

Inhibition of multidrug efflux as a strategy to prevent biofilm formation

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1 **Co-ordinated regulation of multidrug efflux and biofilm formation in**
2 ***Salmonella*.**

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5 10 February 2016

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12

Abstract

Objectives. We have recently shown that inactivation of any of the multidrug efflux systems of *Salmonella* results in loss of ability to form a competent biofilm, the aim of this study was to determine the mechanism linking multidrug efflux and biofilm formation.

Methods. Mutants lacking components of the major AcrAB-TolC system were investigated for their ability to form a biofilm, aggregate and produce biofilm matrix components. The potential for export of a biofilm relevant substrate via efflux pumps was investigated as well as expression of genes that regulate multidrug efflux and production of biofilm matrix components.

Results. Mutants of *Salmonella enterica* serovar Typhimurium which lack TolC or AcrB but surprisingly not AcrA were compromised in their ability to form biofilms. This defect was not related to changes in cellular hydrophobicity, aggregative ability or export of any biofilm specific factor. The biofilm defect associated with inactivation of *acrB* or *tolC* resulted from transcriptional repression of curli biosynthesis genes and consequent inhibition of the production of curli by mutants lacking AcrB or TolC. This repression was associated with up-regulation of the global regulator, *ramA* and artificial over-expression of *ramA*, *marA* and *soxS* each decreased biosynthesis of curli, and inhibited biofilm formation. However, inactivation of these regulators did not rescue the ability of efflux mutants to form a biofilm.

Conclusions. This work shows biofilm formation and multidrug efflux are coordinately regulated, and that transcriptional repression of curli biosynthesis causes a lack of biofilm formation which occurs in response to lack of efflux activity or as a result of over-expression of global regulators *ramA*, *marA* and *soxS*.

Introduction

Bacterial biofilms are a major clinical and industrial problem and eradication of biofilms presents a challenge for antimicrobial chemotherapy.¹⁻³ Bacteria within a biofilm are encased within an extracellular matrix which commonly includes polysaccharides, proteins and other species specific components.^{1,4} Multidrug resistance efflux (MDR) pumps are transporters which can export a wide range of xenobiotics including antibiotics, dyes, biocides and other toxic molecules preventing lethal accumulation within the cell.⁵ The expression of efflux pumps is tightly regulated and efflux genes are usually subject to control by both local and global regulators.⁵ *Salmonella enterica* serovar Typhimurium (*S. Typhimurium* hereafter) has nine MDR systems from four separate protein families; the major system in *Salmonella* is the AcrAB-TolC RND system.⁶ The homologous global transcription regulators MarA, RamA and SoxS can all increase expression of *acrAB* in response to stress.⁷⁻⁸ We have recently described an inability to form a competent biofilm associated with inactivation of any of the MDR systems of *S. Typhimurium* including those which are normally cryptic in standard laboratory conditions.⁹ We found that production of curli, a major component of the *Salmonella* biofilm extracellular matrix was defective in all these strains, suggesting a common mechanism for the lack of biofilm formation in all mutants.

Here, using AcrAB-TolC as a paradigm we investigated the mechanism by which loss of efflux activity results in a lack of curli production. We ruled out export of a factor crucial for biofilm development via AcrAB-TolC and also showed that inactivation of components of AcrAB-TolC did not alter cellular hydrophobicity. However, inactivation of efflux components was found to significantly alter expression of biofilm related genes. We demonstrate that the biofilm defect of

63 mutants lacking AcrB or TolC is due to transcriptional repression of curli biosynthesis
64 in the efflux mutants. Additionally, over-expression of the global regulators *ramA*,
65 *marA* or *soxS* resulted in repression of curli biosynthesis and loss of biofilm
66 formation. This work demonstrates a mechanism whereby loss of MDR efflux pumps
67 impacts production of a biofilm due to co-ordinated regulation of efflux and biofilm
68 formation.

69

Materials and methods

Strains and growth media

All strains used in this study and their origins are shown in Table 1. *S. Typhimurium* ATCC 14028S (L828) was used as a control strain throughout. Isogenic derivatives, L829 (*tolC::cat*) and L830 (*acrB::aph*) have been described previously,⁶ New mutants were created by transduction of mutant alleles into L828, and resistance marker cassettes were removed by plasmid pCP20 as previously described.¹⁰ To select for transductants, 100 µl from each transduction reaction was spread onto LB plates supplemented with 50 mg/L of kanamycin or 25 mg/L of chloramphenicol and incubated overnight at 37°C. Transfer of each mutant allele was verified by PCR and sequencing. Strains were stored at -20°C on Protect™ beads and routinely cultured on Luria-Bertani agar or broth unless stated otherwise. Over-expression and complementation plasmids containing *marA*, *soxS* or *ramA* were constructed in pTRC and pWKS30 as previously described.⁷

Biofilm formation assays

Various models were used to analyse biofilm formation in this study. For crystal violet biofilm assays, overnight cultures of strains were diluted in fresh Luria-Bertani broth without salt to an optical density of 0.1 at 600 nm. 96 well polystyrene microtitre trays (Sterilin) were inoculated with 200 µl of this suspension and incubated at 30°C for 48 hours with gentle agitation. After incubation liquid was removed from all wells and wells were washed with sterile distilled water to remove any unbound cells. Biofilms were stained by adding 200 µl of 1% crystal violet to appropriate wells for 15 minutes. Crystal violet was removed and each well washed with sterile distilled water to remove unbound dye. The stained biofilm was solubilised by adding 200 µl of 70% ethanol and optical density measured at 600 nm

using a FLUOstar Optima (BMG labtech). All biofilm assays were repeated three times with two biological and four technical replicates per repeat.

To determine whether biofilm formation in L829 (*tolC::cat*) and L830 (*acrB::aph*) could be rescued by co-incubation with L828 (wild-type), strains were grown separated by a 0.45 µm membrane and biofilms formed as in the crystal violet assay but in 500 µl volumes in 24 well transwell plates. Assays were repeated with and without the presence of L828 (wild-type) in the upper 'insert' chamber with liquid contiguous between the upper and lower chambers. Biofilms were stained with crystal violet and quantified as above. Assays were repeated with addition of either a mid-logarithmic or stationary phase culture of L828 (wild-type) to assess whether growth phase had an impact upon production of any soluble biofilm promoting factor. Biofilm formation under flow conditions were formed and visualised using a Bioflux microfluidic system (Fluxion) and phase contrast microscopy. Flow channels were inoculated with overnight cultures diluted in LB broth without salt to an optical density of 0.8 at 600 nm, plates were then incubated at 30°C for three hours to allow the bacteria to adhere to the flow channels. Fresh LB broth without salt was then applied to the inlet wells of the plate and pumped through the flow cells at a force of 0.3 dynes at 30°C for 48 hours. Phase contrast microscopy was used to visualise the biofilms formed at x10m, x20 and x40 magnification.

