The Role of Multidrug Efflux Pumps in Biofilm Formation of *Salmonella enterica* serovar Typhimurium

By

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Abstract

Multidrug resistance (MDR) efflux pumps and biofilm formation are two mechanisms by which bacteria can evade the action of many antimicrobials. MDR efflux pumps are able to confer low level multidrug resistance and have found to be over-expressed in MDR clinical isolates. Biofilms are three dimensional, complex, dynamic communities of bacteria encased in a self produced extra cellular matrix. Biofilms protect the bacteria that reside within them both physically by acting as a barrier to any external threats, and metabolically by containing high proportions of persister cells within the biofilm that are metabolically dormant resisting the action of bacteriostatic antimicrobials.

This thesis explores the link between MDR efflux and biofilm formation in *S.* Typhimurium and shows that genetic inactivation of any one of the nine MDR efflux systems results in a biofilm defect. We found that the transcriptional repression of curli, an essential component of the *Salmonella* extracellular matrix, is the reason for the efflux mutants' inability to form a biofilm.

Specifically, the biofilm defect in a strain lacking a functional TolC (outer membrane porin) was able to be rescued by inactivation of a gene encoding a osmolarity sensor, *envZ,* suggesting that activation of a membrane stress response that mediates curli repression is the reason for the efflux mutant's biofilm defect.

Three compounds that are known to inhibit efflux were also found to impart curli repression and cause a subsequent biofilm defect in *S*. Typhimurium. This finding is of clinical relevance as biofilms are a major cause of infection and efflux inhibitors could be a potential novel future anti-biofilm compound.

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List of Abbreviations

Abbreviation	Definition
µg	Micrograms
µl	Microlitres
µM	Micromolar
Ab	Antibiotic
ABC	ATP binding cassette
acr	Acriflavin resistance
aph	Aminoglycoside phosphotransferase
API	Analytical profile index
ATP	Adenosin triphosphate
Bcc	Burkholdeia cepacia complex
bp	Base pairs
cat	Chloramphenicol acetyltransferase
CCCP	Carbonyl cyanide m-chlorophenyl hydrazone
c-di-GMP	Cyclic diguanylic acid
cDNA	Complimentary DNA
CF	Cystic fibrosis
cfu	Colony forming units
CPZ	Chlorpromazine
DHFR	Dihydrofolate reductase
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
EI	Efflux inhibitor
g	Gravity

g	Grams
gfp	Green fluorescence protein
HPA	Health Protection Agency
l	Litres
LB	Lysogeny broth
LPS	Lipopolysaccharide
M	Molar
MATE	Multidrug and toxic compound extrusion family
MDR	Multidrug resistance
MFS	Major facilitator superfamily
mg	Milligrams
min	Minute
MLST	Multilocus sequence typing
MTT	Microtitre tray
NMP	1-(1-naphtylmethyl)-piperazine
NTS	Non typhoidal Salmonellosis
OD	Optical density
PAβN	Phe-arg-beta-napthylamide
PCR	Polymerase chain reaction
PDE	Phosphodiesterase
RATE PCR	Rapid amplifaction of transposon ends PCR
RDAR	Red, dry and rough
RNA	Ribonucleic acid
RND	Resistance nodulation division
rpm	Revolutions per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS-PAGE	Sodium dodecyl xxiiiulphate polyacrylamide gel electrophoresis

SEM	Scanning electron microscopy
SMR	Small multidrug resistance
SOC	Super optimal broth “catabolic repression”
TBE	Tris-borate-EDTA
tol	Colicin tolerance
WHO	World Health Organisation
XLD	Xylose lysine deoxycholate

CHAPTER ONE:

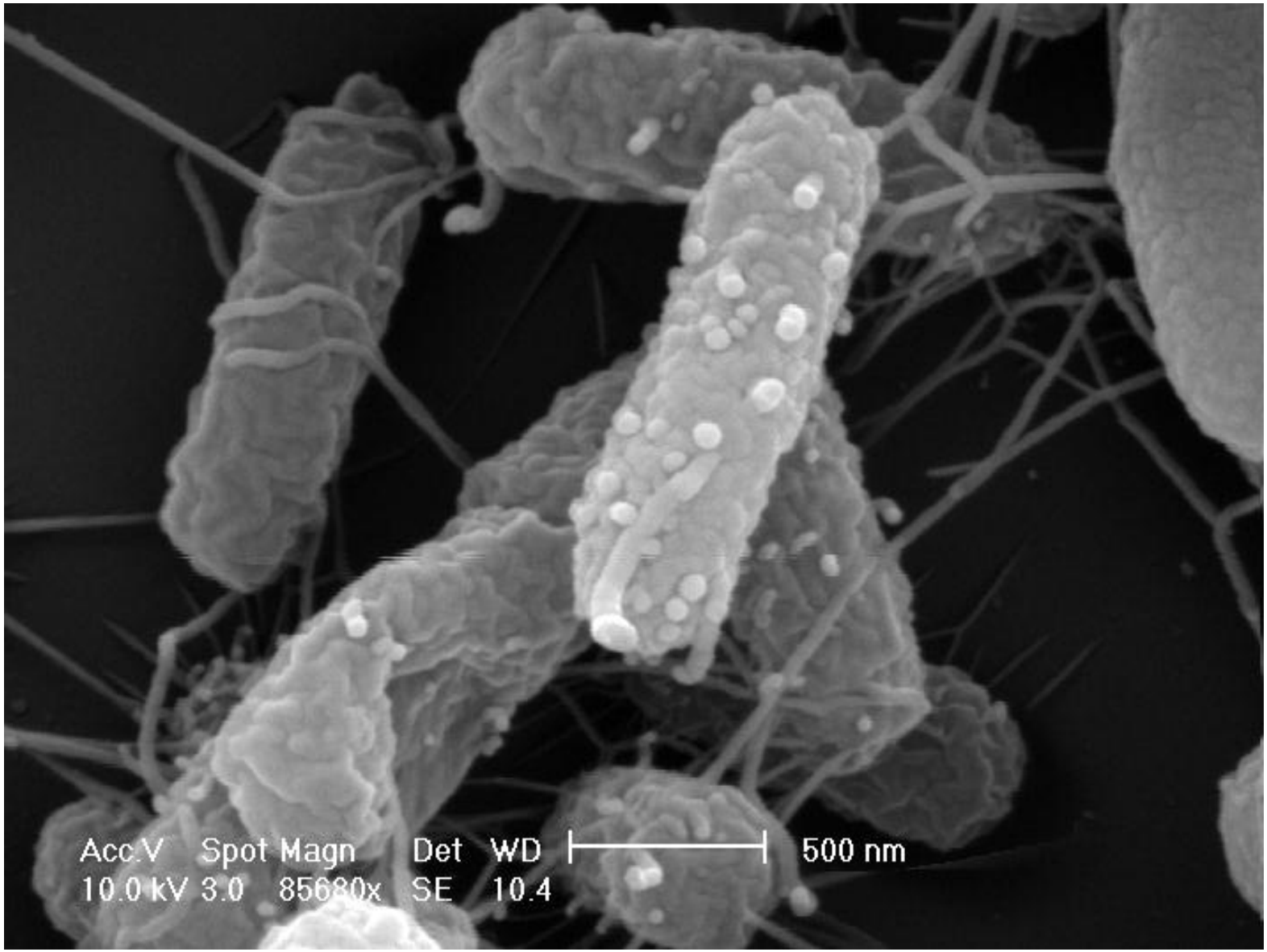
INTRODUCTION

1 Introduction

1.1 *Salmonella*

Theobald Smith first discovered and named *Salmonella* after isolation from pigs, although it was named after his supervisor, Daniel Elmer Salmon. It was first described in the literature in 1885 (Salmon 1885; Salmon 1886). *Salmonella* is a Gram negative rod shaped member of the Enterobacteriaceae family (Figure 1.1) and was discovered by Herbert Durham in 1898 that strains of *Salmonella* can be split into distinct groups using agglutination tests (Mochmann and Kohler 1989; Hardy 2004). Serotyping was used as a means to discriminate between different types or serovars of *Salmonella* for many years and helped study the epidemiology of *Salmonella* infections (Hardy 2004). The introduction of genomic analysis has revealed the *Salmonella* genus contains two distinct species; *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* can be further categorised into six sub-species; *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae* and *indica* (Kauffmann 1966; Crosa *et al.* 1973; Le Minor *et al.* 1982; Le Minor *et al.* 1982; Brenner *et al.* 2000; Fookes *et al.* 2011) and contains over 2500 serovars including those pathogenic to man. These serovars are distinguished serologically by the combinations of somatic, flagella and capsular antigens present on the surface of the outer membrane (Mochmann and Kohler 1989; Tindall *et al.* 2005). The use of multilocus sequence typing (MLST) has recently been shown to be superior to serotyping for the analysis of *Salmonella* population structure as serological characteristics are not fixed and can change in isolation from the genome as a whole (Achtman *et al.* 2012).

Figure 1.1 Scanning electron microscopy image of *Salmonella enterica* serovar Typhimurium



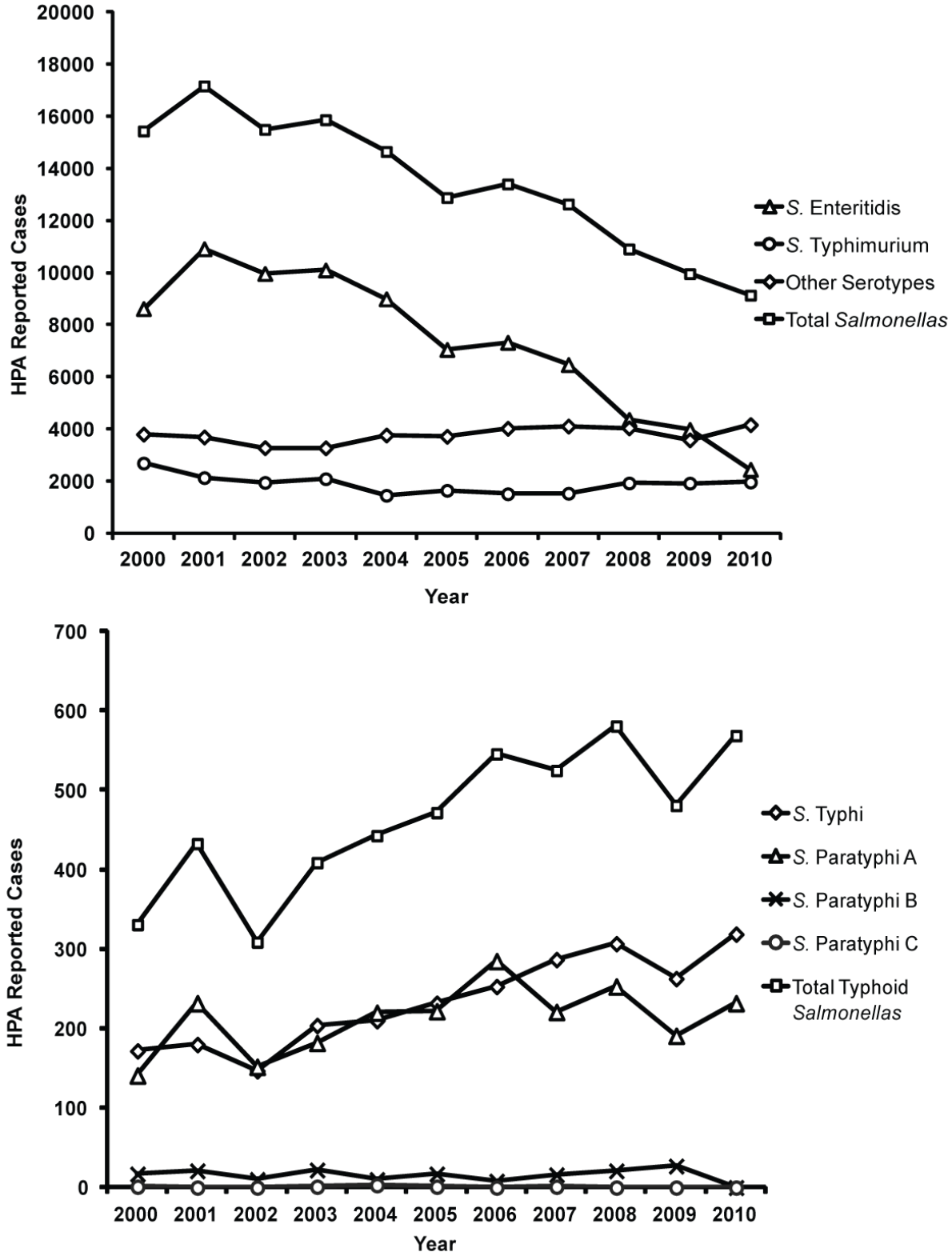
Salmonella has many different natural reservoirs including the gastrointestinal tracts of many animals including chickens and other poultry (Gast *et al.* 2005), pigs (Boyen *et al.* 2008) and rodents (Valdezate *et al.* 2007), the skin of reptiles including lizards, snakes and turtles (Lamm *et al.* 1972; Pedersen *et al.* 2009) and amphibians including frogs (Anon 2010).

1.1.1 *Salmonella* infections

Salmonella infections have been amongst the major causes of food poisoning in the developing world for almost the whole of the 20th century but their importance has only been recognised in the last few decades (Hardy 2004). All infections caused by *Salmonella* are known as salmonellosis, of which there are two main types, non-typhoidal and typhoidal (Valdezate *et al.* 2007). Figure 1.2 shows the number of cases of both non-typhoidal and typhoidal *Salmonella* in England and Wales reported during the last decade; 2000-2010 (http://www.hpa.org.uk).

Non-typhoidal salmonellosis (NTS) is most commonly caused by the serovars *Salmonella enterica* serovar Enteritidis (hereafter *S*. Enteritidis) and *Salmonella enterica* serovar Typhimurium (hereafter *S*. Typhimurium) in human hosts. In England and Wales approximately 2444 and 1959 cases of *S*. Enteritidis and *S*. Typhimurium, respectively, were reported in 2010 making up nearly half of all reported NTS cases (http://www.hpa.org.uk). NTS is the second largest cause of bacterial gastroenteritis in the U.K., the first being *Campylobacter* (Lucey *et al.* 2000; Natrajan and Sheldon 2000; http://www.hpa.org.uk 2010).

Figure 1.2 Public Health England reports of *Salmonella* infections (both non-typhoidal Salmonellosis and typhoidal Salmonellosis,, data shown by serovar) from the last decade, 2000 – 2010. (Latest figures as of July 2013)



Salmonella infections are frequently transmitted via contaminated poultry and eggs (Valdezate *et al.* 2007), however *Salmonella* is a commensal in many animals intestines and so can be transmitted via a variety of different meats (Hardy 2004). It is also thought to be contracted through vegetables or other products contaminated with manure (http://www.who.int 2010). Symptoms of NTS are mainly sickness, diarrhoea, (Valdezate *et al.* 2007) abdominal pain and fever and it is usually a self limiting condition (http://www.emedicine.com 2010). However, the infection can spread to the blood stream where intervention with antibiotics, such as ciprofloxacin or ceftriaxone is required (http://www.emedicine.com; Dutta *et al.* 1995; Lin *et al.* 2003). Reported NTS infections have almost halved in England and Wales in the last decade (from 15,104 cases in 2000 to 8,564 in 2010) although a significant number of people still fall ill because of this organism (Figure 1.2). Enteric fever is caused by *Salmonella enterica* serovar Typhi (Typhoid fever) and *Salmonella enterica* serovar Paratyphi (Paratyphoid fever) (Crump and Mintz 2010). Infections with these serovars are contracted via the faecal-oral route and have no animal or environmental reservoirs; they are entirely adapted to humans (Mandell GL *et al.* 2005). Symptoms include profuse sweating, sickness and a rash of flat rose coloured spots (Mathura *et al.* 2003; Darby and Sheorey 2008). Enteric fever is a more severe disease than NTS, as the infection is likely to become systemic, prompt antibiotic intervention is required for a good prognosis. Approximately 30% of enteric fever cases are fatal when antibiotic treatment is not available, however upon prompt antibiotic treatment with fluoroquinolones such as ofloxacin or ciprofloxacin the prognosis for typhoid fever is good (Bhutta 2006; Crump and Mintz 2010; Effa *et al.* 2011).

Although enteric fever is a more severe disease than NTS, it is very uncommon in the U.K. and therefore doesn't represent a great economic or health burden. However, NTS caused by *S*. Enteritidis and *S.* Typhimurium alone, in the U.K. is estimated to cost £6.5 million per annum, this figure includes medical costs and time lost from work either by the patient, parents or carer (Santos *et al.* 2010).

1.1.2 *Salmonella enterica* serovar Typhimurium

The particular serovar of *Salmonella* that is the focus of this project is *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium). This serovar is of particular clinical relevance as it is not only a major cause of NTS but of the cases reported in the European Union almost half are multi-drug resistant which presents challenges if the infection needs to be treated with antibiotics (Meakins *et al.* 2008). Infections caused by quinolone resistant strains of *S*. Typhimurium have been shown to be three times more likely to become systemic, increasing the risk of fatality, when compared to infections with quinolone susceptible strains (Helms *et al.* 2004).

1.2 Bacterial biofilms

A bacterial biofilm can be defined as a community of bacteria adhered to a surface and each other by an extracellular matrix secreted by the bacterial cells (Stoodley *et al.* 2002). In the mid-twentieth century it was noted that marine bacteria preferred to survive in groups attached to surfaces (Zobell 1943), these bacterial structures would later become known as biofilms and up until 1987 biofilms were seen as simple "chunks" of randomly positioned bacteria within a matrix material (Costerton *et al.* 1987). However with the development of new techniques which have allowed microbiologists to see how bacteria develop in three dimensions, it was discovered

that biofilms are not a stationary, inactive group of bacterial cells but in fact are a complex, diverse community of cells with an ordered and efficient three dimensional structure (Watnick and Kolter 2000; Branda *et al.* 2005). The advantages for bacteria of forming biofilms are many and include a number of adaptations which result from the ability of a community to behave in a way individual cells cannot (Branda *et al.* 2005). Belonging to a biofilm community has one large benefit to an individual bacterium, protection from environmental stress. The manner in which biofilms form means that bacteria can be protected from the environmental conditions via an extracellular matrix that is secreted by the bacterial cells whilst receiving all of the nutrients needed to survive (Watnick and Kolter 2000; Branda *et al.* 2005).

1.2.1 Composition of biofilms

The specificity of biofilm development varies between species and under different conditions, there are however five distinct generalised stages (Figure 1.3). The initial stage of biofilm formation is where a motile planktonic cell initially colonises a surface, these cells attach to a surface via weak reversible bonds (Van der Waals forces of attraction). During this reversible attachment the bacteria start to demonstrate species-specific behaviours such as rolling, creeping, aggregate formation and windrow formation (Korber *et al.* 1997). The attached bacteria begin excreting an extracellular matrix and the initial reversible binding is superseded by much stronger, irreversible covalent bonds which prevent independent movement of cells (Pesci *et al.* 1999). Once the bacterial attachment is established they begin to form a microcolony by recruiting other bacteria from the surrounding environment and replicating. This rapidly growing bacterial community evolves into a fully three dimensional biofilm.

Figure 1.3 A schematic diagram showing the five different stages of biofilm formation (http://woundsinternational.wordpress.com/)

1 – Initial Attachment; **2** – Irreversible Attachment; **3** – Maturation I; **4** – Maturation II; **5** – Dispersal

When the biofilm reaches maturity some cells are released and become planktonic to migrate to other locations suitable for colonisation (Watnick and Kolter 2000; Branda *et al.* 2005). There are many factors that contribute to an ideal biofilm environment, these include; the nature, type and shape of surface, the charge or lack of charge, hydrophobicity and hydrodynamics (Stoodley *et al.* 2002). There are also different features that determine the maturation of the biofilm, including, the physical environment, motility, growth rate, cell signalling ability and extracellular polymeric substance production. Again many of these are species specific (Stoodley et al. 2002). As competent biofilm formation relies upon so many different factors it is not surprising that many diverse bacterial genes have been identified which contribute to the ability to form biofilms in different species.

1.2.2 Genetic differentiation within biofilms

Within a complex, differentiated biofilm local conditions will vary, consequently it has been suggested that biofilms comprise a heterogeneous population of different bacterial cells that have differentiated along distinct developmental pathways (Lazazzera 2005; An and Parsek 2007). Cells within a biofilm express genes in a pattern that varies greatly from their planktonic equivalents and the commitment to a sessile mode of growth involves a switch to a distinct pattern of gene expression (Stoodley *et al.* 2002).

Microarray transcriptomic studies have revealed that biofilms of *P. aeruginosa, V. cholera, E. coli* and *S. aureus* differentially express as much as 10% of their genome in contrast to isogenic planktonic cultures (Schoolnik *et al.* 2001; Whiteley *et al.* 2001; Schembri *et al.* 2003; Beenken *et al.* 2004).

The different stages of biofilm formation are characterised by expression of distinct sets of genes. Initial attachment requires the expression of pili and fimbriae to assist in making contact with the surface and helping the cell overcome electrostatic force that the surface will have upon them. This primary attachment is reversible and cells can easily become detached and return to planktonic state. To adhere irreversibly the bacteria must decrease expression of motility genes and induce expression of genes that encode for extracellular matrix components; curli and cellulose in *Salmonella*. Maturation of a biofilm into a complex three dimensional structure can see further changes in gene expression including quorum sensing molecules in some species. The switch between planktonic and biofilm lifestyles in many species of bacteria can be attributed to the signalling molecule, cyclic diguanylic acid (c-di-GMP). At high levels, c-di-GMP, increases the expression of many genes conducive to forming biofilms and turns down any motility genes required for a planktonic lifestyle. In the same way, low levels of c-di-GMP decrease biofilm genes and increase motility genes. The biosynthesis of c-di-GMP is controlled by diguanylate cyclases (DGCs) and the degradation mediated by phosphodiesterases (PDEs). Bacteria tend to have several DGCs and PDEs which allow the level of c-di-GMP to be adjusted by a complex group of catabolic and anabolic enzymes which can respond to a range of environmental cues (Petrova and Sauer 2012).

The differentiation of structure within a biofilm allows formations to be made which are permissive for life at high density. One example is the maintenance of nutrient channels, which requires communication between individual bacteria to ensure cells grow in the correct direction and the extracellular polymeric substance is secreted

from the cells in the right direction so as to not block the channels that can be an essential source of oxygen and nutrients (Stoodley *et al.* 2002).

1.2.3 Antimicrobial resistance in *Salmonella*

Resistance to antibiotics is an emerging and increasing problem in many pathogenic bacteria and is a huge clinical challenge for modern medicine. Bacteria are increasingly being isolated that are resistant to multiple clinically relevant antibiotics. Methicillin-resistant *Staphylococcus aureus* (MRSA) and carbapenem resistant Gram-negative bacteria are recent examples of antibiotic resistance causing huge problems in hospitals across the world (Reacher et al. 2000; Padmanabhan and Fraser 2005; Grundmann et al. 2006). For *Salmonella* in particular, antibiotic resistance has been seen in both typhoidal and non-typhoidal serovars (Stevens *et al.* 2009; Harish and Menezes 2011). In *Salmonella*, resistance to β-lactams, tetracyclines, phenicols, aminoglycosides and fluoroquinolones is commonly seen. Resistance can be mediated by the acquisition of multiple antibiotic resistance genes on genomic islands (as seen in *S*. Typhimurium DT104) and transmissible elements such as plasmids and integrons. Antibiotic resistance has profound clinical implications and drug resistant infections have a poor prognosis (Helms *et al.* 2004). Although more infections are caused by *S*. Enteritidis in Europe, *S*. Typhimurium is of particular clinical relevance as it is not only the second largest cause of NTS but of the cases reported in the European Union, almost half are multi-drug resistant. This presents challenges if the infection is extra-intestinal and needs to be treated with antibiotics (http://www.enter.net 2008). Infections caused by fluoroquinolone resistant strains of *S*. Typhimurium are three times more likely to become systemic or even fatal when compared to strains without such resistance (Helms *et al.* 2004). As well

as specific antibiotic resistance genes efflux is also an important mechanism of clinically relevant antibiotic resistance in *Salmonella*.

1.2.4 Antibiotic resistance and biofilms – diffusion barrier, gene exchange, dormancy and persistence

As well as bacteria becoming resistant via genetic variation they can also become more antibiotic resistant when adapting the physiologically distinct biofilm mode of growth even though no mutation or acquisition of novel DNA has occurred. Bacteria in a biofilm can be up to 1000 fold more resistant to antibiotics than their planktonic counterparts (Costerton *et al.* 1987). This increased resistance was initially thought to be due to the three dimensional structure of a biofilm dictating that the antibiotics could not penetrate through the extracellular matrix and peripheral cells to reach the cells residing within the biofilm (Costerton *et al.* 1987). More recent research suggests that the difference in metabolism of bacteria within a biofilm compared to planktonic bacteria has a major role in the antibiotic resistance of biofilms. Biofilms contain large numbers of ‘persister’ cells, cells which survive treatment with normally effective antibiotic concentrations due to their metabolic dormancy. This also means that bacteriostatic antibiotics (antibiotics that work by halting division) become ineffective as the cells are not dividing or are only dividing at a low rate. Similarly any target of an antibiotic which is only produced by active cells will not be present at high levels in a metabolically dormant cell (Lewis 2007).

1.2.5 Biofilms in the environment

Bacteria forming a biofilm can survive in varying environments, and adapt to diverse surroundings, the environment has a large impact on biofilm structure (Hall-Stoodley *et al.* 2004). Bacteria in natural aquatic environments are often attached to surfaces

(Zobell 1943), biofilms that dwell in fast flowing water have a tendency to form filamentous streamers, these streamers are attached to the surface by an upstream 'head' which allows the 'tail' oscillate downstream in the current. In calm or slow flowing waters biofilms form stromatolites (mushroom and tower shaped structures), with an isotropic pattern where there is no sign of flow influencing morphology of the biofilm (Hall-Stoodley *et al.* 2004).

1.2.6 Biofilms and food contamination

Bacterial contamination of food leads to many cases of gastroenteritis every year. These infections are not only caused by contamination of meat and poultry products but also from fresh fruit and vegetables (http://www.food.gov.uk; Fatica and Schneider 2011). Bacterial biofilms have been found to exist on surfaces within the food processing plants where produce is packaged (allowing persistence of pathogenic bacteria which is easily transferable to the produce) and also on the surface of the food itself (source of bacterial gastroenteritis for humans) due to the availability of nutrients and moisture. The occurrence of food borne disease outbreaks due to contaminated fresh fruit and vegetables are not unusual, for example; *Escherichia coli* on lettuce leaves, apple cider (Tauxe 1997), radishes (Itoh *et al.* 1998), alfafa (Anon 1999) and beansprouts (Buchholz *et al.* 2011) *Cyclospora cayetanesis* on raspberries (Herwaldt and Ackers 1997), lettuce (Herwaldt 2000; Doller *et al.* 2002), and basil (Lopez *et al.* 2001) and *Shigella* on shredded lettuce (Tauxe 1997). *Salmonella* serovars, in particular, have been associated with many outbreaks in the U.K. including two outbreaks in 2010 associated with beansprouts and salad leaves causing over 250 cases of salmonellosis (http://www.hpa.org.uk 2010).

1.2.7 Mixed population biofilms

Biofilms in nature usually exist as multi species communities. These mixed species environments often contain species which benefit from each other's presence. One example of this was described recently in the human oral microbiome. It was discovered that previously unculturable species that exist as biofilms in the mouth can be grown *in vitro* using a helper bacteria to condition the media (Vartoukian *et al.* 2010). Particular genera of bacteria for which new species have been found using this model of culture include *Catellibacterium*, *Psychrobacter*, *Sphingomonas* and *Symbiobacterium* (Tanaka *et al.* 2004; Bae *et al.* 2005; Kim *et al.* 2008; Nichols *et al.* 2008). Another example of co-operative behaviour in a biofilm is seen with *Pseudomonas putida* and *Acinetobacter sp.* which can form mixed species biofilms in the laboratory when benzyl alcohol is the only source of carbon from the environment. *Acinetobacter* can utilize benzyl alcohol as a carbon source which creates a by product, benzoate, which is excreted into the environment. *P putida* although not being able to catabolise benzyl alcohol can still thrive in an environment where this is the only carbon source in the presence of *Acinetobacter sp*. This is due to *P. putida* utilizing the by product, benzoate, as its source of carbon (Moller *et al.* 1998).

1.2.8 Clinical relevance of bacterial biofilms

1.2.8.1 Medical biomaterials

Infection from biofilm dwelling bacteria can be caused when using an array of different medical biomaterials in clinical practice. Bacteria can form biofilms on prosthetic cardiac valves (Dismukes *et al.* 1973; Blouse *et al.* 1978), cerebrospinal fluid shunts (Callaghan *et al.* 1961; Schoenbaum *et al.* 1975), orthopaedic appliances

(Patterson and Brown 1972; Wilson 1977) and intravascular catheters (Archer 1978; Bender and Hughes 1980). Common bacteria that cause device related biofilm infections include *Staphylococci*, *Escherichia coli*, *Proteus* species*, Serratia marcescens* and *Morganella morganii* (Geipel 2009). These infections are common as artificial materials are often poorly served by the immune system, and are often easier for the bacteria to adhere to than biological surfaces, which create a route of entry into the body (Peel *et al.* 2012).

In the U.S.A, catheter related infections alone are estimated to cost up to £1.4 billion annually with around 250,000 infections and a mortality rate of 12-25% (Rello *et al.* 2000; von Eiff *et al.* 2005)

1.2.8.2 Cystic fibrosis

The lungs of cystic fibrosis sufferers present an attractive setting for *P. aeruginosa* colonization and biofilm formation (Gomez and Prince 2007). As biofilms are less prone to disruption or killing by surfactants, antibiotics, grazing predatory white cells and host defences (including antimicrobial peptides) these chronic *P. aeruginosa* biofilm lung infections are extremely difficult to treat (Costerton 2001). By late adolescence 80% of cystic fibrosis sufferers have chronic lung infection, with *P. aeruginosa* as one of the main causes (Lyczak *et al.* 2002). Once acquired, *P. aeruginosa* is almost impossible to eradicate from the lungs of cystic fibrosis sufferers and eventually this infection will become fatal (Høiby *et al.* 2005).

More recently strains of the *Burkholderia cepacia* complex (Bcc) have been identified as important lung pathogens producing an array of different disease states from asymptomatic to “cepacia syndrome” which is lethal. Whilst colonisation with either

pathogen is detrimental to the patient's health, co-colonisation can exhibit a more severe decline in pulmonary function (Jacques *et al.* 1998). It has been suggested that *in vitro* there is a synergistic relationship between these two microorganisms which allows Bcc strains to increase expression of virulence factors (McKenney *et al.* 1995) and also increase binding to epithelial cells (Saiman *et al.* 1990) in the presence of *P. aeruginosa* conditioned media. This synergy was investigated by Tomlin *et al* who showed dual species biofilm formation was easily achieved *in vitro* and both species readily integrated in close proximity within a biofilm, however it is still unknown if this is the cause of the damaging effects co-colonisation has on the lungs of CF patients (Tomlin *et al.* 2001).

Bacteria readily form biofilms in cystic fibrosis lungs and it is thought that the dense, stagnent mucus present decreases motility of the bacterial cell which results in bacterial cell clusters forming, accumulation of quorum sensing molecules (which trigger biofilm formation) and subsequent biofilm formation. Normal hydrated mucus allows the bacteria to move around freely and prevents bacteria from accumulating in clusters in one area therefore reducing the likelihood of biofilm formation (Matsui *et al.* 2006).

1.2.8.3 Gall stones

Salmonella enterica serovar Typhi (*S*. Typhi) can form biofilms on gall stones in the gall bladder of humans during an active, acute infection and these cells can persist long after the symptoms have subsided and can be the source of recurrent Typhoid infections. This persistence suggests that *S.* Typhi has established a mechanism (biofilm) of evading the antimicrobial action of bile in the gall bladder (Sinnott and

Teall 1987; Vaishnavi *et al.* 2005). Antibiotics are typically ineffective when the patient exhibits both cholesterol gall stones and an *S.* Typhi infection (Lai *et al.* 1992), and there is also a high chance of developing hepatobiliary carcinomas (Dutta *et al.* 2000; Kumar *et al.* 2006). It is currently not known whether other *Salmonella* serovars also form biofilms on gallstones *in vivo*.

1.2.8.4 Legionellosis

Legionella pneumophilia causes 90% of Legionellosis cases (Yu 2002) although there are more than 50 known sub-species of *Legionella* (Diederen 2008). Legionellosis includes two distinct diseases; Legionnaires' disease, a pneumonia that is frequently fatal if not caught early and treated, and Pontiac fever, a milder flu-like illness (Diederen 2008). *L. pneumophilia* rarely causes infection when in a natural water system as conditions do not allow the bacteria to proliferate sufficiently. However if *L. pneumophilia* enters a man made water system where the temperatures are generally higher than ambient the bacteria can colonize existing biofilms formed by other species and proliferate to high numbers if disinfection regimes are not adhered to (Rogers *et al.* 1994). *L. pneumophilia* is extremely particular when it comes to its favoured conditions for growth and when cultured in the laboratory it requires several different nutrients including iron salts and amino acids (George *et al.* 1980). In artificial water systems *L. pneumophilia* survive by parasitizing protozoans which graze on the biofilm (Greub and Raoult 2004). Without this association with other bacterial biofilms *L. pneumophilia* would not be able to proliferate to high enough levels to cause disease in humans.

1.2.8.5 Oral biofilms

Dental plaque is a oral bacterial biofilm which is a dynamic and extremely complex ecosystem (Marsh 2003). Many bacterial species can form plaque; these species have been grouped according to clinical parameters, the disease stage and location in the oral cavity. Several key species can be involved in oral disease, these include; *Porphyromonas gingivalis*, *Treponema denticola*, *Prevotella intermedia*, *Aggregatibacter actinomycetemconitans, Bacteroides* forsythus*, Campylobacter rectus* and *Eubacterium nodatum* (Lovegrove 2004). The biofilms that form in the gingival cavity gives rise to a cellular inflammatory response of the gingiva and surrounding connective tissue. These periodontal diseases are split into two clinical classes; gingivitis and periodontitis. Gingivitis is extremely common and is defined by an inflamed gingival cavity. Periodontitis is a more severe disease where the plaque induced inflammatory response leads to loss of collagen attachment (between the tooth and the bone) and bone loss (Savage *et al.* 2009). Periodontal disease has also been linked with increased susceptibility to other systemic disorders (Amar and Han 2003; Kuo *et al.* 2008) including diabetes mellitus (Kuo *et al.* 2008), Alzheimer's disease (Kamer *et al.* 2008; Kamer *et al.* 2008; Watts *et al.* 2008), cardiovascular disease (Mattila *et al.* 2005), osteoporosis (Kuo *et al.* 2008) and chorioamnionitis leading to premature delivery and low birth weight (Offenbacher *et al.* 1996).

1.3 *Salmonella* biofilms

As well as *Salmonella* Typhi forming biofilms on gallstones during infection (Section 1.2.8.3), non typhoidal *Salmonella* biofilms are a recognized problem in a number of industrial, veterinary and medical settings (Veldman *et al.* 1995). Vestby *et al* showed that the period of persistence of NTS within Norwegian fish meal and food

factories correlated with the ability of strains to form biofilms (Vestby *et al.* 2009). The serovars Agona and Montevideo formed superior biofilms to all other serovars tested and were also found to persist for the longest time period within processing plants. As biofilms are more tolerant to disinfectants than planktonic cells the presence of biofilms in the food industry is a problem requiring very rigorous disinfection regimes. Survival of NTS in biofilms in the food industry increases the risk of food contamination and possible human infections (Veldman *et al.* 1995).

As with other species a number of genes have been shown to contribute to the ability of *Salmonella* to form a biofilm. These include genes required for motility (flagella) and the initial attachment phase (fimbriae, pili etc) and genes which encode the matrix components and promote cell:cell interactions (curli, cellulose etc). Additionally a large number of genes have been identified as having regulatory roles, predominantly by controlling the expression of the matrix components and in particular, *csgD*. Table 1.1 gives a list of genes identified as being involved in biofilm formation of *Salmonella* and an indication of their suggested role in biofilm formation.

1.3.1 Composition of the *Salmonella* biofilm matrix – curli and cellulose

Salmonella biofilms are encased in a matrix largely composed of two major components; curli and cellulose. Curli are thin, hair like filaments which can polymerise to form amyloid fibres protruding from the outer membrane of many bacteria from the Enterobacteriaceae family (Zogaj et al. 2003). They are involved in many functions including adhesion, cell aggregation, environmental persistence and biofilm formation (White *et al.* 2003; Cogan *et al.* 2004; Jonas *et al.* 2007).

Table 1.1 Genes known to be involved in biofilm formation of *Salmonella*

Gene/ Operon	Intermediate gene	Gene/Operon Description	Role in biofilm formation	Reference
csgBA		Major and minor curli subunits	Structural curli proteins (extracellular proteinaceous fibres)	(Grund and Weber 1988)
csgD		Master biofilm regulator	Transcriptional activator of *csgBA*, *adrA*, bapA and *bcsABCD*	(Grund and Weber 1988)
csgEFG		Curli subunit translocation and assembly	Efficient assembly of curli subunits on bacterial cell surface	(Grund and Weber 1988)
bscABCD		Cellulose biosynthesis operon	Cellulose (extracellular polysaccharide) biosynthesis	(Zogaj *et al.* 2001)
bcsEFG		Cellulose biosynthesis operon	Cellulose (extracellular polysaccharide) biosynthesis	(Zogaj *et al.* 2001)
bapA		Large cell surface protein	Pellicle formation (biofilm at liquid-air interface)	(Latasa *et al.* 2005)
dos		cAMP phospohodiesterase	*csgD* repressor	(Tagliabue *et al.* 2010)
rcsBC		two component system	*csgD* repressor	(Latasa *et al.* 2012)
cpxRA		Envelope stress response two component system	*csgD* repressor	(Gerstel and Romling 2003)
fecR		Transmembrane signal transducer	*csgD* repressor	(Brombacher *et al.* 2006)
omrAB		Small RNAs	*csgD* repressor	(Holmqvist *et al.* 2010)
hns		DNA binding protein	*csgD* activator/ repressor	(Ledeboer and Jones 2005)

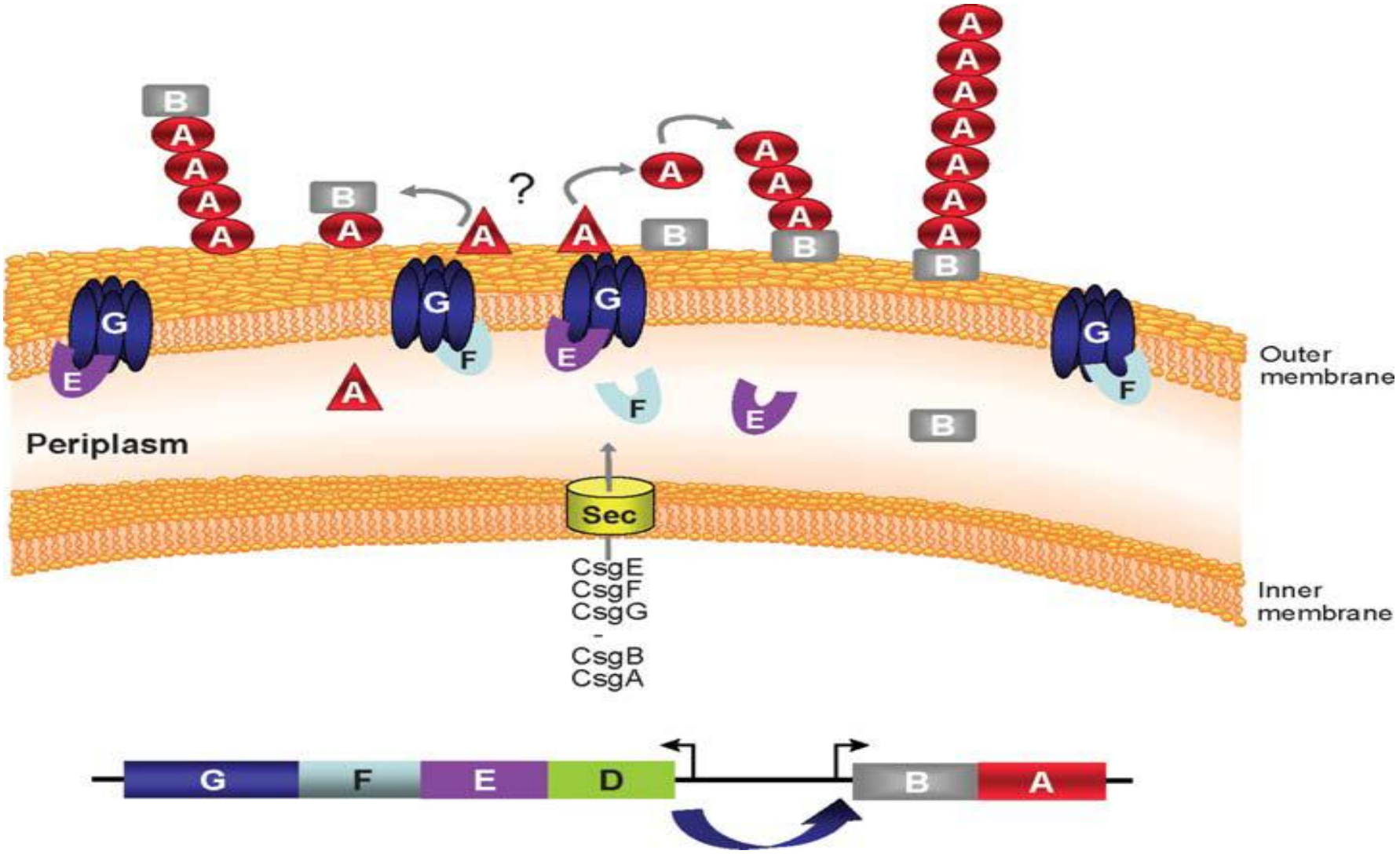
Gene/ Operon	Intermediate gene	Gene/Operon Description	Role in biofilm formation	Reference
ompR/envZ		Osmolarity response two component system	*csgD* activator	
yegE		Putative diguanylate cyclase/phophodiesterase	*csgD* activator	(Kader *et al.* 2006; Simm *et al.* 2007)
yedQ		Diguanylate cyclase	*csgD* activator	(Da Re and Ghigo 2006)
ydaM		Diguanylate cyclase	*csgD* activator	(Mika and Hengge 2013)
yciR		cGMP phosphodiesterase	*csgD* activator	(Garcia *et al.* 2004)
yjcC		Putative diguanylate cyclase/phophodiesterase	*csgD repression*	Kader *et al.* 2006; Simm *et al.* 2007)
mlrA		DNA binding transcriptional activator	*csgD* activator	(Garcia *et al.* 2004)
yddV		Diguanylate cyclase	*csgD* activator	(Tagliabue *et al.* 2010)
ihf		Integration host factor	*csgD* activator	(Garcia-Contreras *et al.* 2008)
mltC		Membrane bound lytic transglcosylase	*csgD* activator	(Monteiro *et al.* 2011)
mltE		Soluble lytic transglcosylase	*csgD* activator	(Monteiro *et al.* 2011)
yoaD		Phosphodiesterase	*bcsABCD* activator	(Romling *et al.* 2000)
adrA		Diguanylate cyclase	*bcsABCD* activator	(Garcia *et al.* 2004)

Gene/ Operon	**Intermediate gene**	**Gene/Operon Description**	**Role in biofilm formation**	**Reference**
rpoS	*yepE* *yedQ* *ydaM* *yciR* *mlrA*	RNA polymerase sigma factor	*yepE/yedQ/ydaM/* *yciR/mlrA* activator	(Cabeza *et al.* 2007)
sdiA	*rpoS*	DNA binding transcriptional activator	*rpoS* activator	(Janssens *et al.* 2008)
crl	*rpoS*	DNA binding transcriptional activator	*rpoS* activator	(Robbe-Saule *et al.* 2006)
fur	*hns*	Ferric uptake regulation	*hns* repressor	(Janssens *et al.* 2008)
csrA	ompR rpoS	Carbon storage regulator	*ompR and rpoS* repressor	(Jackson *et al.* 2002)

In *S*. Typhimurium, curli fimbriae are encoded by two operons; *csgBA* and *csgDEFG* (Hammar *et al.* 1995). *csgA* and *csgB* encode the two structural proteins of identical size and contain similar repeat motifs. Both proteins are needed to form polymerized protein chains on the cellular surface (Collinson *et al.* 1999; White *et al.* 2001). *csgC* is found downstream of *csgBA* but has no reported role in curli biogenesis and no transcript has been detected (Barnhart and Chapman 2006). CsgD, CsgE, CsgF and CsgG are all curli accessory proteins and all are required for efficient curli translocation and assembly. CsgD is a positive transcriptional regulator of the *csgBA* operon. CsgE and CsgF are both periplasmic proteins which interact with CsgG in the outer membrane and are thought to work with CsgG to promote curli assembly, however the exact function of these two proteins is unknown (Hammar *et al.* 1995; Barnhart and Chapman 2006). CsgG is an outer membrane lipoprotein and is essential for the secretion and stability of CsgA and CsgB and has been suggested to be required for CsgA and CsgB to cross the membrane and be translocated into the extracellular milieu where polymerisation can occur (Figure 1.4) (Robinson *et al.* 2006).

Cellulose is a polysaccharide secreted by the bacterial cell that helps cement the cells together and is therefore extremely important in maintaining bacterial multicellular biofilm communities. Cellulose synthesis is encoded by the operon *bcsABZC* (Zogaj *et al.* 2003). As well as their adhesion functions curli and cellulose have also been suggested to have roles in virulence, invasion and chlorine resistance (Sukupolvi *et al.* 1997; Dibb-Fuller *et al.* 1999; Gophna *et al.* 2001).

Figure 1.4 Diagram of the curli translocation and assembly apparatus (Barnhart and Chapman 2006)



Many genes are known to regulate expression of biofilm extracellular matrix components via CsgD, a transcriptional activator of the curli operons as well as other biofilm related operons, *adrA*, *bapA* and *bcsABCD* (Gerstel and Romling 2003). CsgD is the master curli regulator in *Salmonella* and other Gram negative bacteria and *csgD* expression is influenced by a huge number of proteins, small RNAs and signalling molecules. This complex regulation allows *csgD* expression to respond to a number of different extracellular stimuli such as pH, temperature, salt concentration, nutrient supply amongst others (Jubelin *et al.* 2005; Brombacher *et al.* 2006; Ogasawara *et al.* 2010).

1.3.2 'RDAR' phenotype

Expression of the *Salmonella* extra cellular matrix components give *Salmonella* a very distinct phenotype when plated on to LB agar without salt supplemented with a dye called Congo red (a planar hydrophobic diazo dye). Colony morphologies of strains producing a normal matrix appear red, dry and rough (RDAR), both curli and cellulose are bound by Congo red so the colonies also appear dark red (Romling 2005). Mutants lacking the ability to make curli, cellulose or both present with a range of different colony types when stained with Congo red, easily distinguished from 'RDAR' colonies (Solano *et al.* 2002).

1.4 Efflux pumps

1.4.1 Multidrug efflux systems

Efflux pumps are proteins located in the cytoplasmic membrane of all bacteria. Their primary role is to keep cytoplasmic levels of various metabolites and chemicals at a level non detrimental to the cell or to transport molecules to the cell surface or

extracellular milieu to carry out their function. Cytoplasmic efflux pump proteins either work alone or in Gram negative bacteria sometimes as part of a tripartitie system with an additional outer membrane porin protein and periplasmic adapter protein. Some efflux systems are able to extrude antibiotics from within the cell and keep the intracellular concentration of antibiotics at sub lethal levels. If a single efflux pump or system is responsible for conferring even low level resistance to three or more different classes of antibiotics they are known as multidrug efflux systems (Blair and Piddock 2009).

1.4.2 Multidrug resistance efflux pumps in *Salmonella*

Efflux pumps play an essential role in the physiology of bacteria. They do this by mediating the entry and extrusion of essential nutrients, metabolic waste and xenobiotics. As for other Gram negative bacteria there are four classes of solute transporters defined in the Transport Classification (TC) system found within *Salmonella*; channels, secondary transporters, primary active transporters and group translocators. Channels do not depend upon energy but use the gradient of the substrate to drive translocation. Primary active transporters use a source of energy such as the hydrolysis of ATP. Secondary transporters exploit an ion or electrochemical gradient such as the sodium or proton motive force. Group translocators alter their substrates during the transport process (e.g. substrate phoshorylation) (Ren and Paulsen 2007).

A genomic analysis of all membrane transport systems within prokaryotes predicts that *S*. Typhimurium has 350 membrane transporters (73 transporters per Mb of genome). Of these, 83 (23.7%) are primary active transporters (all using ATP as their

energy source), 15 (4.3%) are channels, 29 (8.3%) are group translocators (all using phosphorylation to modify their substrates), 218 (62.3%) are secondary transporters (using either sodium or proton motive force) and 2 (0.6%) are as yet unclassified (Ren and Paulsen 2007).

Of the 350 predicted transport proteins within *Salmonella* there are nine known and experimentally characterised multidrug resistance (MDR) efflux pumps which individually confer low level MDR to three or more different classes of antibiotics, dyes or detergents (Table 1.2) (Baucheron *et al.* 2004; Eaves *et al.* 2004; Buckley *et al.* 2006; Nishino *et al.* 2006). These nine systems belong to four of the five distinct families of efflux pumps known to confer multidrug resistance (Figure 1.5); the ATP binding cassette (ABC) family, the multidrug and toxic compound extrusion (MATE) family, the major facilitator superfamily (MFS), the resistance nodulation division (RND) family and the small multidrug resistance (SMR) family (Piddock 2006). *Salmonella* has at least one MDR pump from each family with the exception of the SMR family of efflux pumps. With the exception of MdsABC (**mds**-**m**ulti**d**rug transporter for ***S****almonella*) which is unique to *Salmonella*, all of the identified MDR efflux systems also exist in *E. coli* (Nishino *et al.* 2006).Their ability to export a very broad range of other toxic compounds including antibiotics and biocides allows the bacteria to survive in hostile environments (Piddock 2006; Blair and Piddock 2009).

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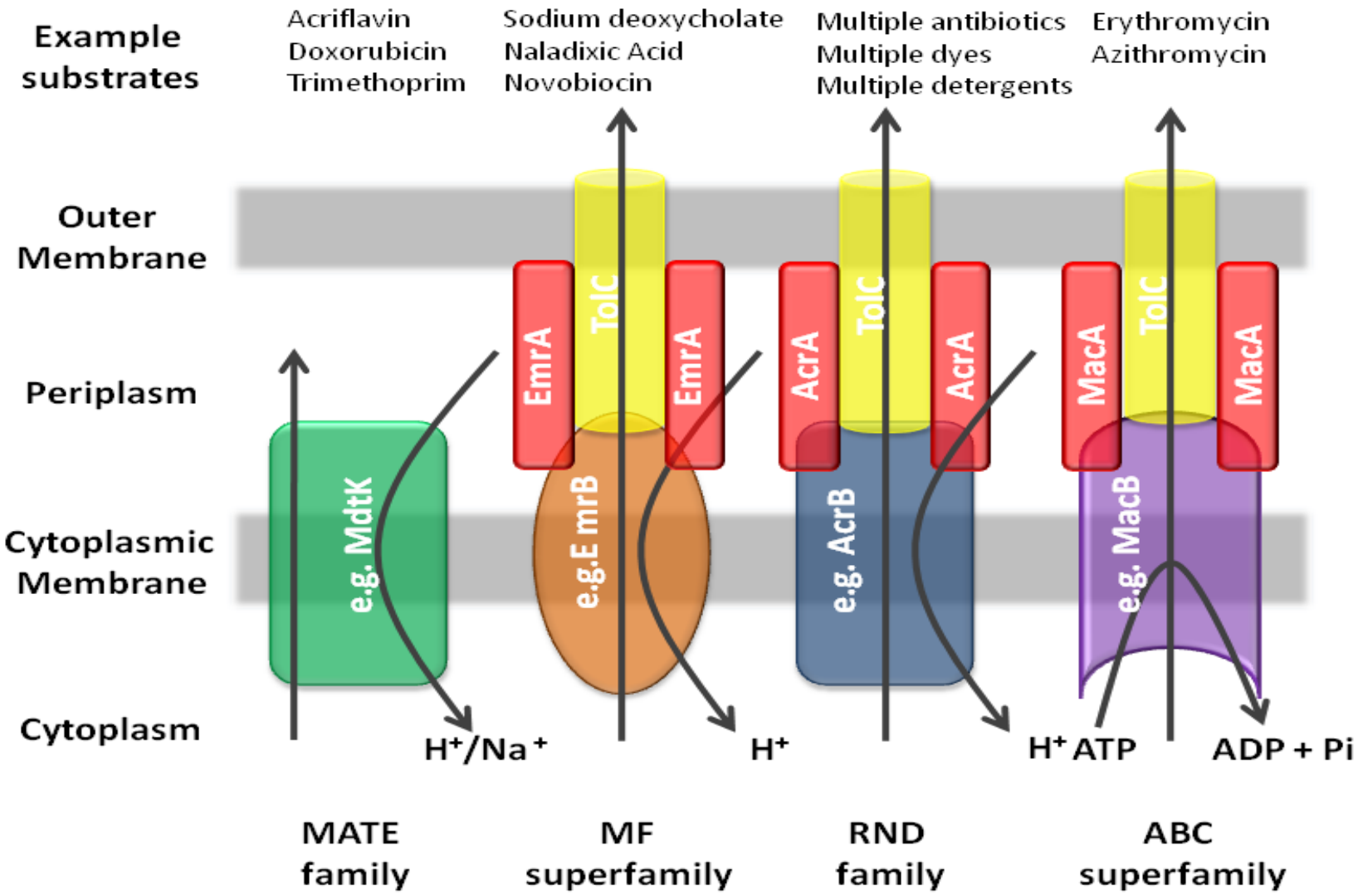
Table 1.2 Nine known MDR efflux pumps of *Salmonella,* their periplasmic adapter protein, outer membrane protein and known substrates.

