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LeuO is a global regulator of gene expression in *Salmonella enterica* serovar Typhimurium

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Summary

We report the first investigation of the binding of the *Salmonella enterica* LeuO LysR-type transcription regulator to its genomic targets *in vivo*. Chromatin-immunoprecipitation-on-chip identified 178 LeuO binding sites on the chromosome of *S. enterica* serovar Typhimurium strain SL1344. These sites were distributed across both the core and the horizontally acquired genome, and included housekeeping genes and genes known to contribute to virulence. Sixty-eight LeuO targets were co-bound by the global repressor protein, H-NS. Thus, while LeuO may function as an H-NS antagonist, these functions are unlikely to involve displacement of H-NS. RNA polymerase bound 173 of the 178 LeuO targets, consistent with LeuO being a transcription regulator. Thus, LeuO targets two classes of genes, those that are bound by H-NS and those that are not bound by H-NS. LeuO binding site analysis revealed a logo conforming to the $TN_{11}A$ motif common to LysR-type transcription factors. It differed in some details from a motif that we composed for *Escherichia coli* LeuO binding sites; 1263 and 1094 LeuO binding site locations were predicted in the *S.* Typhimurium SL1344 and *E. coli* MG1655 genomes respectively. Despite differences in motif composition, many LeuO target genes were common to both species. Thus, LeuO is likely to be a more important global regulator than previously suspected.

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Introduction

The LeuO protein belongs to the family of LysR-type transcriptional regulators (LTTRs), the largest family of prokaryotic DNA binding proteins (Pareja *et al.*, 2006; Momany and Neidle, 2012) with over 55 000 potential members in the Pfam database (PF00126 HTH_1, LysR-_substrate) (Punta *et al.*, 2012). The number of different LTTRs in a given species is often very large; for example, *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) encodes 44 distinct LTTRs (Lahiri *et al.*, 2009). LTTRs are typically 300 to 350 amino acids in length and activate the transcription of operons and regulons involved in diverse cellular functions such as nitrogen fixation, the response to oxidative stress and aspects of bacterial virulence (O'Byrne and Dorman, 1994; Van Rhijn and Vanderleyden, 1995; Sheehan and Dorman, 1998; Hernandez-Lucas *et al.*, 2008; Lahiri *et al.*, 2008; 2009; Maddocks and Oyston, 2008). A typical LysR family member consists of an N-terminal DNA binding domain and a C-terminal sensing domain and it activates or represses the transcription of target genes; some, but not all, are known to bind ligands that influence their DNA binding activity (Schell, 1993; Zaim and Kierzek, 2003).

LeuO is a member of the LTTR family and is found in members of the *Enterobacteriaceae*, including *Escherichia coli*, *Salmonella, Shigella* and *Yersinia* spp. (Maddocks and Oyston, 2008). The designation *leuO* was originally used to describe an open reading frame of unknown function located beside the leucine biosynthesis operon (*leuABCD*) (Henikoff *et al.*, 1988). The *leuO* gene was later found to be part of a complex *cis*-acting promoter relay system that connects the *leuABCD* and *ilvIH* operons (Chen *et al.*, 1992; Wu *et al.*, 1995; Wu and Fang, 2003). More recently it has been shown that the *leuO* gene is activated by the transcriptional regulators RcsB and BglJ and is negatively auto-regulated (Stratmann *et al.*, 2012).

Further work, performed mostly in *E. coli* and *Salmonella* Typhi, has shown that LeuO plays a positive role in the regulation of a number of genes including the *bgl*

operon involved in β-glucoside utilization (Ueguchi *et al*., 1998), the *yjjQ-bglJ* operon encoding LuxR-type transcriptional regulators (Stratmann *et al*., 2008), and the *yjcRQP* operon involved in Sulfa drug efflux (Shimada *et al*., 2009). LeuO also activates the *casABCDE* operon (Hernandez-Lucas *et al*., 2008; Westra *et al*., 2010; Medina-Aparicio *et al*., 2011) that is part of the CRISPR/Cas defence system that provides 'immunity' against mobile genetic elements (Karginov and Hannon, 2010). Furthermore LeuO has been implicated in the bacterial response to stress (Fang *et al*., 2000; Majumder *et al*., 2001) and genetic screens have identified LeuO as a *Salmonella* virulence factor (Tenor *et al*., 2004; Lawley *et al*., 2006). LeuO may in part contribute to *Salmonella* virulence by positively regulating the outer membrane porins OmpS1 and OmpS2 (Fernandez-Mora *et al*., 2004; De la Cruz *et al*., 2007; Hernandez-Lucas *et al*., 2008), which are known virulence factors in the mouse model infection system (Rodriguez-Morales *et al*., 2006). LeuO has also been reported to regulate positively *rovA*, a key regulator of virulence in *Yersinia* spp. (Lawrenz and Miller, 2007).

It is clear that LeuO regulates a diverse set of genes and this has been expanded by a recent SELEX analysis in *E. coli* (Shimada *et al*., 2011). However, we do not have a detailed understanding of the genes that are regulated by LeuO in the important pathogen *S*. Typhimurium. On these grounds, we used a global ChIP-chip approach to identify LeuO-regulated genes to obtain comprehensive information about LeuO binding to its genomic targets in living bacterial cells. This approach allowed us to investigate LeuO binding in the context of other DNA binding proteins that are likely to target the same genes, such as RNA polymerase and the H-NS protein. It also allowed us to study LeuO binding to DNA adopting conformations that are natural to the *in vivo* situation, a factor that is known to influence the binding of several DNA binding proteins (Cameron and Dorman, 2012). In our strategy, we exploited knowledge of LeuO protein expression: the *leuO* gene is expressed maximally in bacteria growing in phosphate-limited minimal medium on entry into stationary phase (VanBogelen *et al*., 1996; Fang *et al*., 2000). We present the first *in vivo* DNA analysis of LeuO binding to the *S.* Typhimurium chromosome using the ChIP-chip technique, and provide evidence that LeuO is a global regulator in *S.* Typhimurium.

Results and discussion

Identification of LeuO target genes in Salmonella *enterica using a ChIP-chip approach*

In order to understand the function of LeuO in *S.* Typhimurium, we identified the network of LeuO gene targets using a chromatin immunoprecipitation (ChIP) assay. The LeuO protein, tagged with the FLAG epitope, was cross-linked to its DNA targets, and then immunoprecipitated using an anti-FLAG antibody. The DNA targets bound by LeuO were then identified by hybridization to a DNA microarray (*Experimental procedures*).

Bacterial cultures to be used in the ChIP assay were grown under conditions known to promote maximal LeuO protein expression: growth in a minimal low-phosphate medium (LPM) to stationary phase (equivalent to an OD_{600} value of 1.4) (Fig. 1A) (VanBogelen *et al*., 1996; Fang *et al*., 2000). The LeuO-bound ChIP DNA fragments were fluorescently labelled with Cy3 dCTP while the genomic DNA control was labelled with Cy5 dCTP. The DNA samples were co-hybridized to a DNA tiling microarray and the intensity of fluorescence of each of the DNA probes was calculated (Fig. 1B). The ChIPOTle peak finding programme (Buck *et al*., 2005) was used to identify LeuO binding sites using a twofold cut-off. This procedure identified 261 binding regions common to two biological replicate experiments. However, as the ChIP-chip procedure often results in the identification of false positive binding events (Waldminghaus and Skarstad, 2010), a control 'mock' ChIP-chip experiment was also performed, in which normal mouse IgG antibodies were used during a ChIP reaction, to identify any DNA sequences that were non-specifically immunoprecipitated. The ChIPOTle programme identified 83 peaks in the control dataset that were also present in the LeuO dataset; consequently these targets were eliminated from the final analysis. Altogether, 178 LeuO binding sites were identified (Fig 1 and Table S1). Previously characterized LeuO target genes from other bacterial species that were found in our dataset include the CRISPR/Cas operon (Fig. 1), *sdiA, ompN/ompS2*, *dnaE*, *cyoABCDE*, *tesB*, *fimD*, *sdhA*, *add*, *cpsG*, *nuoH*, *tdcD*, *treF* and *phoU* (Table S1) (Westra *et al*., 2010; Shimada *et al*., 2011; Turnbull *et al*., 2012). The presence of these targets validated our approach. A large number of new LeuO target genes were also identified in this study, some of which are discussed below (see *Extension of the LeuO regulon*).

