A High-Throughput Method to Examine Protein-Nucleotide Interactions Identifies Targets of the Bacterial Transcriptional Regulatory Protein Fur

Chunxiao Yu1*, Carlos A. Lopez2,3, Han Hu5, Yu Xia5, David S. Freedman4, Alexander P. Reddington2, George G. Daaboul4, M. Selim Ünlü2,4,6, Caroline Attardo Genco1,3*

1 Department of Medicine, Section of Infectious Diseases, Boston University School of Medicine, Boston University, Boston, Massachusetts, United States of America, 2 Department of Electrical and Computer Engineering, Boston University, Boston, Massachusetts, United States of America, 3 Department of Microbiology, Boston University School of Medicine, Boston University, Boston, Massachusetts, United States of America, 4 Department of Biomedical Engineering, Boston University, Boston, Massachusetts, United States of America, 5 Bioinformatics Graduate Program, Boston University, Boston, Massachusetts, United States of America, 6 Physics Department, Boston University, Boston, Massachusetts, United States of America

Abstract

The Ferric uptake regulatory protein (Fur) is a transcriptional regulatory protein that functions to control gene transcription in response to iron in a number of pathogenic bacteria. In this study, we applied a label-free, quantitative and high-throughput analysis method, Interferometric Reflectance Imaging Sensor (IRIS), to rapidly characterize Fur-DNA interactions in vitro with predicted Fur binding sequences in the genome of Neisseria gonorrhoeae, the causative agent of the sexually transmitted disease gonorrhea. IRIS can easily be applied to examine multiple protein-protein, protein-nucleotide and nucleotide-nucleotide complexes simultaneously and demonstrated here that seventy percent of the predicted Fur boxes in promoter regions of iron-induced genes bound to Fur in vitro with a range of affinities as observed using this microarray screening technology. Combining binding data with mRNA expression levels in a gonococcal fur mutant strain allowed us to identify five new gonococcal genes under Fur-mediated direct regulation.

Introduction

The Interferometric Reflectance Imaging Sensor (IRIS) is a photometric biosensing technology, which is designed on the principles of interferometry and has been applied as a microarray-based screening technique for studying the interactions of biomacromolecules [1,2,3,4]. IRIS can be broadly applied to characterize many types of biomolecular interactions, such as DNA-DNA hybridization, protein-protein binding, and protein-DNA interaction in a quantitative format [1,2,3,4]. Specifically, this technique has previously demonstrated utility for probing transcription factor interactions with arrayed oligonucleotides [3]. Bacterial transcription factors control tight regulation of gene expression in response to host specific environmental niches in a number of human pathogenic bacteria. One such pathogen is Neisseria gonorrhoeae, which is the causative agent of the sexually transmitted disease gonorrhea, one of the most common infectious diseases worldwide.

Gonococcal colonization of mucosal surfaces requires tight regulation of gene expression in response to host specific environmental niches including iron-limited host environments. This adaptation is mainly achieved by the up-regulation of iron-acquisition components, which are repressed by a ubiquitous bacterial regulatory protein, the Ferric Uptake Regulator protein (Fur) [6,7,8]. Fur is a conserved 15–17 kDa protein comprised of an amino-terminal DNA binding domain and a carboxyl-terminal dimerization domain and is present in a number of diverse bacterial pathogens [9,10,11,12]. In the presence of iron (II) or other divalent cations [9,10,11,13], Fur forms dimers and binds to dsDNA in a sequence specific manner to manipulate transcription initiation [9,10,11,13,14]. In its most basic form, the iron-bound Fur binds to the -10 and −35 motifs in the promoter region of a gene to prevent binding of RNA polymerase and leads to inhibition of transcription initiation [14]. For example, Fur represses transcription without an iron cofactor, a process termed apo-Fur regulation that has been mainly demonstrated in Helicobacter pylori [15,16]. In its apo-form Fur has also been reported to function as an activator, auto-regulating the fur gene in Vibrio vulnificus [17]. The iron-bound Fur dimer has also been reported to function as a transcriptional activator via direct binding to defined promoter regions [3,8,13,18,19,20,21,22,23,24]. Moreover, Fur influences secondary regulatory components, such as small RNAs and histone-like nucleotide binding protein (H-NS), to regulate a subset of genes indirectly [25,26,27,28,29,30,31,32,33].
The Fur regulon of *N. gonorrhoeae* has not been well defined due to the lack of a *fur* mutant strain and an efficient and rapid method to characterize the interactions between the gonococcal Fur protein and predicted Fur binding sequences. To define the gonococcal Fur regulon more efficiently, we developed a microarray-based, label-free quantitative method utilizing IRIS to screen sequence-specific interactions of Fur to dsDNA in vitro [3,34]. Combining this approach with bioinformatical analysis of the gonococcal genome and transcriptional analysis of *N. gonorrhoeae* wild type, *fur* mutant and *fur* complemented strains, we identified new Fur regulated genes. We also describe potential additional functional roles for Fur mediated regulation in *N. gonorrhoeae*.

**Materials and Methods**

**Prediction of Fur boxes**

The sequences upstream (~400 to +50) of ATG, for each open reading frame (ORF) in the *N. gonorrhoeae* FA1090 genome (GeneBank No. AE004969.1) were retrieved using RSAT (http://rsat.ulb.ac.be/rsat/) [35] to represent the putative promoter regions of each gene. Seven experimentally determined Fur boxes in the two pathogenic Neisseria species, *N. meningitidis* and *N. gonorrhoeae*, were used as the patterns for Fur box predictions (Table S1). Matched sequences in the putative promoter regions were identified using fuzmuc module from EMBOSS by setting the mismatch rate as 0.4 [36]. Each Fur box in Table S1 provided a list of matched sequences in the entire genome. Subsequently, overlapping sequences across the seven lists were kept as the final output of predicted Fur boxes.

