UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE VETERINARIA

DEPARTAMENTO DE SANIDAD ANIMAL

TESIS DOCTORAL

Regulation of the 16S rRNA aminoglycoside resistance methyltransferases: a functional and mutational analysis of the armA 5’ UTR

Regulación de las metiltransferasas del ARNr 16S de resistencia a aminoglucósidos: análisis funcional y mutacional del 5’ UTR de armA

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Andreas Hoefer

DIRECTOR

Bruno González Zorn

Madrid, 2018

TESIS DOCTORAL

Regulation of the 16S rRNA aminoglycoside resistance methyltransferases:

A functional and mutational analysis of the *armA* 5' UTR

Regulación de las metiltransferasas del ARNr 16S de resistencia a aminoglucósidos:

Análisis funcional y mutacional del 5' UTR de *armA*

MEMORIA PRESENTADA PARA OPTAR AL GRADO DE DOCTOR POR

Andreas Hoefer

Bajo la dirección del Profesor

Bruno González Zorn

Madrid, mayo de 2017

UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE VETERINARIA

Departamento de Sanidad Animal

D. Bruno González Zorn, Profesor Titular del Departamento de Sanidad Animal de la Facultad de Veterinaria de la Universidad Complutense de Madrid (UCM),

CERTIFICA:

Que la Tesis Doctoral titulada

"Regulation of the 16S rRNA aminoglycoside resistance methyltransferases: A functional and mutational analysis of the *armA* 5' UTR"

presentada por Don Andreas Hoefer, Licenciado en Microbiología y Máster en Investigación en Ciencias Biomédicas por la Universidad de Warwick, Reino Unido, ha sido realizada bajo mi dirección en las dependencias del Departamento de Sanidad Animal de la Facultad de Veterinaria, y estimamos que cumple todos los requisitos necesarios para optar al grado de Doctor por la Universidad Complutense de Madrid.

De acuerdo con la normativa vigente, firmo el presente certificado, autorizando a su defensa y presentación, como director de la mencionada tesis doctoral.

Madrid, mayo de 2017

Fdo.: Dr. Bruno González Zorn

ACKNOWLEDGEMENTS

An erster Stelle würde ich mich gerne bei meinen Eltern und meiner Schwester bedanken! Meine Eltern die für mich unendlich viel Geduld hatten um mich für die letzten 12 Jahre zu unterstützen! Ohne euch währe diese Arbeit unmöglich gewesen! An meine Schwester, mit der ich immer über alles reden konnte, die ich immer anrufen konnte als ich mich ablenken wollte, auch ohne dich wäre diese Arbeit unmöglich gewesen. Vielen vielen Dank an euch drei, hoffentlich werde ich jetzt mehr zeit haben, die ich mit euch verbringen kann! Ich würde mich auch gerne am Rest der Familie bedanken; an alle die mich hier besucht haben und alle die immer Zeit hatten als ich zu besuch in Deutschland war – Vielen Dank!

Next I would like to send a big thank you to all the close friends that have stood by me through the years, without all those special times in the past I wouldn't be the person I am today. From our earliest experiments designing and building parachutes (that obviously did nothing) to more recent experimentation with fluid dynamics I treasure each and every memory with all of you – you know exactly who you are.

To everyone in the UK, that really shaped me as an – I want to say adult, but I'm not quite sure if I'm there yet – it has been my upmost pleasure to have shared these life defining moments with you. Whether we were out camping, spending endless days walking around outside or up late night discussing the most absurd things, I sincerely thank you! A special thanks to those of you that took the time to come visit me here in Spain and, again, to those that were always ready to go when I stopped by.

Next I would like to thank everyone in Madrid that made this time so special, both in and out of the lab! Bruno, thank you for all the time and effort that you invested in the project and me. Without the late nights, and hard work you put in there wouldn't have even been a project. A very special thank you (and probably an apology as well) goes

to Nata! Without you taking better care of us than we deserve, nothing would move forward. Thank you for the patience and tireless support over the years to both of you.

This environment that we are so lucky to call our 'working' environment is more special than any of us will realize until its in the past. A very special thank you to every single one of you in the lab that really made me feel at home from the first minute. A special thank you to Dani and Laura, who helped me with everything at the beginning, and Dani for literally always being there when you need a hand and always with a smile! Dani, you're a great guy and I really hope that at least one of the applications we wrote together is accepted and you can go do whatever you want! Gabi and Alfonso, for all the nights rollerblading in the garage, throwing things out of my window, the festivals etc. To Belen for teaching me everything I know! A very special thank you to Bosco as well, you've been a great student and I very very much appreciate the time and effort you put into our experiments. Carri for all the times we shared inside and out of the lab! A big thank you to Cristina for all the patience, help and good times! Cris and Jose… the methylase group… its been a real pleasure. And last but definitely not least, thank you to Manu for deleting all the protocols off the thermo cycler ;) and for carefully proofreading my thesis. To each and every one of you, you have been more than friends in my time here and I very much look forward to times with you in the future!

Within the faculty, a very special thank you also goes to Belen and her lab downstairs for her extremely helpful attitude - many of the experiments performed wouldn't have been possible without you! Also, a big thank you to Gustavo and Javi for letting me visualize 1000s of Western Blots in their lab!

Wow… so many people to thank. I can't not mention the TRAIN-ASAP group for all the very excellent times spent around Europe 'Training' – I also very much look forward to times shared with you in the future! Thank you to Mathieu, Carlos, Ali and many many more outside of the lab!

Finally, and most importantly! Thank you Maria! You have been there pretty much throughout my entire thesis! You were calm when I was stressed, you were patient when I was in the lab until the early hours of the morning and you were always there when I needed you! You took a lot of interest in our work even though it is so very different from your work. You spent a lot of great time with me in the lab and with the lab group (that basically all like you more than me – and I don't blame them). Genuinely, without you, this process would have been 100 times harder! I cannot thank you enough! And to your family as well! Your mom, dad, Diego, Helena, Manu and Veronica thank you very much for all the wonderful times we had!

Thank you all so very much!

INDICE

1 TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

2-DOS	2-Deoxystreptamine
AAC	Aminoglycoside acetyltransferase
AMR	Antimicrobial Resistance
ANT	Aminoglycoside
Arm	Aminoglycosde resistance methyltransferase
BHI	Brain Heart Infusion
CDS	Coding sequence
CFU	Colony forming units
CLSI	Clinical and Laboratory Standards Institute
ECDC	European Center for Disease Control
EDP	Energy dependent process
ESBL	Extended spectrum β-lactamases
EUCAST	European Committee on Antimicrobial Resistance Testing
IDSA	Infectious Diseases Society for America
Inc	Incompatibility group
IS	Insertion sequence
ISCR	Insertion sequence common region
Kam	Kanamycin-apramycin methyltransferase
Kgm	Kanamycin-gentamicin methyltransferase
KPC	Klebsiella pneumoniae carbapenemase
LB	Lysogeny broth
MDR	Multi-drug resistance
MH	Mueller-Hinton
MIC	Minimal inhibitory concentration
mRNA	Messenger ribonucleic acid
NDM	New Delhi Metallo-beta-lactamase
NMR	Nuclear magnetic resonance
nt(s)	Nucleotide(s)
ORF	Open reading frame
PAM	Pan-aminoglycoside resistance methyltransferase

PCR	Polymerase chain reaction
RACE	Rapid amplification of cDNA ends
RMTases	Ribosomal methyltransferases
ROS	Reactive oxygen species
RpoD	RNA polymerase sigma factor
rRNA	Ribosomal ribonucleic acid
Rsm	Ribosomal small subunit methyltransferase
RT-PCR	Reverse transcription polymerase chain reaction
RT-qPCR	Qualitative reverse transcription polymerase chain reaction
SAM	S-adenosylmethionine
spp.	Species
Tn	Transposon
tRNA	Transfer ribonucleic acid
UTR	Untranslater region
WHO	World Health Organization
XDR	Extensively drug-resistant

SUMMARY

Antimicrobial resistance remains one of the most serious threats to modern medicine. As such, the selection and administration of antibiotics must be carefully considered, especially since the discovery of novel antimicrobial agents has reduced substantially. Agents that have previously been dismissed for unfavorable secondary activity are becoming increasingly valuable as the resistance prevalence towards the first choice antibiotic increases drastically. The aminoglycosides are a family of antibiotics that belong to this category. Shortly after their introduction to the market, problems with toxicity became apparent and they were no longer considered a drug of choice for physicians. However, increasingly worrisome resistance trends have forced physicians to reevaluate the application of aminoglycosides for life-threatening infections caused by Gram-negative pathogens.

Among other resistance mechanisms, the 16S ribosomal RNA methyltransferases have emerged in Gram-negative pathogenic bacteria as an acquired resistance mechanism conferring high-level resistance to all clinically relevant aminoglycosides, even ones that have not yet been released to the market. Since their discovery in 2003, a total of 13 acquired methyltransferases have been identified including three variants. The rate at which these resistance determinants are spreading, combined with the broad resistance profile they confer to most clinically significant aminoglycosides, is jeopardizing the aminoglycosides as a viable last resort antibiotic.

Initially, the objectives of the here presented doctoral thesis were to perform a comprehensive in silico analysis of the various families of 16S rRNA methyltransferases, including acquired resistance conferring-, intrinsic resistance conferring- and housekeeping- methyltransferases. The aim of this was to identify a putative origin of the acquired resistance conferring methyltransferases, to potentially discovering their ancestral regulatory mechanism. This study revealed that the acquired 16S

methyltransferases most likely convergently evolved from a number of different RNA methyltransferases. Furthermore, this study also demonstrated a high degree of plasticity in the 5' upstream regions between the various acquired resistance conferring methyltransferases, although the region immediately upstream of the coding region was relatively conserved throughout isolate of the same genes. This plasticity is most likely a result of the highly versatile mobile genetic elements in which these acquired resistance determinants are found.

Subsequent promoter predictions performed within a 400 nucleotide region upstream of the CDS of the acquired methyltransferases demonstrated a general conservation of a 'TTGACG' -35 box and a 'TTACACT' -10 box. Using *armA*, the most prevalent 16S aminoglycoside resistance methyltransferase as a model system, we then set out to characterize the region responsible for the expression of *armA*. Sequence alignments demonstrated a very high degree of conservation in the region 221 nucleotides upstream of the resistance gene in all available reference sequences, which harbors the putative promoter elements mentioned. Subsequent rapid amplification of cDNA ends (RACE) identified a 5' UTR of precisely 139 nucleotides. A truncational analysis of this upstream region demonstrated the presence of a domain absolutely necessary for the expression of *armA* immediately upstream of the confirmed 139 nucleotide 5' UTR, as confirmed via the resistance profile and Western Blot analysis by means of a triple FlagTag.

The fusion of the extended *armA* 5' upstream region to a *lacZα* reporter construct demonstrated that under the regulation of the *armA* upstream region the reporter gene was constitutively expressed. Subsequent investigations revealed this constitutive expression was not affected by the presence or absence of neither aminoglycosides nor active methyltransferases in trans. As the *armA* encoding methyltransferase does not appear to be regulated by any known post-transcriptional mechanisms, the study then focused on the upstream promoter region.

We then performed a mutational analysis of this promoter region to identify the specific nucleotides required for the accurate expression of the resistance gene. The results of this study were in-keeping with the model of a sigma factor RpoD recognized promoter region. Substitutions of conserved nucleotides resulted in the complete suppression of *armA* to levels of the susceptible phenotype. Substitutions of less conserved nucleotides also resulted in a three to five fold drop in the MIC conferred by *armA*, which was also demonstrated by Western blot analysis. This suggests that the housekeeping sigma factor RpoD, which preferentially transcribes genes associated with the protein synthesis machinery, induces the transcription of this resistance gene.

Investigations into the expressional profile of *armA* throughout its growth phases indicated an augmented expression that correlates with the exponential phase of bacterial growth. Both Western Blot and RT-qPCR analysis indicated an expressional peak between 2-5 hours of growth as compared to chromosomal reference genes. This further supports the capacity of the promoter region to recruit the RpoD sigma factor to modulate *armA* expression according to expressional levels of other components of the protein synthesis machinery.

As such, this study constitutes an in depth analysis into the putative origins of the acquired 16S rRNA methyltransferases as well as a first mutational study of the promoter region of *armA*. A single nucleotide resolution of this promoter region identifies the key nucleotides responsible for the accurate expression of this prevalent resistance mechanism. Finally, this promoter is likely to ensure adequate resistance levels in the host as it is transcribed using the housekeeping sigma factor RpoD, responsible for the transcription of protein synthesis related gene clusters. This study constitutes a first attempt to elucidate the underlying regulatory mechanisms of the acquired16S rRNA resistance methyltransferases. This resistance mechanism, that results in high level resistance to most clinically significant aminoglycosides, endangers the use of this valuable antibiotic family as a last resort treatment for future medicine.