Pump	Family	Accessory Protein	Outer Membrane Protein	Known substrates	References
AcrB	RND	AcrA	TolC	**Drugs;** chloramphenicol, erythromycin, novobiocin, tetracycline, nalidixic acid, norfloxacin, doxorubicin, acriflavin, tigecycline, minocycline. **Other substrates;** crystal violet, ethidium bromide, methylene blue, rhodamine 6G, tetraphenylphosphonium bromide, benzalkonium chloride, sodium dodecylsulfate, sodium deoxycholate	(Horiyama *et al.*, 2010a; Nishino *et al.*, 2006; Nishino *et al.*, 2009)
AcrD	RND	AcrA	TolC	**Drugs**; novobiocin. **Other substrates;** sodium dodecylsulphate, sodium deoxycholate	(Nishino *et al.*, 2006; Nishino *et al.*, 2009)
AcrF	RND	AcrE	TolC	**Drugs;** erythromycin, novobiocin, tetracycline, nalidixic acid, norfloxacin, doxorubicin, acriflavin, tigecycline, chloramphenicol. **Other substrates;** crystal violet, ethidium bromide, methylene blue, rhodamine 6G, tetraphenylphosphonium bromide, benzalkonium chloride	(Horiyama *et al.*, 2010a; Nishino *et al.*, 2006; Nishino *et al.*, 2009)
MdsB	RND	MdsA	MdsC/TolC	**Drugs;** novobiocin, acriflavin.	(Nishino *et al.*, 2009)
MdtBC	RND	MdtA	TolC	**Drugs**; novobiocin. **Other substrates;** sodium dodecylsulphate, sodium deoxycholate	(Nishino *et al.*, 2009)
EmrB	MF	EmrA	TolC	**Drugs;** novobiocin, naladixic acid. **Other substrates**; sodium dodecylsulphate, rhodamine 6G.	(Nishino *et al.*, 2009)
MdfA	MF	N/A	N/A	**Drugs;** tetracycline, chloramphenicol, norfloxacin, doxorubicin	(Nishino *et al.*, 2009)
MdtK	MATE	N/A	N/A	**Drugs;** acriflavin, norfloxacin, doxorubicin, trimethoprim. **Other substrates;** sodium deoxycholate.	(Nishino *et al.*, 2009)

Figure 1.5 Schematic diagram of the four families of multidrug efflux systems known in *Salmonella* with example inner membrane pump protein, periplasmic adapter protein, outer membrane porin and example substrates for each family.



1.4.3 Resistance Nodulation Division (RND) family

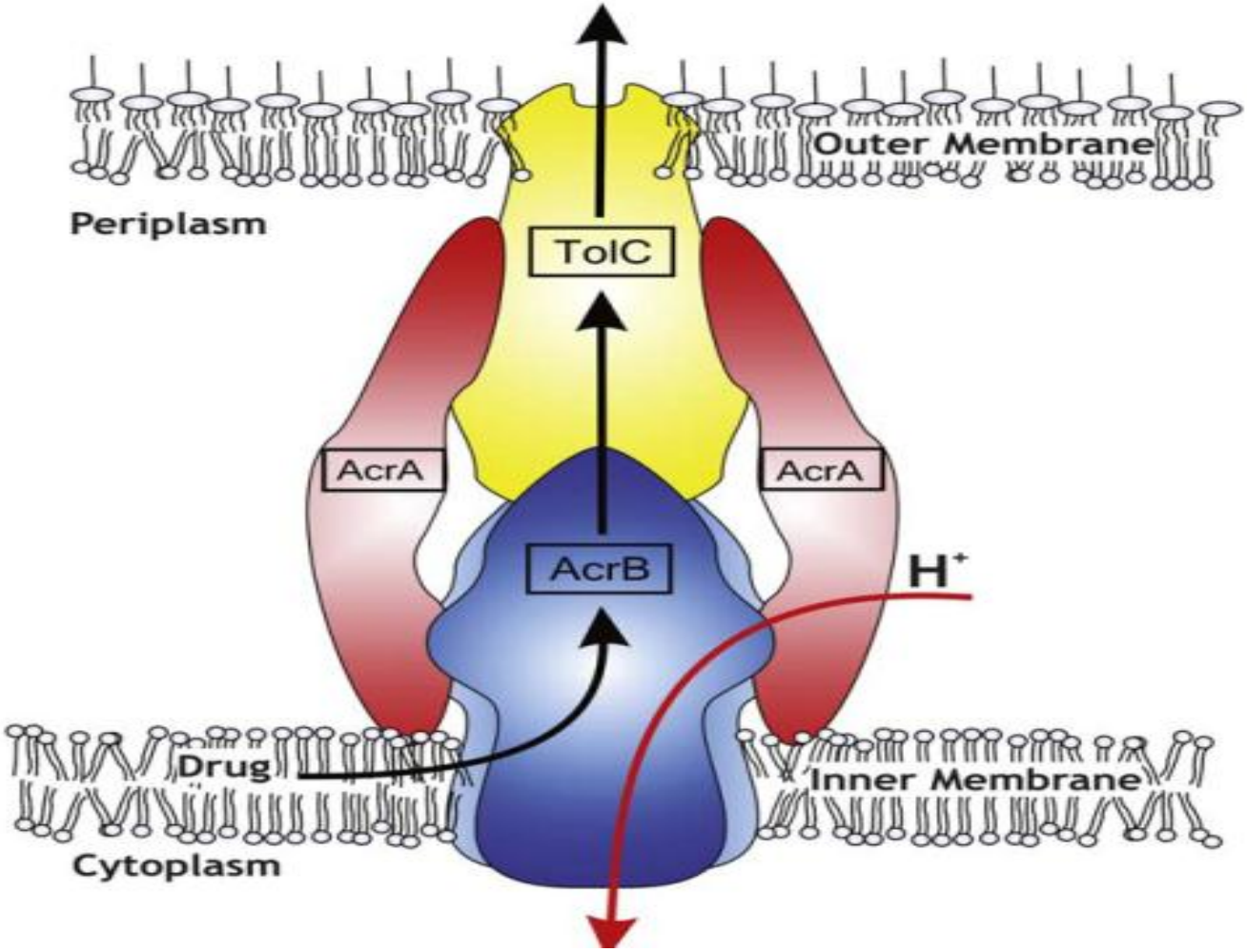
The RND family of efflux pumps are secondary transporters organised as tripartite systems with three components; an inner membrane pump protein, a periplasmic accessory protein and an outer membrane porin protein (Koronakis *et al.* 2004; Ren and Paulsen 2007). The inner membrane pump protein captures substrates from the periplasmic leaflet of the inner membrane phospholipid bilayer and exports them via the outer membrane porin protein into the extracellular milieu (Aires and Nikaido 2005). The RND systems are proton antiporters as they use the proton gradient across the inner membrane to power efflux, substituting one hydrogen ion for one molecule of substrate in an antiport reaction (Paulsen *et al.* 1996). RND pumps assemble as a trimer, linked to an outer membrane channel which is also trimeric (Murakami *et al.* 2002; Murakami *et al.* 2006). Both the pump and porin are stabilised by an adapter protein, the stoichiometry of this adapter protein is as yet uncertain. *Salmonella* possess five different MDR RND efflux proteins, AcrB, AcrD, AcrF, MdsB and MdtBC. AcrB and AcrD associate with AcrA and TolC to form their tripartite systems. AcrF, MdsB and MdtBC all have their own accessory proteins, AcrE, MdsA and MdtA, and all complex with TolC as their outer membrane porin. MdsB as well as associating with TolC in the outer membrane can also associate with its own OMP, MdsC (Nishino *et al.* 2006) (Table 1.2). There is a high level of identity between the RND efflux components of *S.* Typhimurium and the corresponding homologues in *E. coli,* for example AcrA has 94% identity and AcrB has 97% identity between the species (Eaves *et al.* 2004). Although this similarity between *E. coli* and *S.* Typhimurium efflux pumps exists, the substrate profile can differ between them (Nishino *et al.* 2009).

1.4.3.1 AcrAB-TolC

The most well characterised of the RND pumps in *Salmonella* is AcrB and its tripartite complex AcrAB-TolC (Figure 1.6). It comprises the three proteins; AcrB (the inner membrane pump protein), AcrA (the periplasmic protein) and TolC (the outer membrane porin protein). Typically, the genes that encode the three proteins that comprise of RND pump systems are co-localised within an operon with the local regulator followed by the periplasmic adapter protein gene, the pump gene and finally the outer membrane porin gene. However, in *Salmonella* this is not the case for AcrAB-TolC as *acrR* (regulator), *acrA* and *acrB* are located together but *tolC* is elsewhere on the chromosome (Nishino *et al.* 2006).

AcrAB-TolC has many different substrates making this efflux pump (and other RND homologues) a key mediator of multidrug resistance in Gram negative bacteria including many Enterobacteriaceae (Paulsen *et al.* 1996). AcrB can export a range of substrates including cationic dyes (including crystal violet, ethidium bromide, rhodamine 6G), antibiotics (including penicillins, cephalosporins, fluoroquinolones, macrolides, chloramphenicol, tetracyclins, novobiocin, fusidic acid, oxazolidinones and rifampicin) and detergents (including Triton X-100, sodium dodecylsulfate and bile acids). Clearly there is little correlation between the structures of this diverse array of substrates, the only feature that they have in common is the inclusion of lipophilic domains within the molecule (Nikaido 1996; Eaves *et al.* 2004; Nishino *et al.* 2006; Bailey *et al.* 2008). The presence of these lipophilic domains is thought to allow them to associate with the complex binding sites present in the hydrophobic interior of AcrB (Nikaido and Takatsuka 2009).

Figure 1.6 Diagrammatic representation of the AcrAB-TolC efflux system, the archetypal RND efflux pump in Enterobacteriaceae (Blair and Piddock 2009).



All three proteins in the AcrAB-TolC system are essential for efficient efflux of antimicrobial compounds across both membranes, and the lack of any component compromises the activity of the entire complex although not to the same degree (Ma *et al.* 1995; Ma *et al.* 1996; Blair *et al.* 2009).

1.4.4 Major facilitator superfamily (MFS)

MFS pumps also fall under the category of secondary transporters (Ren and Paulsen 2007). Around 25% of all known prokaryotic membrane transporters belong to the MFS, which is the largest and most varied family of transporters containing 58 sub families. There are around 15,000 MFS pumps identified to date (Saier *et al.* 1999). MFS pumps are similar to RND pumps in that they are driven by the proton motive force exchanging one hydrogen ion for one substrate molecule and they span the periplasmic membrane with 12 transmembrane regions (Piddock 2006). However, they do not always form a tripartite complex with an adapter protein and outer membrane porin and can exist as a single protein within the cytoplasmic membrane. MdfA and EmrAB are the two MFS MDR pumps described in *Salmonella*. MdfA exists as a single cytoplasmic efflux protein and exports chloramphenicol, doxorubicin, norfloxacin and tetracycline in *S*. Typhimurium. Unlike MdfA, EmrB can associate with its own periplasmic adapter protein, EmrA, and TolC in the outer membrane to form a tripartite system (Horiyama *et al.* 2010). This EmrAB-TolC complex in *S.* Typhimurium can transport substrates such as novobiocin, naladixic acid, SDS and sodium deoxycholate across both membranes into the extracellular milieu (Nishino *et al.* 2006).

1.4.5 Multidrug and toxic compound extrusion (MATE) family

MATE pumps, similar to the RND and MFS pumps, are also secondary transporters with 12 or 14 transmembrane domains and use the proton motive force as an energy source. However, some MATE pumps use a sodium ion gradient to power efflux. MATE pumps in contrast to RND and MFS pumps never exist as a tripartite system. *Salmonella* has one known MATE MDR efflux pump, MdtK. MdtK is a NorE family MDR MATE transporter that uses the proton motive force to translocate substrates across the periplasmic membrane. MdtK can confer low level resistance to norfloxacin, acriflavin and doxorubicin (Nishino *et al.* 2006).

1.4.6 ATP-binding cassette (ABC) family

ABC transporters are primary active transporters and typically have six transmembrane domains spanning the periplasmic membrane which differs from the 12 domains that RND and MFS pumps possess and assemble as dimers. The characteristic of the ABC family of efflux pumps is the motor domain of the protein that binds and hydrolyses ATP, as it is the only family of transporters that uses ATP as its energy source to drive the export of substrates (Bouige *et al.* 2002). MacB is the only known ABC MDR efflux pump in *Salmonella* and associates with MacA as its accessory protein and TolC as its outer membrane protein. MacB forms the tripartite system, MacAB-TolC, and has been shown to export 14- and 15-membered lactone macrolides, for example erythromycin and azithromycin. MacB is the inner membrane pump protein localised in the cytoplasm and functions as a dimer. MacA is thought to form a hexamer and interacts with the α barrel tip region of TolC to stimulate the activity of the MacB ATPase upon assembly (Tikhonova *et al.* 2007). Expression of

macB is controlled by the PhoPQ system, an essential regulator of virulence in *Salmonella* (Nishino *et al.* 2006).

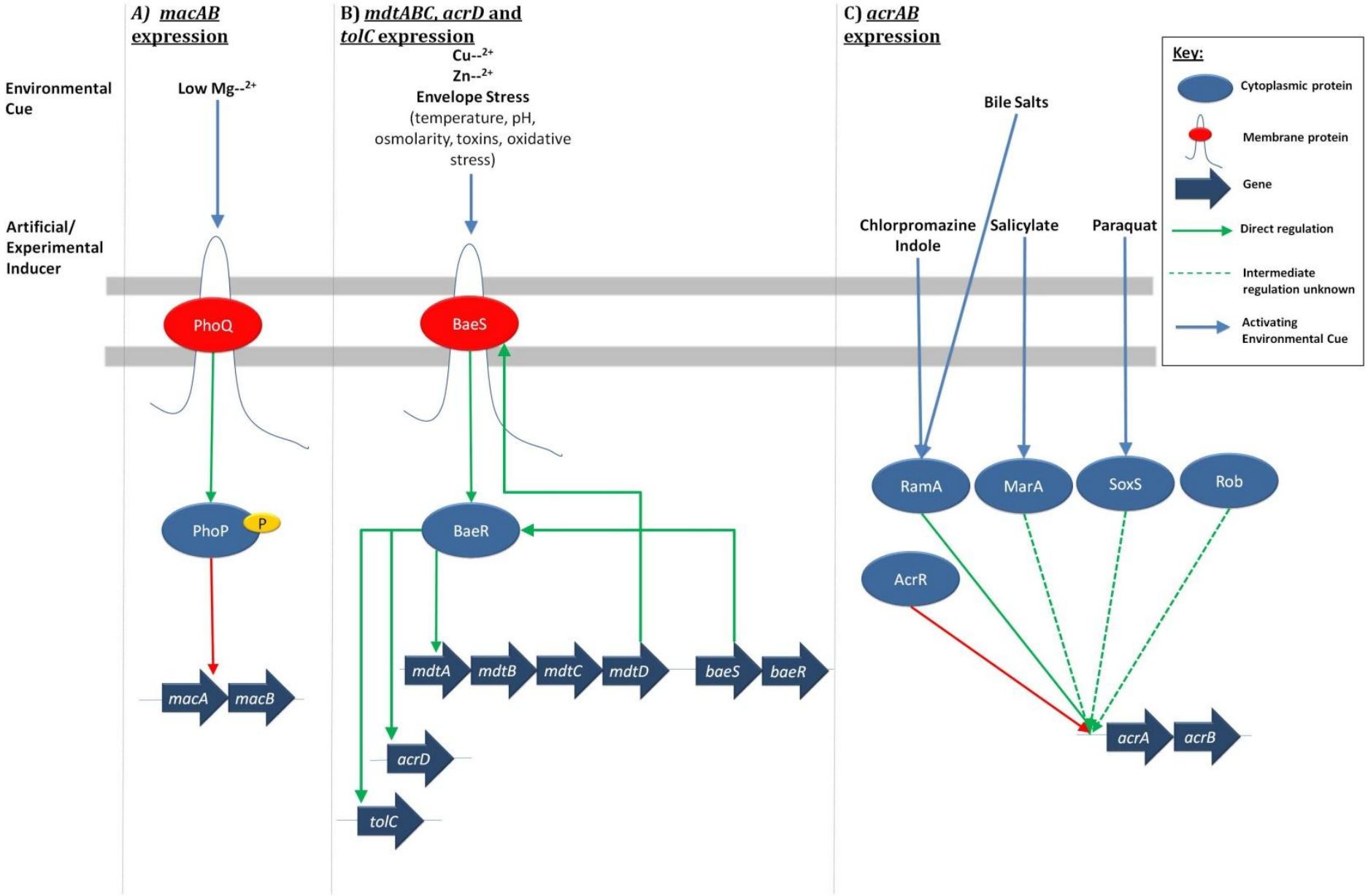
1.4.7 Promiscuity of efflux pump proteins

TolC is not encoded in the same genetic locus as any of the MDR systems of *Salmonella* but acts as an outer membrane porin protein for several of these efflux system. In *S*. Typhimurium, TolC is known to interact with eight multidrug efflux systems including AcrAB (Nishino *et al.* 2006; Horiyama *et al.* 2010), MdsAB (Nishino *et al.* 2006). AcrD (Hirakawa *et al.* 2003; Nishino *et al.* 2003), AcrEF (Nishino *et al.* 2003), MdtABC (Nagakubo *et al.* 2002), EmrAB, (Nishino and Yamaguchi 2002) and MacAB (Kobayashi *et al.* 2001). Clearly TolC is not restricted by the family of efflux pump it associates with as it can form tripartite systems with proteins from RND, MFS and ABC families. AcrA has also been shown to be promiscuous and can combine with three other transporter proteins; AcrD (Elkins and Nikaido 2002), AcrF (Kobayashi *et al.* 2001) and MdtF (Elkins and Nikaido 2002) in addition to AcrB.

1.4.8 Regulation of efflux in *Salmonella*

Multidrug transporters of *Salmonella* and other bacteria are subject to precise and complex transcriptional control (Figure 1.7). In *E. coli* and *S*. Typhimurium, *acrAB* has a local repressor, AcrR. In addition there are global networks of regulation controlled by MarA and SoxS, both members of the AraC/XylS family of transcriptional activators. In many Enterobacteriaceae, including *S*. Typhimurium, another AraC/XylS transcriptional activator is RamA which plays a major role in the regulation of *acrAB* (van der Straaten *et al.* 2004; Bailey *et al.* 2010).

Figure 1.7 Regulation network including environmental cues of efflux pumps in *Salmonella*



These regulators can respond to many environmental stresses to both increase expression of efflux pumps and down-regulate porin expression in order to reduce accumulation of potentially toxic substrates within the cytoplasm. These regulators consist of two components; a repressor and activator gene. In the case of RamA, RamR acts as a repressor of RamA and blocks it's transcription, inducer molecules can then de-repress transcription of *ramA*. Production of elevated levels of RamA results in RamA binding upstream of it's target operons to a recognised amino acid sequence known as the "ram box" where it acts as a transcriptional activator (Bailey *et al.* 2010). As well as controlling efflux pump expression, MarA, SoxS and RamA can all influence relatively large regulons (Martin and Rosner 2002; Bailey *et al.* 2010; Chubiz and Rao 2011). The de-repression of *acrB, acrD* and *acrF* is also co-ordinately regulated and the expression of each other and all respond to these three pumps influences the expression of the global transcriptional activators *marA* and *soxS* (Eaves *et al.* 2004). For example *acrS* is a repressor located upstream of *acrEF* which actually exerts its repressive effect on *acrAB* so if *acrSEF* is induced *acrAB* is repressed (Hirakawa *et al.* 2008). Expression of *acrD* and *mdtABC* are induced by high intracellular levels of copper and zinc (2mM and 1mM respectively) via the regulator two component regulatory system, BaeSR, suggesting that these two efflux systems AcrD and MdtABC have a physiological role in metal homeostatis in *Salmonella* as well as multidrug resistance (Nishino *et al.* 2007)

1.4.9 Inducers of efflux in *Salmonella*

There are a variety of compounds known to induce *acrAB* in *Salmonella* via the global regulator *ramA*. Bile salts, indole, cholic and deoxycholic acid are all thought to induce *acrAB* via *ramA*. This has been shown by mutating *ramA* in *S*. Typhimurium

which has subsequently decreased induction of *acrAB* on addition of these compounds. Indole has also been shown to induce *ramA* directly using a beta-galactosidase reporter plasmid fused to the promoter region of *ramA* (Nikaido *et al.* 2008).

1.4.10 Other physiological roles for MDR efflux pumps in *Salmonella*

Whilst export of antimicrobials is a feature of MDR efflux these pumps have been found to have diverse functional roles. It is thought that the physiological role of efflux pumps in *Salmonella* is to mediate the extrusion of certain molecules produced by the host organism (including bile, hormones, fatty acids and host defence molecules), in order to allow persistence in the face of host factors and invade host cells as well as export toxins, metabolites and other environmental compounds (Piddock 2006; Blair *et al.* 2009). Both *Salmonella* Typhi and Typhimurium are able to export bile salts and other host defence molecules and are able to survive in concentrations of bile much higher than those they would encounter in the animal gastrointestinal tract (van Velkinburgh and Gunn 1999).

Loss of TolC confers a virulence defect in *Salmonella*, *tolC* mutants poorly adhered to *in vitro* embryonic intestinal cells and mouse monocyte macrophages and were also unable to invade these cells (Buckley *et al.* 2006; Nishino *et al.* 2006). *In vivo*, *tolC* mutants colonised the avian gut poorly and also did not persist. However, *acrB* mutants were able to adhere to tissue culture cells *in vitro*, but could not invade (Buckley *et al.* 2006). Other RND family efflux systems were also required for virulence in a mouse model. MdtABC and MdsABC were all required for full virulence and AcrAB and AcrEF mutants showed a delay in the time taken to cause mortality of

the mice (Nishino *et al.* 2006). In the same study it was also found that MacAB, an ABC transporter, was also essential for full virulence of *Salmonella* in the BALB/c mouse model (Nishino *et al.* 2006).

Loss of TolC and AcrB has been shown to result in reduced expression of *Salmonella* Pathogenicity Island 1 genes and loss of AcrA has been linked with reduced expression of *Salmonella* Pathogenicity Island 2 genes (Webber *et al.* 2009). These pathogenicity islands encode Type IV secretion systems used to invade host cells and survive within macrophages.

The involvement of MDR efflux pumps in virulence is not restricted to *Salmonella*. Efflux systems known to be essential for virulence in other species include; AcrAB-TolC in *Erwinia amylovora* (Burse *et al.* 2004), and *Enterobacter cloacae* (Burse *et al.* 2004), MtrCDE in *Neisseria gonorrhoeae* (Jerse *et al.* 2003) and MexAB-OprM in *Pseudomonas aeruginosa* (Hirakata *et al.* 2002). The exogenous addition of efflux inhibitors (EIs) can also re-create similar virulence defects. EIs known to reduce virulence are; PAβN (a known competitive inhibitor of RND pumps) in *Pseudomonas aeruginosa* (Hirakata *et al.* 2009) and *Campylobacter jejuni* (Lin and Martinez 2006) and capsaicin (an inhibitor of the MFS NorA efflux pump) in *Staphylococcus aureus* (Kalia *et al.* 2012) .

1.5 Efflux pumps and biofilm formation

It has previously been shown that inhibition of efflux pumps by EIs, 1-(1-naphtylmethyl)-piperazine (NMP), PAβN and thioridazine reduced biofilm formation of *E. coli*, *Klebsiella*, *S. aureus* and *P. putida* (Kvist *et al.* 2008). Recent work in our laboratory with *S*. Typhimurium has shown a similar biofilm defect results

from inactivation of any of the nine MDR pumps (Baugh *et al.* 2012). Using the well characterised, AcrAB-TolC as a model system, I have shown that *tolC* and *acrB* mutant of *S*. Typhimurium are unable to produce a competent biofilm in many different laboratory models, and that these mutants have repressed transcription of both curli operons. However *acrA* mutants (lacking the periplasmic adapter protein) have a wild-type biofilm phenotype (Baugh *et al.* 2012).

1.6 Hypotheses

This project set out to investigate the link between efflux and biofilm formation in *Salmonella*. Three specific hypotheses which may explain why these are linked were investigated:

1. AcrAB-TolC exports a biofilm crucial factor(s)

2. Inactivation of AcrAB-TolC components alters cell surface hydrophobicity

3. Inactivation of *acrB* or *tolC* leads to expression changes of other biofilm related proteins.

1.7 Aims and Objectives

- Optimise an *in vitro* biofilm assay for *Salmonella enterica* serovar Typhimurium 14028S and its isogenic efflux mutants.

- Determine the mechanism which gives rise to the inability of efflux mutants to form competent biofilms.

- Investigate the utility of efflux inhibitors as anti-biofilm agents in *Salmonella*.

CHAPTER TWO:

MATERIALS & METHODS

2 Materials and Methods

2.1 Bacterial strains, growth, storage and identification

2.1.1 Strains used

This project used a variety of isogenic strains with specific efflux pumps inactivated derived from *S*. Typhimurium 14028S. A number of these have been previously described (Nishino et al., 2006), whilst others were constructed during this project. *S*. Typhimurium 14028S was used as the wild-type in the project as it has previously been characterised, genome sequenced and readily forms a biofilm on a variety of surfaces (Jarvik *et al.* 2010). Full strain details are shown in Table 2.1.

2.1.2 Growth and storage of bacteria

Salmonella strains were kept at -20°C on Protect™ beads (Technical Service Consultants Ltd., U.K.) for long-term storage. All strains were resuscitated from beads on Luria-Bertani (LB) agar plates (Sigma-Aldrich Ltd., U.K.) containing appropriate antibiotics where necessary and incubated at 37°C for 24 hours. Plates were stored at 4°C for up to two weeks. Overnight broth cultures were grown by inoculating a single colony into an appropriate volume of LB broth (Sigma-Aldrich Ltd., U.K.) again containing appropriate antibiotics where needed and incubated overnight at 37°C with shaking (200 rpm).

Table 2.1 Strains used in this study

Lab. ref. no.	Strain	Description	Reference
L133	SL1344 *ramRA::aph*	Mutant lacking functional RamRA	(Bailey *et al.* 2008)
L354	SL1344	Wild-type *Salmonella* Typhimurium	ATCC
L644	SL1344 Δ*acrB*	SL1344 lacking a functional AcrB	This study
L722	14028S *ramA::aph*	14028S lacking functional RamA	Courtesy of V. Der Streaten
L723	14028S *soxS::tet*	14028S lacking functional SoxS	Courtesy of V. Der Streaten
L724	14028S *ramA:aph, soxS::tet*	14028S lacking functional RamA and SoxS	Courtesy of V. Der Streaten
L725	14028S *marA::cat*	14028S lacking functional MarA	Courtesy of V. Der Streaten
L726	14028S *ramA::aph, marA::cat*	14028S lacking functional MarA and RamA	Courtesy of V. Der Streaten
L727	14028S *marA::cat, soxS::tet*	14028S lacking functional MarA and SoxS	Courtesy of V. Der Streaten
L728	14028S *ramA::aph, marA::cat, soxS::tet*	14028S lacking functional RamA, MarA and SoxS	Courtesy of V. Der Streaten
L742	SL1344 Δ*tolC*	SL1344 lacking a functional TolC	This study
L786	SL1344 *ramRA::aph* + pTrc *hisA::ramA*	SL1344 containing inducible *ramA* on a plasmid	Courtesy of V. Der Streaten
L828	14028S	SL1344 *Salmonella* Typhimurium	ATCC

Lab. ref. no.	Strain	Description	Reference
L829	14028S *tolC::cat*	14028S lacking functional TolC	(Nishino *et al.* 2006)
L830	14028S *acrB::aph*	14028S lacking functional AcrB	(Nishino *et al.* 2006)
L832	14028S *acrD::aph*	Mutant lacking functional AcrD	(Nishino *et al.* 2006)
L833	14028S *acrEF::aph*	Mutant lacking functional AcrEF	(Nishino *et al.* 2006)
L834	14028S *mdtABC::aph*	Mutant lacking functional MdtABC	(Nishino *et al.* 2006)
L835	14028S *mdsABC::aph*	Mutant lacking functional MdsABC	(Nishino *et al.* 2006)
L836	14028S *emrAB::aph*	Mutant lacking functional EmrAB	(Nishino *et al.* 2006)
L837	14028S *mdfA::aph*	Mutant lacking functional MdfA	(Nishino *et al.* 2006)
L838	14028S *mdtK::aph*	Mutant lacking functional MdtK	(Nishino *et al.* 2006)
L839	14028S *macAB::aph*	Mutant lacking functional MacAB	(Nishino *et al.* 2006)
L884	SL1344 *acrA::aph*	SL1344 lacking functional AcrA	(Blair *et al.* 2009)
L973	14028S *tolC::cat* +pWKS30-*tolC*	TolC mutant complemented with plasmid containing *tolC*	This study
L974	14028S *acrB::aph* +pWKS30-*acrB*	AcrB mutant complemented with plasmid containing *acrB*	This study

Lab. ref. no.	Strain	Description	Reference
L1019	SL1344+pUA66p*acpP-gfp*	SL1344 with plasmid containing *gfp* gene	This study
L1204	14028S+pUA66p*acpP-gfp*	14028S with plasmid containing *gfp* gene	This study
L1271	14028S *acrA::aph*	14028S lacking functional AcrA	This study
L1303	14028S *ramRA*::*aph*	14028S lacking functional RamRA	This study
L1305	14028S *tolC*::*cat*, *ramRA*::*aph*	14028S lacking functional TolC and RamRA	This study
L1494	SL1344 *ompC::aph*	SL1344 lacking a functional OmpC	Courtesy of A. Lawler
L1496	SL1344 *ompF::aph*	SL1344 lacking a functional OmpF	Courtesy of A. Lawler
L1506	14028S Δ*tolC,* Δ*ramA*	14028S lacking a functional TolC and RamA	This study
L1507	14028S Δ*tolC,* Δ*ramA, marA::aph*	14028S lacking a functional TolC, RamA and MarA	This study
L1508	14028S Δ*tolC,* Δ*ramA,* Δ*soxS*	14028S lacking a functional TolC, RamA and SoxS	This study
L1509	14028S Δ*tolC,* Δ*ramA,* Δ*soxS, marA::aph*	14028S lacking a functional TolC, RamA, SoxS and MarA	This study
L1511	14028S *ramA::cat*	14028S lacking functional RamA	This study
L1561	14028S *envZ*::Tn5	14028S lacking a functional EnvZ	This study

Lab. ref. no.	Strain	Description	Reference
L1562	14028s *tolC::cat*, *envZ*::Tn5	14028S lacking a functional TolC and EnvZ inactivated by P22 transduced from transposon library	This study
L1563	14028S *ompR::aph*	14028S lacking a functional OmpR	This study
L1564	14028S *tolC::cat*, *ompR::aph*	14028S lacking a functional TolC and OmpR	This study
L1575	14028S *tolC::cat*, *envZ*::Tn5	14028S lacking a functional TolC and EnvZ isolated from transposon screen	This study
L1581	14028S *tolC::cat*, *ybaJ*::Tn5	14028S lacking a functional TolC and YbaJ isolated from transposon screen	This study
L1582	14028S + pMW82-*ramA*	14028S harbouring a plasmid with the promoter region of *ramA* fused to *gfp*	This study
L1583	14028S *tolC::cat* + pMW82-*ramA*	14028S lacking a functional TolC harbouring a plasmid with the promoter region of *ramA* fused to *gfp*	This study
L1584	14028S *acrB::aph* + pMW82-*ramA*	14028S lacking a functional AcrB harbouring a plasmid with the promoter region of *ramA* fused to *gfp*	This study
L1585	14028S + pMW82-*marA*	14028S harbouring a plasmid with the promoter region of *marA* fused to *gfp*	This study
L1586	14028S *tolC::cat* + pMW82-*marA*	14028S lacking a functional TolC harbouring a plasmid with the promoter region of *marA* fused to *gfp*	This study
L1587	14028S *acrB::aph* + pMW82-*marA*	14028S lacking a functional AcrB harbouring a plasmid with the promoter region of *marA* fused to *gfp*	This study
L1588	14028S+ pMW82-soxS	14028S harbouring a plasmid with the promoter region of *soxS* fused to *gfp*	This study
L1589	14028S*::cat* + pMW82-*soxS*	14028S lacking a functional TolC harbouring a plasmid with the promoter region of *soxS* fused to *gfp*	This study

Lab. ref. no.	Strain	Description	Reference
L1590	14028S *acrB::aph* + pMW82-*soxS*	14028S lacking a functional AcrB harbouring a plasmid with the promoter region of *soxS* fused to *gfp*	This study
N/A	14028S *tolC::cat* + Tn5	14028S lacking a functional TolC transposon library	This study
N/A	14028S *acrB::aph* + Tn5	14028S lacking a functional AcrB transposon library	This study
N/A	14028S *emrAB::cat* + Tn5	14028S lacking a functional EmrAB transposon library	This study
N/A	14028S *mdtK::cat* + Tn5	14028S lacking a functional MdtK transposon library	This study

2.1.3 Phenotypic identification of strains

Gram staining was used to ensure all strains cultured were Gram negative rods, to further identify the strains, all bacteria were grown on xylose lysine deoxycholate (XLD) agar (Sigma-Aldrich Ltd., U.K.). *Salmonella* were presumptively identified as colonies with black spots in the centre indicating hydrogen sulphite production and a colour change of agar from red to pink indicating xylose fermentation, two characteristic features of *Salmonella*. Analytical profile index (API) tests (Biomérieux) were also used to definitively identify all strains as *Salmonella*. The numerical code obtained from an API reaction of each strain was analysed using the interpretive criteria at https://apiweb.biomerieux.com.

2.1.4 Primer design and genotypic identification

Primers were used to amplify specific DNA fragments for various applications including sequencing, measuring gene expression, complementation, gene inactivation and over-expression (Table 2.2). Primers were designed using the PRIMER v 2.0 software package (Science and Educational Software, Cambridge, U.K.) and made by Invitrogen Life Technologies (Germany) and diluted with sterile distilled water to a concentration of 100 µM and stored at -20°C. When required, primers were diluted with sterile distilled water to a 25 µM working stock and stored at -20°C. The details of all primers used in this study with their applications are shown in Table 2.2.

Table 2.2 Primers used in this study

Primer	Sequence	Description
***16S*-RTF**	CCT CAG CAC ATT GAC GTT AC	16S qRT-PCR forward primer
***16S*-RTR**	TTC CTC CAG ATC TCT ACG CA	16S qRT-PCR reverse primer
***acrA*-checkF**	ACA TCC AGG ATG TGT TGT CG	*acrA* mutant check forward primer
***acrA*-checkR**	CAA TCG TCG GAT ATT GCG CT	*acrA* mutant check reverse primer
***acrB*-checkF**	GGA TCA CAC CTT ATT GCC AG	*acrB* mutant check forward primer
***acrB*-checkR**	TTA ACA GTG ATC GTC GGT CG	*acrB* mutant check reverse primer
***acrD*-checkF**	AAT TGT GCG TGA AGC GGT	*acrD* mutant check forward primer
***acrD*-checkR**	GCT ACA GCG CCA TAG TAA	*acrD* mutant check reverse primer
***acrF*-checkF**	CCG ACG TTA CCG TAG ATG AA	*acrF* mutant check forward primer
***acrF*-checkR**	GTG GTA AGC AAT GCA ACT GG	*acrF* mutant check reverse primer
***bcsA*-RTF**	TGC AGG ACG GTA ACG ATA TG	*bcsA* qRT-PCR forward primer
***bcsA*-RTR**	ATC TTC CGT CAC CGT CTC AA	*bcsA* qRT-PCR reverse primer
***bcsB*-RTF**	AAT CAC CGA TGA TAG CGC TC	*bcsB* qRT-PCR forward primer
***bcsB*-RTR**	TGC ACC AAC AGA TCG ATA CG	*bcsB* qRT-PCR reverse primer
***bcsC*-RTF**	AGC AGT GGA TAA CAC CGA CA	*bcsC* qRT-PCR forward primer
***bcsC*-RTR**	TTA CCG CTA TCC ATA CGC AG	*bcsC* qRT-PCR reverse primer
***bcsZ*-RTF**	GCG ATA TCT GGA TGG CAT GG	*bcsZ* qRT-PCR forward primer
***bcsZ*-RTR**	CGG CAC ATT CAG GAC CTC TT	*bcsZ* qRT-PCR reverse primer
***csgA*-RTF**	AGC ATT CGC AGC AAT CGT AG	*csgA* qRT-PCR forward primer
***csgA*-RTR**	TTA GCG TTC CAC TGG TCG AT	*csgA* qRT-PCR reverse primer
***csgB*-RTF**	ATC AGG CGG CCA TTA TTG GT	*csgB* qRT-PCR forward primer
***csgB*-RTR**	TAC TGG CAT CGT TGG CAT TG	*csgB* qRT-PCR reverse primer
***csgC*-RTF**	AAT TCT CTC TGT GCG CGA CG	*csgC* qRT-PCR forward primer
***csgC*-RTF**	GCA GTG ATT GTC CGT CCG AA	*csgC* qRT-PCR reverse primer
***csgD*-RTF**	GGT ATT CTG CGT GGC GAA TG	*csgD* qRT-PCR forward primer
***csgD*-RTR**	AGT AAT GCG GAC TCG GTG CT	*csgD* qRT-PCR reverse primer
***csgE*-RTF**	ACG CTA TCT GAC CTG GAT TG	*csgE* qRT-PCR forward primer

Primer	Sequence	Description
***csgE*-RTR**	CGT TAT GGT GAT CCA GCT TC	*csgE* qRT-PCR reverse primer
***csgF*-RTF**	GAC GTT CCA GTT CGC TAA TC	*csgF* qRT-PCR forward primer
***csgF*-RTR**	ATC GTT GGT CAC CAT ACG TC	*csgF* qRT-PCR reverse primer
***csgG*-RTF**	CTG GAA CGA CAA GGC TTA CA	*csgG* qRT-PCR forward primer
***csgG*-RTR**	TGA TCC AGC TGA TAC TGC GT	*csgG* qRT-PCR reverse primer
***envZ*-checkF**	GTC ACA GAG CGC ACT TCA GA	*envZ* mutant check forward primer
***envZ*-checkR**	CTC TCG CGA TAA GCT GAT GA	*envZ* mutant check reverse primer
***macA*-checkF**	AAT ACA GCC GGC GCT ATC TT	*macAB* mutant check forward primer
***macB*-checkR**	GAC GTC AGC AAG ATT ACT CC	*macAB* mutant check reverse primer
***mdfA*-checkF**	TTC TTA CAG TGG CTG CTT GG	*mdfK* mutant check forward primer
***mdfA*-checkR**	CAC CTG CAG CAG ACC ATA TT	*mdfK* mutant check reverse primer
***mdsB*-checkF**	GCA GGA TAT CGA ATA CCG GA	*mdsABC* mutant check forward primer
***mdsB*-checkR**	AGG ATC TTC ACG CTC GTC AA	*mdsABC* mutant check reverse primer
***mdtB*-checkF**	GCG CTG GTC GTC ATG ATT AT	*mdtABC* mutant check forward primer
***mdtB*-chackR**	CTG GCA TAC GAT GAC GAT TG	*mdtK* mutant check reverse primer
***mdtK*-checkF**	GTG ATG ATC GTC CTG TGG AA	*mdtK* mutant check forward primer
***mdtK*-checkR**	ATA GCG GTA ACC ACA GCC AT	*mdtABC* mutant check reverse primer
***ompR*-checkF**	CTG TCG ATA TTG CGC ACA CG	*ompR* mutant check forward primer
***ompR*-checkR**	TAA TTG CTG CGC CAT CTG GT	*ompR* mutant check reverse primer
***ramA*- checkF**	CCA TGC GAT AAG CTG TCT CA	ramA mutant check forward primer
***ramA*-checkR**	GCG CGC TGG AAT ATT ATG TC	*ramA mutant check reverse primer*
***ramA* KOF**	ATC AGC CGT TAC GTA TTG ATG ATA TTG CCC GTC ATG CGG G	Forward primer with homology to *ramA* and to *aph/cat* cassette to use with pKD3 or pKD4
***ramA* KOR**	GCA GGT TGA ACG TGC GGG TAA AAA TGC GCG TAA AGG TTT G	Reverse primer with homology to *ramA* and to *aph/cat* cassette to use with pKD3 or pKD4
RATE F	GAA CTT TTG CTG AGT TGA AGG ATC A	RATE forward primer to create transposon insertion site sequencing template
RATE R	ATG GCT CAT AAC ACC CCT TGT ATT A	RATE reverse primer to create transposon insertion site sequencing template

Primer	Sequence	Description
Tn5 F	ACC TAC AAC AAA GCT CTC ATC AAC C	Tn5 specific forward primer for transposon insertion sire sequencing
Tn5 R	GCA ATG TAA CAT CAG AGA TTT TGA G	Tn5 specific reverse primer for transposon insertion sire sequencing
***tolC*-checkF**	CTT CTA TCA TGC CGG CGA CC	*tolC* mutant check forward primer
***tolC*-checkR**	CGC TTG CTG GCA CTG ACC TT	*tolC* mutant check reverse primer

The genotypes of mutant strains were verified as carrying the expected specific disruptions in genes of interest by polymerase chain reaction (PCR). Bacterial cells were lysed to release DNA by boiling a single colony re-suspended in 100 µl of sterile distilled water (SDW) for 10 minutes and immediately placed on ice. This DNA lysate was used as a template for a PCR using specific primers homologous to the upstream and downstream regions of the genes of interest (Table 2.2) using the volumes below:

- 22.5 µl ReddyMix™ PCR Buffer (Thermo Scienctific)
- 0.75 µl upstream primer
- 0.75 µl downstream primer
- 1 µl DNA lysate

PCR parameters varied upon size of expected amplimer and annealing temperature of the primers but a typical PCR cycle for a product of around 1kb is as follows:

• 95°C	5 minute	Initial denature
• 95°C	30 seconds	Denature
• 51°C*	30 seconds	Annealing
• 72°C	1 minute*	Extension
• 72°C	10 minutes	Final Extension

*Variable parameters – annealing temperature is dependent upon GC content of primers. Extension time dependent upon amplimer size, typically 1 minute per kilobase.

1% agarose gels were supplemented with 5 µl of Midori Green (Nippon Genetics, Germany) per 100 ml of agarose to separate PCR amplimers. 5 µl of each reaction along with the appropriate DNA marker (often Hyperladder I (Bioline, U.K.)) were electrophoresed at 100 V for 1 hour. Agarose gels were visualised using a G:Box UV transilluminator and Genesys software (Syngene, U.K.) (Figure 2.1).

If the correct amplimer size was seen Sanger sequencing was used to ensure the amplimer obtained contains the partial sequence of the gene of interest as well as either the correct inactivation cassette (typically *aph* or *cat*) or deletion of a section of the gene (for Δ strains). The sequencing reactions were set up as follows:

- *ng of DNA template (* Amount of template required for a successful sequencing reaction depends upon the nature of the template and if sequencing from PCR the size of the PCR amplimer – Table 2.3)
- 3.2 pM of either upstream OR downstream primer
- Nuclease free water up to 10 µl.

Sequencing reactions were carried out by the Functional Genomics Laboratory (Biosciences, University of Birmingham).

2.2 Mutant construction

To create new mutants with specific genes inactivated, a recombinase mediated insertion of a resistance cassette was used.

Figure 2.1 A typical 1% agarose gel electrophoresed at 100 V for approximately 1 hour

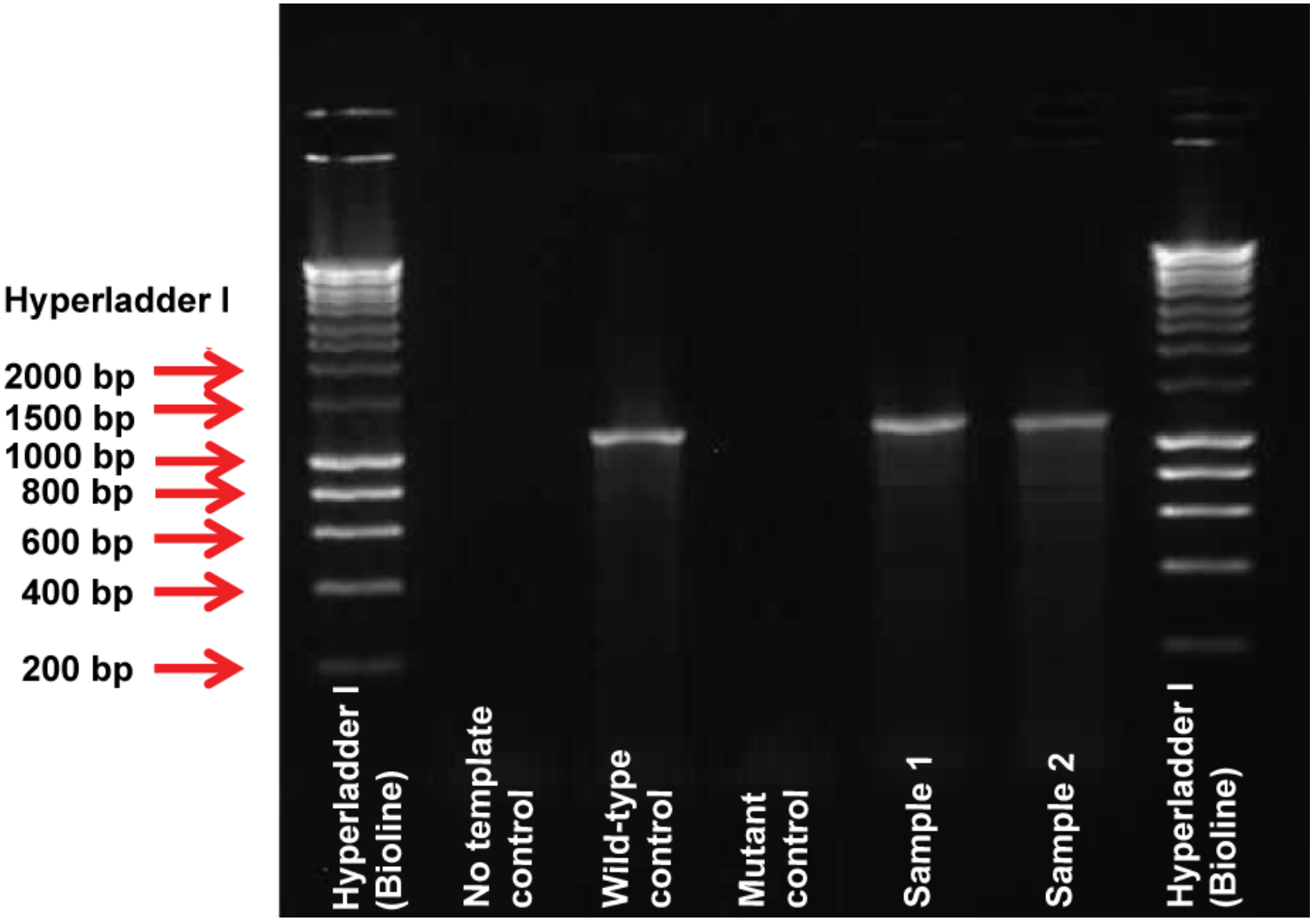


Table 2.3 Types and quantities of DNA template required for an efficient Sanger sequencing reaction

DNA Template	Amplimer size	Quantity required
PCR Amplimer	100 – 200 bp	1-3 ng
	200 – 500 bp	3-10 ng
	500 – 1000 bp	5-10 ng
	1000 – 2000 bp	10-40 ng
	>2000 bp	40-100 ng
Single-stranded DNA	N/A	50-100 ng
Double-stranded DNA	N/A	200-500 ng
Bacterial genomic DNA	N/A	2-3 µg

A 5 ml culture of *S*. Typhimurium SL1344 harbouring pSIM18 (full list of plasmids – Table 2.4), a plasmid carrying the gamma, beta and exo recombinase genes (Chan *et al.* 2007), was incubated overnight at 30°C with shaking in the presence of 150 µg/ml of hygromycin (Sigma-Aldrich Ltd., U.K.). 4 ml of this culture was then used to inoculate 100 ml of fresh LB broth containing 150 µg/ml of hygromycin and incubated at 30°C with shaking until an optical density at 600 nm of 0.3 was achieved. Cells were heat shocked in a water bath at 42°C for 15 minutes and cooled immediately on ice for 5 minutes. Cells were harvested by centrifugation at 3,000 x *g* at 4°C for 10 minutes. Supernatant was discarded and cells re-suspended in 45 ml of sterile ice cold water. Cells were again harvested and the pellet re-suspended as above but in 1.5 ml of ice cold water. Cells were then washed five times by harvesting cells in a microcentrifuge at 10,000 x *g* for 2 minutes and re-suspended again in 1.5 ml of ice cold water after each centrifugation. The final pellets were re-suspended in 100 µl of ice cold water. Primers with 20 bp of homology to a resistance cassette (either *aph* or *cat* genes from pKD4 or pKD3 containing kanamycin or chloramphenicol resistance) and 40 bp of homology to the gene to be inactivated were used to amplify a product via PCR to be inserted into the gene to be inactivated. 40 µl of the competent cells were electroporated with 3-5 µg of the PCR amplimer in pre cooled 2 mm electrocuvettes (Geneflow, U.K.) at 2.5 kV. The cells were recovered in 400 µl of SOC media and incubated at 37°C for 2 hours. Cells were then spread onto agar supplemented with appropriate antibiotic and incubated for up to 48 hours at 37°C. Candidate colonies were verified by PCR using primers flanking the target gene and products sequenced to ensure the correct insertion of the resistance cassette.

Table 2.4 Plasmids used in this study

	Plasmid		Resistance	Reference
Gene inactivation plasmids	pSIM18	Heat inducible recombineering vector	hygromycin/ampicillin	(Lee and Liu 2009)
	pKD3	Template plasmid for gene disruption containing *cat* cassette for chloramphenicol resistance flanked by FRT sites	ampicillin/chloroamphenicol	(Datsenko and Wanner 2000)
	pKD4	Template plasmid for gene disruption containing *aph* cassette for kanamycin resistance flanked by FRT sites	ampicillin/kanamycin	(Datsenko and Wanner 2000)
	pCP20	Temperature sensitive vector containing *flp* (flipase) to remove antibiotic resistance cassettes at the FRT sites	ampicillin	(Cherepanov and Wackernagel 1995)
Over-expression plasmids	pTrc	High-level expression vector template inducible upon addition of IPTG	ampicillin	(Life technologies, UK)
	pTrc *ramA*	High-level expression vector containing *ramA* inducible upon addition of IPTG	ampicillin	Courtesy of Vito Ricci
	pTrc *marA*	High-level expression vector containing *marA* inducible upon addition of IPTG	ampicillin	Courtesy of Vito Ricci
	pTrc *soxS*	High-level expression vector containing *soxS* inducible upon addition of IPTG	ampicillin	Courtesy of Vito Ricci
	pWKS30	Low copy number expression vector template	ampicillin	(Wang and Kushner 1991)
	pWKS30 *acrB*	Low copy number expression vector containing *acrB*	ampicillin	Courtesy of James Wootton
	pWKS30 *tolC*	Low copy number expression vector containing *tolC*	ampicillin	Courtesy of James Wootton
***gfp* reporter plasmids**	pMW82	Medium copy expression vector with promotorless *gfp* (green fluorescence protein) gene	ampicillin	(Bumann and Valdivia 2007)
	pMW82-*ramA*	Medium copy expression vector containing *gfp* (green fluorescence protein) gene fused to *ramA* promoter	ampicillin	(Lawler *et al.* 2013)
	pMW82-*marA*	Medium copy expression vector containing *gfp* (green fluorescence protein) gene fused to *marA* promoter	ampicillin	Courtesy of Vito Ricci
	pMW82-*soxS*	Medium copy expression vector containing *gfp* (green fluorescence protein) gene fused to *soxS* promoter	ampicillin	Courtesy of Vito Ricci

2.3 P22 transduction

Transduction with bacteriophage P22 was used to transfer specific insertion mutations between different strains of *Salmonella*. Transduction was used when transferring mutation between different strains of *S*. Typhimurium (i.e. from SL1344 to 14028S), to create multiple inactivations and to ensure any new inactivations created by the pSIM protocol (section 2.2) are in a 'clean' background.

A 10 ml culture of desired strain containing the inactivation of interest was grown overnight in LB broth at 37°C with shaking (200 rpm). 50 µl of this overnight culture was used to inoculate 5 ml of fresh LB broth supplemented with 500 µl of 100 mM $MgSO_4$ and 50 µl of 500 mM $CaCl_2$ and incubated for 30 minutes at 37°C with shaking (200 rpm). 5 µl of P22 phage stock was added to this culture and incubated overnight at 37°C with shaking (200 rpm). 1 ml of chloroform was added to the overnight culture. Mixed thoroughly by vortexing and pelleted via centrifugation at 3,000 x *g* and 4°C for 10 minutes. The supernatant (only the upper liquid layer) was transferred to a glass bijou and 200 µl of chloroform added and mixed by vortexing briefly. This phage was then kept at 4°C until required.

A 10 ml culture of the recipient strains were grown overnight in LB broth at 37°C with shaking (200 rpm). A pellet was obtained by centrifugation at 2,500 *x g* at room temperature for 10 minutes. The supernatant was discarded and the pellet re-suspended in 1 ml of LB broth containing 10 mM $MgSO_4$ and 5 Mm $CaCl_2$. Transduction reactions were set up by dispensing 100 µl aliquots of cell suspension and adding a volume of P22 phage (containing packaged DNA with desired markers), ranging between 0 and 100 µl, to each sample. These reactions were incubated at 37°C for 15 minutes. 100 µl of sodium citrate and 1 ml of LB broth were

then added to chelate divalent cations and incubated at 37°C for 45 minutes. To select for transductants, 100 µl from each transduction reaction was spread onto LB plates supplemented with appropriate antibiotics and incubated overnight (or 48 hours if required) at 37°C. Any colonies obtained were purity plated onto appropriate antibiotic and transferred mutation verified by PCR and sequencing.

2.4 Removal of resistance cassettes

Resistance cassettes were removed from selected mutant strains to allow transduction of additional mutant alleles harbouring the same resistance cassettes to create strains with two or more mutations. Competent cells of the strains harbouring the resistance cassettes to be removed were made by harvesting 10 ml of a mid-logarithmic culture grown in LB broth via centrifugation at 4°C and 2,250 x *g* for 10 minutes before the resulting supernatant was discarded. Pellets were then re-suspended in 10 ml of ice cold 15% glycerol. This centrifugation and re-suspension step was repeated. Suspensions were harvested again and re-suspended in a final volume of 1 ml of 15% glycerol. Competent cells can be used immediately or stored at -80°C until required. 45 µl of competent cells were then electroporated at 2.5 kV for 5 ms with 1 ng of the plasmid pCP20 (Cherepanov and Wackernagel 1995), and recovered in 1 ml of SOC for one hour at 30°C with shaking (200 rpm). 200 µl of recovered cells were plated onto LB agar plates containing 50 µg/ml of ampicillin and incubated for up to 48 hours at 30°C. Candidate colonies were verified via PCR for loss of resistance cassette and by patching onto LB agar containing appropriate antibiotics. Successful colonies were used to inoculate 10 ml of antibiotic free LB broth and incubated overnight at 43°C with shaking (200 rpm) to allow for loss of the plasmid as pCP20 carries a temperature sensitive origin of

replication. A loopful of culture was streaked onto LB agar and incubated at 37°C overnight. Candidate strains were then patched onto LB agar supplemented with ampicillin and tested for susceptibility (to ensure plasmid has been cured) and kanamycin/chloramphenicol susceptibility (to ensure loss of resistance cassette).

