Supporting Information

Thompson et al. 10.1073/pnas.1201398109

SI Methods

Generation of pET YtgR and YtgCR Expression Vectors. The sequence coding for the final 147 amino acids of CT069 was amplified from *C. trachomatis* serovar D genomic DNA using primers CT1003 and CT1004, which engineered an additional CACC onto the 5′ end of the PCR product for directional insertion into the pET151-D/TOPO vector (Invitrogen) to make pET-YtgR. To generate pET-YtgCR, the full-length *ct069* coding sequence was amplified from pBAD-YtgCR (see below) using primers CT1101 AND CT1102, which engineered an additional 3′ NcoI restriction site. The NcoI restriction site was used to ligate the *ct069* PCR product to a double-stranded oligonucleotide coding for the FLAG epitope, which was generated through the annealing of two custom-synthesized oligonucleotides (CT1033 and CT1034). The ligated sequence was amplified with primers CT1103 and CT1104, which added a 5′ CACC for directional insertion into the pET151-D/TOPO vector.

To generate pBAD-YtgCR, the full-length *ct069* coding sequence was amplified from genomic DNA using primers CT1036 and CT1035, which added an additional 5′ KpnI restriction site. This site was used to ligate a FLAG epitope coding sequence, which was generated through the annealing of two custom synthesized oligonucleotides (CT1033 and CT1034). PCR amplification of the ligated sequence with primers CT1037 and CT1035 generated a PCR product, to which 3′ A-overhangs were added with the addition of Taq polymerase and ATP for 5 min at 72 °C. The PCR product was ligated into the pBAD-TOPO/TA expression vector.

Generation of the pCCT Reporter Plasmids. A nucleotide region of 2,100 bp containing a tetracycline resistance cassette (TetR) and a p15A origin of replication sequence was amplified from pACYC184 using primers CT1018 and CT1019, which incorporated a BglII and XhoI restriction endonuclease sites on the 5′ and 3′ flanks, respectively. A nucleotide region of 4,878 bp was amplified from the pBAD-TOPO/LacZ/V5-His control expression vector using primers CT1020 and CT1021. The resultant PCR product contained an *araC* gene, an arabinose inducible promoter (P_{BAD}), a sequence coding for β-galactosidase tagged C-terminally with V5 and polyhistidine epitopes, rrnB T1 and T2 transcriptional termination sites, and incorporated XhoI and BglII restriction endonuclease sites at the 5′ and 3′ flanks, respectively. The two PCR products were digested with XhoI and BglII simultaneously, before resolution on 0.7% Tris-acetate-EDTA (TAE) agarose gel and purification. Digested products were circularized using T4 ligase treatment overnight at 16 °C to create pCCT100. A KpnI restriction site was inserted into the flanking region of P_{BAD} using inverse PCR with primers CT1022 and CT1023 within the framework of the Site-directed Mutagenesis kit (Invitrogen), to generate pCCT101. A sequence containing the YtgR operator sequence was PCR amplified from *C. trachomatis* serovar D genomic DNA with primers CT1024 and CT1025, which generated KpnI restriction sites on both flanks. Vector and the PCR product were digested with KpnI and ligated together to create pCCT102. Colony PCR verified insert direction, and plasmids were verified by sequencing (GATC).

Expression and Purification of Recombinant YtgR. Overnight cultures of BL21* (DE3) *E. coli* harboring pET-YtgR were subcultured into 200 mL of LB plus carbenicillin, grown to an OD_{600} = 0.5 and supplemented with Isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.75 mM for 5 h before sedimentation by centrifugation (10,000 × *g* for 30 min) and storage at −80 °C.