Aggregation assays

To examine whether loss of *acrB* or *tolC* led to alteration in cellular hydrophobicity or aggregative ability two different assays were used.

To measure the time taken for strains to settle, strains were incubated overnight in 10 ml LB (without salt) broths with shaking (150 rpm) before being placed statically on the bench. Samples (100 µl) were taken periodically from immediately below the

surface of the liquid and the optical density at 600 nm measured and recorded. Enter aggregative *E. coli* O42 was used as a positive control. To determine whether there were any intrinsic differences in aggregative ability of each strain ammonium sulphate was used to induce aggregation of bacterial cells, a 4 M stock of (NH₄)₂SO₄ was made in 1 X PBS and adjusted to a pH 6.8. This stock was then serially diluted and mixed 1:1 (in 100 µl final volume) with bacterial suspensions (adjusted from an overnight culture to an OD 570 nm of 0.8) for each strain. These suspensions were immediately added to a microscope slide and rocked gently for 30 min before aggregation scored visually under a microscope as the presence of a precipitate. The lowest concentration of (NH₄)₂SO₄ required to induce aggregation was recorded for each strain.

Artificial over-expression of *ramA*, *soxS* and *marA*

pTrc-*ramA* carrying an IPTG inducible *ramA* was transformed into L828 (wild-type) to observe the phenotypic effects of over expressing *ramA*.¹¹ pTrc-*soxS* and pTrc-*marA* were constructed in a similar manner and introduced into L828 (wild-type), L829 (*tolC::cat*) and L830 (*acrB::aph*). Plasmid DNA was harvested from 10 ml cultures of strains containing plasmids after overnight incubation in LB broth at 37°C using the QIAprep® Spin Cell Mini Kit (QIAGEN, U.K). The resulting plasmid DNA was analysed by agarose gel electrophoresis and quantified using Gene Tools software (Syngene, Cambridge, U.K). Plasmid DNA was transferred into recipient cells by electroporation. The impact of over-expression of each of the regulators on biofilm formation was investigated in the crystal violet assay; wells containing plasmids were supplemented with 100 mg/L of ampicillin and 1 mM IPTG to induce gene expression.

Determination of gene expression

The temporal and spatial expression of *ramA*, *marA* and *soxS* within biofilms was visualised using promoter-*gfp* fusion reporter constructs in pMW82.¹² Strains carrying reporter plasmids were grown overnight in LB containing 100 mg/L of ampicillin then adjusted in PBS to an optical density of 0.1 at 600nm. Spots (5 µl) were inoculated onto LB – NaCl agar plates containing 100 mg/L of ampicillin and 40 mg/L of Congo red and incubated statically at 30 °C. Fluorescence of colonies was visualised after 24 and 48 h using a Nikon SMZ800 microscope (with Integilight C-HGFI fluorescence module attachment) and representative images captured. The expression of each regulator gene in response to addition of EIs was also inferred from measurements of fluorescence (Ex 487, Em 507) using a FLUOstar OPTIMA (BMG Labtech, U.K). Fluorescence was measured in a wild-type strain every 10 min over a 5 h period after the addition of a range of concentrations of the three EIs; PAβN, CCCP and chlorpromazine. Strains were grown in 100 µl of LB broth (inoculated with ~10⁷ cfu/ml) at 30°C with shaking throughout the experiment. Induction of expression of each gene was calculated as the ration of average expression (based on 8 biological replicates) of induced samples compared to un-induced controls. The students 't' test was used to determine significance of differences in *ramA* expression.

The expression of *marA*, *ramA*, *soxS*, *rob* and 16S rRNA were also determined by reverse-transcriptase PCR as previously described.⁷ The expression of *csgBAC* and *csgDEFG* were all determined using comparative RT-PCR, again as previously described.⁹ All primers used in this study are shown in Table 2.

167 ***Staining of curli fimbriae***

168 Phenotypic differences in curli production were visualised by growing strains on agar
169 containing Congo red (40 mg/L, Sigma-Aldrich Ltd., UK) and incubating them for 48h
170 at 30° C as described previously.⁹

171

Results

Mutants lacking a functional *acrB* or *tolC* do not form competent biofilms whereas a mutant lacking *acrA* is able to biofilm

A high throughput biofilm assay using crystal violet to stain cells adhered to a 96 well plate showed a significant decrease in the biofilm formation ability of L829 (*tolC::cat*) and L830 (*acrB::aph*) (figure 1). However, genetic inactivation of *acrA* (L1271 (*acrA::aph*)), the periplasmic adapter protein, had no negative effect on biofilm formation. The phase contrast microscopy images from biofilms formed in a flow cell under shear stress showed a similar pattern as the crystal violet assay with L828 (wild-type) forming a mature biofilm and L829 (*tolC::cat*) and L830 (*acrB::aph*) adhering as individual cells to the flow cell but unable to form a mature, three dimensional biofilm (figure 2).

Mutants lacking a functional *acrB* or *tolC* do not have an altered aggregative ability

To determine whether inactivation of AcrAB-TolC had altered the intrinsic aggregative nature of the strains lacking *acrB* or *tolC* a settle assay was used, this showed no significant difference in the aggregative ability of L829 (*tolC::cat*) or L830 (*acrB::aph*) (Figure S1A). Salt aggregation tests also showed no defect in the mutants' ability to aggregate. In fact, L829 (*tolC::cat*) cells aggregated in a lower concentration of ammonium sulphate than L828 (wild-type) showing a slightly greater tendency for cells to aggregate than the wild-type (Figure S1B).

