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SUPPLEMENTARY INFORMATION

Comparative effects of Overproducing the AraC-Type Transcriptional Regulators MarA, SoxS, RarA and RamA on Antimicrobial Drug Susceptibility in *Klebsiella pneumoniae*.

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SUPPLEMENTARY METHODS

OMP and Proteomic Analysis

Cells were pelleted by centrifugation (10 min, 4,000 × g, 4˚C) and resuspended in 20 mL of 30 mM Tris-HCl, pH 8 and broken by sonication using a cycle of 1 sec on, 0.5 sec off for 3 min at amplitude of 63% using a Sonics Vibracell VC-505TM (Sonics and Materials Inc., Newton, Connecticut, USA). The sonicated samples were centrifuged at 8,000 rpm (Sorval RC5B PLUS using an SS-34 rotor) for 15 min at 4˚C to pellet intact cells and large cell debris. The supernatant was removed and subjected to centrifugation at 20,000 rpm for 60 min at 4˚C using the above rotor to pellet total envelopes. If OMPs were to be purified, inner membranes and associated proteins were removed from the total envelope pellet by adding 100 µL of 20% w/v n-lauryl sarcosine and incubating at room temperature for 20 min. The sarcosine-insoluble outer membranes were recovered by centrifugation as above, and the pellet solubilised in 100 µL of water. To isolate total envelope proteins, the total envelope pellet (above) was solubilised using 200 µL of 30 mM Tris-HCl pH 8 containing 0.5% (w/v) SDS.

The protein concentration in each sample was quantified using Biorad Protein Assay Dye Reagent Concentrate according to the manufacturer's instructions. Proteins (5 µg/lane for total envelope proteomics; 1 µg/lane for OMP analyses) were separated by SDS-PAGE using 11% acrylamide, 0.5% bis-acrylamide (Biorad) gels and a Biorad Min-Protein Tetracell chamber model 3000X1. For the proteomics experiments only, gels containing total envelope proteins were run at 200 V until the dye front had moved approximately 1 cm into the separating gel; for OMP analyses, the dye front was run off the bottom of the gel. Proteins in all gels were stained with Instant Blue (Expedeon) for 20 min and de-stained in water.

For total envelope proteomics, the one centimeter of gel lane containing total envelope proteins was cut out and subjected to in-gel tryptic digestion using a ProGest automated digestion unit (Digilab UK). The resulting peptides were fractionated using an Ultimate 3000 nanoHPLC system in line with an LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific). In brief, peptides in 1% (v/v) formic acid were injected onto an Acclaim PepMap C18 nano-trap column (Thermo Scientific). After washing with 0.5% (v/v) acetonitrile plus 0.1% (v/v) formic acid, peptides were resolved on a 250 mm × 75 µm Acclaim PepMap C18 reverse phase analytical column (Thermo Scientific) over a 150 min organic gradient, using 7 gradient segments (1-6% solvent B over 1 min., 6-15% B over 58 min., 15-32% B over 58 min., 32-40% B over 3 min., 40-90% B over 1 min., held at 90% B for 6 min and then reduced to 1% B over 1 min.) with a flow rate of 300 nl.min−1. Solvent A was 0.1% formic acid and Solvent B was aqueous 80% acetonitrile in 0.1% formic acid. Peptides were ionized by nano-electrospray ionization at 2.1 kV using a stainless steel emitter with an internal diameter of 30 µm (Thermo Scientific) and a capillary temperature of 250°C. Tandem mass spectra were acquired using an LTQ-Orbitrap Velos mass spectrometer controlled by Xcalibur 2.1 software (Thermo Scientific) and operated in data-dependent acquisition mode. The Orbitrap was set to analyze the survey scans at 60,000 resolution (at m/z 400) in the mass range m/z 300 to 2000 and the top twenty multiply charged ions in each duty cycle selected for MS/MS in the LTQ linear ion trap. Charge state filtering, where unassigned precursor ions were not selected for fragmentation, and dynamic exclusion (repeat count, 1; repeat duration, 30 s; exclusion list size, 500) were used. Fragmentation conditions in the LTQ were as follows: normalized collision energy, 40%; activation q, 0.25; activation time 10 ms; and minimum ion selection intensity, 500 counts.