RESUMEN

La resistencia a antibióticos supone una de las principales amenazas para la medicina moderna. Esto, junto con la drástica disminución del descubrimiento de nuevas moléculas antibióticas, hace que la elección y administración de tratamientos antibióticos deba ser controlada escrupulosamente. Fármacos que anteriormente eran rechazados por sus efectos adversos están comenzando a utilizarse de nuevo debido a los elevados niveles de resistencia a los antibióticos de primera elección. Los aminoglucósidos son un grupo de antibióticos que pertenecen a esta categoría. Poco después de su introducción en el mercado fueron desechados como fármacos de elección debido a su elevada toxicidad. Sin embargo, el alarmante incremento de la resistencia a antibióticos ha obligado a los clínicos a reevaluar el uso de los aminoglucósidos para tratar infecciones graves producidas por patógenos Gram negativos.

Entre otros mecanismos de resistencia, las metiltransferasas del ARNr 16S han emergido en bacterias patógenas Gram negativas como un mecanismo de resistencia adquirido, confiriendo elevados niveles de resistencia frente a todos los aminoglucósidos clínicamente relevantes, incluso también frente a nuevos aminoglucósidos que todavía no han salido al mercado. Desde su descubrimiento en el año 2003, se han identificado un total de 13 metiltransferasas, incluyendo tres variantes. La velocidad a la que estos determinantes de resistencia se están diseminando, junto con el amplio espectro de aminoglucósidos frente a los que confieren resistencia, está poniendo en peligro el empleo de este grupo de fármacos como antibióticos de último recurso.

Inicialmente, los objetivos de esta Tesis Doctoral consistieron en realizar un estudio in silico en profundidad de las diferentes familias de metiltransferasas del ARNr 16S, que incluyen a las metiltransferasas de resistencia, tanto adquiridas como intrínsecas, y a las metiltransferasas endógenas. El objetivo de este análisis fue poder identificar el origen putativo de las metiltransferasas de resistencia adquiridas, con la finalidad de

descubrir el origen de su mecanismo de regulación. Este estudio reveló que las metiltransferasas adquiridas del ARNr 16S probablemente evolucionaron de manera convergente a partir de las metilasas intrínsecas del ARN. Además, este análisis también mostró el alto grado de plasticidad en la región 5´ no codificante de las metiltransferasas adquiridas, aunque en distintos aislados de cada uno de los genes codificantes pudimos observar que la región inmediatamente corriente arriba de la secuencia codificante presentaba un alto grado de conservación. Esta plasticidad es debida, presumiblemente, a la versatilidad de los elementos genéticos móviles en los que estos determinantes de resistencia se localizan.

Seguidamente, se estudió la presencia de regiones promotoras comprendidas en los 400 nucleótidos situados corriente arriba de las secuencias codificantes de las metilasas adquiridas. Este análisis reveló la existencia de un promotor putativo conservado, siendo la caja -35 "TTGACG" y la caja -10 "TTACACT". Usando como modelo ArmA, la metiltransferasa 16S de resistencia a aminoglucósidos más prevalente, se caracterizó la región responsable de la expresión del gen *armA*. El alineamiento de las secuencias descritas de *armA* mostró un alto grado de conservación de los primeros 221 nucleótidos corriente arriba del comienzo del gen entre todas las secuencias de referencia disponibles, estando situada en esta secuencia la región promotora putativa descrita previamente. Posteriormente, mediante la técnica de rapid amplification of cDNA ends (RACE), se identificó una región 5´UTR de exactamente 139 nucleótidos. El análisis mediante truncamientos de esta región de 221 nucleótidos demostró la presencia de un dominio, situado inmediatamente corriente arriba del 5´UTR, absolutamente necesario para la expresión de *armA*, que también se confirmó analizando el perfil de resistencia y con la técnica de Western Blot con tripe FlagTag.

La fusión de la región extendida 5´ de *armA* al gen reportero *lacZα* reveló que, bajo la regulación de esta región situada corriente arriba de *armA*, el gen reportero se expresaba de manera constitutiva. El análisis en profundidad de esta construcción también

demostró que la expresión constitutiva de *lacZα* no se veía afectada por la presencia o ausencia de aminoglucósidos en el medio ni por la presencia de metiltransferasas activas en trans. Dado que estos resultados sugerían que el gen codificante de ArmA no está regulado por ningún mecanismo post-transcripcional, nuestro estudio se centró en el análisis de la región promotora situada corriente arriba del gen.

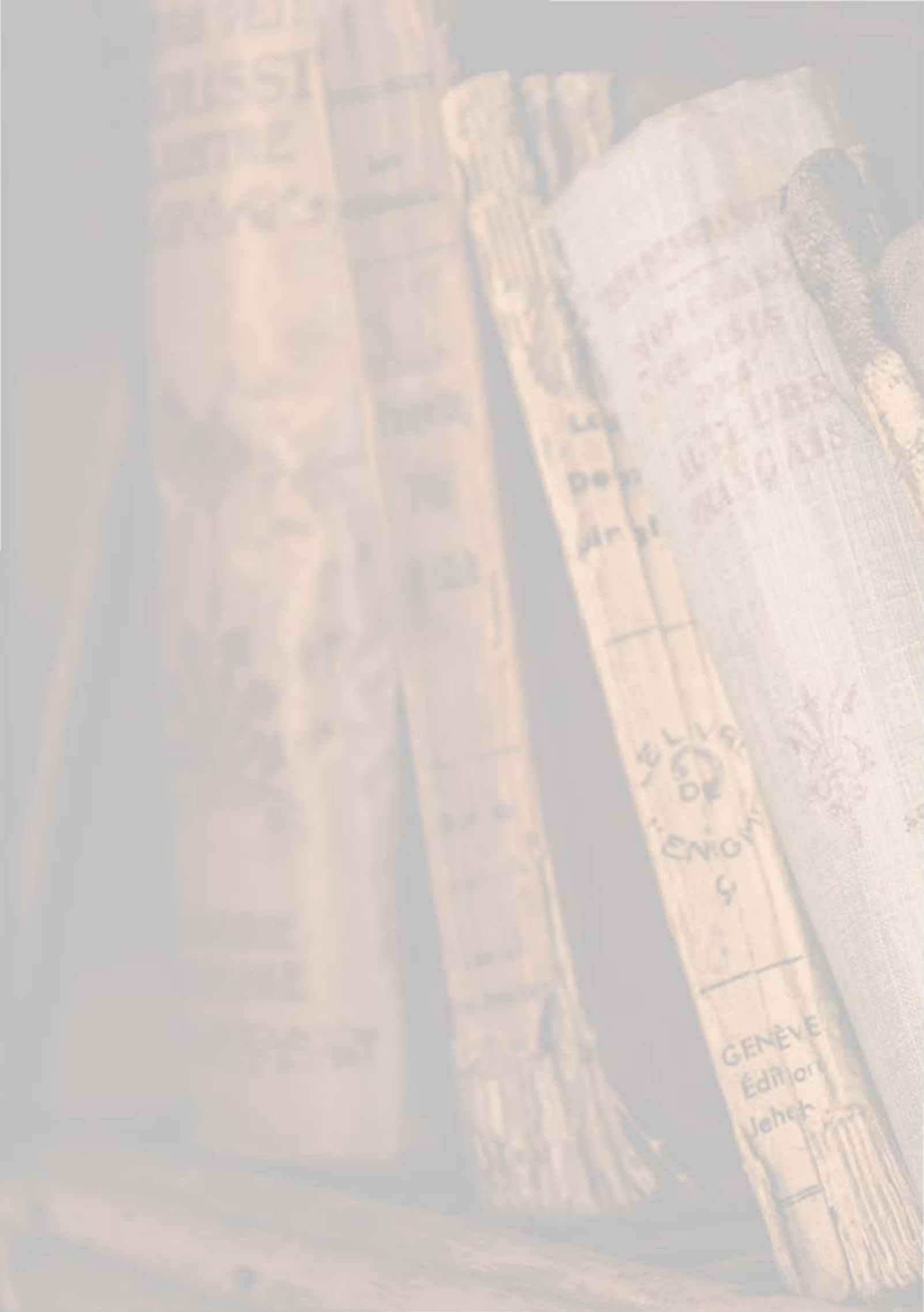
A continuación realizamos un análisis mutacional de esta región promotora para identificar los nucleótidos específicos requeridos para la correcta expresión del gen de resistencia. Los resultados de este estudio se correspondieron con el modelo de la región promotora que es reconocida por el factor sigma RpoD. La sustitución de los nucleótidos conservados dio lugar a la supresión completa de la expresión de *armA*, alcanzando los niveles del fenotipo susceptible. Por otro lado, sustituciones en nucleótidos menos conservados dieron lugar a una reducción de 3 a 5 veces de la Concentración Mínima Inhibitoria conferida por *armA*, lo cual también se comprobó mediante Western Blot. Esto sugiere que el factor sigma RpoD, que preferentemente inicia la transcripción de genes asociados con la síntesis proteica, también induce la transcripción del gen de resistencia *armA*.

El análisis del perfil de expresión de *armA* a lo largo de una curva de crecimiento bacteriana indicó un aumento en la expresión durante la fase de crecimiento exponencial. Tanto el Western Blot como la RT-PCR cuantitativa indicaron la presencia de un pico en la expresión de *armA* entre las 2 y 5 horas de la curva de crecimiento, en comparación con los genes cromosómicos de referencia. Esto, una vez más, sugiere la capacidad de la región promotora para reclutar el factor sigma RpoD y modular así la expresión de *armA* de acuerdo a los niveles de expresión de otros componentes relacionados con la síntesis proteica.

En conclusión, este estudio lleva a cabo un análisis en profundidad de los orígenes putativos de las metiltransferasas adquiridas del ARNr 16S y un primer análisis mutac-

ional de la región promotora de *armA*. El análisis nucleótido a nucleótido de esta región promotora ha permitido identificar los nucleótidos claves para la correcta expresión de este mecanismo de resistencia tan prevalente. Por último, los resultados de nuestro trabajo sugieren que este promotor es el responsable de asegurar unos niveles de resistencia adecuados en el hospedador, gracias a su transcripción por parte del factor sigma RpoD, responsable de la transcripción de clusters génicos relacionados con la síntesis proteica.

Este estudio constituye la primera aproximación para dilucidar los mecanismos regulatorios de las metiltransferasas adquiridas del ARNr 16S, un mecanismo que confiere altos niveles de resistencia frente a todos los aminoglucósidos de relevancia clínica y que, a día de hoy, ya limita la utilidad de esta familia de antibióticos de importancia crítica.



INTRODUCTION

3

AMINOGLYCOSIDES AND AMINOGLYCOSIDE RESISTANCE 3

3.1 The origins and discovery of antibiotics

It is a well-established fact that antibiotics revolutionized modern medicine in the 20th century. That being said, the use of mixtures containing antibiotic properties ranges back over 2000 years, with ancient Greeks and Egyptians using specially selected moulds and plants to treat infections (Forrest, 1982).

In 1928, Alexander Fleming accidentally discovered the first antibiotic, penicillin, when a plate containing *Staphylococcus* was mistakenly contaminated by what was eventually identified as the fungus *Penicillium notatum* (Fleming, 1929).

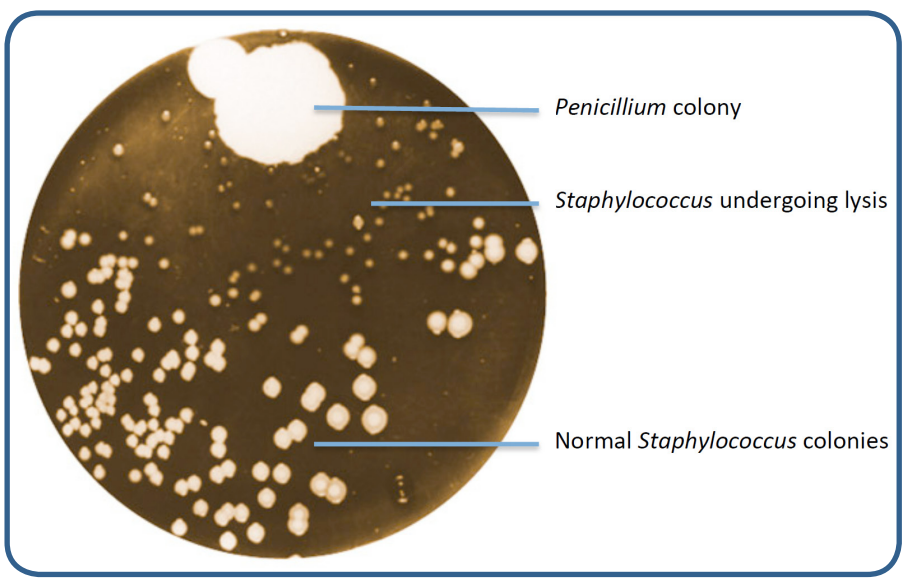


Figure 1. A photograph of a plate demonstrating the inhibition of Staphylococcus growth around a Penicilium colony (Figure from Fleming 1929)

Although this phenomenon had been described with a number of moulds in the late 19th century, researchers at the time were unable to discover the mechanism underlying this growth prevention. When Fleming noticed the inhibition zones around the fungus, it lead him to conclude that something was being released by the fungus that

results in the lysis of the bacterial cells (Figure 1) (Fleming, 1929). In as early as 1930, the English physician C.G. Paine registered the first cure using crude penicillin (Wainwright *et al.*, 1986). Over the years, the discovery of penicillin would finally provide humanity with a strong therapeutic option to combat infectious diseases, which was of dire need at the time. Due to its effectiveness and relatively easy access, penicillin and a number of other antibiotics discovered in the following years were used to treat a very large number of infections, especially during World War II (Figure 2) (Bud, 2007).