The majority of the bacterial transcription factors that have been studied by genome-wide location analysis have been found to bind predominantly to non-coding DNA sequences (Grainger *et al*., 2004; 2005; 2006; 2007; Cho *et al*., 2008; Shimada *et al*., 2011; Wei *et al*., 2012). This is not surprising because most transcription factors regulate transcription by binding to DNA sites that are located upstream of open reading frames (ORFs). In general, LTTRs bind to several intergenic sites located upstream of their regulated gene(s) (Maddocks and Oyston, 2008). While they often bind close to promoter regions (–55 bp to +20 bp), sites located more than 200 bp upstream of the promoter have been detected, as have binding sites inter-

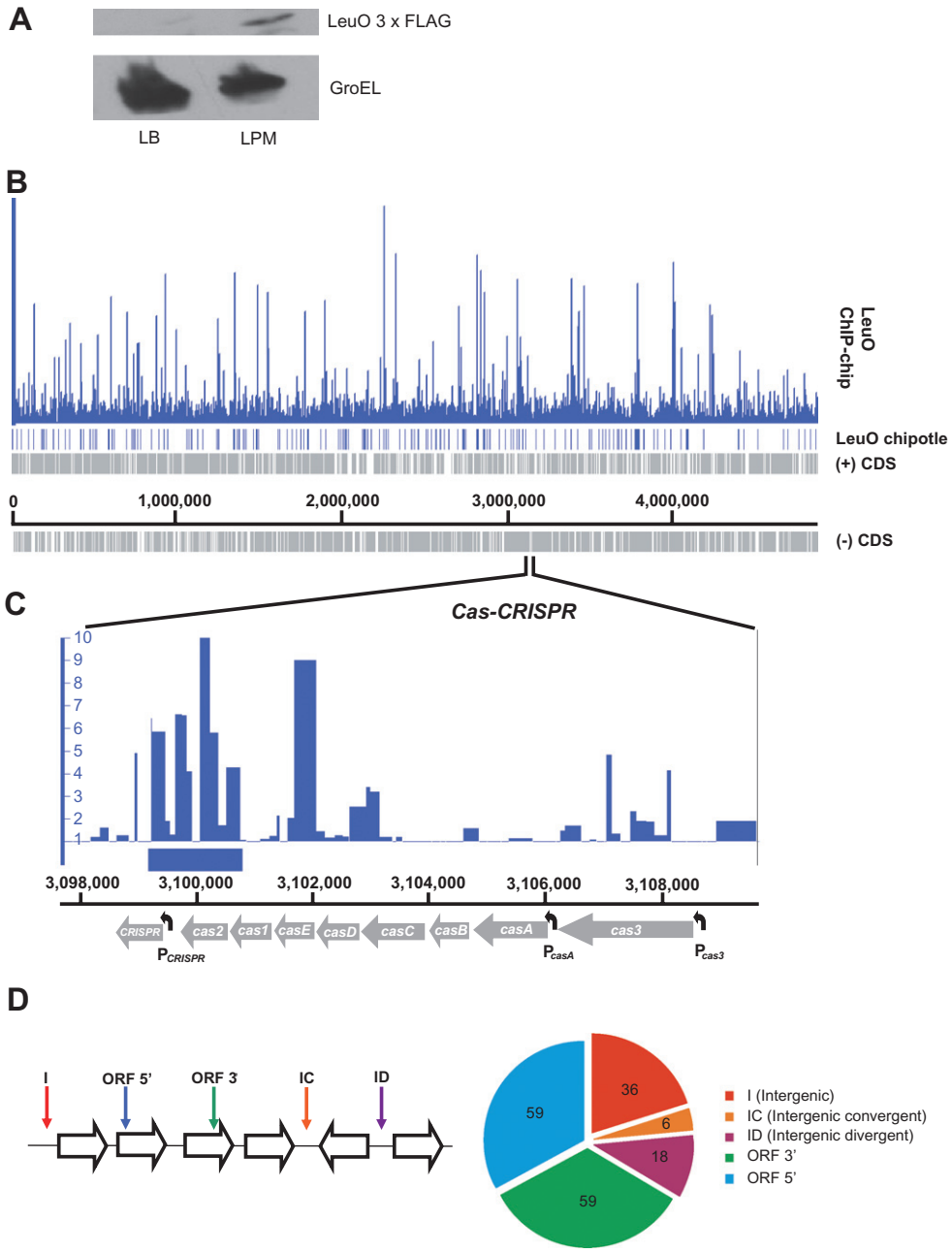


Fig. 1. Global identification and categorization of LeuO binding sites by ChIP-chip.
A. Western immunoblot analysis of LeuO protein levels in SL1344 cells grown to stationary phase in LB or LPM are shown in the top panel. GroEL loading controls are indicated in the bottom panel.
B. Visualization of LeuO ChIP-chip data using the Integrated Genome Browser (IGB) for *S.* Typhimurium SL1344. The locations of LeuO binding sites, as defined by the ChIPOTle algorithm, are indicated by horizontal bars in the LeuO ChIPOTle track. The locations of known coding sequences (CDS) on the plus (+) and minus (–) DNA strands and SL1344 chromosome co-ordinates are also shown.
C. Detailed view of the *S.* Typhimurium CRISPR/Cas locus. LeuO ChIP-chip data are presented quantitatively, with enrichment ratios shown on the *y*-axis. The binding site identified by ChIPOTle is depicted by the blue rectangle and known promoter locations are indicated by bent arrows. The *cas3* transcription start site was determined by Kröger *et al.* (2012).
D. Schematic representation of LeuO binding site classification as outlined in the text. The pie chart shows the relative distribution of LeuO binding sites among the location classes summarized in the genetic diagram on the left, with horizontal open arrows used to represent ORFs and their relative orientations. The colours of the vertical arrows correspond to the colours used in the pie chart segments.

nal to the ORF (+350 bp) (Wilson *et al*., 1995; Viswanathan *et al*., 2007). The evidence that LTTRs can exert their regulatory influence through binding to a wide variety of locations prompted us to examine the location of *S.* Typhimurium LeuO binding sites in detail.

LeuO binding sites were classified based on their location, i.e. intergenic or within an ORF. Intergenic and intra-ORF sites were further subcategorized into Intergenic (I, located upstream of an individual gene), Intergenic Convergent (IC, located between two convergently transcribed genes), Intergenic Divergent (ID, located between two divergently transcribed genes), ORF 3′ (located within the 3′ promoter-distal half of an ORF) and ORF 5′ (located within the 5′ promoter-proximal half of the ORF) (Fig. 1D). Surprisingly we found that only about 33% of binding sites were located in intergenic regions with the remaining ~ 66% of binding sites being located within ORFs (Fig. 1D). Thirty-six of the 60 intergenic binding events were located upstream of an individual gene, 18 were located between divergently transcribed genes and six binding events were located between convergently transcribed genes, making target gene predictions based on binding site location difficult. The same number (59) of ORF binding events were distributed equally between the 5′ and 3′ regions of ORFs. It is possible that a proportion of these intra-ORF binding events have been incorrectly classified in the case of adjacent genes that share short intergenic regions. This is because the resolution capacity of the ChIP-chip method is limited by the average size of the sonicated DNA fragments (~ 500 bp). However, most represent intra-ORF LeuO binding sites of the type that have been documented previously for LeuO and other LysR-like regulators (Wilson *et al*., 1995; Viswanathan *et al*., 2007; Shimada *et al*., 2011). The significance of intra-ORF binding is not clear but because of their location they may have a repressive effect on transcription as in some instances LeuO has been shown to act as a negative regulator (Hernandez-Lucas *et al*., 2008; Shimada *et al*., 2009). Alternatively, as H-NS can bind within coding regions to form transcriptionally repressive nucleoprotein complexes (Nagarajavel *et al*., 2007), these intra-ORF binding events could simply reflect LeuO-mediated antagonism of H-NS binding to internal gene regions. It is also possible that they may reflect an architectural role for the LeuO protein in determining the structure of the nucleoid (Chen and Wu, 2005).

Extension of the LeuO regulon

Our ChIP-chip analysis greatly extended the number of known LeuO target genes in *S.* Typhimurium. Prior knowledge of LeuO targets in *Salmonella* extended only to relatively few loci, including genes in *S.* Typhi rather than *S.* Typhimurium (De la Cruz *et al*., 2007; Hernandez-Lucas *et al*., 2008; Medina-Aparicio *et al*., 2011; Turnbull *et al*., 2012). The 164 new *S.* Typhimurium regulon members identified here are involved in a variety of cellular processes and include, for example, inner/outer membrane proteins, transport proteins, motility factors, cell division proteins, oxidative stress response proteins and also other LTTRs (Table S1).

LeuO binding has been mapped previously to the CRISPR-associated *casA* and *cas3* promoters in *S.* Typhi (Medina-Aparicio *et al*., 2011); here, we observed high levels of LeuO binding at the promoter for the CRISPR repeats with little or no binding at the *casA* and *cas3* promoters in *S.* Typhimurium (Fig. 1C). However, while P_{casA} and P_{cas3} were not classified as LeuO targets in LPM growth conditions, subsequent LeuO-binding-site motif analysis predicted LeuO binding in the 5′ regulatory region of these genes (see *Genome-wide prediction and validation of LeuO binding sites*).

Other notable LeuO target genes are *sopA*, encoding an effector protein that is translocated by the *Salmonella* pathogenicity island (SPI) 1 type III secretion system, and *sifA,* the SPI-2 translocated effector gene. These are important virulence determinants of *S.* Typhimurium and their detection is consistent with the previous characterization of LeuO as a *Salmonella* virulence factor required for host-pathogen interactions (Tenor *et al*., 2004).

The *rcsA* gene was also identified as a LeuO target. Its product, RcsA, is an auxiliary regulator for the Rcs (regulation of capsular polysaccharide biosynthesis) two-component phosphophorelay system that senses alterations in the outer membrane and the peptidoglycan layer of the cell envelope (Majdalani and Gottesman, 2005). Interestingly, RcsA can form heterodimers with the response regulator RcsB, which activates *leuO* transcription in conjunction with BglJ, counteracting H-NS repression of *leuO* transcription (Stratmann *et al*., 2012).

The *rssB* gene, which encodes a response-regulator-like adaptor protein (RssB) for ClpXP proteolytic degradation of the RpoS stress and stationary phase sigma factor (Klauck *et al*., 2001), was found to be a LeuO target. This LeuO–RssB link is significant in the context of an earlier discovery that a Tn*10* insertion that activated *leuO* expression led to a reduction in RpoS levels (Klauck *et al*., 1997). It is possible that in addition to the established effect of LeuO on DsrA regulatory RNA expression, and hence DsrA-sensitive RpoS mRNA translation, LeuO may also influence RpoS protein levels by modulating the expression of the RssB adaptor protein.

