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How does growth in biofilm affect the phenotype of antibiotic resistance genes?

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Abstract

Biofilms are a distinct form of microbial life with emergent properties. These include reduced susceptibility to antibiotics and persistence in chronic infections both within the human body and by formation on medical devices. Biofilm infections are hard to treat due to their tolerant nature but also due to the ever-increasing problem of antibiotic-resistant bacteria present, particularly in a hospital setting. These bacteria may be resistant to certain classes of antibiotics via different resistance mechanisms however, the resistance phenotype expressed by bacteria in planktonic cultures may not be directly comparable to the resistance phenotype expressed within a biofilm. This means that antibiotic resistance observed in planktonic cultures of bacteria may be due to the expression of different resistant phenotypes and different resistance mechanisms than that of resistant bacteria within a biofilm. This project addressed whether the relative level of resistance associated with a target mutation versus resistance dependent on the synthesis of an enzyme (target or drug modifying) is identical or different between bacteria in the planktonic versus the biofilm growth state. To investigate this, genes encoding two different resistance enzymes *aac* (a drug modifying enzyme) and *rmtB* (a target modifying enzyme) and a chromosomal mutation in *rpsL* (conferring intrinsic target resistance), were engineered into the chromosome of the biofilm-forming *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) strain ATCC14028. The constructed mutants were exposed to different concentrations of aminoglycoside antibiotics in both planktonic and biofilm cultures. The results highlighted that the resistance phenotype of *rmtB* is significantly less efficient in biofilm compared to planktonic culture. In contrast, the resistance phenotypes of *aac,* and the *rpsL* mutation are expressed as effectively in biofilm as in planktonic culture. These results suggest that the relative expression of resistance phenotypes in biofilm versus planktonic culture deserves to be explored further with the aim of assessing whether this knowledge could be leveraged to improve antibiotic therapy.

Key words: *biofilm, planktonic, phenotype, resistance mechanism*

Popular scientific summary

Bacteria are single-celled organisms that have been present in all environments since the beginning of cellular life on earth. They vary in shape, size and habitat they can be found in, from inhabiting the gut microbiota of mammals to extreme places no other organism can survive. Bacteria can be commensal or 'helpful' bacteria that protect humans against colonization by more harmful species. These harmful species of bacteria are capable of causing significant infectious diseases. The discovery of Penicillin in 1938 was an incredible achievement in the medical world and allowed for treatment of what were previously fatal bacterial infections. The discovery of antibiotics has saved countless lives. However, although resistance occurs naturally in the microbial environment, antibiotic resistance has become a worldwide problem threatening effective antimicrobial therapy in human and animal health sectors through the overuse and misuse of many drugs. Strains of bacteria that are resistant to antibiotics, such as Methicillin-Resistant *Staphylococcus aureus,* are a huge threat within the hospital setting. They represent a challenging group of organisms, much like bacteria that form a biofilm on medical implants and in persistent in chronic infections.

Biofilms are formed by communities of microorganisms such as bacteria, that are enclosed within a self-produced, matrix of proteins, lipids and polysaccharides. The biofilm can be attached to a living or non-living surface such as a medical prosthetic device, or as dental plaque on human teeth. The matrix can surround multiple species of bacteria, which engage in complex interactions that impact health and disease worldwide.

Biofilms are relatively tolerant to antibiotics due to several factors including (i) the physical barrier to antibiotic entry provided by the matrix and (ii) the physiology of bacteria within the biofilm where their slower growth and metabolism increases their tolerance, and (iii) the expression of distinctive resistance mechanisms utilized by bacteria within the biofilm compared to those expressed by planktonic bacteria. Bacteria within a biofilm are thought to have a different physiology and gene expression pattern compared to planktonic bacterial cultures. This is important as most of the antibiotic susceptibility testing, such as measurement of the Minimum Inhibitory Concentration is done on bacteria in planktonic cultures, and not bacteria growing in a biofilm. The question is whether resistance to antibiotics via the variety of known resistance mechanisms is identical or different between bacteria in the planktonic versus the biofilm growth state.

Aminoglycoside antibiotics effectively inhibit bacterial protein synthesis by binding to their target on the 30S ribosome. A resistance mutation in the ribosomal protein S12 will result in all ribosomes that are synthesized being resistant to the aminoglycoside streptomycin. However, resistance could also be caused by the expression of an aminoglycoside modifying enzyme, or by the expression of a ribosome modifying enzyme, and the question is whether these enzyme-based resistance mechanisms are equally affecting in planktonic cultures and in biofilms. The study investigated this question by measuring relative resistance in a modified time-kill assay which is based on measuring bacterial survival after exposure to a wide range of antibiotic concentrations over a defined time period.

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1. Introduction

1.1. Biofilm Structure and Physiology

Biofilms, a ubiquitous form of microbial life, are formed by communities of microorganisms encompassed within a self-produced, highly structured extracellular polymeric matrix adhered to a living or non-living surface or pellicle[1]. The matrix can surround single or multiple species of microorganisms including bacteria and some species of protozoa and fungi. Biofilms are complex systems, differing greatly in terms of microbial cell growth rate and physiology to that of planktonic or "free-living" microbes.[1] Many species of Gram-negative and Gram-positive bacteria form biofilms, including *Pseudomonas aeruginosa, Escherichia coli* and *Salmonella* species. In a biofilm, bacteria are embedded within a matrix of extra polymeric substances (EPS), which is composed of proteins, polysaccharides, lipids and extracellular DNA (eDNA).[1] The EPS provides mechanical stability to the biofilm structure and mediates adhesion to surfaces whilst also providing protection for bacteria and allowing for exchange of oxygen and nutrients.[1,4] The mechanisms that regulate biofilm formation and determine it structural features vary between and even within different species of bacteria.[2] The initial attachment of planktonic bacteria to a surface is initially reversible, but as cells aggregate detachment becomes irreversible. Biofilm formation is regulated by signaling cascades and quorum sensing – a form of communication system between cells generated by self-produced signaling molecules.[3] Quorum sensing allows bacteria to coordinate gene expression based on cell density and can regulate many metabolic processes within the biofilm.[2] The general formation of a biofilm can be simplified into four stages; 1 – attachment of planktonic cells to a surface or pellicle, 2 – microcolony formation, 3 – maturation of the biofilm and 4 – dispersal of cells into the surrounding environment. [3, 4] The eDNA is thought to be a stabilizing feature of the biofilm structure, either by forming crosslinks like in *Pseudomonas aeruginosa*, or by secondary stabilization via DNA binding proteins within the matrix.[1,4] Biofilms have a contrasting phenotype to free-living bacteria in terms of growth rate and gene expression and the characteristics of a biofilm cannot be directly compared to that of planktonic cells.[5] The growth rate of bacteria in a biofilm is comparable to that of stationary phase bacteria in a lab, cells are confined to a limited space and nutrient availability is lower in the biofilm, leading to some cells becoming dormant or metabolically inactive.[6]