2.5 Plasmid purification, construction and transformation

2.5.1 Plasmid purification

Plasmids were isolated from strains to over-express genes of interest, allow complementation and to introduce fluorescent reporter proteins for imaging. Plasmid DNA was harvested using the QIAprep® Spin Cell Mini Kit (QIAGEN) as follows. 10 ml overnight cultures in LB broth of strains of interest (containing plasmids) were incubated and pellets harvested by centrifugation for 10 minutes at 2,250 x *g*. Pellets were re-suspended in 250 µl of buffer P1 (QIAGEN) and transferred to a microcentrifuge tube. 250 µl of buffer P2 (QIAGEN) was then added and the tube was immediately mixed by inversion to ensure the solution was mixed thoroughly. This was followed by addition of 350 µl of buffer N3 (QIAGEN) and again the tubes were immediately mixed by inversion. The tubes were centrifuged at 4,500 x *g* in a table-top centrifuge for 10 minutes and the supernatants transferred to spin columns using a pipette. Spin columns were placed in clean microcentrifuge tubes and centrifuged again for 1 minute at 4,500 x *g* and the flow-through discarded. Columns were washed with 500 µl of buffer PB (QIAGEN), centrifuged for 1 minute and the flow-through discarded. Columns were again washed by adding 750 µl of buffer PE (QIAGEN), centrifuged for 1 minute and the flow-through discarded before being centrifuged for an additional minute to remove residual wash buffer. Columns were placed into clean microcentrifuge tubes and plasmid DNA eluted by adding 50 µl of

buffer EB (QIAGEN) and centrifuging for 1 minute. The resulting plasmid DNA was analysed by agarose gel electrophoresis using a 1% agarose gel. The plasmid DNA was quantified by analysing gel images using Gene Tools (Syngene, U.K.). Plasmid DNA was stored at -20°C.

2.5.2 Plasmid transformation

Competent cells of recipient strains were prepared (see section 2.4) and chilled on ice until transformation. 50 µl of competent cells and around 1 ng of plasmid DNA were added to a microcentrifuge tube and chilled on ice for 20 minutes. Suspensions were transferred to a 2 mm electrocuvette and electroporated immediately with 2.5 kV for 5 ms. Cells were recovered by adding 950 µl of SOC media and incubated at 37°C with shaking (200 rpm) for 1.5 hours. Two volumes of the recovered cells (100 and 200 µl) were spread onto LB agar containing appropriate antibiotics and incubated overnight at 37°C. Colonies obtained were verified by purity plating on LB agar supplemented with appropriate antibiotics and plasmid purification (as in section 2.5.1) to ensure plasmid has been successful inserted.

2.6 Growth Kinetics

To determine whether any of the mutants studied had a growth deficit which could impact on their ability to form a biofilm, growth kinetics of each were investigated. Overnight cultures of strains were diluted 1 in 25 in fresh pre-warmed LB broth without salt. For each strain two biological and two technical replicates were used. A 96 well microtitre tray was inoculated with 100 µl of this bacterial suspension and incubated at 30°C with intermittent agitation. The optical density at 600 nm was measured every 10 minutes for 16 hours using a FLUOstar OPTIMA (BMG, U.K.). Results were analysed by plotting average values for each strain on a logarithmic

graph and calculating standard deviations to use as error bars. Generation times were also calculated using the logarithmic growth phase of the line graph and the following equation;

$$\mathbf{g} = \frac{\mathbf{T}\ \ln 2}{\ln (OD_{end}/OD_{start})}$$ (where **g** is generation [doubling] time and **T** is time)

Values which appeared different to the parent strain were evaluated for significance at defined time points using a students 't' test.

2.7 Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was used to define the impact of gene inactivation and as another method to verify the phenotype of all mutant strains. The agar doubling dilution method was used to determine the minimum inhibitory concentration (MIC) of antimicrobials according to the method of the British Society of Antimicrobial Chemotherapy (Andrews 2007). Antibiotic stocks were prepared and diluted and labelled universal tubes and Petri dishes were prepared for each final test concentration of each antibiotic required. The appropriate volume of antibiotic stock was added to a universal to give the desired concentration when added to 20 ml of molten iso-sensitest agar (using a dilution table – Table 2.5). A perifill peristaltic pump was set up aseptically and used to dispense 20 ml of molten agar into the universals containing the antibiotics, swirled and poured into the corresponding Petri dish. Two antibiotic free Petri dishes were also set up to become a 'start' and 'finish' plate to act as inoculation controls. The agar plates were left on the bench to set for 15 minutes before being dried at 50°C. 100 µl of overnight LB broth cultures were diluted in 10 ml of sterile LB broth to give approximately 10^7 cfu/ml.

Table 2.5 Antibiotic stocks required to prepare final antibiotic concentrations in 20 ml of agar

	Final concentration of antibiotic in 20 ml of agar					
Final required concentration	**1024**	**512**	**256**	**128**	**64**	**32**
Volume of 10,000 µg/ml stock required	2048	1024	512	256	128	64
Final required concentration	**16**	**8**	**4**	**2**	**1**	
Volume of 1,000 µg/ml stock required	320	160	80	40	20	
Final required concentration	**0.5**	**0.25**	**0.12**	**0.06**		
Volume of 100 µg/ml stock required	100	50	25	13		
Final required concentration	**0.03**	**0.015**	**0.008**			
Volume of 10 µg/ml stock required	60	30	15			

From this bacterial suspension, 100 µl was added to the wells of a 21 or 100 pin template. All agar plates were inoculated using a multipoint inoculator with 1 µl of the diluted suspension to give a final inocula of ~10^4 cfu starting with the 'start' plate and finishing with the 'finish' plate. Inoculated agar plates were incubated overnight at 37°C. Plates were read by first ensuring that all strains grew on the antibiotic free 'start' and 'finish' plates. The lowest concentration of an antibiotic at which the each strain showed no growth was recorded and defined as the minimum inhibitory concentration (MIC) of that drug against the relevant strain.

2.8 Metabolomics

In parallel with this project a metabolomics investigation was used to explore the hypothesis that AcrAB-TolC may export metabolites whose accumulation is toxic and deleterious to biofilm formation, differences in intracellular metabolites present within L828 (wild-type) and L830 (*acrB::aph*) were investigated. The results from this work were made available for interrogation in this project; the methodology was briefly as follows:

Six replicate cultures (25 ml, defined MOPS minimal media) were grown to logarithmic phase (OD600 of 0.7) after incubation with shaking (170 rpm) at 37°C. Ten ml of each culture was withdrawn and metabolites quenched by adding the sample to 10 ml of -46°C 60% methanol (+ 70 mM HEPES). Cells were then harvested by centrifugation at 3250 x *g* at -4°C. The supernatant was discarded and cell pellets stored at -80°C overnight. The pellets were then re-suspended in 5ml of -46°C 60% methanol (+ 70 mM HEPES) and the OD (600 nm) measured for each and additional -46°C 60% methanol (+ 70 mM HEPES) added to standardize the concentration of metabolites before being harvested again. Supernatants were

discarded again and pellets stored at -80°C until metabolite extraction. Metabolite extraction and analysis by mass spectrometry was completed by the functional genomics unit (School of Biosciences, University of Birmingham) according to their protocols. Analysis of metabolites present and differences between samples used a principal component analysis, Benjamini Hochberg correction for removal of false positives and calibration of the experimental data against mass results for known metabolites. All bioinformatic analysis was in collaboration with the group of Dr Mark Viant (School of Biosciences, University of Birmingham).

2.9 Biofilm formation assays

A variety of different assays were used to evaluate formation of biofilms over time, in different media, on various materials, at liquid air interfaces and under different flow conditions.

2.9.1 Optimising the crystal violet biofilm formation assay

A simple, high throughput biofilm formation assay to allow screening of large numbers of strains or inhibitors was used as follows; overnight broths were diluted in 5 ml of appropriate media to an optical density at 600 nm of 0.1. A 200 µl (or 500 µl) aliquot of this dilution was dispensed into a well of a microtitre tray. The pattern of inoculation of the microtitre tray was recorded. A number of variables were assessed when defining optimum conditions:

- 24 (inoculated with 500 µl of bacterial suspension rather than 200 µl) or 96 well microtitre trays were used
- Temperature was altered between 30°C and 37°C

- Different media was used – LB broth, MOPS minimal media (Teknova, USA) and M9 minimal media
- Varying concentrations of NaCl were added to the media
- Gentle agitation or static incubation
- Time – biofilms were analysed between 24 and 96 hours of growth.

Once the desired incubation time had ended biofilms were washed in sterile distilled water to remove unattached cells. Next, 200 µl of 1% crystal violet was added to the wells and incubated for 15 minutes at room temperature to stain the cells associated with the biofilm. The crystal violet was removed from the wells which were washed again using sterile distilled water. The crystal violet retained was solubilised by addition of 200 µl of 70% ethanol. The OD (600 nm) of each well was then measured using a FLUOstar OPTIMA (BMG labtech). At least two biological replicates of each strain and four technical replicates of each were used in a run, repeat runs were included before final analysis of data. Average biofilm formation for each strain was determined and plotted on a bar chart, as well as standard deviations and values used as error bars. Differences between strains or addition of inhibitors was analysed with the students 't' test.

2.9.2 Biofilm formation assay with EIs

An assay was developed to observe the effects of different efflux inhibitors (EIs) on the formation of biofilms. The EIs used in this study were carbonyl cyanide m-chlorophenylhydrazone (CCCP), chlorpromazine (CPZ) and phenyl-arginine-β-naphthylamide (PAβN), each thought to have a different mechanism of action. CCCP abolishes the proton motive force (Pages *et al.* 2005) which provides the energy

used for the majority of MDR efflux systems (with ABC transporters being the exception). CPZ has been shown to stop serotonin uptake in human cells and also inhibits bacterial efflux and is thought to work via inducing *ramA* and repressing *acrB* (Bailey *et al.* 2008). PAβN is a substrate and acts as a competitive inhibitor of AcrAB-TolC (Lomovskaya *et al.* 2001). All wells of a 96 well MTT were filled with 50 µl of appropriate media. 50 µl of EI (at twice the desired highest final concentration) was added to each well of column one and mixed by pipetting up and down gently. 50 µl of this solution was removed from column 1 and transferred to column 2, this doubling dilution was repeated across the MTT leaving column 12 free from EI. Overnight broths were diluted with appropriate broth to an optical density at 600 nm of 0.1. 50 µl of this suspension was then used to inoculate appropriate rows of the MTT. Each MTT was then incubated for 48 hours at 30°C with gentle agitation. Biofilms were stained and analysed (see section 2.9.1).

2.9.3 Biofilm formation assay with exogenous polyamine

This assay was used to observe the effects of exogenous polyamines on the ability of *Salmonella* to form biofilms. Polyamines have been linked with biofilm formation in *Yersinia pestis* (Patel *et al.* 2006) and were also identified as over-accumulated within L830 (*acrB::aph*) relative to L828 (wild-type) in metabolic experiments carried out in this laboratory. Polyamines used were; acetyl-spermidine, cadaverine, putrescine and spermidine.

100 mM stocks of the appropriate polyamine were prepared in broth and each row of a 96 well MTT was loaded with 100 µl of a different concentration of polyamine (each at twice the final desired concentration), i.e. row 1 – 20 mM, row 2 – 2 mM, row 3 – 0.2 mM etc. Overnight broths cultures prepared in LB broth of desired bacterial

strains were diluted in sterile LB broth without salt to an optical density at 600 nm of 0.2 and 100 µl used to inoculate appropriate wells of the MTT. Broth controls (containing no bacteria) and polyamine controls (containing bacteria but no polyamine) were included. Two biological and two technical repeats were used for each strain. MTTs were incubated at 30°C for 48 hours with gentle agitation. Biofilms were stained and analysed (see section 2.9.1).

2.9.4 Membrane stress biofilm formation assay

Membrane stress two component systems have been shown to evoke repression of biofilm related genes (Jubelin *et al.* 2005). An assay to artificially stress the bacterial membrane through changes in osmolarity and pH of the extracellular environment was used to assess whether a membrane stress response was relevant to biofilm repression in the efflux mutants. 10 ml overnight cultures were diluted to an OD (600 nm) of 0.1 in LB broth without salt adjusted to appropriate pH levels and LB broth without salt supplemented with 20% glucose. Two biological and four technical replicates were used for each strain or pH level. A 200 µl aliquot of these dilutions were dispensed into the wells of a 96 well MTT and incubated at 30°C for 48 hours. Biofilms were stained and analysed (see section 2.9.1).

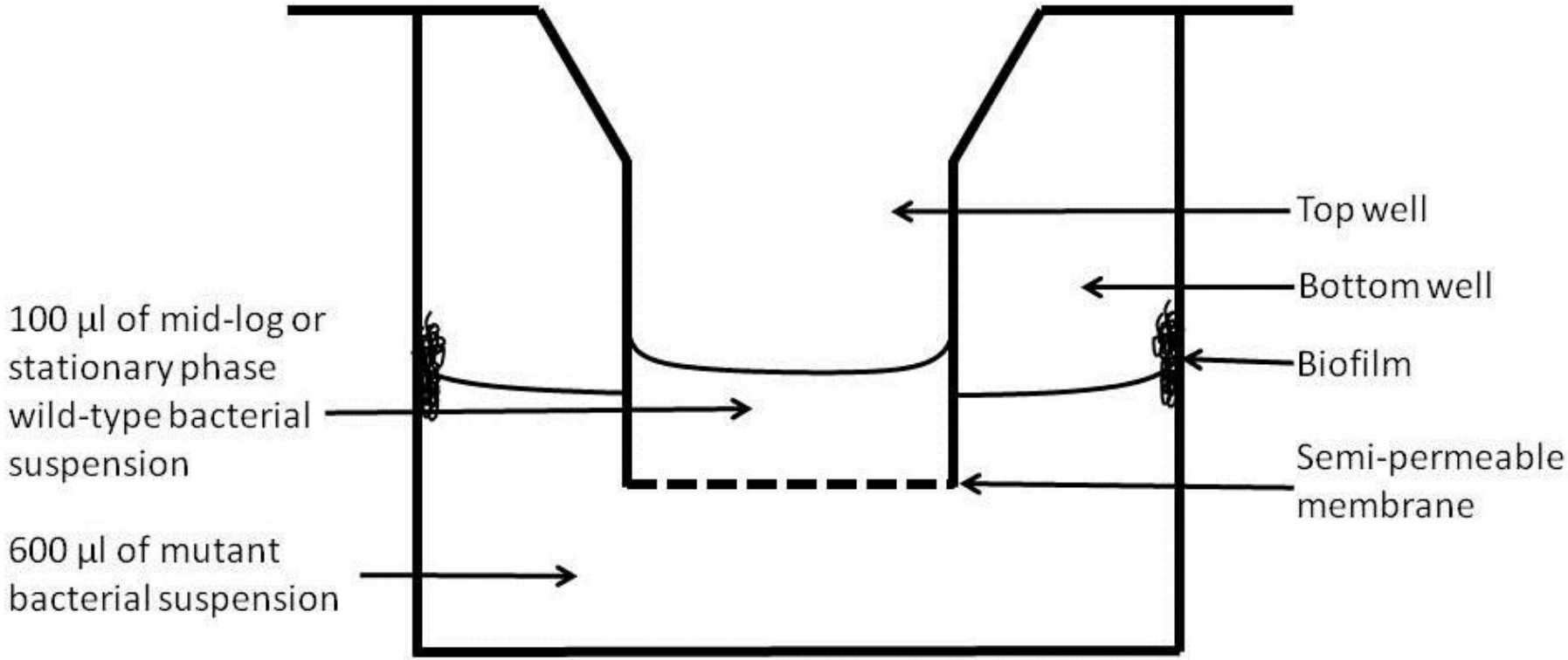
2.9.5 Formation of floating biofilm mats

10 ml overnight cultures grown in LB broth were diluted to an OD (600 nm) of 0.1 in LB broth without salt. 1 ml of this bacterial suspension was transferred into appropriate wells of a 24 well MTT. The MTT was incubated statically, at room temperature for 5 days. By incubating in this manner floating biofilm mats were formed on the surface of the liquid and easily removed with sterile tweezers allowing intact biofilms to be harvested for further analysis.

2.9.6 Transwell biofilm assay

A transwell assay was used to assess if the mutants' ability to form a biofilm can be rescued by the presence of wild-type supernatant. Transwell plates (ThermoScientific, U.K.) were used to co-incubate both wild-type and mutant separated by a permeable membrane to allow wild-type supernatant (but not wild-type cells) into the well where the mutant cells are forming a biofilm. 10 ml overnight cultures were prepared of both wild-type and efflux mutant strains in LB broth, these cultures were diluted to an optical density at 600 nm of 0.1 in fresh LB broth without salt. 600 µl of mutant bacterial suspension was transferred into the bottom well and 100 µl of wild-type suspension into the top well (Figure 2.2). Control wells were also set with wild-type suspension in both top and bottom wells. Transwell plates were then incubated at 30°C for 48 hours with gentle agitation. Top wells were removed from the transwell plate and planktonic culture from the bottom well discarded. Biofilms formed in the bottom well were stained with 1 ml of 1% crystal violet and incubated at room temperature for 15 minutes. Any unbound dye was removed from the wells and washed with sterile distilled water. Bound dye was solubilised with 1 ml of 70% ethanol. The optical density at 600 nm was measured on a FLUOstar, averages plotted on a bar chart and standard deviation calculated. Statistically significant differences were calculated using a student's t test with $p<0.05$ as the threshold.

Figure 2.2 Schematic cross section diagram of a Transwell co-incubation well showing where wild-type and mutant suspensions were incubated, the positioning of the semi-permeable membrane that allows supernatant from with well to permeate and the location of the biofilm growth that was quantified via crystal violet staining.



2.9.7 Biofilm co-incubation assay

In an attempt to determine whether the efflux mutants' could be incorporated into a biofilm when grown with the wild-type strain, direct co-incubation experiments were used. 10 ml overnight cultures were diluted to an optical density at 600nm of 0.1 in LB broth without salt. Two biological and four technical replicates of each strain were tested. 1 ml of bacterial suspension was used to inoculate control wells containing only one strain and the co-incubation wells were inoculated with 0.5 ml of each strain (Figure 2.3). The strains were also incubated in isolation to ensure that all strains were viable under assay conditions, after a 96 hour incubation period a mat of biofilm floating on the surface of the media should be visible. The viable count of the planktonic culture (to ensure the co-incubated strains were able to grow to a similar cfu/ml) and the biofilm mat of each well were calculated after 24, 48, 72 and 96 hour incubation periods for planktonic cfu/ml and 96 hours for biofilm mat. This was achieved for the planktonic culture by diluting 20 µl of culture from the well in 180 µl sterile PBS. For the biofilm mat, the mat was extracted with sterile tweezers and transferred into 1 ml of sterile PBS in a microcentrifuge tube and sonicated on ice four times at 30 seconds. Both the planktonic and biofilm mat suspensions were then further double diluted along a 96 well microtitre tray so 12 dilutions were made. Each dilution was used to aliquot three, 20 µl spots onto an LB agar plate supplemented with appropriate antibiotic. Co-incubation bacterial dilutions were plated onto both antibiotic free LB agar plates and either 25 µg/ml chloramphenicol LB agar plates (L829 (*tolC::cat*)) or 50 µg/ml kanamycin LB plates (L830 (*acrB::aph*)). Only the mutant strains could grow on the antibiotic agar plate so colony counts from antibiotic agar plates were taken to represent mutant numbers

Figure 2.3 Co-incubation biofilm assay 24 well microtitre tray template showing pattern of inoculation for wild-type and mutant bacterial suspensions including media quality controls, all strain incubated alone to ensure planktonic growth is consistent and both co-incubation tests.

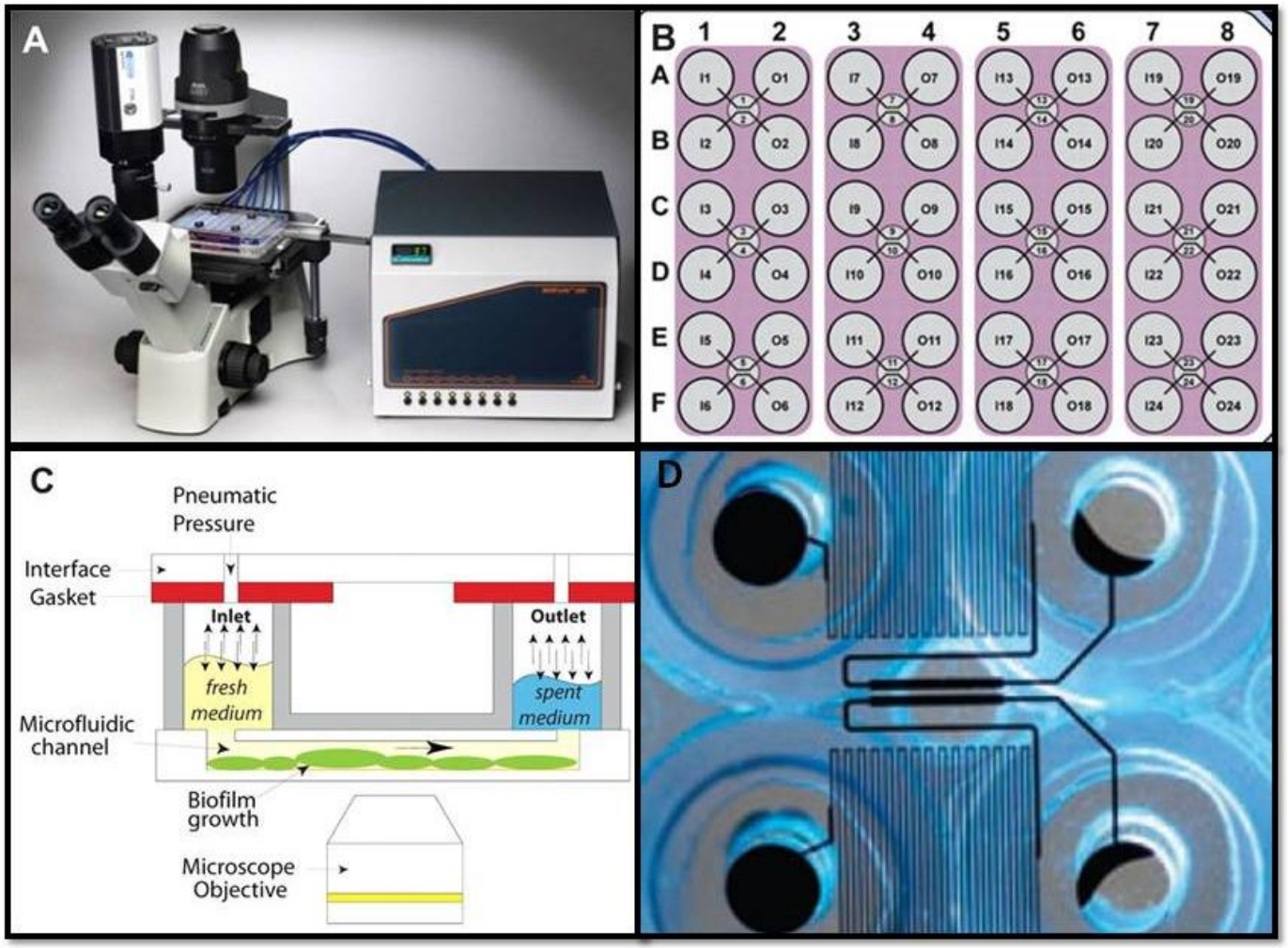
	1	2	3	4	5	6
A	LB broth without salt control	L828 (**wild-type**)	L829 (*tolC::cat*)	L830 (*acrB::aph*)	L828 (**wild-type**) + L829 (*tolC::cat*)	L828 (**wild-type**) + L830 (*acrB::aph*)
B	LB broth without salt control	L828 (**wild-type**)	L829 (*tolC::cat*)	L830 (*acrB::aph*)	L828 (**wild-type**) + L829 (*tolC::cat*)	L828 (**wild-type**) + L830 (*acrB::aph*)
C	LB broth without salt control	L828 (**wild-type**)	L829 (*tolC::cat*)	L830 (*acrB::aph*)	L828 (**wild-type**) + L829 (*tolC::cat*)	L828 (**wild-type**) + L830 (*acrB::aph*)
D	LB broth without salt control	L828 (**wild-type**)	L829 (*tolC::cat*)	L830 (*acrB::aph*)	L828 (**wild-type**) + L829 (*tolC::cat*)	L828 (**wild-type**) + L830 (*acrB::aph*)

The wild-type count was calculated by subtracting the mutant count (i.e. the count on the antibiotic agar plate) from the total count (i.e. the count on the antibiotic free LB agar plate). Data was analysed by plotting average cfu/ml on a logarithmic graph and standard deviations calculated.

2.9.8 Microfluidic flow cell biofilm assay

A Bioflux (Fluxion, US) was used to visualise the effect to the biofilm phenotype under flow conditions. To prime the flow cells with media, 200 µl of LB broth without salt was added to the inner circle of the output wells of a 48 well Bioflux flow cell plate (Fluxion, US) (Figure 2.4). A Bioflux manifold (Fluxion, US) was attached to the 48 well plate along with the relevant Bioflux tubes. The Bioflux software was used to select media (LB broth), temperature (20°C) and flow rate (5 dynes). The required wells were selected on the software to start the flow and flow stopped after 5 minutes. Any residual media was removed, apart from the media in the inner pockets of the inlet and outlet wells. Overnight cultures diluted in fresh LB broth without salt were diluted to an optical density at 600 nm of 0.1 and the flow channels were inoculated by transferring 50 µl of this bacterial suspension to the output wells. To balance the flow cell, 50 µl of fresh LB broth without salt was added to the inlet well. The bacteria were then introduced into the flow channel at 3 dynes for approximately 3 seconds until the bacterial cells reach the start of the flow cell. This can be seen by placing the plate onto a LTSi-1000 inverted microscope (Labtech, US) so that the neck of the flow cell is in field, once cells can be seen the flow must be stopped to ensure the sterility of the serpentine (tubing leading up to the inlet well). Even coverage of the bacterial cells in the flow channel needs to be observed before allow the bacteria to attach at 30°C for 3 hours.

Figure 2.4 High-throughput biofilm flow cell system (**A**) Bioflux system set up consisting of pneumatic pump attached to a manifold via tubing which is placed on top of a 48 well Bioflux plate and an inverted microscope. (**B**) Diagram of a 48 well Bioflus plate showing 24 inlet and 24 outlet well connected by 24 independent flow cells. (**C**) Cross-section diagram of one inlet-flow cell-outlet set up with annotation showing fresh and spent medium, biofilm growth and where the pneumatic pressure is placed. (**D**) Two microfluidoc flow cells; black circles represent fresh medium in the inlet wells and the black line shows the path of the media through the serpentine then the flow cell and in the outlet well (Benoit *et al.* 2010).



Once bacterial cells have attached 1 ml of fresh LB broth without salt was added to the inlet well and flow rate set at 0.3 dynes for 16 hours. Camera images can be taken at various intervals at x10, x20 and x40 magnification. Estimation of areas of flow cells covered used the Bioflux EZ software (Labtech, U.K) and triplicate flow cells were measured multiple times to give average values for coverage in defined areas which were then compared between strains or treatments.

2.10 Visualisation and quantification of biofilm matrix components

2.10.1 Scanning electron microscopy

5 ml overnight cultures in universal tubes were incubated at 37°C overnight with shaking (200 rpm) before addition of a 10 mm square glass cover slip at 30°C for 48 hours to allow a biofilm to form on the surface of the cover slip. The cover slip was removed and submerged in a primary fixative of 2.5% gluteraldehyde in phosphate buffer. Subsequent dehydration and mounting steps were carried out at the Centre for Electron Microscopy, University of Birmingham. Dehydration of samples was achieved by using a graded ethanol series as follows;

- 70% ethanol for 15 minutes (twice)
- 90% ethanol for 15 minutes (twice)
- 100% ethanol for 15 minutes (twice)
- 100% dried ethanol with molecular sieve for 15 minutes (twice)

The samples were then dried in a Polaron E3000 CPD critical point drier (Quorum Technologies Ltd, U.K.). Samples were mounted on stubs and sputter coated with platinum in an EMScope SC500 sputter coater (Quorum Technologies Ltd, U.K.).

Scanning electron microscopy images were viewed at a variety of magnifications on a Philips XL30 FEG ESEM (FEI, U.S.A.).

2.10.2 Quantitative Congo red staining

To assess production of the aggregated amyloid fibre, curli a dye called Congo red was used. Strains were streaked onto LB agar plates without salt and incubated at 30°C overnight. Bacterial suspensions were then made by harvesting a 1 cm patch of bacterial growth from a densely inoculated section of the plate in 5 ml of PBS and optical density at 600 nm measured. All samples were corrected to the lowest OD obtained by diluting with PBS. 500 µl of each bacterial suspension was added to 500 µl of a 20,000 µg/ml Congo red solution and left to stain for 20 minutes. Pellets were obtained by centrifugation at 13,000 rpm for 5 minutes, washed with 1 ml of sterile distilled water and harvested again. The pellets were then re-suspended in 200 µl of PBS and the optical density at 492 nm and fluorescence (Ex 492, Em 600) measured in a FluoSTAR OPTIMA (BMG, U.K.).

2.10.3 Congo red agar

The presence of curli proteins on the surface of the outer membrane of strains of interest were detected by diluting overnight cultures of strains of interest grown in LB broth to a final dilution of 1:10,000 in sterile PBS. 5 µl of this final cell suspension was dispensed onto LB agar without salt supplemented with 40 µg/ml of Congo red (Sigma-Aldrich Ltd., U.K.). Red, dry and rough colonies were observed for strains producing both curli and cellulose. Pink colonies were observed for strains producing cellulose but no curli. Smooth and white colonies were observed for strains producing neither curli or cellulose (Jonas et al., 2007).

2.10.4 Calcoflour agar

The presence of cellulose in the extracellular matrix was detected (see section 2.10.3) using 200 µg/ml of Calcofluor (fluorescence brightener 28 - Sigma-Aldrich Ltd., U.K.) in the agar instead of 40 µg/ml of Congo Red. Agars plates were visualised on a UV transilluminator and difference in fluorescence levels compared to wild-type under ultra violet indicates differing phenotypic cellulose expression.

2.11 Cell surface hydrophobicity and aggregative ability

2.11.1 Settle assay

Any changes in cellular hydrophobicity which allows aggregative ability could impact the ability of strains to form a biofilm, settle assays were used to measure this. 10 ml LB without salt broths were incubated overnight with shaking (150 rpm) and the following morning placed on a laboratory bench around a Bunsen burner. Three biological replicates and two technical replicates of each strain were used. A 100 µl sample was taken from immediately below the surface of each overnight broth, at 0 hours, two hours, four hours, six hours and 24 hours, and the optical density at 600 nm measured. The data was then analysed by converting each hourly optical density reading to a percentage of the initial optical density (at time 0) and the average of the six replicates plotted on a bar graph. Standard deviations were measured and used as error bars and any differences to wild-type analysed using the students ‘t’ test.

2.11.2 Salt aggregation test

Bacterial cell aggregation can also be measured by observing the lowest concentration of a salt solution required to induce aggregation (Ljungh and Wadstrom 1982). A 4 M stock of $(NH_4)2SO_4$ was prepared in phosphate buffered saline (PBS) and the pH of the solution adjusted to 6.8. The 4 M stock was double diluted in sterile

PBS to create a range of concentrations from 4 M to 0.002 M. Overnight cultures were diluted to an optical density at 600 nm of 0.8 in PBS. 50 µl of bacterial suspension was transferred onto a microscope slide and mixed with 50 µl of 4 M (NH_4) $2SO_4$. This was repeated for each concentration of (NH_4) $2SO_4$. The microscope slides were gently rocked for 2 minutes and left on the bench for 1 hour. A visual reading was taken against a black background with a light microscope. Cell surface hydrophobicity was quantified by using the lowest (NH_4) $2SO_4$ concentration needed for aggregation to be seen as the salt aggregation test (SAT) value. The lower the SAT value the greater the cell surface hydrophobicity of the bacterial cell.

2.12 Membrane component preparations

2.12.1 Lipopolysaccharide preparation

To determine whether disruption of efflux pumps altered lipopolysaccharide (LPS) production, LPS was prepared and analysed. A 5 ml of culture of bacterial strains of interest were grown in LB broth at 37°C overnight with shaking (200 rpm). The optical density of the overnight culture were measure at 600 nm, ideally the overnight cultures will have reached an optical density at 600nm of 3.5 or higher. 250 µl of the overnight culture was dispensed into a microcentrifuge tube and pelleted via centrifugation at 14,000 x *g* for 10 minutes. All supernatant was discarded and pellet re-suspended in cracking buffer (SDS-Page loading buffer), the volume of which is calculated by multiplying the optical density at 600nm of the overnight culture by 25 (e.g if the optical density at 600 nm of the overnight culture is 4, then 100 µl of cracking buffer would be used to re-suspend the pellet). This suspension was then boiled for 4 minutes and frozen at -80°C for 5 minutes. The frozen lysates were then defrosted quickly and boiled again for 4 minutes. Pellets were again obtained via

centrifugation in a microcentrifuge at 14,000 x *g* for 2 minutes. Around 80 µl of the supernatant was discarded and 5 µl of 5 mg/ml of Proteinase K to digest any protein (A 5 ml/mg stock of Proteinase K was prepared by diluting a 20 mg/ml stock 1 in 4 with cracking buffer). This mixture was incubated at 60°C for 1 hour and then heated to 95°C for 5 minutes. 4 µl of loading buffer was added to 10 µl of LPS sample heated to 95°C for 5 minutes and then loaded onto a 10% Bis-Tris Bolt™ gel (Life Technologies, U.K.) and electrophoresed at 80 V for around 1 hour. Bis-Tris Bolt™ gels were stained using SimplyBlue™ SafeStain (Life Technologies, U.K.) (see section 2.12.4).

2.12.2 Outer membrane protein preparation

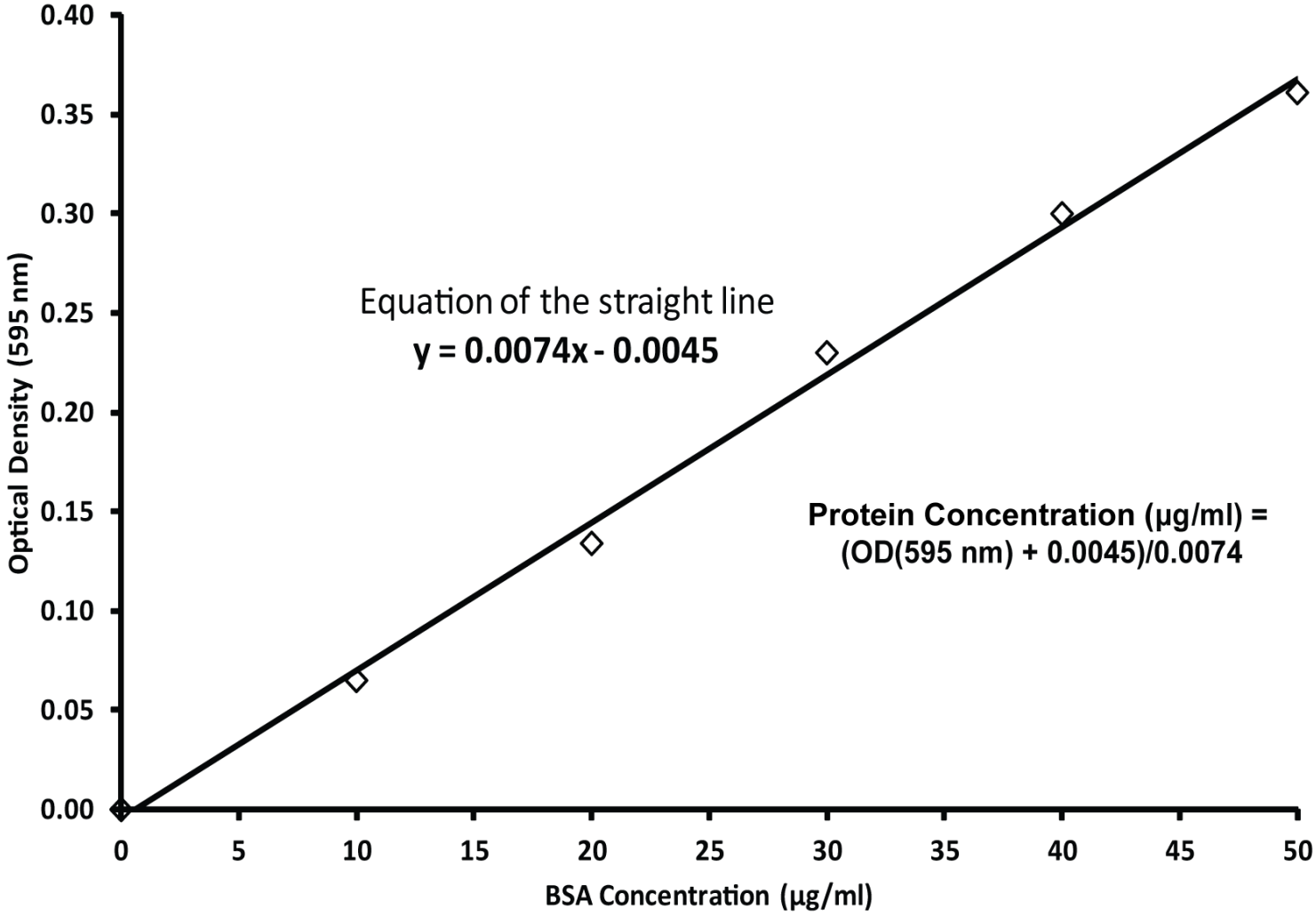
3 ml overnight cultures of strains were prepared in LB broth and incubated at 37°C with shaking (200 rpm). 50 ml of pre-warmed LB broth without salt was inoculated with a 4% inoculums of the overnight culture and incubated at 30°C until mid to late logarithmic phase is reached (an optical density at 600 nm of 0.7-1.0 for *Salmonella*). Around 25 ml of this culture was transferred to a sterile universal tube and pelleted via centrifugation at 3000 x *g* at 4 °C for 15 minutes. The supernatant was discarded and the remainder of the culture transferred on top of the pellet and centrifugation repeated. The supernatant was once again discarded and the pellet was re-suspended in 10 ml of ice cold sterile PBS to wash cells. Pellets were again obtained via centrifugation at 3000 x *g* at 4°C for 10 minutes. Samples were kept on ice between steps. The supernatent was once again discarded and pellet re-suspended in 1 ml of ice cold sterile PBS, the sample was then transferred to a sterile glass bijou tube and stored at -20°C overnight. The following day samples were thawed on ice and sonicated with a Soniprep 150 microprobe (Sanyo, Japan)

four times at 30 seconds with a 30 second rest on ice. Sample was then transferred to a labelled ice cold microcentrifuge tube and pelleted via centrifugation at 5,000 x *g* at 4°C for 5 minutes. The supernatant was discarded and the pellet was re-suspended by vortexing in 200 µl of a 2% sarcosyl solution and incubated at room temperature for 30 minutes. The sample was once again pelleted via centrifugation at 10,000 x *g* at room temperature for 30 minutes. The supernatant was again discarded and pellet air dried for 15 minutes to ensure the pellet is completely dry. The dry pellet was re-suspended by vortexing in 50 µl of sterile PBS. The sample was peletted via centrifugation in a microcentrifuge at room temperature at 10,000 x *g* for 10 minutes to wash off any remaining sarcosyl. The supernatant was discared for the final time and the pellet re-suspended in 50 µl of sterile PBS and can be stored at -20°C prior to estimating the amount of protein present (see section 2.12.3) and analysing on a 10% Bis-tris Bolt™ gel (see section 2.12.4).

2.12.3 Bradford assay for protein quantification

A set of 5 bovine serum albumin (BSA) (Sigma, U.K.) standards were prepared; 0.1, 0.2,0.3, 0.4 and 0.5 mg/ml. Transfer 20 µl of each standard into clean microcentrifuge tubes and add 1 ml of Bradford reagent (Sigma, U.K.) (Bradford 1976). Samples were incubated at room temperature for 5 minutes. A no protein control (1 ml of Bradford regent and 20 µl of water) in a cuvette was used to calibrate a spectrophotometer at 595 nm. Each sample was transferred to a cuvette and optical density at 595 nm was then measured in a spectrophotometer and plotted on a graph of optical density against BSA concentration (Figure 2.5) on Microsoft Excel 2010 (Microsoft, USA). A line of best fit was added to the graph and equation of the straight line calculated.

Figure 2.5 A representative Bradford assay standard curve for protein quantification using standards of Bovine Serum Albumin (BSA)of 10, 20, 30, 40 and 50 µg/ml and corresponding optical density values at 595 nm plotted on a line graph. Line of best fit and equation of a straight line calculated using Microsoft Excel 2010 (Microsoft, US) and equation rearranged and annotated to allow estimation of unknown protein samples from their optical density value.



20 µl of the protein sample to be quantified was added to 1 ml of Bradford reagent in a cuvette and optical density at 595 nm measured on a spectrophotometer. To calculate the quantity of protein in the sample put the optical density obtained into the equation of the line of best fit from the graph. Each protein preparation was diluted the match the protein sample of the lowest concentration before being analysed on a 10% Bis-tris Bolt gel (see section 2.12.4)

2.12.4 OMP/LPS gel staining

OMP and LPS Bolt™ gels were stained with SimplyBlue™ SafeStain (Invitrogen, U.K.). Gels were transferred into a square petri dish and washed with 100 ml of Ultrapure water with gentle agitation for 5 minutes. This wash step was repeated a further 2 times. The gel was then stained with 20 ml of SimplyBlue SafeStain for 1 hour with gentle agitation. The stain was then removed and washed with 100 ml of Ultra pure water with gentle agitation for 1 hour. Gels were visualised on a transilluminator with a white light converter.

2.13 Gene expression analysis

Expression of genes thought to be relevant to biofilm formation was measured under various conditions.

2.13.1 RNA preparation from stationary phase planktonic cells

10 ml cultures in LB broth were prepared for strains of interest and incubated overnight at 37°C. These cultures were the used to inoculate 50 ml of fresh LB without salt broth in 250 ml glass flasks and incubated at 30°C for 6 hours with shaking (200 rpm) to attempt resemble conditions used for biofilm formation. 4 ml of each suspension was added to 1 ml of ice cold 5% phenol, 95% ethanol and stood

on ice for 30 minutes. The Promega 'SV40 total RNA kit' protocol was then followed according to the manufacturer's instructions. After incubation on ice for 30 minutes, pellets were obtained by centrifugation at 4°C (2,250 x *g*, 10 minutes) and the supernatant discarded. The resulting pellets were re-suspended in residual supernatant and transferred to a 1.5 ml microcentrifuge tube. Pellets again were obtained via centrifugation in a table top centrifuge at 14,000 x *g* and the supernatant discarded. Pellets were re-suspended using 100 µl of TE buffer containing 50 mg/ml lysozyme and incubated at room temperature for 5 minutes. 75 µl of lysis reagent (Promega, U.K.) was added and samples were mixed by inversion followed by addition of 350 µl of RNA dilution buffer (Promega, U.K.) and again mixed well by inversion. Samples were heated at 70°C for 3 minutes, mixed by vortexing and centrifuged at 4,500 x *g* in a table-top centrifuge. The supernatant (containing RNA) was transferred to a clean microcentrifuge tube. 200 µl of 100% ethanol was added to the supernatant, mixed and transferred to spin columns (Promega, U.K.). Columns were centrifuged for 30 seconds, then washed with 600 µl wash buffer (Promega, U.K.) and centrifuged for 30 seconds. DNAse mix was prepared (using reagents supplied with kit, Promega, U.K.): 5 µl 90 mM MnCl2, 40 µl DNAse core buffer and 5 µl DNAse. 50 µl of this DNAse mix was added to each spin column and incubated at room temperature for 15 minutes. The reaction was stopped by adding 200 µl of DNAse stop buffer (from kit) and centrifuged for 30 seconds. Columns were washed with 600 µl wash buffer, centrifuged and the eluate discarded. Columns were washed again with 250 µl of wash buffer and centrifuged. Columns were transferred into sterile microcentrifuge tubes and 100 µl of RNAse-free distilled water was added and allowed to stand for 1 minute. Columns were centrifuged at 4,500 x *g* for 2 minutes to

elute RNA before being discarded. Three, 3 µl aliquots of RNA were retained and the rest of the RNA stored at -80°C. One aliquot was used as a template for a PCR used to ensure there was no DNA contamination, if DNA was present RNA samples were again treated with DNase. A second aliquot was analysed by agarose gel electrophoresis and a third analysed by a NanoDrop spectrophotometer (NanoDrop Technologies, USA) to quantify total RNA and estimate protein and organic compound contamination. Figure 2.6 shows a typical RNA preparation electrophoresed for approximately 1 hour on a 1% agarose gel at 100 V.

2.13.2 cDNA synthesis

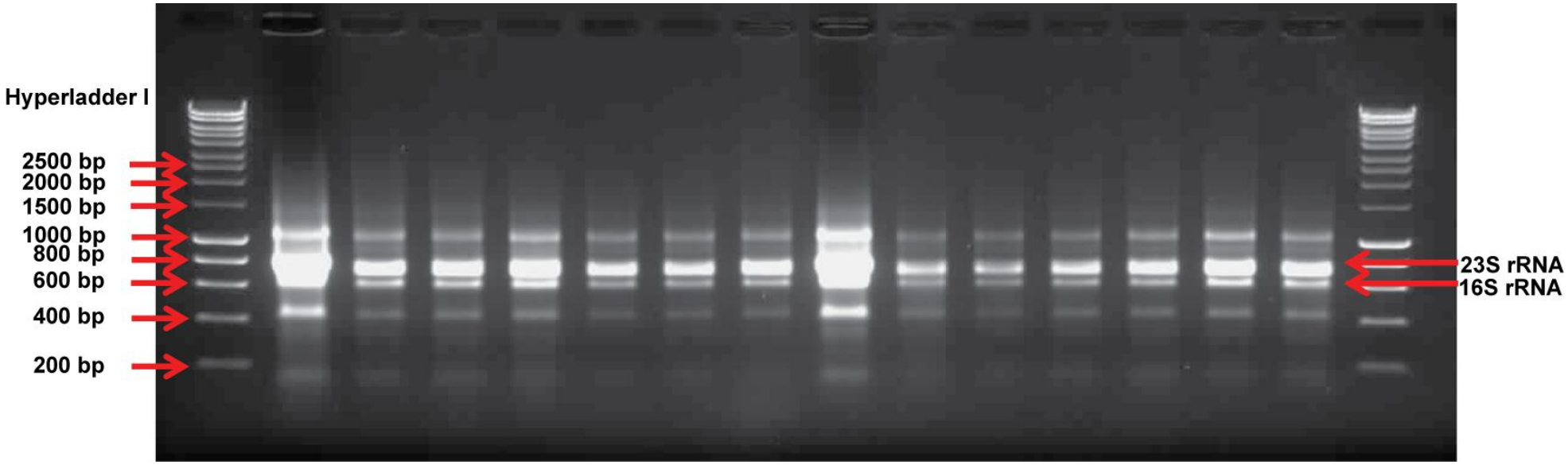
The RNA prepared using (see section 2.13.1) was used as a template from which to synthesise cDNA. The following component solutions were added together in a microcentrifuge tube for each reaction:

- 1 µl of 50 ng/µl of random primers (Life Technologies, U.K.)
- 1 µl of 10 mM dNTP mix (Life Technologies, U.K.)
- 500 ng of RNA
- Sterile distilled water to 13 µl.

This mixture was heated at 65°C for 5 minutes and incubated for 1 minute on ice and centrifuged to collect the contents of the tubes and the following components added:

- 4 µl of 5x First-strand buffer (Life Technologies, U.K.)
- 1 µl of 0.1 M DTT (Life Technologies, U.K.)
- 1 µl of RNaseOUT (Life Technologies, U.K.)
- 1 µl of SuperScript III RT (220 units/µl) (Life Technologies, U.K.).

Figure 2.6 A typical RNA preparation separated by electrophoreses for 1 hour on a 1% agarose gel at 100 V.



The contents of the tube were mixed by gently pipetting up and down. They were then incubated at 25°C for five minutes followed by 50°C for one hour. The SuperScript III enzyme was then inactivated by heating to 70°C for 15 minutes. cDNA was stored at -20°C until required.

2.13.3 Comparative reverse transcriptase PCR (cRT-PCR)

cRT-PCR was used to compare expression of different genes between strains or conditions (Eaves *et al.* 2004).

cRT-PCR mixtures contained the following components in a microcentrifuge tube:

- 45 µl of PCR Mastermix (ABgene®, U.K.)
- 1.5 µl of forward primer
- 1.5 µl of reverse primer
- 2 µl of cDNA (as made in section 2.13.2)

Two technical replicates of cDNA were used from three biological replicates of RNA (six replicates in total). All primers were used at a concentration of 25 µM. To avoid saturation of reactions and ensure final product concentrations were a reflection of initial template concentrations cDNA was used at a 1:10 dilution (of original cDNA obtained) except when using 16SrRNA primers where a 1:1000 dilution was used. The dilutions needed to avoid reaction saturation were experimentally determined for each gene using cDNA dilutions before ‘test’ reactions were completed. All reactions were completed in a PCR machine with appropriate PCR conditions:

- 95°C 5 minutes
- 95°C 30 seconds
- ??°C* 30 seconds
- 72°C ??* seconds

x 30

- 72°C 10 minutes

*Appropriate annealing temperature or extension times were used according to specific primers/amplimers.

Successful PCR amplification was verified by agarose gel electrophoresis and bands quantified using GeneTools (SynGene, U.K.) with Hyperladder I (Bioine, U.K.) as a standard. Average values for each gene/condition were calculated and normalised to the corresponding 16S values. Normalised data was plotted on a bar chart, standard deviation calculated and any variation between the data measured for statistical significance using a student's '*t*' test. Differences with a $p<0.05$ were considered as significant.

2.14 Expression of global regulators of efflux using fluorescent markers

To determine whether the stress response regulators *marA*, *ramA*, *soxS* and *rob* showed differences in i) temporal and ii) spatial expression between wild-type and efflux mutants a set of promoter *gfp* reporter constructs were used. Reporter constructs were previously constructed in pMW82 and included the promoter of each gene cloned upstream of an unstable *gfp* (Table 2.4) (Lawler *et al.* 2013). Each plasmid was electroporated into competent cells (section 2.4).

2.14.1 Temporal expression of global regulators in wild-type and efflux mutants

Overnight cultures prepared in 10 ml of LB broth containing 50 µg/ml of ampicillin (to retain the reporter plasmid) were used to inoculate fresh 5 ml of LB broth without salt again containing 50 µg.ml of ampicillin 1:100. Expression of each gene in the wild-type and efflux mutants was determined by monitoring fluorescence (Ex 492, Em 520 nm) in a FLUOstar OPTIMA. Three biological and three technical replicates of each strain were used and 100 µl aliquots transferred to black 96 well microtitre trays. Fluorescence was then measure every 180 seconds for 16 hours whilst being incubated at 30°C.

2.14.2 Spatial expression of global regulators in wild-type and efflux mutants

Overnight cultures and bacterial dilutions were prepared as in section 2.14.1. Congo red agar assay was used as in section 2.10.3 with the addition of the Congo red agar being supplemented with 50 µg/ml ampicillin to ensure the plasmid is retained. Plates were incubated as in section 2.10.3. Colonies were observed under both light microscopy to see Congo red morphology (indicating the spatial expression of curli within the colony) and fluorescence microscopy (to observe spatial expression of *ramA*, *marA*, *soxS* and *rob*) using a SM2 400 (Nikon, U.K.).

2.15 Induction assay

To identify whether efflux mutants differed in their ability to respond to inducers of *marA*, *ramA*, *soxS* and *rob* a high throughput fluorescence induction assay was used to measure the *gfp* expression of *gfp* reporter plasmids (section 2.14 and Table 2.3). Fresh pre-warmed broth was inoculated with a 4% inoculum of an overnight culture and supplemented with appropriate antibiotic. Cultures were incubated at appropriate temperature with shaking until an OD 600 of 0.9 was reached. Stocks of appropriate

inducer at 100 x final required concentration were prepared. 10 µl of inducer stock was added to 990 µl of cells. Also 10 µl of inducer solvent was added to 990 µl of cells as an un-induced control. 100 µl aliquots of un-induced and induced cultures were loaded into a black 96 well MTT. Fluorescence intensities (Ex 492, Em 520) were measured every 180 seconds for 5 hours. Three biological and three technical repeats were used for each strain, an average taken and standard deviation calculated.