AcrAB-TolC does not export a factor required for biofilm formation

If a soluble biofilm promoting factor was exported by AcrAB-TolC, addition of culture supernatant conditioned by growth with L828 (wild-type) should be able to rescue the ability of the *tolC* and *acrB* mutant strains to form a biofilm. However, two co-

incubation assays with wild-type and mutant strains suggested that there is no 'biofilm factor' exported by AcrAB-TolC. Transwell assays showed the same poor ability to form a biofilm of the *acrB* and *tolC* mutants when incubated alone or co-incubated with L828 (wild-type) (Figure S2). In addition, no rescue of the biofilm defect was observed when co-incubated with logarithmic or stationary phase cultures of L828 (wild-type) (Figure S2). Similarly, biofilm mat assays co-inoculated with an equal ratio of wild-type and mutants showed that mutant cells did not comprise any of the biofilm mats formed, whereas the corresponding planktonic culture comprised an equal mixture of mutant and wild-type cells (data not shown).

Expression of efflux and biofilm regulator genes differs between *acrB* and *tolC* mutants and an *acrA* mutant

To explore the key observation that L1271 (*acrA::aph*) was not compromised in its ability to form a biofilm we compared the expression of genes known to regulate efflux gene and curli gene expression between this strain and the *acrB* and *tolC* mutants.^{9, 13} This analysis revealed that *ramA* is significantly over-expressed in *acrB* and *tolC* mutants but not in the *acrA* mutant.¹³ Expression of *ramA* in these strains was also measured by RT-PCR and a *ramA* promoter-*gfp* fusion and results were consistent in showing up-regulation of *ramA* in the *acrB* (average ~2 fold) and *tolC* (average ~4 fold) mutants but not in the *acrA* mutant. The transcriptome of a *ramA* over-expressing strain was also investigated to identify alterations in expression of biofilm relevant genes; L786 (SL1344 pTrc::*ramA*) showed repression (two to five fold) of all the curli genes, including *csgDEFG*.^{7, 11} This observation suggested that the biofilm defect in the *acrB* and *tolC* mutants was mediated by repression of curli biosynthesis and that this may be mediated by up-regulation of *ramA*.

The lack of curli production in mutants lacking *acrB* and *tolC* is due to transcriptional repression

Congo red supplemented agar and Congo red staining of bacterial suspensions showed qualitatively and quantitatively that curli expression is repressed in the *tolC* and *acrB* mutants and produced at wild-type levels in the *acrA* mutant.⁹ This lack of curli production was found to result from transcriptional repression of various genes in the curli biosynthetic loci, as measured by cRT-PCR including the regulator *csgD* and all the structural and assembly genes also needed to produce curli. Expression of these genes was repressed in the *tolC* and *acrB* mutants but not in the *acrA* mutant (Figure 3).

Role of RamA, MarA and SoxS in repression of curli production and a loss of biofilm formation

To investigate whether RamA, MarA and SoxS are able to repress biofilm formation each was over-expressed in L828 (wild-type) and the consequences investigated. Artificial over-expression of *ramA* in L828 (wild-type) resulted in a complete loss of the ability of the strain to form a biofilm, over-expression of *marA* and *soxS* also resulted in a loss of biofilm formation although to a lesser extent than that seen with *ramA* (Figure 4). Production of curli on Congo red agar was repressed in each over-expression strain.

Spatial expression of *ramA*, *soxS* and *marA* within colonies of L828 (wild-type), L829 (*tolC::cat*) and L830 (*acrB::aph*) was visualised by fluorescence using regulator-*gfp* reporter plasmids. Figure 5 shows the pattern of expression seen for both *ramA* and *marA* was the inverse of where curli was being produced in each colony on Congo red agar. In wild-type colonies the highest *ramA* and *marA* expression was seen at the perimeter, where curli expression was at it's lowest. In the *tolC* and *acrB* mutant,

a higher level of *ramA* and *marA* expression is seen dispersed throughout the colony, again inversely correlating with phenotypic curli expression.

Inactivation of the global regulators does not restore the ability of a *tolC* mutant to form a competent biofilm

As all three regulators have the ability to repress biofilm formation when overexpressed and both *marA* and *soxS* are up-regulated upon inactivation of *ramA*, all three genes were inactivated to establish if rescue of biofilm formation would occur in *tolC* and *acrB* mutants. Inactivation of each of the regulators alone in the *tolC* and *acrB* mutants failed to rescue curli production and biofilm formation, however loss of each of the regulator genes was followed by consequent up-regulation of the others which may compensate for their inactivation (Figure 6). A series of multiple mutants lacking combinations of the three regulators also failed to rescue biofilm formation (Figure 7 shows the lack of rescue of the *tolC* mutant by loss of *marA*, *soxS* and *ramA*).

Discussion

Multidrug efflux pumps have a central role in the biology of bacteria with roles in drug resistance, cell division, pathogenicity and as recently described the formation of biofilms.^{9,14} Here, we investigated the mechanism which explains the inability of mutants lacking AcrB and TolC, constituents of the major AcrAB-TolC system of *Enterobacteriaceae*, to form a competent biofilm. Mutants of *Salmonella* lacking a functional *tolC* or *acrB* were unable to form biofilms under various conditions and this was not related to any defect in growth, cellular hydrophobicity/aggregative ability or export of a biofilm promoting substrate. Surprisingly a mutant lacking a functional AcrA (but still expressing AcrB),¹⁰ was not defective in its ability to form a biofilm. Loss of *acrA* has previously been shown to result in hyper-susceptibility to various drugs and a decreased ability to attach to and invade epithelial cells in tissue culture.¹⁰ The phenotype of an *acrA* mutant was, however, distinct from that of mutants lacking *acrB*.¹⁰ One major difference between *acrA* and *acrB/tolC* mutants is expression of the global regulator *ramA*,¹³ known to positively regulate expression of *acrAB* and *tolC* as well as other genes. Expression of *ramA* was up-regulated in both *acrB* and *tolC* mutants but not in an *acrA* mutant and analysis of microarray data showed that *ramA* up-regulation was associated with decreased expression of curli biosynthetic genes,¹¹ this led us to hypothesise that curli repression was the reason that the efflux mutants did not form a biofilm and that *ramA* has a role in the co-ordinated regulation of efflux and biofilm formation. We confirmed the absence of curli production in mutants lacking *tolC* or *acrB* is due to strict repression of all the curli biosynthetic genes. We also confirmed that *ramA* over-expression repressed curli production and completely abolished biofilm formation. However, inactivation of *ramA* in the *tolC* and *acrB* mutants did not rescue their ability to form a biofilm. To