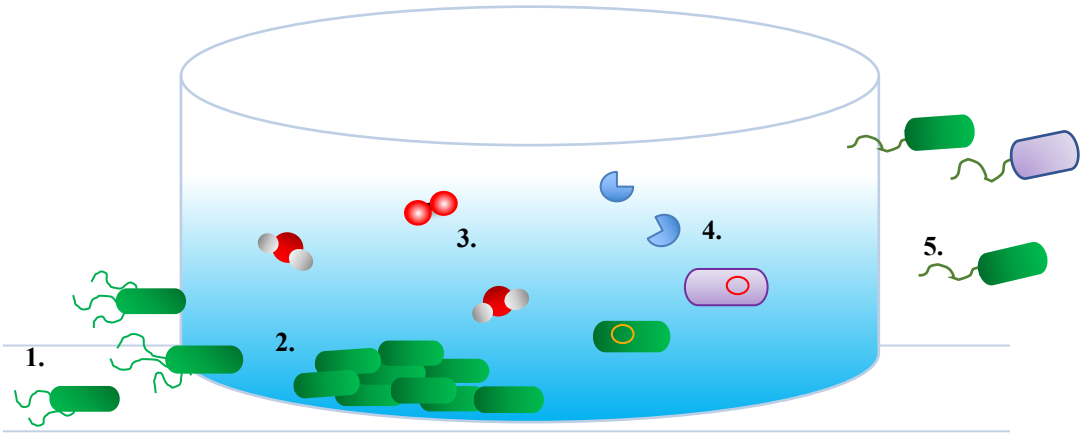

Figure 1. Biofilm formation on a surface. **1.** Planktonic bacteria attach and aggregate on a surface or pellicle. **2** Bacteria form microcolonies and reside within niche in the biofilm. **3.** Limited nutrient availability means that some cells enter a dormant lifestyle. **4.** Biofilm-specific pattern of gene expression where enzyme-based resistance may not be expressed as effectively as in planktonic culture. **5.** Bacteria can detach and disperse from the biofilm into the surrounding environment.

1.2 Biofilm Resistance to Antibiotics

Antibiotic resistance is a serious global issue that threatens human and animal health. Resistance occurs naturally, however the overuse and misuse of antibiotics has resulted in a healthcare problem seen now in every corner of the world.[7] Along with increased mortality, antibiotic resistance results in longer hospital stays and increased use of resources with many infections, such as pneumonia and salmonellosis, which are becoming harder to treat due to the reduced efficacy of antibiotic treatment on resistant strains of bacteria.[7]

The relatively antibiotic-resistant nature of biofilms can be attributed to several factors including the physical barrier to antibiotic entry provided by the EPS, the physiology of bacteria within the biofilm where their slower growth and metabolism increases their intrinsic resistance, and the expression of distinctive resistance mechanisms utilized by bacteria within the biofilm compared to those expressed by planktonic bacteria.[8] Part of the bacterial population within a biofilm can enter a 'dormant' state where they become less susceptible to antibiotics that act by inhibiting growth or metabolic processes.[7,8] These cells are called 'persister' cells and are highly tolerant to antibiotics, even though they have not mutated or acquired any resistance genes to produce a resistant phenotype.[9] The exact mechanism of persister cell generation is not fully understood, however they are thought to arise in response to numerous factors within the biofilm such as the limited oxygen and nutrient supply, oxidative stress and exposure to antibiotics.[9] These tolerant cells may comprise around 1% of the population within the biofilm and although they do not grow in the presence of antibiotics, they remain viable and may return to growth phase in the absence of antibiotics and other environmental stressors.[9,10] Gene expression regulates the formation of the biofilm, and may affect the expression of other genes such as the expression of genes causing antibiotic resistance.[11] Along with tolerant persister cells, there are populations expressing a resistant phenotype caused by genetic alterations such as, or cells that are resistant because of the acquisition of resistance gene cassettes. Bacteria in a biofilm reside in microcolonies where cells are in close proximity to one another and there is evidence for increased horizontal gene transfer within the biofilm compared to planktonic bacteria of the same genotype.[12] In these microcolonies, transformation of DNA is said to occur at higher frequencies, and conjugative plasmid transfer is also more efficient than in planktonic populations.[13] The increased opportunities for transfer of genetic information within a biofilm may contribute to the fact that bacteria can more rapidly become multidrug-resistant to bactericidal antibiotic classes such as aminoglycosides, fluoroquinolones and β-lactams.[11] They may become resistant via conventional mechanisms such as mutation of a target with an altered binding site, synthesis of an enzyme that modifies the drug or modifies the target, or by a mutation that causes overexpression of efflux pumps.[11,12] Antibiotic resistance genes, and mutations causing antibiotic resistance, are routinely studied in planktonic cultures. It is currently unknown to what extent the resistance phenotypes in planktonic cultures translate into resistance phenotypes in the very different environment of a biofilm. Knowledge of whether these antibiotic resistance genes and mutations generate a similar or significantly different resistance phenotype within a biofilm is therefore of particular importance in terms of biofilm treatment with respect to the increased occurrence of antibiotic-resistant bacterial populations seen globally today.

1.3 Aminoglycoside resistance

Aminoglycosides are a class of bactericidal antibiotics that act on Gram-negative and some Gram-positive bacteria by inhibiting protein translation by binding to the bacterial ribosome.[14] They act by binding to the 30S ribosomal subunit, specifically to the 16S rRNA in the vicinity of the A-site which leads to inhibition of translation by interference with polypeptide chain synthesis.[15] There are several ways in which resistance can be generated to aminoglycosides. Resistance mechanisms include: enzymatic modifications of the ribosomal RNA by ribosomal methyltransferases (RMT's) thus preventing binding of the drug to the target; enzymatic modification of the drug by acetyltransferases, phosphotransferases, or nucleotidyltransferases thus reducing the concentration of active drug; a variety of mutations that decrease drug passage across the membrane, thus reducing the intracellular concentration of the drug.[15] In addition, point mutations in the *rpsL* gene which encodes the ribosomal protein S12, part of the 30S ribosomal subunit, generate resistance to the aminoglycoside Streptomycin.[16] This latter case is an example of intrinsic resistance, because all ribosomes that are synthesized should be resistant, and resistance is not dependent on the synthesis of an enzyme for breakdown of the drug or modification of the target. Concerning enzymatic resistance mechanisms: acetyltransferases are enzymes that belong to the GNAT family of proteins (GCN5-*N*-acetyltransferases) which work by acetylation of the amine (NH_2) groups using acetyl coenzyme A as a donor substrate;[17] and ribosomal methyltransferases are enzymes that post-transcriptionally methylate the N-7 position of nucleotide G1405 or N-1 position of A1408 of the16S rRNA.[16]