We also identified the genes coding for three other LysR-like regulators as LeuO targets: *metR*, *yeeY* and *stm2180*. While the functions of YeeY and STM2180 are uncharacterized, MetR is known to be involved in the regulation of methionine biosynthesis (Maxon *et al*., 1989). The possibility that bacteria may link their amino acid

biosynthetic pathways through gene regulation is intuitively appealing, since mounting appropriate responses to metabolic challenges is essential for survival. It may be also significant during infection: the *Salmonella* containing vacuole in an infected host cell is a nutrient limiting environment (simulated by growth in LPM), and control of amino acid biosynthesis by LeuO may facilitate survival during the infection process.

The observed binding of LeuO to its known target *ompS2*/*ompN* but not to *ompS1*/*ompS* provided an important insight. LeuO is known to induce *ompS2* expression at a lower concentration than required for the induction of *ompS1* (De la Cruz *et al*., 2007), consistent with LeuO having a higher affinity for the regulatory region of *ompS2*. The low intracellular concentration of LeuO when cultured in LPM may not allow LeuO to occupy lower affinity sites such as the regulatory region of *ompS1*. To investigate if the genome-wide binding pattern of LeuO was altered upon an increase in the intracellular concentration of LeuO, we used the inducible pBAD system to overexpress 3x FLAG tagged LeuO and monitored its binding pattern using the ChIP-chip technique. This analysis revealed that LeuO bound to 331 chromosomal locations (after removal of any false positives also present in a mouse IgG control ChIP-chip) (Table S1). We observed LeuO binding to other known targets including *ompS1* and *cas3*, which we did not detect previously. This is consistent with LeuO having a lower affinity for these sites so that a higher intracellular concentration of LeuO is required before full binding is achieved. LeuO concentration is very low in exponentially growing cells (~ 200 molecules per cell) but is believed to reach up to 2000–4000 molecules per cell in stationary phase *E. coli* cultures (Shimada *et al*., 2011). If LeuO binds as a dimer, there may be as many as 1000–2000 LeuO binding sites (see *Genome-wide prediction and validation of LeuO binding sites*). It is not inconceivable that under appropriate growth conditions *Salmonella* synthesizes large amounts of LeuO to occupy even more binding sites than documented here as we are only beginning to understand the growth conditions under which LeuO plays a regulatory role (Gallego-Hernandez *et al*., 2012).

LeuO binding in close proximity to H-NS

Enteric bacteria encode the abundant DNA binding H-NS protein which acts as a global repressor of ~ 20% of genes in *S.* Typhimurium (Dorman, 2004; Dillon and Dorman, 2010). H-NS represses transcription of these genes by binding to curved AT-rich DNA sequences and mediates its repressive function by preventing RNA polymerase binding or by trapping RNA polymerase at promoters (Dame *et al*., 2006; Lucchini *et al*., 2006; Navarre *et al*., 2006; Walthers *et al*., 2011). Counteracting the repressive function of H-NS is important if cells are to express H-NS regulated genes; not surprisingly, a number of H-NS antagonism mechanisms have been described (Stoebel *et al*., 2008). LeuO has recently emerged as an important antagonist of H-NS (Hernandez-Lucas *et al*., 2008; Shimada *et al*., 2009; 2011) and it may exert this function by simply competing with H-NS for binding to DNA (Shimada *et al*., 2011) or acting as a barrier to H-NS polymerization (Chen *et al*., 2003; 2005; Chen and Wu, 2005). While LeuO is known to antagonize H-NS, its own gene is repressed by H-NS (Klauck *et al*., 1997; Chen *et al*., 2001; Stratmann *et al*., 2012). Deletion of *hns* in *Salmonella* Typhimurium strain SL1344 resulted in a dramatic increase in LeuO protein levels (Fig. 2A), confirming the repressive action of H-NS at the *leuO* gene in SL1344. LeuO binding was observed in close proximity to a previously mapped H-NS binding site at *leuO* (Dillon *et al*., 2010), consistent with LeuO functioning as an H-NS antagonist in *S*. Typhimurium.

It was important to determine which of the 178 *S.* Typhimurium LeuO binding events were associated with H-NS and to ascertain whether LeuO mediated its function by displacing H-NS or by another mechanism. To address these questions, H-NS binding to the SL1344 chromosome was examined by ChIP-chip analysis under the same growth conditions that are known to promote *leuO* expression (i.e. grown to stationary phase in LPM) (Fig. 2B). In addition, previously published data (Dillon *et al*., 2010) on H-NS binding under standard laboratory growth conditions in which LeuO is undetectable [i.e. grown to exponential phase in Luria–Bertani (LB)] were analysed and the findings were integrated with those from the present investigation.

The ChIPOTle algorithm identified 496 H-NS binding regions in SL1344 grown in LPM (Table S2) (456 binding regions were identified in LB, Dillon *et al*., 2010). The locations of these H-NS binding regions were compared with the LeuO binding sites and those LeuO binding sites that overlapped with, or were located within 200 bp of, an H-NS binding region were classified as LeuO + H-NS sites; the remaining sites were classified as LeuO sites (Fig. 2C) (Table S1). We identified 68 LeuO sites that met our criteria for classification as LeuO + H-NS sites; the remaining 110 LeuO sites were not associated with H-NS colocalization and so were designated as LeuO sites.

LeuO colocalization with H-NS at 68 locations is consistent with a global H-NS antagonism function. However, it is important to consider that LeuO may also repress some of its target genes, perhaps in conjunction with H-NS. This possibility is supported by the observation that LeuO and H-NS are both known to repress the *fimAICDFGH* operon in *E. coli* (Shimada *et al*., 2011). The 68 LeuO + H-NS sites represent only 38% of the total number of LeuO binding events; in contrast, Shimada *et al*. (2011) found that 95% of LeuO sites in *E. coli* overlapped with H-NS sites. We then examined average LeuO and H-NS occupancy at the two

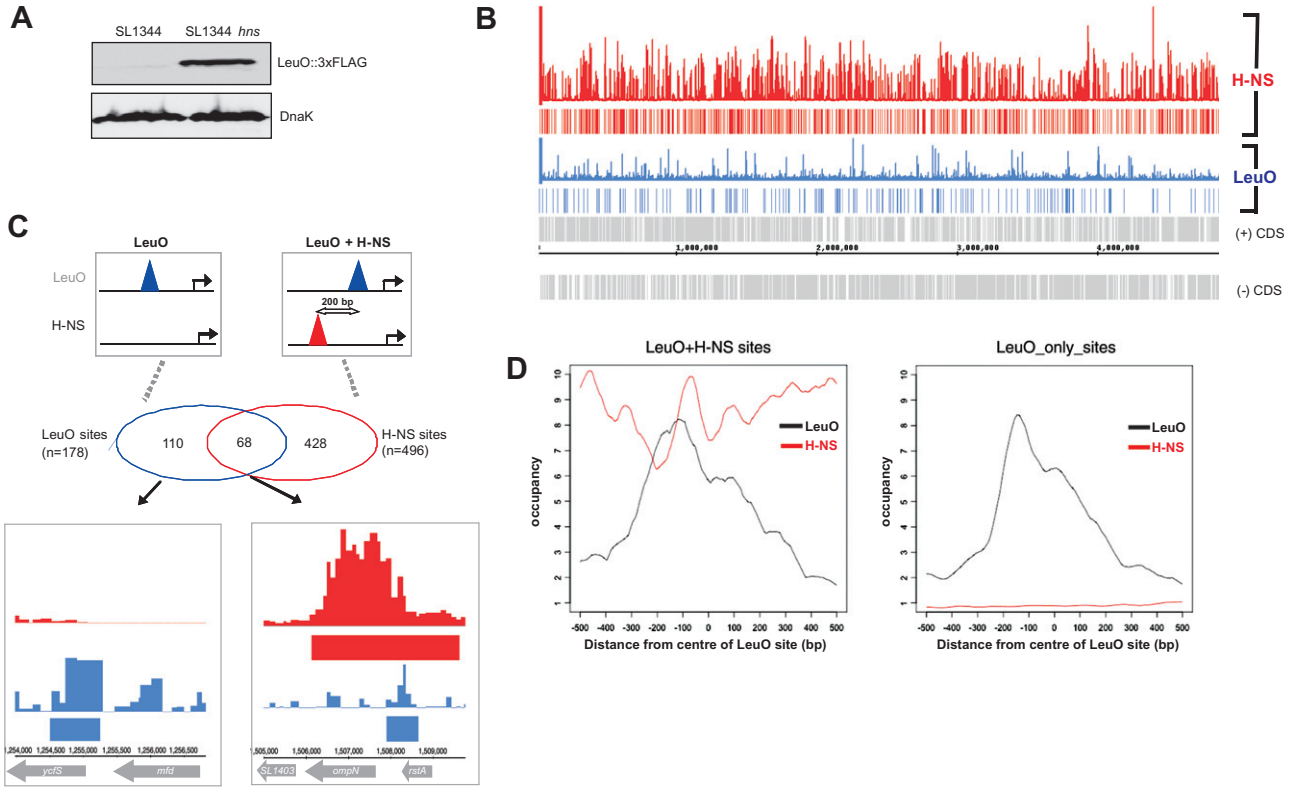


Fig. 2. Integration of H-NS and LeuO genome-wide binding data.
A. Western immunoblot analysis of LeuO protein levels in wild-type SL1344 and SL1344 *hns* are shown in the top panel. DnaK loading controls are indicated in the bottom panel.
B. Visualization of H-NS (red) and LeuO (blue) ChIP-chip data in the IGB with ChIPOTle identified binding sites depicted below each lane as horizontal bars. The locations of known CDS on the plus (+) and minus (–) DNA strands and SL1344 chromosome co-ordinates are also shown.
C. Schematic representation of LeuO and H-NS overlap analysis. The Venn diagram illustrates the number of LeuO sites that did (LeuO + H-NS) and did not (LeuO) overlap with an H-NS binding site. Quantitative LeuO (blue) and H-NS (red) ChIP-chip data are shown for representative examples of LeuO and LeuO + H-NS binding sites.
D. Average plots of LeuO and H-NS occupancy (ChIP-chip enrichment ratios) at LeuO + H-NS and LeuO-only sites. Averaged LeuO and H-NS data were plotted in 100 bp windows with respect to the centre of ChIPOTle-defined LeuO binding sites.

classes of LeuO binding sites. Average H-NS and LeuO ChIP occupancies were calculated +/–500 bp with respect to the centre of the LeuO binding sites. These surveys showed that the peak of LeuO binding was offset by 100 bp from the ChIPOTle peak centre (Fig. 2D). However, an interesting pattern emerged: close analysis of regions of LeuO and H-NS co-occupancy revealed that the LeuO binding peak coincided consistently with a trough in the H-NS binding landscape (Fig. 2D). The significance of this is not clear but may indicate that LeuO functions as an H-NS barrier or antagonist, taking up a position interposed between two consecutive H-NS binding peaks. It is also possible that the intracellular concentrations of LeuO might not be high enough to displace H-NS completely.