After determining recombinant YtgR remained predominantly insoluble, we used a purification process previously described for the purification of other insoluble transcription factors (1, 2), with slight modifications. Pellets of *E. coli* harboring pET-YtgR were thawed on ice, and all subsequent steps were carried out at 4 °C or on ice. The thawed pellet was resuspended in 20 mL of Buffer A [50 mM Tris HCl, 50 mM NaCl, 0.1 mM DTT, 5% glycerol, 0.1 mM EDTA, 2% (wt/vol) deoxycholate (pH 7.0)] supplemented with 0.2 mg/mL lysozyme for 30 min. The bacterial suspension was then sonicated and centrifuged at 12,000 × *g* for 30 min. The insoluble pellet was washed twice with buffer A (without lysozyme), with each wash lasting 1 h with gentle agitation. After the second wash step, the insoluble fraction was sedimented by centrifugation at 16,000 × *g* for 30 min. The YtgR recombinant was liberated from the insoluble fraction by resuspending the pellet in buffer B [50 mM Tris HCl, 50 mM NaCl, 0.1 mM DTT, 5% glycerol, 0.25% (wt/vol) sarkosyl (pH 7.0)] for 1 h with gentle agitation. The soluble fraction was collected after centrifugation (16,000 × *g*), and another round of protein liberation was performed. Soluble fractions were pooled and recombinant YtgR was purified using the Ni-NTA kit (QIAGEN) as described by the instructions of the manufacturer for nondenaturing purification. Samples from each step in this process were analyzed by SDS/PAGE. Elutions containing a single product by Coomassie staining were pooled, and dialyzed into a buffer suitable for low-temperature storage (50 mM Tris HCl, 50 mM NaCl, 0.1 mM DTT, 5% glycerol, pH = 7.0). Half of the sample was incubated with Chelex-100 resin (Bio-Rad) for 2 h at 4 °C. Non-Chelex-treated (referred to as purified YtgR) and Chelex treated (referred to as apo-YtgR) were aliquoted, flash frozen, and stored at −80 °C. Protein concentrations were determined using the BCA Protein Assay Kit (Pierce).

Generation of Biotin-Tagged DNA Oligonucleotides. The region upstream of the *ytgA* coding sequence, containing the putative promoter elements and YtgR operator sequence was amplified from genomic DNA purified from *C. trachomatis* Serovar D EBs using primers CT1011 and CT1012 (Dataset S1). Primer CT1011 was engineered with a 5′ Biotin-tag (Custom ordered from Invitrogen). A single amplified PCR product was ethanol precipitated, resuspended in nuclease free water and stored at −20 °C.

Dot-Blot Assay. Whatman Optitron BA-5 83 0.2 μM NC (Millipore) membrane was soaked in 0.4 M KOH for 5 min, before 3× 5-min washes with distilled deionized H_2O. The membrane was equilibrated in wash buffer [20 mM Tris-HCl (pH 8.0), 50 mM KCl, 5% glycerol, 0.5 mM DTT, 2 mM $MgCl_2$, 150 μM ascorbic acid] for at least 15 min at 4 °C. The apo-YtgR was diluted into 38-μL reaction buffer (wash buffer plus 150 μM $FeSO_4$ or $MnCl_2$) and allowed to equilibrate for 15 min at room temperature. A 2-μL volume of a 20 nM concentration of the biotinylated-DNA sequence was added to the reaction, which was then incubated at 30 °C for 30 min. The NC membrane was placed on top of filter paper within the dot-blot hybridization manifold (DHM-48; Fischer), and wells were flushed with wash buffer two times. Without application of the vacuum, samples were added to respective wells. The vacuum was then applied and each used well was washed two more times with wash buffer. The apparatus was disassembled, and the NC membrane was blocked in TBS plus 0.1% Tween-20 containing 3% BSA

(Sigma). The NC blot was probed with HRP-conjugated streptavidin in the same blocking buffer at a dilution of 1:20,000 for 1 h at room temperature. Biotinylated DNA retention to the NC blot was detected using the Immobilon HRP-substrate chemiluminescent detection kit (Millipore) and images were captured using the FUJI-3000 LAS imager at −30 °C. Dot-blot intensities were measured from a single image, using ImageJ software. Values were transformed by the average intensity of an unused well. Results displayed in Fig. 2 *A* and *B* are one representative experiment of three showing the same trend.

Biolayer Interferometry. Biolayer interferometry kinetic studies of DNA:protein interactions were performed using the Octet QKe (ForteBio). Target DNA was PCR amplified with a 5′ biotinylated oligo, as described above. A 25 nM final concentration of purified recombinant YtgR was equilibrated in Kinetics Buffer (KB) (ForteBio) supplemented with the various metal solutions ($FeSO_4$, $MnCl_2$, $ZnCl_2$, $CuCl_2$, or $CoCl_2$) at a final concentration of 200 μM for ∼20 min. During this time, the biotinylated PCR product was loaded onto the streptavidin-coated sensors for 15 min, which was ample time to achieve tip saturation. After washing in KB) which contains BSA to negate nonspecific protein binding, sensors were incubated in the recombinant $YtgR^{+/-}$ various metal supplements. The change in internally reflected light attributable to DNA:protein interactions was collected in real time for 10 min, at which point the tips were washed once in KB and incubated for an additional 20 min in KB to collect dissociation data. Binding kinetics were calculated based on the change in the reflected light over time (measured as a change in distance between a reference reflection point and the bound material on the tip) using the software provided with the Octet QKe.