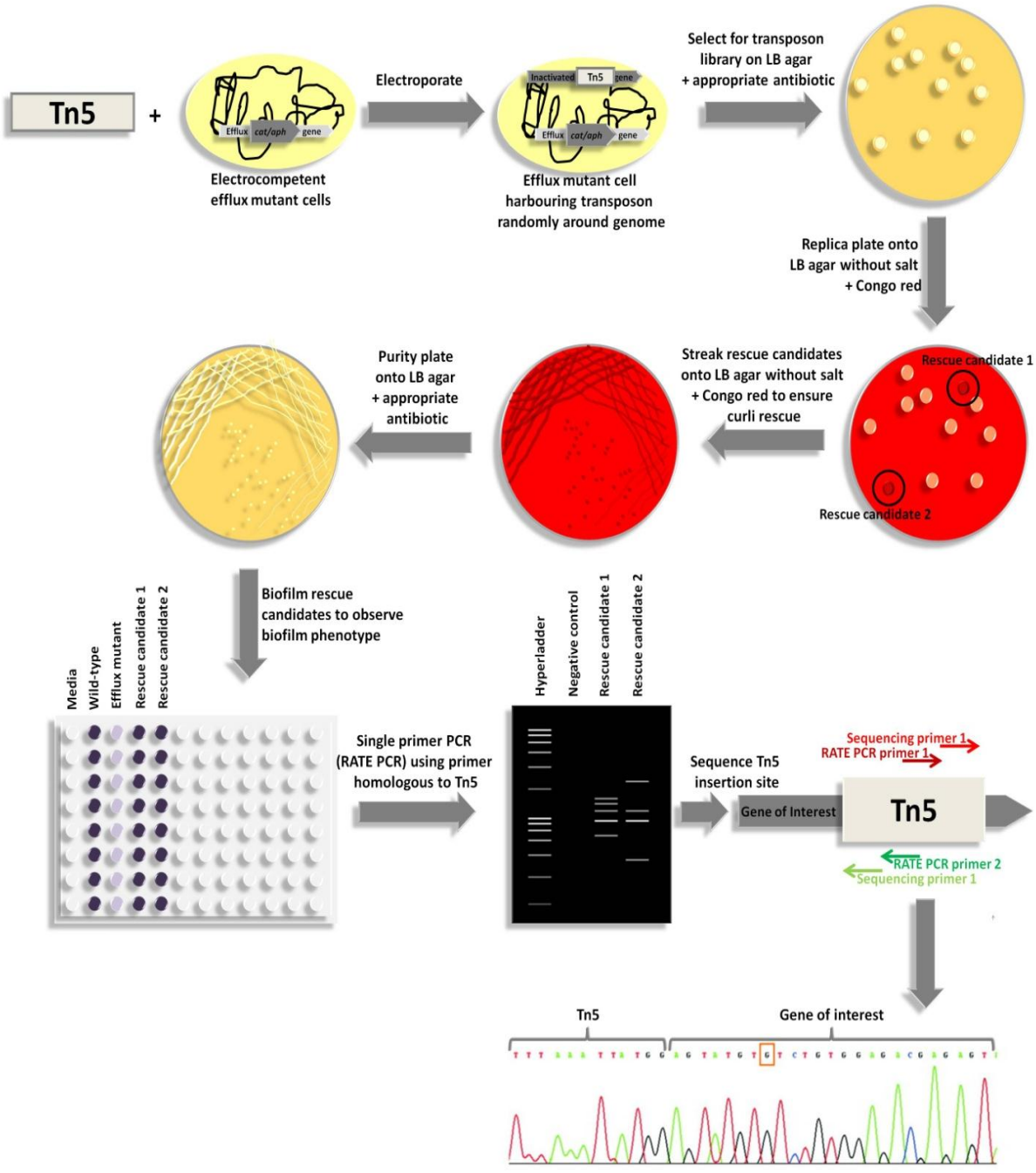
2.16 Transposon mutagenesis to identify curli 'rescue' mutants

Transposon mutagenesis was used to create libraries of mutants in *S*. Typhimurium 14028S with efflux pump mutations (Figure 2.7). This method allowed the libraries to be screened for mutants with the ability to produce curli and in theory form a biofilm. If the reason for the efflux mutants' biofilm/curli defect is over-expression of a gene/protein that is repressing curli genes this approach could identify the gene responsible.

2.16.1 Creating a random transposon library

To create transposon libraries either the EZ-Tn5^{TM}<DHFR-1>Tnp Transposome^{TM} or the EZ-Tn5^{TM}<KAN-2>Tnp Transposome^{TM} Kits (Epicentre® Biotechnologies, U.S.A.) were used. This allowed versatility in choosing selective markers and avoided conflict with those used in the efflux pump mutant construction. Competent cells of the recipient strain were made as in section 2.4. 1 µl of transposome was electroporated with 50 µl of competent cells at 2.5 kV in a two mm electrocuvette. Cells were recovered by the addition of 1 ml of SOC medium and incubated at 37°C for 30-60 minutes. Transposon mutants were selected on appropriate antibiotic plates (either 50 µg/ml of kanamycin or 50 µg/ml of trimethoprim).

Figure 2.7 Diagram of the transposon mutagenesis protocol showing electroporation, recovery of transformed cells, Congo red screening, subsequent Congo red phenotypic testing, crystal violet biofilm formation assay and sequencing of transposon insertion site.



All transposon mutant colonies were collected on a loop, re-suspended in 20% glycerol and stored at -20°C until screening.

2.16.2 Identifying curli 'rescue mutants' on Congo red agar

Transposon libraries of efflux mutants were diluted to approximately 1000 cfu/ml and 100 µl aliquots of this dilution spread onto LB agar containing appropriate antibiotics (either 50 µg/ml of kanamycin or 50 µg/ml of trimethoprim) and incubated overnight at 37°C. Colonies obtained were then replica plated with velveteen squares onto LB agar without salt supplemented with 40 µg/ml of Congo red and incubated at 30°C for 48 hours. Plates were visualised and any colonies displaying a red and rough morphology were selected and purity plated. These 'rescue' candidates were verified as still containing the expected efflux mutation. Congo red agar assays were used to verify the candidates ability to produce curli (section 2.10.3) and crystal violet biofilm assays to see if 'curli rescue' correlated to 'biofilm rescue' (section 2.9.1).

2.16.3 DNA sequencing of transposon insertion site

Bacterial lysates (section 2.1.4) of candidate transposon mutants were used as a template for a single primer PCR using a primer specific for the Tn5 transposon DNA (Table 2.2). An extended PCR programme was performed to amplify specific and non-specific fragments using the PCR parameters as follows (Karlyshev *et al.* 2000):

95°C	5 minutes	
95°C	30 seconds	x 30
55°C	30 seconds	
72°C	3 minutes	
95°C	30 seconds	x 30
30°C	30 seconds	
72°C	2 minutes	
95°C	3- seconds	x 30
55°C	30 seconds	
72°C	2 minutes	

The resulting PCR amplimers were then used as a template for DNA sequencing (see section 2.1.4 and Table 2.3), again using a nested primer specific to the Tn5 transposon. The resulting sequence was analysed using Chromas software (Tecchnelysium. U.K.) and aligned against the Tn5 transposon sequence to identify the junction with chromosomal sequence. Insert sites were identified using the Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov).

CHAPTER THREE:

BASELINE RESULTS

3 Optimisation of biofilm models, test conditions and strain selection

3.1 Introduction

The main focus of this project was initially the AcrAB-TolC efflux pump (Table 1.2) as an exemplar and the best studied efflux system in *Salmonella* and *E. coli*. Whilst there was a panel of efflux mutants available at the start of this project (Nishino et al, 2006) there was no *acrA* mutant in the culture collection. A mutant lacking *acrA* was recently found to have a distinct phenotype to *acrB* or *tolC* mutants (Blair *et al.* 2009). Therefore an isogenic *acrA* mutant in *S*. Typhimurium 14028S needed to be constructed. *S*. Typhimurium 14028S was chosen as the model parent strain as it has a full genome sequence (Jarvik *et al.* 2010), is widely used as a model *S*. Typhimurium and will readily form biofilms *in vitro*.

All the strains used in the project needed to be verified genotypically by PCR to confirm the presence of the expected genetic mutations and growth kinetics analysed to ensure the various efflux mutants did not possess any growth defect which may influence their ability to form a biofilm.

A wide range of different experimental protocols to study biofilm formation have been described in the literature. The crystal violet microtitre assay was used a relatively rapid, inexpensive and semi high throughput assay to routinely monitor biofilm formation in this project. A number of variables (media, temperature, time) needed to be investigated to define standard conditions under which different mutants or treatments could be assessed.

3.2 Strain verification

Before being used in any experiment, all strains were phenotypically examined by Gram staining, plating onto XLD agar and API 20E biochemical tests to verify strains were *Salmonella*. Genetic disruptions in mutant strains were verified by PCR and sequencing. All results from these tests were consistent with that expected for each strain.

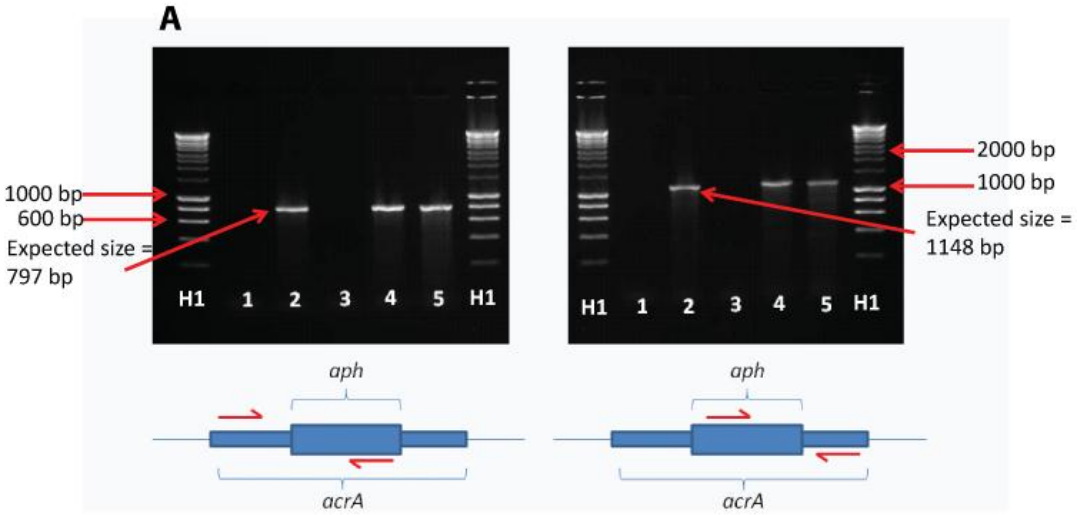
3.3 Inactivation of *acrA*

Recently a mutant with a specific disruption of *acrA* by insertion of the *aph* kanamycin resistance gene was created in *Salmonella* Typhimurium SL1344 (Blair *et al.* 2009). This mutant *acrA* allele was transferred into 14028S (L828) by transduction using bacteriophage P22. Colonies were obtained on selective agar (50 µg/ml kanamycin) for all transduction reactions. Two colonies were chosen, purity plated onto LB supplemented with kanamycin and XLD agar and subjected to an API20E biochemical test to ensure the transductants obtained were *Salmonella*. Specific PCR using primers flanking *acrA* ensured the transductants carried the correct mutation (Figure 3.1). These candidates were named L1270 and L1271, in subsequent experiments L1271 was the *acrA::aph* mutant used.

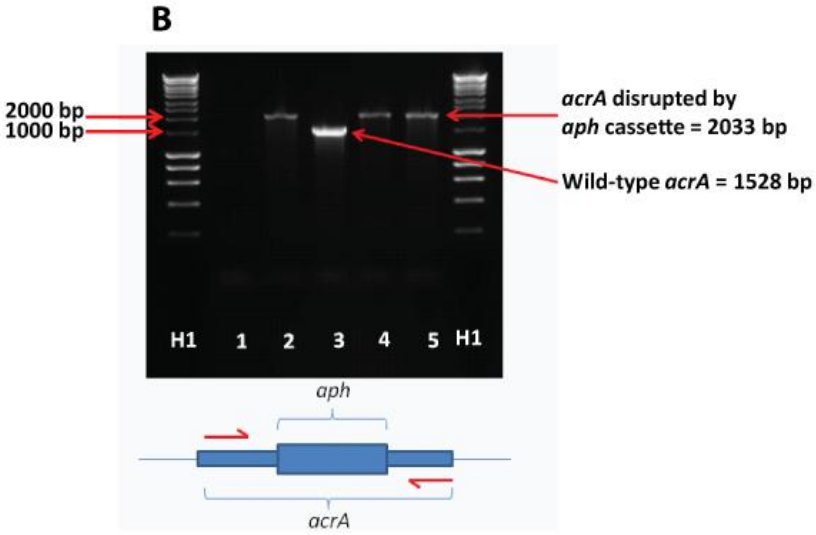
3.4 Growth kinetics

Growth kinetics were monitored over 16 hours for L828 (wild-type), 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*). Growth kinetics were carried out at 30°C and in LB broth without salt to mimic conditions used in the biofilm formation assays. All mutants displayed similar growth pattern to wild-type over the duration of the experiment (Figure 3.2).

Figure 3.1 PCR verification of 14028S (*acrA::aph*) transductants candidates 1 and 2 A PCR verification using primers homologous to both *acrA* and the *aph* cassette. B PCR verification using primers within *acrA* upstream and downstream of the aph cassette.



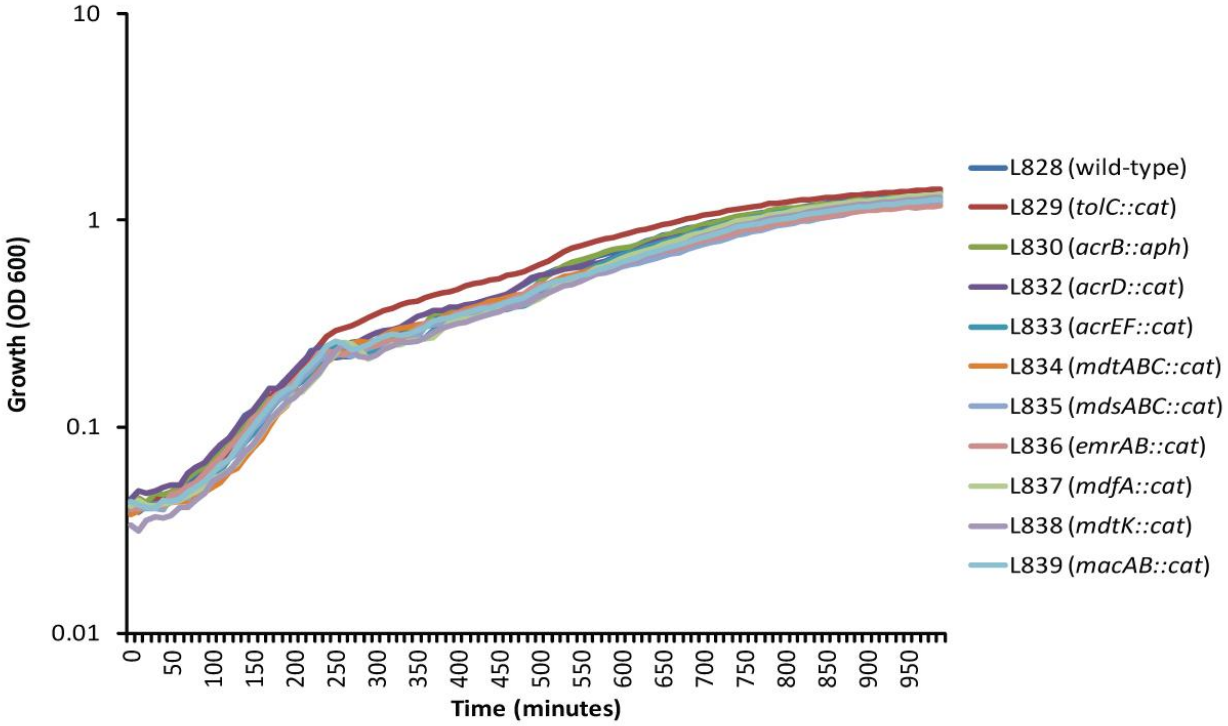
Lane:	Product:
H1	Hyperladder I (Bioline)
1	negative control
2	SL1344 (*acrA::aph*)
3	14028S
4	14028S (*acrA::aph*) candidate 1
5	14028S (*acrA::aph*) candidate 2



Lane:	Product:
H1	Hyperladder I (Bioline)
1	negative control
2	SL1344 (*acrA::aph*)
3	14028S
4	14028S (*acrA::aph*) candidate 1
5	14028S (*acrA::aph*) candidate 2

Figure 3.2 A Growth kinetics and **B** generation times of L828 (wild-type), 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*).

A



B

	Generation time (minutes)	Standard Deviation	P value
L828 (wild-type)	111.84	14.60	
L829 (*tolC::cat*)	169.08	78.40	0.28
L830 (*acrB::aph*)	129.91	19.51	0.45
L832 (*acrD::cat*)	121.73	2.77	0.51
L833 (*acrEF::cat*)	143.65	17.55	0.10
L834 (*mdtABC::cat*)	179.80	33.78	0.18
L835 (*mdsABC::cat*)	172.56	66.59	0.87
L836 (*emrAB::cat*)	121.80	7.67	0.26
L837 (*mdfA::cat*)	203.30	16.50	0.00
L838 (*mdtK::cat*)	146.90	39.54	0.08
L839 (*macAB:cat*)	182.07	40.33	0.34

.

There was no statistically significant difference of the generation times of the efflux mutants when compared to wild-type with the exception of L837 (*mdfA::cat*) which had an average generation time of 203 minutes compared to the 112 minutes generation time of wild-type. A comparison of the final OD value achieved by all strains showed no significant difference for any of the mutants compared to wild-type.

3.5 Optimisation of the Crystal Violet biofilm assay

Formation of competent biofilms depends upon the media used (Figure 3.3). L828 (wild-type) formed most biofilm in LB without salt, followed by minimal media and least biofilm in LB broth with salt. L829 (*tolC::cat*) in contrast formed most biofilm in minimal media followed by LB without salt and again least in normal LB media. L830 (*acrB::aph*) formed relatively poor biofilms in all media conditions.

Various temperatures were also tested to establish the optimum temperature at which *Salmonella* will biofilm, it was found that L828 (wild-type), L829 (*tolC:cat*) and L830 (*acrB::aph*) all formed more biofilm at 30°C when compared to 37°C. Subsequently, all biofilm assays were performed at 30°C in LB without salt and on 96 well polystyrene microtitre trays to allow for high throughput.

3.6 Biofilm assay of all efflux mutants

All efflux mutants tested (Figure 3.4) were compromised in their ability to form biofilms when grown in LB broth without salt except for L1271 (*acrA::aph*) which biofilms to wild-type levels. L829 (*tolC::cat*) the only strain without a functional outer membrane channel for the majority of the efflux system is the most compromised at forming a biofilm, forming around six fold less biofilm than wild-type.

Figure 3.3 Optimisation of the crystal violet biofilm assay **A** Crystal violet biofilm assay of L828 (wild-type) with two representative efflux mutants L829 (*tolC::cat*) and L830 (*acrB::aph*) with various media; Minimal, LB and LB without salt. **B** Crystal violet biofilm assay of L828 (wild-type), L829 (*tolC::cat*) and L830 (*acrB::aph*) in LB without salt at 30°C and 37°C. Statistical significance between strains and temperatures are calculated using a student's t test ($p<0.05$) and indicated with an asterisk.

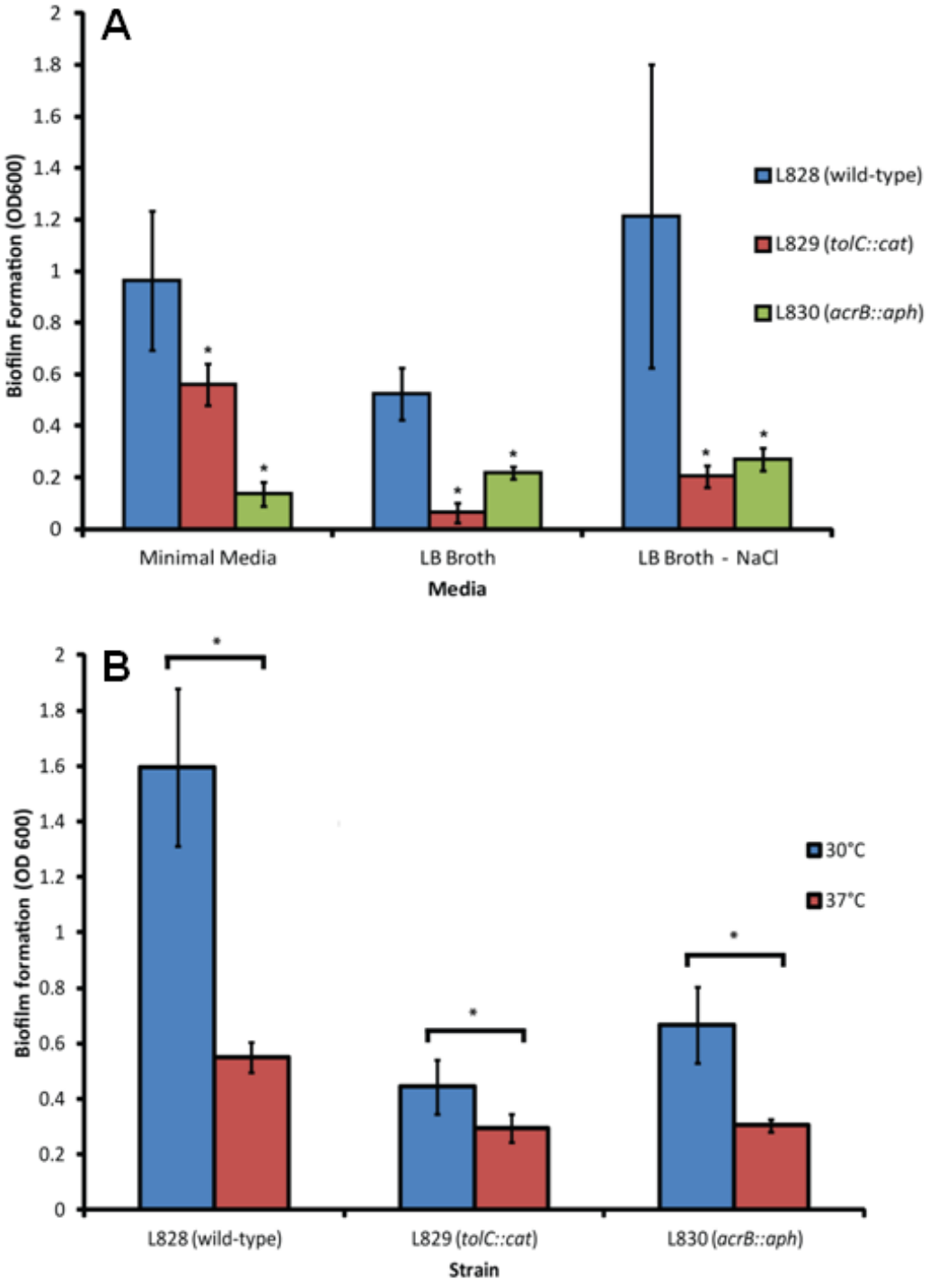
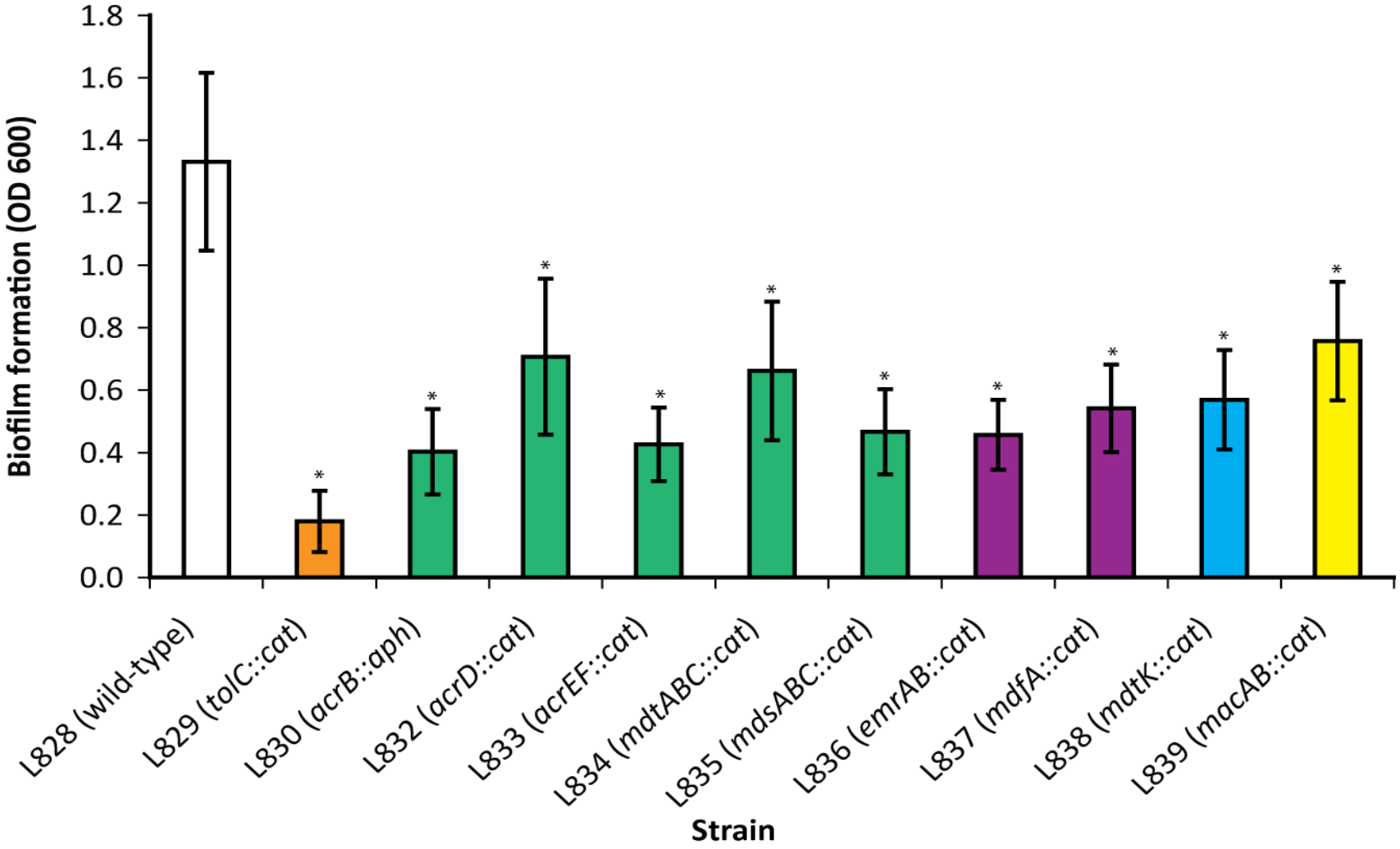


Figure 3.4 Crystal violet biofilm assay of L828 (wild-type) and all MDR efflux mutants of *S*. Typhimurium. White bar - L828 (wild-type); orange bar - outer membrane porin mutant, L829 (*tolC::cat*); green bars - RND efflux mutants; purple bars - MFS efflux mutants; blue bar - MATE mutant; yellow bar - ABC transporter mutant. Statistically significant differences between L828 (wild-type) and efflux mutants are calculated using a student's t test ($p<0.05$) and indicated with an asterisk.



L830 (*acrB::aph*), L833 (*acrEF::cat*) L835 (*mdsABC:cat*), L836 (*emrAB::cat*), L837 (*mdfA::cat*) and L838 (*mdtK::cat*) have slightly better biofilm phenotype forming around three fold less biofilm than wild-type. L832 (*acrD::cat*), L834 (*mdtABC::cat*) and L839 (*macAB::cat*) are the least affected but still only form around half of the amount of biofilm formed by wild-type.

3.7 Biofilm 'mat' formation

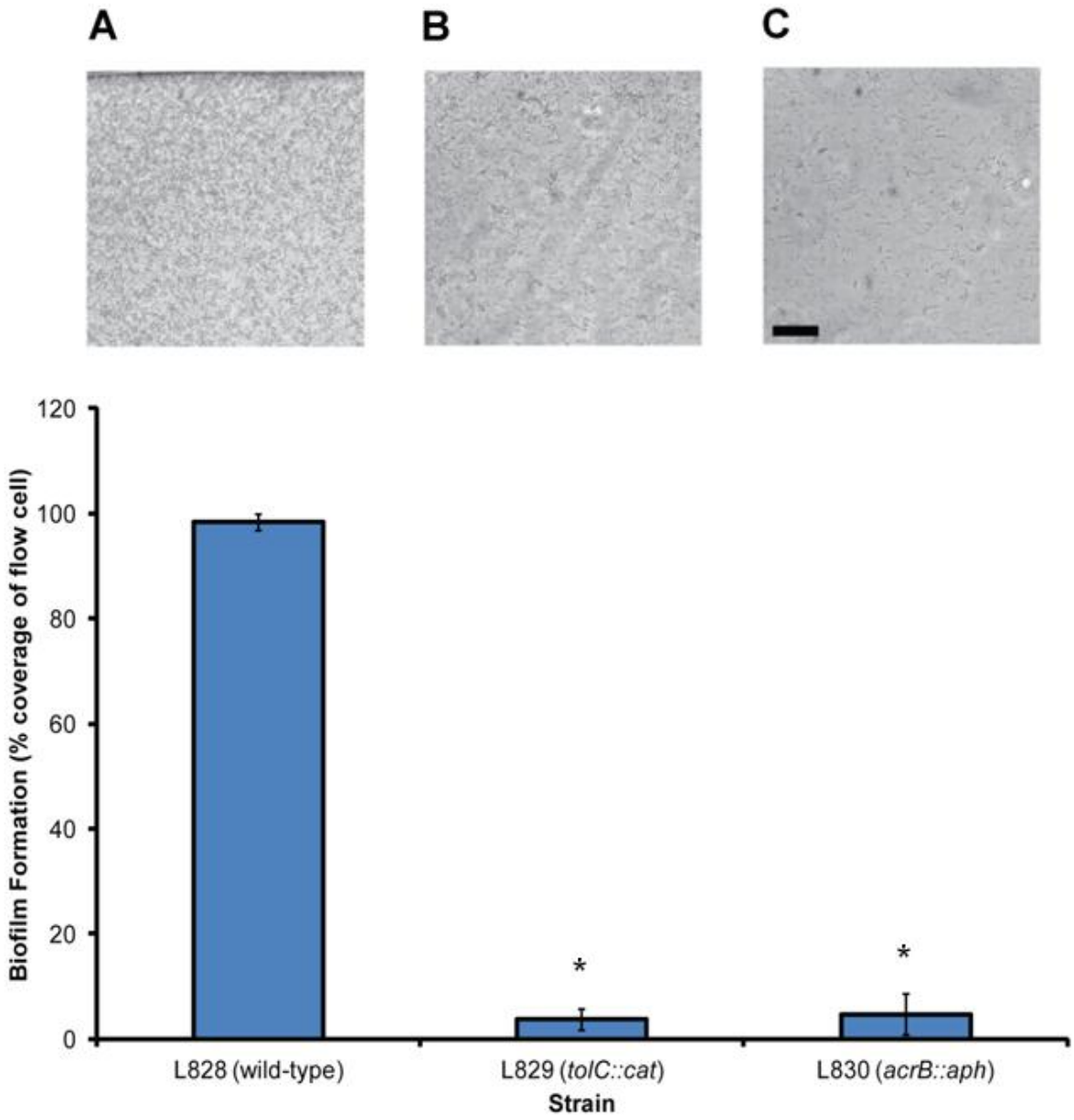
Whilst *S*. Typhimurium form a biofilm at the air-liquid interface in a 96 well microtitre tray it has recently been shown that they will form a 'mat' of biofilm on the surface of the liquid if incubated statically in a 24 well microtitre tray (Romling and Rohde 1999). The trend observed in the crystal violet biofilm assay is also observed when using the 'floating mat model' of biofilm formation. Only L828 (wild-type) forms a biofilm mat on the surface of the media after 3 days. The panel of ten efflux mutants did not produce a floating mat of biofilm even when left for 5 days.

3.8 Biofilm flow cell model

To ensure the defect seen is not just a static biofilm phenomenon, L828 (wild-type), L829 (*tolC::cat*) and L830 (*acrB::aph*) were incubated at room temperature within a flow cell with LB broth without salt flowing through the cell at around 0.3 dynes. The reason for using a flow biofilm model was to try and replicate some real life biofilm situations as biofilm will form in waterways, stream and under flow on medical devices. The cells were allowed to adhere for 3 hours on the flow cell before the flow was started and again only L828 (wild-type) formed a mature three dimensional biofilm. L829 (*tolC::cat*) and L830 (*acrB::aph*) although cells have initially adhered during the 3 hour seeding incubation no further maturation has occurred (Figure 3.5).

Figure 3.5 A Phase contrast microscopy images using x 40 objective of L828 (wild-type), L829 (*tolC::cat*) and L830 (*acrB::aph*) forming a biofilm under flow conditions under 0.3 dynes using a Bioflux pneumatic pump and 48 well plate system. Black bar depicts a 5 µm scale.

B Average biofilm coverage of the microfluidic flow cell quantified using the Bioflux EZ software. Statistically significant differences in biofilm formation in comparison to their parent strain are indicated by an asterisk ($p<0.05$).



3.9 Discussion

Before any phenotypic characterisation was carried out, an isogenic *acrA::aph* mutant was constructed to complete the panel of *S*. Typhimurium 14028S efflux mutants. A similar mutant made in *S*. Typhimurium SL1344 had a distinct phenotype from *acrB* or *tolC* mutants, warranting investigation of the impact of *acrA* on biofilm formation. Initial baseline experiments showed that inactivation of efflux pumps of *S*. Typhimurium did not have an adverse effect on the growth kinetics and in particular the generation time of the bacteria. Growth kinetics were carried out at 30°C and in LB without salt, in optimal biofilm conditions. This shows that the biofilm defect observed in these efflux mutants is specific and is not a result of any generic growth defect.

Various biofilm formation conditions were tested to elucidate the optimal conditions for *S*. Typhimurium biofilm formation *in vitro*. When comparing biofilm assays incubated in minimal media, LB and LB without salt, the optimal media for biofilm formation by the wild-type was LB without salt. The presence of salt in media reduces the cell surface hydrophobicity of the bacteria and decreases the cells ability to aggregate and adhere to a surface reducing the amount of biofilm formed (Kuntiya *et al.,* 2005; Giaoris *et al*, 2005). Therefore removing salt from the media helps the bacteria to form a more competent mature biofilm, but still providing rich nutrition for the bacterial cells. Two temperatures were also tested for optimal biofilm formation, 30°C (a temperature used widely in the literature for *in vitro Salmonella* biofilm assays) and 37°C (optimal temperature for *Salmonella* planktonic growth). L828 (wild-type), L829 (*tolC::cat*) and L830 (*acrB::aph*) mutants all formed more biofilm at 30°C than 37°C. Although *Salmonella* grows best at 37°C as it is mammalian body

temperature, in this context it will invade cells and biofilm formation may not be important. However, environmentally *Salmonella* will tend to exist in a biofilm, therefore lower temperatures are conducive to induction of the biofilm mode of growth (Giaoris *et al*, 2005).

Efflux mutants first observed to have a biofilm defect carried defined a single genetic inactivation of components of the well characterised AcrAB-TolC system. L829 (*tolC::cat*) and L830 (*acrB::aph*) have a significant biofilm defect however L1271 (*acrA::aph*) forms a biofilm as well as L828 (wild-type). A complete panel of efflux mutants representing all the other efflux systems in *Salmonella* were tested and all were found to have a significant biofilm defect in various laboratory biofilm models (Baugh *et al*, 2012). This is a surprising observation as many of the efflux pumps have overlapping substrate specificity (Nishino *et al.* 2006; Nishino *et al.* 2009) and when individual efflux systems are inactivated other systems can compensate for the loss. Also some of these efflux systems are cryptic under laboratory conditions therefore it seems surprising that they should have such a striking effect upon the ability of *S*. Typhimuirum to form a biofilm.

CHAPTER FOUR:

HYPOTHESIS ONE –

EXPORT OF A BIOFILM FACTOR

4 Hypothesis One – AcrAB-TolC exports a crucial biofilm factor

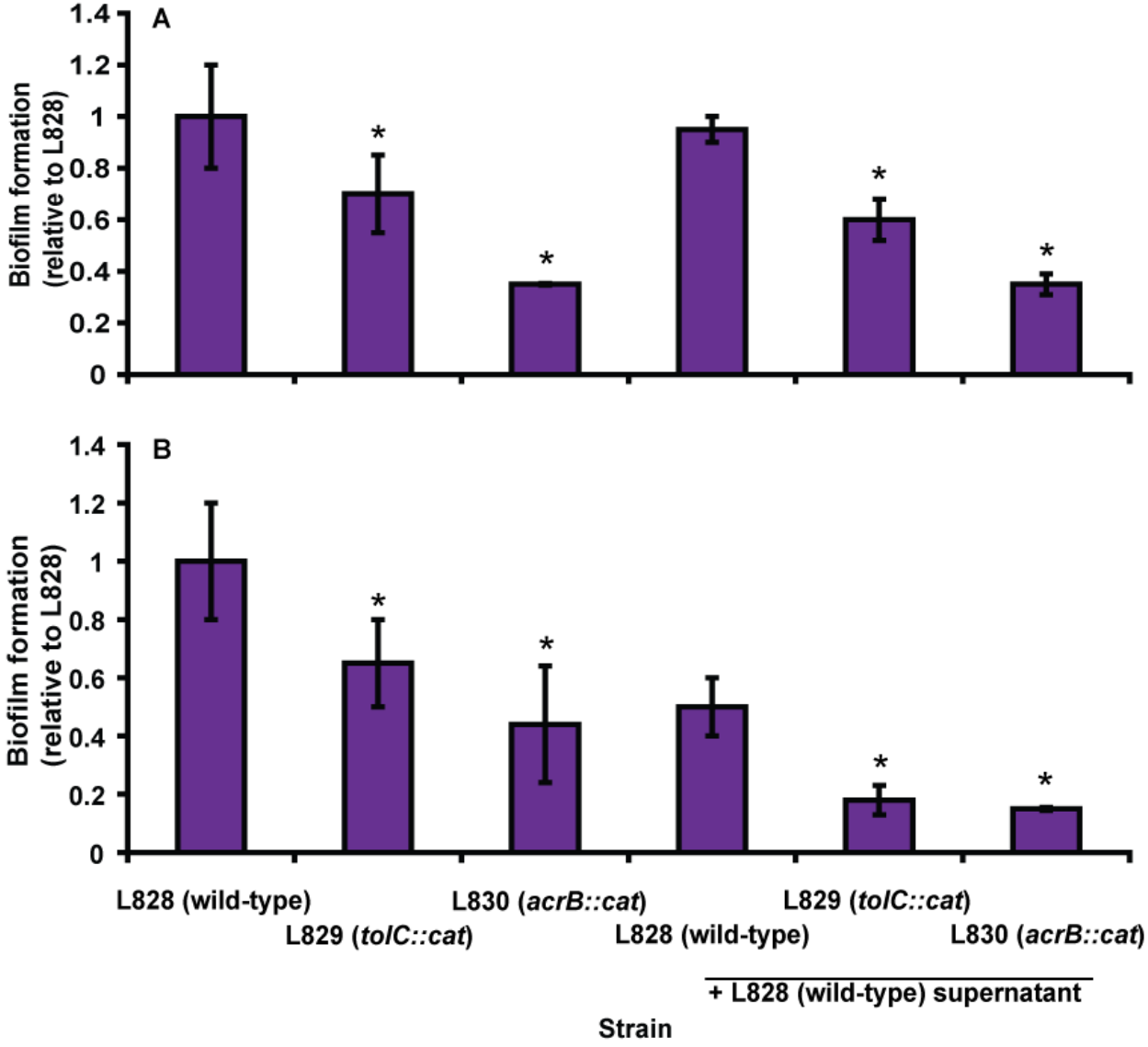
4.1 Background

AcrAB-TolC has been well characterised, is known to have a wide substrate specificity and can efflux various antibiotics, dyes, detergents, solvents and peptides. Knowing this I hypothesised that AcrAB-TolC may be either exporting a molecule essential for biofilm formation or a metabolite that inhibits biofilm formation if it remains intracellular. Various assays were used to investigate whether an exported substrate of AcrAB-TolC is required to form a biofilm.

4.2 Transwell biofilm assay of wild-type with efflux mutants

Transwell assays were used to grow wild-type and mutant strains in the wells of a 24 well microtitre tray with contiguous media separated by a membrane which prevents bacteria but not solute movement. Co-incubation of mutants with a mid-logarithmic phase culture of L828 (wild-type) showed no ability to restore or improve the ability of L829 (*tolC::cat*) or L830 (*acrB::aph*) to form biofilms (Figure 4.1). When the experiment was repeated with an overnight, stationary phase culture of L828 (wild-type) as the supernatant 'donor' again no rescue of the ability of the mutants to form biofilms was observed, conversely biofilm formation was inhibited to some degree (Figure 4.1). This experiment indicates that no soluble factor exported by AcrAB-TolC by the wild-type strain during logarithmic growth or in stationary phase is able to restore biofilm formation in mutants lacking a functional *acrB* or *tolC*.

Figure 4.1 Transwell biofilm assay of L828 (wild-type) with L829 (*tolC::cat*) or L830 (*acrB::aph*). **A** Co-incubation of L829 (*tolC::cat*) and L830 (*acrB::aph*) with L828 (wild-type) – wells inoculated with wild-type and mutant simultaneously **B** Co-incubation of L829 (*tolC::cat*) and L830 (*acrB::aph*) with a stationary phase culture of L828 (wild-type). Statistically significant differences in biofilm formation compared to L828 (wild-type) exposed to the same conditions as the mutant tested were analysed using a student's t test and indicated with an asterisk ($p<0.05$).



4.3 Biofilm co-incubation assay

The biofilm mat assay was used as another way to investigate whether the wild-type strain (L828) could complement the mutants' biofilm defect. Biofilm mats formed on the surface of all co-incubated wells of 24 well microtitre trays, the mats were removed and disrupted to release individual cells and wild-type and mutant cells enumerated by viable counting. Although mutant strains were able to grow well planktonically in competition with L828 (wild-type) when co-incubated, the mutants were unable to form a mat biofilm or intergrate into the wild-type biofilm (Figure 4.2). L829 (*tolC::cat*) cannot associate with wild-type biofilm to any degree and L830 (*acrB::aph*) cells comprised only 1 in 10^6 of the total biofilm formed.

4.4 Metabolomics

Data from a metabolomics experiment comparing metabolites present within the *acrB* mutant were analysed to look for differences which may suggest molecules exported by AcrB that impact on biofilm formation. Approximately 750 peaks representing distinct metabolites were detected by mass spectrometry from all samples and the principle component analysis (PCA) indicated that mutant and wild-type replicates separated into two distinct clusters (one replicate from the wild-type was a consistent outlier, analysis was repeated with and without this sample, little differences to overall results ensued). Approximately 1.5% of the peaks were significantly different in abundance between the wild-type and mutant samples. Of the metabolites which could be assigned an identity the metabolite with the greatest difference between the two strains was spermidine which was found to be in the mutant samples at levels 33 fold higher than the wild-type and was the metabolite responsible for the majority of the discrimination of the samples by the PCA (Table 4.1 and Figure 4.3)

Figure 4.2 A Diagrammatic representation of the inoculation pattern of the co-incubation mat biofilm assay and viable count enumeration of L828 (wild-type), L829 (*tolC::cat*) and L830 (*acrB::aph*) of **B** planktonic liquid and **C** proportion of mixed strain biofilm comprised of mutant cells. Statistically significant differences in cfu/ml compared to L828 (wild-type) at each time point in panel B were analysed using a student's t test and indicated with an asterisk ($p<0.05$).

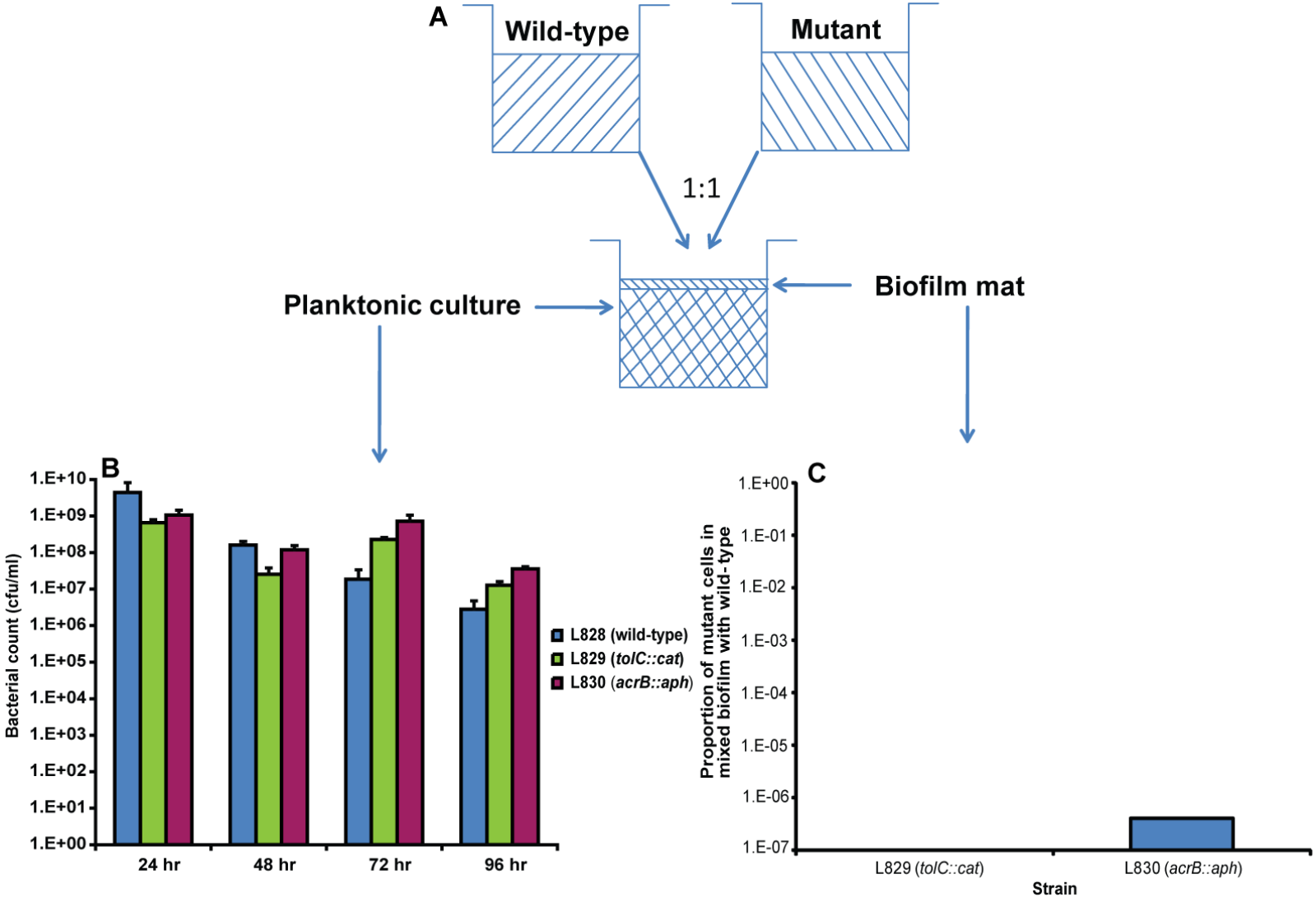
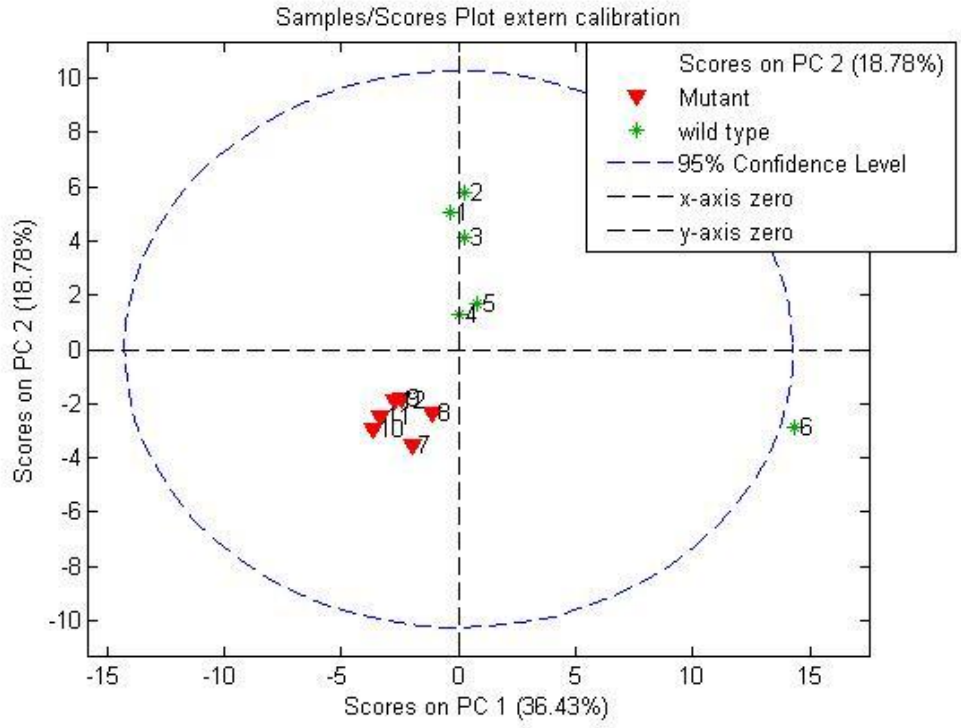


Table 4.1 Metabolites identified with different abundance from within L828 (wild-type) and L830 (*acrB::aph*) cells

mz	Metabolite	Change mutant/wild-type	P value
146.1651	Spermidine	33.7421	0.002867
171.0417	Dihydrocoumarin plus others	0.549924	0.148721
156.0421	Valine, betaine, etc	0.247751	0.388728
221.0361	Xanthan	1.66932	0.001798
352.2246	steroid/alkaloid	0.156018	0.350022
183.0781	Diisopropyl phosphate plus fatty acid	1.393881	0.019276
424.2822	['Myxalamid B']	0.661969	0.034707

Figure 4.3 Principle component analysis (PCA) of samples from showing separation of replicates. The circled sample consistently has an outlier, analysis was repeated with and without this sample



4.5 Biofilm formation with addition of exogenous polyamines

As the metabolimics data suggested excess accumulation of spermidine within efflux mutants the impact of adding exogenous polyamines on biofilm formation was analysed. The addition of exogenous polyamines to crystal violet biofilm assays had no effect on biofilm formation by L828 (wild-type), L829 (*tolC::cat*) or L830 (*acrB::aph*), the polyamines neither hindered nor rescued biofilm formation of wild-type or mutants (Figure 4.4).

4.6 Biofilm formation assay with exogenous indole, tryptophan and casamino acids

Recent work has suggested that amino acid metabolism is increased in biofilm formation and that indole can regulate efflux pump regulation (Hamilton *et al.* 2009; Nikaido *et al.* 2011). Biofilm assays with the addition of either exogenous tryptophan or casamino acids showed no impact on biofilm rescue of the mutants biofilm defect. Increasing the concentration of exogenous indole seems to have a negative effect of biofilm formation. (Figure 4.5).

4.7 Discussion

The hypothesis examined in this chapter investigated whether inactivation of AcrAB-TolC prevents export of a substrate of the pump which promotes biofilm formation. Both the transwell and co-incubation assays suggest that this is an unlikely explanation for the defect of mutant strains as the presence of wild-type biofilms were unable to rescue the biofilm phenotype of the *tolC* and *acrB* mutants, either in trans or when directly co-incubated.

Figure 4.4 Crystal violet biofilm formation assay of L828 (wild-type), L829 (*tolC::cat*) and L830 (*acrB::aph*) with addition of increasing concentrations of three different polyamines putrescine (**A**), cadaverine (**B**) and spermidine (**C**).

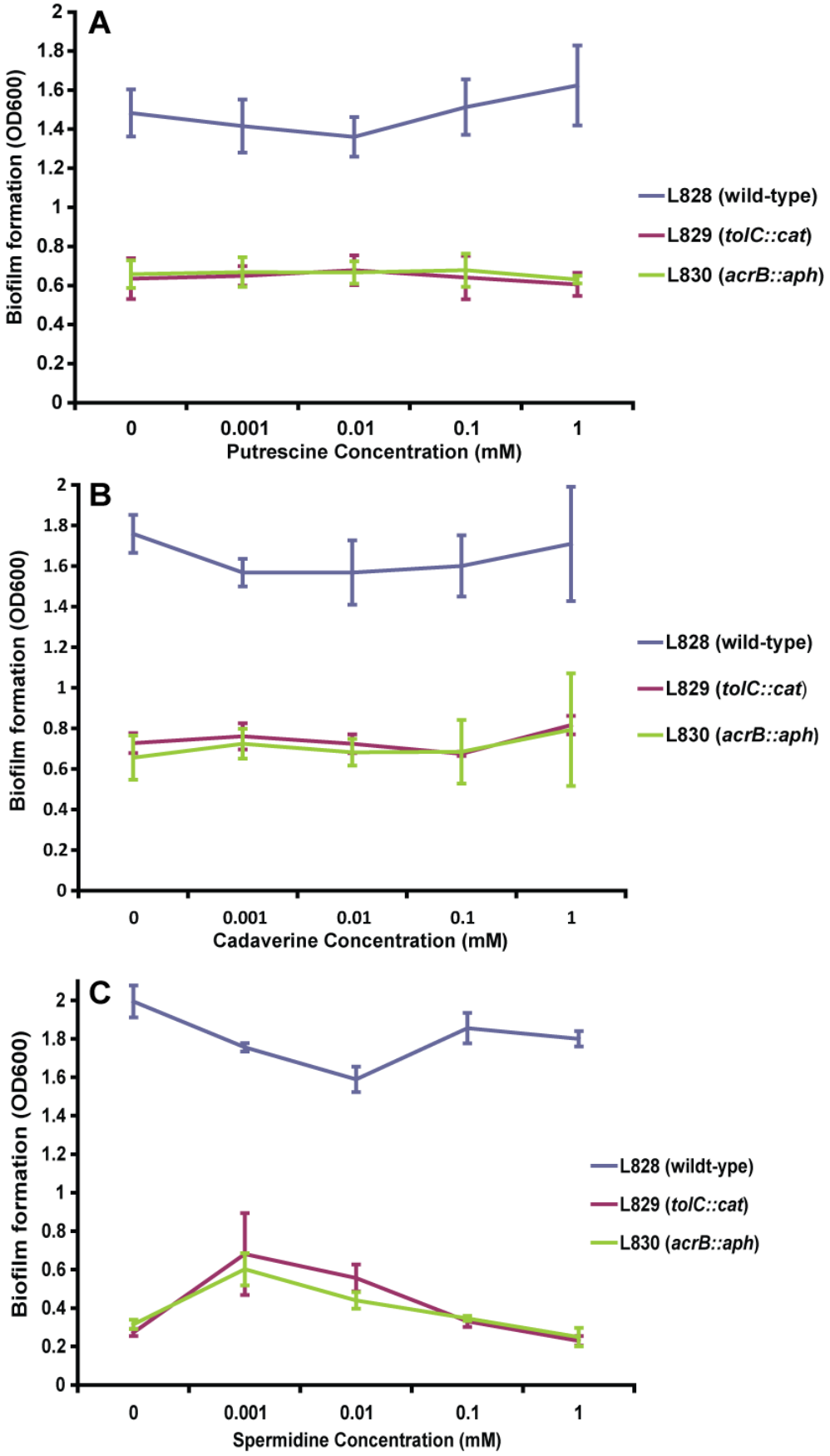
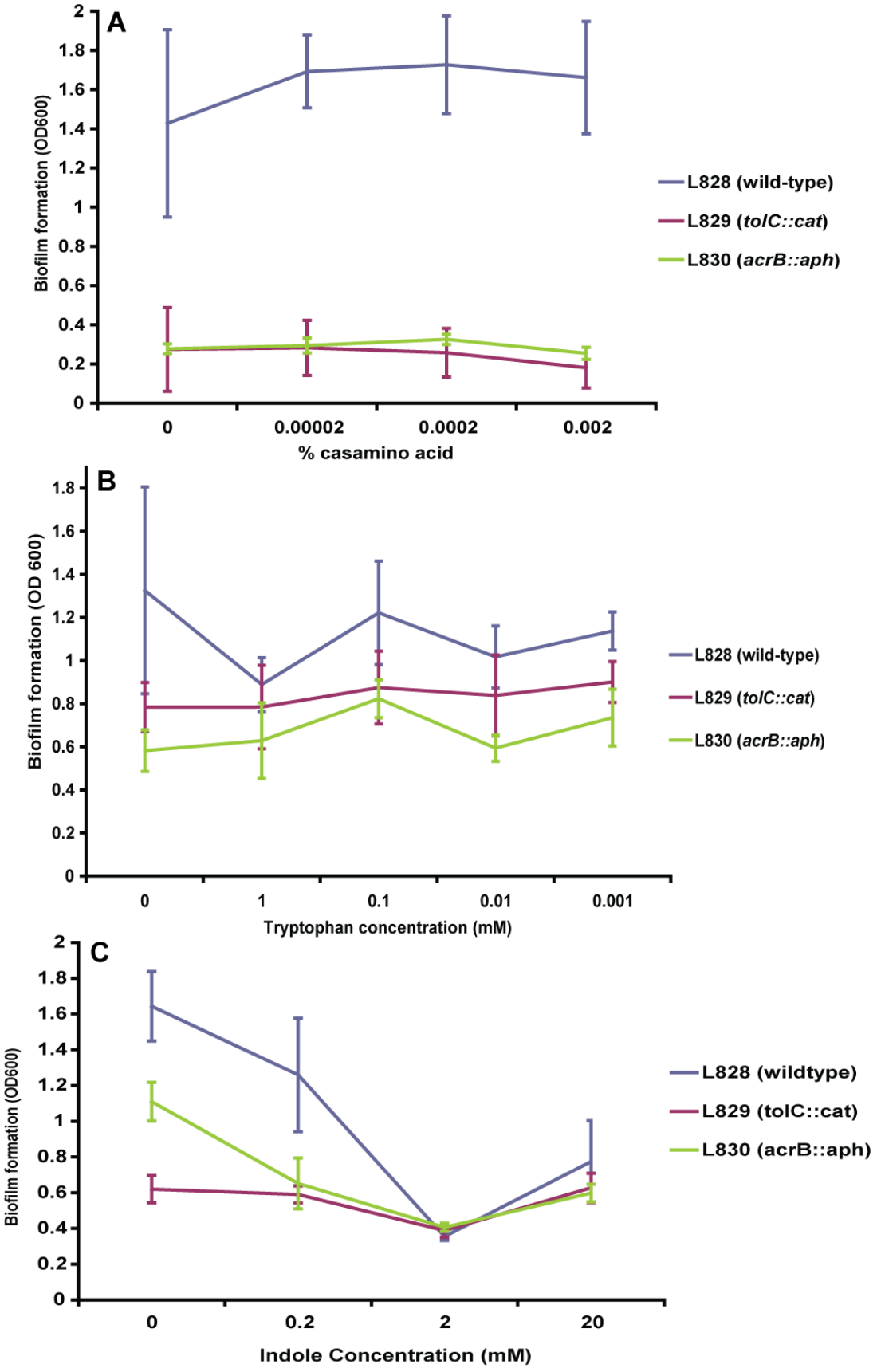


Figure 4.5 Crystal violet biofilm formation assay of L828 (wild-type), L829 (*tolC::cat*) and L830 (*acrB::aph*) with addition of exogenous casamino acids (**A**), tryptophan (**B**) and indole (**C**)



The second part of this hypothesis considered the idea that by inactivating AcrAB-TolC, a toxic metabolite accumulates in the bacterial cell that inhibits the cells ability to live at high density. Metabolomics data showed that spermidine (or another closely related polyamine) accumulated in the cell in the absence of a functional AcrB, polyamines have also been linked with biofilm formation in a number of different bacteria (Karatan *et al.* 2005; Patel *et al.* 2006; Lee *et al.* 2009). However the addition of spermidine, cadaverine or putrescine had no significant effect on the biofilm formation of either wild-type or the mutant strains. If a polyamine(s) was accumulating to toxic levels within the mutant strains it may have been expected that addition of exogenous polyamines would inhibit wild-type biofilm formation. After these experiments were completed with the *acrB* and *tolC* mutants it was observed that a much wider panel of efflux mutants showed a biofilm defect (Section 3.6). Some of the efflux systems lacking in this panel of mutants are considered cryptic under laboratory conditions and there are no known substrates common to all the MDR efflux systems in *Salmonella*. These data further suggest it is unlikely a biofilm promoting substrate is exported. In conclusion it appears unlikely that direct export of a biofilm relevant solute can account for the biofilm defect of efflux mutants.

