THE ROLE OF SUPERCOILING IN ALTERING CHROMOSOME STRUCTURE, GENE EXPRESSION AND ANTIBIOTIC RESISTANCE IN BACTERIA

by

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Abstract

Antibiotic resistance is a major problem estimated to cost $100 trillion and cause 10 million deaths per year by 2050. Despite novel molecules targeting Gram-positive bacteria, there are no new antibiotics active against Gram-negatives. To prolong use of current drugs, we need to understand mechanisms of resistance to inform prescribing practices and drug discovery. Quinolone resistance is primarily conferred by mutations in the target loci: DNA gyrase (*gyrA*) and topoisomerase IV. Quinolone resistance arising from *gyrA* mutations has also been shown to confer a low level of protection against a range of non-quinolone drugs. This thesis investigated the hypotheses that altered supercoiling levels, resulting from *gyrA* mutations, alter expression of stress response genes and confer a generic protective effect against other antibiotics and chemicals. The effects of equivalent *gyrA* mutations in *Salmonella* and *E. coli* upon supercoiling were analysed. Both GyrA Ser83Phe and GyrA Asp87Gly substitutions resulted in altered topoisomer profiles, although these were different between the species. When exposed to stresses, *Salmonella gyrA* mutants maintain supercoiling in a relatively fixed manner, providing a degree of antimicrobial protection but possibly limiting flexibility in response to environmental change. Fluorescent reporter assays showed a modest elevation of stress responses in *Salmonella* GyrA Asp87Gly cells, but highly upregulated stress responses in *E. coli* GyrA Asp87Gly cells. This correlated with a competitive fitness benefit of *E. coli* GyrA Asp87Gly cells vs the parent in the presence of low levels of triclosan. The elevated stress responses likely result from supercoiling-induced changes in promoter accessibility, and are probably responsible for the generic protective effect *gyrA* mutation confers against other chemicals and antibiotics. Non-quinolone antimicrobials can provide a selective pressure that favours *gyrA* mutants, although this is highly dependent on condition and species.

To: Mum, Dad, Hannah, Nana and Grandad.

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“We’re throwing science at the wall here to see what sticks. No idea what it’ll do - probably nothing.”

Cave Johnson, *Portal Two*

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Abbreviation	Definition
Å	Ångström
AHVLA	Animal Health & Veterinary Laboratory Agency
Amp	Ampicillin
ARG	Antimicrobials Research Group
Atn	Aztreonam
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
BMJ	British Medical Journal
BRIG	BLAST Ring Image Generator
BSAC	British Society for Antimicrobial Chemotherapy
CARD	Comprehensive Antibiotic Resistance Database
Caz	Ceftazidime
cDNA	Complementary DNA
Chl, Chlor	Chloramphenicol
Cip, Cipro	Ciprofloxacin
CPZ	Chlorpromazine
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic acid
EHEC	Enterohaemorrhagic *E. coli*
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
EUCAST	European Committee of Antimicrobial Susceptibility Testing
ExPEC	Extraintestinal pathogenic *E. coli*
FabI	Enoyl-Acyl Carrier Protein Reductase
FIS	Factor for Inversion Stimulation
g	Grams
g	Gravity
Gent	Genatmicin
GFP	Green Fluorescent Protein
Gyr(A/B)	DNA Gyrase (subunit A/B)
h	Hours
H-NS	Histone-like Nucleoid Structuring Protein
IHF	Integration Host Factor
Kan	Kanamycin
kb	Kilobases
kV	Kilovolts
L	Litres
LB (agar/broth)	Luria-Bertani (agar/broth)
M	Molar
MFS	Major Facilitator Superfamily (efflux pump)
mg	Milligrams
MIC	Minimum Inhibitory Concentration
mL	Millilitres
mM	Milimolar
Mnd	Menadione

Abbreviation	Definition
mRNA	Messenger RNA
ms	Milliseconds
MS	Microsoft
n/a	Not applicable
n/t	Not tested
Nal	Nalidixic Acid
NCBI	National Center for Biotechnology Information
ng	Nanograms
nm	Nanometres
Nov	Novobiocin
ºC	Degrees Centigrade
Par(C/E)	Topoisomerase IV (subunit C/E)
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
pmol	Picomoles
PMQR	Plasmid Mediated Quinolone Resistance
Pqt	Paraquat
QRDR	Quinolone Resistance Determining Region
RNA	Ribonucleic Acid
RND	Root Nodulation Division (efflux pump)
rpm	Revolutions per minute
rRNA	Ribosomal RNA
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SDW	Sterile distilled water
SHX	Serine hydroxamate
SPI	*Salmonella* Pathogenicity Island(s)
Sucr	Sucrose
TAE	Tris-Acetate-EDTA
TBE	Tris-Borate-EDTA
Tet	Tetracycline
Topo	Topoisomerase
Trc	Triclosan
tRNA	Transfer RNA
UK	United Kingdom
UPEC	Uropathogenic *E. coli*
USA	United States of America
US$	United States dollar(s)
V	Volts
WT	Wild type
XLD	Xylose Lysine Deoxycholate
µg	Micrograms
µL	Microlitres
-ve	Negative

Chapter One

Introduction

1. Introduction

1.1 Antibiotics

The name “antibiotic” comes from the Greek term “*αντι-βιος*”, meaning “against life”, and in practice describes compounds that are used to treat bacterial infections. While the term “antimicrobial” can be applied to antibiotics, it is an umbrella term that also includes agents that have activity against viruses (antivirals and/or antiretrovirals) and/or fungi (antifungals). In common parlance, antibiotics are chemical compounds that specifically target bacteria and either kill them (termed “bacteriocidal” antibiotics), or prevent their growth (termed “bacteriostatic” antibiotics) (Pankey and Sabath, 2004). Antibiotics act by targeting and interfering with essential cellular processes, or the mechanisms controlling said processes (Zaffiri *et al.*, 2012). Antibiotics are used in medicine and animal husbandry for the treatment and prevention of bacterial disease. The majority of antibiotics have been derived from molecules that occur naturally, and have been isolated from microbes - primarily the Actinomycetes (Barka *et al.*, 2016). In their natural environment, antibiotics are produced by bacteria to prevent the growth of competing organisms (Baltz, 2008).

1.1.1 Early Discovery – Fleming, Penicillin & Other Early Antibiotics

Penicillin, while not the first modern antibiotic, is arguably the most famous antibiotic. A product of *Penicillium notatum* accidentally discovered by Alexander Fleming in the 1920s (Fleming, 1929), penicillin is a broad-spectrum beta lactam that acts by binding to Penicillin Binding Protein to disrupt bacterial cell wall synthesis (Spratt, 1977). Fleming was awarded the Nobel Prize in Physiology or Medicine in 1945 for the discovery of penicillin, an award that he shared with Florey and Chain, who

realised its therapeutic benefits (Raju, 1999). However, penicillin was not easily purified, and the earliest mention in the literature of it being produced in large quantities was not until 1943 (Clifton, 1943), with a patent for its industrial purification being granted in 1947 (Jacques L Watchell, 1947). The first widely used antibiotics were the sulphonamides (Bickel, 1988), which were derivatives of a dye and first described by Domagk (1935). Marketed as Prontosil, the first sulphonamide in use was effective against Gram-positive cocci (Gibberd, 1937) and was used by Domagk to cure his daughter of an infection (Bentley, 2009). He was awarded the Nobel Prize in Physiology or Medicine in 1939 for his work (Nobel Foundation, 1965), but was unable to accept it because of the political situation in his native Germany at the time (Nobel Media AB, 2014). Since these initial discoveries were made, a wide range of antibiotics belonging to different classes were discovered allowing new treatments for many infections to be introduced.

1.1.2 Uses of Antibiotics

Antibiotics are used to treat bacterial infections, both in humans and animals. They are also used prophylactically, to prevent patients undergoing surgery from acquiring infections (Kavanagh *et al.*, 2014), and this usage has been shown to reduce mortality in immunocompromised individuals, *e.g.* cancer patients (Schlesinger *et al.*, 2009). Antibiotics are also used to promote growth in livestock (Phillips *et al.*, 2004) in some parts of the world, including the United States of America. The use of antibiotics for this purpose was banned in Sweden in 1986, followed by Denmark a decade later, and then banned throughout the European Union by 1999 (Casewell *et al.*, 2003). After Denmark stopped using antibiotics for growth promotion in livestock,

the amount of antibiotics used to treat infections in livestock increased each year until 2009. However, the amount of antibiotics used for the treatment of infections each year after the ban was enacted was still less than the total amount of antibiotics used each year before the ban was put in place (Statens Serum Institut *et al.*, 2015).

Antibiotic use is controlled in Northern Europe and North America, requiring a prescription from a medical professional. However, in developing countries, many antibiotics can be obtained over-the-counter without a prescription (Morgan *et al.*, 2011). Antibiotics can also be obtained without a prescription online (Boyd *et al.*, 2017). Unregulated antibiotic use contributes to the development and spread of antibiotic resitance, as patients may take wrong or unnecessary antibiotics, or a dose which is too low to clear the infection (Review on Antimicrobial Resistance, 2015a).

1.1.3 Overview of Antibiotic Targets

Antibiotics act by interfering with a process and/or component essential to bacterial viability, either killing cells or stopping them from growing any further. Ideally, antibiotics should target components which are specific to bacteria, so as to minimise the possibility of negative effects, *e.g.* toxicity, affecting the patient (Duffield *et al.*, 2010), The main ways antibiotics function are as follows (Lange *et al.*, 2007):

- Disruption of the cell envelope's integrity – *e.g.* penicillin and other beta lactam antibiotics, which bind to penicillin binding proteins and prevent peptidoglycan from being incorporated into the cell wall. The cell continues to attempt to grow, but as it cannot remodel the cell wall, this leads to a build-up of internal pressure inside the cell and, ultimately, lysis (Spratt, 1980). Daptomycin and

colistin are two antibiotics that disrupt the integrity of the cell membrane by triggering a loss of membrane potential, leading to the leakage of the cell's internal contents (Falagas and Kasiakou, 2005; Silverman *et al.*, 2003). It has been shown that exposure of bacteria to polymixins ultimately leads to cell lysis (David and Rastogi, 1985; Mohamed *et al.*, 2016).

- Protein synthesis inhibition is a viable antibacterial strategy as protein production is an essential process that is targeted by various antibiotics – for example, mupirocin inhibits the tRNA synthetase that loads isoleucine onto its respective tRNA molecules, thus preventing isoleucine from being incorporated into polypeptide chains (Nakama *et al.*, 2001). Chloramphenicol and tetracycline stop tRNAs from entering the A site of the ribosome, which stops the amino acids loaded on them from being added to nascent polypeptide chains (Connell *et al.*, 2003; Schlünzen *et al.*, 2001).
- Inhibition of nucleic acid synthesis – rifampicin and other rifamycins target the β subunit of the bacterial RNA polymerase and physically block it from being able to lengthen the nascent chain of nucleotides (Floss and Yu, 2005). Quinolones target the complexes formed between Type II topoisomerases and DNA, which are involved in the decatenation of daughter chromosomes and relaxing supercoils introduced into the chromosome by replication and transcription forks, and lead to an accumulation of double stranded breaks in the DNA (Redgrave *et al.*, 2014). As they are one of the focal points of this work, quinolones will be discussed in more detail later.
- It is also possible to inhibit essential enzymes. Two examples are the inhibition of fatty acid biosynthesis and folate biosynthesis. Triclosan is a widely-used

biocide that targets FabI, and inhibits fatty acid biosynthesis (McMurry *et al.*, 1998). Although a biocide and not an antibiotic, it is of relevance as it has been shown that mutations in *gyrA* confer reduced susceptibility to triclosan (Webber *et al.*, 2008). Trimethoprim is an example of an antibiotic that prevents folate biosynthesis in bacteria by inhibiting the dihydrofolate reductase enzyme (Quinlivan *et al.*, 2000). The sulphonamides target a different enzyme in the folate biosynthesis pathway (Mondal *et al.*, 2015). Folate is a cofactor for the enzymes used in the synthesis of a variety of essential cellular components, such as nucleic acids and amino acids. Although folate is required by all forms of life, mammals cannot synthesise their own folate and instead acquire it through their diet, making the bacterial enzymes involved in its synthesis good drug targets (Bermingham and Derrick, 2002; Hawser *et al.*, 2006).

1.1.4 Discovery Void

Most of the classes of antibiotics in use today were discovered in the 1950s through to the mid-1970s (Silver, 2011). Traditionally, antibiotics have been isolated from screening the products made by soil microorganisms (Butler *et al.*, 2016). There has, however, been a subsequent “discovery void” with only four new classes of antibiotic having been discovered since. This is demonstrated graphically in Figure 1.1.4.1. It has been proposed that one of the reasons for the lack of new classes of compounds is that all the major potential targets have been discovered and utilised, so there are no new targets, and thus no new classes of compounds, that could be developed effectively (Bumann, 2008; Silver, 2011). It has also been observed that the

continued use of a natural product screening approach has led to a high rate of rediscovery of existing antibiotics (Baltz, 2006). Other factors contributing to the stalling of the antibiotic development pipeline are the time, financial cost and low success rate. The development of a new antibacterial agent by a company that has the workforce and financial ability to invest in doing so, such as GlaxoSmithKline, can take at least a decade going from high-throughput screening of compounds all the way through to optimisation, clinical trials and the agent's launch (Payne *et al.*, 2007).

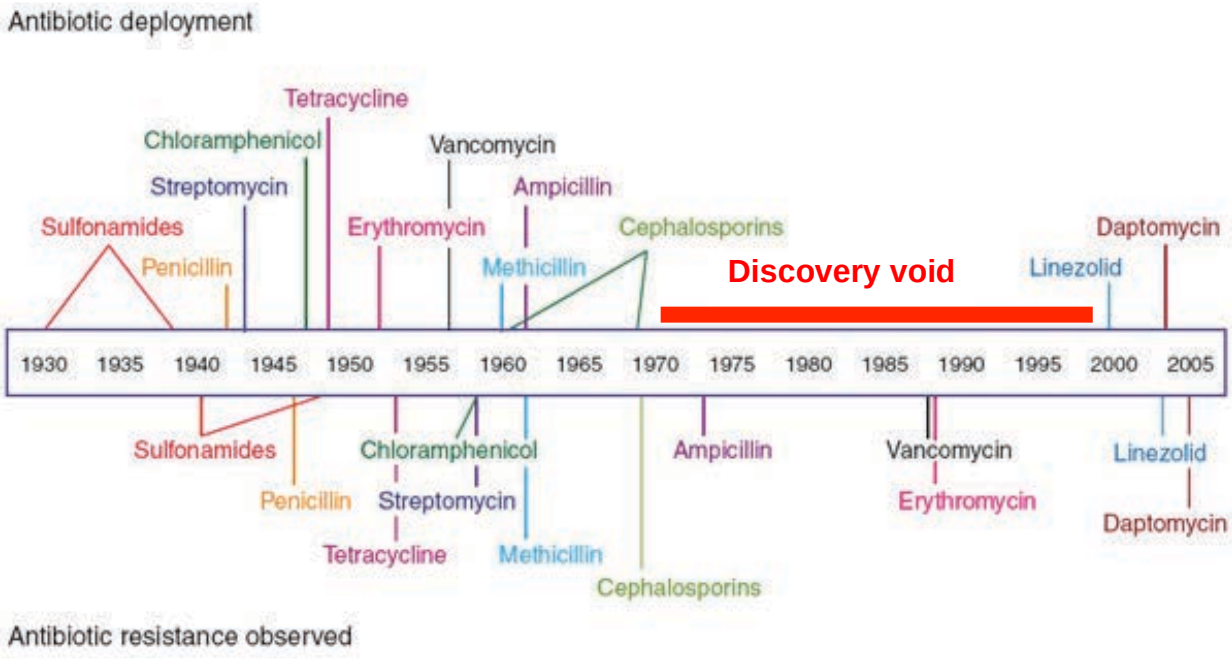


Figure 1.1.4.1: Timeline of antibiotic development, introduction and resistance

Timeline showing when major antibiotics were introduced into clinical use (top), as well as when clinical resistance (bottom) to those antibiotics was first reported. Resistance to linezolid and daptomyicn was observed witin five years of their introduction. The section marked in red, between 1970 and 2000, marks a time often referred to as the “discovery void”, when no new classes of antibiotics were discovered or deployed (adapted from Clatworthy *et al.*, 2007). There have since been new classes of antimicrobial agents discovered, such as teixobactin, but these require further development before they may be used in a clinical setting.

1.2 Antibiotic Resistance

Antibiotic resistance occurs naturally and has evolved to protect bacteria against antimicrobial threats in the environment. Metagenomic analysis of 30,000-year-old permafrost from Canada has identified a vancomycin resistance locus similar to that found in modern *Staphylococcus aureus* (D'Costa *et al.*, 2011), and phylogenetic analyses have shown serine beta lactamase enzymes to be over two billion years old (Hall and Barlow, 2004). A recent genomic analysis of a *Shigella flexneri* strain, the first strain to be stored in the National Collection of Type Cultures and which was isolated from a soldier who died of bacillary dysentery in World War 1, showed it to be naturally resistant to penicillin and erythromycin, despite these antibiotics not having been discovered or used in a clinical setting until several decades later (Baker *et al.*, 2014). The reliance on naturally derived antibiotics in human medicine results in a high likelihood that resistant strains will already exist in nature.

According to the European Committee on Antimicrobial Susceptibility Testing, bacteria are defined as being resistant to antibiotics when they possess a resistance mechanism against the agent that is being tested, and if the minimum inhibitory concentration of that antibiotic agent exceeds a defined value, or "breakpoint", and is "associated with a high likelihood of therapeutic failure" (European Committee on Antimicrobial Susceptibility Testing, 2013). Antibiotic resistance is a term based on clinical definitions and should only really be used in such contexts, but organisms defined as "wild type" may also intrinsically resist treatment from particular antibiotics if they lack the components which are targeted by the antibiotics in question (European Committee on Antimicrobial Susceptibility Testing, 2013).

Whilst resistant strains that pre-exist in nature can be selected for through the use of antibiotics, it is also possible for resistance to emerge *de novo.* Upon being awarded the Nobel Prize for Physiology or Medicine in 1945, Fleming said in his acceptance speech that, “it is not difficult to make microbes resistant to penicillin in the laboratory by exposing them to concentrations not sufficient to kill them, and the same thing has occasionally happened in the body”, and also feared of a time penicillin would be available over the counter, as it would increase the potential for its misuse (Fleming, 1999).

A more recent antibiotic family, the oxazolidinones, were approved for use in the early 2000s, although they were originally patented in the 1970s (Silver, 2011) and the development of resistance was observed not long after their introduction into use, even allowing for modern pharmacokinetic guidelines (Marchese and Schito, 2001).

Rises in numbers of resistant isolates have altered therapeutic options. Colistin, a polymyxin that kills bacteria by disrupting the structure and stability of their membranes, was retired from clinical use in the 1970s because of concerns over the toxicity of antibiotics of this class (Yu *et al.*, 2015). However, it was brought back into use in the 1990s because of increasing levels of resistance to other antibiotics amongst Gram-negative bacteria, and the dearth of novel antibiotics (Zavascki *et al.*, 2007). As a result, it is widely regarded as the last line of defence against some antibiotic-resistant Gram-negatives. Colistin resistance has long been known to be possible, and is classically associated with chromosomal mutations in genes

controlling lipopolysaccharide structure. However, towards the end of 2015, plasmid-mediated polymyxin resistance alleles were identified in south-eastern China, and the plasmid on which they are contained, pHNSHP45, is capable of transforming *E. coli, K. pneumoniae* and *P. aeruginosa* with relative ease in a laboratory setting. Subsequently, the *mcr1* gene has been seen in isolates from patients in the USA, showing the possibility for this plasmid to spread easily in the "wild" (Liu *et al.*, 2016). These examples illustrate the dynamic nature of the evolution of resistance mechanisms.

1.2.1 Problem, Scale and Outlook

It has been suggested that, unless something is done about antibiotic resistance, the inability to treat bacterial infections with antibiotics would render surgery and chemotherapy, amongst other procedures, highly risky as the chance of infection would be greatly elevated (Davies, 2013). Dame Sally Davies, the Chief Medical Officer for Her Majesty's Government, has gone on record to say that the threat of global antimicrobial resistance is as severe as the threats from terrorism and global warming (McCarthy, 2013). In their 2016 report on global risks and their impacts, the World Economic Forum considered the impact of the spread of infectious disease to be as severe as those of another global financial meltdown, cyber-terrorism attacks and global food shortages, but about as likely as a "critical information infrastructure breakdown". One of the main concerns raised by the World Economic Forum was that the mechanisms by which infections spread do not respect political borders (World Economic Forum, 2016).

Although antibiotics have been a major driver in the advancement of healthcare and are arguably the cornerstone of modern medicine, there have been other factors contributing to improved clinical outcomes, such as improved hygiene and vaccination programmes (Edwards, 2016). Politicians, pharmaceutical companies and investors have started taking action to address the problem – action which is considered too late in some circles (Smith and Coast, 2013).

The European Commission was encouraged to foster co-operation between the different agencies and members of the European Union by coming up with suitable incentives for the development of new antimicrobials. The European Parliament later declared an official stance of antimicrobial resistance being an issue of major concern, and recommended that the European Commission come up with a plan to deal with the problem. The Commission presented a twelve-point five-year plan of action, which, in broad strokes, encompasses improving the regulation of antibiotic use, preventing the unnecessary use of antibiotics in animals, improving hygiene levels to prevent infection, and making a concerted effort to monitor the levels of antibiotic resistance throughout the European Union (European Commission, 2011).

A recent review by Lord O'Neill of Gatley analysed the problem of antibiotic resistance from an economic standpoint and present recommendations on how to solve the crisis. Lord O'Neill's committee has published a series of reports, stating that around 700,000 people currently die per year worldwide as a result of antimicrobial-resistant infections, and that, left unchecked, this will likely increase to 10,000,000 deaths per annum in the year 2050 (Review on Antimicrobial Resistance,

2014). This would also lead to a global loss of productivity to the tune of US$100 trillion. The main recommendations of the committee are to increase investment in antibiotic research (Review on Antimicrobial Resistance, 2015b), to incentivise pharmaceutical companies to develop more antibiotics (Review on Antimicrobial Resistance, 2015c), and to be more protective of the antibiotics we currently have (Review on Antimicrobial Resistance, 2015b).

Whilst novel drugs are not likely to be introduced into clinical use in the immediate short term, increased efforts in researching lead compounds are promising. For example, teixobactin, a novel antimicrobial compound, has been discovered using the “iChip” method to culture microorganisms in their native environments as opposed to the lab, This allows around 50% of soil-borne bacteria to grow, a fifty-fold increase on conventional methods (Ling *et al.*, 2015). However, it will be many years before any novel drugs pass through all the stages of drug development that come in-between the initial discovery phase of a compound and its deployment as a medicine – even in well-funded pharmaceutical companies, this can take around fourteen years (Payne *et al.*, 2007). Teixobactin itself shows good activity against Gram-positive bacteria with a low rate of selection for mutants with reduced susceptibility (Ling *et al.*, 2015). Unfortunately, teixobactin is inactive against *E. coli* and other Gram-negative bacteria (Piddock, 2015). Its inactivity against them is a concern as the majority of pathogens that concern the National Health Service are Gram-negative organisms (Davies, 2013; Davies and Gibbens, 2014) and there is consequently less demand for agents that are active against Gram-positive organisms.

Although new drugs are needed, improved diagnostics could greatly reduce antibiotic misuse and ensure the appropriate use of antibiotics is improved. The Longitude prize, originally set up in the 18th Century to incentivise people to solve the problem of how to tell the time at sea, is, in its current incarnation, a competition to develop a cheap, fast, simple and effective test to distinguish between bacterial and viral infections, and ideally also identify the pathogen's antibiotic susceptibility profile (Nesta, 2014).

Other proposed ways of combatting the problem of antibiotic resistance include:

- Combination therapies, *e.g.* treating an infection with both a beta lactam and a beta lactamase inhibitor at the same time (Nesta, 2014). Combination therapy has been shown to not select for the development of resistance (Yeh *et al.*, 2009), and is considered to be one of the only options left for treating extensively-drug resistant *Enterobacter cloacae* infections (Cai *et al.*, 2016). However, combination therapy is likely little more than a "stop-gap" solution – there have been reports of failure to successfully treat *Neisseria gonorrhoeae* through the simultaneous use of ceftriaxone and azithromyocin in a clinical setting (Fifer *et al.*, 2016).
- "Antibiotic cycling" – sequentially treating an infection with a number of different antibiotics, starting and ending with the same drug. The idea behind this is that it is energetically unfavourable for bacteria to maintain mechanisms to reduce their susceptibility towards agents to which they are not currently being exposed, meaning that if they tolerate antibiotic X and are exposed to

antibiotic Y and then antibiotic Z, they will no longer tolerate antibiotic X when they are treated with it again (Fuentes-Hernandez *et al.*, 2015; Imamovic and Sommer, 2013). Computer simulations of this approach have appeared promising, but it has not been shown to be any more effective in real life than current treatments (Cobos-Trigueros *et al.*, 2016). The revival of old antibiotics (Bergen *et al.*, 2012) could be interpreted as using this principle on a longer-term scale.

- Vaccination (Review on Antimicrobial Resistance, 2016) – by challenging the immune system with fragments of bacterial antigens, antibodies against said antigen are produced. This leads to the development of memory cells that will produce the same antibody should the antigen be encountered again (Hajj Hussein *et al.*, 2015). Some examples of vaccination against bacterial infections include: the diphtheria vaccine was introduced in the UK in 1940 and led to a reduction in reported cases, from over 61,000 cases and 3,283 deaths in 1940, to 38 cases and six deaths in 1957 (Sailsbury and Ramsay, 2013); vaccines are used to inoculate poultry against *Salmonella* infections (Preisler *et al.*, 2006); trials of an *Acinetobacter baumannii* vaccine in mice have shown promise (Shu *et al.*, 2016); vaccines against the common microbial cell surface antigen poly-N-acetyl glucosamine have undergone phase I clinical trials in humans (Skurnik *et al.*, 2016), and the UK has vaccinated against some forms of *Neisseria meningitidis* since 1999 (Ladhani *et al.*, 2016).
- Phage therapy - lytic bacteriophages were discovered in the early 20th century and were used to treat dysentery and cholera (Domingo-Calap *et al.*, 2016).

They were largely forgotten by the West in the wake of the Second World War and the discovery of antibiotics, but remained a popular field of study in the Soviet Union and its allied nations. However, the clinical trials conducted in the Eastern Bloc are considered to have been mismanaged in hindsight (Sulakvelidze *et al.*, 2001). Bacteriophages invade a host cell, utilise the host's replication machinery to make copies of their genome, surround the resulting copies of the genome within capsids, and then cause the host cell to lyse. Phages can also propagate their genome lysogenically, incorporating their genome into that of their host (Sulakvelidze *et al.*, 2001). Modern studies have shown the potential applications of phage therapy include, but are not limited to, the treatment of urinary tract infections (Sybesma *et al.*, 2016) and the prevention and treatment of bacterial infections in food animals (Atterbury *et al.*, 2007). It has been suggested that, as bacteria can evolve to be less susceptible to antibiotics, they can also evolve to be less susceptible to treatment with phage, but there is a counter-argument that phages themselves can in turn evolve to overcome this (Koskella and Brockhurst, 2014). Although the T4 bacteriophage has been shown to be safe for human consumption (Bruttin and Brüssow, 2005), there remains no officially approved phage treatment for human use in the West (Lu and Koeris, 2011).

- In a similar vein to bacteriophages, *Bacteriovorax* is a genus of bacteria that are obligate predators of other bacteria. *Bdellovirbio bacteriovorus,* a member of this genus (Chen *et al.*, 2015), has been shown to prey on *Escherichia*, *Acinetobacter*, *Salmonella*, *Campylobacter*, *Pseudomonas*, *Klebsiella* and *Shigella* species, amongst others (Dashiff *et al.*, 2011). It has also been shown

to have activity against *Staphylococcus aureus* biofilms (Monnappa *et al.*, 2014). The lipopolysaccharides of *B. bacteriovorus* have been shown to be less effective at inducing the human immune response compared with *Escherichia coli* (Schwudke *et al.*, 2003), leading some groups to suggest that *Bdellovibrio* could be exploited therapeutically (Dwidar *et al.*, 2012).

1.2.2 Overview of Resistance Mechanisms

There are many factors that can influence the selection and expansion of antibiotic resistant bacteria (Stokes and Gillings, 2011). Resistance often develops in response to selective pressure in a classically Darwinian manner; mutants within large populations resistant to an antibiotic will proliferate when exposed to the antibiotic, at the expense of susceptible strains (Rodríguez-Rojas *et al.*, 2013; Sykes, 2010). More recently, the powerful selective pressure exerted by low-levels of antimicrobials such as those found in the environment has been shown to select for resistance alleles and resistant strains (Andersson and Hughes, 2012; Andersson and Hughes, 2014). The carriage of antibiotic resistance can have an associated fitness cost, particularly if mediated by alterations to target sites which encode essential enzymes (Deris *et al.*, 2013; Spratt, 1996). Subsequent additional mutations are often able to compensate for the fitness cost (Björkman *et al.*, 2000), restoring growth levels to normality and antibiotic resistant isolates from patients are often able to grow as well as susceptible strains.

Resistance to a drug may occur as a result of a number of different methods:

- Enzymes may develop which degrade or alter drugs and prevent their function. For example, penicillin and other beta lactams can be degraded by

beta lactamases, preventing their binding to the target penicillin binding proteins (Jovetic *et al.*, 2010).

- The target site of the antibiotic can be altered or protected. Mutations can arise in the target site for an antibiotic (Spratt, 1994), preventing the drug from interacting with the target and thus having any effect. These are often single nucleotide changes that alter amino acids at critical positions. Mutations conferring quinolone resistance, such as aspartate to glycine substitutions at codon 87 (GAC to GGC) of GyrA in *S.* Typhimurium are an example of this (Brown *et al.*, 1996). Targets may be protected by the addition of different groups. Examples include the methylation of ribosomes to protect against aminoglycosides, and the decoration of topoisomerases with pentapeptide repeats to block quinolone activity. This stops the antibiotic from having an effect, but does not prevent the target from carrying out its normal function (Blair *et al.*, 2015b).
- Cells may prevent access of drugs to targets, either by changes to membrane structure, in particular loss of porin expression in Gram-negative bacteria or by increasing expression of multi-drug or drug-specific efflux pumps, such as the AcrAB-TolC system, both resulting in a reduced amount of antibiotic accumulation at target sites (Nikaido *et al.*, 2008).
- Finally, bacteria can acquire new alleles that replace the function of an antibiotic target, but are themselves resistant to the action of the antibiotic. For example, the *mecA* gene, encoding an alternative penicillin binding protein and conferring methicillin resistance to *Staphylococcus aureus*, is a classic example of this ‘metabolic bypass’ (Hao *et al.*, 2012).

For some antibiotics, multiple mechanisms of resistance are relevant.

1.2.3 Quinolones

Quinolones are synthetic compounds derived from 1,8-naphthyridine that possess antimicrobial activity. The earliest description of a quinolone was in 1949, as a modification of melicopine (Price, 1949). Quinolones with antibiotic activity were not discovered until a few years later, as impure by-products arising from the production process of quinines (Mitscher, 2005). The activity of these molecules against Gram-negative bacteria looked promising, and the first paper describing their *intentional* synthesis and their effectiveness was published in 1962, with particular interest being given to 1-ethyl-7-methyl-1,8-naphthyridin-4-one-3-carboxylic acid, now more commonly known as nalidixic acid (Lesher *et al.*, 1962). Since the synthesis of nalidixic acid, a large number of derivatives have also been synthesised. Table 1.2.3.1 shows a number of quinolones from each of the four existing generations, their chemical formulae and their uses, and Figure 1.2.3.1 shows the structures of seven quinolones.

Gen.	Drug	Formula	Usage
I	Nalidixic acid	$C_{12}H_{12}N_2O_3$	Urinary tract infections and gastroenteritis (Hopkins *et al.*, 2005)
	Cinoxacin	$C_{12}H_{12}N_2O_5$	Discontinued (Hopkins *et al.*, 2005).
II	Norfloxacin	$C_{16}H_{18}FN_3O_3$	Withdrawn in Europe (Committee for Medicinal Products for Human Use, 2009).
	Ciprofloxacin	$C_{17}H_{18}FN_3O_3$	Urinary tract infections, respiratory infections, gastroenteritis and joint infections (BMJ Group and Royal Pharmaceutical Society of Great Britain, 2016a).
	Levofloxacin	$C_{18}H_{20}FN_3O_4$	Respiratory infections (BMJ Group and Royal Pharmaceutical Society of Great Britain, 2016a).
III	Sparfloxacin	$C_{19}H_{22}F_2N_4O_3$	Withdrawn; linked to heart failure (Ball, 2003; Qureshi *et al.*, 2011).
	Gatifloxacin	$C_{19}H_{22}FN_3O_4$	Withdrawn; linked to dysglycemia (Mehlhorn and Brown, 2007; Qureshi *et al.*, 2011).
	Grepafloxacin	$C_{19}H_{22}FN_3O_3$	Withdrawn; linked to cardiac arrests (Ball, 2003; Qureshi *et al.*, 2011).
	Tosufloxacin	$C_{19}H_{15}F_3N_4O_3$	Mainly used in Japan to treat a variety of conditions – some side effects (Niki, 2002).
IV	Trovafloxacin	$C_{20}H_{15}F_3N_4O_3$	Withdrawn; linked to liver failure (Ball, 2003; Qureshi *et al.*, 2011).
	Moxifloxacin	$C_{21}H_{23}FN_3O_4$	Respiratory, skin and intra-abdominal infections, and pelvic inflammation (Tulkens *et al.*, 2012).
	Gemifloxacin	$C_{18}H_{20}FN_5O_4$	Not approved in Europe (European Medicines Agency, 2009).

Table 1.2.3.1: Examples of quinolones. Table showing examples of quinolones and their uses, ordered by generation. Nalidixic acid acid has a much narrower range of targets than later quinolones, as it only displayed effectiveness against Gram negative organisms causing urinary tract infections and gastroenteritis. The main structural differences between first generation quinolones and later quinolones is the addition of a fluorine atom at the C-6 position, and additional inclusions at the C-7 position. Quinolones which have a fluorine atom at the C-6 position are referred to as fluoroquinolones.

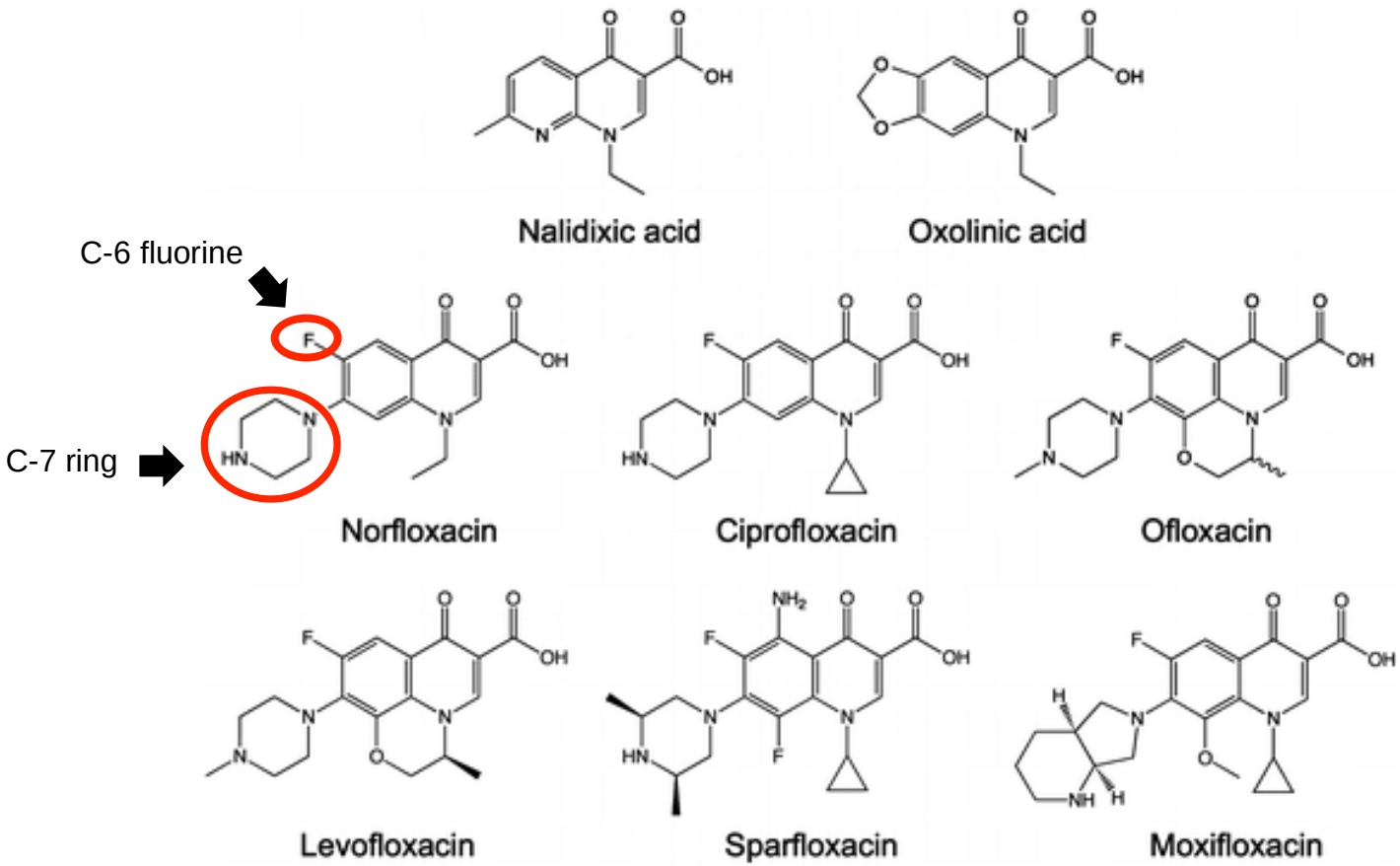


Figure 1.2.3.1: Chemical structures of a selection of quinolone antibiotics. The structures of the first-generation quinolone, nalidixic acid, and a selection of fluoroquinolones are shown. The core structure common to all quinolones is a naphthalene-like structure of two fused six-carbon rings, with a nitrogen atom replacing the carbon at position 1, a carboxyl group at position C-3, and a ketone group at position C-4. The key differences between nalidixic acid and later quinolones are the addition of the C-7 ring, and the fluorine atom at position C-6, as circled on norfloxacin. Figure adapted from Aldred *et al.*, 2014.

1.2.3.1 Uses and Importance

Quinolones are widely used in both human and veterinary medicine for the treatment of bacterial infections (Aldred *et al.*, 2014; Redgrave *et al.*, 2014). They are broad-spectrum antibiotics that disrupt the ability of bacterial Type IIa topoisomerases to disentangle DNA (Drlica, 1999; Redgrave *et al.*, 2014) . The use of quinolones to treat children and pregnant women is not recommended as a link to joint problems has been observed in young animals, nor is their use recommended in cases where the patient has pre-existing heart complaints (BMJ Group and Royal Pharmaceutical Society of Great Britain, 2016a). A number of quinolones have been withdrawn from use, and others have failed to pass the clinical trial stage, either because of toxicity concerns or because they have since been supplanted by better medicines (Ball, 2003; Hopkins *et al.*, 2005; Mehlhorn and Brown, 2007; Qureshi *et al.*, 2011) - see Table 1.2.3.1 for more details. Nalidixic acid, the first quinolone to be used, only displayed effectiveness against Gram-negative bacteria causing urinary tract infections. Subsequent modifications, including the addition of a fluorine molecule to the carbon at the sixth position of the core aromatic ring structure (Bolon, 2011), have broadened the range of pathogens against which the antibiotics are effective. Fluorinated quinolones are referred to as fluoroquinolones. The division of quinolones into generations is largely based on shared components of their structures, and the bacteria against which they were effective upon their introduction (Andriole, 2005; Ball, 2000; Bolon, 2011).

Ciprofloxacin, a second-generation fluoroquinolone, is considered by the World Health Organization to be an essential medicine for the treatment of a wide range of

bacterial infections. The British National Formulary advises that ciprofloxacin may be used for the treatment of respiratory tract infections, urinary tract infections, prostatitis, gonorrhoea, anthrax, the prevention of meningococcal meningitis and the prevention of the development of infections during surgery, and the treatment of most other infections (BMJ Group and Royal Pharmaceutical Society of Great Britain, 2016b). It may also be used to treat some cases of perianal fistulae that can occur as a result of Crohn's disease (BMJ Group and Royal Pharmaceutical Society of Great Britain, 2016c), and corneal infections (BMJ Group and Royal Pharmaceutical Society of Great Britain, 2016d). Levofloxacin is also considered an essential medicine; it may be used in the treatment of tuberculosis (World Health Organization, 2015). The World Health Organization defines essential medicines as those that address the healthcare needs of a population to a satisfactory standard (World Health Organization, 2016). Levofloxacin is considered a second-line medicine for the treatment of a range of respiratory infections in Britain (BMJ Group and Royal Pharmaceutical Society of Great Britain, 2016a).

As discussed in my review (Redgrave *et al.*, 2014), although prescribing guidelines advise that fluoroquinolones should be reserved, resistance rates continue to rise. In the United Kingdom, 7% of *E. coli* isolates were classified as resistant in 2001, and this figure had risen to 23% by 2006. This eventually fell to 19% by 2011. In *Klebsiella* spp., approximately 10% of isolates were classified as resistant to fluoroquinolones in 2001, rising to 18% in 2007, and falling again to 10% by 2009 (Livermore *et al.*, 2013).

1.2.3.2 Mechanism of Action

Quinolones target the GyrA subunit of the Type IIa topoisomerase, DNA gyrase, in most Gram-negative bacteria, and the equivalent protein subunit in Topoisomerase IV of most Gram-positive bacteria, ParC, though this can vary depending on the quinolone and/or bacterial species. They inhibit the ability of the enzymes to reseal double stranded DNA breaks created during the relaxation of DNA by intercalating with the enzyme-DNA complex to form an enzyme-drug-DNA complex, or “ternary complex”. This ultimately leads up to an accumulation of double stranded DNA breaks, and cell death (Drlica *et al.*, 2008). Quinolones specifically target GyrA, forming ionic bonds with Ser83 and Asp87, or the amino acids at the equivalent positions in ParC. This interaction is mediated by a water-metal ion bridge (Aldred *et al.*, 2014). The amino acids in GyrA targeted by the quinolones are very close to important residues in the enzyme, with the residues His78 and His80 having been shown to be key determinants of the ability of GyrA to cleave DNA in *E. coli* (Hockings and Maxwell, 2002). The crystal structure of the enzyme-drug-DNA complex has been solved at a resolution of 2.4 Å for DNA gyrase from *Mycobacterium tuberculosis*, in a complex with DNA and the quinolone moxifloxacin (Blower *et al.*, 2016). This is shown in Figure 1.2.3.2.1.

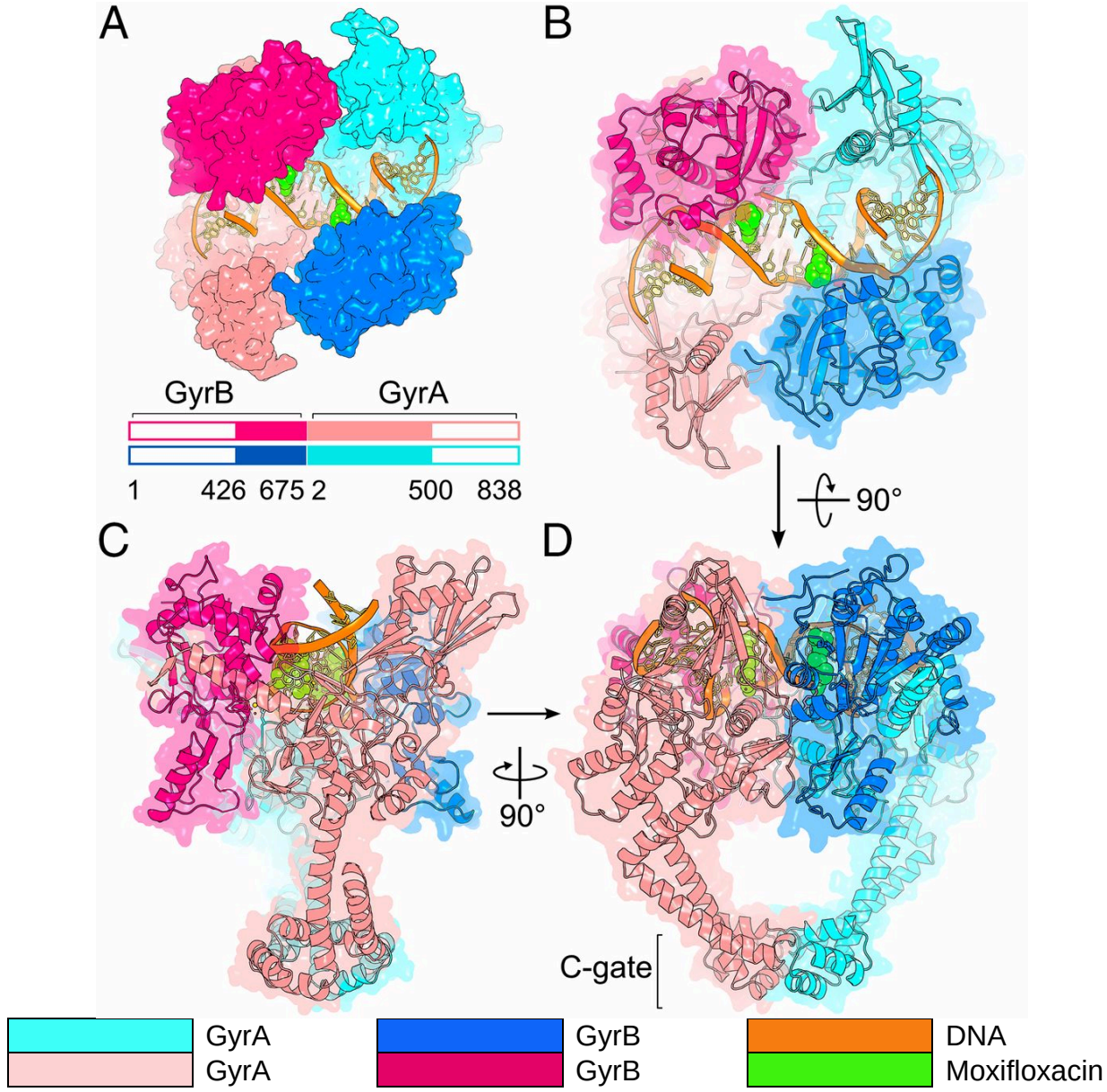


Figure 1.2.3.2.1: Structure of DNA Gyrase in complex with DNA and moxifloxacin. A) Space-filling model of two *Mycobacterium tuberculosis* GyrBA protein fusions (blue and pink; the pale colours represent GyrA, and the dark colours are GyrB) in complex with DNA (orange) and the quinolone moxifloxacin (green). B) Ribbon model of part A). Parts C) and D) provide additional views of part B), rotated 90º about the axes shown. The C gate, which is labelled in part D), is the region of the enzyme through which DNA is passed after being cleaved and religated. Figure from Blower *et al.* (2016).

1.2.4 Quinolone Resistance Mechanisms

The main mechanism of conferring reduced susceptibility to quinolones is the acquisition of mutations in *gyrA* and *parC*, resulting single amino-acid substitutions in the active site of the enzyme. The most common clinically-relevant substitutions are found at GyrA Ser83 and GyrA Asp87 in *Escherichia coli* and *Salmonella* spp. (Everett *et al.*, 1996; Webber *et al.*, 2013), though these are not the only substitutions that can arise (S. Baker *et al.*, 2013) and the precise positions of the amino acids can vary depending on the species in question. Table 1.2.4.1 shows the impacts different quinolone resistance mechanisms have upon the minimum inhibitory concentration of ciprofloxacin for *E. coli* and *S. aureus*.

Resistance mechanism	Fold change in ciprofloxacin MIC	Source(s)
Gram-negative species (*Escherichia coli*)		
Topoisomerase substitutions		
gyrA	10-16	Everett *et al.*, 1996;
parC	0	Lindgren *et al.*, 2005;
gyrA (x2) + *parC*	60	Morgan-Linnell and Zechiedrich, 2007; Piddock, 1999
Permeability changes		
Efflux upregulation	2-8	Lindgren *et al.*, 2005
Porin loss	4	Strahilevitz *et al.*, 2009
Plasmid mediated quinolone resistance		
Carriage of *qnr* alleles	>30	Briales *et al.*, 2012; Martínez-Martínez *et al.*, 1998
Carriage of *qepA*	32	Yamane *et al.*, 2007
Carriage of *oxqAB*	16	Hansen *et al.*, 2007
Carriage of *aac(6')Ib-cr*	4	Robicsek *et al.*, 2006
Gram-positive species (*Staphylococcus aureus*)		
Topoisomerase substitutions		
grlA	4-8	Hooper, 2000; Hooper, 1999
grlB	4-8	
gyrA	0	
grlA + *gyrB*	64-128	
Permeability changes		
Efflux upregulation	4	Hooper, 2000; Hooper, 1999; Kaatz *et al.*, 1999

Table 1.2.4.1: Quinolone resistance mechanisms and their impact on ciprofloxacin MIC. Table, adapted from Redgrave *et al.*, 2014, showing the impact of various quinolone resistance mechanisms on the minimum inhibitory concentration of ciprofloxacin for a Gram-negative species (*Escherichia coli*) and a Gram-positive species (*Staphylococcus aureus*). Mutations in the genes encoding the target proteins result in a greater change in MIC than alterations to the genes regulating efflux pumps, but antibiotics can overcome target site mutations if efflux is inhibited completely.

The two most commonly occurring amino acid substitutions that confer reduced quinolone susceptibility are GyrA Ser83 and GyrA Asp87, using the numbering of the amino acids in the *E. coli* version of the protein (Hooper and Jacoby, 2015). The development of a *gyrA* mutation precedes that of the development of a *parC* mutation in Gram-negative organisms, and *vice versa* in Gram-positive organisms (Zhao and Drlica, 2001). The additional mutations allow extremely high levels of resistance to be achieved although single topoisomerase mutations can confer clinically relevant resistance. This order has been shown in *Neisseria gonorrhoeae*, where selection experiments resulted in mutations conferring substitutions equivalent to GyrA Ser83Phe and GyrA Asp87Asn in *E. coli*, followed by ParC Ser88Pro and ParC Glu91Lys, in that order (Belland *et al.*, 1994).

The Quinolone Resistance Determining Region (QRDR) is a region in the GyrA protein that, when the amino acids therein are altered, confers a reduction in quinolone susceptibility. This was initially defined as spanning the region between the 67^{th} and 106^{th} amino acids in GyrA in *E. coli* (Yoshida *et al.*, 1990), or the equivalent amino acids in different species, though other substitutions that confer reduced quinolone susceptibility have since been found outside this range and its borders have moved to account for this (Friedman *et al.*, 2001; Griggs *et al.*, 1996). The amino acid substitutions in *E. coli* GyrA that confer reduced quinolone susceptibility identified so far can occur at the following positions (their wild type residues are shown): Ala51, Ala61, Gly81, Asp82, Ser83, Ala84, Asp87, Gln106 and Ala196 (Hopkins *et al.*, 2005). The QRDR concept is not confined to GyrA and a QRDR also exists within ParC (Choi *et al.*, 1998). The active site of GyrA in *E. coli* is Tyr122

(Horowitz and Wang, 1987), though the residues Arg32, Arg47, His78 and His80 have been shown to play roles in the DNA gyrase-mediated DNA breakage-reunion reaction (Hockings and Maxwell, 2002). The proximity of these key residues to amino acids in positions that, when substituted, confer reduced quinolone susceptibility therefore interferes with the ability of DNA gyrase to function efficiently. The detrimental effects of amino acid substitution on DNA gyrase efficiency have been shown experimentally although the fitness cost is not prohibitive as mutations within *gyrA* are commonly recovered in isolates from patients (Webber *et al.*, 2013).

It was initially proposed that, because quinolones are synthetic compounds and were not known to be naturally produced by microorganisms, resistance to them would be uncommon and there would be no horizontally transferrable resistance loci. It was instead thought that resistance would be confined to vertically transferred gene mutations. Rather naively, it was also thought that there would be no enzymes dedicated to quinolone degradation (Burman, 1977).

A number of plasmid-mediated quinolone resistance (PMQR) genes have been identified. The first confirmed transferable PMQR determinant was reported in *Shigella dysenteriae* ten years later (Munshi *et al.*, 1987). Although other potential PMQR determinants were described previously (Panhotra *et al.*, 1985), the authors were unable to confirm that they were plasmid-mediated at that time. The Qnr proteins, of which there are five families (Vetting *et al.*, 2011), are transferable PMQR determinants first described in 1998, with the archetypal locus, *qnrA*, having been found on the plasmid pMG252 in a clinical isolate of *Klebsiella pneumoniae* (Martínez-Martínez *et al.*, 1998). However, they are not exclusively plasmid-

mediated, with chromosomal copies having been found more recently in some Gram-positive bacteria (Rodríguez-Martínez *et al.*, 2008). They take the form of pentapeptide repeat proteins and QnrA is similar to the protein McbG (Tran and Jacoby, 2002), which confers immunity to microcin B17, a DNA replication inhibitor (Garrido *et al.*, 1988). Structural analysis of QnrB1 has suggested that the Qnr proteins bind to and destabilise the topoisomerase/DNA/quinolone complex, removing the quinolone and restoring topoisomerase function (Vetting *et al.*, 2011). Additionally, the QepA efflux system is another plasmid-mediated resistance mechanism. It takes the form of a 14-transmembrane MFS-type efflux pump that increases the minimum inhibitory concentration of ciprofloxacin 32-fold in comparison to strains of *E. coli* lacking the *qepA* gene (Yamane *et al.*, 2007). OqxAB is another plasmid-mediated efflux pump and a member of the RND multidrug efflux pump family, and its expression has been shown to confer reduced susceptibility to both fluorinated and non-fluorinated quinolones (Hansen *et al.*, 2007).

In addition to the PMQR genes, chromosomally encoded multidrug efflux pumps, such as the tripartite RND-type AcrAB-TolC complex, are able to remove quinolones and other drugs from Gram-negative bacteria in exchange for importing hydrogen ions (Piddock, 2006). Fluoroquinolones exert selective pressure that favours the emergence of mutants that constitutively express the loci which encode for these proteins, usually through the de-repression of the *mar*, *sox* or *ram* global regulatory systems (Sun *et al.*, 2011; Zheng *et al.*, 2011, 2009), as well as single nucleotide mutations that result in an AcrB Gly288Asp substitution, as shown in *Salmonella* (Blair *et al.*, 2015a). Combinations of mutations in the efflux pump loci and the genes

which encode the pump's regulators can have a synergistic effect, further reducing quinolone susceptibility (Blair *et al.*, 2015a). Figure 1.2.4.1, adapted from Aldred *et al.*, 2014, represents these and other quinolone resistance mechanisms diagrammatically.

It should be noted that exposure to other antimicrobial agents is able to confer reduced quinolone susceptibility. It has been shown that the use of biocides, such as the widely-used triclosan (Schweizer, 2001), selects for increased expression of genes encoding efflux pump components and regulators, leading to increased antibiotic tolerance (Randall *et al.*, 2007; Webber *et al.*, 2015, 2008). Biocide exposure has also been shown to select for point mutations in *gyrA*, reducing the effectiveness of quinolones (Randall *et al.*, 2004). The precise reason why this occurs is unknown, since triclosan targets FabI rather than GyrA (McMurry *et al.*, 1998), but it is thought that *gyrA* mutations confer a reduction in triclosan susceptibility by changing supercoiling levels. This then alters gene expression, a consequence of which would be differences in the expression of outer membrane proteins (Randall *et al.*, 2007). This could manifest as either increased efflux pump activity and the removal of biocides, or reduced levels of porins, preventing biocides from entering the cell.

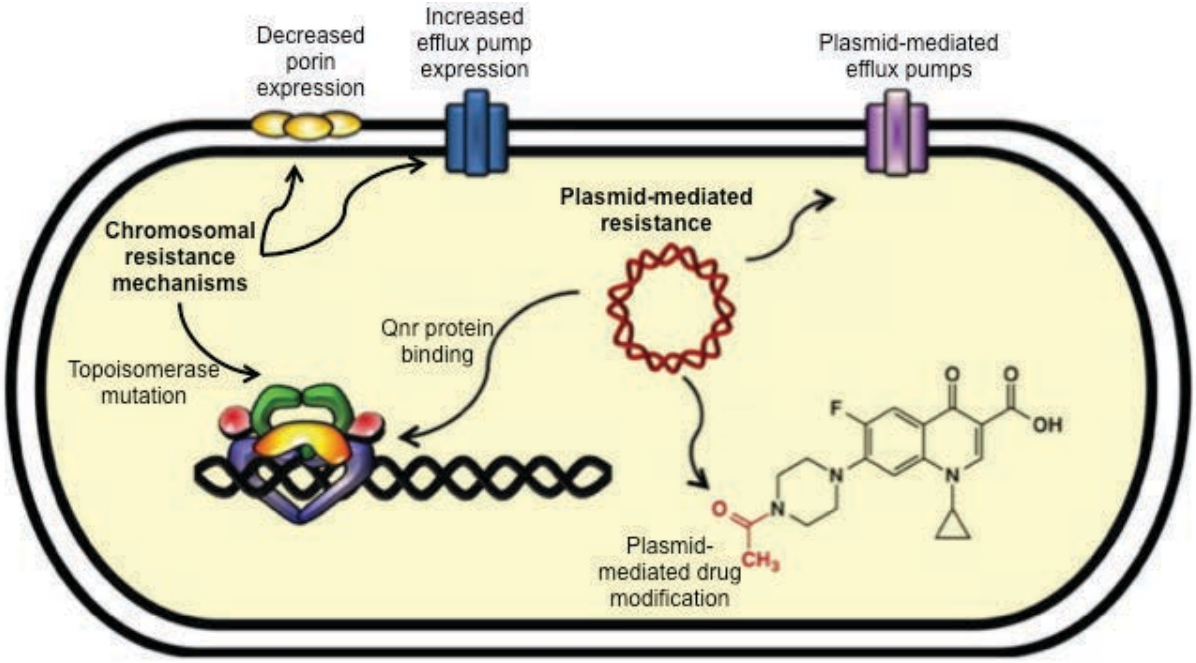


Figure 1.2.4.1: Overview of quinolone resistance mechanisms.

Figure showing chromosomal and plasmid-mediated mechanisms of quinolone resistance. Adapted from Aldred *et al.* (2014). Chromsomal resistance mechanisms include mutations occurring within the topoisomerase that reduce the effectiveness of the quinolone, decreasing porin expression so that the drug cannot enter the cell, and increasing efflux pump expression so that the drug is unable to remain in the cells should it enter. Plasmid-mediated quinolone resistance mechanisms include the enzyme-mediated modification of the quinolone to stop it from functioning, the introduction of efflux pumps that can transport the drug out of the cell, or destabilisation of the topoisomerase/DNA/quinolone complex by Qnr proteins.

1.2.5 Resistance and Fitness

It has been shown by experimentation that having an antibiotic resistance mechanism can incur a fitness cost to the cell (Marcusson *et al.*, 2009), though this has usually been shown through competition assays with isogenic strains in a laboratory setting, and does not necessarily translate to what is seen in clinical practice (Andersson, 2006). One experiment showed that, after eight isogenic single mutants that conferred reduced norfloxacin susceptibility were derived from *Pseudomonas aeruginosa* PA01, only three of the eight mutants had reduced fitness compared with the parent strain; these were GyrA Thr83Ile, GyrA Asp87Tyr and GyrB Glu469Asp. The other mutants carried substitutions which conferred a neutral fitness cost (Kugelberg *et al.*, 2005). Additionally, isogenic mutants in the *gyrA* and *gyrB* loci of *Clostridium difficile* that resulted in reduced fluoroquinolone susceptibility did not incur a fitness cost *in vivo* and persisted in the absence of selective pressure (Wasels *et al.*, 2015). It was also shown that GyrA Ser91Phe and GyrA Asp95Asn substitutions in *Neisseria gonorrhoeae* reduce fluroquinolone susceptibility without a noticeable impact on cell fitness, but that the subsequent acquisition of a ParC Asp86Asn substitution is detrimental. The fitness cost of the ParC substitution is, however, ameliorated through the acquisition of further compensatory mutations (Kunz *et al.*, 2012).