Western Blots. *C. trachomatis* D/UW-3/CX was obtained originally from Ted Hacksadt (Rocky Mountain Laboratory, National Institute of Allergy and Infectious Diseases, Hamilton, MT), and propagated/purified in HeLa cells as described (3). Infected HeLa cell monolayers (multiplicity of infection of 2) were grown for 1, 6, or 24 h postinvasion before being lysed into RIPA buffer. Samples were mixed with 5× SDS sample buffer and resolved on a 12% TGS/polyacrylamide gel. Protein transfer and immunoblotting were performed with standard methods, using rabbit polyclonal antisera, raised against the full-length CT069, which was a kind gift of Guangming Zhong (University of Texas Health Sciences Center, San Antonio, TX).

Top10 *E. coli* harboring the pBAD-YtgCR expression vector, or the pBAD-empty vector, were grown overnight in 5 mL of LB broth containing 0.2% (wt/vol) D-glucose and 50 μg/mL carbenicillin. The next day, 1 mL of the cultures were pelleted and rinsed with LB broth twice, to remove traces of D-glucose. Each bacterial culture was then subcultured to an OD_{600} of 0.1 in LB supplemented with carbenicillin, in triplicate. After 2 h, cultures were either induced for expression of the YtgCR [addition of 0.002% (wt/vol) L-arabinose], repressed for expression [addition of 0.2% (wt/vol) D-glucose], or were left unchanged (uninduced). After 4 h of additional growth, 0.1 OD units were pelleted and resuspended in 100 μL of 1× SDS sample buffer. Samples were boiled for 10 min at 100 °C and resolved by SDS/PAGE. Proteins were transferred, immunoblotted, and detected using standard techniques, in this case using a 1:2,000 dilution of an anti-V5 epitope monoclonal antibody that was conjugated to HRP (AbCam). To determine the affinity of the CT069 antisera, *E. coli* harboring pBAD-YtgCR were subcultured as before and supplemented with 0.02% (wt/vol) L-arabinose (induced) or 0.2% (wt/vol) D-glucose (repressed) for 4 h.

Reporter Gene Expression Assay. Chemically competent BL21* *E. coli* were initially transformed with pCCT101 or pCCT102 and plated onto LB agar containing tetracycline (15 μg/mL). Selected colonies were verified for the presence of the reporter plasmid, grown in selective LB broth culture, made electrocompetent, transformed with pET151-EV, pET-YtgR, or pET-YtgCR by electroporation and were plated onto LB agar containing tetracycline (15 μg/mL), carbenicillin (50 μg/mL), and X-gal (40 μg/mL). Single transformants of either set of plasmids did not grow on double selective media. Colonies of cotransformants were grown overnight in double selective LB broth containing D-glucose (0.2% wt/vol) at 37 °C and 200 rpm. The next day, cultures were pelleted and resuspended in LB broth (to remove D-glucose) and subcultured to an OD_{600} of 0.1 in 5 mL of double-selective LB broth supplemented with 5 μL of a 50 mM $FeSO_4$ solution. Samples were grown at 37 °C at 200 rpm. After 1 h, cultures were supplemented with 200 mM IPTG, to induce expression of the pET vectors. Following an additional hour of induction, L-arabinose was supplemented to a final concentration of 0.2% (wt/vol) to abrogate AraC repression of the P_{BAD} promoter. Samples were collected 2 h after L-arabinose supplementation, and LacZ expression was measured via the Miller assay, as described (4). The experiment was performed three times independently, with each trial including the full complement of cotransformant samples. Interexperimental error was corrected by transforming the values of each trial, such that the mean of all samples within each batch were identical. The mean MU of each sample from the three batch-corrected experiments is shown. Error bars represent 1 SD from the mean.