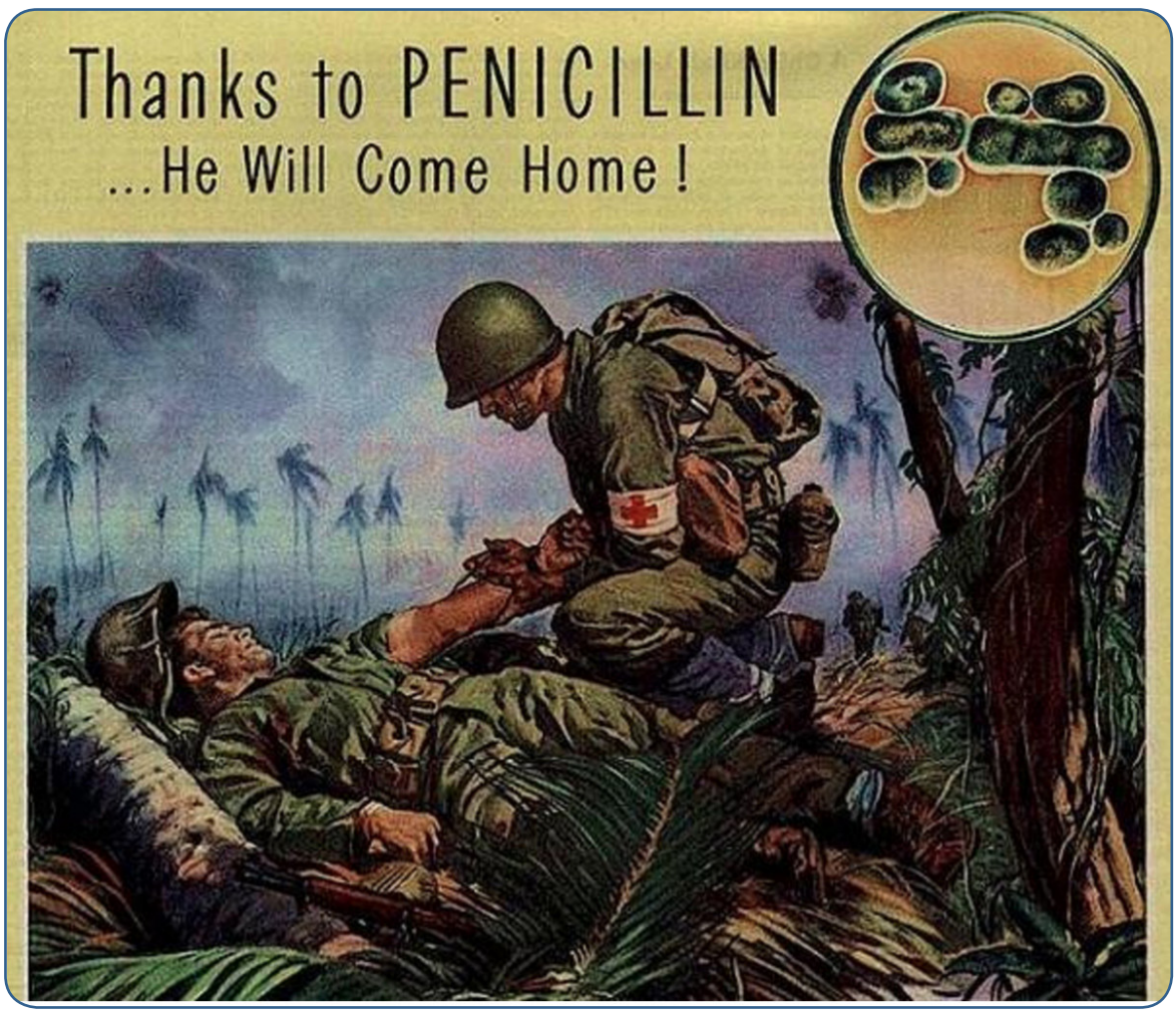


Figure 2: Illustration from a war-time advertisement for the products of Shenley Laboratories (Life Magazine)

As penicillin was inactive against *Mycobacterium tuberculosis*, a very common and extremely dangerous pathogen at the time, in the early 1940's Selman Waksman started the first systematic screening of soil bacteria in the search for agents exhibiting inhib-

itory activity to this deadly pathogen. In 1944, one of Waksman's PhD students, Albert Schatz, discovered such an inhibitory activity, mediated by the bacterium *Streptomyces griseus* (Waksman, 1944). What would later commonly be known as streptomycin, was the first antibiotic compound to exhibit inhibitory effects towards *M. tuberculosis*, and would result in Waksman being awarded a Nobel Prize in Medicine in 1952. However, with the discovery of streptomycin, Albert Schatz did not only discover an antibiotic effective against tuberculosis but also the first of a large group of antibiotics known as the aminoglycosides.

3.1.2 Aminoglycosides

In 1949, not long after the discovery of streptomycin, Waksman and another of his students discovered the antibiotic neomycin, isolated from the bacterium *Streptomyces fradiae* (Waksman *et al.*, 1949). Both streptomycin and neomycin found numerous applications in the treatment of infectious diseases. However, the discovery of aminoglycosides was far from over. The increasing resistance towards streptomycin combined with unfavourable levels of toxicity when treating life-threatening infections added pressure to discover more aminoglycosides. During this early struggle with antibiotic resistance, a large number of aminoglycosides were discovered from soil bacteria of the *Streptomyces* and *Micromonospora* genera. Among them were agents such as gentamicin in 1963, which constituted a significant advance in the treatment of aerobic gram-negative bacterial infections (Weinstein *et al.*, 1967). The emerging resistance as well as their relative toxicity are responsible for an array of medicinal biochemistry approaches attempting to extend their antibacterial spectrum and potency while reducing their troubling toxicity. These efforts have resulted in semi-synthetic agents such as dibekacin and amikacin (derived from kanamycin), or more recently arbekacin (derived from dibekacin) among others, which have demonstrated that compounds obtained by this method are not only capable of overcoming certain resistance mechanisms but they also display distinct toxicological profiles (Jana *et al.*, 2006). Today,

aminoglycosides remain highly potent, broad-spectrum antibiotics that are considered a critically needed last resort antimicrobial by the WHO (WHO, 2007). Especially, as gram-negative bacterial strains acquire more advanced antimicrobial resistance profiles, physicians have had to re-consider the application of aminoglycosides.

3.1.2.1 Nomenclature and structure of aminoglycosides

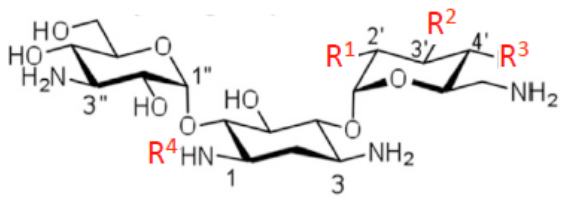
As previously mentioned, all naturally occurring aminoglycosides were isolated from bacteria of either the *Streptomyces* (bearing the suffix -micin) genus or *Micromonospora* (bearing the suffix –mycin).

Aminoglycoside activity is highly dependent on their chemical structure, which relies on the presence of one or more aminated sugars joined to a dibasic cyclitol backbone via pseudoglycosidic linkages (Figure 3)(Vincens *et al.*, 2002). The nature of this backbone is the foundation of aminoglycoside function, as both the number of attached sugars and their precise location drastically alter the biological activity (Magnet *et al.*, 2005; Benveniste *et al.*, 1973). Furthermore, it has been shown that the presence of at least one amino group in these sugars is required for biological activity, although exceptions such as spectinomycin (an aminocyclitol not linked to amino sugars) and compounds like astromicin that bear the aminocyclitol fortamine, are also included in this family (Jana *et al.*, 2006).

The naturally occurring aminoglycosides identified to date are therefore categorized based on the nature of their aminocyclitol ring. As such, most literature divides this family of antibiotics into two groups: 2-deoxystreptamine (2-DOS) and non-2-deoxystreptamine (non-2-DOS) (Figure 3). Based on the subtle differences within the backbone of antibiotics in these groups some choose to define the aminoglycosides based on the position of their glycosidic bonds e.g. 4,6 2-deoxystreptamine, 4,5 2-deoxystreptamine and non-2-DOS agents (Figure 3)(Magnet *et al.*, 2005).

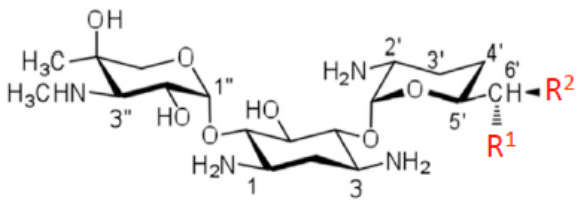
4,6-Disubstituted 2-deoxystreptamines

Kanamycin Subgroup



	R¹	R²	R³	R⁴
Kanamycin A	-OH	-OH	-OH	-H
Kanamycin B	-NH_2	-OH	-OH	-H
Tobramycin	-NH_2	-H	-OH	-H
Dibekacin	-NH_2	-H	-H	-H
Amikacin	-OH	-OH	-OH	-X*
Arbekacin	-NH_2	-H	-H	-X*

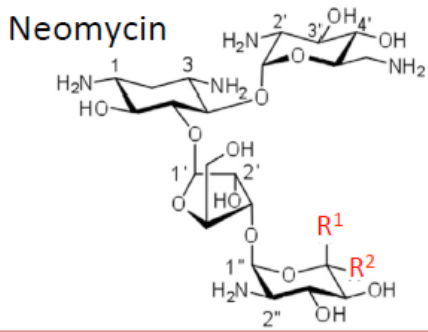
Gentamicin Subgroup



	R¹	R²
Gentamicin C_1	-$NHCH_3$	-CH_3
Gentamicin C_{1a}	-NH_2	-H
Gentamicin C_2	-NH_2	-CH_3

4,5-Disubstituted 2-deoxystreptamines

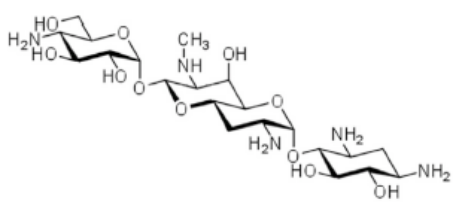
Neomycin



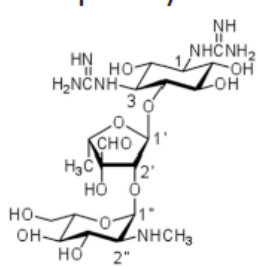
	R¹	R²
Neomycin B	-CH_2NH_2	-H
Neomycin C	-H	-CH_2NH_2

Others

Apramycin



Streptomycin



Spectinomycin

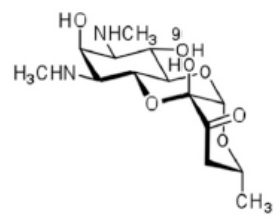


Figure 3. Representation of 4,6 2-DOS, 4,5 2-DOS and other aminoglycosides organized by their aminocyclitol ring and the positions of their glycosidic bonds (adapted from Doi et al., 2016)

Aminoglycosides belonging to the 4,6 2-DOS family are among the most clinically significant aminoglycosides including agents such as kanamycin and gentamicin (WHO, 2007). As these compounds were extremely successful, they were frequently used as templates for semi-synthetic drug development (Park et al, 2013). Within this group of antibiotics, there are two major subgroups. The first group consists of the aminoglycosides kanamycin A and its derivatives (dideoxy-kanamicin and amikacin), kanamycin B and its derivatives (dibekacin and arbekacin) as well as tobramycin. The second subgroup of the 4,6 2-DOS aminoglycosides consists of gentamicin related compounds, which were first described in 1963, including the naturally occurring gentamicin, gentamicin B, gentamicin C, sisomicin and geneticin. As with the kanamycin subgroup, the gentamicin related aminoglycosides were potent antimicrobials that were used as templates for derivatives such as netilmicin and isepamicin (Magnet *et al.*, 2005). Due to its low cost and stability, gentamicin is the number one choice aminoglycoside in hospitals with low levels of resistance among Enterobacteriaceae and *Pseudomonas aeruginosa*.