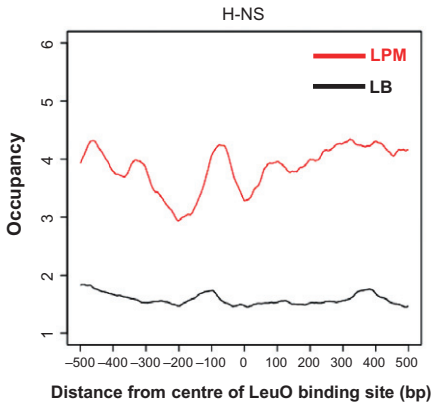
We wished to know if LeuO could antagonize H-NS binding by competing with and displacing this protein from DNA. We examined H-NS binding at the 110 LeuO-only sites in LB-grown SL1344 and found that none of these 110 locations was occupied by H-NS (Table S1). Therefore, the presence of LeuO had not simply resulted in the complete displacement of H-NS in LPM since these locations lacked H-NS binding in the absence of detectable levels of LeuO. Next we examined H-NS occupancy at the 68 LeuO + H-NS sites in LB-grown cultures and found that fewer LeuO target genes were bound by H-NS in LB when compared with LPM (41 of the 68 genes were bound by H-NS in LB) (Fig. 3A). Therefore, the presence of LeuO appeared to correlate with H-NS binding to more LeuO target genes, which would not be expected if LeuO simply displaced H-NS from its cognate binding sites. However, these results did not rule out the possibility that LeuO influenced the pattern of H-NS occupancy without completely displacing H-NS. Therefore, we calculated the average H-NS binding levels at LeuO binding sites in both LPM and LB (Fig. 3B). This analysis revealed much higher levels of H-NS binding at LeuO target genes in LeuO-inducing (i.e. LPM) conditions compared with repressive (LB) conditions. Our data are inconsistent with

A

LeuO LPM target genes (n=178)

	H-NS only	H-NS + RNAP	RNAP only	None
LPM	5	63	105	5
LB	30	11	50	46

B



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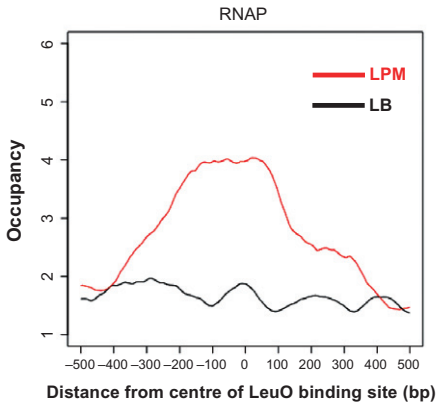


Fig. 3. LeuO does not simply displace H-NS from DNA and may be involved in the recruitment of RNA Polymerase to target genes.
A. The number of H-NS, RNA Polymerase (RNAP) and LeuO colocalization events in LeuO-inducing conditions (LPM) is indicated in the first row of the table. The second row indicates the number of H-NS and RNAP binding events at the LPM defined LeuO sites in non-inducing conditions (LB).
B. An average plot of H-NS occupancy with respect to LeuO binding site location in LeuO inducing conditions (LPM) and non-inducing conditions (LB).
C. An average plot of RNAP occupancy with respect to LeuO binding site location in LeuO inducing conditions (LPM) and non-inducing conditions (LB).

a simplistic model in which LeuO overcomes H-NS transcriptional repression activity simply by displacing H-NS. They are more suggestive of a subtle remodelling of the nucleoprotein complex that overcomes H-NS-mediated repression without stripping H-NS from the DNA. A mechanism of this kind has been proposed for other H-NS antagonists in *S.* Typhimurium, such as the SlyA protein and OmpR (Perez *et al.*, 2008; Cameron and Dorman, 2012). The ability of LeuO to form DNA-protein-DNA bridges, analogous to those created by LacI, might also allow LeuO to interfere with H-NS activity without removing the protein from the DNA. Significantly, the LacI protein can replace LeuO as an H-NS antagonist at *leuO* (Chen and Wu, 2005), a finding that is consistent with the two proteins operating through a common mechanism. Here, the LeuO/LacI proteins erect a DNA–protein–DNA bridge between two binding sites that contains H-NS behind a LeuO/LacI barrier. This barrier protects a nearby promoter from encroachment by H-NS polymerization without the need to displace H-NS from the DNA.

RNA polymerase recruitment to LeuO target genes

LTTRs are known to bind sites overlapping or adjacent to the target promoter to repress or activate transcription (Maddocks and Oyston, 2008). It has been suggested that LTTRs activate transcription by interacting with the C-terminal domain of the α subunit of RNA polymerase (αCTD). For example, the LTTR family member OxyR increases RNA polymerase binding to OxyR-dependent promoters, suggesting that OxyR activates transcription partly by recruiting RNA polymerase (Kullik *et al.*, 1995). To determine whether LeuO might recruit RNA polymerase, we examined RNA polymerase binding at LeuO target genes under LeuO-inducing growth conditions (LPM) and non-inducing growth conditions (LB). ChIPO-Tle analysis of both datasets and comparison with the location of LeuO binding sites revealed that 173 of the 178 LeuO binding sites were also associated with RNA polymerase binding in LPM (co-occupancy was defined as a LeuO binding region located within 200 bp of an RNA polymerase binding region), whereas only 61 of the LeuO binding sites were occupied by RNA polymerase upon growth in LB (Fig. 3A and Table S1).

This analysis provided three striking observations. First, LeuO appears to be associated with active transcription, as the vast majority of LeuO target genes are associated with RNA polymerase binding in LPM. However, it cannot be excluded that some of these colocalization events represent transcriptionally repressive events as LeuO may prevent promoter clearance by trapping RNA polymerase at promoters, a property already described for H-NS (Dame *et al.*, 2002). Second, the presence of RNAP at 105 LeuO target genes in inducing conditions (and its absence in non-inducing conditions) suggests that LeuO recruits RNA polymerase. Lastly the observation that 63 of the 68 LeuO + H-NS co-occupancy sites are also associated with RNA polymerase binding is intriguing as binding of H-NS and RNA polymerase are believed to be mutually exclusive in *Salmonella* (Lucchini *et al.*, 2006). As discussed above this may represent trapping of RNA polymerase by LeuO and H-NS. Alternatively, LeuO may

remodel H-NS oligomers and allow RNA polymerase to bind to promoters without the need to displace H-NS. We cannot discount the other possibility that all three factors do not in fact colocalize in individual cells as ChIP quantifies protein occupancy across a population of cells.

Next we plotted the mean RNA polymerase occupancy in both growth conditions with respect to the location of the LeuO binding sites (Fig. 3C). RNA polymerase occupancy was plotted for a distance of +/–500 bp from the centre of the LeuO binding sites and was found to peak with respect to the centre of LeuO binding sites in both growth media. However, the average RNA polymerase occupancy in LeuO-inducing conditions (LPM) was much higher than in non-inducing conditions (LB) and the peak of binding was also broader. This is consistent with LeuO promoting the recruitment of RNA polymerase to target genes, and the broader peaks of binding are consistent with the detection of elongating RNA polymerase.

Identification of an AT-rich LeuO DNA binding motif

The ability of proteins to recognize specific DNA sequences is a key feature of many biological processes. Recognition of a specific DNA sequence by a protein often involves the formation of amino acid and nucleotide-specific hydrogen bonds (Garvie and Wolberger, 2001). For LysR-like proteins, a DNA sequence known as the LTTR box has been identified (Maddocks and Oyston, 2008). The consensus sequence of the LTTR box is T-N_{11}-A and often displays imperfect dyad symmetry (Parsek *et al*., 1994). However, this motif is highly degenerate and does not give an accurate understanding of the DNA sequences with which LeuO interacts. Therefore, we wanted to determine if LeuO binding was associated with a specific DNA motif in our ChIP-chip binding sites. The recent SELEX study of LeuO binding in *E. coli* (Shimada *et al*., 2011) also gave us information on the location of LeuO binding sites in a related species and we were able to incorporate this information into our analysis.