The majority of *gyrA* point mutations that confer reduced quinolone susceptibility have been shown to have an associated fitness advantage in *Salmonella* Typhi when grown in minimal media, and strains carrying multiple mutations can show a further increase in fitness (S. Baker *et al.*, 2013). Work from our research group has shown

that, in the absence of any selective pressure, *S.* Typhimurium SL1344 grows faster in LB media than isogenic strains with *gyrA* mutations (Webber *et al.*, 2013) and additionally out-competes *gyrA* mutant strains when grown in co-culture (Webber *et al.*, manuscript under review). The GyrA Ser83Phe and GyrA Asp87Gly proteins have both been shown to be less efficient than wild type GyrA *in vitro,* each with a 3-fold and 6-fold drop in gyrase activity compared with the wild type, respectively (Webber *et al.*, 2017). However, in the presence of various antibiotics or biocides, the *gyrA* mutants outperform the wild type strains, demonstrating a fitness advantage in the presence of antimicrobials (Webber *et al.*, 2013).

1.3 DNA Topology

Bacteria do not have a membrane-bound nucleus containing multiple paired, linear chromosomes like most eukaryotes, but instead usually possess a single circular chromosome, which is found in a membrane-free region known as the nucleoid (Kellenberger *et al.*, 1958; Thanbichler *et al.*, 2005). Additional genetic information may be conveyed on plasmids, which are transmissible (Meynell *et al.*, 1968). When extruded, the bacterial chromosome is approximately ten times longer than the length of the cell in which it resides, famously demonstrated in Ruth Kavenoff's electron micrograph of the *E. coli* chromosome, and shown in Figure 1.3.1 (Wang *et al.*, 2013). In the case of *E. coli*, the chromosome has to be compacted down to approximately a thousandth of its relaxed size to be able to fit within the cell (Holmes and Cozzarelli, 2000).

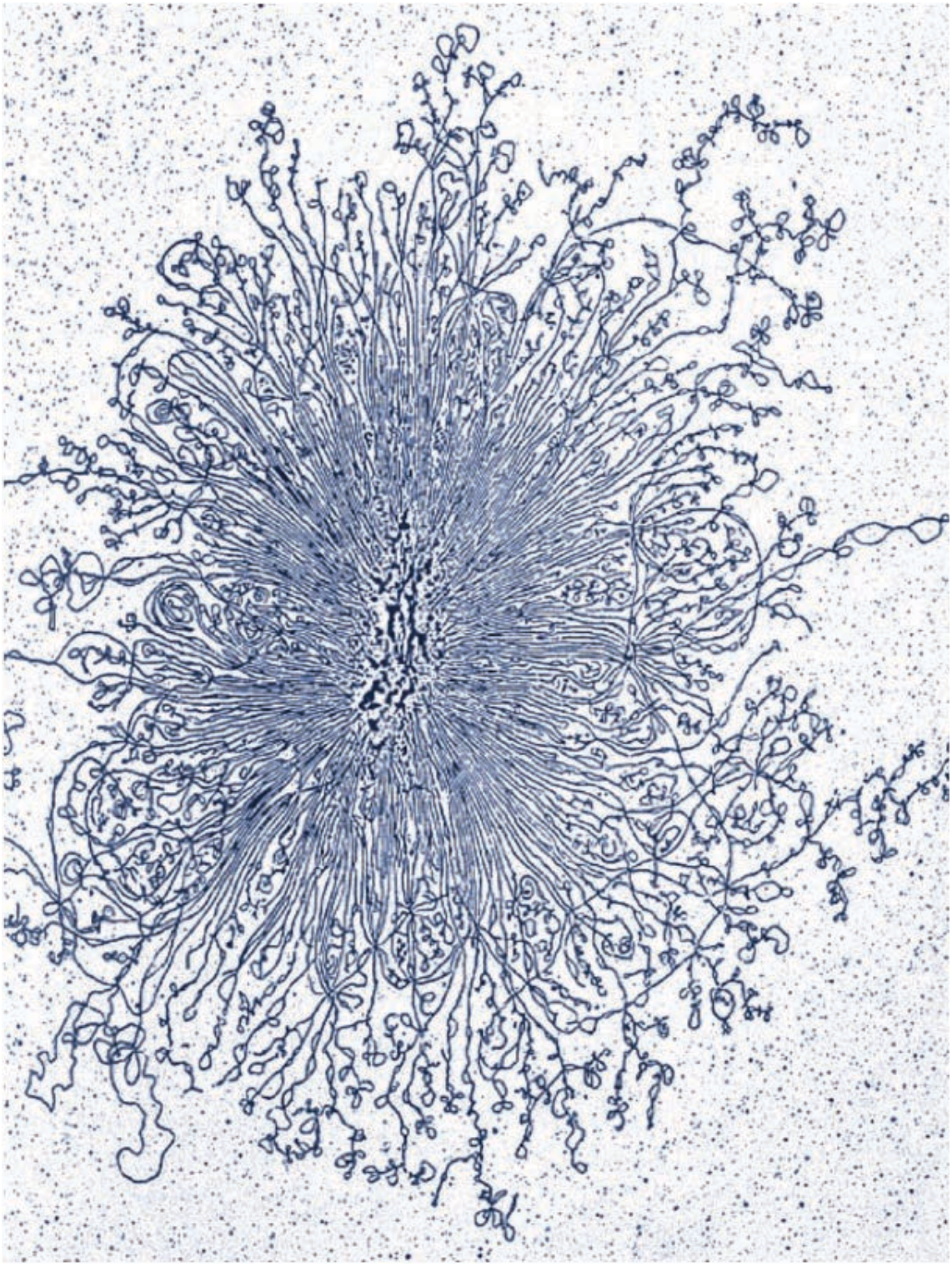


Figure 1.3.1: The extruded bacterial nucleoid. Figure from Kavenoff, via Wang *et al.*, 2013, showing the extruded nucleoid of *E. coli*, bound to cytochrome C and visualised using transmission electron microscopy. The nucleoid is approximately an order of magnitude greater (about 20 μm) than the average *E. coli* cell (around 2 μm).

Fluorescent *in situ* hybridisation experiments, as well as recombination experiments, have identified four major regions of the *E. coli* chromosome, termed macrodomains (Niki *et al.*, 2000; Valens *et al.*, 2004). These are the ori, ter, left and right macrodomains, and they localise to different parts of the cell as the cell cycle progresses. Macrodomains are highly ordered structures, approximately 1 megabase in size in *E. coli* (Dame *et al.*, 2011), and are defined by the regions in which long-range DNA interactions do and do not occur (Thiel *et al.*, 2012; Valens *et al.*, 2004).

The macrodomains are sub-divided into supercoiled regions, known as plectonemic loops, microdomains, or supercoiled domains (Worcel and Burgi, 1972). Plectonemic loops are transient regions of overwound DNA, changing their size and location as the cell grows, but average a size of about 10 kilobases (Postow *et al.*, 2004; Stein *et al.*, 2005). Changes in supercoiling in one loop are confined to that loop (Higgins *et al.*, 1996; Postow *et al.*, 2004). This is different to what is observed in plasmids, where changes in supercoiling affect the whole plasmid (Wang *et al.*, 2013). The ability of the change in supercoiling to spread throughout the rest of the chromosome is limited by microdomains, and additional supercoils can be introduced by the effects of transcription (Deng *et al.*, 2004; Scheirer and Higgins, 2001).

Nucleoid-associated proteins are another important aspect of bacterial chromosomal architecture (Dillon and Dorman, 2010), and they are the closest equivalent bacteria have to histones – when they were first discovered, many were initially described as “histone-like proteins” (Drlica and Rouvière-Yaniv, 1987). They are able to form physical links between parts of the chromosome that would otherwise be distant if the

chromosome were purely linear (Dorman, 2013), and are involved in defining the limits of supercoiling domains (Hardy and Cozzarelli, 2005). It has been suggested that nucleoid-associated proteins may play more of a role in regulating gene expression levels than they do in terms of controlling nucleoid structure (Zimmerman, 2006). The expression levels and binding profiles of nucleoid-associated proteins can change in response to environmental stimuli and depending on the growth phase of a cell, and consequently alter global chromosome structure (Cameron *et al.*, 2011). The changes in expression levels are represented in Figure 1.3.2.

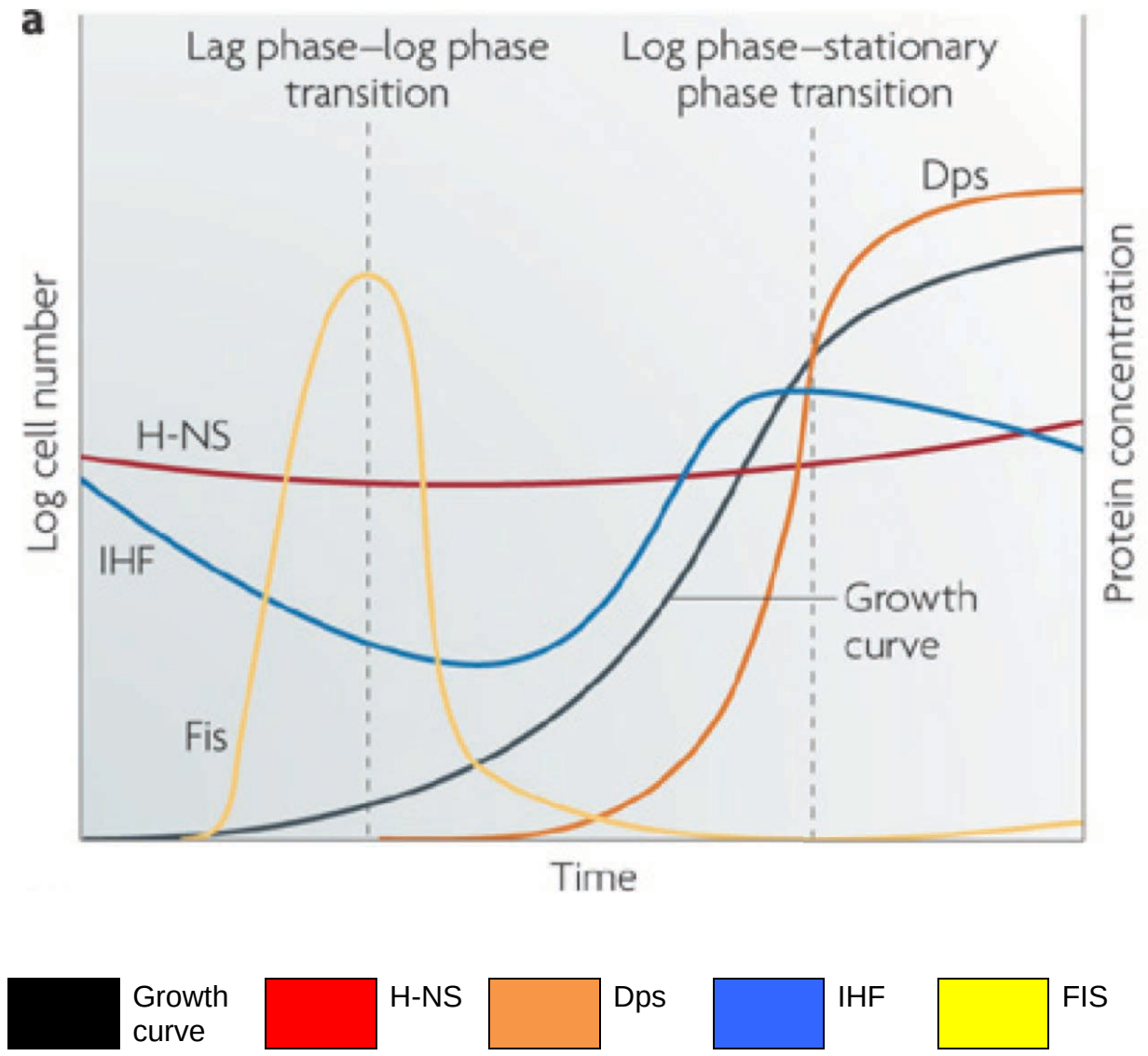


Figure 1.3.2: Nucleoid associated protein levels change with growth.

Graph showing the relative protein concentrations of the major nucleoid associated proteins, Fis (yellow), H-NS (red), IHF (blue) and Dps (orange) and how they change as a population of phase progresses through its growth cycle (black). H-NS is relatively constant, Fis levels peak at the transition between lag phase and logarithmic phase, IHF falls, then rises again, and Dps gradually increases. Figure from Dillon and Dorman, 2010.

Some of the more important nucleoid-associated proteins found in Gram-negative bacteria (Dillon and Dorman, 2010) are discussed below. Each can bind DNA and therefore alter chromosome structure, although this can happen in response to different stresses at different times.

- HU, a histone-like protein named after *E. coli* strain U93 (Oberto *et al.*, 2009), has two subunits, HUα and HUβ – these form dimers, but whether they form heterodimers or homodimers is dependent upon which growth phase the cell is currently going through (Claret and Rouvière-Yaniv, 1997). HUα and HUβ have both been implicated in the ability of *S.* Typhimurium to invade epithelial cells (Mangan *et al.*, 2011); double mutants of *hupA* and *hupB* were shown to be less able to tolerate extremes of temperature (Wada *et al.*, 1988) and to have altered mini chromosome stability, but were still able to initiate replication, possibly by being compensated for by other histone-like proteins (Ogawa *et al.*, 1989).
- H-NS (Histone-like Nucleoid Structuring protein) favours binding to AT-rich sequences, which are usually acquired from other organisms, and has been shown to compete with the sigma subunit of the RNA polymerase complex for transcription start sites, silencing genes (Dame *et al.*, 2002; Fang and Rimsky, 2008; Lang *et al.*, 2007). H-NS is capable of forming filaments and bridge structures with DNA; these are both different ways in which H-NS can polymerise, and which structure is formed depends upon the cellular magnesium concentration (Lim *et al.*, 2014). There are some loci that H-NS is unable to repress when acting alone, but it can do so by forming complexes with Hha and YdgT, two small proteins only found in enteric bacteria, *e.g.*

Salmonella (Ali *et al.*, 2013). The expression of H-NS is relatively constant and does not change substantially as the cell grows (Hinton *et al.*, 1992).

- FIS (Factor for Inversion Stimulation) forms dimers that bind to non-specific regions of DNA and ultimately interact with one another to form filamentous structures (Skoko *et al.*, 2006), but more recent evidence has shown it binds to AT rich regions of DNA (Cho *et al.*, 2008), and that it has a 15 bp core binding sequence of GTTTGAATTTTGAGC (Hancock *et al.*, 2016). Its expression is highest during exponential phase (Ó Cróinín and Dorman, 2007). It was originally described as being involved in site-specific recombination (Osuna *et al.*, 1995), but has since been shown to control expression of the Type III secretion systems involved in virulence in *S.* Typhimurium (Kelly *et al.*, 2004).
- IHF (Integration Host Factor) forms complexes with Dps (DNA-binding Protein from Starved cells), another nucleoid associated protein (Lee *et al.*, 2015; Mangan *et al.*, 2006). Both are most commonly found in stationary phase, with Dps being the most commonly occurring of the two. IHF also has an AT-rich consensus sequence (Goodrich *et al.*, 1990). Interestingly, HU and IHF have been shown to able to substitute for one another, owing to an amino acid sequence similarity of around 60% (Segall *et al.*, 1994).
- MatP, which is not shown in Figure 1.3.2, binds to a repeated motif found in the Ter domain (Mercier *et al.*, 2008). It interacts with ZapA and ZapB to link the Ter domain to the divisome (Männik *et al.*, 2016), and is able to interact with MukBEF to ensure that topoisomerase IV segregates daughter chromosomes at the correct time (Nolivos *et al.*, 2016).

DNA topology can be expressed in terms of “linking number” – this is a mathematical way of saying how many times one DNA strand crosses over another, and is the sum of the “twist” and the “writhe” (Mirkin, 2001). Twist is a measure of the number of turns in a piece of DNA; the most common form of DNA, B-DNA (Du *et al.*, 2013), has 10.5 base pairs to every turn (Watson and Crick, 1953). Writhe is a measure of how many times a strand of DNA crosses over itself. Linking number can only change through the action of topoisomerases - without them, it is static and a change in twist has to be compensated for by a change in writhe (Mirkin, 2001).

1.3.1 Topoisomerases and Supercoiling

Topoisomerases are highly conserved enzymes found in all kingdoms of life that create and repair breaks in DNA to alter DNA tension. They have roles in DNA replication and cell division, and are critical to cell survival (Champoux, 2001), making them an excellent target for antibiotics (Collin *et al.*, 2011). Supercoiling can be used as a measure for how over or under-wound the DNA of a cell is. Over-wound DNA is described as being positively supercoiled, and under-wound DNA as negatively supercoiled. Supercoiling is not in a fixed state, and differs between micro-domains within a bacterial chromosome and between species, which maintain their chromosomes at different basal levels of supercoiling. The degree of supercoiling within a cell can be altered in response to a number of different stimuli, including environmental factors (Cameron *et al.*, 2011) and locally in response to DNA replication or the transcriptional state of a gene (Booker *et al.*, 2010; Dorman and Corcoran, 2009). DNA is inherently negatively supercoiled in *S. enterica* and

Escherichia coli (Cameron *et al.*, 2011) and this provides a store of potential energy which helps drive cellular processes (Mirkin, 2001).

1.3.2 Type IIa Topoisomerases

Topoisomerases are categorised as Type I or Type II depending on how many strands of DNA they cut. Type I topoisomerases cut one strand of DNA and are subdivided into further categories based on the precise mechanisms involved. Type II topoisomerases cut two strands of DNA (Champoux, 2001). Type II topoisomerases can be divided into Type IIa and Type IIb topoisomerases, based on their sequence similarities (Forterre *et al.*, 2007).

The Type IIa topoisomerases that are the focus of this work are DNA gyrase and Topoisomerase IV. Topoisomerase IV separates catenated daughter chromosomes and relaxes supercoils of both directions, whilst DNA gyrase is unique in its ability to introduce negative supercoils into DNA (Corbett *et al.*, 2005); this is represented diagrammatically in Figure 1.3.2.1. These enzymes are highly conserved and essential, thus making good drug targets – the complexes which they form with DNA are targeted by quinolones (Giraud *et al.*, 2006). Both topoisomerases are heterotetrameric protein complexes, comprising two copies of each of the A subunit, encoded by *gyrA* in DNA gyrase and *parC* in Topoisomerase IV, and the B subunit, encoded by *gyrB* in DNA gyrase and *parE* in Topoisomerase IV (Higgins *et al.*, 1978; Peng and Marians, 1993). Figure 1.3.2.2 shows the structure of the *E. coli* DNA gyrase complex. Topoisomerase IV separates daughter chromosomes during the DNA replication process and relaxes supercoils of both directions, whereas DNA

gyrase increases the level of negative supercoiling. Gyrase does not favour any particular binding motifs (Messerschmidt and Waldminghaus, 2015), but preferentially localises to the Ori domain (Sobetzko *et al.*, 2013).

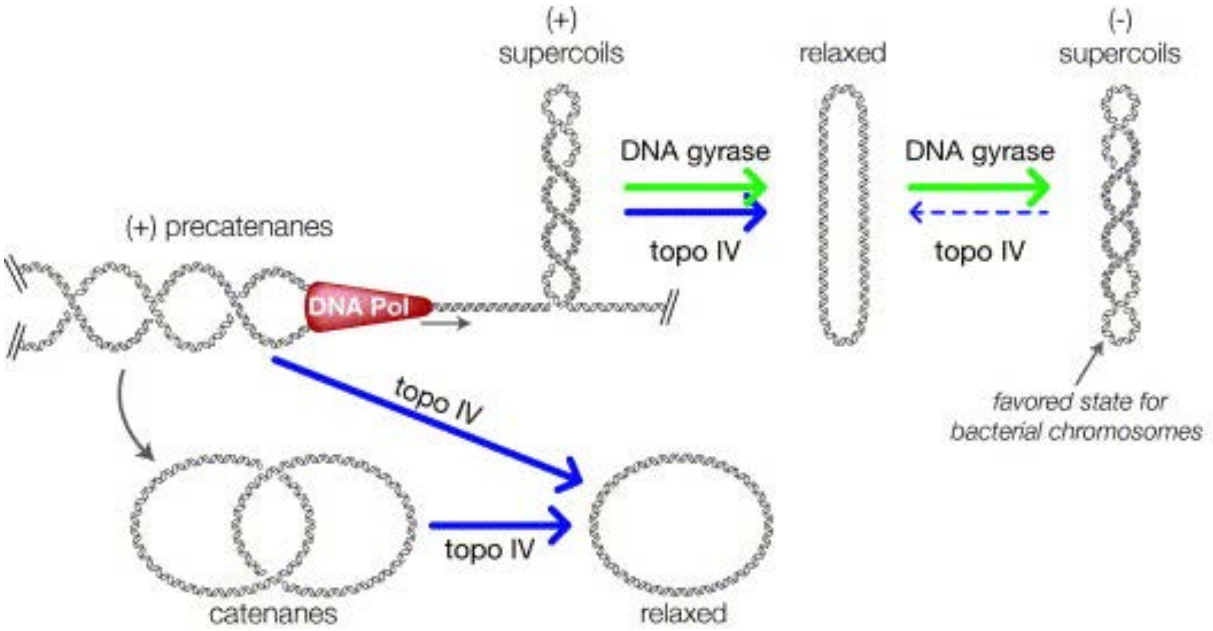


Figure 1.3.2.1: The roles of Type IIa topoisomerases in altering chromosome dynamics. This figure, from Corbett *et al.*, 2005, shows the processes in which Type IIa topoisomerases are involved in altering genomic supercoiling. Topoisomerase IV relaxes supercoils of both directions and separates catenated daughter chromosomes, which arise as a result of the DNA replication process. DNA gyrase, however, introduces negative supercoils into DNA.

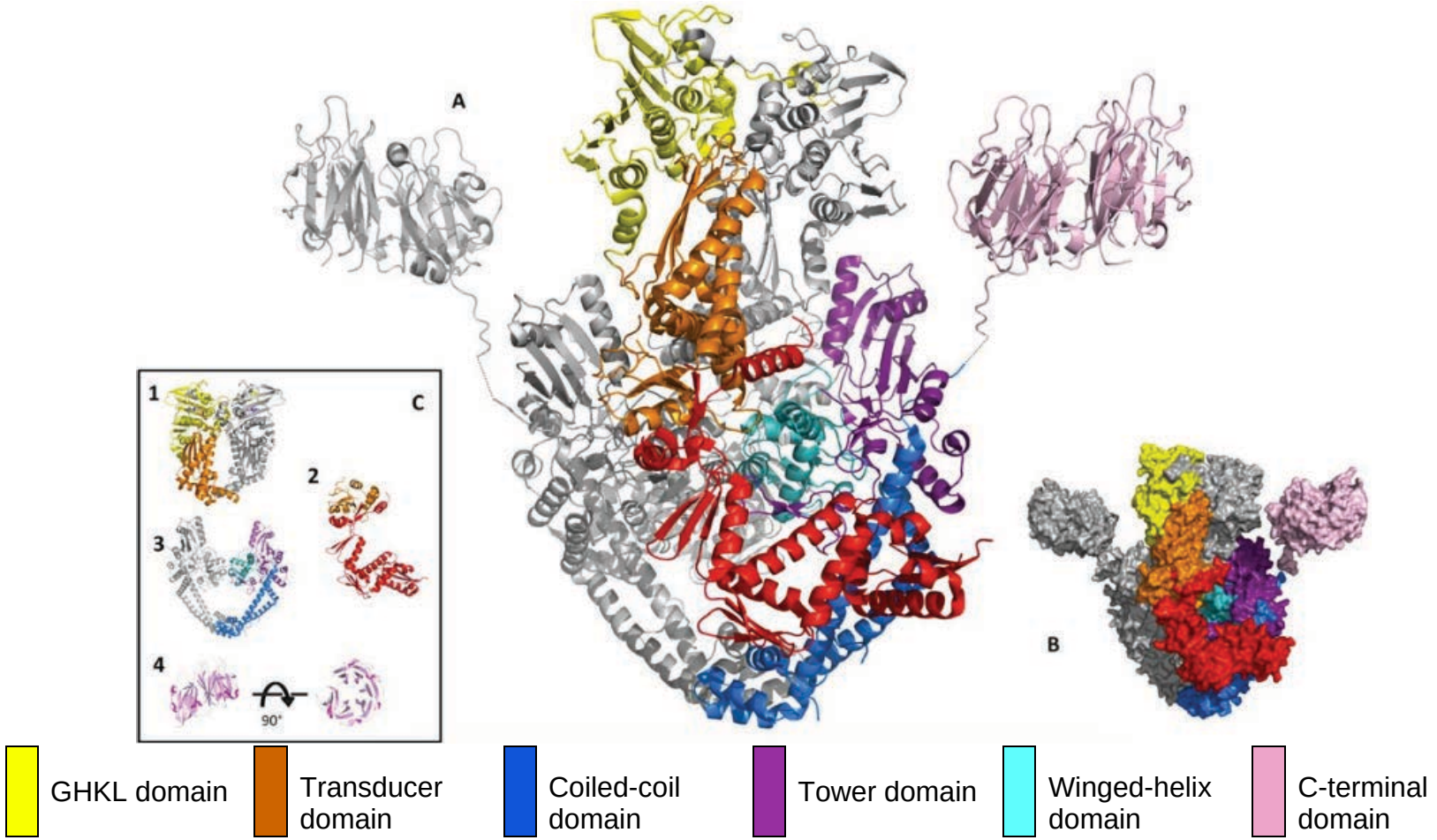


Figure 1.3.2.2: Structure of *Escherichia coli* DNA gyrase. A) Ribbon model of the DNA gyrase holoenzyme. The enzyme is comprised of two copies of each of the GyrA and GyrB subunits. B) Space-filling model of the DNA gyrase holoenzyme. C) Subunits of *Escherichia coli* DNA gyrase. 1: GyrB subunit and ADPNP. 2: GyrB TOPRIM domain. 3: GyrA subunit. 4: Two views of the C-terminal domain of the GyrA subunit. (Figure from Bush *et al.*, 2015).

A comprehensive review of topoisomerase structure and mechanism was written by Schoeffler and Berger (2008); Type IIa topoisomerases are symmetrical, possess three gates and function in an ATP-dependent manner. A double-stranded segment of DNA, termed the G segment, is captured by the DNA gate of the enzyme, then the N gate is closed and another double-stranded segment of DNA, termed the T segment, is captured upon the binding of two ATP molecules to the enzyme. One ATP is then hydrolysed and both strands of the G segment of DNA are cut, allowing the T segment to be passed through. The fragmented G segment is re-ligated and the C gate opens. The T segment can then be released from the enzyme, and the second ATP is hydrolysed (Schoeffler and Berger, 2008). This process is shown in Figure 1.3.2.3.

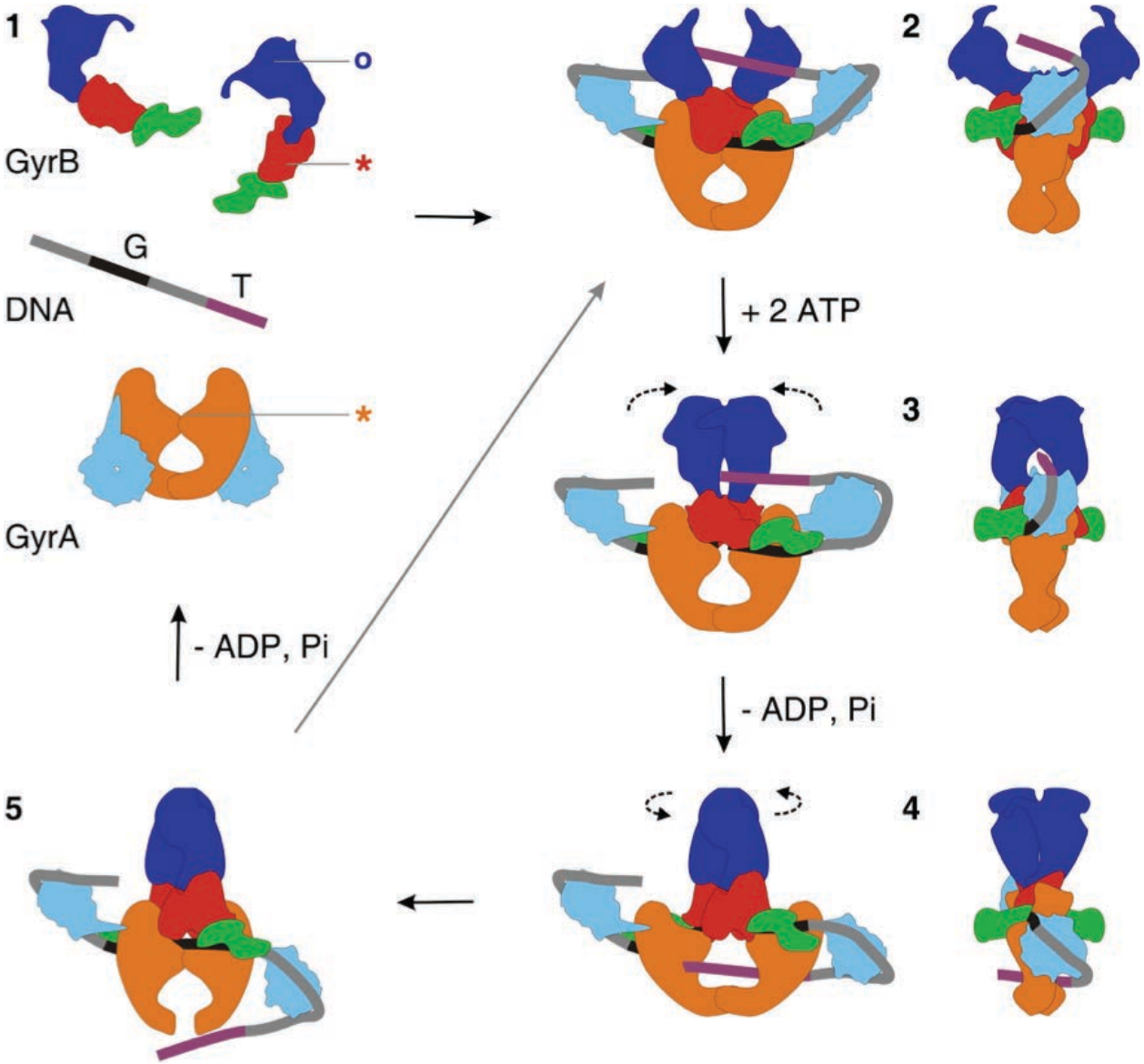


Figure 1.3.2.3: Cleavage of double-stranded DNA by DNA Gyrase.
1) Free-floating GyrA homodimer, GyrB monomers and DNA.

2) Assembly of the holoenzyme and capture of DNA.

3) Initial ATP-mediated cleavage of the G segment of DNA.

4) ATP hydrolysis and passing of the T segment of DNA through the cleaved G segment.

5) Religation of the G segment. The enzyme can then either capture and cut more DNA, or be disassembled.

(Figure from Costenaro *et al.*, 2007)

GyrA N-terminal domain

GyrA C-terminal domain

GyrB N-terminal domain

GyrB Toprim domain

GyrB tail domain

1.3.3 The Impact of Location and Structure of DNA on Function

The fact that the chromosomal location of a gene can affect its expression has been known about in eukaryotes for some time; its occurrence in bacteria is a comparatively recent discovery (Feuerborn and Cook, 2015). Through the use of fluorescent reporters controlled by the *lac* promoter in *E. coli*, it has been shown that the position of a gene in the chromosome can have an effect on how strongly it is expressed in bacteria, and that the transcription of one gene can cause the repression of its neighbours (Bryant *et al.*, 2014). Location-dependent changes in promoter activity have also been observed in *S. pneumoniae* (Ferrándiz *et al.*, 2014). It has also been shown that transcriptionally silent extensive protein occupancy domains, regions of the genome which are transcriptionally silent because of bound proteins blocking access by transcriptional machinery (Kröger *et al.*, 2012; Zarei *et al.*, 2013), correlate with regions bound and supressed by H-NS, with the activity of promoters in these regions and near the borders of macrodomains being more heavily repressed than promoters found elsewhere (Brambilla and Sclavi, 2015).

The base pair content of a gene can also affect its expression. It has been shown that, although there are multiple mRNA triplets encoding for the same amino acid, transcripts containing specific triplets encoding for a given amino acid tend to be enriched, and this appears to be influenced by the hydrophobicity of the mRNA (Boël *et al.*, 2016). Additionally, the nucleoid-associated protein H-NS preferentially binds AT-rich regions of DNA (Lang *et al.*, 2007; Navarre *et al.*, 2006), preventing them from being able to be accessed by RNA polymerase – though the precise mechanism by which RNA polymerase is occluded by H-NS is unclear (Landick *et*

al., 2015). The strength of the binding of H-NS to AT-rich regions is because of these regions' inherent curvature (Dame *et al.*, 2001). In *Salmonella*, horizontally acquired DNA sequences often have a higher AT content than "native" DNA, so are bound and supressed by H-NS to prevent any potential detrimental impacts upon fitness the genes contained within may have upon the cell (Ali *et al.*, 2014; Navarre *et al.*, 2006), and a similar role has been observed in *E. coli*, where H-NS prevents the expression of intragenic DNA sequences from spurious promoters (Singh *et al.*, 2014).

1.4 *Salmonella* Typhimurium as a Model Organism

1.4.1 *Salmonella* Genus and Phylogeny

Salmonella is a genus of Gram-negative bacteria (Heinrichs *et al.*, 1998) within the order Enterobacteriales, within the class Gammaproteobacteria. Other well-known members of Enterobacteriales include *Escherichia coli* and *Yersinia pestis* (Williams *et al.*, 2010), and the last common ancestor of *Salmonella* and *Escherichia* is thought to have existed around 100 million years ago, based on molecular clock analyses (Doolittle *et al.*, 1996). The genus *Salmonella* was given its name by Lignières in 1901 (St John-Brooks, 1934) in honour of the discovery of *Bacillus choleraesuis* (now *Salmonella enterica* serovar Choleraesuis) by Smith and Salmon in the 1880s (Schultz, 2008).

There were formerly approximately 2,500 species defined in the *Salmonella* genus, the organisation of which was based on serotyping (Kauffmann and Edwards, 1952). The genus has more recently been reorganised into two species, *Salmonella bongori* and *Salmonella enterica,* with almost all former species now being recognised as

serovars of *S. enterica* (Brenner *et al.*, 2000). Public Health England has moved to using a combination of whole genome sequencing and multi-locus sequence typing to categorise serovars (Ashton *et al.*, 2016). Various serovars of *S. enterica* can be pathogenic to humans and animals. The serovar *Salmonella* Typhimurium was first isolated by Löffler from a diseased population of field mice in Thessaly, but was named *Bacillus typhi-murium* at the time (H. J. Baker *et al.*, 2013).

1.4.2 Relevance as a Pathogen

S. Typhimurium is a non-typhoidal serovar of *Salmonella* that causes gastroenteritis in humans, but causes typhoid fever in mice ("Typhimurium" is Latin for "mouse typhoid"). Mouse models of *S.* Typhimurium have historically been used to research *S.* Typhi, which causes typhoid fever in humans (Carter and Collins, 1974), but the role of *S.* Typhimurum as a major human gastrointestinal pathogen is still relevant.

Despite the vaccination of chickens against *Salmonella* in the United Kingdom, there were approximately 90,000 reported cases of Salmonellosis in the European Union in 2012, making it the second most common gastrointestinal infection (European Centre for Disease Prevention and Control, 2014). However, this figure will be less than the real value, as salmonellosis is widely underreported, both in Europe and throughout the rest of the world (Gibbons *et al.*, 2014; Hall *et al.*, 2008; Marzel *et al.*, 2014; Mellou *et al.*, 2013). Additionally, multiple outbreaks of *Salmonella* in the United Kingdom in the past decade have been caused by cheap eggs and poultry imported from countries which have lower food hygiene standards and do not vaccinate as thoroughly, if at all, such as Spain (Janmohamed *et al.*, 2011; O'Brien *et al.*, 2004;

Public Health England, 2015). Seven hundred and twenty one isolates of *Salmonella* from chickens were reported in Great Britain in 2014, none of which were found as a result of investigations under the Zoonoses Order (Animal and Plant Health Agency, 2015).

1.4.3 Infection Cycle and Treatment

Salmonella usually enters the body through the digestive tract and can tolerate the acidic conditions of the stomach (Garcia-del Portillo *et al.*, 1993). When *Salmonella* enters the small intestine, it invades macrophages (Gahring *et al.*, 1990) or crosses the epithelial barrier through M cells within the Peyer's patches (Hohmann *et al.*, 1978). *Salmonella* are able to secrete effectors via the Type 3 Secretion System (Galán, 2001; Zhou and Galán, 2001), and these effectors alter host cell behaviour and disrupt the water, ion and salt content of the intestine. This results in diarrhoea and the bacteria are ultimately shed into the intestine, either to re-invade the intestine or to be passed out of the digestive system (Haraga *et al.*, 2008).

Typhoidal serovars of *Salmonella*, such as Typhi, Sendai and Paratyphi, cause typhoidal and paratyphoidal fever. They are restricted to human hosts and incubate over a two-week period. Symptoms, mainly a high fever, last for three weeks, but can also include dehydration, rashes, nausea, headaches, a swollen liver and spleen, and either diarrhoea or constipation, depending on the patient. Untreated, this can be fatal (Gal-Mor *et al.*, 2014). It is possible to be infected with a typhoidal strain and not exhibit symptoms (Del Bel Belluz *et al.*, 2016) - Mary Mallon infamously was an asymptomatic carrier of typhoid in the late 1800s and, working as a cook, managed

to infect upwards of 122 people, five of whom died (Marineli *et al.*, 2013). Typhoid should not be confused with typhus, which is caused by *Rickettsia* spp (Osterloh *et al.*, 2016).

Non-typhoidal *Salmonella* infections are caused by most other serovars of *Salmonella* to those aforementioned and present after six to twelve hours in the form of a fever and diarrhoea, and last about a week (Gal-Mor *et al.*, 2014). The use of oral rehydration therapy is recommended to replenish liquids, salts and sugars lost as a consequence of diarrhoea (Sentongo, 2004). Non-typhoidal salmonellosis is usually self-limiting, but can become complicated. Fluoroquinolones or cephalosporins are the first choices of antibiotics in cases requiring antibiotic treatment (Gal-Mor *et al.*, 2014). The British National Formulary recommends the use of ciprofloxacin or cefotaxime for the treatment of non-typhoidal *Salmonella* if, and only if, the infection is severe or there is a strong likelihood of the infection becoming invasive – for example, if the patient is immunocompromised, has a defective form of haemoglobin, or is under six months old (BMJ Group and Royal Pharmaceutical Society of Great Britain, 2016e). As of this writing, there are currently seven known programmes to develop a vaccine for non-typhoidal *Salmonella*, mainly targeting components of the outer membrane. Of the seven programmes, only one has passed the pre-clinical stage and is undergoing Phase I clinical trials (Tennant *et al.*, 2016).

1.4.4 Genome Organisation

Salmonella Typhimurium strain LT2 was the first representative of the serovar Typhimurium to have its complete genome sequence published. It has a 4.86

megabase circular chromosome and a 94 kilobase virulence plasmid, with the two containing a total of approximately 4,500 coding sequences and both having a GC content of 53% (McClelland *et al.*, 2001).

The *S.* Typhimurium strain SL1344, which is the primary subject of this study, has a 4.88 megabase circular chromosome and three plasmids, pSLT, pCol1b9 and pRSF1010, which are 94, 87 and 9 kilobases in size, respectively. The two smaller plasmids are not present in LT2. The SL1344 genome has a GC content of 52.3% and contains approximately 4,700 coding sequences (Kröger *et al.*, 2012). Strain 14028S has a 4.87 megabase genome and is 92% identical to strain LT2 (Jarvik *et al.*, 2010).

The *Salmonella* Pathogenicity Islands are AT-rich regions of DNA acquired from foreign sources, and are usually silenced by H-NS to counteract the negative fitness costs resulting from the acquisition of foreign DNA (Ali *et al.*, 2014). SPI-I and SPI-II, the main islands, encode Type III secretion systems and other components that are involved in virulence (Figueira and Holden, 2012; Lostroh and Lee, 2001). There are at least nine known such pathogenicity islands (Velásquez *et al.*, 2016). SPI-I is 40 kilobases in length and absent from the *E. coli* chromosome (Mills *et al.*, 1995), forming one of the main genetic differences between the two species (Ali *et al.*, 2014). The *Salmonella* genome is more negatively supercoiled than that of *E. coli* (Cameron *et al.*, 2011; Higgins, 2016), and the levels of FIS, which has been shown to repress *gyrA* and *gyrB* expression in *S. enterica* (Keane and Dorman, 2003), vary between the two species (Cameron *et al.*, 2011).

1.4.5 Quinolone Resistance in *Salmonella*

Quinolone resistance in *Salmonella* is a major concern as quinolones and fluoroquinolones are the antibiotic of choice in human and animal medication to treat severe cases of gastroenteritis (Aarestrup *et al.*, 2003), in addition to salmonellosis and bacteraemia. The Cochrane Collaboration, the medical meta-analysis and systematic review organisation, does not recommend the use of antibiotics to treat diarrhoea caused by non-typhoidal *Salmonella* as it can select for clinical resistance to antibiotics (Onwuezobe *et al.*, 2012), and as the infection is usually self-limiting (Gal-Mor *et al.*, 2014), there is no clinical benefit to their use (Onwuezobe *et al.*, 2012). The Cochrane Collaboration has also noted that "generally, fluoroquinolones performed well" when used to treat typhoid, but could not give a strong recommendation for or against their use because of fears of the development of resistance (Effa *et al.*, 2011).

In 2012, there were just over 91,000 confirmed cases of human salmonellosis in Europe (European Food Safety Authority and European Centre for Disease Prevention and Control, 2014a), and 5% of isolates were classified as resistant to ciprofloxacin (European Food Safety Authority and European Centre for Disease Prevention and Control, 2014b). This decreased to 83,000 reported human cases in 2013 (European Food Safety Authority and European Centre for Disease Prevention and Control, 2015a), 4% of which were classified as resistant to ciprofloxacin (European Food Safety Authority and European Centre for Disease Prevention and Control, 2015b). In 2014, this figure increased to approximately 90,000 confirmed cases of human salmonellosis (European Food Safety Authority and European

Centre for Disease Prevention and Control, 2015c) and 9% of isolates were classified as resistant to ciprofloxacin (European Food Safety Authority and European Centre for Disease Prevention and Control, 2016). Eighty-four per cent of *S.* Kentucky isolates were found to be resistant to quinolones (European Food Safety Authority and European Centre for Disease Prevention and Control, 2016); this is owing to the massive expansion of the ST198 strain since having been isolated in the 1990s and its acquisition of substitutions in GyrA in the early 2000s (Le Hello *et al.*, 2013).

The first two cases of ciprofloxacin-resistant clinical isolates of *Salmonella* ever reported in the literature were published in 1990 (Piddock *et al.*, 1990), three years after its introduction into clinical use in 1987 (Török *et al.*, 2009). The first patient presented with a urinary tract infection that was caused by *Salmonella* Typhimurium and was initially susceptible to nalidixic acid and other antimicrobials. A combined treatment of amoxicillin and clavulanic acid did not clear the infection, so a two-week course of ciprofloxacin was prescribed instead. After a month, the MIC of nalidixic acid had increased 32-fold from 8 μg/mL at the start of treatment to 256 μg/mL, and the MIC of ciprofloxacin had increased approximately 64-fold from 0.03 μg/mL at the start of treatment to 2 μg/mL at the end of the month (Piddock *et al.*, 1990). As a reference point, the MICs for nalidixic acid and ciprofloxacin against the type strain, *Salmonella* Typhimurium NCTC74 (Stanley *et al.*, 1993; Public Health England, n.d.) were 2 μg/mL and 0.015 μg/mL, respectively (Piddock *et al.*, 1990).

The second patient had undergone aortic aneurysm surgery and *S.* Typhimurium was isolated from a haematoma. These isolates were initially sensitive to

ciprofloxacin, so the infection was treated with ciprofloxacin. After a week, two populations of *S.* Typhimurium were isolated from the wound; one was ciprofloxacin resistant and the other was ciprofloxacin susceptible. The MIC of ciprofloxacin required to kill the resistant isolate was within levels that were, at the time, considered acceptable. Treatment failed and the infection was ultimately cleared with a twelve-week course of aztreonam (Piddock *et al.*, 1990). The timeline for this treatment is shown in Figure 1.4.5.1, and the MICs for a number of antibiotics against the clinical isolates are shown in Table 1.4.5.1.

It is interesting to note that, since these cases were reported, the European Committee for Antimicrobial Susceptibility Testing has officially decided that the clinically acceptable breakpoint concentration of quinolone antibiotics for *Salmonella* is lower than that of other members of the Enterobacteriaceae by a factor of approximately 16-fold – 0.06 µg/mL compared with 2 µg/mL (European Committee on Antimicrobial Susceptibility Testing, 2016), after it had been observed that a common theme in the treatment of *Salmonella* infections was that doses of quinolones within this range of concentrations were ineffective (Aarestrup *et al.*, 2003).

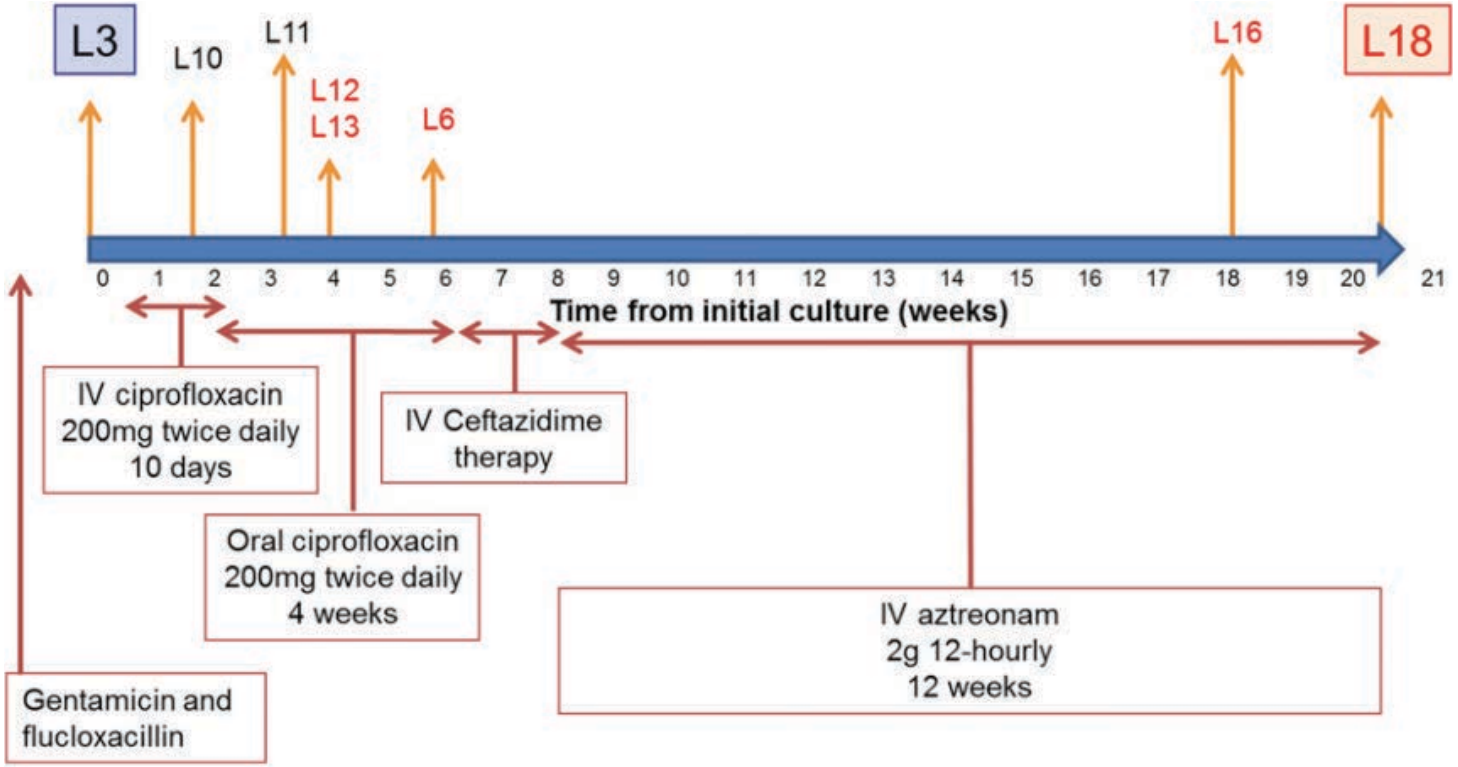


Figure 1.4.5.1: Progression of treatment for a patient with one of the earliest reported cases of quinolone-resistant *Salmonella* Typhimurium. Figure, taken from Blair *et al.*, (2015a) showing the course of treatment for Patient B, a 52-year old male with a *S.* Typhimurium infection as described in Piddock *et al.*, 1990. Bacterial isolates were obtained from the patient over a 21-week period. The patient was given a combination of gentamicin and flucloxacillin before the first isolate was taken. He then received intravenous ciprofloxacin for 10 days, followed by four weeks of oral ciprofloxacin. He was then administered intravenous ceftazidime for two weeks, followed by three months of treatment with intravenous aztreonam. L*x* = Antimicrobials Research Group internal culture collection numbers. The minimum inhibitory concentrations of the agents used to treat this infection are shown in Table 1.4.5.1, overleaf.

Strain	Timepoint isolated after therapy	Minimum Inhibitory Concentration (μg/mL)					
		Cip	Nal	Chl	Tet	Atm	Caz
L3	0 weeks	0.015	2	2	1	0.06	0.12
L10	1 week	0.06	8	8	2	0.12	0.5
L11	3 weeks	0.03	16	8	2	0.25	0.25
L12	3 weeks	0.5	64	32	8	0.5	1
L13	3 weeks	0.5	64	16	8	0.5	0.5
L6	5 weeks	0.5	64	32	8	0.5	0.5
L16	17 weeks	0.5	64	32	8	0.5	1
L18	19 weeks	0.5	64	32	8	0.5	2

Table 1.4.5.1: MICs of various antibiotics for bacterial isolates taken from a patient with one of the earliest reported cases of quinolone-resistant *Salmonella* Typhimurium. Table, taken from Blair *et al.*, (2015a) showing the MICs for antibiotics tested against isolates of *Salmonella* Typhimurium taken from Patient B, a 52-year old male with a *S.* Typhimurium infection as described in Piddock *et al.*, 1990. The minimum inhibitory concentrations of all the antibiotics used, bar ceftazidime, reach a plateau around three weeks and do not increase further. Cip = ciprofloxacin; Nal = nalidixic acid; Chl = chlorpromazine; Tet = tetracyclone; Atm = aztreonam; Caz = ceftazidime.

1.5 Work Preceding This Study

The work in this project is based on previous work from this laboratory (Webber *et al.*, 2013). The previous work observed that a clinically-relevant amino acid substitution within GyrA conferring reduced fluoroquinolone susceptibility (Asp87Gly) in S. Typhimurium improved survival compared with the parent strain when challenged with a range of non-quinolone drugs. The presence of this substitution has been reported in quinolone-resistant isolates of *Salmonella* taken from both humans and animals (Griggs *et al.*, 1996). The non-quinlone drugs to which the GyrA Asp87Gly susbsitution decreased susceptibility included beta-lactams, folate synthesis inhibitors, protein synthesis inhibitors and toxic anions (Webber *et al.*, 2013). The GyrA Asp87Gly substitution also resulted in a more negatively supercoiled topoisomer profile than both wild type *S.* Typhimurium and GyrA Ser83Phe cells. Based on the protective effects of the GyrA Asp87Gly substitution against other antibiotics (Webber *et al.*, 2013), the fact that supercoiling changes in response to environmental stresses (Cameron *et al.*, 2011), and the increasing build-up of low levels of antibiotics in the environment (Andersson and Hughes, 2014), the next step would be to investigate what effect, if any, exposure to low-level stresses (antibiotic-mediated or otherwise) had upon supercoiling, how these impact upon gene expression and antibiotic resistance and the role of *gyrA* mutation in these processes.

1.6 Aims, Objectives and Hypotheses

The hypotheses to be investigated during this thesis are:

- Mutations within gyrase alter the supercoiling levels of *Salmonella* due to changes in chromosome structure, thereby influencing the expression of numerous genes around the chromosome;
- The expression of genes involved in antimicrobial tolerance and environmental stresses are sensitive to supercoiling;
- Analogous mutations within *gyrA* will have similar impacts in both *Salmonella* and *E. coli*;
- Supercoiling-sensitive genes will be influenced by chromosomal location.

The aims of this project are:

- To precisely identify the repertoire of supercoiling-sensitive genes in *Salmonella* Typhimurium;
- To determine the effect of supercoiling on chromosome structure, DNA binding protein distribution and promoter locations of supercoiling-sensitive genes, and to determine the dynamics of supercoiling changes;
- To define the contribution of alterations in supercoiling to antibiotic resistance.

Chapter Two

Materials and Methods

2. Materials and Methods

2.1 Growth of Bacterial Strains

The strains used in this study are listed in Table 2.1.1. This study used a number of strains of *Salmonella* Typhimurium SL1344 and *Escherichia coli* MG1655 with defined, clinically relevant mutations in *gyrA*. The *S.* Typhimurium strain SL1344 was obtained from the ARG Culture Collection, but was originally acquired from the AHVLA. The *E. coli* strain MG1655 was acquired from the Coli Genetic Stock Center at Yale University. *Salmonella* strains with separate GyrA Ser83Phe and GyrA Asp87Gly substitutions were previously made using site-directed mutagenesis and have already been characterised (Webber *et al.*, 2013); they were obtained from the ARG Culture Collection. The *E. coli* GyrA Ser83Leu strain was made by an intercalating medical student using site-directed mutagenesis (Sutton, 2014), and the *E. coli* GyrA Asp87Gly strain was a kind gift from Tony Maxwell of the University of East Anglia. A range of plasmid constructs were also made and transformed into these strains as part of this study.

In routine growth, cells were grown aerobically on Luria-Bertani (LB) agar (Sigma-Aldrich, Poole, UK), supplemented with 50 µg/mL of ampicillin where necessary, at 37 °C overnight without shaking unless otherwise stated. For liquid cultures, overnight cultures were grown aerobically at 37 °C for 16 hours in 5 mL of LB broth (Sigma-Aldrich, Poole, UK), supplemented with 50 µg/mL ampicillin where necessary. Cells were periodically grown on xylose lysine deoxycholate (XLD) medium (Oxoid, Basingstoke, UK) under the same conditions, to presumptively identify strains as *Salmonella* and to verify that contamination had not arisen. The

genotypes of strains were also verified by PCR followed by DNA sequencing for specific loci. API(20E) strips were also used to confirm the species of each strain (Biomerieux, France).

***Salmonella enterica* serovar Typhimurium**			
Lab code	**Parent**	**Description**	**Source**
L354	SL1344	Wild type strain	Wray & Sojka (1978)
L821	SL1344	GyrA Ser83Phe	Webber *et al.* (2013)
L825	SL1344	GyrA Asp87 Gly	Webber *et al.* (2013)
L1650	SL1344	Wild type carrying pBR322	This study
L1651	SL1344	GyrA Ser83Phe/pBR322	This study
L1652	SL1344	GyrA Asp87Gly/pBR322	This study
L1781	SL1344	Wild type/pMW82 p*recA*:*gfpmut2*	This study
L1782	SL1344	Wild type/pMW82 p*gabD*:*gfpmut2*	This study
L1783	SL1344	Wild type/pMW82 p*glnA*:*gfpmut2*	This study
L1784	SL1344	Wild type/pMW82 p*bamA*:*gfpmut2*	This study
L1785	SL1344	Wild type/pMW82 p*opdA*:*gfpmut2*	This study
L1786	SL1344	GyrA Ser83Phe/pMW82 p*recA*:*gfpmut2*	This study
L1787	SL1344	GyrA Ser83Phe/pMW82 p*gabD*:*gfpmut2*	This study
L1788	SL1344	GyrA Ser83Phe/pMW82 p*glnA*:*gfpmut2*	This study
L1789	SL1344	GyrA Ser83Phe/pMW82 p*bamA*:*gfpmut2*	This study
L1790	SL1344	GyrA Ser83Phe/pMW82 p*opdA*:*gfpmut2*	This study
L1791	SL1344	GyrA Asp87Gly/pMW82 p*recA*:*gfpmut2*	This study
L1792	SL1344	GyrA Asp87Gly/pMW82 p*gabD*:*gfpmut2*	This study
L1793	SL1344	GyrA Asp87Gly/pMW82 p*glnA*:*gfpmut2*	This study
L1794	SL1344	GyrA Asp87Gly/pMW82 p*bamA*:*gfpmut2*	This study
L1795	SL1344	GyrA Asp87Gly/pMW82 p*opdA*:*gfpmut2*	This study
L644	SL1344	Δ*acrB*	Eaves *et al.* (2004)
L1749	SL1344	Δ*acrB*/pBR322	This study
L742	SL1344	Δ*tolC*	Buckley *et al.* (2006)
L1750	SL1344	Δ*tolC*/pBR322	This study
L1232	SL1344	Wild type/pMW82 p*ramA:gfpmut2*	Lawler *et al.* (2013)
L1233	SL1344	ΔramA/pMW82 p*ramA:gfpmut2*	Lawler *et al.* (2013)
L1234	SL1344	ΔramRA/pMW82 p*ramA:gfpmut2*	Lawler *et al.* (2013)
L1407	SL1344	Wild type/pMW82 (no promoter)	Lawler *et al.* (2013)

Table continues on next page

Escherichia coli			
Lab code	**Parent**	**Description**	**Source**
I113	NCTC10418	Reference strain used for minimum inhibitory concentration assays	National Collection of Type Cultures
I364	MG1655	Wild type strain	Coli Genetic Stock Center, Yale
I980	MG1655	GyrA Ser83Leu	Sutton (2014)
I1042	MG1655	GyrA Asp87Gly	Tony Maxwell, University of East Anglia
I974	MG1655	Wild type strain/pBR322	Sutton (2014)
I975	MG1655	GyrA Ser83Leu/pBR322	Sutton (2014)
I1070	MG1655	GyrA Asp87Gly/pBR322	Iddles (2016)
I1074	MG1655	Wild type/pMW82 p*recA*:*gfpmut2*	This study
I1075	MG1655	Wild type/pMW82 p*gabD*:*gfpmut2*	This study
I1076	MG1655	Wild type/pMW82 p*glnA*:*gfpmut2*	This study
I1077	MG1655	Wild type/pMW82 p*bamA*:*gfpmut2*	This study
I1078	MG1655	Wild type/pMW82 p*opdA*:*gfpmut2*	This study
I1079	MG1655	GyrA Ser83Leu/pMW82 p*recA*:*gfpmut2*	This study
I1080	MG1655	GyrA Ser83Leu/pMW82 p*gabD*:*gfpmut2*	This study
I1081	MG1655	GyrA Ser83Leu/pMW82 p*glnA*:*gfpmut2*	This study
I1082	MG1655	GyrA Ser83Leu/pMW82 p*bamA*:*gfpmut2*	This study
I1083	MG1655	GyrA Ser83Leu/pMW82 p*opdA*:*gfpmut2*	This study
I1084	MG1655	GyrA Asp87Gly/pMW82 p*recA*:*gfpmut2*	This study
I1085	MG1655	GyrA Asp87Gly/pMW82 p*gabD*:*gfpmut2*	This study
I1086	MG1655	GyrA Asp87Gly/pMW82 p*glnA*:*gfpmut2*	This study
I1087	MG1655	GyrA Asp87Gly/pMW82 p*bamA*:*gfpmut2*	This study
I1088	MG1655	GyrA Asp87Gly/pMW82 p*opdA*:*gfpmut2*	This study
I825	DH5α	Sub-cultured commercial electrocompetent cells	Bioline, USA
n/a	DH5α	Alpha-select gold efficiency chemically competent cells	Bioline, USA
n/a	DH5α	Alpha-select electrocompetent cells (discontinued by manufacturer)	Bioline, USA

Table 2.1.1: Strains used in this study. Wild type strains and *gyrA* mutants were obtained from the ARG Culture Collection, but their original sources are shown here. They were transformed with pMW82-based *recA*, *gabD, glnA, bamA* and *opdA* reporters as part of this project.

