Loss of or inhibition of all multidrug resistance efflux pumps of Salmonella enterica serovar Typhimurium results in impaired ability to form a biofilm

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Objectives: To investigate the contribution of multidrug efflux pump systems of Salmonella enterica serovar Typhimurium to the formation of a competent biofilm.

Methods: Biofilm formation by a wild-type strain and 10 efflux mutant strains was quantified using crystal violet biofilm assays and visualized using scanning electron microscopy. Curli expression was investigated qualitatively and quantitatively by measuring binding of the dye Congo red to polymerized curli and by comparative RT–PCR.

Results: All efflux mutants of Salmonella Typhimurium were compromised in their ability to form biofilms. Scanning electron microscopy images showed that the mutants were able to adhere to a surface but were unable to form a complex three-dimensional biofilm. Congo red assays demonstrated an inability of the efflux mutants to produce curli, a proteinaceous filament present on the cell surface and an essential component of the Salmonella biofilm extracellular matrix. Mutants expressed significantly less csgB or csgD than wild-type. Chemical inactivation of efflux in wild-type Salmonella Typhimurium with the efflux inhibitors (EIs) phenylarginine-β-naphthylamide, carbonyl cyanide m-chlorophenylhydrazone and chlorpromazine also repressed biofilm formation.

Conclusions: Our data demonstrates a link between all efflux systems of Salmonella Typhimurium and biofilm formation. Loss of functional efflux gives rise to a lack of curli expression. Biofilm formation was also inhibited by addition of a variety of EIs with differing mechanisms of action, suggesting a novel role for EIs as anti-biofilm compounds.

Keywords: curli, RND, MFS, ABC, MATE, efflux inhibitor

Introduction

Biofilms comprise complex, diverse communities of bacteria with an ordered three-dimensional structure,1,2 it has been estimated that >90% of bacteria in nature exist as a biofilm.3 Biofilms are highly differentiated communities that show widespread changes in gene expression and protein production dependent upon location within the biofilm.4,5 Whilst common in nature, biofilms are clinically significant and there are many examples of human infections caused by biofilms, including Pseudomonas aeruginosa in the lungs of cystic fibrosis patients,6 Legionella pneumophila causing legionellosis, many bacterial species involved in periodontal diseases and various device-associated infections of temporary and permanent medical devices.7–13 Biofilms are difficult to eradicate due to their inherent drug resistance; the concentration of many antibiotics needed to kill bacteria when residing in a biofilm can be up to 1000 times greater than that required to kill cells from corresponding planktonic cultures.3 Various factors have been proposed to account for this, including the impermeability of biofilms, metabolic inactivity and the presence of persister cells residing deep within the biofilm that have an extremely slow metabolic rate and thereby escape the action of bacteriostatic antibiotics.14

Salmonella enterica can form biofilms on diverse surfaces (including biofilms formed on gallstones in the gall bladder by Salmonella Typhi), where bacteria can persist and act as a reservoir for re-infection.15,16 Recently, Salmonella Typhimurium has been shown to form biofilms in vivo on tumours in a murine cancer model.17 Many serovars of S. enterica, including Salmonella Typhimurium, are capable of forming biofilms and this

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ability has been implicated in the persistence of non-typhoidal Salmonella in the environment and in industrial, veterinary and medical settings. Biofilm formation on foodstuffs is a source of Salmonella infections and several recent food poisoning outbreaks have been linked to contaminated salad leaves. A wide range of genes have been implicated in the ability of Salmonella to form a biofilm, including genes needed for adhesion and matrix production. Three major genes involved in biofilm formation by Salmonella Typhimurium are csgD, adeA and bapA. CsgD and AdeA are involved in the regulation of curli and cellulose production, the two major components of Salmonella biofilm extracellular matrix. BapA is a large protein expressed on the surface of the outer membrane that is also required for Salmonella to form competent biofilms.

Another key mechanism of antibiotic resistance is efflux. The AcrAB–ToIC efflux system, found in Salmonella Typhimurium and other Gram-negative bacteria, is the best characterized MDR system and is the archetype of the RND family. It comprises AcrB, an inner membrane efflux pump, AcrA, a periplasmic adapter protein, and TolC, an outer membrane channel. AcrAB–ToIC can export a wide variety of substrates, including biocides, dyes and detergents, and has been found to have roles in multidrug resistance and virulence. AcrEF (a homologue of AcrAB) has also been shown to have a role in cell division. We have recently shown that a number of MDR efflux systems, including AcrAB–ToIC, have a role in the formation of competent biofilms of Salmonella Typhimurium. Kvist et al. have also described a biofilm defect for various efflux mutants in Gram-negative species, including E. coli and Klebsiella.