determine whether loss of *ramA* expression is compensated by other transcriptional activator genes, *marA*, *soxS* and *rob* expression was measured in mutant strains lacking *ramRA*, *marRA* or *soxRS*.^{8,11,15-17} Loss of *ramRA* resulted in increased expression of *marA* and *soxS*, both of which have some known functional overlap with RamA.¹⁸⁻¹⁹ Furthermore, over-expression of each of *ramA*, *marA* and *soxS* resulted in repression of curli production and biofilm formation. This suggests repression of biofilm formation and curli expression may be a core role for transcriptional activators that respond to stress and co-ordinate efflux up-regulation. The regulation of curli expression is extremely complex with multiple pathways known to impact curli production. Amongst these pathways are two component systems which respond to membrane stress (*cpxRA*, *rscCB*, *envZ/ompR*), *rpoE* and the lytic transglycosylases *mltC* and *mltE*.²⁰ All these systems have membrane or periplasmic bound components demonstrating that curli synthesis is sensitive to changes in the membrane. Whether RamA/MarA/SoxS) act directly to repress curli synthesis or via one of the other currently known pathways which can influence curli repression is not known. Although there is a suggested RamA/MarA/SoxS consensus binding site, there is no good match for this sequence within the curli locus suggesting that the action of these regulators in mediating curli repression is indirect.^{7,21} Whilst we demonstrate here that over-expression of each of *ramA*, *marA* and *soxS* can repress biofilm we were unable to rescue the *acrB* and *tolC* mutants biofilm and curli production defect by inactivation of these genes individually or in combination. Therefore, while we have shown these regulators can repress biofilm formation in a manner phenotypically identical to that seen in response to loss of efflux it is unclear how important they are in mediating the biofilm defect seen in efflux mutants.

311 The demonstration that biofilm formation and efflux are co-ordinately but inversely
312 regulated with loss of function of multidrug efflux resulting in repression of biofilm is
313 interesting. Both de-repression of AcrAB-TolC and formation of a biofilm are in
314 themselves protective against antibiotic action, the inverse regulation observed here
315 seems counterintuitive. This relationship may act as a paradigm for other systems in
316 other species where a link between efflux and biofilm formation exists. It is possible
317 that conditions where efflux is up-regulated in response to stress represent a
318 hazardous environment where formation of a biofilm and the subsequent attachment
319 to a single site is a poor survival strategy explaining the evolution of a genetic switch
320 between the two.

321

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329 **Transparency declaration**

330 None to declare.

331

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Figure legends

Figure 1. Crystal violet biofilm assay quantifies biofilm formation of L828 (wild-type), L829 (*tolC::cat*), L830 (*acrB::aph*) and L1271 (*acrA::aph*), showing genetic inactivation of *tolC* or *acrB* creates an inability to form a biofilm. Asterisks indicate significantly different average values to wild-type ($p < 0.05$).

Figure 2. Phase contrast microscopy images of L828 (wild-type, panel A), L830 (*acrB::aph*, panel B) and L829 (*tolC::cat*, panel C) at 40 X magnification after 48 hours incubation under flow conditions.

Figure 3. Expression of curli genes. A schematic of the curli biosynthesis genes with average expression values determined by RT-PCR used to colour each gene showing repression in the *tolC* and *acrB* mutants. All expression values less than 50% of the wild-type were statistically significantly different ($p < 0.05$).

Figure 4. Over-expression of *ramA*, *marA* or *soxS* represses biofilm formation. The bar chart shows biofilm formation in the crystal violet assay by L828 carrying pTrc-*marA* or pTrc-*soxS* or pTrc-*ramA* without or with induction with 1mM IPTG.

Figure 5. Expression of *ramA* and *marA* is up-regulated in efflux mutants and differentiated spatially within colonies shown by gfp reporter plasmids and correlates with a lack of curli production shown phenotypically on Congo red agar.

Figure 6. Inactivation of the *ram*, *mar* or *sox* loci results in compensatory up-regulation of redundant regulators. The graph shows average expression data from RT-PCR in each mutant and shows that when *ramA* is inactivated in L828 (wild-type) there is a large increase in expression of *marA* and *soxS* to compensate.

Figure 7. Crystal violet biofilm assay shows loss of *ramA*, *marA*, *soxS* and combinations thereof does not rescue *tolC* mutant's biofilm defect. Asterisks indicate significantly different average values to wild-type ($p < 0.05$).

