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Elizabeth Joyce
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Professor Nicholas Loman and Professor Neil Thomas

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Abbreviations

AMR – Anti-Microbial Resistance
MDR – Multi drug resistant
MRSA – Methicillin resistant *Staphylococcus aureus*
VRE – Vancomycin resistant *Enterococcus*
HGT – Horizontal gene transfer
MGE – Mobile genetic elements
PBP – Penicillin binding protein
NAG – N-acetylglucosamine
NAM – N-acetylmuramic acid
SNP – Single nucleotide polymorphism
RCA – Rolling circle amplification
NEB – New England BioLabs
PCR – Polymerase chain reaction
RT - Room temperature
NF - Nuclease free
LB – Luria Broth
SDS-PAGE – Sodium dodecyl sulfate – polyacrylamide gel electrophoresis
RPM – Rotations per minute
TEV – Tobacco etch virus
SOC - Super optimal broth with catabolite repression
IPTG - Isopropyl β -D-1-thiogalactopyranoside
BSS – Bismuth subsalicylate
DMSO - Dimethylsulphoxide
ICPMS - Inductively Couple Plasma Mass Spectrometry

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1. Introduction

Antimicrobial Resistance

Infectious diseases have been our constant competitor throughout history. As we develop means to combat them, they evolve the means to evade. However, with the discovery of antibiotics, medicine and science has been revolutionised, making possible incredible advancements that would otherwise be beyond our capabilities. This is due largely to ‘magic bullet’ properties of antibiotics, in that they are able to target and kill specific microbes without causing significant harm to the host, and as such can cure diseases that have been plaguing humanity for centuries. Because of antibiotics, medical procedures such as surgery, organ transplantation and cancer therapies that were once thought of as impossible are regularly performed with success, transforming the lives of people who undergo these procedures (1,2).

They have also moulded the way in which microbiology is studied within the lab and have facilitated the discovery and isolation of a plethora of microbes, acting as an essential tool for scientists to utilise. However, due to a number of reasons, antimicrobial resistance is becoming rapidly more commonplace, rendering many classes of antibiotic useless. An overdependence on and overexploitation of antibiotics is leading inexorably towards a global health crisis that we are currently poorly equipped to cope with: one that has been described as ‘Apocalyptic’(2). In this chapter I aim to outline the history and development of antibiotics, as well as the rise of antimicrobial resistance and possible actions that could be taken to mitigate the disaster of worldwide antimicrobial resistance.

Antibiotics are defined as substances that are able to kill or inhibit the growth of bacteria, and are used particularly to treat bacterial infections (3). They are often biological in origin, derived from the secondary metabolites produced by fungi and other bacteria, although more recently antibiotics have been developed that are either partially or fully synthetic (3). Although the concept of antibiotics as we know it today is tied to the development of germ theory by Louis Pasteur (1822-1895) and Robert Koch (1843-1910) (4), humans have been using antimicrobial compounds for centuries to treat diseases and preserve food. This was usually performed without awareness of their mechanisms of action, for example the application of mouldy bread to open wounds in ancient Egypt to abate infection (5). The potential of using mould to treat infections had been noted several times by scientists throughout the years, including John Scott Burdon-Sanderson (1828-1905) and Joseph Lister (1827-1912), culminating in the famous discovery of penicillin by Alexander Fleming in 1928 (4). It was Fleming that recognised the significance and potential applications of the discovery, and Howard Florey and Ernst Chain who developed the purification technique allowing the first commercial antibiotic, penicillin, to be developed (6). Following this came the ‘Golden Age’ of antibiotic discovery, and many of the antibiotic families established then are still mainstays of medical therapy, such as the beta-lactams and glycopeptides (7).

It is safe to say that antibiotics have made possible a multitude of procedures that would otherwise prove impossible, and their applications are countless. Within medicine, effective treatment of infection allows for surgical treatments to have a much lower infection rate, and they can be used prophylactically within organ transplantation, joint replacements, and within cancer therapy (8,9). They are invaluable for those with compromised immune systems. Microbiological lab work is also dependent upon antibiotic selective media to grow the bacteria of choice for study, as resistance plasmids can be inserted into the bacteria of interest

it to allow it to grow unhindered. Within the agriculture industry, antibiotics can also be used for growth promotion, to improve the meat yield of farm animals, although this practice is now illegal in many places (10).

Some of the early antibiotics such as vancomycin and cephalosporin were derived from soil microbes that had developed antibiotic biosynthesis likely to allow them to outcompete bacterial contenders within their ecological niche (11). This unfortunately however has primed many bacteria with an innate resistance to many antibiotics due to this evolutionary arms race, and so advantageous resistance genes are already present within the environment (known as the environmental resistome) and are easily passed on to other surrounding bacteria (12). Even from the first experiments performed using penicillin by Alexander Fleming, it was almost immediately apparent that use of antibiotics encouraged formation of resistance against them, and hence it was necessary to obtain new and diverse classes of antibiotics to ensure their continued effective use. To begin with, this was relatively simple, and within the first 20 years after the discovery of penicillin, almost all of the major groups of antibiotics that we still use today had been discovered. Unfortunately, the antimicrobial pipeline has somewhat dried up, the reasons being multifaceted (4,7).

It is still difficult to culture the majority of bacterial and fungal species within lab conditions, and as such their secondary metabolites which are the main source of antibiotics, are difficult to produce and test, and so only those that survive well in lab conditions have been identified (13). As well as this, negative results are more rarely published, so the same ground is prone to being re-trodden many times. Legislation for approval of drugs for human use is stringent, and as such many promising compounds do not meet criteria to pass clinical testing, due to unacceptable side effects. One of the largest barriers to antibiotic development is lack of interest from pharmaceutical companies, as antibiotics are seen as unprofitable investments. This is due to the fact that they are usually short-term curative treatments as opposed to long term therapies i.e. for cancer, and as such are less lucrative (14). In addition, an effective antibiotic must act specifically on prokaryotic cells, without incurring severe negative effects on eukaryotic cells, limiting the targets the antibiotics can act on. Antibiotics for the most part work by exploiting the physiological differences between the host and bacterial cells, and antibiotics are generally grouped by which of the three main differences they target (3).

Mechanisms of Action

In general, antibiotics will target either protein synthesis, nucleic acid synthesis, or the cell walls of the bacteria. They tend to act either bactericidally (killing the cell) or bacteriostatically (stopping growth and division of the bacteria). Penicillin is one such compound that disrupts the cell wall and is of the class of antibiotics known as beta-lactams, along with methicillin, flucloxacillin, and ampicillin. They are so called because of the characteristic beta-lactam ring in the chemical composition of each of these drugs (5). These drugs target and disrupt cell wall biosynthesis, specifically by competitively binding the penicillin binding protein (PBP), an enzymatic component of the peptidoglycan layer within the cell walls of bacteria. The peptidoglycan layer is formed primarily of a backbone of two molecules; N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM). These units are joined by chains of pentapeptides that are connected to the NAG and NAM molecules by processes of transglycosylation and transpeptidation, carried out by transpeptidase enzymes such as PBP. These enzymes form the cross-links that stabilises the cell wall during biosynthesis. The beta-lactam ring is structurally similar to the D-alanine-D-alanine component of the cross-linking peptides. These peptides are enzymatically split apart during

cell growth to enable new sections of cell wall to be constructed, but in the presence of beta-lactams, the PBP enzymes are competitively bound to the antibiotics, reducing their availability, and stopping them from catalysing the cross-linking of the cell wall components. This stalls the cell wall production, and the cell membrane begins to bleb out of the gaps in the wall, leading to cell lysis (15).

Another type of antibiotic that targets cell wall biosynthesis are the glycopeptides, which include teicoplanin and vancomycin. Glycopeptides work by binding to the two terminal amino acids in the pentapeptide chain, D-alanyl-D-alanine, and forms 5 hydrogen bonds with them. This blocks the action of the cross-linking enzyme PBP, and prevents new cell wall synthesis from occurring, disrupting the growth of the cell. In addition to this, bacteria also facilitate their own degradation of the cell wall to allow for growth, and so enhance the action of the antibiotic. Changes in osmotic pressure in and around the cell can also lead to lysis at the points of the weakened cell wall; hence glycopeptides are very effective at producing a bacteriostatic, and in some cases, bactericidal mechanism of action (16).

Protein biosynthesis is also an essential process for bacterial cells to grow and proliferate. The process works by the transcription of RNA from DNA, and then the translation of that RNA into proteins using bacterial ribosomes, which consist of a 50S and a 30S subunit (17). These subunits are the targets of aminoglycosides and tetracyclines (30S), and chloramphenicol, macrolides, lincosamides, and oxazolidinones (50S) (3).

Macrolides interfere with and cause the premature termination of the peptide chain as it is being synthesised, causing the proteins to become truncated and have reduced function (17). Aminoglycosides are bactericidal, broad spectrum antibiotics that bind to the ribosomal RNA 16S site on the 30S, which leads to a conformational change of the ribosome, resulting in misreading of the codon and the incorporation of incorrect amino acids into the protein. These aberrant proteins may then damage the other cellular components such as the cell membrane (18). Another broad-spectrum family of antibiotics is the tetracyclines. First derived from soil samples in the 1940's, many tetracyclines have been synthetically modified to increase their efficacy, although the development of resistance has curtailed their use somewhat. The mechanism of actions of these drugs is related to its ability to bind multiple sites on the 30S and 50S ribosomes, with the highest affinity binding site on the 30S subunit, which blocks the binding of aminoacyl tRNA to the subunit and so halts proteins synthesis (19,20). A family of fully synthetic antibiotics called the Oxazolidinones, including linezolid, also interferes with protein synthesis, largely within Gram-positive bacteria. The antibiotics bind solely to the 50S subunit, and blocks the formation of the ribosomal complex, and thus the ability of the bacterial cell to synthesize proteins (21).

The third target exploited by antibiotics is nucleic acid synthesis. The main contender in this category are the fluoroquinolones, as well as nalidixic acid, that act by inhibiting the bacterial enzyme DNA gyrase. This enzyme creates nicks in the DNA and facilitates supercoiling, before resealing the nick to reduce stress on the DNA strands during replication (22). The fluoroquinolones act by preventing the nicking and resealing of the strands. This has a bactericidal effect, and is largely effective against Gram-negative bacteria (23). Also worth noting are the sulphonamides, a synthetic family of antibiotics that disrupts the cellular biosynthesis of folic acid, a compound essential for bacterial nucleic acid synthesis. The sulphonamides are structurally analogous to para-aminobenzoic acid, a precursor of folic acid, and compete with it in the metabolic pathway and thus disrupt it (24).

The Development of Resistance

Anti-microbial resistance is the development of methods by microbes (for this review, usually bacteria) to circumvent or reduce the action of antibiotics against them. Resistance can occur as either an innate or as an acquired trait, which can be shared within bacterial populations. Innate resistance occurs because the target for the antibiotic does not exist within the bacteria, or the antibiotic is unable to reach the target site. For example many glycopeptide antibiotics (e.g. vancomycin) are ineffective against Gram-negative bacteria, as the drugs are not able to permeate into the cell through the outer membrane (25,26). Acquired resistance refers to cases where bacteria that were originally susceptible to an antibiotic have attained means to reduce the effect of the drug, either through a mutation or the uptake of genes from external sources (27).

Resistance arises because antibiotics exert a selective pressure on susceptible microbes, so the few within a population that have acquired resistance will be the only ones to survive an encounter with the antibiotics and will then be free to proliferate even when treated with said antibiotic (27). The rise of these resistant pathogens means that treatments of bacterial diseases are becoming ineffective, particularly in cases of multi-drug resistant (MDR) pathogens such as methicillin resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus* species (VRE) particularly *E. faecium* and *E. faecalis*, which are causing a marked increase in patient mortality and morbidity (27–29). MDR pathogens are defined as being non-susceptible to at least one compound in three or more classes of antibiotics (30), and are associated with higher morbidity and mortality. Pathogens that become resistant to a wide range of antibiotics can become essentially untreatable, and are a looming threat within the medical community (25).