Aminoglycosides bearing an aminocyclitol ring substituted at positions 4 and 5 constitute the second subgroup of 2-DOS aminoglycosides (Figure 3). This family is made up of a number of naturally occurring agents isolated from the *Streptomyces* genus, including (but not limited to) neomycins, paromomycins, and butirosins. Another member of this group, ribostamycin, isolated from *Streptomyces ribosidificus*, is thought to be the precursor of the 4,5-2-DOS aminoglycosides (Kudo *et al.*, 2016).

All other members of the aminoglycoside family that do not bear a 2-DOS aminocyclitol, belong to the non-2-DOS subgroup (Figure 3). This includes agents such as the original aminoglycoside streptomycin, which possesses a streptidine ring as the central aminocyclitol, and its derivatives (dihydrostreptomycin), fortimicin A and its derivatives (dactimicin) and apramycin (Magnet *et al.*, 2005).

3.1.2.2 The ribosome as a drug target

Before detailing the biochemical properties of aminoglycosides, it is important to highlight the prokaryotic ribosome and its features as an antimicrobial drug target (Figure 4).

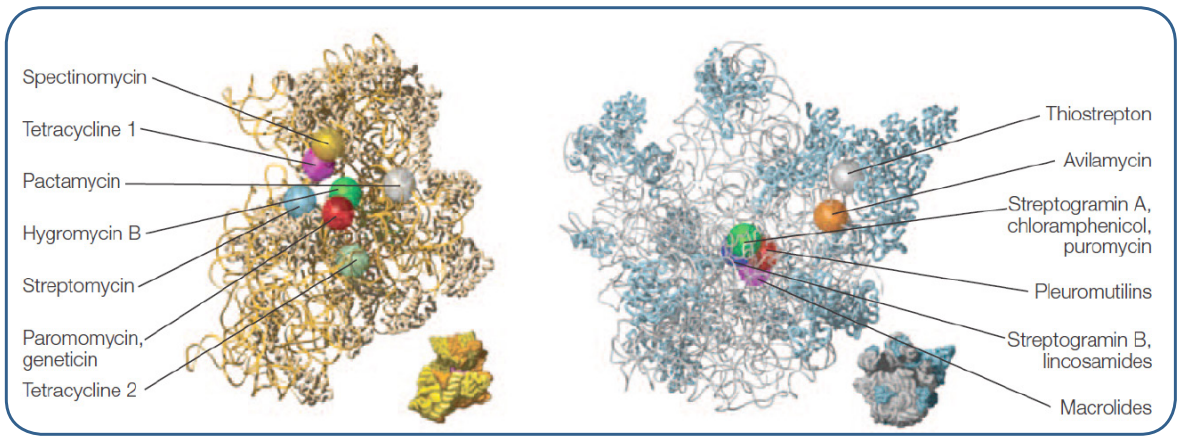


Figure 4. Binding sites of antibiotics on the prokaryotic ribosomal subunits. The 30S subunit is shown on the left and the 50S subunit on the right. These sites were initially identified via biochemical and genetic techniques and later confirmed by means of X-ray crystallography. The ribosomal RNA is shown in yellow and grey, the ribosomal proteins (some of which have been removed for clarity) in bronze and blue. (Poehlsgaard et al., 2005)

Since the structure of the prokaryotic ribosome was resolved by X-ray crystallography (Tischendorf *et al.*, 1975), researchers have worked tirelessly to unravel the precise mechanisms of antibiotics that act on this intricate molecular machine that is so highly conserved throughout all living cells. While being highly conserved, subtle differences enable for the development of compounds with a surprising degree of specificity (Böttger *et al.*, 2001).

Prokaryotic and eukaryotic cells last shared a common ancestor a staggering 3.5 billion years ago, and since then the evolutionary changes have significantly diversified ribosomes while maintaining the same function in their various hosts (Poehlsgaard *et al.*, 2005). Ribosomal activity is a very precisely regulated task that relies on a vast array of supporting proteins, methylations and other post-transcriptional modifications to function accurately. The prokaryotic ribosome consists of two subunits: the smaller 30S subunit and the larger 50S subunit. The large component of the bacterial ribosome

consists of a 120 ribonucleotide 5S, a 2900 ribonucleotide 23S and a total of 31 proteins. The smaller component of the prokaryotic ribosome contains a 1540 ribonucleotide 16S rRNA subunit that is bound to a further 21 proteins (Melnikov *et al.*, 2012).

Ribosomal fidelity is dependent on a highly orchestrated assembly process, requiring the precise regulation of ribosomal proteins and methylations. As such, even small modifications in certain functionally conserved regions of the ribosome (e.g. the A-site) can be fatal to the cell.

To summarize, the strength of the ribosome as an antimicrobial drug target can be attributed to both its conservation and its diversification. The high degree of conservation means that even subtle changes of this extremely complex machinery can result in cell death, while its diversification permits for the design of therapeutic agents that target elements of the ribosome that are not present (or sufficiently altered) in eukaryotic cells.

3.1.2.3 Mode of action

The cellular internalization of highly polar aminoglycosides begins with a self-promoted process requiring the drug-induced disruption of Mg^{2+} bridges between adjacent lipopolysaccharide molecules of the outer membrane (Vaara, 1992). Thereafter, aminoglycosides pass through the cytoplasmic membrane via an energy dependent process (EDP-I) utilizing the electron transport chain (Bryan *et al.*, 1983). This sensitive process is rate limiting and can be inhibited by divalent cations, hyperosmolarity, low pH and anaerobiosis (Xiong *et al.*, 1996). Based on the biochemical properties of aminoglycosides, once within the cytosol, they exhibit a high affinity for the A-site within the 30S subunit of prokaryotic rRNA (Figure 5).

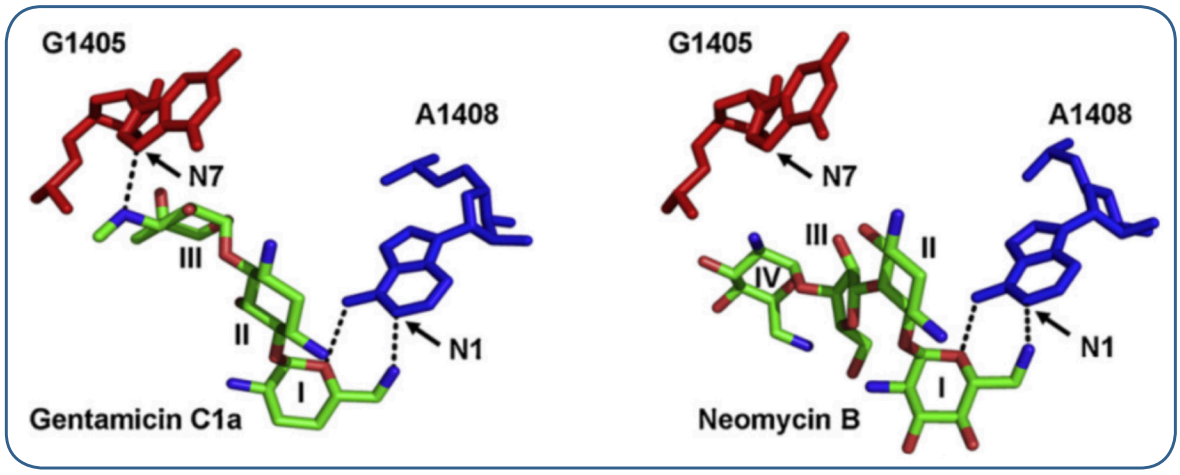


Figure 5. Molecular representation of a 4,6 2-DOS (left) and a 4,5 2-DOS (right) aminoglycoside interacting with specific residues within their ribosomal target (Dashed lines represent possible hydrogen bonds, N1 and N7 refer to the position of the methyl group). The aminoglycosides are depicted in green whereas the ribosomal residues G1405 and A1408 are depicted in red and blue respectively. (Figure from Wachino et al., 2012)

The binding to their target in the A-site of the 16S rRNA is the second energy dependent process (EDP-II) of aminoglycoside action (Bryan *et al.*, 1983). The interaction between aminoglycosides and the ribosome they act on has been the subject of comprehensive biochemical research using nuclear magnetic resonance (NMR) and X-ray crystallization. The application of X-ray diffraction methods in 2000 shed considerable light on the exact mechanism that is being disrupted by the presence of aminoglycosides in the A-site (Yoshizawa *et al.*, 1998). Studies performed in the presence and absence of aminoglycosides bound to their target site demonstrated that aminoglycosides interrupt a crucial proofreading step required for translocation and further translation. When functioning correctly, there is an initial recognition of the mRNA codon and its charged tRNA counterpart (the anticodon), after which a proofreading step is required prior to translocation (Melançon *et al.*, 1992; Green *et al.*, 1997, Jenner *et al.*, 2010). A hydrogen bond network formed by A1492 and A1493 (of helix 44) with the ribose 2'-hydroxyl groups of the first two bases of both the codon and anticodon allows for the discrimination of correct codon-anticodon matches (Cate *et al.*, 1999). If and when the cognate mRNA-tRNA complex is recognized, the two adenine residues 1492 and 1493 flip out of the helix, which results in an increased affinity for the cognate tRNA and thereby stabilizes the complex (Vicens *et al.*, 2002).

The aminoglycoside-binding site is located at the 16S tRNA acceptor A-site (aminoacyl site), which includes portions of loop 530, helix 34 and helix 44 (involved in codon anticodon matching) as well as a less described secondary site in helix 69 of the 23S portion of the ribosome, involved in ribosomal recycling. The cationic amine groups of aminoglycosides bind specifically to the major grove of helix 44, in a negatively charged pocket on the 16S rRNA (Figure 6)(Moazed *et al.*, 1987; Woodcock *et al.*, 1991).

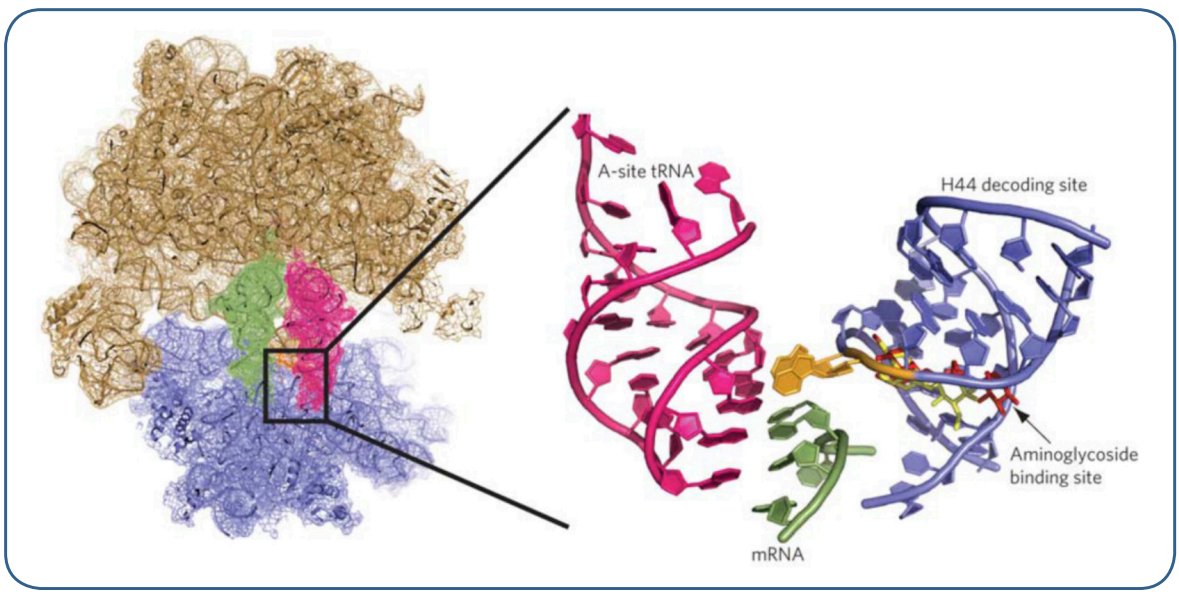


Figure 6. Representation of the prokaryotic 70S ribosome, with the large 50S subunit (tan) and the small 30S subunit (light blue) with an enlarged view of the decoding site which forms the aminoglycoside binding pocket within helix 44 of the 16S rRNA. Bases A1492-A1493 (orange) flip out upon cognate codon-anticodon recognition or constitutively in the presence of aminoglycosides. Figure depicts gentamicin (yellow) and neomycin (red) bound to the decoding site. (Figure from Feldman et al., 2010)

These interactions result in a constitutional flip-out within helix 44, which not only decreases the rate of A-site tRNA dissociation but also increases the tRNA binding affinity (Fourmy *et al.*, 1996; Recht *et al.*, 1996). Recent literature expands our traditional understanding of this process, demonstrating that some aminoglycosides (such as apramycin) but not all, do not displace A1492 and A1493, and that their main activity is a result of blocking translocation (Figure 6)(Feldman *et al.*, 2010; Tsai *et al.*, 2013). As such, the consequence of aminoglycosides binding to the A-site constitutively and indiscriminately stabilizes both cognate and noncognate mRNA-tRNA complexes and translation can continue. Subsequent peptide synthesis will thus be faulty and/or trun-

cated, due to the loss of translational fidelity; eventually, the build-up of faulty proteins results in cell death. Additionally, the incorporation of these faulty proteins into the cytoplasmic membrane facilitates further aminoglycoside uptake (Busse *et al.*, 1992).

