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Kettles, Rachel A; Tschowri, Natalia; Lyons, Kevin J; Sharma, Prateek; Hengge, Regine; Webber, Mark A; Grainger, David C

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The Escherichia coli MarA protein regulates the ycgZ-ymgABC operon to inhibit biofilm formation

Rachel A. Kettes,1 Natalia Tschowri,2 Kevin J. Lyons,1 Prateek Sharma,1 Regine Hengge,2 Mark A. Webber3 and David C. Grainger 1,4*
1 School of Biosciences, Institute of Microbiology and Infection, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK.
2 Institut für Biologie/Mikrobiologie, Humboldt-Universität zu Berlin, 10115 Berlin, Germany.
3 Quadram Institute Bioscience, Norwich Research Park, Norwich NR4 7UQ, UK.

Summary

The Escherichia coli marRAB operon is a paradigm for chromosomally encoded antibiotic resistance. The operon exerts its effect via an encoded transcription factor called MarA that modulates efflux pump and porin expression. In this work, we show that MarA is also a regulator of biofilm formation. Control is mediated by binding of MarA to the intergenic region upstream of the ycgZ-ymgABC operon. The operon, known to influence the formation of curli fibres and colanic acid, is usually expressed during periods of starvation. Hence, the ycgZ-ymgABC promoter is recognised by σ38 (RpoS)-associated RNA polymerase (RNAP). Surprisingly, MarA does not influence σ38-dependent transcription. Instead, MarA drives transcription by the housekeeping σ70-associated RNAP. The effects of MarA on ycgZ-ymgABC expression are coupled with biofilm formation by the rcsCDB phosphorelay system, with YcgZ, YmgA and YmgB forming a complex that directly interacts with the histidine kinase domain of RcsC.

Introduction

The Escherichia coli multiple antibiotic resistance (mar) locus was discovered as a genetic element providing resistance to tetracycline (George and Levy, 1983). The region encodes an operon designated marRAB and also provides resistance to quinolones, β-lactams and a range of phenolic compounds (George and Levy, 1983; Ariza et al., 1994; White et al., 1997). Usually transcribed stochastically, constitutive marRAB expression can result from mutation (Cohen et al., 1993; Ariza et al., 1994; El-Meouche et al., 2016). Hence, clinical levels of drug resistance are associated with the inactivation of marR that encodes an auto repressor (Cohen et al., 1993; Ariza et al., 1994). Salicylic acid, and related phenolic molecules can also reduce repression by altering the conformation of MarR (Duval et al., 2013; Hao et al., 2014). The ability of the operon to provide resistance against antimicrobial compounds is dependent on marA that encodes a transcriptional activator (Ariza et al., 1994; Rhe et al., 1998). MarA plays an important role in drug resistance by activating the expression of the acrAB-toIC encoded efflux pump (White et al., 1997; Zhang et al., 2008).

Many bacterial transcription factors act as dimers at palindromic DNA sequences (Robison et al., 1998; Aravind et al., 2005). In contrast, MarA binds to its DNA site, the marbox, as a monomer (Rhee et al., 1998). Hence, MarA–DNA complexes are asymmetrical with defined orientation (Martin et al., 1999). Promoters regulated by MarA can be divided into two classes. At class I promoters the marbox (5′-GCAHWWWWTGYYAAA-3′) is usually in the reverse orientation and located between ~50 and 70 base pairs (bp) upstream of the transcription start site (Martin et al., 1999). Consequently, MarA contacts the RNA polymerase (RNAP) α subunit C-terminal domain (αCTD) to activate transcription (Martin et al., 1999). This interaction requires a surface of MarA comprising residues D18, W19, D22 and R36 (Dangi et al., 2004). At class II promoters, the marbox is in the forward orientation and overlaps the promoter ~35 element (Martin et al., 1999). Hence, a contact with region 4 of the RNAP σ subunit may be involved (Zafar et al., 2011). In recent work, we identified more than 30 transcription units directly targeted by MarA (Sharma et al., 2017). A current aim is to understand the regulation and physiological functions of these targets.

Biofilms are populations of bacterial cells coalesced within a complex matrix of DNA, proteins and polysaccharides (Hall-Stoodley et al., 2004; Flemming, et al., 2016). As well as being structural, the matrix helps to

*For correspondence. Email d.grainger@bham.ac.uk; Tel. +44 (0)121 414 5437.

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protect cells from damage (Hall-Stoodley et al., 2004; Flemming, et al., 2016). Hence, biofilms may permit cell survival upon antibiotic treatment (Stewart and Costerton, 2001). In E. coli, the ability to form biofilms is regulated by the second messenger cyclic-di-GMP (Simm et al., 2004). The downstream signalling pathway enhances the expression of a transcriptional activator called CsgD (Hammar et al., 1995; Weber et al., 2006). Subsequently, curli fibres are produced (Hammar et al., 1995). These amyloid fibres facilitate surface adhesion, cell aggregation and are a major component of the biofilm matrix (Serra et al., 2013; Hobley et al., 2015). Curli expression can be inhibited by products of the ycgZ-ymgABC operon. Briefly, these proteins induce the rcsCDB-encoded phosphorelay system that reduces the levels of CsgD via the RprA sRNA (Tschowri et al., 2009; Mika et al., 2012; Tschowri et al., 2012). In this work, we showed that, in addition to controlling the expression of efflux pumps, MarA directly activates the ycgZ-ymgABC operon and so represses the formation of curli fibres and biofilms. Activation of ycgZ-ymgABC proceeds via a class I mechanism whereby MarA binds to the 62 bp upstream of the ycgZ-ymgABC promoter. Unusually, for class I promoters, the marbox is in the forward orientation and this is essential for activation. Stimulation of ycgZ-ymgABC by MarA is σ factor specific. Hence, MarA drives transcription by RNAP associated with σ32 but not σ38. Consistent with regulation via the RcsCDB system, we show that rcsB is required for the effects of MarA on biofilm production mediated by ycgZ-ymgABC. We also show that YcgZ, YmgA and YmgB form a complex that directly interacts with the histidine kinase (HK) domain of RcsC, presumably altering its phosphorylation state.