Resistance to beta-lactams was observed soon after the discovery of penicillin itself, with Alexander Fleming noting that the misuse of the compound could lead to the selection of resistance within bacterial populations (26). Bacteria have a great capacity to both modify their genomes and to alter their gene expression to adapt to the environment around them. Bacterial genomes are highly malleable and tolerate mutations well, and many bacterial species exploit this genetic plasticity, invoking mechanisms to increase the rate of mutagenesis when under stress conditions (31). This creates a larger pool of genetic diversity that can be utilised to allow survival in a range of challenging conditions (25). Additionally, bacteria are able to incorporate foreign genetic material into their genome through horizontal gene transfer (HGT). HGT can work via transduction (phage borne), transformation (uptake of exogenous DNA) or conjugation (transferal of DNA from one bacterium to another, usually through a ‘sex’ pilus) (32). The genetic material that is shared via conjugation is usually in the form of mobile genetic elements (MGE), for instance small, circular, extra-chromosomal rings of DNA called plasmids, or transposons: both of which can carry AMR genes between species of bacteria (26).

Resistance mutations function in one of several ways. By altering the genes, the bacteria may produce a target protein that has a reduced affinity with the antibiotic, and renders it ineffective, or it may bypass the production of the antibiotic target with alternative metabolic pathways. The bacteria may also produce additional enzymes that can degrade the antibiotic (as is the case with beta-lactamases), or produce efflux pumps that remove the drug from the cell and away from the target site (25). In the case of the bacteria *E. faecium*, a single base pair change from a G to a T in the 23S rRNA gene (which codes for the 50S ribosome) is enough to reduce the effectiveness of the antibiotic linezolid. Linezolid usually acts by

binding to the 50S ribosome subunit and thus blocks protein synthesis, however the mutation is enough to reduce the binding of the antibiotic to the ribosome, and so confer resistance to it (21). Because many antibiotics are derived from environmental microbes, it is also important to recognise that many resistance genes are present innately in the environment, being a product of the co-evolution of the antibiotic producing microbe and those competing with it to occupy the same ecological niche. Resistance genes are also often present within the microbe that produces the antibiotic itself, so that it is not killed by its own metabolites (25).

Overcoming Anti-Microbial Resistance

Antimicrobial resistance is devastating both from a healthcare perspective and from a socioeconomic perspective. Each year, an estimated 50, 000 deaths are associated with AMR disease in the US and Europe, with a further 10 million deaths per year predicted by 2050 if no action is taken. The cost of failed treatments, prolonged illness, work hours missed, and time spent within the healthcare system negatively impacts a countries economic output, and the global cost is estimated to be in the trillions of US dollars (33). A key aspect of reducing the spread of AMR is to prevent its development in the first place.

Reducing the reservoir of AMR genes available in the environment to pathogens would limit their capacity to uptake those genes, and as such the pathogens would be dependent upon spontaneous mutations in order to develop resistance (34). As the selective pressures of antibiotics expedites the development of resistance, reducing the amount of antibiotics they are exposed to would be a logical initial step. However, antibiotics are so prevalent in the environment (particularly within areas of agriculture, aquaculture, and healthcare) that this would be a difficult task (26). Carrying AMR genes sometimes incurs a fitness cost on the organism, because of energy requirements to replicate and maintain the increased chromosome size or the presence of a plasmid, as well as the energy needed to express the protein that confers the resistance. An example of this is the AMR mutation in the ribosome of *Escherichia coli* (*E. coli*), *rspL*, which confers resistance to streptomycin but slows the rate of peptide elongation (35). However, this fitness cost is not always apparent (10). Nonetheless, it has been shown that removing the selection pressure of environmental antibiotics has been shown to reduce the proliferation of AMR in bacteria (36).

This reduction of antibiotic use is a complex and difficult problem, and any solution would require cooperation between medical, educational, and legislative bodies to enable to coordinated approach. Education of patients and healthcare providers, as well as legislation to limit the type and quantity of antibiotics prescribed would be a solid foundation, and improved management of currently available antibiotics could slow the progression of AMR (37). The discovery of new antibiotics and disease treatments would be a benefit, but without proper precautions would suffer the same fate as current drugs. A relatively innocuous approach would involve working to improve health and sanitation of the general population, as this would prevent disease development in some cases, and so would negate the need for antibiotic treatments. Implementation of vaccine schemes where available for diseases would also work prophylactically to reduce the need for disease treatment (37). Using several antibiotics in combination with a range of modes of action could also limit AMR development, however this approach would need to be carefully crafted to avoid discouraging patients from complying with treatment if undesirable treatment schedules and side effects were to be exacerbated (38). Another approach would involve treating the virulence of a pathogen instead of attempting to kill it outright, which would make the bacteria less damaging to the host and may also alleviate the selective pressure that is imposed by

antibiotics. While this would not remove infection, it would likely reduce mortality linked to, and the emergence of, antimicrobial resistance (39).

Within the agricultural and veterinary industries, antibiotic use and misuse is rife. Within the US, antibiotics are administered as growth promoters to livestock to increase yield, with as much as 70% of all antibiotics used in the US used for this purpose. Much of the drug is excreted from the animal unaltered, and contaminates soil and groundwater around the facilities (26,34). Antibiotics that are medically important for the treatment of humans (e.g. penicillin and tetracycline) should be excluded from use in agriculture, particularly in the egregious cases when they are used on healthy animals (34).

One promising avenue of research into AMR concerns using new technologies such as the Oxford Nanopore sequencer to identify resistance genes within patient infections. This formed the subject of the research rotation undertaken at the University of Birmingham. Rapid genome sequencing using the nanopore allows for early diagnosis of the bacterial agents causing an infection in patients, as well as identification of any AMR genes that it might have and would allow for real-time monitoring of disease progression. This would inform the decision of what the most effective course of action would be and would allow the tailoring of specific treatment plans for the patient to cure them quickly and efficiently. It would also reduce side effects of unnecessary antibiotic use for the patient such as antibiotic associated diarrhoea. Whole genome sequencing data from these infections could then be deposited in a database to add to the bank of information available for future studies, and could lead to the identification of new targets that could be exploited for antibiotic treatments. The project involved using rolling circle amplification to allow for more accurate genome reads to be acquired, which would allow for the identification of single nucleotide polymorphisms (SNPs), particularly the mutation conferring resistance to the antibiotic linezolid on the 23S gene of the pathogen *Enterococcus faecium* (*E. faecium*) (40). Identifying this mutation would prevent treatment of patients with ineffective drugs, helping to cure patients more quickly and reduce the development of AMR.

Another area of research worth exploring involves developing improved drug delivery of antibiotics to increase their effectiveness at treating diseases. In the second research rotation undertaken this year, the effectiveness of encapsulating bismuth salts in a protein shell of apoferritin was examined. Bismuth salts such as bismuth subsalicylate have been used for the treatment of *Helicobacter pylori* (*H. pylori*) infections for decades. *H. pylori* is a bacterial pathogen that can lead to stomach ulcers that can eventually progress to gastric cancer if not treated, however antibiotic resistance to the first line treatment, clarithromycin, has been observed more frequently in recent years (41). Ferritin is a protein found ubiquitously throughout prokaryotic and eukaryotic life, which is usually used to contain iron when not needed by the cell to prevent oxidative damage. In this rotation project, the iron core was removed to form hollow apoferritin, and attempts were made to encapsulate the bismuth salts. Apoferritin-drug complexes have previously been used to target drug delivery to the cells of interest, and provide a more concentrated dose of the drug to the target tissue alone (42). If this process was successful with antibiotic therapy, it would allow antibiotics to be delivered solely to the site of infection and require smaller doses of antibiotic to reach minimum inhibitory concentration against *H. pylori*. Bismuth administered with antibiotics has been shown to reduce the development of AMR in *H. pylori* (43), and in the future this approach could be applied to other pathogens. There is no one single solution for the antibiotic crisis. It will require years of research on many different fronts to avert disaster, but it must be done.

Antimicrobial resistance is a problem without borders, and so the solution should be a global effort.

2. Evaluating Molecular Methods for Antimicrobial Resistance (AMR) Detection Using Singlemolecule Real-Time Nanopore Sequencing

Abstract

Diagnosis of diseases can be hindered by slow and imprecise blood cultures, and incorrect treatment can help spread antimicrobial resistance (AMR) genes. Using modern, high-throughput genetic sequencing technologies such as the MinION can increase the speed of identification of the causative pathogens, and inform treatment decisions by highlighting the presence of resistance genes at point of care. However, MinION sequencing is impeded by low coverage rates that obscure the presence of mutations that can confer AMR. By using rolling circle amplification of the 23S gene of the opportunistic pathogen *Enterococcus faecium* (*E. faecium*), this study presents a protocol for DNA sequencing and processing that improves coverage and identification of mutations, as well as allowing >99% species level identification of bacteria.

Introduction

Antimicrobial resistance (AMR) is an emerging problem that threatens to render useless many of the treatments and procedures we take for granted. Bacteria are able to circumvent the actions of the antibiotics that would usually be effective against them, a process which is often facilitated by mutations in the bacterial genomes (44). In some cases, a single nucleotide polymorphism (SNP) within the genome can confer resistance, whereby a change in a single base pair can mean that a particular antibiotic treatment becomes ineffective. One such example is the antibiotic linezolid, (used to treat Gram-positive bacterial infections) that functions by binding to the 50S ribosomal subunit within bacteria, and inhibiting protein synthesis (21). The 50S ribosomes are in part encoded by the 23S rRNA gene, but a mutation in domain V at site G2576T (*Escherichia coli* numbering convention) reduces the interaction between the drug and the ribosome and makes it ineffective.

The Gram-positive bacteria *Enterococcus faecium* (*E. faecium*) is usually a part of the gut microflora but can cause a variety of diseases, including bacteraemia and urinary tract infections (45). Infections are often spread within hospitals, being particularly prevalent within immunosuppressed transplant patients. These infections can be challenging to treat due to the inherent resistance the bacteria has to many antibiotics (46), and the emergence of vancomycin resistant enterococcus (VRE) means that linezolid is often used as a last-resort antibiotic for treatments. However, the recent rise in incidences of linezolid resistant *E. faecium* infections is making effective treatments more difficult to implement (47), and the G2576T SNP (present at base 2589 within *E. faecium*) appears to be prevalent within these cases (48).

Quick and effective treatments of infections is crucial in terms of recovery. Currently, microbiological cultures from patient infections are used for identification and diagnosis, and for informing what treatments should be prescribed to treat infections, however it is necessary to allow time for cultures to grow. *E. faecium* can also exist normally within the

gastrointestinal tract of humans as a commensal, non-pathogenic organism. This means that a positive microbiological culture test may be uninformative, as the presence of the bacteria does not necessarily indicate that it is responsible for the infection in question. The results of microbiological culture tests can also be unclear in cases of polymicrobial infections (49). Inaccurate diagnosis, particularly in cases where a patient requires urgent treatment, can lead to the prescription of ineffective or broad-spectrum antibiotics, which may result in the selection for and spread of resistant bacterial populations. Therefore, finding ways to quickly and accurately diagnose infections and detect the presence of resistance genes is of vital importance.

Sequencing

Sequencing the genome of bacteria present in patient samples is an alternative diagnostic method to blood cultures, and may be advantageous for several reasons. Sequencing could not only detect exactly which strains of pathogen are infecting the patient, but it could also inform treatment choices based on the resistance markers the bacteria may present. This would allow for tailored treatments that would have fewer side effects such as disturbing the patient's commensal microflora, and would reduce the spread of AMR (50). However, drawbacks include the comparatively high cost of sequencing compared to culturing, different skill sets required, and time taken to provide results. Advances in Next-Generation sequencing may abate these setbacks (51), but further work is required.