433 **Table 1. List of strains used in this study.**

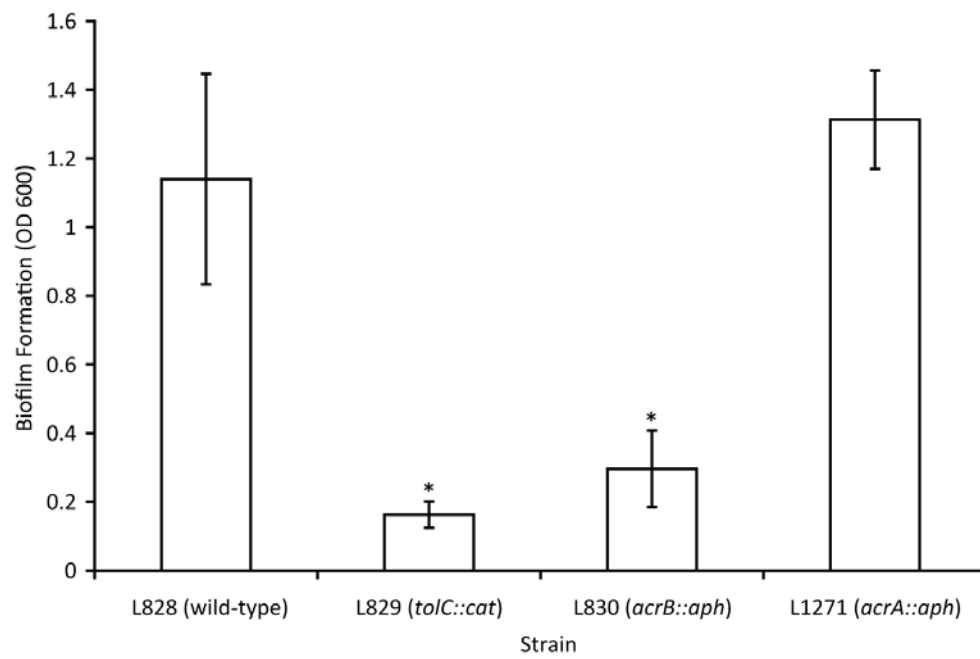
Strain	Genotype	Description	Reference
L828	14028S	Wild-type <i>Salmonella</i> Typhimurium	ATCC
L829	14028S <i>tolC::cat</i>	Mutant lacking TolC	6
L830	14028S <i>acrB::aph</i>	Mutant lacking AcrB	6
L1271	14028S <i>acrA::aph</i>	Mutant lacking AcrA	This study
L1303	14028S <i>ramRA::aph</i>	Mutant lacking RamRA	This study
L1306	14028S <i>tolC::cat, ramRA::aph</i>	Mutant lacking TolC and RamRA	This study
L1506	14028S $\Delta tolC$, $\Delta ramA$	Mutant lacking TolC and RamA	This study
L1507	14028S $\Delta tolC$, $\Delta ramA$, $\Delta soxS$	Mutant lacking TolC, RamA and SoxS	This study
L1508	14028S $\Delta tolC$, $\Delta ramA$, <i>marA::aph</i>	Mutant lacking TolC, RamA and MarA	This study
L1509	14028S $\Delta tolC$, $\Delta ramA$, $\Delta soxS$, <i>marA::aph</i>	Mutant lacking TolC, RamA, SoxS and MarA	This study
L1511	14028S <i>ramA::cat</i>	Mutant lacking RamA	This study
L1512	14028S <i>acrB::aph, ramA::cat</i>	Mutant lacking AcrB and RamA	This study
N/A	<i>E. coli</i> 042	Enterotoaggregative <i>E. coli</i>	ATCC
Plasmid			
<i>pTrc-marA</i>		<i>marA</i> over-expression plasmid	This study
<i>pTrc-ramA</i>		<i>ramA</i> over-expression plasmid	7
<i>pTrc-soxS</i>		<i>soxS</i> over-expression plasmid	This study
<i>pWKS30-marA-gfp</i>		<i>marA</i> gfp reporter plasmid	This study
<i>pWKS30-ramA-gfp</i>		<i>ramA</i> gfp reporter plasmid	This study
<i>pWKS30-soxS-gfp</i>		<i>soxS</i> gfp reporter plasmid	This study

435 **Table 2. Primers used in this study.**

Primer	Sequence	Description
<i>acrB</i> -checkF	GGATCACACCTTATTGCCAG	<i>acrB</i> mutant check forward primer
<i>acrB</i> -checkR	TTAACAGTGATCGTCGGTCG	<i>acrB</i> mutant check reverse primer
<i>tolC</i> -checkF	CTTCTATCATGCCGGCGACC	<i>tolC</i> mutant check forward primer
<i>tolC</i> -checkR	CGCTTGCTGGCACTGACCTT	<i>tolC</i> mutant check reverse primer
<i>acrA</i> -checkF	ACATCCAGGATGTGTTGTCTG	<i>acrA</i> mutant check forward primer
<i>acrA</i> -checkR	CAATCGTCGGATATTGCGCT	<i>acrA</i> mutant check reverse primer
<i>pTrc-ramA</i> F	ATGACCATTTCGCTCAGGT	<i>pTrc-ramA</i> cloning forward primer
<i>pTrc-ramA</i> R	TCAATGCGTACGGCCATGCT	<i>pTrc-ramA</i> cloning reverse primer
<i>pTrc-marA</i> F	ATGTCCAGACGCAACACTGA	<i>pTrc-marA</i> cloning forward primer
<i>pTrc-marA</i> R	CTAGTAGTTGCCATGGTTCA	<i>pTrc-marA</i> cloning reverse primer
<i>pTrc-soxS</i> F	ATGTCGCATCAGCAGATAAT	<i>pTrc-soxS</i> cloning forward primer
<i>pTrc-soxS</i> R	CTACAGGCGGTGACGGTAAT	<i>pTrc-soxS</i> cloning reverse primer
<i>marA</i> -RTF	CGCAACACTGACGCTATTAC	<i>marA</i> qRT-PCR forward primer
<i>marA</i> -RTR	TTCAGCGGCAGCATATAC	<i>marA</i> qRT-PCR reverse primer
<i>ramA</i> -RTF	TCCGCTCAGGTTATCGACAC	<i>ramA</i> qRT-PCR forward primer
<i>ramA</i> -RTR	AGCTTCCGTTACGCACGTA	<i>ramA</i> qRT-PCR reverse primer
<i>soxS</i> -RTF	CATATCGACCAACCGCTA	<i>soxS</i> qRT-PCR forward primer
<i>soxS</i> -RTR	CGGAATACACGCGAGAAG	<i>soxS</i> qRT-PCR reverse primer
16S-RTF	CCTCAGCACATTGACGTTAC	16S qRT-PCR forward primer
16S-RTR	TTCCTCCAGATCTCTACGCA	16S qRT-PCR reverse primer

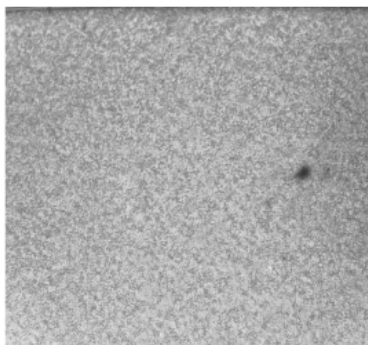
<i>csgA</i> -RTF	AGCATTCGCAGCAATCGTAG	<i>csgA</i> qRT-PCR forward primer
<i>csgA</i> -RTR	TTAGCGTTCCACTGGTCGAT	<i>csgA</i> qRT-PCR reverse primer
<i>csgB</i> -RTF	ATCAGGCGGCCATTATTGGT	<i>csgB</i> qRT-PCR forward primer
<i>csgB</i> -RTR	TACTGGCATCGTTGGCATTG	<i>csgB</i> qRT-PCR reverse primer
<i>csgC</i> -RTF	AATTCTCTCTGTGCGCGACG	<i>csgC</i> qRT-PCR forward primer
<i>csgC</i> -RTR	GCAGTGATTGTCCGTCCGAA	<i>csgC</i> qRT-PCR reverse primer
<i>csgD</i> -RTF	GGTATTCTGCGTGGCGAATG	<i>csgD</i> qRT-PCR forward primer
<i>csgD</i> -RTR	AGTAATGCGGACTCGGTGCT	<i>csgD</i> qRT-PCR reverse primer
<i>csgE</i> -RTF	ACGCTATCTGACCTGGATTG	<i>csgE</i> qRT-PCR forward primer
<i>csgE</i> -RTR	CGTTATGGTGATCCAGCTTC	<i>csgE</i> qRT-PCR reverse primer
<i>csgF</i> -RTF	GACGTTCCAGTTCGCTAATC	<i>csgF</i> qRT-PCR forward primer
<i>csgF</i> -RTR	ATCGTTGGTCACCATACGTC	<i>csgF</i> qRT-PCR reverse primer
<i>csgG</i> -RTF	CTGGAACGACAAGGCTTACA	<i>csgG</i> qRT-PCR forward primer
<i>csgG</i> -RTR	TGATCCAGCTGATACTGCGT	<i>csgG</i> qRT-PCR reverse primer