Results

MarA binds to a specific target site at the ycgZ-ymgABC promoter

Previously, we used chromatin immunoprecipitation (ChIP) coupled with sequencing (ChIP-seq) to map MarA binding across the E. coli genome (Sharma et al., 2017). Locations bound by MarA included the intergenic region upstream of the ycgZ-ymgABC operon. Figure 1A shows the ChIP-seq data for MarA binding, the DNA sequence of the intergenic region, and the predicted marbox. Our first aim was to determine if MarA bound at the proposed site. Hence, we generated a 119 bp DNA fragment corresponding to

![Fig. 1. Binding of MarA to the ycgZ-ymgABC intergenic region.](image_url)

A. ChIP-seq data for MarA binding at the ycgZ-ymgABC locus. Genes are shown as blue arrows and the ChIP-seq data for MarA binding is in green (ArrayExpress accession number E-MTAB-5521). The sequence of the intergenic region, corresponding to the ycgZ.1 DNA fragment, is shown below the ChIP-seq profile. The sequence of the predicted marbox is in green and the centre of the ChIP-seq peak for MarA is denoted by an asterisk. The ycgZ-ymgABC transcription start site is indicated by a bent arrow and the promoter extended −10 and −35 elements are underlined. Bases in italic are important for conferring recognition by σ32. Mutations introduced in the ycgZ.1m DNA fragment are shown above the wild type DNA sequence in red. The 5' ends of the ycgZ.1 and ycgZ.2 DNA fragments are indicated by inverted triangles.

B. Binding of MarA to the ycgZ-ymgABC intergenic region in vitro requires the predicted marbox. The results of electrophoretic mobility shift assays are shown for different derivatives of the ycgZ-ymgABC intergenic region. Where present, MarA was used at concentrations of 0.4, 1.2, or 2.0 μM.
the sequence in Fig. 1A. This DNA fragment was named ycgZ.1. We also prepared mutated (ycgZ.1m) and truncated (ycgZ.2) derivatives. The mutations and site of truncation, both predicted to abolish MarA binding, are indicated alongside the ycgZ.1 DNA sequence in Fig. 1A. The ability of MarA to bind each of the DNA fragments was tested in vitro using electrophoretic mobility shift assays (Fig. 1B). As expected, MarA bound to the ycgZ.1 DNA fragment. However, MarA did not bind to ycgZ.1m or ycgZ.2. Hence, MarA binds to the predicted site 62 bp upstream of the ycgZ-ymgABC transcription start site.

The ycgZ-ymgABC promoter is recognised by σ70- and σ38-associated RNA polymerase in vitro

The ycgZ-ymgABC operon is transcribed from a single promoter denoted PycgZ-ymgABC (Tschowri et al., 2012) (Fig. 1A). Previous work noted reduced PycgZ-ymgABC activity in cells lacking σ38, the alternative RNAP sigma factor from starved cells (Tschowri et al., 2009). Consistent with this, PycgZ-ymgABC exhibits features that specifically enhance σ38-mediated transcription (bases italicised in Fig. 1) (Typas et al., 2007a). We and others have previously shown that promoters recognised by σ38 can also be targets for the housekeeping σ70 factor (Ttypas et al., 2007b; Grainger et al., 2008; Singh et al., 2011). Furthermore, even when utilising the same promoter, the two σ factors may respond differently to adjacently bound regulatory proteins (Colland et al., 2000; Germer et al., 2001; Grainger et al., 2008; Singh et al., 2011). To understand the ability of each RNAP derivative to utilise the ycgZ-ymgABC promoter we used in vitro transcription assays. To facilitate this, the 119 bp ycgZ.1 DNA fragment was cloned in plasmid pSR upstream of the λ oop transcription termination signal. Hence, transcripts generated from the ycgZ-ymgABC promoter are 128 nt in length and can be detected following electrophoresis. The result of the experiment is shown in Fig. 2A. The smaller RNAI transcript originates from the plasmid replication origin and serves as an internal control. As expected, σ38-associated RNAP stimulated transcription from the ycgZ-ymgABC promoter (lane 1). An identically sized transcript was produced by the σ70-associated RNAP but with 4-fold lower efficiency (lane 8). Hence, the ycgZ-ymgABC promoter can be recognised by both RNAP derivatives.

Binding of MarA at the ycgZ-ymgABC promoter stimulates transcription by σ70-associated RNA polymerase in vitro

We next sought to understand if MarA could alter transcription from the ycgZ-ymgABC promoter. Hence, we added increasing concentrations of MarA to our in vitro transcription incubations. For reactions with σ70, there was no detectable change in transcription at any of the MarA concentrations tested (Fig. 2B, lanes 1–4). Conversely, σ70-dependent transcription increased 3-fold in the presence of MarA (Fig. 2B, lanes 8–11). In equivalent experiments, using the ycgZ.1m or ycgZ.2 DNA sequences, the loss of the marbox prevented activation by MarA (Fig. 2B).

Stimulation of σ70-dependent transcription at the ycgZ-ymgABC promoter requires MarA side chain W19

The position of the ycgZ-ymgABC marbox suggests activation by a contact with the RNAP α CTD. Previously, Dangi and co-workers (2004) identified a surface of MarA, including key amino acid residue W19, which mediates α CTD interactions. Hence, we purified MarAW19A and tested its ability to stimulate transcription from PycgZ-ymgABC. The data are shown in Fig. 2A. As expected, there was no effect on the MarA independent transcription driven by σ38-associated RNAP (lanes 5–7). Conversely, stimulation of σ70-dependent transcription by MarA required residue W19 (compare lanes 8–11 with 12–14). Taken together, the position of the MarA binding site, and role of residue W19, are consistent with PycgZ-ymgABC stimulation involving a MarA contact with α CTD.