The raw data files were processed and quantified using Proteome Discoverer software v1.2 (Thermo Scientific) and searched against the UniProt K. pneumoniae strain ATCC 700721 / MGH 78578 database (5126 protein entries; UniProt accession 272620) using the SEQUEST (Ver. 28 Rev. 13) algorithm. Peptide precursor mass tolerance was set at 10 ppm, and MS/MS tolerance was set at 0.8 Da. Search criteria included carbamidomethylation of cysteine (+57.0214) as a fixed modification and oxidation of methionine (+15.9949) as a variable modification. Searches were performed with full tryptic digestion and a maximum of 1 missed cleavage was allowed. The reverse database search option was enabled and all peptide data was filtered to satisfy false discovery rate (FDR) of 5 %. The Proteome Discoverer software generates a reverse "decoy" database from the same protein database used for the analysis and any peptides passing the initial filtering parameters that were derived from this decoy database are defined as false positive identifications. The minimum cross-correlation factor filter was readjusted for each individual charge state separately to optimally meet the predetermined target FDR of 5 % based on the number of random false positive matches from the reverse decoy database. Thus each data set has its own passing parameters. Protein Area measurements were calculated from peptide peak areas using the Top 3 method [S1] and were then used to calculate the relative abundance of each protein between pBAD transformants. Proteomic analysis was repeated at least three times, each using a separate batch of cells.

Quantitative Reverse-Transcriptase PCR.

RNA in cultures was stabilised using RNAprotect Bacteria Reagent (Qiagen, Crawley, UK) according to the manufacturer's instructions, before 1.5 mL of stabilised culture was centrifugation for 5 min at 4000 rpm (Function Line Labofuge 400R centrifuge, Heraeus, Hanau,

Germany) to pellet cells. Following aspiration of the supernatant, 200 µL of TE buffer containing lysozyme (50 mg/mL) was used to disrupt the pellet, and the mixture was left for 10 min at room temperature, vortexing every 2 min before addition of 700 µL RLT buffer (Qiagen) containing 150 mM β-mercaptoethanol. The mixture was transferred to a tube containing acid washed silica lysing matrix B (MP Biochemicals, Eschwege, Germany) and cells were disrupted in a Ribolyser (Hybaid, Basingstoke, UK) (setting 6.0; 2 cycles lasting 45 s each). Following disruption, RNA was purified using a Qiagen RNeasy RNA purification kit, according to the manufacturer's instructions. RNA from 1.5 mL of bacterial culture was dissolved in 50 µL RNase free water, contaminating genomic DNA was digested using a TURBO DNA-free™ Kit (Ambion, Foster City, CA, USA) following the manufacturer's instructions. The concentration of RNA in each sample was measured using a NanoDrop ND-100 spectrophotometer (Labtech, UK). One microgram of total RNA was converted into cDNA using qScript^{TM} reverse transcriptase (Quanta Biosciences, Gaithersburg, MD, USA) in a 20 µL reaction which included random hexamer primers. The reaction was incubated for 5 min at 25°C, 30 min at 42°C and 5 min at 85°C. cDNA samples were stored at -20°C until used as templates for gene-specific real-time qPCR. The reference gene in each case was *rrsE*. Each real-time qPCR reaction was prepared using 100 ng of cDNA (quantified by Nanodrop) as template in a 20 µL reaction with PowerUp™ SYBR Green Master Mix (Applied Biosystems, Waltham, Massachusetts, USA) and 500 nM of each primer (Table S1). The amplification and quantification of cDNA copies was performed using a StepOnePlus™ Real-Time PCR System (Applied Biosystems). Samples were run as 4 technical replicates and 3 biological replicates were used for each comparison, each from a separate RNA purified from a separate batch of cells. The PCR amplification cycles consisted of initial denaturation at 95°C for 2 min followed by 40 cycles of denaturation at 95°C for 2 s and

annealing/extension at 60°C for 30 s. The ratio of target to reference cDNA in a sample of comparator B was calculated relative to that in a sample of comparator A according to the $\Delta\Delta C^T$ method.[S2] In each case, comparator A was a transformant carrying the pBAD control plasmid and comparator B was a transformant carrying the pBAD plasmid overexpressing the appropriate regulator gene.