1. Nguyen LH, Jensen DB, Burgess RR (1993) Overproduction and purification of sigma 32, the Escherichia coli heat shock transcription factor. *Protein Expr Purif* 4:425–433.
2. Posey JE, Hardham JM, Norris SJ, Gherardini FC (1999) Characterization of a manganese-dependent regulatory protein, TroR, from Treponema pallidum. *Proc Natl Acad Sci USA* 96:10887–10892.
3. Caldwell HD, Kromhout J, Schachter J (1981) Purification and partial characterization of the major outer membrane protein of Chlamydia trachomatis. *Infect Immun* 31:1161–1176.
4. Miller JH (1972) *Experiments in Molecular Genetics* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

Consensus XXXFLTXXGXXXAXXXXRXHRLWEXYLXXXXXXXXXXXVHXXAXXXEHXXXXXLXXXLXXXLXXPXXXXXXXPMPXXXDPHXXXIPXXXXXXXXXXX

Identity

Iron-dependent repressor protein, dimerization domain

1. O84072|Y069_CHLTR extraction
2. C0QMH4|C0QMH4_DESAH extraction
3. B5ECZ6|B5ECZ6_GEOBB extraction
4. C6E6W6|C6E6W6_GEOSM extraction
5. E8WRP7|E8WRP7_GEOS8 extraction
6. D4H2B6|D4H2B6_DENA2 extraction
7. A3ZXJ0|A3ZXJ0_9PLAN extraction
8. D5SYP0|D5SYP0_PLAL2 extraction
9. E8R461|E8R461_ISOPI extraction
10. D2R7Y8|D2R7Y8_PIRSD extraction
11. F0SLH1|F0SLH1_PLABD extraction
12. F2AP05|F2AP05_RHOBT extraction
13. Q7UIM9|Q7UIM9_RHOBA extraction
14. A6C9P2|A6C9P2_9PLAN extraction
15. E6SJ31|E6SJ31_THEM7 extraction
16. E4M786|E4M786_9FIRM extraction
17. F3B501|F3B501_9FIRM extraction
18. E6LLW2|E6LLW2_9FIRM extraction
19. G5GEW3|G5GEW3_9FIRM extraction
20. D6YSH0|D6YSH0_WADCW extraction
21. F8LF66|F8LF66_9CHLA extraction
22. F8KVM5|F8KVM5_PARAV extraction
23. D1R644|D1R644_9CHLA extraction
24. Q6MEL7|Q6MEL7_PARUW extraction
25. F8L6P3|F8L6P3_SIMNZ extraction
26. Q9Z8J6|Y347_CHLPN extraction
27. D1A0Z6|D1A0Z6_CHLPP extraction
28. F6FAP8|F6FAP8_CHLPS extraction
29. E5AHT0|E5AHT0_CHLP1 extraction
30. F3NV98|F3NV98_CHLPS extraction
31. F6F5J6|F6F5J6_CHLPS extraction
32. F0T3X1|F0T3X1_CHLP6 extraction
33. F6FD68|F6FD68_CHLPS extraction
34. F6F882|F6F882_CHLPS extraction
35. Q5L653|Q5L653_CHLAB extraction
36. Q254E8|Q254E8_CHLFF extraction
37. Q823H1|Q823H1_CHLCV extraction
38. B0B9H7|B0B9H7_CHLT2 extraction
39. F9YDB8|F9YDB8_CHLTC extraction
40. B0BB56|B0BB56_CHLTB extraction
41. D3UTN1|D3UTN1_CHLTS extraction
42. D6YP98|D6YP98_CHLT1 extraction
43. D6YCH7|D6YCH7_CHLT5 extraction
44. G4NM41|G4NM41_CHLTR extraction
45. D6YEM3|D6YEM3_CHLT7 extraction
46. D6YXA0|D6YXA0_CHLT9 extraction
47. D6YJW4|D6YJW4_CHLTG extraction
48. D7DFW0|D7DFW0_CHLTL extraction
49. D6YH87|D6YH87_CHLT0 extraction
50. D7DCP1|D7DCP1_CHLTD extraction
51. Q3KMV2|Q3KMV2_CHLTA extraction
52. C4PLS2|C4PLS2_CHLTZ extraction
53. Q9PKX0|Y341_CHLMU extraction
54. F4DIR0|F4DIR0_CHLPE extraction
55. Q7UIM8|Q7UIM8_RHOBA extraction
56. F2AP06|F2AP06_RHOBT extraction
57. A6C9P1|A6C9P1_9PLAN extraction
58. D5EQF0|D5EQF0_CORAD extraction
59. B3ET27|B3ET27_AMOA5 extraction
60. G8R720|G8R720_9FLAO extraction
61. Q11TH1|Q11TH1_CYTH3 extraction
62. E4TRG1|E4TRG1_MARTH extraction
63. Q31DN8|Q31DN8_THICR extraction
64. B5JMV0|B5JMV0_9BACT extraction
65. D5SYP1|D5SYP1_PLAL2 extraction

Fig. S1. Permease-repressor fusion proteins are highly conserved at metal-coordinating residues. All of the fusion proteins with predicted DtxR iron-dependent repressor domains in the C terminus were subjected to multiple sequence alignment. The region aligning to the dimerization domain was extracted, and a >75% sequence identity was used to generate a consensus sequence. The six metal-coordinating residues, as determined by the DtxR crystal structure, are marked with a closed circle above the consensus sequence.