1.4 Clinical relevance

Biofilms can form in numerous settings and are extremely relevant in modern medicine in a number of ways. Biofilm formation within the human body can be common, such as dental plaque formation on teeth. These microbial aggregates can also form on medical devices like urinary catheters, prosthetic heart valves and cardiac pacemakers.[18] Biofilm formation on medical implants can cause severe chronic infections and become particularly life-threatening upon dispersal into the bloodstream, which can result in sepsis. Medical implant infections account for around 70% of nosocomial infections and single antibiotic treatments are often ineffective due to the complex physiochemical reactions occurring within the biofilms themselves.[19] Even with the refined biotechnical production of most medical implant devices today, they are still susceptible to biofilm adhesion and this represents a major problem for modern medicine. There are a number of persistent diseases associated with biofilm formation such as urethritis, colitis, and vaginitis amongst others.[20] Biofilms are particularly predominant in oral health, with dental plaque being a prevalent and visible biofilm formation. Biofilms have reduced susceptibility to the host immune defense systems and many biofilm infections are also associated with chronic inflammation in conditions such as cystic fibrosis and prostatitis.[21] Biofilms can be difficult to detect using traditional culture-based methods because it is hard to break and detect biofilm fragments, so microscopy and molecular methods are routinely used [22] A rapid and decisive method of detection of a biofilm would be advantageous for clinicians to catch and possibly prevent a chronic infection early on but there are still multiple hurdles before this is possible. Developing drugs and therapeutics to target the biofilm structure and phenotype, or drugs that interfere with the cell signaling that promotes biofilm formation or drugs that would induce detachment of cells from biofilms could be potential strategies to alleviate or even prevent persistent biofilm infections.[22]

2. Aims of the project

Antibiotic resistance is routinely measured using bacteria growing in the planktonic state and thus current knowledge of whether a resistance gene or resistance mutation expresses the same relative level of resistance in a biofilm is still limited. This project addressed whether the relative level of resistance associated with a target mutation versus resistance dependent on the synthesis of an enzyme (target or drug modifying) was identical or different between bacteria in the planktonic versus the biofilm growth state. Aminoglycoside antibiotics effectively inhibit bacterial protein synthesis by binding to their target on the 30S ribosome. A point mutation in the ribosomal protein S12 will result in all ribosomes that are synthesized being resistant to the aminoglycoside streptomycin. However, resistance could also be caused by the expression of an aminoglycoside modifying enzyme, or by the expression of a ribosomal modifying enzyme. A point mutation leading to the change to the structure of the S12 protein should result in all subsequently synthesized ribosomes being intrinsically resistant. However, it is unknown to what degree the expression of a gene conferring resistance via an enzyme that modifies the target or drug will be effective within a biofilm environment compared to a planktonic environment.

To address this question, genes encoding two different resistance enzymes (one drug modifying, the other target modifying), and a chromosomal mutation conferring intrinsic target resistance, were engineered into the chromosome of the biofilm-forming *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) strain ST14028 and the constructed mutants were exposed to different concentrations of aminoglycoside antibiotics in both planktonic and biofilm cultures. Relative resistance was measured in an assay based on measuring bacterial survival after exposure to a wide range of antibiotic concentrations over a defined time period.

3. Materials and Methods

3.1 Bacterial strains

A biofilm-forming *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) strain ST14028s was used as the background for the construction of genetic mutants. Genes conferring resistance to gentamicin (GEN) were PCR amplified from clinical *E. coli* isolates for the purpose of transformation into ST14028s. The *E. coli* isolates were: EN1111 carrying the ribosome methyltransferase gene *rmtB* conferring GEN resistance; EN1077 carrying *aac (3')-IIa* encoding an acetyltransferase which confers resistance to GEN. In addition, a point mutation in *rpsL* (Lysine 42 to Arginine) was engineered into 14028s which confers streptomycin (STR) resistance by synthesis of intrinsically resistant enzymes.

3.2 Growth Conditions for Bacteria

All bacteria were grown in Luria Bertani broth composed of 10g/L Tryptone, 10g/L Sodium Chloride and 5g/L Yeast Extract. Luria agar (LA) was made using LB components with 15g/L agar. Solid medium for selection was made by adding antibiotic to LA before pouring, the volume of antibiotic added depended on desired final concentration in selective plates. All liquid cultures were grown as 2 mL cultures in 15 mL glass tubes for standard overnight cultures or in 15 mL Falcon tubes for time-kill experiments. A single colony was selected and inoculated in 2 mL LB or MHII overnight in a shaking incubator at 37°C. Strains used in lambda red recombineering were grown at 30°C and under selective antibiotic pressure to maintain the plasmid needed for recombination functions.

3.3 Polymerase Chain Reaction

PCR amplification was used to amplify resistance genes for recombination into the *S. typhimurium* strains TH11521 and TH11522. Resistance genes from several clinical *E. coli* strains (EN1111, EN0798, EN1077), were amplified using PCR. The 25µl reactions contained 12.5µl Phusion polymerase, 1.25µl forward primer (fw) and 1.25µl reverse primer (rv), 2.5µl dimethyl sulfoxide (DMSO), 7.5µl dH_2O and 1µl DNA. The SacB program was used to amplify the resistance cassettes from *E. coli* and ran as follows on a Bio-Rad thermo-cycler.

98°C - 30 seconds

98°C - 10 seconds

55 °C - 30 seconds

72 °C - 1minute 30 seconds (adjusted for expected product size)

Back to 98 °C 10 seconds x 30

72 °C - 7 minutes

4°C - forever

3.4 Minimum Inhibitory Concentration Assay Conditions

Minimum inhibitory concentration (MIC) of antibiotic was measured in cation-adjusted Mueller-Hinton II broth. An 8x12 format 96-well round bottomed microtiter plate was used to assay 8 strains with 10 antibiotic concentrations, plus one medium control well and one antibiotic control well per row. To prepare the plate, 50µl of MHII was added into wells 2 to 11 and 100µl into well 12 for the antibiotic control. Antibiotic concentration was prepared from a stock concentration and diluted by MHII to 128µg/mL and vortexed. Next,100µl of the 128µg/mL antibiotic was transferred into well 1 and from this 50µl serial dilutions were performed until well 10. To prepare the inoculum, fresh colonies that were grown on LA plates at 37°C were suspended in 0.9% sodium chloride (NaCl) to an optical density of 0.5 McFarland (measured using a Nephelometer). Bacteria were transferred to 10mL MHII to obtain a final concentration of approximately $1x10^6$ cfu/mL and 50⌈l of this suspension was transferred into wells 1 to 11 to create a final assay volume of 100µl per well. All MIC assay plates were incubated for 16-20 hours at 37°C and inhibition of growth was detected visually.

