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The multiple antibiotic resistance operon of enteric bacteria controls DNA repair and outer membrane integrity

Prateek Sharma1, James R.J. Haycocks1, Alistair D. Middlemiss1, Rachel A. Kettles1, Laura E. Sellars1, Vito Ricci2, Laura J.V. Piddock2 & David C. Grainger1

The multiple antibiotic resistance (mar) operon of *Escherichia coli* is a paradigm for chromosomally encoded antibiotic resistance in enteric bacteria. The locus is recognised for its ability to modulate efflux pump and porin expression via two encoded transcription factors, MarR and MarA. Here we map binding of these regulators across the *E. coli* genome and identify an extensive mar regulon. Most notably, MarA activates expression of genes required for DNA repair and lipid trafficking. Consequently, the mar locus reduces quinolone-induced DNA damage and the ability of tetracyclines to traverse the outer membrane. These previously unrecognised mar pathways reside within a core regulon, shared by most enteric bacteria. Hence, we provide a framework for understanding multidrug resistance, mediated by analogous systems, across the Enterobacteriaceae. Transcription factors MarR and MarA confer multidrug resistance in enteric bacteria by modulating efflux pump and porin expression. Here, Sharma et al. show that MarA also upregulates genes required for lipid trafficking and DNA repair, thus reducing antibiotic entry and quinolone-induced DNA damage.
The Escherichia coli multiple antibiotic resistance (mar) locus was identified as a determinant for cross-resistance to tetracyclines, quinolones and β-lactams. Widespread among enteric bacteria, the system confers clinically relevant antimicrobial resistance in E. coli. The mar phenotype results from induction of an operon designated marRAB. In wild-type cells, marRAB expression is stochastic and enhanced by many antimicrobial compounds. Conversely, MarA and marbox containing promoters. It is likely that Rob behaves and porin production.

Palindromic DNA targets. This DNA sequence, called the ‘marbox’, is conserved in Gram-negative bacteria. Proteins related to MarA, such as SoxS, Rob and RamA, also recognise the marbox and have overlapping regulatory effects. However, interplay between these factors is complex. For example, Rob binds DNA with low specificity, but high affinity, and is usually found in the non-functional state. Conversely, MarA and SoxS have a lower affinity for the marbox but form ternary complexes with RNA polymerase that enhance recognition of marbox-containing promoters. It is likely that Rob behaves differently to other marbox binding proteins because Rob has a multimerisation domain and can interact with DNA using only one of its two helix-turn-helix motifs.

Models that depend on drug efflux and reduced porin production cannot explain all mar phenotypes. For example, substantial resistance to minocycline is retained in ΔmarR cells lacking tolC or acrAB. Such observations have stimulated attempts to define the complete MarA regulon. Transcriptome analyses identified hundreds of genes putatively controlled by MarA. However, only three genes were common to independent studies. Similarly, while 10,000 copies of the marbox occupy the E. coli genome, most are non-functional. Consequently, only a handful of experimentally confirmed MarA binding sites are listed in the Ecocyc database (Supplementary Table 1).

In this work, we have mapped genome-wide DNA binding by both MarR and MarA using chromatin immunoprecipitation and DNA sequencing (ChiP-seq). We show that the mar regulon is extensive and encompasses both DNA repair and lipid trafficking systems. These regulatory events allow the mar locus to combat quinolone-induced DNA damage and penetration of the outer membrane by antimicrobial compounds. Although previously unrecognised, these pathways reside within a core mar regulon shared by many enteric bacteria.