Two of the main technologies for Next-Generation sequencing are Illumina and the Oxford Nanopore Technologies (ONT) MinION. Briefly, Illumina sequencing involves reading short fragments of a section of DNA multiple times on a flowcell so that a highly accurate consensus can be reached (52). However, these short reads must be reassembled after sequencing to determine the whole length of the sequence, and this can obscure epistatic mutations. These are defined as when the fitness of the organism is dictated by two or more genes that interact with one another. However, as these genes do not necessarily need to be near each other on the genome, the initial act of fragmenting the DNA for Illumina sequencing can render them undetectable (53). Conversely, the MinION sequences individual long molecules of DNA to procure lengthier reads, albeit ones that are more prone to errors. This is particularly the case when the sequence contains more than 3 of the same base-pair in a row, as it is difficult to distinguish how many times the base is repeated. The coverage is also lower for any given part of the DNA, and so an accurate consensus is harder to arrive at (54). SNPs may be mistaken as read errors, and vice-versa. Many AMR mutations have an associated fitness cost, but epistatic mutations may facilitate AMR by negating that cost (55).

Using Rolling Circle Amplification to Produce Concatemers

Rolling circle amplification (RCA) involves producing amplicons of the gene of interest via PCR and circularising them using the enzyme Blunt/TA ligase, into plasmid-like structures. The bacteriophage derived enzyme polymerase $\phi 29$ can then be used to synthesize a linear copy of the circular DNA amplicon. The enzyme can repeatedly copy the circular DNA fragment in consecutive replication events, producing a long linear tandem repeat of the sequence of interest, called a linear concatemer. This repeating length of DNA can then be sequenced via MinION, and the tandem repeats extracted and used to provide better coverage of the gene sequence (54). The aim of this study is to produce linear concatemers of the 23S gene of three strains of *E. faecium* via RCA, and to use this data to determine if it is possible to

differentiate between SNPs and incorrect base calls when identifying possible epistatic mutations.

Methods

Primer Design and 23S rRNA Gene PCR Amplification

The protocol was based on the NanoAmpli-Seq Workflow outlined by Calus *et al.* (56), with modifications outlined below. Primers 23SF (5' - GATTTCCGAATGGGGGAACCC) and 23SR (5' - AGTCCTCGACCGATTAGTATTGG) were designed using Benchling (<https://benchling.com>) based on the *E. faecium* genome (NCBI Accession: NC_017960) that would incorporate the 23S rRNA coding region; specifically including base 2589, which can contain the G to T AMR conferring mutation. 8 PCR reactions were set up in a 200µl flat top PCR strip tube, with each containing 5µl of 5x Q5 Reaction Buffer (New England Biolabs - NEB), 0.5µl of 10mM dNTPs, 0.25µl Q5 Polymerase (NEB), and nuclease free (NF) water to 25µl. 5µl template DNA from three samples of *Enterococcus faecium* (strain numbers 1310, 1319, and 1802, which had all previously demonstrated resistance to linezolid), with 5µl NF water for the control. This was done in duplicate, resulting in 8 samples. Each tube had 0.8µl of 10µM primers 23SF (forward) and 23SR (reverse) primers added. PCR reactions underwent an initial denaturation step at 98°C for 30s, followed by 30 cycles of 98°C for 5s, 60°C for 10s, and 72°C for 2:30 minutes, and a final extension of 72°C for 2 minutes.

Product Clean Up and Quantification

Unless otherwise stated, after each of the following reaction steps, Agencourt AMPure XP magnetic beads (Beckman Coulter) were mixed with PCR product at a ratio of 1:1 volume:volume, then samples put on a magnetic rack, and the supernatant removed. The DNA/XP bead mixture was then washed twice with 200µl 80% ethanol to remove contaminants and then the DNA eluted in 50µl EB. DNA was then quantified using a Qubit High Sensitivity assay (Thermo Fisher).

Ligation and Rolling Circle Amplification

To circularise, samples were diluted to 3ng/µl at a volume of 60µl, and 5µl Blunt/TA ligase (NEB) was added, then samples were incubated for 1 hour at room temperature (RT), and cleaned. Remaining linear DNA fragments were removed by digestion by adding the following to each 23µl DNA sample: 3µl 10x NEBuffer 4 (NEB), 3µl 10mM ATP, and 1µl Exonuclease V (NEB). Samples were incubated at 37°C for 30 minutes. RCA was performed using protocol and reagents from the REPLI-g Mini Kit (QIAGEN), containing φ29 REPLI-g Polymerase. AMPure XP clean-up required an additional 1.5 hour 37°C incubation step before DNA elution in order to homogenise the clumped DNA product, and eluted product was then diluted to ~100ng/µl DNA per sample tube.

Enzymatic Debranching and Barcoding

After RCA was complete, branching DNA strands were removed. 50µl reactions were assembled containing 5µl NEBuffer 2, 1µl T7 endonuclease 1, and 44µl of DNA product, and incubated at RT for 5 minutes. Samples were cleaned and eluted with 20µl EB, and diluted to a volume of 18.5µl at a concentration of 51.2ng/µl. Each sample was assigned a barcode,

which was ligated to each sample using 18.5µl DNA, 2.5µl NB barcode, 6µl Quick Ligation Reaction Buffer (5x), and 3µl Quick T4 DNA Ligase, and incubated at RT for 30 minutes. After clean-up, 5µl (150ng) of DNA from each sample was pooled together and made up to 60µl with EB. This was combined with 5µl BAM 1D adapters, 25µl Ligation Buffer, and 10µl Quick T4 DNA ligase, and incubated at 20 minutes at RT.

Sequencing

Pooled samples were prepared for nanopore sequencing according to the Oxford Nanopore 1D Genomic DNA by Ligation protocol, with library prep adjusted to include 20µl Loading Beads (LB) and 18.5µl DNA. Samples were sequenced using ONT MinION, on a R9.4.1 flowcell. Sequencing was run for 48 hours and basecalling was performed using Guppy.

Bioinformatics Analysis

Seqtk (<https://github.com/lh3/seqtk>) was used to extract subsequences from the raw data. The longest reads were queried on NCBI BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to confirm identity, and compared against an *E. faecium* 23S reference genome and visualised using minidot maps (<https://github.com/thackl/minidot>). The sequences were demultiplexed, and adapters removed using Porechop (<https://github.com/rrwick/Porechop>). Minimap2 (<https://github.com/lh3/minimap2>) was used to convert the remaining .FASTA files into .sam files, and Samtools used to convert these into bam.fai files so that sequences could be visualised in IGV (<https://github.com/igvteam/igv>). A script was then developed that utilised Racon (<https://github.com/isovic/racon>) to take the longest of the repeated sequences from each concatemer and use this to aid alignment of the other reads from that same individual sequence. Previously obtained Illumina sequencing data of the same samples was also processed in the same way for comparative purposes.

Results

PCR, Ligation, and Rolling Circle Amplification

The PCR and subsequent ligation of the amplicon of interest using Blunt/TA ligase (NEB) produced circularised fragments of DNA. Comparing Agilent TapeStation analysis of the same PCR sample from before and after self-ligation (Fig. 1A) and nuclease treatment (Fig. 1B), shows a change in DNA fragment size in base pairs (bp) in the sample between treatments. DNA that has been circularised migrates more slowly through TapeStation ScreenTapes during gel electrophoresis. Therefore, the post-ligation (circular) DNA sample should appear to have a slightly bigger size (bp) on the Agilent TapeStation image (Fig. 1A), in comparison to the pre-ligation (linear) DNA samples. This would indicate that some of the DNA in the sample has indeed been circularised. In accordance with this principle, the post-nuclease digestion sample in Figure 1B should appear larger than the pre-nuclease digestion sample, due to the linear DNA fragments in the sample being digested and only the circularised fragments remaining. After performing rolling circle amplification on the circularised DNA samples, the branching fragments were removed enzymatically, to leave singular double stranded DNA samples. Agilent TapeStation analysis of the samples before and after enzymatic debranching (Fig. 2) demonstrate that after debranching, samples appear to be comparatively shorter (bp) due to the removal of branches that will have slowed down the migration of the sample through the gel, due to being longer and the multiple branches hindering sample movement.

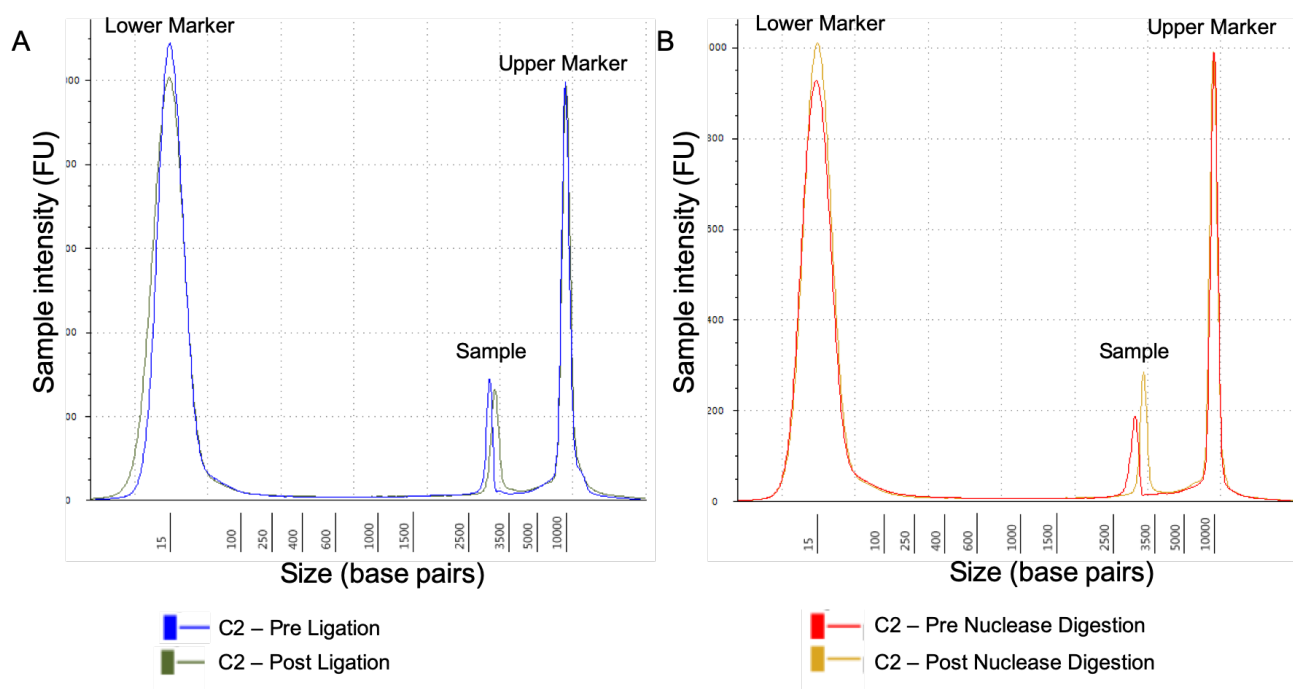


Figure 1. Aligent 4200 TapeStation analysis of 1 μ l 0.16 ng/ μ l of amplicons of *E. faecium* S23 DNA run on High Sensitivity D5000 Screen Tape. Large peaks to the left and right are markers used to align results, and samples are represented by the smaller peaks in the middle. A) Sample C2 (*E. faecium* sample 1319) was measured before and after ligation (circularization) of the PCR amplicons using Blunt/TA Ligase (NEB). B) Compares the same sample before and after enzymatic digestion of the remaining linear amplicons using Exonuclease V (NEB).

MinION Sequencing of DNA

After MinION sequencing of the RCA DNA, NanoPlot read data showed that 70,767 strands of DNA had been sequenced, with a mean read length of 5,802bp and a mean Phred read quality score of 8 (~90% base call accuracy) (Table 1). NCBI BLAST results of the longest high quality read returns a 99% query cover score with *E. faecium* isolate Ef_DMGI500501 (Accession no. LT603678 Region: 2583283..2586075), in the region of the 23S gene. This sequence was taken and used as a reference for mapping subsequent reads. Read data was filtered to remove sequences smaller than 28000bp (i.e. shorter than ~10 amplicons in length), and imported into Microsoft Excel. The length of the read was divided by 2798 (the length in bp of an individual amplicon), to provide an initial quantification of the number of amplicon repeats a sequence could contain, and the frequency at which they occur (Fig. 3).