SUPPLEMENTARY REFERENCES

1. Silva JC, Gorenstein MV, Li G-Z, et al. Absolute quantification of proteins by LCMSE: a virtue of parallel MS acquisition. *Mol Cell Proteomics* 2006; **5**: 144-156.
2. Pfaffl, MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 2001; **29**: e45

SUPPLEMENTARY TABLES

Table S1: PCR and qRT-PCR primers used in this study

Primer	Sequence (5' to 3')
ramA+	ATGACGATTTCCGCTCAG
ramA-	AGCTGGTTGAGACATTCC
marA+	ATGTCCAGACGTAATAATGAC
marA-	TCATGATTTCCTCAATACGTC
rarA+	ATGATGAACACTGGCGC
rarA-	ATCGCTGACGAGATGTC
soxS+	ATGTCCCATCAGGATATTATTC
soxS-	TCACGCGGAGATCTGATG
ompK35+ (NcoI site underlined)	CCATGGTGAAGCGCAATATTC
ompK35-	CACTTCGATGTATTTAACCAG
ompK36+ (NcoI site underlined)	CCATGGAAGTTAAAGTACTGT
ompK36-	CAGCTTGCAACTTAGAACTG

Table S2. Disc susceptibility assay for *K. pneumoniae* SM transformants expressing regulator genes

Antibiotic (µg in disc)	pBAD(control) Zone (mm)	pBAD(*marA*) Zone [Difference from control] (mm)	pBAD(*soxS*) Zone [Difference from control] (mm)	pBAD(*rarA*) Zone [Difference from control] (mm)	pBAD*(ramA*) Zone [Difference from control] (mm)
Amikacin (30)	23 (S)	NC	NC	NC	-2 (S)
Gentamicin (10)	23 (S)	NC	NC	NC	-2 (S)
Tobramycin (10)	20 (S)	NC	NC	NC	NC
Cefoxitin (30)	26 (S)	NC	-2 (S)	-3 (S)	-5 (S)
Cefuroxime (30)	25 (S)	NC	-2 (S)	-3 (S)	-6 (S)
Ceftriaxone (30)	31 (S)	NC	NC	-2 (S)	NC
Cefotaxime (30)	35 (S)	NC	-3 (S)	-3 (S)	-4 (S)
Ceftazidime (30)	30 (S)	-2 (S)	-3 (S)	-4 (S)	-7 (S)
Cefepime (30)	32 (S)	NC	NC	NC	-2 (S)
Aztreonam (30)	33 (S)	NC	-2 (S)	-4 (S)	-3 (S)
Imipenem (10)	25 (S)	NC	NC	NC	NC
Meropenem (10)	30 (S)	NC	NC	NC	NC
Doripenem (10)	26 (S)	NC	NC	NC	NC
Ciprofloxacin (5)	34 (S)	NC	-2 (S)	-4 (S)	-8 (S)
Norfloxacin (10)	31 (S)	NC	-3 (S)	-3 (S)	-7 (S)
Ofloxacin (5)	31 (S)	NC	-3 (S)	-4 (S)	-6 (S)
Tigecycline (15)	21	NC	NC	NC	-4
Minocycline (30)	20 (S)	NC	NC	-2 (S)	-8 (R)
Chloramphenicol (30)	28 (S)	NC	-2 (S)	-4 (S)	-8 (S)
Trimethoprim/ Sulfamethoxazole (1.25/23.75)	30 (S)	NC	NC	NC	-2 (S)

Assays were performed using Muller Hinton agar with 0.2 % w/v arabinose to stimulate expression of the cloned genes and 50 mg/L ampicillin to select for the pBAD plasmids. Otherwise, the assay was performed according to the CLSI protocol.[17] Abbreviations used are S: Susceptible; I: intermediate Resistant; NC: No Change in zone diameter versus control. Values reported are the means of at least three repetitions rounded to the nearest integer. Mean changes <2 mm are reported as NC. Susceptibility breakpoints are as set by the CLSI.[20]