Bacteria were stored on Protect™ beads (Technical Service Consultants, Ltd., Heywood, UK) for long-term storage, *Salmonella* strains were stored at -20 °C and *E. coli* at -80 °C. When required for experiments, bacteria were resuscitated by being grown overnight on LB agar plates supplemented with the appropriate antibiotics, as described in Section 2.1. To create fresh beads, a sterile loop was used to collect colonies from approximately half a freshly incubated plate and these were introduced to tubes of Protect™ beads. The tubes were shaken briefly and the glycerol was removed. Two sets of beads were made per strain; one was maintained for routine use and the other was stored in the main ARG culture collection, if required, after having been assigned a strain number.

2.2 Antibiotics and Chemicals

Antibiotics were used to select for antibiotic-resistant bacteria, determine susceptibility profiles, induce stress responses and ensure plasmid retention within populations. Working stocks of antibiotics were routinely made at a concentration of 10,000 µg/mL by dissolving 0.1 g of antibiotic in 10 mL of the appropriate solvent, and then further diluted as necessary. Other chemicals were used as solvents for antibiotics or to induce stress responses. All antibiotics and chemicals were purchased from Sigma-Aldrich (Poole, UK) unless stated otherwise, and are listed in Table 2.2.1.

Name	Mode of action	Solvent
Ampicillin	Cell wall synthesis inhibition	Sodium bicarbonate
Nalidixic acid	DNA gyrase inhibition	Sterile distilled water
Chlorpromazine	Efflux pump inhibition	Sterile distilled water
Ciprofloxacin	DNA gyrase inhibition	Acetic acid
Gentamicin	30S ribosome subunit inhibition	Sterile distilled water
Triclosan (Irgasan)	Fatty acid synthesis inhibition	70% methanol
Boric acid	Toxic ion shock	Sterile distilled water
Sulphamethoxazole	Folate biosynthesis inhibition	Sterile distilled water
Acriflavine	DNA intercalation	Sterile distilled water
Serine hydroxamate	Amino acid starvation	Sterile distilled water
Sucrose	Osmotic shock	Sterile distilled water
Paraquat	Superoxide stress generator	Sterile distilled water
Menadione	Superoxide stress generator	Dimethyl sulphoxide
Kanamycin	30S ribosomal subunit inhibition	Sterile distilled water

Table 2.2.1: Antibiotics and chemicals. Table showing the antibiotics and other chemicals that were used in this study as selective agents or as a means of inducing cellular stresses. Solutions were usually made as 10,000 µg/mL stocks and diluted as required. The majority of agents listed above were purchased from Sigma-Aldrich (Poole, United Kingdom). The triclosan was from Ciba, now part of BASF (Germany).

2.3 The Polymerase Chain Reaction

The polymerase chain reaction (PCR) was used to amplify regions of interest in the genome for further manipulation, to verify that the size of DNA inserts was as expected, to produce a sufficient quantity of DNA for sequencing, or to quantify the abundance of specific templates present in diluted cDNA samples.

Primers, as listed in Table 2.3.1, were either obtained from the ARG primer collection, or designed using the genomic context of the sequence to be amplified and the Primer 3 Plus software (Untergasser *et al.*, 2012). Newly synthesised primers were ordered from Life Technologies (ThermoFisher Scientific, USA). Reactions were performed in a Veriti 96-Well Thermal Cycler (ThermoFisher Scientific, USA) in 25 µL, using 11.25 µL 2x MyTaq Red Mix (Bioline, USA), 11.25 µL Gibco nuclease-free water (ThermoFisher Scientific, USA), 0.75 µL forward primer, 0.75 µL reverse primer and 1 µL template DNA unless stated otherwise. Samples were heated to 95 °C for 1 minute, followed by 25-35 cycles of denaturing at 95 °C for 15 seconds, annealing at 54 °C for 15 seconds, and extending at 72 °C for 10 seconds, though the annealing temperature and extension times were changed if necessary. There was no final extension and samples were held at 4 °C after the last step of the cycle until they were ready for further processing.

No.	Name	Sequence	Product size (bp)	Tm (ºC)
n/a	*recA* F	TCGAAATTTACGGGCCGGAA	599	60.0
n/a	*recA* R	CTGGAACTCGGCCTGCTTAA	599	60.0
n/a	*lexA* F	GTTAACGGCCAGGCAACAAG	594	60.0
n/a	*lexA* R	CATTCCCCGTTGCGAATGAC	594	59.9
238	K1	CAGTCATAGCCGAATAGCCT	n/a	57.3
239	K2	CGGTGCCCTGAATGAACTGC	n/a	61.4
324	16sRNAstm1	CCTCAGCACATTGACGTTAC	246	57.3
325	16sRNAstm2	TTCCTCCAGATCTCTAGGCA	246	57.3
981	*gfp* mut2 F	TCCCATGGCCAACACTTGTC	373	72.0
982	*gfp* mut2 R	GCTAGTTGAACGCTTCAATC	373	68.0
1341	pMW82 GFP *Acc*65I	TGCGGTACCGATAGTCATGCCCCGCGCCC	1304	51.0
1477	*gyrA*_ST_int_F	TGGATTATGCGATGTCGG	475	55.0
1478	*gyrA*_ST_int_R	GTCGCCATACCTACTGCG	475	55.0
1511	pMW82 p-GFP *Sca*I	TGCAGTACTGATAGTCATGCCCCGCGCCC	n/a	54.0
1914	RecA_*gfp*_F	AAAGGATCCATGAAGCCAAAGCGCAGAT	383	60.9
1916	GabD_*gfp*_F	AAAGGATCCCTTATCGGCTGTGCAGGAC	370	60.4
1918	GlnA_*gfp*_F	AAAGGATCCTCGATCACAACTTTGCCTCA	354	60.4
1920	YaeT_*gfp*_F	AAAGGATCCCTCGAAGCCACGGATAAGAC	351	59.8
1930	RecA_v2_R	CGCTCTAGATGAACCGGATAGTGAATCGT	383	59.4
1931	GabD_v2_R	CGCTCTAGATGCACAGCGAGTTTTTCATC	370	60.0
1932	GlnA_v2_R	CGCTCTAGAGGTCGTCGTGGTAACGAGAT	354	60.0
1933	YaeT_v2_R	CGCTCTAGAGAAAAGTGCAAGCCCCATTA	351	60.1
2090	*opdA*_prom_f_*Bam*HI	CGGAAGGATCCATGTTAAAGGGGCGGCTTAT	210	59.8
2091	*opdA*_prom_r	ACATGCTCCGTTTTAATTGC	210	60.0
2094	aphFwd for pMW82	AGCTAGCTGCAGAGCTGTGTAGGCTGGAGCTGCTTC	1500	53.0
2095	aphRev for pMW82	GACTTCAAGCTTAGCTGGGAATTAGCCATGGTCCATATGA	1500	53.0
2216	2216_pyaeT_BamHI_NEW	AAAGGATCCGAAATAACGCCCAACTCGAT	936	59.0

Table 2.3.1: Primers used in this study. The *recA*, *lexA* and 16sRNAstm primer pairs were used for quantifying the expression of *recA*, *lexA* and the 16S RNA using RT-PCR. The *gyrA*_ST_int pair was used for plasmid-to-profile sequencing of the QRDR. All other primers listed were used in the construction of fluorescent reporter plasmids.

PCR products were separated by electrophoresis on 1% agarose gels, made by combining 1 g agarose (Sigma-Aldrich, Poole, UK) and 100 mL 1x Tris/Borate/EDTA (TBE) buffer (Sigma-Aldrich, Poole, UK) and heating in a microwave at 50% power until dissolved. Upon cooling, 5 µL Midori Green (Nippon Genetics Europe, Germany) was added. The gel was loaded with 5 µL of each sample. In the event that a master mix that did not already contain loading dye was used, samples were combined with 2 µL 5x DNA Loading Buffer Blue (Bioline, USA). Samples were separated alongside a HyperLadder 1 kilobase molecular weight marker (Bioline, USA). Electrophoresis used 100 V for 30 minutes. Gels were visualised using a G:BOX F3 gel imaging cabinet (Syngene UK, Cambridge, UK) and analysed with GeneSys and GeneTools software (Syngene UK, Cambridge, UK).

2.4 Susceptibility Testing

The minimum inhibitory concentration of a range of antibiotics was determined using two different methods. Agar-based assays were performed in accordance with the standardised British Society for Antimicrobial Chemotherapy (BSAC) agar doubling dilution protocol which has now been harmonised with recommendations of the European Committee of Antimicrobial Susceptibility Testing (EUCAST) (Andrews and BSAC Working Party on Susceptibility Testing, 2006), and microtitre tray-based MIC assays again used the EUCAST/BSAC microdilution method (Andrews, 2001).

2.5 DNA Sequencing

Genes and genomes were sequenced to confirm expected genotypes, and to check the results of PCRs and ligations. Sequencing of small fragments was carried out

using the plasmid-to-profile service provided by the University of Birmingham Functional Genomics Facility. Primers upstream and downstream of the region to be sequenced were diluted to 0.8 pmol. One diluted primer, at a volume of 4 µL, was combined with 4 µL of nuclease free water and 2 µL of the DNA of interest. This reaction was then taken to the Functional Genomics Facility. The Functional Genomics team added 10 µL of Big Dye Terminator Reaction Mix and sequenced the DNA using their ABI 3730 DNA Analyser (ThermoFisher Scientific, USA). Sequences were visualised using 4Peaks (Griekspoor and Groothius, 2006). They were analysed by aligning the obtained sequence data against the reference gene sequence, obtained from the NCBI Gene database (Brown *et al.*, 2015). This alignment was performed using Clustal W2 (Larkin *et al.*, 2007).

2.6 Growth Kinetics Assays

To determine the impacts of the addition of antibiotics and/or chemicals to bacterial cultures upon growth rate, growth kinetics assays were performed as described in Webber et al 2013. Overnight cultures were diluted 1:100, with 50 µL of culture being added to 5 mL LB broth. A clear plastic 96-well plate was inoculated with 200 µL aliquots of the cultures of interest and antibiotics or chemicals were added to a final concentration of half of the MIC. The plates were incubated overnight in a Fluostar Optima (BMG Labtech, Germany) at 37 ºC, and the optical density at 600 nm was recorded every three minutes. Data were exported to an .xls file and analysed in MS Excel for Mac 2011 (Microsoft Corportation, USA).

2.7 Perturbation of Cultures to Examine Impacts on Supercoiling and Gene Expression

A central aim of this project was to determine if there was a relationship between alterations in stress response expression and supercoiling. To examine this the impact of a range of stress conditions on the supercoiling state of pBR322 or expression of stress responsive promoters was determined. Cultures were grown to mid-logarithmic phase before being perturbed. An example is determining the effects of induction of the SOS response; *Salmonella* Typhimurium or *E. coli* cells with and without *gyrA* mutations were grown in liquid culture (5 mL) to logarithmic phase at 37 ºC under aerobic conditions. The cultures were divided into two groups, one of which had nalidixic acid added to a final concentration of 80 µg/mL, and the other had the equivalent volume of sterile distilled water added. These were then returned to the incubator for a further 45 minutes. Subsequently, either RNA or the plasmid pBR322 was extracted (the use of fluorescent reporter fusions to determine gene expression is detailed later in Section 2.15), depending on the subsequent processes that were to be used. Plasmid extraction is described in Section 2.8, and RNA extraction is described in Section 2.13. This same scheme was used for perturbing supercoiling with other antibiotics and chemicals to assay supercoiling. The agents used for perturbation are described in Table 2.2.1, and the concentrations used to provoke perturbations were based on the results of susceptibility testing or the literature.

2.8 Plasmid Extraction

Plasmids were extracted from cells for a number of reasons, including acquiring a template for cloning, constructing fluorescent reporters, and for assaying

supercoiling. Overnight cultures carrying plasmids were harvested by centrifugation at 3,300 x *g* for 10 minutes in a Heraeus Megafuge 40R centrifuge (ThermoFisher Scientific, USA). Plasmids were subsequently extracted using a GeneJET Plasmid Miniprep kit (ThermoFisher Scientific, USA) according to the manufacturer's instructions. This kit uses a combination of alkaline lysis and spin columns to isolate plasmid DNA of an appropriate size. The presence of products, and their sizes, were verified by separation on a 1% agarose gel for 30 minutes at 100 V alongside a HyperLadder 1 kb molecular weight marker (Bioline, USA), and visualised as described in Section 2.3. The concentrations of extracted plasmids were estimated using GeneTools (Syngene UK, Cambridge) and determined by measuring the ratio of absorbance at 260 nm and at 280 nm using a NanoDrop 1000 spectrophotometer (ThermoFisher Scientific, USA).

2.9 Transformation of Strains with pBR322

The plasmid pBR322, as shown in Figure 2.9.1, was transformed into cells so that it could be used as a proxy to assay genomic supercoiling. The plasmid was obtained from a pre-existing host using an alkaline lysis kit, as described in Section 2.8. Cells initially had to be made either electrocompetent or chemically competent before the plasmid could be transformed into them.

To make cells electrocompetent, the strains of interest were grown to an optical density of 0.6 (600 nm), roughly equivalent to mid-logarithmic phase, in 5 mL LB broth supplemented with antibiotics as necessary. The mid-logarithmic phase cells were harvested in a centrifuge, cooled to 4 ºC, at 3,300 x *g* for ten minutes. The

supernatant was discarded and the pellet was re-suspended in 10 mL ice-cold 10% glycerol. The process of centrifugation and re-suspension was repeated twice more. The cells were harvested again under the same conditions and then re-suspended in 500 µL ice-cold 10% glycerol. Cells were then stored at -20 ºC until required.

Figure 2.9.1: The plasmid pBR322. Figure showing the plasmid pBR322, adapted from Bolivar *et al.,* (1977) and Watson (1988), which was used as a proxy for genomic supercoiling. The plasmid is 4,361 bp in length, and contains a tetracycline resistance cassette (*tet*), an ampicillin resistance cassette (*bla*), an origin of replication (*ori*) and a restrictor of plasmid copy number *(rop)*. The ampicillin resistance cassette was used to select for successful transformants by culturing cells on media containing ampicillin.

To make cells chemically competent, 0.5 mL of an overnight culture of cells was diluted in 50 mL of LB broth in a 500 mL bottle and growing aerobically at 37ºC to an optical density of between 0.4-0.6 at 600 nm. These cells were then harvested by centrifugation at 3,600 x *g* for 10 minutes at 4 ºC. The supernatant was removed and cells were re-suspended in 25 mL 0.1 M calcium chloride solution (Sigma-Aldrich, Poole, UK) and chilled on ice for half an hour before being harvested again. The supernatant was removed and the pellet was re-suspended in 2 mL 0.1 M calcium chloride. The cells were then heat shocked as described above. If the cells were to be used at a later date, 0.4 mL of 50% glycerol was added per millilitre of re-suspended cells and they were then stored at -80 ºC.

To transform plasmids into electrocompetent cells, 2 µL of the plasmid of interest, at a concentration of approximately 50 ng/µL, was combined with 50 µL of the cells of interest in a pre-cooled 2 mm gap electroporation cuvette and kept on ice for five minutes. *Salmonella* were subjected to a pulse of 2.5 kV for 5 ms and *E. coli* were subjected to a pulse of 1.5 kV. To transform plasmids into chemically competent cells, 50 µL aliquots of cells were combined with 2 µL of the plasmid of interest and kept on ice for half an hour. These were then placed in a water bath at 42 ºC for 45 seconds and then placed on ice for two minutes.

After transformation, cells were then transferred to universal tubes containing 1 mL pre-warmed LB broth and grown for an hour at 37 ºC and shaken at 200 rpm. The cultures were harvested and the supernatant was poured off, with the pellet being re-suspended in the remaining supernatant. Spread plates were then made with 100 µL

aliquots of the recovery cultures on LB agar plates supplemented with an appropriate selective agent, and incubated overnight at 37 ºC without shaking.

Recovery cultures were retained in the event that there was no growth on the plates and, in this case the remaining culture was harvested after recovery, most of the supernatant removed and the pellet re-suspended in the remainder before this was used to inoculatea plate. Plates were examined for growth and colonies were chosen and sub-cultured onto fresh LB agar plates containing an appropriate selective agent, as well as XLD agar plates, to verify they were the correct species and to give a quantity of bacteria that was suitable to work with. If the colonies on XLD agar were the correct colour (black for *Salmonella* and yellow for *E. coli*), bead stocks were made from the colonies grown on LB agar.

2.10 One-Dimensional Topoisomer Separation

To assay genomic supercoiling, the plasmid pBR322 was used as a proxy due to the difficulty in measuring chromosomal supercoiling directly and the need to complete a large number of supercoiling measurements in this project. Initially, a protocol adapted from Cameron *et al.* (2011) was utilised. After plasmids had been extracted from cells of interest as described in Section 2.8, a 1% agarose gel was made with 2x TBE supplemented with 2.5 µg/mL >=98% chloroquine (Sigma-Aldrich, Poole, UK), and the running buffer was made of the same components bar the agarose. Aliquots of the plasmid containing 1 µg DNA were combined with 4 µL of 5x Loading Buffer Blue (Bioline, USA) and loaded into the gel alongside 5 µL of HyperLadder 1 kilobase molecular weight marker (Bioline, USA). Topoisomers were then separated

by electrophoresis at a voltage of 3 V per centimetre of the length of the gel (*e.g.* a 15 centimetre gel would be subjected to 45 V) for 16 hours. Subsequently, the gel was washed in a container of tap water on a shaking platform for 3 hours to remove the chloroquine, with the water being changed every 20 minutes. The gel was then stained with 1 µg/mL ethidium bromide or the same concentration Midori Green (Nippon Genetics Europe, Germany) by washing in water supplemented with the dye for 1 hour, followed by a brief wash in tap water before being imaged as described in Section 2.3.

It was later found to be more efficient to run the gels at a higher voltage for a shorter time, producing comparable results whilst saving significant amounts of time. The revised method used 1x TBE gels containing 1.5% agarose, 5 µL Midori Green (Nippon Genetics Europe, Germany) per 100 mL of TBE and 2.5 µg/mL chloroquine. A volume of sample equivalent to 300 ng was combined with 5 µL 5x Loading Buffer Blue (Bioline, USA), and separated alongside 5 µL of HyperLadder 1 kilobase molecular weight marker (Bioline, USA). Electrophoresis was performed in a running buffer of 1x TBE supplemented with 2.5 µg/mL chloroquine at 80 V for 4 hours, and gels were imaged as described in Section 2.4. This protocol was adapted from prior work by Maxwell *et al.* (2006) after exploring various combinations of pre- and post-run staining, with both Midori Green and ethidium bromide, no appreciable difference between pre-stained Midori Green gels and post-stained ethidium bromide gels was observed. Adapting the protocol involved topoisomers being separated on 1%, 1.5% and 2% agarose gels for 4h at 80 V and stained with different protocols. To compare methods, four 50 g 1% agarose gels were set up, two of which had 2.5 µL Midori

Green added before they solidified, and the remaining two were washed for an hour in tap water, had their water changed and washed again in tap water containing 20 µL Midori Green. One gel from each pair also had 2.5 µg/mL of chloroquine added. Loading dye and 5 µL of the same preparation of plasmid pBR322 were combined and separated on each gel for 4 hours at 80 V. Subsequently, 1.5% and 2% 50 mL agarose gels were set up containing 2.5 µL Midori Green and no chloroquine; topoisomers of pBR322 from the same preparation were again separated on these gels for 4 hours at 80 V and the gels were imaged as described previously.

2.11 Two-Dimensional Topoisomer Separation

To separate topoisomers in two dimensions and thus differentiate between positively supercoiled and negatively supercoiled DNA, a number of methods were examined. Initially, 1% agarose gels were made with 1x TBE and 3 µL Midori Green (Nippon Genetics Europe, Germany) per 100 mL TBE. The gel and running buffer were supplemented with 2.5 µg/mL of chloroquine. (Sigma-Aldrich, Poole, UK) Approximately 10 µL of concentrated plasmid was mixed with 5 µL 5x Loading Buffer Blue (Bioline, USA), and topoisomers were separated by electrophoresis at 120 V for an hour and a half. Gels were then rotated 90º clockwise (so that the wells were on the right-hand side) and more chloroquine was added, to a final concentration of 2.5 mL per litre of TBE (including the TBE in the gel). Separation then continued for 45 minutes at the same voltage, and gels were then imaged as described in Section 2.3. This initial protocol was unsuccessful; gels were frequently blank when imaged.

Different combinations of staining the gel before running it, and staining and washing it afterwards were attempted as to address concerns that the gels were blank when imaged because they were being overstained resulting in a high amount of background stain on each gel. Changes to the duration of the steps during the washing section of the protocol were tried. The samples were also loaded with non-commercial loading dye. However, these variations on the protocol did not make significant improvements and gels were still blank upon imaging. Ultimately, the most effective “quick” method was to make a 1% agarose gel with 1x Tris-Acetate-EDTA (TAE), supplemented with 3 µL Midori Green per 100 mL and poured to a thickness of 5 mm. TAE was made as a 50x stock by gradually adding 242 g of Tris (Sigma-Aldrich, Poole, UK) and 18.6 g of EDTA (Sigma-Aldrich, Poole, UK) to 700 mL deionised water. This mixture was stirred on a magnetic stirring plate and then combined with 57.1 mL of glacial acetic acid (Sigma-Aldrich, Poole, UK), after which it was made up to a final volume of 1 L with deionised water. The 50x TAE stock was diluted in deionised water as required and 1x TAE was used for both the gel and the running buffer. Electrophoresis occurred at 120V for 2 hours in the first dimension, with imaging being carried out hourly in the G:BOX imaging cabinet as described in Section 2.3. The buffer was supplemented with 5 µL Midori Green upon the replacement of the gel in the tank. After the second hour, gels were rotated 90º clockwise in the tank and the buffer was supplemented with 1.5 mL 10,000 µg/mL chloroquine. Topoisomers were separated by electrophoresis for a further 15 minutes and gels were then imaged as described in the G:BOX imaging cabinet as described in Section 2.3.

2.12 RNA Extraction, cDNA Synthesis and RT-PCR

RNA was extracted from cells grown in the presence and absence of nalidixic acid, to determine the level of induction of the SOS response. Centrifuge tubes with a capacity of 50 mL were pre-cooled on ice and then had 1 mL of ice cold 95% ethanol: 5% phenol added. After reaching an appropriate optical density, 5 mL of bacterial culture was added to each 50 mL tube. The tubes were then left to incubate on ice for half an hour, before being harvested at 3,000 x *g* for 10 minutes in a centrifuge that had been pre-cooled to 4 ºC. The supernatant was then disposed of accordingly, and the tubes were stored at -80 ºC. The samples were later defrosted and re-suspended in the residual liquid, then transferred to microfuge tubes. These were harvested at 10,000 x *g* for 60 seconds in a refrigerated centrifuge, and the supernatant was discarded. The pellets were then re-suspended in 100 µL Tris-EDTA buffer containing 50 µg/mL lysozyme. This mixture was incubated at room temperature for five minutes, and then 75 µL SV40 Total RNA Isolation System lysis reagent (Promega, USA) was added and the tubes were mixed by inversion. Then, 350 µL RNA dilution buffer (Promega, USA) was added and the samples were mixed by inversion. Samples were then heated at 70 ºC for 3 minutes, and spun in a microcentrifuge for 10 minutes at maximum speed.

While the samples were being spun, a DNAse mixture was made by combining 50 µL 90 mM manganese(II) chloride, 40 µL DNAse core buffer and 5 µL DNAse, each multiplied by the number of samples plus one. The reagents for making the DNAse mixture were, like the other buffers, obtained from the SV40 Total RNA Isolation System kit (Promega, USA).

Once the centrifugation had finished, the supernatant was transferred to a clean tube, combined with 200 µL 95% ethanol, and mixed by inversion. This was then transferred to a spin column and harvested in a refrigerated desktop centrifuge at full speed for 30 seconds. The elutate was discarded, and 600 µL wash buffer (also from the RNA isolation system kit) was added to each spin column before the columns were spun again under the same conditions.

Subsequently, 50 µL of the DNAse mixture was added to each spin column and the columns were incubated at room temperature (approx. 22 ºC) for quarter of an hour. The reaction was then stopped through the addition of 200 µL DNAse stop mix (Promega, USA) and the spin columns were spun in a desktop centrifuge fro 30 seconds. The columns had 600 µL wash buffer added to them and were spun again, with the eluate being discarded. The wash step was repeated with 250 µL buffer, then the spin filter was returned to the collection tube and spun for a minute at full speed. The columns were then transferred to sterile microfuge tubes with 1 µL RNase OUT (Promega, USA) and 100 µL nuclease-free distilled water. After being left to stand for 1 minute, the tubes were spun at 10,000 rpm for 2 minutes in a desktop centrifuge. The column was discarded and the tubes were immediately placed on ice.

Subsequently, 5 µL of RNA was combined with 2 µL of 5x Loading Buffer Blue (Bioline, USA), and separated on a 1 % agarose gel at 100 V, alongside 5 µL of HyperLadder 1 kb molecular weight marker (Bioline, USA). The gel was visualised

using a G:BOX F3 gel imaging cabinet (Syngene UK, Cambridge, UK) and analysed with GeneSys and GeneTools (Syngene UK, Cambridge, UK) to determine an approximate RNA concentration. A more accurate measure of RNA concentration was determined by using a NanoDrop 1000 spectrophotometer (ThermoFisher Scientific, USA).

To make cDNA, RNA was reverse transcribed by combining 5 µg RNA, 100 ng of Random Primers (ThermoFisher Scientific, USA), 1 µL 10 mM dNTP Mix (ThermoFisher Scientific, USA) and 5 µL of nuclease-free sterile distilled water to a final volume of 13 µL. This mixture was heated at 65 ºC for 5 minutes and then incubated on ice for 5 minutes. The mixture was briefly spun in a desktop centrifuge. After the spin had finished, 4 µL 5x First Strand Buffer, 1 µL 0.1 M dithiothreitol, 1 µL RNase Inhibitor and 1 µL Superscript III Reverse Transcriptase (all ThermoFisher Scientific, USA) were added to the mixture. This was then incubated at room temperature for 5 minutes, then at 50 ºC for an hour, and finally at 70 ºC for 10 minutes. The resulting cDNA was then used for a reverse transcription PCR, with the remainder stored at -80 ºC. The aim of the reverse transcription PCR to determine the levels of transcripts identified as being part of the SOS response. Reactions were set up as per Section 2.3, using the cDNA and the *recA* F/R, *lexA* F/R and 16SstmRNA1/2 primer pairs – the sequences of which are listed in that section. The resulting PCR products were analysed on an agarose gel, as described in Section 2.3.

2.13 Identification of Sigma Factor Responsive Promoters and Construction of Fluorescent Reporters

The regulons and binding motifs of a series of sigma factors implicated as being up-regulated in gyrase mutants were identified via a literature survey and examination of available biochemical data in the Ecocyc database (Keseler *et al.*, 2017). Promoters were chosen as representatives of each sigma regulon and those with promoters essentially identical in *E. coli* and *Salmonella* were selected as proxies for activation of the regulon as a whole. These are shown in Table 2.13.1, below. Once a promoter was chosen the coding sequence of the gene and 500 nucleotides upstream of the first members of each operon were obtained from the NCBI. Primers were designed using Primer 3 Plus so as to amplify the appropriate sigma factor binding sites, and then tagged with an *Xba*I site and a *Bam*HI site so that the amplimers could be digested and inserted into the plasmid pMW82. This is represented diagrammatically in Figure 2.13.1.

Plasmid pMW82 lacking a promoter upstream of the *gfpmut2* locus was obtained from a strain of *S.* Typhimurium that was already carrying the plasmid, using a GeneJET Miniprep kit (ThermoFisher Scientific, USA) according to the manufacturer's instructions, as described in Section 2.8. The previously identified promoter regions of interest were amplified from genomics DNA of SL1344 by PCR. Separate restriction digests were then set up for the plasmid and promoters, using *Xba*I and *BamH*I FastDigest restriction enzymes (ThermoFisher Scientific, USA) in accordance with the manufacturer's instructions. The restriction enzymes were then deactivated by incubating the reaction mixtures at 80 ºC for five minutes. The

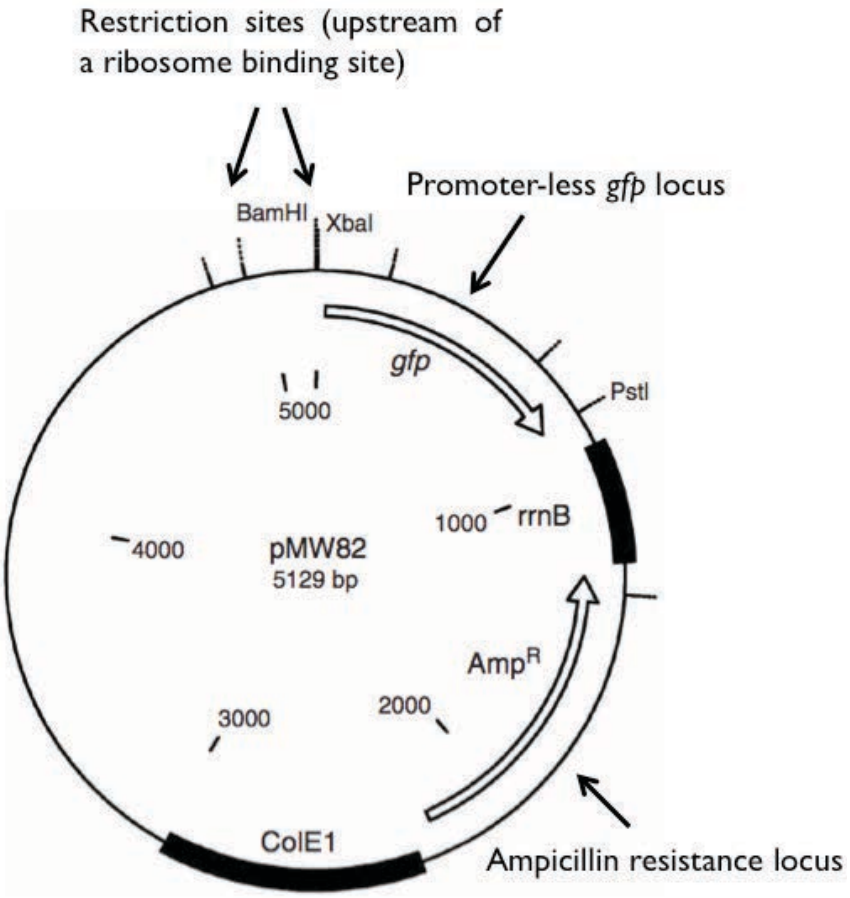
reaction mixtures were then treated with 4 µL Calf Intestinal Alkaline Phosphatase (Promega, USA) and 4 µL Alkaline Phosphatase 10x Buffer (Promega, USA), and then incubating at 37 ºC for half an hour so as to prevent autoreligation. A further 4 µL Calf Intestinal Alkaline Phosphatase was added and the reactions were incubated at 37 ºC for another half an hour.

The digested promoter sequences were ligated with separate aliquots of the digested plasmid by combining them in the molar ratio of 3 parts insert to 1 part vector and adding this to 1 µL of Ligase 10x buffer (Promega, USA) and 1 µL of T4 DNA ligase (Promega, USA). This was then made up to a final volume of 10 µL with nuclease-free water, and the reaction was incubated at 4 ºC overnight. The correct insertion of the promoters into the plasmid was verified by PCR, as described in Section 2.3, with the primers being the appropriate forward primer for amplifying the promoter sequence and the *gfpmut2* reverse primer. The PCR products were then separated on a gel and imaged as described in Section 2.3. Cells were made competent and transformed with the pMW82 constructs using the same method for transforming cells with pBR322, as described in Section 2.10.

Stress condition	Sigma factor	Target locus	Source	Inducer used	Amount
Stationary phase	RpoS	*gabD*	Bang *et al.* (2005)	Time	n/a
Osmotic shock	RpoE	*bamA*	Namdari *et al.* (2012); Skovierova *et al.* (2006)	Sucrose	20% solution
Heat shock	RpoH	*opdA*	Conlin and Miller (2000)	Growth at 42 ºC	n/a
Nitrogen stress	RpoN	*glnA*	Samuels *et al.* (2013)	Serine hydroxamate	100 µg/mL
SOS response	n/a	*recA*	Cox (2007)	Nalidixic acid	80 µg/mL

Table 2.13.1: Sigma factors and target loci that were used to design fluorescent reporter constructs. Sigma factors and the conditions in which they are incorporated into the RNA polymerase were identified on the basis of a literature survey. This literature survey also identified loci that were part of the regulons of these sigma factors, and the promoters of these loci were used to create a series of pMW82-derived fluorescent reporter constructs.

A)



B)

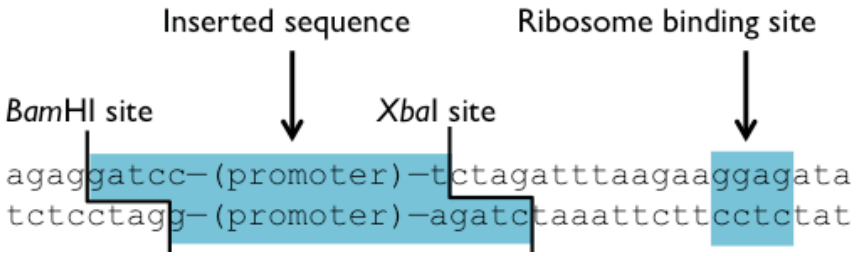


Figure 2.13.1: The plasmid pMW82, with detail of the *Bam*HI and *Xba*I restriction sites. Part A) shows the fluorescent reporter plasmid pMW82. This is a 5.1 kb plasmid with a promoterless GFP locus, and an ampicillin cassette that can be used to selection transformants. Part B) shows the sequence surrounding the *Bam*HI and *Xba*I sites upstream of the *gfpmut2* locus. Digesting the plasmid with these two enzymes allows for the insertion of a promoter sequence upstream of the *gfpmut2*, provided it is flanked with the appropriate sequences. The site of promoter insertion and the ribosome binding site are both highlighted in blue. Figure adapted from Bumann and Valdivia (2007).

2.14 Fluorescence Induction Assays

To assay the effects of different stresses on the expression of *gfpmut2* as controlled by the inserted promoters, a protocol adapted from that described in Lawler *et al.* (2013) was utilised. All strains (WT and *gyrA* mutants) were stratified into groups determined by which construct they were carrying (*i.e.* for the p*recA:gfpmut2* reporter, a group consisted of SL1344 and the two *gyrA* mutants each carrying pMW82 with p*recA:gfpmut2*). Each group were grown overnight as described in Section 2.1. Sub-cultures were grown aerobically at 37 ºC the following morning in the absence of any antibiotics until they reached an optical density of 0.3 at 600 nm. They were incubated in the absence of antibiotics as ampicillin has been shown to be auto-fluorescent under some conditions (Brittain, 2005) and can interfere with the behaviour of fluorescent probes (Renggli *et al.*, 2013). Aliquots with a volume of 190 µL were taken and inoculated on a black, clear-bottomed 96-well plate (ThermoFisher Scientific, USA), and then had varying concentrations of different chemicals added to make a final volume of 200 µL, using the layout shown in Figure 2.14.1. A Breathe-Easy sealing membrane (Sigma-Aldrich, Poole, UK) was then applied to the top of the plate. Plates were incubated overnight for 16-19 hours at 37 ºC in a Fluostar Optima or Fluostar Omega (BMG Labtech, Germany), depending on whichever was available, with fluorescence (excitation 492 nm, emission 520 nm) and absorbance (600 nm) measured approximately every three minutes. The data for each time point was merged and combined into a spreadsheet, which was analysed in MS Excel for Mac 2011 (Microsoft Corporation, USA).

The fluorescence reading for each well at each time point was then divided by the

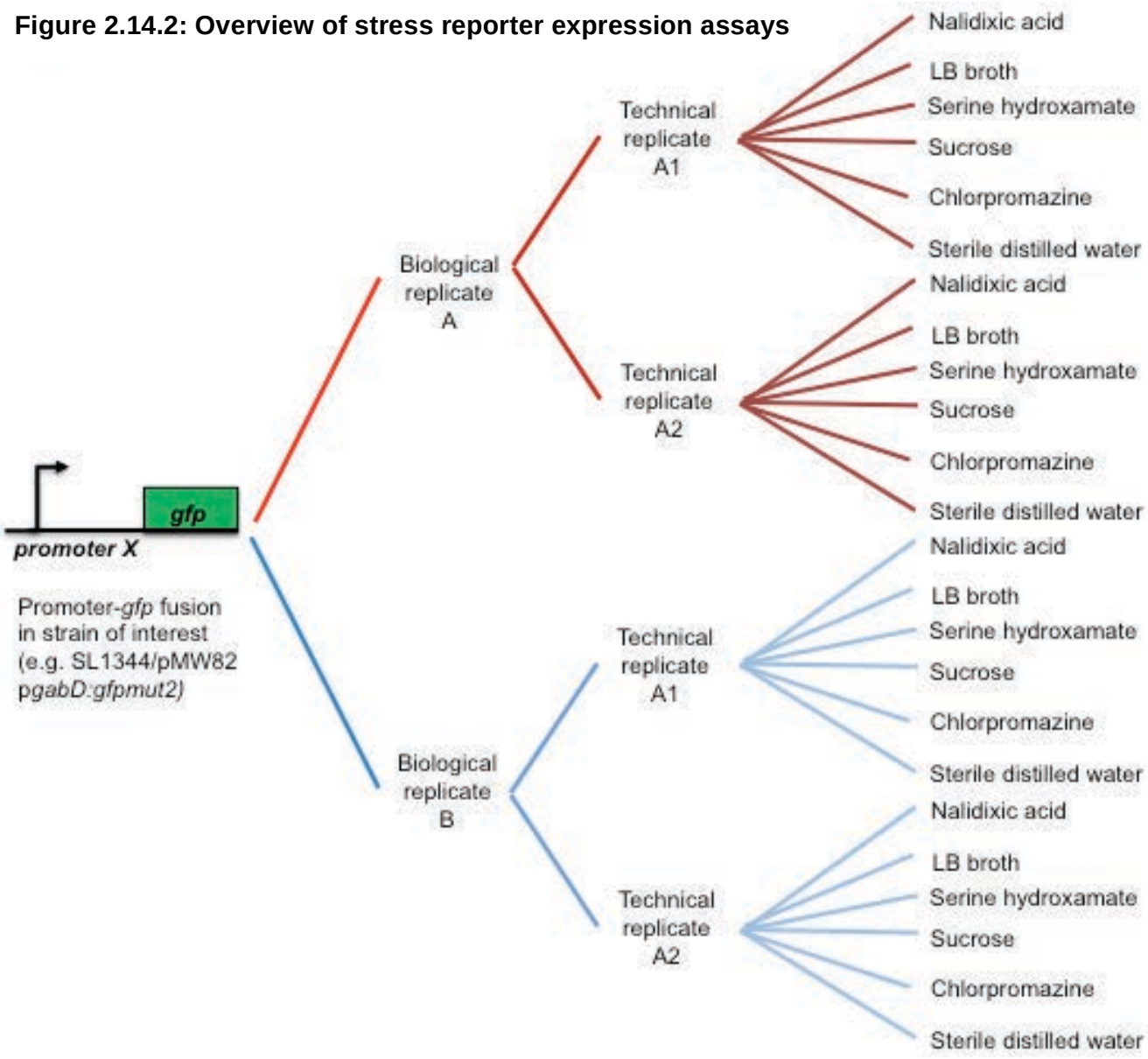
corresponding absorbance reading to give the relative fluorescence with respect to absorbance. This reading was then pooled with wells containing the same combination of strain and inducer, and the average was taken. These data were then plotted on a graph. Additional graphs were plotted wherein the relative fluorescence readings for each strain under each condition were normalised to the reading for the wild type strain exposed to each of the inducers at time zero.

The experiments were repeated with the pMW82 constructs of interest transformed into *Escherichia coli* MG1655-derived backgrounds. *E. coli* MG1655 itself was used as the wild type. *E. coli* MG1655 GyrA Ser83Leu and *E. coli* MG1655 GyrA Asp87Gly were used in place of *S.* Typhimurium SL1344 GyrA Ser83Phe and *S.* Typhimurium SL1344 GyrA Asp87Gly, respectively. The *S.* Typhimurium strains that were used as fluorescence controls (*i.e.*, SL1344, SL1344/pMW82, SL1344/pMW82 p*ramA*:*gfpmut2* and SL1344 Δ*ramRA*/pMW82 p*ramA:gfpmut2*) were still used in that capacity. Data were generated and analysed in the same way as when the experiments were done with the constructs in *Salmonella* backgrounds. The strategy used for generating data is represented diagrammatically in Figure 2.14.2.

	1	2	3	4	5	6	7	8	9	10	11	12
A	LB broth	Salmonella Typhimurium SL1344/pMW82 p*ramA:gfpmut2* + 50 µg/mL chlorpromazine	WT + 50 µg/mL chlorpromazine	WT + 80 µg/mL nalidixic acid	WT + 20% sucrose	GyrA Ser83(Phe/Leu) + 50 µg/mL chlorpromazine	GyrA Ser83(Phe/Leu) + 80 µg/mL nalidixic acid	GyrA Ser83(Phe/Leu) + 20% sucrose	GyrA Asp87Gly + 50 µg/mL chlorpromazine	GyrA Asp87Gly + 80 µg/mL nalidixic acid	GyrA Asp87Gly + 20% sucrose	
B												
C	*Salmonella* Typhimurium SL1344	Salmonella Typhimurium SL1344 Δ*ramRA*/pMW82 p*ramA:gfpmut2*										
D												
E	*Salmonella* Typhimurium SL1344/ pMW82	*Escherichia coli* MG1655 (only used when constructs in columns 3-11 were in an *E. coli* background)	WT + 100 µg/mL serine hydroxamate	WT + 10 µL LB broth	WT + 40 µL sterile distilled water	GyrA Ser83(Phe/Leu) + 100 µg/mL serine hydroxamate	GyrA Ser83(Phe/Leu) + 10 µL LB broth	GyrA Ser83(Phe/Leu) + 40 µL sterile distilled water	GyrA Asp87Gly + 100 µg/mL serine hydroxamate	GyrA Asp87Gly + 10 µL LB broth	GyrA Asp87Gly + 40 µL sterile distilled water	
F												
G	*Salmonella* Typhimurium SL1344/pMW82 p*ramA:gfpmut2*											
H												

Figure 2.14.1: Fluorescence reporter assay plate layout. Layout used for fluorescence reporter assays in a black, clear-bottomed 96-well plate as read in a Fluostar Optima or Omega (BMG Labtech, Germany). WT = the fluorescent reporter constructs in either a *Salmonella* Typhimurium SL1344 background or an *Escherichia coli* MG1655 background, depending on which species was being assayed. Columns 6 through 8 contained either cells with a GyrA Ser83Phe or GyrA Ser83Leu substitution, depending on which species was being assayed. GyrA Ser83Phe cells were used when the species assayed was *S.* Typhimurium, and GyrA Ser83Leu cells were used when the species assayed was *E. coli*.

Figure 2.14.2: Overview of stress reporter expression assays



Five pMW82 reporters were made (p*recA*, p*gabD*, p*glnA*, p*bamA* and p*opdA*). Each was transformed into three strains (WT GyrA, GyrA Ser83[Phe/Leu], GyrA Asp87Gly), in two species (*Salmonella* Typhimurium and *E. coli*). The reporters were assayed under six conditions. Experiments used two biological replicates, with two technical replicates each, per reporter in each each background under each condition. 6 conditions x 2 technicals x 2 biologicals x 5 constructs x 3 strains = 360 data points at each time point per species = 360 data points (720 for both species).
Data were generated over the course of 19 hours, with recordings made every 3 minutes (*i.e.* 20 per hour). This equates to over a quarter of a million total data points (19x20x720 = 273,600). This number does not include data generated from control strains, which were also included in every run.

2.15 Cladistic Analysis of Topoisomerase Protein Sequences

Toposiomerase genes are conserved across the Enterobacteriaceae although this is not complete. As some of the data generated in this project indicated that analogous mutations within *gyrA* had different impacts on DNA gyrase in *E. coli* and *Salmonella* the potential for lineage specific adaptations in *gyrA* was investigated. To determine if there was a correlation between the components of the bacterial topoisomerases and the pathogenic niche occupied by strains, a cladistic analysis of their protein sequences was performed.

A number of extraintestinal pathogenic *E. coli* (ExPEC), enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC) and uropathogenic *E. coli* (UPEC) strains were identified based on a literature survey and are listed in Table 2.15.1. The protein sequences of GyrA, GyrB, ParC, ParE and TopA from these strains, as well as the non-pathogenic *E. coli* strains MG1655 and BL21, and the *S.* Typhimurium strains SL1344, LT2 and 14028S, were obtained from the NCBI protein database. The protein sequences for the topoisomerases of *Staphylococcus aureus* strain 55-2053 were also obtained as they were expected to be an appropriate out-group.

Multiple sequence alignments for each set of protein sequences were performed using Clustal Omega (Sievers *et al.*, 2014) using the default parameters. Cladograms and percentage identity matrices were created by uploading the output file from Clustal Omega into Clustal W2 (Larkin *et al.*, 2007) using the default settings, save for changing the Percentage Identity Matrix setting to “on”.

Strain	Pathotype	Source
O157:H7	Enterohaemorrhagic	Yoon and Hovde, 2008
O104:H4	Enterohaemorrhagic	Monecke *et al.*, 2011
ST95	Extraintestinal	Ciesielczuk *et al.*, 2016
ST131	Extraintestinal	Ciesielczuk *et al.*, 2016
O103:H2	Enteropathogenic	Witsø *et al.,* 2016
O127:H6	Enteropathogenic	Iguchi *et al.*, 2009
UTI89	Uropathogenic	Hagan *et al.*, 2009
CFT073	Uropathogenic	Hagan *et al.*, 2009
NA114	Uropathogenic	Avasthi *et al.*, 2011
O139:H28	Enterotoxigenic	Jordi *et al.*, 1991
H10407	Enterotoxigenic	Yamamoto and Yokota, 1983
UMNF18	Enterotoxigenic	Shepard *et al.*, 2012
MG1655	Non-pathogenic	Bereswill *et al.*, 2013
BL21	Non-pathogenic	Wu *et al.*, 2003

Table 2.15.1: List of *Escherichia coli* strains subjected to cladistic analysis of topoisomerase sequences. A list showing the *Escherichia coli* strains that were used to investigate the relationship between topoisomerase protein sequence and pathotype. A range of strains from a number of different pathotypes were chosen on the basis of a literature survey, and their topoisomerase protein sequences were then obtained from the NCBI Protein database. The analysis also included the *Salmonella* Typhimurium strains SL1344, 14028S and LT2, and the *Staphylococcus aureus* strain 55-2053; the *Salmonella* strains were expected to cluster together, and the *Staphylococcus aureus* strain was chosen to serve as an outgroup.

2.16 Flow Cytometry

To assay the levels of fluorescence of the pMW82 reporter constructs on a per cell basis, rather than at a population level, cells with and without the reporter constructs were analysed using an Attune NxT Flow Cytometer (ThermoFisher Scientific, USA).

Subcultures of cells were set up by adding 50 µL of an overnight culture to 5 mL of LB broth, with appropriate antibiotics added where necessary. Upon reaching a desired optical density, 200 µL aliquots were taken and harvested in a desktop centrifuge at full speed for 2 minutes. The supernatant was discarded, and then the pellet was re-suspended in 50 µL filtered, autoclaved phosphate-buffered saline solution (Sigma-Aldrich, Poole, UK). The re-suspended cells were then diluted further in 1 mL of filtered, autoclaved phosphate-buffered saline.

Samples were loaded, one at a time, into the flow cytometer using the syringe pump. Phosphate-buffered saline, a non-fluorescent bacterial strain and a constitutively fluorescing bacterial strain, *Salmonella* Typhimurium Δ*ramAE* carrying the pMW82 p*ramA*:*gfp* reporter construct, were used to define appropriate gates based on their forward scatter, side scatter and fluorescence. GFP was excited using the 488 nm blue laser, set to a voltage of 500 V. After the gates had been defined, the cells of interest were analysed. Ten thousand fluorescent cells were sampled at a rate of 50 µL per minute. The data were exported as a .csv file and analysed in MS Excel 2016 (Microsoft Corportation, USA). The level of expression within each population was compared as well as the numbers of individual cells producing high levels of *gfp*.

Chapter Three

Mutations in *gyrA* Alter Supercoiling and Antibiotic Tolerance

3.1 Introduction

Bacterial DNA is highly condensed so as to be able to fit inside the cell, as the bacterial chromosome is many times larger than the cell itself when unfurled. DNA supercoiling is one mechanism by which DNA can be compacted. In *Escherichia coli* and *Salmonella* Typhimurium, the supercoiling state of the chromosome has been shown to change after exposure to different conditions, *e.g.* temperature, acid stress, salt concentration, and also varies greatly depending on the growth phase of the cell (Cameron *et al.*, 2011).

Topoisomerases are enzymes that can modify the supercoiling state of DNA by relaxing or introducing supercoils. The Type II topoisomerases can introduce negative supercoils through strand-passage (Bush *et al.*, 2015). They are highly conserved and make good targets for antibiotics (Redgrave *et al.*, 2014). Quinolones and fluoroquinolones are antibiotics that disrupt the function of the Type IIa topoisomerases, DNA gyrase and topoisomerase IV, by intercalating with the complex the enzymes form with DNA (Aldred *et al.*, 2014). Modern fluoroquinolones are broad-spectrum agents and are widely used in clinical settings (Aldred *et al.*, 2014), though clinical resistance to quinolones was first observed in the early 1990s (Piddock *et al.*, 1990) and has since become widespread. The major mechanism of quinolone resistance is the mutation of the genes that encode the proteins that comprise DNA gyrase and topoisomerase IV (Redgrave *et al.*, 2014). It has been shown that clinically relevant mutations in the quinolone-resistance determining region of *gyrA* in *S.* Typhimurium reduce susceptibility to quinolones, but also influence susceptibility to a number of other antimicrobials, and alter the supercoiling

profile of the cell (Webber *et al.*, 2013). An explanation proposed for this phenotype is that mutations in *gyrA* can alter the supercoiling state of the genome and change the cells ability to respond to various stresses. This is investigated in this chapter.

It was also felt that the effects of equivalent *gyrA* substitutions in *Escherichia coli* should be investigated. This was because *Escherichia coli* and *Salmonella* Typhimurium are both Gram-negative, rod-shaped Gammaproteobacteria (Williams *et al.*, 2010) capable of causing intestinal illness. They are evolutionarily closely related and their core genomes are a similar size, with the *E. coli* genome having 4.6 megabases (Blattner *et al.,* 1997), and the *S.* Typhimurium genome having 4.8 megabases (McClelland *et al.,* 2001). Figure 3.1.1, created using PhyloPhlAn (Segata *et al.*, 2013), shows a phylogenetic tree of bacterial relationships, with the positions of *E. coli* and *S.* Typhimurium highlighed. Some major differences between the core genomes of both species relate to horizontally acquired islands characterised by the presence of large AT-rich regions. Examples of such AT-rich regions in the *Salmonella* genome are the *Salmonella* Pathogenicity Islands, SPI-1 and SPI-2, which encode for Type III Secretion System components involved in the infection pathway (Hansen-Wester and Hensel, 2001). The last common ancestor of the two species was estimated to be around 100 million years ago (Doolittle *et al.*, 1996), though the use of a constant molecular clock rate to determine this has been questioned by some, owing to differences between *in vitro* and *in vivo* mutation rates (Achtman, 2016; Ochman *et al.*, 1999).

Whilst the core genomes are very similar between the two species, it has previously been shown that the basal supercoiling levels of *Escherichia coli* and *Salmonella* Typhimurium differ, and that the nucleoid associated protein FIS has greater influence over supercoiling in *E. coli* than in *S.* Typhimurium (Cameron *et al.*, 2011). Topoisomerases are highly conserved between species, sharing common domains, owing to their essentiality for cell survival (Huang, 1996). For example, the *gyrB* sequences of the *Salmonella* Typhimurium strain 14028S and the *E. coli* strain ATCC 25922 are 90.9% identical (Fukushima *et al*., 2002).

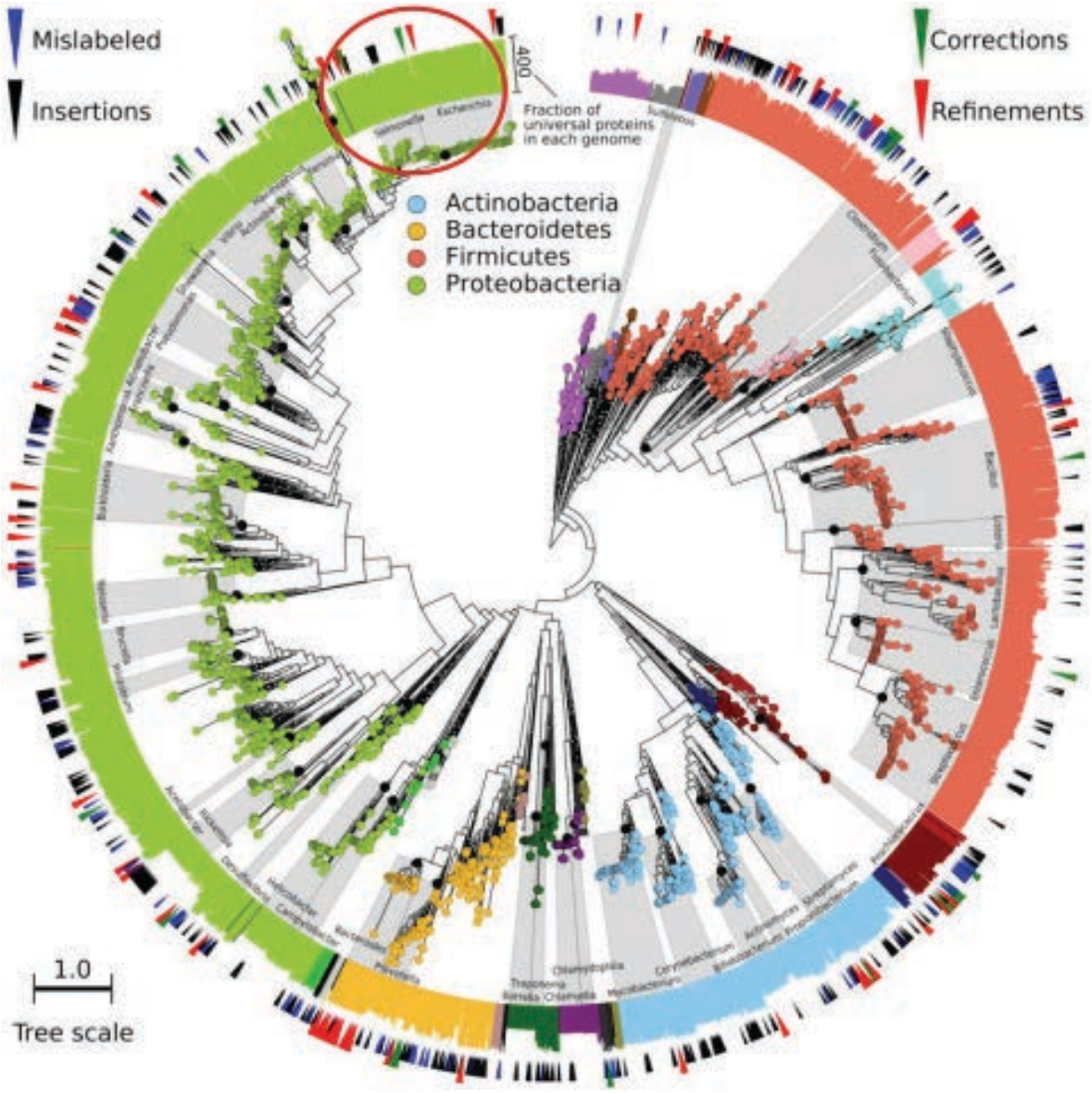


Figure 3.1.1: A representation of bacterial phylogeny created using "PhyloPhlAn". Phylogenetic relationships inferred computationally from predicted sequences of 400 broadly conserved proteins in over 3,700 whole genome sequences. These proteins are highly similar in *Salmonella* and *E. coli,* and the position of these genera is highlighted in a red circle.

Triangles correspond to sequence errors. Red arcs correspond to Firmicutes, blue arcs to Actinobacteria, yellow arcs to Bacteriodetes and green arcs correspond to Proteobacteria. Other coloured arcs correspond to smaller groups of species. Figure adapted from Segata *et al.* (2013).

It has previously shown that mutations in *gyrA* alter supercoiling in *Salmonella* (Webber *et al.*, 2013), having the knock-on effects of altering gene expression and stress responses, thus conferring a generic protective effect against some antibiotics and stresses. Given the difference in basal supercoiling between the two species we aimed to determine whether alterations in DNA gyrase impacted both species in a similar way.

The hypotheses tested in this chapter were:

- Mutations in *gyrA* will cause changes in supercoiling in both *Salmonella* Typhimurium and *Escherichia coli*
- Topoisomerase protein sequences will cluster based on pathotype;
- Any changes in supercoiling conferred by *gyrA* mutations will alter the expression of stress response loci, in a manner similar to that seen in *Salmonella.*
- Changes in supercoiling afford protection against various antibiotics;
- Treatment with non-quinolone antibiotics will alter supercoiling;
- Mutations in *gyrA* will “override” inducible changes in supercoiling.

The aims for this chapter were:

- To validate the impacts that GyrA Ser93Phe and GyrA Asp87Gly substitutions have on the supercoiling profile of *Salmonella* Typhimurium
- To validate the impacts that GyrA Ser83Leu and GyrA Asp87Gly substitutions have on the supercoiling profile of *Escherichia coli;*
- To compare and contrast any differences in supercoiling in the two species that are a consequence of equivalent substitutions;

- To determine if *Escherichia coli* topoisomerase protein sequences cluster based on pathotype;
- To determine the effects of *gyrA* mutations on the relationship between stresses and supercoiling in multiple conditions in *Salmonella;*
- To determine if mutations in *gyrA* alter levels of supercoiling in response to treatment with antibiotics compared with wild type cells in *Salmomella*;
- To determine whether the nature of the response to antibiotic treatment varies depending upon the class of antibiotic in *Salmonella.*

3.2 Results

3.2.1 Verification of Strains by Phenotypic Analysis and Sequencing

The *Salmonella* Typhimurium strains SL1344, SL1344 GyrA Ser83Phe and SL1344 GyrA Asp87Gly were obtained from the ARG Culture Collection. The *Salmonella* parent strain SL1344 was previously obtained from the AHVLA and has been sequenced in house on multiple occasions, and specific *gyrA* mutants were created prior to the start of this project by site-directed mutagenesis and described previously (Webber *et al.,* 2013).

The *Escherichia coli* strain MG1655 was also obtained from the ARG Culture Collection, though it was originally obtained from the Coli Genetic Stock Center at Yale University. The GyrA Ser83Leu strain was created by site-directed mutagenesis by an intercalating medical student as part of his project (Sutton, 2014), and the GyrA Asp87Gly strain was a kind gift from Professor Tony Maxwell of the University of East Anglia.

The species were confirmed as being either *Salmonella enterica* or *Escherichia coli* by Gram staining, growth on chromogenic XLD agar, and verifying their metabolic profile with API(E) strips (Biomerieux, France). Growth kinetics assays, shown in Figure 3.2.1.1, were performed in control conditions and showed that *gyrA* mutation in *Salmonella* did not have an adverse effect on growth rate in drug free media, or in the presence of ampicillin for strains carrying pBR322 (used in supercoiling assays).

At the start of this project the Quinolone Resistance Determining Region of the *gyrA* genes from all six strains were sequenced, and compared to the NCBI records of the sequences of the *gyrA* genes from each species' respective parent strains. The expected sequences were present in all strains, with the *gyrA* mutant strains containing single-nucleotide changes that resulted in the expected amino acid substitutions. Cells were then transformed with the plasmid pBR322 so that it could be used as means of measuring supercoiling.