Marbox position is important for σ70-dependent activation of the ycgZ-ymgABC promoter

The forward orientation of the marbox at the ycgZ-ymgABC regulatory region is unexpected; all other class I MarA activated promoters contain a marbox in the reverse orientation (Martin et al., 1999). The only exception is the zwf promoter where the marbox is positioned unusually close to the promoter −35 element (Martin et al., 1999). The only exception is the zwf promoter where the marbox is positioned unusually close to the promoter −35 element (Martin et al., 1999). Hence, we next sought to understand the importance of marbox orientation and position upstream of PycgZ-ymgABC. To do this, we created a series of ycgZ.1 derivatives in plasmid pSR. The full DNA sequences are shown in Fig. S1 and schematic illustrations are in Fig. 3A. In each case, either the position or orientation of the marbox was been altered (Fig. 3A). The consequences were measured using in vitro transcription assays (Fig. 3B). Whereas σ70-dependent transcription from the starting ycgZ.1 fragment was enhanced by MarA (Fig. 3B, lanes 1–5), MarA could not stimulate transcription when the marbox was in the reverse orientation (lanes 6–10). Activation by MarA was also abolished when the marbox was moved upstream by 1 bp (lanes 11–15), 5 bp (lanes 16–20) or 10 bp (lanes 21–25). Positioning the marbox closer to the promoter was better tolerated. Thus, activation was observed when the marbox was −61 (lanes 26–30) or −52 (lanes 36–40) bp upstream of the transcription start site. Moving the marbox 5 bp closer to PycgZ-ymgABC was
MarA activates σ^38-dependent transcription from a ycgZ-ymgABC promoter derivative with a repositioned marbox.

Interestingly, the ycgZ-ymgABC promoter has a distal (i.e. not abutting the −35 hexamer) UP-element half-site (Fig. 1A). Previously, we showed that RNAP associated with σ^38 is able to utilise such sequences. Conversely, σ^70 containing holoenzyme is often defective at such promoters (Typas and Hengge, 2005). The PycgZ-ymgABC promoter derivatives described above have different UP-element configurations (Fig. 3A). Hence, we also measured the ability of σ^38 bound RNAP to utilise the variants (Fig. 3C).

Inverting or moving the marbox further upstream left the UP-element half-site intact. These changes had little impact on σ^38-dependent transcription (Fig. 3C, lanes 1–30). Moving the marbox 5 bp closer to the −35 element simultaneously deleted 5 bp of UP-element DNA. This promoter derivative was poorly able to drive transcription by σ^38 holoenzyme (lanes 31–35). Strikingly, when the marbox was positioned 10 bp further downstream, replacing the UP-element half-site, σ^38-dependent transcription was reduced (lane 36) but could be stimulated ~4-fold by MarA (lanes 36–40).

The ycgZ-ymgABC promoter marbox is required for maximal activity in vivo

To understand the role that MarA might play in controlling ycgZ-ymgABC transcription in vivo we fused various...
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PyCGZ-ymgABC DNA fragments to lacZ in the reporter plasmid pRW50. The plasmid constructs were used to transform E. coli Δlac strain JCB387 and cells were grown to either mid-log phase or stationary phase in Luria Broth. The cells were then lysed and β-galactosidase activities determined using the lysates. The data are shown in Fig. 4A. In the presence of the marbox, β-galactosidase activities were similar for growing and stationary phase

Fig. 3. Spacing and orientation requirements for activation of the ycgZ-ymgABC promoter by MarA.

A. The schematics show the ycgZ.1 DNA fragment and derivatives. The marbox is shown as a green arrow to depict orientation. The centre of the marbox with respect to the transcription start site (black bent arrow) is indicated. Promoter elements are in grey and labelled. Note that the UP-element half-site is labelled ‘UP’ for brevity.

B. Activation of transcription from different derivatives of ycgZ.1 by σ70-associated RNA polymerase. The data are images of gels used to separate products from in vitro transcription assays. The DNA template was plasmid pSR carrying the different ycgZ.1 derivatives. RNA polymerase was used at a concentration of 0.4 μM and MarA was used at concentrations of 0.4, 0.8, 1.2, or 2.0 μM.

C. Activation of transcription from different derivatives of ycgZ.1 by σ38-associated RNA polymerase. Data are otherwise as described for panel B.
cells (solid green bars). Deletion (open bars) or mutation (striped bars) of the marbox caused larger decreases in transcription for growing cells compared to starved cells (Fig. 4A). Recall that our in vitro transcription assays showed stimulation of $\sigma^{70}$- but not $\sigma^{38}$-dependent transcription from $P_{ycgZ-ymgABC}$ by MarA (Fig. 2A). Hence, the reduced requirement for the marbox in starved cells is probably due to an increase in $\sigma^{38}$-dependent transcription.

The $ycgZ$-$ymgABC$ promoter marbox is required for induction by salicylic acid in vivo

Promoters activated by MarA can be induced with sodium salicylate (Duval et al., 2013) because salicylic acid relieves repression of the marRAB operon by MarR (Duval et al., 2013). We reasoned that transcription from the $ycgZ$-$ymgABC$ promoter should increase in the presence of salicylic acid. Furthermore, any such increase should require the marbox. Hence, we repeated our measurements of $ycgZ$-$ymgABC$ promoter activity in growing cells with or without the addition of 5 mM sodium salicylate (Fig. 4B). As expected, $ycgZ$-$ymgABC$ transcription increased upon the addition of sodium salicylate (compare solid bars). Conversely, little or no increase was observed when the marbox was deleted (open bars) or changed by mutation (striped bars).

Curl fibre formation is inhibited by the $ycgZ$-$ymgABC$ operon in a marbox dependent manner

The $ycgZ$-$ymgABC$ operon inhibits the formation of biofilms by indirectly reducing the formation of curli fibres (Tschowri et al., 2009; Mika et al., 2012; Tschowri et al., 2012). Briefly, expression of $ycgZ$-$ymgABC$ ultimately reduces the abundance of CsgD; a positive regulator of curli production. To understand the role of MarA, we made derivatives of plasmid pBR322Δbla. These DNA constructs encoded $ycgZ$-$ymgABC$ under the control of its own promoter and the upstream marbox. We also made variants of the plasmid where the marbox was mutated or deleted as in Fig. 1A. The plasmids were used to transform E. coli JCB387 or a Δ$ycgZ$-$ymgABC$ derivative. Production of curli was then monitored in macrocolonies grown on agar plates containing Congo red dye that binds the fibres (Reichhardt et al., 2015). Results are shown in Fig. 5. First, we compared macrocolonies formed by JCB387 or a Δ$ycgZ$-$ymgABC$ derivative, carrying the control pBR322Δbla with no cloned insert. Wild type colonies had a pale pink appearance and a red ring at their periphery (panel A). Conversely, Δ$ycgZ$-$ymgABC$ colonies were red with a narrow pink ring just inside the border of the colony (panel E). As expected, the introduction of the plasmid encoding $ycgZ$-$ymgABC$, under the control of $P_{ycgZ-ymgABC}$ and the upstream marbox, reduced
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Biofilm formation is inhibited by the ycgZ-ymgABC operon in a marbox dependent manner