CHAPTER FIVE:

HYPOTHESIS TWO –

CELL SURFACE

HYDROPHOBICITY

5 Hypothesis Two – Inactivation of AcrAB-TolC impacts upon cell surface hydrophobicity

5.1 Background

Removal of large membrane proteins from both the inner and outer membrane of Gram negative bacteria has the potential to create instability of the cell envelope. The second hypothesis investigated if a potential membrane change impacts upon the cells surface hydrophobicity and ultimately the cells ability to aggregate to surfaces and each other.

5.2 Settle assay

The settle assay suggested that the mutants are not compromised in their ability to aggregate in comparison to the wild-type (Figure 5.1), if anything the mutants aggregated slightly more than the wild-type, in particular L830 (*acrB::aph*) however changes were not statistically significant (Baugh *et al.* 2013).

5.3 Salt aggregation test

L828 (wild-type) and L830 (*acrB::aph*) both showed a similar ability to aggregate; the lowest ammonium sulphate molarity causing aggregation was 2 M (Figure 5.2). L829 (*tolC::cat*) again demonstrated a higher aggregation ability as the lowest ammonium sulphate molarity required to cause aggregation was 1 M, however at only one dilution difference the results are not conclusive (Baugh *et al.* 2013).

Figure 5.1 Settle assay of L828 (wild-type), L829 (*tolC::cat*) and L830 (*acrB::aph*). Statistically significant differences in cfu/ml compared to L828 (wild-type) at each time point were analysed using a student's t test and indicated with an asterisk ($p<0.05$).

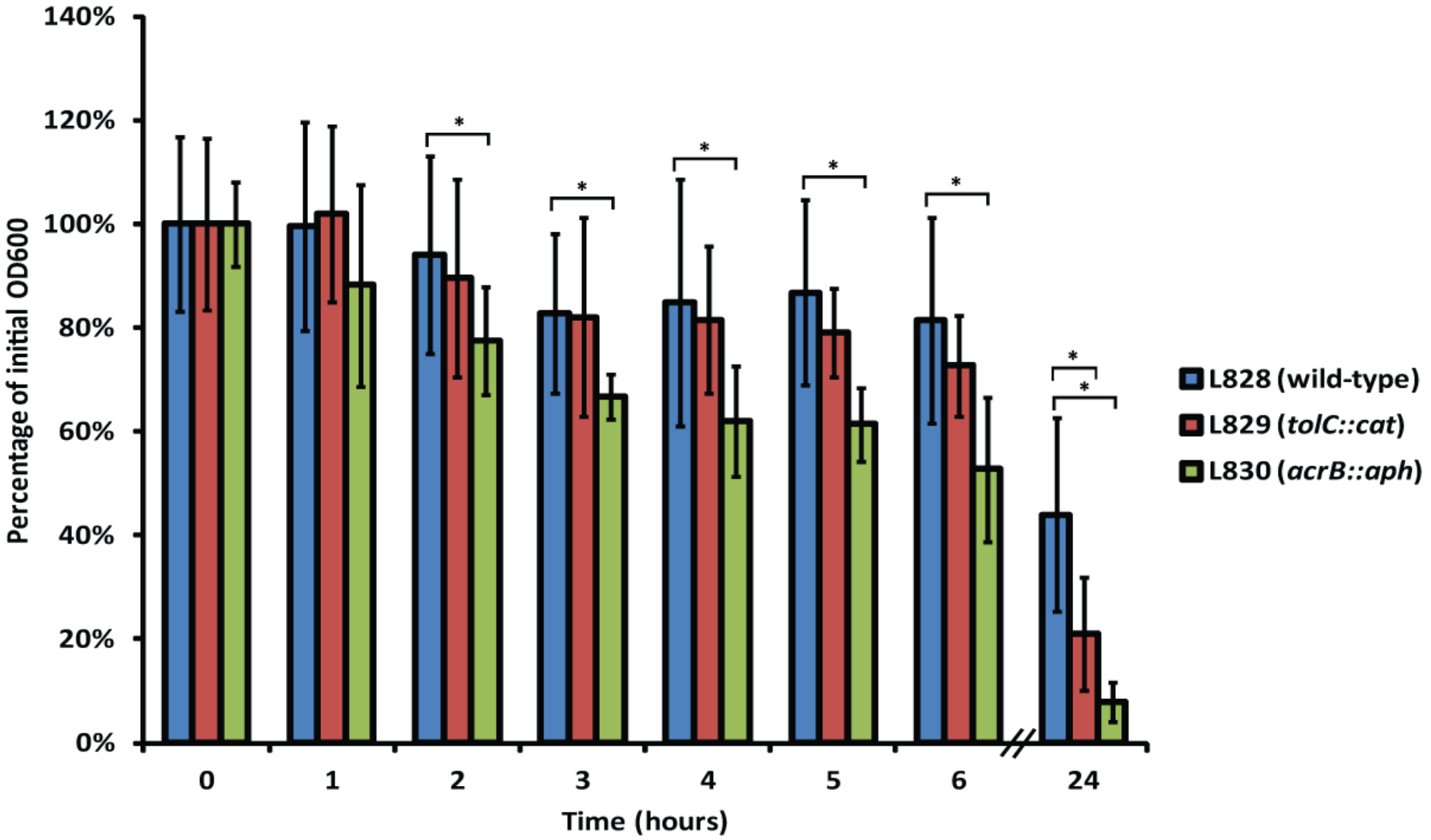
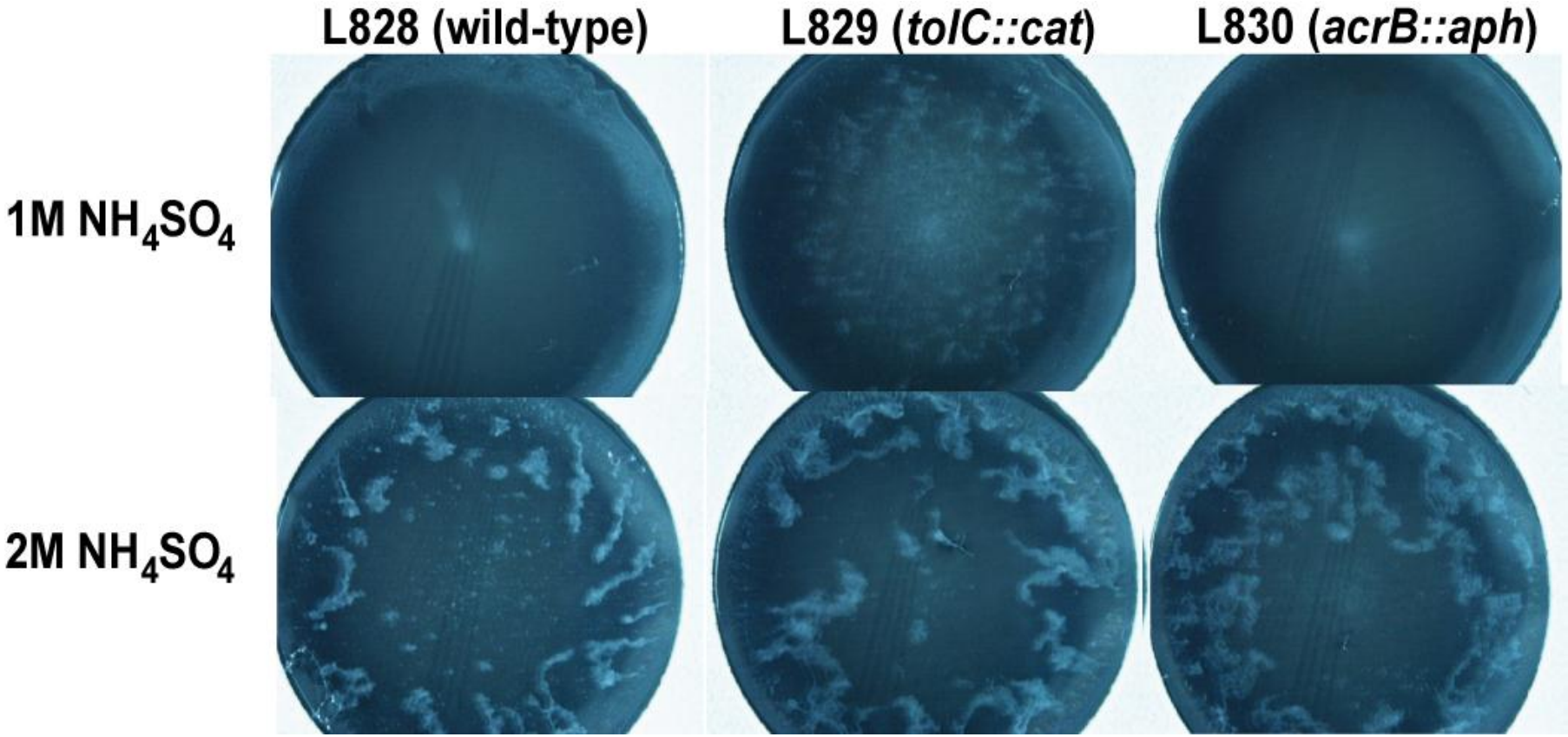


Figure 5.2 Salt aggregation test images of L828 (wild-type), L829 (*tolC::cat*) and L830 (*acrB::aph*) in 1M and 2M ammonium sulphate. Presence of a white precipitate indicates bacterial aggregation.



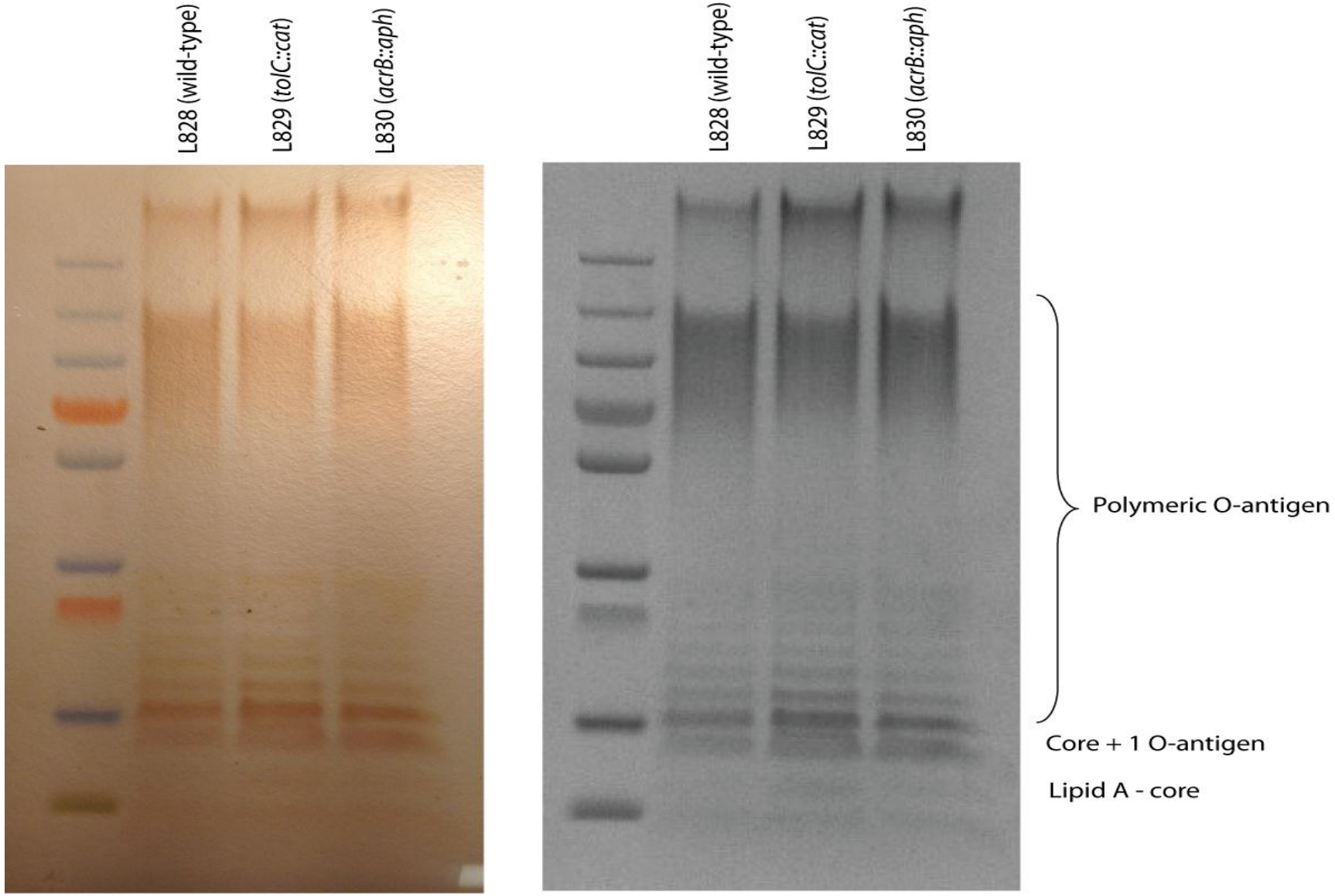
5.4 LPS preparation

A crude LPS preparation method was used to extract LPS from L828 (wild-type), L829 (*tolC::cat*) and L830 (*acrB::aph*) to determine if there were any change in LPS pattern resulting from inactivating *tolC* or *acrB*. No difference in the band pattern was observed between wild-type and mutants when the LPS preparations were run on an SDS-PAGE gel (Figure 5.3).

5.5 Discussion

The second hypothesis considered the idea that deletion of large membrane proteins (AcrB and TolC) physically altered the properties of the bacterial cell envelope and perhaps disrupted the ability of the bacteria to aggregate with each other or to a surface. All assays that tested either aggregation, hydrophobicity or membrane structure suggest there is no defect in the *tolC* and *acrB* mutants cell surface hydrophobicity or ability to aggregate. These data suggest that the reason for the mutants biofilm defect is not a decreased ability to aggregate. This conclusion is consistent with chemical inactivation of AcrAB-TolC with various EIs where L828 (wild-type) is inhibited in its ability to form biofilms but still has a physically intact efflux system.

Figure 5.3 Analysis of LPS preparations of L828 (wild-type), L829 (*tolC::cat*) and L830 (*acrB::aph*) separated on 12% SDS PAGE gel by electrophoresis



CHAPTER SIX:

HYPOTHESIS THREE –

INACTIVATION OF COMPONENTS

OF AcrAB-TolC ALTERS

EXPRESSION OF BIOFILM

GENES/PROTEINS

6 Hypothesis three – Inactivation of components of AcrAB-TolC alters expression of biofilm related genes or proteins

6.1 Background

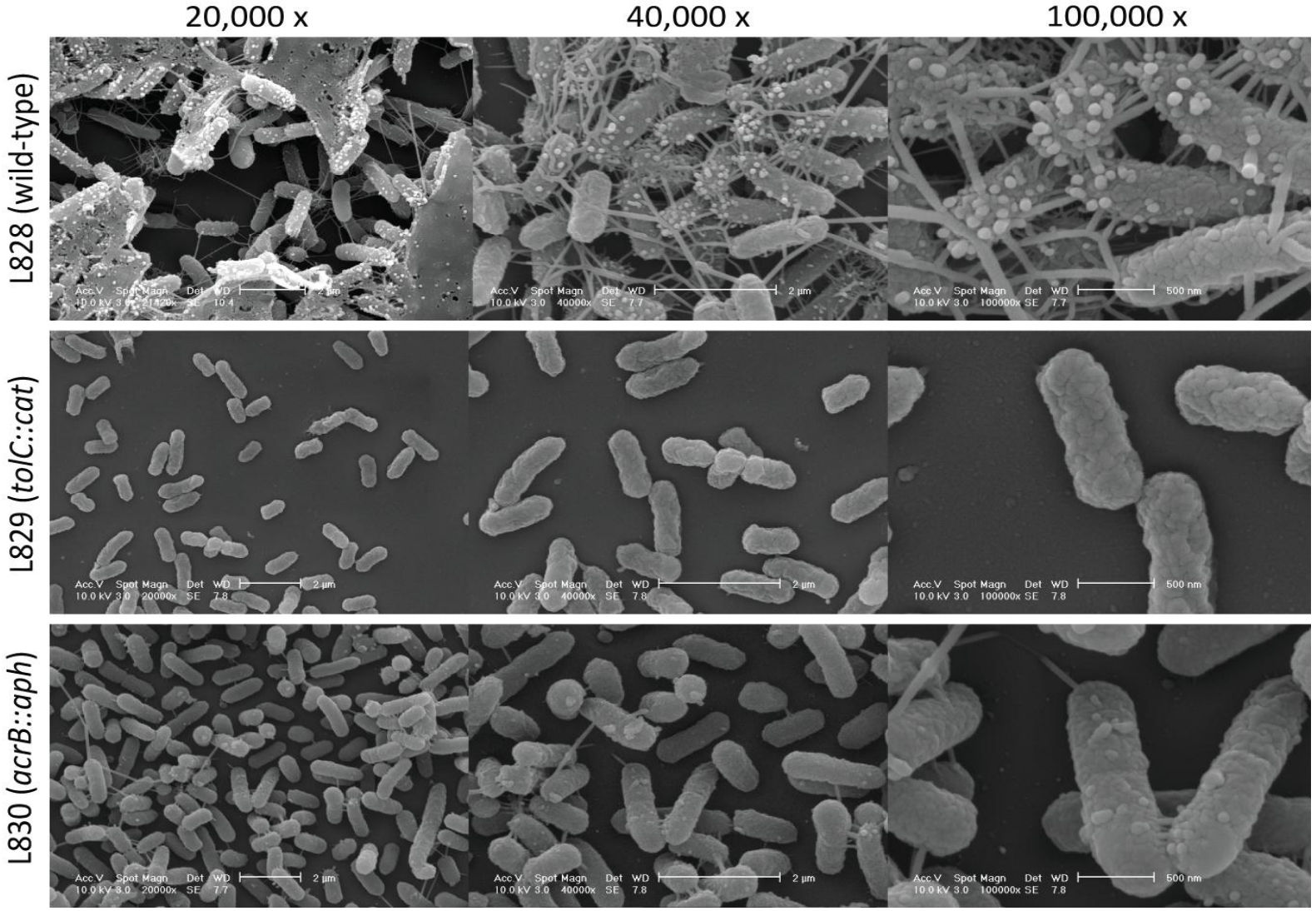
Many genes are involved in the efficient biofilm formation of *Salmonell*a; two *csg* operons encoding curli proteins, the *bsc* operon encoding cellulose biosynthesis, *bapA* encoding a membrane protein involved in pellicle formation and many other genes that regulate the expression of these crucial biofilm genes. *csgD* is the master biofilm regulator gene in *Salmonella* and is involved in the up-regulation of *csgBA* (curli structural subunits), *bscABCD* (cellulose biosynthesis) and *bapA* (pellicle formation). Curli and cellulose expression was investigated in this chapter as they are the two major components of the *Salmonella* biofilm extracellular matrix.

6.2 Characterisation of extra cellular matrix components of *tolC* and *acrB* mutants

6.2.1 Scanning Electron microscopy

In order to determine whether efflux pump mutants were unable to adhere to surfaces at all or were adherent but unable to form a biofilm, cultures were incubated on glass slides (submersed in LB without salt for 48 hours) before being fixed for SEM. Images obtained from the SEM showed L828 (wild-type) forming a complex three dimensional biofilm where you can see clearly the extracellular matrix as well as some spherical structures putatively identified as outer membrane vesicles (OMVs) (Figure 6.1). Images of L829 (*tolC::cat*) and L830 (*acrB::aph*) showed no multicellular communities only individual bacterial cell.

Figure 6.1 Scanning electron microscopy images of L828 (wild-type), L829 (*tolC::cat*) and L830 (*acrB::aph*) at 20,000, 40,000 and 100,000 X magnification.



The outer membrane of L829 (*tolC::cat*) appeared relatively smooth with no curli structures or OMVs whereas L830 (*acrB::aph*) appeared to have no curli but did have OMVs associated with the bacterial outer membrane.

6.2.2 Transcriptomic and phenotypic cellulose characterisation

Production of cellulose can be phenotypically characterised by plating strains out onto LB agar without salt and supplemented with 200 µg/ml of Calcofluor which fluoresces under UV light when bound to cellulose. This method showed no disruption of cellulose production for any of the mutants (Figure 6.2). Comparative RT-PCR confirmed this observation showing no significant difference in the genetic expression of the *bcsABCZ* cellulose biosynthesis operon in either L829 (*tolC::cat*) or L830 (*acrB::aph*) (Figure 6.3).

6.2.3 Transcriptomic and phenotypic curli characterisation

Curli production was visualised on Congo red agar by examining morphological appearance. L828 (wild-type) showed a red and rough morphology indicating the presence of curli in contrast to L829 (*tolC::cat*) and L830 (*acrB::aph*) which presented with a pink and smooth morphology indicating the absence of curli (Figure 6.4). This trend was also confirmed by staining a bacterial suspension with Congo red and measuring both the absorbance and fluorescence stained cells (Figure 6.4).

To ascertain if this curli repression was occurring at the transcriptional level, RNA was isolated from L828 (wild-type), L829 (*tolC:cat*), L830 (*acrB::aph*) and L1271 (*acrA::aph*) and cDNA synthsised which was used as the template for cRT-PCR of the genes within both curli operons. Expression of all curli genes were significantly down regulated in both L829 (*tolC::cat*) and L830 (*acrB::aph*) (Figure 6.5).

Figure 6.2 Images taken under UV light to assess cellulose expression of L828 (wild-type), L829 (*tolC::cat*) and L830 (*acrB::aph*) using Calcofluor staining.

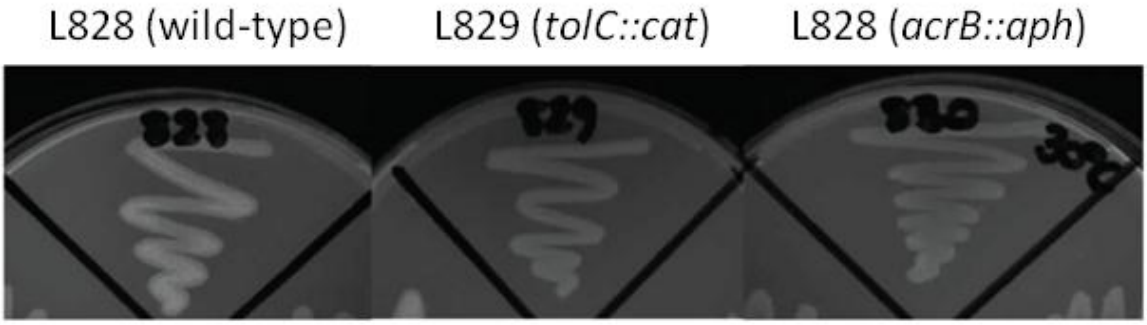


Figure 6.3 Cellulose synthesis gene expression of L828 (wild-type), L829 (*tolC::cat*) and L830 (*acrB::aph*) using comparative RT-PCR. Statistically significant differences in cellulose expression in comparison to L828 (wild-type) are indicated by an asterisk ($p<0.05$).

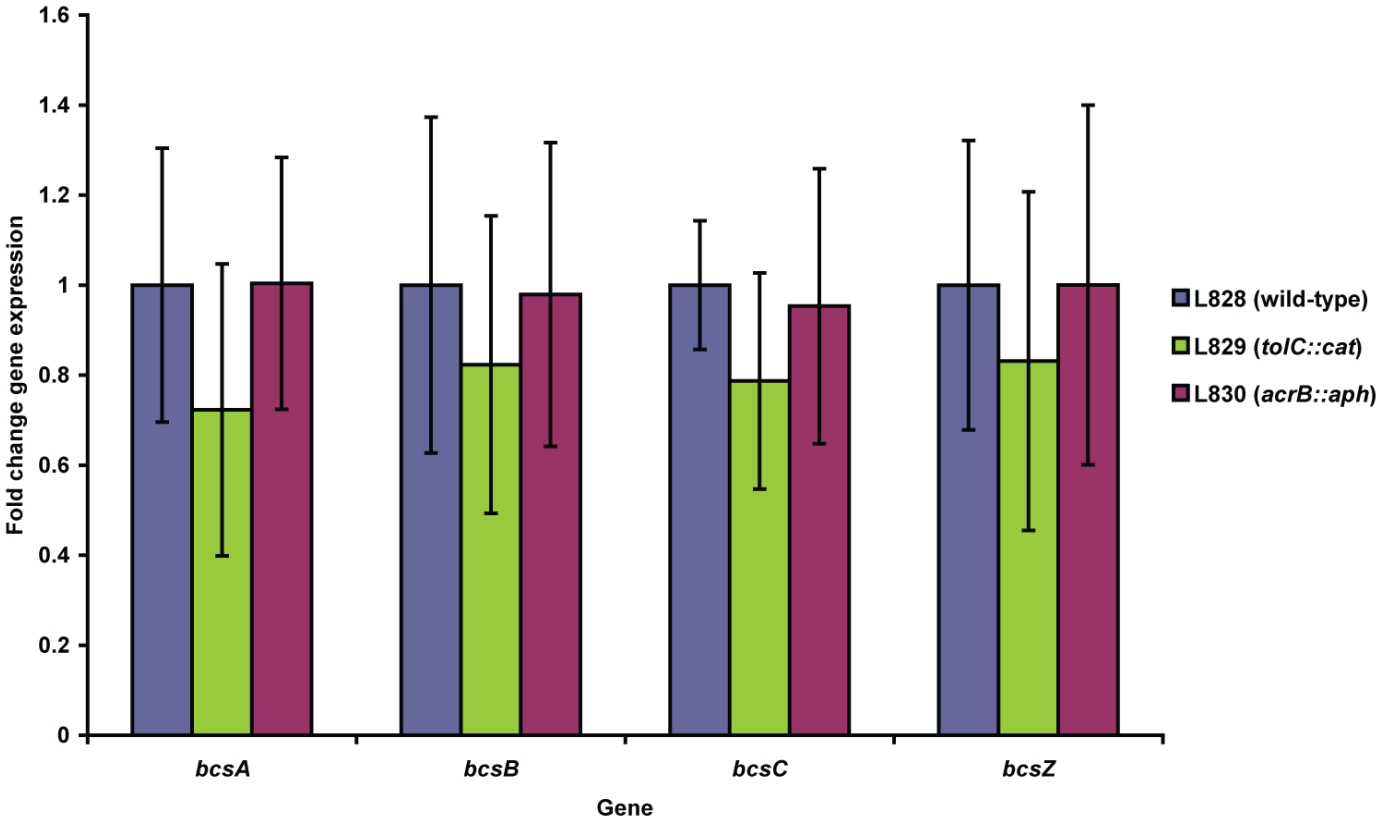


Figure 6.4 Phenotypic curli expression of L828 (wild-type), L829 (*tolC::cat*) and L830 (*acrB::aph*) using various Congo red assays and quantification; Congo red agar morphologies (**A)**, Congo red staining of bacterial suspension (**B**) quantified by both optical density (**C**) and fluorescence (**D**) In panels C and D Statistically significant differences in curli expression in comparison to L828 (wild-type) are indicated by an asterisk ($p<0.05$).

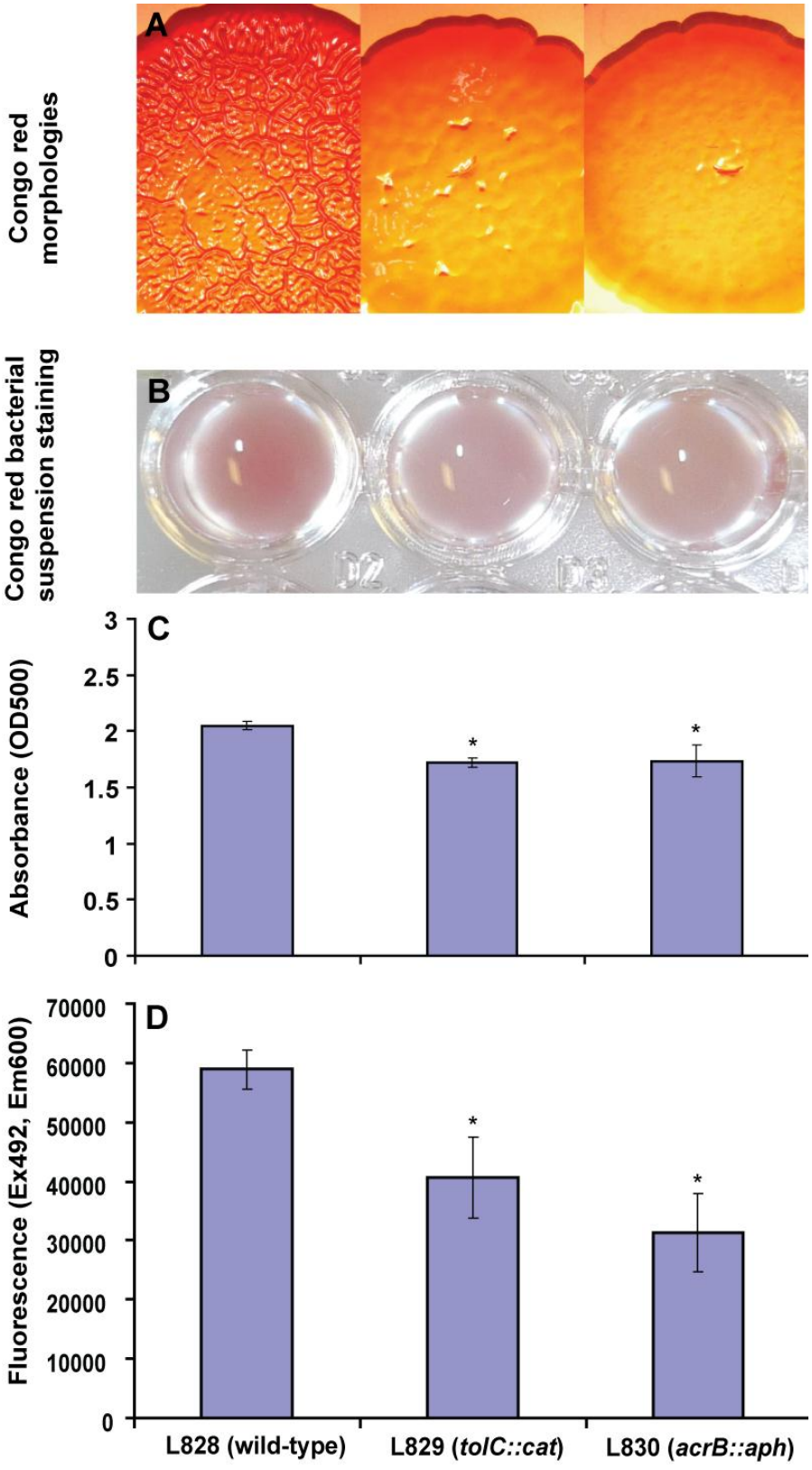
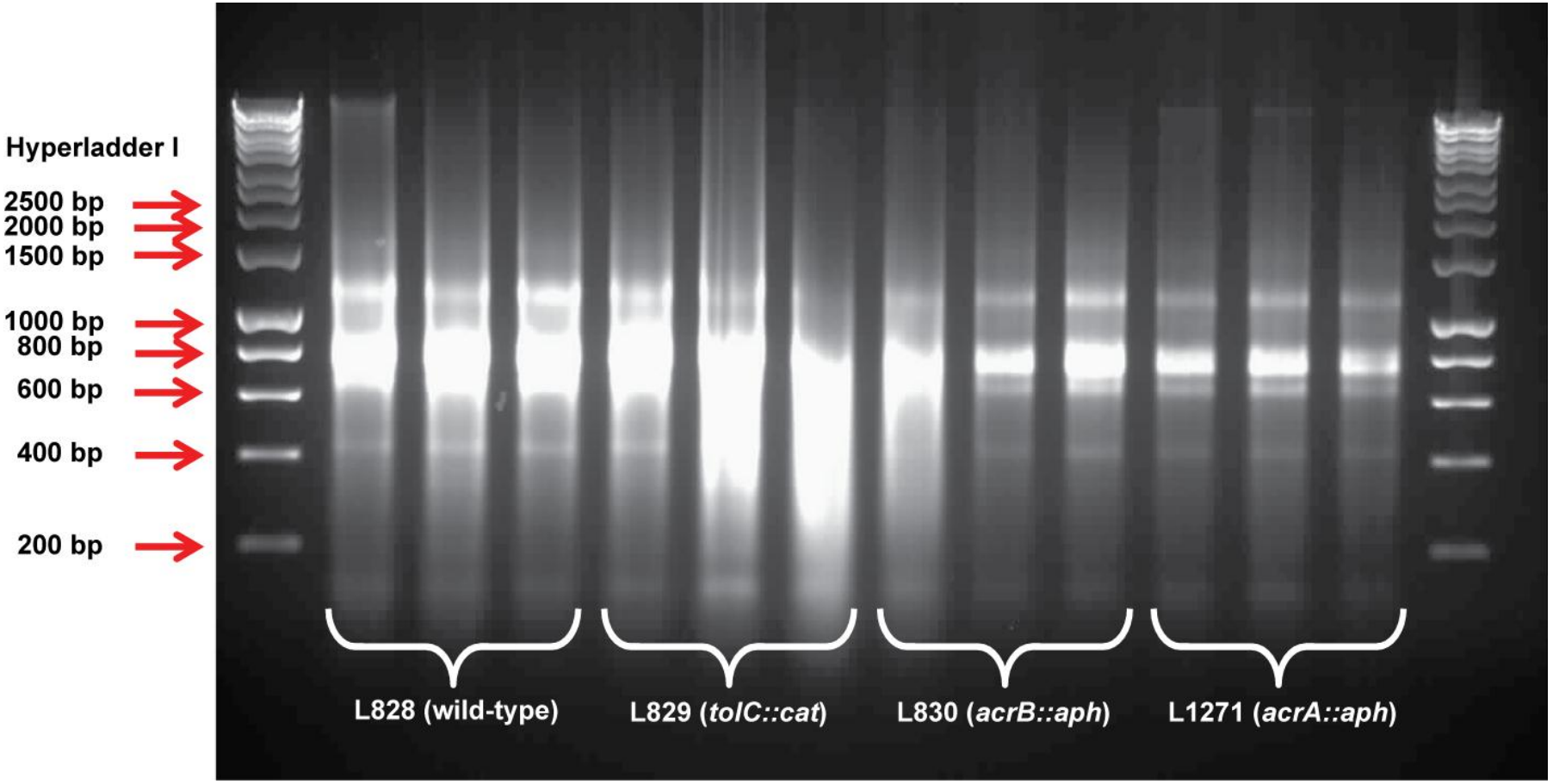


Figure 6.5 RNA preparation of L828 (wild-type), L829 (*tolC::cat*), L830 (*acrB::aph*) and L1271 (*acrA::aph*) grown to stationary phase in LB without salt broth at 30°C. 5 µl of RNA preparation was mixed with 1 µl of DNA loading buffer, loaded onto a 1% agarose gel along with DNA marker Hyperladder I and separated by electrophoresis at 100 V for 1 hour.



The master regulator, *csgD*, was down regulated 10 fold for both mutants. The three genes required for efficient assembly and translocation of the structural subunits were also all down regulated to varying degrees; *csgE* was down regulated 2 and 4 fold in the *acrB* and *tolC* mutants respectively, *csgF* was down regulated by 1.5 fold in both mutants and *csgG* down regulated by 5 and 10 fold in the *acrB* and *tolC* mutants respectively (Figure 6.6 and Figure 6.7) (Baugh *et al.* 2013).

6.3 Curli is down-regulated in all multidrug resistance efflux mutants

Congo red agar revealed that the entire panel of efflux mutants tested for biofilm formation (section 3.6) were lacking curli on the surface of their outer membrane (Figure 6.8). cRT-PCR of *csgB* (the major structural curli subunit) and *csgD* (the mastor curli regulator) confirmed that this repression was occurring at the mRNA level as in the L829 (*tolC:cat)* and L830 (*acrB::aph)* (Figure 6.9). Both genes were significantly down-regulated in L829 (*tolC::cat*) and all five RND efflux mutants; L830 (*acrB::aph*), L832 (*acrD::cat*), L833 (*acrEF::cat*), L834 (*mdtABC::cat*) and L835 (*mdsABC::cat*). *csgB* expression was down-regulated in the four mutants belonging to the MFS, MATE and ABC efflux families; L836 (*emrAB::cat*), L837 (*mdfA::cat*), L838 (*mdtK::cat*) and L839 (*macAB::cat*) however *csgD* expression remains unchanged.

Figure 6.6 cRT-PCR of *csgG* with L828 (wild-type), L829 (*tolC::cat*), L830 (*acrB::aph*) and L1271 (*acrB::aph*) cDNA (synthesised from RNA in Figure 6.5). 5 µl of each cRT-PCR sample was loaded onto a 1% agarose gel and electrophoresed at 100 V for 1 hour.

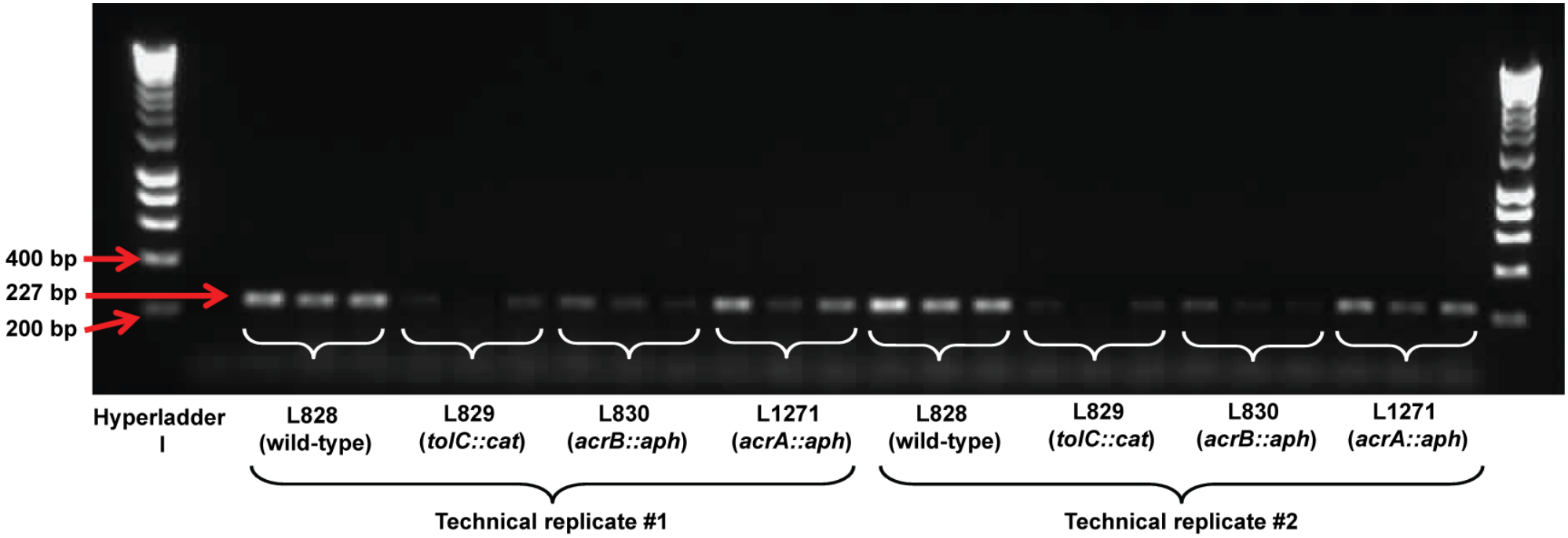


Figure 6.7 Gene expression of genes in both curli operons of L828 (wild-type), L829 (*tolC::cat*) and L830 (*acrB::aph*) using comparative RT-PCR on **A** bar chart. Statistically significant differences in gene expression in comparison to L828 (wild-type) are indicated by an asterisk ($p<0.05$).and **B** a heat map of transcription of the two curli loci.

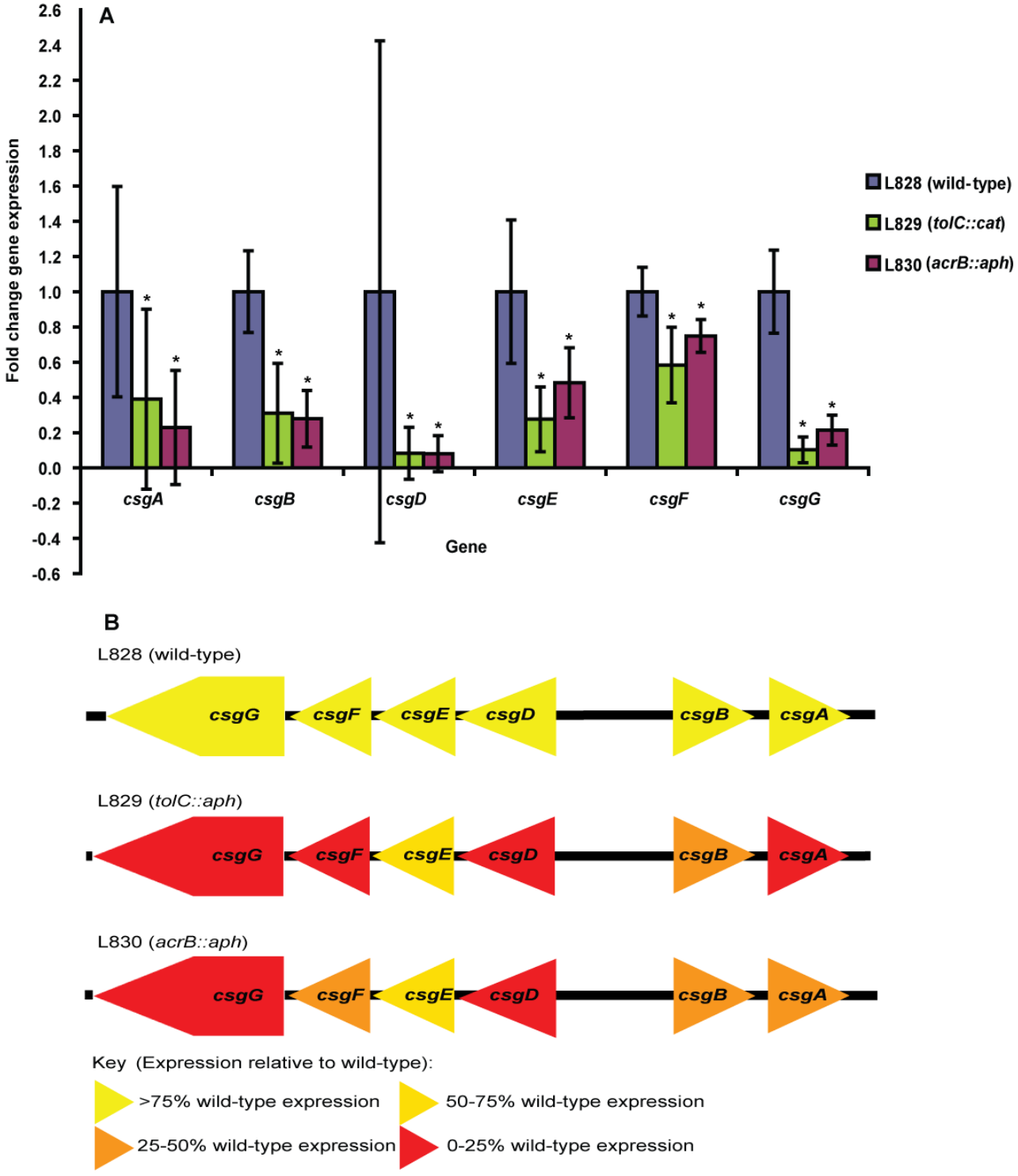


Figure 6.8 Congo red morpholgies of L828 (wild-type), L832 (*acrD:cat*), L833 (*acrEF:cat*), L834 (*mdtABC::cat*), L835 (*mdsABC::cat*), L836 (*emrAB::cat*), L837 (*mdfA::cat*), L838 (*mdtK;::cat*) and L839 (*macAB::cat*) showing absence of curli by the pink and smooth morphology in all efflux mutants.

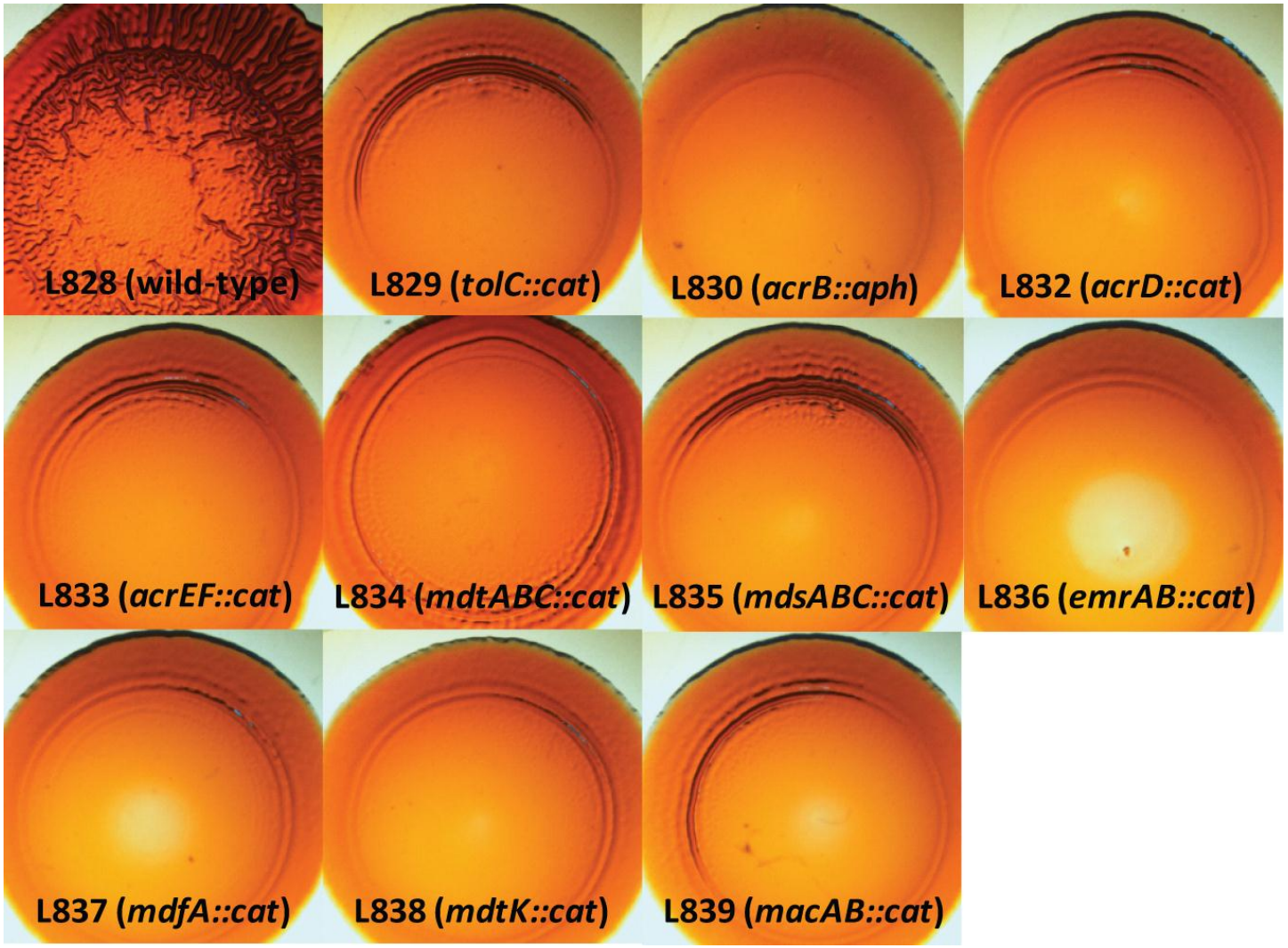


Figure 6.9 Gene expression of *csgB* and *csgD* in L828 (wild-type), L832 (*acrD:cat*), L833 (*acrEF:cat*), L834 (*mdtABC::cat*), L835 (*mdsABC::cat*), L836 (*emrAB::cat*), L837 (*mdfA::cat*), L838 (*mdtK;:cat*) and L839 (*macAB::cat*) using comparative RT-PCR. Statistically significant differences gene expression in comparison to L828 (wild-type) are indicated by an asterisk ($p<0.05$).