3.1.2.4 therapeutic properties and clinical applications

One of the most important features of any therapeutic agents is the specificity of its action. Achieving the adequate bioavailability of a therapeutic agent in the correct location is the key to a successful treatment. Aminoglycoside toxicity levels have been well described since the first applications of aminoglycosides (Heilman *et al.*, 1945; Hettig, 1946). However, in the early days of the aminoglycosides, streptomycin was the first, and at the time, only antibiotic to show activity against *M. tuberculosis* and, as such, the advantages of its application overshadowed issues with toxicity, especially in life threatening situations. Today aminoglycosides are still a very significant last resort therapeutic agent despite their unfavourable side effects.

Aminoglycosides are water-soluble and have a relatively low molecular weight of around 500-800 g/mol. They are highly stable at room temperature and a pH between 7.5 and 8.5, giving them a shelf life of several months in solution (Vining, 1990). This stability also makes them heat resistant, which amplifies their clinical potential. Members of this family of antimicrobials, such as gentamicin, have a number of key positive attributes including their broad spectrum, low cost as well as their capacity to be administered synergistically with other antibiotics. When aminoglycosides are administered with β-lactams or other cell wall synthesis inhibiting agents, their activity is increased due to augmented cellular uptake, especially in intrinsically resistant microbes unable to internalize the aminoglycosides (Eliopoulos, 1989).

Aminoglycosides are considered to be broad-spectrum antimicrobial agents that act in a concentration dependent fashion. With the exception of spectinomycin and kasug-

amycin (inhibitors of translocation), they act in a bactericidal manner (Levitan, 1967; Tsai *et al.*, 2013). Currently, the 4,6 2-DOS aminoglycosides (e.g. gentamicin and amikacin) are the most clinically significant aminoglycosides. They exhibit high levels of activity against a wide range of mostly aerobic gram-negative bacteria such as *Pseudomonas* spp., while they have also been shown to display bactericidal activity on some mycobacteria and staphylococci (Jana et al, 2006). Due to their biochemical properties aminoglycosides are poorly absorbed in the gut and are therefore usually administered via injection or infusion (Kadurugamuwa *et al.*, 1993; Edson *et al.*, 1999). Conventionally, aminoglycosides have been administered in multiple doses per day, although it has been shown that single daily doses achieve a similar antimicrobial efficacy (Freeman *et al.*, 1997). As mentioned, their action is concentration based, whereby typically ≤ 25 µg/ml of clinically applied aminoglycosides are required to inhibit protein synthesis (Takano *et al.*, 1994). Although the clinical application of aminoglycosides has largely been replaced by cephalosporins, their broad spectrum of activity makes them ideal agents for emergency treatments.

3.1.2.5 Aminoglycoside toxicity

Unfortunately, it is known that aminoglycosides also exhibit activity on eukaryotic 80S ribosomes - although they have at least 10 times more affinity for the prokaryotic ribosome - leading to toxicity and secondary effects during treatment (Recht *et al.*, 1999). Specifically, they result in ototoxicity, nephrotoxicity and, in very rare cases, neurotoxicity, all of which can be mitigated based on the choice of drug, duration of treatment and the patients kidney function (Jiang *et al.*, 2007). While the nephrotoxicity of aminoglycosides can be counteracted, the drug induced hearing loss is typically permanent. Side effects such as hearing loss are usually associated with long-term treatments. There are isolated cases in which a single injection has led to hearing loss, but a significant proportion of those cases have been linked to a guanosine to adenosine substitution at position 1555 of the human mitochondrial ribosomes (Forge *et*

al., 2000). The mechanism underlying aminoglycoside toxicity has been the focus of many studies. It has been described that this toxicity originates due to the interruption of mitochondrial ribosomes and the subsequent release of reactive oxygen species (ROS) (Gutell *et al.*, 1994). The ototoxicity of aminoglycosides has been linked to their metal-chelating capacities, as demonstrated using metal complexes with gentamicin and neomycin (Xie *et al.*, 2011). The, in some cases, permanent side effects of aminoglycoside toxicity is a major drawback of this entire family of antibiotics, which must be taken into careful consideration when selecting an appropriate therapeutic agent. Although in many cases clinicians prefer antibiotics with less toxicological side effects, rising resistance levels to other antibiotics and their efficacy in emergency situations make aminoglycosides a crucial therapeutic option.

3.1.2.6 Antibiotic resistance

Antibiotic resistance is the ability of a microbe to survive otherwise lethal concentrations of an antimicrobial agent. Such resistance can be achieved in three ways: (i) a bacterium may be intrinsically resistant; (ii) resistance can be achieved via genetic mutation; (iii) a resistance determinant can be acquired horizontally from other bacteria (Davies, 1997).

As antibiotics are produced by microbes themselves, resistance genes preventing the suicidal action of these agents to their producers are equally ancient mechanisms. Intrinsic resistance in producers is generally mediated by one of three main pathways: (i) inactivation of the antibiotic, (ii) efflux of the antibiotic and (iii) modification of the susceptible molecular target. However, intrinsic resistance is not limited to antibiotic producers. Depending on the species of bacteria, for example, differences in the composition of the membrane may naturally prevent the internalization of the compound, rendering the bacterium resistant (Nikaido, 1994).

Although resistance can appear spontaneously because of random genetic mutations, factors such as the excessive misuse of antibiotics greatly facilitate the prevalence of resistance (Fair *et al.*, 2014). Since antibiotics have been in ever-widening therapeutic use, the development of antibiotic resistance has followed. Researchers and clinicians were quick to realize that, every time a novel antibiotic, a derivative of an existing antibiotic or a broader spectrum variant of an antibiotic is released to the market, clinically significant resistance emerges rapidly. For a number of antibiotics, such as penicillin, resistance was discovered almost immediately after the introduction of the agent to the market, as early as 1940 (Abraham *et al.*, 1940), whereas in other cases such as vancomycin, resistance took almost 30 years to be reported (1987) (Schwalbe *et al.*, 1987). This has largely been associated with the limited use of vancomycin compared to penicillin. Furthermore, the resistance mechanisms to various antibiotics differ substantially. While β-lactam resistance can be achieved by the action of a single gene product, the hydrolytic β-lactamase, the vancomycin resistant *Enterococcus* (VRE) requires a 5-gene resistance cassette (Bugg *et al.*, 1991). Hospital environments are especially troubling for antibiotic resistance dissemination, as there is an intensive virtually constant exposure of bacteria to antibiotics. In such conditions there is a very strong selective pressure for microbes to maintain resistance determinants, compensate for their biological burden and disseminate them (Schulz zur Wiesch *et al*., 2010).

The troubling rate of increased resistance prevalence is also in large part due to the horizontal transfer of resistance determinants (Courvalin, 1994). Resistance conferring genes are often found within genetic elements capable of mobilizing resistance genes between genetic environments and even between bacteria of different families (Barlow, 2009). Such mobile genetic environments greatly facilitate the dissemination of resistance genes as multiple determinants can be quickly acquired and integrated by a receiving strain. Many resistance genes exclusively exist in such highly variable genetic environments (Martinez, 2008).

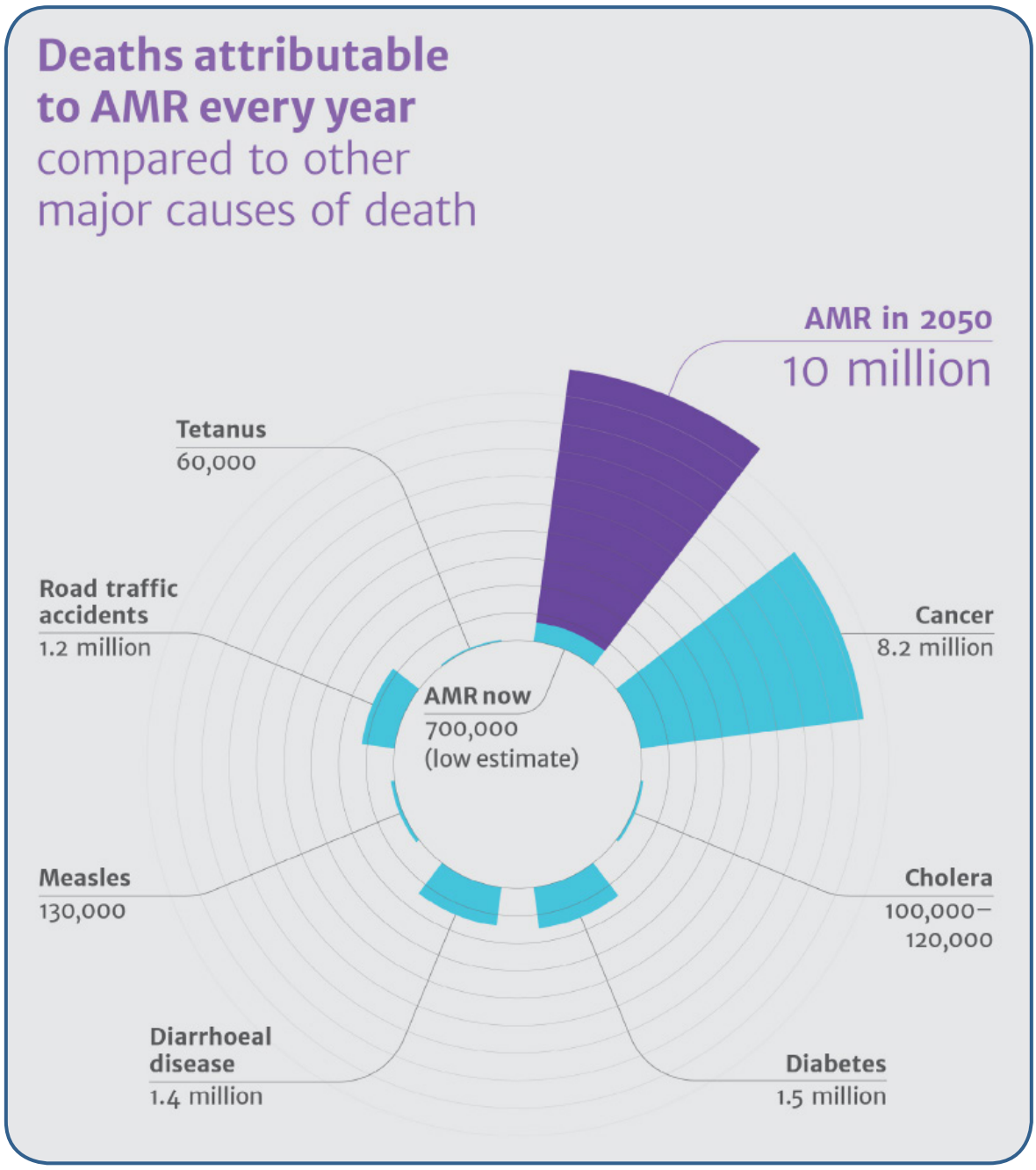


Figure 7 Mortality impact of antimicrobial resistance in relationship to other major diseases between now and 2050 (Figure from O'Neill, 2014)

The rate at which antimicrobial resistance is disseminating is truly alarming for the future of modern medicine. It is estimated that prior to the introduction of antibiotics, approximately 30% of all deaths in the United States were as a result of bacterial infections (tuberculosis, pneumoniae and gastrointestinal infections) (Department of Commerce and Business administration, 1906). In 2014, an estimated 700,000 deaths were associated with antimicrobial resistance and in the same review on AMR it was

published that, by 2050, mortality rates associated with antimicrobial resistance will rise to around 10 million per year (Figure 7)(O'Neill *et al.*, 2014). While this dilemma is spiralling out of control, pharmaceutical policies and new regulatory hurdles are making the antimicrobial market increasingly unattractive (Figure 8).