**Results**

**Genome-wide distribution of marRAB-encoded transcription factors.** We used ChiP-seq to map global DNA binding by the marRAB-encoded transcription factors and the RNA polymerase σ70 subunit. Experiments were done using the enterotoxigenic E. coli strain H10407. The strain shares 3766 genes with E. coli K-12 and has 599 additional genes encoded by 25 discrete chromosomal loci and 4 plasmids (p948, p666, p58 and p52). We expected to identify a primary mar regulon, shared by most E. coli species, and additional targets specific to toxigenic strains. The MarA, MarR and σ70 binding profiles of are shown in Fig. 1A. In each plot, genes are illustrated by blue lines (tracks 1 and 2), MarA binding is in green (track 3), σ70 binding is in orange (track 4) and MarR binding is in black (track 5). As expected, MarR bound only to the marRAB promoter while MarA and σ70 bound at many loci. We used MEME to identify sequence motifs associated with the 33 MarA binding peaks. Only one statistically significant (E-value < 1e−12) motif was found (Fig. 1B, top panel). The motif closely resembles known MarA binding sites (Fig. 1B, compare top and bottom panel). For all peaks we determined the distance to the nearest start codon and sorted these distances into 100 bp bins. The distribution of peaks among the bins is illustrated in Fig. 1C; MarA and σ70 most frequently bind the 100 bp preceding the 5’ end of a gene. Of the 33 MarA binding peaks, 15 were within 150 bp of a binding peak for σ70 (Fig. 1C, inset). To support our ChiP-seq analysis, we tested binding of purified MarA to DNA fragments overlapping all 33 peaks. As a control, we also tested five DNA fragments from elsewhere in the genome. All but one of the DNA sequences derived from MarA ChiP-seq peaks bound MarA in vitro (Supplementary Fig. 1A). However, there was no binding to any of the control sequences (Supplementary Fig. 1B). The ChiP-seq and in vitro DNA binding data are summarised in Table 1. Five MarA targets were specific to E. coli H10407. Of these targets, one did not bind MarA in vitro, four were within prophase remnants, and only one was near to the 5’ end of a gene. Note that our list of MarA targets will not include sites occluded by other proteins in vivo. For example, most known marboxes preferentially bind SoxS or Rob; we did not expect to isolate these loci (Supplementary Table 1). Consistent with this, our ChiP-seq identified only the known marboxes with a high affinity for MarA (Supplementary Table 1).

**PxseA is a MarA-activated promoter.** Figure 2A shows co-binding of MarA and RNA polymerase to the xseA locus. The sequence of the regulatory region is in Fig. 2B. The putative marbox (green) is immediately adjacent to the xseA promoter (PxesA; orange). To confirm binding at the marbox, we used electrophoretic mobility shift assays (EMSA) and DNASel footprinting. For the EMSA experiments we used the xseA1 and xseA2 DNA fragments; the 5’ end of each fragment is denoted by an inverted triangle in Fig. 2B. The –36C mutation, present only in xseA2, ensures marbox inactivation. The EMSA experiment is shown in Fig. 2C. As predicted, MarA bound to the xseA1 fragment (top panel) but not the xseA2 fragment (bottom panel). In DNASel footprinting, MarA protects the marbox from digestion and induces hypersensitivity at adjacent sites (Fig. 2D). The xseA1 and xseA2 fragments were also fused to lacZ in the reporter plasmid pRW50. Measurements of β-galactosidase activity suggest a role for the marbox in transcription activation (Fig. 2E). Consistent with this, raising the intracellular concentration of MarA stimulates PxseA only in the presence of the marbox (Supplementary Fig. 3). Note that this approach avoids deletion of marA and the possibility of compensatory regulation by SoxS or Rob.
Fig. 1 Global analysis of MarA and its target genes. a Genome-wide distribution of MarA, MarR and RNA polymerase in enterotoxigenic Escherichia coli strain H10407. Plots are shown for the H10407 chromosome and four plasmids. In each plot, the tick mark at the 12 o’clock position represents the first base pair (bp) of the DNA element. Subsequent tick marks are spaced by 1 Mbp (chromosome) 10 Kbp (p948 and p666) or 1 Kbp (p58 and p52). In each plot, tracks 1 and 2 (blue lines) show the position of genes, track 3 (green) is the MarA binding profile, track 4 (orange) is the RNA polymerase σ70 subunit binding profile and track 5 (black) is the MarR binding profile. b DNA sequence motifs recovered from MarA binding peaks. The top panel shows a DNA sequence motif identified by MEME present in DNA sequences associated with MarA in ChIP-seq analysis. The bottom panel shows a DNA sequence motif generated by aligning experimentally verified MarA binding sites listed in Ecocyc. c Location of MarA and RNA polymerase binding peaks with respect to genes. A histogram depicting the distance between ChIP-seq binding peaks and the nearest 5’ end of a gene; data for MarA binding are in green and data for σ70 binding are in orange. Each binding peak was allocated to a series of 100 bp bins. The inset is a Venn diagram that illustrates the number of MarA and σ70 binding peaks that overlap. d Phenotypic landscape of the MarA regulon. The heatmap illustrates fitness scores29 of strains lacking MarA target genes (y-axis) compared to the wild-type parent strain. Strains were grown in the presence of different antibiotics (x-axis). The antibiotics are clustered according to the cellular process targeted (labelled above heatmap). Drugs are further divided into classes i through x by yellow dotted lines. The classes are as follows: i quinolones, ii non-quinolone topoisomerase inhibitors, iii antifolates, iv macrolides; v aminoglycosides, vi tetracyclines, vii penicillins, viii cephalosporins, ix other cell envelope antibiotics, x miscellaneous. Individual row and column names are provided in Supplementary Fig. 2
** Activation of xseA mediates innate ciprofloxacin resistance.** Consistent with previous observations, the minimum inhibitory concentration (MIC) of ciprofloxacin decreased in cells lacking xseA (Supplementary Table 2). To determine if the ciprofloxacin hypersensitivity phenotype was dependent on both xseA and the marbox, we used genetic complementation. Hence, we constructed derivatives of plasmid pBR322 encoding xseA downstream of P seA. This plasmid was able to rescue growth of an fl oxacin-resistant strain in the presence of ciprofloxacin (Supplementary Fig. 1b). Consistent with this, Nichols et al. also concluded that both components of the xseA determinant required for exonuclease VII function, including the interaction between XseA and XseB, result in hypersensitivity to ciprofloxacin (Supplementary Fig. 5b). Consistent with this, Nichols et al. also concluded that both components of exonuclease VII were required for innate levels of ciprofloxacin resistance. We conclude that XseA acts via its described exonuclease VII activity in our assays.