Here, we demonstrate a biofilm defect for mutants lacking any of the multidrug resistance efflux pumps of Salmonella Typhimurium. We also show that this is due to a lack of production of curli fibres on the surface of the cell. Chemical inhibitors of efflux pumps also repressed biofilm formation, indicating a possible novel application for efflux inhibitors (EIs) in addition to their known ability to reduce drug resistance and inhibit pathogenicity.

**Materials and methods**

**Strains and growth media**

All strains used in this study and their origins are shown in Table 1. Salmonella Typhimurium ATCC 14028S (L828) was used as a control strain throughout. Isogenic derivatives, where each MDR efflux system is inactivated, have been described previously. Mutants of all classes of the MDR system were used in this study: RND mutants lacked a functional acrB, acrD, acrEF, mdtABC or mdsABD, MFS mutants lacked emrAB or mdtA, a MATE mutant lacked mdtK, an ABC mutant lacked macAB and a TolC mutant lacked the outer membrane efflux channel that partners most of these systems. Strains were stored at −20 °C on ProtecTM beads and routinely cultured on Luria–Bertani (LB) agar or broth unless stated otherwise.

**Biofilm formation assays**

A variety of models were used to analyse biofilm formation in this study.

**Crystal violet biofilm assay**

Overnight cultures of strains were diluted in fresh, antibiotic-free LB broth without salt to an optical density (OD) of 0.1 at 600 nm. Ninety-six-well polystyrene microtitre trays (Sterilin) were inoculated with 200 μL of this suspension and incubated at 30 °C for 48 h with gentle agitation. After incubation, liquid was removed from all wells and the 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 min. Crystal violet was removed and each well washed with sterile distilled water to remove any unbound dye. The stained biofilm was solubilized by adding 200 μL of 70% ethanol and the OD was measured at 600 nm using a FLUOstar Optima (BMG Labtech). All biofilm assays were performed three times with two biological and four technical replicates per repeat. Student’s t-test was used to compare the statistical significance of results from each mutant to L828 (wild-type).

**Biofilm mat model**

Bacterial suspensions were prepared as above, and 1 mL of this suspension was used to inoculate 24-well polystyrene microtitre trays (Falcon). Trays were then incubated statically at room temperature, 30 °C or 37 °C for 3–5 days until thick biofilm mats were formed on the surface of the liquid. The biofilm mats could then be harvested for subsequent analyses.

**Biofilm assay with the addition of exogenous EIs**

Phenyl-arginine-β-naphtylamide (PAβN), carbonyl cyanide m-chlorophenylhydrazone (CCCP) and chlorpromazine (CPZ) were used as EIs. The assay was performed as described above, with the addition of 200 μL of each EI at 250 μM to each well prior to the inoculation of the biofilm mat model. Student’s t-test was used to compare the statistical significance of results from each mutant to L828 (wild-type).

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>L828</td>
<td>14028S (wild-type)</td>
<td>wild-type</td>
<td>ATCC</td>
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<tr>
<td>L829</td>
<td>14028S tolC::cat</td>
<td>mutant lacking functional ToIC</td>
<td>31</td>
</tr>
<tr>
<td>L830</td>
<td>14028S acrB::aph</td>
<td>mutant lacking functional AcrB</td>
<td>31</td>
</tr>
<tr>
<td>L831</td>
<td>14028S acrD::cat</td>
<td>mutant lacking functional AcrD</td>
<td>31</td>
</tr>
<tr>
<td>L832</td>
<td>14028S acrEF::cat</td>
<td>mutant lacking functional AcrEF</td>
<td>31</td>
</tr>
<tr>
<td>L833</td>
<td>14028S mdtABC::cat</td>
<td>mutant lacking functional MdtABC</td>
<td>31</td>
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<tr>
<td>L834</td>
<td>14028S mdsABC::cat</td>
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<td>31</td>
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<td>mutant lacking functional EmrAB</td>
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<td>31</td>
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<td>31</td>
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<tr>
<td>L838</td>
<td>14028S macAB::cat</td>
<td>mutant lacking functional MacAB</td>
<td>31</td>
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<td>L971</td>
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<td>complemented tolC::cat</td>
<td>this study</td>
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<td>14028S acrB::aph + pWKS30-acrB</td>
<td>complemented acrB::aph</td>
<td>this study</td>
</tr>
</tbody>
</table>
to observe the effect of EIs on biofilm formation. Biofilms were established as above in the crystal violet biofilm assay but with the addition of doubling dilutions of exogenous EI from 1 to 2048 mg/L.