First, the *S.* Typhimurium LeuO + H-NS binding regions described above in which LeuO binding overlapped with or was close to an H-NS binding location were selected for DNA sequence motif analysis. We also created a list of DNA sequences bound by LeuO in *E. coli* by extracting 500 bp DNA sequences that centred on the genomic co-ordinate provided for each LeuO binding site by Shimada *et al*. (2011). Recall that almost all of the *E. coli* sites were of the LeuO + H-NS type. The details of these datasets are provided in Fig. 4A and in the *Experimental procedures*. Next we used the unbiased motif-finding algorithm Meme to search the two datasets for significantly over-represented sequence motifs (Bailey *et al*., 2009). Meme identified a 28 bp motif in both datasets (Fig. 4B). Two striking features of the LeuO motifs are their imperfect dyad symmetry and their A + T richness. While some dyad symmetry is discernable in the *E. coli* logo, it is much harder to detect in its *S.* Typhimurium counterpart. Furthermore both motifs contain a central region matching the T-N_{11}-A LTTR box motif and alignment of the central T-N_{11}-A motifs of the sequence logos shows significant overlap between the two motifs (Fig. 4B). However, the *E. coli* LTTR box displays a much stronger nucleotide preference at most positions, a sequence divergence that may explain why only 15 of the *E. coli* LeuO target genes were common to *S.* Typhimurium (Table S1). Regulon divergence is not uncommon even in closely related species and this is reflected in differences in the presence and nature of regulatory protein binding sites (Perez and Groisman, 2009). While the *E. coli* and *S.* Typhimurium LeuO proteins are highly related (87% amino acid identity) there are a number of amino acid differences in the N-terminal DNA binding domain which may have altered DNA binding site specificity. Furthermore selective pressure associated with the acquisition and regulatory integration of horizontally acquired SPIs that contain a large number of predicted LeuO binding sites (see following section), may have also altered DNA binding site preference.

We know that the LTTR box motif is often associated with dyad symmetry (Schell, 1993; Grob *et al*., 1997; Sheehan and Dorman, 1998) and this property appears to be a general feature of the extended 28 bp motifs identified here, albeit weakly in the case of the *S.* Typhimurium example. The presence of dyad symmetry is consistent with individual LeuO subunits binding to half-sites to form a dimer. However, LTTRs are known to be functionally active as tetramers that protect large regions of DNA (50–60 bp) (Maddocks and Oyston, 2008). Tetramer formation by LeuO would lend itself to DNA–protein–DNA bridging, as it is the case with tetrameric LacI (Chen and Wu, 2005). This would allow LeuO to participate in both short-range and long-range protein–DNA interactions, facilitated by DNA looping. Supporting this hypothesis are data from our genome-wide prediction of LeuO binding sites that identified many examples of clustered binding sites (see the *Genome-wide prediction and validation of LeuO binding sites* section below).

The extremely high A + T content of both logos (Fig. 4B) is consistent with the proposed role of LeuO as an H-NS antagonist as H-NS binds to A + T rich sequences (Lucchini *et al*., 2006; Navarre *et al*., 2006; Dillon *et al*., 2010). Furthermore both logos contain a central A-tract at nucleotide positions 15–17. These A-tracts are intriguing because DNA structural studies have determined that A-tracts are associated with narrowing of the DNA minor groove (Beveridge *et al*., 2004; Rohs *et al*., 2009). Variation in DNA shape, in particular DNA minor groove width and DNA twist, is emerging as an important 'indirect'

A

Species	Dataset	Number of Sequences	Average Size (bp)	Total Size (bp)	% A+T	Source
Salmonella Typhimurium	LeuO+ HNS	64	653	41825	50.5	This study
Escherichia coli	E. coli LeuO	119	501	59619	59.9	Shimada *et al.* (2011)

B

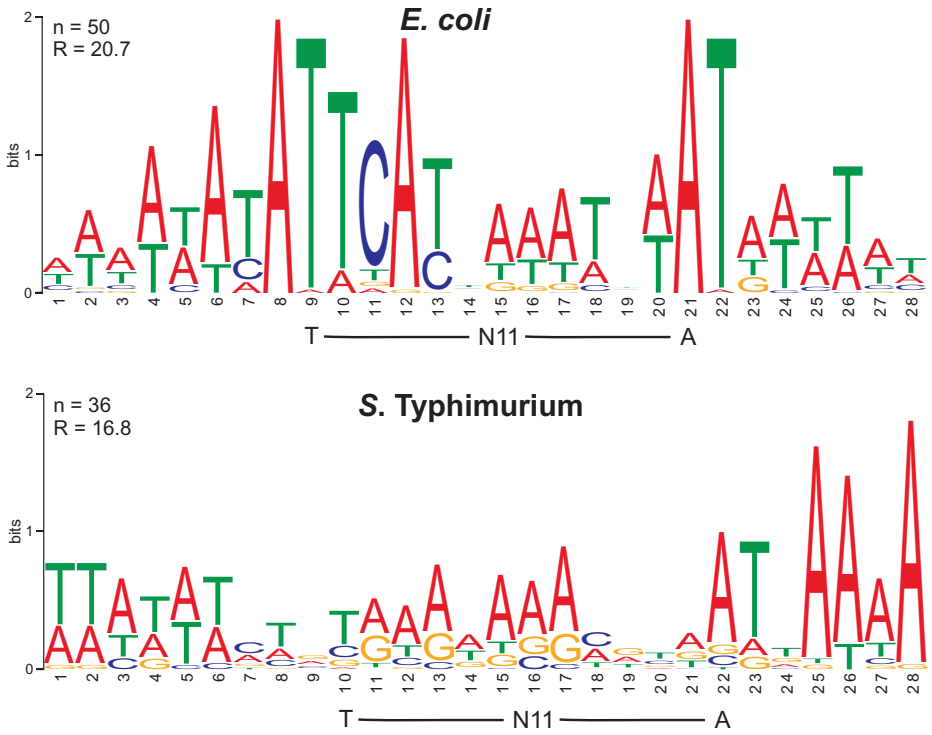


Fig. 4. Similarities and differences in the LeuO binding site motifs of *S.* Typhimurium and *E. coli*.
A. Details of the manually curated *S.* Typhimurium and *E. coli* LeuO datasets used to derive the LeuO binding motifs.
B. Alignment of sequence logos illustrating the *E. coli* and *S.* Typhimurium motifs identified by Meme. The location of the LTTR T-$_{N11}$-A box is indicated below each Logo.

mechanism used by proteins to achieve DNA binding specificity in the absence of base-specific contacts (Rohs *et al.*, 2009; Cameron and Dorman, 2012). In this mechanism the bases are not necessarily involved in contacting the protein but in allowing the DNA to assume a conformation that facilitates protein binding (Rohs *et al.*, 2009). These flexible bases are often located in linker sequences that connect two half-sites that are directly bound by protein subunits (Hizver *et al.*, 2001; Rohs *et al.*, 2009). The quasi-palindromic nature and presence of A-tracts in the LeuO motifs suggests that a combination of direct amino-acid-base-pair interactions and DNA shape may be important features in determining LeuO binding specificity.

Genome-wide prediction and validation of LeuO binding sites

The identification of the 28 bp LeuO DNA binding motifs suggested that accurate prediction of LeuO binding sites in *E. coli* and *S.* Typhimurium genome sequences would be possible. We used the Motif Alignment and Search Tool (MAST), which is part of the MEME suite of tools used for motif discovery and searching (Bailey *et al.*, 2009), to generate a position-specific scoring matrix (PSSM) from the LeuO sequence logos. This PSSM was used to search for sequence matches in the *S.* Typhimurium SL1344 and *E. coli* K-12 MG1655 genome sequences as described in the *Experimental procedures* section. This analysis resulted in the identification of 1263 and 1094 matches in the *S.* Typhimurium and *E. coli* genome sequences respectively (Fig. 5A and B; Table S3). These predicted sites were often located in intrinsically curved A + T rich regions and are also associated with H-NS binding (Fig. 5A and Table S4). Eight hundred and eighty-nine of the 1263 predicted LeuO binding sites in *S.* Typhimurium were associated with H-NS binding *in vivo*, suggesting that LeuO may function as a more global antagonist of H-NS than previously thought. While the PSSMs used to search

for binding sites differed between the species, there were many instances of homologous genes being associated with a predicted binding site(s) in *both* species. For example, predicted LeuO binding sites were identified for the *yjiAXY* operon and in the nearby *yiiQ-bglJ* operon in both species (Fig. 5C and D). LeuO is known to activate *bglJ* in *E. coli* (Stratmann *et al*., 2008) while RcsB-BglJ heterodimers activate *leuO* transcription (Stratmann *et al*., 2012). Furthermore *rcsB* is also associated with a predicted LeuO site in both species, illustrating the complexity of the LeuO regulatory network.

In order to validate our genome-wide prediction of LeuO binding sites we searched for sites in other known LeuO-regulated genes that were not identified in our ChIP-chip study. We correctly predicted sites in the 5′ regulatory region of *leuO* itself (Chen and Wu, 2005), in the *yjjQ-bglJ* operon (Stratmann *et al*., 2008), in *ompS1*, *assT* (*stm3192*), and in the CRISPR-associated *casA* and *cas3* genes (Westra *et al*., 2010; Medina-Aparicio *et al*., 2011; Gallego-Hernandez *et al*., 2012). Furthermore LeuO binding sites have been precisely mapped in the regulatory regions of *Salmonella* Typhi *casA* (Medina-Aparicio *et al*., 2011) and *ompS1* (De la Cruz *et al*., 2007) and our predicted binding sites map to these locations.