Biofilms are complex structures involving many extracellular components in addition to curli fibres (Tschowri et al., 2009; Hobley et al., 2015; Flemming et al., 2016). Hence, we next investigated the role of ycgZ-ymgABC, and upstream marbox, in controlling biofilm formation in cell culture plates. As described above, we tested different combinations of wild type E. coli JCB387, and the ΔycgZ-ymgABC derivative, carrying plasmid-encoded ycgZ-ymgABC with variants of the upstream regulatory DNA. Crystal violet dye was used to detect biofilms formed and the amount of dye bound by the biofilm was quantified by spectrophotometry. We first compared biofilms formed by JCB387, or the ΔycgZ-ymgABC derivative, carrying the control pBR322Δbla with no cloned insert (Fig. 6A and 6B, grey bars). There was a small increase in the ΔycgZ-ymgABC strain (P = 1.1e−5). As expected, the introduction of the plasmid encoding ycgZ-ymgABC, under the control of PycgZ-ymgABC and the upstream marbox, reduced biofilm formation (Fig. 6A and 6B, green bars). Removal or mutation of the marbox triggered an increase in biofilm production (Fig. 6A and 6B, open and striped bars). Surprisingly, differences were most pronounced for the wild type JCB387 strain (Fig. 6A). We speculate that deleting chromosomal ycgZ-ymgABC may have additional uncharacterised downstream consequences.

Regulation of ycgZ-ymgABC by MarA is uncoupled from biofilm formation in cells lacking rcsB

Recall that the ycgZ-ymgABC operon exerts its effect on biofilms by activating the RscCDB phosphorelay system (Tschowri et al., 2009; Mika et al., 2012; Tschowri et al., 2012). Briefly, RscC is an inner membrane sensor kinase that can phosphorylate the phosphotransferase RscD. In turn, RcsD phosphorylates the response regulator RcsB that activates the expression of a sRNA called RprA. The sRNA inhibits the translation of CsgD; a positive regulator of curli production and biofilm formation (Tschowri et al., 2009; Mika et al., 2012). Hence, we reasoned that effects of MarA on biofilms, mediated by ycgZ-ymgABC, should be abolished in cells lacking RcsB. To test this prediction, we repeated our assays of biofilm production in derivatives of the E. coli JCB387 strain lacking rcsB. As expected, deletion of rcsB increased the production of biofilms twofold (compare grey bars in Fig. 6A and 6C). In this genetic background introducing the plasmid encoding ycgZ-ymgABC, under the control of PycgZ-ymgABC and the upstream marbox, had no effect (Fig. 6C, green, open and striped bars).

Fig. 4. The ycgZ-ymgABC promoter marbox is required for maximal activity in vivo during exponential growth and in the presence of salicylic acid.

A. The graphs show levels of β-galactosidase activity measured in lysates of E. coli strain JCB387 carrying different ycgZ::lacZ fusions in plasmid pRW50. Cultures in M9 minimal media were grown to exponential phase or stationary phase as indicated. The value of P was calculated using a two-tailed student’s t-test. B. Levels of β-galactosidase activity measured in lysates of cells grown in the presence of 5 mM sodium salicylate. The value of P was calculated using a two-tailed student’s t-test.

curli production. Hence, both wild type (panel B) and ΔycgZ-ymgABC (panel F) colonies had the same pale pink appearance. Removal or mutation of the plasmid marbox triggered an increase in curli production. In wild type cells, this was evident as a solid red macrocolony (panels C and D). Similarly, cells lacking ΔycgZ-ymgABC exhibited a red ring on the periphery of the colony and a deeper interior pink colour (panels G and H).
Fig. 5. Expression of the ycgZ-ymgABC operon reduces curli fibre production in a marbox dependent manner. Each of the panels (A) through (H) shows a macrocolony grown on Congo red agar plates. Images in panels A–D are of wildtype E. coli strain JCB387 transformed with empty plasmid vector (A), plasmid vector encoding ycgZ-ymgABC under the control of its native promoter (B), or derivatives lacking (C) or having a mutated (D) ycgZ-ymgABC marbox. Equivalent data are shown in panels E–H for a JCB387 derived strain lacking the chromosomal ycgZ-ymgABC operon.

Fig. 6. Expression of the ycgZ-ymgABC operon reduces biofilm formation in a marbox and rcsB dependent manner. The figure shows results from assays of biofilm formation. Briefly, biofilms grown in culture plates were stained with crystal violet dye. After washing away excess dye, biofilms were dried and the dye solubilised. Spectrophotometry was then used to quantify the amount of dye bound by each biofilm. Data are shown for (A) wildtype E. coli strain JCB387 and derivatives lacking (B) ycgZ-ymgABC or (C) rcsB. The strains were transformed with different plasmids. The plasmid derivatives are indicated by the key at the bottom of the figure. Each data point is the mean value from four independent biofilms and error bars indicate standard deviation. For each panel, the value of \( P \) was calculated using a two-tailed student’s t-test.
Putative interactions between ycgZ-ymgABC and rcsCDB-encoded proteins are revealed by two-hybrid analysis