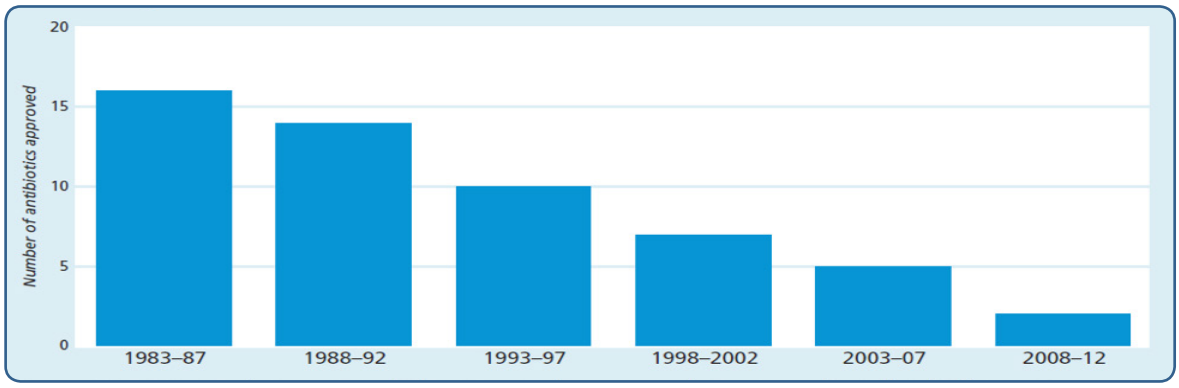


Figure 8. Representation of the antimicrobial drugs approved in the United States (Figure from Spellberg, 2011)

In 2004, only 1.6% of drugs in clinical developments by the world's 15 largest pharmaceutical companies were antibiotics (Shlaes, 2010). This is due to a variety of factors. Primarily, antibiotic treatment is typically administered for a short time-period and then no longer necessary, making this market much less profitable than medicines related to chronic conditions. Additionally, in order to prevent the rapid emergence of antimicrobial resistance to new agents, recently approved antibiotics are typically reserved for complicated infections that have shown to be resistant to older first-choice antibiotics. While this is an effective strategy to conserve the functionality of these drugs, it greatly diminishes the initial investment return for the pharmaceutical companies. However, depending on the type of resistance mechanism (e.g. target modification), bacteria may even harbor resistance to antimicrobial agents that have not yet been released to the market, which also greatly limits the return (e.g. plazomicin) (Doi *et al.*, 2016).

To make matters worse, regulatory measures grow increasingly tight for antibiotics (Fair *et al.*, 2014). While the tolerance for side effects grows smaller, pharmaceutical companies are now required to demonstrate superiority of novel agents rather than non-inferiority, as was the case in the past (Spellberg *et al.*, 2008). As a result of the aforementioned complications, currently the cost of developing a single antimicrobial agent has skyrocketed to around $1.7 billion and takes between 12 and 15 years. As such, it is not surprising, but very concerning, that as of 2013 there are only 4 multinational pharmaceutical companies that produce antibiotics (Fair et al, 2014).

While there are government initiatives to introduce novel antibiotics, no such entity has ever discovered and developed an antibiotic and as of now, major contributions from this sector are not expected. In response to the diminishing antimicrobial market, policies have been enacted and incentives have been offered by international organizations such as the World Health Organisation (WHO), the European Center for Disease Control (ECDC), and the Infectious Diseases Society for America (IDSA) to revitalize this market (Fair *et al*., 2014). Additionally, the US senate and the European Union, among others, are placing increasing emphasis on tackling antimicrobial resistance by offering funds and scholarships to educate the next generation of antimicrobial resistance researchers (European Medicines Agency, 2010; WHO, 2014).

There are four acquired mechanisms by which microbes resist antibiotic activity: (i) mutation/modification of the drug target site (ii) enzymatic drug modification or inactivation (iii) decreased permeability of the drug across the membrane and (iv) increased efflux of the drug out of the cell (Figure 9).

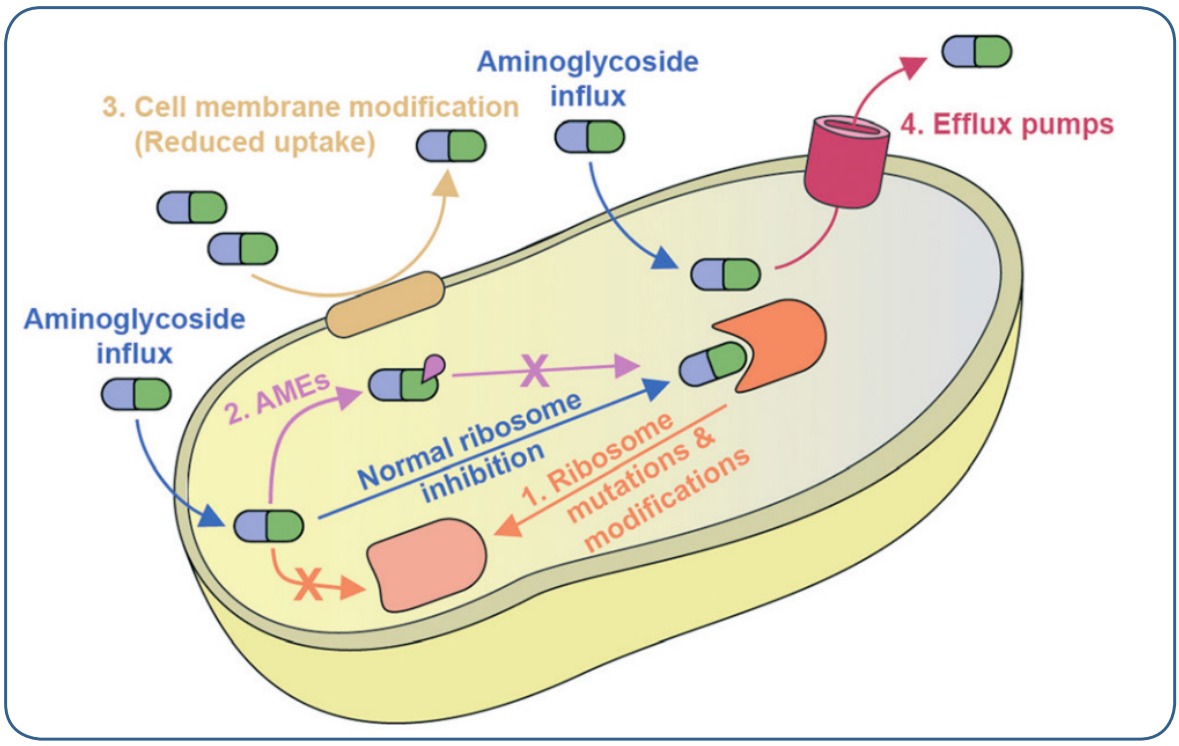


Figure 9. Representation of the normal activity of aminoglycosides (blue) and the four mechanisms of resistance: target modification (orange), enzymatic modification (purple), decreased drug permeability (tan) and efflux pumps (pink). (Figure from Garneau-Tsodikova et al., 2016)

3.1.2.7 Aminoglycoside resistance

Shortly after the discovery of streptomycin as an antimicrobial agent, Waksman himself recognized that bacteria grown in the presence of streptomycin quickly became resistant to its effects (Waksman *et al.*, 1945). As previously described, this early struggle with antibiotic resistance lead to the discovery of a large number of antibiotics, including aminoglycosides, from soil bacteria such as *Streptomyces* and *Micromonospora* species as well as the formulation of semi-synthetic derivatives (Park et al, 2013). Unfortunately, no matter how many new antibiotics are discovered, resistance will always follow shortly. In the following sections, I will briefly introduce the three major pathways by which microbes can become aminoglycoside resistant.

Decrease in intracellular drug concentration

This resistance mechanism is most commonly found in *Pseudomonas spp*. as well as other non-fermenting gram-negative bacilli and is associated with membrane impermeabilization (Taber *et al.*,1996). Although this mechanism only results in intermediate resistance levels, it is clinically significant as it is a stable phenotype that affects all aminoglycosides. Additionally, it has been shown that prolonged aminoglycoside treatments of aerobic gram-negative bacteria, gradually renders previously susceptible microbes more resistant (Karlowsky *et al.*, 1997; Xiong *et al.*, 1997). This is most likely a result of gradually acquiring membrane modifications that reduce aminoglycoside uptake into the cell.

Energy dependent bacterial efflux of aminoglycosides is another significant cause of antibiotic resistance and has been shown to confer resistance to agents such as kanamycin, neomycin and hygromycin A (Hayashi *et al.*, 1997). Recently it has been demonstrated that aminoglycosides are substrates for a number of multidrug efflux pumps, including members of the five major families of bacterial transporters (Poole, 2005). This is interesting because initially it was assumed that multidrug transporters were specific for either hydrophobic or amphiphilic compounds and that, as such, aminoglycosides would not be affected (Putman et al, 2000). Fortunately, at times, this resistance mechanism can be overcome by the synergistic application of aminoglycosides with cell wall destabilizing agents such as the β- lactams (Ioannis *et al.*, 2005).

Aminoglycoside-modifying enzymes

Aminoglycoside modifying enzymes constitute the most prevalent and widely distributed form of resistance to this family of antibiotics, as they are typically found within mobile genetic elements such as transposons and plasmids (Tolmasky, 2007). Although levels of resistance do not reach those achieved by target modification, there are many different enzymes capable of modifying specific aminoglycosides and multiple such en-

zymes can reach significant levels of resistance to a broad range of aminoglycosides. Deactivating enzymes such as *N*-acetyltransferases (AAC), *O*-nucleotidyltransferases (ANT) and *O*-phosphotransferases (APH) are commonly found in both gram-positive and gram-negative microbes (Ramirez *et al.*, 2010). These enzymes typically catalyse the covalent modifications of specific amino or hydroxyl residues. The resulting modified agent then displays a much lower binding capacity for its ribosomal target site (Shaw *et al.*, 1993). Additionally, these modifications also interrupt the second energy dependent process (EDP-II) of aminoglycoside activity, which prevents the binding of the aminoglycosides to the A-site of the ribosome (Mingeot-Leclercq *et al.*, 1999). Although not entirely clear, these enzymes most likely originate from intrinsic enzymes of the bacteria that, under normal conditions, do not result in a resistance phenotype to aminoglycosides. However, small modifications or the overexpression of such an enzyme (especially under the pressure of aminoglycoside treatment) could lead to low levels of resistance (Wright *et al.*, 1997). Even minor alterations in the amino acid sequence of these proteins have a profound effect on the substrate specificity (Wu *et al.*, 1997). Therefore, it has also been speculated that a number of the current deactivating enzymes have originated from one or a few common ancestors (Magnet *et al.*, 2005). The variety of molecules in combination with their substrate plasticity are concerning because even if newly derived aminoglycosides can avoid the action of such an enzyme, simple modifications under antibiotic pressure may render this new agents ineffective (Jana *et al.*, 2006).

Target modification

There are two types of aminoglycoside resistance mechanisms that are mediated by modifications of the ribosomal target site. The first, although uncommon (due to the high degree of conservation within this region), are point mutations in the ribosomal 16S RNA as well as mutations in the *rpsL* gene, which codes for the ribosomal protein S12 that has been associated with aminoglycoside resistance (Cooksey *et al.*, 1996).

Mutations of the 16S rRNA are only clinically relevant for streptomycin resistance of *M. tuberculosis*, as Mycobacteria are the only genus of eubacteria with a single copy of the ribosomal operon, implying that a single mutation can confer resistance (Finken *et al.*, 1993). The second, and clinically much more significant mechanism of resistance conferring target modification, is the post-transcriptional methylation of the 16S A-site. Such methyltransferases are very common in aminoglycoside producing *Streptomyces spp.* and *Micromonospora spp*. but are becoming increasingly relevant, as there is an ever-growing number of acquired resistance methyltransferases isolated from mobile genetic elements, facilitating their dissemination to clinically relevant bacteria (Wachino *et al.*, 2012). The details of both the intrinsic and acquired aminoglycoside resistance conferring 16S rRNA methyltransferases will be the subject of the following section.

3.2 16S rRNA methyltransferase mediated aminoglycoside resistance

The 16S rRNA methyltransferases, whether acquired or intrinsic, confer extremely high levels of resistance to a broad range of aminoglycosides, including the most clinically relevant. This section will outline the key characteristics of both groups of methyltransferases.

3.2.1 Intrinsic aminoglycoside resistance conferring 16S rRNA methyltransferases

Antibiotics are naturally occurring secondary metabolites that have been produced by microbes long before man discovered their potential applications in medicine. As such, antibiotic resistance is an equally ancient phenomenon. The first resistance conferring mechanisms were likely to those protecting antibiotic producers from the toxic effects of these agents. While this can be achieved in many ways, as outlined in the previous section, target modification is a very effective way of ensuring that the expression of these secondary metabolites does not kill the producer (Cundliffe, 1989).

Especially for antibiotics that act on the ribosome, target modification is a very widespread and effective form of self-defence. This is usually achieved by site-specific methylation of ribosomal residues within the target site of the antibiotic in question, and results in high levels of resistance to agents sharing that target site. Microorganisms producing agents that act on the risobome such as macrolides, lincosaminides or thiopeptides have been shown to use ribosomal target modification to protect themselves from antibiotic action (Cundliffe *et al.*, 2010). That being said, not all producers of antibiotics that act on the ribosome rely on this mechanism of resistance. However, producers of agents such as neomycin, streptomycin and tetracycline have not been found to carry intrinsic resistance conferring rRNA methyltransferases (Table 1)(Cundliffe *et al.*, 2010).