**Interferometric Reflectance Imaging Sensor (IRIS), substituting EMSA**

IRIS functions on the principles of interferometry [1,2,3,4,37]. Briefly, the optical path length of surface-reflected light is affected by changes in accumulated biomass on that surface. Differences in the optical path lengths (OPD) between the top layer and buried silica surface can be measured very precisely, which allows optical height information to be converted to accumulated mass (density) on the surface. With this technique, biomass accumulation can easily be quantified on a surface with a sensitivity of ~10 pg/mm² for a large field-of-view (FOV) to study multiple binding interactions simultaneously.

**Microarray preparation**

Amino-labeled forward DNA strands (50 μM) (Table S2) were mixed with excess complementary strands to create dsDNA through standard hybridization methods: mixed DNA solutions were heated to 80°C for 10 min and then allowed to cool to room temperature in a slow, controlled manner (<1°C/min). IRIS sensor chips were functionalized with a copolymeric surface chemistry to immobilize the amino-functionalized DNA strands in high density [38,39] as previously demonstrated. The dsDNA solutions were spotted onto the pre-functionalized sensor chips using a non-contact piezo-driven spotter, the sciFLEXARRAYER S3 (Sciencion, Inc, Berlin, Germany). After a 12 hr immobilization period, spotted chips were washed with Saline Sodium Citrate (SSC) buffer in decreasing concentrations (2x, 0.2x, and 0.1x) to produce a microarray of different covalently-attached dsDNA probes. IRIS images were taken for each chip to determine the initial dsDNA densities corresponding to each spot in the microarray. The chips were then incubated with 0, 200, 400, and 800 nM of purified gonococcal Fur protein [8,40,41,42] in 2 mL of binding buffer (20 mM Tris-Cl pH 7.9, 5 mM MgCl₂, 40 mM KCl, 0.125 mM MnCl₂, 2 mM DTT, 10% Glycerol, 0.19 μg/μL poly dIdC), respectively at RT for 2 hr. Subsequently, chips were washed three times (3 min for each wash) using 10 mL binding buffer followed by a 5 sec rinse in 0.1x binding buffer, and images were taken of the post-incubation array.

**Binding interaction analysis**

Increases in mass density due to Fur binding were determined by subtracting initial mass densities, determined for each spot from the pre-incubation images, from the final mass density measurements made after incubation [6] Figure S1. On a spot by spot basis, the number of immobilized dsDNA molecules was determined from knowledge of the measured OPD, a known IRIS conversion factor for OPD (nm) to mass surface density (ng/mm²) for dsDNA, and the molecular weight of each dsDNA oligomer. For the post-incubation measurements, an increase in the measured OPD was attributed solely to Fur binding and this difference was converted to the number of bound Fur dimers using similar information as described above for calculating the number of immobilized dsDNA probes. In this way, the number of bound Fur dimers could be calculated for each spot within the microarray to effectively determine the number of protein dimers present per dsDNA molecule that was immobilized. For these measurements, between 6 and 10 spots were used to derive statistics on mass density calculations among the same condition.

**Purification of gonococcal Fur**

Purification of gonococcal Fur protein was performed as described previously [8]. Briefly, the ORF of the *fur* gene was PCR amplified from genomic DNA of *N. gonorrhoeae* F62 and cloned into a PET15b vector (Novagen, San Diego, CA) by the restriction sites NdeI and BamHI. The 6xHis-tagged Fur protein was over-expressed in a *E. coli* fur mutant strain, HBMBV119 [43] with 0.1 mM IPTG overnight at RT and purified using Ni-charged resin according to the manufacturer’s protocol (Novagen) [44,45]. Purified Fur protein was dialyzed against buffer [50 mM Tris-Cl, 500 mM NaCl, 100 μM MnCl₂, 10% glycerol, pH 7.9]. The 6xHis tag was cleaved using a bintiylated thrombin (Novagen) and removed by flowing through another Ni-charged resin [40,46,47].

**Electrophoretic mobility shift assay (EMSA)**

The probes used in EMSAs had the same sequences as those probes used in IRIS (Table S2). The dsDNA probes were obtained by mixing with its complementary strand at equal concentrations (5 μM), which were subsequently labeled with [γ-³²P]-ATP using T4 DNA kinase (Applied Biosystems, Ambion, Carlsbad, CA). The radio-labeled probes were purified using a G25 Sephadex QuickSpin column (GE healthcare, Pittsburgh, PA). EMSA was performed as described previously [8,41,42]. Labeled dsDNA Probes (12.5 nM) were incubated with purified gonococcal Fur in binding buffer (20 mM Tris-Cl pH 7.9, 5 mM MgCl₂, 40 mM KCl, 0.125 mM MnCl₂, 2 mM DTT, 10% Glycerol, 0.19 μg/μL poly dIdC, 3.125 μg/μL BSA) at RT for 30 min. For cold competition assays, unlabeled competitor DNA, as indicated (50 to 1000 fold), was added to the reaction. Each reaction mixture was electrophoresed on a native 6% polyacrylamide gel (acrylamide/bisacrylamide ratio, 37.5:1 [wt/wt]) (Bio-Rad, Hercules, CA), dried at 80°C on a filter paper for 1 hr and detected by autoradiography. The integrated density of bound and unbound DNA bands was quantified using ImageJ software and the percentage of bound DNA corresponding to a Fur concentration was calculated accordingly in each lane. The binding affinity (KD, equilibrium dissociation constant) of Fur to each
DNA probe was calculated using GraphPad Prism Software by plotting the percentage of bound DNA (Y axis) with the Fur concentration (X axis) and using the equation Y = (Bmax • X) / (KD + X). In this equation, Bmax is the total number of receptors expressed in the same units as the Y values.