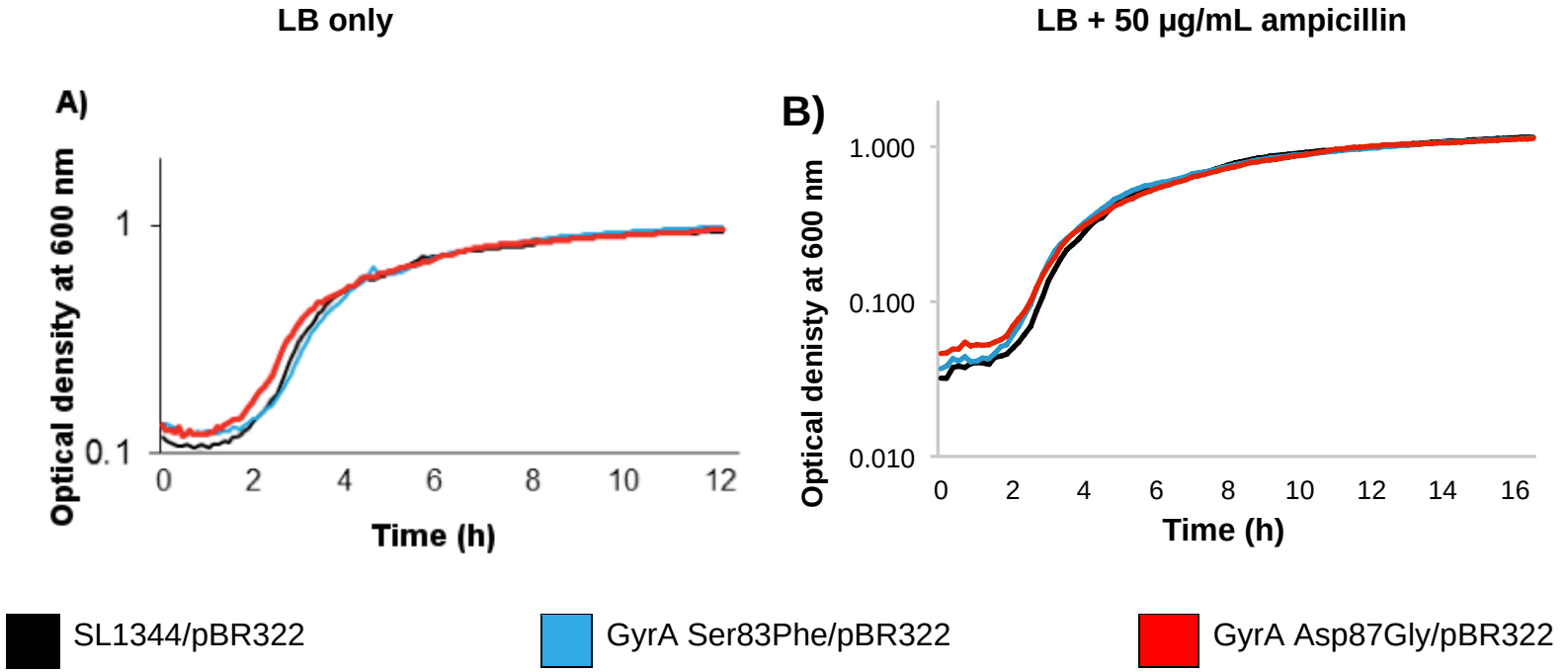


Figure 3.2.1.1: Growth kinetics data for SL1344 and *gyrA* mutant strains. Key: black = *Salmonella* Typhimurium SL1344/pBR322; blue = *S.* Typhimurium SL1344 GyrA Ser83Phe/pBR322; red = *S.* Typhimurium SL1344 GyrA Asp87Gly/pBR322. Part A) shows growth kinetics data for the strains carrying the plasmid pBR322 when grown in LB broth, the averages of readings from eight independent replicates. Part B) shows growth kinetics data for the strains carrying the plasmid pBR322 in LB broth supplemented with ampicillin. The lines shown are the averages of readings from 16 independent replicates. There is no appreciable difference in generation times between strains.

3.2.2 Susceptibility Testing

The minimum inhibitory concentrations (MICs) of various antibiotics were determined for the strains in this study and are shown in Table 3.2.1. The MIC values for nalidixic acid, ciprofloxacin and triclosan were in agreement with those presented previously (Webber *et al.*, 2013). The SL1344 cells were more susceptible to nalidixic acid and ciprofloxacin (a quinolone and fluoroquinolone, respectively), than the strains with GyrA substitutions, as expected. Both the GyrA Ser83Phe substitution and the GyrA Asp87Gly substitution had an MIC of nalidixic acid that was at least 64-fold higher than that for SL1344, increasing from 4 μg/mL to >256 μg/mL. Cells carrying these substitutions grew in the presence of 256 μg/mL nalidixic acid, but this was also the upper limit of the range of concentrations used. The presence of these substitutions also increased the MIC of ciprofloxacin eight-fold, from 0.03 μg/mL to 0.25 μg/mL, and increased the MIC of triclosan four-fold, from 0.06 μg/mL to 0.25 μg/mL. However, for all strains tested, the MICs for gentamicin and ampicillin were higher than previously reported. The acceptable difference in dilution from previously reported values is a two-fold difference (either higher or lower), but the difference in MIC values for gentamicin and ampicillin exceeded this acceptable range.

Strain	Minimum inhibitory concentration (μg/mL)										
	Nal	**Cip**	**Trc**	**Gent**	**Amp**	**Chlor**	**Nov**	**Tet**	**Pqt**	**Mnd**	**Kan**
S. Typhimurium SL1344	4	0.03	0.06	0.5	0.5	2	>256	1	512	128	2
S. Typhimurium SL1344 GyrA Ser83Phe	>256	0.25	0.25	0.5	1	4	>256	1	256	192	2
S. Typhimurium SL1344 GyrA Asp87Gly	>256	0.25	0.25	0.5	1	2	>256	1	512	128	2
S. Typhimurium SL1344/pBR322	8	0.03	0.06	0.5	0.5	2	>256	1	n/t	n/t	2
S. Typhimurium SL1344 GyrA Ser83Phe/pBR322	>256	0.25	0.25	0.5	1	2	>256	1	n/t	n/t	2
S. Typhimurium SL1344 GyrA Asp87Gly/pBR322	>256	0.25	0.25	0.5	1	2	>256	1	n/t	n/t	2
E. coli NCTC 10418	8	0.015	0.06	1	1	1	>256	0.5	256	192	1

Table 3.2.2.1: Minimum inhibitory concentrations for a range of compounds as tested against *Salmonella* Typhimurium. Table showing the minimum inhibitory concentrations of antibiotics tested against the *Salmonella* Typhimurium strains used in this study. *E. coli* NCTC 10418 is included as a control. Mutations in *gyrA* confer an over 64-fold increase for the MIC of nalidixic acid and an approximately 8-fold increase in the MIC of ciprofloxacin, and are also associated with an approximately 4-fold increase in the MIC of triclosan.

Nal = nalidixic acid; Cip = ciprofloxacin; Trc = triclosan; Gent = gentamicin; Amp = ampicillin; Chlor = chloramphenicol; Nov = novobiocin; Tet = tetracycline; Ery = erythromycin; Pqt = paraquat; Mnd = menadione; Kan = kanamycin; n/t = not tested.

3.2.3 Effects of GyrA Substitutions on Tolerance to Triclosan in *Salmonella*

It has previously been shown that *gyrA* mutation confers a protective benefit against the biocide triclosan, with both the GyrA Ser83Phe and GyrA Asp87Gly substitutions increasing the minimum inhibitory concentration three-fold, from 0.06 µg/mL to 0.25 µg/mL. Additionally, growth kinetics assays in the presence of triclosan showed that strains carrying the GyrA Asp87Gly substitution grew better than those that carried either the GyrA Ser83Phe substitution, or *Salmonella* Typhimurium SL1344 without any substitution in GyrA (Webber *et al.*, 2013).

These previous observations suggested that, in competition with a wild type strain exposure of GyrA mutants to low levels of triclosan may promote their expansion. To test this hypothesis competition assays were performed over the course of a week between SL1344 and the GyrA Ser83Phe strain, as well as between SL1344 and the GyrA Asp87Gly strain. Whilst I helped design these experiments, they were completed by Dr M Buckner during my absence on account of a period of extended illness. The data are shown in Figure 3.2.3.1. In the absence of triclosan, SL1344 was able to outcompete both of the *gyrA* mutant strains. In the presence of 0.03 µg/mL triclosan (half the MIC for SL1344), both the *gyrA* mutant strains were still outcompeted by SL1344.

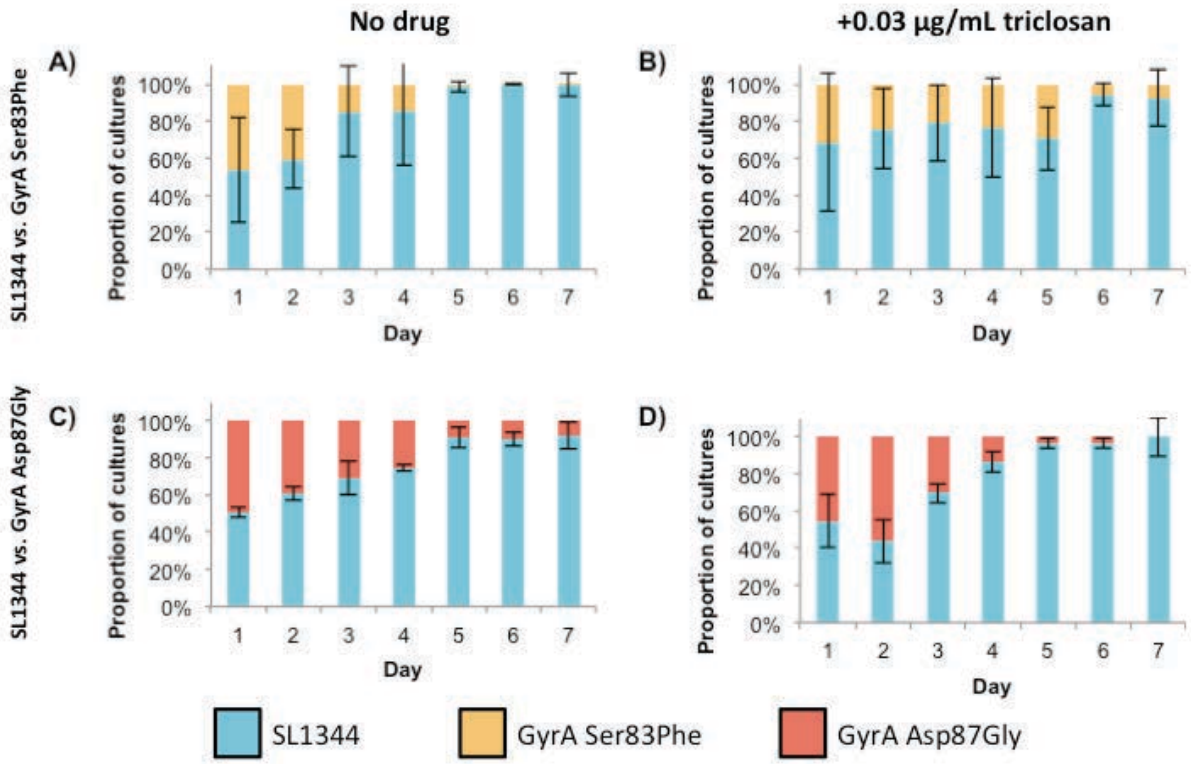


Figure 3.2.3.1: Competition assays between SL1344 and the *gyrA* mutant strains in the presence of absence of triclosan. Part A) shows *Salmonella* Typhimurium SL1344 (blue) vs. the GyrA Ser83Phe strain (yellow) in the absence of triclosan, and SL1344 is able to completely dominate the population after a week. Part B) shows SL1344 vs. GyrA Ser83Phe in the presence of 0.03 µg/mL triclosan. Part C) shows SL1344 vs. GyrA Asp87Gly (red) in the absence of triclosan, and part D) shows these two strains in the presence of 0.03 µg/mL triclosan. In all cases, the majority of cells throughout the course of a week isolated were SL1344; despite *gyrA* mutations being less susceptible to triclosan in MIC assays, they did not appear to confer a selective advantage in the presence of the biocide at the concentration used. The proportion of each colour in the bars is representative of the proportion of cells isolated that belong to each strain in the experiment. Each bar is the average of four independent replicates, and the error bars show the standard deviation from the mean. These experiments were performed in my absence by Dr. Michelle Buckner.

3.2.4 Effects of GyrA Substitutions on Tolerance to Triclosan in *E. coli*

It has been shown that, as in *Salmonella, gyrA* mutation in *E. coli* decreases susceptibility to the biocide triclosan in addition to conferring resistance to quinolones (Webber *et al.*, 2017).

To test the hypothesis that the exposure of GyrA mutants to low levels of triclosan may promote their expansion in *E. coli*, competition experiments were performed over the course of a week between MG1655 and the GyrA Ser83Leu strain, as well as between MG1655 and the GyrA Asp87Gly strain. The data from these experiments were generated by Dr Michelle Buckner in my absence, and are shown in Figure 3.2.4.1. In the absence of triclosan, populations were evenly split between MG1655 and the mutant strains. In the presence of 0.03 µg/mL triclosan, the GyrA Ser83Leu strain was outcompeted by MG1655. However, MG1655 was markedly outcompeted by the GyrA Asp87Gly strain in the presence of this concentration of triclosan. This is in contrast to the results from *Salmonella* seen in Figure 3.2.3.1.

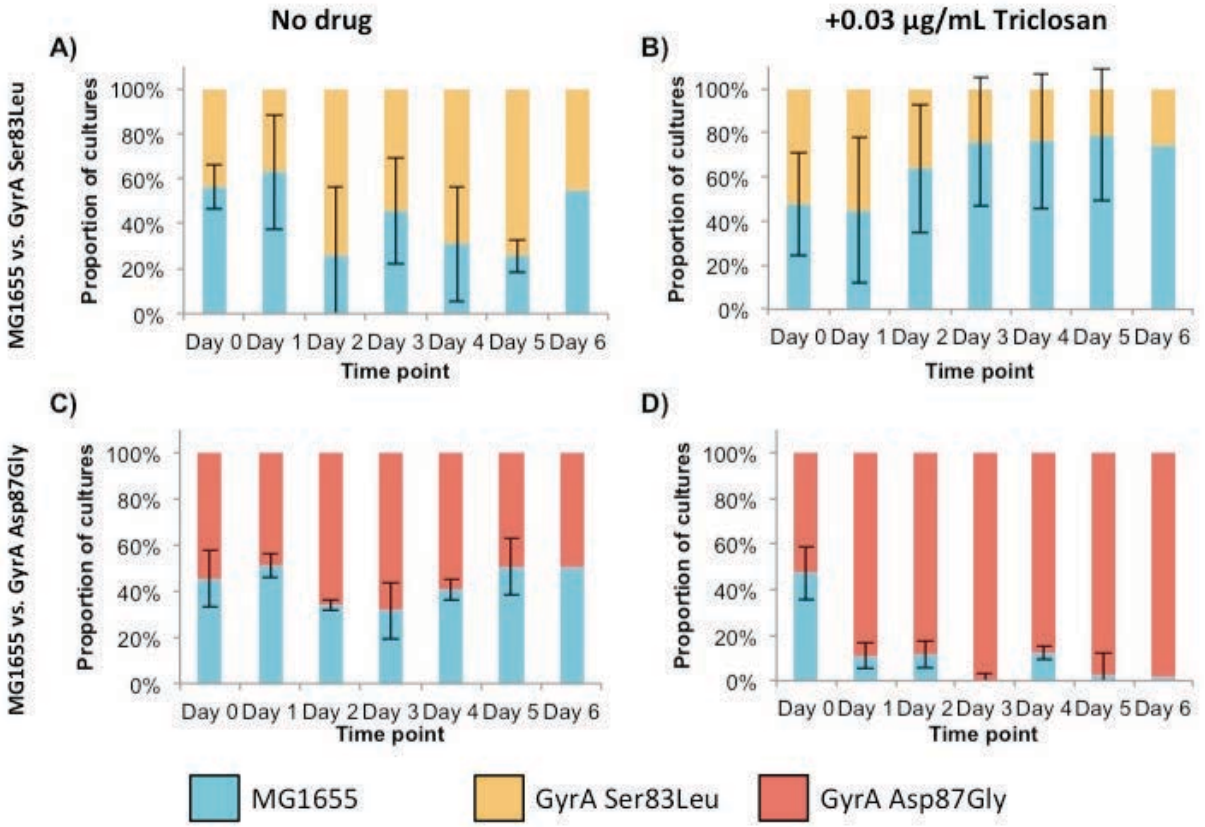


Figure 3.2.4.1: Competition assays between MG1655 and the *gyrA* mutant strains in the presence and absence of triclosan. Part A) shows *Escherichia coli* MG1655 (blue) vs. the GyrA Ser83Leu strain (yellow) in the absence of triclosan, and although the GyrA Ser83Leu strain is more abundant in the middle of the week, the distribution of strains is roughly even by the end of it. Part B) shows MG1655 vs. GyrA Ser83Leu in the presence of 0.03 µg/mL triclosan – MG1655 is the most abundant strain. Part C) shows MG1655 vs. GyrA Asp87Gly (red) in the absence of triclosan, and the population is roughly evenly split by between the two strains at the end of the week. Part D) shows these two strains in the presence of 0.03 µg/mL triclosan; the GyrA Asp87Gly strain was the only strain isolated at the end of the week, suggesting that the presence of the GyrA Asp87Gly substitution confers a selective advantage at the concentration of triclosan tested. The proportion of each colour in the bars is representative of the proportion of cells isolated that belong to each strain in the experiment. Each bar is the average of four independent replicates, and the error bars show the standard deviation from the mean. These experiments were performed in my absence by Dr. Michelle Buckner.

3.2.5 One-Dimensional Topoisomer Separation from Control Conditions

To determine the baseline levels of supercoiling in cells, the plasmid pBR322 was extracted from stationary phase cultures and the topoisomers were separated overnight on chloroquine-containing gels. As shown in Figure 3.2.5.1 and using the wild type as a point of reference, the GyrA Asp87Gly strain had a much more supercoiled topoisomer profile, and the GyrA Ser83Phe strain had a topoisomer profile which was more supercoiled than the wild type, but less supercoiled than that of the GyrA Asp87Gly strain. This was in accordance with the data presented previously by Webber *et al.* (2013).

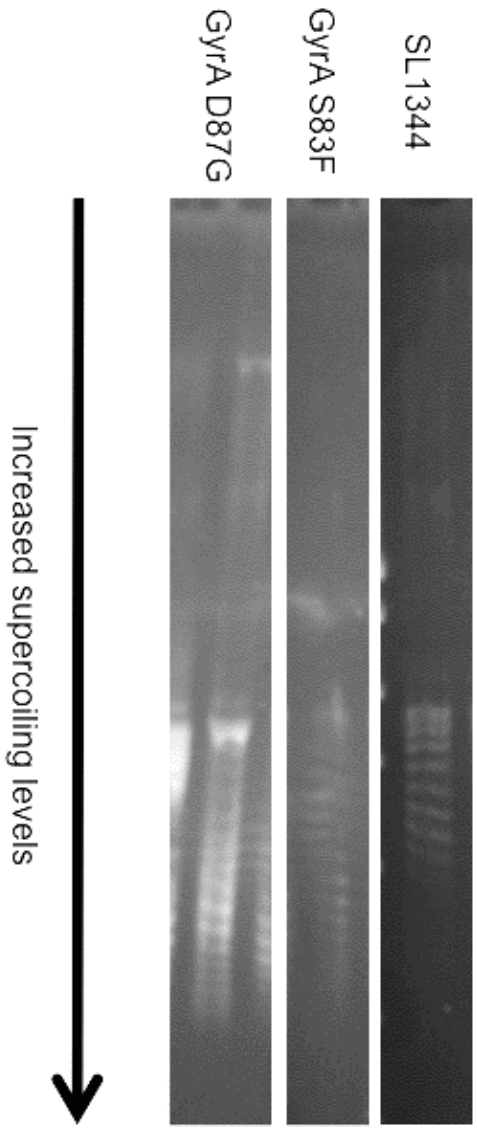


Figure 3.2.5.1: Images of separated topoisomers of pBR322 from stationary phase wild type, GyrA Ser83Phe and GyrA Asp87Gly cells, taken from the same gel. The more supercoiled a plasmid is, the further it travels down the gel. The plasmid from the GyrA Ser83Phe strain (middle) is more supercoiled than the wild type (right), and the profile of band distribution of the plasmid from the GyrA Asp87Gly strain (left) is supercoiled further still. The gel on which these topoisomers were separated is shown in the appendix, as Figure A.1.1.

3.2.6 Impact of Treatment with Nalidixic Acid on Supercoiling in *Salmonella enterica*

Experiments were designed to examine the effects of nalidixic acid exposure on supercoiling in the strains. Nalidixic acid is known to induce the SOS response (Piddock and Wise, 1987), and the expression of the SOS response genes *recA* and *lexA* was increased in SL1344 GyrA Asp87Gly in previous microarray experiments (Webber *et al.*, 2013). As these strains have differing supercoiling phenotypes and because supercoiling can be altered in response to stresses (Cameron *et al.*, 2011), a potential link between SOS response induction and the genomic supercoiling profile was suspected.

The *Salmonella* strains SL1344/pBR322, SL1344 GyrA Ser83Phe/pBR322 and SL1344 GyrA Asp87Gly/pBR322 were grown to late logarithmic/early stationary phase and then split. Half of the samples were incubated with nalidixic acid (80 µg/mL) for 45 minutes; the remainder were not. This concentration of nalidixic acid was chosen as it would induce a strong SOS response phenotype (Piddock and Wise, 1987; Walters *et al.*, 1989). Plasmids were extracted and the topoisomers were separated on a 2xTBE/chloroquine gel. Topoisomers obtained from SL1344/pBR322 which had been treated with nalidixic acid had lost negative supercoiling compared with untreated controls and the supercoiling pattern now resembled that seen in the *gyrA* mutants (Figure 3.2.6.1). This is consistent with loss of full functionality of gyrase. This was repeated with stationary phase cultures and similar results obtained showing the impact of *gyrA* mutation on supercoiling was similar at different growth phases.

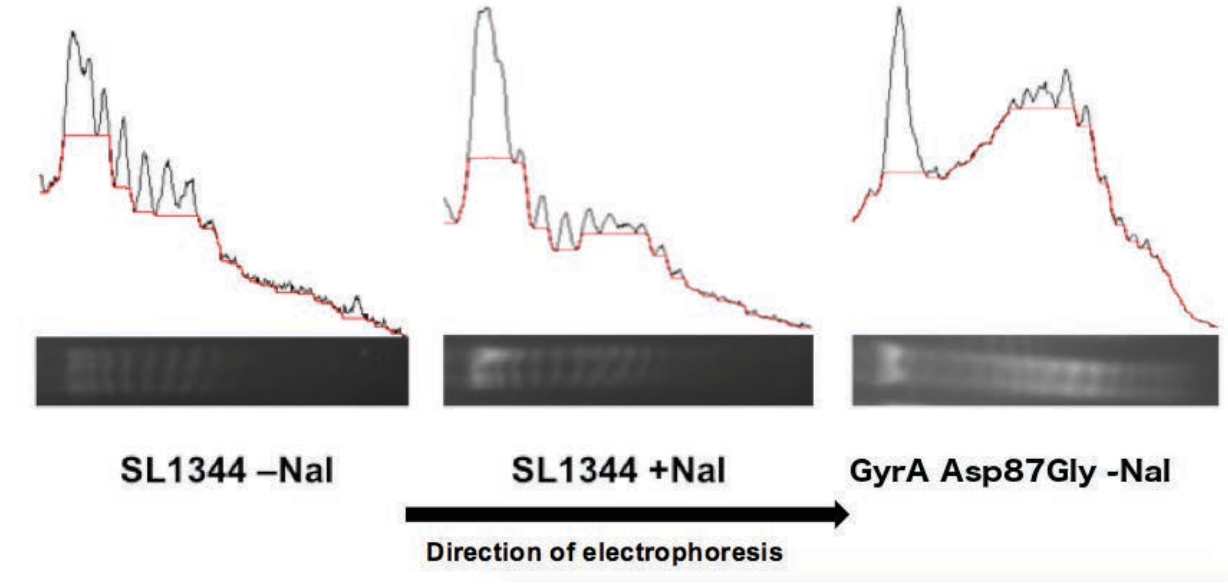


Figure 3.2.6.1: Densitometry plots of the levels of supercoiling in the pBR322 plasmid before and after treatment of SL1344 with nalidixic acid. The black pattern of peaks and troughs show the position of bands of topoisomers within the lanes, with the height of the peaks representing the brightness (and thus amount of plasmid) of each band. The topoisomers in the nalidixic acid-treated sample from the wild type SL1344 cells are more widely distributed than the untreated sample from the same strain, and more topoisomers have migrated further down the gel. Therefore, it appears that nalidixic acid increases the level of supercoiling in SL1344. The GyrA Asp87Gly strain is included for comparative purposes.

The red line shows the shortest distance between two troughs and is an artefact of the imaging software. It is not relevant to this discussion, but unfortunately it cannot be removed without disrupting the plot as a whole.

3.2.7 One-Dimensional Separation of Topoisomers from *E. coli* and Comparison with Topoisomers from *Salmonella* Typhimurium

To investigate the impacts of separate GyrA Ser83Leu and GyrA Asp87Gly substitutions on MG1655, plasmid pBR322 was isolated from these strains and used as a proxy for genomic supercoiling. They were separated by electrophoresis on a gel containing chloroquine, alongside plasmids isolated from *Salmonella* strains containing the equivalent GyrA substitutions. These experiments were done by Robyn Iddles in my absence and the gel is shown in Figure 3.2.7.1.

The “baseline” level of supercoiling appears to be similar for the two species – *i.e.* the topoisomers from the wild type strains, *E. coli* MG1655 (Lane B) and *S.* Typhimuirum SL1344 (Lane E), have migrated about the same distance and appear to be distributed in a similar manner to one another. The GyrA Ser83Leu (Lane C) and GyrA Ser83Phe (Lane F) substitutions appear to cause a similar shift in supercoiling and the topoisomer distribution skews slightly less negatively supercoiled in both species by roughly the same amount. However, the effects of the GyrA Asp87Gly substitution appear to be different in the two species. In *Salmonella*, the supercoiling state of the GyrA Asp87Gly (Lane G) strain was similar to that of SL1344. In *E. coli*, the GyrA Asp87Gly (Lane D) was more supercoiled than MG1655.

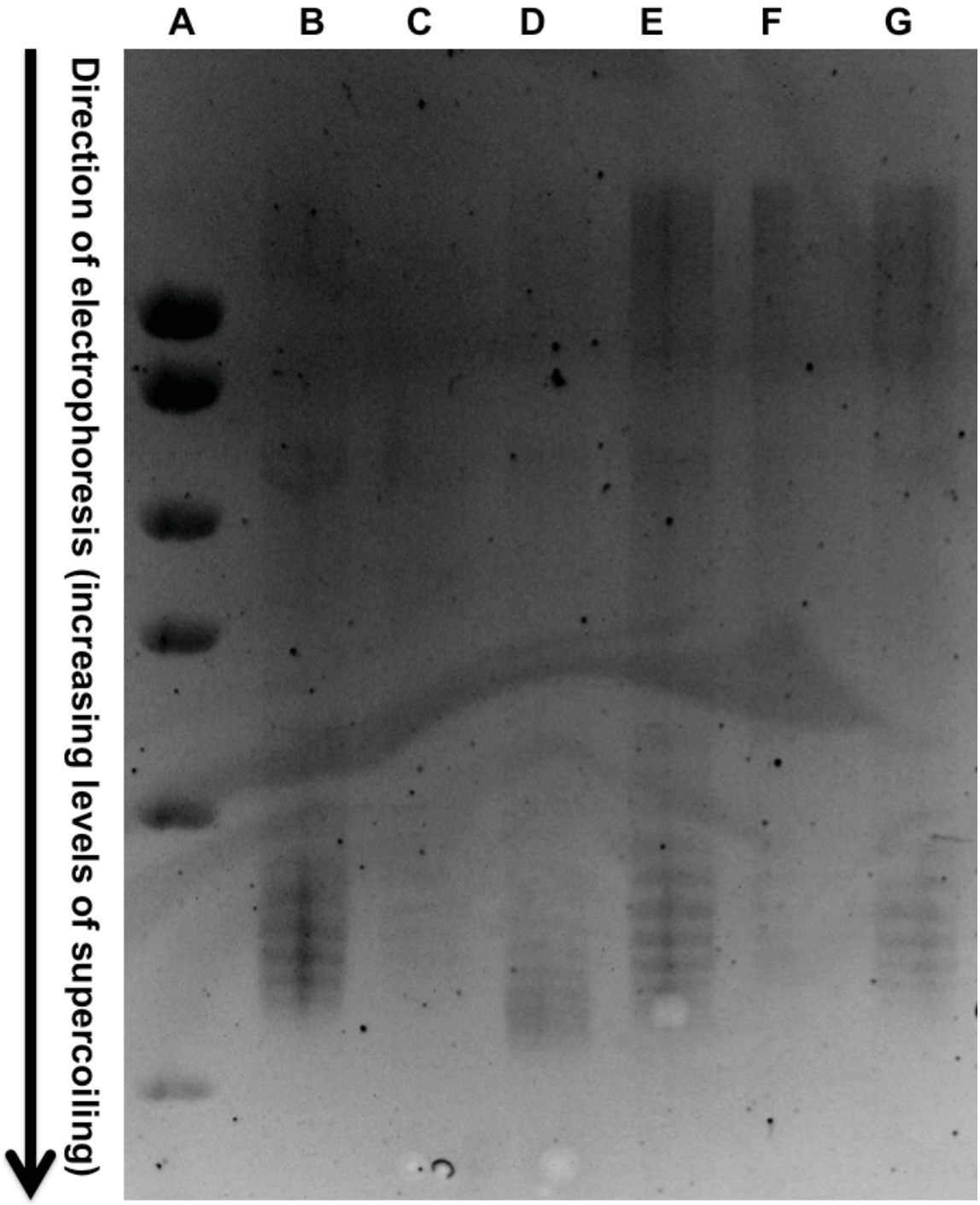


Figure 3.2.7.1: Supercoiling gel of pBR322 extracted from *Escherichia coli* and *Salmonella* Typhimurium wild type and *gyrA* mutant cells under control conditions. A) HyperLadder 1 kb B) *E. coli* MG1655 C) *E. coli* GyrA Ser83Leu D) *E. coli* GyrA Asp87Gly E) *S.* Typhimurium SL1344 F) *S.* Typhimurium GyrA Ser83Phe G) *S.* Typhimurium GyrA Asp87Gly. Topoisomers from *E. coli* GyrA Asp87Gly cells appear to migrate further than the MG1655 cells or the equivalent mutant in *Salmonella.* Topoisomers from MG1655 cells appear more densely packed than those from SL1344 cells. Those from cells with substitutions at the position GyrA Ser83 appear too faint to deterime effectively. These data were generated by Robyn Iddles as part of her BMedSci intercalation project.

3.2.8 Optimisation of “Quick” Supercoiling Gels

Whilst the gold standard for measuring supercoiling requires extended electrophoretic separation, this is time consuming and not amenable to comparing large numbers of conditions for impacts on supercoiling. It is possible to carry out topoisomer separation assays within the space of one day (Lee *et al.*, 2013; Mustaev *et al.*, 2014). This is generally achieved by subjecting 1% agarose gels to electrophoresis at 80 V for four hours. Four 50 g gels were set up and the separation of pBR322 topoisomers were assayed under those conditions. To examine the impacts of pre- and post staining and the use of chloroquine to stabilise topoisomers two had 2.5 µL Midori Green added, one of which also contained 2.5 µg/mL chloroquine. The other two gels were identical, except for the fact that they did not contain Midori Green and were instead washed for an hour in water, and washed for another hour in water containing 20 µL Midori Green after the completion of electrophoresis. All the conditions tested resulted in similar results and banding patterns of toposiomers were visible in 1% agarose gels with Midori Green pre-added and without chloroquine. Further permutations of these conditions showed a 1.5% agarose gel containing Midori Green to be optimal, and this is shown in Figure 3.2.8.1, below. A 1.5% agarose gel was therefore used for initial screens of large numbers of plasmids isolated from strains in different conditions.

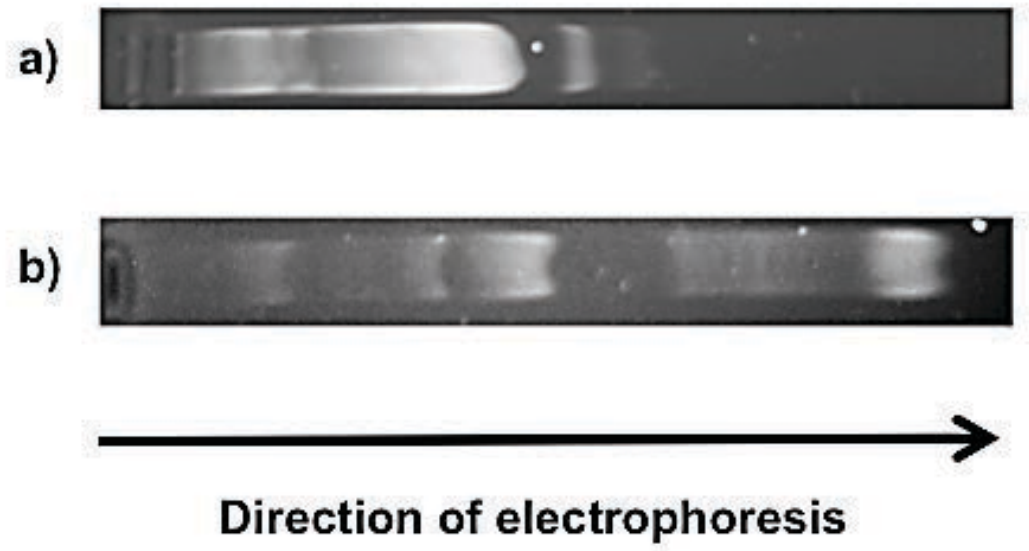


Figure 3.2.8.1: Topoisomers separated using "quick" supercoiling gel procedures. Topoisomers of the plasmid pBR322 separated for 4 hours at 80 V on either a) a 2% agarose gel; or, b) a 1.5% agarose gel, both of which contain Midori Green, but no chloroquine. The purpose of this experiment was to deterime if there was a method of determining supercoiling using an agarose gel that was faster than the electrophoresis assay that lasted 16 hours, yet still produced comparable results. The plasmids were isolated from the same sample: a stationary phase culture of *Salmonella* Typhimurium SL1344, and 300 ng was loaded on each gel. The pattern of topoisomers is more distinct in the 1.5% agarose gel, so this is what was used for the rest of this study.

3.2.9 Analysis of Supercoiling Changes in Various Conditions

To determine the effects of different stresses upon topoisomer profiles, wild type and *gyrA* mutant cells carrying the plasmid pBR322 were grown to mid-logarithmic phase and exposed to various stresses for 45 minutes. These were heat shock at 42 ºC; growth in the presence of 8 µg/mL nalidixic acid; and growth in the presence of 1 µg/mL gentamicin. Plasmids were also extracted from cells grown to early-logarithmic and stationary phase.

Topoisomers were initially separated from the overnight cultures, mid-logarithmic controls and heat-shocked cells. This is shown in Figure 3.2.9.1. The pattern of supercoiling in the wild type and GyrA Asp87Gly cells was observed to be similar no matter the condition. Additionally, GyrA Ser83Phe cells consistently had a slightly more supercoiled profile than those from the other two strains.

Subsequently, further topoisomers were separated from a wider range of conditions so as to determine how low-level stresses impacted topoisomer behaviour. The conditions chosen were based on either previous biolog data indicating gyrase mutants have a growth benefit under these conditions, or known inducers of stress responses seen to be up-regulated in the gyrase mutants. These were heat shock at 42 ºC and exposure to gentamicin, nalidixic acid, triclosan, ethanol, acriflavine, sulphamethoxazole, acetone and boric acid. The topoisomer profiles of each strain under these conditions are shown in Figure 3.2.9.2, and are grouped by strain.

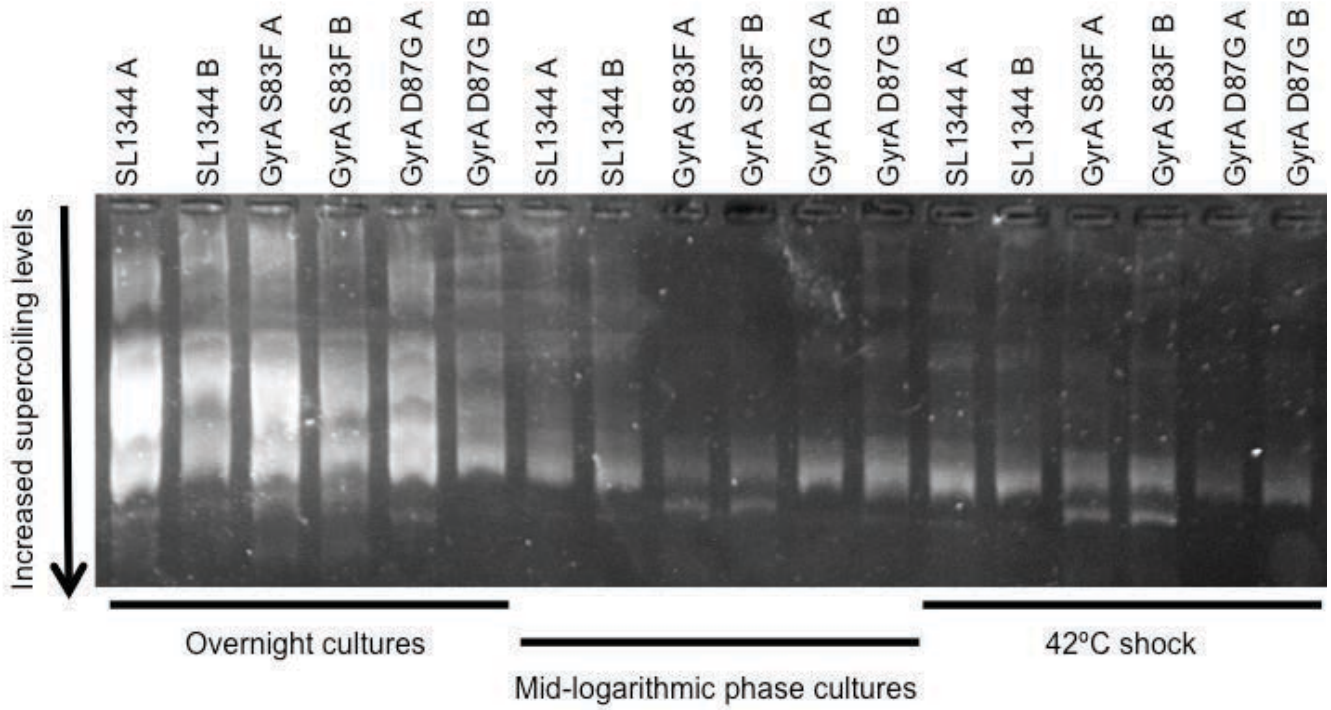


Figure 3.2.9.1: Plasmids extracted from *S.* Typhimurium under a range of conditions and subsequently separated. Topoisomer profiles for the plasmid pBR322, extracted from all three *S.* Typhimurium strains investigated in this study. 'A' and 'B' reflect separate, independent plasmid extractions, patterns were essentially identical between replicates. Samples from overnight cultures are too smeared to allow for the determination of topoisomer distribution. SL1344 and GyrA Asp87Gly cells appear to have responded similarly to the 42 ºC shock and being grown to mid-logarithmic phase as they possess a similar pattern of separated topoisomers. GyrA Ser83Phe cells have a more supercoiled phenotype (*i.e.* the topoisomers have migrated further down the gel) in response to these conditions, but also appear more supercoiled in control conditions as well.

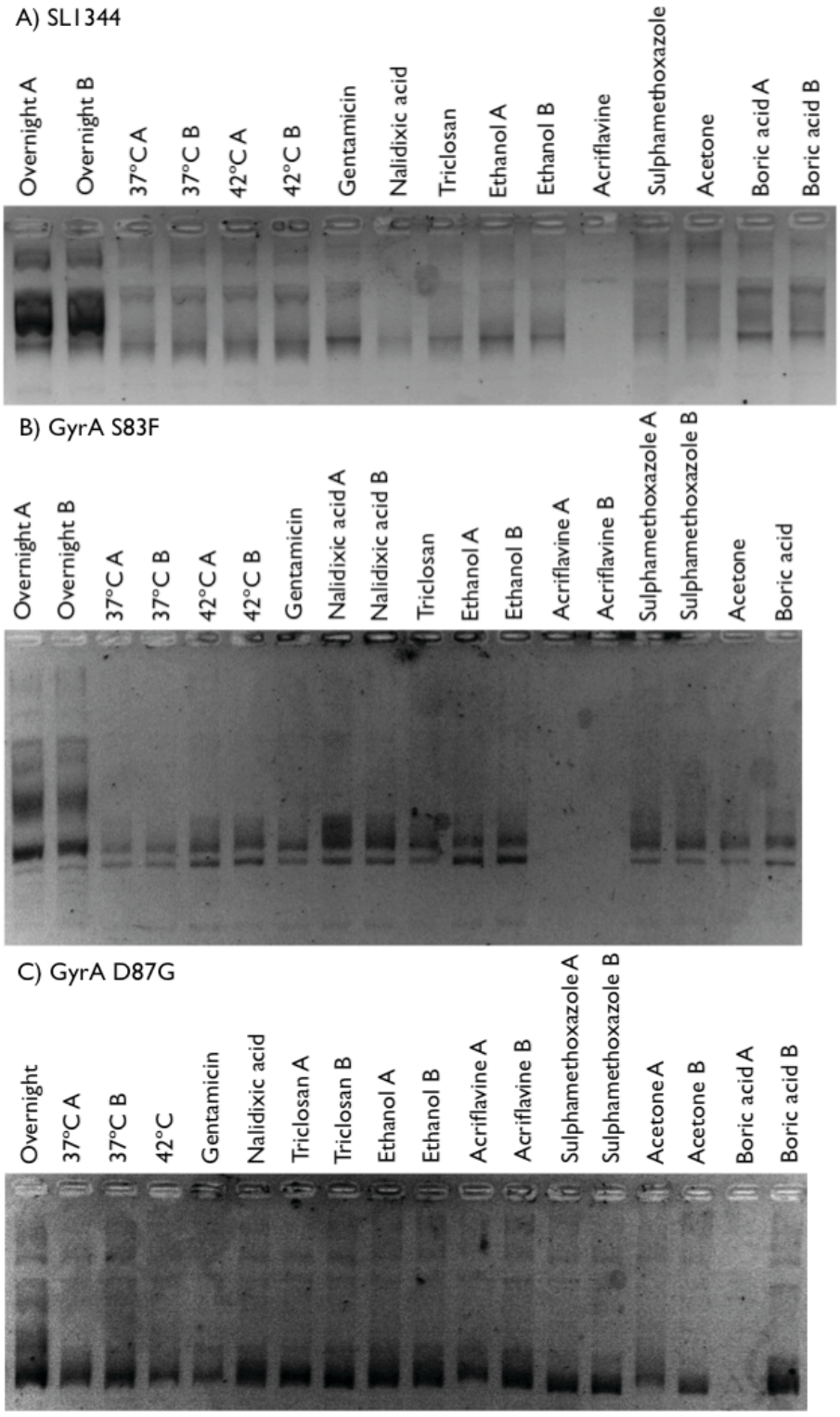


Figure 3.2.9.2: Plasmids extracted from *S.* Typhimurium under additional conditions and separated. One-dimensional gels of pBR322 samples extracted from SL1344 and the *gyrA* mutants after their having been exposed to a range of conditions. Patterns of topoisomer distribution roughly appear similar within strains, but differ in intensity. It appears as though plasmids do not migrate as far after treatment with chemicals in the gel of plasmids isolated from SL1344 cells, suggesting they are less supercoiled than those from control conditions but this could be artefactual. The migration of plasmids from *gyrA* mutants, and thus the levels of supercoiling, appears unchanged after treatment with chemicals or antibiotics.

In the SL1344 samples, shown in part A), samples from overnight cultures, those from samples grown at 37 ºC and those from samples grown at 42 ºC all appear to show a very similar banding pattern, with two distinct bands. This suggests that exposure to a raised temperature for 45 minutes does not appear to have much of an impact on supercoiling. After treatment with antibiotics and chemicals, the topoisomer patterns appeared to have not migrated as far down the gel as those isolated from cells grown in control conditions, regardless of the antibiotic or chemical used, and are thus less supercoiled after treatment with drugs than in the untreated 37 ºC samples,. Admittedly the gel is at a slight angle, but even once this is corrected for, samples from cells treated with drugs still have not migrated as far down the gel as those from cells that were not.

The intensity of the bands appears to vary. Some samples, such as those isolated from cells treated with gentamicin or ethanol, have a much stronger band towards the bottom of the gel. Although this has not migrated as far as the bands isolated from cells grown at 37 ºC, it does appear more intense. This would suggest that there are a greater proportion of supercoiled topoisomers within samples from cells treated with ethanol or gentamicin, even if these topoisomers are not as highly supercoiled as those from cells grown in control conditions.

The acriflavine sample cannot be seen; this could be because of acriflavine being a DNA-binding agent (Tubbs *et al.*, 1964; Chan and Van Winkle, 1969) and possibly competing with the Midori Green, which functions similarly to ethidium bromide (Nippon Genetics Europe, n.d.), but this does not explain why it is visible in the GyrA

Asp87Gly samples. A more likely explanation is that, despite having calculated the volume of sample needed to load 300 ng of DNA onto the gel, the calculations were incorrect.

Part B) shows samples from the GyrA Ser83Phe strain. In contrast to the wild type strain, there is little difference between the drug-treated samples and the non-treated samples. With the exception of those from overnight cultures, samples exhibit two very strong, clear bands close together. There are again differences in the intensity of the bands. Topoisomers from cells treated with nalidixic acid appear as a thick smear; the gel might benefit from being run for longer so as to make these appear more distinct. Topoisomers from cells treated with acriflavine still cannot be seen.

In part C), samples which have been isolated from GyrA Asp87Gly cells exhibit subtile differences in the topoisomer distribution pattern when treated and untreated samples are compared. While there is some variation in the acriflavine and sulphamethoxazole-treated samples, this appears inconsistent between replicates. As the majority of topoisomers from each seem to be collected in one band fairly low down the gel, the pattern displayed is more similar to that topoisomers from SL1344 cells, as opposed those from the GyrA Ser83Phe cells.

Samples obtained from GyrA Asp87Gly cells that have been exposed to gentamicin, acriflavine and acetone are subtly different to the other samples. The topoisomer distributions in the samples from the remaining conditions appear to be broadly similar to one another, but this is likely a consequence of the concentration of DNA

being too high to distinguish different bands from one another. Topoisomers from GyrA Asp87Gly cells generally appear as thick black smears. Ideally a current could have been applied to the gel for longer, separating out the topoisomers further, or a lower concentration of DNA could have been used.

In all samples taken from all strains when grown at 42 ºC, the patterns appeared identical to the samples taken from the same strain at 37 ºC. Densitometry plots were made from each sample and these were then directly compared across mutants in each condition (Figure 3.2.9.3).

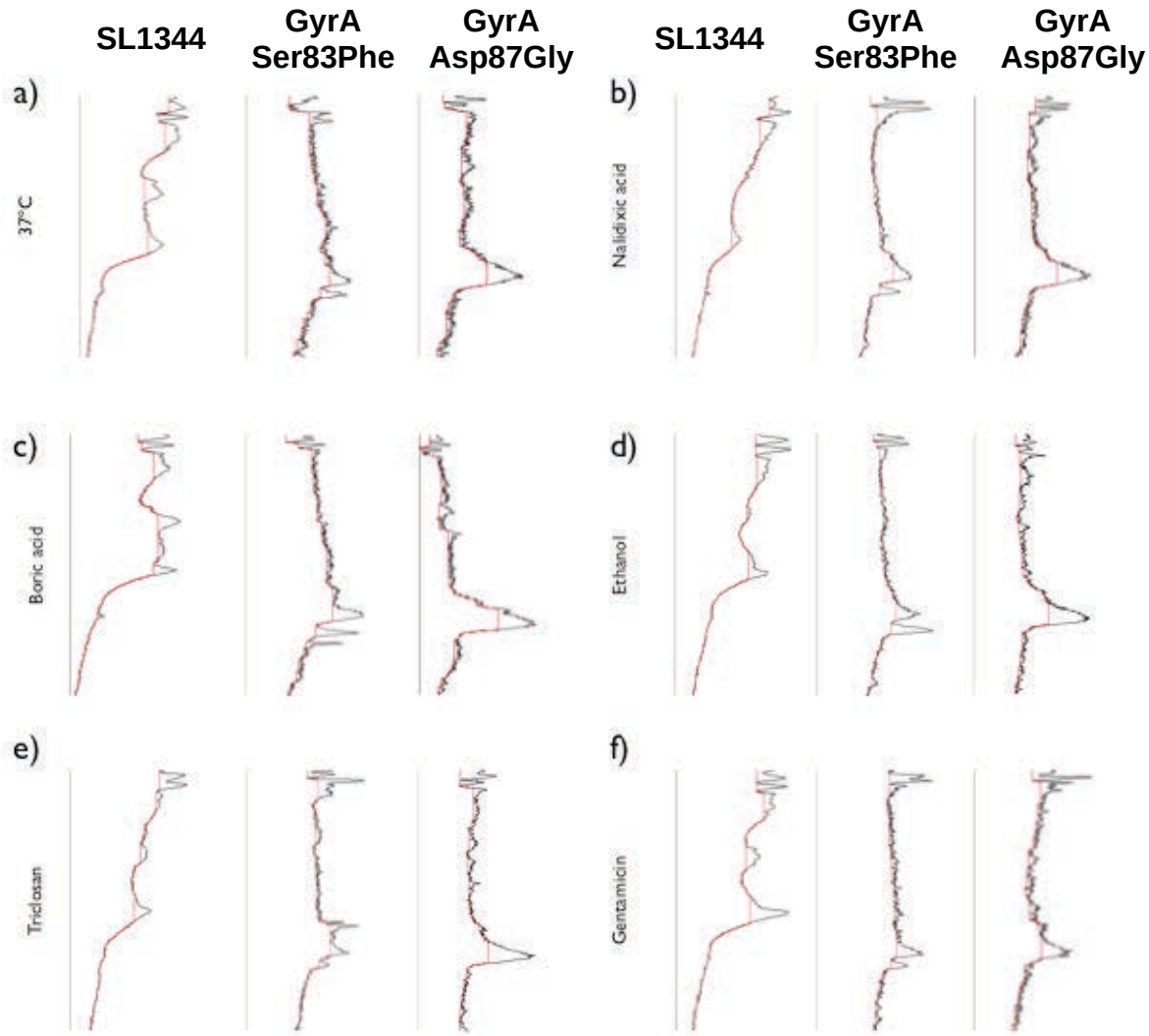


Figure 3.2.9.3: Examples of topoisomer densitometry plots. In each triplet, the plots are from samples taken from SL1344, GyrA Ser83Phe and GyrA Asp87Gly cells, going left to right. A) Distribution of topoisomers in samples taken from mid-logarithmic phase cultures grown at 37 ºC. b) Distribution of topoisomers in samples from mid-logarithmic phase cultures perturbed with nalidixic acid. C) Topoisomer distribution from samples treated with boric acid. D) Topoisomer distribution from samples treated with ethanol. E) Topoisomer distribution from samples treated with triclosan. F) Topoisomer distribution from samples treated with gentamicin.
The pattern of supercoiling appears to change for SL1344 cells in response to drug treatment; the trace appears to smooth out after treatment with nalidixic acid, or bunch up into one peak after ethanol treatment. The pattern of supercoiling appears to be fixed for the GyrA Ser83Phe cells, with two peaks at roughly the same position about three quarters of the way down the gel, only varying in their intensity. The pattern of supercoiling appears to be fixed for the GyrA Asp87Gly cells, as there is one noticeably large peak visible in each trace, that appears at approximately the same distance down the gel no matter the condition in which the cells were grown.

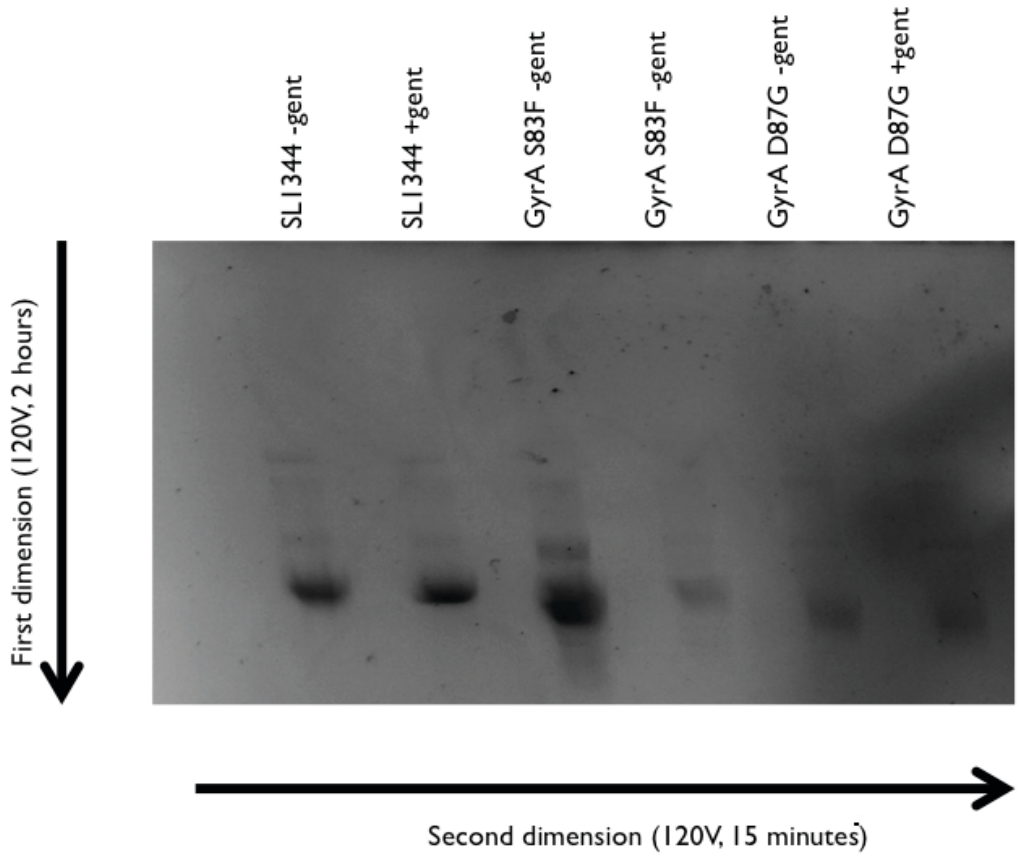
Figure 3.2.9.3 shows the topoisomer distribution patterns from all three strains under a series of representative conditions from those examined. Using the 37 ºC samples in part A) as a baseline, in the majority of cases treatments only resulted in changes in supercoiling in SL1344, whereas patterns of supercoiling the two GyrA strains were unchanged - this suggests that the mutations in *gyrA* override any condition-induced change in supercoiling. Samples taken from SL1344 appear to be more supercoiled in other conditions. The GyrA Ser83Phe cells consistently show a pattern with two peaks of varying intensity, and the GyrA Asp87Gly cells show a pattern with one strong peak, at a distance intermediate to the peaks of the wild type and GyrA Ser83Phe cells. In the majority of conditions, the supercoiling states of topoisomers from the two mutants are unchanged compared with those taken from cells grown at 37 ºC in the absence of any stressor.

3.2.10 Two-Dimensional Separation of Topoisomers

Supercoiling can be positive or negative, to determine if the changes in overall supercoiling levels were a result of changes in positive and/or negative supercoiling, topoisomers were separated in the second dimension in the presence of a greater chloroquine concentration. Figure 3.2.10.1 shows the gel photo from this assay, and the densitometry plots obtained from the gel. When separated in the second dimension, the migration of samples appeared to be uniform, suggesting that a difference in proportions of negatively and positively supercoiled topoisomers is not present. The patterns observed for wild type cells are in agreement with those seen previously (Webber *et al.*, 2013) and in Sections 3.2.7 and 3.2.9; topoisomers isolated from GyrA Ser83Phe cells under control conditions appear to migrate further

than those isolated from wild type cells. Incubation of samples in the presence of gentamicin appears to have no impact on the directionality of supercoiling, though topoisomers from GyrA Ser83Phe cells treated with gentamicin do not appear to migrate as far as those from their untreated counterparts.

A)



B)

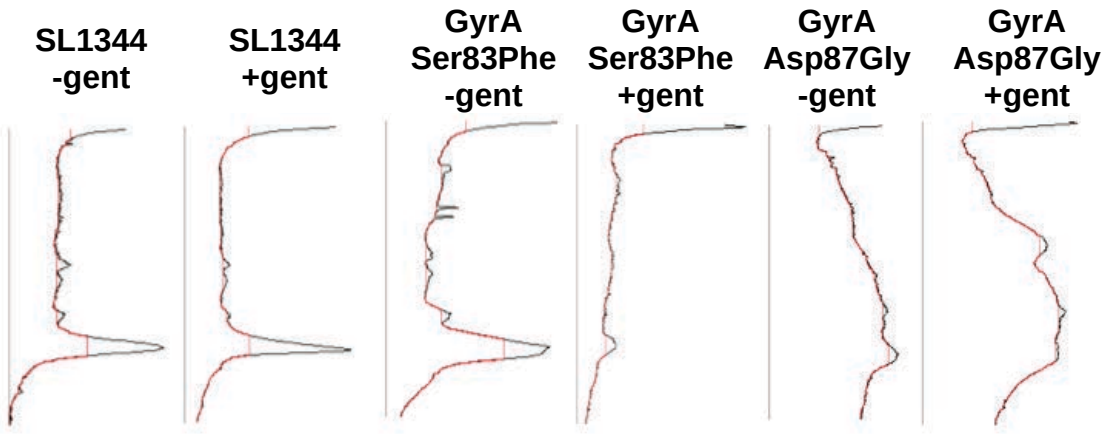


Figure 3.2.10.1: Two-dimensional separation of topoisomers from *S.* Typhimurium. Part A) shows a two-dimensional gel of pBR322 samples isolated from *S.* Typhimurium, both with and without GyrA substitutions, grown in the presence and absence of gentamicin. The slanted pattern of the bands is a consequence of the gel being run in one direction for 2 hours, then being rotated 90º and run for a further 15 minutes. The large accumulation of sample in the GyrA Ser83Phe –gent lane is analogous to those seen in Figure 3.2.9.2, and does not appear to be anything special. A ladder was included but ran off the gel. Part B) shows densitometry plots of the traces from part A). The densitometry plots for wild type cells and untrated GyrA Ser83Phe cells appear identical. The GyrA Asp87Gly plots have been distorted by the massive buildup of dye at that side of the gel. The red lines are again not relevant.

3.2.11 Relationship Between *E. coli* Topoisomerase Protein Sequences and Pathotype

As *E. coli* comprises a broad range of strains adapted into separate pathotypes, differences in DNA gyrase sequence between strains of each pathotype were investigated. To determine if there was any relationship between topoisomerases and *E. coli* pathotypes, a number of *E. coli* strains from a range of pathotypes were identified through a literature survey. The protein sequences of GyrA, GyrB, ParE, ParC and TopA were then obtained from the NCBI database for these strains and used to construct cladograms.

The cladogram for GyrA is shown in Figure 3.2.11.1. There is no apparent clustering based on pathotype. This was also observed for the other proteins (data not shown). Sequences from a selection of *Salmonella* strains were included as a positive control and these do cluster together.