**Innate ciprofloxacin resistance requires canonical XseA activity.** Exonuclease VII comprises large and small subunits; XseA and XseB, respectively. However, MarA binding was only detected upstream of xseA (Table 1). Hence, XseA could act independently of XseB to increase quinolone tolerance. Briefly, XseA consists of four functional regions: an N-terminal OB-fold, a central catalytic domain, a coiled-coil and a short C-terminal domain (Supplementary Fig. 5a). The OB-fold is responsible for DNA binding, the catalytic domain mediates exonuclease activity, the coiled-coil binds XseB and the C-terminal domain contains three α-helices. To investigate the role of these activities, we introduced point mutations at key positions in xseA encoded by pBR322. The mutations, previously characterised by Poleszak et al. are described in Supplementary Fig. 5a. Inactivation of any xseA determinant required for exonuclease VII function, including the interaction between XseA and XseB, results in hypersensitivity to ciprofloxacin (Supplementary Fig. 5b). Consistent with this, Nichols et al. also concluded that both components of exonuclease VII were required for innate levels of ciprofloxacin resistance. We conclude that XseA acts via its described exonuclease VII activity in our assays.

**Cells lacking MarA-controlled xseA acquire DNA strand breaks.** Exonuclease VII is known to influence DNA repair. Hence, the simplest interpretation of our data is that MarA activates xseA to reduce DNA damage. We tested this prediction by visualising DNA damage in Hoechst-stained E. coli cells. As expected, in the presence of ciprofloxacin, xseA::kan cells were

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**Table 1 MarA binding sites identified by ChIP-seq**

| Chromosomal targets | MEME site centre | Site sequence (5′-3′) | H10407 genes | MG1655 genes | MarA binding in vitro |
|---------------------|----------------|---------------------|--------------|---------------|---------------------|
| 206                 | 161            | gcacagacagataaa     | ETECO001     | thrL          | +++                 |
| 87300               | 87344          | gcacattgtaaat      | ETECO0074<ETECE0075 | leuL<leuO     | +++                 |
| 18421              | 184180         | gcgttatcgttaaat    | ETECO157     | degP          | ++                  |
| 428898             | 428876         | gcataattgtaaat     | ETECO4000    | locZ          | ++                  |
| 529310             | 529338         | gcacaaattgtaaat    | ETECO500     | yboO          | ++                  |
| 655542             | 655557         | gcacattgtaaat      | ETECO604     | phoP          | ++                  |
| 846408             | 846418         | ccacctacagtgaac    | ETEC765<acrZ | modD<acrZ     | +++                 |
| 963300             | 963348         | cctatatcgtgaataaa  | ETEC889      | yboV         | +                   |
| 994036             | 994060         | gcattaattgtaaat    | ETEC9016<ETECE0917 | grxA<ibjC     | +++                 |
| 1354006            | 1354020        | gcattaattgtaaat    | ETEC1264<ETECE1265 | ycgf<ycgZ    | +++                 |
| 1536992            | 1537038        | gcacaaattgtaaat    | ETEC1438     | fmr             | ++                  |
| 1717096            | 1717112        | gcacaaattgtaaat    | ETEC1580     | yneO          | +++                 |
| 1739002            | 1739016        | ccacctacagtgaac    | ETEC1599<ETECE1600 | marC<marR     | +++                 |
| 2321000            | 2321026        | gcacattgtaaat      | ETEC2315     | yef             | ++                  |
| 2538709            | 2538641        | gcacattgtaaat      | ETEC2344<micF | ampc<micF    | ++                  |
| 2727941            | 2727994        | gcacctacagtgaac    | ETEC2509     | ygee           | +++                 |
| 2755444            | 2755459        | gcacaaattgtaaat    | ETEC2533<ETECE2534 | yfsS<ycaM    | +++                 |
| 2887268            | 2887307        | gcacctacagtgaac    | ETEC2665<ETECE2666 | guaB>xxeA | +++                 |
| 3455714            | 3455708        | ccaatcctcgaaac     | ETEC3200     | ETEC specific  | -                   |
| 3569696            | 3569763        | gcacaaattgtaaat    | ETEC3306<ETECE3307 | nudF<tolC    | ++                  |
| 3695690            | 3695710        | gcacaaattgtaaat    | ETEC3426     | (ybV)         | +++                 |
| 3733124            | 3733195        | ccacctacagtgaac    | ETEC3460<ETECE3461 | mla<ylbG     | +++                 |
| 4289772            | 4289787        | gcacaaattgtaaat    | ETEC3977<ETECE3978 | ibpA<ydiQ    | ++                  |
| 4348148            | 4348176        | gcacattgtaaat      | ETEC4032     | mmmG          | ++                  |
| 4499984            | 4495025        | ccacctacagtgaac    | ETEC4151     | (ybHT)        | ++                  |
| 4510208            | 4510184        | gcacctacagtgaac    | ETEC4166     | (yigG)        | ++                  |
| 4685066            | 4685041        | gcacctacagtgaac    | ETEC4304     | ETEC specific  | ++                  |
| 4686377            | 4686370        | ccaacaaactgtaaat  | ETEC4307     | ETEC specific  | ++                  |
| 4737304            | 4737238        | gcacaaattgtaaat    | ETEC4370<ETECE4371 | yjc<yjcC    | ++                  |
| 5066070            | 5066105        | gcacaaattgtaaat    | ETEC4666<ETECE4667 | yji<yjiQ    | +++                 |
| 5093964            | 5093988        | gcacaaattgtaaat    | ETEC4702     | ETEC specific  | ++                  |
| 5132420            | 5132347        | gcacaaattgtaaat    | ETEC4739     | deBo           | ++                  |