RNA purification and quantitative RT-PCR

*N. gonorrhoeae* F62 wild type, fur mutant and fur complemented strains [48] were plated on GCB agar plates (Remel, Thermo Scientific) and grown overnight at 37°C with 5% CO₂ and used to inoculate CDM medium [49] containing 0.042% Na₂CO₃ with shaking for 2 hr. The cultures were diluted into fresh CDM inoculate CDM medium [49] containing 0.042% Na₂CO₃ with

| Predicted Fur box | Gene     | Function                              | Fur binding | Fur-regulation |
|-------------------|----------|---------------------------------------|-------------|---------------|
| TTAATATAGAATTATAC  | NGO0037  | Fe-S oxidoreductases family 1         | –¹         | NE            |
| TATATACGCCACGATTTC | NGO0037  | Fe-S oxidoreductases family 1         | –¹         | NE            |
| TTAATATAGAATTATAC  | NGO0073  | phosphoglycerate phosphatase           | +           | NR            |
| TATATATATATATATAT  | NGO0101  | cytochrome c4                          | +           | NR            |
| TATTATATATATATATAT  | NGO0155  | hypothetical DNA binding protein       | +           | Repressed     |
| GAACACAGGATTTTTTTTC| NGO0302  | hypothetical protein                   | –           | NE            |
| TTAATATATATATATATA  | NGO0304  | phenylalanyl-IRNA synthetase beta subunit | +         | NR            |
| CAAACACACACACACTTTT | NGO0337  | NadC family protein, transporter       | –           | NE            |
| TACACTAGATCTCTTT    | NGO0436  | putative 3-methyl-2-oxobutanoate hydroxymethyltransferase | +         | Activated     |
| TATATAATACATCTT     | NGO0641  | type III restriction/modification system modification methylase | +         | Repressed     |
| ACAATAGGTCTTCTTATA   | NGO0711  | alcohol dehydrogenase                  | –²         | NE            |
| TTATTTTTAATATTTT     | NGO0899  | transcription elongation factor, GreA  | +           | NR            |
| TGAAAAGAATCATATC     | NGO1189  | chaperonin, HslO                       | +           | NR            |
| TATATAATATATATGATT   | NGO1284  | hypothetical protein                   | +           | NR            |
| ACAAGAAGTATATCTT     | NGO1419  | hypothetical protein                   | +           | NR            |
| TATATAAGCCGCCTATT    | NGO1684  | hypothetical protein                   | –           | NE            |
| CATAACATTATATATATTTTC| NGO1738  | NADH dehydrogenase I subunit M         | +           | Activated     |
| TAACACGCACGCACTTCATGC| NGO1745  | NADH dehydrogenase I subunit G         | –           | NE            |
| TCAAATAGAATCGTTATC   | NGO1751  | NADH dehydrogenase I subunit A         | –²         | NE            |
| AAGAGAGATGATTATTTT   | NGO1845  | 30S ribosomal protein S12              | +           | NR            |
| TATATATATATATATATT    | NGO1948  | hypothetical protein                   | +           | NR            |
| TAAAGAGAACCCTACTC    | NGO1957  | putative export protein                | +           | Repressed     |

¹Iron regulation as previously determined by microarray analyses of *N. gonorrhoeae* grown in defined medium (-Fe) or CDM with 10 μM ferric nitrate (+Fe) [50,51].

²Fur regulation was determined by comparing mRNA levels in *N. gonorrhoeae* WT, fur mutant and fur complemented strains grown under iron-replete and iron-deplete conditions at 1h after addition of iron and desferal using qRT-PCR. NR, not regulated by Fur. Activated: mRNA level of the gene was increased in the wild type strain. Repressed: mRNA level of the gene was decreased in the fur mutant strain under either iron-replete or iron-deplete conditions compared to that in the wild type strain. NE, not examined; transcriptional regulation was not tested in this study.

³Fur regulation of the genes as previously determined [8].

⁴In vitro Fur binding to the 500 bp upstream sequence of ATG of these genes was determined using EMSA and footprinting in previous studies [8].

Table S1. Predicted Fur binding sequences within the promoter regions of *N. gonorrhoeae* iron-induced genes.