Figure 1

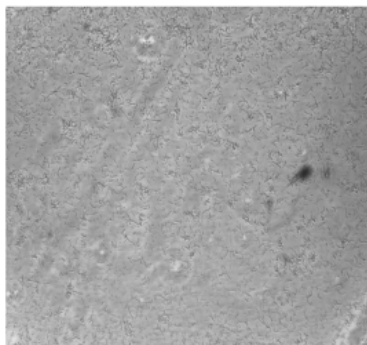


440 **Figure 2**

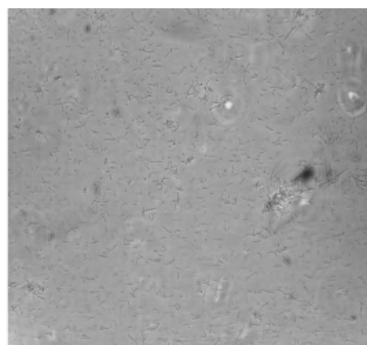
A



B



C



441

442 **Figure 3**

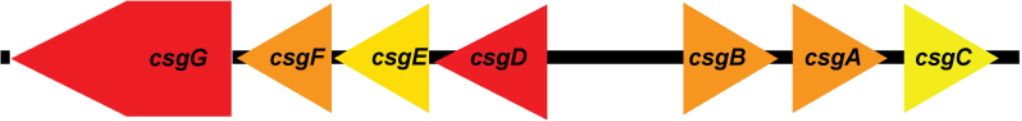
L828 (wild-type)



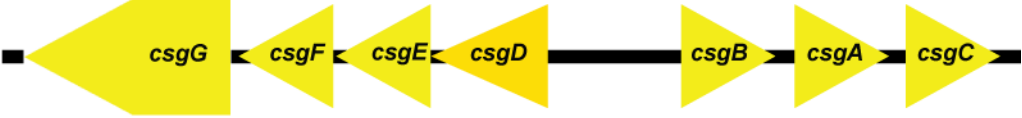
L829 (*tolC::aph*)



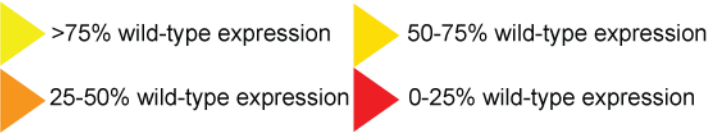
L830 (*acrB::aph*)



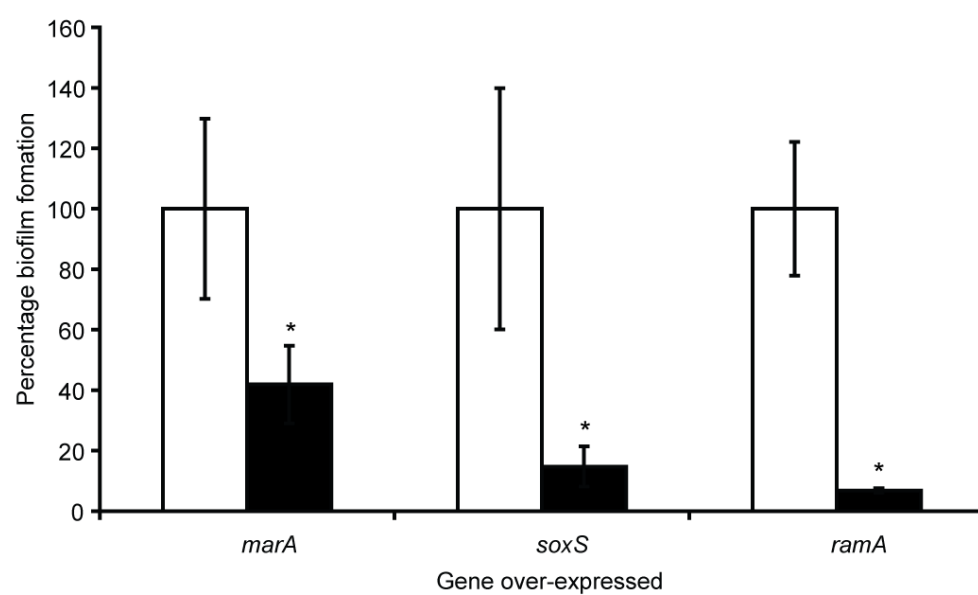
L1271 (*acrA::aph*)



Key (Expression relative to wild-type):