We next aimed to better understand how the ycgZ-ymgABC gene products impact the RcsCDB phosphorelay system. In particular, we wondered if direct protein–protein interactions were involved. To test this, we utilised the BacterioMatch two-hybrid system (Fig. 7A, Dove and Hochschild, 2004). The assay detects interactions between ‘bait’ and ‘target’ proteins fused to the λcl transcription factor and RNAP α subunit respectively. If fused proteins interact, the λcl derivative recruits modified RNAP to a semisynthetic promoter. This allows expression of the downstream yeast HIS3 gene, required for histidine biosynthesis. Hence, interactions between ‘bait’ and ‘target’ allow E. coli to grow on media containing 3-amino-1,2,4-triazole (3-AT), an inhibitor of histidine production (Dove and Hochschild, 2004). Figure 7B shows the growth of E. coli harbouring different combinations of YcgZ, YmgA and YmgC, fused to λcl or RNAP αNTD, in plasmids pBT and pTRG respectively. To check reproducibility, five individual colonies of each strain were ‘patched’ on both selective (with 3-AT) and nonselective (no 3-AT) media. As expected, cells were able to grow on nonselective media regardless of the plasmid combination used (Fig. 7B, i–v). Conversely, growth on selective media was only permitted by certain combinations of the various fusions. (Fig. 7B, vi–x). Specifically, all combinations of fusions containing YcgZ, YmgA and YmgB allowed growth on selective plates (Fig. 7B, vi–vii, top two rows). Hence, these three factors can all interact and might form a complex. In contrast, fusions with YmgC did not reproducibly stimulate growth in any combination (Fig. 7B, viii). Next, we examined interactions between ycgZ-ymgABC- and rcsCDB-encoded proteins. We did not detect any interactions involving YcgZ or YmgA (data not shown). Conversely, a reproducible interaction was detected between YmbG and RcsC (Fig. 7C, vi, top row). For YmgB in combination with RcsB or RcsD, the data were erratic; growth was sparse and inconsistent (Fig. 7C, vi, middle two rows). Such ambiguities are not unusual and suggest a weak or artefactual interaction barely sufficient to permit survival (Tschowri et al., 2012). A reproducible interaction was detected between YmcG and RcsC (Fig. 7C, vii, top row). We were also able to detect interactions between RcsC and RcsD (Fig. 7C, vii, bottom row). To better define interactions, we tested the ability of YmbG or YmcG to interact with individual RcsC domains. Hence, we cloned the HK, alpha-beta-loop (ABL) or phosphoreceiver (PR) domains of RcsC in pTRG. The data show that both YmbG and YmcG can contact the cytoplasmic HK domain of RcsC (Fig. 7C, top row of panels viii and ix). We were unable to obtain a reproducible result for the interaction of YmcG and RcsC-ABL (Fig. 7C, ix, second row).

Validation of protein–protein interactions by affinity purification and coelution

The two-hybrid analysis suggests that YmcA, YmbG and YmcZ can all interact with each other (Fig. 7B). Furthermore, both YmbG and YmcC interact with RcsC (Fig. 7C). To independently validate these interactions we used in vivo coelution assays. Hence, we constructed plasmids encoding RcsC, RcsB, YmcA or YmbG with either a His6- or Strep-tag. The plasmids were used to transform strains expressing Flag-tagged YmcG, YmcA or YmbG. After cell lysis, His6- or Strep-tagged proteins were purified by affinity chromatography. Copurification of FLAG-tagged proteins was probed by western blotting. The data show copurification of YmcG-FLAG with His6-RcsC (Fig. 7D, lane 1), YmcA-FLAG with YmbG-Strep (lane 8) and YmcZ-Flag with His6-YmcA (lane 12). To check interactions between YmbG and RcsB or RcsC we coexpressed YmbG-Strep with His6-RcsC or RcsC. The His6 proteins were purified from cell lysates and the presence of YmbG-Strep probed by western blotting. The YmbG-Strep copurified with His-RcsC (Fig. 7E, lane 1) but not RcsC (lane 3).

Discussion

In this work, we show that MarA is a positive regulator of the ycgZ-ymgABC promoter in E. coli (Figs 2 and 3). We also demonstrate that activation of ycgZ-ymgABC by MarA reduces biofilm production in a manner requiring rcsB (Fig. 6). Hence, the simplest explanation is that MarA exerts its effect via the known ability of ycgZ-ymgABC to stimulate the RcsCDB phosphorelay system. We show that ycgZ-ymgABC targets RcsCDB directly; YmbG forms a complex with YmcG and YmcA that contacts the HK domain of RcsC (Fig. 7). Since activation of the RcsCDB system triggers the production of the RprA sRNA, which inhibits CsgD expression, the production of curli fibres is reduced (Fig. 5) (Tschowri et al., 2009; Mika et al., 2012). Our model is summarised in Fig. 8. Note that the regulation of ycgZ-ymgABC likely impacts other aspects of biofilm formation beyond curli production. For instance, the bdm (biofilm-dependent modulation) gene is also subjected to regulation by the RcsCDB cascade (Francez-Charlot et al., 2005). It is initially counterintuitive that increased MarA production should inhibit biofilm formation; the biofilm mode of life is considered favourable for surviving treatment with antibiotics (Stewart and Costerton, 2001; Hall-Stoodley et al., 2004; Hobley et al., 2015; Flemming et al., 2016).
However, for growing planktonic cells, 24 h are required to establish a biofilm (Elvers et al., 2002; Adamus-Białek et al., 2015). Clearly, a biofilm must already exist to provide protection (Stewart and Costerton, 2001; Hall-Stoodley et al., 2004; Hobley et al., 2015; Flemming et al., 2016). Hence, nascent biofilm formation seems
The *E. coli* MarA protein regulates the *ycgZ-ymgABC* operon.