3.5 λ-Red Recombineering

Gene cassettes from several clinical *E. coli* strains (EN1111, EN0798, EN1077) were amplified via PCR for transformation into *Salmonella* 14028s to create mutant strains with different resistance cassettes. Firstly, pSIM5 with a chloramphenicol ($pSIM5_{CAT)}$ resistance marker was transformed into14028s as follows; 1ml of overnight culture of 14028s was washed with de-ionised water (dH_2O) three times and then resuspended in 100µl dH_2O. From this, 50µl was mixed with 5µl $pSIM5_{CAT}$ and 50µl used as a negative control. The mixture of cells was electroporated and resuspended into 1mL pre-warmed LB. The negative control had 1mL added and all cells were recovered at 30°C overnight. The next day 100µl of all the cells were plated on LA+Cam25 and incubated overnight 30°C. Colonies were then pure streaked and from this an overnight culture was made to be frozen at -80°C.

3.6 Planktonic Time Kill Experiments

Susceptibility of constructed strains to aminoglycosides was analyzed by performing two different time-kill experiments to measure the rate of killing as a function of time and antibiotic exposure. In the initial time-kill experiment all constructed strains and wild-type *Salmonella* 14028s were exposed to gentamicin and streptomycin at 0, 1, 2 and 8 x MIC. Bacteria were grown in Mueller-Hinton II broth and on Mueller-Hinton II agar. The constructed mutant strains were grown in 15mL Falcon tubes in 2ml MHII and incubated overnight at 37°C in a shaking water bath. From the overnight cultures, a starting culture was prepared by 100-fold dilution followed by growth at 37°C for 1.5 hours. During this time, the different antibiotic stock solutions to be used in the time-kill assay were prepared. Stock concentrations of 0x, 1x, 2x and 8x the MIC were prepared by diluting the stock antibiotic by Mueller Hinton II to obtain the desired concentrations relative to the MIC of each strain.

A follow-up second set of time-kill experiments involved exposure of bacterial strains to a larger range of antibiotic concentrations but for 4 hours only. The experimental set-up was identical to that used for the first time-kill assays, however the concentrations 0, 1, 2, 8, 16, 32, 128, and 1024µg/ml were used for exposure and dilutions were plated at time point 0 and after 4 hours of antibiotic exposure.

3.7 Biofilm Time Kill Experiments

Overnight independent cultures were grown in Mueller-Hinton II broth in 15ml Falcon tubes in a shaking water bath at 37°C. The cultures were diluted 10^{-3} (1µl culture in 1000µl of Mueller-Hinton). Next, 200µl was plated into a 96-well microtiter biofilm plate with hydroxyapatite-coated pegs. The biofilm was allowed to grow for 72 hours to form a mature biofilm, and then the lid with the pegs was transferred into 200µl of NaCl for washing off of unbound cells. After 72 hours, five pegs were broken off and suspended in 600µl of NaCl and vortexed for 1 minute, and these replicates served to measure cfu at time point zero before the drug was added. The biofilm plate lid with the remaining pegs was then transferred onto a fresh plate with Mueller-Hinton and different antibiotic concentrations in the wells according to the scheme illustrated in the figure below. The biofilms on these pegs were incubated in the presence of antibiotic for 4 hours, then suspended in NaCl and vortexed to disperse the cells. Dilutions were plated on Mueller-Hinton agar with the actual dilutions employed based on the MIC for each strain and how they had performed in the planktonic time kill. For each dilution,100µl was plated and incubated for 16-20 hours in the 37°C incubator. All strains were exposed to the same concentration range (from zero up to 1024µg/ml) as in the planktonic time-kill experiment.

0	1	2	8	16	32	128	1024

Figure 2. Biofilm plate layout with rows of different concentrations (0g/ml -1024g/ml) of aminoglycoside antibiotic.

3.8 Whole Genome Sequencing

Bacterial genomic DNA was prepared for whole genome sequencing using an Epicentre Genomic DNA Preparation kit as follows. For the preparation of the cell samples, 1µl of 50mg/mL Proteinase K was diluted into 300µl of Tissue and Cell Lysis solution for each sample. The cells were pelleted by centrifugation and supernatant discarded, leaving 25µl of liquid. Cells were resuspended by vortexing for 10 seconds. The Tissue and Cell Lysis solution with Proteinase K was added to the pelleted cells and mixed thoroughly. This was incubated at 65°C for 15 minutes with the mixture vortexed briefly every 5 minutes. Thereafter, samples were cooled to 37°C and 1µl of 5mg/ml RNase A was added and mixed. Samples were then incubated for 30 minutes at 37°C. The next step was precipitation of total DNA. 175µl of MPC Protein Precipitation Reagent was added to 300µl of lysed sample and vortexed for 10 seconds. The debris was pelleted by centrifugation for 20 minutes at 4°C and 10,000g in a microcentrifuge. The supernatant was transferred to a clean microcentrifuge tube and the pellet discarded. Next, 500µl of isopropanol was added to the supernatant and tubes were inverted 30-40 times. The DNA was pelleted by centrifugation at 4°C for 10 minutes in the microcentrifuge. The isopropanol was carefully poured off and samples were washed twice with 200µl of 75% ethanol. Residual ethanol was removed with a pipette and DNA resuspended in 50µl of elution buffer (Qiagen)and placed in 4°C fridge in preparation for Dr. Douglas Huseby, who performed the WGS on a Miseq System from Illumina, California. All WGS data were analysed using the CLC Genomics Workbench (Qiagen Bioinformatics).

3.9 Primers

Table 1. Primers used for amplification of gene cassettes and validation of inserts.