Table 1. Intrinsic 16S rRNA methyltransferases harboured by aminoglycoside producers.

Intrinsic Resistance Methyltransferases of Aminoglycoside Producers			
RMTase Family	Methyltransferase	Aminoglycoside	Producer
Kgm Family (N7-G1405)	*gmrA*	Gentamicin	*M. purpurea*
	kmr	Kanamycin	*S. kanamyceticus*
	kgmB	Nebramycin	*S. tenebrarius*
	fmrO	Fortimicin	*M. olivasterospora*
Kam Family (N1-A1408)	*kamA*	Istamycin	*S. tenjimariensis*
	kamB	Nebramycin	*S. tenebrarius*
	kamC	Spiramycin	*S. hirsuta*

Actinomycetes are ground dwelling gram-positive microorganisms that are, in large part, made up of *Streptomyces spp*. and *Micromonospora spp*., that naturally produce aminoglycosides as secondary metabolites. This is achieved by a post-transcriptional methylation of either the N-7 position of nucleotide G1405 or the N1 position of nucleotide A1408 (Figure 10) (Beauclerk *et al.*, 1987).

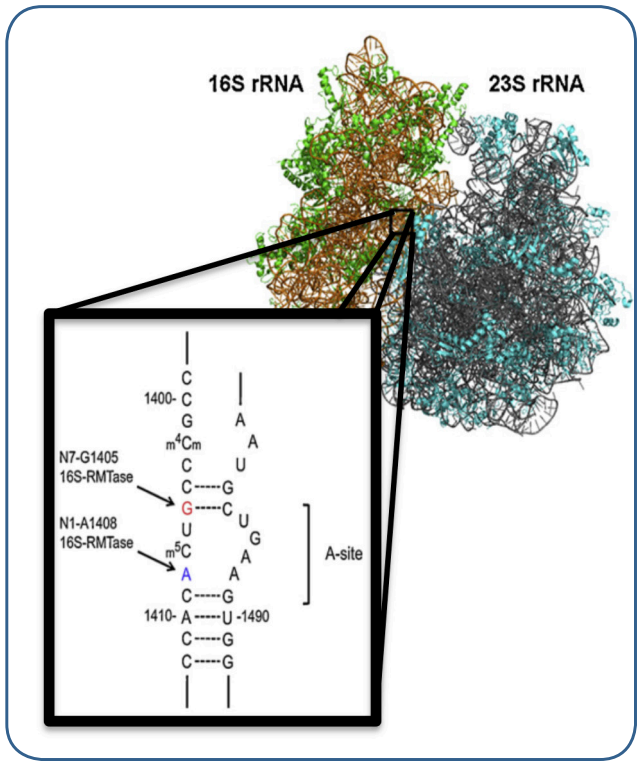


Figure 10. Representation of the ribosomal A-site, the target site of aminoglycosides and both intrinsic and acquired aminoglycoside resistance conferring 16S methyltransferases (Figure adapted from Magnet et al., 2005)

While methyltransferases acting on G1405 have been identified in both *Streptomyces* spp. and *Micromonospora* spp., methylations of A1408 have only been described in *Streptomyces* spp. (Wachino et al, 2012). Methyltransferases acting on both of these residues require S-adenosyl-L-methionine (SAM) as the source of the methyl group to generate 7-methylguanosine or 1-methyladenosine, respectively. These intrinsic resistance determinants can be divided into two categories: the Kgm family and the Kam family (Holmes *et al.*, 1991a Husain *et al.*, 2010). Methyltransferases pertaining to the Kgm (kanamycin gentamicin methyltransferase) family, methylate position N7 of residue G1405 whereby they confer high levels of resistance (>1000mg/L) to 4,6-2-DOS aminoglycosides such as kanamycin, gentamicin and tobramycin, but remain susceptible to other aminoglycosides (Cundliffe, 1989; Holmes *et al.*, 1991a). The specificity of this resistance is based on the specific structure of the 4,6-2-DOS aminoglycosides that form a hydrogen bond with G1405. Due to structural differences in the 4,5-2-DOS group of aminoglycosides, they do not require such an interaction and therefore microbes methylating G1405 remain susceptible.

The second family of intrinsic resistance methyltransferases is known as the Kam (kanamycin apramycin methyltransferases) family, which methylate position N1 of residue A1408 (Holmes et al., 1991b). Like the Kgm family, this family of intrinsic methyltransferases confers equally high levels of resistance (>1000mg/L), but to neomycin and apramycin as well as kanamycin but less so to gentamicin.

Producing microbes must ensure that this methylation takes place prior to the production of the aminoglycosides. As such, both the Kgm and Kam family of intrinsic methyltransferases are commonly found within gene clusters responsible for the biosynthetic production of the respective aminoglycoside (Mak *et al.*, 2014). This is an elegant form of ensuring the producers safety after the production of this potentially lethal secondary metabolite while minimizing the biological burden incurred.

3.2.2 Acquired aminoglycoside resistance conferring 16S rRNA methyltransferases

3.2.2.1 History and nomenclature

In the year 2000, a *Klebsiella pneumoniae* isolated from a urinary tract infection in France displayed unusually high levels of resistance to all 4,6-2-DOS aminoglycosides (Galimand *et al.*, 2003). The genetic fragment bearing the resistance determinant was cloned and sequenced, before comparing it to previously uploaded sequences in Genbank. This unknown determinant was later shown to be identical to a sequence uploaded from a *Citrobacter freundii* strain bearing a pCTX-M3 (Accession number AF550415) plasmid in Poland (Golebiewski *et al.*, 2007). Interestingly, the sequence of pCTX-M3 was already 'completely' annotated, however this methyltransferase like gene was missed. In 2003 Galimand *et al.* characterized and named this protein ArmA (aminoglycoside resistance methyltransferase A) (Galimand *et al.*, 2003). It is also noteworthy that even the first isolate bearing *armA* was discovered alongside the ESBL gene $bla_{CTX\text{-}M\text{-}3}$. It did not take long to realize that *armA* was just the tip of the iceberg. Only 4 months after the publication describing *armA*, Yokoyama *et al.* published an article describing another 16S

rRNA methyltransferase from a 1997 *P. aeruginosa* isolate, *rmtA* (ribosomal RNA methyltransferase A) (Yokoyama *et al.*, 2003). Like *armA,* this gene conferred unusually high resistance to 4,6-2-DOS aminoglycosides.

Since the discovery of ArmA and RmtA until now, more than ten distinct acquired 16S rRNA methyltransferases have been published, in addition to three variants (Doi *et al.*, 2016). The acquired 16S RMTases can be divided into two families, Arm (aminoglycoside resistance methyltransferase) and Pam (pan-aminoglycoside resistance methyltransferase). All of the previously described RMTases (ArmA and RmtA-H) belong to the Arm family of methyltransferases and confer resistance to the most clinically relevant aminoglycosides. Interestingly, the only known acquired 16S methyltransferase that belongs to the Pam family is NpmA, which methylates position N1 of A1408 resulting in resistance to structurally diverse aminoglycosides such as kanamycin, neomycin or apramycin (Wachino *et al.*, 2007).

3.2.2.2 Mode of action

The acquired aminoglycoside resistance conferring 16S rRNA methyltransferases function very similarly to the intrinsic resistance RMTases. In fact, based on their GC content (*rmtA* has a GC content of >55%), it has been speculated that these acquired resistance methyltransferases originated from ancestral intrinsic resistance RMTases within high GC content species (e.g. actinomycetes)(Liou *et al.*, 2006; Doi *et al.*, 2007). Their action also depends on SAM as a source of the methyl group. Furthermore, these 16S RMTases also act on the N-7 position of residue G1405 or the N1 position of residue A1408 to produce 7-methylguanosine or 1-methyladenosine, respectively (Kotra *et al.*, 2000, Gutierrez *et al.*, 2012). The respective methylation greatly reduces the affinity of aminoglycosides for their target-site, without affecting the function of the ribosome, resulting in high levels of resistance to a number of aminoglycosides. This enzymatic activity has been further characterized in vitro with purified ArmA, RmtB and RmtC, demonstrating that the SAM dependent methylation occurs once the 30S subunit of the ribosome is fully assembled

(Wachino *et al.*, 2010). As this methylation does not occur with 16S rRNA alone, it is clear that the affinity of these enzymes requires the full 3D binding pocket to function. Experiments conducted using Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) have also confirmed that the methylation site of these enzymes is position N7 of residue G1405 (Gutierrez *et al.*, 2012). Similar experiments have also demonstrated that the affinity of NpmA for the N1 position of residue A1408 also requires the mature 30S subunit of the ribosome (Wachino *et al.*, 2010). In summary, these precise target modifications are capable of preventing aminoglycoside binding, without interfering with the proof reading mechanisms in place to allow for the accurate translation of proteins. The methylation of G1405 does however interfere with the housekeeping methylation of C1407 by the *rsmF* methyltansferase (Gutierrez *et al.*, 2012). Despite displacing this highly conserved methylation, the deletion of this intrinsic methylation does not incur effects on the fitness of the bacterium (Gutierrez *et al.*, 2012). Lioy et al, however, demonstrated that the expression of *armA* did incur a biological cost on the host. By generating mutants incapable of interacting with the ribosomal RNA they demonstrated that this fitness cost is a result of the methylation rather than the transcription/translation of *armA* (Lioy *et al.*, 2014).

3.2.2.3 Clinical significance and prevalence

As aminoglycosides are typically not a first line antibiotic, until recently, the clinical impact of this resistance mechanism was thought to be relatively limited (Doi *et al.*, 2016). However, the increasing prevalence of this resistance determinant alongside other clinically relevant resistance genes is troubling. Furthermore, the nature of this resistance mechanism is very concerning. A single methylation of residue G1405, for example, causes an unusually high-level resistance to almost all clinically significant (4,6 2-DOS) aminoglycosides (Magnet *et al.*, 2005). As previously outlined in the clinical applications of aminoglycosides, their broad-spectrum activity towards Gram-negative microbes makes them an ideal agent for emergency treatments of multidrug-resistant (MDR) and

extensively drug-resistant (XDR) microbial infections, which makes this resistance mechanism even more concerning (WHO, 2007).

Clinically, the most prevalent mechanism of aminoglycoside resistance is mediated by enzymatic modification; however, strategies are currently being employed to ensure the continued efficacy of the aminoglycosides (Magnet *et al.*, 2005). Amikacin, for example, is typically reserved for pathogens that exhibit resistance to both the first line antibiotic and aminoglycosides. As amikacin is, for the most part, immune to enzymatic modifications, it can be utilized despite the presence of this resistance mechanism (Ramirez *et al.*, 2010). A key difference between 16S rRNA methyltransferases and resistance mechanisms like the modifying enzymes is the level of MIC they achieve. Deactivating enzymes are also typically less broad spectrum and as such confer resistance to some aminoglycosides, but others can still be used in their place, as is the case for amikacin (Garneau-Tsodikova *et al.*, 2016).

Since their discovery in the early 2000's, 16S rRNA methyltransferases have been increasingly identified alongside other resistance determinants, such as carbapenemase genes, which confer resistance to other last resort antibiotics (Doi *et al.*, 2016). The global spread of carbapenem-resistant Enterobacteriaceae has greatly accelerated since the appearance of KPC and NDM-type carbapenemases, demonstrating a 30% increase in carbapenem resistant *A. baumannii* from 1995-2004 (Fair *et al.*, 2014). Although the 16S RMTases are a relatively new resistance mechanism, they appear to be converging with the carbapenemase epidemic, which is greatly facilitating the appearance of extensively drug-resistant and even pandrug-resistant microbes that cause untreatable life-threatening infections (Fair *et al.*, 2014). In pathogens where carbapenems have already been ruled out due to resistance, the presence of 16S methyltransferases remove key aminoglycosides such as gentamicin, amikacin and tobramycin as a last resort option (Garneau-Tsodikova *et al.*, 2016). For example, plazomicin, which is a new aminoglycoside under development specifically for the treatment of carbape-

nem-resistant Enterobacteriaceae, cannot be applied to treat infections caused by bacteria that also harbor 16S methyltransferases (Doi *et al.*, 2016). Currently, in Europe, Japan and Argentina, the prevalence of 16S rRNA methyltransferases remains relatively low, around 1% of Enterobacteriaceae have been shown to harbor these methyltransferases (Doi *et al.*, 2016). That being said, several single-center surveillance studies have placed 16S RMTase prevalence in Enterobacteriaceae significantly higher, especially in Asia. In Korea prevalence rates have been published to be as high as 11.4% in Enterobacteriaceae (Doi *et al.*, 2016). A more comprehensive study of Chinese isolates between 2006 and 2008 demonstrated that 5.4% and 6.2% of *E. coli* and *K. pneumoniae* clinical isolates harbored either *armA* or *rmtA* (Livermore *et al.*, 2011). Studies concerning methyltransferace prevalence in Enterobacteriaceae demonstrated that out of 1000 consecutive clinical isolates, collected in an Indian hospital between 2010 and 2011, a total of 14% carried at least one 16S rRNA methyltransferase. However, even higher rates have been reported: in Saudi Arabia, 37% of 330 Enterobacteriaceae isolates carried at least one 16S RMTase, in 2011 (Al Sheikh *et al.*, 2014).