Fig. 7. A complex formed by YmgA, YmgB and YcgZ interacts with RcsC.
A. Schematic representation of the BacterioMatch two-hybrid system. Plasmids pBT and pTRG are used to fuse prey proteins to λCI or replace the RNA polymerase α C-terminal domain with target proteins. Upon expression *in vivo*, interactions between λCI-prey and α-bait fusions allow sufficient expression of His3 for growth in the presence of the His3 inhibitor 3-AT.
B. BacterioMatch two-hybrid system reporter cells were cotransformed with derivatives of the pBT and pTRG plasmids and vector-only controls. YcgZ, YmgA, YmgB and YmgC were expressed either as hybrid proteins fused to α-NTD from pBT or as fusions to RNAP alpha-NTD from pTRG. For experiments with RcsC or RcsD we cloned the entire cytosolic part of the protein or the indicated histidine kinase (RcsC-HK), alpha-beta-loop (RcsC-ABL) and phosphoreceiver domain (RcsC-PR). Full-size RcsB was expressed from pTRG. Interactions were detected by growth in the presence of the His3 inhibitor 3-AT (selective) at 37°C for 24 h following incubation at 28°C for 48 h. Each row on the plates shows patches of five independent cotransformants.
C. The *E. coli* strain MC4100 encoding chromosomal *ymgC-Flag*, *ymgA-Flag* or *ycgZ-Flag* was transformed with pBAD33-derivatives expressing Hisα-RcsC, Hisα-RcsB, or Hisα-YmgA. YmgB-Strep was expressed from pBAD18 and the empty vectors served as negative controls. Flag-tagged proteins coeluting in Ni-NTA or Strep-Tactin Sepharose based chromatography of cellular lysates were detected using immunoblot analysis and the monoclonal anti-Flag antibody.
D. Wild-type MC4100 was cotransformed with pBAD18 or pBAD18-ymgB-Strep and either pBAD33-His-rcsC (cytosolic part) or pBAD33-His-rcsB. YmgB-Strep coeluting with His-RcsC or His-RcsB from extracts subject to Ni-NTA chromatography was detected using the anti-Strep-Tactin antibody. Lanes labelled (M) contain size markers.

Fig. 8. Model for repression of biofilm formation by MarA. Genes are shown as block arrows and proteins are shown as spheres or ovals. Stimulatory and inhibitory interactions are shown by arrows and bar-headed lines respectively. Nucleic acids are shown as double (DNA) or single (sRNA) wavy lines. The inner membrane (IM), outer membrane (OM) and curli fibres are labelled.

to be a poor strategy for surviving immediate threats. We suggest that, during planktonic growth, induction of the mar response inhibits biofilm formation and favours short term survival strategies including drug efflux, altered outer membrane permeability and DNA repair (White *et al.*, 1997; Sharma *et al.*, 2017).
Activation of the ycgZ-ymgABC promoter requires MarA residue W19 (Fig. 2). Furthermore, MarA can exert its effect from different positions (Fig. 3). This suggests a contact with the RNAP αCTD (Gaston et al., 1990; Wing et al., 1995; Dangi et al., 2004). Surprisingly, activation only occurs when RNAP is associated with the σ70 subunit; σ38-dependent transcription from PycgZ-ymgABC is not stimulated by MarA. Hence, activation by MarA is sigma factor specific but does not require a direct interaction with σ70. It is well established that σ70 and σ38 have vastly different DNA binding capacities (Shin et al., 2005). Furthermore, we have shown previously that the holoenzyme variants interact with UP-elements differently; complexes with σ38 preferentially utilise promoter distal UP-element half-sites. At PycgZ-ymgABC, one such element is amongst several sequence characteristics that favour basal transcription initiation involving σ38 (Fig. 1A). The –13C base is directly contacted by σ38 K173 whilst nonoptimal spacers and AT-rich discriminators are better tolerated by σ70 (Becker and Hengge-Aronis, 2001; Typas and Hengge, 2006; Typas et al., 2007a). The different interactions that αCTD makes in the context of σ38 holoenzyme also explain selective activation by MarA. Hence, the σ38 derivative preferentially uses the distal UP-element half-site whilst MarA provides a point of contact for αCTD in the context of σ70 holoenzyme. Consistent with this, moving the marbox 10 bp downstream, to replace the UP-element, negates basal preference of the promoter for σ38 and permits MarA to activate σ38-dependent transcription. To our knowledge, selective regulation has not previously been demonstrated for AraC family regulators. However, other transcription factors are known to behave in this way (Colland et al., 2000; Germer et al., 2001; Typa et al., 2007b; Grainger et al., 2008; Singh et al., 2011). For example, OxyR, Fis and H-NS regulate σ70 but not σ38-dependent transcription at the dps locus (Grainger et al., 2008). Similarly, at the proP2 promoter, Fis activates σ38, but not σ70-dependent transcription (Typas et al., 2007b). Hence, PycgZ-ymgABC fits the general rule that σ38 can act more autonomously than σ70 (Typas et al., 2007a).

Interestingly, Vila and Soto previously noted that MarA could inhibit biofilm formation in uropathogenic E. coli (UPEC) (Vila and Soto, 2012). It was suggested that the reduced production of type I fimbriae was responsible and that this was mediated via repression of fimB by MarA (Vila and Soto, 2012) We suggest that any such repression must be indirect since the fimB promoter does not contain a marbox (Sharma et al., 2017). Conversely, UPEC strains do encode the ycgZ-ymgABC operon and the position and sequence of the MarA binding site are conserved. Hence, an alternative explanation is that MarA repression of biofilm formation in UPEC involves the mechanism outlined here. We speculate that production of type I fimbriae may be modulated in response to expression of the ycgZ-ymgABC operon (Fig. 8). We also note that Duval and coworkers reported the accumulation of ycgZ-ymgABC-encoded proteins in strains lacking the Lon protease (Duval et al., 2017). This is due to Lon targeting MarA for degradation and levels of MarA thus increasing substantially in Lon deficient cells (Martin et al., 2008). Hence, our work is concordant with, and provides an explanation for, several previous observations linking MarA, biofilm formation and control of ycgZ-ymgABC.