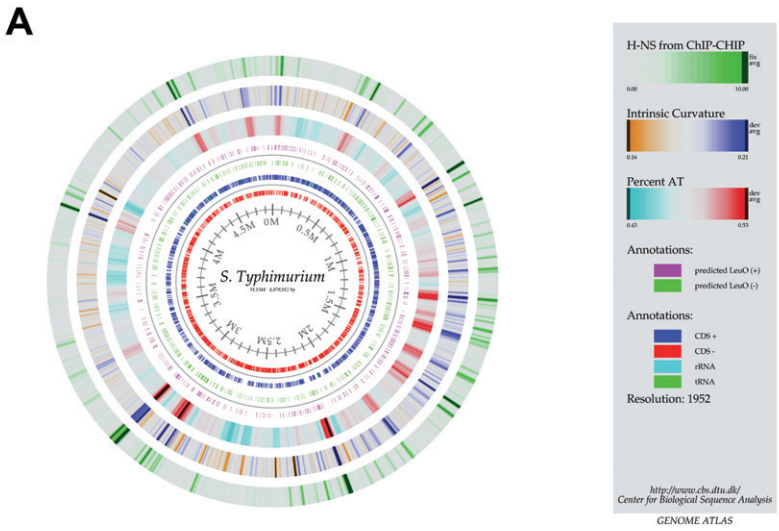
To further validate our genome-wide prediction of LeuO binding sites, three *S.* Typhimurium regions were tested for *in vitro* binding of purified LeuO protein by electrophoretic mobility shift assays (EMSA). The *pipA* and *envR* genes each contain one and two predicted LeuO sites respectively, while *SL3361,* which is located beside *envR* and does not contain a predicted LeuO binding site motif, was used as a negative control (Fig. 6A). Both *pipA* and *envR* DNA probes showed a clear pattern of retarded migration after incubation with increasing concentrations of purified LeuO while LeuO did not bind to the *SL3361* DNA probe (Fig. 6A). The *envR* 5′ regulatory region (P_{envR}) contains two predicted LeuO binding sites located in close proximity to each other (81 bp spacing) and displayed a higher affinity for LeuO binding than the *pipA* region. The presence of two LeuO binding sites in close proximity and in helical register along the DNA may lead to LeuO oligomerization and DNA bending (Hryniewicz and Kredich, 1994), which may account for the apparently higher affinity observed for this DNA probe.

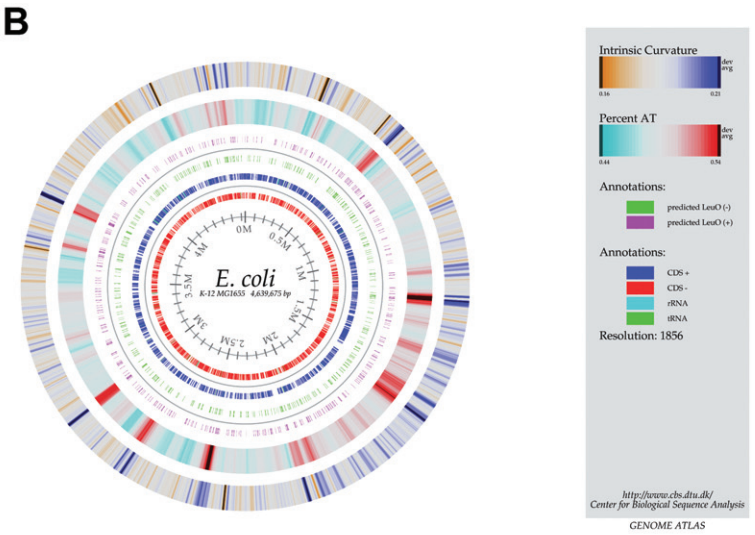
Next we used primer extension to resolve DNase I footprints on PCR amplified DNA templates (Cameron and Dorman, 2012). This approach can be used to more accurately map LeuO binding sites and validate the location of predicted LeuO binding sites. A 400 bp DNA probe encompassing two predicted sites in P_{envR} was used as the target in our experiments. We identified five regions that were protected from DNase I digestion by LeuO, two of which overlapped with the location of the predicted binding sites (Fig. 6B). Three other protected sites were identified further upstream of the *leuO* ORF which did not contain a predicted site but were located in a 60 bp region of high A + T content (66%), consistent with LeuO binding to A + T rich sequences.

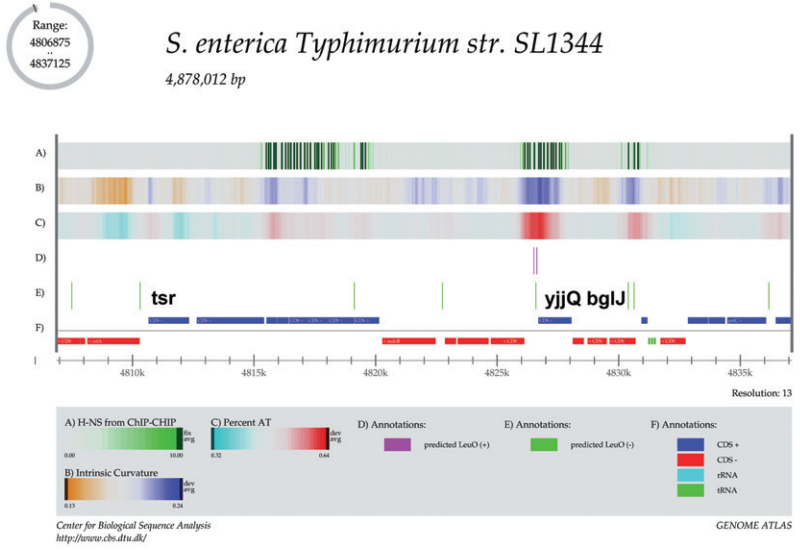
Finally we used quantitative RT-PCR to examine the effect of deleting *leuO* on the transcript levels of four predicted LeuO target genes which are also bound by H-NS (Table S4) – *envR*, *pipA*, *sifA* and *sopA*. We examined transcript levels for these genes in (i) a strain that harbours a T-POP transposon (Lee *et al*., 2007) upstream of *leuO* in its native chromosomal location, so that *leuO* is overexpressed when tetracycline is added to the culture (SL1344 Tpop-*leuO*) and (ii) a strain that harbours a T-POP transposon upstream of *leuO* but the *leuO* gene is deleted (SL1344 Tpop-*ΔleuO*). Deletion of *leuO* dramatically decreased the level of *envR* transcript (Fig. 6C) and increased the levels of *sifA*, *sopA* and *pipA* transcripts (Fig. 6C). The results of this analysis suggest that LeuO activates transcription of *envR*, perhaps by antagonizing H-NS, but appears to function as a repressor at the other target genes. It is possible that LeuO and H-NS function together to repress transcription of these genes. The location of the LeuO binding sites may be important as the predicted binding sites near *envR* are located upstream of the *envR* start codon, consistent with LeuO functioning as a transcriptional activator of this promoter. Conversely the predicted LeuO binding sites for *sifA*, *sopA* and *pipA* are all located downstream of the start codons, where LeuO binding is likely to have a repressive effect on transcription. Thus it appears that LeuO has a dual role as activator and repressor of transcription. Transcriptional activation by LeuO is well documented but LeuO has also been shown to repress the acid stress regulator *cadC*, the small RNA *dsrA* and the *fimAICDFGH* operon in *E. coli* (Shi and Bennett, 1995; Repoila and Gottesman, 2001; Shimada *et al*., 2011). Furthermore, LeuO has a complex relationship with its own gene, antagonizing H-NS-mediated *leuO* repression and antagonizing RcsB-BglJ-mediated *leuO* activation (Chen and Wu, 2005; Stratmann *et al*., 2012).

Many of the genes on the A + T-rich SPIs 1 and 2 are repressed by H-NS (Dillon *et al*., 2010) and our analysis identified 25 predicted LeuO sites in SPI1 and 11 in SPI2. Notably, sites were predicted in the promoter regions of key regulators *hilA*, *hilC*, *hilD*, and *ssrAB* (Table S3). This may explain why LeuO was identified as a virulence factor in a *S.* Typhimurium host-pathogen model system (Tenor *et al*., 2004) and in a long-term systemic infection mouse model system (Lawley *et al*., 2006).

It is also important to point out that 24 of 44 genes encoding *S.* Typhimurium LTTRs (Lahiri *et al*., 2009) contain one or more predicted LeuO binding site(s) in their regulatory region. These include the genes encoding TdcA, which is involved in the metabolism of L-serine and L-threonine (Kim *et al*., 2009), and NhaR, which regulates







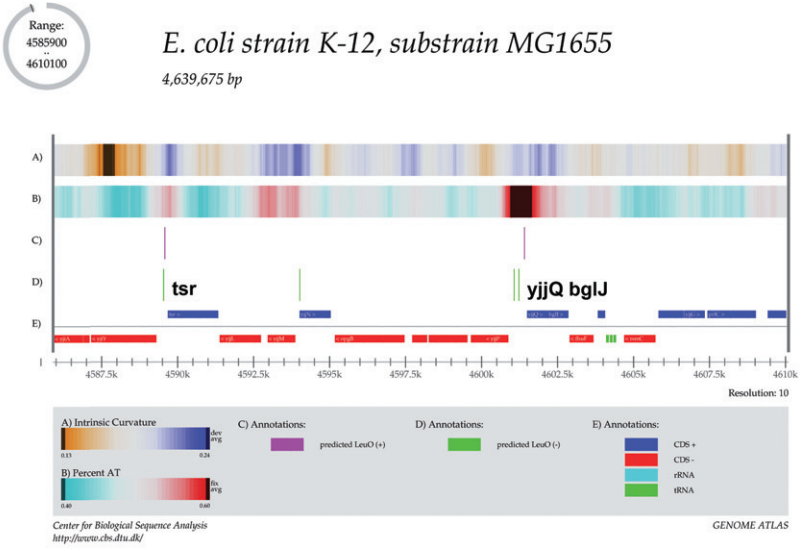


Fig. 5. Predicted LeuO binding sites in the context of genome atlases for the *S.* Typhimurium and *E. coli* chromosomes.
A + B. The locations of predicted LeuO binding sites on both strands of the *S.* Typhimurium SL1344 (A) *and E. coli* MG1655 (B) chromosomes are shown using coloured concentric circles. In addition, we show published H-NS ChIP-chip data from SL1344 grown in LPM together with DNA intrinsic curvature measurements and percentage A + T content (http://www.cbs.dtu.dk/services/GenomeAtlas). The colour code used in each concentric circle is explained in the key at the right of each diagram. Numbers on the inside of the innermost circle are locations relative to position zero measured in millions of base pairs (Mbp).
C + D. A detailed view of a predominantly syntenic region of the *S.* Typhimurium (C) and *E. coli* (D) chromosomes illustrating the similarities and differences of predicted LeuO binding sites in the two species. The locations of predicted LeuO binding sites on the plus and negative strands are indicated by purple and green bars in tracks D and E. The locations of CDS on the plus and negative strands are indicated by blue and red rectangles respectively.