Primer name and function	**Sequence in 5'- 3' direction**
KP122 fw primer for PCR amplification of *rmtB*	TTTAAAACACGTCTGGAAACCCGCTCGCCCGCAGCG TGGTATCAACGGGTTGGGG
KP123 rv primer for PCR amplification of *rmtB*	GCCACGCGTCGCCACCAGGCATCGGGGTATTTTTTA ACGCAAAAAGCCCCGAGCGGTTAAACTCAGGGCTTT ATTTTTTCCAAGAAAATGAGTGCC
KP126 fw primer for PCR amplification of *aac(3)-Iia*	TTTAAAACACGTCTGGAAACCCGCTCGCCCGCAGCA CCAAAATCCCTTAACGTGA
KP127 rv primer for PCR amplification of *aac(3)-Iia*	GCCACGCGTCGCCACCAGGCATCGGGGTATTTTTTA ACGCAAAAAGCCCCGAGCGGTTAAACTCAGGGCTTT ATTTTGTCGACGGCCTCTAACCGG
LP3 ss lambda red for insertion of rpsL point mutation in 14028s	CGAACACGGCAAACTTTACGCAGTGCGGAGTTCGGT TTTcTAGGAGTGGTAGTATATACACGAGTACATACG CCACGTTT
Bio 14 fw primer for 14028s lambda red recipient	GCGAATGTGGACGTGAAA
Bio 15 rv primer for 14028s lambda red recipient	CGGGTAGGCCTGATAAGA

4. Results

4.1 Strain Construction using λ-Red Recombineering

Strain 14028s was transformed with the plasmid pSIM5 carrying a chloramphenicol resistance marker and the λ genes required to promote efficient recombination of linear DNA into the chromosome. Successful transformation of $pSIM5_{CAT}$ was validated by the selection of colonies on LA + chloramphenicol. Colonies pure streaked on the same medium. The PCR products carrying resistance gene cassettes of *rmtB* and *aac* were then transformed by electroporation into 14028s + $pSIM5_{CAT}$, the λ-red genes were induced, and recombinants were selected by plating the culture on LA+ gentamicin. The *rpsL* mutation was introduced in 14028s + $pSIM5_{CAT}$ by transforming a single-stranded oligonucleotide and selecting recombinants on LA+streptomycin after induction of the λ-red genes. Colonies from each selection were pure streaked on the same medium and stocks of each strain were stored frozen at -80°C. All recombinants were validated by diagnostic PCR and by DNA sequencing to confirm the correct gene sequence in each strain.

4.2 Minimum Inhibitory Concentration Assay

After validating successful strain construction, the minimum inhibitory concentration was determined for all constructed mutant strains and the wild-type 14028s. An assay was set up where wild-type, *rmtB, aac* and *rpsL* constructed strains were exposed to gentamicin and streptomycin at a concentration range of 0μg/mL up to 1024 μg/mL.

Table 2. Minimum Inhibitory Concentration values for Gentamicin and Streptomycin.

Strain	**MIC gentamicin (μg/ml)**	**MIC streptomycin (μg/ml)**
wild-type 14028s	1 μg/ml	8 μg/ml
rpsL mutant	1 μg/ml	>1024 μg/ml
aac mutant	16 μg/ml	8 μg/ml
rmtB mutant	>1024 μg/ml	8 μg/ml

Wild-type 14028s had a MIC value of 1μg/ml for gentamicin and 8μg/ml for streptomycin. The *rpsL* mutant had a value of 1μg/ml for gentamicin, and as expected, more than 1024 μg/ml for streptomycin. The *aac* mutant had a value of 8μg/ml for streptomycin and an intermediate value of 16μg/ml for gentamicin, the aminoglycoside it is known to confer resistance to. The *rmtB* mutant had a MIC value of 8μg/ml for streptomycin and an off-the-scale value of more than 1024μg/ml for gentamicin.
For *Salmonella* the current clinical susceptible breakpoint value for gentamicin is ≤ 4μg/ml and the resistant breakpoint ≥ 16μg/ml. For streptomycin, the susceptible breakpoint is ≤ 16μg/ml and the resistant breakpoint is ≥ 32μg/ml.

4.3 Time Kill Experiments

4.3.1 Planktonic Time Kills

To validate that the mutant strains confer resistance to their respective antibiotic and grow in planktonic culture, they, along with wild-type 14028s were exposed to concentrations of 0x, 1x, 2x, and 8x their respective MIC values for up to 24 hours and samples were taken to count surviving cfu's at defined time points. For the *rpsL* and *rmtB* mutants, concentrations of 0µg/ml and 128µg/ml were used as both had MIC values of beyond 1024µg/ml and so a concentration well above the MIC for the wild-type but well below the MIC for each strain was used to demonstrate relative resistance of the strain compared to the no drug control.