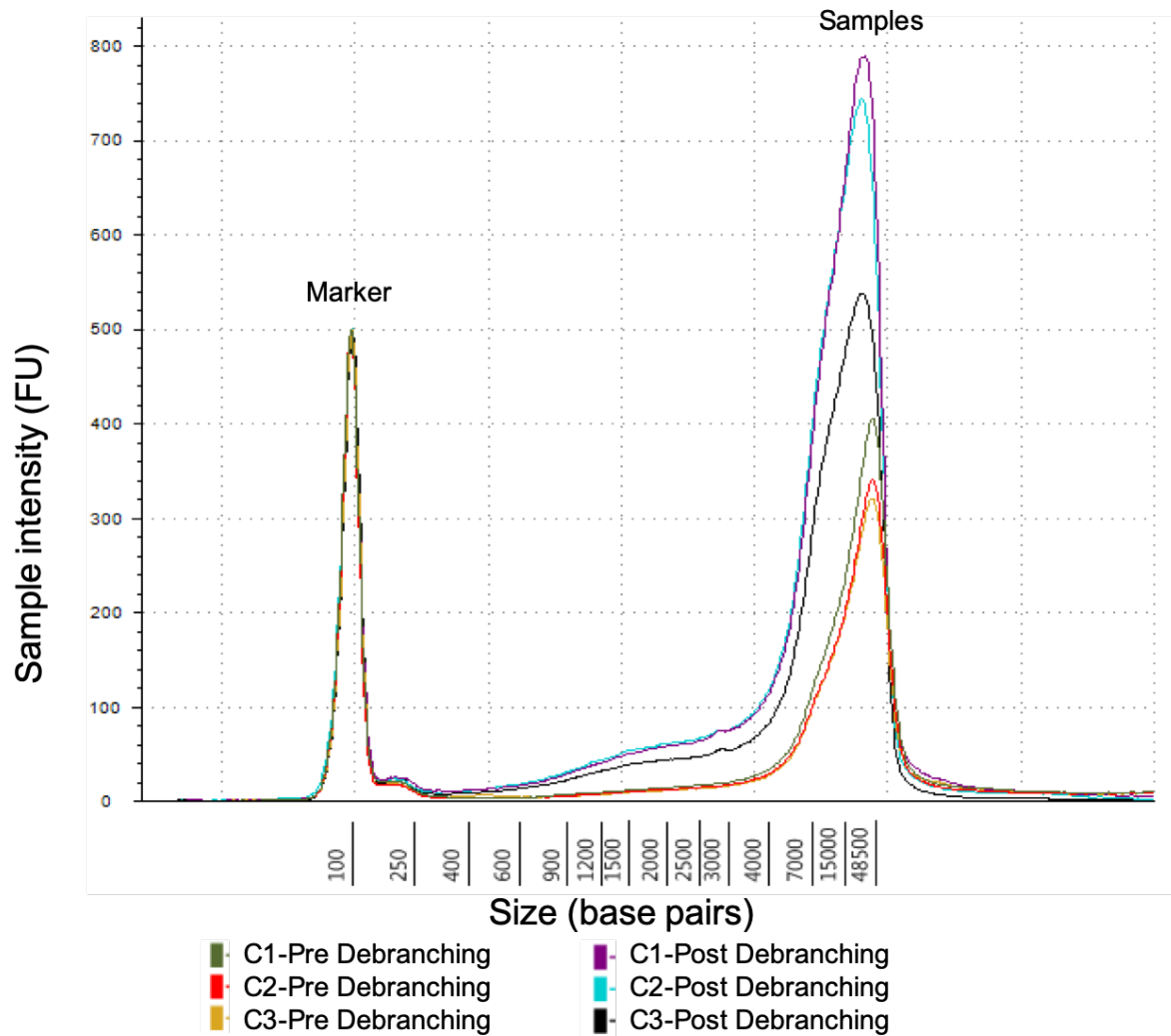


Figure 2. Aligent 4200 TapeStation analysis of 1µl 0.16 ng/µl of amplicons of *E. faecium* S23 DNA RCA product, before and after enzymatic debranching, run on High Sensitivity Genomic DNA Screen Tape. Samples C1, C2, and C3 (Pre Debranching) (*E. faecium* samples 1310, 1319, and 1802 respectively) were taken from PCR amplicon RCA product before T7 endonuclease 1 (NEB) debranching was performed, and samples C1 to C3 (Post Debranching) are the same samples after treatment. Peaks indicates sample intensity, indicating the amount of DNA present for any given DNA strand length in base pairs.

Table 1. NanoPlot Report Summary Statistics of the sequencing run of the rolling circle amplification DNA product.

Active channels:	142
Mean read length:	5,802.70
Mean read quality:	8
Median read length:	3,092.00
Median read quality:	8.7
Number of reads:	70,767.00
Read length N50:	11,442.00
Total bases: 410,638,765.0	410,638,765

The DNA library was prepared using the 1D genomic DNA by ligation protocol, and sequencing was performed on an ONT MinION using a R9.4.1 flowcell.

1480 sequences were long enough to include 10 or more concatemers. Shorter reads occurred at a higher frequency than longer reads. Mapping the 50 longest sequences against the 23S reference sequence (Fig. 4) allowed identification of multiple sequences or concatemers. After discounting multiple sequence reads, 29 of the 50 longest reads remained. When only counting full amplicon repeats (i.e. not including partial repeats), 17 of the 29 longest concatemers had the same number of amplicons as was predicted by their length, i.e. the full length of the sequence was comprised of the repeating amplicon sequence, with a maximum repeat number of 28 out of an estimated 28 sequences (Fig. 5).

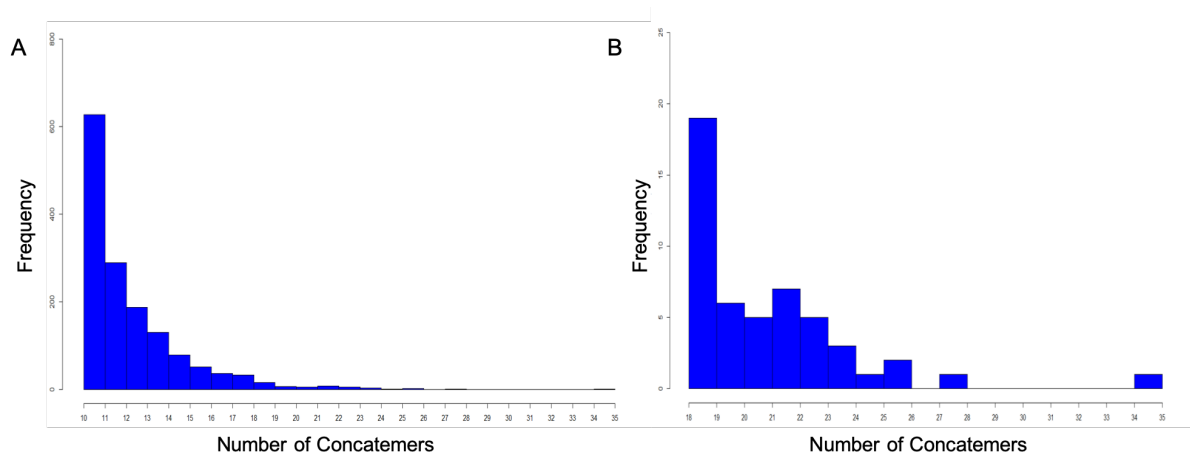


Figure 2. A 15% SDS-PAGE of fractions collected from his-tag purification of *E. coli* Rosetta with *H. pylori* apoferritin gene insert plasmid, induced with IPTG and incubated at 20°C for four hours. Each lane had a sample from different fractions collected during the akta prime purification process (Lane 1) PageRuler™ Low Range Unstained Protein Ladder 2) Waste flow through collected during the loading of the protein onto the column 3) 1st fraction collected using 10% buffer B (elution buffer) 4) 2nd fraction collected using 10% buffer B 5) 1st fraction collected using 60% buffer B 6) 2nd fraction collected using 60% buffer B 7) 1st fraction collected using 100% buffer B 8) 2nd fraction collected using 100% buffer B. The red box indicated a protein present of ~19kDa, matching the profile of the *H. pylori* apoferritin subunit 9) Insoluble fraction collected from the *H. pylori* apoferritin protein sample before his-tag purification, dissolved in 1ml 8M urea. 10) filtered centrifuged flow through.

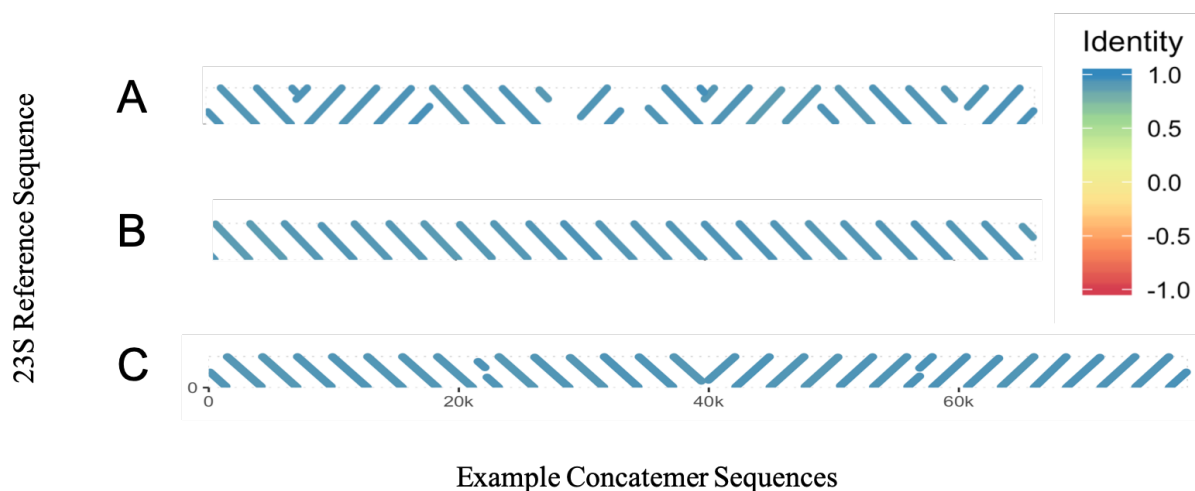


Figure 4. A Minidot map images of 3 example concatemer sequences mapped against the 23S reference sequence. Each sample displays a high identity score with the reference (1.0). A) Multiple mixed orientation sequences on a single concatemer. B) A concatemer of 23 full repeats of the target sequence, with incomplete partial sequence repeats at the start and end. C) A naturally occurring 1D2 sequence, with an insertion included in the 8th full repeat, and in its complementary strand running in the opposite direction on the 21st full repeat.

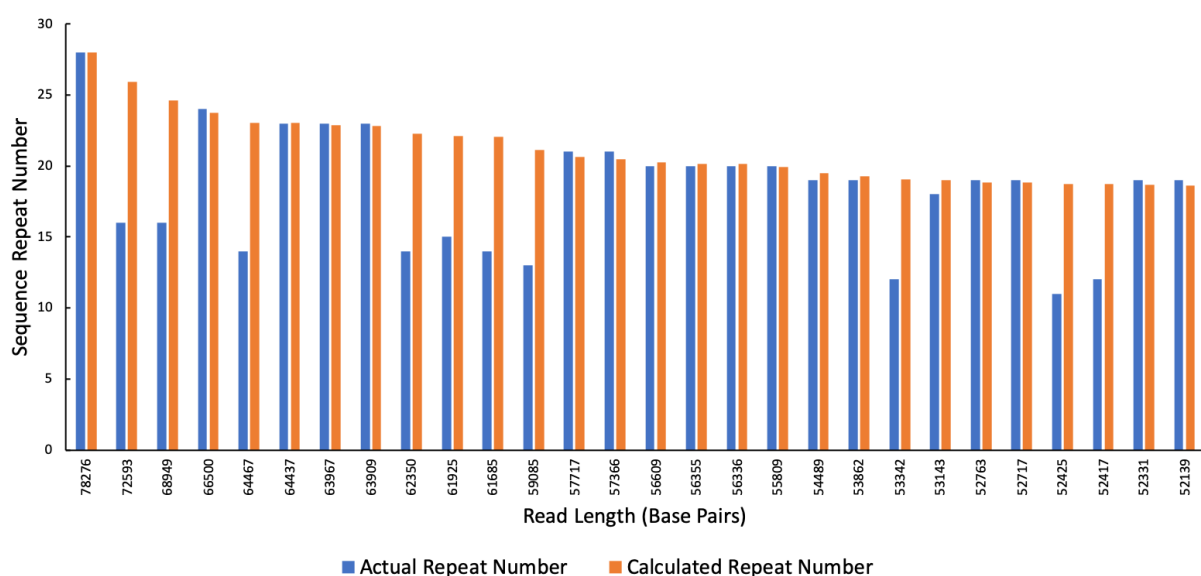


Figure 5. A histogram displaying the calculated number of repeats vs the actual number of repeats for the longest 28 concatemers present from MinION sequencing of *E. faecium* DNA, produced by rolling circle amplification. Calculated repeat number was length of sequence in base pairs divided by 2798 (the length of one amplicon). Actual repeat number was obtained by creating Minidot maps of the 50 longest sequences, and discarding any chimeric multisequences or natural 1D2 sequences. The full number of repeats were then counted.