a sodium proton antiporter (Rahav-Manor *et al.*, 1992). These LTTRs and their neighbouring regulatory targets are also repressed by H-NS (Table S2), suggesting a complex regulatory interplay between LeuO, other LTTRs and H-NS. As LTTRs often auto-regulate their own expression (Maddocks and Oyston, 2008), it is possible that LeuO establishes a heterotypic interaction with the corresponding LTTR family member to facilitate this auto-regulation

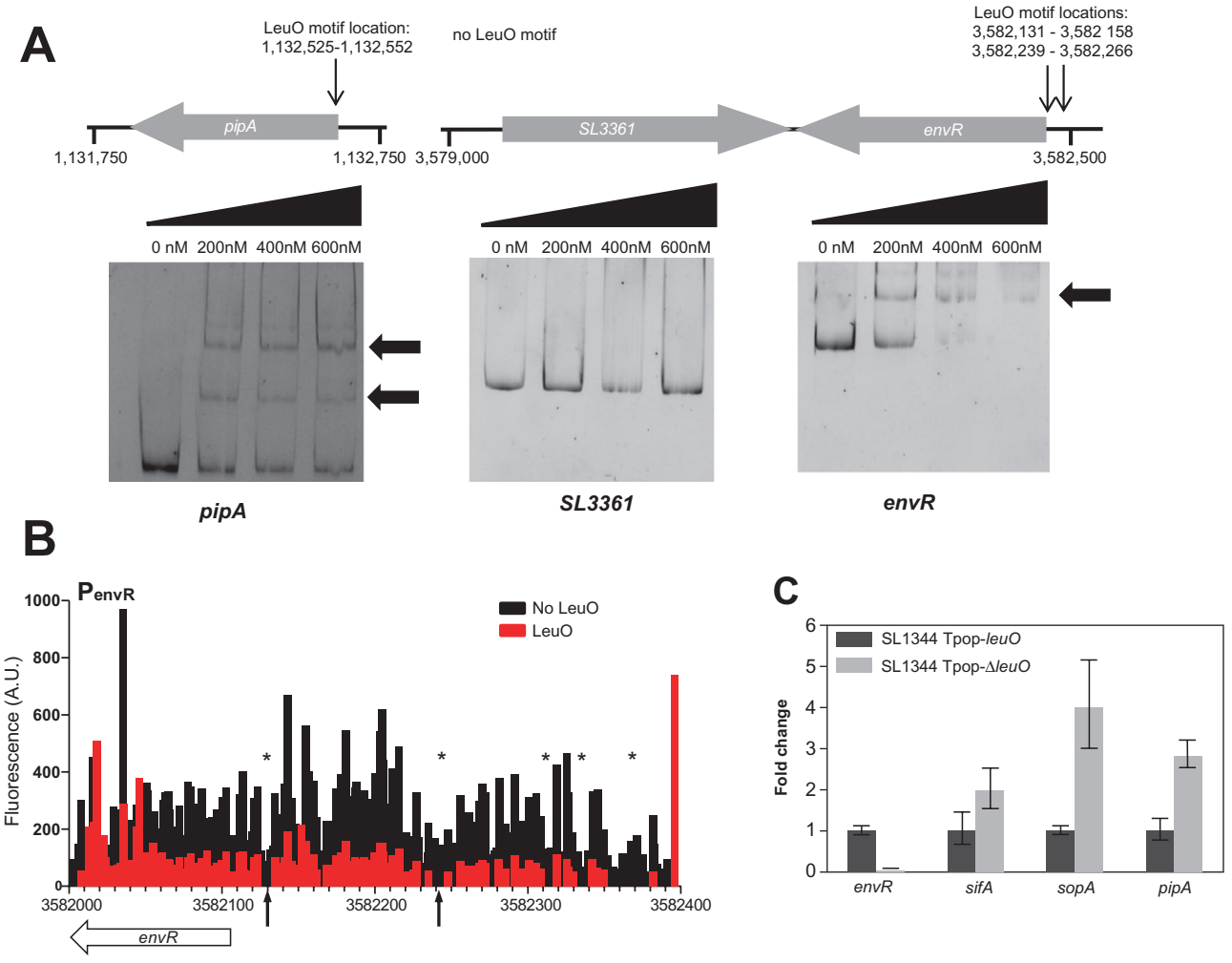


Fig. 6. Validation of predicted LeuO binding sites.
A. EMSA analysis was used to validate predicted LeuO binding sites. *pipA* and *envR* were associated with one and two predicted binding sites respectively (indicated by thin black arrows) while *SL3361* was not associated with a predicted site. DNA probes were incubated with increasing amounts of LeuO and complex formation is indicated by thick black arrows.
B. DNase I footprinting of LeuO binding to P_{envR} using end-labelled DNA fragments. The size and quantity of 6-FAM-labelled DNase I digestion products were measured by fluorescent DNA sequencing with capillary electrophoresis. The locations of predicted LeuO binding sites are indicated by arrows and protected regions are indicated by asterisks. SL1344 chromosome co-ordinates are indicated on the *x*-axis.
C. qRT-PCR was used to monitor transcript levels for *envR, sifA, sopA* and *pipA* following *leuO* overexpression (SL1344 *Tpop-leuO*) and deletion of *leuO* (SL1344 *Tpop-ΔleuO*). Fold changes in transcript levels are expressed relative to strain SL1344 *Tpop-leuO*, which is normalized to 1.

(Knapp and Hu, 2010). The presence of LeuO binding sites at so many LTTR genes shows that LeuO also has the potential to co-ordinate their expression within a LeuO-dependent regulatory network.

Many genes that contain a predicted LeuO binding site were not detected by our ChIP-chip approach. The tetrameric structure of LTTRs permits simultaneous binding to two sites. The nucleoprotein complex upstream of a given target gene can include a variety of distal sites, bridged by the LTTR to a common target-gene-proximal site. Distal site availability may be determined stochastically or may reflect changes in nucleoid structure, dictating which distal sites are likely to be brought into a position adjacent to the target-gene-proximal site such that LeuO-mediated bridging becomes possible. These considerations are not only relevant to a consideration of LeuO as a regulator of transcription, but also apply to its potential role as a nucleoid-structuring element.

Experimental procedures

Bacterial strains and culture conditions

The bacterial strains used in this study were *S.* Typhimurium strain SL1344 (*his,* a histidine auxotroph) (Hoiseth and Stocker, 1981); CJD1034scd (SL1344 *leuO*::3xFLAG); CJD1028scd (SL1344 *hns*::3xFLAG); SL1344 *hns*::*kan*; SL1344 *hns*::*kan leuO*::*cat*, SL1344 pBAD*leuO*::3xFLAG; SV7424 (SL1344 Tpop-*leuO*), SV7425 (SL1344 Tpop-Δ*leuO*. *E. coli* K-12 strain BL21 (plZ1871) was used to overexpress and purify the LeuO protein. *S.* Typhimurium strain SL1344 and its derivatives were grown in 250 ml flasks in LPM (5 mM KCl, 7.5 mM $(NH_4)_2SO_4$, 0.5 mM K_2SO_4, 0.1% casamino acids, 0.34% glycerol, 80 mM MES, after autoclaving 337.5 μM K_2HPO_4/KH_2PO_4 and 10 mM $MgCl_2$ were added, pH 7) or Luria broth at 37°C and at 200 r.p.m. in a C76 water bath shaker (New Brunswick Scientific). Where necessary, antibiotics were used at the following final concentrations: carbenicillin (100 μg ml^{-1}), kanamycin (50 μg ml^{-1}), streptomycin (50 μg ml^{-1}) and chloramphenicol (25 μg ml^{-1}).

Construction of SL1344 derivatives expressing 3xFLAG tagged LeuO and H-NS

The *leuO*::3xFLAG and *hns*::3xFLAG derivatives of *S.* Typhimurium SL1344 were generated using a modified version of the λ Red recombination method (Uzzau *et al.*, 2001). The primers used for these constructions are listed in Table S5. The *leuO*::3xFLAG and *hns*::3xFLAG strains were marked with the kanamycin antibiotic resistance cassette, which was transduced into a clean background using phage P22 HT *int105*. Green agar plates were used to screen for colonies free from phage (Sternberg and Maurer, 1991). The kanamycin resistance cassette was flanked by FRT sites (FLP recombinase recognition targets), which allowed its removal by site-specific recombination using the pCP20 plasmid (Cherepanov and Wackernagel, 1995).

ChIP assay

Cultures of *S.* Typhimurium SL1344, SL1344 *leuO*::3xFLAG, SL1344 *hns*::3xFLAG were inoculated 1:100 into fresh Luria broth or LPM and grown at 37°C until stationary phase. SL1344 pBAD*leuO*::3xFLAG was inoculated 1:100 into fresh Luria broth and grown to exponential phase (OD_{600} ~ 0.2) and then L-arabinose was added to a final concentration of 0.2% for 1 h before harvesting cells. For all experiments 25 ml of culture was harvested and resuspended in 50 ml of PBS. DNA–protein interactions were cross-linked for 30 min by adding formaldehyde (Sigma-Aldrich, catalogue number F8775) to a final concentration of 1%. Glycine was then added to a final concentration of 0.125 M to stop the cross-linking. The ChIP assay was then performed as detailed in Dillon *et al.* (2010). The following antibodies were used in this study: mouse monoclonal to the RNA polymerase β' subunit (Neoclone catalogue number W0001), mouse monoclonal to the FLAG epitope (Sigma-Aldrich, catalogue number F3165) and normal mouse IgG (Millipore, catalogue number 12-371).