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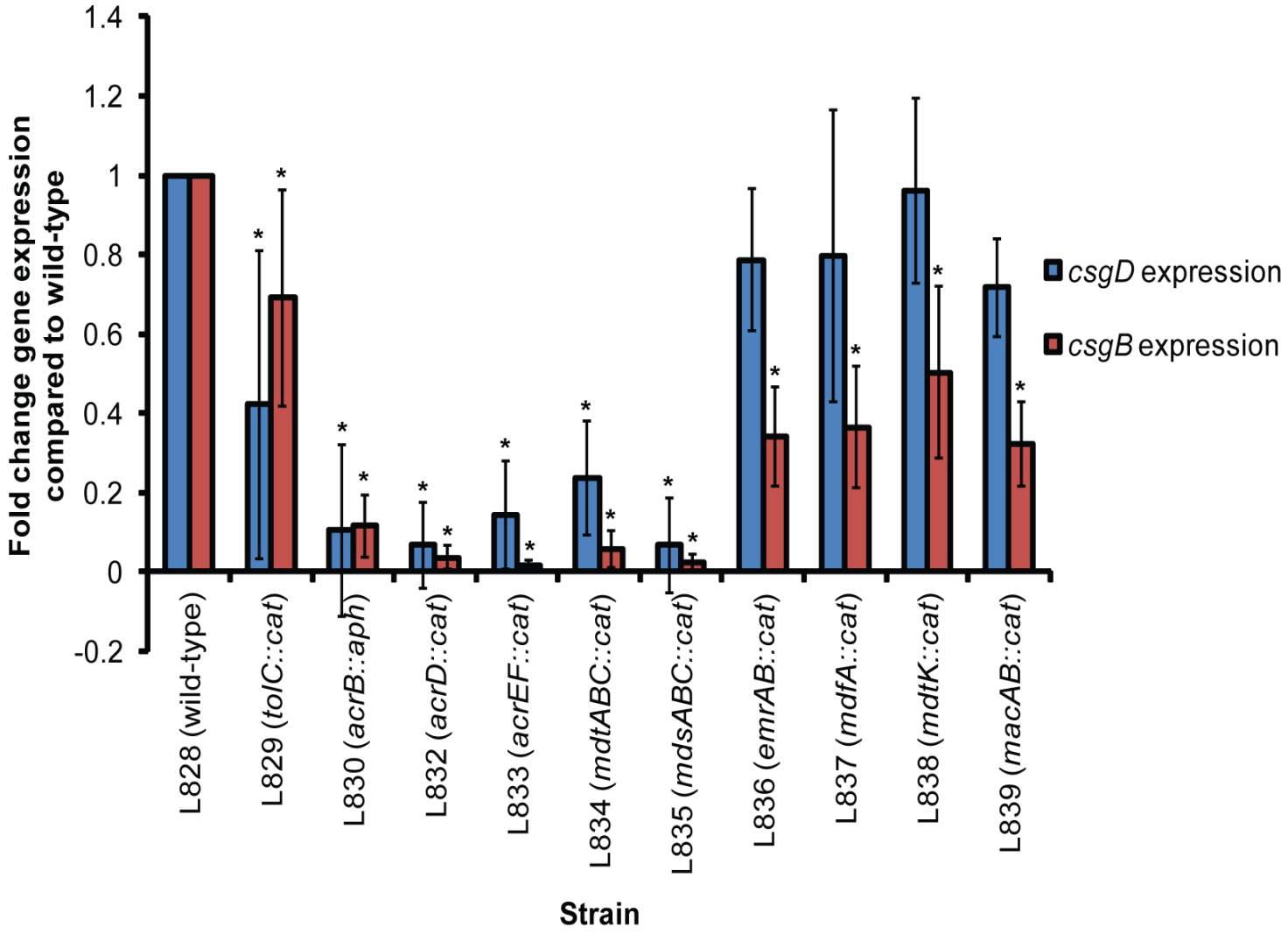


6.4 Discussion

SEM images showed L828 (wild-type) *Salmonella* forming a mature biofilm and the two efflux mutants, L829 (*tolC::cat*) and L830 (*acrB::aph*) adhering as individual cells but not forming three dimensional biofilms as the wild-type does. Phenotypic and genotypic characterisation of cellulose showed no significant difference between wild-type and the two efflux mutants. However, there is a significant difference in Congo red morphology between wild-type (red, dry and rough) and the two mutants (smooth and pink) suggesting curli is not expressed in the mutants and this is confirmed by cRT-PCR of both curli operons. Further investigation into expression of curli of the entire panel of MDR efflux mutant revealed all mutants lack curli and the repression is at the transcriptional level. Both mutants were significantly down regulated although the error is large for the *csgD* expression of wild-type. This could be explained by *csgD* being a phase variable gene, meaning the gene is either turned on or off (Grantcharova *et al.* 2009), if a much larger proportion of the population have *csgD* turned ‘on’ but there is still a subset with *csgD* turned ‘off’ the error will appear large.

This chapter suggests that repression of curli seen in efflux mutants is the mechanism underpinning the observed biofilm defect. It also confirms the mechanism is regulatory and the this third hypothesis is most likely to be correct.

CHAPTER SEVEN:

THE INFLUENCE OF GLOBAL REGULATORS OF AcrAB-TolC ON BIOFILM FORMATION

7 Global regulators of AcrAB-TolC repress curli synthesis

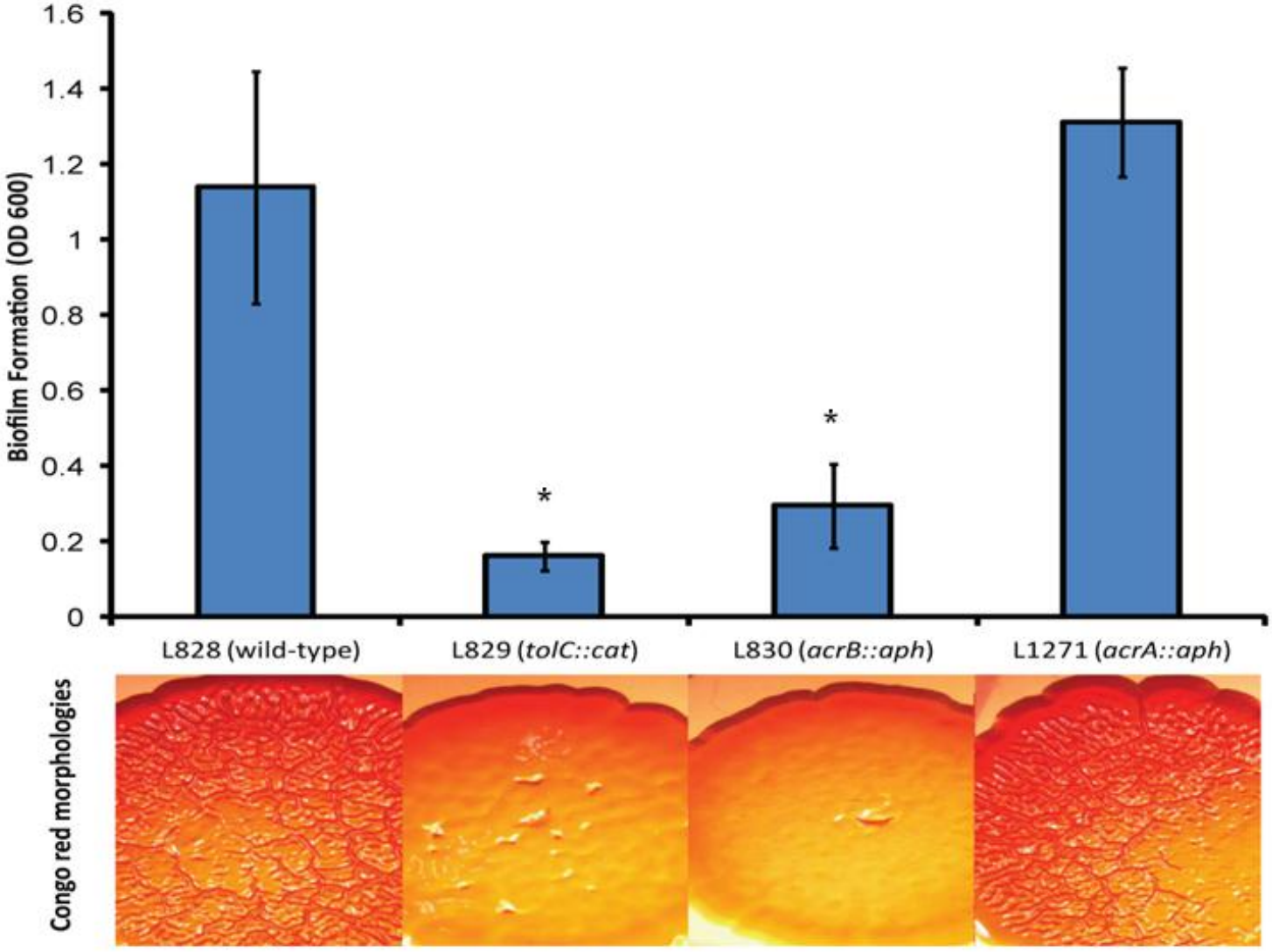
7.1 Background

Chapter 6 shows that the link between inactivation of efflux and inhibition of biofilm formation is due to repression of both curli operons. This suggests that there is a regulatory switch being invoked upon inactivation of efflux components that directly or indirectly represses curli. There are a known group of inducible transcriptional regulators belonging to the AraC family in *Salmonella* that have previously been identified as being able to control expression of AcrAB-TolC; *ramA*, *marA* and *soxS*. In this chapter these regulators were investigated to elucidate if they play a role in curli expression in *S*. Typhimuirum.

7.2 Inactivation of *acrA* has no effect on biofilm formation or curli expression

Genetic inactivation of both *tolC* or *acrB* creates a biofilm defect via repression of both curli operons, however inactivation of *acrA* does not have an effect on biofilm formation or curli expression and the *acrA* mutant can biofilm to wild-type levels (Figure 7.1). Previous transcriptomic data from our laboratory has shown that inactivation of *tolC* or *acrB* results in greatly increased expression of *ramA* (Webber *et al.* 2009), however inactivation of *acrA* has no effect on levels of *ramA* (Bailey *et al.* 2010). Furthermore, transcriptomic data for an *S*. Typhimurium wild-type carrying a plasmid that results in over-expression of *ramA* showed that curli expression was down regulated in response to over expression of *ramA* (Bailey *et al.* 2010).

Figure 7.1 Crystal violet biofilm assay and Congo red morphologies of L828 (wild-type), L829 (*tolC::cat*), L830 (*acrB::aph*) and L1271 (*acrA::aph*). Statistically significant differences in biofilm formation in comparison to L828 (wild-type) are indicated by an asterisk ($p<0.05$).



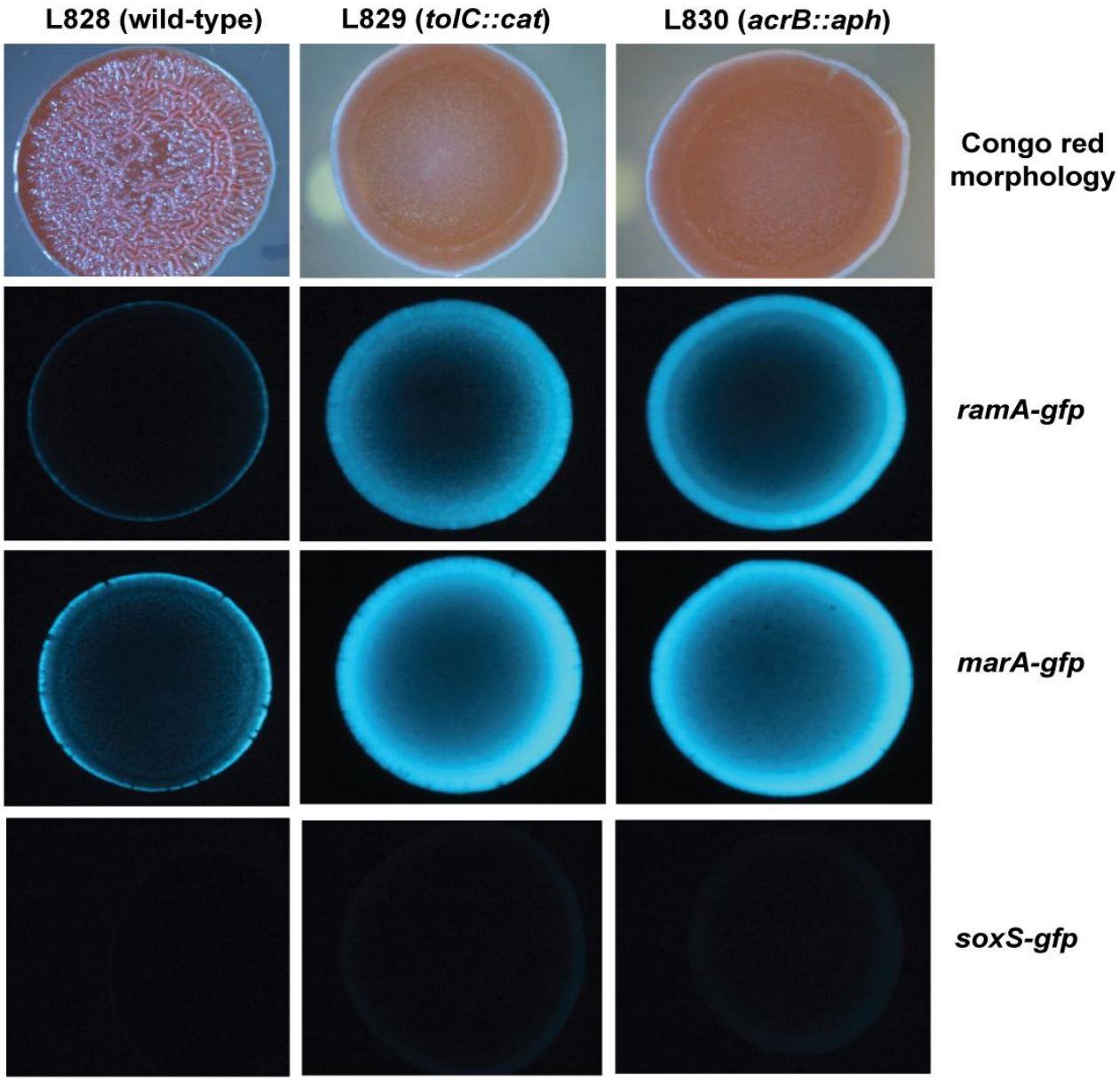
7.3 Spatial expression of *ramA*, *marA* and *soxS* inversely correlates to curli expression

To visualise the expression of *ramA*, *marA* and *soxS*, the promoter region of each gene was cloned into the *gfp* reporter plasmid pMW82. The construct was then electroporated into L828 (wild-type), L829 (*tolC::cat*) and L830 (*acrB::aph*). The strains harbouring these plasmids were plated onto Congo red plates so curli expression could be visualised as well as *ramA*, *marA* and *soxS* expression using fluorescence microscopy.

The inverse correlation between *ramA*/*marA* expression and curli expression is visually quite striking (Figure 7.2). The leading edge of the colony is not expressing curli, shown by the pink and smooth morphology, presumably as the outer most part of the colony is still growing and therefore not reached stationary phase yet which is when curli is expression is most apparent. The centre of the colony presents with a red and rough morphology indicating curli expression. The corresponding wild-type colonies harbouring *ramA* and *marA* *gfp* reporter plamsids under fluorescence microscopy show very high expression of both *ramA* and *marA* at the leading edge of the colony where curli is not expressed and much less throughout the colony where curli is expressed. Again this inverse correlation is seen in the both efflux mutants, Congo red morphology is pink and smooth throughout the colony, indicated absence of curli, which again correlated with high levels of *ramA* and *marA* expression seen across the whole colony.

The correlation between absence of curli and high *soxS* expression is less obvious, perhaps because *soxS* is not expressed at the same kind of levels as *ramA* and *marA*.

Figure 7.2 Congo red morphologies and fluorescence images of L828 (wild-type), L829 (*tolC::cat*) and L830 (*acrB::aph*) containing *gfp* reporter plasmids for *ramA*, *marA* and *soxS*.



However, there is still an increase in *soxS* expression in both L829 (*tolC::cat*) and L830 (*acrB::aph*) throughout the colony, but the ring of fluorescence indicated high expression at the leading edge of the colony seen with *ramA* and *marA* expression is absent.

7.4 Addition of efflux inhibitor increases *ramA* expression and decreases biofilm formation in wild-type *S*. Typhimurium

ramA can be induced in a dose dependent manner using the efflux inhibitor, PAβN. PAβN is a competitive inhibitor of RND efflux pumps, and has a particularly strong affinity for AcrB (Lomovskaya *et al.* 2001), thus triggering *ramA*, one of the global regulators of *acrB,* to become up regulated. Upon increasing concentrations of PAβN, *ramA* expression increased in a dose dependent manner (Figure 7.3). There is again a striking inverse correlation with biofilm formation, as *ramA* begins to be up regulated, biofilm formation decreases and again upon increasing concentrations of PAβN decreases in a dose dependent manner.

7.5 Artificial over-expression of the global regulators; *ramA*, *marA* and *soxS* represses curli expression

Given the link seen in the transcriptomic data between inactivation of *tolC* or *acrB*, subsequent over-expression of *ramA* and further repression of curli genes and up regulation of regulators in association with efflux inhibitor exposure, I investigated the impact of over expression of *ramA*, *marA* and *soxS* on biofilm formation by wild-type *S*. Typhimurium. The pTrc expression vector was used to over-express *ramA*, *marA* or *soxS* in wild-type *S*. Typhimurium. Over-expression of all three regulators decreased biofilm formation significantly in a static crystal violet biofilm assay upon induction with 1 mM IPTG (Figure 7.4)

Figure 7.3 Crystal violet biofilm assay of L828 (wild-type) (blue bars) and *ramA* induction assay upon addition of increasing concentrations of the efflux inhibitor, PAβN (red line). Statistically significant decreases in biofilm formation and increases gene expression in comparison to absence of PAβN are indicated by a blue and red asterisk respectively ($p<0.05$).

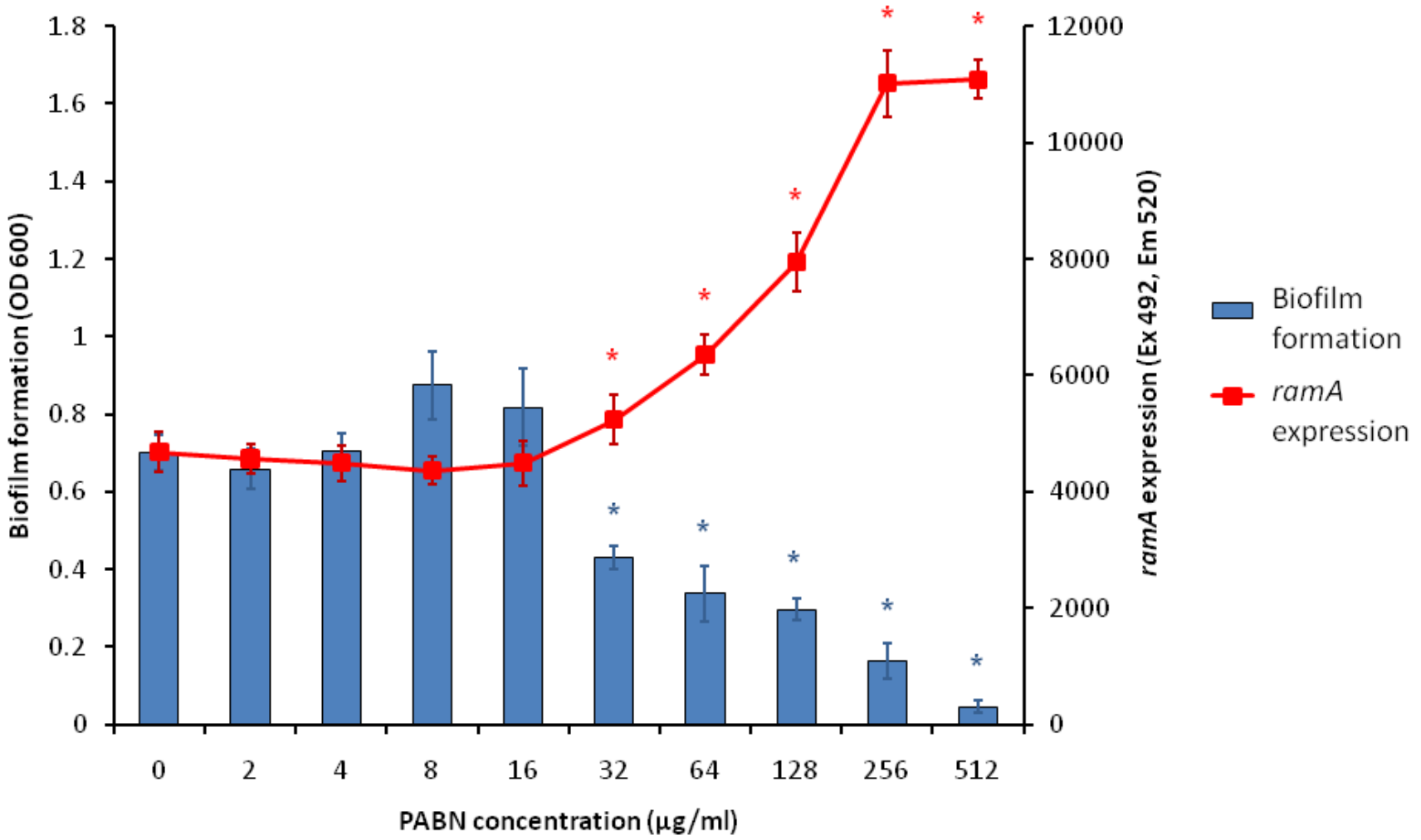
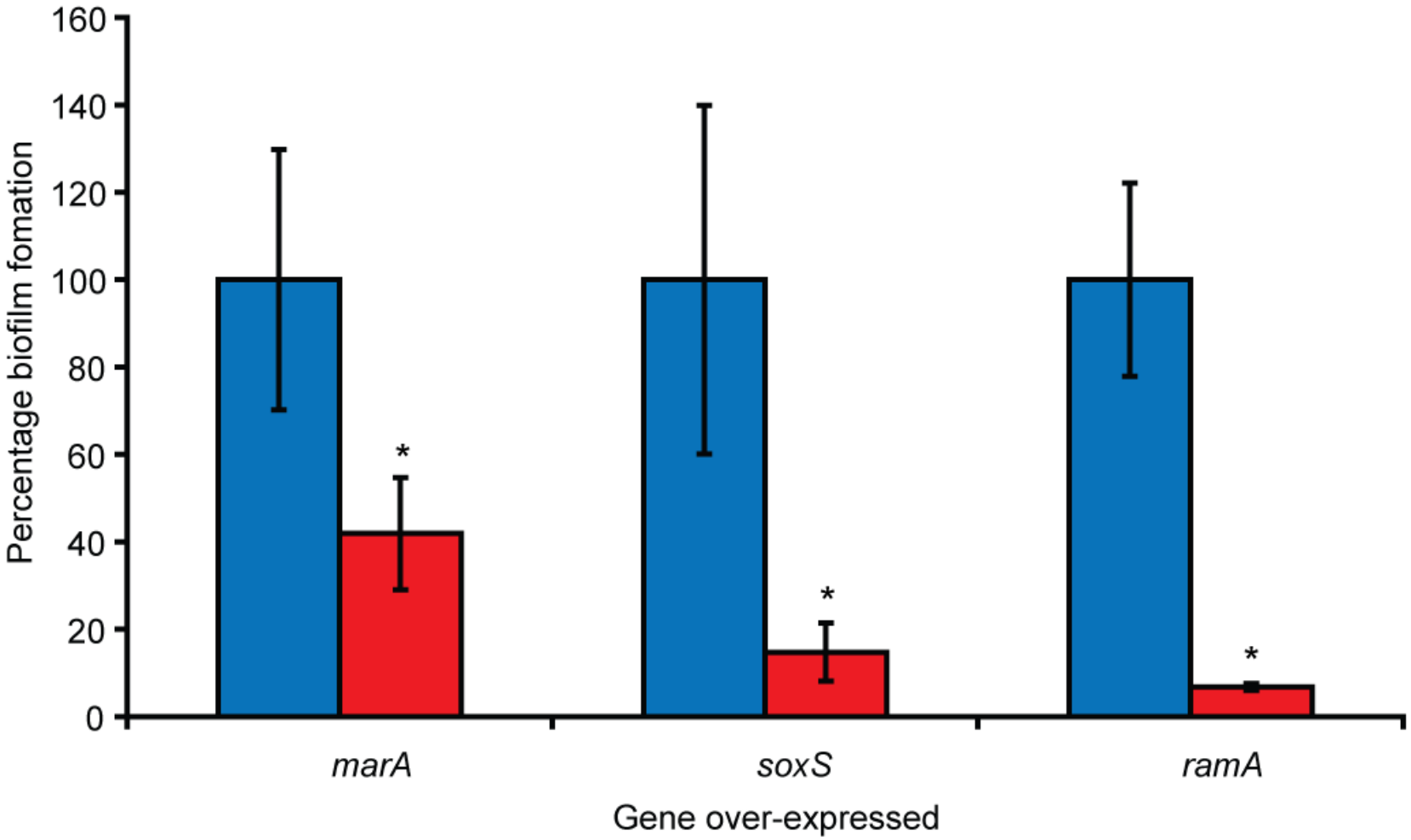


Figure 7.4 Crystal violet biofilm assay of L828 (wild-type) harbouring pTrc-*marA*, pTrc-*soxS* or pTrc-*ramA*; both uninduced (blue bars) and upon induction with 1mM IPTG (red bars). Statistically significant differences in biofilm formation of strains with induced regulators in comparison to uninduced are indicated by an asterisk ($p<0.05$).



Furthermore, over-expression of these three regulators also induces a curli defect supporting the transcriptomic data of the *ramA* over expressing wild-type and supporting the idea that the regulators prevent biofilm formation by repression curli expression.

7.6 Inactivation of global regulators in a *tolC* mutant does not rescue biofilm formation

To investigate whether over-expression of *ramA*, *marA* or *soxS* is the mechanism by which loss of efflux activity results in curli repression, various combinations of regulator inactivation mutations were transduced into L829 (*tolC::cat*) (the mutant with the most significant biofilm defect) and biofilm formation quantified to establish if the biofilm defect of the *tolC* mutant can be rescued. Inactivation of either *ramA* or *marA* alone evoked no defect in biofilm formation, inactivation of *marA* had a slight but significant increase in biofilm formation in *S*. Typhimurium 14028S (Figure 7.5). However, inactivation of *soxS* resulted in a significant decrease in biofilm formation. Inactivating multiple regulators in the same strain had no effect on biofilm formation apart from in the *marA/soxS* double mutant where biofilm formation was slightly yet significantly decreased. When inactivating these same regulators in the *tolC::cat* background it again had no impact on biofilm formation (Figure 7.6). Deletion of multiple global regulators was required as there is functional redundancy between them and if one is inactivated the other two are over-expressed in response (Figure 7.7). All mutation combinations tested were again not able to rescue the biofilm defect of the *tolC* mutant (Figure 7.6).

Figure 7.5 Biofilm formation of L828 (wild-type) and single, double and triple inactivation mutations in *ramA*, *marA* and *soxS*. Statistically significant differences in biofilm formation in comparison to L828 (wild-type) are indicated by an asterisk ($p<0.05$).

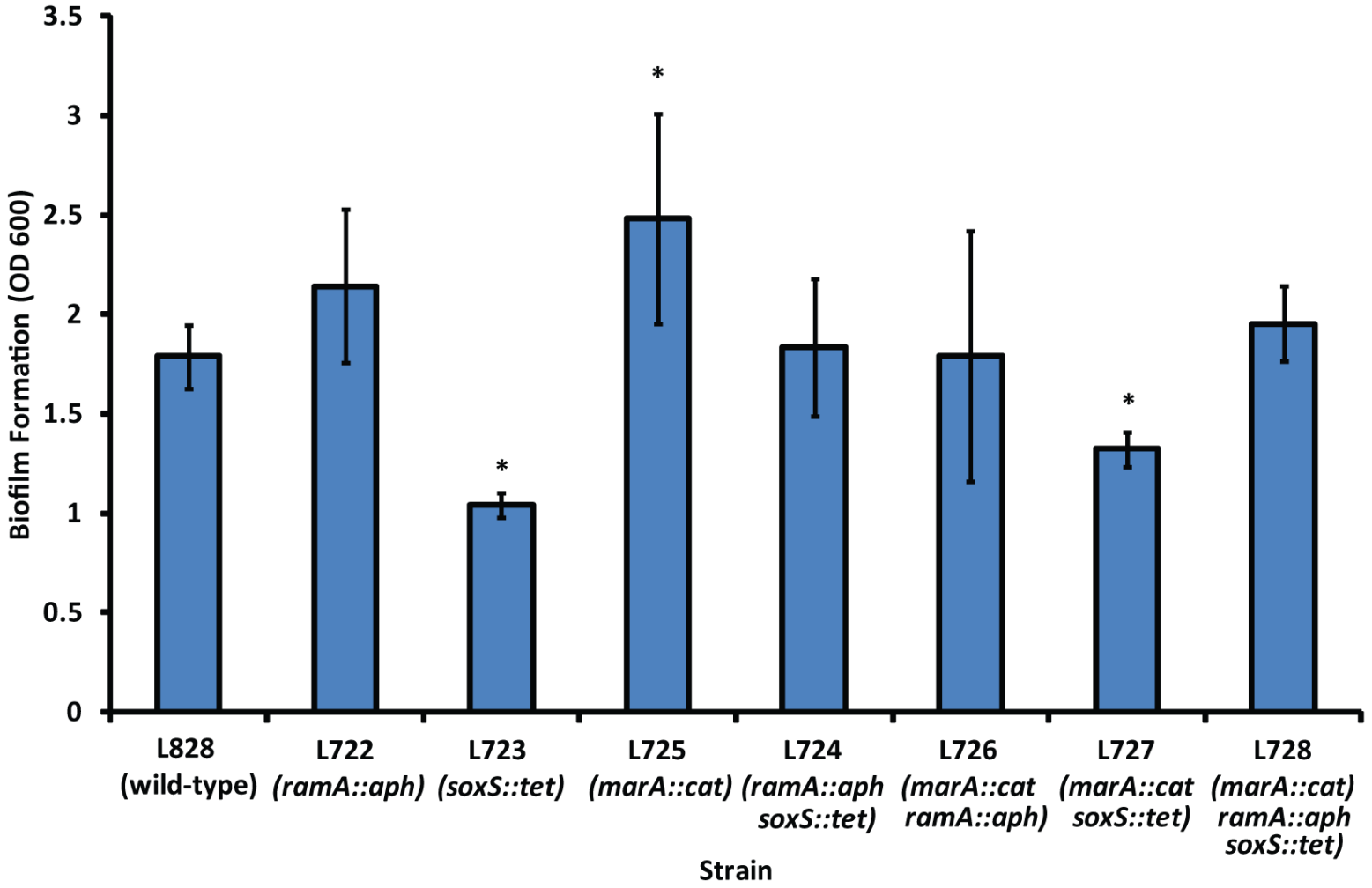


Figure 7.6 Crystal violet biofilm assay of wild-type *S*. Typhimurium and mutants lacking various combinations of *tolC*, *ramA*, *marA* and *soxS*. Statistically significant differences in biofilm formation in comparison to L828 (wild-type) are indicated by an asterisk ($p<0.05$).

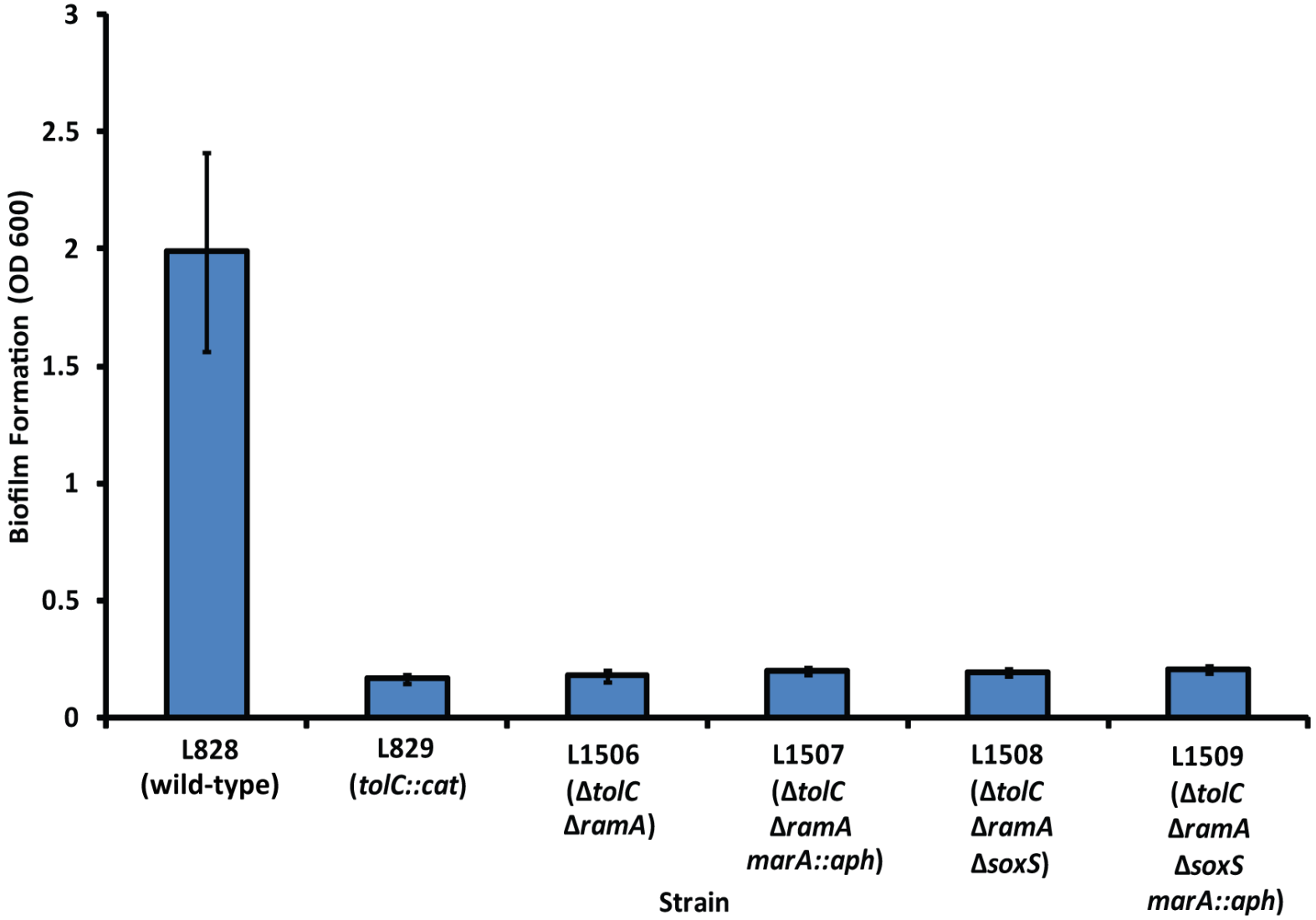
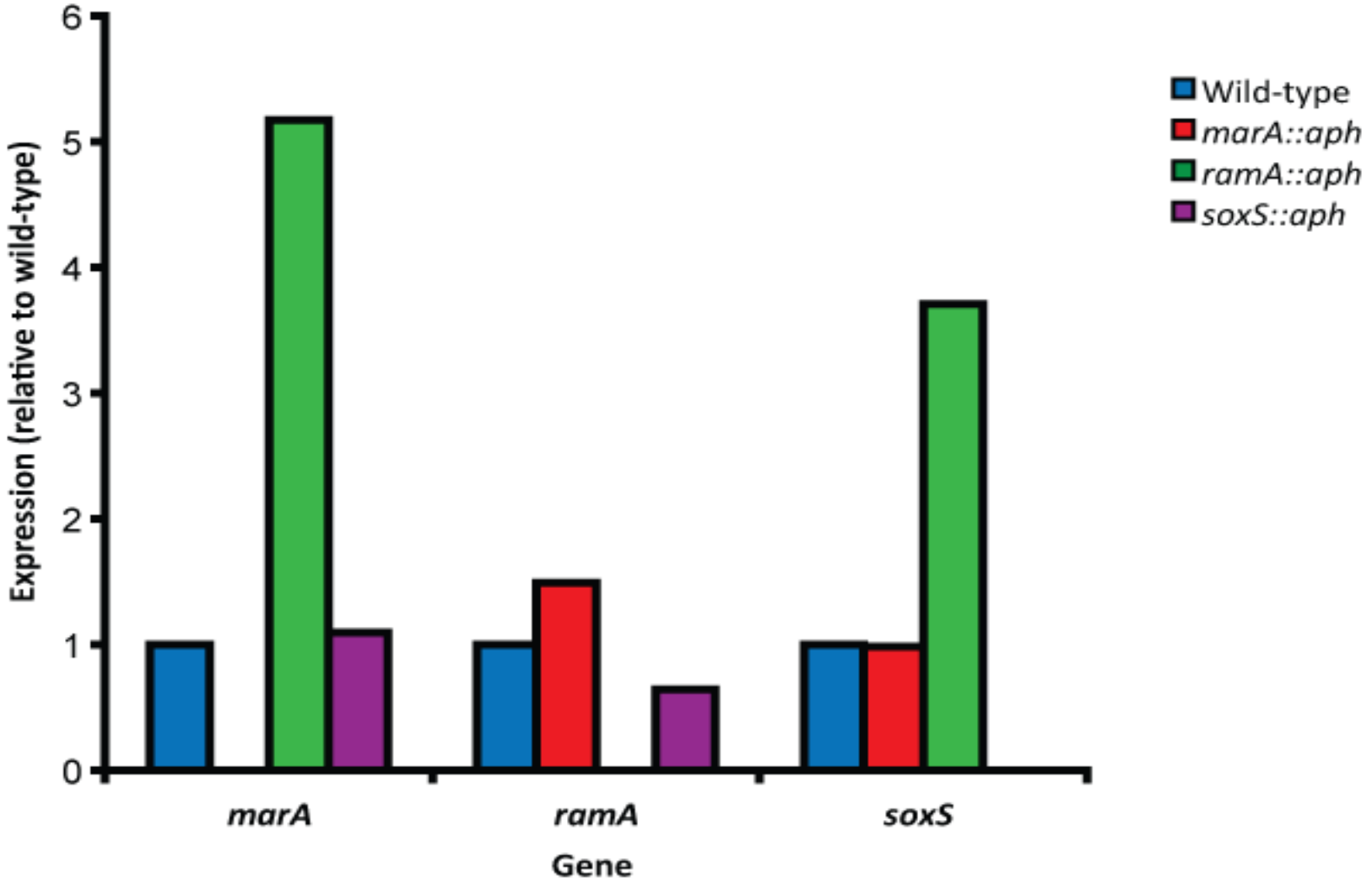


Figure 7.7 Compensatory gene expression of global regulators of AcrAB-TolC upon inactivation of a single regulator showing functional redundancy of *ramA*, *marA* and *soxS* in *S*. Typhimurium (courtesy of Amelia Lawler).



7.7 Discussion

The global regulators of AcrAB-TolC, *ramA*, *marA* and *soxS* can all negatively regulate curli expression and therefore biofilm formation revealing a novel role for these proteins. The mechanism for this is likely to be repression occurring either directly or indirectly via *csgD* as this is the major biofilm regulator in *Salmonella*. These three regulators are known transcriptional activators and increase expression by binding upstream of the target gene to allow for more efficient transcription, they are not known to directly repress any members of their regulons therefore the negative regulation of curli is likely to be indirect. There are also no obvious *mar*/*sox*/*ram* boxes (the sequence where these regulators bind) around the *csg* operons. Efflux inhibitors, such as PAβN used in the assay in this chapter, can also induce these regulators as well as repress biofilm formation, and the dose dependent correlation between induction of *ramA* and repression of biofilm formation is striking. It is currently unclear how these regulators influence curli expression and no known genes belonging to all these regulons is known to repress curli. However, inactivation of these regulators in a *tolC* mutant does not rescue the ability to form a biofilm. As there is redundancy between the three regulators and over expression of each regulator leads to a biofilm defect all three genes were inactivated. This did not rescue efflux but this may be as inactivation of four separate genes may have a negative effect on the physiology of the cell.

Following this very specific approach to try and rescue efflux mutants' ability to form a biofilm, a broader, more unbiased approach needed to be taken to identify regulators blocking biofilm formation. Therefore a random transposon library of the mutants was created and screened for restoration of curli expression.

CHAPTER EIGHT:

TRANSPOSON MUTAGENESIS TO RESCUE BIOFILM DEFECT OF EFFLUX MUTANTS

8 Transposon mutagenesis to rescue biofilm defect of efflux mutants

8.1 Background

As inactivation of *ramA*, *marA*, *soxS* or combinations thereof did not rescue curli repression and biofilm defect in the *tolC* mutant, a literature search was carried out to identify all genes known to regulate curli expression in *Salmonella* and *E. coli* (Figure 8.1). However, this literature search highlighted that curli regulation is highly complex and involves many different genes, proteins, small RNAs and signalling molecules which can act to promote or repress curli expression. Therefore a generalised, unbiased approach was required to identify genes responsible for curli repression in efflux mutants. If the curli repression was mediated via a gene or protein that is over-expressed in the efflux mutants then a transposon screen should identify the gene(s) or protein(s) involved.

8.2 Transposon mutagenesis

A literature search showed no previously established method for using a transposon library to screen for changes in curli phenotype, therefore a method needed to be established (Figure 8.2). The EZ-Tn5™ <KAN-2> and EZ-Tn5™<DHFR-1> Insertion Kits (Epicentre® Biotechnologies – Madison, U.S.) were used to randomly insert a transposon around the genome giving rise to either a library of kanamycin resistant (EZ-Tn5™ <KAN-2>) or trimethoprim resistant mutants (EZ-Tn5™<DHFR-1>). Electrocompetent cells (as in section 2.4) of efflux mutants of interest were prepared by glycerol re-suspension on ice to ensure a high electroporation efficiency and insertion of the transposon introduced into the bacterial cell by electroporation (as In section 2.2).

Figure 8.1 Complex regulation of curli operons using literature for both *Salmonella* and *E. coli.* (Vidal *et al.* 1998; Adams and McLean 1999; Brown *et al.* 2001; Batchelor *et al.* 2005; Jonas *et al.* 2007; Troxell *et al.* 2007; Lee *et al.* 2009; Sommerfeldt *et al.* 2009; Holmqvist *et al.* 2010; Tagliabue *et al.* 2010; Monteiro *et al.* 2011).

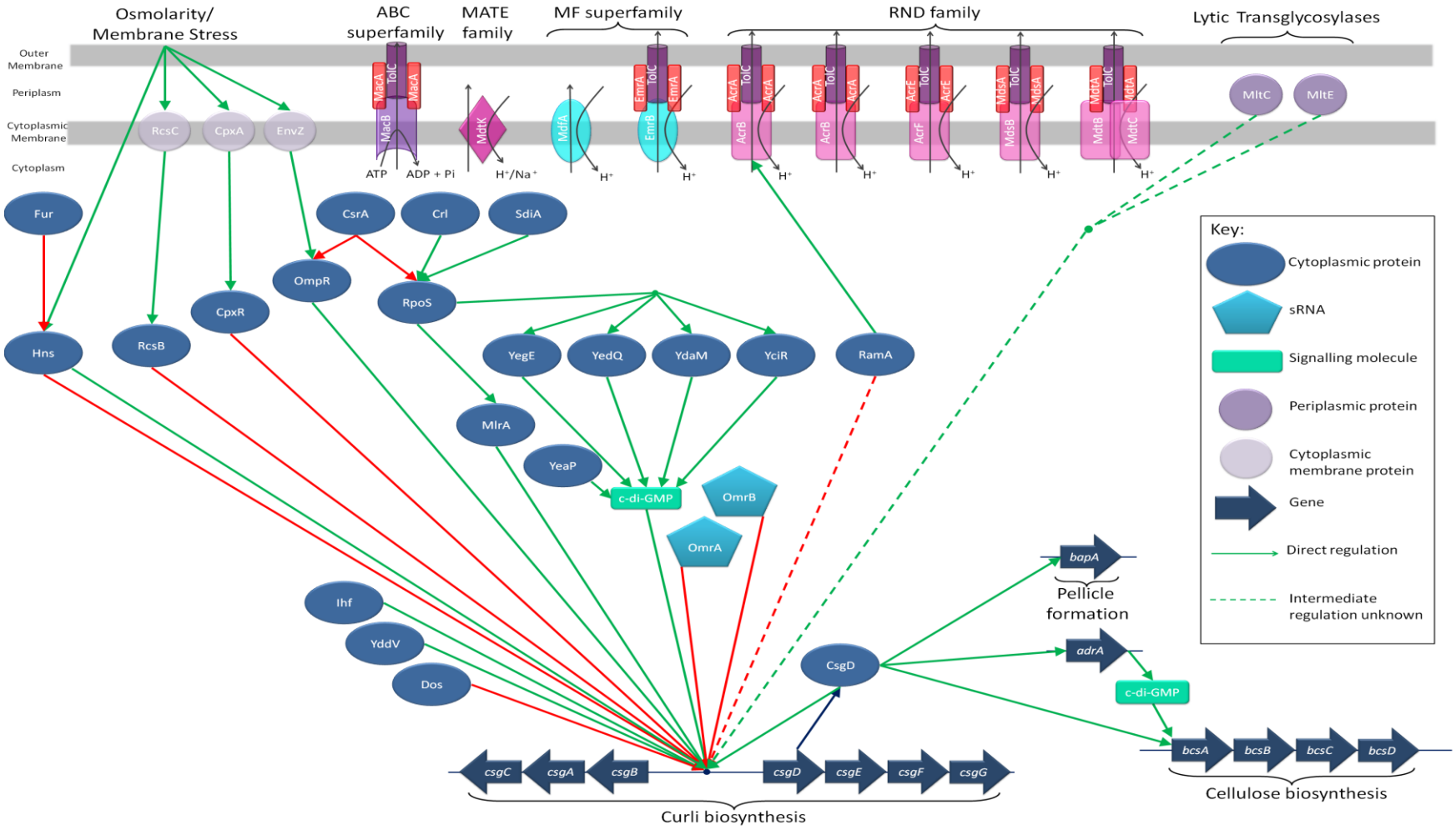
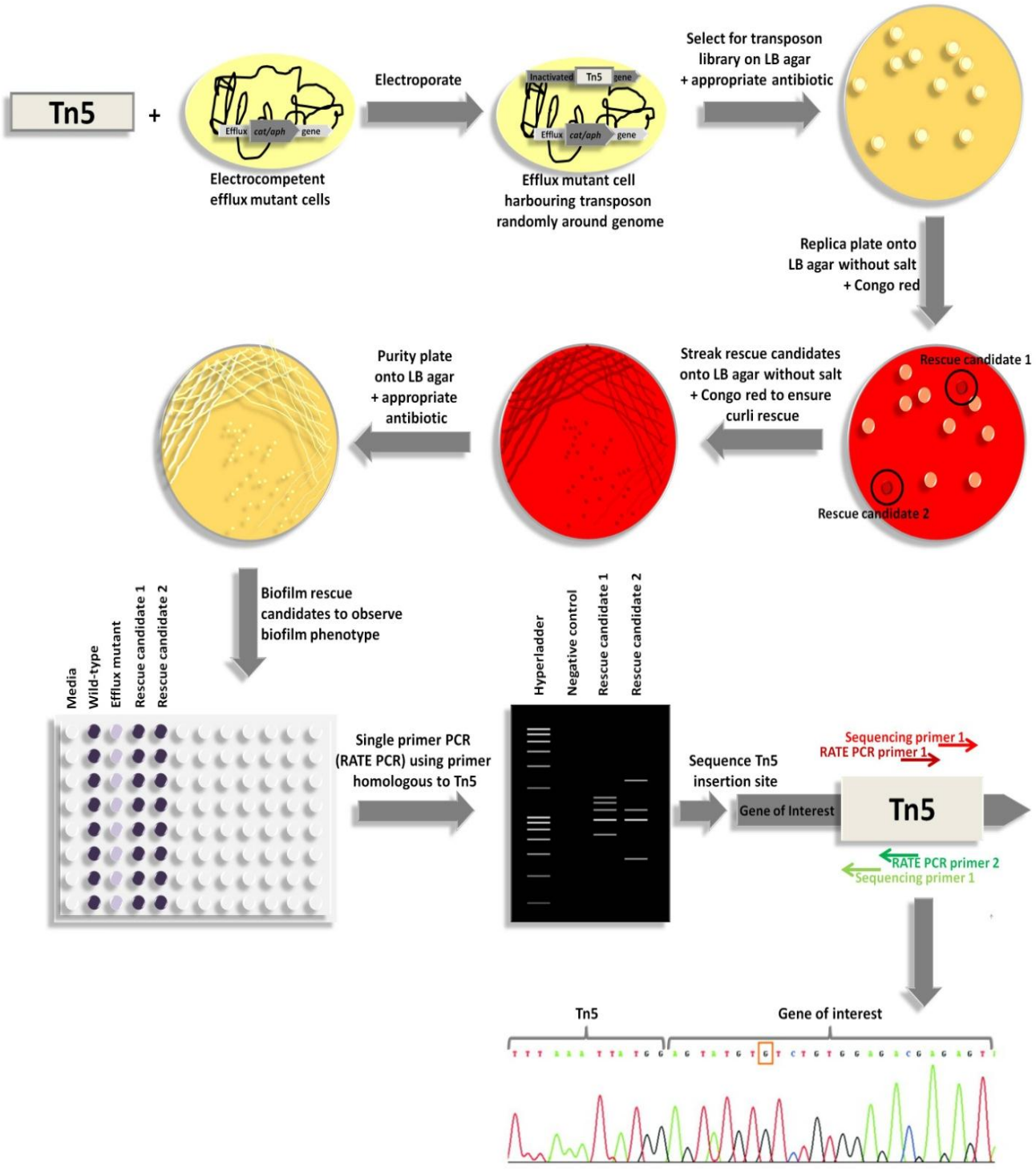


Figure 8.2 Diagram of method devised for transposon insertion, screening and sequencing.



Libraries were then plated onto LB agar supplemented with the appropriate antibiotic. Drug free control plates were also used to assess the total number if viable cells. All colonies were harvested and stored at -20°C in 50% glyercol. For screening, each transposon library was diluted to around 1000 cfu/ml and 100 μl aliquots spread onto LB agar plates supplemented with the appropriate antibiotic to ensure all colonies obtained were harbouring the transposon within their genome. Colonies obtained were replica plated onto LB agar without salt supplemented with 40 μg/ml of Congo red (Figure 8.3). As curli positive/negative phenotypes are easily distinguishable on Congo red plates, any rescue of curli could be detected by the morphology of the colonies; red, dry and rough for curli positive and pink and smooth for curli negative. L828 (wild-type) was used as a curli positive control and L829 (*tolC::cat*) was used as a curli negative control. Any potential curli rescue mutants were re-streaked onto Congo red to ensure a curli positive phenotype (Figure 8.4) and subsequently purity plated onto LB supplemented with the appropriate antibiotic.

The rescue candidates then underwent genotypic characterisation. Firstly, the original efflux mutation that the strain was harbouring was checked by PCR to ensure the strain is correct. Secondly, the transposon insert site needed to be identified to establish the insert site. The EZ-Tn5™ <KAN-2> and EZ-Tn5™<DHFR-1>; Insertion kits claim Sanger sequencing can be successful directly from a genomic DNA preparation as a template. However this method failed to work reliably with the candidates in this study with only one attempt being successful. As an alternative method for sequencing transposon insertion sites named RATE (Rapid Amplification of Transposon Ends) that uses a PCR amplimer as the sequencing template (Karlyshev et al. 2000).

Figure 8.3 Replica plate of L829 (*tolC::cat*) transposon library onto Congo red agar to distinguish between different curli phenotypes.

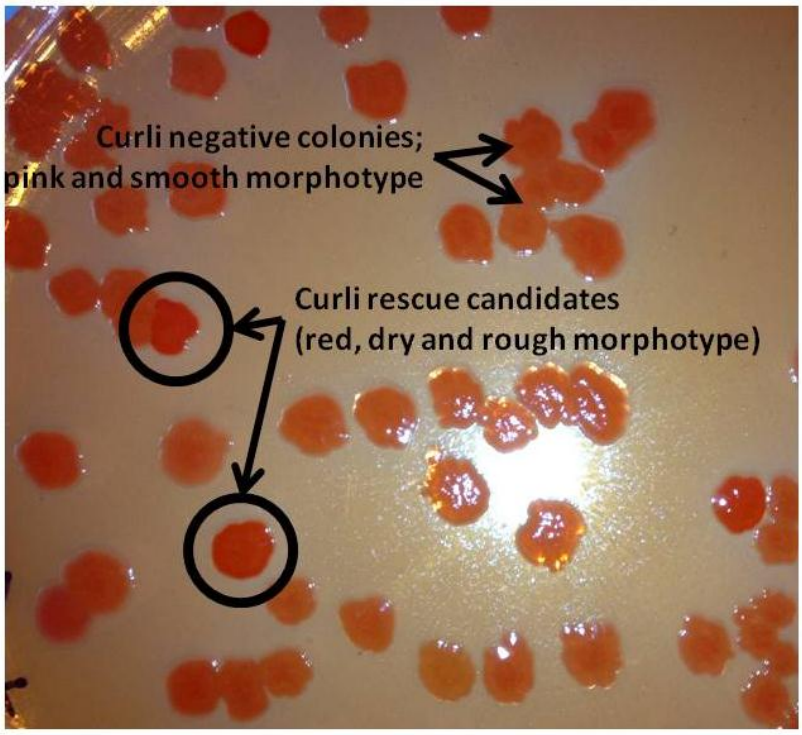
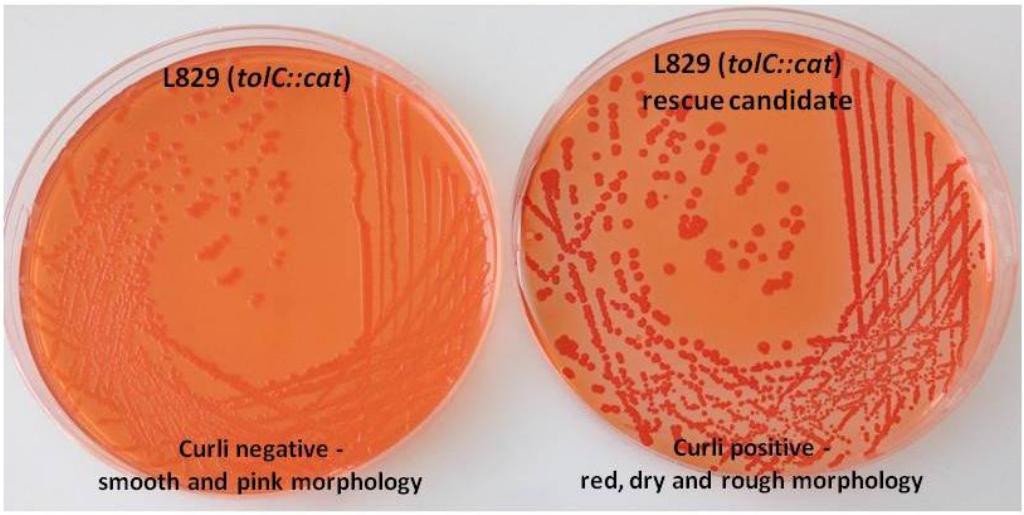


Figure 8.4 Congo red agar streak plates of a curli negative strain, L829 (*tolC::cat*), and a transpsoson mutant where curli expression has been restored.



RATE PCR uses a single primer homologous to the transposon and a non-specific PCR to create both the specific and non-specific amplimers (Figure 8.5). The initial amplimers were then sequenced with a second, transposon specific nested primer to ensure sequencing of the specific RATE amplimers only. Sequences were compared against the transposon sequence and the junction identified. Insert sites were identified by comparing the chromosomal sequence against the *S*. Typhimurium 14028S genome using the BLAST algorithm. Specific primers flanking the genes identified from sequencing were designed and specific PCRs used as another check to ensure the transposon has been inserted within that gene (Figure 8.6).

8.3 Inactivation of various genes rescues curli/biofilm defect in efflux mutants

A candidate efflux mutant representing each class of efflux pump were chosen to determine if curli rescue could be achieved by inactivation of genes with the Tn5 transposon. The mutants chosen were; L829 (*tolC::cat*) – outer membrane porin, L830 (*acrB::aph*) – RND transporter, L836 (*emrAB::cat*) – MFS transporter and L838 (*mdtK::cat*) – MATE transporter. Transposon libraries were successfully created and colonies screened for each strain were approximately; 4000 colonies (15 curli positive) for L829 (*tolC::cat*), 3500 colonies (five curli positive) for L830 (*acrB::aph*), 3000 colonies (seven curli positive) for L836 (*emrAB::cat*) and 3700 colonies (six curli positive) for L838 (*mdtK::aph*). Some tranpososon insertions may be intergenic therefore not inactivating any gene, some genes are unable to be viably inactivation as they are essential and there is a possibility that the same gene has the transposon inserted into the same gene multiple times.