Processing of MinION Sequences to Produce Consensus Data

The reads were again filtered to remove sequences of less than 35000bp, approximately 12 times the 23S amplicon length. Data was processed as described in the methods, allowing the 3 *E. faecium* strains to be separated, and the concatemers separated and aligned to themselves. This was performed on the reads both with and without data undergoing a ‘correction’ process using Racon. For uncorrected reads, average coverage for a position is approximately 16,000 reads, and corrected reads have a coverage of approximately 1800. It was then possible to visualise the aligned sequences on IGV and extract a consensus sequence. The three variants of the consensus sequences (Corrected, Uncorrected, and Illumina) of each of the three *E. faecium* strains were then aligned to the 23S reference data on Benchling to identify any discrepancies between them (Table 2). Identity between the 4 samples (Reference, Corrected, Uncorrected, and Illumina) for each bacterial strain was more than 99%, with only a maximum of 4 bases differing between the data sets. Each strain contained the same base 2 variants, at positions 11 (T in Illumina and reference data, C in MinION data) and 2536 (T in Illumina and reference data, K in MinION data, indicating an ambiguous T or G base). This study may have benefited from performing a MinION sequence of 23S without RCA, to allow for comparison of levels of coverage.

Table 2. Variations in sequence consensus data of the three strains of *E. faecium*, compared to the 23S reference gene.

	Nucleotide Position	Reference	Corrected	Uncorrected	Illumina
Strain 1310	1	-	T	T	T
	11	T	C	C	T
	319	C	M (C or A)	M (C or A)	A
	2536	T	K (T or G)	K (T or G)	T
Strain 1319	1	-	T	T	T
	11	T	C	C	T
	2536	T	K (T or G)	K (T or G)	T
Strain 1802	1	-	T	T	T
	11	T	C	C	T
	2536	T	K (T or G)	K (T or G)	T

Nucleotide position is given as base pairs starting at 1 from one base before the start of the 23S reference gene, as it is not present in the reference gene. Data is only shown if a consensus between all samples was not found. Uncorrected alignment data is consensus reached before processing via Racon, and Corrected is from after. Where letters M and K appear shows ambiguity in base identity. The '-' character in the reference gene indicates no base present at that position.

Allele Variance Frequency and Epistatic Mutations.

Identification of discrepancies in base variation was performed in IGV. Allele frequency threshold was set at 0.2 (bases with more than 20% discrepancy between base identity would be flagged). For the corrected data, strain 1310 had 9 allele variances, sample 1319 had 7, and 1802 had 9. Identified SNPs did not appear to be at locations of low fidelity i.e. after multiple repeats of the same nucleotide. Sequences of DNA with more than 4 of the same base in sequence appeared as low coverage within IGV on the last repeated base. One of the longest repeating sequences in strain 1802 position 1105 contained 5 consecutive T bases within both the reference sequence and the Illumina data sequence, but less than half of the corrected MinION reads displayed this as a base (990 reads indicated a deletion, 795 reads indicated a T).

Discussion

The diagnosis of diseases, and the informed choices of treatments are of vital importance, particularly in cases of antimicrobial resistant pathogens. Quickly and accurately identifying what (if any) antibiotics to prescribe can save lives and help halt the spread of AMR. While blood cultures are currently the most widespread means of diagnosing diseases such as *E. faecium*, these can be time consuming and uninformative. Sequencing the DNA of the bacteria is an attractive alternative; however further work is required to reduce time taken and improve accuracy, particularly for MinION sequencing. Here I present findings that show that it is possible to use rolling circle amplification to produce concatemers of the 23S gene, which have a comparative sequence identity of >99%. Furthermore, SNPs can be readily identified. The use of RCA means that the coverage is comparable to that of Illumina sequencing, allowing an accurate consensus sequence to be reached. This study may have benefited from performing a MinION sequence of 23S without RCA, to allow for comparison

of levels of coverage. Although further refinement is needed, this may lead towards the use of MinION sequencing as a viable diagnostic tool.

Rolling Circle Amplification of Circularised Amplicons Produces Concatemers that can be Sequenced Using a MinION

The process of amplifying and circularising the 23S gene from *E. faecium* was successful, and the subsequent rolling circle amplification produced large amounts of DNA, although this process took somewhat longer than in (14). The majority of the longest MinION concatemers produced contained repeated reads of the gene of interest, and *in silico* processing of the data after the fact meant that these could be isolated and processed accordingly. By separating and mapping the repeated reads from the concatemer sequence data, it was possible to create high coverage alignments, and from those extract a consensus sequence comparable to that of the Illumina data for each sample, allowing species level identification. The sequence identity from the samples matched the reference sample at >99%, comparable to previous studies (54).

Although in this study all sequencing was performed on pure samples, it is possible to perform RCA and MinION sequencing to correctly identify constituents of a mixed microbial population, as demonstrated by Calus *et al.*, (56) using the 16S rRNA sequence. Other research performed by Leggett *et al.* (57), determined that even mixed microbial population samples with a low abundance of DNA for each species (as may be the case in clinical samples) allow for species identification and AMR gene profile detection using MinION sequencing. Aside from the 1st amplification step, none of the steps in this protocol were sequence specific and could theoretically be applied to a mixed microbial population. However, because the first step amplified only the 23S gene within the *E. faecium* bacteria, where it is known that the linezolid resistance mutation occurs, careful consideration would need to be taken before applying this protocol to a mixed population, with the aim of detecting AMR genes in other, unknown locations in the genome. Particularly, further work would need to be done to determine if RCA can still be performed on low abundance DNA. As well as this, the upper boundaries of the size of the circularised DNA that can still be used for RCA would need to be determined. If long sequences of low abundance DNA were able to successfully undergo RCA as well as the subsequent steps of this protocol, then the PCR amplification step (including the sequence specific primers) would not be necessary, and the protocol could be used on clinical, mixed microbial population samples. Alternatively, 23S primers could be designed that are specific to a wide range of pathogenic organisms, as the 23S sequence is highly conserved across many bacterial species (58), although this would limit AMR gene identification to those within the 23S sequence.

Identification of Polymorphisms and Epistatic Mutations.

Visualising the stacked concatemer data in IGV after mapping to the 23S reference gene allowed for quick identification of SNPs present within the samples. Although several G to T allele polymorphisms were identified, it could not be definitively stated whether or not any of them were the linezolid resistance mutation, because the sequences were numbered starting from the first base in the 23S reference gene, and identifying the mutation by base number was not possible. The problem of reduced identification of repeated bases due to the MinION sequencing chemistry is still present. However, although the coverage for a 5 times repeated base was less than half of the total coverage for that particular base, the coverage was still over 700 times for the corrected data,

which is still higher than the coverage for the corresponding base in the Illumina data. The comparatively lower coverage for this base did not seem to negatively affect the consensus building, as the base was either not detected or detected as the correct base (i.e. there was no discrepancy between two different reads at that position, only the presence or absence of the correct base). This study has therefore demonstrated that using RCA, it is possible to both detect SNPs within a DNA sample with high certainty, as well as sequence those samples as long reads. This represents the ‘best of both worlds’ of Illumina and MinION sequencing.

The *E. faecium* samples used in this study were chosen because they previously demonstrated resistance to linezolid, which is denoted by an SNP. Antimicrobial resistance genes such as the mutation in the S23 gene in *E. faecium* may also be accompanied by epistatic mutations, i.e. mutations in other areas of the genome that interact with and potentially stabilise the resistance gene, in effect offsetting the fitness cost associated with carrying an AMR gene. However, as these genes do not need to necessarily be near each other in the genome to still exert effects on each other, any epistatic link between mutations may be obscured by the fragmentation of the DNA samples involved in Illumina sequencing. Therefore, with further work, this protocol of RCA and MinION sequencing could allow for the identification of SNPs that confer AMR, as well as other mutations within the DNA that allow the AMR gene to function with a reduced fitness cost.

Conclusion

Using rolling circle amplification and MinION sequencing, it is possible to identify bacteria in a sample, and detect with more certainty the presence of SNPs, compared to minION sequencing alone. This study also demonstrates that long read sequencing of RCA products enables the detection of multiple SNPs at different points along the same DNA strand, which is often obscured when using Illumina sequencing. However, further research would need to be carried out to improve this protocol to reduce time taken from sample collection to production of results. If this process was to be of use for diagnostics, it would also be necessary to have professionals trained in bioinformatics to process and interpret the data. The study was also limited by time constraints and lack of suitable programmes for performing data processing. That said, several protocol improvements and bioinformatic programme developments have been made over the course of this project that would significantly decrease time taken to repeat the project in the future. AMR continues to be an emerging problem around the world, however with further advancements in diagnostics and treatment, it may be possible to limit the damage caused by this threat.

3. Apoferitin-Bismuth complexes for the treatment of *Helicobacter pylori* infections.

Abstract

Helicobacter pylori (*H. pylori*) is a pathogen that infects the lining of the human stomach and is associated with peptic ulcers and gastric cancer. Treatment consists of triple therapy with antibiotics and proton pump inhibitors, however increasing resistance worldwide is reducing the effectiveness of therapy. An additional therapy of bismuth salts has been found to be effective at treating antibiotic resistant strains but fails to eradicate infections alone. Apoferitin is a protein cage made of 24 subunits that can be used to encapsulate drugs and target their delivery to specific sites. This study successfully purified *H. pylori* apoferitin subunits and demonstrated the assembly of the protein into its cage form, as well as attempting the

encapsulation of bismuth compounds. Bismuth salts encapsulated in apoferritin may work as a novel alternative to antimicrobial therapies in treating *H. pylori* infection, and so reduce the incidence of antimicrobial resistance.

Introduction

Since the case was made for a link between *Helicobacter pylori* and gastric ulcers in 1984 (59), the scientific community has been working to find effective treatments for those infected. *H. pylori* is a Gram-negative bacterium that is rare in its ability to colonise the human stomach, by occupying the protective mucus layer of the stomach lining (60); and by utilising its ability to maintain a pH of around 7 within the periplasm regardless of extracellular conditions (61).

It has been found that *H. pylori* is present in approximately 50% of the world's population, and once the bacteria colonise the stomach, they can cause chronic morbidity if not treated (62). Infection by *H. pylori* is associated with chronic gastritis, however prolonged infection can develop into the formation of gastric ulcers, as well as stomach cancers such as gastric adenocarcinoma or MALT lymphoma, which have significant mortality associated with them (63). Because of the causal relationship between colonisation with this pathogen and development of gastric cancers, *H. pylori* has been declared a class one carcinogen by the World Health Organisation (64). Therefore, it is essential that *H. pylori* infections are treated effectively before late stage disease development can occur.

Treatment currently involves a 'triple therapy' of drugs, which includes a combination of two antibiotics and an acid suppressing proton pump inhibitor (PPI) (65). This approach has been hindered in recent years due to the increased occurrence of antimicrobial resistance (AMR) within *H. pylori* (63), particularly in regards to clarithromycin resistance, which has been increasing worldwide (66). An additional component (a quadruple therapy) that includes bismuth salts has been found to be effective at treating antibiotic resistant strains of the disease, as these compounds are capable of working in synergy with antibiotics – particularly beta lactams - to eradicate *H. pylori* infections in the stomach (67). Additionally, it has been found that the inclusion of bismuth compounds in the treatment of *H. pylori* reduces the development of AMR, compared to treatment with an antibiotic alone (43).