**Plasmid p948 targets**

| 65178              | 65159         | gcacctacagtgaac    | ETECp9480770 | ETEC specific  | +++                 |

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* Genome coordinate of MarA ChIP-seq peak centre in H10407. Bold type indicates peaks within 150 bp of a a 67 binding peak
* Genome coordinate of MarA binding site predicted by MEME
* Sequence of MarA binding site predicted by MEME
* Nearest gene to MarA binding site. Some MarA targets were between divergent (>) and convergent (<) genes. Genes in parentheses indicate that the ChIP-seq peak is located within that gene
* E. coli K-12 homologues of ETEC genes in the previous column. E. coli K-12 MarA binding sites, listed in the Ecocyc database, are highlighted according to experimental confirmation (solid line) or prediction (dashed line)
* In vitro binding of purified MarA observed at a concentration of 0.3 μM (+++), 1.0 μM (+++) or 1.7 μM (+) (Supplementary Fig. 1a). Note that five control DNA fragments (cyoD, yboS< yboT, ETEC2117, cyOA and PseA) did not bind MarA at any of these concentrations (Supplementary Fig. 1b)

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filamentous with abnormal nucleoid morphology (Fig. 2g, compare top two panels). Plasmid pBR322 encoding xseA was able to negate this phenotype, while deletion of the P\textsubscript{xseA} marbox blocked complementation (Fig. 2g, bottom two panels). We confirmed that the unusual nucleoid morphology was indicative of DNA fragmentation using pulse field gel electrophoresis (PFGE) (Fig. 2h). Chromosomal DNA ran as a tight band (lanes 1–3) but smearing was evident upon ciprofloxacin treatment of cells lacking xseA (lanes 4, 5). As in the microscopic analysis, genetic complementation required an intact marbox (lanes 6, 7). For direct comparison, lanes 3, 5, 6 and 7 equate to the top through bottom panels in Fig. 2g, respectively.

**Transcription of the *mla* operon is activated by *MarA*.** We next turned our attention to the *mlaFEDCB* locus that also co-binds MarA and $\sigma^70$ (Fig. 3a). We confirmed binding of MarA to the *mlaFEDCB* regulatory DNA using EMSA and DNAse I

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**Figure 2:**

(a) Schematic representation of the *mla* operon and its interaction with MarA and $\sigma^70$. (b) Sequence of the *xseA* promoter showing the binding sites for MarA and $\sigma^70$. (c) Electrophoretic mobility shift assay (EMSA) showing the binding of MarA to the *xseA* promoter. (d) Northern blot analysis of degraded chromosomal DNA. (e) Quantification of mRNA levels using qRT-PCR. (f) Western blot analysis of proteins involved in DNA repair. (g) Fluorescence microscopy of cells treated with and without ciprofloxacin. (h) Diagram showing the size distribution of degraded chromosomal DNA.
footprinting assays (Fig. 3c, d). The experiments were done using the mlaF1 and mlaF2 DNA fragments. The 5’ end of each fragment is marked by an inverted triangle in Fig. 3b. The results are consistent with MarA binding to the predicted marbox (green in Fig. 3b). However, the location of nearby promoters is unknown.