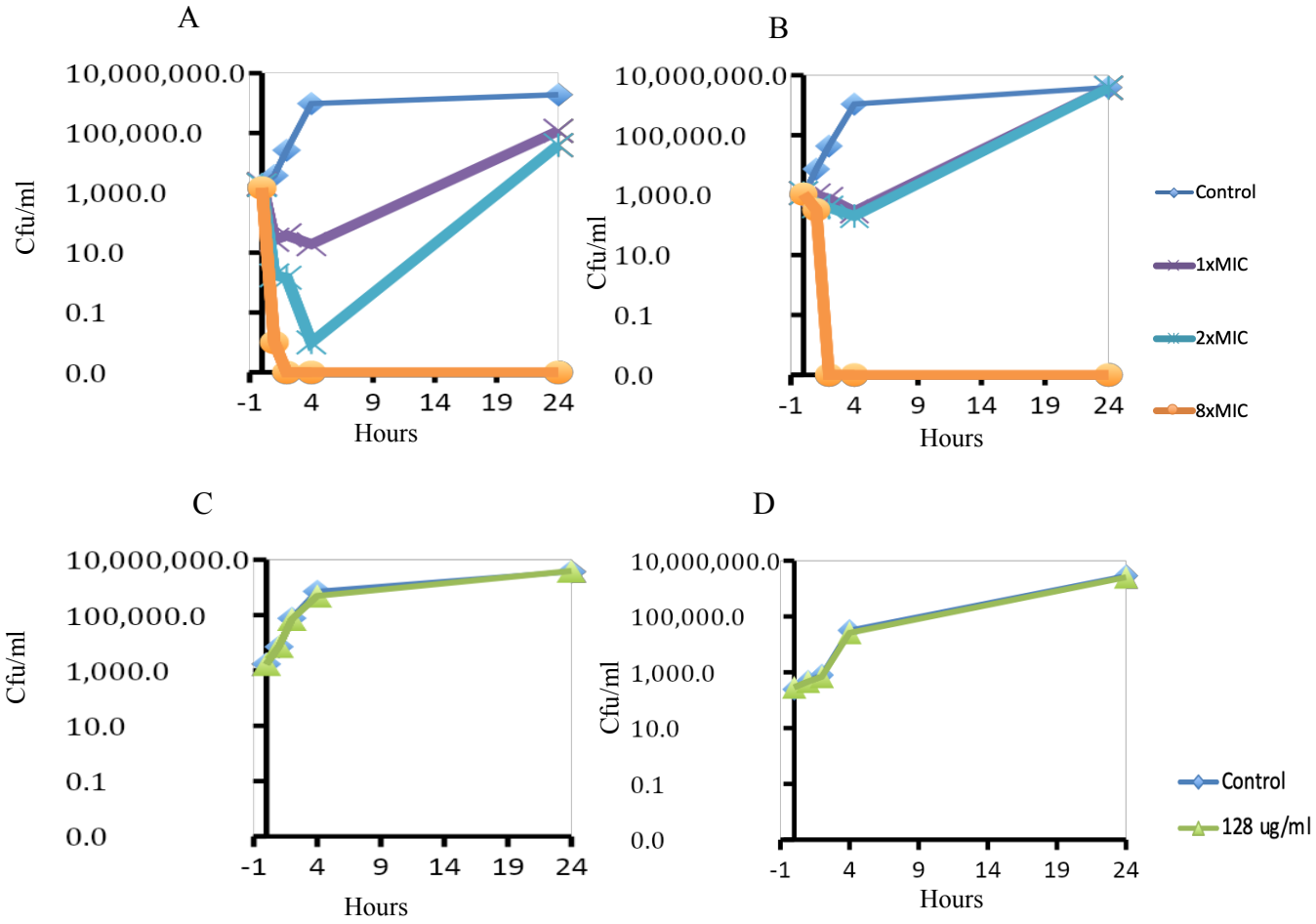

Figure 3. Planktonic time kill experiments with (A) wild-type, (B) *aac*, (C) *rmtB* and GEN and (D) *rpsL* and STR. Susceptible wild-type is killed after 4 hours at 1x and 2x MIC and killed after 1 hour at 8x MIC exposure (A). The *aac* mutant is killed after 4 hours at 1x and 2x MIC and after 2 hours at 8x MIC. Cultures exposed to 1x and 2x MIC recover to control population numbers after 24 hours whereas cultures exposed to 8x MIC do not recover. The *rmtB* mutant (C) and *rpsL* mutant (D) both grow as well as the growth control with no drug at all time points.

Viable colonies that grew on the Mueller-Hinton plated were counted for each drug concentration and colony-forming units determined and plotted to form the graphs shown in Figure 3. The wildtype 14028s (Fig.3 A) was killed after 1 hour of exposure to 8x MIC (8µg/ml) GEN, and after 4 hours at 1x MIC (1µg/ml) and 2x (2µg/ml). The *aac* mutant (Fig.3 B) was killed after 2 hours of exposure to 8x MIC (128µg/ml) and some killing occurred at 1xMIC (8µg/ml) and 2xMIC (16µg/ml) although not to the extent of wildtype population reduction. Both the *rmtB* (Fig.3 C) and *rpsL* (Fig.3 D) mutants grew equally well in the presence of drug at 128µg/ml (128x wildtype MIC and 8x *aac* MIC for GEN).

4.3.2 Planktonic vs Biofilm Time Kill Experiments

Relative resistance of the mutants and wild-type was measured in both planktonic and biofilm time-kill assays which measured bacterial survival after 4 hours during which they were exposed to an antibiotic concentration range of 0 - 1024µg/ml. Planktonic time kill assays were set up whilst the biofilm grew for 72 hours to reach maturity. Each strain was grown for 4 hours at 37°C in the following tubes; 0, 1, 2, 8, 16, 32, 128 and 1024 µg/ml. Samples were taken at time point 0, before drug was added, and again after 4 hours exposure. For the biofilm, after 72 hours the pegs were transferred to NaCl to wash and one row of pegs broken off to replicate time point 0 as in planktonic. Next, the remaining pegs with the formed biofilms was transferred into a fresh plate with rows containing the different final concentrations of drug 0 - 1024µg/ml. Dilutions were plated on non-selective Mueller-Hinton plates and colonies counted the following day.

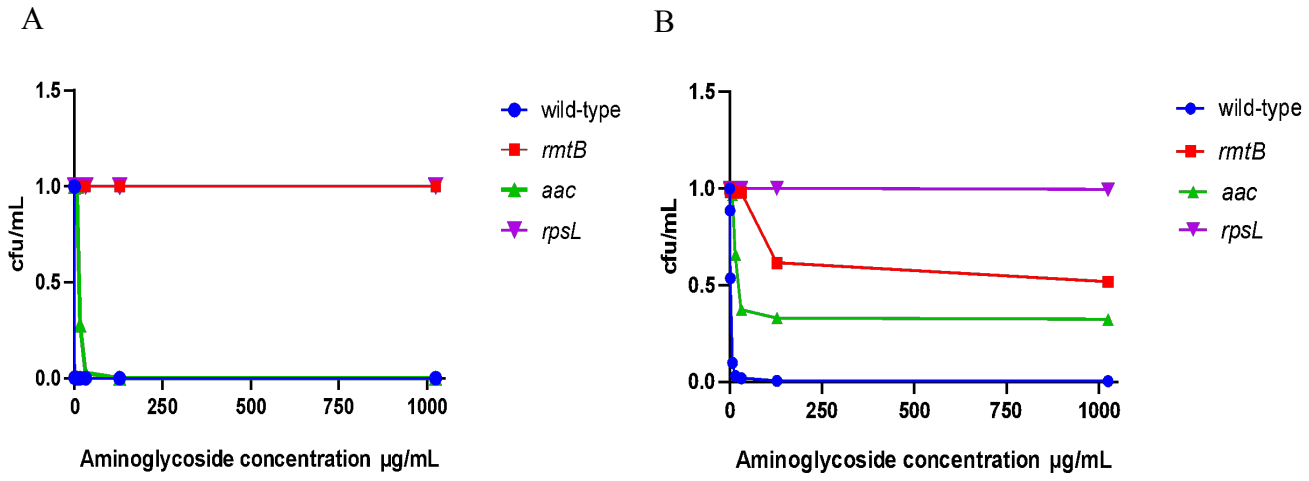

Figure 4. Time kill results show bacterial killing as a function of antibiotic concentration after 4 hours of exposure for bacteria growing in planktonic culture (A), and in a biofilm (B). The wildtype is killed at low antibiotic concentrations in both planktonic and in a biofilm whereas the *rpsL* mutant grows equally well in planktonic and in biofilm at high antibiotic concentrations. The *aac* mutant shows reduced killing in a biofilm in the presence of antibiotics compared to in planktonic culture. The *rmtB* mutant population decreased after 4 hours in the biofilm in the presence of high antibiotic concentrations but was resistant to all tested concentrations in the planktonic culture.