| Predicted Fur box | Gene     | Function                              | Fur binding | Fur-regulation |
|-------------------|----------|---------------------------------------|-------------|---------------|
| TTAATATAGAATTATAC  | NGO0037  | Fe-S oxidoreductases family 1         | –¹         | NE            |
| TATATACGCCACGATTTC | NGO0037  | Fe-S oxidoreductases family 1         | –¹         | NE            |
| TTAATATAGAATTATAC  | NGO0073  | phosphoglycerate phosphatase           | +           | NR            |
| TATATATATATATATAT  | NGO0101  | cytochrome c4                          | +           | NR            |
| TATTATATATATATATAT  | NGO0155  | hypothetical DNA binding protein       | +           | Repressed     |
| GAACACAGGATTTTTTTTC| NGO0302  | hypothetical protein                   | –           | NE            |
| TTAATATATATATATATA  | NGO0304  | phenylalanyl-IRNA synthetase beta subunit | +         | NR            |
| CAAACACACACACACTTTT | NGO0337  | NadC family protein, transporter       | –           | NE            |
| TACACTAGATCTCTTT    | NGO0436  | putative 3-methyl-2-oxobutanoate hydroxymethyltransferase | +         | Activated     |
| TATATAATACATCTT     | NGO0641  | type III restriction/modification system modification methylase | +         | Repressed     |
| ACAATAGGTCTTCTTATA   | NGO0711  | alcohol dehydrogenase                  | –²         | NE            |
| TTATTTTTAATATTTT     | NGO0899  | transcription elongation factor, GreA  | +           | NR            |
| TGAAAAGAATCATATC     | NGO1189  | chaperonin, HslO                       | +           | NR            |
| TATATAATATATATGATT   | NGO1284  | hypothetical protein                   | +           | NR            |
| ACAAGAAGTATATCTT     | NGO1419  | hypothetical protein                   | +           | NR            |
| TATATAAGCCGCCTATT    | NGO1684  | hypothetical protein                   | –           | NE            |
| CATAACATTATATATTTTC  | NGO1738  | NADH dehydrogenase I subunit M         | +           | Activated     |
| TAACACGCACGCACTTCATGC | NGO1745  | NADH dehydrogenase I subunit G         | –           | NE            |
| TCAAATAGAATCGTTATC   | NGO1751  | NADH dehydrogenase I subunit A         | –²         | NE            |
| AAGAGAGATGATTATTTT   | NGO1845  | 30S ribosomal protein S12              | +           | NR            |
| TATATATATATATATATT    | NGO1948  | hypothetical protein                   | +           | NR            |
| TAAAGAGAACCCTACTC    | NGO1957  | putative export protein                | +           | Repressed     |
via microarray analyses [50,51] of *N. gonorrhoeae* cultured under iron-replete and iron-deplete conditions. Forty-five iron-repressed genes with predicted Fur boxes in their promoter regions (Table S3) were identified, which included 18 out of 21 of the previously identified Fur-repressed genes/operons in *N. gonorrhoeae* [52]. This consistency validated our prediction of Fur binding sequences in the gonococcal genome. Twenty-two Fur binding sequences were predicted in the promoter regions of 21 iron-induced genes in this study (Table 1) including 2 previously identified Fur-activated genes, NGO0711 and NGO1751 [8].

**Interactions of Fur with predicted consensus sequences in *N. gonorrhoeae***

We next characterized *in vitro* binding of the Fur protein to predicted Fur boxes in the promoter regions of iron-induced genes using our high-throughput, label-free technology-IRIS. As shown in **Figure 1A**, Fur binding to the probes containing a predicted Fur box demonstrated a concentration dependent increase in differential spot height (DSH). The Fur boxes in the promoter regions of fur, norB and nspA, which have different binding affinities to Fur (26.1 nM, 1.2 nM and 724.8 nM, respectively) were utilized as positive controls and the aniA promoter region was used as a negative control in this IRIS assay. (A) Differential spot height for each dsDNA probe after incubation with 0 nM, 200 nM, 400 nM and 800 nM Fur protein, respectively. (B) The number of Fur dimers bound per dsDNA molecule of each probe as calculated from initial and post-incubation mass density measurements. The gene designations of *N. gonorrhoeae* F62 were assigned according to their homologues in *N. gonorrhoeae* FA1090.
doi:10.1371/journal.pone.0096832.g001

**Figure 1.** Identification of Fur binding to predicted Fur boxes by label-free IRIS screening. A set of ~50 bp dsDNA probes containing the predicted Fur box in the middle of the probes were designed for IRIS screening (Table S4). Each dsDNA probe was immobilized on a chip to produce a spot with a diameter of approximately 100 μm. Three concentrations of Fur protein, 200 nM (red bars), 400 nM (blue bars) and 800 nM (orange bars), were incubated with the individually prepared arrays (in addition to a 0 nM control incubation (black bars) and the binding of Fur protein to dsDNA spots was measured. A mass increase for each spot was represented as a differential spot height (DSH). The known Fur boxes in fur, norB and nspA promoter regions were used as positive controls, and the aniA promoter region was used as negative control in this IRIS assay. (A) Differential spot height for each dsDNA probe after incubation with 0 nM, 200 nM, 400 nM and 800 nM Fur protein, respectively. (B) The number of Fur dimers bound per dsDNA molecule of each probe as calculated from initial and post-incubation mass density measurements. The gene designations of *N. gonorrhoeae* F62 were assigned according to their homologues in *N. gonorrhoeae* FA1090.