**Scanning electron microscopy of biofilms**

To visualize biofilms using a scanning electron microscope, biofilms of L828 (wild-type) and efflux mutant strains were grown on 10 mm square glass slides submerged in LB broth without salt for 72 h. Slides were then removed from the broth, immersed in fixative and visualized under a scanning electron microscope at a range of magnifications.

**Determination of growth kinetics**

The growth kinetics of all strains were determined as previously described by measuring the OD of at least three biological replicate cultures automatically in a FLUOstar Optima. Growth was analysed over a 16 h period at 30 °C in fresh LB broth without salt.

**Staining of curli fimbriae**

Phenotypic differences in curli expression were visualized by staining with Congo red (Sigma-Aldrich Ltd, UK) as follows. The presence of curli protein on the surface of the outer membrane was detected by diluting overnight cultures 1:10000 in PBS and dispensing 5 μL of this final cell suspension onto LB agar without salt supplemented with 40 mg/L Congo red and incubating for 48 h at 30 °C as described previously. Red, dry and rough colonies were observed for strains producing curli and cellulose. Pink colonies were observed for strains producing cellulose but no curli. Comparisons were made between wild-type and mutants and photographs were taken from the same plates.

**Expression of curli biosynthesis genes**

In order to determine whether loss of curli production was transcriptional or due to an assembly defect, the expression of csgB and csgD, the first genes of any of the two curli biosynthetic operons, was determined for all mutants. Comparative RT–PCR was used to determine the average expression from RNA harvested from at least six independent replica cultures of each strain. RNA extraction, cDNA preparation and PCR quantification were as described previously. Cultures were grown to stationary phase overnight at 30 °C as for the biofilm experiments (see above) before cells were harvested. Primers used were as follows: csgB, ATCAGGGCGGCAATTTGCTT; csgD, GTATCGCGTCCGAGGCTGCT. Expression of 16S rRNA was used as a control as previously described. Student’s t-test was used to compare results statistically.

**Vector construction**

Complementation vectors pacrB and ptolC, derived from the low copy number plasmid pWKS30, have been described previously. Overexpression of components of AcrAB–TolC was achieved using the pTRCHis2–TOPO vector (Invitrogen). Primers were designed to amplify the entire coding sequence of acrB or tolC, including the native stop codons, to prevent production of a recombinant protein containing the c-myc epitope and polyhistidine tags present in the vector. The PCR was used to amplify each specific insert, and these inserts were then cloned into the pTRCHis2–TOPO vector according to the manufacturer’s recommendations. Restriction digests with EcoRI (acrB construct) or PvuI (tolC construct) followed by agarose gel electrophoresis and sequencing of products were used to verify correct construction of each vector. After plasmids had been introduced into the efflux mutants and selected by growth on ampicillin (50 mg/L), subsequent biofilm assays used antibiotic-free media (pWKS30 is retained stably for >50 generations).

**Results**

**Mutants lacking MDR efflux systems are compromised in their ability to form biofilms**

Both the crystal violet biofilm assay (Figure 1) and the biofilm mat model were used to determine the ability of L828 (wild-type) and mutants [L829 (tolC::cat), L830 (acrB::aph), L832 (acrD::cat), L833 (acrEF::cat), L834 (mdtABC::cat), L835 (mdsABC::cat), L836 (emrAB::cat), L837 (mdfA::cat), L838 (mdtK::cat) and L839 (macAB::cat)] to form biofilms. All strains lacking any of the different efflux pumps were unable to form a competent biofilm in the crystal violet model. Scanning electron microscopy images of biofilm mutants showed a stark difference in ability to form extracellular matrix. Figure 2 shows scanning electron microscopy images of representative mutants after growth on glass. L828 (wild-type) was able to produce large amounts of extracellular matrix; however, L829 (tolC::cat) and L830 (acrB::aph) were able to adhere to the glass slides but did not produce any extracellular matrix and did not form mature biofilms (Figure 2). None of the mutants formed a biofilm in the mat model at any of the temperatures tested, whereas L828 (wild-type) formed a thick biofilm after 48 h (data not shown). Mutants were unable to form a biofilm in either model even after extended incubation times up to 96 h.