It has been found that bismuth compounds such as bismuth subsalicylate (i.e. Pepto-Bismol©) and bismuth citrate have a bactericidal effect on *H. pylori*, resulting in a reduced bacterial burden and a decrease in disease symptoms and severity. Bismuth salts function by disrupting some of the essential processes of *H. pylori*, such as inhibiting metabolic enzymes, binding histidine-rich and heat shock proteins, and inducing oxidative stress (68), however the specifics of the actions of these compounds against *H. pylori* are not fully understood (69). Nonetheless, despite the capability of these compounds in infection treatment, bismuth quadruple therapy is still not always effective at eradicating the infection fully, and the disease can reoccur. This is a particular risk factor in the development of antimicrobial resistance (70).

Despite numerous reports of the development of antimicrobial resistance within *H. pylori* to a range of antibiotic treatments (71), there have as yet been no known cases of the bacteria becoming resistant to bismuth treatments (67), making them a good candidate for further treatment development. If a process could be devised that improved the method of delivery, or rate of uptake of the bismuth salts by the bacteria, it might eliminate the need for any antibiotics to be used in treatments for the disease. Thus, it could reduce the development of AMR, as well

as leading to more effective treatments for the disease. It would also eliminate the side effects associated with antimicrobial therapy and so increase patient compliance.

One possible method for achieving this would be to encapsulate the bismuth salts in a protective protein shell. Ferritin is a protein found ubiquitously within both eukaryotic and prokaryotic life forms and is an essential component of iron homeostasis - in that it allows for the chelation and storage of iron in a non-reactive state (72). *H. pylori* apoferritin is a protein made up of 24 identical 19 kDa subunits, which assemble into a cage-like structure. The subunits of the cage have an enzymatic domain that leads to the oxidation of iron to form a biologically unreactive ferric-oxide core, (73) giving ferritin. With a central cavity of 8 nano meters (nm) in diameter, it is capable of containing around 4500 iron atoms, or can exist in an iron-free, hollow form called apoferritin (74). Apoferritin can be used to encapsulate other materials and drugs, which works to protect them and facilitate their delivery (42).

A bismuth compound encapsulated in *H. pylori* apoferritin could be used in the process of targeted drug delivery to the site of infection within the stomach, and perhaps lead to more efficient, bismuth-compound only based therapy. This would mean a reduction in the amount of antibiotics used, which in turn would reduce the associated side effects, i.e. the development of AMR, disruption to the patients gut microbiota, and disease resurgence due to poor patient compliance. Therefore, the aim of this study is to express and purify the *H. pylori* protein apoferritin and test its applications as a vehicle for more effective drug delivery of bismuth salts for the treatment of *H. pylori* infections; and thus, reduce the incidence of antimicrobial resistance.

Methods

Bacterial Transformation and Growth conditions

All bacteria were grown in Luria broth (LB) or on LB agar plates with 100mg/ml ampicillin, and incubated at 37°C, with flasks shaking at 200 rotations per minute (rpm). The *H. pylori* *pfr* sequence with attached N-terminal pentaglycine, TEV cleavage site (Tobacco Etch Virus), and His-tag regions was ordered in a plasmid backbone pJ414:361494 from Atum. Plasmid stock was rehydrated in 100µl nuclease free (NF) water to give 13.96ng/µl and transformed via electroporation into the *Escherichia coli* (*E. coli*) expression strains Rosetta (DE3) Competent Cells (Novagen #709554) and BL21 (DE3) Competent Cells (NEB #C25271). Transformation was performed using 5µl plasmid added to 1ml electrocompetent cells in a cuvette and pulsed for ~2.6 milliseconds at 2.1kilovolts, then immediately added to 950µl SOC (super optimal broth with catabolite repression) media, and incubated at 200 rpm shaking at 37°C for 1 hour, before plating onto selective ampicillin LB plates (100mg/ml) and incubated overnight to allow successful transformants to form colonies.

Protein expression

Protein expression tests were performed using transformed Rosetta and BL21 colonies, grown overnight in 10ml LB. Each strain was transferred into 3x250ml LB flasks with an initial optical density at 600nm of ~0.002 for each flask, and then incubated for a further 4 hours until optical density at 600nm reached between 0.6 and 1. Each of the 6 flasks then had 250µl 1M Isopropyl β-D-1-thiogalactopyranoside (IPTG) added to induce protein expression. One flask for each strain was then incubated in the following conditions: 37°C for 16 hours, 30°C for four hours, and 20°C for four hours. Subsequent Rosetta protein expressions were performed

in the same way, in 1L LB with 1ml 1M IPTG added, and incubated at 20°C for four hours. The culture was then centrifuged at 4000g for 20 minutes and resuspended in 6ml degassed buffer A (35mM imidazole, 20mM Tris, pH 8.2).

Protein Purification and Characterisation

Cell suspension was sonicated in ice at an amplitude of 14 microns 5 times at 1-minute intervals, totalling 10 minutes, and centrifuged at 16,000g for 35 minutes. The soluble phase was passed through a 0.45µm Durapore® Membrane Filter, and a scraping of the pellet was dissolved in 1ml 8M urea. His-tag purification of the filtered protein was achieved with a nickel 5ml Hitrap Chelating Chromatography column on an Akta Prime. Protein sample was loaded with buffer A, and eluted using 10%, 60%, and 100% concentrations of buffer B, (700mM imidazole, 20mM tris, pH 8.2). Fractions were collected from each and run on an SDS-PAGE (sodium dodecyl sulfate – polyacrylamide gel electrophoresis) (5% stacking and 15% resolving gels). Fractions containing the target protein were dialysed in 20mM tris overnight, and concentrated from ~30ml down to 5ml in a Vivaspin™ 10,000 Da molecular weight cut-off protein concentration column and quantified using a Bradford assay. Samples from before and after spin concentration were run on a 6% percent native PAGE to visualise apoferritin cages. The amount of protein present was quantified using a Bradford assay, with absorbance measured at 595nm using a UV-vis TECAN plate reader.

Bismuth salt assays and Encapsulation

An absorbance scan was performed on the TECAN microplate reader of 100mg bismuth subsalicylate (BSS) dissolved into 1ml dimethylsulphoxide (DMSO). The apoferritin encapsulation protocol was modified from Breen *et al.* (75). 100 equivalents (0.11µl) of BSS in DMSO (100mM stock) was added every half hour for 5 hours to 1ml of 0.06mg/ml *H. pylori* apoferritin, to total 1000 equivalents. The same treatment was applied to a 1ml sample of 62.4µM human apoferritin, adding a total of 1000 equivalents over 5 hours (2.5µl of 100mM BSS/DMSO every half hour). Samples were slowly rotated at 4°C throughout, then dialysed in 20mM HEPES buffer (pH 7.4) overnight to remove any non-encapsulated BSS. Serial dilutions of BSS DMSO solution in HEPES buffer were made, and absorbance measured with a TECAN plate reader to create a calibration curve, in order to determine bismuth quantity. Absorbance of the samples were then measured at 900nm and 595nm to determine protein/bismuth concentrations.

Results

Protein expression tests in *E. coli*

The electrocompetent *E. coli* cells (Rosetta and BL21 strains) both successfully took up the expression plasmid via transformation, and sequencing confirmed that the *pfr* gene insert was present and had not mutated. General protein expression was higher for both strains when incubated at 20°C for 16 hours after induction with IPTG compared to the other treatments, likely due to higher cell count after a longer incubation period. A higher protein yield was obtained from the soluble phase compared to the insoluble phase for both bacterial strains. Although a protein band was present in all samples at the expected size of the apoferritin subunit (19kDa), it was not possible to tell if this was the Pfr protein (Fig. 1). Therefore, based on previous findings within the lab of suitable expression conditions for apoferritin, the Rosetta

expression strain was selected, and the protein expressed as described in the methodology, and incubated at 20°C for four hours after the addition of IPTG.

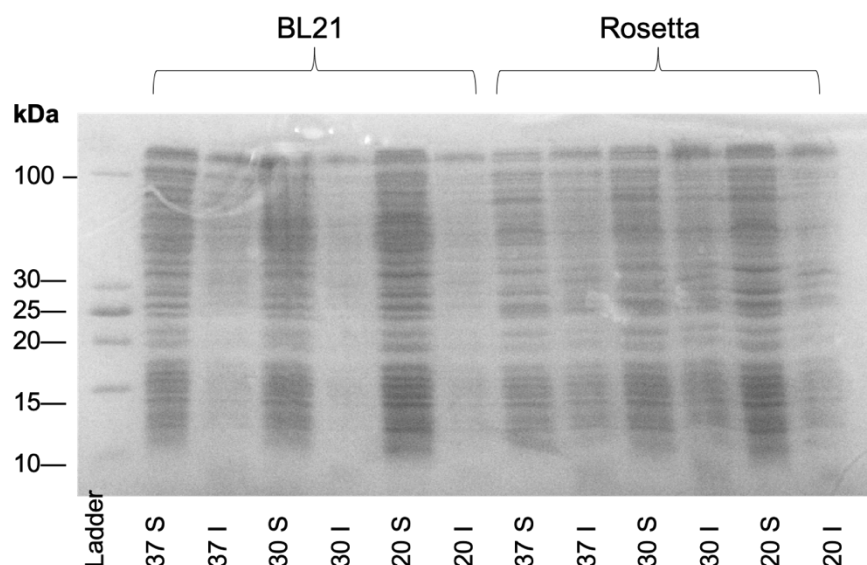


Figure 1. Protein expression of *E. coli* Rosetta and BL21 under a range of conditions, analyzed on a 15% SDS-PAGE. (Lane 1) PageRuler™ Low Range Unstained Protein Ladder. Lanes 2-7 contain lysed cell product from BL21 samples, and lanes 8-13 are from Rosetta cells. Number indicates temperature of incubation after induction with IPTG, and S or I indicates if the lysed cell product was from the soluble or insoluble fractions, respectively. Insoluble cell pellet was suspended in 1ml 8M urea before running on the gel. Proteins were visualized using Coomassie blue staining.

Pfr protein expression and purification

Fractions collected during the protein purification were run on an SDS-PAGE. Little protein was visible in the 10% and 60% column eluate fractions, and no significant amount of target protein was present within the insoluble fraction gel profile. The second 100% buffer B eluent fraction contained a single band of 19kDa in size, matching the profile of the target protein (Fig. 2). A Bradford assay was performed and scanned on a TECAN plate reader using an absorbance of 535nm. The output of this indicated that a low level of protein was present, calculated as 0.0637mg/ml. A native PAGE of the product exhibited a small amount of protein of approximately 510 kDa (Fig. 3), the size of a 24-subunit apoferritin cage.