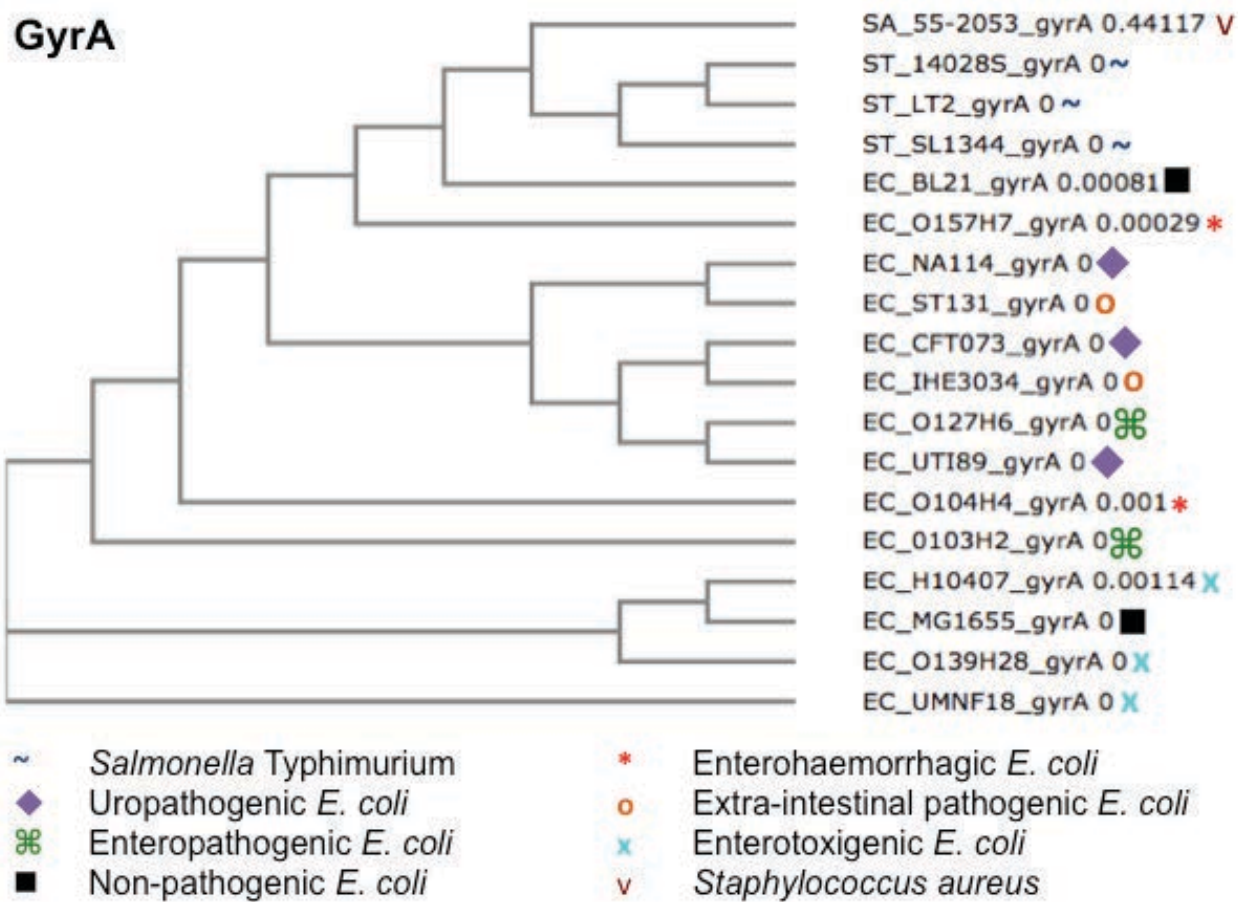


Figure 3.2.11.1: Cladogram of GyrA protein sequences. Cladogram showing the relationship between the protein sequences of GyrA from *Staphylococcus aureus*, a range of *Salmonella* strains and a variety of *Escherichia coli* strains of different pathotypes. As this is a cladogram, branch lengths are arbitrary. The *Salmonella* sequences cluster together and the *Staphylococcus* sequence forms an outgroup, but, contrary to expectations, the *E. coli* sequences do not cluster based on pathotype.

3.3 Discussion

3.3.1 Effects of GyrA Substitution on Topoisomerase Distribution, and Changes in Topoisomerase Distribution in Response to Stress

In accordance with what was previously shown in the literature, it was confirmed that amino acid substitutions in GyrA resulted in alterations to baseline supercoiling levels and minimum inhibitory concentrations for these strains were, for the most part, in agreement with those previously published (Webber *et al.*, 2013). Topoisomers from *S.* Typhimurium cells with GyrA substitutions were observed to migrate further than those from cells with the wild type form of the enzyme, suggestive of a the topoisomers from the *gyrA* mutants being more supercoiled than those from the wild type cells. It was not always consistent between experiments, but the largest change in supercoiling was usually seen in the GyrA Ser83Phe strain, though *in vitro* experiments have shown that the GyrA Asp87Gly substitution results in a larger reduction in supercoiling activity, as shown in Table 3.3.1 (Webber *et al.*, 2017). Although GyrA Asp87Gly is less active than either the wild type or GyrA Ser83Phe, the similarity of its behaviour as compared with that of the wild type enzyme in the data presented in this chapter suggests that it is possibly more efficient than GyrA Ser83Phe. If the reduction in activity were coupled with a reduction in efficiency, one might perhaps expect to see DNA extracted from GyrA Asp87Gly cells under control condtions be less supercoiled than DNA extracted from other cells, given gyrase introduces supercoils into DNA.

The plasmid pBR322 was isolated from *E. coli* and *Salmonella* and topoisomers separated out on a gel. This was used as a proxy measurement for levels of genomic

supercoiling. It has been shown in the literature that there should be differences in supercoiling between the two species in the absence of any stress or mutation (Cameron *et al.*, 2011), but the data presented here suggest that the supercoiling in MG1655 and SL1344 was similar under such conditions. Differences in the migration of topoisomers were observed in strains with GyrA substitutions both between and within species, suggesting that these GyrA substitutions alter genomic supercoiling in both species, and do so differently.

Work done as part of a collaboration with Tony Maxwell's group at the University of East Anglia (Webber *et* al., 2017) has shown that gyrase with an Asp87Gly substitution is much less active than either the wild type version of the enzyme or the Ser83Leu version of the enzyme, as shown in Table 3.3.1. The data suggest that the GyrA Ser83Phe substitution has a more detrimental effect on the activity of GyrA in *Salmonella* than it does in *E. coli*, with the activity of the enzyme being reduced to 30% that of the wild type level in the former species, and to 90% of the wild type level in the latter. The GyrA Ser83Phe substitution is associated with a greater fold-change in MIC in *E. coli* as well. In both *E. coli* and *Salmonella*, the GyrA Asp87Gly substitution reduces supercoiling activity by 85% relative to the wild type version of the enzyme and results in a 10-fold change of ciprofloxacin IC_{50}. However, the GyrA Ser83Phe substitution reduces activity by 70% in *Salmonella,* conferring a 5-fold increase in ciprofloxacin IC_{50}, and reduces activity by 10% in *E. coli*, conferring a 14-fold increase in ciprofloxacin IC_{50}.

These differences in supercoiling activity in the two species suggest a greater potential for differences in the transcriptome of the *E. coli* GyrA Asp87Gly cells compared with MG1655, and this could be investigated thoroughly with RNA sequencing. It would be useful to investigate how the supercoiling profile changes in *E. coli* and its related strains carrying GyrA substitutions in response to stresses, as was done with *Salmonella.* The impact on changes to supercoiling levels on pathogenicity and metabolic capacity in each species could also be compared with help understand how supercoiling is influencing the biology of each species and how changes to their evolved optimum influence each.

Species	GyrA substitution	Supercoiling activity relative to WT (%)	Fold change in cipro IC_{50}
S. Typhimurium	Wild type	100	n/a
	Ser83Phe	30	5
	Asp87Gly	15	10
	Ser83Phe+Asp87Gly	30	>14
E. coli	Wild type	100	n/a
	Ser83Phe	90	14
	Asp87Gly	15	10
	Ser83Phe+Asp87Gly	20	>200

Table 3.3.1: Comparison of changes in *in vitro* supercoiling activity and IC_{50} values for ciprofloxacin as a result of GyrA substitution in *S.* Typhimurium and *E. coli.* Table, adapted from (Webber *et al.*, 2017), showing how different GyrA substitutions alter gyrase activity changes in *Salmonella* Typhimurium, as shown through *in vitro* experiments. Data are shown as percentages relative to the activity of the wild type version of the enzyme. All substitutions listed result in reduced activity relative to the wild type enzyme. The GyrA Asp87Gly substitution is less active than the GyrA Ser83Phe substitution. However, the GyrA Ser83Phe Asp87Gly double substitution is equally as active as the GyrA Ser83Phe substitution.

The *Salmonella* Typhimurium *gyrA* mutants had their genomes sequenced by MicrobesNG, with the intention of using the sequence data to identify single nucleotide polymorphisms. The genome sequence data was unfortunately of too poor quality for such an analysis to be meaningful.

Despite previous results, and those presented here, showing that *gyrA* mutation confers reduced susceptibility to triclosan, this did not appear to provide a competitive advantage against the parental strain when each of the two *gyrA* mutant strains were grown in co-culture with SL1344. Only one concentration of triclosan (0.03 µg/ml) was used in these experiments, representing a sub-lethal exposure to mimic low levels of accumulation seen in the environment. When competition assays were performed in the absence of any drug, both gyrase mutants appeared to be less fit than SL1344. The most likely explanation for a lack of benefit in the triclosan experiments is that the concentration of triclosan used in these assays is too low to select against the parental strain sufficiently to advantage the gyrase mutants. Triclosan has been found at a concentration of 2.7 µg/mL in human urine from healthy volunteers (Pirard *et al.*, 2012). This value is two orders of magnitude higher than the concentration used in the competition assays. This could be investigated further by repeating the competition assays using a range of concentrations of triclosan.

Various permutations on the “quick” supercoiling gel protocol were investigated, leading to the conclusion that the optimal conditions were to run the gel for 4 hours at 80 volts. It was observed that the presence or absence of chloroquine, a topoisomer

relaxation agent, made no appreciable difference to the supercoiling profile. It appeared as though the topoisomers in some samples were more concentrated than others, as evidenced by differences in band intensity. For example, in the gel comparing samples from GyrA Ser83Phe cells, the nalidixic acid samples were much darker than the samples from control conditions, suggesting that the topoisomers were more concentrated, and the samples from cells that were grown in the presence of acriflavine were completely invisible. Although samples were supposed to have been normalised to 300 ng of DNA per lane, this might not necessarily have worked in practice.

3.3.2 Impacts of GyrA Substitution on Tolerance to Triclosan

It has previously been shown that GyrA substitution confers reduced susceptibility to triclosan in *Salmonella*, and this is also the case in *E. coli*. To investigate the impact of this reduction in susceptibility on cell survival, competition assays were performed over the course of a week between wild type and *gyrA* mutant cells in the presence and absence of 0.03 µg/mL triclosan. In *Salmonella*, the wild type strain was able to outcompete both the GyrA Ser83Phe and GyrA Asp87Gly strains in both the presence and absence of triclosan. However, for *Escherichia coli*, the wild type and mutant strains were found to be in equal proportion after a week in the absence of triclosan. In the presence of triclosan, wild type cells out-competed the GyrA Ser83Leu cells, but were out-competed by GyrA Asp87Gly cells. This suggests that the GyrA Asp87Gly substitution confers a much greater protective effect against low levels of triclosan in *E. coli* than it does in *Salmonella.* This could perhaps be a consequence of the same *gyrA*_mutation having different impacts on gyrase

efficiency and consequently gene expression, but further investigation would be needed to determine the actual reason.

This has implications for the potential evolution of *gyrA* mutants in each species – it may be that in *E. coli* there are benefits to carrying *gyrA* mutations in the presence of low levels of antimicrobial stress which will promote persistence of these strains. Fluoroquinolone resistance is relatively more common in *E. coli* than it is in *Salmonella* – data from isolates taken from broiler chickens in the European Union showed that 65.7% of *E. coli* isolates were being clinically resistant to ciprofloxacin, whereas only 53.5% of *Salmonella* isolates from broiler chickens were clinically resistant to ciprofloxacin (European Food Safety Authority and European Centre for Disease Prevention and Control, 2016).

3.3.3 Analysis of *E. coli* Topoisomerase Protein Sequences and Their Relationship to Pathotype

To determine if the protein sequence of topoisomerases was in any way linked to pathotype, the GyrA, GyrB, ParC, ParE and TopA protein sequences from *Staphylococcus aureus, Salmonella* Typhimurium and a range of *Escherichia coli* strains were analysed using bioinformatics. Phylogenetic trees were then constructed for each protein. It was expected that the protein sequences from *E. coli* would cluster together based on the strains' pathotypes, but this was not observed. As expected, the *Salmonella* sequences clustered together, and the *Staphylococcus* sequence was noticeably an out-group. However, the sequences from enterohaemmorhagic *E. coli* did not cluster together, those from uropathogenic *E.*

coli sequences did not cluster together, *et cetera*. Therefore there is no evidence that alterations in supercoiling arising from topoisomerase genotypes have contributed to the divergent evolution of *E. coli* pathotypes.

The impacts of exposure to different classes of antibiotics and a range of stresses had upon supercoiling were investigated, leading to the observations that the supercoiling profile of cells carrying the GyrA Asp87Gly substitution was ‘fixed’ under all conditions investigated and not sensitive to any of the conditions examined. This suggests that any change in gene expression that relates to supercoiling will be relatively constant and not a transient effect, which supports the phenotypes seen in this mutant.

Transcriptomic data has previously shown the expression levels of a number of stress response regulators to be elevated in the GyrA Asp87Gly strain relative to SL1344, and it is hypothesised that this is a result of altered supercoiling (Webber *et al.*, 2013). The evidence presented suggests that any benefit to the cell conferred by altered supercoiling, such as heightened stress responses, will be seen all the time in GyrA Asp87Gly cells as supercoiling appears to be fixed no matter what condition these cells are subjected to. To investigate this further, fluorescent reporter constructs were created. These constructs were designed to fluoresce when the cells were exposed to conditions that trigger specific stress responses, and they will be discussed in Chapter Four.

Chapter Four

The Relationship Between GyrA Substitutions and Stress Responses

4.1 Introduction

Changes in supercoiling as a consequence of *gyrA* mutation have previously been correlated with an altered transcriptome (Webber *et al.*, 2013). It is also well established that supercoiling can change in response to exposure to various environmental factors and stresses and that changes in supercoiling can have global impacts on gene expression (Cameron *et al.*, 2011).

Transcription relies on RNA polymerase and the sigma factors are subunits of the RNA polymerase holoenzyme that guide it to specific promoter sequences. Cells have a catalogue of sigma factors available to them and the sigma factor incorporated into the holoenzyme can vary. This switching can be in response to changes in environment, growth phase, *etc*. currently being experienced by the cell. The common “housekeeping” sigma factor is sigma 70; other sigma factors control expression of genes that respond to envelope stress, nitrogen deficiency and adverse temperatures, to name a few. The incorporation of different sigma factors into RNA polymerase alters the transcriptome of the cell by changing which promoters are recognised, and, consequently, which genes are expressed (Feklístov *et al.*, 2014; Österberg *et al.*, 2011; Paget, 2015). Promoter availability can influence the rate at which a RNAP holoenzyme can bind a promoter and initiate transcription, the supercoiling state of DNA can influence this availability and hence influence transcription.

As shown in the previous chapter, *gyrA* mutations confer a generic protective benefit against stresses. To test the hypothesis that this phenotype is a result of changes in stress response expression the purpose of this chapter was to investigate whether

expression of stress response operons was altered in the gyrase mutants using fluorescent reporter constructs controlled by different sigma factors.

The specific hypotheses tested in this chapter are:

- Changes in supercoiling conferred by *gyrA* mutations will alter the expression of stress response loci;
- Alterations in stress response loci expression will provide a protective effect against quinolone and non-quinolone stresses;
- Mutations in *gyrA* will “override” inducible changes in supercoiling.

The aims for this chapter are:

- To determine the impacts that separate GyrA Ser83Phe and GyrA Asp87Gly substitutions have on the expression of the SOS response loci *recA* and *lexA*;
- To construct fluorescent reporter plasmids controlled by a range of sigma factors;
- To determine the effects of *gyrA* mutations on the relationship between stresses and supercoiling in multiple conditions.

4.2 Results

4.2.1 Expression of SOS Response Regulators

To determine the expression levels of genes involved in the SOS response and how this varied between cells with different *gyrA* mutations, cells were incubated with nalidixic acid. RNA was then extracted and used as a template for reverse transcription PCR of the *lexA* and *recA* mRNAs, and the 16S rRNA.

The expression levels of *recA* increased in all strains after the addition of nalidixic acid. There was a large but non-significant increase (Student's *t*-test, p = 0.1) of *recA* mRNA levels in the GyrA Ser83Phe strain after the addition of nalidixic acid, relative to *recA* mRNA levels in untreated SL1344 cells, as shown in Figure 4.3.1.1. No significant change was observed in the levels of 16S rRNA or *lexA* mRNA in either the wild type strain or the two *gyrA* mutants after the addition of nalidixic acid (data not shown).

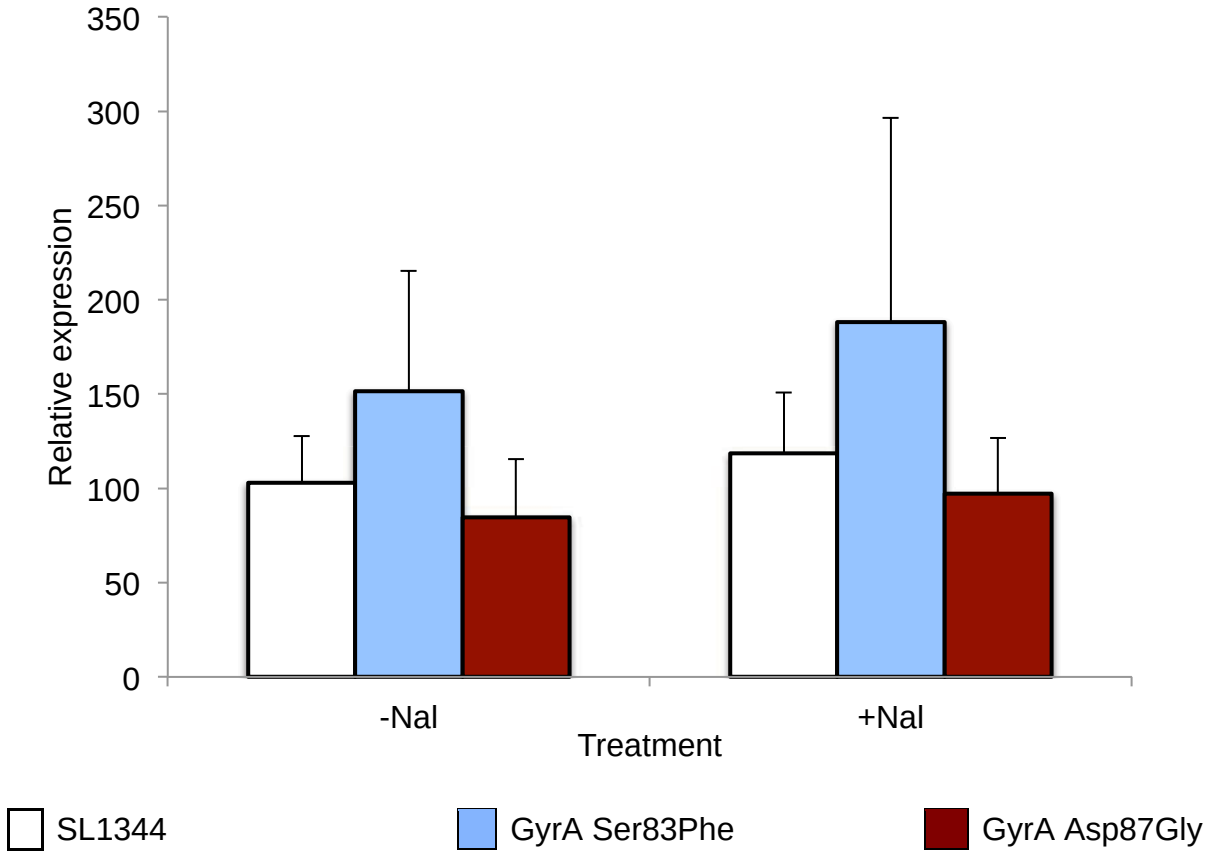


Figure 4.2.1.1: RT-PCR of *recA* after the exposure, or lack thereof, of *Salmonella* Typhimurium to nalidixic acid. Relative expression of the SOS inducer *recA* in treated (+Nal) and untreated (-Nal) cells, as compared with untreated SL1344 cells. Key: white = SL1344, blue = GyrA Ser83Phe, red = GyrA Asp87Gly. Error bars = 95% confidence intervals. Data shown are the averages of six replicates.

4.2.2 Fluorescent Reporter Construction

To determine the impacts of *gyrA* mutation on stress responses, fluorescent reporter constructs were made using pMW82, a plasmid containing an unstable *gfp* locus that lacked a promoter, and then transformed into SL1344 and the two *gyrA* mutant strains. The constructs had the promoters for either *recA, gabD, glnA, bamA* or *opdA* inserted upstream of the *gfpmut2* locus. Promoters that had completely identical -10 and -35 sequences in *Salmonella* and *E. coli* were chosen. The fluorescence of these constructs was used as a proxy for gene expression, which in turn was used as a proxy for the activity of the sigma factors responsible for their transcription.

Positive control conditions to induce expression from each promoter were identified from literature searches and also the expression levels of the corresponding genes from which the promoters were amplified were queried using the SalCom dataset (Kröger *et al.*, 2013). This dataset shows both the relative and absolute expression levels of over 85% of all known loci in *Salmonella* Typhimurium in an array of twenty-two infection-relevant conditions. Of these conditions, the expression level of *recA* was found to be increased 6.18-fold when cells were exposed to peroxide shock. This is unsurprising, as peroxide is known to trigger oxidative stress, which in turn activates RecA as part of the SOS response. Its expression is also upregulated 2.1-fold when cells were exposed to an oxygen shock. The dataset showed the expression of *glnA* was increased in mid-exponential phase and during oxygen shock (3.42 and 4.33-fold, respectively). It was downregulated in the all other conditions. The locus *yaeT* (*bamA*) was only upregulated during oxygen shock in the conditions

in the SalCom dataset (Kröger *et al.*, 2013). Its expression was unchanged in mid-exponential phase and it was downregulated under all other conditions.

The SalCom dataset (Kröger *et al.*, 2013) showed the expression of the *opdA* gene (*prlC*, in *Escherichia coli* (Conlin *et al.*, 1992)) was up-regulated 3.02-fold in peroxide shock, 2.12-fold during sodium chloride shock, 2-fold during low iron shock and 2.26-fold during mid-exponential phase. It was increasingly downregulated from late exponential phase onwards. Although it has previously been shown to be part of an operon which is activated in response to heat shock (Conlin and Miller, 2000), this condition was not part of the bank of conditions tested. During a typhoid fever infection, the afflicted will run a temperature of around 39 to 40 ºC (Parry *et al.*, 2002), which is lower than the standard laboratory heat shock condition of 42 ºC.

The expression of *gabD* was unchanged in the set of conditions in the SalCom database (Kröger *et al.*, 2013). This is not consistent with what has otherwise been suggested by the literature, as it should be active during stationary phase (Bang *et al.*, 2005), which was included in the range of conditions tested.

The expression levels of the above genes under the conditions tested in the SalCom dataset are shown in Table 4.2.2.1, below.

	Early Exponential	Mid Exponential	Late Exponential	Early Stationary	**Mid exponential**	15 ºC shock	pH 3 shock	pH 5.8 shock	NaCl shock	Bile shock	Low Fe2+ shock	Anaerobic shock	**Anaerobic growth**	Oxygen shock	**Induced SPI2**	Induced SPI2 & low Mg2+	Induced SPI2 & peroxide shock	Induced SPI2 & itric oxide shock	**No SPI2**	Induced SPI2
recA	**1**	1.39	0.97	0.53	**1**	1.47	0.29	0.52	0.91	1.2	0.97	0.41	**1**	2.1	**1**	1.08	6.18	1.51	**1**	0.9
gabD	**1**	1	2.4	1.29	**1**	1	1	1	1	1	1	1	**1**	1	**1**	1	1	1	**1**	1
glnA	**1**	3.42	0.53	0.24	**1**	0.86	0.28	0.1	0.15	0.84	0.67	0.14	**1**	4.33	**1**	0.07	0.92	0.3	**1**	0.99
yaeT	**1**	1	0.76	0.35	**1**	0.76	0.35	0.75	0.53	0.96	0.98	0.39	**1**	2.12	**1**	0.82	0.33	0.43	**1**	0.71
opdA	**1**	2.26	0.63	0.61	**1**	1.33	0.16	0.39	2.12	1.05	2	0.64	**1**	1.38	**1**	0.91	3.02	1.62	**1**	1.05

Relative to expression in early exponential phase

Relative to expression during anaerobic growth

Relative to expression in the absence of SPI2

Relative to expression in mid-exponential phase

Relative to expression during SPI2 induction

Table 4.2.2.1: Summary of changes in gene expression during various conditions experienced by *Salmonella* spp. during the infection cycle. This table summarises the data from the SalCom dataset (Kröger *et al*, 2013) as discussed in Section 4.2.2. It shows the relative changes in expression for the genes of which the promoters were used to create the pMW82 reporter constructs used in this study. Blue = expression relative to early exponential phase; green = expression relative to mid-exponential phase; orange = expression relative to during anaerobic growth; red = expression relative to during SPI2 induction; purple = expression relative to during the absence of SPI2. Not all the conditions tested as part of the fluorescence induction assays are found within the SalCom dataset. The gene *gabD* is only upregulated during late exponential and early stationary phase, whereas the expression of the other genes is affected by a wider range of conditions.

4.2.3 Green Fluorescent Protein Assays

The fluorescence levels of each of the constructs were read in a fluorescent plate reader under a range of conditions giving a total of approximately 270,000 datapoints. Readings were normalised with respect to the cultures' optical density, so as to determine the activity of the promoters, and thus the activity of various sigma factors, in real time. Figure 4.3.4.1 shows that the control strains fluoresced as expected: SL1344 with no plasmid showed a very low level of autofluorescence, SL1344/pMW82 p*ramA*:*gfpmut2* fluoresced strongly when induced by chlorpromazine but not without induction and SL1344 Δ*ramRA*/pMW82 p*ramA:gfpmut2* constitutively fluoresced at a high level.

Data from a small selection of experiments are shown graphically in Figure 4.3.4.2. The rest can be found in the appendices. For the *Salmonella* mutants, there was no consistent up-regulation of any of the stress response pathways in any background. There was variation between fluorescence from different constructs and mutants but no consistent correlation with genotype was obvious. Examples of some conditions with different expression patterns are shown in Figure 4.3.4.2. The fluorescence levels increased over time for the p*gabD* construct in the SL1344 background in the presence of nalidixic acid, but remained low in the other backgrounds, as shown in part B). Additionally, the fluorescence levels of the p*glnA* construct in the GyrA Ser83Phe background remained elevated in the presence of chlorpromazine over the 16-hour measurement period, as shown in part E).

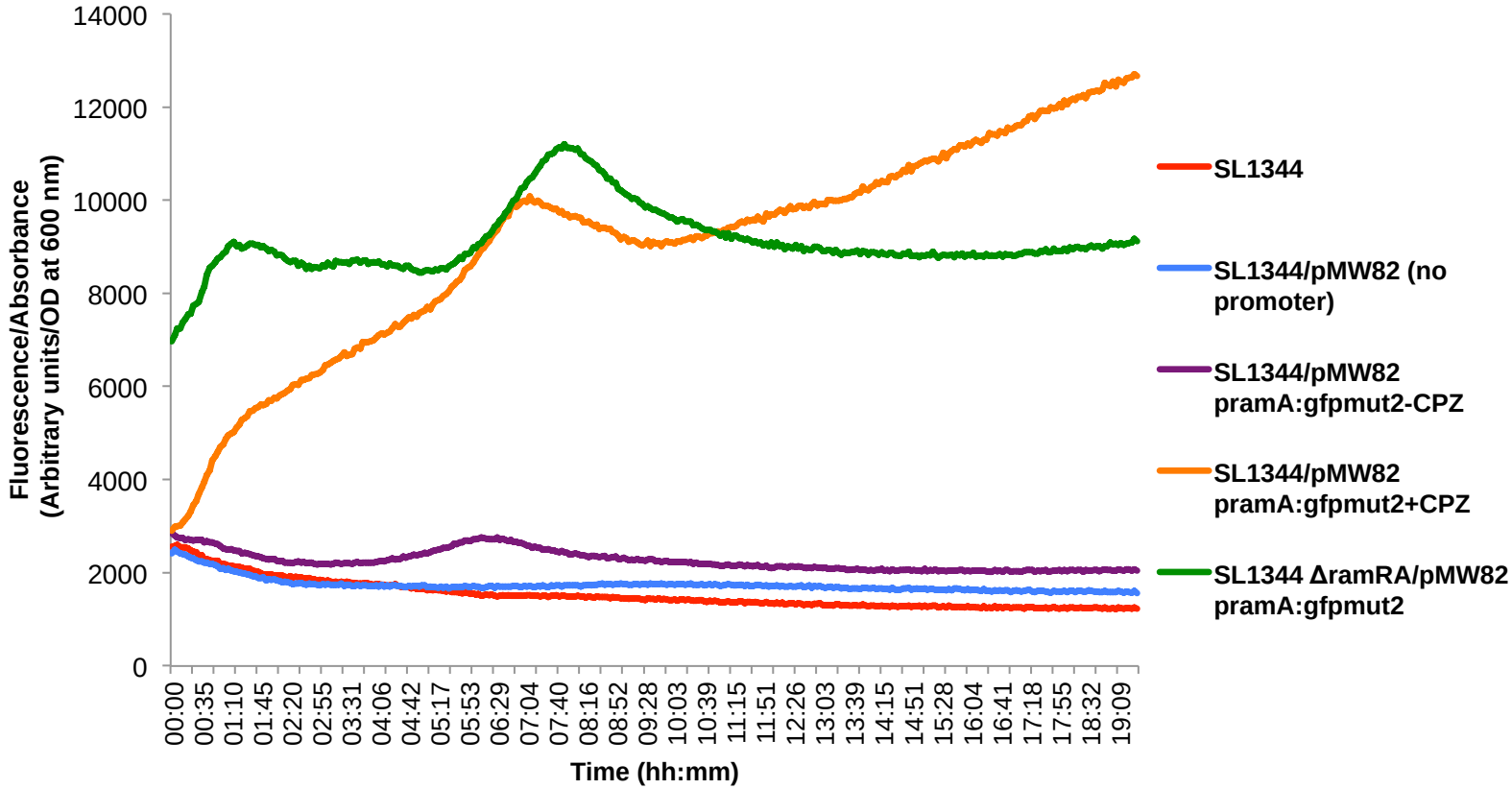


Figure 4.2.3.1: Fluorescence levels of control strains in reporter assay experiments. Representative example of fluorescence/absorbance data from control strains in a time course assay. Similar readings were observed for all experiments. As expected, the fluorescence of cells with the constitutive reporter plasmid increases over time, and the fluorescence of cells with the inducible reporter plasmid in the presence of the inducer is at a consistently high level. No fluorescence is observed in cells lacking the reporter, cells with a promoterless version of the reporter plasmid, or cells with the inducible reporter plasmid in the absence of the inducer.
Key: SL1344 = red; SL1344/pMW82 = blue; SL1344/pMW82 p*ramA*:*gfpmut2* (without chlorpromazine) = purple; SL1344/pMW82 p*ramA*:*gfpmut2* (with chlorpromazine) = orange; SL1344 Δ*ramRA*/pMW82 p*ramA*:*gfpmut2* = green. Data shown are the averages of two independent replicates.

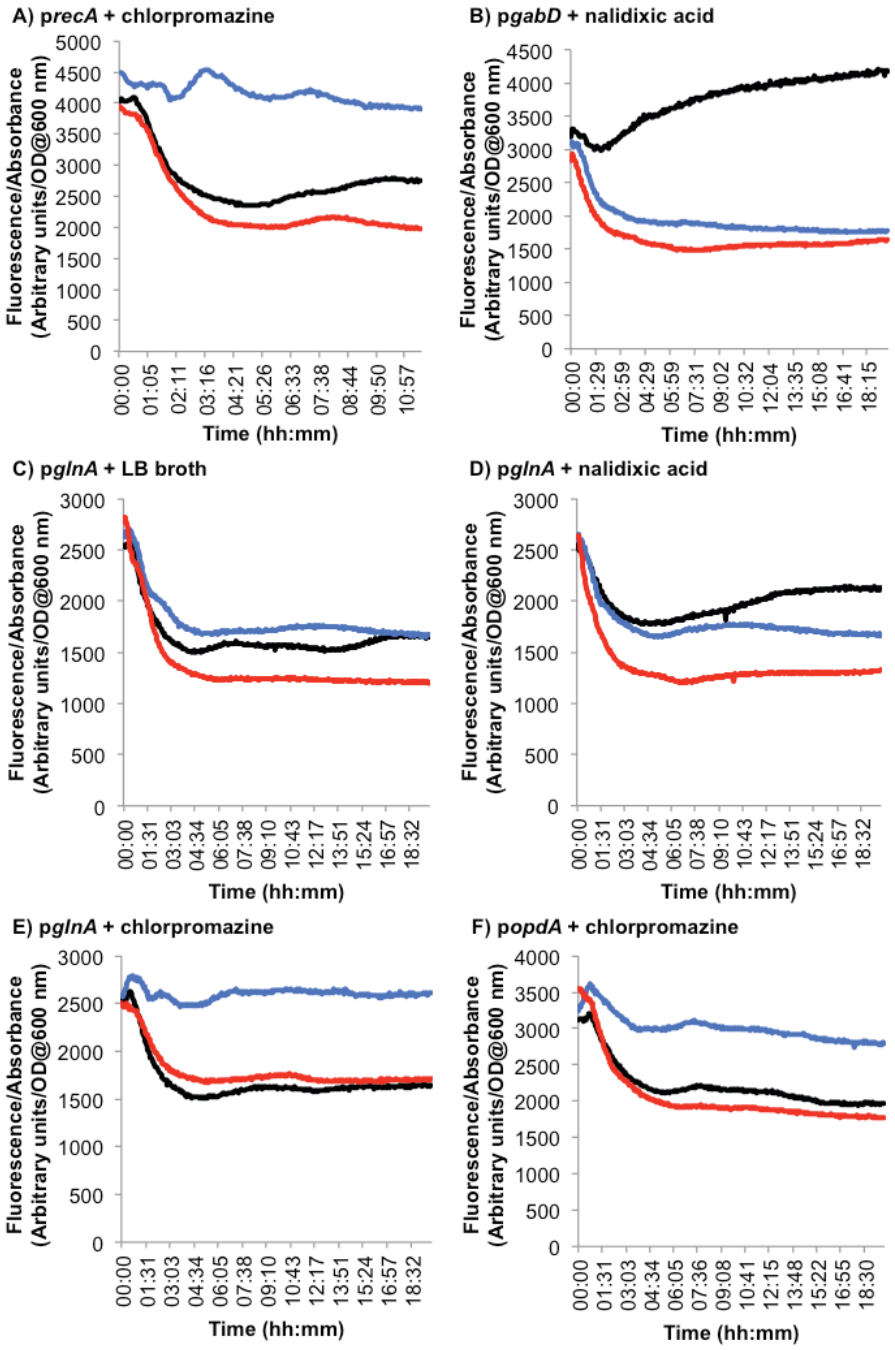


Figure 4.2.3.2: Optical density-adjusted fluorescence levels of a number of reporter constructs under a selection of conditions. Data shown are the averages of four independent replicates. Parts A), E) and F) show the fluorescence of various reporters (p*recA*, p*glnA* and p*opdA,* respectively) in response to chlorpromazine exposure. The expression of these reporters in the GyrA Ser83Phe background appears to persist at a higher level than in the other backgrounds. Part B) shows the p*gabD* construct in the presence of nalidixic acid; its expression increases over time in SL1344 but does not increase in the other backgrounds.. Part C) shows p*glnA* in the presence of LB broth and is included for control purposes. The p*glnA* construct is also shown in part D), in the presence of nalidixic acid.

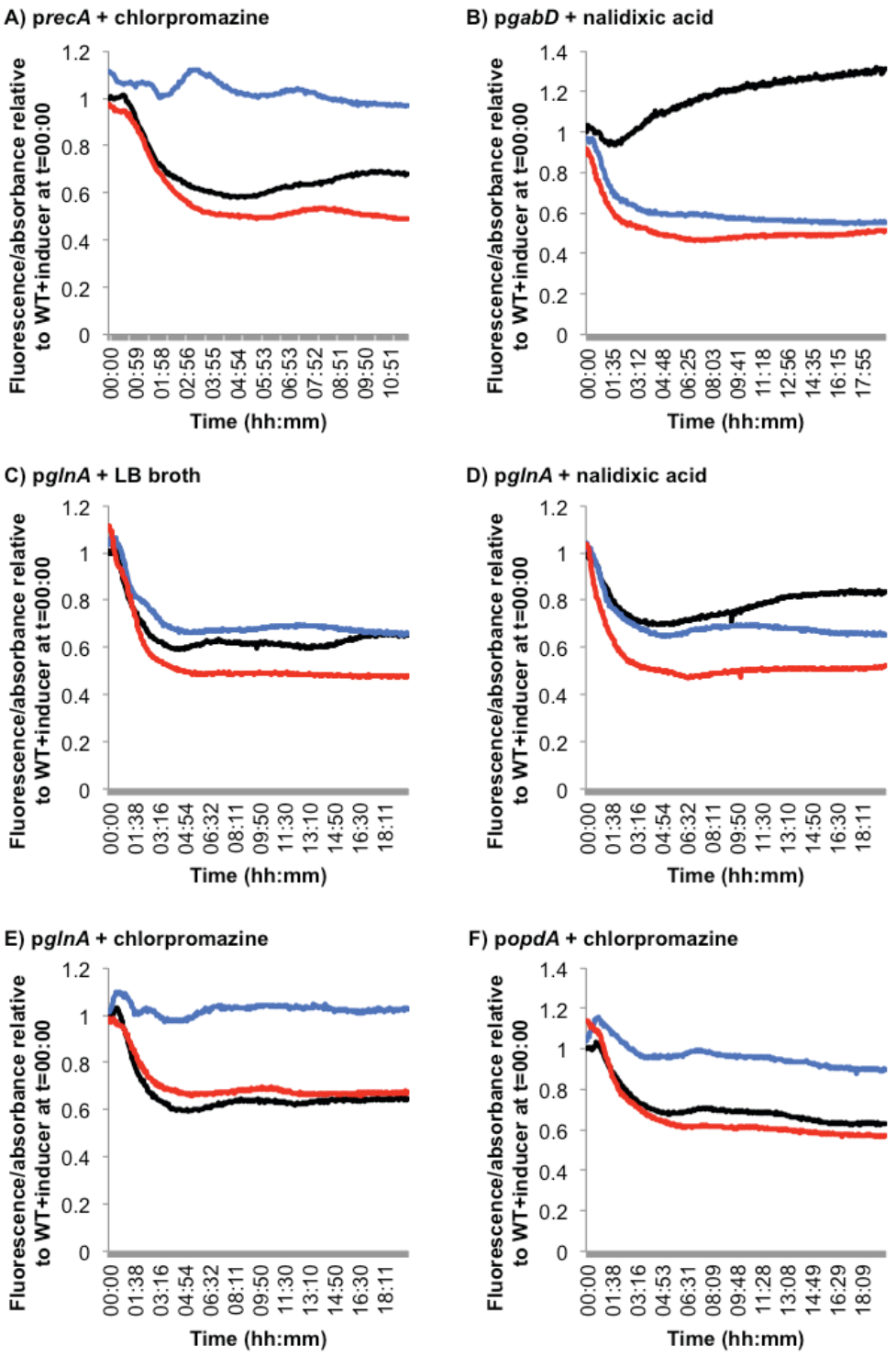


Figure 4.2.3.3: Optical density-adjusted fluorescence levels recalculated relative to the reading for SL1344 carrying the reporter of interest exposed to the condition of interest at time zero. These are the same data as shown in Figure 4.2.3.2, but corrected to account for any differences in initial inoculum.

The relative fluorescence readings were also normalised to the level observed in the wild type background at time zero, for each of the six inducers. An example of this is shown in Figure 4.2.3.3. Patterns of the data were similar after normalisation. In the GyrA Ser83Phe background, fluorescence appeared to relatively constant for the p*recA*, *popdA* and p*glnA* constructs in the presence of chlorpromazine, remaining at a consistently high level throughout. The fluorescence of the p*gabD* construct in the SL1344 background increased over time in the presence of nalidixic acid. However, the fluorescence of most reporter constructs fell over time, no matter the strain they were in or condition to which they were exposed, reaching a plateau at around one and a half hours after measurements started. Therefore, although the fluorescence of some reporters increased in some backgrounds, there was no consistent upregulation of stress responses based on strains or conditions.

4.2.4 Transformation of *E. coli* with pMW82 Constructs, and Subsequent Fluorescence Induction Assays

To determine how sigma factor activity changes in *E. coli* with respect to *gyrA* mutation and in response to stresses, the pMW82 reporters described earlier were transformed into *E. coli* MG1655 and the two *gyrA* mutant strains. A literature survey showed that the promoters used to make the pMW82 constructs were strongly conserved within Gammaproteobacteria (data from Conlin and Miller, 2000; Merrick, 1993; Nonaka, 2006; Rhodius *et al.*, 2005; Skovierova *et al.*, 2006; Weisemann and Weinstock, 1991) so the reporters were expected to work in *E. coli* without needing modification. Figure 4.2.4.1 shows, for example, the similarity of the promoter sequences recognised by σ^H in both species, and a comparison of these with the

sequence inserted into pMW82 to make the p*opdA* construct. However, it should be noted that even small variations in promoter sequence, such as a single nucleotide change, or slight changes in the number of repeat sequences, can have dramatic impacts upon gene expression (Browning and Busby, 2016). This could potentially explain the observed differenes in reporter expression between *E. coli* and *S.* Typhimurium.

Figure 4.2.4.1: Promoter sequences recognised by σ^H in *Salmonella* and *E. coli*. This figure shows the conserved -35 and -10 promoter sequences recognised by σ^H in *Salmonella* and *E. coli*, highlighted in colour. Both species have a shared -35 sequence of TTGAAA and both have species a conserved CCCAT sequence at -10. These sequences are shown alongside the 5' UTR of *opdA,* which was inserted into pMW82 to make the p*opdA* reporter construct, with the -35 and -10 sequences again highlighted in colour. The σ^H recognition sequences in the insert and the canonical σ^H recognition sequences are largely identical.

The fluorescence levels of the reporter constructs were read in a fluorescent plate reader under a range of conditions, in the same manner as in *Salmonella*. Readings were again normalised with respect to the cultures' optical density, so as to determine the activity of the promoters, and thus the activity of various sigma factors, in real time.

Figure 4.2.4.3 shows the relative fluorescence readings for the p*recA,* p*gabD*, p*glnA* and p*bamA* constructs in the absence of stress, normalised to the level observed in the wild type strain at time zero. Data from a selection of other experiments are shown in Figure 4.2.4.4, and are normalised to the wild type at time zero in Figure 4.2.4.5. Whilst as with *Salmonella* there was variation of expression across the various conditions it was evident that, in the absence of stress, the GyrA Asp87Gly strain demonstrated increased fluorescence from four of the reporters, suggesting constitutive de-repression of these pathways in this strain.

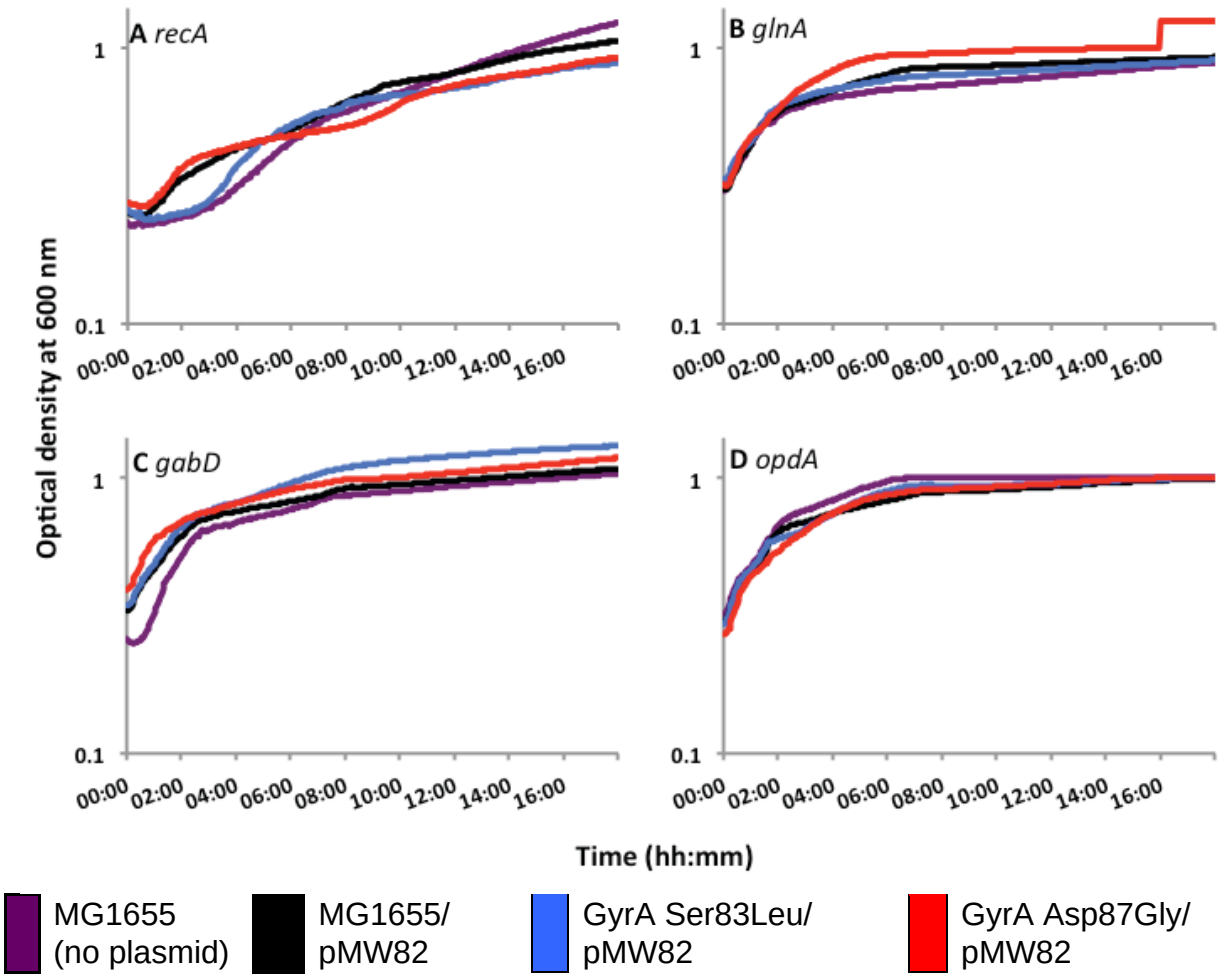


Figure 4.2.4.2: Growth curves for *E. coli* strains carrying the pMW82 reporter constructs under control conditions. Cells were grown in unsupplemented LB broth at 37 ºC overnight. In most cases, plasmid carriage does not appear to adversely affect growth rates, nor does *gyrA* mutation. However, cells with a GyrA Asp87Gly substitution and carrying the *glnA* reporter construct appeared to remain in logarithmic phase for longer than other cells carrying this reporter construct. Data shown are the averages of four independent replicates.

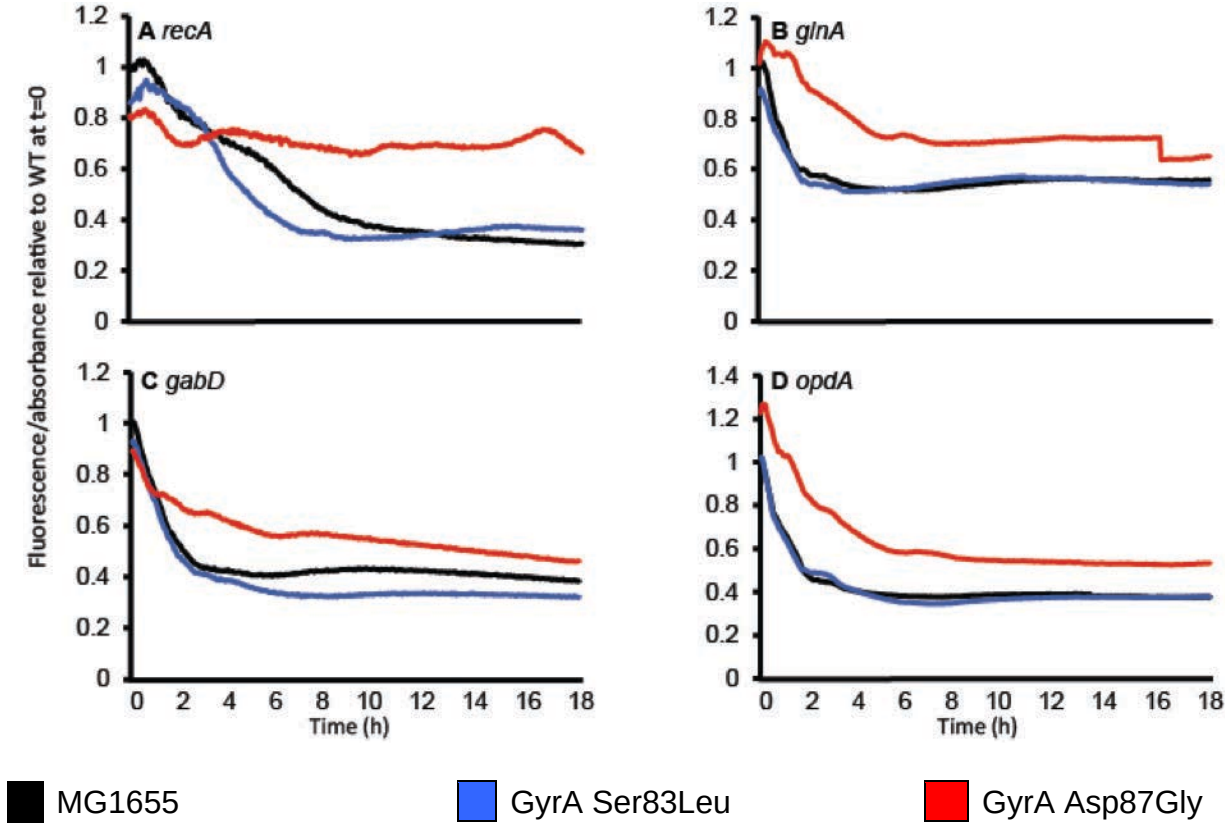


Figure 4.2.4.3: Fluorescence of reporter constructs in *E. coli* under control conditions. Data shown are the averages of four independent replicates, and have been recalculated relative to the fluorescence of the reporter assayed in MG1655 at time zero. Expression of *gfpmut2* from the *recA* promoter is constant in the GyrA Asp87Gly background, and its expression from the other promoters is elevated in that background compared with the others. When in the MG1655 and the GyrA Ser83Leu cells, the reporters appear to follow a similar pattern of expression irrespective of the background.

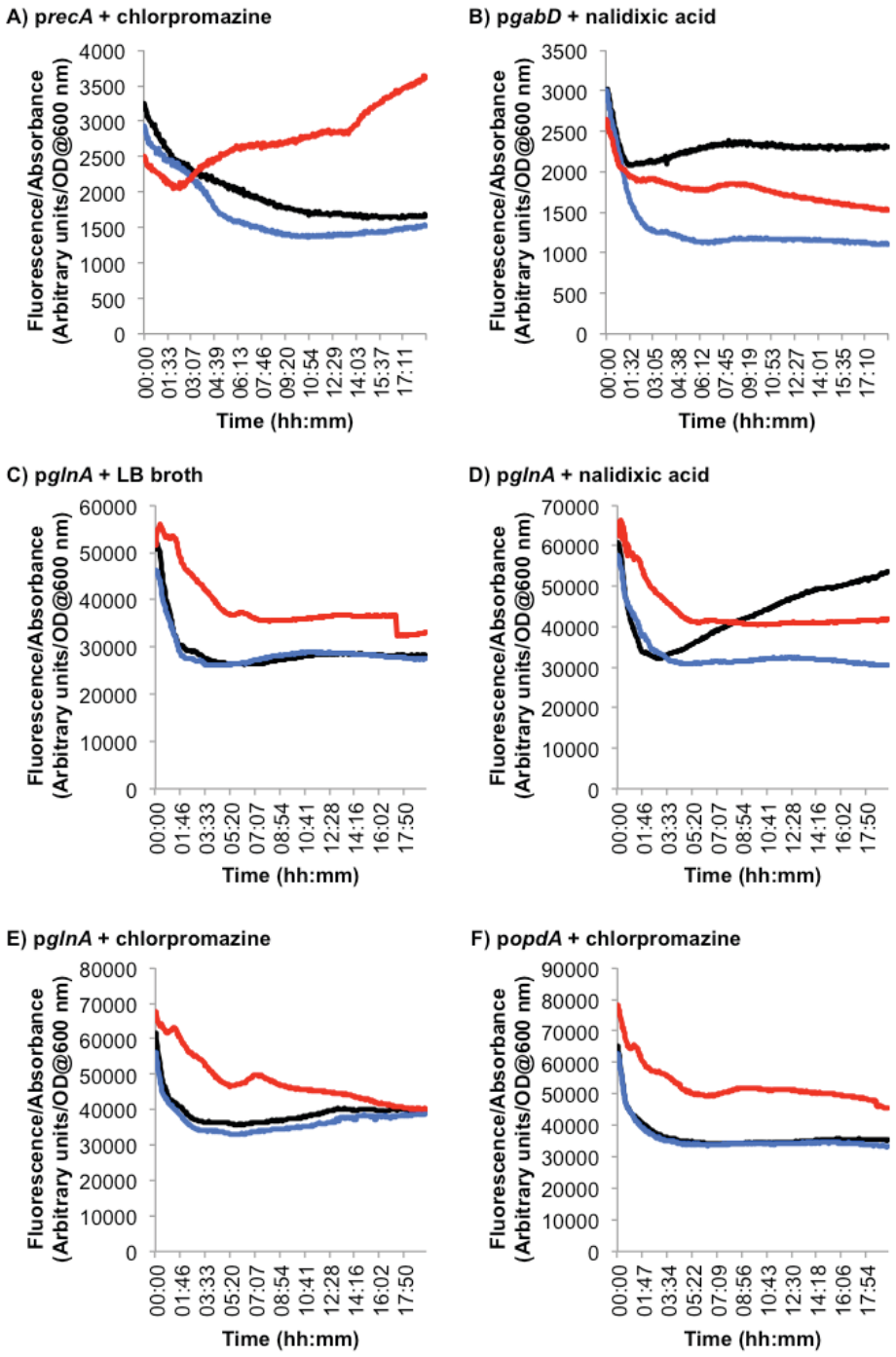


Figure 4.2.4.4: Optical density-adjusted fluorescence levels of a number of reporter constructs under a selection of conditions in *E. coli*. Data shown are the averages of 4 independent replicates. Parts A), E) & F) show the fluorescence of various reporters (p*recA*, p*glnA* and p*opdA,* respectively) in response to chlorpromazine exposure. Part B) shows the p*gabD* construct in the presence of nalidixic acid. Part C) shows p*glnA* in the presence of LB broth and is included for control purposes. The p*glnA* construct is also shown in part D), in the presence of nalidixic acid. With the exception of Part B), where the expression of the reporter in the MG1655 background is highest, and part D), where the expression of the reporter in the MG1655 background increases over time, the expression of the reporters is highest in the GyrA Asp87Gly background, which was not expected.

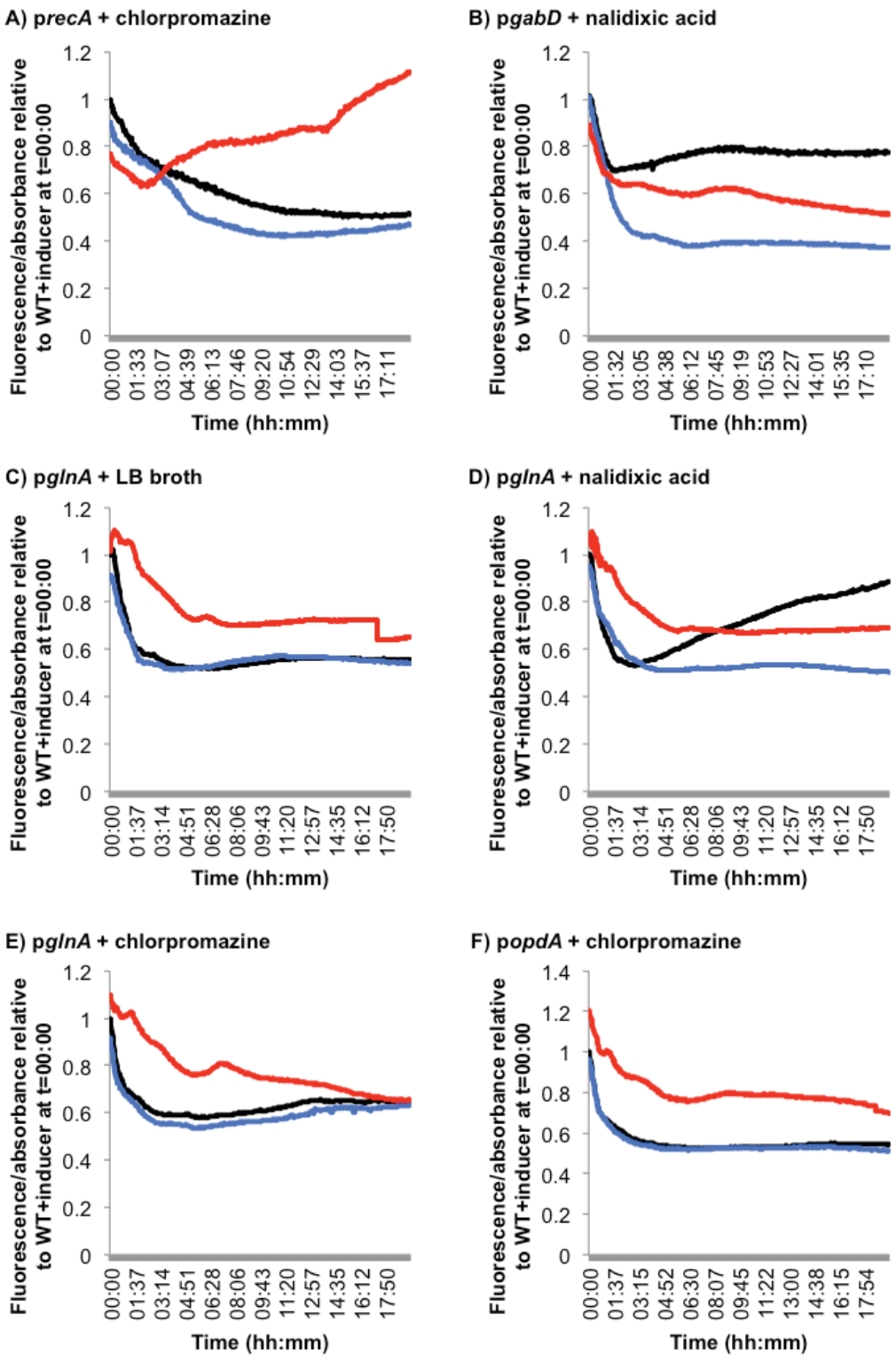


Figure 4.2.4.5: Optical density-adjusted fluorescence levels recalculated relative to the reading for MG1655 carrying the reporter of interest exposed to the condition of interest at time zero. These are the same data as shown in Figure 5.2.5.3, but corrected to account for any differences in initial inoculum.

4.2.5 Flow Cytometry

To determine fluorescence levels in *Salmonella* on a per cell basis, flow cytometry was used. The same reporter constructs were used as in the FluoStar assays described above and in the wild type and gyrase mutant backgrounds. Initial experiments carried out by Robyn Iddles in my absence as part of her BMedSci intercalation project (data not shown) showed that the proportion of fluorescent cells in each background was around 20-25% in the absence of any stressor. This was irrespective of the reporter construct being used, though the fluorescence intensity of *gfp*-positive cells carrying the p*gabD* and p*glnA* constructs appeared to be higher (data not shown). Subsequently, the fluorescence levels of the p*gabD* and p*glnA* constructs in the absence of any stress were determined in more detail using flow cytometry. Samples containing 50,000 cells were analysed on four separate occasions, with each repeat containing one sample from each of the three backgrounds. The data are represented graphically in Figure 4.2.5.1. These data supported the initial screen showing a greater proportion of GyrA Ser83Phe cells (and to a lesser extent Asp87Gly cells) expressed the p*gabD* construct than SL1344 cells. However, whilst a larger number of the gyrase mutants were expressing *gabD*, the level of fluorescence of those cells that were producing GFP was lower than for those that were expressing *gabD* in SL1344. To summarise, *gyrA* mutants were more likely to be expressing *gabD* than SL1344, but when a wild type cell was expressing *gabD* the level of fluorescence was higher than in a mutant cell.