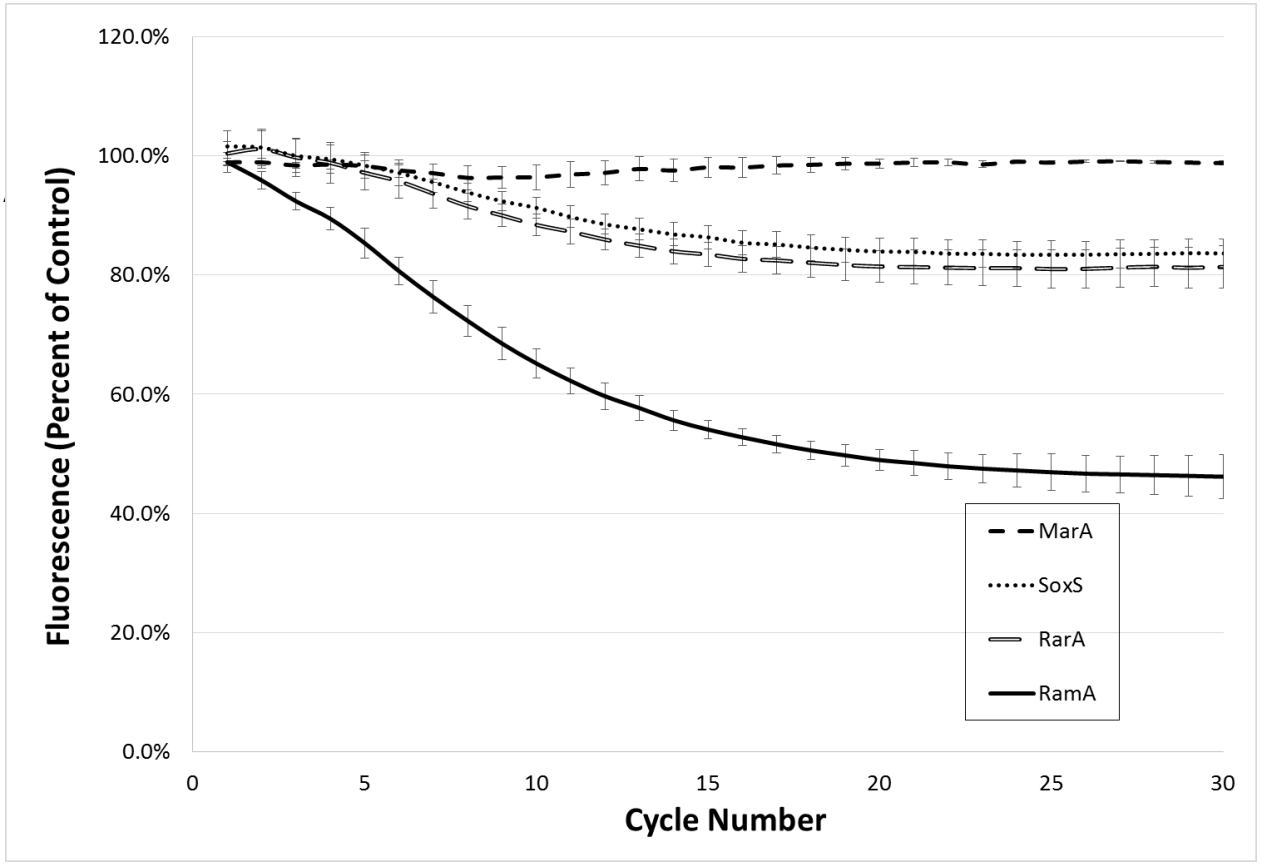
SUPPLEMENTARY FIGURES

Figure S1: Effect of AraC-type Regulator Over-Production in *K. pneumoniae* SM on Envelope Permeability and OMP profile.

A: The accumulation of H33342 dye by *K. pneumoniae* SM transformants overexpressing *ramA*, *rarA*, *soxS* or *marA* is represented as a percentage relative to accumulation in the plasmid-only control transformant (set to 100%) over a 30 cycle (45 minute) incubation period. Each graph shows mean data for four biological replicates with 8 technical replicates in each, and error bars define the standard error of the mean (SEM). **B**: OMPs were purified from *K. pneumoniae* SM transformants carrying the control plasmid or those overexpressing *ramA, rarA, soxS or marA*. All OMPs were separated using SDS-PAGE and stained as set out in Materials & Methods. These data are representative of three biological replicates.