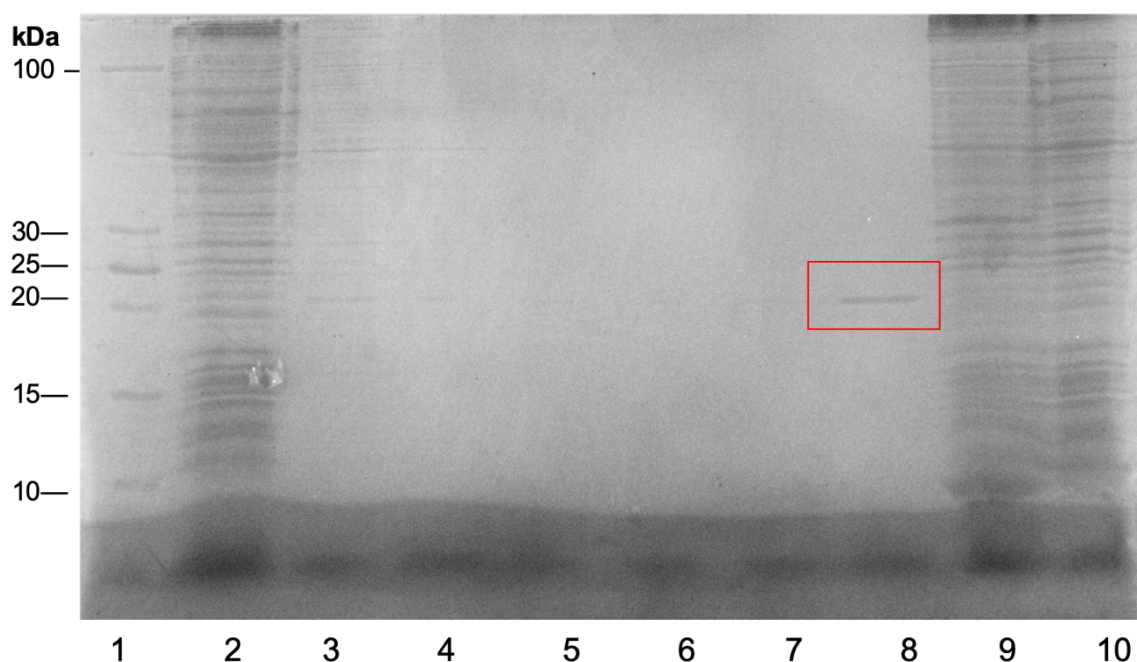


Figure 2. A 15% SDS-PAGE of fractions collected from his-tag purification of *E. coli* Rosetta with *H. pylori* apoferritin gene insert plasmid, induced with IPTG and incubated at 20°C for four hours. Each lane had a sample from different fractions collected during the akta prime purification process (Lane 1) PageRuler™ Low Range Unstained Protein Ladder 2) Waste flow through collected during the loading of the protein onto the column 3) 1st fraction collected using 10% buffer B (elution buffer) 4) 2nd fraction collected using 10% buffer B 5) 1st fraction collected using 60% buffer B 6) 2nd fraction collected using 60% buffer B 7) 1st fraction collected using 100% buffer B 8) 2nd fraction collected using 100% buffer B. The red box indicated a protein present of ~19kDa, matching the profile of the *H. pylori* apoferritin subunit 9) Insoluble fraction collected from the *H. pylori* apoferritin protein sample before his-tag purification, dissolved in 1ml 8M urea. 10) filtered centrifuged flow through.

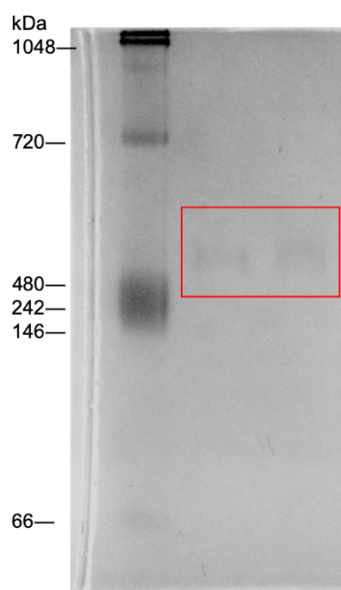


Figure 3. A 6% native PAGE of purified *E. coli* Rosetta expressed *H. pylori* apoferritin eluted in 100% buffer B (elution buffer). Lane 1) Ladder is Native Mark TM Protein standard – Invitrogen. Lanes 2 and 3 contain the concentrated protein samples after dialysis, and match the profile of a 24 subunit apoferritin cage (510kDa).

Encapsulation of BSS in apoferritin

BSS was suspended in 1M DMSO to form a 100mM BSS/DMSO suspension, which appeared as a cloudy white liquid. BSS was considered encapsulated by apoferritin if it remained within the apoferritin after overnight dialysis. After encapsulation and overnight dialysis of BSS in both human and *H. pylori* apoferritin, the protein in the human sample had precipitated out and formed two discrete fractions, whereas the *H. pylori* apoferritin remained soluble. Previous research performed in the lab had demonstrated that apoferritin has an absorbance wavelength of 280 nm due to the presence of tyrosine and tryptophan within the protein. It was not possible to determine BSS/DMSO absorbance from the TECAN plate readings (Fig. 4). Initially, it was thought that absorbance values of the bismuth subsalicylate suspended in DMSO alone, without protein, appeared to show a slight absorbance increase between wavelengths of 900 and 1000nm (Fig. 4 A). This seemed to correspond to a slight absorbance peak between wavelengths of 900 and 1000nm in the human upper fraction and *H. pylori* encapsulations (although not human lower fraction of apoferritin) (Fig. 4 B, C and D), which was incorrectly at first thought to indicate that some encapsulation had occurred. However, the 900nm absorption peak detected was more likely an artefact of the upper ranges of the TECAN plate reader. Using the protein and bismuth calibration values (Fig. 5), concentrations of apoferritin and BSS were calculated, and used to determine drug encapsulation and loading efficiencies, and average molecules of drug per cage (Table 1). Although the data collected from the bismuth/DMSO serial dilution curve had a positive correlation of BSS concentration to absorbance, as would be expected if the BSS absorbance was being detected, it could not be determined if the calibration curve was accurate due to the uncertainty of the 900nm absorbance value. This uncertainty was compounded by the unlikely figures returned by the calculations returned in Table 1.

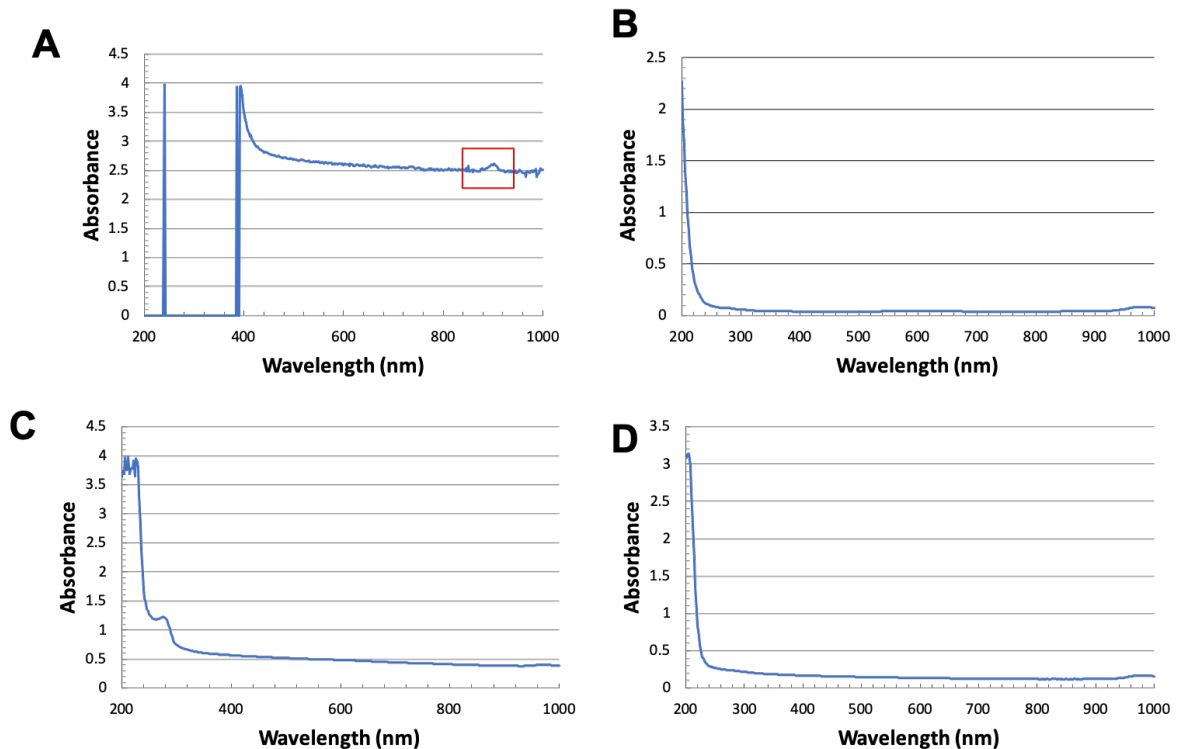


Figure 4. Graphical output from TECAN plate reader absorbance of apoferritin and bismuth against wavelength. A) Absorbance of bismuth subsalicylate in DMSO at a range of wavelengths (200-1000nm). The red square indicates a suspected absorbance peak at 900nm. B) Human apoferritin and encapsulated bismuth subsalicylate, upper fraction after overnight dialysis C) human apoferritin encapsulated bismuth subsalicylate lower fraction after overnight dialysis D) *H. pylori* apoferritin and encapsulated bismuth subsalicylate sample after overnight dialysis.

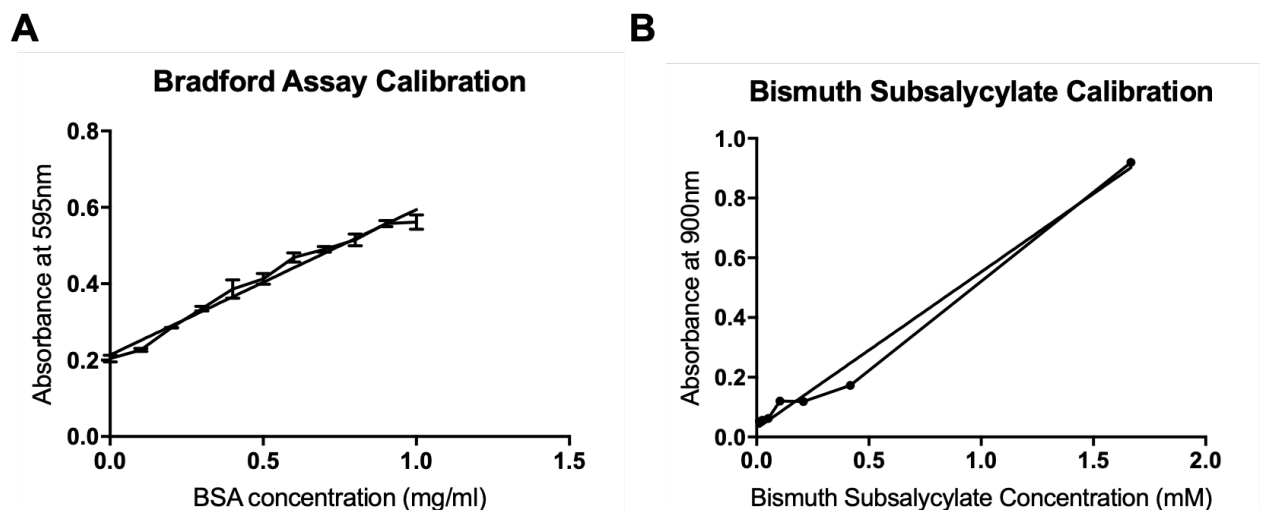


Figure 5. Calibrations of absorbance on TECAN plate reader vs sample concentration. A) Linear regression of absorbance at 595nm of bovine serum albumin (BSA) for a Bradford assay N=3, giving $Y = 0.3807 \cdot X + 0.2137$ B) Linear regression of absorbance at 900nm of 30 fold dilution of 100mM Bismuth Subsalsalicylate DMSO suspension in 20mM HEPES buffer, giving $Y = 0.5239 \cdot X + 0.02861$. A stock solution of 100 μ l 100mM bismuth subsalicylate in DMSO was added to the first well of a 96 well plate with 100 μ l DMSO. Each subsequent well (a total of 8) had half the amount of bismuth stock added, and brought up to 200 μ l volume with DMSO. A second row of 145 μ l 20mM HEPES per well was made, and 5 μ l of the corresponding stock solution from the well above added. One outlying data point was removed.

Table 1. Calculated values for human and *H. pylori* apoferritin encapsulation of bismuth subsalicylate. Human apoferritin precipitated and formed an upper and lower fraction during encapsulation protocol. Concentration of apoferritin and bismuth subsalicylate per sample calculated using respective calibration equations (fig. 5). Molecules of bismuth subsalicylate per cage given by number of bismuth molecules/number of cages. Encapsulation efficiency given by (amount of drug encapsulated (mol)/total amount of drug loaded (mol))*100%. Drug loading efficiency given by (amount of drug encapsulated (mol)/amount of drug encapsulated (mol) + amount of apoferritin * 100%.