To identify MarA regulated promoters, we used in vitro transcription assays. A DNA fragment containing the mlaFEDCB intergenic region was cloned upstream of the lacZ terminator in plasmid pSR. The resulting plasmid was used as a template for RNA synthesis (Fig. 3e). Note the 108 nt RNAI transcript is derived from the plasmid replication origin and serves as an internal control. Transcripts of 128, 148 and 157 nt in length were also observed (Fig. 3e). These initiate within the mlaFEDCB intergenic DNA at sites denoted by a bent arrow (Fig. 3b). Each messenger RNA start site maps downstream of promoter −10 and −35 elements. We refer to the promoters as mlaP1, mlaP2 and mlaP3 (Fig. 3b). MarA activates mlaP2 by binding adjacent to the −35 hexamer (Fig. 3b, e). Hence, deletion of the marbox reduces transcription derived from the mlaFEDCB intergenic region in lacZ fusion assays (Fig. 3). Similarly, MarA overproduction activated mlaFEDCB only in the presence of the marbox (Supplementary Fig. 3). Inactivation of mlaP1, which overlaps mlaP2 and the marbox, increased the stimulatory effect of the marbox on mlaP2 activity (Supplementary Fig. 6).

Activation of mlaFEDCB mediates innate doxycycline resistance. We quantified an eight-fold reduction in doxycycline MIC for mlaE::kan compared to wild-type cells (Supplementary Table 2). To confirm that doxycycline hypersensitivity was dependent on both mlaFEDCB and the marbox, we used genetic complementation. Thus, we constructed derivatives of plasmid pBR322 carrying mlaFEDCB and the upstream regulatory DNA. This plasmid was able to rescue growth of BW25113 mlaE::kan cells in the presence of doxycycline (Supplementary Fig. 7). As expected, deletion of the marbox had little effect in the absence of doxycycline (Fig. 3g, left). Conversely, in the presence of doxycycline, the marbox was essential for growth (Fig. 2g, right).

Cells lacking mlaFEDCB have defective barrier function. In Gram-negative bacteria, cell surface barrier function relies on outer membrane asymmetry; the inner and outer leaflets comprise phospholipids and lipopolysaccharides, respectively. The ABC transport system encoded by mlaFEDCB removes unwanted phospholipids from the outer leaflet. Hence, mlaFEDCB could enhance drug resistance by improving outer membrane barrier function. Alternatively, ABC transport activity could support drug efflux. To assess these hypotheses, we compared drug accumulation and efflux in wild-type and mlaE::kan cells. Accumulation increased dramatically upon disruption of mlaE (Fig. 3h), but efflux was identical in both strains (Fig. 3i). Hence, our data are consistent with increased cell surface permeability but not defective efflux.

Cells lacking mlaFEDCB have increased surface hydrophobicity. Our model predicts reduced barrier function due to accumulation of phospholipids in the outer leaflet of the outer membrane. This should coincide with increased cell surface hydrophobicity. To test this, we measured partitioning of wild-type and mlaE::kan cells in a two solvent system (aqueous PUM buffer and p-xylene). In this assay, cells with increased surface hydrophobicity migrate to the organic phase. Consequently, turbidity of the aqueous phase is reduced. Consistent with this, mlaE::kan cells were retained in aqueous suspension less efficiently than the parent strain (Fig. 3j). Similar results were obtained in crystal violet adsorption assays; the hydrophobic dye was bound more efficiently by the mlaE::kan strain (Fig. 3k).

SoxS and Rob binding to the xseA and mlaFEDCB promoters. The SoxS and Rob proteins share 42 and 43% sequence identity with MarA across their helix-turn-helix determinants, respectively. Rob usually binds marboxes with a higher affinity than either MarA or SoxS. Indeed, Rob can bind DNA fragments containing no marbox more tightly than MarA and SoxS bind canonical targets. Hence, we next sought to better understand the comparative affinity of MarA, SoxS and Rob for the xseA and mlaFEDCB promoters. In control experiments, we confirmed that Rob had appreciable non-specific DNA binding activity (Supplementary Fig. 9a) and also bound a known marbox with higher affinity than MarA or SoxS (Supplementary Fig. 9b). Interestingly, most well-defined marboxes preferentially bind SoxS rather than MarA (Supplementary Table 1). This was not the case for the xseA or mlaFEDCB promoters, which bound MarA but not SoxS (Supplementary Fig. 10).