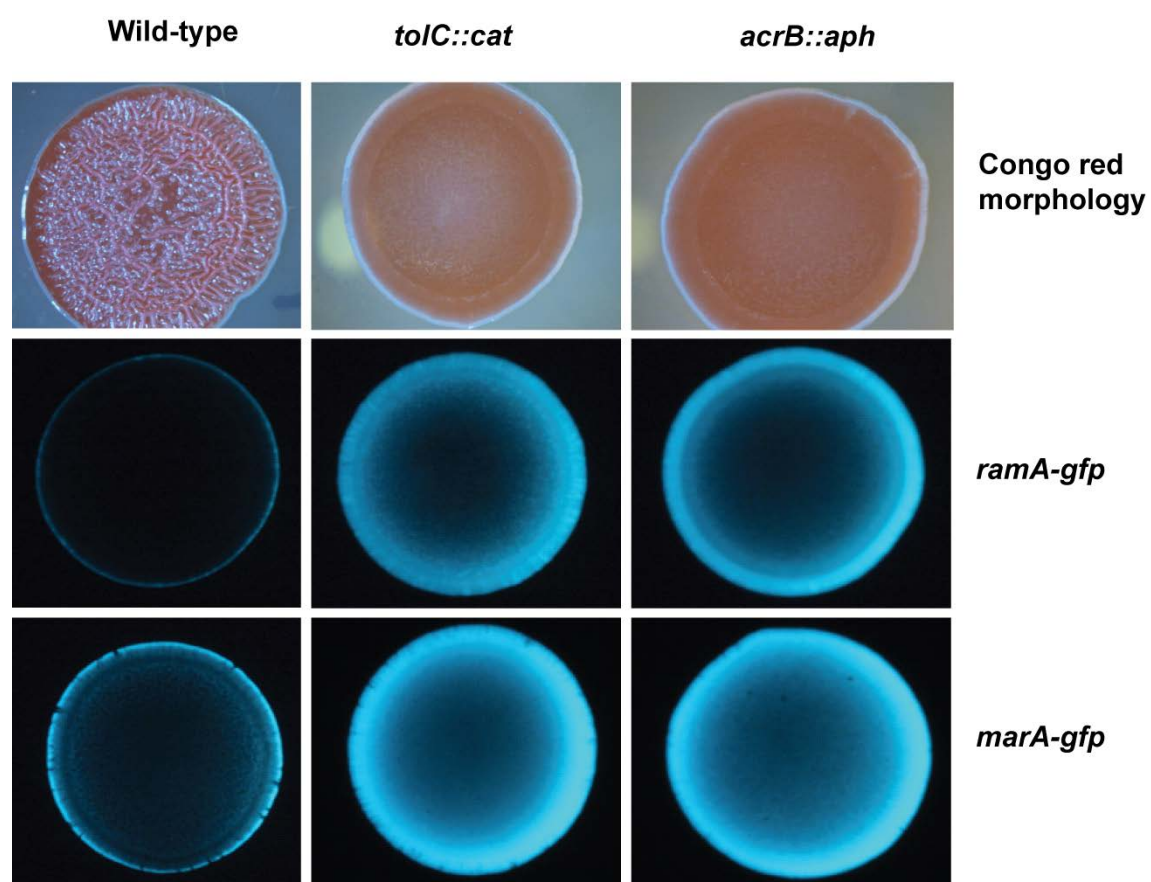


444 **Figure 4**



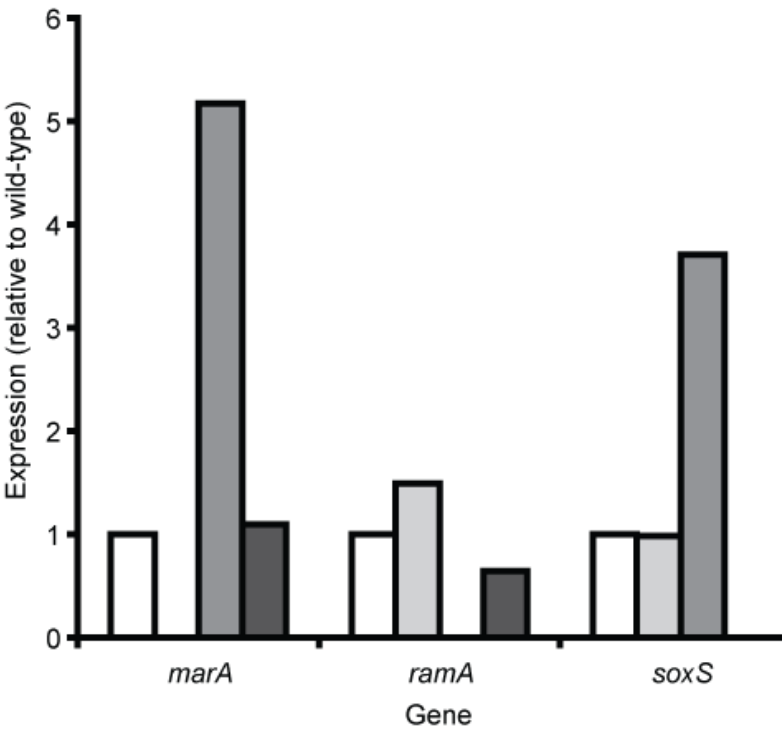
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446 **Figure 5**



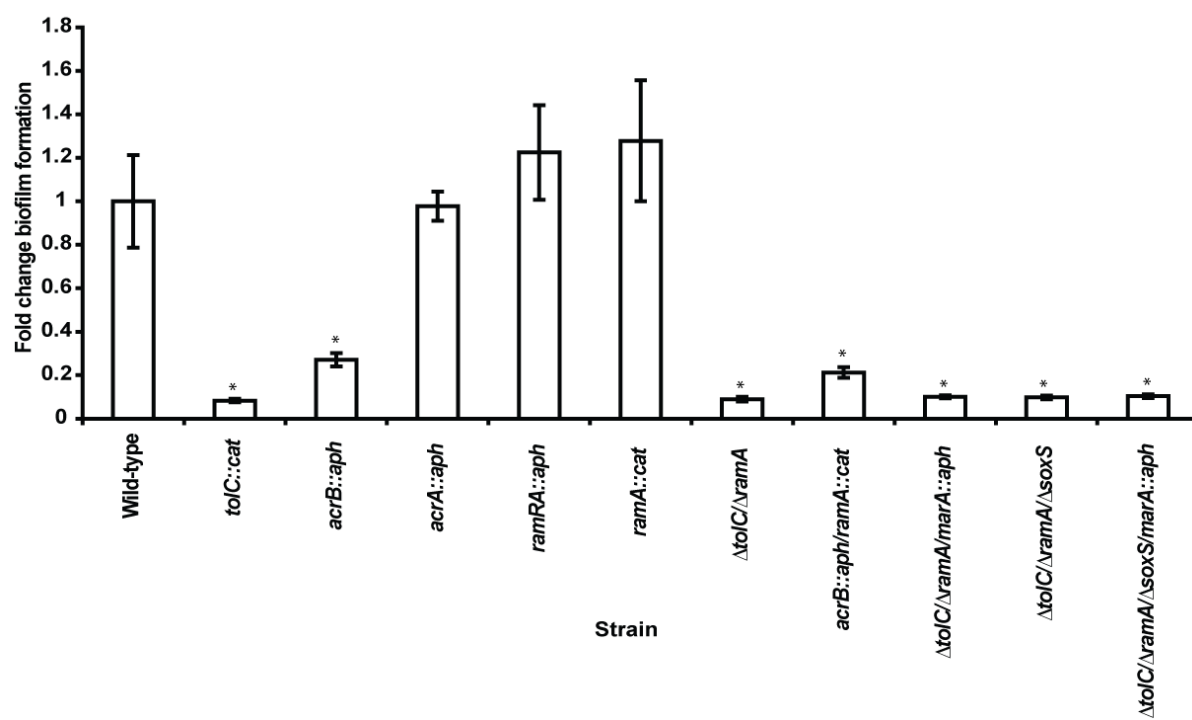
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448 **Figure 6**



449

450 **Figure 7**



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SUPPLEMENTARY FIGURES

Figure S1.