A preliminary experiment to investigate how nalidixic acid affected the fluorescence of the p*gabD* and p*glnA* constructs in all three backgrounds was carried out and the

results are shown in Figure 4.2.5.2. Fluorescence appeared to increase in all strains after incubation with nalidixic acid, except for the p*gabD* construct in SL1344.

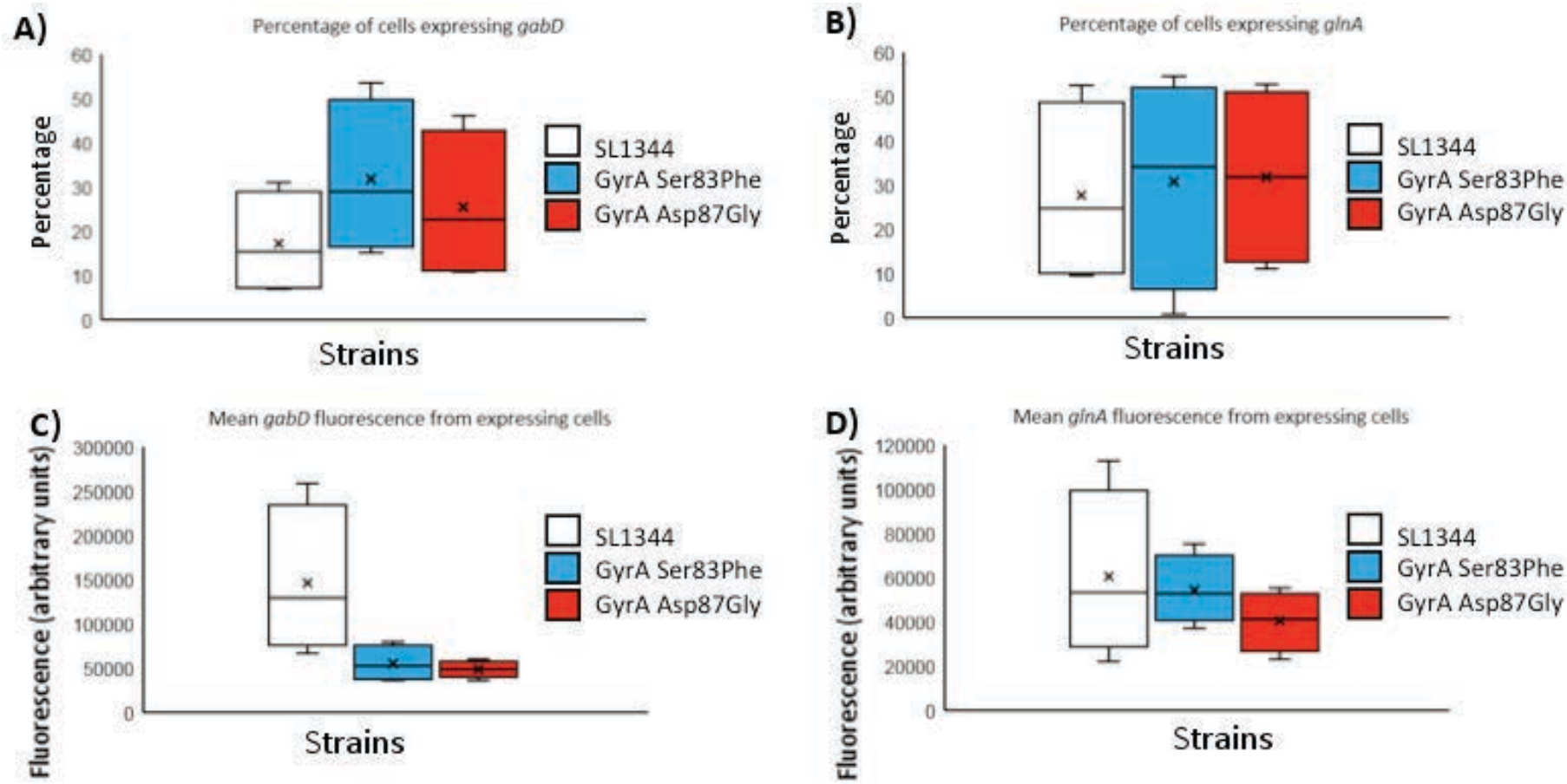


Figure 4.2.5.1: Flow cytometry data for *Salmonella* p*gabD* and p*glnA* constructs. Key (all parts): White = SL1344; Blue = GyrA Ser83Phe; Red = GyrA Asp87Gly. Part A) shows the percentage of cells in a population of 50,000 expressing *gfpmut2* from the *gabD* construct. Part B) shows the percentage of cells in a population of 50,000 expressing *gfpmut2* from the p*glnA* construct. Part C) shows the mean fluorescence level of cells that were expressing *gfpmut2* from the p*gabD* construct. Part D) shows the mean fluorescence level of cells that were expressing *gfpmut2* from the p*glnA* construct. A greater proportion of cells within the mutant populations are expressing GFP from the reporter plasmids compared with the wild type populations, although the overall fluorescence level of the wild type population is higher. Data are taken from four independent replicates.

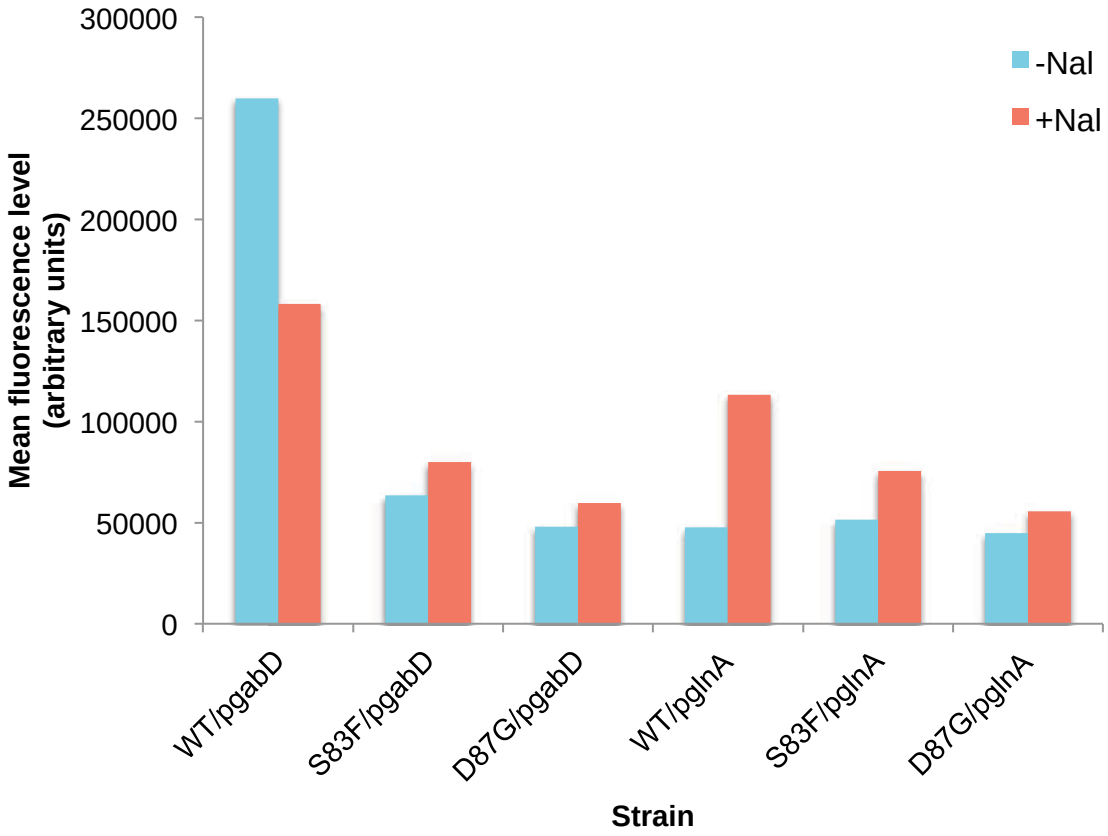


Figure 4.2.5.2: Preliminary flow cytometry data from *Salmonella* nalidixic acid exposure experiments. Figure showing the mean fluorescence level of p*gabD* and p*glnA* reporters in SL1344 (WT) and the two *gyrA* mutant backgrounds that had been grown to mid-logarithmic phase and incubated in the presence of absence of nalidixic acid for 45 minutes. Aside from the p*gabD* reporter in the SL1344 background, fluorescence appeared to increase after nalidixic acid exposure in all cases, though this experiment was only repeated once. Data are from samples of 50,000 cells.

4.3 Discussion

4.3.1 Expression of SOS Response Regulators in *Salmonella*

One possible mechanism by which mutation in *gyrA* may provide antimicrobial protection is by initiating the SOS response which helps the cell repair DNA damage. To examine this *recA* expression was measured in the presence and absence of the inducer, nalidixic acid. The data show that the *gyrA* mutants do not show a constitutive up-regulation of *recA* and so this is unlikely to be the mechanism underpinning their broad antimicrobial tolerance. The data show a non-significant increase in the expression of the SOS response regulator *recA* after exposure of all three of *S.* Typhimurium SL1344, GyrA Ser83Phe and GyrA Asp87Gly cells to nalidixic acid. It has previously been shown multiple times by multiple different authors that nalidixic acid would be expected to induce *recA* in the wild type (Piddock and Wise, 1987; Power and Phillips, 1993; Ysern *et al.*, 1990).

The large but non-significant increase in *recA* expression in the *Salmonella* GyrA Ser83Phe strain, compared with the lower increases in SL1344 and the GyrA Asp87Gly strain, suggests that nalidixic acid exposure is sensed more easily by GyrA Ser83Phe cells, despite the increase in minimum inhibitory concentration the substitution confers. The concentration of nalidixic acid used here is ten-fold higher than that used as an inducer in published work; this higher concentration was used as it was expected that there would be a dose-dependent shift in RNA levels – a dose-dependent change in the levels of SOS proteins in response to nalidixic acid has previously been observed (Piddock and Wise, 1987). A lack of a change in *recA* expression would be understandable in SL1344, given the MIC for nalidixic acid

against this strain is 4 μg/mL, as shown in previously (Webber *et al.*, 2013), but the MIC against GyrA Ser83Phe and GyrA Asp87Gly is at least 256 μg/mL. It would be pertinent to see how the SOS response changes for the *gyrA* mutant strains at nalidixic acid concentrations approaching the MIC although *recA* expression does not appear to be key to the phenotype of the gyrase mutants.

4.3.2 Fluorescent Reporter Construction and *Salmonella* Typhimurium Induction Assays

A very large set of data was assembled of fluorescence from various stress responsive promoter-*gfp* reporters measured in the three strain backgrounds in the presence of various stresses. In the *Salmonella* strains, there was no consistent up-regulation of any of the stresses associated with a particular genotype, and the reporters were not necessarily always induced by the agents that the literature survey suggested would induce them. For example, it was expected that the RpoN reporter, with the *glnA* promoter, should have been induced by serine hydroxamate, but it was difficult to tell if this had actually occurred. It might perhaps have been more appropriate to use a fluorescent plate reader with an injector function; this would have allowed for the fluorescence of the cell populations to be measured before the addition of any supposed inducer. As it stands, readings were only taken after the addition of inducing agents, owing to how the plate reader that was used works; measuring fluorescence from a point prior to the addition of an inducer would mean that one would more easily be able to determine if any changes in fluorescence were a genuine response to the inducer. The current setup leaves some ambiguity as to whether or not the reporters are functioning correctly.

There were some interesting alterations in expression of some reporters. For example, the apparent constant fluorescence of the p*recA* and p*glnA* constructs in the GyrA Ser83Phe background in the presence of chlorpromazine suggests that, given the *gfpmut2* found in pMW82 is unstable (Bumann and Valdivia, 2007), more GFP is being produced on account of continual transcription of the mRNA from those promoters in that condition. This suggests that the SOS response and nitrogen stress response are both elevated in GyrA Ser83Phe cells under this condition. It is unlikely that this increased expression is purely the consequence of induction by chlorpromazine, as one would expect the reporter constructs to be equally as active in the other backgrounds if that were the case. It has been shown in *Pseudomonas aeruginosa* that heightened stress responses are correlated with antibiotic resistance, and could contribute to resistance phenotypes (Poole, 2014); it might be possible that something similar is occurring here, though if this were the case, one would also expect to see increased expression of the reporter constructs in the GyrA Asp87Gly cells in a wide range of conditions, which does not appear to be the case either. The RpoS reporter also appeared to have elevated expression levels in SL1344 in the presence of nalidixic acid. This is perhaps unsurprising as RpoS has been shown to become activated in response to DNA damage, as well as a range of other conditions (Bougdour *et al.*, 2008). Additionally, the nucleoid-associated proteins H-NS and Fis have been shown to prevent transcription by RNA polymerases containing the housekeeping sigma factor and encourage transcription by RNA polymerases containing the stationary phase sigma factor (Grainger *et al.*, 2008).

4.3.3 Fluorescent Reporter Induction in *E. coli*

Fluorescent reporter induction assays in *E. coli* showed consistently higher fluorescence when constructs were transformed into the GyrA Asp87Gly background, suggesting elevated stress responses in this strain. This is in contrast to the data from *Salmonella*, where there was no consistent upregulation of stress responses based on either strain or condition. The constant level of expression of the p*recA* construct in the *E. coli* GyrA Asp87Gly background suggests that the SOS response loci are expressed at a semi-constant elevated level compared to in other backgrounds. The constant level of expression is important given the GFP used is unstable; it is thus being replenished. The increased expression of GFP from this promoter is likely a consequence of global changes in gene expression caused by the altered supercoiling state of this strain, given the equivalent substitution in *Salmonella* has been shown to result in heightened gene expression (Webber, *et al*. 2013). Another way of verifying this would be to use RNA seq or an RT-PCR based approach to determine transcript levels as was used in *Salmonella.* The increased expression levels of stress response loci in this strain could explain why it is better able to tolerate exposure to triclosan and other stresses than either the GyrA Ser83Leu strain or MG1655.

One further avenue of interest would be to compare the effects of *gyrA* mutation in *E. coli* and *Salmonella* with the effects of equivalent *parC* mutations in a Gram-positive species. The equivalent mutations in *parC* in a Gram-positive species would be of greater interest than the identical mutations in *gyrA* on account of how quinolones are usually more active against topoisomerase IV than gyrase in Gram-positive

organisms (Drlica *et al.*, 2008). Additionally, time did not allow for the exposure of cells to various stresses and then assaying how the topoisomer profile changes as a result, as was done in *Salmonella*, which would be included if this line of questioning was pursued further in future.

4.3.4 Flow Cytometry

Whilst the data from assays measuring expression from whole populations allowed a large number of conditions to be assayed the resolution of the data is not able to inform responses within a population which may differ. Therefore, for selected conditions flow cytometry was also used. The flow cytometry data suggest that the proportion of cells in the population that are fluorescent is higher in the GyrA Ser83Phe and GyrA Asp87Gly backgrounds, for both the p*gabD* and p*glnA* constructs, than in SL1344. However, the mean fluorescence levels appear to be higher in the SL1344 background. Therefore, more of the *gyrA* mutant populations are producing these stress responses constitutively although the range of expression appears to be curtailed. This pattern of expression was not obvious from the whole population fluorescence assays and shows that the gyrase mutants are more likely to be producing a potentially protective stress response at any given time.

Previously published data based on a transcriptomic experiment has shown that a range of stress response genes had significantly increased expression in GyrA Asp87Gly cells compared with SL1344, including but not limited to *rpoE, rpoS, rpoD, rpoN* and *recA* (Webber *et al.*, 2013). The flow cytometry data here support this data and provide a more detailed view of the expression dynamics.

The exposure of the p*gabD* and p*glnA* constructs to nalidixic acid and their subsequent analysis with flow cytometry lacks sufficient replicates to be able to draw an adequate conclusion. If this experiment were to be repeated again, it could be expanded to include the other promoter constructs and a wide range of stressors.

The data presented in this chapter show that the gyrase mutants appear to be pre-disposed to expression of *glnA* and *gabD* suggesting these stress response pathways are constitutively expressed in a significant fraction of cells. This may help explain the antimicrobial tolerance in these strains. The data was however not conclusive and the up-regulation of stress responses was not universally conserved in different conditions. The specific impact of these stress responses on the antimicrobial tolerance of gyrase mutants could be investigated by inactivating these pathways and measuring the impact on phenotype.

One interesting question relates to the mechanism by which *gyrA* mutation could impact on expression of stress responses. To test the impact of supercoiling another avenue for investigation would be to see if there is any interplay between positional effects on gene expression, as described by Bryant *et al.* (2014), and *gyrA* mutation-mediated changes in gene expression. This could be achieved by recombining the promoter:*gfpmut2* fusions into differently supercoiled locations in the chromosomes of SL1344 and the two *gyrA* mutant strains.

Chapter Five

Overall Discussion, Future Work and Conclusions

5. Overall Discussion, Future Work and Conclusions

5.1 Discussion

The experiments presented in this thesis set out to investigate the impacts of two separate clinically relevant GyrA substitutions, GyrA Ser83Phe and GyrA Asp87Gly, on supercoiling in *Salmonella* Typhimurium and to determine what effects any changes in supercoiling had on antibiotic resistance and gene expression. The impacts of equivalent substitutions in *Escherichia coli*, GyrA Ser83Leu and GyrA Asp87Gly, were also investigated.

The data presented herein agree with the previously published observation that *gyrA* mutation reduces susceptibility to the quinolones and fluoroquinolones, antibiotics that inhibit topoisomerase function. These data also agree with the observation that *gyrA* mutation decreases susceptibility to the biocide triclosan (Webber *et al.*, 2013). However, GyrA substitution did not confer any advantages to *Salmonella* in competition assays using 0.03 μg/mL triclosan. This is below the wild type MIC of triclosan and was chosen to see if low concentrations of triclosan would promote emergence of *gyrA* mutants which would represent a “real-world” threat given these concentrations are now common in the environment. Further experiments at higher concentrations may perhaps show otherwise; the concentration used is likely insufficient to fall within what Drlica (2003) has described as the “mutant selection window”. Conversely, in *E. coli,* GyrA Asp87Gly cells were able to outcompete MG1655 cells when this concentration of triclosan was used showing a much greater impact of the GyrA Asp87Gly substitution in *E. coli*. Previous selection experiments have shown that exposure of *Salmonella* to triclosan does not increase the frequency

with which mutations occur, but does enrich the mutants that are already present in the population (Birošová and Mikulášová, 2009).

Both the *gyrA* mutations examined changed the MIC of triclosan against both *Salmonella* and *E. coli* from 0.06 µg/mL to 0.25 µg/mL (Webber *et al.*, 2017), so it might be of interest to perform competition assays between different *gyrA* mutants in the same species, or the same *gyrA* mutants in different species. Despite *S.* Typhimurium SL1344 having a lower triclosan MIC than either of the two *gyrA* mutants in that species, it still managed to outcompete them when the concentration of triclosan was sub-inhibitory demonstrating that, in *Salmonella* the growth deficit incurred by *gyrA* mutation is greater than the relative increase in triclosan tolerance at low concentrations.

It has been shown in the literature that GyrA substitutions have an associated fitness cost (S. Baker *et al.*, 2013), but this was not seen in the growth kinetics data presented here, as growth kinetics are not the most sensitive measure of fitness and small changes are not necessarily reported using this method. The competition assays used did show a reduction in fitness for the *gyrA* mutants in *Salmonella* although this was smaller in the GyrA Asp87Gly strain than it was in the GyrA Ser83Leu strain, smaller costs were seen in *E. coli*. The impacts of mutations on fitness are highly condition specific. It has been suggested through a combination of mathematical modelling and selection experiments that, in *E. coli*, in the presence of ciprofloxacin the most favourable route for acquiring substitutions that reduce quinolone susceptibility is an initial change to GyrA Ser83Leu, followed by additional

substitutions of GyrA Ser83Leu ParC Ser80Ile, then finally GyrA Ser83Leu Asp87Asn ParC Ser80Ile. This is considered to be the case because other substitutions are less energetically favourable and are outcompeted by the ones mentioned (Huseby *et al.*, 2017). However, in *Salmonella* Typhi, the equivalent triple substitution (GyrA Ser83Phe Asp87Gly ParC Ser80Ile), despite conferring the largest observed change in norfloxacin MIC, is energetically unfavourable in control conditions. However, another two-step substitution, GyrA Ser83Phe Asp87Asn confers sufficient change in norfloxacin MIC to make the cells clinically resistant to the antibiotic, and has a comparatively high selection coefficient (S. Baker *et al.*, 2013). In my experiments, we did not focus on the impact of quinolones as selective agents – the GyrA Asp87Gly substitution is seen in non-laboratory settings but was of interest here as it has previously been repeatedly selected by exposure to non-quinolone biocides. It has been shown here that this substitution confers a greater protective effect to triclosan (particularly in *E. coli*) than the GyrA Ser83Leu substitution that is favoured under quinolone selection alone. Therefore, the local selective conditions will greatly impact the fitness of different mutations making predictions from laboratory data alone about selection in the field difficult.

Using the plasmid pBR322 as a proxy for genomic supercoiling, experiments presented herein have reconfirmed the previously published observation that *gyrA* mutation alters supercoiling in *Salmonella* (Webber *et al.,* 2013) – the GyrA Ser83Phe substitution increases supercoiling levels in comparison to SL1344, whereas the GyrA Asp87Gly substitution results in a similar topoisomer distribution to that seen in the wild type. It has also previously been shown that there are

differences in basal levels of supercoiling between *Salmonella* and *E. coli* (Cameron *et al.*, 2011), with which these data are in agreement. The experiments here have shown for the first time that the impacts of equivalent substitutions in *E. coli* upon supercoiling differ significantly to those seen in *Salmonella,* with the GyrA Asp87Gly substitution resulting in a much more supercoiled profile in *E. coli* than either the MG1655 strain, the *E. coli* GyrA Ser83Leu strain, or any of the *Salmonella* strains. This large difference in impact is interesting given the close relationship of *E. coli* and *Salmonella* and, in concert with the previous observations that the two species maintain a different basal level of supercoiling suggests changes to supercoiling affect global regulatory networks and phenotypic outputs which result differently.

Other methods of investigating supercoiling which directly analyse the chromosome tend to be *in vitro* and strip DNA from the cellular context, *e.g.* using “magnetic tweezers” and fusing DNA to a glass slide (Lebel *et al.*, 2014). A recent study used fluorescence resonance energy transfer to measure supercoiling, by inserting sequences tagged with a fluorophore and a quencher into a plasmid, such that they were located far apart (and thus fluorescent) when the plasmid was relaxed, and adjacent (and thus not fluorescent) when the plasmid was supercoiled (Gu *et al.*, 2016). This approach could perhaps be applied in future experiments and used to monitor changes in supercoiling in real time, without having to extract the plasmid from the cell.

When mid-logarithmic phase *Salmonella* were incubated with antibiotics, how they responded appeared to be linked to whether or not the cells carried a *gyrA* mutation.

Plasmids from cells lacking *gyrA* mutations generally appeared to become less supercoiled after antibiotic treatment, which appears consistent with the observed relaxation of topoisomers observed when *S. enterica* was incubated with varying concentrations of sodium chloride (Cameron *et al.*, 2011). Conversely, the supercoiling state did not appear to change for plasmids isolated from cells with either a GyrA Ser83Phe or a GyrA Asp87Gly substitution after incubation with an antibiotic or other chemical. It therefore appears that *gyrA* mutation confers a generic protective effect against antibiotics, in terms of preserving the supercoiling state.

The use of fluorescent reporter constructs showed that there was a general elevation of stress responses in the GyrA Asp87Gly background in *E. coli* under all conditions. There was a much less obvious pattern in *Salmonella* where more cells were found to be expressing the *gabD* and *glnA* reporters in the GyrA Asp87Gly background although the level of expression from these cells appeared to be capped compared with those wild type cells that were expressing these stress responses. These data are consistent with our hypothesis that the phenotypic tolerance to non-quinolone antibiotics seen in the GyrA Asp87Gly mutant relates to altered expression of stress responses. The addition of various stresses did not change the picture and there was no consistent difference in response of these stress response pathways in different conditions in the *gyrA* mutants compared with the parent in both species. Therefore the mutants do not appear to respond to stress better than their parents, but to be in a generic state of enhanced protection.

The wider impact on the transcriptome has been suggested in *Salmonella* (Webber *et al.*, 2013). This could be followed up under multiple conditions in both species to get a more comprehensive picture of expression changes by RNA sequencing.

5.2 Future Work

It has previously been shown that supercoiling varies between regions of the chromosome (Higgins *et al.*, 1996; Postow *et al.*, 2004). It follows that, if a gene is moved from its native region to a different part of the chromosome, the supercoiling state of this gene will be dictated by the supercoiling state of the surrounding region into which it is inserted, and that this will alter the its expression. Therefore, supercoiling has been suggested to be a “master regulator” of gene expression. This has been shown in *E. coli* through the insertion of a GFP locus into differentially supercoiled chromosomal macrodomains and measuring fluorescence as a proxy for gene expression. It has also been shown that gene expression can be altered by disturbing the supercoiling profile with novobiocin, a gyrase inhibitor (Bryant *et al.*, 2014). One might therefore expect that inserting a *gfp* reporter into different regions of the *S.* Typhimurium genome should result in different expression levels depending on where the reporter is inserted. It would also be expected that the expression of the *gfp* reporter in *S.* Typhimurium should differ from its expression in *E. coli,* because of the inherent differences in the supercoiling state of the genomes of the two species, and that the expression of the *gfp* reporter should be altered by differences in the supercoiling state that are a consequence of GyrA substitutions. One way to address these hypotheses would be to create *aph* fusions with the existing pMW82 reporter constructs, and then transduce these into different sites on the chromosome using

the Datsenko-Wanner λ-Red phage recombination method (Datsenko and Wanner, 2000). It is primarily used to create gene knockouts, but could be used for the insertion of sequences. This would work by flanking the *aph* locus and promoter:*gfp* fusion with FLP recognition target sites and sequences homologous to sections of the chromosome, and introducing them into various positions in the chromosome using the recombinase λ-Red. Kanamycin ould then be used to select for successful recombinants. The fluorescence of the resulting cells could then be measured under a variety of conditions. It might be possible to achieve this aim in another way, such as by using a more targeted method, such as the CRISPR/Cas9 system (Choi and Lee, 2016; Doudna and Charpentier, 2014) or no-SCAR system (Reisch and Prather, 2015). These methods eliminate the need for the introduction of an antibiotic resistance marker, cutting down on the number of steps needed to edit the genome. However, the λ-Red method has been used in this laboratory previously (Eaves *et* al., 2004) and thus the materials and expertise needed to use this method are more readily available and affordable than CRISPR/Cas9.

Previous work by Webber *et al* (2013) used microarrays to examine changes in the transcriptome of *Salmonella* as a result of *gyrA* mutation. This experiment could be repeated in *E. coli* in a similar manner. Other sets of experiments that would allow a more thorough comparison of the effects of *gyrA* mutation in *E. coli* compared to *Salmonella* would be to repeat the flow cytometry assays in *E. coli*, as well as the topoisomer separation assays performed after exposing mid-logarithmic phase cells to a stress. These experiments would use similar methods to those described in this thesis.

The experiments presented herein investigated the impacts of *gyrA* mutation in *Escherichia coli* and *Salmonella* Typhimurium, which are Gram negative bacteria. It might be interesting to investigate the effects of equivalent substitutions in *gyrA* upon a Gram positive species, such as *Staphylococcus aureus* or *Streptococcus pneumoniae.* One possible approach would be to create isogenic mutants by taking a transformable laboratory strain of *S. aureus* or *S. pneumoniae* and introducing separate substitutions equivalent to GyrA Ser83Phe and GyrA Asp87Gly using an appropriate homologous recombination method. These could then be used to introduce the plasmid pBR322 into cells, and then the experiments presented herein could be repeated in those species.

The experiments presented do not show the impacts of *gyrA* mutation in an immunological context. Examples of questions one might pose include “Does *gyrA* mutation alter virulence?”, “How is invasion affected?”, and “Does *gyrA* mutation improve survival after uptake by macrophages?”. Traditionally, mouse models might be used to do this (Masopust *et al.*, 2017), but this approach is costly and an ethical quagmire (Ramarao *et al.*, 2012). An experiment that could be performed to assess impacts on virulence would be to feed *Gallerica mellonnella* larvae, which have a similar innate immune response to vertebrates (Tsai *et al.*, 2016), nutrient agar enriched with either wild type *Salmonella* or *Salmonella* with a *gyrA* mutation, and monitor the survival of the larvae over a 96 hour period, as used by Wang-Kan *et al.*, (2017). Impacts on invasion and survival in macrophages could be assessed using a tissue culture model. The model used by Wang-Kan *et al* (2017) to investigate the

effects of the loss of efflux pump function in *Salmonella* on invasion and macrophage survival uses Caco2 intestinal epithelial cells and J774 murine macrophages, so these cells would be appropriate for a similar study about *gyrA* mutation.

5.3 Conclusions

In conclusion, *gyrA* mutation alters supercoiling in both *Salmonella* and *E. coli*, decreasing susceptibility to quinolones and triclosan. This impact was much greater for the GyrA Asp87Gly substitution in *E. coli,* which outcompetes its parent in low triclosan concentrations. This was not seen in *Salmonella*. The data also show that the GyrA Asp87Gly substitution confers a generic protective effect against a range of chemicals and antibiotics, as the supercoiling does not appear to change in this strain. There is also a general upregulation of stress responses, which in response to non-specific stresses in *Salmonella,* but occurs under control conditions for the GyrA Asp87Gly strain in *E. coli*. The upregulation of stress responses is likely a result of changes in supercoiling causing widespread alterations in gene expression by changing the accessibility of promoters, and these elevated stress responses are responsible for the generic protective effect *gyrA* mutation confers against quinolone and non-quinolone antibiotics.

Taken together these data show that mutations in *gyrA* can influence susceptibility to non-quinolone antibiotics but that this impact is species and condition dependent – the potential impacts on fitness of such mutations are hard to predict in a non-laboratory setting. More work is needed to assess the potential of non-quinolone

antimicrobials, including those that are now common in the environment, to act as drivers of clinically important quinolone resistance.

References

Aarestrup, F.M., Wiuff, C., Molbak, K., Threlfall, E.J., 2003. Is It Time To Change Fluoroquinolone Breakpoints for *Salmonella* spp.? *Antimicrob. Agents Chemother.* **47**, 827–829. doi:10.1128/AAC.47.2.827-829.2003

Achtman, M., 2016. How old are bacterial pathogens? *Proc. R. Soc. B Biol. Sci.* **283**, 20160990. doi:10.1098/rspb.2016.0990

Aldred, K.J., Kerns, R.J., Osheroff, N., 2014. Mechanism of Quinolone Action and Resistance. *Biochemistry (Mosc.)* **53**, 1565–1574. doi:10.1021/bi5000564

Ali, S.S., Soo, J., Rao, C., Leung, A.S., Ngai, D.H.-M., Ensminger, A.W., Navarre, W.W., 2014. Silencing by H-NS Potentiated the Evolution of *Salmonella*. *PLoS Pathog.* **10**, e1004500. doi:10.1371/journal.ppat.1004500

Ali, S.S., Whitney, J.C., Stevenson, J., Robinson, H., Howell, P.L., Navarre, W.W., 2013. Structural Insights into the Regulation of Foreign Genes in *Salmonella* by the Hha/H-NS Complex. *J. Biol. Chem.* **288**, 13356–13369. doi:10.1074/jbc.M113.455378

Andersson, D.I., 2006. The biological cost of mutational antibiotic resistance: any practical conclusions? *Curr. Opin. Microbiol.* **9**, 461–465. doi:10.1016/j.mib.2006.07.002

Andersson, D.I., Hughes, D., 2014. Microbiological effects of sublethal levels of antibiotics. *Nat. Rev. Microbio*l. **12**, 465–78. doi:10.1038/nrmicro3270

Andersson, D.I., Hughes, D., 2012. Evolution of antibiotic resistance at non-lethal drug concentrations. *Drug Resist. Updat.* **15**, 162–172. doi:10.1016/j.drup.2012.03.005

Andrews, J.M., 2001. Determination of minimum inhibitory concentrations. *J. Antimicrob. Chemother.* **48**, 5–16. doi:10.1093/jac/48.suppl_1.5

Andrews, J.M., BSAC Working Party on Susceptibility Testing, 2006. BSAC standardized disc susceptibility testing method (version 5). *J. Antimicrob. Chemother.* **58**, 511–529. doi:10.1093/jac/dkl277

Andriole, V.T., 2005. The Quinolones: Past, Present, and Future. *Clin. Infect. Dis*. **41**, S113–S119. doi:10.1086/428051

Animal and Plant Health Agency, 2015. Chapter 6: Reports of *Salmonella* in Chickens, in: *Salmonella in Livestock Production in Great Britain, 2014.* Animal and Plant Health Agency, London.

Ashton, P.M., Nair, S., Peters, T.M., Bale, J.A., Powell, D.G., Painset, A., Tewolde, R., Schaefer, U., Jenkins, C., Dallman, T.J., de Pinna, E.M., Grant, K.A., *Salmonella* Whole Genome Sequencing Implementation Group, 2016. Identification of *Salmonella* for public health surveillance using whole genome sequencing. *PeerJ* **4**, e1752. doi:10.7717/peerj.1752

Atterbury, R.J., Bergen, M.A.P.V., Ortiz, F., Lovell, M.A., Harris, J.A., Boer, A.D., Wagenaar, J.A., Allen, V.M., Barrow, P.A., 2007. Bacteriophage Therapy To Reduce *Salmonella* Colonization of Broiler Chickens. *Appl. Environ. Microbiol.* **73**, 4543–4549. doi:10.1128/AEM.00049-07

Avasthi, T.S., Kumar, N., Baddam, R., Hussain, A., Nandanwar, N., Jadhav, S., Ahmed, N., 2011. Genome of Multidrug-Resistant Uropathogenic *Escherichia coli* Strain NA114 from India. *J. Bacteriol.* ***1*93**, 4272–4273. doi:10.1128/JB.05413-11

Baker, H.J., Lindsey, J.R., Wesibroth, S.H. (Eds.), 2013. *The Laboratory Rat: Biology and Diseases.* Elsevier.

Baker, K.S., Mather, A.E., McGregor, H., Coupland, P., Langridge, G.C., Day, M., Deheer-Graham, A., Parkhill, J., Russell, J.E., Thomson, N.R., 2014. The extant World War 1 dysentery bacillus NCTC1: a genomic analysis. *The Lancet* **384**, 1691–1697.

Baker, S., Duy, P.T., Nga, T.V.T., Dung, T.T.N., Phat, V.V., Chau, T.T., Turner, A.K., Farrar, J., Boni, M.F., 2013. Fitness benefits in fluoroquinolone-resistant *Salmonella* Typhi in the absence of antimicrobial pressure. *eLife* **2**, e01229. doi:10.7554/eLife.01229

Ball, P., 2003. Adverse drug reactions: implications for the development of fluoroquinolones. *J. Antimicrob. Chemother.* **51**, 21–27. doi:10.1093/jac/dkg209

Ball, P., 2000. Quinolone generations: natural history or natural selection? *J. Antimicrob. Chemother.* **46**, 17–24.

Baltz, R.H., 2008. Renaissance in antibacterial discovery from actinomycetes. *Curr. Opin. Pharmacol.* **8**, 557–563. doi:10.1016/j.coph.2008.04.008

Baltz, R.H., 2006. Marcel Faber Roundtable: Is our antibiotic pipeline unproductive because of starvation, constipation or lack of inspiration? *J. Ind. Microbiol. Biotechnol.* **33**, 507–513. doi:10.1007/s10295-005-0077-9

Bang, I.-S., Frye, J.G., McClelland, M., Velayudhan, J., Fang, F.C., 2005. Alternative sigma factor interactions in *Salmonella*: σE and σH promote antioxidant defences by enhancing σS levels. *Mol. Microbiol.* **56**, 811–823. doi:10.1111/j.1365-2958.2005.04580.x

Barka, E.A., Vatsa, P., Sanchez, L., Gaveau-Vaillant, N., Jacquard, C., Klenk, H.-P., Clément, C., Ouhdouch, Y., van Wezel, G.P., 2016. Taxonomy, Physiology, and Natural Products of Actinobacteria. *Microbiol. Mol. Biol. Rev.* **80**, 1–43. doi:10.1128/MMBR.00019-15

Belland, R.J., Morrison, S.G., Ison, C., Huang, W.M., 1994. *Neisseria gonorrhoeae* acquires mutations in analogous regions of *gyrA* and *parC* in fluoroquinolone-resistant isolates. *Mol. Microbiol.* **14**, 371–380.

Bentley, R., 2009. Different roads to discovery; Prontosil (hence sulfa drugs) and penicillin (hence β-lactams). *J. Ind. Microbiol. Biotechnol.* **36**, 775–786. doi:10.1007/s10295-009-0553-8

Bereswill, S., Fischer, A., Dunay, I.R., Kühl, A.A., Göbel, U.B., Liesenfeld, O., Heimesaat, M.M., 2013. Pro-inflammatory potential of *Escherichia coli* strains K12 and Nissle 1917 in a murine model of acute ileitis. *Eur. J. Microbiol. Immunol.* **3**, 126–134. doi:10.1556/EuJMI.3.2013.2.6

Bergen, P.J., Landersdorfer, C.B., Lee, H.J., Li, J., Nation, R.L., 2012. 'Old' antibiotics for emerging multidrug-resistant bacteria: *Curr. Opin. Infect. Dis.* **25**, 626–633. doi:10.1097/QCO.0b013e328358afe5

Bermingham, A., Derrick, J.P., 2002. The folic acid biosynthesis pathway in bacteria: evaluation of potential for antibacterial drug discovery. *BioEssays* **24**, 637–648. doi:10.1002/bies.10114

Bickel, M.H., 1988. The Development of Sulfonamides (1932-1938) as a Focal Point in the History of Chemotherapy. *Gesnerus* **45**, 67–86.

Birošová, L., Mikulášová, M., 2009. Development of triclosan and antibiotic resistance in *Salmonella enterica* serovar Typhimurium. *J. Med. Microbiol.* **58**, 436–441. doi:10.1099/jmm.0.003657-0

Björkman, J., Nagaev, I., Berg, O.G., Hughes, D., Andersson, D.I., 2000. Effects of Environment on Compensatory Mutations to Ameliorate Costs of Antibiotic Resistance. *Science* **287**, 1479–1482. doi:10.1126/science.287.5457.1479

Blair, J.M.A., Bavro, V.N., Ricci, V., Modi, N., Cacciotto, P., Kleinekathöfer, U., Ruggerone, P., Vargiu, A.V., Baylay, A.J., Smith, H.E., Brandon, Y., Galloway, D., Piddock, L.J.V., 2015a. AcrB drug-binding pocket substitution confers clinically relevant resistance and altered substrate specificity. *Proc. Natl. Acad. Sci.* **112**, 3511–3516. doi:10.1073/pnas.1419939112

Blair, J.M.A., Webber, M.A., Baylay, A.J., Ogbolu, D.O., Piddock, L.J.V., 2015b. Molecular mechanisms of antibiotic resistance. *Nat. Rev. Microbiol.* **13**, 42–51. doi:10.1038/nrmicro3380

Blattner, F.R., Plunkett, G., Bloch, C.A., Perna, N.T., Burland, V., Riley, M., Collado-Vides, J., Glasner, J.D., Rode, C.K., Mayhew, G.F., Gregor, J., Davis, N.W., Kirkpatrick, H.A., Goeden, M.A., Rose, D.J., Mau, B., Shao, Y., 1997. The Complete Genome Sequence of *Escherichia coli* K-12. *Science* **277**, 1453–1462. doi:10.1126/science.277.5331.1453

Blower, T.R., Williamson, B.H., Kerns, R.J., Berger, J.M., 2016. Crystal structure and stability of gyrase–fluoroquinolone cleaved complexes from *Mycobacterium tuberculosis*. *Proc. Natl. Acad. Sci.* **113**, 1706–1713. doi:10.1073/pnas.1525047113

BMJ Group, Royal Pharmaceutical Society of Great Britain, 2016a. *5.1.12 Quinolones : British National Formulary* [WWW Document]. Br. Natl. Formul. URL https://www.evidence.nhs.uk/formulary/bnf/current/5-infections/51-antibacterial-drugs/5112-quinolones (accessed 5.19.16).

BMJ Group, Royal Pharmaceutical Society of Great Britain, 2016b. *Ciprofloxacin : British National Formulary* [WWW Document]. Br. Natl. Formul. URL http://www.evidence.nhs.uk/formulary/bnf/current/5-infections/51-antibacterial-drugs/5112-quinolones/ciprofloxacin (accessed 8.8.16).

BMJ Group, Royal Pharmaceutical Society of Great Britain, 2016c. *Inflammatory bowel disease : British National Formulary* [WWW Document]. Br. Natl. Formul. URL http://www.evidence.nhs.uk/formulary/bnf/current/1-gastro-intestinal-system/15-chronic-bowel-disorders/inflammatory-bowel-disease#PHP482 (accessed 8.8.16).

BMJ Group, Royal Pharmaceutical Society of Great Britain, 2016d. *11.3.1 Antibacterials : British National Formulary* [WWW Document]. Br. Natl. Formul. URL http://www.evidence.nhs.uk/formulary/bnf/current/11-eye/113-anti-infective-eye-preparations/1131-antibacterials (accessed 8.8.16).

BMJ Group, Royal Pharmaceutical Society of Great Britain, 2016e. *Gastro-intestinal system infections, bacterial: British National Formulary* [WWW Document]. Med. Complete. URL https://www.medicinescomplete.com/mc/bnf/current/PHP78125-gastro-intestinal-system-infections-bacterial.htm (accessed 7.30.16).

Boël, G., Letso, R., Neely, H., Price, W.N., Wong, K.-H., Su, M., Luff, J.D., Valecha, M., Everett, J.K., Acton, T.B., Xiao, R., Montelione, G.T., Aalberts, D.P., Hunt, J.F., 2016. Codon influence on protein expression in *E. coli* correlates with mRNA levels. *Nature* **529**, 358–363. doi:10.1038/nature16509

Bolivar, F., Rodriguez, R.L., Greene, P.J., Betlach, M.C., Heyneker, H.L., Boyer, H.W., Crosa, J.H., Falkow, S., 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. *Gene* **2**, 95–113.

Bolon, M.K., 2011. The Newer Fluoroquinolones. *Med. Clin. North Am.* **95**, 793–817. doi:10.1016/j.mcna.2011.03.006

Booker, B.M., Deng, S., Higgins, N.P., 2010. DNA topology of highly transcribed operons in *Salmonella enterica* serovar Typhimurium. *Mol. Microbiol.* **78**, 1348–1364. doi:10.1111/j.1365-2958.2010.07394.x

Bougdour, A., Cunning, C., Baptiste, P.J., Elliott, T., Gottesman, S., 2008. Multiple pathways for regulation of σS (RpoS) stability in *Escherichia coli* via the action of multiple anti-adaptors. *Mol. Microbiol.* **68,** 298–313. doi:10.1111/j.1365-2958.2008.06146.x

Boyd, S.E., Moore, L.S.P., Gilchrist, M., Costelloe, C., Castro-Sánchez, E., Franklin, B.D., Holmes, A.H., 2017. Obtaining antibiotics online from within the UK: a cross-sectional study. *J. Antimicrob. Chemother.* **72**, 1521-1528 doi:10.1093/jac/dkx003

Brambilla, E., Sclavi, B., 2015. Gene Regulation by H-NS as a Function of Growth Conditions Depends on Chromosomal Position in *Escherichia coli. G3 Genes Genomes Genet.* **5**, 605–614. doi:10.1534/g3.114.016139

Brenner, F.W., Villar, R.G., Angulo, F.J., Tauxe, R., Swaminathan, B., 2000. *Salmonella* Nomenclature. *J. Clin. Microbiol*. **38**, 2465–2467.

Briales, A., Rodríguez-Martínez, J.M., Velasco, C., de Alba, P.D., Rodríguez-Baño, J., Martínez-Martínez, L., Pascual, A., 2012. Prevalence of plasmid-mediated quinolone resistance determinants qnr and aac(6′)-Ib-cr in *Escherichia coli* and *Klebsiella pneumoniae* producing extended-spectrum β-lactamases in Spain. *Int. J. Antimicrob. Agents* **39**, 431–434. doi:10.1016/j.ijantimicag.2011.12.009

Brittain, H.G., 2005. Solid-state fluorescence of the trihydrate phases of ampicillin and amoxicillin. *AAPS PharmSciTech* **6**, E444–E448.

Brown, G.R., Hem, V., Katz, K.S., Ovetsky, M., Wallin, C., Ermolaeva, O., Tolstoy, I., Tatusova, T., Pruitt, K.D., Maglott, D.R., Murphy, T.D., 2015. Gene: a gene-centered information resource at NCBI. *Nucleic Acids Res*. **43**, D36–D42. doi:10.1093/nar/gku1055

Brown, J.C., Thomson, C.J., Amyes, S.G.B., 1996. Mutations of the *gyrA* gene of clinical isolates of *Salmonella typhimurium* and three other *Salmonella* species leading to decreased susceptibilities to 4-quinolone drugs. *J. Antimicrob. Chemother*. **37**, 351–356. doi:10.1093/jac/37.2.351

Browning, D.F., Busby, S.J.W., 2016. Local and global regulation of transcription initiation in bacteria. *Nat. Rev. Microbiol*. **14**, 638–650. doi:10.1038/nrmicro.2016.103

Bruttin, A., Brüssow, H., 2005. Human Volunteers Receiving *Escherichia coli* Phage T4 Orally: a Safety Test of Phage Therapy. *Antimicrob. Agents Chemother*. **49**, 2874–2878. doi:10.1128/AAC.49.7.2874-2878.2005

Bryant, J.A., Sellars, L.E., Busby, S.J.W., Lee, D.J., 2014. Chromosome position effects on gene expression in *Escherichia coli* K-12. *Nucleic Acids Res*. **42**, 11383–11392. doi:10.1093/nar/gku828

Buckley, A.M., Webber, M.A., Cooles, S., Randall, L.P., La Ragione, R.M., Woodward, M.J., Piddock, L.J.V., 2006. The AcrAB–TolC efflux system of

Salmonella enterica serovar Typhimurium plays a role in pathogenesis. *Cell. Microbiol*. **8**, 847–856. doi:10.1111/j.1462-5822.2005.00671.x
Bumann, D., 2008. Has nature already identified all useful antibacterial targets? *Curr. Opin. Microbiol*. **11**, 387–392. doi:10.1016/j.mib.2008.08.002
Bumann, D., Valdivia, R.H., 2007. Identification of host-induced pathogen genes by differential fluorescence induction reporter systems. *Nat. Protoc*. **2**, 770–777. doi:10.1038/nprot.2007.78
Burman, L.G., 1977. Apparent absence of transferable resistance to nalidixic acid in pathogenic Gram-negative bacteria. *J. Antimicrob. Chemother.* **3**, 509–516. doi:10.1093/jac/3.5.509
Bush, N.G., Evans-Roberts, K., Maxwell, A., 2015. DNA Topoisomerases. *EcoSal Plus* **3**. doi:10.1128/ecosalplus.4.4.9
Butler, M.S., Blaskovich, M.A., Owen, J.G., Cooper, M.A., 2016. Old dogs and new tricks in antimicrobial discovery. *Curr. Opin. Microbiol*. **33**, 25–34. doi:10.1016/j.mib.2016.05.011
Cai, Y., Lim, T.-P., Teo, J., Sasikala, S., Lee, W., Hong, Y., Chan, E.C.Y., Tan, T.Y., Tan, T.-T., Koh, T.H., Hsu, L.Y., Kwa, A.L., 2016. In vitro Activity of Polymyxin B in Combination with Various Antibiotics against Extensively-Drug Resistant *Enterobacter cloacae* with Decreased Susceptibility to Polymyxin B. *Antimicrob. Agents Chemother.* **60**, 5238–5246. doi:10.1128/AAC.00270-16
Cameron, A.D.S., Stoebel, D.M., Dorman, C.J., 2011. DNA supercoiling is differentially regulated by environmental factors and FIS in *Escherichia coli* and *Salmonella enterica. Mol. Microbiol.* **80**, 85–101. doi:10.1111/j.1365-2958.2011.07560.x
Carter, P.B., Collins, F.M., 1974. Growth of Typhoid and Paratyphoid Bacilli in Intravenously Infected Mice. *Infect. Immun.* **10**, 816–822.
Casewell, M., Friis, C., Marco, E., McMullin, P., Phillips, I., 2003. The European ban on growth-promoting antibiotics and emerging consequences for human and animal health. *J. Antimicrob. Chemother.* **52,** 159–161. doi:10.1093/jac/dkg313
Champoux, J.J., 2001. DNA Topoisomerases: Structure, Function, and Mechanism. *Annu. Rev. Biochem*. **70**, 369–413. doi:10.1146/annurev.biochem.70.1.369
Chan, L.M., Van Winkle, Q., 1969. Interaction of acriflavine with DNA and RNA. *J. Mol. Biol.* **40**, 491–495. doi:10.1016/0022-2836(69)90167-3
Chen, H., Brinkac, L.M., Mishra, P., Li, N., Lymperopoulou, D.S., Dickerson, T.L., Gordon-Bradley, N., Williams, H.N., Badger, J.H., 2015. Draft genome sequences for the obligate bacterial predators *Bacteriovorax* spp. of four phylogenetic clusters. *Stand. Genomic Sci.* **10**, 11. doi:10.1186/1944-3277-10-11
Cho, B.-K., Knight, E.M., Barrett, C.L., Palsson, B.Ø., 2008. Genome-wide analysis of Fis binding in *Escherichia coli* indicates a causative role for A-/AT-tracts. *Genome Res.* **18**, 900–910. doi:10.1101/gr.070276.107
Choi, H., Lee, H.-J., Lee, Y., 1998. A Mutation in QRDR in the ParC Subunit of Topoisomerase IV was Responsible for Fluoroquinolone Resistance in Clinical Isolates of *Streptococcus pneumoniae*. *Yonsei Med. J*. **39**, 541–545.
Choi, K.R., Lee, S.Y., 2016. CRISPR technologies for bacterial systems: Current achievements and future directions. *Biotechnol. Adv.* **34**, 1180–1209. doi:10.1016/j.biotechadv.2016.08.002

Ciesielczuk, H., Jenkins, C., Chattaway, M., Doumith, M., Hope, R., Woodford, N., Wareham, D.W., 2016. Trends in ExPEC serogroups in the UK and their significance. *Eur. J. Clin. Microbiol. Infect. Dis.* **35**, 1661–1666. doi:10.1007/s10096-016-2707-8

Claret, L., Rouvière-Yaniv, J., 1997. Variation in HU Composition During Growth of *Escherichia coli:* the Heterodimer is Required for Long Term Survival. *J. Mol. Biol.* **273**, 93–104.

Clatworthy, A.E., Pierson, E., Hung, D.T., 2007. Targeting virulence: a new paradigm for antimicrobial therapy. *Nat. Chem. Biol.* **3**, 541–548. doi:10.1038/nchembio.2007.24

Clifton, C.E., 1943. Large-Scale Production of Penicillin. *Science* **98**, 69–70. doi:10.1126/science.98.2533.69

Cobos-Trigueros, N., Solé, M., Castro, P., Torres, J.L., Rinaudo, M., Lazzari, E.D., Morata, L., Hernández, C., Fernández, S., Soriano, A., Nicolás, J.M., Mensa, J., Vila, J., Martínez, J.A., 2016. Evaluation of a Mixing versus a Cycling Strategy of Antibiotic Use in Critically-Ill Medical Patients: Impact on Acquisition of Resistant Microorganisms and Clinical Outcomes. *PLoS ONE* **11**, e0150274. doi:10.1371/journal.pone.0150274

Collin, F., Karkare, S., Maxwell, A., 2011. Exploiting bacterial DNA gyrase as a drug target: current state and perspectives. *Appl. Microbiol. Biotechnol.* **92**, 479–497. doi:10.1007/s00253-011-3557-z

Committee for Medicinal Products for Human Use, 2009. *Norfloxacin containing medicinal products (International non-proprietary name: norfloxacin) (Opinion following an article 31 referral No. EMEA/660192/2008).* European Medicines Agency, London.

Conlin, C.A., Miller, C.G., 2000. *opdA*, a *Salmonella enterica* serovar Typhimurium gene encoding a protease, is part of an operon regulated by heat shock. *J. Bacteriol.* **182**, 518–521.

Conlin, C.A., Trun, N.J., Silhavy, T.J., Miller, C.G., 1992. *Escherichia coli prlC* encodes an endopeptidase and is homologous to the *Salmonella typhimurium opdA* gene. *J. Bacteriol*. **174**, 5881–5887.

Connell, S.R., Tracz, D.M., Nierhaus, K.H., Taylor, D.E., 2003. Ribosomal Protection Proteins and Their Mechanism of Tetracycline Resistance. *Antimicrob. Agents Chemother*. **47**, 3675–3681. doi:10.1128/AAC.47.12.3675-3681.2003

Corbett, K.D., Schoeffler, A.J., Thomsen, N.D., Berger, J.M., 2005. The Structural Basis for Substrate Specificity in DNA Topoisomerase IV. *J. Mol. Biol.* **351**, 545–561. doi:10.1016/j.jmb.2005.06.029

Costenaro, L., Grossmann, J.G., Ebel, C., Maxwell, A., 2007. Modular Structure of the Full-Length DNA Gyrase B Subunit Revealed by Small-Angle X-Ray Scattering. *Structure* **15**, 329–339. doi:10.1016/j.str.2007.01.013

Cox, M.M., 2007. Regulation of Bacterial RecA Protein Function. *Crit. Rev. Biochem. Mol. Biol.* **42**, 41–63. doi:10.1080/10409230701260258

Dame, R.T., Kalmykowa, O.J., Grainger, D.C., 2011. Chromosomal Macrodomains and Associated Proteins: Implications for DNA Organization and Replication in Gram Negative Bacteria. *PLoS Genet.* **7**, e1002123. doi:10.1371/journal.pgen.1002123

Dame, R.T., Wyman, C., Goosen, N., 2001. Structural basis for preferential binding of H-NS to curved DNA. *Biochimie* **83**, 231–234.

Dame, R.T., Wyman, C., Wurm, R., Wagner, R., Goosen, N., 2002. Structural Basis for H-NS-mediated Trapping of RNA Polymerase in the Open Initiation Complex at the *rrnB* P1. *J. Biol. Chem.* **277**, 2146–2150. doi:10.1074/jbc.C100603200

Dashiff, A., Junka, R. A., Libera, M., Kadouri, D. E., 2011. Predation of human pathogens by the predatory bacteria *Micavibrio aeruginosavorus* and *Bdellovibrio bacteriovorus. J. Appl. Microbiol.* **110**, 431–444. doi:10.1111/j.1365-2672.2010.04900.x

Datsenko, K.A., Wanner, B.L., 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci.* **97**, 6640–6645. doi:10.1073/pnas.120163297

David, H.L., Rastogi, N., 1985. Antibacterial action of colistin (polymyxin E) against *Mycobacterium aurum. Antimicrob. Agents Chemother.* **27**, 701–707.

Davies, S.C., 2013. *Annual Report of the Chief Medical Officer, Volume Two, 2011, Infections and the rise of antimicrobial resistance.* Department of Health, London.

Davies, S.C., Gibbens, N., 2014. *UK 5 Year Antimicrobial Resistance (AMR) Strategy 2013-2018: Annual progress report and implementation plan, 2014.* Department of Health, London.

D'Costa, V.M., King, C.E., Kalan, L., Morar, M., Sung, W.W.L., Schwarz, C., Froese, D., Zazula, G., Calmels, F., Debruyne, R., Golding, G.B., Poinar, H.N., Wright, G.D., 2011. Antibiotic resistance is ancient. *Nature* **477**, 457–461. doi:10.1038/nature10388

Del Bel Belluz, L., Guidi, R., Pateras, I.S., Levi, L., Mihaljevic, B., Rouf, S.F., Wrande, M., Candela, M., Turroni, S., Nastasi, C., Consolandi, C., Peano, C., Tebaldi, T., Viero, G., Gorgoulis, V.G., Krejsgaard, T., Rhen, M., Frisan, T., 2016. The Typhoid Toxin Promotes Host Survival and the Establishment of a Persistent Asymptomatic Infection. *PLoS Pathog.* **12**, e1005528. doi:10.1371/journal.ppat.1005528

Deng, S., Stein, R.A., Higgins, N.P., 2004. Transcription-induced barriers to supercoil diffusion in the *Salmonella typhimurium* chromosome. *Proc. Natl. Acad. Sci. U. S. A*. **101**, 3398–3403. doi:10.1073/pnas.0307550101

Deris, J.B., Kim, M., Zhang, Z., Okano, H., Hermsen, R., Groisman, A., Hwa, T., 2013. The Innate Growth Bistability and Fitness Landscapes of Antibiotic-Resistant Bacteria. *Science* **342**, 1237435. doi:10.1126/science.1237435

Dillon, S.C., Dorman, C.J., 2010. Bacterial nucleoid-associated proteins, nucleoid structure and gene expression. *Nat. Rev. Microbiol.* **8**, 185–195. doi:10.1038/nrmicro2261

Domagk, G., 1935. Ein Beitrag zur Chemotherapie der bakteriellen Infektionen. *Dtsch. Med. Wochenschr.* **61**, 250–253. doi:10.1055/s-0028-1129486

Domingo-Calap, P., Georgel, P., Bahram, S., 2016. Back to the future: bacteriophages as promising therapeutic tools: Bacteriophage-based therapy. *HLA* **87**, 133–140. doi:10.1111/tan.12742

Doolittle, R.F., Feng, D.-F., Tsang, S., Cho, G., Little, E., 1996. Determining divergence times of the major kingdoms of living organisms with a protein clock. *Science* **271**, 470–477.

Dorman, C.J., 2013. Genome architecture and global gene regulation in bacteria: making progress towards a unified model? *Nat. Rev. Microbiol*. **11**, 349–355. doi:10.1038/nrmicro3007

Dorman, C.J., Corcoran, C.P., 2009. Bacterial DNA topology and infectious disease. *Nucleic Acids Res.* **37**, 672–678. doi:10.1093/nar/gkn996

Doudna, J.A., Charpentier, E., 2014. The new frontier of genome engineering with CRISPR-Cas9. *Science* **346**, 1258096. doi:10.1126/science.1258096

Drlica, K., 2003. The mutant selection window and antimicrobial resistance. *J. Antimicrob. Chemother.* **52**, 11–17. doi:10.1093/jac/dkg269

Drlica, K., 1999. Mechanism of fluoroquinolone action. *Curr. Opin. Microbiol.* **2**, 504–508. doi:10.1016/S1369-5274(99)00008-9

Drlica, K., Malik, M., Kerns, R.J., Zhao, X., 2008. Quinolone-Mediated Bacterial Death. *Antimicrob. Agents Chemother.* **52**, 385–392. doi:10.1128/AAC.01617-06

Drlica, K., Rouvière-Yaniv, J., 1987. Histone-like proteins of bacteria. *Microbiol. Rev.* **51**, 301.

Du, X., Wojtowicz, D., Bowers, A.A., Levens, D., Benham, C.J., Przytycka, T.M., 2013. The genome-wide distribution of non-B DNA motifs is shaped by operon structure and suggests the transcriptional importance of non-B DNA structures in *Escherichia coli. Nucleic Acids Res*. **41**, 5965–5977. doi:10.1093/nar/gkt308

Duffield, M., Cooper, I., McAlister, E., Bayliss, M., Ford, D., Oyston, P., 2010. Predicting conserved essential genes in bacteria: in silico identification of putative drug targets. *Mol. Biosyst.* **6**, 2482. doi:10.1039/c0mb00001a

Dwidar, M., Monnappa, A.K., Mitchell, R.J., 2012. The dual probiotic and antibiotic nature of *Bdellovibrio bacteriovorus. BMB Rep*. **45**, 71–78. doi:10.5483/BMBRep.2012.45.2.71

Eaves, D.J., Ricci, V., Piddock, L.J.V., 2004. Expression of *acrB*, *acrF*, *acrD*, *marA*, and *soxS* in *Salmonella enterica* Serovar Typhimurium: Role in Multiple Antibiotic Resistance. *Antimicrob. Agents Chemother.* **48**, 1145–1150. doi:10.1128/AAC.48.4.1145-1150.2004

Edwards, M., 2016. Antibiotic resistance and longevity improvements, in: *Longevity Bulletin: Antimicrobial Resistance.* Institute and Faculty of Actuaries, pp. 14–16.