When exposed to antibiotic at 1µg/ml and above the wildtype was killed after 4 hours in both planktonic and biofilm assay. The *rpsL* mutant maintained the same population numbers after 4 hours at every concentration of antibiotic in both planktonic and biofilm. After 4 hours, the *aac* mutant population was reduced to a quarter of the starting population when exposed to 16µg/ml and was killed at higher concentrations in the planktonic assay. In the biofilm, the *aac* mutant population was reduced to just below half of the starting population, but remained stable at higher concentrations of antibiotic. In the planktonic assay, the *rmtB* mutant was unaffected by exposure to high concentrations of antibiotic and no killing was observed after 4 hours. However, in the biofilm, the *rmtB* population almost reduced by half at exposure to 128µg/ml antibiotic, which is almost ten-fold less than the >1024 µg/ml MIC value for this mutant.

4.4 Efficiency of Gene Protection in Planktonic and Biofilm

The next step was to analyze the data to assess the efficiency of protection the gene may provide compared to the wild-type in planktonic and biofilm culture. Data was first normalized to time point 0 in the planktonic culture assay, so the number of colonies at each concentration was divided by the number of colonies at the start of the time kill. For the biofilm, data was normalized to the no drug control and colony count after four hours was divided by the no drug value after four hours. Protection of the gene or mutation was calculated on a log scale by dividing the number of colonies for each concentration of drug by the value of the wild-type at that concentration in either the biofilm or planktonic culture.

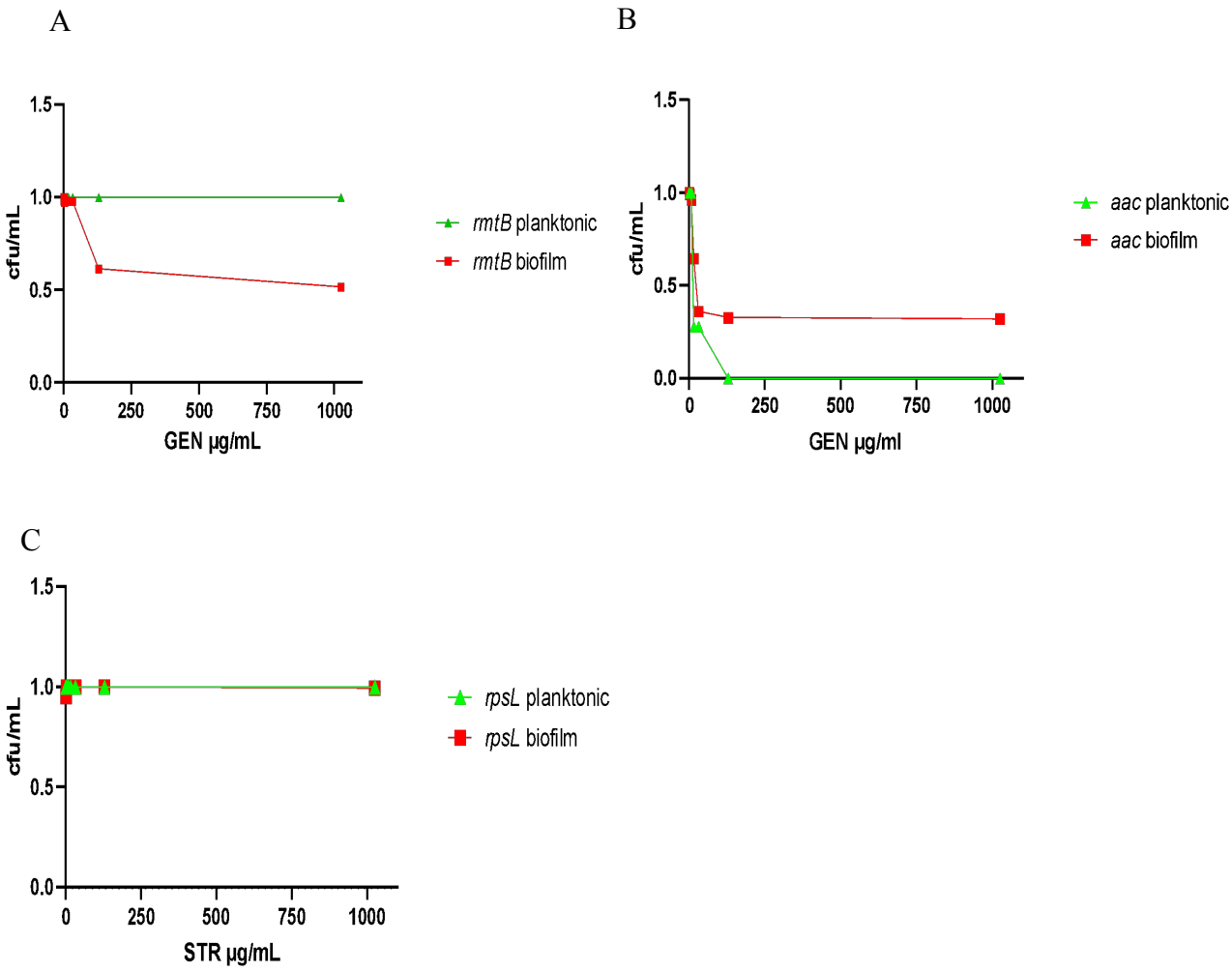

Figure 5. The relative antibiotic-protective phenotype of each gene relative to wildtype susceptibility.
The relative protective phenotype of *rmtB* gene is less efficient in biofilm compared to planktonic culture (A). The protective phenotype of the *aac* gene is more effective in biofilm compared to planktonic culture (B)
The relative protective phenotype of *rpsL* mutation is equally efficient in biofilm and planktonic culture (C)

The relative resistance phenotype for *rmtB* which encodes a ribosome target modifying enzyme (Fig.5, A) is shown to be much more efficient in planktonic growth compared to biofilm growth, even at concentrations well below the MIC value. The *rmtB* population is almost halved at 128µg/ml when grown in a biofilm compared to the full protection seen in planktonic growth. The resistant phenotype of *aac,* which encodes an aminoglycoside modifying enzyme, is somewhat effective at protection in a biofilm as the population is not reduced to the same extent at concentrations above the MIC of 16µg/ml compared to reduction of planktonic growth (Fig.5, B). The *rpsL* which alters the target ribosomes conferring intrinsic resistance is equally effective at protecting in both planktonic and biofilm culture.