**A High-Throughput Method for Detecting Protein-DNA Interactions**

PLOS ONE | www.plosone.org 4 May 2014 | Volume 9 | Issue 5 | e96832
According to the above analytical criteria, a large number of tested predicted Fur boxes (14 out of 18) in the promoter regions of iron-induced gonococcal genes displayed specific binding properties to Fur. Specifically, NGO1683 and NGO1745 did not show increased DSH for any concentration of Fur protein (Figure 1A). The DSHs of predicted Fur boxes in NGO302 and NGO377 promoter regions were only ~1 nm (1.0±0.06 nm and 1.0±0.02 nm, respectively) when 800 nM Fur protein was added (Figure 1A), suggesting that these two predicted sequences did not bind to Fur according to the defined criteria. In contrast, the other 14 predicted Fur boxes showed variable binding properties to Fur. The predicted Fur boxes in the NGO1436 and NGO1738 promoter regions displayed DSH values of 1.4±0.05 nm and 2.0±0.2 nm, respectively when 800 nM Fur was added (Figure 1A), corresponding to 0.5 dimer/dsDNA molecule and 1.6 Fur dimers/dsDNA molecule, respectively (Figure 1B). When 400 nM Fur was added, the predicted Fur boxes in the promoter regions of NGO1284, NGO1845 and NGO1948 showed DSH values of 1.7±0.03 nm, 1.7±0.3 nm and 1.7±0.07 nm, respectively, which all corresponded to 0.5 Fur dimers/dsDNA molecule (half amount of dsDNA is bound by Fur dimers) (Figure 1A and B). The Fur boxes in the promoter regions of NGO155, NGO304, NGO899 and NGO1189 had DSH values of 1.9±0.1 nm, 2.0±0.1 nm, 2.2±0.1 nm and 2.0±0.03 nm, respectively (Figure 1A). These DSH values corresponded to approximately one Fur dimer/dsDNA molecule (Figure 1B). When 200 nM Fur was added, the predicted Fur boxes in NGO0101, NGO0641 and NGO1419 promoter regions had DSH values of 1.9±0.4 nm, 2.0±0.1 nm and 1.9±0.4 nm, respectively which correlated to one Fur dimer/dsDNA (Figure 1A and B). The two highest binding affinities were observed for predicted Fur boxes in NGO0073 and NGO1957 promoter regions. When 200 nM Fur was added, DSH values of these two Fur boxes were 3.5±0.4 nm and 2.9±0.1 nm, respectively, corresponding to 3 Fur dimers and 1.5 Fur dimers/dsDNA molecule, respectively (Figure 1A and B).

To further validate Fur binding properties of the predicted Fur boxes characterized with IRIS, we performed traditional EMSA on two of the newly identified Fur bound probes, NGO0073 and NGO0101. Fur binding to the predicted Fur boxes in NGO0073 and NGO0101 promoter regions was specific as determined by a cold competition assay (Figure 2A). Consistent with the binding affinity trend estimated using IRIS, the apparent K_D of Fur binding to Fur boxes of fur gene, NGO0073 and NGO0101 calculated using EMSA results were 31.8±7.9 nM, 39.8±6.3 nM and 406.1±144.8 nM, respectively (Figure 2B). The combination of these results supports IRIS as an accurate method to determine the specificity and affinity of Fur binding to an array of immobilized dsDNA sequences.

Of interest among the 18 tested predicted Fur boxes in the promoter regions of iron-induced genes, fourteen were shown to bind to Fur specifically in vitro (Table 1). No information about Fur binding to these promoter regions was known previously.

![Figure 2](image-url)
highlighting the power of IRIS in identifying novel promoters bound by Fur.

New targets directly regulated by Fur

We next examined the regulatory role of Fur in transcription of iron-induced genes containing a functional Fur box in the promoter regions using quantitative RT-PCR of *N. gonorrhoeae* wild type, *fur* mutant, and *fur* complemented strains. This analysis revealed 5 new Fur regulated genes [NG00155, NGO0436, NGO0641, NGO1738 and NGO1957] (Figure 3, Table 1). An iron bound Fur-repressed gene, *fbpA* [6,53], was used as a control for Fur and iron regulation. We observed a statistically significant increase (5-fold) in *fbpA* transcript levels in the *fur* mutant strain grown under iron-replete conditions as compared to the wild type strain grown under iron-replete conditions. As expected we observed restoration of Fur mediated repression in the *fur* complemented strain (Figure 3). Transcription of NGO0155 was up-regulated in the *fur* mutant strain compared to the wild type strain under iron-replete (+Fe) conditions, and was partially restored in the *fur* complemented strain, suggesting that iron-bound Fur directly represses transcription of NGO0155. Transcriptional levels of NGO0436 and NGO1738 were reduced in the *fur* mutant strain compared to those in the wild type strain and partially restored in the *fur* complemented strain grown under iron-replete conditions (Figure 3), suggesting that NGO0436 and NGO1738 are directly activated by iron-bound Fur. Transcription levels of NGO0641 and NGO1957 were up-regulated in the *fur* mutant strain compared to the wild type strain under iron-deplete conditions (-Fe) and were restored in the *fur* complemented strain...
The used growth conditions in this study are termed silent Fur binding. Transcription of NGO0155 target genes. A subset of genes contain a Fur box in their putative promoter regions but were not regulated by Fur under transcription of NGO0155, encoding a methyltransferase (triangle), results in alteration of DNA methylation (*) and subsequently alters transcription of a subset of genes. Transcriptional repression of NGO0155, encoding a putative transcriptional regulator, results in alteration of transcription of NGO0155 target genes. A subset of genes contain a Fur box in their putative promoter regions but were not regulated by Fur under the used growth conditions in this study are termed silent Fur binding.

doi:10.1371/journal.pone.0096832.g004

Figure 4. Schematic depicting Fur mediated control mechanisms in N. gonorrhoeae as revealed by IRIS. Solid lines with arrowheads indicate direct activation of transcription via Fur binding to promoter regions of gonococcal genes. Solid lines with bars indicate direct repression of transcription via binding of Fur to promoter regions of gonococcal genes. Ellipsoids indicate transcriptional regulatory proteins other than Fur. Transcriptional repression of NGO0641, encoding a methyltransferase (triangle), results in alteration of DNA methylation (*) and subsequently alters transcription of a subset of genes. Transcriptional repression of NGO0155, encoding a putative transcriptional regulator, results in alteration of transcription of NGO0155 target genes. A subset of genes contain a Fur box in their putative promoter regions but were not regulated by Fur under the used growth conditions in this study are termed silent Fur binding.

doi:10.1371/journal.pone.0096832.g004

(Figure 3). This transcriptional pattern was similar to that previously observed in apo-Fur mediated repression [15,16].