Effa, E.E., Lassi, Z.S., Critchley, J.A., Garner, P., Sinclair, D., Olliaro, P.L., Bhutta, Z.A., 2011. *Fluoroquinolones for treating typhoid and paratyphoid fever (enteric fever)*. Cochrane Database Syst. Rev.

European Centre for Disease Prevention and Control, 2014. *Annual epidemiological report 2014: Food- and waterborne diseases and zoonoses*. ECDC, Stockholm.

European Commission, 2011*. Action plan against the rising threats from Antimicrobial Resistance (Communication from the Commission to the European Parliament and the Council No. COM (2011) 748)*. European Commission, Brussels.

European Committee on Antimicrobial Susceptibility Testing, 2016. *Breakpoint tables for interpretation of MICs and zone diameters, Version 6.0.* [WWW Document]. URL http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_6.0_Breakpoint_table.pdf (accessed 6.22.16).

European Committee on Antimicrobial Susceptibility Testing, 2013. *EUCAST definitions of clinical breakpoints and epidemiological cut-off values* [WWW Document]. EUCAST Clin. Break. URL http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/EUCAST_SOPs/EUCAST_definitions_of_clinical_breakpoints_and_ECOFFs.pdf (accessed 6.15.16).

European Food Safety Authority, European Centre for Disease Prevention and Control, 2016. The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2014: EUSR on AMR in zoonotic and indicator bacteria from humans, animals and food 2014. *EFSA J*. **14**, 4380. doi:10.2903/j.efsa.2016.4380

European Food Safety Authority, European Centre for Disease Prevention and Control, 2015a. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2013: EU summary report on zoonoses, zoonotic agents and food-borne outbreaks 2013. *EFSA J*. **13**, 3991. doi:10.2903/j.efsa.2015.3991

European Food Safety Authority, European Centre for Disease Prevention and Control, 2015b. EU Summary Report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2013: EUSR on AMR in zoonotic and indicator bacteria from humans, animals and food 2013. *EFSA J.* **13**, 4036. doi:10.2903/j.efsa.2015.4036

European Food Safety Authority, European Centre for Disease Prevention and Control, 2015c. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2014: EU summary report on zoonoses, zoonotic agents and food-borne outbreaks 2014. *EFSA J.* **13**, 4329. doi:10.2903/j.efsa.2015.4329

European Food Safety Authority, European Centre for Disease Prevention and Control, 2014a. The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2012: EU summary report on zoonoses, zoonotic agents and food-borne outbreaks 2012. *EFSA J.* **12**, 3547. doi:10.2903/j.efsa.2014.3547

European Food Safety Authority, European Centre for Disease Prevention and Control, 2014b. The European Union Summary Report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2012: EU summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food 2012. *EFSA J*. **12**, 3590. doi:10.2903/j.efsa.2014.3590

European Medicines Agency, 2009. *Withdrawal Assessment Report for Factive - International non-proprietary name: gemifloxacin (No. EMEA/H/C/995)*. European Medicines Agency, London.

Everett, M.J., Jin, Y.F., Ricci, V., Piddock, L.J.V., 1996. Contributions of individual mechanisms to fluoroquinolone resistance in 36 *Escherichia coli* strains isolated from humans and animals. *Antimicrob. Agents Chemother*. **40**, 2380–2386.

Falagas, M.E., Kasiakou, S.K., 2005. Colistin: The Revival of Polymixins for the Management of Multidrug-Resistant Gram-Negative Bacterial Infections. *Clin. Infect. Dis*. **40**, 1333–1341. doi:10.1086/429323

Fang, F.C., Rimsky, S., 2008. New Insights into Transcriptional Regulation by H-NS. *Curr. Opin. Microbiol.* **11**, 113. doi:10.1016/j.mib.2008.02.011
Feklístov, A., Sharon, B.D., Darst, S.A., Gross, C.A., 2014. Bacterial Sigma Factors: A Historical, Structural, and Genomic Perspective. *Annu. Rev. Microbiol*. **68**, 357–376. doi:10.1146/annurev-micro-092412-155737
Ferrándiz, M.-J., Arnanz, C., Martín-Galiano, A.J., Rodríguez-Martín, C., Campa, A.G. de la, 2014. Role of Global and Local Topology in the Regulation of Gene Expression in *Streptococcus pneumoniae. PLoS ONE* **9**, e101574. doi:10.1371/journal.pone.0101574
Feuerborn, A., Cook, P.R., 2015. Why the activity of a gene depends on its neighbors. *Trends Genet.* **31**, 483–490. doi:10.1016/j.tig.2015.07.001
Fifer, H., Natarajan, U., Jones, L., Alexander, S., Hughes, G., Golparian, D., Unemo, M., 2016. Failure of Dual Antimicrobial Therapy in Treatment of Gonorrhea. *N. Engl. J. Med.* **374**, 2504–2506. doi:10.1056/NEJMc1514294
Figueira, R., Holden, D.W., 2012. Functions of the *Salmonella* pathogenicity island 2 (SPI-2) type III secretion system effectors. *Microbiology* **158**, 1147–1161. doi:10.1099/mic.0.058115-0
Fleming, A., 1999. Penicillin, in: *Nobel Lectures, Physiology Or Medicine, 1942-1962.* World Scientific Publishing Co. Pte. Ltd., Singapore, pp. 83–93.
Fleming, A., 1929. On the antibacterial action of cultures of a penicillium, with special reference to their use in the isolation of *B. influenzae. Br. J. Exp. Pathol.* **10**, 226–236.
Floss, H.G., Yu, T.-W., 2005. Rifamycin: Mode of Action, Resistance, and Biosynthesis. *Chem. Rev.* **105**, 621–632. doi:10.1021/cr030112j
Forterre, P., Gribaldo, S., Gadelle, D., Serre, M.-C., 2007. Origin and evolution of DNA topoisomerases. *Biochimie* **89**, 427–446. doi:10.1016/j.biochi.2006.12.009
Friedman, S.M., Lu, T., Drlica, K., 2001. Mutation in the DNA Gyrase A Gene of *Escherichia coli* That Expands the Quinolone Resistance-Determining Region. *Antimicrob. Agents Chemother*. **45**, 2378–2380. doi:10.1128/AAC.45.8.2378-2380.2001
Fuentes-Hernandez, A., Plucain, J., Gori, F., Pena-Miller, R., Reding, C., Jansen, G., Schulenburg, H., Gudelj, I., Beardmore, R., 2015. Using a Sequential Regimen to Eliminate Bacteria at Sublethal Antibiotic Dosages. *PLoS Biol*. **13**, e1002104. doi:10.1371/journal.pbio.1002104
Fukushima, M., Kakinuma, K., Kawaguchi, R., 2002. Phylogenetic Analysis of *Salmonella*, *Shigella*, and *Escherichia coli* Strains on the Basis of the *gyrB* Gene Sequence. *J. Clin. Micro.* **40**, 2779-2785. doi:10.1128/jcm.40.8.2779-2785.2002
Gahring, L.C., Heffron, F., Finlay, B.B., Falkow, S., 1990. Invasion and replication of *Salmonella typhimurium* in animal cells. *Infect. Immun.* **58**, 443–448.
Galán, J.E., 2001. *Salmonella* Interactions with Host Cells: Type III Secretion at Work. *Annu. Rev. Cell Dev. Biol.* **17**, 53–86. doi:10.1146/annurev.cellbio.17.1.53
Gal-Mor, O., Boyle, E.C., Grassl, G.A., 2014. Same species, different diseases: how and why typhoidal and non-typhoidal *Salmonella enterica* serovars differ. *Front. Microbiol.* **5**. doi:10.3389/fmicb.2014.00391

Garcia-del Portillo, F., Foster, J.W., Finlay, B.B., 1993. Role of acid tolerance response genes in *Salmonella typhimurium* virulence. *Infect. Immun*. **61**, 4489–4492.

Garrido, M.C., Herrero, M., Kolter, R., Moreno, F., 1988. The export of the DNA replication inhibitor Microcin B17 provides immunity for the host cell. *EMBO J*. **7**, 1853–1862.

Gibberd, G.F., 1937. Prontosil etc. in puerperal *Streptococcus* infections. *Br. Med. J*. **2**, 695.

Gibbons, C.L., Mangen, M.-J.J., Plass, D., Havelaar, A.H., Brooke, R.J., Kramarz, P., Peterson, K.L., Stuurman, A.L., Cassini, A., Fèvre, E.M., others, 2014. Measuring underreporting and under-ascertainment in infectious disease datasets: a comparison of methods. *BMC Public Health* **14**, 1.

Giraud, E., Baucheron, S., Cloeckaert, A., 2006. Resistance to fluoroquinolones in *Salmonella*: emerging mechanisms and resistance prevention strategies*. Microbes Infect*. **8**, 1937–1944. doi:10.1016/j.micinf.2005.12.025

Goodrich, J.A., Schwartz, M.L., McClure, W.R., 1990. Searching for and predicting the activity of sites for DNA binding proteins: compilation and analysis of the binding sites for *Escherichia coli* integration host factor (IHF). *Nucleic Acids Res*. **18**, 4993–5000.

Grainger, D.C., Goldberg, M.D., Lee, D.J., Busby, S.J.W., 2008. Selective repression by Fis and H-NS at the *Escherichia coli dps* promoter. *Mol. Microbiol*. **68**, 1366–1377. doi:10.1111/j.1365-2958.2008.06253.x

Griekspoor, A., Groothius, T., 2006. *4Peaks version 1.7.1.*

Griggs, D.J., Gensberg, K., Piddock, L.J.V., 1996. Mutations in *gyrA* gene of quinolone-resistant *Salmonella* serotypes isolated from humans and animals. *Antimicrob. Agents Chemother.* **40**, 1009–1013.

Gu, M., Berrido, A., Gonzalez, W.G., Miksovska, J., Chambers, J.W., Leng, F., 2016. Fluorescently labeled circular DNA molecules for DNA topology and topoisomerases. *Sci. Rep.* **6,** 36006. doi:10.1038/srep36006

Hagan, E.C., Donnenberg, M.S., Mobley, H.L.T., 2009. Uropathogenic *Escherichia coli. EcoSal Plus* **3**. doi:10.1128/ecosalplus.8.6.1.3

Hajj Hussein, I., Chams, N., Chams, S., El Sayegh, S., Badran, R., Raad, M., Gerges-Geagea, A., Leone, A., Jurjus, A., 2015. Vaccines Through Centuries: Major Cornerstones of Global Health. *Front. Public Health* **3**. doi:10.3389/fpubh.2015.00269

Hall, B.G., Barlow, M., 2004. Evolution of the serine β-lactamases: past, present and future. *Drug Resist. Updat*. **7**, 111–123. doi:10.1016/j.drup.2004.02.003

Hall, G., Yohannes, K., Raupach, J., Becker, N., Kirk, M., 2008. Estimating Community Incidence of *Salmonella, Campylobacter* and Shiga Toxin-producing *Escherichia coli* Infections, Australia. *Emerg. Infect. Dis*. **14**, 1601–1609.

Hancock, S.P., Stella, S., Cascio, D., Johnson, R.C., 2016. DNA Sequence Determinants Controlling Affinity, Stability and Shape of DNA Complexes Bound by the Nucleoid Protein Fis. *PLOS ONE* **11**, e0150189. doi:10.1371/journal.pone.0150189

Hansen, L.H., Jensen, L.B., Sørensen, H.I., Sørensen, S.J., 2007. Substrate specificity of the OqxAB multidrug resistance pump in *Escherichia coli* and

selected enteric bacteria. *J. Antimicrob. Chemother.* **60**, 145–147. doi:10.1093/jac/dkm167
Hansen-Wester, I., Hensel, M., 2001. *Salmonella* pathogenicity islands encoding type III secretion systems. *Microbes Infect.* **3**, 549–559. doi:10.1016/S1286-4579(01)01411-3
Hao, H., Dai, M., Wang, Y., Huang, L., Yuan, Z., 2012. Key genetic elements and regulation systems in methicillin-resistant *Staphylococcus aureus. Future Microbiol.* **7**, 1315–1329. doi:10.2217/fmb.12.107
Haraga, A., Ohlson, M.B., Miller, S.I., 2008. *Salmonellae* interplay with host cells. *Nat. Rev. Microbiol.* **6**, 53–66. doi:10.1038/nrmicro1788
Hardy, C.D., Cozzarelli, N.R., 2005. A genetic selection for supercoiling mutants of *Escherichia coli* reveals proteins implicated in chromosome structure. *Mol. Microbiol*. **57**, 1636–1652. doi:10.1111/j.1365-2958.2005.04799.x
Hawser, S., Lociuro, S., Islam, K., 2006. Dihydrofolate reductase inhibitors as antibacterial agents. *Biochem. Pharmacol*. **71**, 941–948. doi:10.1016/j.bcp.2005.10.052
Heinrichs, D.E., Yethon, J.A., Whitfield, C., 1998. Molecular basis for structural diversity in the core regions of the lipopolysaccharides of *Escherichia coli* and *Salmonella enterica. Mol. Microbiol*. **30**, 221–232. doi:10.1046/j.1365-2958.1998.01063.x
Higgins, N.P., 2016. Species-specific supercoil dynamics of the bacterial nucleoid. *Biophys. Rev.* **8**, 113–121. doi:10.1007/s12551-016-0207-9
Higgins, N.P., Peebles, C.L., Sugino, A., Cozzarelli, N.R., 1978. Purification of subunits of *Escherichia coli* DNA gyrase and reconstitution of enzymatic activity. *Proc. Natl. Acad. Sci. U. S. A.* **75**, 1773–1777.
Higgins, N.P., Yang, X., Fu, Q., Roth, J.R., 1996. Surveying a supercoil domain by using the gamma delta resolution system in *Salmonella typhimurium. J. Bacteriol*. **178**, 2825–2835.
Hinton, J.C.D., Santos, D.S., Seirafi, A., Hulton, C.S.J., Pavitt, G.D., Higgins, C.F., 1992. Expression and mutational analysis of the nucleoid-associated protein H-NS of *Salmonella typhimurium. Mol. Microbiol*. **6**, 2327–2337. doi:10.1111/j.1365-2958.1992.tb01408.x
Hockings, S.C., Maxwell, A., 2002. Identification of Four GyrA Residues Involved in the DNA Breakage–Reunion Reaction of DNA Gyrase. *J. Mol. Biol.* **318**, 351–359. doi:10.1016/S0022-2836(02)00048-7
Hohmann, A.W., Schmidt, G., Rowley, D., 1978. Intestinal Colonization and Virulence of *Salmonella* in Mice. *Infect. Immun*. **22**, 763–770.
Holmes, V.F., Cozzarelli, N.R., 2000. Closing the ring: links between SMC proteins and chromosome partitioning, condensation, and supercoiling. *Proc. Natl. Acad. Sci.* **97**, 1322–1324.
Hooper, D.C., 2000. Mechanisms of Action and Resistance of Older and Newer Fluoroquinolones. *Clin. Infect. Dis*. **31**, S24–S28. doi:10.1086/314056
Hooper, D.C., 1999. Mechanisms of fluoroquinolone resistance. *Drug Resist. Updat*. **2**, 38–55. doi:10.1054/drup.1998.0068
Hooper, D.C., Jacoby, G.A., 2015. Mechanisms of drug resistance: quinolone resistance. *Ann. N. Y. Acad. Sci.* **1354**, 12–31. doi:10.1111/nyas.12830

Hopkins, K.L., Davies, R.H., Threlfall, E.J., 2005. Mechanisms of quinolone resistance in *Escherichia coli* and *Salmonella*: Recent developments. *Int. J. Antimicrob. Agents* **25**, 358–373. doi:10.1016/j.ijantimicag.2005.02.006

Horowitz, D.S., Wang, J.C., 1987. Mapping the Active Site Tyrosine of *Escherichia coli* DNA Gyrase. *J. Biol. Chem*. **262**, 5339–5344.

Huang, W.M., 1996. Bacterial Diversity Based on Type II DNA Topoisomerase Genes. *Annu. Rev. Genet.* **30**, 79–107. doi:10.1146/annurev.genet.30.1.79

Huseby, D.L., Pietsch, F., Brandis, G., Garoff, L., Tegehall, A., Hughes, D., 2017. Mutation supply and relative fitness shape the genotypes of ciprofloxacin-resistant *Escherichia coli. Mol. Biol. Evol.* **5**, 1029-1039. doi:10.1039/molbev/msx052.

Iddles, R.G., 2016. *Supercoiling and stress responses: the impact of changing DNA structure on antibiotic resistance in Salmonella and E. coli (BMedSc Clinical Sciences)*. University of Birmingham, Birmingham.

Iguchi, A., Thomson, N.R., Ogura, Y., Saunders, D., Ooka, T., Henderson, I.R., Harris, D., Asadulghani, M., Kurokawa, K., Dean, P., Kenny, B., Quail, M.A., Thurston, S., Dougan, G., Hayashi, T., Parkhill, J., Frankel, G., 2009. Complete Genome Sequence and Comparative Genome Analysis of Enteropathogenic *Escherichia coli* O127:H6 Strain E2348/69. *J. Bacteriol.* **191**, 347–354. doi:10.1128/JB.01238-08

Imamovic, L., Sommer, M.O.A., 2013. Use of Collateral Sensitivity Networks to Design Drug Cycling Protocols That Avoid Resistance Development. *Sci. Transl. Med*. **5**, 204ra132. doi:10.1126/scitranslmed.3006609

Jacques L Watchell, 1947. *Method for the Isolation of Penicillin*. US2432638.

Janmohamed, K., Zenner, D., Little, C., Lane, C., Wain, J., Charlett, A., Adak, B., Morgan, D., 2011. National outbreak of *Salmonella* Enteritidis phage type 14b in England, September to December 2009: case-control study. *Eurosurveillance* **16**, 19840.

Jarvik, T., Smillie, C., Groisman, E.A., Ochman, H., 2010. Short-Term Signatures of Evolutionary Change in the *Salmonella enterica* Serovar Typhimurium 14028 Genome. *J. Bacteriol.* **192**, 560–567. doi:10.1128/JB.01233-09

Jordi, B.J.A.M., van Vliet, A.H.M., Willshaw, G.A., van der Zeijst, B.A.M., Gaastra, W., 1991. Analysis of the first two genes of the CS1 fimbrial operon in human enterotoxigenic *Escherichia coli* of serotype O139:H28. *FEMS Microbiol. Lett.* **80**, 265–270.

Jovetic, S., Zhu, Y., Marcone, G.L., Marinelli, F., Tramper, J., 2010. β-Lactam and glycopeptide antibiotics: first and last line of defense? *Trends Biotechnol*. **28**, 596–604. doi:10.1016/j.tibtech.2010.09.004

Kaatz, G.W., Seo, S.M., Foster, T.J., 1999. Introduction of a *norA* Promoter Region Mutation into the Chromosome of a Fluoroquinolone-Susceptible Strain of *Staphylococcus aureus* Using Plasmid Integration. *Antimicrob. Agents Chemother*. **43**, 2222–2224.

Kauffmann, F., Edwards, P.R., 1952. Classification and nomenclature of Enterobacteriaceae. *Int. J. Syst. Evol. Microbiol*. **2**, 2–8.

Kavanagh, K.T., Calderon, L.E., Saman, D.M., Abusalem, S.K., 2014. The use of surveillance and preventative measures for methicillin-resistant *Staphylococcus aureus* infections in surgical patients. *Antimicrob. Resist. Infect. Control* **3**, 18. doi:10.1186/2047-2994-3-18

Keane, O.M., Dorman, C.J., 2003. The *gyr* genes of *Salmonella enterica* serovar Typhimurium are repressed by the factor for inversion stimulation, Fis. *Mol. Genet. Genomics* **270**, 56–65. doi:10.1007/s00438-003-0896-1

Kellenberger, E., Ryter, A., Séchaud, J., 1958. Electron microscope study of DNA-containing plasms II. Vegetative and mature phage DNA as compared with normal bacterial nucleoids in different physiological states. *J. Biophys. Biochem. Cytol.* **4**, 671–678.

Kelly, A., Goldberg, M.D., Carroll, R.K., Danino, V., Hinton, J.C.D., Dorman, C.J., 2004. A global role for Fis in the transcriptional control of metabolism and type III secretion in *Salmonella enterica* serovar Typhimurium. *Microbiology* **150**, 2037–2053. doi:10.1099/mic.0.27209-0

Keseler, I.M., Mackie, A., Santos-Zavaleta, A., Billington, R., Bonavides-Martínez, C., Caspi, R., Fulcher, C., Gama-Castro, S., Kothari, A., Krummenacker, M., Latendresse, M., Muñiz-Rascado, L., Ong, Q., Paley, S., Peralta-Gil, M., Subhraveti, P., Velázquez-Ramírez, D.A., Weaver, D., Collado-Vides, J., Paulsen, I., Karp, P.D., 2017. The EcoCyc database: reflecting new knowledge about *Escherichia coli* K-12. *Nucleic Acids Res*. **45**, D543–D550. doi:10.1093/nar/gkw1003

Koskella, B., Brockhurst, M.A., 2014. Bacteria–phage coevolution as a driver of ecological and evolutionary processes in microbial communities. *FEMS Microbiol. Rev.* **38**, 916–931. doi:10.1111/1574-6976.12072

Kröger, C., Colgan, A., Srikumar, S., Händler, K., Sivasankaran, S.K., Hammarlöf, D.L., Canals, R., Grissom, J.E., Conway, T., Hokamp, K., Hinton, J.C.D., 2013. An Infection-Relevant Transcriptomic Compendium for *Salmonella enterica* Serovar Typhimurium. *Cell Host Microbe* **14**, 683–695. doi:10.1016/j.chom.2013.11.010

Kröger, C., Dillon, S.C., Cameron, A.D.S., Papenfort, K., Sivasankaran, S.K., Hokamp, K., Chao, Y., Sittka, A., Hébrard, M., Händler, K., Colgan, A., Leekitcharoenphon, P., Langridge, G.C., Lohan, A.J., Loftus, B., Lucchini, S., Ussery, D.W., Dorman, C.J., Thomson, N.R., Vogel, J., Hinton, J.C.D., 2012. The transcriptional landscape and small RNAs of *Salmonella enterica* serovar Typhimurium. *Proc. Natl. Acad. Sci.* **109**, E1277–E1286. doi:10.1073/pnas.1201061109

Kugelberg, E., Löfmark, S., Wretlind, B., Andersson, D.I., 2005. Reduction of the fitness burden of quinolone resistance in *Pseudomonas aeruginosa. J. Antimicrob. Chemother.* **55**, 22–30. doi:10.1093/jac/dkh505

Kunz, A.N., Begum, A.A., Wu, H., D'Ambrozio, J.A., Robinson, J.M., Shafer, W.M., Bash, M.C., Jerse, A.E., 2012. Impact of Fluoroquinolone Resistance Mutations on Gonococcal Fitness and *In Vivo* Selection for Compensatory Mutations. *J. Infect. Dis.* **205**, 1821–1829. doi:10.1093/infdis/jis277

Ladhani, S.N., Ramsay, M., Borrow, R., Riordan, A., Watson, J.M., Pollard, A.J., 2016. Enter B and W: two new meningococcal vaccine programmes launched. *Arch. Dis. Child.* **101**, 91–95. doi:10.1136/archdischild-2015-308928

Landick, R., Wade, J.T., Grainger, D.C., 2015. H-NS and RNA polymerase: a love–hate relationship? *Curr. Opin. Microbiol*. **24**, 53–59. doi:10.1016/j.mib.2015.01.009

Lang, B., Blot, N., Bouffartigues, E., Buckle, M., Geertz, M., Gualerzi, C.O., Mavathur, R., Muskhelishvili, G., Pon, C.L., Rimsky, S., Stella, S., Babu, M.M.,

Travers, A., 2007. High-affinity DNA binding sites for H-NS provide a molecular basis for selective silencing within proteobacterial genomes. *Nucleic Acids Res*. **35**, 6330–6337. doi:10.1093/nar/gkm712

Lange, R.P., Locher, H.H., Wyss, P.C., Then, R.L., 2007. The targets of currently used antibacterial agents: lessons for drug discovery. *Curr. Pharm. Des*. **13**, 3140–3154.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., Higgins, D.G., 2007. Clustal W and Clustal X version 2.0. *Bioinformatics* **23**, 2947–2948. doi:10.1093/bioinformatics/btm404

Lawler, A.J., Ricci, V., Busby, S.J.W., Piddock, L.J.V., 2013. Genetic inactivation of *acrAB* or inhibition of efflux induces expression of *ramA*. *J. Antimicrob. Chemother*. **68**, 1551–1557. doi:10.1093/jac/dkt069

Le Hello, S., Bekhit, A., Granier, S.A., Barua, H., Beutlich, J., Zając, M., Münch, S., Sintchenko, V., Bouchrif, B., Fashae, K., Pinsard, J.-L., Sontag, L., Fabre, L., Garnier, M., Guibert, V., Howard, P., Hendriksen, R.S., Christensen, J.P., Biswas, P.K., Cloeckaert, A., Rabsch, W., Wasyl, D., Doublet, B., Weill, F.-X., 2013. The global establishment of a highly-fluoroquinolone resistant *Salmonella enterica* serotype Kentucky ST198 strain. *Front. Microbiol*. **4**. doi:10.3389/fmicb.2013.00395

Lebel, P., Basu, A., Oberstrass, F.C., Tretter, E.M., Bryant, Z., 2014. Gold rotor bead tracking for high-speed measurements of DNA twist, torque and extension. *Nat. Methods* **11**, 456–462. doi:10.1038/nmeth.2854

Lee, I., Dong, K.C., Berger, J.M., 2013. The role of DNA bending in type IIA topoisomerase function. *Nucleic Acids Res*. **41**, 5444–5456. doi:10.1093/nar/gkt238

Lee, N., Yuen, K.-Y., Kumana, C.R., 2003. Clinical Role of β-Lactam/β-Lactamase Inhibitor Combinations. *Drugs* **63**, 1511–1524. doi:10.2165/00003495-200363140-00006

Lee, S.Y., Lim, C.J., Dröge, P., Yan, J., 2015. Regulation of Bacterial DNA Packaging in Early Stationary Phase by Competitive DNA Binding of Dps and IHF. *Sci. Rep*. **5**, 18146. doi:10.1038/srep18146

Lesher, G.Y., Froelich, E.J., Gruett, M.D., Bailey, J.H., Brundage, R.P., 1962. 1,8-Naphthyridine Derivatives. A New Class of Chemotherapeutic Agents. *J. Med. Pharm. Chem.* **5**, 1063–1065. doi:10.1021/jm01240a021

Lim, C.J., Kenney, L.J., Yan, J., 2014. Single-molecule studies on the mechanical interplay between DNA supercoiling and H-NS DNA architectural properties. *Nucleic Acids Res*. **42**, 8369–8378. doi:10.1093/nar/gku566

Lindgren, P.K., Marcusson, L.L., Sandvang, D., Frimodt-Møller, N., Hughes, D., 2005. Biological Cost of Single and Multiple Norfloxacin Resistance Mutations in *Escherichia coli* Implicated in Urinary Tract Infections. *Antimicrob. Agents Chemother*. **49**, 2343–2351. doi:10.1128/AAC.49.6.2343-2351.2005

Ling, L.L., Schneider, T., Peoples, A.J., Spoering, A.L., Engels, I., Conlon, B.P., Mueller, A., Schäberle, T.F., Hughes, D.E., Epstein, S., Jones, M., Lazarides, L., Steadman, V.A., Cohen, D.R., Felix, C.R., Fetterman, K.A., Millett, W.P., Nitti, A.G., Zullo, A.M., Chen, C., Lewis, K., 2015. A new antibiotic kills pathogens without detectable resistance. *Nature* **517**, 455–9. doi:10.1038/nature14098

Liu, Y.-Y., Wang, Y., Walsh, T.R., Yi, L.-X., Zhang, R., Spencer, J., Doi, Y., Tian, G., Dong, B., Huang, X., Yu, L.-F., Gu, D., Ren, H., Chen, X., Lv, L., He, D., Zhou, H., Liang, Z., Liu, J.-H., Shen, J., 2016. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *Lancet Infect. Dis*. **16**, 161–168. doi:10.1016/S1473-3099(15)00424-7

Livermore, D.M., Hope, R., Reynolds, R., Blackburn, R., Johnson, A.P., Woodford, N., 2013. Declining cephalosporin and fluoroquinolone non-susceptibility among bloodstream Enterobacteriaceae from the UK: links to prescribing change? *J. Antimicrob. Chemother*. **68**, 2667–2674. doi:10.1093/jac/dkt212

Lostroh, C.P., Lee, C.A., 2001. The *Salmonella* pathogenicity island-1 type III secretion system. *Microbes Infect.* **3**, 1281–1291. doi:10.1016/S1286-4579(01)01488-5

Lu, T.K., Koeris, M.S., 2011. The next generation of bacteriophage therapy. *Curr. Opin. Microbiol.* **14**, 524–531. doi:10.1016/j.mib.2011.07.028

Mangan, M.W., Lucchini, S., Danino, V., Croinin, T.O., Hinton, J.C.D., Dorman, C.J., 2006. The integration host factor (IHF) integrates stationary-phase and virulence gene expression in *Salmonella enterica* serovar Typhimurium. *Mol. Microbiol.* **59**, 1831–1847. doi:10.1111/j.1365-2958.2006.05062.x

Mangan, M.W., Lucchini, S., Ó Cróinín, T., Fitzgerald, S., Hinton, J.C.D., Dorman, C.J., 2011. Nucleoid-associated protein HU controls three regulons that coordinate virulence, response to stress and general physiology in *Salmonella enterica* serovar Typhimurium. *Microbiology* **157**, 1075–1087. doi:10.1099/mic.0.046359-0

Männik, J., Castillo, D.E., Yang, D., Siopsis, G., Männik, J., 2016. The role of MatP, ZapA and ZapB in chromosomal organization and dynamics in *Escherichia coli. Nucleic Acids Res*. **44**, 1216–1226. doi:10.1093/nar/gkv1484

Marchese, A., Schito, G.C., 2001. The oxazolidinones as a new family of antimicrobial agent. *Clin. Microbiol. Infect.* **7**, 66–74. doi:10.1046/j.1469-0691.2001.00060.x

Marcusson, L.L., Frimodt-Møller, N., Hughes, D., 2009. Interplay in the Selection of Fluoroquinolone Resistance and Bacterial Fitness. *PLoS Pathog*. **5**, e1000541. doi:10.1371/journal.ppat.1000541

Marineli, F., Tsoucalas, G., Karamanou, M., Androutsos, G., 2013. Mary Mallon (1869-1938) and the history of typhoid fever. *Ann. Gastroenterol. Q. Publ. Hell. Soc. Gastroenterol.* **26**, 132.

Martínez-Martínez, L., Pascual, A., Jacoby, G.A., 1998. Quinolone resistance from a transferable plasmid. *The Lancet* **351**, 797–799. doi:10.1016/S0140-6736(97)07322-4

Marzel, A., Desai, P.T., Nissan, I., Schorr, Y.I., Suez, J., Valinsky, L., Reisfeld, A., Agmon, V., Guard, J., McClelland, M., Rahav, G., Gal-Mor, O., 2014. Integrative Analysis of Salmonellosis in Israel Reveals Association of *Salmonella enterica* Serovar 9,12:l,v:− with Extraintestinal Infections, Dissemination of Endemic *S. enterica* Serovar Typhimurium DT104 Biotypes, and Severe Underreporting of Outbreaks. *J. Clin. Microbiol.* **52**, 2078–2088. doi:10.1128/JCM.00399-14

Masopust, D., Sivula, C.P., Jameson, S.C., 2017. Of Mice, Dirty Mice, and Men: Using Mice To Understand Human Immunology. *J. Immunol.* **199**, 383–388. doi:10.4049/jimmunol.1700453
Maxwell, A., Burton, N.P., O'Hagan, N., 2006. High-throughput assays for DNA gyrase and other topoisomerases. *Nucleic Acids Res.* **34**, e104. doi:10.1093/nar/gkl504
McCarthy, M., 2013. *Chief Medical Officer Dame Sally Davies: Resistance to antibiotics.* The Independent.
McClelland, M., Sanderson, K.E., Spieth, J., Clifton, S.W., Latreille, P., Courtney, L., Porwollik, S., Ali, J., Dante, M., Du, F., Hou, S., Layman, D., Leonard, S., Nguyen, C., Scott, K., Holmes, A., Grewal, N., Mulvaney, E., Ryan, E., Sun, H., Florea, L., Miller, W., Stoneking, T., Nhan, M., Waterston, R., Wilson, R.K., 2001. Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. *Nature* **413**, 852–856. doi:10.1038/35101614
McMurry, L.M., Oethinger, M., Levy, S.B., 1998. Triclosan targets lipid synthesis. *Nature* **394**, 531–532.
Mehlhorn, A.J., Brown, D.A., 2007. Safety Concerns with Fluoroquinolones. *Ann. Pharmacother.* **41**, 1859–1866. doi:10.1345/aph.1K347
Mellou, K., Sideroglou, T., Kallimani, A., Potamiti-Komi, M., Pervanidou, D., Lillakou, E., Georgakopoulou, T., Mandilara, G., Lambiri, M., Vatopoulos, A., Hadjichristodoulou, C., 2013. Evaluation of underreporting of salmonellosis and shigellosis hospitalised cases in Greece, 2011: results of a capture-recapture study and a hospital registry review. *BMC Public Health* **13**, 875. doi:10.1186/1471-2458-13-875
Mercier, R., Petit, M.-A., Schbath, S., Robin, S., Karoui, M.E., Boccard, F., Espéli, O., 2008. The MatP/*matS* Site-Specific System Organizes the Terminus Region of the *E. coli* Chromosome into a Macrodomain. *Cell* **135**, 475–485. doi:10.1016/j.cell.2008.08.031
Merrick, M.J., 1993. In a class of its own — the RNA polymerase sigma factor σ;54 (σN). *Mol. Microbiol*. **10**, 903–909. doi:10.1111/j.1365-2958.1993.tb00961.x
Messerschmidt, S.J., Waldminghaus, T., 2015. Dynamic Organization: Chromosome Domains in *Escherichia coli. J. Mol. Microbiol. Biotechnol.* **24**, 301–315. doi:10.1159/000369098
Meynell, E., Meynell, G.G., Datta, N., 1968. Phylogenetic relationships of drug-resistance factors and other transmissible bacterial plasmids. *Bacteriol. Rev.* **32**, 55.
Mills, D.M., Bajaj, V., Lee, C.A., 1995. A 40 kb chromosomal fragment encoding *Salmonella typhimurium* invasion genes is absent from the corresponding region of the *Escherichia coli* K-12 chromosome. *Mol. Microbiol*. **15**, 749–759.
Mirkin, S.M., 2001. DNA Topology: Fundamentals, in: *Encyclopedia of Life Sciences*. Wiley-Blackwell.
Mitscher, L.A., 2005. Bacterial Topoisomerase Inhibitors: Quinolone and Pyridone Antibacterial Agents. *Chem. Rev.* **105**, 559–592. doi:10.1021/cr030101q
Mohamed, Y.F., Abou-Shleib, H.M., Khalil, A.M., El-Guink, N.M., El-Nakeeb, M.A., 2016. Membrane permeabilization of colistin toward pan-drug resistant Gram-negative isolates. *Braz. J. Microbiol.* **47**, 381–388. doi:10.1016/j.bjm.2016.01.007

Mondal, S., Mandal, S.M., Mondal, T.K., Sinha, C., 2015. Structural characterization of new Schiff bases of sulfamethoxazole and sulfathiazole, their antibacterial activity and docking computation with DHPS protein structure. *Spectrochim. Acta. A. Mol. Biomol. Spectrosc.* **150**, 268–279. doi:10.1016/j.saa.2015.05.049

Monecke, S., Mariani-Kurkdjian, P., Bingen, E., Weill, F.-X., Balière, C., Slickers, P., Ehricht, R., 2011. Presence of Enterohemorrhagic *Escherichia coli* ST678/O104:H4 in France Prior to 2011. *Appl. Environ. Microbiol*. **77**, 8784–8786. doi:10.1128/AEM.06524-11

Monnappa, A.K., Dwidar, M., Seo, J.K., Hur, J.-H., Mitchell, R.J., 2014. *Bdellovibrio bacteriovorus* Inhibits *Staphylococcus aureus* Biofilm Formation and Invasion into Human Epithelial Cells. *Sci. Rep.* **4**. doi:10.1038/srep03811

Morgan, D.J., Okeke, I.N., Laxminarayan, R., Perencevich, E.N., Weisenberg, S., 2011. Non-prescription antimicrobial use worldwide: a systematic review. *Lancet Infect. Dis.* **11**, 692–701. doi:10.1016/S1473-3099(11)70054-8

Morgan-Linnell, S.K., Zechiedrich, L., 2007. Contributions of the Combined Effects of Topoisomerase Mutations toward Fluoroquinolone Resistance in *Escherichia coli. Antimicrob. Agents Chemother.* **51**, 4205–4208. doi:10.1128/AAC.00647-07

Munshi, M.H., Sack, D.A., Haider, K., Ahmed, Z.U., Rahaman, M.M., Morshed, M.G., 1987. Plasmid-mediated resistance to nalidixic acid in *Shigella dysenteriae* Type 1. *The Lancet* **330**, 419–421.

Mustaev, A., Malik, M., Zhao, X., Kurepina, N., Luan, G., Oppegard, L.M., Hiasa, H., Marks, K.R., Kerns, R.J., Berger, J.M., Drlica, K., 2014. Fluoroquinolone-Gyrase-DNA Complexes: Two Modes of Drug Binding. *J. Biol. Chem*. **289**, 12300–12312. doi:10.1074/jbc.M113.529164

Nakama, T., Nureki, O., Yokoyama, S., 2001. Structural Basis for the Recognition of Isoleucyl-Adenylate and an Antibiotic, Mupirocin, by Isoleucyl-tRNA Synthetase. *J. Biol. Chem.* **276**, 47387–47393. doi:10.1074/jbc.M109089200

Namdari, F., Hurtado-Escobar, G.A., Abed, N., Trotereau, J., Fardini, Y., Giraud, E., Velge, P., Virlogeux-Payant, I., 2012. Deciphering the Roles of BamB and Its Interaction with BamA in Outer Membrane Biogenesis, T3SS Expression and Virulence in *Salmonella. PLoS ONE* **7**, e46050. doi:10.1371/journal.pone.0046050

Navarre, W.W., Porwollik, S., Wang, Y., McClelland, M., Rosen, H., Libby, S.J., Fang, F.C., 2006. Selective Silencing of Foreign DNA with Low GC Content by the H-NS Protein in *Salmonella. Science* **313**, 246–238. doi:10.1126/science.1128794

Nesta, 2014. *Longitude Prize 2014: Prize Rules*. Nesta, London.

Nikaido, E., Yamaguchi, A., Nishino, K., 2008. AcrAB Multidrug Efflux Pump Regulation in *Salmonella enterica* serovar Typhimurium by RamA in Response to Environmental Signals. *J. Biol. Chem*. **283**, 24245–24253. doi:10.1074/jbc.M804544200

Niki, H., Yamaichi, Y., Hiraga, S., 2000. Dynamic organization of chromosomal DNA in *Escherichia coli. Genes Dev.* **14**, 212–223. doi:10.1101/gad.14.2.212

Niki, Y., 2002. Pharmacokinetics and safety assessment of tosufloxacin tosilate. *J. Infect. Chemother.* **8**, 1–18.

Nippon Genetics Europe, n.d. *Midori Green Advance* [WWW Document]. URL http://www.nippongenetics.eu/dnarna-electrophoresis/dna-stains/midori-green-advance/ (accessed 3.25.15).
Nobel Foundation, 1965. *Nobel Lectures, Physiology or Medicine* 1922-1941. Elsevier Publishing Company, Amsterdam.
Nobel Media AB, 2014. *Gerhard Domagk - Facts* [WWW Document]. URL http://www.nobelprize.org/nobel_prizes/medicine/laureates/1939/domagk-facts.html (accessed 4.7.16).
Nolivos, S., Upton, A.L., Badrinarayanan, A., Müller, J., Zawadzka, K., Wiktor, J., Gill, A., Arciszewska, L., Nicolas, E., Sherratt, D., 2016. MatP regulates the coordinated action of topoisomerase IV and MukBEF in chromosome segregation. *Nat. Commun*. **7**, 10466. doi:10.1038/ncomms10466
Nonaka, G., 2006. Regulon and promoter analysis of the *E. coli* heat-shock factor, 32, reveals a multifaceted cellular response to heat stress. *Genes Dev.* **20**, 1776–1789. doi:10.1101/gad.1428206
Ó Cróinín, T., Dorman, C.J., 2007. Expression of the Fis protein is sustained in late-exponential- and stationary-phase cultures of *Salmonella enterica* serovar Typhimurium grown in the absence of aeration. *Mol. Microbiol*. **66**, 237–251. doi:10.1111/j.1365-2958.2007.05916.x
Oberto, J., Nabti, S., Jooste, V., Mignot, H., Rouviere-Yaniv, J., 2009. The HU Regulon Is Composed of Genes Responding to Anaerobiosis, Acid Stress, High Osmolarity and SOS Induction. *PLoS ONE* **4**, e4367. doi:10.1371/journal.pone.0004367
O'Brien, S., Gillespie, I., Charlett, A., Adak, B., Threlfall, J., Ward, L., 2004. National case-control study of *Salmonella* Enteritidis Phage Type 14b infection in England and Wales implicates eggs used in the catering trade. *Eurosurveillance* **8**, pii=2388.
Ochman, H., Elwyn, S., Moran, N.A., 1999. Calibrating bacterial evolution. *Proc. Natl. Acad. Sci.* **96**, 12638–12643. doi:10.1073/pnas.96.22.12638
Ogawa, T., Wada, M., Kano, Y., Imamoto, F., Okazaki, T., 1989. DNA replication in *Escherichia coli* mutants that lack protein HU. *J. Bacteriol.* **171**, 5672–5679.
Onwuezobe, I.A., Oshun, P.O., Odigwe, C.C., 2012. Antimicrobials for treating symptomatic non-typhoidal *Salmonella* infection, in: The Cochrane Collaboration (Ed.), *Cochrane Database of Systematic Reviews.* John Wiley & Sons, Ltd, Chichester, UK.
Österberg, S., Peso-Santos, T. del, Shingler, V., 2011. Regulation of Alternative Sigma Factor Use. *Annu. Rev. Microbiol.* **65**, 37–55. doi:10.1146/annurev.micro.112408.134219
Osterloh, A., Papp, S., Moderzynski, K., Kuehl, S., Richardt, U., Fleischer, B., 2016. Persisting *Rickettsia typhi* causes fatal central nervous system inflammation. *Infect. Immun*. **84**, 1615–1632. doi:10.1128/IAI.00034-16
Osuna, R., Lienau, D., Hughes, K.T., Johnson, R.C., 1995. Sequence, regulation, and functions of *fis* in *Salmonella typhimurium*. *J. Bacteriol.* **177**, 2021–2032.
Paget, M.S., 2015. Bacterial Sigma Factors and Anti-Sigma Factors: Structure, Function and Distribution. *Biomolecules* **5**, 1245–1265. doi:10.3390/biom5031245
Panhotra, B.R., Desai, B., Sharma, P.L., 1985. Nalidixic-acid-resistant *Shigella dysenteriae* I. *The Lancet* **325**, 763.

Pankey, G.A., Sabath, L.D., 2004. Clinical Relevance of Bacteriostatic versus Bactericidal Mechanisms of Action in the Treatment of Gram-Positive Bacterial Infections. *Clin. Infect. Dis.* **38**, 864–870. doi:10.1086/381972

Parry, C.M., Hien, T.T., Dougan, G., White, N.J., Farrar, J.J., 2002. Typhoid Fever. *N. Engl. J. Med.* **347**, 1770–1782. doi:10.1056/NEJMra020201

Payne, D.J., Gwynn, M.N., Holmes, D.J., Pompliano, D.L., 2007. Drugs for bad bugs: confronting the challenges of antibacterial discovery. *Nat. Rev. Drug Discov.* **6**, 29–40. doi:10.1038/nrd2201

Peng, H., Marians, K.J., 1993. *Escherichia coli* Topoisomerase IV: Purification, Characterization, Subunit Structure, and Subunit Interactions. *J. Biol. Chem*. **268**, 24481–24490.

Phillips, I., Casewell, M., Cox, T., Groot, B.D., Friis, C., Jones, R., Nightingale, C., Preston, R., Waddell, J., 2004. Does the use of antibiotics in food animals pose a risk to human health? A critical review of published data. *J. Antimicrob. Chemother*. **53**, 28–52. doi:10.1093/jac/dkg483

Piddock, L.J., Wise, R., 1987. Induction of the SOS response in *Escherichia coli* by 4-quinolone antimicrobial agents. *FEMS Microbiol. Lett.* **41**, 289–294.

Piddock, L.J.V., 2015. Teixobactin, the first of a new class of antibiotics discovered by iChip technology? *J. Antimicrob. Chemother.* **70**, 2679–2680. doi:10.1093/jac/dkv175

Piddock, L.J.V., 2006. Clinically Relevant Chromosomally Encoded Multidrug Resistance Efflux Pumps in Bacteria. *Clin. Microbiol. Rev.* **19**, 382–402. doi:10.1128/CMR.19.2.382-402.2006

Piddock, L.J.V., 1999. Mechanisms of fluoroquinolone resistance: an update 1994-1998. *Drugs* **58** Suppl 2, 11–18.

Piddock, L.J.V., Whale, K., Wise, R., 1990. Quinolone resistance in *Salmonella*: clinical experience. *The Lancet* **335**, 1459. doi:10.1016/0140-6736(90)91484-R

Pirard, C., Sagot, C., Deville, M., Dubois, N., Charlier, C., 2012. Urinary levels of bisphenol A, triclosan and 4-nonylphenol in a general Belgian population. *Environ. Int.* **48**, 78–83. doi:10.1016/j.envint.2012.07.003

Poole, K., 2014. Stress responses as determinants of antimicrobial resistance in *Pseudomonas aeruginosa*: multidrug efflux and more. *Can. J. Microbiol.* **60**, 783–791. doi:10.1139/cjm-2014-0666

Postow, L., Hardy, C.D., Arsuaga, J., Cozzarelli, N.R., 2004. Topological domain structure of the *Escherichia coli* chromosome. *Genes Dev*. **18**, 1766–1779.

Power, E.G.M., Phillips, I., 1993. Correlation between *umuC* induction and *Salmonella* mutagenicity assay. *FEMS Microbiol. Lett.* **112**, 251–254.

Preisler, A., Mraheil, M.A., Heisig, P., 2006. Role of novel *gyrA* mutations in the suppression of the fluoroquinolone resistance genotype of vaccine strain *Salmonella* Typhimurium *vacT* (*gyrA* D87G). *J. Antimicrob. Chemother.* **57**, 430–436. doi:10.1093/jac/dki475

Price, J.R., 1949. Some reactions of 1-methyl-4-quinolone-3-carboxylic acid, a degradation product of the alkaloids. *Aust. J. Sci. Res.* **217**, 272–281.

Public Health England, 2015. *Outbreak of Salmonella Enteritidis phage type 14b, May-September 2014.* Public Health England.

Public Health England, n.d. *Bacteria Collection: NCTC 74 Salmonella enterica subsp. enterica* [WWW Document]. URL https://www.phe-

culturecollections.org.uk/products/bacteria/detail.jsp?refId=NCTC%2074&collection=nctc (accessed 6.22.16).
Quinlivan, E.P., McPartlin, J., Weir, D.G., Scott, J., 2000. Mechanism of the antimicrobial drug trimethoprim revisited. *FASEB J*. **14**, 2519–2524.
Qureshi, Z.P., Seoane-Vazquez, E., Rodriguez-Monguio, R., Stevenson, K.B., Szeinbach, S.L., 2011. Market withdrawal of new molecular entities approved in the United States from 1980 to 2009: DISCONTINUATION OF NMES. *Pharmacoepidemiol. Drug Saf.* **20**, 772–777. doi:10.1002/pds.2155
Raju, T.N., 1999. The Nobel Chronicles. *The Lancet* **353**, 936. doi:10.1016/S0140-6736(05)75055-8
Ramarao, N., Nielsen-Leroux, C., Lereclus, D., 2012. The insect *Galleria mellonella* as a powerful infection model to investigate bacterial pathogenesis. *J. Vis. Exp.* e4392. doi:10.3791/4392
Randall, L.P., Cooles, S.W., Coldham, N.G., Penuela, E.G., Mott, A.C., Woodward, M.J., Piddock, L.J.V., Webber, M.A., 2007. Commonly used farm disinfectants can select for mutant *Salmonella enterica* serovar Typhimurium with decreased susceptibility to biocides and antibiotics without compromising virulence. *J. Antimicrob. Chemother*. **60**, 1273–1280. doi:10.1093/jac/dkm359
Randall, L.P., Cooles, S.W., Piddock, L.J.V., Woodward, M.J., 2004. Effect of triclosan or a phenolic farm disinfectant on the selection of antibiotic-resistant *Salmonella enterica. J. Antimicrob. Chemother.* **54**, 621–627. doi:10.1093/jac/dkh376
Redgrave, L.S., Sutton, S.B., Webber, M.A., Piddock, L.J.V., 2014. Fluoroquinolone resistance: mechanisms, impact on bacteria, and role in evolutionary success. *Trends Microbiol*. **22**, 438–445. doi:10.1016/j.tim.2014.04.007
Reisch, C.R., Prather, K.L.J., 2015. The no-SCAR (Scarless Cas9 Assisted Recombineering) system for genome editing in *Escherichia coli. Sci. Rep.* **5**, 15096. doi:10.1038/srep15096
Renggli, S., Keck, W., Jenal, U., Ritz, D., 2013. Role of Autofluorescence in Flow Cytometric Analysis of *Escherichia coli* Treated with Bactericidal Antibiotics. *J. Bacteriol.* **195**, 4067–4073. doi:10.1128/JB.00393-13
Review on Antimicrobial Resistance, 2016. *Vaccines and alternative approaches: Reducing our dependence on antimicrobials.* London.
Review on Antimicrobial Resistance, 2015a. *Safe, secure and controlled: Managing the supply chain of antimicrobials.* London.
Review on Antimicrobial Resistance, 2015b. *Tackling a global health crisis: Initial steps.* London.
Review on Antimicrobial Resistance, 2015c. *Securing new drugs for future generations: The pipeline of antibiotics.* London.
Review on Antimicrobial Resistance, 2014. *Antimicrobial Resistance: Tackling a crisis for the health and wealth of nations.* London.
Rhodius, V.A., Suh, W.C., Nonaka, G., West, J., Gross, C.A., 2005. Conserved and Variable Functions of the σE Stress Response in Related Genomes. *PLoS Biol.* **4**, e2. doi:10.1371/journal.pbio.0040002
Robicsek, A., Strahilevitz, J., Jacoby, G.A., Macielag, M., Abbanat, D., Hye Park, C., Bush, K., Hooper, D.C., 2006. Fluoroquinolone-modifying enzyme: a new adaptation of a common aminoglycoside acetyltransferase. *Nat. Med.* **12**, 83–88. doi:10.1038/nm1347

Rodríguez-Martínez, J.M., Velasco, C., Briales, A., García, I., Conejo, M.C., Pascual, A., 2008. Qnr-like pentapeptide repeat proteins in Gram-positive bacteria. *J. Antimicrob. Chemother.* **61**, 1240–1243. doi:10.1093/jac/dkn115

Rodríguez-Rojas, A., Rodríguez-Beltrán, J., Couce, A., Blázquez, J., 2013. Antibiotics and antibiotic resistance: A bitter fight against evolution. *Int. J. Med. Microbiol*. **303**, 293–297. doi:10.1016/j.ijmm.2013.02.004

Sailsbury, D., Ramsay, M. (Eds.), 2013. Diptheria: the green book, chapter 15, in: *Immunisation against Infectious Disease.* The Stationery Office, London, pp. 109–125.

Samuels, D.J., Frye, J.G., Porwollik, S., McClelland, M., Mrázek, J., Hoover, T.R., Karls, A.C., 2013. Use of a promiscuous, constitutively-active bacterial enhancer-binding protein to define the σ54 (RpoN) regulon of *Salmonella* Typhimurium LT2. *BMC Genomics* **14**, 602. doi:10.1186/1471-2164-14-602

Scheirer, K.E., Higgins, N.P., 2001. Transcription induces a supercoil domain barrier in bacteriophage Mu. *Biochimie* **83**, 155–159.

Schlesinger, A., Paul, M., Gafter-Gvili, A., Rubinovitch, B., Leibovici, L., 2009. Infection-control interventions for cancer patients after chemotherapy: a systematic review and meta-analysis. *Lancet Infect. Dis*. **9**, 97–107. doi:10.1016/S1473-3099(08)70284-6

Schlünzen, F., Zarivach, R., Harms, J., Bashan, A., Tocilj, A., Albrecht, R., Yonath, A., Franceschi, F., 2001. Structural basis for the interaction of antibiotics with the peptidyl transferase centre in eubacteria. *Nature* **413**, 814–821.

Schoeffler, A.J., Berger, J.M., 2008. DNA topoisomerases: harnessing and constraining energy to govern chromosome topology. *Q. Rev. Biophys*. **41**, 41–101. doi:10.1017/S003358350800468X

Schultz, M., 2008. Theobald Smith. *Emerg. Infect. Dis.* **14**, 1940–1942. doi:10.3201/eid1412.081188

Schweizer, H.P., 2001. Triclosan: a widely used biocide and its link to antibiotics. *FEMS Microbiol. Lett.* **202**, 1–7. doi:10.1111/j.1574-6968.2001.tb10772.x

Schwudke, D., Linscheid, M., Strauch, E., Appel, B., Zahringer, U., Moll, H., Muller, M., Brecker, L., Gronow, S., Lindner, B., 2003. The Obligate Predatory *Bdellovibrio bacteriovorus* Possesses a Neutral Lipid A Containing -D-Mannoses That Replace Phosphate Residues: SIMILARITIES AND DIFFERENCES BETWEEN THE LIPID As AND THE LIPOPOLYSACCHARIDES OF THE WILD TYPE STRAIN *B. BACTERIOVORUS* HD100 AND ITS HOST-INDEPENDENT DERIVATIVE HI100. *J. Biol. Chem.* **278**, 27502–27512. doi:10.1074/jbc.M303012200

Segall, A.M., Goodman, S.D., Nash, H.A., 1994. Architectural elements in nucleoprotein complexes: interchangeability of specific and non-specific DNA binding proteins. *EMBO J*. **13**, 4536.

Segata, N., Börnigen, D., Morgan, X.C., Huttenhower, C., 2013. PhyloPhlAn is a new method for improved phylogenetic and taxonomic placement of microbes. *Nat. Commun.* **4**, 2304. doi:10.1038/ncomms3304

Sentongo, T.A., 2004. The use of oral rehydration solutions in children and adults. *Curr. Gastroenterol. Rep.* **6**, 307–313.

Shepard, S.M., Danzeisen, J.L., Isaacson, R.E., Seemann, T., Achtman, M., Johnson, T.J., 2012. Genome Sequences and Phylogenetic Analysis of K88-

and F18-Positive Porcine Enterotoxigenic *Escherichia coli. J. Bacteriol.* **194**, 395–405. doi:10.1128/JB.06225-11
Shu, M.-H., MatRahim, N., NorAmdan, N., Pang, S.-P., Hashim, S.H., Phoon, W.-H., AbuBakar, S., 2016. An Inactivated Antibiotic-Exposed Whole-Cell Vaccine Enhances Bactericidal Activities Against Multidrug-Resistant *Acinetobacter baumannii. Sci. Rep.* **6**, 22332. doi:10.1038/srep22332
Sievers, F., Wilm, A., Dineen, D., Gibson, T.J., Karplus, K., Li, W., Lopez, R., McWilliam, H., Remmert, M., Soding, J., Thompson, J.D., Higgins, D.G., 2014. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol. Syst. Biol*. **7**, 539–539. doi:10.1038/msb.2011.75
Silver, L.L., 2011. Challenges of Antibacterial Discovery. *Clin. Microbiol. Rev*. **24**, 71–109. doi:10.1128/CMR.00030-10
Silverman, J.A., Perlmutter, N.G., Shapiro, H.M., 2003. Correlation of Daptomycin Bactericidal Activity and Membrane Depolarization in *Staphylococcus aureus. Antimicrob. Agents Chemother*. **47**, 2538–2544. doi:10.1128/AAC.47.8.2538-2544.2003
Singh, S.S., Singh, N., Bonocora, R.P., Fitzgerald, D.M., Wade, J.T., Grainger, D.C., 2014. Widespread suppression of intragenic transcription initiation by H-NS. *Genes Dev*. **28**, 214–219. doi:10.1101/gad.234336.113
Skoko, D., Yoo, D., Bai, H., Schnurr, B., Yan, J., McLeod, S.M., Marko, J.F., Johnson, R.C., 2006. Mechanism of Chromosome Compaction and Looping by the *Escherichia coli* Nucleoid Protein Fis. *J. Mol. Biol.* **364**, 777–798. doi:10.1016/j.jmb.2006.09.043
Skovierova, H., Rowley, G., Rezuchova, B., Homerova, D., Lewis, C., Roberts, M., Kormanec, J., 2006. Identification of the σE regulon of *Salmonella enterica* serovar Typhimurium. *Microbiology* **152**, 1347–1359. doi:10.1099/mic.0.28744-0
Skurnik, D., Cywes-Bentley, C., Pier, G.B., 2016. The exceptionally broad-based potential of active and passive vaccination targeting the conserved microbial surface polysaccharide PNAG. *Expert Rev. Vaccines* **15**, 1041–1053. doi:10.1586/14760584.2016.1159135
Smith, R., Coast, J., 2013. The true cost of antimicrobial resistance. *BMJ* **346**, f1493. doi:10.1136/bmj.f1493
Sobetzko, P., Glinkowska, M., Travers, A., Muskhelishvili, G., 2013. DNA thermodynamic stability and supercoil dynamics determine the gene expression program during the bacterial growth cycle. *Mol. Biosyst*. **9**, 1643–1651. doi:10.1039/C3MB25515H
Spratt, B.G., 1996. Antibiotic resistance: Counting the cost. *Curr. Biol.* **6**, 1219–1221. doi:10.1016/S0960-9822(96)00700-2
Spratt, B.G., 1994. Resistance to antibiotics mediated by target alterations. *Science* **264**, 388–393. doi:10.1126/science.8153626
Spratt, B.G., 1980. Biochemical and genetical approaches to the mechanism of action of penicillin. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **289**, 273–283.
Spratt, B.G., 1977. Properties of the Penicillin-Binding Proteins of *Escherichia coli* K12. *Eur. J. Biochem*. **72**, 341–352.
St John-Brooks, R., 1934. The Genus *Salmonella* Lignières, 1900. *J. Hyg. (Lond.)* **34**, 333–350.

Stanley, J., Baquar, N., Threlfall, E.J., 1993. Genotypes and phylogenetic relationships of *Salmonella typhimurium* are defined by molecular fingerprinting of IS200 and 16S *rrn* loci. *Microbiology* **139**, 1133–1140.

Statens Serum Institut, National Veterinary Institute, Technical University of Denmark, National Food Institute, Technical University of Denmark, 2015. *DANMAP 2014 - Use of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, food and humans in Denmark.* DANMAP.

Stein, R.A., Deng, S., Higgins, N.P., 2005. Measuring chromosome dynamics on different time scales using resolvases with varying half-lives. *Mol. Microbiol.* **56**, 1049–1061. doi:10.1111/j.1365-2958.2005.04588.x

Stokes, H.W., Gillings, M.R., 2011. Gene flow, mobile genetic elements and the recruitment of antibiotic resistance genes into Gram-negative pathogens. *FEMS Microbiol. Rev.* **35**, 790–819. doi:10.1111/j.1574-6976.2011.00273.x

Strahilevitz, J., Jacoby, G.A., Hooper, D.C., Robicsek, A., 2009. Plasmid-Mediated Quinolone Resistance: a Multifaceted Threat. *Clin. Microbiol. Rev.* **22**, 664–689. doi:10.1128/CMR.00016-09

Sulakvelidze, A., Alavidze, Z., Morris, J.G., 2001. Bacteriophage Therapy. *Antimicrob. Agents Chemother.* **45**, 649–659. doi:10.1128/AAC.45.3.649-659.2001

Sun, Y., Dai, M., Hao, H., Wang, Y., Huang, L., Almofti, Y.A., Liu, Z., Yuan, Z., 2011. The Role of RamA on the Development of Ciprofloxacin Resistance in *Salmonella enterica* Serovar Typhimurium. *PLoS ONE* **6**, e23471. doi:10.1371/journal.pone.0023471

Sutton, S.B., 2014. *The role of supercoiling in the evolution of superbugs (BMedSc Clinical Sciences).* University of Birmingham, Birmingham.