The mar regulon is conserved among the Enterobacteriaceae. Overexpression of MarA-like regulators is associated with clinically relevant resistance to quinolones and tetracyclines in many enteric bacteria. Hence, we quantified conservation of MarA targets among the Enterobacteriaceae. The result of the analysis is illustrated in Fig. 4. As expected, MarA binding sites associated with efflux systems and porin expression were found in most species. The xseA marbox was similarly distributed. However, the best conserved marbox occurred upstream of mlaFEDCB; this...
sequence was present in all but the most divergent *Cedecea neteri* genome. Overall, the *E. coli* MarA regulon is best conserved among *Escherichia* spp. and *Shigella* spp. However, a core regulon is shared by many Enterobacteriaceae. For example, *Salmonella*, *Citrobacter*, *Klebsiella*, *Enterobacter* and *Raoultella* spp. share a similar subset of MarA target genes including *tolC*, *micF*, *xseA* and *mlaFEDCB*.

**Discussion**

MarA-like proteins are frequently implicated in the development of clinical resistance to quinolone and tetracycline family antibiotics.\(^{40, 41}\) For example, constitutive MarA production reduces the rate of killing by norfloxacin and ofloxacin in *E. coli*.\(^{42}\) This provides an opportunity for subsequent beneficial mutations to arise.\(^{4, 40}\) Of the MarA target genes, we identified
loss of xseA, rather than tolC, had the biggest impact on quinolone activity (Fig. 1d). Hence, we demonstrate a direct molecular link between MarA expression and reduced DNA damage (Fig. 2). This overlooked feature of the mar system in E. coli is likely to be widespread; the position and sequence of the marbox at PseA is conserved in many enteric bacteria (Fig. 4). Deregulation of the mar operon is also associated with evolution of resistance to tetracyclines43. Indeed, clinically relevant levels of tetracycline resistance have been attributed solely to overproduction of MarA in E. coli44. Interestingly, substantial resistance to some tetracycline family antibiotics is retained in marR mutants lacking tolC or acrAB25. Hence, the mar regulon must encode other determinants for drug resistance. Our data show that the mlaFEDCB operon is of particular importance; the locus couples MarA to the control of lipid trafficking and outer membrane integrity (Figs. 1, 3). We note that tetracycline family antibiotics vary in their properties; doxycycline and minocycline

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Klebsiella pneumoniae

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MarA controls outer membrane barrier function via activation of the mlaFEDCB operon. a ChIP-seq data for MarA and σ70 binding at mlaFEDCB. Data are smoothed in a 100 bp window. b DNA sequence upstream of mlaF (start codon in blue). DNA elements are labelled and arrows indicate orientation. Transcription start sites identified in vitro are highlighted by bent arrows. The 5‘-end of the mlaF1 and mlaF2 DNA fragments are indicated by inverted black triangles. c Electrophoretic mobility shift assays with the mlaF1 fragment (+marbox) and the mlaF2 fragment (~marbox). Concentrations of MarA are 0.3, 1.0 and 1.7 μM. d DNASEI footprinting experiment, using the mlaF1 DNA fragment, calibrated with a Maxam–Gilbert GA sequencing ladder. Positions relative to the xseA transcription start site (+1) are labelled. Concentrations of MarA are 0.3, 1.0, 1.7, 2.4 and 3.3 μM. e Results of in vitro transcription assays with the mlaF1 DNA fragment cloned in plasmid pSR. The gel is calibrated with a GA sequencing ladder. The RNAII transcript, derived from the pSR promoter, acts as an internal control. Concentrations of MarA are 0.3 or 1.0 μM. f G-galactosidase assay with lysates of JC5837 transformed with a reporter plasmid where lacZ is controlled by either mlaF1 (~marbox) or mlaF2 (~marbox). Error bars show standard deviation (n = 3). g The graph shows OD600 values for cultures of strain BW25113 mlaE::kan grown with or without 1.0 μg/ml doxycycline. The BW25113 mlaE::kan cells were transformed with pBR322 derivatives encoding mlaFEDCB under the control of either the mlaF1 fragment (~marbox) or the mlaF2 fragment (~marbox). Error bars show standard deviation (n = 3). h, i Accumulation of doxycycline or efflux of ethidium bromide as a function of time for BW25113 (solid line) or the mlaE::kan derivative (dashed line). j Percentage absorbance of the aqueous phase at equilibrium after mixing with p-xylene. Data are for BW25113 (solid line) and the mlaE::kan derivative (dashed line). k The graph shows crystal violet adsorption by BW25113 (solid bar) or the mlaE::kan derivative (open bar). Data are normalised relative to BW25113 cells. Error bars show standard deviation (n = 3).
To determine the distance between peak centres for σ70 and MarA, we used the fetch closest non-overlapping feature tool in Galaxy57. Peaks for σ70 and MarA described as overlapping were those centred within 150 bp of each other.