Figure 8.5 RATE PCR amplimers of various strains harbouring Tn5 were separated alongside Hyperladder I (Bioline) on a 1% agarose gel at 100 V for 1 hour.

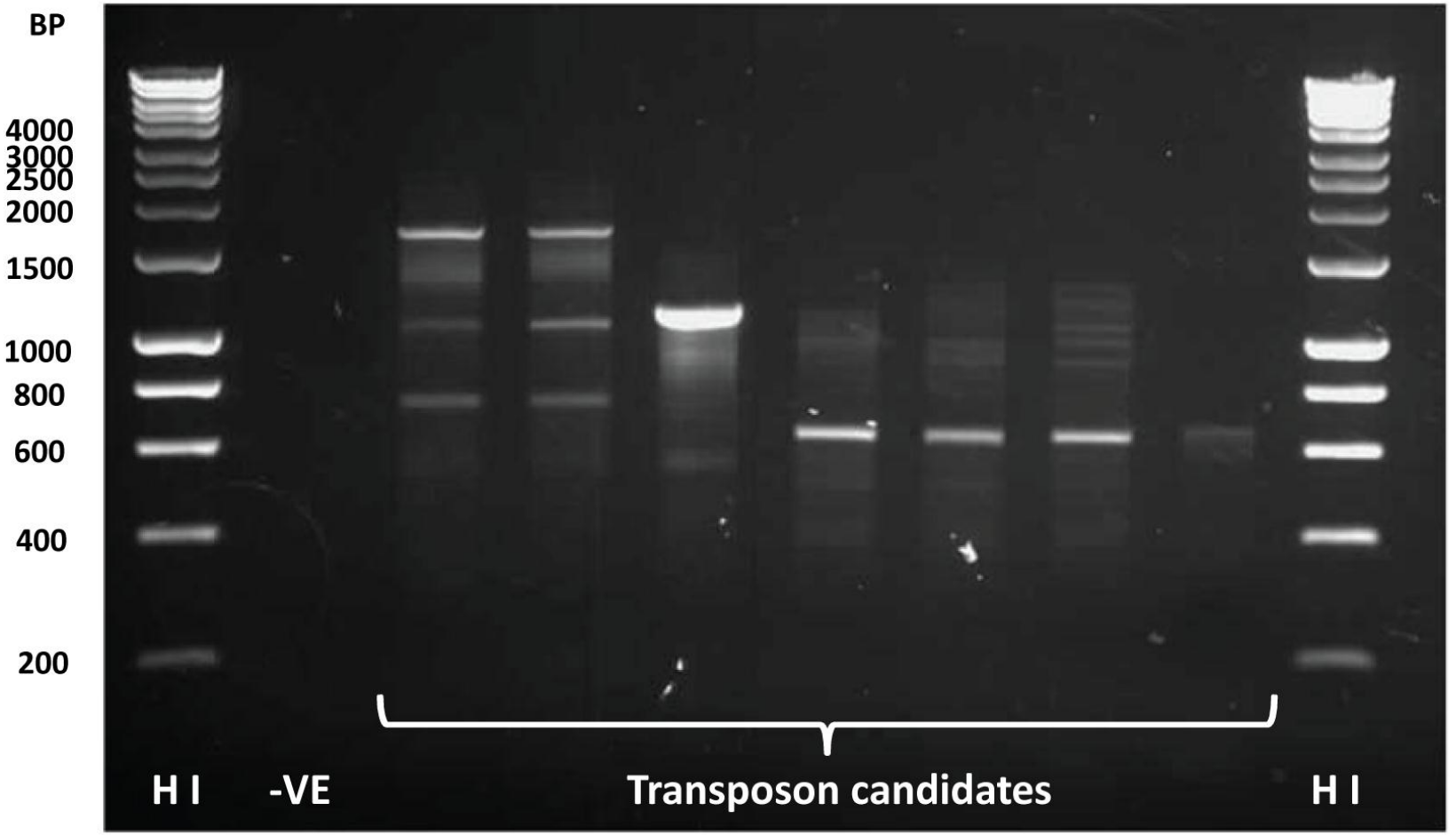
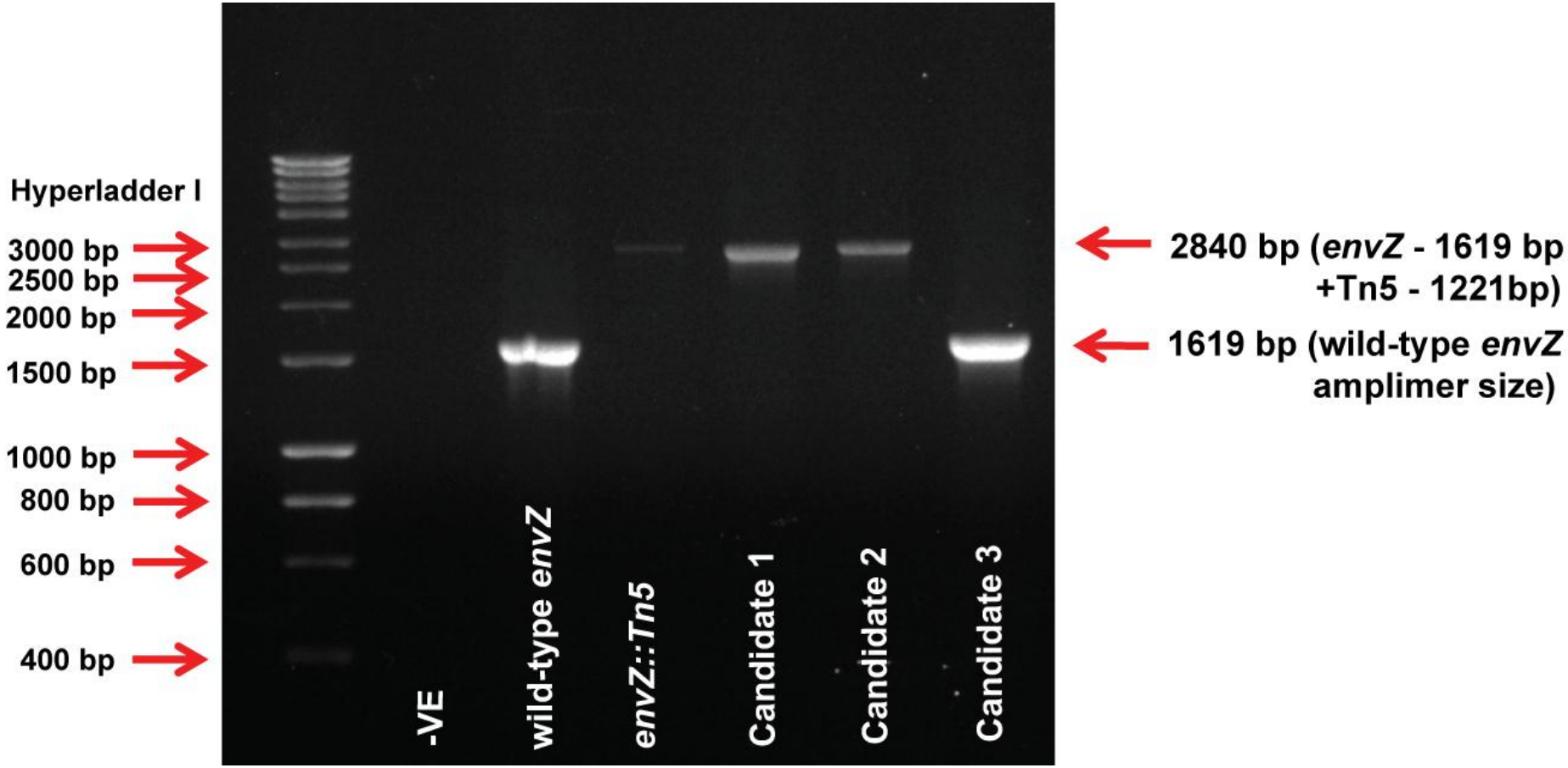


Figure 8.6 PCR using *envZ* flanking primers and electrophoresed on a 1% agarose gel for 1 hour at 100 V revealing two out of the three curli positive candidates obtained in the second transposon screen as *envZ*::Tn5 mutants.



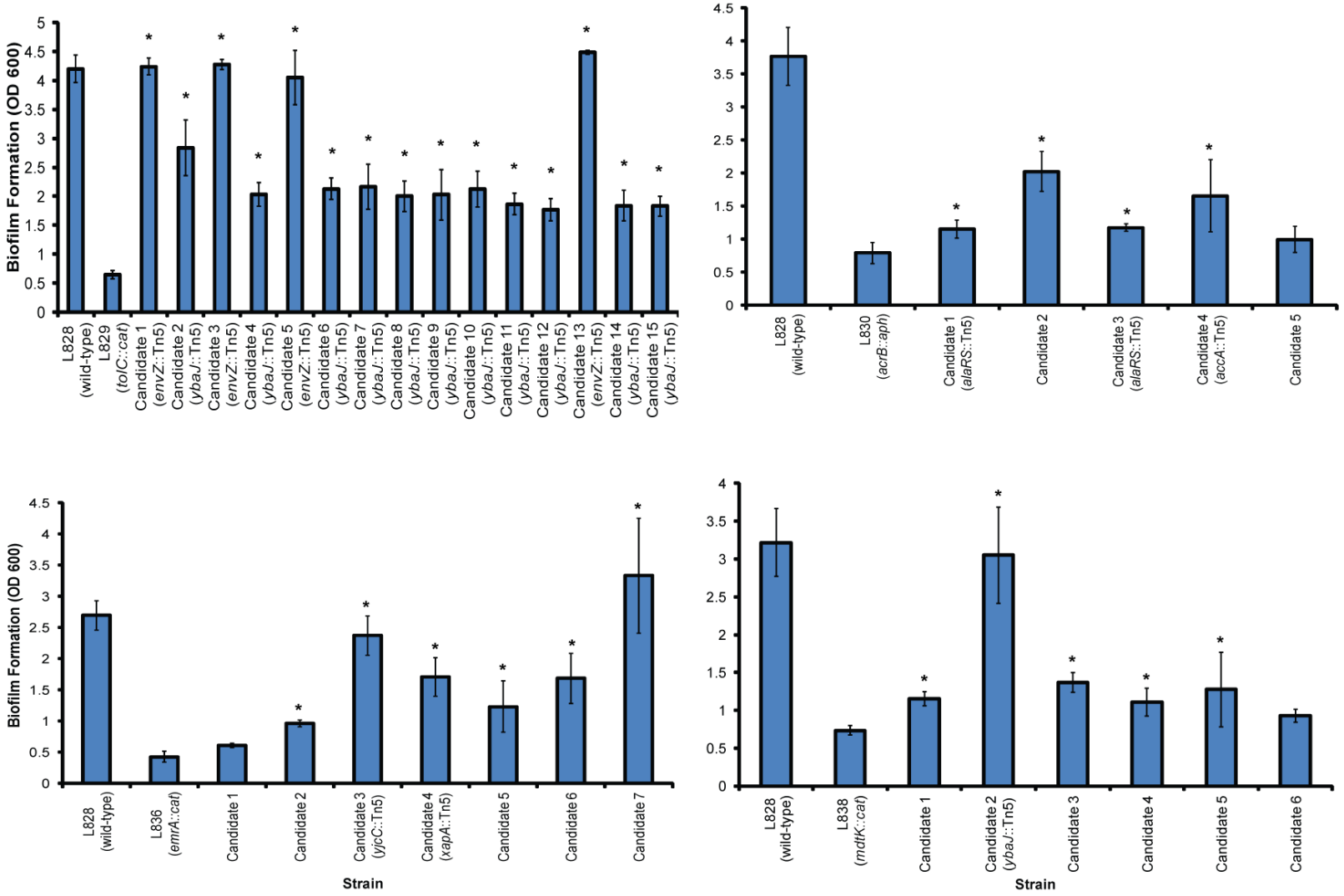
For full coverage of the *S*. Typhimurium genome, containing around 4,500 genes, it would be ideal to screen around 10,000 colonies. Crystal violet biofilm assays were carried out for all curli positive candidates to observe their biofilm phenotype in a static laboratory model (Figure 8.7). Three out of the four selected efflux mutants had a fully rescued curli and biofilm phenotype; L829 (*tolC::cat*), L836 (*emrAB::cat*) and L838 (*mdtK::cat*). The insert sites that brought about full curli and biofilm rescue were all within the genes *envZ* (in L829 (*tolC::cat*)), *yjcC* (in L836 (*emrAB::cat*)) and *ybaJ* (in L838 (*mdtK::cat*)).

Other genes identified as having a positive effect on the efflux mutants ability to express curli and form a biofilm are *alaRS* (in L830 (*acrB::aph*)), *accA* (in L830 (*acrB::aph*)) and *xapA* (in L836 (*emrAB::cat*)).

8.4 Discussion

Successful transposon libraries were created for four efflux mutants harbouring mutations in efflux genes belonging to varying efflux families. Also a high throughput screen for curli rescue has been developed that allowed us to identify curli phenotypes of thousands of colonies at once. The Congo red agar plates used to assess the curli phenotype of the strains was highly successful and curli rescue candidates were isolated for each efflux mutant screened. The curli positive candidates did for the most part correlated with an increase in biofilm formation in relation to the parent strain, however not all candidates biofilm ability rescued to wild-type levels. The biofilm defect phenotype seen in the efflux mutants was rescued in all but one, L830 (*acrB::aph*). In the three remaining mutants the biofilm and curli phenotypes were rescued fully to wild-type levels by transposon insertion.

Figure 8.7 Crystal violet biofilm formation assays of L829 (*tolC::cat*), L830 (*acrB::aph*), L836 (*emrAB::cat*) and L838 (*mdtK::cat*) transposon library curli positive strains. Location of the transposon is stated where known. Statistically significant differences in biofilm formation in comparison to parent strains are indicated by an asterisk ($p<0.05$).



The genes inactivated for this rescue to occur were different in all three cases, *envZ* to rescue *tolC*, *yjcC* to rescue *emrAB* and *ybaJ* to rescue *mdtK.* All the 'rescue' genes identified are not known to interact or be part of the same regulatory network suggesting that repression of curli in the different efflux mutants is mediated by different mechanisms, or that all these genes contribute to the same pathway in a currently unknown way.

yjcC is a diguanylate cyclase also known to be important in biofilm repression (Rouf *et al.* 2011), and when inactivated is known to enhance biofilm formation in *S.* Typhimuirum (Kim and Wei 2009). *yjcC* expression is dependent upon SoxS (Huang *et al.* 2013) which is a global regulator of AcrAB-TolC (Chapter 7) and upon artificial over-expression represses curli expression and induces a biofilm defect.

ybaJ is important in biofilm formation and control of aggregation and motility (Barrios *et al.* 2006). It is a suggested antitoxin in a toxin-antitoxin complex with Hha (Garcia-Contreras *et al.* 2008); an osmolarity- and temperature-dependent global modulator of toxin alpha-hemolysin synthesis (Mourino *et al.* 1996; Nieto *et al.* 2002) and *ybaJ* expression. It is located only 496 base pairs downstream of the *acrB*, the gene that encodes for the major efflux pump of *S*. Typhimuirum. In a parallel project this *ybaJ*::Tn5 mutation was transduced into the *acrB* mutant to see if this gene is responsible for the *acrB* mutant's biofilm defect. However, biofilm formation and curli expression of the *acrB* mutant was not rescued upon introduction of the *ybaJ* mutation. The positioning of *ybaJ* could be important in the intermediate rescue phenotype caused when inactivated *ybaJ* in the *tolC* mutant. Inactivation of *tolC* is known to induce expression of global regulators of AcrAB-TolC, which in turn induces

expression of *acrB*, this up-regulation of *acrB* could cause up-regulation of genes downstream of it due to transcriptional carry through. This mechanism would also work for the phenotype seen in the *ramA*, *marA* and *soxS* over expressing strains. This hypothesis would need to be further investigated to confirm or rule out its validity.

envZ is part of a two component system (*envZ/ompR*) that recognises environmental changes in pH and osmolarity and changes expression of many stress response genes as a result (Prigent-Combaret *et al.* 1999), *csgD* (master curli regulator) being one of them. As *envZ* rescues the ability of the *tolC* mutant to form a biofilm and was already on our list of genes known to control *csgD* (the master curli regulator) and is a membrane bound protein (therefore located in the same part of the cell as efflux proteins) we decided to investigate further into the potential link between efflux and biofilm formation and the contribution of membrane stress.

Other genes found to have a positive effect on the biofilm formation of the efflux mutants were accA, alaRS (inactivation increased biofilm formation of *acrB* mutant) and xapA (inactivation increased biofilm formation of *emrAB* mutant). Although these increases were significant they were only slight.

CHAPTER NINE:

BIOFILM/CURLI RESCUE OF *tolC* MUTANT VIA INACTIVATION OF *envZ*

9 Biofilm and curli rescue of *tolC* mutant via inactivation of *envZ*

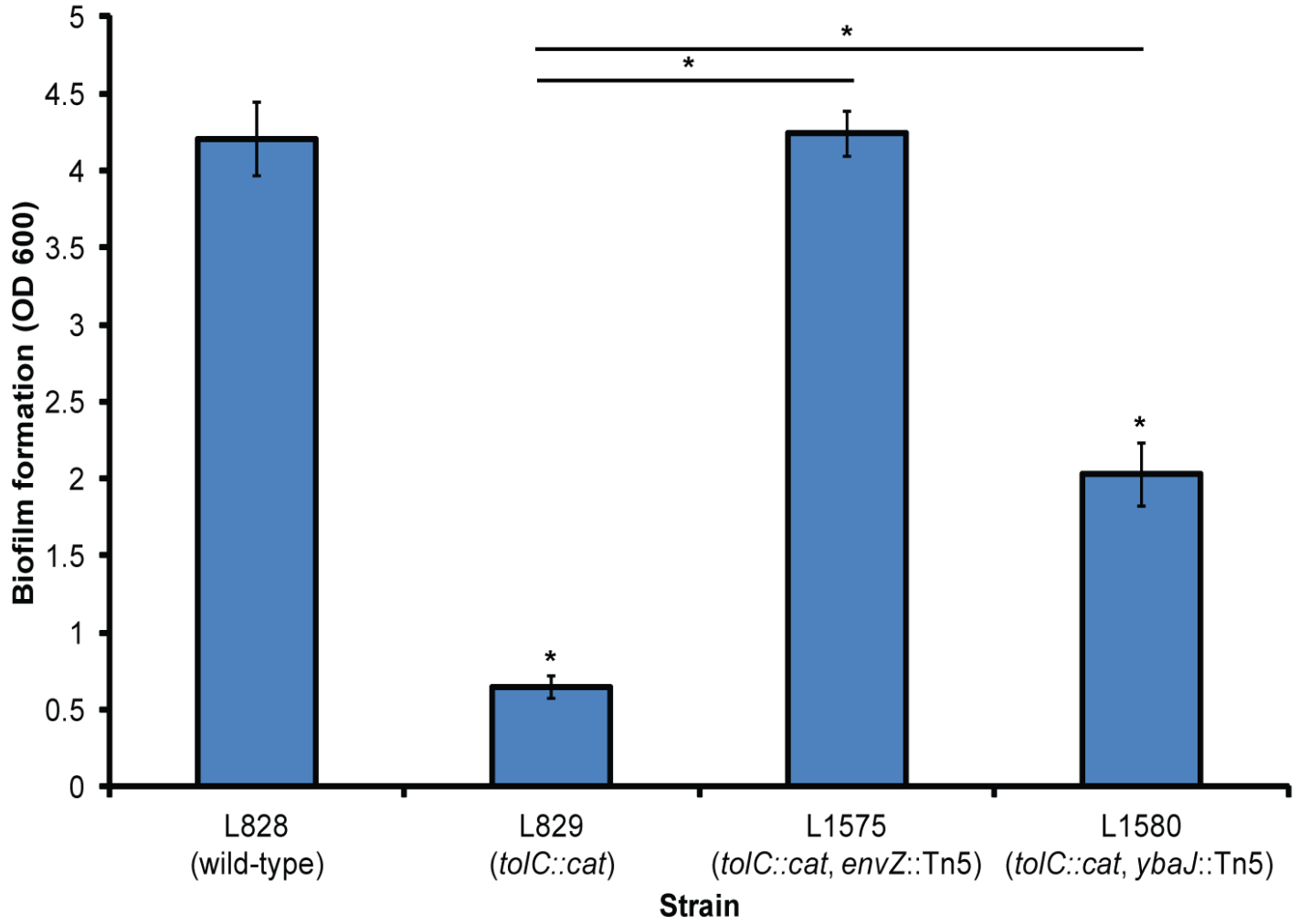
9.1 Background

Transposon screening of L829 (*tolC::cat*) identified that inactivation of *envZ*, one part of a two component regulatory system (with *ompR*), rescued the ability of the strain to express curli and subsequently form a biofilm. The EnvZ/OmpR two component system is important in regulating a response to membrane stress. EnvZ is an inner membrane bound protein and detects changes in the cells environment such as osmolarity and pH and relays this message to by phosporylating OmpR and subsequently promotes or represses expression of it's effector genes (Cai and Inouye 2002; Parkinson 2003). *ompC* and *ompF* are the two main effector genes, the expression of which depends upon the ratio of phosphorylated and unphosphorylated OmpR. A reduced level of phosphorylated OmpR favours *ompF* transcription and an elevated level of phosphorylated OmpR allows activation of *ompC* transcription and repression of *ompF* (Parkinson 2003).

9.2 L829 (*tolC::cat*) transposon screen reveals two groups of rescue mutants

An initial transposon library screen of over 4,000 L829 (*tolC::cat*) Tn5 colonies was carried out on Congo red plates exposing 15 curli positive colonies (as in Figure 8.3). All 15 colonies were purity plated and checked again for curli rescue of which all candidates possessed the red, dry and rough morphology in strains expressing curli. To observe if 'curli rescue' translated to 'biofilm rescue' a crystal violet biofilm assay was carried out for these strains and revealed two classes of rescue candidates; 'full rescue' and 'intermediate rescue' candidates (Figure 9.1). The 'full rescue' mutants were able to produce the same level of biofilm in the standard crystal violet biofilm assay as L828 (wild-type).

Figure 9.1 Crystal violet biofilm assay of L828 (wild-type), L829 (*tolC::cat*) and a representative of two different rescue candidates isolated from the transposon screen; the 'full rescue' *envZ*::Tn5 and 'partial rescue' *ybaJ*::Tn5 mutations. Statistically significant differences in biofilm formation are indicated by an asterisk in comparison to L828 (wild-type) or comparing the two bars below the ends of the black lines ($p<0.05$).



This suggests that the transposon mutagenesis procedure had released all repression of the curli operons seen in *tolC* mutant or over-expression of curli via an unrelated pathway had overridden the *tolC* mutant's curli repression. Sequencing of the transposon insertion site was carried out by RATE PCR (Figure 9.2) and Sanger sequencing. All four candidates exhibiting the 'full rescue' biofilm phenotype had the transposon insertion site in the same location. The transposon was inserted 935 base pains from the start codon of *envZ* (Figure 9.3) inhibiting translation of the last 200 amino acids of the 451 amino acid protein causing disruption of nearly half the gene. *envZ* is one part of a two component system involved in sensing membrane stress and known to regulate curli synthesis (Jubelin *et al.* 2005). The 'intermediate rescue' mutants all harboured the transposon between base pair 170 and 171 of a 375 base pair gene, *ybaJ*, a known biofilm regulator (Barrios *et al.* 2006). The inserted transposon was inserted within the 57th amino acid, inhibiting translation of 69 amino acids, over half of the proteins primary structure would be affected. The identical insert sites suggest clonal expression of the two separate events.

The *envZ*::Tn5 mutation was transduced using P22 phage into a 'clean' L829 (*tolC::cat*) background to observe the biofilm phenotype. However, this 're-transduced' strain, L1562 (*tolC::cat*, *envZ*::Tn5), was unable to form a biofilm as the strain isolated from the transposon library (Figure 9.4).

To determine if loss of *envZ* function could rescue other mutants the *envZ*::Tn5 mutation was transduced into L828 (wild-type) and the entire panel of efflux mutants.

Figure 9.2 Single primer RATE PCR used as a template for Sanger sequencing of the transposon insertion site, 5 µl of each reaction was run on a 1% agarose gel and along with DNA marker, Hyperladder I and electrophoresed at 100 V for 1 hour. The ladder pattern observed is a mixture of specific transposon fragments and non specific DNA fragments due to the PCR parameters used.

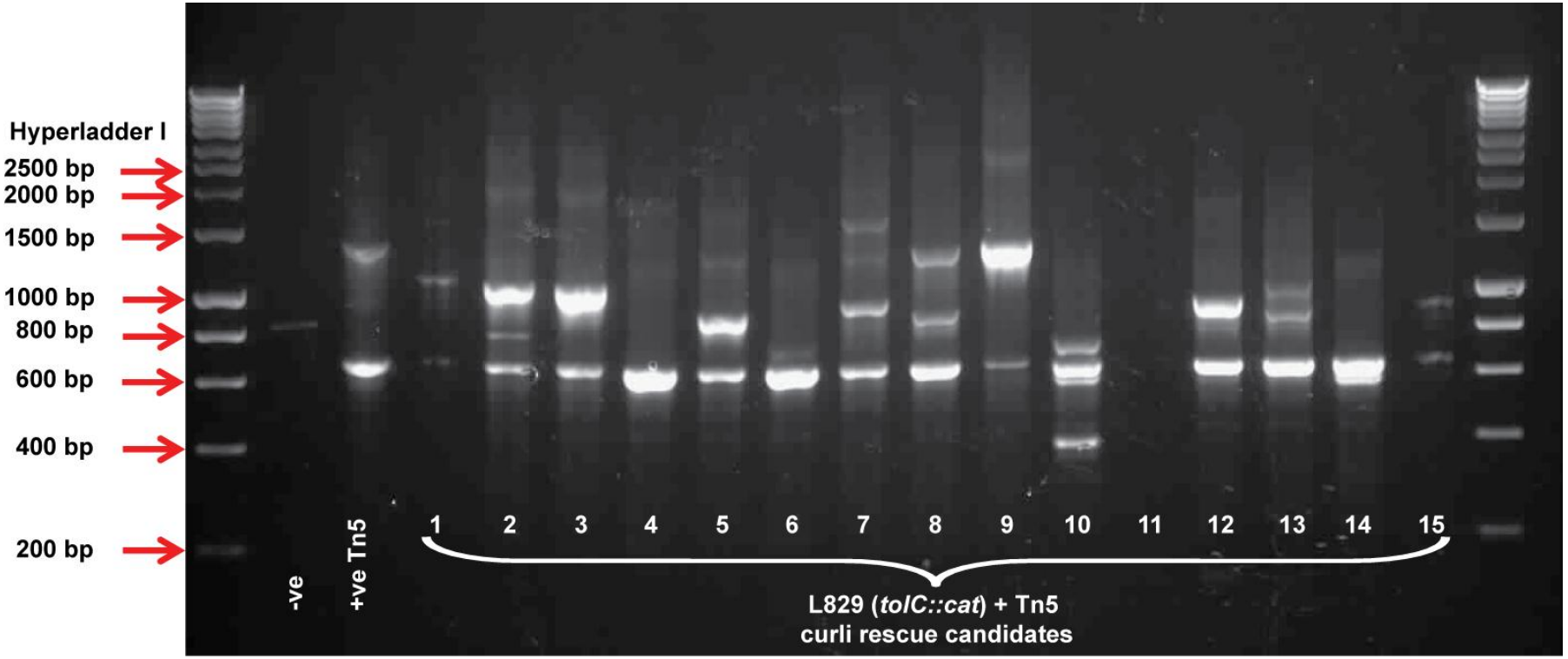
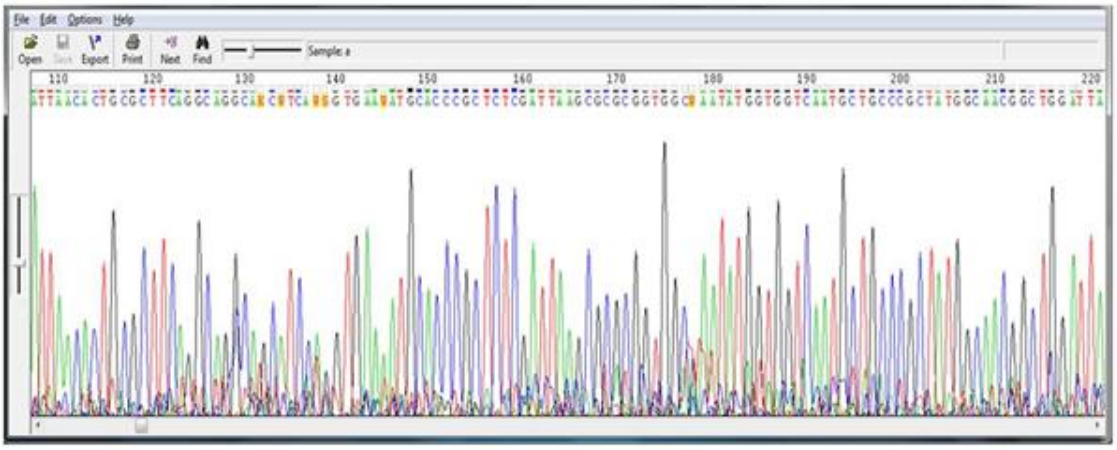


Figure 9.3 A Sanger sequencing output from Chromas of L829 (*tolC::cat*) transposon mutant candidate 3. **B** Sanger sequencing read out of candidate 3 transposon insertion site showing regions of homology with both Tn5 transposon sequence and *envZ* and **C** A diagrammatic representation of where the transposon has been inserted within the *envZ/ompR* locus.

A

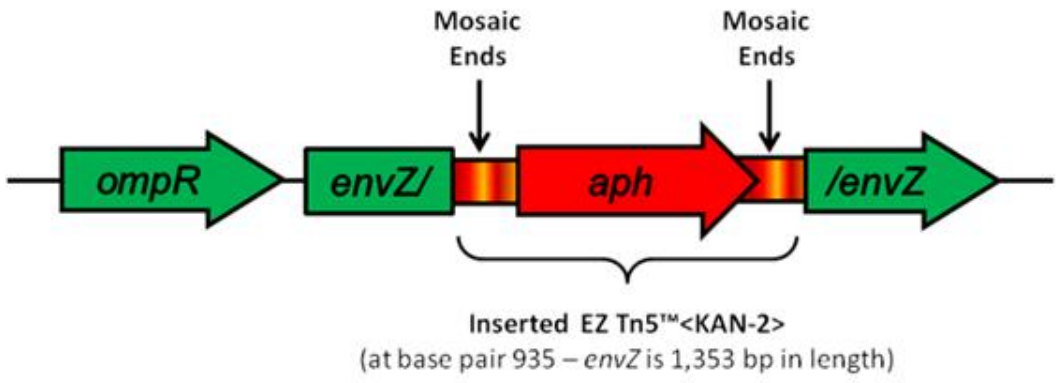


B

GGTTGAKATGTGTATAAGAAKGATTGCGGGGAAAAAAGCTATGA
GCGTGAGATTAACACTGCGCTTCAGGCAGGCAKCWTCAWSGTGA
AWATGCACCCGCTCTCGATTAAGCGCGCGGTGGCWAATATGGTG
GTCAATGCTGCCCGCTATGGCAACGGCTGGATTAAGGTCARCAG
CGGCACCGAGTCGCATCGCGCCTGGTTTCAGGTAGAAGATGACG
GGCCGGGCATTAWSCCGGAGCAGCGTAAMCATCTGTTTCAGCCT
TTTGTGCGTGGCGACAGCGCCCGTARCACCAGCGGCACWGG

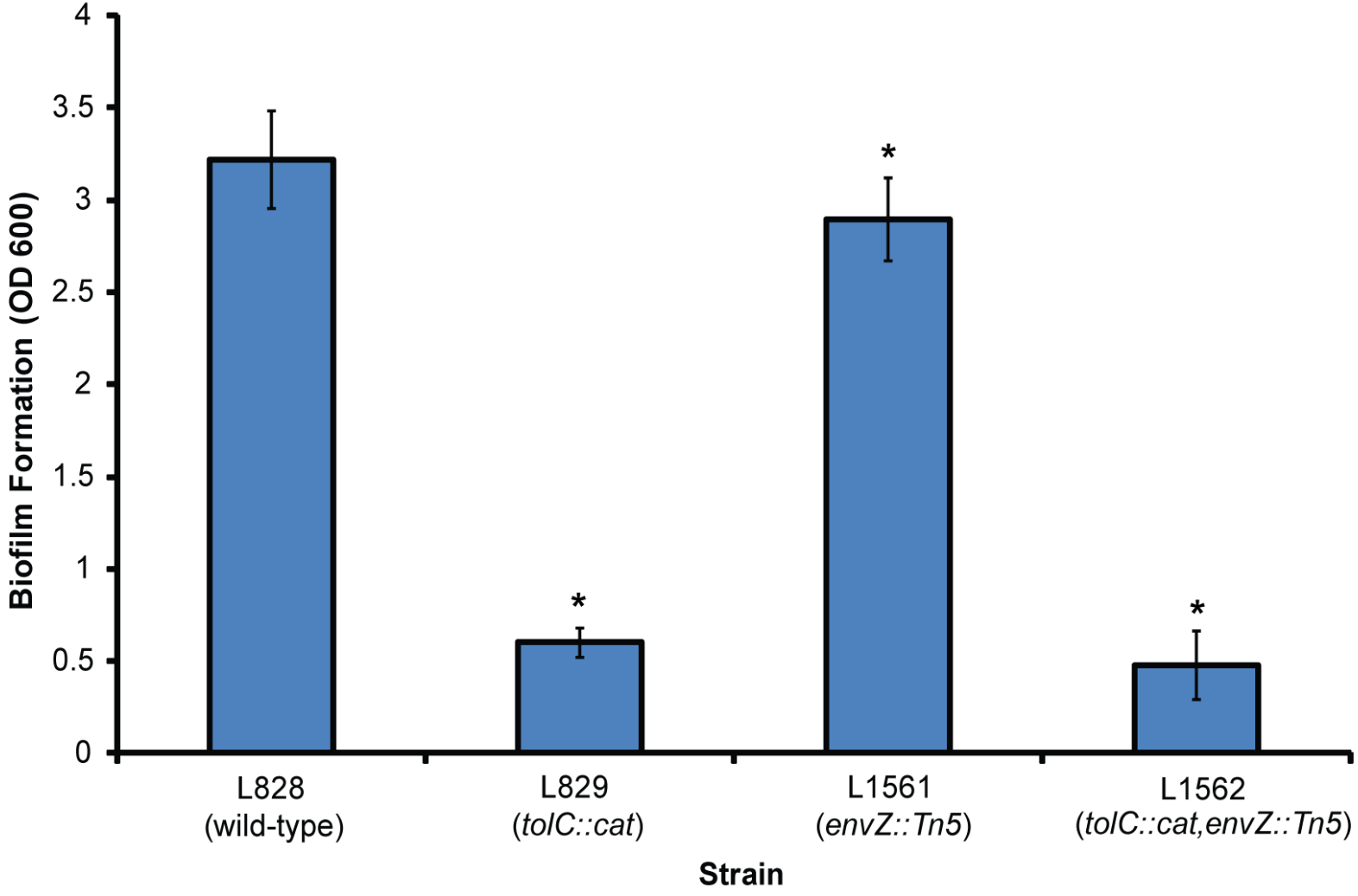
Red text - Homologous to Tn5 transposon
Green text - Homologous to *envZ*

C



Inserted EZ Tn5™<KAN-2>
(at base pair 935 – *envZ* is 1,353 bp in length)

Figure 9.4 Crystal violet biofilm assay of L828 (wild-type), L829 (*tolC::cat*), L1561 (*envZ::Tn5*) and L1562 (*tolC::cat, envZ::Tn5*). Statistically significant differences in biofilm formation in comparison to L828 (wild-type) are indicated by an asterisk ($p<0.05$).



The *envZ*::Tn5 mutation alone reduced the biofilm formation in comparison to L828 (wild-type) slightly but significantly. Transduction of the *envZ*::Tn5 mutation into any of the other efflux mutants saw no rescue of biofilm formation (Figure 9.5).

9.3 Artificial membrane stress decreases biofilm formation in *S*. Typhimurium

Membrane stress can be artificially induced upon the exogenous addition of 20% sucrose or increasing the pH of the broth (Wood 1999) To investigate the hypothesis that EnvZ/OmpR is being activated in the *tolC* mutants in response to a membrane stress and repressing curli, L828 (wild-type) was artificially stressed to attempt to replicate the phenotype. Both types of stimuli caused a defect in biofilm formation of both L828 (wild-type) and L829 (*tolC::cat*) (Figure 9.6). 20% sucrose caused L828 (wild-type) to form six times less biofilm and L829 (*tolC::cat*) to form around three times less biofilm. A statistically significant difference in biofilm formation was seen at pH 7.5 for L828 (wild-type) and pH 8 for L829 (*tolC::cat*). As L828 (wild-type) biofilm formation can be disrupted upon artificial membrane stress it suggests that the membrane stress seen in the *tolC* mutant is not caused by an accumulation of a metabolite inducing a regulatory cascade but a physical membrane stress.

9.4 Inactivation of *ompR* does not rescue biofilm formation

EnvZ acts as a membrane stress sensor protein that interacts with its partner response protein OmpR which directly promotes or represses expression of target genes. *ompR* was inactivated in the *tolC* mutant to observe if this also resulted in rescue of biofilm formation. Inactivation of *ompR* in L829 (*tolC::cat*) did not have any impact on biofilm formation, relative to L829 (*tolC::cat*), inactivation of *ompR* alone decreased biofilm formation four fold in comparison to L828 (wild-type) (Figure 9.7).

Figure 9.5 Crystal violet biofilm assay of L828 (wild-type), 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*) with and without the *envZ*::Tn5 mutation. Statistically significant differences in biofilm formation in comparison to L828 (wild-type) are indicated by an asterisk ($p<0.05$).

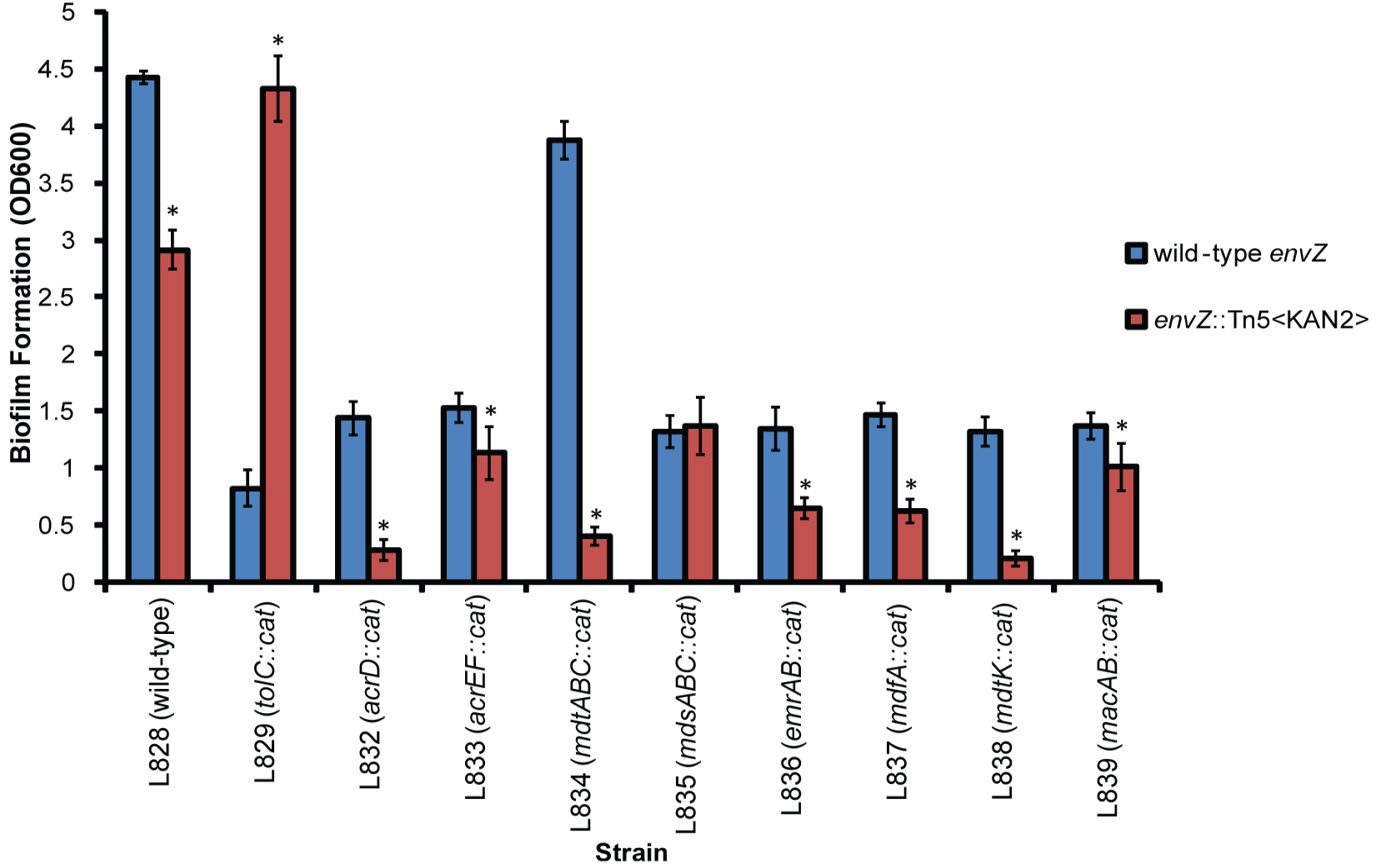


Figure 9.6 Crystal violet biofilm assay showing artificial membrane stress of L828 (wild-type) and L829 (*tolC::cat*) under membrane stress caused by exogenous addition of sucrose and increasing pH. Statistically significant differences in biofilm formation in comparison to their parent strain are indicated by an asterisk ($p<0.05$).

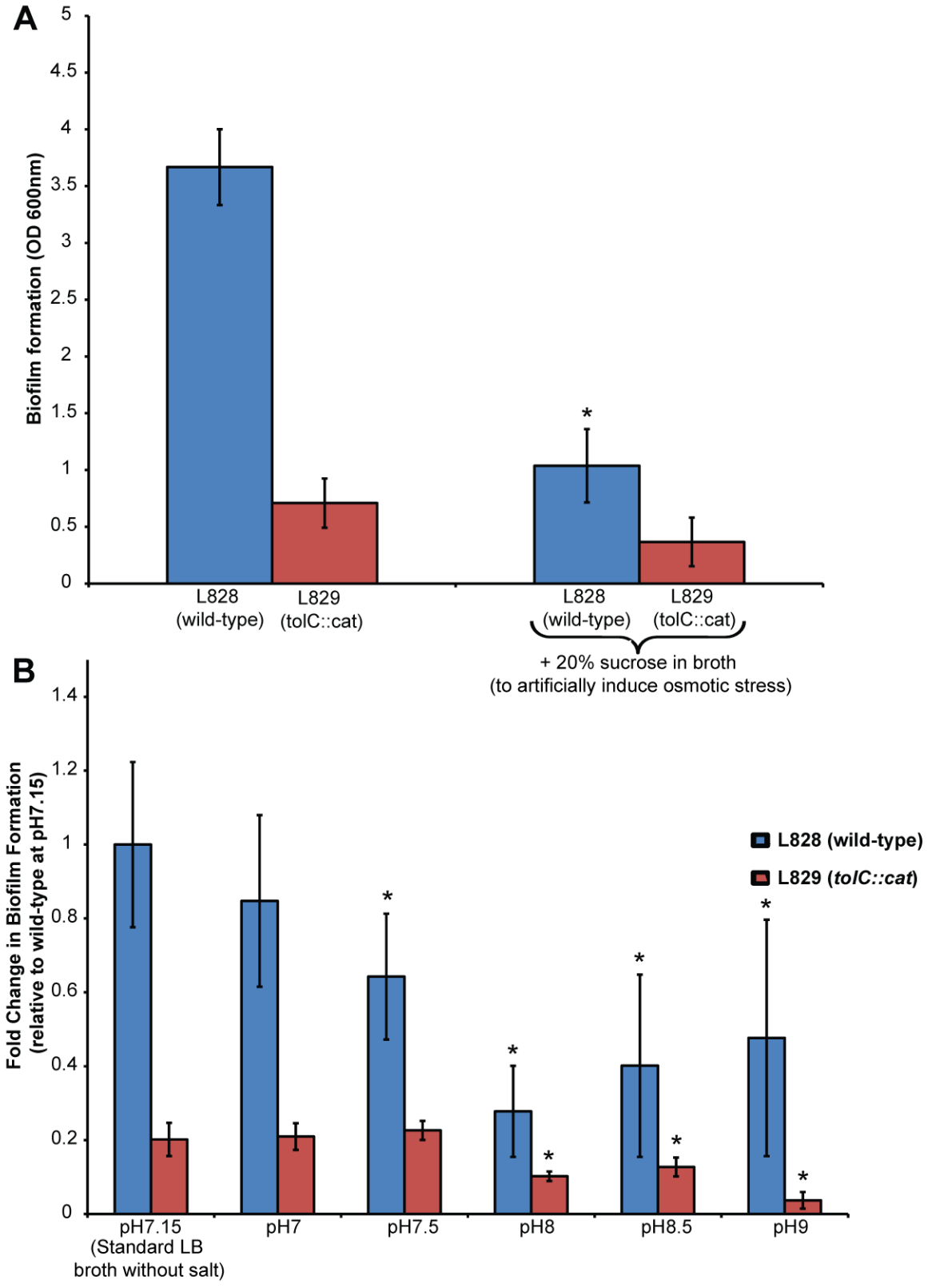
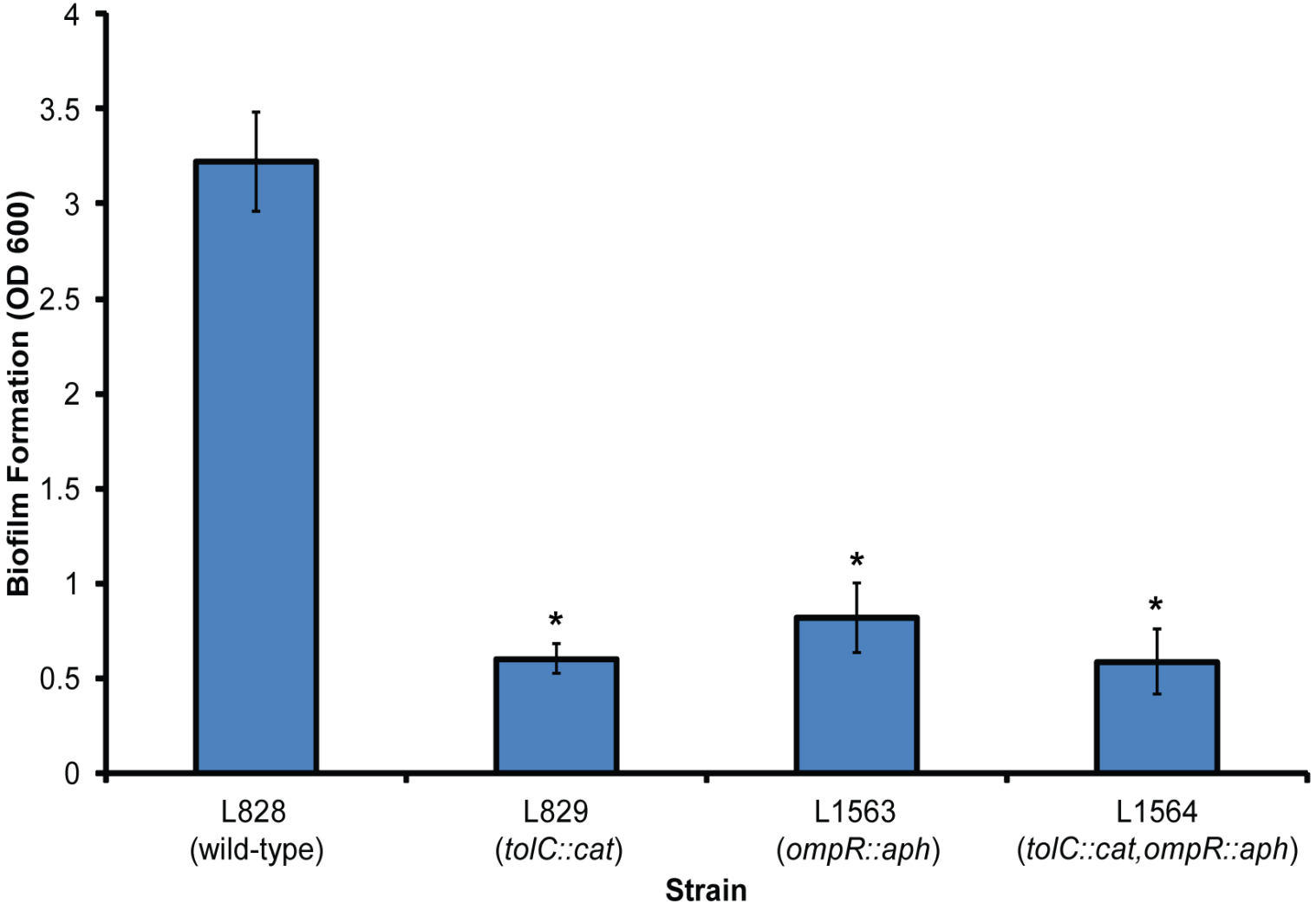


Figure 9.7 Crystal violet biofilm assay of L828 (wild-type), L829 (*tolC::cat*), L1563 (*ompR::aph*) and L1564 (*tolC::cat*, *ompR::aph*) showing no rescue of the *tolC* biofilm phenotype upon inactivation of *ompR*. Statistically significant differences in biofilm formation in comparison to their parent strain are indicated by an asterisk ($p<0.05$).



These results suggest that either curli repression in the *tolC* mutant is not occurring via *ompR* (the usual regulation pathway from *envZ*) or that rescue of the *tolC* mutant by inactivation of *envZ* requires *ompR* to be in a particular state in order to release the transcriptional repression on the curli operons.

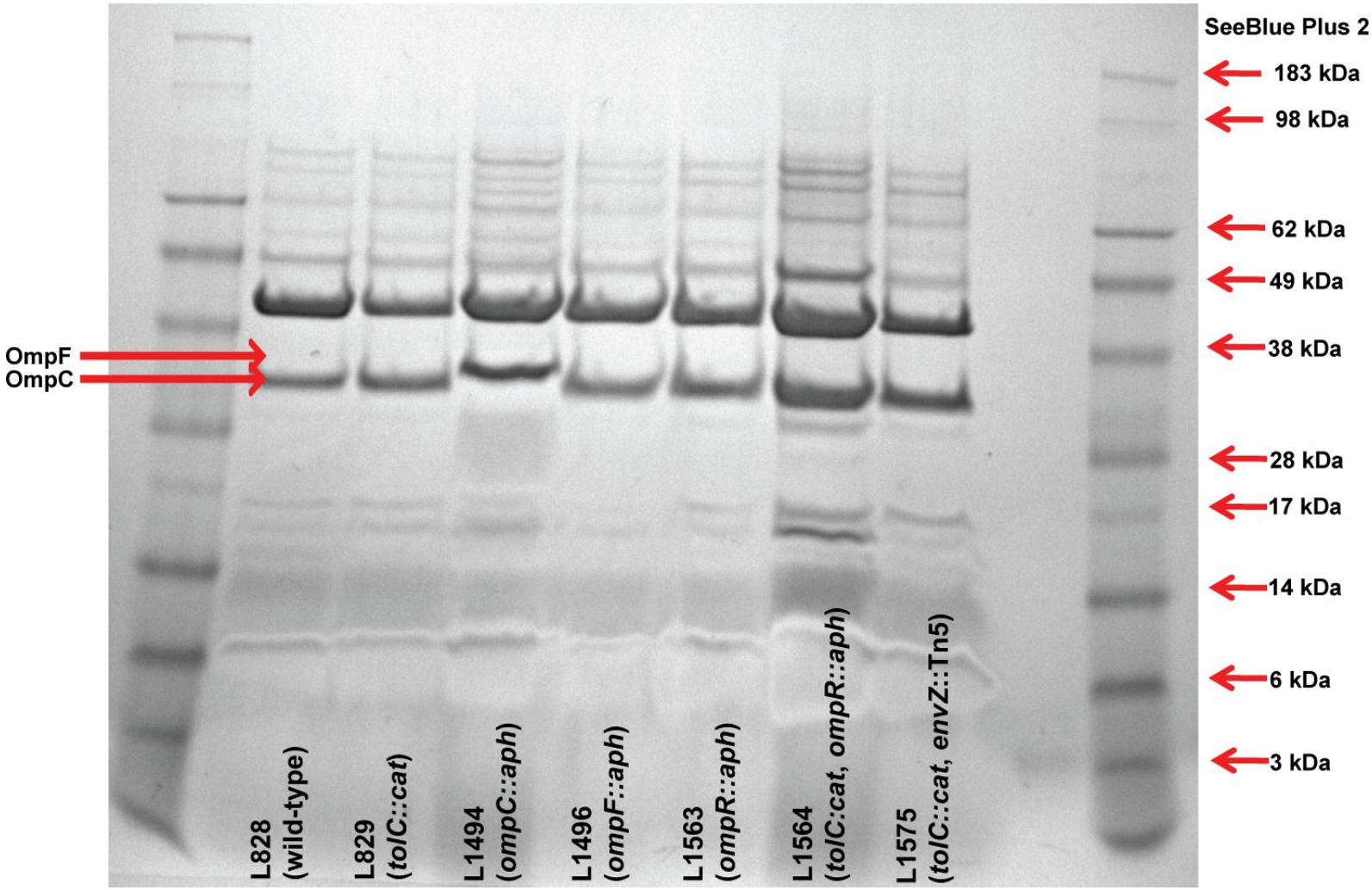
9.5 Outer membrane protein preparation shows no difference in OMP profile

As an alternative way to investigate the contribution of OmpR, the balance of OmpC/OmpF expression was investigated as this is sensitive to the state of OmpR. An outer membrane protein preparation was electrophoresed on a Bolt™ Bis-Tris 4-12% gel for to assess if there is a difference in the OMP profile of L829 (*tolC::cat*) and L1575 (*tolC::cat*, *envZ*::Tn5) in comparison to L828 (wild-type) (Figure 9.8). This information can give us an insight to the activity of OmpR, as depending on the state OmpR is in (i.e. polymerised or phosphorylated) the OMP profile will change. OmpC was the predominantly expressed porin protein which is seen expressed at similar levels throughout the strains tested, with the exception of L1494 (*ompC::aph*) which expresses OmpF but no OmpC. The unchanged OMP profile suggests that OmpR activity remains unchanged in L829 (*tolC::cat*) and L1575 (*tolC::cat*, *envZ*::Tn5).