Transcriptional levels of nine genes, NGO0073, NGO0101 (Figure 3), NGO0304, NGO0899, NGO1189, NGO1284, NGO1419, NGO1845 and NGO1948, showed no difference in expression among the three strains (Table 1, Figure S2). This suggests that these genes are not regulated by Fur under the growth conditions in this study. However, due to the fact that Fur specifically binds to promoter regions of these genes with high affinities, we termed this phenomenon “silent Fur binding” in N. gonorrhoeae.

Discussion

IRIS provides a simple microarray screening technique for fast identification of in vitro protein-nucleotide interactions, which substitutes for onerous gel shift assay. This method is able to circumvent complicated and tedious operations such as DNA manipulation that may be hindered due to limited availability of genetic tools for some organisms and can be a complementary method for ChIP-seq [54]. Further application of this microarray screening method includes optimization of biochemical conditions for protein-nucleotide binding, determination of essential nucleotides in the conserved binding sequences, as well as determination of the dissociation constant of a protein-nucleotide interaction through the development of a real-time/dynamic assay.

In this study we used Fur, a global transcriptional protein in N. gonorrhoeae to demonstrate the application of IRIS in determining the protein-nucleotide interactions. As a successful application of IRIS in this study, we identified 14 new Fur binding sequences out of 18 tested predicted Fur boxes in the intergenic regions within the gonococcal genome and 5 of these 14 genes were shown to be regulated by Fur under the experimental conditions used (Figure 3). NGO00155 encodes a small protein of 4.7 kDa that contains a DNA binding domain. NGO0436 and NGO1738 encode a putative hydroxymethyltransferase and a NADH dehydrogenase I subunit M, respectively, which may be important for the metabolism of N. gonorrhoeae. NGO1957 encodes a putative export protein and NGO0641 encodes a DNA methyltransferase (ModA13) of a Type III R-M system. ModA13 methyltransferase recognizes 5’-AGAAA-3’ and methylates the third adenine [55]. Deletion of NGO0641 or phase OFF of NGO0641 in N. gonorrhoeae resulted in transcriptional alteration of 17 genes, which suggests that the methyltransferase NGO0641 is involved in epigenetic regulation in N. gonorrhoeae [55]. While the relationship between Fur and phase variation needs further investigation, it is plausible that transcriptional repression of NGO0641 by Fur may change the methylation profile of the gonococcal genome and results in epigenetic regulation of the gonococcal transcriptome. We also hypothesize a new function for the Fur protein (silent Fur binding), based on the observation that ~64% of gonococcal genes containing a functional Fur box in promoter regions were not regulated by Fur under the used experimental conditions (Figure 4). This phenomenon of silent Fur binding has been recently demonstrated with a novel gonococcal phage repressor protein, Npr [48]. Under In vivo conditions, some Fur binding sites may not be accessible due to the binding of other transcriptional regulators and nucleoid-associated proteins including H-NS (histone-like nucleoid structuring protein), IHF (integration host factor), HU (heat unstable protein) and Fis (factor for inversion stimulation) [56]. Thus it is possible that a number of gonococcal genes that contain a Fur box in the promoter regions are not primarily regulated by Fur, but regulated by other transcriptional regulators that may respond to different stimuli. In this scenario, cross-talk of Fur with other regulatory proteins would result in co-regulation of the same targets in response to multiple environmental signals encountered during gonococcal infection. Such condition-specific expression phenomenon was also discovered in the FNR regulon in E. coli via a genome-scale ChIP-seq study [54].

Previous studies on Fur regulated genes have been designed to identify Fur regulated genes first in a fur mutant strain followed by in vitro Fur binding assays to confirm the direct regulatory role of Fur in gene transcription [8,24,57,58,59]. By design, this type of analysis identifies genes that are either directly or indirectly regulated by Fur. In contrast, IRIS allows for the rapid identification of Fur binding DNA sequences in vitro first, followed by subsequent transcriptional analyses. IRIS microarray screening of protein-nucleotide interaction will be a powerful high-through-
put tool to complement transcriptome analysis for the identification of regulatory networks of a broad array of bacterial transcriptional proteins.

Supporting Information

Figure S1 Mass density image of microarray surface produced using IRIS. [Left] Following incubation with Fur protein (800 nM), an image of the oligonucleotide array is produced quantifying the surface mass density across the entire surface. By comparing the post-incubation densities to those of the pre-incubation image, mass changes attributed to Fur binding can be easily determined on a spot by spot basis. [Right] The differential spot heights (DSHs) were determined from the mean of between 500 and 800 total pixels, depending on spot size, used for comparison of the circular spot region (green area) and an outer background annulus region (red region). Pixels in the image which returned large residual error during the data fitting process (ex: salt residue left in the center of each spot – shown here as missing pixels within the green spot region) were eliminated using a threshold value.

Figure S2 Transcriptional regulation patterns of genes determined by quantitative real-time PCR. The RNA samples were purified from cultures of the wild-type (WT), +fur mutant and +fur complemented strains under iron-replete (+Fe, grey bars) or iron-deplete (-Fe, white bars) conditions 1 h after addition of 100 μM iron or 150 μM desferal. The mRNA levels observed for the five conditions (WT strain under –Fe conditions, +fur mutant strain under +Fe and –Fe conditions, and +fur complemented strain under +Fe and –Fe conditions) were compared to the value of WT strain under +Fe conditions. The final results were represented as mean ± standard deviation. A * indicates significantly different compared to the mRNA level of WT+Fe. The gene designations of N. gonorrhoeae F62 were assigned according to their homologues in N. gonorrhoeae FA1090.