Panel A. Settle assay of L828 (wild-type), L829 (*tolC::cat*) and L830 (*acrB::aph*). *E. coli* O42 was used as a positive, aggregative control. Values indicate the percentage of an initial absorbance from readings taken immediately below the surface of the liquid of a broth which was incubated statically over a 24h period.

Panel B. Salt aggregation test images of L828 (wild-type), L829 (*tolC::cat*) and L830 (*acrB::aph*) in 1M and 2M ammonium sulphate. Aggregation was recorded as formation of a visible precipitate and the lowest concentration of ammonium sulphate to prompt precipitation recorded for each strain.

Figure S2. Transwell assays show no rescue of biofilm formation by mutants when co-incubated with wild-type. **(A)** shows strains incubated with and without the presence of L828 inoculated at the same density as the mutants, **(B)** shows the same experiment but with co-incubation with an overnight, undiluted culture of L828.

468 **Figure S1**

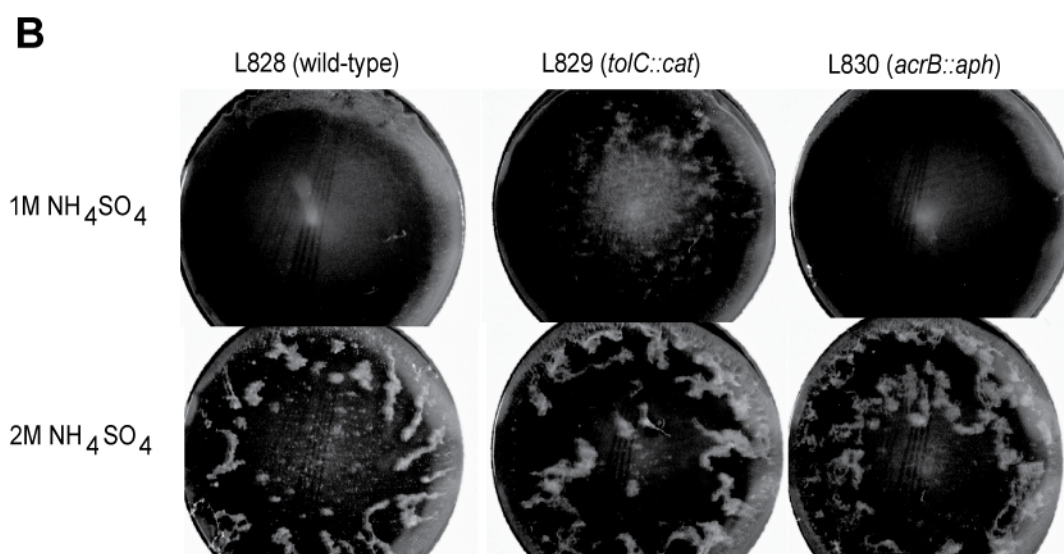
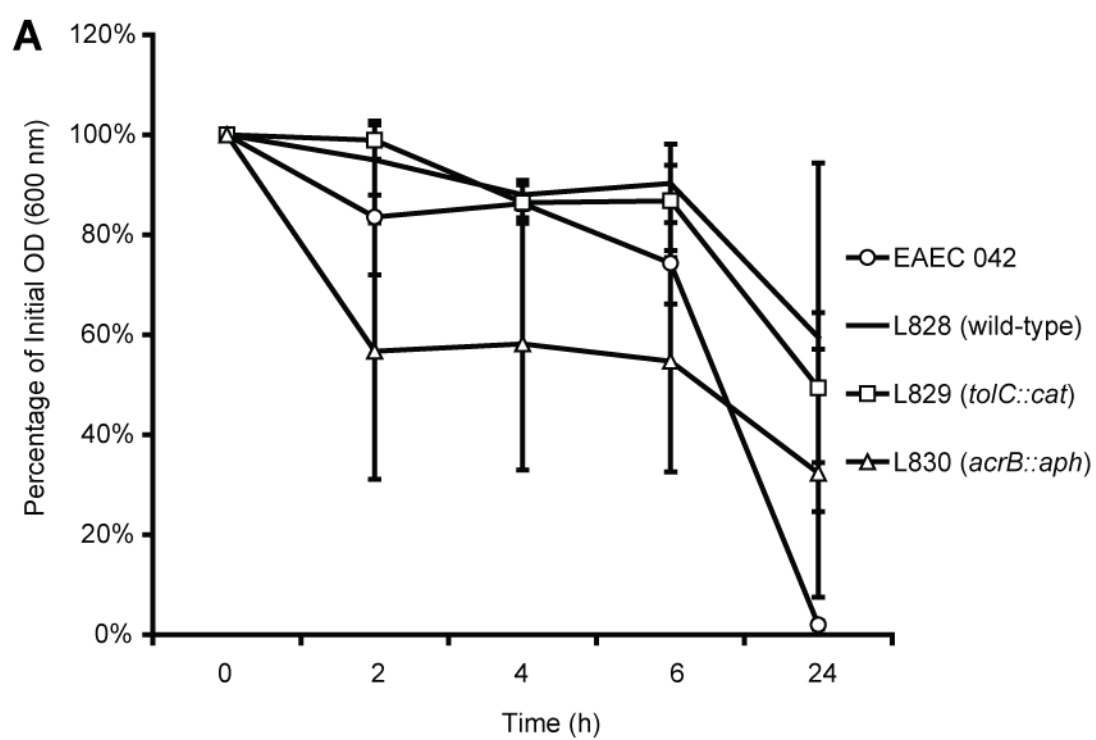


Figure S2