**Phylogenetic analysis of the marbox.** Excluding *E. coli* species, we used BLASTp to search for genomes encoding MarA. We manually removed genomes encoding MarA with changes in the amino-acid sequence of either DNA recognition helix; this is indicative of altered DNA binding specificity. Many of the resulting 161 genes were derived from closely related strains of the same species. These were omitted so that 29 representative genomes remained. Phylogeny was determined using the sequence of the polA gene from each organism and BLASTn pairwise alignments. We used BLASTn to search the 29 genome sequences for a match to the 201 bp DNA sequence derived from each MarA ChIP-seq peak. If multiple hits were obtained from a single genome, only the best match was used. If required, pairwise alignments were optimised manually to remove alignment gaps within marbox sequences. If no sequence match was identified, this was scored as ‘region absent’. An identified marbox was scored as ‘conserved’ if it matched either the equivalent *E. coli* sequence, or the consensus marbox (5′-gcactaattgctaa-3′) in at least 14 of the 15 possible positions. Sites were scored as ‘conserved with mismatches’ if the above criteria were satisfied at 13 of the 15 marbox positions. Sequences falling below this threshold we scored as ‘not conserved’.

**Identification of H10407 MarA targets shared with K-12.** To locate regions of the *E. coli* K-12 genome equivalent to those in strain H10407, we used BLASTn. We compared the sequence of the marbox, intergenic region (defined as 200 bp upstream of the target gene or entire region between convergent genes) and target gene. Of the 28 shared marboxes, all but 6 were within identical intergenic regions. Of the six intergenic regions that differed, four contained a single base change and two had differences in just two positions. None of the intergenic regions contained insertions or deletions, consistent with identical juxtaposition of promoter elements and regulator binding sites between strains. Target genes were a minimum of 97% identical at the nucleotide level and 13 genes had 100% identity.

**Proteins.** Purified MarA, SoxS and Rob were a gift from Lee Rosner and Bob Martin (NIH, Bethesda). RNA polymerase was purified using a method derived from Burgess and Jendrisak58. Briefly, *E. coli* strain MG1655 was grown overnight in 31 of LB broth. Cells were harvested by centrifugation and resuspended in 100 ml of lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM MgCl2, 0.1 mM DTT). One protease inhibitor cocktail tablet (Roche) was added per 20 ml of buffer. Cell lysis and DNA shearing was done using 4 × 30 s pulses, at 20% output, with a Misonix XL2020 tip sonicator. Lysates were cleared by centrifugation at 13,000 × g for 45 min at 4 °C. Following filtration (0.45 μm filter) Polynin P and ammonium sulphate precipitation were done as described in Burgess and Jendrisak58. Precipitated protein was resuspended in TGED buffer (10 mM Tris-HCl pH 7.9, 5 mM magnesium, 1 mM EDTA and 0.1 mM DTT) containing 100 mM NaCl and passed through a HiPrep Heparin FF column (GE Healthcare). The column was washed with 0.1 M NaCl TGED and RNA polymerase was eluted in TGED using a gradient to 1 M NaCl. RNA polymerase containing fractions were pooled and protein precipitated using ammonium sulphate. After resuspension in TGED, RNA polymerase was further purified using a Mono Q HR column (GE Healthcare). Column washing and protein elution were as described in the previous step. RNA polymerase containing fractions were pooled and dialysed against −80 °C storage buffer (TGED, 0.1 M NaCl, 50% glycerol).

**DNA binding and in vitro transcription assays.** For EMSA experiments, DNA fragments were prepared using PCR as described by Shimada et al.66 with oligonucleotides listed in Supplementary Table 3. Protein binding and subsequent electrophoresis were done as described by Chintakayala et al.60 For footprinting experiments, DNA fragments were prepared as described by Grainger et al.61 Protein binding, DNA digestion and electrophoresis were done as described by Singh and Grainger82. Briefly, DNA fragments were labelled at one end using [γ-32P]-ATP and T4 polynucleotide kinase and used at a final concentration of ~10 nM in footprinting reactions. All reactions contained excess of herring sperm DNA (12.5 μg/ml) as a non-specific competitor. Our in vitro transcription assays were done as described by Haycock et al.55 Briefly, supercoiled pSR plasmid carrying promoter inserts (16 μg/ml) was pre-incubated with MarA in buffer containing 20
mM Tris pH 7.9, 5 mM MgCl₂, 500 µM DTT, 50 mM KCl, 100 µg/mL BSA, 200 µM ATP, 200 µM GTP, 200 µM CTP, 10 µM UTP and 5 µg [α-32P]-UTP. Purified ϕC31 RNA was added to start reactions. DNAasel digested DNA and in vitro generated transcripts were analysed on 6% DNA sequencing gels (molecular dynamics). The results were visualised using a Fuji phosphor screen and Bio-Rad Molecular Imager FX. Raw gel images are in Supplementary Fig. 11.