Table S1 Experimentally determined Neisserial Fur boxes used as templates for prediction of Fur boxes in the genome of N. gonorrhoeae.

Table S2 Predicted Fur boxes in the promoter regions of N. gonorrhoeae iron-repressed genes.

Table S3 Double strand DNA probes used in IRIS.

Table S4 Primers used for quantitative RT-PCR.

Table S5 Predicted Fur boxes in the genome of N. gonorrhoeae.

Acknowledgments

We thank Dr. Kenneth Barth and Dr. Ryan McClure for helpful discussions of the manuscript.

Author Contributions

Conceived and designed the experiments: CY CAG. Performed the experiments: CY CAL HH. Analyzed the data: CY CAL CAG. Contributed reagents/materials/analysis tools: YX APR GGD MSU. Wrote the paper: CY CAG CAG MSU.

References

1. Daaboul GG, Vedula RS, Ahn S, Lopez CA, Reddington A, et al. (2011) LED-based interferometric reflectance imaging sensor for quantitative dynamic monitoring of biomolecular interactions. Biosens Bioelectron 26: 2221–2227.
2. Lopez CA, Daaboul GG, Vedula RS, Ozkumur E, Bergstein DA, et al. (2011) Label-free multiplexed virus detection using spectral reflectance imaging. Biosens Bioelectron 26: 3432–3437.
3. Ozkumur E, Ahn S, Yalcin A, Lopez CA, Cevik E, et al. (2010) Label-free microarray imaging for direct detection of DNA hybridization and single-nucleotide mismatches. Biosens Bioelectron 25: 1789–1795.
4. Ozkumur E, Needham PV, Bergstein DA, Gonzalez R, Cabodi M, et al. (2008) Label-free and dynamic detection of biomolecular interactions for high-throughput microarray applications. Proc Natl Acad Sci U S A 105: 7988–7992.
5. Ahn S, Huang CL, Ozkumur E, Zhang X, Chinnala J, et al. (2012) TATA binding proteins can recognize nontraditional DNA sequences. Biophys J 103: 1510–1517.
6. Agarwal S, King CA, Klein EK, Soper DE, Rice PA, et al. (2005) The gonococcal Fur-regulated dbpA and dbpB genes are expressed during natural mucosal gonococcal infection. Infect Immun 73: 4201–4207.
7. Agarwal S, Sebastian S, Szmulwajsk S, Rice PA, Genco CA (2008) Expression of the gonococcal global regulatory protein Fur and genes encompassing the Fur and iron regulon during in vitro and in vivo infection in women. J Bacteriol 190: 3129–3139.
8. Yu C, Genco CA (2012) Fur-mediated activation of gene transcription in the human pathogen Neisseria gonorrhoeae. J Bacteriol 194: 1730–1742.
9. Bag A, Neilands JB (1987) Ferric uptake regulation protein acts as a repressor, employing iron (II) as a cofactor to bind the operator of an iron transport operon in Escherichia coli. Biochemistry 26: 5471–5477.
10. Mills SA, Marletta MA (2005) Metal binding characteristics and role of iron oxidation in the ferric uptake regulator from Escherichia coli. Biochemistry 44: 13533–13539.
11. Sherlock MA, Taylor GL (2009) Crystal structure of the Vibrio cholerae ferric uptake regulator (Fur) reveals insights into metal co-ordination. Mol Microbiol 72: 1208–1220.
12. Pohl E, Haller JC, Mijovicovich A, Meyer-Klaucke W, Garman E, et al. (2003) Architecture of a protein central to iron homeostasis: crystal structure and spectroscopic analysis of the ferric uptake regulator. Mol Microbiol 47: 903–915.
13. de Lorenzo V, Woo S, Herrero M, Neillands JR (1987) Operator sequences of the aerobactin operon of plasmid CoV-K30 binding the ferric uptake regulation (fur) repressor. J Bacteriol 169: 2624–2630.
14. Escolar L, Perez-Martin J, de Lorenzo V (1998) Binding of the ferric uptake regulator (fur) repressor of Escherichia coli to arrays of the GATAAT sequence. J Mol Biol 283: 537–547.
15. Miles S, Carpenter BM, Gance H, Merrill DS (2010) Helicobacter pylori apo-Fur regulation appears unconserved across species. J Microbiol 48: 378–386.
16. Carpenter BM, Whimire JM, Merrill DS (2009) This is not your mother’s repressor: the complex role of fur in pathogenesis. Infect Immun 77: 2590–2601.
17. Lee H, Bang SH, Lee KH, Park SJ (2007) Positive regulation of fur gene expression via direct interaction of fur in a pathogenic bacterium, Vibrio vulnificus: J Bacteriol 189: 2629–2636.
18. Butcher BG, Bronstein PA, Myres CR, Soodhill PV, Bolon J, et al. (2011) Characterization of the Fur Regulon in Pseudomonas syringae pv. tomato DC3000. J Bacteriol 193: 4598–4611.
19. Ledala N, Sengupta M, Mulhajan A, Wilkinson BJ, Jayawal RK (2010) Transcriptomic response of Listeria monocytogenes to iron limitation and Fur mutation. Appl Environ Microbiol 76: 406–416.
20. Merrill DS, Thompson LJ, Kim CC, Mitchell H, Tompkins LS, et al. (2003) Growth phase-dependent response of Helicobacter pylori to iron starvation. Infect Immun 71: 6510–6525.
21. Whithy PW, Scale TW, VanWagoner TM, Morton DJ, Stall TL (2009) The iron/heme regulated genes of Haemophilus influenzae: comparative transcriptional profiling as a tool to define the species core modulon. BMC Genomics 10: 6.
22. Whithy PW, VanWagoner TM, Scale TW, Morton DJ, Stall TL (2006) Transcriptional profile of Haemophilus influenzae: effects of iron and heme. J Bacteriol 188: 5640–5645.
23. Zhou D, Qin L, Han Y, Qu J, Chen Z, et al. (2006) Global analysis of iron regulon during in vitro and in vivo infection in women. J Bacteriol 188: 5640–5645.
24. Ledala N, Sengupta M, Mulhajan A, Wilkinson BJ, Jayawal RK (2010) Transcriptomic response of Listeria monocytogenes to iron limitation and Fur mutation. Appl Environ Microbiol 76: 406–416.
25. Delany I, Rappuoli R, Scarlato V (2004) Fur functions as an activator and as a repressor in Neisseria meningitidis. Mol Microbiol 52: 1091–1098.
26. Davis BM, Quinones M, Pratt J, Ding Y, Waldor MK (2005) Characterization of the small untranslated RNA RybB and its regulon in Vibrio cholerae. J Bacteriol 187: 4005–4014.
PLOS ONE | www.plosone.org 9 May 2014 | Volume 9 | Issue 5 | e96832