### Experimental procedures

#### Strains, plasmids and oligonucleotides

Strains and plasmids are listed in Table 1 and oligonucleotides in Table S1. To construct pET28a-MarA, a DNA fragment encoding marA was generated by PCR using the MarA-OE-F and Mar-OE-R oligonucleotides. Following digestion with BamHI and NdeI, the DNA was ligated downstream of the T7-lac promoter in pET28a. The pBR322::bla plasmid was made by the digestion of pBR322 with AatI and VspI to excise the β-lactamase gene. A small linker with terminal AatI and VspI sites was used to recircularise the plasmid. Fragments encoding ycgZ-ymgABC operon, with variants of the upstream DNA, were then cloned in pBR322::bla via the EcoRI and AatI restriction sites. The ΔycgZ-ymgABC derivative of E. coli strain JCB387 was created using the gene the expression of ycgZ-ymgABC operon. For in vivo interaction assays, BacterioMatch II Two-Hybrid System vectors pBT and pTRG were used (Stratagene, Agilent Technologies). The relevant genes were cloned using primers listed in Table S1 to generate C-terminal fusions either to the lambda cI repressor from pBT or to the N-terminal domain of the E. coli RNAP from pTRG and fusion proteins were tested for interaction in histidine auxotrophic XL1-Blue MRF’-λ-red-based recombination procedure (Uzzau et al., 2001). For in vivo interaction assays, BacterioMatch II Two-Hybrid System vectors pBT and pTRG were used (Stratagene, Agilent Technologies). The relevant genes were cloned using primers listed in Table S1 to generate C-terminal fusions either to the lambda cI repressor from pBT or to the N-terminal domain of the α subunit of E. coli RNAP from pTRG and fusion proteins were tested for interaction in histidine auxotrophic XL1-Blue MRFl-derivative E. coli strain (Stratagene, Agilent Technologies). For in vivo coelution experiments, the soluble cytosolic parts of RcsC, RcsB and YmgA, respectively were N-terminally fused to a 6xHis-tag and cloned into pBAD33. YmgB carrying a Strep-tag at the C-terminus was cloned into pBAD18. pBAD33-derived RcsC (cytosolic moiety only), RcsB and YmgA were either expressed in strains containing ymgC::Flag, ymgA::Flag and ycgZ::Flag were constructed in E. coli K-12 strain MC4100 using pSUB11 (Uzzau et al., 2001) as a template for PCR and oligonucleotides listed in Table S1 following λRED-based recombination procedure (Uzzau et al., 2001). For in vivo interaction procedures and subsequent purification by liquid chromatography,
were grown to an OD600 of 0.8 and expression of MarA was
Jair
factor at room temperature for 20 minutes prior
excess of
was generated by incubating the core enzyme with a 4-fold

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Table 1. Strains and plasmids.

| Name          | Description                                                                 | Source               |
|---------------|------------------------------------------------------------------------------|----------------------|
| **Bacterial strains**                                      |                                                                           |                      |
| JCB387        | ΔnirB, Δlac                                                                  | Page et al. (1990)   |
| JCB387ΔycgZ-ymgABC ΔrcsB                                 | This work                                                               |
| JCB387ΔycgZ-ymgABC ΔrcsB                                 | This work                                                               |
| BL21 DE3      | T7 RNAPol + F-ompT rib-ma- fhuA2                                             | Studier (1991)       |
| T7 Express    | lacZ:T7 gene1 [lon ompT gal sulA11 R(mcr-T3::miniTn10::TcS)]2 [dcm]          | NEB                  |
|               | R(zgb-210::Tn10::TcS) endA1 Δ(mcrC-mrn)14::IS10                             |                      |
| MC4100        | E.coli K12 F- araD139 O(argF-lac)U169 deoC lllB5301 relA1 rpsL150 ptsF25 trbR | Casadaban (1976)     |
| NAT239        | MC4100 ycgZ::Flag                                                             | This work            |
| NAT240        | MC4100 ymgA::Flag                                                             | This work            |
| NAT242        | MC4100 ymgC::Flag                                                             | This work            |
| XL1-Blue MRF  | Δ(xcrA183 Δ(xcrCB-hsdSMR-mn)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac [F’ proAB lacIq endA supE -1 96 gyrA1 th] | AgilentTechnol.     |
|               |                                                                              |                      |
| **Plasmids**                                           |                                                                           |                      |
| pRW50        | Low copy number 16 kb plasmid for making lacZ fusions. Contains the RK2 origin of replication and encodes TetR | Lodge et al. (1992) |
| pSR          | 4.8 kb pBR322 derivative that encodes AmpR. Contains an EcoRI-HindIII cloning site upstream of the lacI promoter terminator and rop for restriction of plasmid copy number | Kolb et al. (1995)  |
| pBR322       | 4.4 kb, encodes TetR and AmpR. Contains the pMB1 origin of replication       | Bolivar et al. (1977) |
| pBR322Δbla    | pBR322 lacking the bla gene                                                  | This work            |
| pET-28a      | 5 kb, encodes KanR. Contains the T7 lac promotor for high-level IPTG-inducible expression of recombinant proteins with N- or C-terminal His tags and a thrombin cleavage site | Novagen               |
| pDOC-C       | 5 kb, encodes AmpR, derived from pEX100T. Used as a cloning vector for gene sequencing. Features a cloning region flanked by two I-SceI recognition sites | Lee et al. (2009)   |
| pDOC-K       | Derived from pEX100T and contains a kanamycin resistance cassette between two Fip recombinant recognition sites | Lee et al. (2009)   |
| pACBSR       | 7.3 kb, encodes CamR. Recombination plasmid for gene sequencing; carries arabinose inducible λ-Red and I-SceI endonuclease genes | Herring et al. (2003) |
| pCP20        | 9.4 kb, encodes CamR and AmpR. Encodes yeast FLP recombinase                  | Cherepanov and Wackernagel (1995) |
| pSUB11       | 3.5 kb, encodes KanR. Used to amplify Fip recombinant target (FRT)-flanked kanamycin resistance cassette with 3xFlag | Uzzau et al., 2001  |
| pKD46        | 6.3 kb, encodes AmpR. Used to express λ Red recombinase                       | Uzzau et al., 2001  |
| pBT          | 3.2 kb, contains CamR. BacterioMatch II Two-Hybrid System vector.             | Agilent Technol.     |
| pTRG         | 4.4 kb, encodes TeR. BacterioMatch II Two-Hybrid System vector.               | Agilent Technol.     |
| pBAD18       | 4.6 kb, encodes AmpR. Carries arabinose-inducible araBAD promoter, pBR322 origin | Guzman et al. (1995) |
| pBAD33       | 5.3 kb, encodes CamR. Carries arabinose-inducible araBAD promoter, pACYC184 origin | Guzman et al. (1995) |

as described by Grainger et al. (2008). The RNAP core
enzyme was purchased from NEB. The RNAP holoenzyme
was generated by incubating the core enzyme with a 4-fold
efficiency of σ factor at room temperature for 20 minutes prior
to use. MarA purification was based on that described by
Jair et al. (1995). T7 Express cells containing pET28a-Mara
were grown to an OD600 of 0.8 and expression of MarA was
induced with 0.4mM IPTG for 3 h. Cells were harvested by
centrifugation and washed with a buffer containing 50M
Tris–HCl (pH 7.5), 1mM EDTA, 1m NaCl before lysis using an
AVESTIN EmulsiFlex C3 high pressure motorised homoge-

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using thrombin sepharose beads (BioVision) for 5 h at room temperature. Beads were removed by centrifugation and the His-tag was removed by a second round of affinity chromatography. Untagged MarA that did not bind the HisTrap™ column was transferred into 1 M NaCl, 5 mM HEPES, 1 mM dithiothreitol, 5 mM EDTA, 0.1 mM Triton X-100 and 20% (v/v) glycerol by dialysis. Samples were stored at −80 °C until use.