Fluorescent labelling of DNA and microarray hybridization

Fluorescent labelling of ChIP DNA samples for microarray experiments was carried out as described in Dillon *et al.* (2010). The microarrays used in this study were designed and produced by Oxford Gene Technology. The microarrays consisted of 43,453 60-mer oligonucleotides tiled throughout the *S.* Typhimurium SL1344 chromosome and pSLT plasmid. Microarrays were hybridized for 24 h in a hybridization oven (Agilent Technologies) and washed according to instructions provided by Oxford Gene Technology.

Microarray data acquisition, analysis and data access

The microarray slides were scanned using an Agilent G2505C scanner. Cy3 and Cy5 images were acquired at 3-micron resolution. Scanned images were analysed using Agilent Feature extraction software. This software package was used to quantify the fluorescent intensities of each spot representing an array element. Background subtracted fluorescence values were reported for each spot in the Cy3 and Cy5 channels and used to calculate a background subtracted Cy3/Cy5 ratios. The baseline levels of each dataset were normalized to a value of one, allowing all experiments to be directly compared from this baseline value. The data centring was performed by calculating the median ratio for each experiment and dividing all the Cy3/Cy5 ratios (obtained in that experiment) by this number. The ChIPOTle algorithm (Buck *et al.*, 2005) was used to define regions of enrichment in ChIP-on-chip datasets by using a sliding window approach. ChIPOTle calculates the average $\log_2$ ratio within each window and the fold cut-off chosen was $\log_{21}$. A window size of 500 bp and a step size of 125 bp were used for analysing the datasets, the rationale being that the ChIP procedure produces DNA fragments of approximately 500 bp in size. The raw ChIP-on-chip datasets have been submitted to the GEO database (Accession number GSE35826).

Quantitative reverse transcriptase PCR (qRT-PCR)

RNA was extracted from *S.* Typhimurium strain SL1344 stationary phase cultures (OD_{600} ~ 2) using the SV total RNA isolation system (Promega, Madison, WI, USA) as described at http://www.ifr.ac.uk/safety/microarrays/protocols.html. The quantity and quality of the extracted RNA were determined using a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). To diminish genomic DNA contamination, the preparation was treated with DNase I (Turbo DNA free, Applied Biosystems/Ambion, Austin, TX, USA). An aliquot of 0.6 μ*g* of DNase-I-treated RNA was used for cDNA synthesis using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). Quantitative RT-PCR reactions were performed in LightCycler 480 II (Roche). Each reaction was carried out in a total volume of 10 μl on a 480-well optical reaction plate (Roche) containing 5 μl SYBR, 0.5 μl DYE II (Takara), 4.6 μl cDNA (1/10 dilution) and two gene-specific primers at a final concentration of 0.2 mM each. Real-time cycling conditions were as follows: (i) 95°C for 10 min and (ii) 40 cycles at 95°C for 15 s, 60°C for 1 min. A non-template control was included for each primer set. Melting curve analysis verified that each reaction contained a single PCR product. Gene expression levels were normalized to transcripts of *rfaH* that served as an internal control. Gene-specific primers were designed with PRIMER3 software (http://primer3.sourceforge.net) and are listed in Table S5.

Purification of LeuO protein

The *leuO* gene was PCR-amplified using oligonucleotide primers pET21-leuO-BamHI and pET21-leuO-SalI (Table S5). The amplification product was digested with BamHI and SalI, and cloned in plasmid pET21a to yield pIZ1871. For LeuO protein purification, *E. coli* BL21 (pIZ1871) was grown in YT broth, and adding 1 mM IPTG induced LeuO expression. After 4 h of induction, cells were centrifuged and resuspended in lysis buffer [20 mM Tris, 300 mM NaCl, 10 mM imidazole, 1 mM PMSF, 5 μl ml^{-1} protein inhibitor cocktail (Sigma Chemical, St Louis, MO, USA), 10% glycerol] and lysed by sonication. The suspension was centrifuged and the supernatant was mixed with Ni-agarose beads (QIA express Type ATG Kit, Qiagen) and incubated overnight with gentle mixing on a rotating wheel at 4°C. Agarose beads were washed with washing buffer (20 mM Tris, 300 mM NaCl, 20 mM imidazole, 10% glycerol). Protein elution was performed with 500 μl of elution buffer (20 mM Tris, 300 mM NaCl, 200 mM imidazole, 10% glycerol). Imidazole was removed by washing with storage buffer (20 mM Tris, 300 mM NaCl, 10% glycerol) and centrifuged using Amicon® Ultra centrifugal filters. LeuO-6xHis protein was stored at −80°C.

Gel mobility shift assays

DNA probes labelled with 6-caroxyfluorescein (6-FAM) were prepared by PCR amplification and primer pairs used are listed in Table S5. PCR products were purified with the Wizard® SV Clean-Up-System (Promega, Madison, WI, USA). For gel shift assays, 50 ng of each FAM-labelled probe was incubated at room temperature for 30 min with increasing concentrations of LeuO-6xHis in a final volume of 20 μl. The binding buffer L10× contained 20 mM HEPES, 100 mM KCl, 2 mM $MgCl_2$, 0,1 mM EDTA and 20% glycerol (De la Cruz *et al.*, 2007). Protein–DNA complexes were subjected to electrophoresis at 4°C in a 6% non-denaturing acrylamide-: bisacrylamide (29:1) gel in 0.5 Tris-borate-EDTA buffer. DNA fragments were visualized with a FLA-5100 Imaging system (Fujifilm, Tokyo, Japan).

DNase I footprinting of LeuO binding to PCR-amplified DNA

The P_{envR} DNA probe labelled with 6-caroxyfluorescein (6-FAM) was prepared by PCR amplification and the primers used are listed in Table S5. DNase I footprinting was performed as described in Cameron and Dorman (2012) with some small changes. The DNase I footprinting reactions were conducted in 15 μl reaction volumes containing 1× DNase I buffer (Roche) (40 mM Tris-HCl, 10 mM NaCl, 6 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.9) 0.01 mM DTT, 100 ng μl^{-1} BSA, 50 nM bait DNA, and 50 nM LeuO-6xHis. LeuO-DNA binding was allowed to equilibrate at 37°C for 20 min, then 1 μl (0.04 units) of pre-warmed DNase I was added and mixed gently, then incubated at 37°C for 5 min. Reactions were stopped by addition of 2 μl EDTA (100 mM) followed by vigorous vortex mixing and heat denaturation at 95°C for 10 min. Digestion products were desalted using MicroSpin G-25 columns (GE Healthcare) and were analysed on an ABI 3730 DNA Analyzer along with GeneScan 500-LIZ size standards (Applied Biosystems).

Western immunoblotting

Total proteins extracts were prepared from bacterial cultures grown at 37°C in LPM or LB medium until stationary phase was reached (OD_{600} ~ 1.4 and OD_{600} ~ 2 respectively). Bacterial cells contained in 1 ml of culture were collected by centrifugation (16 000 *g*, 2 min, 4°C) and suspended in 50 μl of Laemmli sample buffer [1.3% SDS, 10% (v/v) glycerol, 50 mM Tris-HCl, 1.8% β-mercaptoethanol, 0.02% bromophenol blue, pH 6.8]. Proteins were resolved by Tris–Tricine–PAGE, using 12% gels. Conditions for protein transfer have been described elsewhere (Balbontin *et al.*, 2006). Primary antibodies were anti-FLAG M2 monoclonal antibody (1:5000, Sigma Chemical Co, St Louis, MO, USA), anti-DnaK monoclonal antibody (1:5000, MBL International, MA, USA), and anti-GroEL polyclonal antibody (1:10 000, Sigma Chemical Co, St Louis, MO, USA). Goat anti-mouse horseradish peroxidase-conjugated antibody (1:5000, Bio-Rad, Hercules, CA, USA) was used as secondary antibody. Proteins recognized by the antibodies were visualized by chemiluminiscence using luciferin–luminol.

DNA sequence analysis

To test for the presence of over-represented motifs in DNA sequences bound by LeuO in *E. coli* and *S.* Typhimurium, the

SELEX screening (Shimada *et al*., 2011) and ChIPOTle datasets were manually curated to define short binding regions that could be analysed by the Meme motif-finding program. For the SELEX data this involved extracting 500 bp of DNA sequence centred on the genomic co-ordinate presented for each LeuO binding site in Shimada *et al*. (2011). For *S.* Typhimurium this involved selecting the highest-scoring probes from the broader binding regions identified by ChIPOTle and the corresponding DNA sequences were extracted using the Artemis genome viewer (Rutherford *et al*., 2000). The unbiased motif-finding program Meme (Bailey *et al*., 2009) was used to search the curated datasets. Meme parameters were set as follows: motifs could range in size from 10 to 50 bp, each DNA sequence could contain multiple or no motif sites, and both palindromic and non-palindromic motifs could be found. The MAST program (Bailey *et al*., 2009) was used to generate PSSM from the *E. coli* LeuO and *S.* Typhimurium motifs. The PSSMs were used to scan the *E. coli* K-12 MG1655 and *S.* Typhimurium SL1344 genome sequences for matches with an *E*-value < 0.1 and a position *P*-value < 0.0001. The matching sequences are listed in Table S3.

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