Sybesma, W., Zbinden, R., Chanishvili, N., Kutateladze, M., Chkhotua, A., Ujmajuridze, A., Mehnert, U., Kessler, T.M., 2016. Bacteriophages as Potential Treatment for Urinary Tract Infections. *Front. Microbiol.* **7**, 465. doi:10.3389/fmicb.2016.00465

Sykes, R., 2010. The 2009 Garrod Lecture: The evolution of antimicrobial resistance: a Darwinian perspective. *J. Antimicrob. Chemother.* **65**, 1842–1852. doi:10.1093/jac/dkq217

Tennant, S.M., MacLennan, C.A., Simon, R., Martin, L.B., Khan, M.I., 2016. Nontyphoidal *Salmonella* disease: Current status of vaccine research and development. *Vaccine* **34**, 2907–2910. doi:10.1016/j.vaccine.2016.03.072

Thanbichler, M., Wang, S.C., Shapiro, L., 2005. The bacterial nucleoid: A highly organized and dynamic structure. *J. Cell. Biochem.* **96**, 506–521. doi:10.1002/jcb.20519

Thiel, A., Valens, M., Vallet-Gely, I., Espéli, O., Boccard, F., 2012. Long-Range Chromosome Organization in *E. coli* : A Site-Specific System Isolates the Ter Macrodomain. *PLoS Genet.* **8**, e1002672. doi:10.1371/journal.pgen.1002672

Török, M.E., Cooke, F.J., Moran, E., 2009. Antimicrobials, in: *Oxford Handbook of Infectious Diseases and Microbiology.* Oxford University Press, Oxford.

Tran, J.H., Jacoby, G.A., 2002. Mechanism of plasmid-mediated quinolone resistance. *Proc. Natl. Acad. Sci.* **99**, 5638–5642. doi:10.1073/pnas.082092899

Tsai, C.J.-Y., Loh, J.M.S., Proft, T., 2016. *Galleria mellonella* infection models for the study of bacterial diseases and for antimicrobial drug testing. *Virulence* **7**, 214. doi:10.1080/21505594.2015.1135289

Tubbs, R.K., Ditmars Jr., W.E., Van Winkle, Q., 1964. Heterogeneity of the interaction of DNA with acriflavine. *J. Mol. Biol*. **9**, 545–557. doi:10.1016/S0022-2836(64)80226-6

Tulkens, P.M., Arvis, P., Kruesmann, F., 2012. Moxifloxacin safety. *Drugs RD* **12**, 71–100.

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B.C., Remm, M., Rozen, S.G., 2012. Primer3—new capabilities and interfaces. *Nucleic Acids Res.* **40**, e115. doi:10.1093/nar/gks596

Valens, M., Penaud, S., Rossignol, M., Cornet, F., Boccard, F., 2004. Macrodomain organization of the *Escherichia coli* chromosome. *EMBO J*. **23**, 4330–4341. doi:10.1038/sj.emboj.7600434

Velásquez, J.C., Hidalgo, A.A., Villagra, N., Santiviago, C.A., Mora, G.C., Fuentes, J.A., 2016. SPI-9 of *Salmonella enterica* serovar Typhi is constituted by an operon postively regulated by RpoS and contributes to adherence to epithelial cells in culture. *Microbiology* **162**, 1367–1378. doi:10.1099/mic.0.000319

Vetting, M.W., Hegde, S.S., Wang, M., Jacoby, G.A., Hooper, D.C., Blanchard, J.S., 2011. Structure of QnrB1, a Plasmid-mediated Fluoroquinolone Resistance Factor. *J. Biol. Chem.* **286**, 25265–25273. doi:10.1074/jbc.M111.226936

Wada, M., Kano, Y., Ogawa, T., Okazaki, T., Imamoto, F., 1988. Construction and characterization of the deletion mutant of *hupA* and *hupB* genes in *Escherichia coli*. *J. Mol. Biol*. **204**, 581–591.

Walters, R.N., Piddock, L.J.V., Wise, R., 1989. The effect of mutations in the SOS response on the kinetics of quinolone killing. *J. Antimicrob. Chemother*. **24**, 863–873. doi:10.1093/jac/24.6.863

Wang, X., Llopis, P.M., Rudner, D.Z., 2013. Organization and segregation of bacterial chromosomes. *Nat. Rev. Genet.* **14**, 191–203. doi:10.1038/nrg3375

Wang-Kan, X., Blair, J.M.A., Chirullo, B., Betts, J., Ragione, R.M.L., Ivens, A., Ricci, V., Opperman, T.J., Piddock, L.J.V., 2017. Lack of AcrB Efflux Function Confers Loss of Virulence on *Salmonella enterica* Serovar Typhimurium. *mBio* **8**, e00968-17. doi:10.1128/mBio.00968-17

Wasels, F., Kuehne, S.A., Cartman, S.T., Spigaglia, P., Barbanti, F., Minton, N.P., Mastrantonio, P., 2015. Fluoroquinolone Resistance Does Not Impose a Cost on the Fitness of *Clostridium difficile In Vitro*. *Antimicrob. Agents Chemother.* **59**, 1794–1796. doi:10.1128/AAC.04503-14

Watson, J.D., Crick, F.H.C., 1953. Molecular Structure of Nucleic Acids: A Structure for Deoxyribose Nucleic Acid. *Nature* **171**, 737–738.

Watson, N., 1988. A new revision of the sequence of plasmid pBR322. *Gene* **70**, 399–403.

Webber, M.A., Buckner, M.M.C., Redgrave, L.S., Ifill, G., Mitchenall, L.A., Webb, C., Iddles, R.G., Maxwell, A., Piddock, L.J.V., 2017. Quinolone-resistant gyrase mutants demonstrate decreased susceptibility to triclosan. *J. Antimicrob. Chemother.* dkx201. doi:10.1093/jac/dkx201

Webber, M.A., Randall, L.P., Cooles, S., Woodward, M.J., Piddock, L.J.V., 2008. Triclosan resistance in *Salmonella enterica* serovar Typhimurium. *J. Antimicrob. Chemother.* **62**, 83–91. doi:10.1093/jac/dkn137

Webber, M.A., Ricci, V., Whitehead, R., Patel, M., Fookes, M., Ivens, A., Piddock, L.J.V., 2013. Clinically Relevant Mutant DNA Gyrase Alters Supercoiling, Changes the Transcriptome, and Confers Multidrug Resistance. *mBio* **4**, e00273-13. doi:10.1128/mBio.00273-13

Webber, M.A., Whitehead, R.N., Mount, M., Loman, N.J., Pallen, M.J., Piddock, L.J.V., 2015. Parallel evolutionary pathways to antibiotic resistance selected by biocide exposure. *J. Antimicrob. Chemother*. **70**, 2241–2248. doi:10.1093/jac/dkv109

Weisemann, J.M., Weinstock, G.M., 1991. The promoter of the *recA* gene of *Escherichia coli. Biochimie* **73**, 457–470.

Williams, K.P., Gillespie, J.J., Sobral, B.W.S., Nordberg, E.K., Snyder, E.E., Shallom, J.M., Dickerman, A.W., 2010. Phylogeny of Gammaproteobacteria. *J. Bacteriol.* **192**, 2305–2314. doi:10.1128/JB.01480-09

Witsø, I.L., Rukke, H.V., Benneche, T., Scheie, A.A., 2016. Thiophenone Attenuates Enteropathogenic *Escherichia coli* O103:H2 Virulence by Interfering with AI-2 Signaling. *PLoS ONE* **11**, e0157334. doi:10.1371/journal.pone.0157334

Worcel, A., Burgi, E., 1972. On the structure of the folded chromosome of *Escherichia coli. J. Mol. Biol.* **71**, 127–147.

World Economic Forum, 2016. *The Global Risks Report 2016 - 11th Edition (Insight Report No. 080116)*. World Economic Forum, Geneva.

World Health Organization, 2016. *Essential medicines* [WWW Document]. WHO Essent. Med. URL http://www.who.int/medicines/services/essmedicines_def/en/ (accessed 6.15.16).

World Health Organization, 2015. *WHO Model List of Essential Medicines: 19th List (April 2015) -- Amended November 2015* [WWW Document]. WHO WHO Model Lists Essent. Med. URL http://apps.who.int/iris/handle/10665/70640 (accessed 6.15.16).

Wu, C.-F., Valdes, J.J., Bentley, W.E., Sekowski, J.W., 2003. DNA microarray for discrimination between pathogenic 0157:H7 EDL933 and non-pathogenic *Escherichia coli* strains. *Biosens. Bioelectron*. **19**, 1–8. doi:10.1016/S0956-5663(03)00118-0

Yamamoto, T., Yokota, T., 1983. Plasmids of enterotoxigenic *Escherichia coli* H10407: evidence for two heat-stable enterotoxin genes and a conjugal transfer system. *J. Bacteriol*. **153**, 1352–1360.

Yamane, K., Wachino, J., Suzuki, S., Kimura, K., Shibata, N., Kato, H., Shibayama, K., Konda, T., Arakawa, Y., 2007. New Plasmid-Mediated Fluoroquinolone Efflux Pump, QepA, Found in an *Escherichia coli* Clinical Isolate. *Antimicrob. Agents Chemother.* **51**, 3354–3360. doi:10.1128/AAC.00339-07

Yeh, P.J., Hegreness, M.J., Aiden, A.P., Kishony, R., 2009. Drug interactions and the evolution of antibiotic resistance. *Nat. Rev. Microbiol*. **7**, 460–466.

Yoon, J.W., Hovde, C.J., 2008. All blood, no stool: enterohemorrhagic *Escherichia coli* O157: H7 infection. *J. Vet. Sci.* **9**, 219–231.

Yoshida, H., Bogaki, M., Nakamura, M., Nakamura, S., 1990. Quinolone resistance-determining region in the DNA gyrase *gyrA* gene of *Escherichia coli*. Antimicrob. Agents Chemother. 34, 1271–1272. doi:10.1128/AAC.34.6.1271

Ysern, P., Clerch, B., Castańo, M., Gibert, I., Barbé, J., Llagostera, M., 1990. Induction of SOS genes in *Escherichia coli* and mutagenesis in *Salmonella*

typhimurium by fluoroquinolones. *Mutagenesis* **5**, 63–66. doi:10.1093/mutage/5.1.63
Yu, Z., Qin, W., Lin, J., Fang, S., Qiu, J., 2015. Antibacterial Mechanisms of Polymyxin and Bacterial Resistance. *BioMed Res. Int*. **2015**, 1–11. doi:10.1155/2015/679109
Zaffiri, L., Gardner, J., Toledo-Pereyra, L.H., 2012. History of Antibiotics. From Salvarsan to Cephalosporins. *J. Invest. Surg.* **25**, 67–77. doi:10.3109/08941939.2012.664099
Zarei, M., Sclavi, B., Cosentino Lagomarsino, M., 2013. Gene silencing and large-scale domain structure of the *E. coli* genome. *Mol. Biosyst.* **9**, 758. doi:10.1039/c3mb25364c
Zavascki, A.P., Goldani, L.Z., Li, J., Nation, R.L., 2007. Polymyxin B for the treatment of multidrug-resistant pathogens: a critical review. *J. Antimicrob. Chemother*. **60**, 1206–1215. doi:10.1093/jac/dkm357
Zhao, X., Drlica, K., 2001. Restricting the selection of antibiotic-resistant mutants: a general strategy derived from fluoroquinolone studies. *Clin. Infect. Dis*. **33**, S147–S156.
Zheng, J., Cui, S., Meng, J., 2009. Effect of transcriptional activators RamA and SoxS on expression of multidrug efflux pumps AcrAB and AcrEF in fluoroquinolone-resistant *Salmonella* Typhimurium. *J. Antimicrob. Chemother.* **63**, 95–102. doi:10.1093/jac/dkn448
Zheng, J., Tian, F., Cui, S., Song, J., Zhao, S., Brown, E.W., Meng, J., 2011. Differential Gene Expression by RamA in Ciprofloxacin-Resistant *Salmonella* Typhimurium. *PLoS ONE* **6**, e22161. doi:10.1371/journal.pone.0022161
Zhou, D., Galán, J., 2001. *Salmonella* entry into host cells: the work in concert of type III secreted effector proteins. *Microbes Infect*. **3**, 1293–1298. doi:10.1016/S1286-4579(01)01489-7
Zimmerman, S.B., 2006. Cooperative transitions of isolated *Escherichia coli* nucleoids: Implications for the nucleoid as a cellular phase. *J. Struct. Biol.* **153**, 160–175. doi:10.1016/j.jsb.2005.10.011

Appendix

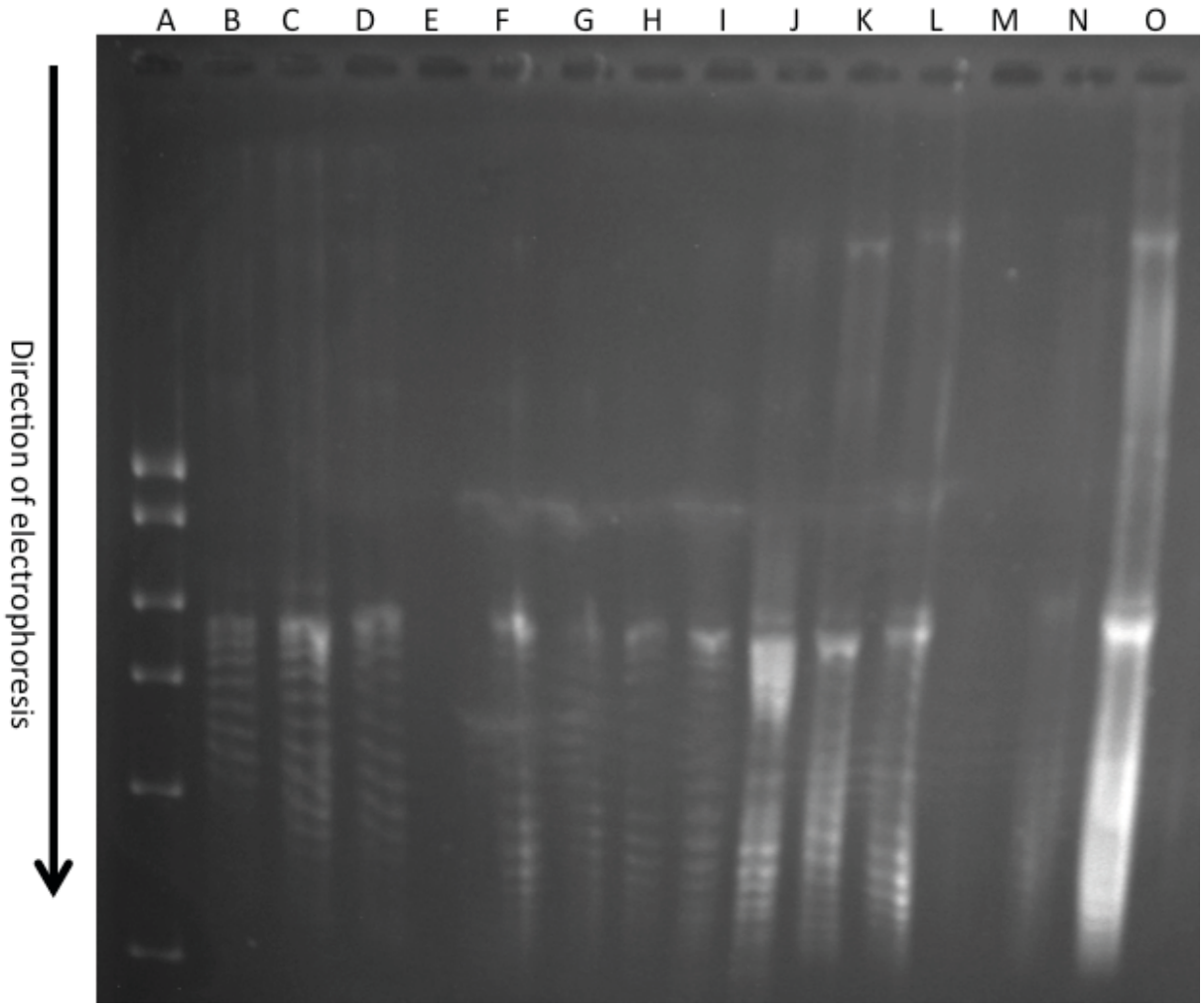


Figure A.1.1: Gel photo from which Figure 3.2.5.1 was formed.
Gel photo showing topoisomers distribution of the plasmid pBR322, extracted from cell cultures that were grown to stationary phase and then incubated for a further 45 minutes in either the presence or absence of nalidixic acid. The more supercoiled DNA is, the further it migrates down the gel. There are two samples of treated (+Nal) and untreated (-Nal) cells from each background. All samples are from *Salmonella* Typhimurium. Lanes B, G, and K were used to create Figure 3.2.5.1, and the contrast was adjusted to aid in visibility.
Lane A: HyperLadder 1kb. **Lane B:** SL1344, -Nal (1) **Lane C:** SL1344, +Nal (1) **Lane D:** SL1344 -Nal (2) **Lane E:** Empty **Lane F:** SL1344 +Nal (2) **Lane G:** GyrA Ser83Phe -Nal (1) **Lane H:** GyrA Ser83Phe +Nal (2) **Lane I:** GyrA Ser83Phe -Nal (2) **Lane J:** GyrA Ser83Phe +Nal (2) **Lane K:** GyrA Asp87Gly, -Nal (1) **Lane L:** GyrA Asp87Gly +Nal (1) **Lane M:** empty **Lane N:** GyrA Asp87Gly, -Nal (2) **Lane O:** GyrA Asp87Gly +Nal (2).

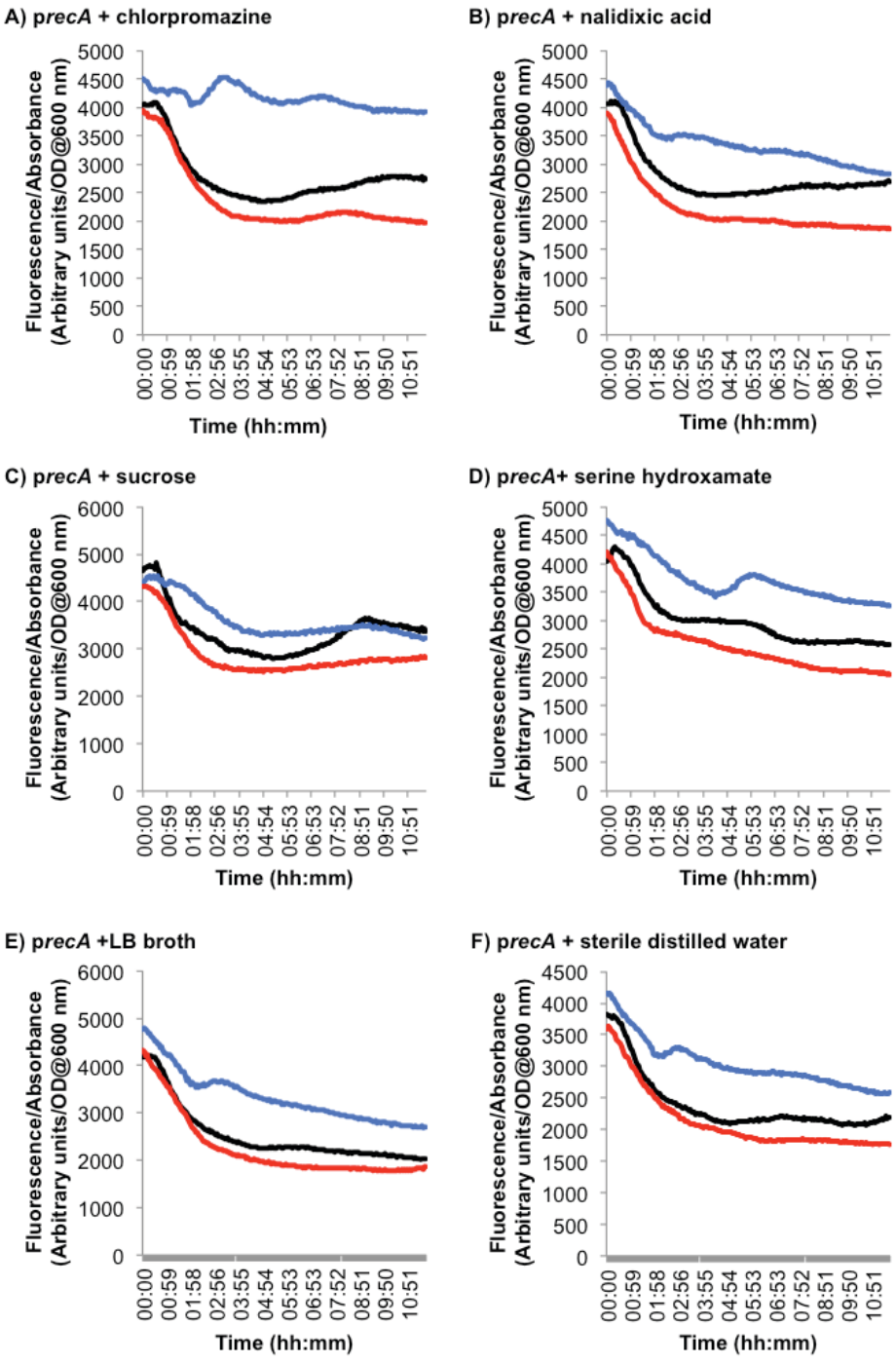


Figure A.1.2: Optical density-adjusted fluorescence levels of *precA* reporter constructs under a selection of conditions in *Salmonella.* Data shown are the averages of four independent replicates. Fluorescence appears to be higher in the GyrA Ser83Phe background than other backgrounds in all conditions tested, with fluorescence after chlorpromazine exposure appearing to be roughly constant. In the SL1344 and GyrA Asp87Gly backgrounds, the fluorescence level falls over time.

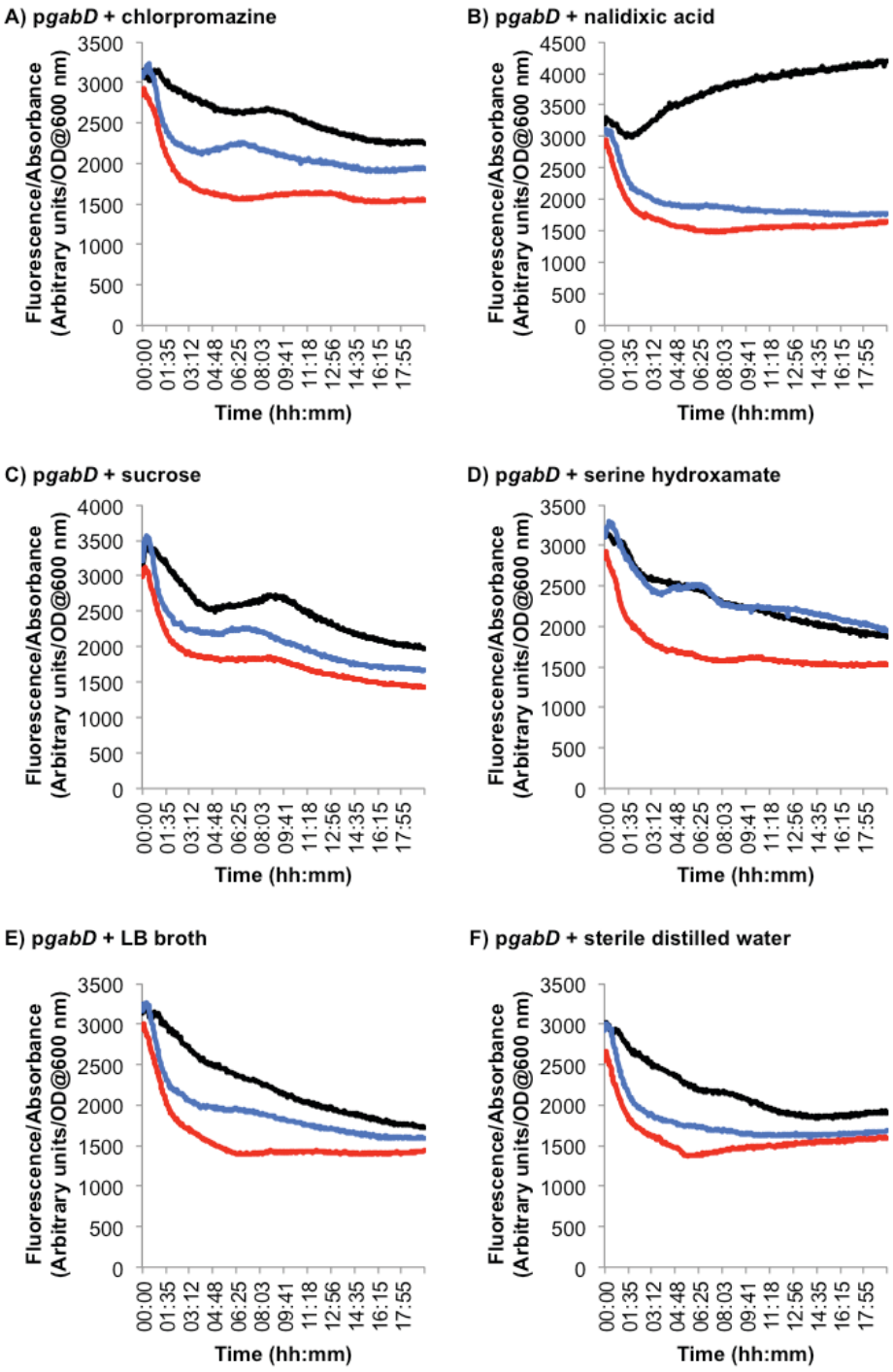


Figure A.1.3: Optical density-adjusted fluorescence levels of p*gabD* reporter constructs under a selection of conditions in *Salmonella.* Data shown are the averages of four independent replicates. Fluorescence from this reporter is highest in the SL1344 background, but reduces over time in all backgrounds in all conditions except for when the SL1344 cells were exposed to nalidixic acid, where it increases. Fluorescence in the GyrA Asp87Gly background is lowest. The fluorescence in the SL1344 and GyrA Ser83Phe backgrounds appears to be identical after exposure to serine hydroxamate.

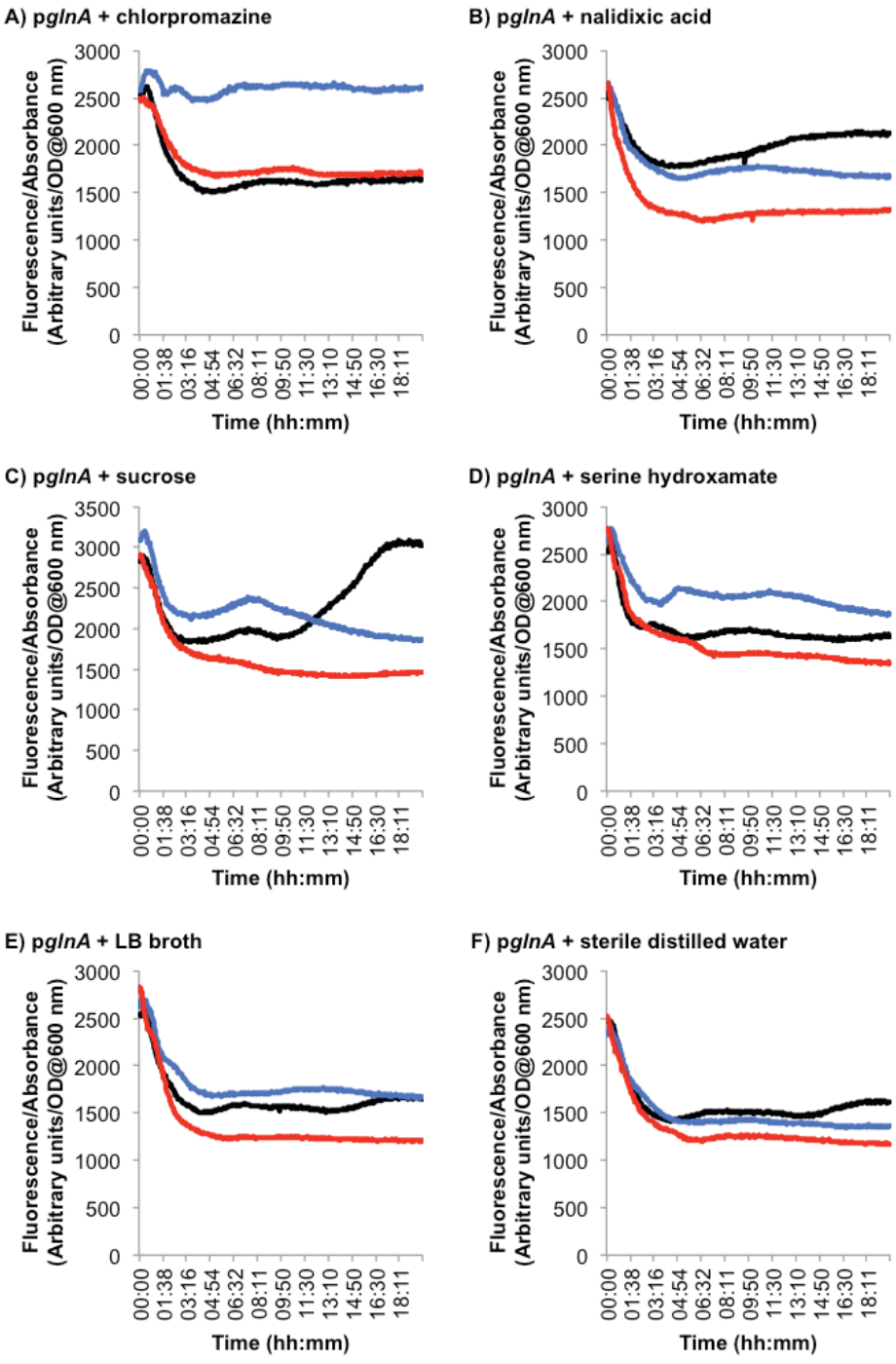


Figure A.1.4: Optical density-adjusted fluorescence levels of p*glnA* reporter constructs under a selection of conditions in *Salmonella.* Data shown are the averages of four independent replicates. Fluorescence appears to be highest in the GyrA Ser83Phe background in most conditions, though still declines and reaches a plateau after about an hour and a half in most cases. It appears to remain elevated in the GyrA Ser83Phe background after exposure to chlorpromazine. After around 10 hours of sucrose exposure, fluorescence increases in the SL1344 background.

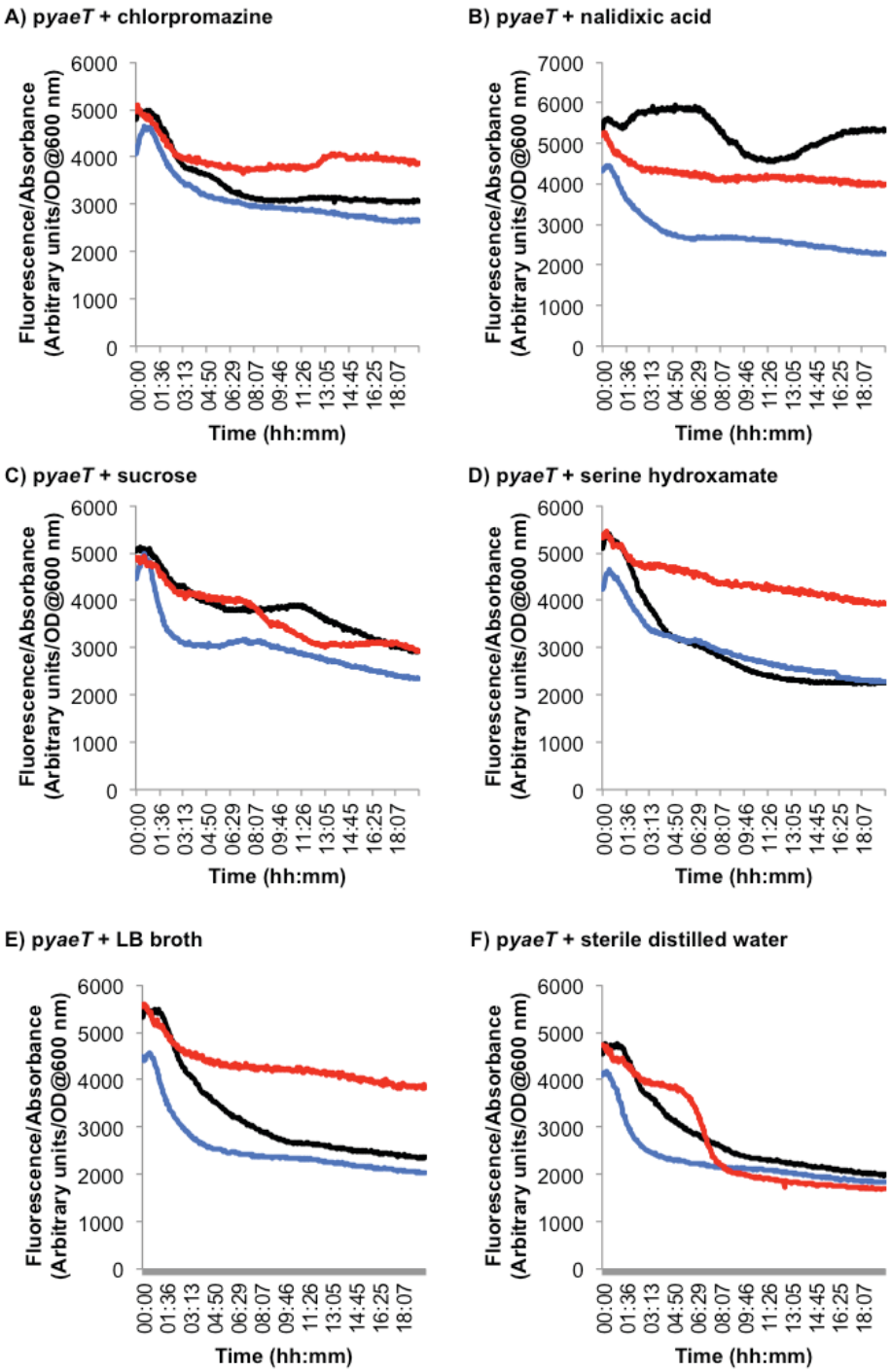


Figure A.1.5: Optical density-adjusted fluorescence levels of *pyaeT* reporter constructs under a selection of conditions in *Salmonella.* Data shown are the averages of four independent replicates. Fluorescence appears to decline much less sharply in the GyrA Asp87Gly background than others, except when cells were exposed to sucrose or sterile distilled water. Fluorescence is generally highest in this background, though it is similar in SL1344 cells after sucrose exposure, and is higher in SL1344 cells after exposure to nalidixic acid. Fluorescence is lowest in GyrA Ser83Phe cells.

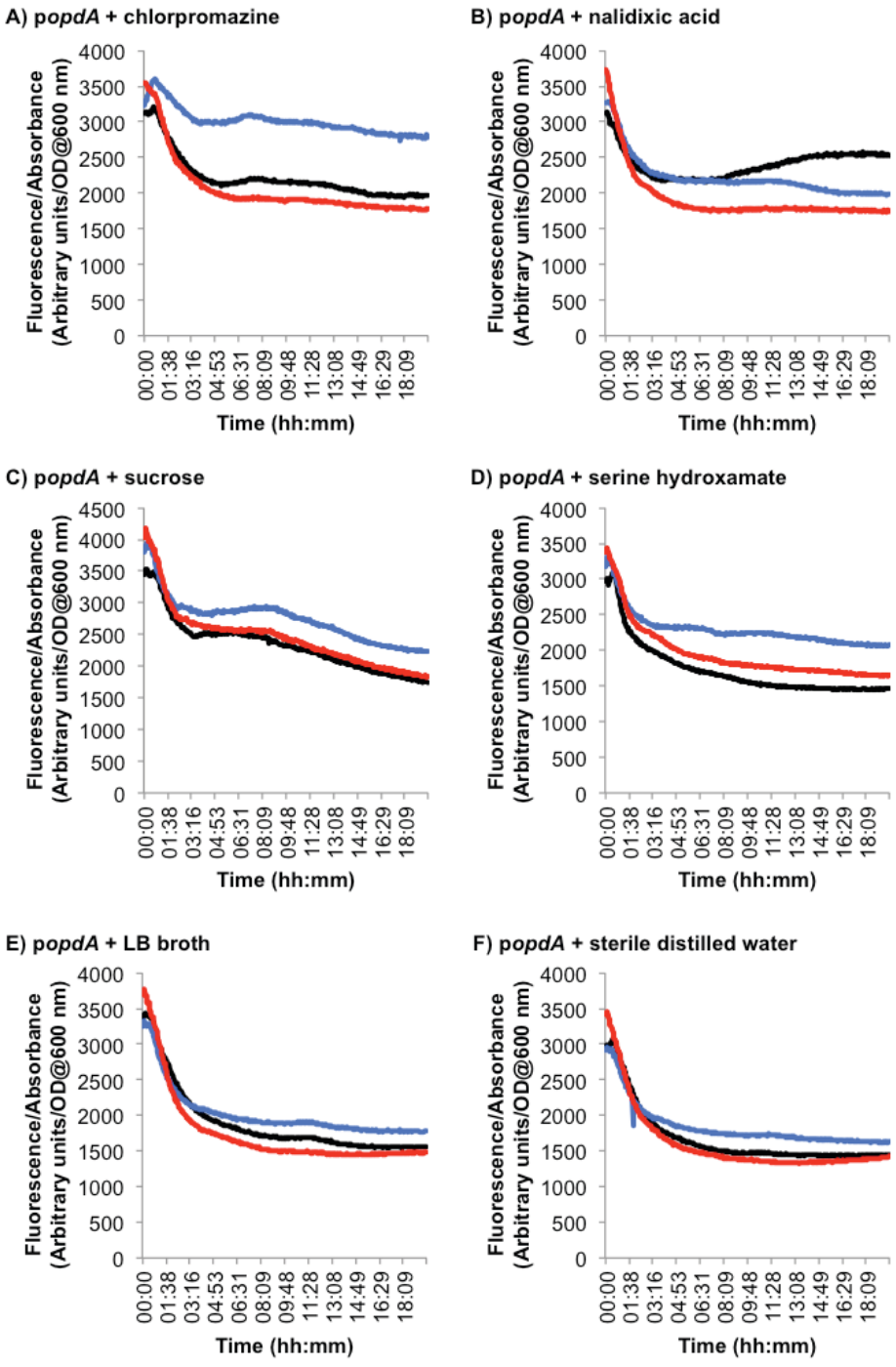


Figure A.1.6: Optical density-adjusted fluorescence levels of *popdA* reporter constructs under a selection of conditions in *Salmonella.* Data shown are the averages of four independent replicates. Fluorescence declines over time in all backgrounds in all conditions, though declines more slowly in GyrA Ser83Phe cells after exposure to chlorpromazine. There is also an increase in fluorescence levels in the SL1344 background after nalidixic acid exposure, at around 8 hours.

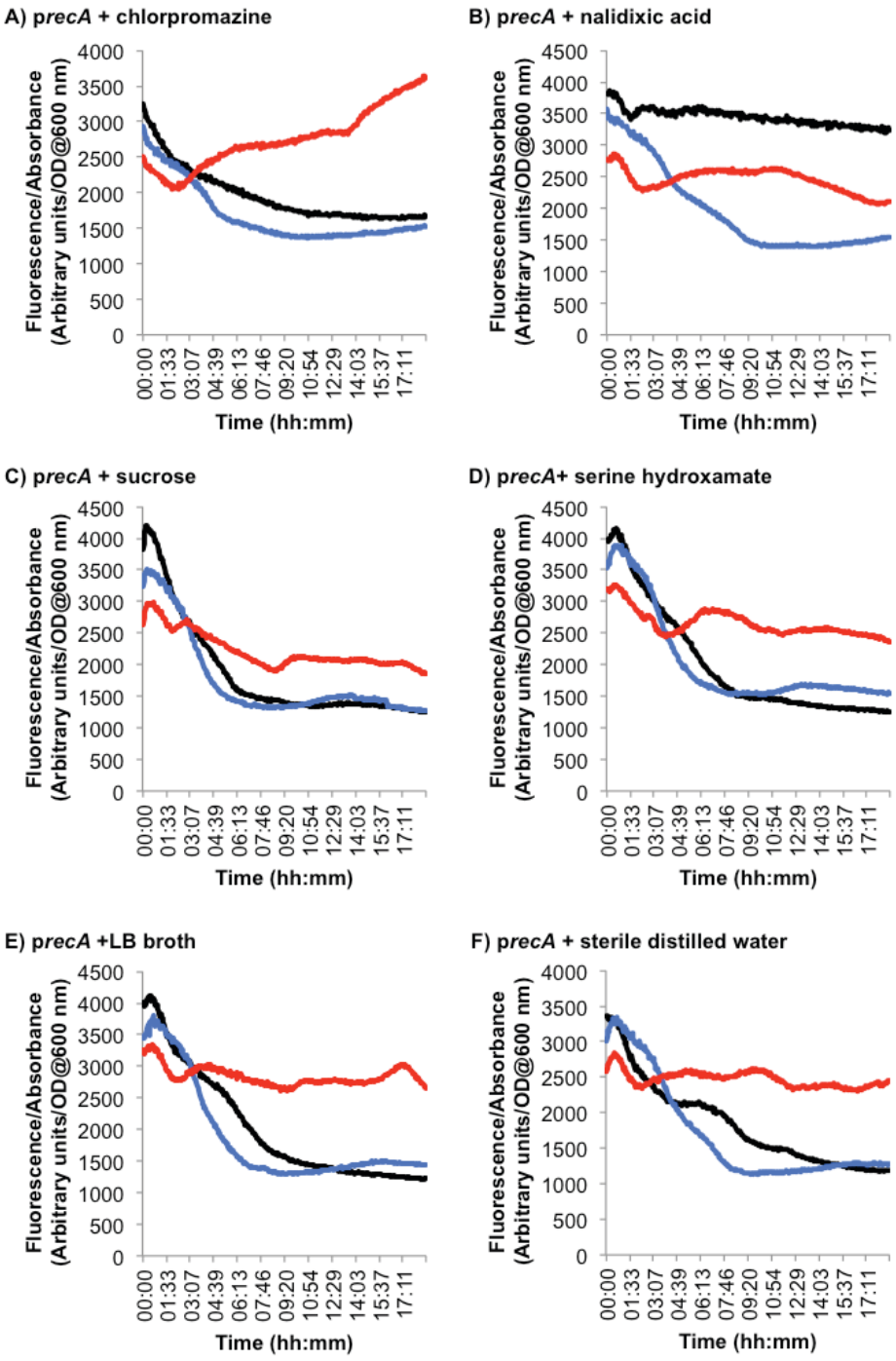


Figure A.1.7: Optical density-adjusted fluorescence levels of *precA* reporter constructs under a selection of conditions in *E. coli*. Data shown are the averages of four independent replicates. Fluorescence remains roughly constant over the experimental period in the GyrA Asp87Gly background, except after exposure to chlorpromazine, where it increases over time. Fluorescence also remains at a high level and declines slowly in the MG1655 cells after exposure to nalidixic acid. In all other cases, fluorescence in MG1655 and GyrA Ser83Phe cells declines over time.

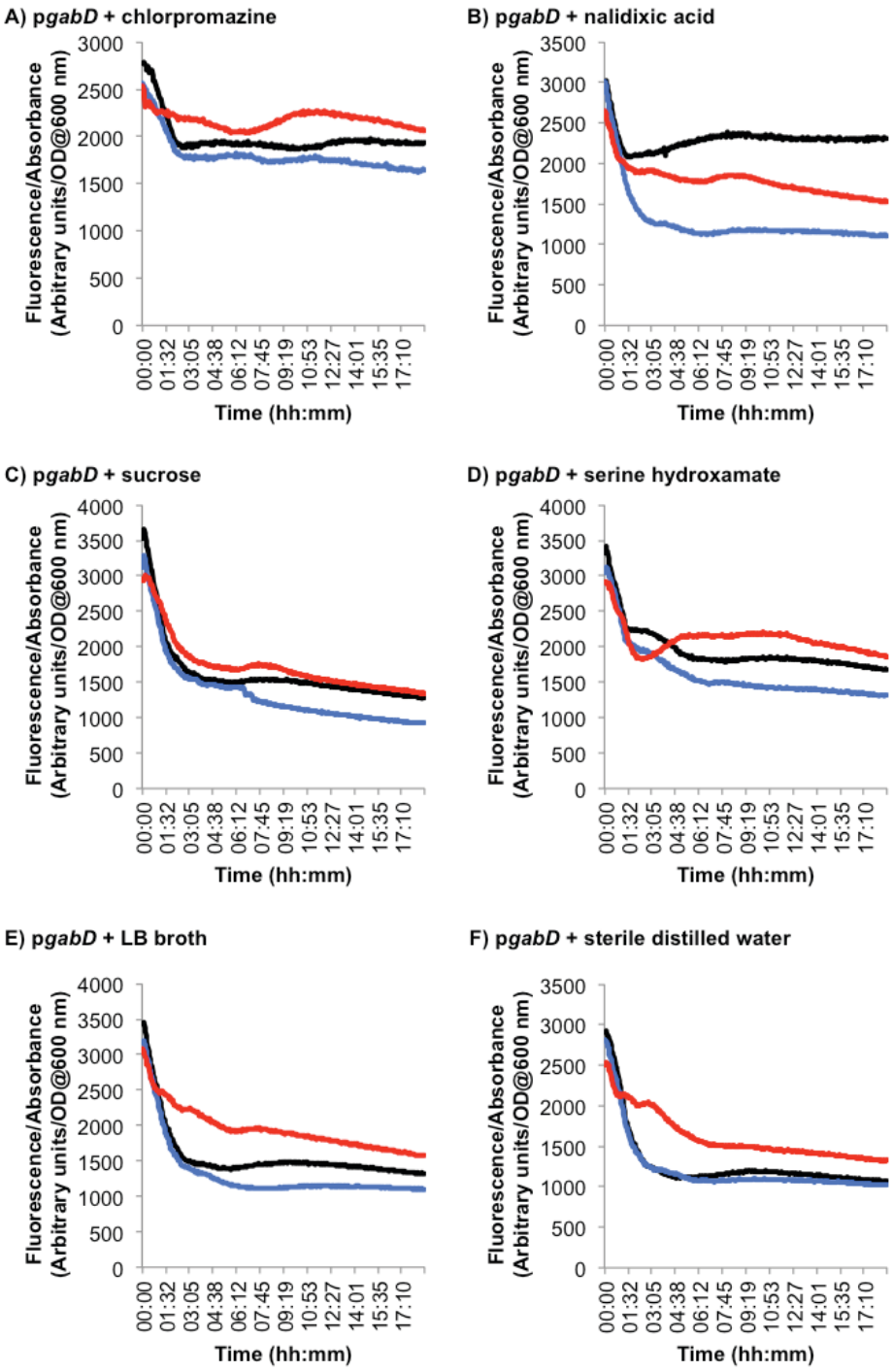


Figure A.1.8: Optical density-adjusted fluorescence levels of p*gabD* reporter constructs under a selection of conditions in *E. coli*. Data shown are the averages of four independent replicates. Fluorescence reaches a plateau in all backgrounds after chlorpromazine exposure, but is highest in GyrA Asp87Gly cells. Fluorescence decreases over time in most other conditions in all backgrounds, and fluorescence in the GyrA Asp87Gly background is usually highest. The exception is nalidixic acid exposure, where fluorescence in the MG1655 background is highest and reaches a plateau higher than other backgrounds after 6 hours.

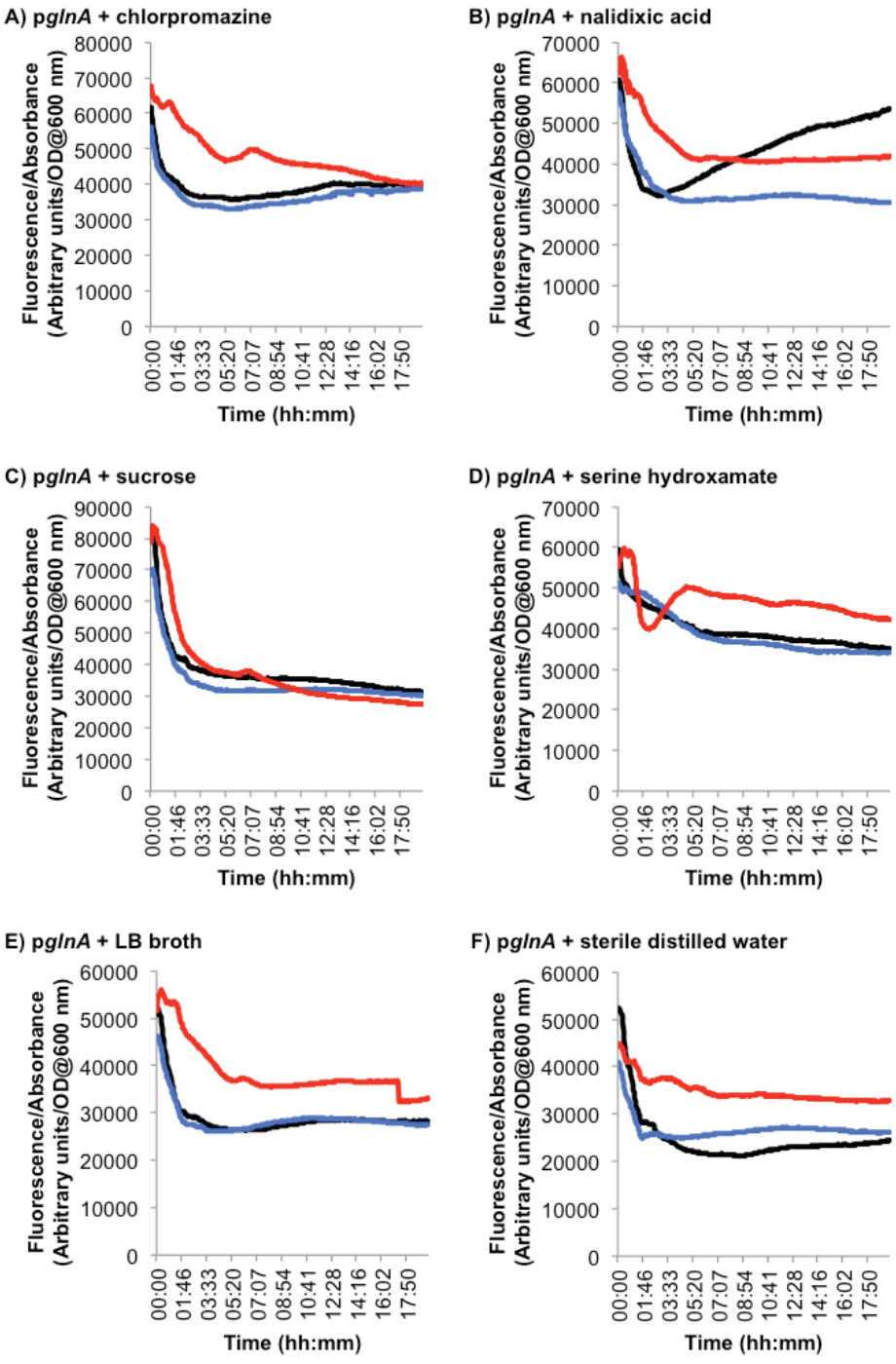


Figure A.1.9: Optical density-adjusted fluorescence levels of p*glnA* reporter constructs under a selection of conditions in *E. coli.* Data shown are the averages of four independent replicates. Fluorescence in GyrA Asp87Gly cells is highest and lowest in GyrA Ser83Leu cells in most conditions. However, fluorescence increase over time in MG1655 cells after exposure to nalidixic acid, after reaching a nadir at around 2 hours.

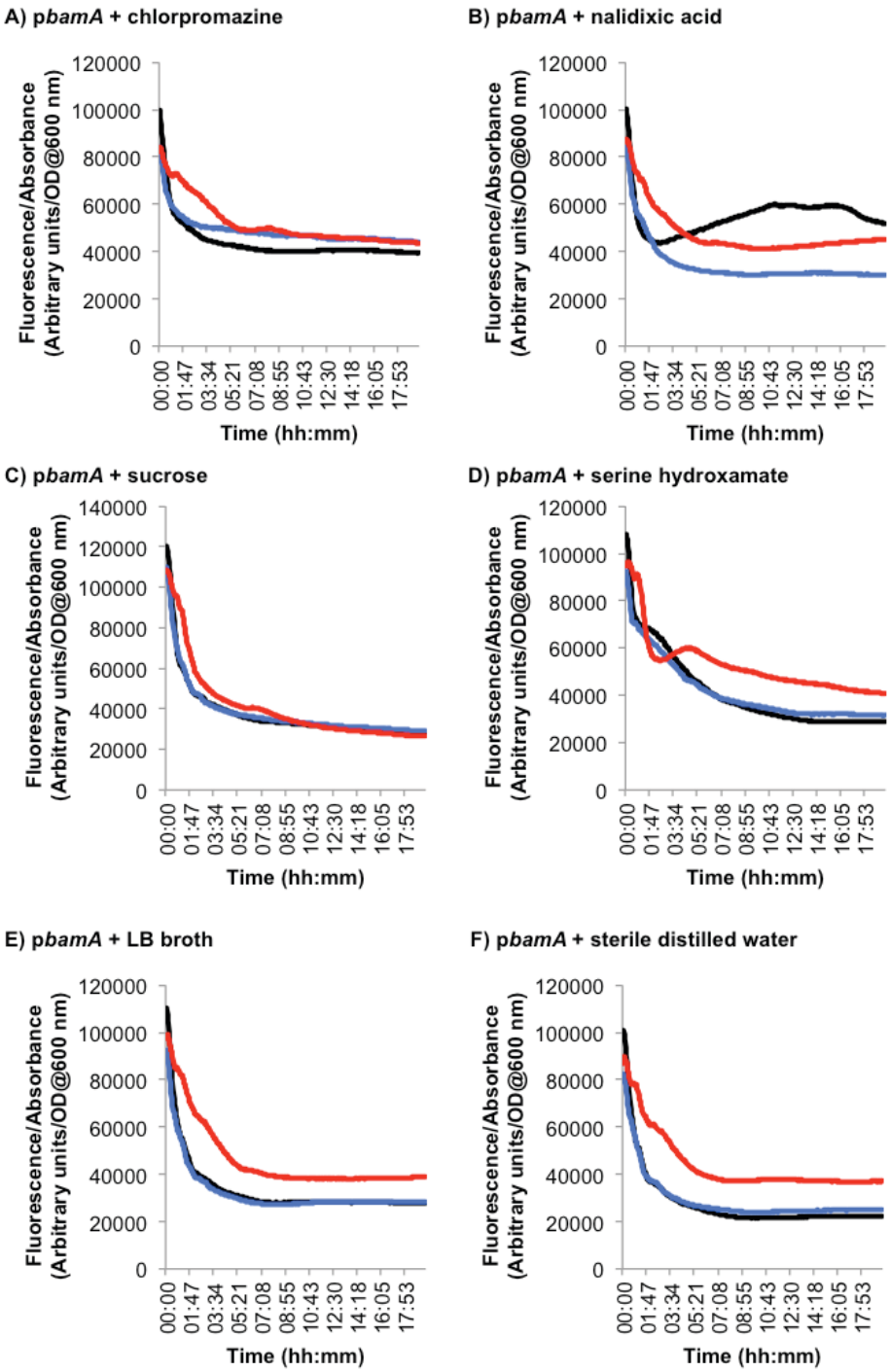


Figure A.1.10: Optical density-adjusted fluorescence levels of p*bamA* reporter constructs under a selection of conditions in *E. coli.* Data shown are the averages of four independent replicates. Fluorescence appears to be highest in GyrA Asp87Gly cells in all conditions, with the exception of after nalidixic acid exposure, where it is suprassed by MG1655 cells at around 5 hours. In most conditions, fluorescence levels in MG1655 cells and GyrA Ser83Phe cells appears identical.

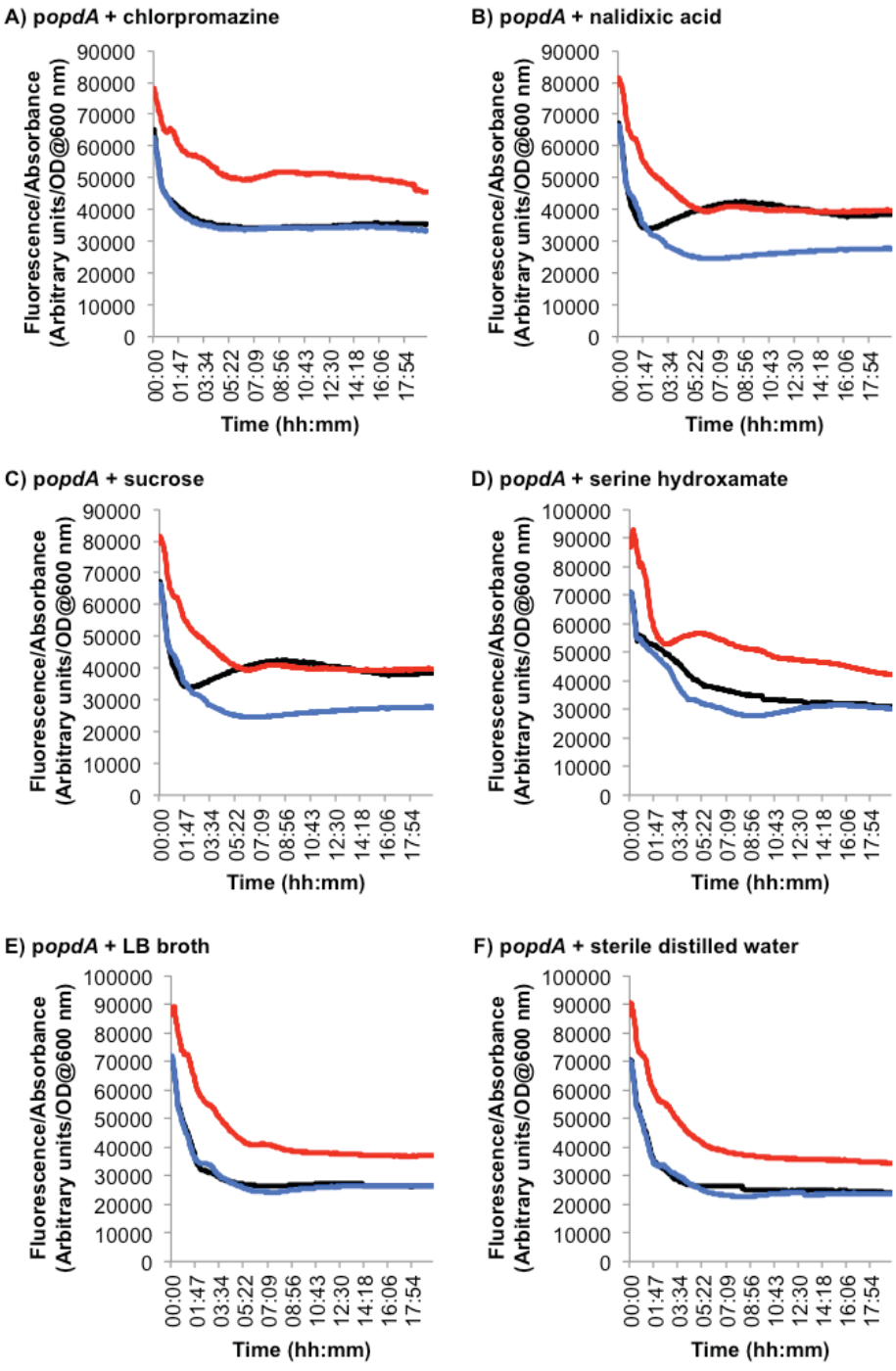


Figure A.1.11: Optical density-adjusted fluorescence levels of p*opdA* reporter constructs under a selection of conditions in *E. coli.* Data shown are the averages of four independent replicates. Fluorescence is generally highest in GyrA Asp87Gly cells. It increases over time in MG1655 cells exposed to sucrose and nalidixic acid, after reaching a nadir at around 2 hours, ultimately reaching similar levels of fluorescence to GyrA Asp87Gly cells exposed to the same inducers. Fluorescence in GyrA Ser83Leu cells generally follows the same downward trend as MG1655 cells, with the exception of the two aforementioned conditions.