9.6 Inactivation of *envZ* in different strain of *S*. Typhimurium lacking a functional TolC also rescues biofilm formation above wild-type levels

A different strain of *S*. Typhimurium (SL1344) lacking a functional TolC or AcrB has the same relative biofilm defect observed in the wild-type used in this project (*S.* Typhimurium14028S) although it intrinsically is a poorer biofilm forming strain.

Figure 9.8 Outer membrane protein preparation of L828 (wild-type), L829 (*tolC::cat)*, L1494 (*ompC::aph*), L1496 (*ompF::aph*), L1563 (*ompR::aph*), L1564 (*tolC::cat, ompR::aph*), L1575 (*tolC::cat*, *envZ*::Tn5) showing the OMP profiles of biofilm deficient and rescued strains to be unchanged.



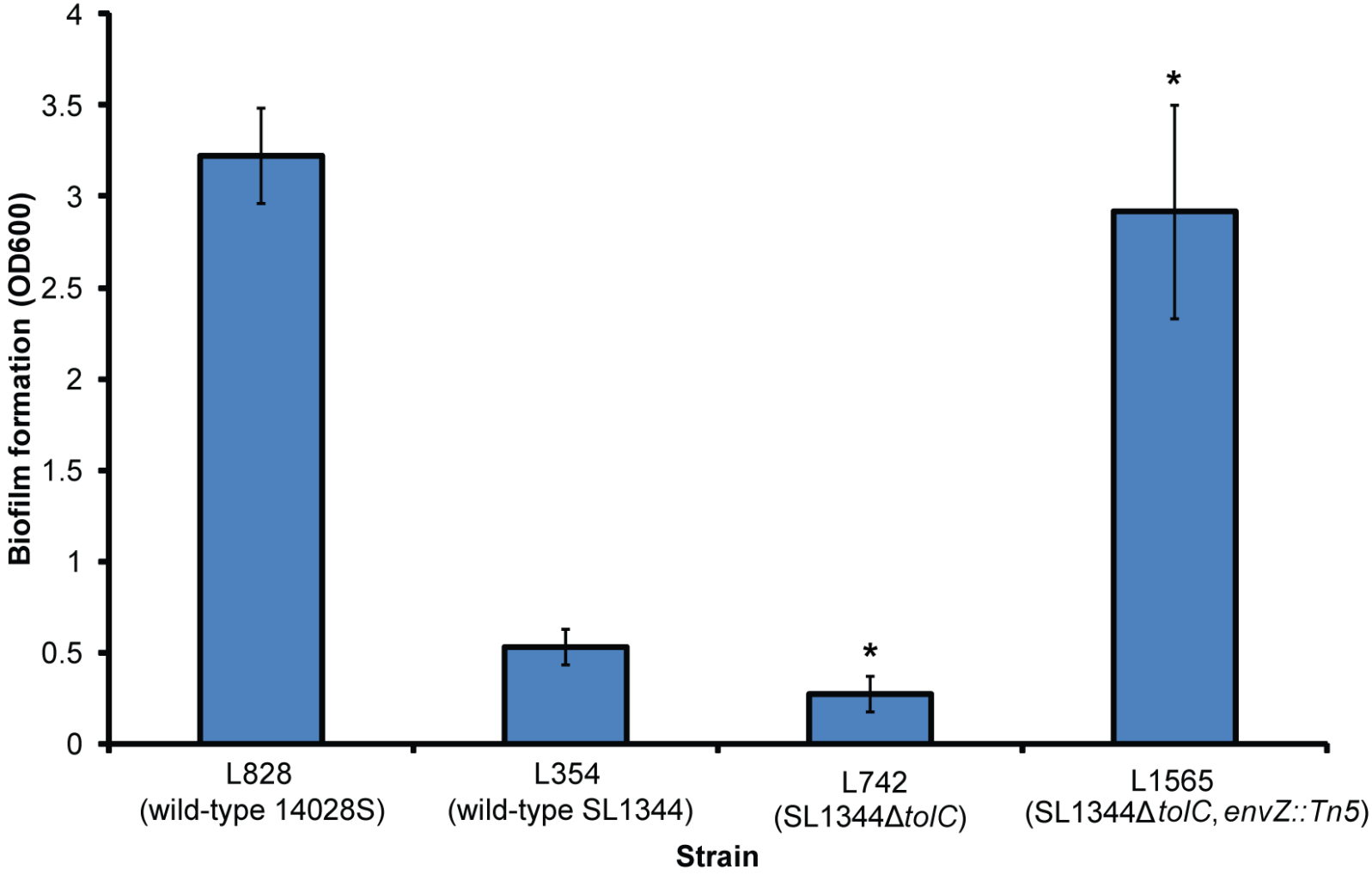
To see if this rescue is strain specific *envZ* was inactivated via P22 transduction in *S*. Typhimurium SL1344, a crystal violet biofilm assay of SL1344 (Δ*tolC*, *envZ*::Tn5) along side the wild-type and Δ*tolC* show a huge increase in biofilm formation compared to both Δ*tolC* and wild-type (Figure 9.9). Unlike the 14028S *tolC::cat* transposon library rescue mutant which was rescued up to L828 (wild-type 14028S) biofilm levels, the L1565 (SL1344 Δ*tolC, envZ::*Tn5) rescue mutant biofilms at six times the L354 (SL1344 wild-type) levels.

9.7 Discussion

The curli repression and biofilm defect seen in L829 (*tolC::cat*) is rescuable via inactivation of the osmolarity sensor *envZ*, located in the inner membrane. The *envZ*::Tn5 mutation did not rescue any of the other efflux mutants suggesting that the efflux and curli link is not completely generic. The tolC mutant was chosen for study as it is the most affected mutant due to inactivation of the one outer membrane porin effects multiple efflux systems.

To determine if induction of a membrane stress response in L828 (wild-type) can replicate the defect in L829 (*tolC::cat*) an artificial membrane stress assay was carried out. The results showed that artificial membrane stress (induced either by addition of exogenous 20% sucrose or increasing pH of the media) confers a biofilm defect in both L828 (wild-type) and L829 (*tolC::cat*). L828 (wild-type) was more susceptible to the increase in pH of the broth than L829 (*tolC:cat*) possibly because this strain is already under physical membrane stress. Inactivation of *tolC* has also recently been implicated in induction of at least five different stress response systems including, BaeRS and CpxARP (Rosner and Martin 2013).

Figure 9.9 A crystal violet biofilm assay showing a different strain of *S*. Typhimurium SL1344, SL1344 (Δ*tolC*) and SL1344 (Δ*tolC*, *envZ*::Tn5). *S*. Typhimurium wild-type 14028S (the wild-type used throughout this project) is included as a point of reference. Statistically significant differences in biofilm formation in comparison to wild-type SL1344 are indicated by an asterisk ($p<0.05$).



EnvZ is known to regulate genes via OmpR, however inactivation of *ompR* in the *tolC::cat* mutant did not rescue biofilm or curli expression as inactivation of *envZ* did. This however does not mean that EnvZ is not working via OmpR in this situation as OmpR has various configurations (i.e. monomer, polymer and phosphorylated) and each of these configurations can regulate the EnvZ/OmpR regulon in different ways. Depending upon the configuration of OmpR the expression of OmpA, OmpC and OmpF differ, the OMP preparation I performed showed no variation in the expression of these three proteins between the *tolC::cat* and *tolC::cat*, *envZ*::Tn5 mutants suggesting inactivation of *envZ* is not having an effect upon the configuration of OmpR or at least not that results in a known regulatory output in relation to Omp expression. Whilst EnvZ is thought to be highly specific for OmpR (Siryaporn and Goulian 2008) it is possible EnvZ is regulating curli expression via a different, novel pathway.

To investigate the EnvZ mediated biofilm and curli expression rescue seen in *S*. Typhimurium 14028S was strain specific, the *envZ*::Tn5 mutation was transduced into another *S*. Typhimuirum *tolC* mutant in the SL1344 background with a biofilm defect, L742 (SL1344 Δ*tolC*). Not only did inactivation of *envZ* in L742 (Δ*tolC*) increase biofilm formation this was to six times above the level of the parent L354 (SL1344 wild-type). This shows the EnvZ inactivation can resuce a *tolC* mediated biofilm defect in various strains and in SL1344 can promote biofilm formation above the level of the parent considerably.

CHAPTER TEN:

CHEMICAL INACTIVATION OF EFFLUX CAUSES A BIOFILM DEFECT IN A RANGE OF SPECIES

10 Chemical Inactivation of efflux causes a biofilm defect in a range of species

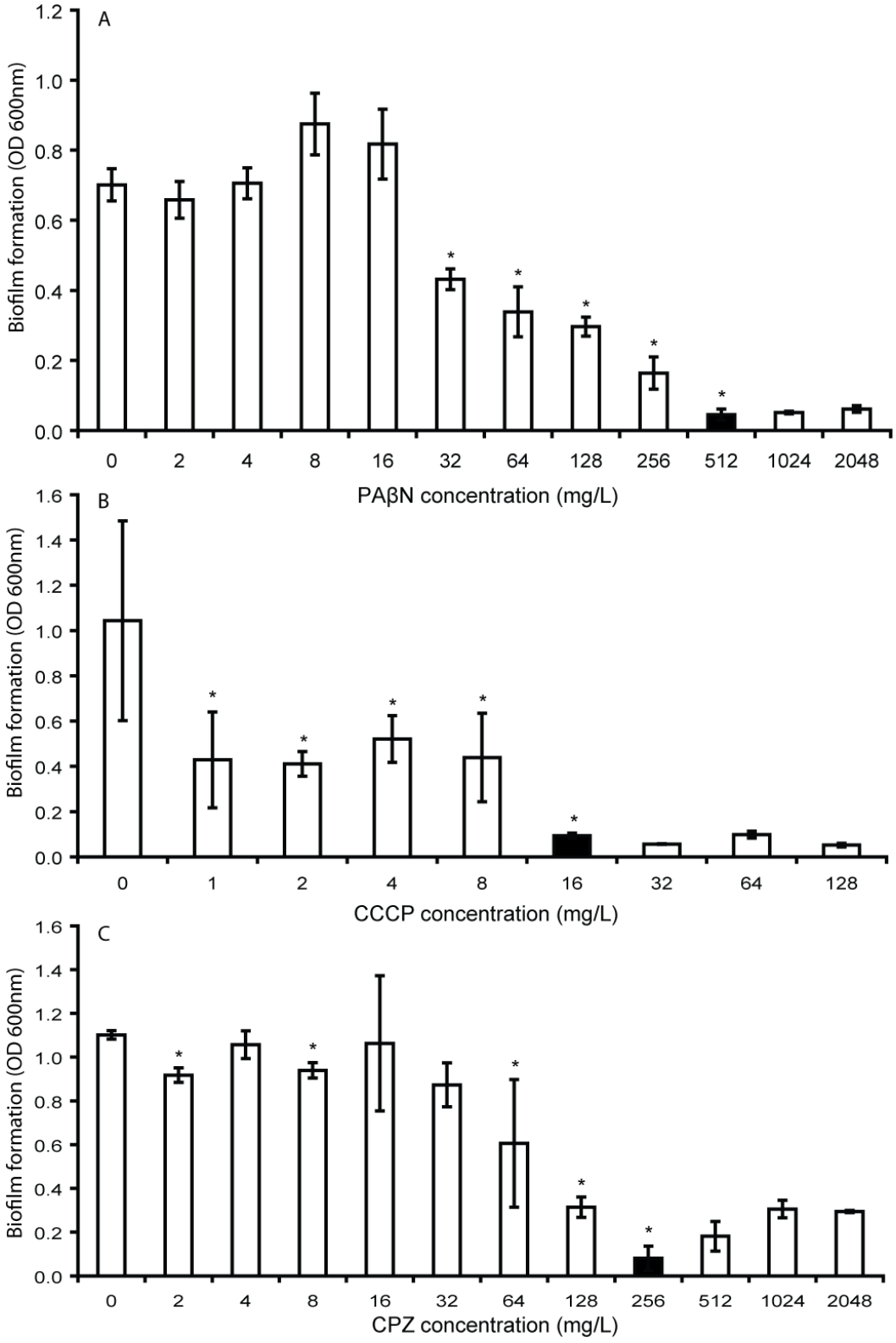
10.1 Exogenous addition of efflux inhibitors induces a biofilm defect of L828 (wild-type) in the crystal violet biofilm assay

To see if the biofilm defect can be induced via chemical efflux inhibition, addition of three different efflux inhibitors with various mechanisms of action were added exogenously to a crystal violet biofilm assay. PAβN, CCCP and CPZ all cause a biofilm defect in L828 (wild-type), the concentrations required to cause an anti-biofilm effect are all below minimum inhibitory concentration (Figure 10.1). The most effective EI for preventing L828 (wild-type) from forming a biofilm was CCCP where the effect was seen at 1 µg/ml of exogenous CCCP. Both PAβN and CPZ seem to have similar efficiencies, where the start of the dose dependent anti-biofilm effect was at 32 µg/ml and 64 µg/ml respectively. There was a small but significant effect with 2 and 8 µg/ml of CPZ but the dose dependent effect not seen until 64 µg/ml of CPZ (Baugh *et al.* 2012).

10.2 Efflux inhibitors also induce a biofilm defect in wild-type *S. aureus*, *E. coli* and *P. aeruginosa*

To assess whether chemical inhibition of efflux using efflux inhibitor compounds has impact as an anti-biofilm approach in other bacterial species we evaluated the ability of the same three inhibitors used with *S*. Typhimurium (section 10.1); CPZ, CCCP and PAβN to prevent biofilm formation by representative strains of *E. coli* (NCTC MG1655), *P. aeruginosa* (NCTC PA01) and *S. aureus* (NCTC 8532) (Baugh *et al.* 2013).

Figure 10.1 Crystal violet biofilm assays of L828 (wild-type) with increasing concentrations of exogenous efflux inhibitor **A** PAβN, **B** CCCP or **C** CPZ. Statistically significant differences in biofilm formation in comparison to absence of efflux inhibitor are indicated by an asterisk ($p<0.05$). Minimum inhibitory concentration of L828 (wild-type) to each efflux inhibitor marked by a black bar.



These particular species were chosen as they are the source of biofilm related infections either in the food industry or hospital setting (Archer *et al.* 2011; Joo and Otto 2012; Romling and Balsalobre 2012). These inhibitors act in different ways; disruption of the proton motive force, competitive substrate of the pump or transcriptional repression) therefore we hypothesise that any anti-biofilm effect common to all species investigated is due to inhibition of efflux and not specific effects of the inhibitors themselves. The crystal violet biofilm assay shows all three inhibitors significantly reduced the biofilm formed ($p<0.05$) by all three species however inhibitor concentrations that provoked the greatest effect varied between species (Figure 10.2). The most susceptible strain to CCCP was *S. aureus* with 2 mg/L causing a 5 fold reduction in biofilm formation; PAβN had the greatest impact on *E. coli* with 16 mg/L preventing formation of a normal biofilm. *P. aeruginosa* was least susceptible to the inhibitors with 128 mg/L of CCCP and 512 mg/L of the other inhibitors needed to prevent biofilm formation. Irrespective of the species, biofilm inhibitory concentrations were all below the MIC of each compound under the test conditions demonstrating a specific anti-biofilm effect rather than a generalised growth inhibition.

10.3 The same efflux inhibitor dependent biofilm defect was seen under flow conditions

After an initial attachment period, efflux inhibitors were added to the appropriate media and flowed over the cells that have initially attached to the flow cell to observe if a mature biofilm can develop in the presence of efflux inhibitors. All efflux inhibitors repressed all strains ability to form a mature biofilm under flow conditions. Bacterial coverage in the flow cell by *S. aureus* and biofilm formation was severely reduced or

absent after addition of CCCP (4 mg/L, resulted in a 6-fold reduction in coverage of the flow cell), CPZ (64 mg/L, resulted in a 6-fold reduction in coverage of the flow cell) and PAβN (64 mg/L, resulted in a 500 fold reduction in coverage of the flow cell) (Figure 10.3). All all EIs were effective against all species under flow conditions, representative phase contrast microscopy images show this inhibition for wild-type *P. aeruginosa*, *S. aureus*, *S*. Typhimurium and *E. coli* incubated alone and with appropriate concentrations of CCCP, i.e. below the MIC of the specific strain but within the range where an anti-biofilm effect is seen in the crystal violet biofilm assays (Figure 10.4) (Baugh *et al.* 2013).

10.4 The mechanism controlling the biofilm defect of L828 (wild-type) with efflux inhibitors is repression of curli genes

Phenotypic studies of L828 (wild-type) curli production with EI were difficult as Congo red, the dye used to stain curli, is exported by the efflux pumps addition of an EI into the agar prevented the bacteria from growing properly as Congo red is toxic at high concentrations. Therefore, comparative RT-PCR was used to investigate whether addition of efflux inhibitors induce the same curli repression as genetic efflux inactivation. Both *csgB,* the major structural curli subunit and *csgD,* the master curli regulator were down-regulated upon addition of 32 and 64 μg/ml of PAβN (Figure 10.5).

10.5 Discussion

Chemical inactivation of efflux in *S*. Typhimurium 14028S causes a biofilm defect in both static and flow conditions. Three different efflux inhibitors with various mechanisms of action were used to ensure any effect seen was not an artefact the efflux inhibitor but was in fact an effect of stopping the efflux pump from working.

Figure 10.2 Crystal violet biofilm assays of *S. aureus*, *E. coli*, *P. aeruginosa* with various sub MIC concentrations of the three efflux inhibitors, CCCP, PAβN and CPZ. Statistically significant differences in biofilm formation in comparison to their parent strain are indicated by an asterisk ($p<0.05$).

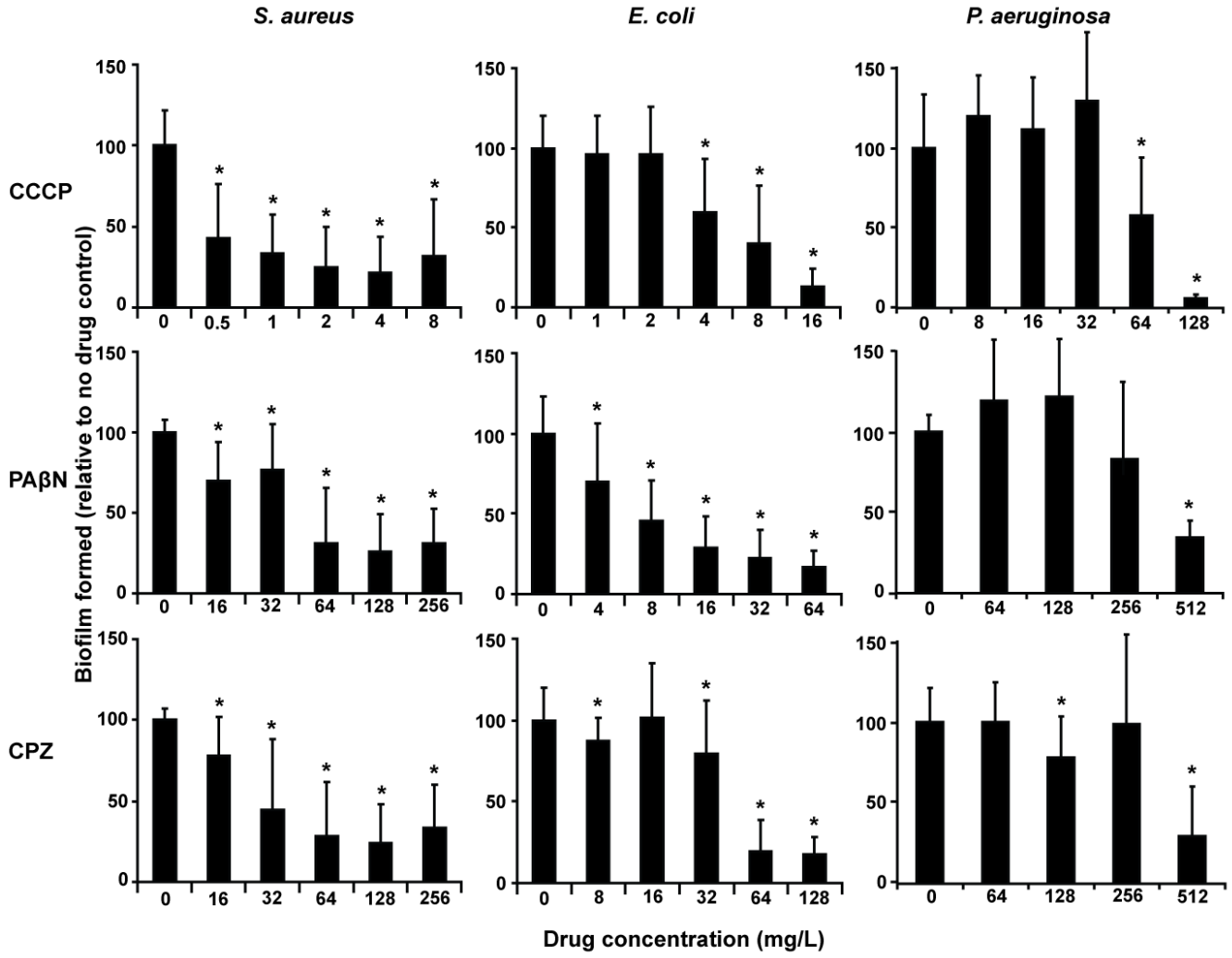


Figure 10.3 Percentage of flow cell covered by biofilm of F77 (*S. aureus* wild-type) with and without efflux inhibitors (64 µg/ml CPZ, 1 µg/ml CCCP and 64 µg/ml PAβN) after 16 hours under a flow rate of 0.3 dynes. Statistically significant differences in biofilm formation in comparison to F77 (*S. aureus* wild-type) without efflux inhibitor are indicated by an asterisk ($p<0.05$).

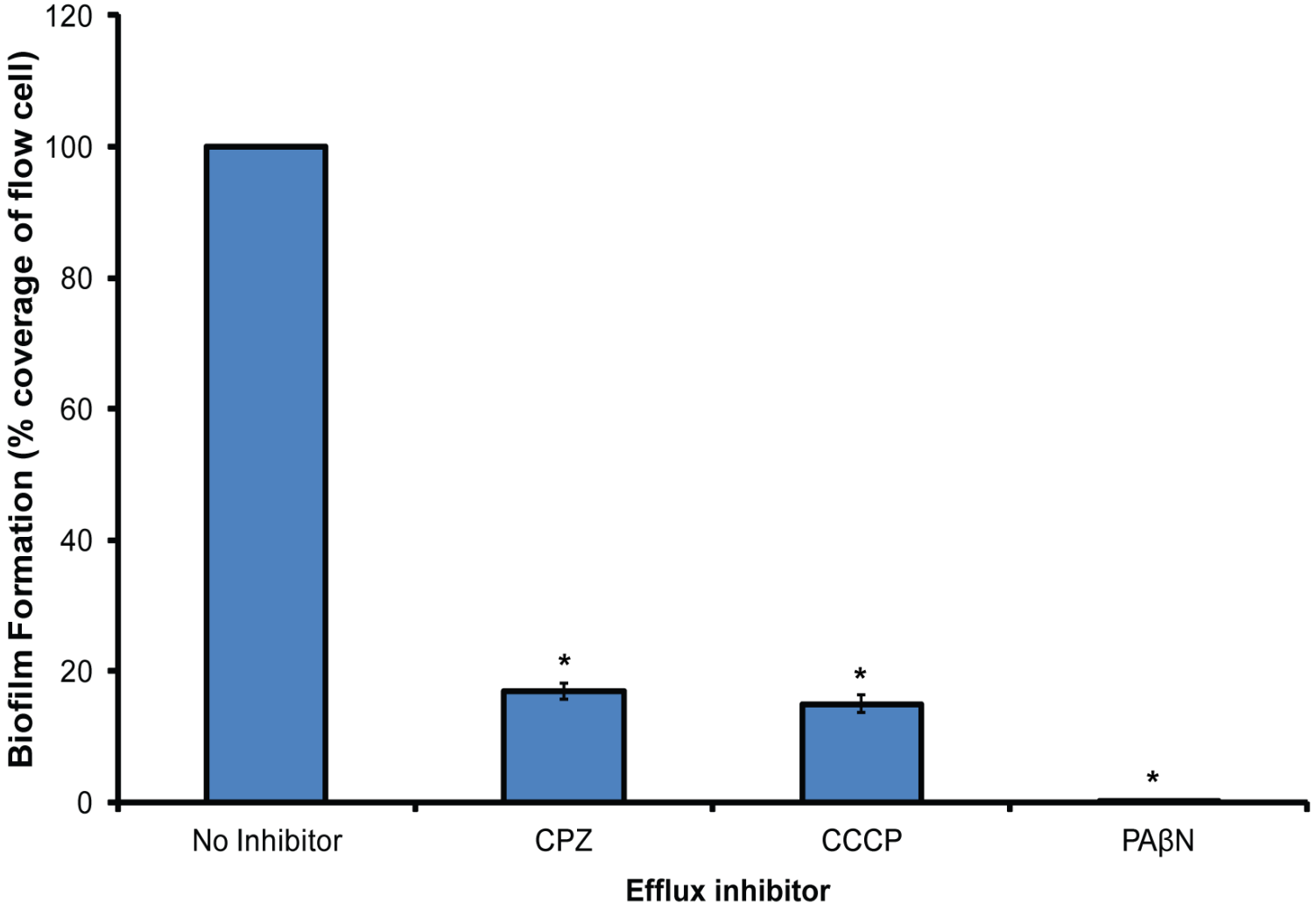


Figure 10.4 Phase contrast microscopy images (x10 magnification) of *P. aeruginosa*, *S. aureus*, *S*. Typhimurium and *E. coli* biofilms after 16 hours incubation under flow conditions. with and without appropriate concentrations of CCCP for the specific strain. Concentrations of CCCP used; *P. aeruginosa* – 64 μg/ml, *S. aureus* – 1 μg/ml, *S.* Typhimurium – 2 μg/ml and *E. coli* – 4 μg/ml.

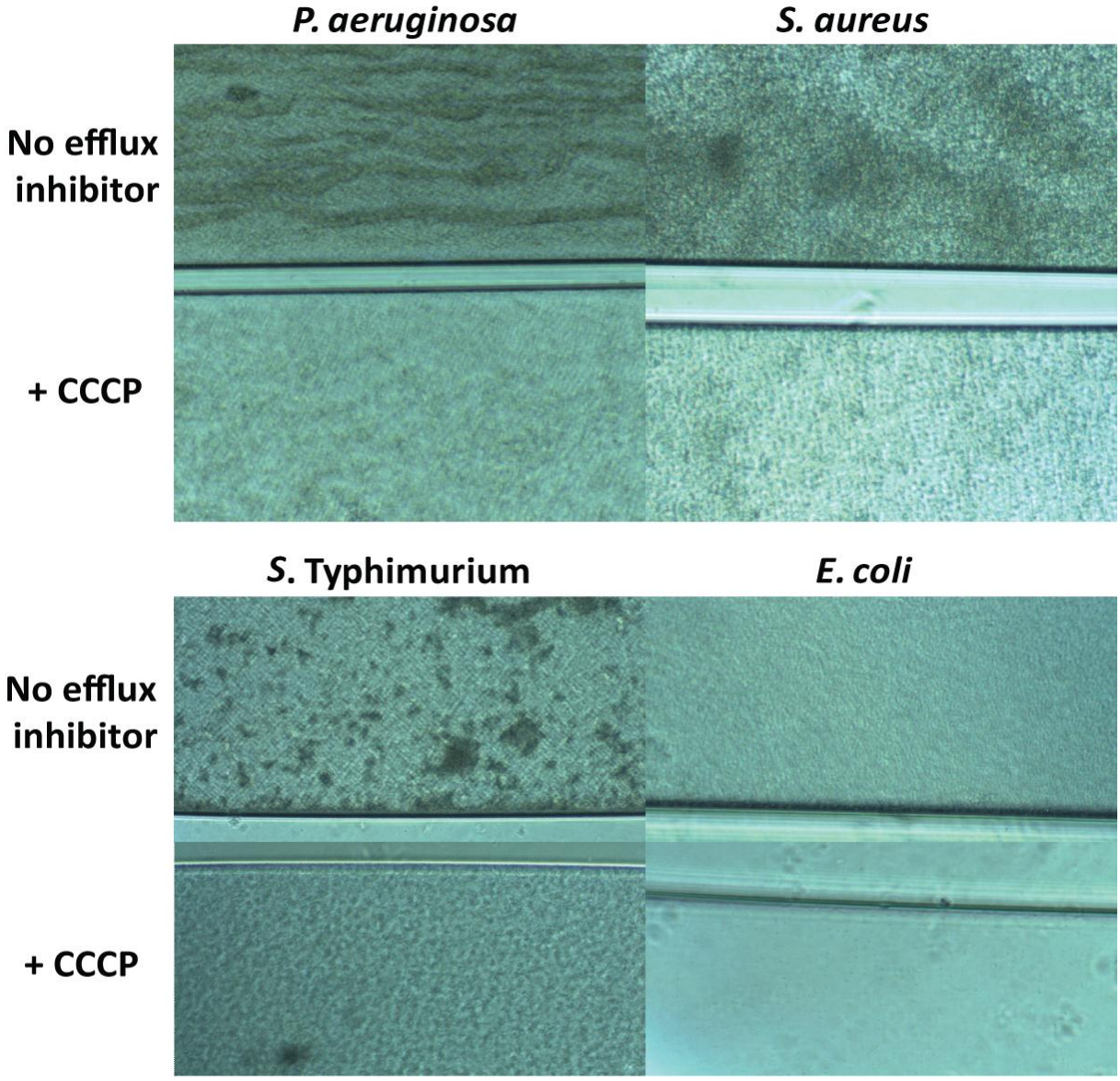
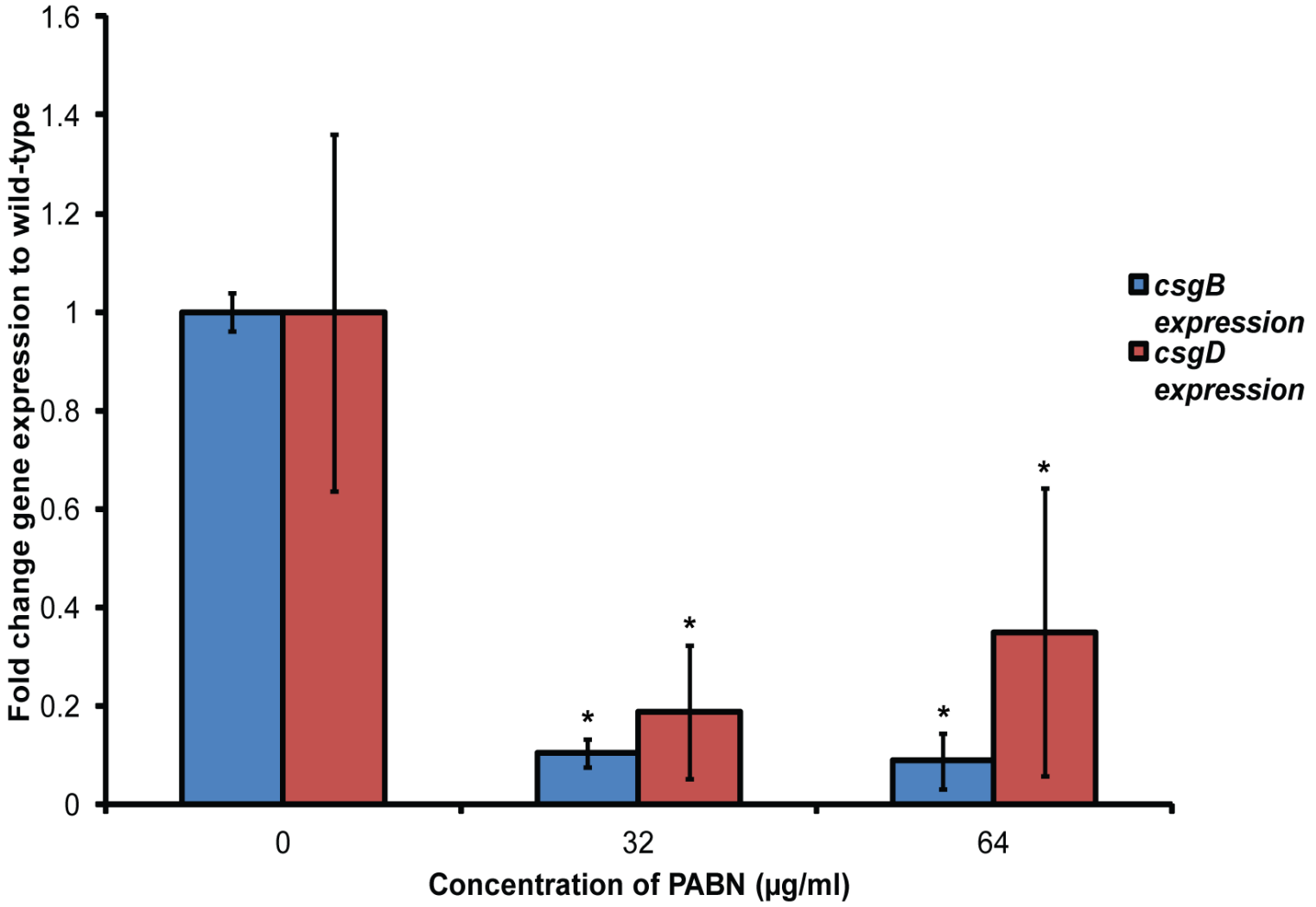


Figure 10.5 L828 (wild-type) mRNA expression of *csgB* and *csgD* with exogenous addition of 32 and 64 μg/ml of PAβN. Statistically significant differences in gene expression of wild-type compared to absence of efflux inhibitor are indicated by an asterisk ($p<0.05$).



The three EIs all caused an anti-biofilm effect well below the MIC level, this shows that the EIs are acting as anti-biofilm agents and the biofilm defect in not due to the EI acting as an antimicrobial and preventing growth. Each EI worked to varying degrees with CCCP being most effective followed by PAβN then CPZ. This could make sense as CCCP abolishes the proton motive force (Pages *et al.* 2005) so will effect eight out of the nine multidrug efflux pumps in *Salmonella* (Nishino *et al.* 2003). PAβN is a known substrate of RND pumps and may have an effect on up to five of the nine MDR pumps (Lomovskaya *et al.* 2001). CPZ is thought to be a transcriptional repressor of global regulators of certain pumps (Bailey *et al.* 2008).

To see if this biofilm defect caused by addition of EIs is *Salmonella* specific, EIs were added to crystal violet static biofilm assays of *S. aureus*, *E. coli* and *P. aeruginosa*. All three EIs caused an anti biofilm effect for all three species to varying degrees; CCCP was most effective against *S. aureus* biofilms, CCCP and PAβN worked best against *E. coli* biofilms and CCCP was most effective by far against *P. aeruginosa* biofilms. All EIs also had an anti-biofilm effect on all species under flow conditions, where cells were incubated without EI for around 3 hours to initially attach to the flow cell after which EI was added to see if a mature biofilm could be prevented from forming.

EIs have previously been shown to decrease biofilm formation in *Pseudomonas putida* (*P. putida*) and *S. aureus*, the EIs used in that study were thioridazine, 1-(1-naphtylmethyl)-piperazine (NMP) and PAβN (Kvist *et al.* 2008). A direct comparison that can be drawn from this study is the effect of PAβN against prevention formation of *S. aureus*.biofilms. Kvist et al showed that 20 μg/ml of PAβN

reduced the amount of biofilm formed by around 60%, this study substantiates this finding as we showed that both 16 and 32 µg/ml of PAβN reduces biofilm formation to 60% of the non EI control. Another comparison we can draw from the Kvist study is the use of phenothiazines as EIs, the Kvist study used thioridazine which is in the same class of drugs as the chlorpromazine used in this study. 20 µg/ml of thioridazine caused a decrease in biofilm formation causing *S. aureus* to form 30% of biofilm formed by the no EI control, in our study 32 µg/ml of chlorpromazine had a very similar effect where biofilm formation of *S. aureus* decreased to around 40%.

The use of EIs in a clinical setting could be a therapeautic avenue although two of the EIs used in this study, CCCP and PAβN, are toxic to humans due to them affecting the efflux pumps in human cells. However, chlorpromazine is part of a group of drugs called phenothiazines which are already used in medicine as anti-psychotics, so there is a possibility that these type of drugs could be used therapeutically as a classic antimicrobial and ant-biofilm drug either alone or in conjunction with an antibiotic. Another use of EIs could be impregnated medical biomaterials such as catheters, joint prostheses and pacemakers to prevent the formation of biofilms on the surface of these devices which are sources of human infection.

The ability of EIs to repress curli expression is also another line of evidence for the mechanism linking efflux and biofilm formation being regulatory and a result of loss of efflux function.

CHAPTER ELEVEN:

OVERALL DISCUSSION

11 Overall discussion

11.1 Genetic inactivation of multidrug resistant efflux pumps causes a biofilm defect

The initial findings of this project identified a link between multidrug resistance efflux pumps and biofilm formation in *Salmonella*. This finding was corroborated by anothyer study by Kvist *et al* with *E. coli*, *K. pneumonia*, *P. putida* and *S. aureus* (Kvist *et al.* 2008). The work in our laboratory showed that genetic inactivation of the individual components of the major efflux system of *Salmonella*, AcrAB-TolC, creates a biofilm defect in a simple static crystal violet biofilm assay. The Kvist study showed both genetic and chemical inactivation of efflux pumps of a variety of species creates a biofilm defect, but did not propose a mechanism for the phenotype observed or investigate the input of deletion of multiple pumps per species.

Further investigation into which MDR efflux pumps are involved in biofilm formation highlighted that inactivation of all nine MDR efflux pumps known to be present in *Salmonella* created a biofilm defect to varying degrees. The nine MDR efflux pumps in *Salmonella* belong to four different efflux families; five RND, two MFS, one MATE and one ABC. This shows that the biofilm defect we see is not specific to a single efflux pump or indeed a single class of efflux pump, no previous consistent phrnotype has been attributed to loss of all efflux systems.

Various biofilm assays were tested and optimised, and the biofilm defect was seen in all assays with both static and flowing media. The flow cell biofilm model was important as many ‘real-world’ biofilms will form and mature under flow conditions both in the environment such as waterways and in human disease such as on catheters.

11.2 Three different hypotheses were investigated

Three different hypotheses were formulated; AcrAB-TolC exports a crucial biofilm factor, inactivation of AcrAB-TolC impacts upon cell surface hydrophobicity and inactivation of components of AcrAB-TolC alters expression off biofilm related genes or proteins. Experiments were chosen and designed to investigate each hypothesis.

The first hypothesis, that AcrAB-TolC exports a crucial biofilm factor, was tested by using various co-incubation assays to assess if presence of the wild-type cells could rescue the *tolC* and *acrB* mutants' ability to form a biofilm. Transwell assays, where wild-type and mutant cells were separated with a semi-permeable membrane which allows solutes through but restricts movement of the cells, showed no rescue of either *tolC* or the *acrB* mutants. Both wild-type mid-logarithmic phase and stationary phase cultures were used as the metabolites excreted can vary depending upon the growth phase of the bacteria. Another co-incubation biofilm assay used involves the formation of wild-type biofilm mats which grow on the surface of the media and enumerating the number of *acrB* or *tolC* mutant cells that are incorporated in the biofilm mat. Again, this co-incubation assay showed that presence of wild-type cells were not able to rescue the ability of the either the *tolC* or *acrB* mutants' to form a biofilm. Metabolomics data revealed differences between the accumulated metabolomic profile of wild-type cells and the *acrB* mutant, the biggest difference seen is the accumulation of the polyamine, spermidine. Polyamines have previously been shown to be an essential metabolite in the formation of biofilms of *Yersinia pestis* (Patel *et al.* 2006). To assess if lack of export of polyamines prevents the *tolC* or *acrB* mutants' ability to biofilm, various concentrations of spermidine, acetyl-spermidine cadaverine and putrescine were added exogenously to the simple crystal

violet biofilm assay. Addition of any of the polyamines did not positively affect either mutant's ability to form a biofilm. If accumulation of polyamines is occurring to toxic levels when cells are residing within a biofilm then exogenous addition of polyamines would not answer this question. However, you might see that exogenous addition of polyamines have a negative effect on wild-type biofilm formation, which is not seen. These assays suggest that polyamines are not involved in the link between MDR efflux and biofilm formation but these experiments are not definitive. Another piece of evidence to suggest that it is not export or accumulation of a specific metabolite is that inactivation of all MDR efflux pumps causes the same biofilm defect, albeit to varying degrees, therefore you would expect the other eight efflux pumps to compensate. However, inactivation of each efflux system could cause accumulation of a different metabolite causing the same biofilm defect mediated by accumulation of different substrates although this seems highly unlikely.

The second hypothesis investigated whether inactivation of AcrAB-TolC causes a change in cell surface hydrophobicity or membrane composition which can affect cellular aggregation and therefore could impact upon biofilm formation. Various aggregation assays were used to test this hypothesis. The simplest assay used was the settle assay which measures the speed at which the cells sink to the bottom of a tube, this showed no decrease in the aggregation ability of the *tolC* or *acrB* mutants but in fact it showed a slight increase in the *acrB* mutant's ability to aggregate. Another simple assay used various concentrations of ammonium sulphate which upon addition of a bacterial suspension causes aggregation observed by the presence of a white precipitate. The lower the concentration of ammonium sulphate required for a precipitate to form the higher the cell surface hydrophobicity. Again, no

decrease in cell surface hydrophobicity was seen for either the *tolC* or *acrB* mutant however, a slight increase was seen for the *tolC mutant*. The composition of the outer membrane was also investigated by isolating both the lipoploysaccharide and outer membrane proteins and visualising on a Bis-Tris gel. No apparent differences in the banding pattern of the LPS or OMP preparations were seen in the *tolC* or *acrB* mutants. None of the assays used highlighted a decrease in cell surface hydrophobicity of the cell or change in the composition of the outer membrane.

The final hypothesis tested was that inactivation of components of the AcrAB-TolC efflux system changes expression of biofilm related genes or proteins. To test this hypothesis we used phenotypic assays to assess the expression of components of the *Salmonella* extracellular matrix, essential for formation of mature biofilms. Cellulose, the polysaccharide used to help adhere cells to each other within a biofilm was assessed on agar supplemented with Calcofluor, a dye that binds to cellulose and fluoresces. No difference was seen between wild-type and the *tolC* or *acrB* mutants was seen .This was confirmed by cRT-PCR where expression of the mRNA of the four genes responsible for cellulose biosynthesis remained unchanged in both mutants. The other extracellular matrix component essential for biofilm formation is a proteinaceous filament that protrudes from the outer membrane of the cell called curli. Agar supplemented with Congo red, a dye used to stain amyloid fibres (curli when assembled on the surface of the outer cell membrane is an amyloid fibre) (Romling 2005) revealed that both the *tolC* and *acrB* did not display the red, dry and rough phenotype indicative of strain expressing curli. cRT-PCR confirmed that the repression of curli was occurring at the transcriptional level. All genes in both of the curli operons, *csgBA* and *csgDEFG* in the *tolC* and *acrB* mutant were down regulated

in comparison to wild-type. Congo red agar assays and cRT-PCR of *csgB* and *csgD* exposed curli repression in the entire panel of efflux mutants strongly suggesting that the biofilm defect seen in all mutants is due to absence of this essential proteinaceous extracellular matrix component.

11.3 Artificial overexpression of global regulators of AcrAB-TolC also induces a biofilm defect

A previous study found that increase of *marA* decreases curli expression and subsequent biofilm formation (Guinta 2010). This finding led us to hypothesise that upon inactivation of efflux pumps, the up-regulation of global regulators of efflux was the reason for curli repression and subsequent biofilm formation. Artificial over-expression of the global regulators of efflux, *marA*, *ramA* and *soxS* inhibits biofilm formation by wild-type *S*. Typhimurium. The spatial expression of the three global regulators visualised using a *gfp* fusion reporter plasmid also correlates with the absence of curli visualised on Congo red agar. Various combinations of all three regulator genes in the *tolC* mutant were inactivated, however, rescue of the *tolC* mutant's ability to biofilm or express curli was not achieved. These data does not discount the hypothesis that regulators are the reason for the efflux mutants biofilm defect but does not prove it either.

11.4 Membrane stress response systems are involved in curli repression of the *tolC* mutant

To further explore the regulatory mechanism controlling the curli repression of the genetically inactivated efflux mutants a transposon mutagenesis screen was developed and optimised during this study. Transposon libraries in representative mutants of various classes of efflux pumps were created and screened on Congo red

agar to expose any candidates with curli expression restored. The *tolC* mutant library was investigated in more detail as the *tolC* mutant's biofilm formation was most effected and any curli or biofilm rescue would perhaps be more noticeable. It was discovered that insertion of the transposon into the membrane bound osmolarity sensor *envZ* restores that ability of the *tolC* mutant to express curli and form a biofilm. The *envZ*::Tn5 mutation was transduced into all other MDR efflux mutants and also back into the *tolC* mutant to investigate if the same mechanism was responsible for the curli repression and biofilm defect. None of the other efflux mutants formed a biofilm in the crystal violet biofilm assay.

The *envZ* mutation was transduced into a *tolC* mutant of a different strain of *S*. Typhimurium (SL1344) to see if this curli/biofilm rescue was strain specific. However, surprisingly the SL1344 *tolC*/*envZ* mutant did not just rescue biofilm formation to wild-type levels but greatly beyond that of the parent.

The EnvZ/OmpR two component system is already known to be a regulator of the curli operons (Prigent-Combaret *et al.* 1999) along with two other two component systems, CpxA/CpxR and RcsB/RcsC. All three systems respond to changes in the environment including pH, osmolarity and membrane stress (Dorel *et al.* 1999; Huang *et al.* 2006). This response to environmental or membrane stress makes sense because if the cell is sensing stress in the environment it is residing in, it would not be conducive to the cell to express curli and form a biofilm. Artificial stressing of the membrane using sucrose or increasing the of the pH media also reduced biofilm formation of the wild-type again supporting membrane stress as the mechanism.

Inactivation of *tolC* in *E. coli* has recently been implicated in induction of at least five different stress response systems including, BaeRS and CpxARP (Rosner and Martin 2013). The study suggested that accumulation of a metabolite usually exported via efflux systems using TolC as their outer membrane porin is detected by these stress response genes. However, the metabolite responsible was not found, although accumulation of indole, acetate and ethanol were all ruled out (Rosner and Martin 2013). As accumulation of polyamines within the cell was not ruled as the cause of the biofilm defect in the *tolC* and *acrB* mutant, it is possible that the metabolite responsible could be one of spermidine, acetyl-spermidine, putrescine or cadaverine. However, to investigate this hypothesis mutants lacking genes in the polyamine biosynthetic pathways would have to be constructed in the *tolC* mutant background. If this hypothesis were correct these double mutants may gain back the ability to express curli and form a competent biofilm.

11.5 Efflux inhibitors cause an anti-biofilm effect in various species

In addition to genetic inactivation of efflux creating a biofilm defect, chemical efflux inhibition using various efflux inhibitor molecules also induces the same defect. Three different efflux inhibitors were incubated with wild-type *S*. Typhimurium at various concentrations in a simple crystal violet biofilm assay. PAβN, a competitive inhibitor of RND efflux pumps, CCCP, a proton motive force de-coupler, and chlorpromazine which down regulates the expression of MDR efflux pumps. All three induced a dose dependent biofilm defect in the wild-type strain. This biofilm inhibition was observed well below the MIC values for each EI suggesting the inhibition is anti-biofilm in nature and that the EIs are not acting as generic antimicrobials. CCCP seems to have the biggest effect causing a significant decrease in biofilm formation as low as 1

μg/ml which would make sense as CCCP would inactivate eight out of the nine MDR efflux pumps, all with the exception of the ABC pump, MacABC, which uses ATP as its energy source. PAβN is the next effective EI with the dose dependent effect starting at 32 μg/ml, this EI would inhibit up to five of the nine efflux pumps as there are five RND MDR pumps known in *S*. Typhimurium. Chlorpromazine is the least effective with the dose dependent effect starting at 64 μg/ml, chlorpromazine is thought to act as a transcriptional repressor which would affect just *acrAB-tolC*.

Comparitive RT-PCR of *csgB*, the major structural curli subunit and *csgD*, the master curli regulator, revealed that the reason for the biofilm defect of wild-type *S*. Typhimurium upon addition of PAβN is the down regulation of curli genes. This data again suggests that accumulation of a metabolite could be responsible for the curli repression seen in the efflux mutants as chemical inhibition of efflux leaves efflux pumps *in situ* but inhibits export of substrates.

As biofilms are a major clinical problem that are a hugely antibiotic resistant, novel anti-biofilm agents are desperately needed. Biofilms are a problem both as a source of infection within the body such as *P. aeruginosa* and *B. cepacia* (Govan and Deretic 1996) in the lungs of cystic fibrosis patients and on medical biomaterials such as *S. aureus* on catheters (Zimmerman *et al.* 1988), cannulae (Thomas and Morris 2005) and hip joint prostheses (Lebeaux and Ghigo 2013). Both CCCP and PAβN, as with the majority of EIs, are toxic to human cells due to all cells including human cells containing efflux pumps. Toxic EIs would be dangerous if administered systemically however, chlorpromazine is one of a group of molecules called phenothiazines that are already used in human medicine as anti-psychotic drugs. Non-toxic EIs could be

used alone or in conjunction with standard antimicrobials to increase the efficacy as the antimicrobial will no longer be exported from the cell and will be able to work more effectively. EIs could possibly be used to coat medical biomaterials to prevent biofilms forming on their surface and subsequently prevent human infections.

Other species found to be susceptible to efflux inhibitors when forming a biofilm in the Kvist study were *E. coli*, *K. pneumonia*, *P. putida* and *S. aureus* (Kvist *et al.* 2008). A variety of other species were also tested in this project including *S. aureus*, *E. coli* and *P. aeruginosa*. All three species' ability to form a biofilm in both flow and static models were affected by exogenous addition of all three EIs tested (CCCP, PAβN and CPZ) to varying degrees. These data shows that the link between efflux and biofilm formation is not specific to *Salmonella* but seems to be a generic phenomenon effecting both Gram negative and Gram positive bacteria. The mechanism causing the biofilm defect in each species is unknown, although the genes encoding curli are seen throughout various bacterial species however curli is not an essential biofilm component in all bacteria (Dueholm *et al.* 2013).

11.6 Overall conclusions and future work

Multidrug resistance efflux and biofilm formation in *Salmonella* and a variety of other species is definitely co-regulated. Both genetic and chemical inactivation of efflux pumps, as well as artificial over expression of the pumps results in repression of the curli operons and a subsequent biofilm defect. For the *tolC* mutant the mechanism by which curli repression is occurring seems to be via a membrane stress response, already known to be responsible for curli regulation. However, whilst EnvZ inactivation rescued biofilm formation in the tolC mutant, inactivation of OmpR did

not. Further investigation into this mechanism would help develop understanding of how and why the bacteria down-regulate biofilm genes when under membrane stress. I would liked to have sequenced the genome of the *tolC* rescue mutant obtained from the transposon screen to ensure all mutations were known, as *envZ* does not seem solely responsible for curli and biofilm rescue. The obvious explanation is that upon inactivation of the pump the cell accumulates a metabolite which is sensed by a stress response gene. If the cell senses that the membrane is under stress, it would not be favourable for the bacteria to form a biofilm under those conditions.

A possible clinical application of this project is the use of chemical efflux inhibitors as anti-biofilm agents, either administered systemically with or without an antibiotic or impregnated into medical biomaterials. Although we know that addition of EIs prevent biofilms forming, future work could elucidate if EIs can disrupt already established mature biofilms. This information could again have a clinical application for disinfection of surfaces or tissues on which biofilms have already formed.

Publications associated with this study

Baugh S., Ekanayaka A.S., Piddock L.J.V. and Webber M.A. (2012) Loss of or inhibition of all multidrug resistance efflux pumps of *Salmonella enterica* serovar Typhimurium results in impaired ability to form a biofilm*. J Antimicrob Chemother.* 2012 Oct;67(10):2409-17.

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Poster presentations at international conferences

Baugh S., Ekanayaka A.S., Piddock L.J.V. and Webber M.A. Chemical Inhibition of Multidrug Resistance Efflux Abolishes Biofilm Formation by *Salmonella enterica* serovar Typhimurium. 52nd ICAAC, San Francisco, 2012, poster number C1-674.

Baugh S., Piddock L.J.V. and Webber M.A. The contribution of AcrAB-TolC to biofilm formation in *Salmonella enterica* serovar Typhimurium. 462nd WE Heraeus Seminar, Bremen, 2010.

Poster presentations at national conferences

Baugh S., Ekanayaka A.S., Piddock L.J.V. and Webber M.A. Co-ordinated regulation of multidrug efflux pumps and biofilm formation in *Salmonella* Typhimurium involves curli biosynthesis and membrane stress SGM, Manchester, 2013.

Baugh S., Piddock L.J.V. and Webber M.A. New approaches to targeting bacterial infection: Blocking biofilm formation. SET for BRITAIN, London, 2012.

Baugh S., Ekanayaka A.S., Piddock L.J.V. and Webber M.A. Co-ordinated regulation of multidrug efflux pumps and biofilm formation in *Salmonella* Typhimurium involves curli biosynthesis and membrane stress. SfAM Summer Conference, Edinburgh, 2012

Baugh S., Piddock L.J.V. and Webber M.A. Inactivation of efflux pumps inhibits biofilm formation of *S.* Typhimurium as a result of curli repression. 5th BSAC, ARM, Birmingham, 2011.

Oral presentations at international conferences

Baugh S., Ekanayaka A.S., Piddock L.J.V. and Webber M.A. Multidrug resistance efflux pumps are required for curli expression and biofilm formation in *Salmonella.* 22nd ECCMID, London, 2012.

Baugh S., Piddock L.J.V. and Webber M.A. Control of multidrug efflux pumps and biofilm formation in *Salmonella.* 21st ECCMID, Milan, 2011.

Oral presentations at national conferences

Baugh S., Piddock L.J.V. and Webber M.A. Control of multidrug efflux pumps and biofilm formation in *Salmonella.* SfAM Summer Conference, Dublin, 2011.

Baugh S., Piddock L.J.V. and Webber M.A. Control of multidrug efflux pumps and biofilm formation in *Salmonella.* Microbiology Symposium, University of Birmingham, 2011.

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