	Human upper fraction	Human lower fraction	<i>H. pylori</i>
Apoferitin cage size (kDa)	584	584	510
Apoferitin per sample (μmol)	0.02	0.17	0.01
Bismuth subsalicylate per sample (μmol)	0.03	0.57	0.15
Ave. molecules per cage	124.21	295.71	2073.83
Encapsulation efficiency (%)	1.22	22.86	135.18
Drug Loading efficiency (%)	99.20	99.66	99.95

Discussion

The quick and effective treatment of *H. pylori* infections can prevent recurrence of the disease, reduce risk of cancer development, and discourage the development of antimicrobial resistance, and is therefore of the utmost importance. Current frontline therapy for *H. pylori* has a high failure rate, often due to antibiotic resistance and poor patient compliance, and although bismuth salts have been shown to improve therapy success, they are not effective alone as a treatment. As such, a novel drug delivery system was devised using apoferritin to encapsulate bismuth salts, in an attempt to improve targeted delivery and drug efficacy, and therefore reduce the amount of antibiotic required for therapy and increase cure rates. This study was successful in expressing and purifying the *H. pylori* Pfr protein, in both individual subunit and 24-mer cage configurations and had further success in using this to encapsulate the compound BSS. This is a first step towards a novel therapy for *H. pylori* infection.

Apoferitin Expression and Purification

It was found that protein expression of *H. pylori* apoferritin was successful, and that the modified protein was able to self-assemble into a 24-subunit cage. Purification was also successful, although not without difficulty, and expression levels of the Pfr protein in *E. coli* strains were not pronounced under any of the test conditions. Low expression levels of the protein meant that downstream procedures were hindered, i.e. being unable to determine the precise quantity needed for nanoreactor encapsulation. A more robust initial set of protein expression tests may have helped overcome this issue, and several factors could be optimised for future experimentation. Particularly, when comparing the *E. coli* BL21 and the Rosetta strains, for each temperature condition, a sample should be incubated without the addition of IPTG as a control, to determine if the IPTG induction is working. A range of IPTG concentrations could then be tested to determine the optimum amount needed for expression. Additionally, when checking for overexpression of the protein of interest, the protein concentration in the collected cell pellet could be measured and equilibrated between samples before running on the SDS-PAGE gel in order to allow for comparable visualisation of overexpression between the treatment conditions. A control of non-induced protein growth

should also be included for comparison. If this was unsuccessful, anti his-tag antibodies in a western blot could be used to visualise the Pfr protein, or a different promotor system considered if this too was unsuccessful.

Encapsulation and Quantification of Bismuth

The results suggest that BSS encapsulation in both human and *H. pylori* ferritin is achievable, however this preliminary data should be met with caution. While the results are encouraging and warrant further investigation, more robust verification of the results are needed. During the drug loading, the human apoferritin precipitated out likely due to coating of the protein in the bismuth compound, which altered its charge. An alternative drug loading approach (pH dependent disassembly and assembly) could also be attempted, and the stability of *H. pylori* ferritin at stomach pH characterised.

It was initially thought that an absorbance peak for BSS in DMSO at 900nm (fig. 4A) would allow the concentration of the drug to be determined, however data in table 1 calls this into question, particularly in regard to the *H. pylori* sample encapsulation efficiency of 135%. The calculated figures of average molecules per cage are within an acceptable range for the human upper and lower fractions (124 and 295 molecules per cage, respectively), but while the figure of 2073 molecules per cage for the *H. pylori* sample is not outside the realm of possibility, it is still higher than previously obtained figures for encapsulation (75). Similarly, the drug loading efficiency (99% for all samples) is higher than previous studies (76), indicating that the data used in this study to calculate the results may be flawed. As the protein quantification using the Bradford assay has been used successfully before for apoferritin quantification (75), it is reasonable to work under the assumption that the calculated amount of apoferritin per sample was correct. However, the study would benefit from reassessing the method by which bismuth concentration is calculated. As mentioned in the results, the TECAN plate absorption readings shown in Figure 4 are not appropriate for determining bismuth subsalicylate encapsulation rates in apoferritin. The aromatic ring in the structure of the BSS would suggest a peak of between 250-320nm, which would overlap with the protein tyrosine peak of 280nm and as such would not be suitable for use. A future attempt at quantification could use inductively coiled plasma mass spectrometry (ICPMS) to ensure a more accurate assessment of BSS encapsulation.

Apoferritin as a Drug Delivery System

Within mammals, ferritin acts as a storage and transportation device for iron within the body, and as such exists both intracellularly and within blood serum (77). Cells that are low in iron upregulate the expression of the TtR1 receptor, which mediates uptake into the lysosome, the low pH of which mediates cage disassembly and allows the iron or encapsulated drug to be released, making it a candidate for anti-cancer therapies (78). It was therefore considered a possibility that *H. pylori* apoferritin would be taken up by the bacteria in the same way. However, current literature suggests that ferritin in bacteria acts only as an intracellular protein (79)(72), and is not endocytosed from extracellular sources. Nevertheless, the functionality of apoferritin as a targeted delivery system is still worth considering.

Bismuth acts locally within the stomach, not requiring absorption into the body to be effective (80). Although bismuth salts are able to penetrate the mucus lining of the stomach to reach the site of infection, the high turnover rate of the gut mucosa (~3 hours) means that mucus bismuth concentrations are quickly reduced to below the minimum inhibitory concentration for *H. pylori* (67). *H. pylori* attaches to the lining of the stomach by binding to the MUC5AC receptor

on epithelial cells (81). It has been found that it is possible to modify the outer surface of the ferritin cage to target specific cell types (77), and so if it were possible to attach a MUC5AC ligand to the exterior surface of the apoferritin cage, it could facilitate movement of the caged drug to the area of infection and allow a concentrated release of the drug. As the encapsulated bismuth salts would be taken in combination with the current therapy of proton pump inhibitors, which markedly reduce the production of stomach acid (from ~pH 2 to ~pH 6) (82), the acidic conditions of the stomach would not likely cause the early disassembly of the apoferritin cage, and so it would reach the site of infection intact. Even without cage disassembly, apoferritin cages can allow the slow release of their drug cargo due to small pores in the protein shell (83). Therefore, if the apoferritin/bismuth cages were able to reach the site of infection and bind to the local MUC5AC receptors, they could potentially release the bismuth salts to the mucus lining over a longer, sustained period of time compared to the ~3 hour window currently demonstrated by the bismuth salts alone. The rate of bismuth salt release once at the site of infection would need to be assessed in the future in order to determine if the amount of drug released would be therapeutically useful.

Conclusion and Further Work

H. pylori is a pathogen ubiquitous around the world that colonises the human stomach. The rise in antibiotic resistant strains globally have caused first line therapies to fail in ever more cases, and so alternative therapies are urgently needed. Both BSS and apoferritin are effective therapeutics in their own right, and this study forms the basis of combining those therapies to make more efficient treatment, which if successful, would diminish the need for antibiotics and reduce recurrent infections. This study demonstrates that *H. pylori* apoferritin can be purified and used in encapsulation protocols. However, further work would need to be done to properly determine the loading potential of bismuth compounds and determine the efficacy of using encapsulated bismuth in treating *H. pylori*, both *in vivo* and *in vitro*. Considerations for further work would be to determine if *H. pylori* apoferritin is immunogenic, or if had the ability to enter human cells. A successful therapy based on the combined properties of bismuth and apoferritin would drastically improve the lives of countless people living with *H. pylori*, and could reduce the spread of antimicrobial resistance worldwide.

4. Prospects for PhD Development

Conclusion

The use of antibiotics is under threat from the global rise of antimicrobial resistance. The pipeline for antibiotics in development has all but dried up, and investment into the discovery of new antibiotics has little interest. The crisis of the rise antibiotic of resistant bacteria has reached a critical point, and work must be done to ensure that disaster is averted. Without functional antibiotics, infections that were once easily treatable will prove lethal, and safe operations and surgeries will be near impossible to perform. The death toll associated with antibiotic resistance is already at catastrophic levels, and the economic impact of AMR if allowed to escalate unhindered would be ruinous. There are several approaches that could be taken to try and mitigate this threat, one of which involves developing methodologies to improve the efficacy of the antimicrobials we currently have in our arsenal, and attempt to stop the development of AMR with more effective treatments. This was the aim of the second rotation project and could be developed further to be undertaken as a full PhD.

The bacteria *H. pylori* is a human pathogen that has been included on the International Agency for Research on Cancer list of carcinogens, due to infection leading to the development of stomach cancer in some individuals. Up to half of the global population are estimated to have been colonised with the bacteria, which proliferate within the mucous lining of the stomach and produce urease enzymes to neutralise the pH of the stomach acid in order to persist there (84). The recommended treatments include a triple therapy of the antibiotics clarithromycin and metronidazole, as well as a proton pump inhibitor. The addition of bismuth to this therapy is recommended if resistance to one of these antibiotics is observed, as the *H. pylori* appear to become susceptible to the antibiotics when combined with bismuth treatment even if they are genetically resistant (67,71).

Study Design

The study undertaken was based on the notion of novel drug delivery systems to target the site of the infection, using apoferritin as a vehicle to transport the bismuth to the *H. pylori*. While apoferritin has shown success in the targeting of cancer cells, this concept could not be fully adapted to function with *H. pylori* during the timeframe of the project. The crux of the success of apoferritin lies in the fact that within humans, ferritin binds to receptors on the cell surface and is actively taken up into the cell, where it disassembles within the low pH of the lysosome to release the drug payload (42). Conversely, while *H. pylori* does indeed produce and utilise ferritin, it is not released from the cell, and research suggests that it has no mechanism by which the bacteria can uptake external ferritin, which means its capacity to enter the cell to deliver the drug is reduced. However, there may be other applications for apoferritin in *H. pylori* that could be explored. The external surface of the apoferritin cage can be modified to bind to particular target receptors and could potentially be made to bind to MUC5A receptors present on the epithelial cells of the mucous layer of the human stomach that apoferritin colonises (81). This could aid permeation of the drug to the site of infection and allow for a longer exposure of the infection to the drug between doses, aiding in the increased effectiveness of bismuth salts at treating the disease.

The project 'Apoferritin-Bismuth complexes for the treatment of *Helicobacter pylori* infections' was an interesting study, but limited in some respects. As with many research projects, more lab time would have allowed for further exploration of the questions posed in the project brief, however good progress was made despite time constraints. Delays were also caused by the need for off-site facilities for more robust quantification of experimental results, such as using the ICPMS (Inductively Couple Plasma Mass Spectrometry) machine on Sutton Bonington campus to quantify bismuth content of the apoferritin, albeit this would not pose an issue in a long-term project. The basis of the project theorising that *H. pylori* would take up apoferritin in the same way that mammalian cells do was unsupported in the literature, however the concept of using apoferritin in antimicrobial therapy still has merit and is worth exploring further.

Future of the Project

The problem of AMR, particularly in *H. pylori*, is a major worldwide concern, and would be worth addressing. A PhD project exploring these issues could take several approaches for long- and short-term progress in results, with the focus being increasing the efficacy of current antimicrobials used for treatment, with aims to reduce the development of AMR and increase the success rates of treatments for patients. In the short term, it would be interesting to continue exploring apoferritin as a candidate for drug delivery, particularly by modifying

the outer shell of the protein to confer various attributes to it, such as altering the pH at which the cage disassembles in order to survive the acidic environment of the stomach. Bismuth is also a promising alternative and addition to antibiotic therapy. Literature suggests that bismuth can be used to circumvent AMR within resistant *H. pylori* infections to increase treatment success (65,67), however little is known of the *in vivo* effects of bismuth as the sole therapeutic agent. The mode of action for bismuth against *H. pylori* is also little understood, and further work could be performed to elucidate the mechanisms by which it is bactericidal. Finally, a study has been conducted that highlights a link between the long-term use of aspirin and the increased treatment success of antibiotics against *H. pylori* (85). As of yet, the reasons behind this association remain unclear, but considering aspirin is a drug already approved for human use, it would be worth examining further that non antibiotic compounds could be the key to overcoming antimicrobial resistance, by strengthening the arsenal we already have at our disposal for treating diseases.

Antimicrobial resistance is a multifaceted problem, and as such the solution will need to be approached from multiple angles. It will be by the effort of collaborators all around the world that the looming threat of resistant bacteria and untreatable infection is overcome.

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