YtgR apo-YtgR

25-
20-

Fig. S2. Recombinant YtgR is purified to a single product by Coomassie staining. Aliquots of purified, recombinant YtgR, or apo-YtgR (Chelix-100-treated) were resolved on a 12% TGS/polyacrylamide gel and stained by Coomassie blue. After destaining, only one product was visible.

YtgR (nM) 150 15 1.5 0.15 0
mock
Fe^{2+}
Mn^{2+}

Fig. S3. Manganese does not activate apo-YtgR DNA-binding. Various concentrations of apo-YtgR were mixed with $FeSO_4$ or $MnCl_2$ (150 μM) before incubation with a biotin-labeled dsDNA molecule that corresponded to the region upstream of *ytgA*. Reactions were filtered through a NC membrane and washed once, before detection of biotinylated DNA retained on the NC. Iron-supplemented protein samples retained the DNA oligonucleotide, whereas no appreciable retention over the no protein control (NPC) in the manganese-supplemented samples was observed.

MW purified YtgR
20 kDa -

Fig. S4. CT069 antisera recognize purified recombinant YtgR. An aliquot of recombinant YtgR was resolved by TGS/PAGE and transferred to NC membrane. The membrane was blotted using the CT069 antisera and chemiluminescence detected with the Millipore HRP detection kit. Chemiluminescence was observable without use of the Western blot imager. Visible light picture is shown.

Table S1. Plasmids used in this study

Plasmid	Relevant details	Source
pACYC184	Subcloning vector	New England Biolabs
pBAD-TOPO/TA	Expression vector	Invitrogen
pBAD-LacZ-V5-pHis	Control Expression vector	Invitrogen
pET151-D/TOPO	Expression vector	Invitrogen
pET151-EV	circularized empty vector	This paper
pET-YtgR	Expression of the YtgR domain with an N-terminal pHis tag	This paper
pET-YtgC-YtgR	Expression of recombinant full-length YtgC-YtgR with an N-terminal pHis tag	This paper
pBAD-YtgC-YtgR	Expression of YtgC-YtgR with a C-terminal V5 and pHis tag	This paper
pCCT100	LacZ reporter plasmid with arabinose inducible promoter	This paper
pCCT101	pCCT100 with KpnI RE site next to promoter	This paper
pCCT102	pCCT101 with YtgR operator containing sequence inserted into the KpnI site	This paper

Table S2. Oligonucleotide primers used in this study

Primer ID	Sequence (5′ to 3′)
CT1003	CACCATGCATCTATTGAAAATCTTTGGTATTTAC
CT1004	CTAGCAACCATCCGACTTCCTTG
CT1018	CCTCCTCTCGAGGCGCATTCACAGTTCTCCGC
CT1019	CCTCCTAGATCTCGGAGATTTCCTGGAAGATGCCA
CT1020	TATAAGATCTCCGGCGGATTTGTCCTACTCAGGAG
CT1021	ATATCTCGAGGCGAAGGCGAAGCGGCATG
CT1022	ACGGGTATGGAGAAACAGTAGAGAGTTGCG
CT1023	TACTGTTTCTCCATACCCGTGGTACCTTTTTGGGCTAG
CT1024	TAATGGTACCAGATAATCGATACGGGGACATAC
CT1025	ATATGGTACCGAATAGCTCCTTAGTGGGTAG
CT1033	TAATCCATGGACTACAAGGACGATGACAAGGGTACCTAAT
CT1034	ATTAGGTACCCTTGTCATCGTCCTTGTAGTCCATGGATTA
CT1035	GCAACCATCCGACTTCCTTGTTCG
CT1036	ATATGGTACCCTGAGTTGTATATTTCAGGACACTATCTTTCTATCCAG
CT1037	CCATGGACTACAAGGACGATGACAAGGG
CT1101	ATGCTGAGTTGTATATTTCAGGACACTATC
CT1102	ATACCATGGCGCAACCATCCGACTTCCTTGTT
CT1103	CACCATGCTGAGTTGTATATTTCAGGACACTATC
CT1104	CTAGGTACCCTTGTCATCGTCCT

Other Supporting Information Files

Dataset S1 (XLSX)