Figure S1

A


120.0%
100.0%
80.0%
60.0%
40.0%
20.0%
0.0%
Fluorescence (Percent of Control)
0
5
10
15
20
25
30
Cycle Number
MarA
SoxS
RarA
RamA

B

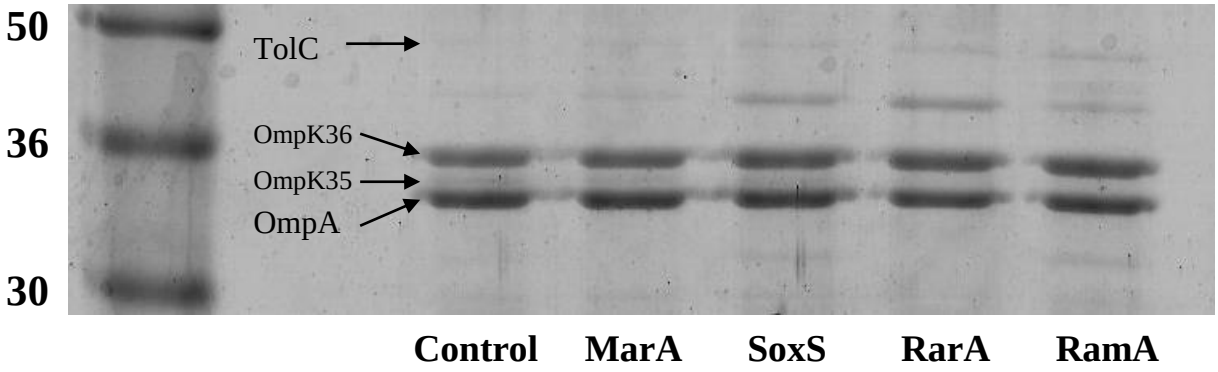
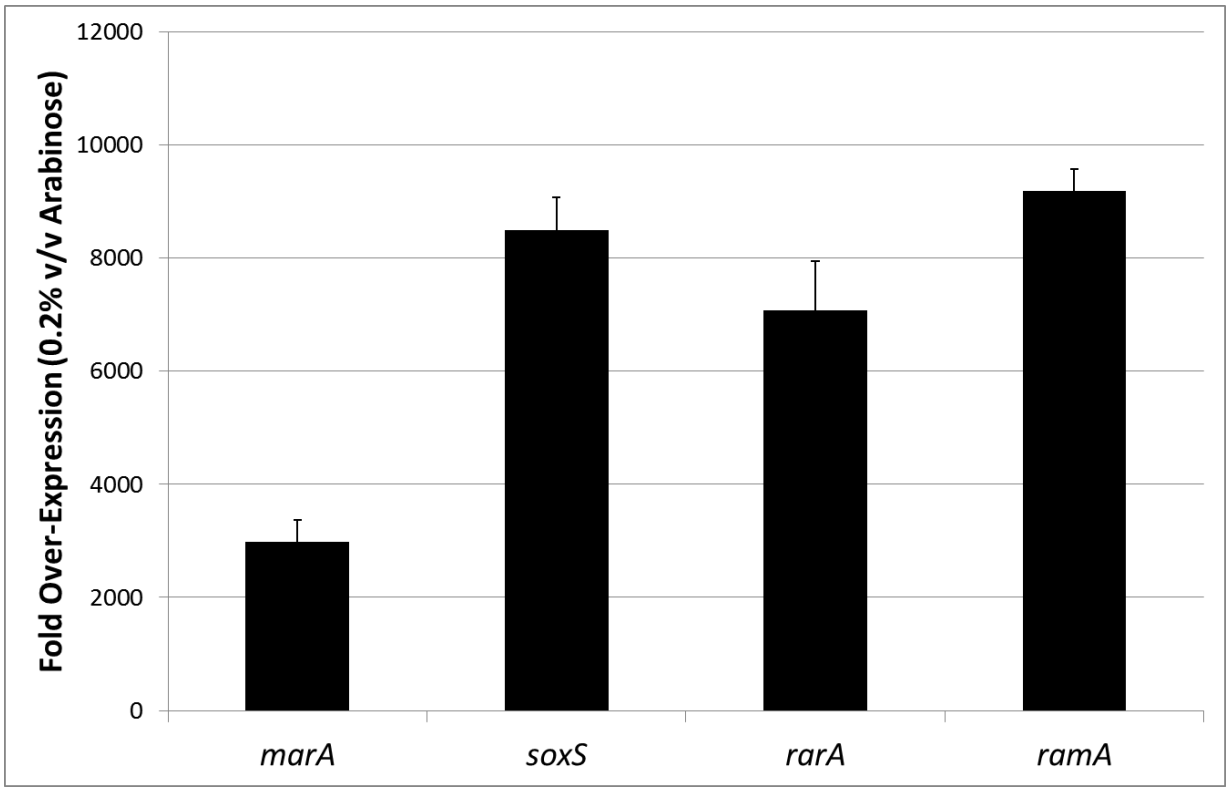

50
36
30
TolC
OmpK36
OmpK35
OmpA
Control
MarA
SoxS
RarA
RamA

Figure S2: Changes in Cefoxitin Zone Diameter Following Overproduction of AraC-type regulators in *K. pneumoniae* NCTC5055.