42. Yang Q, Gilmartin GM, Doublie S (2010) Structural basis of UGUA recognition by the Nadix protein CFlim25 and implications for a regulatory role in mRNA 3' processing. Proc Natl Acad Sci U S A 107: 10062–10067.

43. Baumlter AJ, Tsolis RM, van der Velden AW, Soulijnck I, Anic S, et al. (1996) Identification of a new iron regulated locus of Salmonella typhi. Gene 183: 207–213.

44. Yang Q, Bruschweiler S, Chou JJ (2014) Purification, crystallization and preliminary X-ray diffraction of the N-terminal calmodulin-like domain of the human mitochondrial ATP-Mg/Pi carrier SCA5MC1. Acta Crystallogr F Struct Biol Commun 70: 68–71.

45. Yang Q, Bruschweiler S, Chou JJ (2014) A Self-Sequestered Calmodulin-like Ca2+ Sensor of Mitochondrial SCA5MC Carrier and Its Implication to Ca(2+)-Dependent ATP-Mg/Pi Transport. Structure 22: 209–217.

46. Yang Q, Faucher F, Coseno M, Heckman J, Double S (2011) Purification, crystallization and preliminary X-ray diffraction of a disulphide crosslinked complex between bovine poly(A) polymerase and a chemically modified 15-mer oligo(A) RNA. Acta Crystallogr Sect F Struct Biol Cryst Commun 67: 241–244.

47. Yang Q, Nausch LW, Martin G, Keller W, Double S (2014) Crystal structure of human poly(A) polymerase gamma reveals a conserved catalytic core for canonical poly(A) polymers. J Mol Biol 436: 43–50.

48. Yu C, Genco CA (2012) Fur-mediated global regulatory circuits in pathogenic Neisseria: the requirement for hypoxanthine. Can J Microbiol 58: 13–20.

49. Ducey TF, Carson MB, Orvis J, Stintzi AP, Dyer DW (2005) Identification of the iron-responsive genes of Neisseria gonorrhoeae by microarray analysis in defined medium. J Bacteriol 187: 4663–4674.

50. Jackson LA, Ducey TF, Doy MW, Zaintshik JB, Orvis J, et al. (2010) Transcriptional and functional analysis of the Neisseria gonorrhoeae Fur regulon. J Bacteriol 192: 77–85.

51. Yu C, Genco CA (2012) Fur-mediated global regulatory circuits in pathogenic Neisseria species. J Bacteriol 194: 6372–6381.

52. Myers KS, Yuan H, Ong EM, Chang D, Liang K, et al. (2013) Genome-scale analysis of escherichia coli FNR reveals complex features of transcription factor binding. PLoS Genet 9: e1003563.

53. Srikantha VN, Dowideit SJ, Edwards JL, Falsetta ML, Wu HJ, et al. (2009) Phasevarions mediate random switching of gene expression in pathogenic Neisseria. PLoS Pathog 5: e1000400.

54. Delany I, Grifantini R, Bartolini E, Rappuoli R, Scarlato V (2006) Identification of small RNA-encoding genes in the intergenic regions of Escherichia coli. Curr Microbiol 53: 365–370.

55. Srikhanta YN, Dowideit SJ, Edwards JL, Falsetta ML, Wu HJ, et al. (2009) Phasovarion mapping and transcriptional regulation of the iron-regulated Neisseria gonorrhoeae fhpA gene. J Bacteriol 191: 3047–3052.

56. Dame RT (2005) The role of nucleoid-associated proteins in the organization and compaction of bacterial chromatin. Mol Microbiol 56: 838–870.

57. Delany I, Grifantini R, Bartolini E, Rappuoli R, Scarlato V (2006) Effect of Neisseria meningitidis fur mutations on global control of gene transcription. J Bacteriol 188: 2483–2492.

58. Grifantini R, Sebastian S, Frigimelica E, Draghi M, Bartolini E, et al. (2003) Identification of iron-activated and -repressed Fur-dependent genes by transcriptionome analysis of Neisseria meningitidis group B. Proc Natl Acad Sci U S A 100: 9542–9547.

59. Sebastian S, Agarwal S, Murphy JR, Genco CA (2002) The gonococcal fur regulon: identification of additional genes involved in major catalytic, recombination, and secretory pathways. J Bacteriol 184: 3965–3974.