**Complementation of AcrB or TolC rescues mutants’ ability to form a biofilm**

Complementation vectors were constructed for the tolC::cat and acrB::aph mutants to restore expression of the inactivated genes, and the resulting strains returned to wild-type biofilm phenotype with the addition of wild-type tolC or acrB on a plasmid (Figure 3). Complementation of the tolC and acrB mutants with wild-type alleles carried on either pWKS30 or pTRC resulted in restoration of biofilm formation back to wild-type levels.

**Growth rates of efflux mutants are not compromised**

To determine whether the inability of efflux mutants to form biofilms was related to a general growth deficit, the ability of each strain to grow was compared with that of L828 (wild-type). The results showed that all mutants displayed similar growth kinetics to the wild-type parent strain over the duration of the experiment (Figure 4).

**Mutants lacking MDR efflux systems do not express curli genes**

Use of Congo red-supplemented agar showed qualitatively that curli production was significantly reduced in all efflux mutants (Figure 5) that fail to present the classic ‘RDAR’ (red, dry and rough) phenotype observed when curli is present. Mutant colonies were smooth and pink, indicating a lack of curli production but normal production of cellulose. Production of cellulose was also assessed using calcofluor agar and expression of cellulose biosynthesis genes was measured by cRT–PCR; no decreased
Figure 1. Crystal violet biofilm assay of all strains. Biofilm formation by L828 (wild-type), L829 (acrC::cat), L830 (acrB::aph), L832 (acrD::cat), L833 (acrEF::cat), L834 (mdtABC::cat), L835 (mdsABC::cat), L836 (emrAB::cat), L837 (mdfA::cat), L838 (mdtK::cat) and L839 (macAB::cat). Biofilms were formed in polystyrene microtitre trays and quantified by measuring the OD (at 600 nm) of dissolved crystal violet after 48 h. Values marked with an asterisk indicate those significantly different from L828 using Student’s t-test (*P<0.05).

Figure 2. Scanning electron microscopy images of L828 (wild-type), L829 (acrC::cat) and L830 (acrB::aph). All strains were incubated on glass slides for 72 h in LB without salt and biofilms viewed at ×20000, ×40000 and ×100000 magnification.
production or expression of cellulose was apparent in the mutants (data not shown), consistent with the RDAR phenotype seen on Congo red plates.

The expression of \textit{csgB} and \textit{csgD}, the first genes in both the curli biosynthetic pathways, was also determined in all strains (Figure 6). Expression of \textit{csgB} was significantly reduced, by between 2- and 60-fold, in all the efflux mutants (an average of 13-fold reduction in expression compared with L828) (Figure 6). Expression of \textit{csgD} was also significantly reduced in nine of the ten mutants studied, with between 2- and 13-fold reduced expression (an average of 4-fold repression) compared with L828. The expression of \textit{csgD} was lowest in mutants lacking an RND class efflux system. These mutants showed very low expression of both \textit{csgB} and \textit{csgD}, whereas mutants of other classes showed smaller reductions in \textit{csgD} expression.

\textbf{Biofilm formation is impeded by chemical inhibition of efflux}

To determine the effect of chemical inhibition of efflux, crystal violet biofilm assays of L828 (wild-type) were carried out in the presence of three EIs with differing mechanisms of action.

![Figure 3](https://academic.oup.com/jac/article-abstract/67/10/2409/723887)

\textbf{Figure 3.} Crystal violet biofilm formation assay showing the rescued biofilm phenotype upon complementation of L829 (\textit{tolC::cat}) and L830 (\textit{acrB::aph}). Complementation of \textit{tolC} or \textit{acrB} mutations restores the ability of mutant strains to form biofilm. Asterisks indicate significantly different average values to the mutant after complementation.