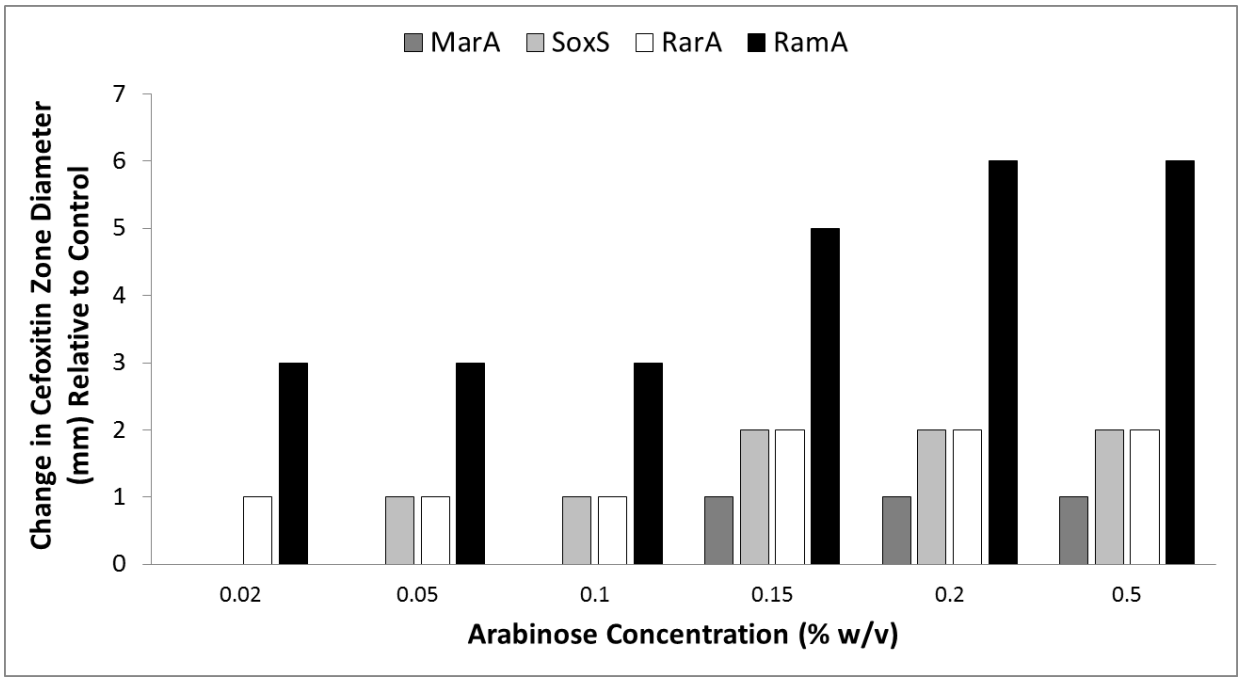
A: qRT-PCR analysis of AraC-type regulator gene expression following over-production using the pBAD vector with 0.2 % w/v Arabinose as inducer. Samples were collected from Nutrient Broth cultures incubated with arabinose for 2 h. **B**: CLSI antibiotic disc susceptibility testing [17] was performed using agar containing different amounts of arabinose designed to induce expression of *ramA*, *rarA*, *soxS* or *marA* in *K. pneumoniae* NCTC5055 transformants to different degrees. The zone diameter across a cefoxitin disc was measured for each transformant and at each arabinose concentration and compared with the zone diameter for NCTC5055 carrying the control plasmid when grown at the same arabinose concentration. Differences in zone diameter in the transformants relative to control are reported in the graph. Data are modes of three biological replicates.

Figure S2

A


Fold Over-Expression (0.2% v/v Arabinose)
12000
10000
8000
6000
4000
2000
0
marA
soxS
rarA
ramA

B


MarA
SoxS
RarA
RamA
Change in Cefoxitin Zone Diameter (mm) Relative to Control
7
6
5
4
3
2
1
0
0.02
0.05
0.1
0.15
0.2
0.5
Arabinose Concentration (% w/v)