DPS SJG. Performed the experiments: DPS JBK TRC MSS LS CDN. Analyzed the data: DPS ADN SJG.

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