![Figure 4](https://academic.oup.com/jac/article-abstract/67/10/2409/723887)

\textbf{Figure 4.} Growth kinetics of mutant strains of each class of efflux pump. Lines show the average OD for each strain at each timepoint over a 16 h period. Mutants of different classes of efflux pump are shown in different panels: (a) RND class mutants; (b) MFS class mutants; (c) MATE class mutants; and (d) ABC class mutants.
Figure 5. Colony morphologies of all efflux mutants on Congo red agar. Mutants unable to form a biofilm do not produce curli, as shown by morphologies on Congo red agar. L828 (wild-type) shows a red and rough morphology, indicative of curli production. L829 (tolC::cat), L830 (acrB::aph), L832 (acrD::cat), L833 (acrEF::cat), L834 (mdtABC::cat), L835 (mdsABC::cat), L836 (emrAB::cat), L837 (mdfA::cat), L838 (mdtK::cat) and L839 (macAB::cat) show a smooth, pink morphology indicative of a lack of curli production. Pictures show colonies grown on the same plate.

Figure 6. Expression of csgB and csgD by all mutants. Mutants unable to form a biofilm show decreased expression of curli genes. Expression of csgB (white bars) and csgD (black bars) was determined by comparative RT–PCR for all strains. Bars indicate average expression values relative to L828, error bars show standard deviations and results significantly different from L828 are marked with asterisks.
PAβN is a competitive inhibitor of RND pumps, CCCP abolishes the proton motive force and the efflux inhibition mechanism of chlorpromazine is as yet still unclear. The same effect was observed for all three EIs: increasing EI concentration decreased biofilm formation (Figure 7). This effect was significant, with the degree of inhibition of the amount of biofilm formed by L828 (wild-type) resulting from addition of the EIs being similar to that seen as a result of genetic inactivation of the efflux pumps. PAβN and CCCP were most effective at preventing biofilm formation, with CCCP in particular fully repressing biofilm formation at a concentration of only 1 mg/L. The inhibition of biofilm formation was not a result of antibacterial
action as the anti-biofilm effect occurred at significantly lower concentrations than the MIC of each EI.

Discussion

Multidrug efflux pumps have been recognized as playing a central role in the biology of bacteria and have roles in drug resistance, cell division, pathogenicity and, as recently described, the formation of biofilms. Here, we demonstrate the inability of mutants lacking any of the nine multidrug resistance efflux pumps of Salmonella to form a mature biofilm. Efflux mutants of Salmonella were unable to form biofilms under various conditions and this biofilm defect was not due to any decrease in growth rates. Here we show phenotypically that the biofilm defect in all the efflux mutants is associated with a lack of curli production. The fact that all the MDR efflux pumps of Salmonella can influence biofilm formation is interesting because these pumps are known to have different roles in terms of substrate export and contribution to virulence.

The AcrAB–ToLC system is usually considered the major efflux system in Salmonella, with many of the others cryptic in laboratory conditions. The demonstration that loss of any of the MDR efflux systems results in a common phenotype is novel and demonstrates coordinated regulation of efflux and biofilm formation. Curli is an essential component of the Salmonella extracellular matrix and without it biofilm formation is greatly reduced. We show here that transcriptional repression of the two curli biosynthetic operons is responsible for loss of curli production in the efflux mutants and thereby biofilm formation. The mechanisms of regulation of curli are complex, and in another article we describe the regulatory relationship between multidrug efflux and biofilm formation and show a mechanism explaining coordinated regulation of efflux and curli biosynthesis that is mediated by global regulators of efflux (S. Baugh, A. S. Ekanayaka, L. J. V. Piddock and M. A. Webber, unpublished results).

We observed that, in addition to genetically disrupting any of the MDR efflux systems in Salmonella Typhimurium, chemical inactivation of MDR efflux using EIs, with different mechanisms of inhibitory action, all repressed biofilm formation. Of the EIs tested, PAuN, a competitive inhibitor of RND efflux pumps, and CCCP, a proton motive force uncoupler, had the greatest ability to repress biofilm formation. This suggests it is the function of the pumps that is important for biofilm formation rather than any structural role. The EIs were able to repress biofilm to the same extent as that seen in efflux mutants, demonstrating a good degree of potency of all three EIs tested. This indicates a new potential application for EIs as anti-biofilm agents. The development of compounds that can inhibit efflux and be therapeutically valuable is an area of active research. The demonstration that EIs can prevent biofilm formation whilst also potentiating antibiotics and possibly reducing the pathogenic potential of virulent bacteria suggests there may be value in developing EIs for incorporation into materials for surfaces that require sterility to be maintained.

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Transparency declarations

None to declare.

References