4.5 Whole Genome Sequencing

The resistance gene cassettes were inserted via λ-red recombination at nucleotide position 2412157 of the *Salmonella* 14028s chromosome between the genes *eco* and *yojl*. The *rpsL* point mutation was conferred via single-stranded λ-red which led to an amino acid change of AAA -AGA conferring a change from Lysine to Arginine at position 42.

5. Discussion

As biofilm physiology and growth is quite distinct from planktonic growth, it is reasonable to assume that resistance gene expression will differ between them. The reduced susceptibility of biofilms to antibiotics can be viewed as a phenotypic trait as cells within a biofilm are often compared to those in planktonic stationary phase, and these planktonic stationary phase cells have been shown to regain antibiotic susceptibility upon dilution into fresh media which may reduce cell-cell signaling.[23] This reversible susceptibility highlights that tolerance is a phenotype rather than strictly due to genetic alterations. However, biofilm specialized cells can arise from differences in gene expression but potentially not composition and so the questions asked are whether these differences determine resistance gene expression and phenotype. There have been many studies investigating gene expression patterns in biofilms and comparison with planktonic cultures. Fux *et al*.showed that gene expression in biofilms differed from that of planktonic cells by 1% in *P. aeruginosa* and 6% in *B. subtilis* using DNA microarray assays which measures the expression of genes.[24] Furthermore, a study by Whiteley *et al*. in 2001 used a microarray to compare gene expression between planktonic and biofilm growth in *Pseudomonas aeruginosa.* They found few genes that expressed differentially in biofilms compared to planktonic but remarked that their analysis averaged the whole gene expression of the biofilm, and did not consider the heterogenous nature of biofilms and the fact that many microcolonies could exhibit different gene expression profiles to an adjacent subpopulation within the same biofilm, so some microcolonies could exhibit largely different profiles than homogenous planktonic cultures.[25]
As predicted, the strain carrying the mutated *rpsL* gene conferring resistance by a target mutation grew equally well in planktonic and biofilm cultures at a range of antibiotic concentrations (Fig.4 and Fig.5, C). This highlights that a chromosomal mutation conferring intrinsic resistance by a mutationally altered antibiotic target (the ribosome in this case) provides protection for bacteria in planktonic and a biofilm states equally well. Another example of an intrinsic resistance mechanism that can be expressed in the biofilm is through the mutationally altered membrane-proteins or porin genes that affect the permeability of bacteria to antibiotics.[23] Mutations in *ompF* or *ompC* which encode outer membrane porins OmpF and OmpC have been shown to increase resistance to β-lactam antibiotics.[24] Interestingly, the *rmtB* mutant did not grow as well in a biofilm and expressed a weaker resistance phenotype compared to planktonic culture, even at a concentration ten-fold below its MIC value (Fig. 4 and Fig.5 A). From the results it seems the expression of a gene leading to synthesis of a resistance enzyme that post-transcriptionally alters the target is less effective at protecting against the antibiotic in the biofilm. The *brlR* gene which stands for biofilm resistance locus regulator is a transcriptional activator of biofilm-specific resistance in

Pseudomonas aeruginosa and the protein product of this gene BrlR is unique as it is found in biofilms but not planktonic culture.[27] However, in a study by Stewart *et al* in 2015 there was no difference in antibiotic resistance to tobramycin between a *blrR* mutant and the wild-type, but further studies suggest that *brlR* may not be expressed under the growth conditions used in the study.[27] Suboptimal growth conditions may be a factor that influenced the *rmtB* mutant, however, the weaker resistance phenotype observed in the biofilm may be due to the upregulation of biofilm-specific functional genes and the down-regulation of *rmtB*. A study by Becker *et al.* in 2001 using a micro-representational-difference analysis (micro-RDA) identified five genes in *Staphylococcus aureus* that are differentially expressed in the biofilm compared to planktonic cultures. The genes encoded enzymes of the glycolysis/fermentation pathway which is essential in regard to the limited oxygen availability and gradients within the biofilm.[28] The gene expression within an *E.coli* biofilm has been shown to be altered due to oxygen deprivation.[28] In this sense, upregulation of genes encoding enzymes focused on survival aspects such as nutrient or oxygen acquisition could take precedence over the expression of a gene which codes for an enzyme that has to bind to a transcriptional target may be less expressed.

The *aac* mutant demonstrated a weaker resistance phenotype in the planktonic culture compared to in the biofilm, this may be due to the tolerant nature of biofilms previously described. In Figure.5, B it is shown that after exposure to a concentration of 16μg/ml onwards of gentamicin, the population of *aac* mutants remains stable and does not drop to population numbers seen in the planktonic culture. This could be due to the presence of slow growing, metabolically inactive cells within the population in response to antibiotic stress, and improved survival in biofilm may be explained by an altered reaction to cell damage, however whether these dormant cells arise within 4 hours is unclear. It could again also be due to the heterogenous nature within a biofilm and a subpopulation may be experiencing a different environment with different nutrient gradients and so growing at a different rate.[29] The level of resistance between the two enzyme-synthesizing mutants *rmtB* and *aac* may be important in terms of biofilm resistance phenotype. A study by Pozzi *et al.* in 2012 highlighted the importance of the level of resistance of beta-lactams for biofilm phenotype in methicillin-resistant and susceptible *Staphylococcus aureus* (MRSA and MSSA) and their expression of either low-level heterogenous resistance or high-level homogenous resistance and found that hospital-acquired MRSA displayed an overall downregulation of resistance or virulent gene expression.[31,32]

Overall, from the time-kill experiments it can be concluded that the resistance mechanism that may be very efficient in producing a successful resistance phenotype in planktonic culture may not always translate to success within a biofilm, such as the case of *rmtB*, which had a MIC value of > 1024 μg/ml and grew equally as well as the control in the planktonic assay, but when subjected to antibiotic exposure in the biofilm, the *rmtB* population was inhibited/killed at a concentration of 128μg/ml. On the other hand, the drug modifying *aac* seemed to show somewhat protective efficiency within the biofilm after the MIC of 16μg/ml, but this may be due to the protective physiological nature of the biofilm. The intrinsically resistant *rpsL* mutant demonstrated that some resistance mechanisms are equally effective in both planktonic and biofilm growth, regardless of outside factors. These results may highlight the importance of implementing accurate susceptibility testing for bacteria that are in a biofilm as well as the routinely measured planktonic cultures, as they can provide two very different environments for bacteria to express resistance phenotypes.

Future studies could incorporate such time-kill assays to other resistance genes and mutations such as the *gyrA* mutation conferring resistance to fluoroquinolones to elucidate whether the same effects are observed between planktonic and biofilm antibiotic resistance phenotype.

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