**Electrophoretic mobility shift assays**

Assays were performed as described previously (Cosgriff et al., 2010). Briefly, DNA fragments were generated by PCR amplification from an E. coli genomic DNA template. Following purification, PCR products were cut with HindIII and EcoRII prior to being end-labelled with [γ-32P]-ATP and polynucleotide kinase. The DNA fragments were incubated with MarA in buffer containing 20 mM Tris pH 7, 10 mM MgCl₂, 100 mM EDTA and 120 mM KCl. Reactions were analysed by electrophoresis through a 5% polyacrylamide gel. Raw gel images are shown in Fig. S2.

**β-galactosidase assays**

DNA fragments containing the desired derivative of the ycgZ-ymgABC regulatory region were cloned in plasmid pRW50 to generate promoter::lacZ fusions. The β-galactosidase levels in lysates of cells carrying these recombinants were measured by the Miller method (Miller, 1972). Activities are the average of three or more independent experiments.

**Congo red binding assays**

Bacterial strains were cultured overnight in lysogeny broth (LB) lacking salt (10 g/L of tryptone and 5 g/L of yeast extract). Curli fibres were detected by spotting 5 μl of overnight culture onto LB agar lacking salt and supplemented with 40 μg/ml of Congo red. The agar plates were then incubated at 37°C overnight. The morphology and colour of colonies were recorded by digital photography. The experiments were done multiple times to check that colony phenotypes were reproducible and images shown are representative. The raw image is shown in Fig. S2.

**Crystal violet binding assays**

The crystal violet assay described in Baugh et al. (2014) was used to quantify biofilm production between bacterial strains. Two independent overnight cultures per strain were diluted in LB to an OD₆₀₀ of 0.1. A 200 μl aliquot was added to a flat-bottomed 96-well microtitre plate, with four replicate wells per culture. The plate was incubated at 30°C for 48 h. Wells were washed with water to remove unattached cells and 200 μl of 0.1% w/v crystal violet was added for 15 minutes. Wells were then washed with water again to remove unbound crystal violet and 200 μl of 70% ethanol was added to solubilise the retained crystal violet. The A₆₀₀ was then measured using a CLARIOStar plate reader (BMG Labtech) to give a quantitative measure of biofilm formation.

**Bacterial two-hybrid assays**

In vivo protein–protein interactions were detected using BacterioMatch II Two-Hybrid System (Dove and Hochschild, 2004). Interaction of coexpressed hybrid proteins linked to the NTD of lambda cI (from pBT) and to the bacterial RNAP alpha-NTD (from pTRG) activates HIS3 gene expression suppressing histidine auxotrophy of the reporter strain (E. coli XL1-Blue MRF' derivative). The assay was performed according to the instruction manual (Stratagene, Agilent technologies). Cotransformants were obtained on nonselective plates and five independent clones were patched on both, nonselective and selective medium respectively. Growth on selective medium containing the His3 inhibitor 3-amino-1,2,3-triazole (3-AT) indicates the interaction of the tested hybrid proteins leading to increased expression of HIS3 gene. Plates were incubated for 24 h at 37°C and for an additional 48 h at 28°C.

**In vivo coelution and immunoblot analysis**

In vivo protein–protein interactions were also analysed using coelution (‘pull-down’) assays. pBAD33-6xHis-rcsC (cytosolic part only), pBAD33-6xHis-rcsB, pBAD-6xHis-ymgA and pBAD18-ymgB-Strep were transformed into E. coli K-12 MC4100 containing chromosomally Flag-tagged ymgC, ymgA and ycgZ respectively. Expression of pBAD-encoded genes was induced with 0.1% arabinose at OD₆₀₀ of 0.8. Cells were grown overnight at 28°C and cell pellets were lysed using a French press. Cells expressing His-tagged genes were lysed in ‘His-lysis buffer’: 50 mM NaH₂PO₄, 150 mM NaCl, 20 mM imidazole, pH 8. Strep lysis buffer (100 mM Tris–HCl pH8, 150 mM NaCl, 1 mM EDTA) was used for lysis of cells expressing ymgB-Strep. Ni-NTA agarose (Qiagen) was used for affinity chromatography of His-tagged proteins. Strep-Tactin Sepharose (IBA, Gottingen) was served for affinity purification of YmgB-Strep. Chromatography columns were washed with the respective lysis buffer and elution was performed using 50 mM NaH₂PO₄, 150 mM NaCl, 250 mM imidazole, pH 8 for His-tagged proteins and buffer E (IBA) for YmgB-Strep. Copurified Flag-tagged proteins were detected using monoclonal anti-FLAG antibody (Sigma) following SDS polyacrylamide gel electrophoresis. To detect protein–protein interactions, samples were boiled in loading buffer and separated on a 10% polyacrylamide gel. Proteins were transferred to nitrocellulose membrane and detected using a polyclonal anti-Myc antibody (BD Biosciences).
interactions between YmgB-Strep and either 6xHis-RcsC (cytosolic part) or 6xHis-RcsB, respectively, pBAD33-6xHis-rcsC or pBAD33-6xHis-rcsB were cotransformed and expressed with pBAD18-ymgB-Strep in MC4100. The cells were grown and treated as described above. His-tagged proteins were purified using Ni-NTA. Coeluted YmgB-Strep was detected using anti-Strep-Tactin antibody (IBA).

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Data availability statement

All data are available within the manuscript figures or within the cited references.

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Supporting Information
Additional supporting information may be found online in the Supporting Information section at the end of the article