β-galactosidase assays. β-galactosidase assays were done as described previously12,16 using the yeast protocol of Miller64. All assay values are the mean of three independent experiments with a standard deviation equivalent to <10% of the mean β-galactosidase activity. Cells were grown aerobically at 37°C to mid-log phase in LB medium unless stated otherwise.

Growth assays and MIC determination. A single colony of each bacterial strain was used to inoculate 5 ml of LB broth that was incubated overnight at 37°C. The OD₆₀₀ was recorded so that equivalent OD₆₀₀ units could be used for sub-culturing each strain in fresh LB medium. The sub-cultures were then placed in a shaking incubator at 37°C and, at 40 min intervals, 200 µl was transferred to a 96-well plate to measure OD₆₀₀ units. We used the MIC broth microtitre double dilution method to assess antibiotic sensitivity30,36. These assays were done in a final volume of 100 µl of LB medium in a 96 well round bottomed microtitre plate. Each well contained 10 colony forming units of E. coli, 100 µl of LB medium and antibiotics as required. The microtitre plate was covered with a sterile lid and kept in a gently shaking incubator for 24 h at 37°C. The MIC was the lowest antibiotic concentration that prevented bacterial growth. Results were only accepted if the observed MIC for the control NCTC E. coli 10418 and ATCC E. coli 25922 strains was within one doubling dilution of the expected result.

Microscopy. Cells were grown in 1.5 ml of LB medium in the presence or absence of 0.005 µg/ml ciprofloxacin for 36 h at 37°C. Cells were harvested by centrifugation and washed three times with PBS (140 mM NaCl, 2 mM KCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄). After washing, cells were resuspended in 10 µl of PBS containing Hoechst 33258 (5 µM poly-L-lysine) at a concentration of 2 × 10⁶ cells/ml. The suspension was incubated at 25°C for 10 min and mixed with an equal volume of PIV at a concentration of 0.005 µg/ml poly-L-lysine (10 mg/ml; Sigma) onto the centre of a slide. After drying, the slide was coated with 5 µl of the cells suspension and a cover slip applied. The slides containing the cells were imaged using a Nikon Eclipse 90i microscope containing a Nikon Eclipse C2 + ORCA ER camera (pixel size 6.45 µm) and Nikon Plan Apo VC ×100 Oil immersion lens (Numerical Aperture 1.4). The magnification was ×100 and a DAPI filter set allowed detection of Hoechst 33258-stained nucleoids. Exposure time was 90 ms, the excitation filter range was 340–380 nm and barrier filter range was 435–485 nm. Microscopy was done at room temperature and slides were imaged within 30 min of preparation. Images were analysed using the Nikon’s NIS elements software.

Pulse field gel electrophoresis. Preparation of DNA for PFGE was based on the method described by Heath et al.66 Overnight cultures of BW25113 and derivative strains were grown in presence or absence of 0.005 µg/ml ciprofloxacin and 40% (v/v) glycerol and left at room temperature for 10 min. Microscope slides were prepared by spreading 5 µl poly-L-lysine (10 mg/ml; Sigma) onto the centre of a slide. After drying, the slide was coated with 5 µl of the cells suspension and a cover slip applied. The slides containing the cells were imaged using a Nikon Eclipse 90i microscope containing a Nikon Eclipse C2 + ORCA ER camera (pixel size 6.45 µm) and Nikon Plan Apo VC ×100 Oil immersion lens (Numerical Aperture 1.4). The magnification was ×100 and a DAPI filter set allowed detection of Hoechst 33258-stained nucleoids. Exposure time was 90 ms, the excitation filter range was 340–380 nm and barrier filter range was 435–485 nm. Microscopy was done at room temperature and slides were imaged within 30 min of preparation. Images were analysed using the Nikon’s NIS elements software.

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Author contributions

P.S., A.D.M. and R.A.K. did the experimental work. J.R.J.H., L.E.S., V.R. and L.J.V.P. provided support with experimental work and participated in discussion. D.C.G. conceived the study and wrote the paper with input from all co-authors.

Additional information

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