While the prevalence of the Arm family of acquired resistance conferring 16S methyltransferases is consistently augmenting, there has still only been one isolated case of a Pam family N1-A1408 methyltransferase, *npmA*, in a Japanese *E. coli* isolated in 2003 (Wachino *et al.*, 2007). This is interesting, as a widespread dissemination of a pan-aminoglycoside resistance methyltransferase could be a serious problem for the future of aminoglycosides. While recent reports of *npmA* in *K. pneumoniae* spp. and *Enterobacter* spp. in Saudi Arabia are troubling, the dissemination of *npmA* is virtually non-existent when compared to the dissemination of the most prominent Arm family 16 methyltransferases (Doi *et al.*, 2016). The 16S rRNA methyltransferases are steadily increasing in more and more clinically significant bacteria, including in some of the ESKAPE pathogens (***E****nterococcus faecium,* ***S****taphylococcus aureus,* ***K****lebsiella pneumoniae,* ***A****cinetobacter baumannii,* ***P****seudomonas aeruginosa and* ***E****nterobacter* species), which are highly resistant bacteria responsible for 2/3 of the deaths caused by resistant pathogens worldwide (Rice., 2008).

3.2.3 The genetic environment of the 16S rRNA methyltransferases and its role in their dissemination

Due to the potential impact of this resistance mechanism and the fast rate of its dissemination, a number of studies have focused on the genetic environment that underlies the dissemination of 16S rRNA methyltransferases (Galimand *et al.*, 2003; Gonzalez-Zorn *et al.*, 2005; Toleman *et al.*, 2006). As is the case with many troubling resistance mechanisms, the acquired 16S rRNA methyltransferases are exclusively found within mobile genetic elements (Doi *et al.*, 2016). While most 16S methyltransferases are encoded on plasmids (both conjugative and non-conjugative) alongside a number of other resistance mechanisms, recent literature also describes these acquired methyltransferases within the chromosomes of multi-drug resistant pathogens (Rahman *et al.*, 2015). As such, the rate of their dissemination is greatly facilitated by both horizontal and vertical gene transfer. In the following section I will outline the genetic environments, plasmid incompatibility groups and any resistance determinants found alongside the acquired 16S rRNA methyltransferases.

3.2.3.1 *armA*

ArmA, the most disseminated 16S methyltransferase has been almost exclusively identified on plasmids, although very recent publications now describe *armA* within the chromosome (Karah *et al.*, 2016). Initially it was discovered alongside the ESBL gene $bla_{\text{CTX-M3}}$ on the IncL/M plasmids pCTX-M3 and pIP1204 in *C. freundii* and *K. pneumoniae* respectively (Galimand *et al.*, 2005). The *armA* gene has exclusively been identified within the 16.6kb transposon Tn1548. Specifically *armA* is located downstream of an insertion sequence (ISCR1) and followed by a class 1 integron containing the resistance genes *dhfrXII* (a dihydrofolate reductase conferring trimethoprim resistance), *aadA2* (an adenyltransferase conferring streptomycin resistance) as well as *sul1* (conferring sulfonamide resistance). Typically, the macrolide resistance conferring genes *mel* and *mph2* aswell as *trpA* (a transposase-like gene) have been identified downstream of

armA. While the genetic environment of *armA* within the class 1 integron have been shown to vary slightly this global genetic context of *armA* appears to be very well conserved despite being widely disseminated amongst Enterobacteriaceae and *Acinetobacter baumannii* (Doi *et al.*, 2007; Bercot *et al.*, 2008; Granier *et al.*, 2011).

Within Tn1548 *armA* was shown to be flanked by two copies of IS6, which have been experimentally shown to readily transpose *armA* between genetic environments (Galimand *et al.*, 2005). This in combination with the vast array of resistance genes *armA* can be co-selected with, have greatly facilitated the global dissemination of *armA*, making it, alongside *rmtB*, the most prevalent 16S rRNA methyltransferase. The prevalence of both *armA* and *rmtB* has been repeatedly associated with the co-selection of these methyltransferases alongside the global emergence of $bla_{\text{NDM-1}}$ harboring Enterobacteriaceae (Carattoli *et al.*, 2012).

3.2.3.2 *rmtA*

To date, *rmtA* has also only been identified within transferable plasmids or more specifically in transferable genetic elements such as transposons. The genetic environment harboring *rmtA* in *P. aeruginosa* was found to be flanked by two copies of a kappa-gamma element (predicted to be a relic of mobile genetic elements) that was previously found within the composite transposon Tn5041 that was previously associated with harboring mercury resistance determinant in *Pseudomonas* spp. (Yamane *et al.*, 2004). Historically, the isolation of *rmtA* has been more infrequent than *armA*, with only a limited number of *P. aeruginosa* isolates harboring *rmtA* from Japan and Korea (Yamane *et al.*, 2004; Jin *et al.*, 2009).

3.2.3.3 *rmtB/B2*

rmtB was first reported on a plasmid downstream of a Tn3-like transposon flanked by two IS26 elements. The downstream region of *rmtB* has been shown to be quite variable, although typically associated with the fluoroquinolone efflux gene *qepA1* or a variation (Perichon *et al.*, 2007; Yamane *et al.*, 2007). More recently, *rmtB* has been identified within a multidrug resistant region on an IncI1 plasmid. In these plasmids, *rmtB* was identified alongside $bla_{\text{TEM-1}}$, , a class 1 integron cassette array (intI1-dfrA12-orfF-aadA2-qacEΔ1-sul1) and the aminoglycoside N-acetyltransferase *aacC2*.

Not unlike *armA*, the distribution of *rmtB* has been associated with the world-wide emergence of bla_{NDM-1} harboring Enterobacteriaceae with which both methyltransferases are commonly associated (Carattoli *et al.*, 2012). RmtB is also the first acquired methyltransferase with an identified variant. Based on a single amino acid variation within a conserved domain, RmtB 2 is classified as a distinct variant. Based on the similarities among *rmtB* and *rmtB* 2, it is not surprising that their genetic environment has maintained a high degree of conservation (Doi *et al.*, 2016).

3.2.3.4 *rmtC*

When *rmtC* was initially discovered in a clinical strain of *P. mirabilis*, it was located on a non-conjugative plasmid adjacent to an ISEcp1-like element, which has been shown to also provide the promoter region of the methyltransferase (Wachino *et al.*, 2006). While this ISEcp1 has been frequently associated with β-lactamases, it is expected that *rmtC* dissemination will be greatly facilitated among the Enterbacteriaceae via the transposition activity of this genetic element (Toleman *et al.*, 2011). Although currently *rmtC* is not as globally disseminated as neither *armA* nor *rmtB*, its coproduction alongside $bla_{\text{NDM-1}}$ is very worrisome, as it is believed that the global emergence of this resistance gene has facilitated the dissemination of the most prevalent 16S rRNA methyltransferases (Poirel *et al.*, 2011). Interestingly, *rmtC* is one of the few acquired 16S methyltransferases that has been identified in the chromosome of *S. enterica* clinical isolates in the United Kingdom (Hopkins *et al.*, 2010)

3.2.3.5 *rmtD/D2*

rmtD has, as of now, only been described in South America, and its general prevalence is considered to be quite low. In the Brazilian strain discovered, *rmtD* followed by an *orf494* (a putative transposase gene) and a 3' conserved region harboring *qacEΔ1* and *sul1* of the class 1 integron In163 (Doi *et al.*, 2008). In the area immediately upstream of *rtmD* an *orfA* (encoding a putative ribosyltransferase gene), Δ*groEL* and a futher copy of *orf494* were identified. Both *orf494* copies were found to be truncated by an IS6. These flanking open reading frames, have now been proposed as the putative transposase ISCR14 (Doi *et al.*, 2008). More recent variants of *rmtD* have been isolated in *K. pneumoniae* from Brazil alongside bla_{KPC-2} (Beuno *et al.*, 2013).

The genetic context of *rmtD2* was highly similar to that of *rmtD*, however, a large portion of the 5' extreme of the Δ*groEL* gene was deleted as compared to *rmtD* (Tijet *et al.*, 2011). Although *rmtD* and *rmtD2* have been identified alongside β-lactamase resistance determinants, as of now, they have never been identified on the same plasmid as the *bla* gene. That being said, methyltransferases found alongside β-lactamase genes, such as bla_{NDM-1} and $bla_{CTX-M3,}$ are among the most prevalent 16S methyltransferases in the world. Therefore the close proximity of *rmtD* and *rmtD2* with, in this case bla_{KPC}, could suggest a wider future dissemination of these methyltransferases.

3.2.3.6 *rmtE/E2*

Both *rmtE* and *rmtE2* are among the least described methyltransferases. Currently, there are only two reported cases of *rmtE* from a cow and a human isolate in the United States. In most cases *rmtE* was borne on a class 1 integron harbored by a self-conjugative 199kb IncA/C plasmid designated pYDC637 (Lee *et al.*, 2015). This plasmid has been shown to be composed of a 144kb core region followed by a 55kb acquired region. Within this acquired region there are two class 1 integrons, one of which harbors *rmtE* bound by

an ISCR20-like element and an IS1294-like insertion sequence that are commonly associated with resistance genes and 16S rRNA methyltransferases (e.g. *rmtD*/*D2* and *rmtF*) (Lee *et al.*, 2015).

3.2.3.7 *rmtF*

Of all the 16S rRNA methyltransferase, *rmtF* is perhaps the most closely associated with the $bla_{\text{NDM-1}}$ resistance gene. The first strain identified to express *rmtF* (a *K. pneumoniae* from the Reunion Islands) harbored the 16S methyltransferase downstream of $bla_{\text{NDM-1}}$ on a class 1 integron (Galimand *et al.*, 2012). In this case *rmtF* was harbored on a 40kb non-self transferable plasmid pIP849 and it was cotranscribed with the *aac(6')-IB* resistance gene (Galimand *et al.*, 2012). Furthermore the *rmtF* gene was bracketed by a 3' portion of an ISCR5 element including *oriIS* and by a truncated *insE* gene for the ISCR5 transposase together with *oriIS (*Galimand *et al.*, 2012). Galimand *et al.*, further stipulate that the genetic environment of *rmtF* suggests that it was recruited either by ISCR transposition or homolgous recombination similar to that proposed for *rmtD1* and *rmtD2* and the associated ISCR14 (Toleman *et al.*, 2006; Tijet *et al.*, 2011).

3.2.3.8 *rmtG*

Like *rmtD*, *rmtG* has been almost exclusively described in South America. As of now, *rmtG* has been identified in colistin resistant *K. pneumoniae* in the United States (Miami), Brazil and in Chile (Bueno *et al.*, 2013; Poirel *et al.*, 2014; Hu *et al.*, 2014) alongside a number of extended spectrum b-lactamase genes (e.g. $bla_{\text{CTX-M2}}$, $bla_{\text{TEM-1}}$, $bla_{\text{SHV-11}}$) (Poirel *et al.*, 2014). While the epidemiological information pertaining to this methyltransferase is limited, currently it has only been identified in *K. pneumoniae*. Carattoli *et al.* have confirmed via replicon typing that *mtG* was harbored on an 80-kb IncA/C plasmid (Carattoli *et al.*, 2005).

3.2.3.9 *rmtH*

In 2006 a highly resistant *K. pneumoniae* strain was isolated from the wound of a soldier in Iraq. This strain, which presented very high levels of aminoglycoside resistance was later shown to harbor the methyltransferase *rsmH,* which shares a 64% identity with Arm family methyltransferases *rmtB* and *rmtB 2* (O'Hara *et al.*, 2013). This isolate also presented resistance to cephalosporins, aztreonam and ciprofloxacin. Furthermore, as previously seen with the other 16S rRNA methyltransferases, *rmtH* was co-harbored alongside $bla_{\text{CTX-M-15}}$ $bla_{\text{SHV-1}}$ and $bla_{\text{OXA-1}}$ (O'Hara *et al.*, 2013). *rmtH* was bracketed by two copies of ISCR2 which is an IS91-like transposable element that has been found to be associated with a number of antibiotic resistance mechanisms (Toleman *et al.*, 2006).

As demonstrated above the 16S rRNA methyltransferases are all harbored within mobile genetic elements readily capable of translocating the resistance gene, which have been summarized in figure 11.

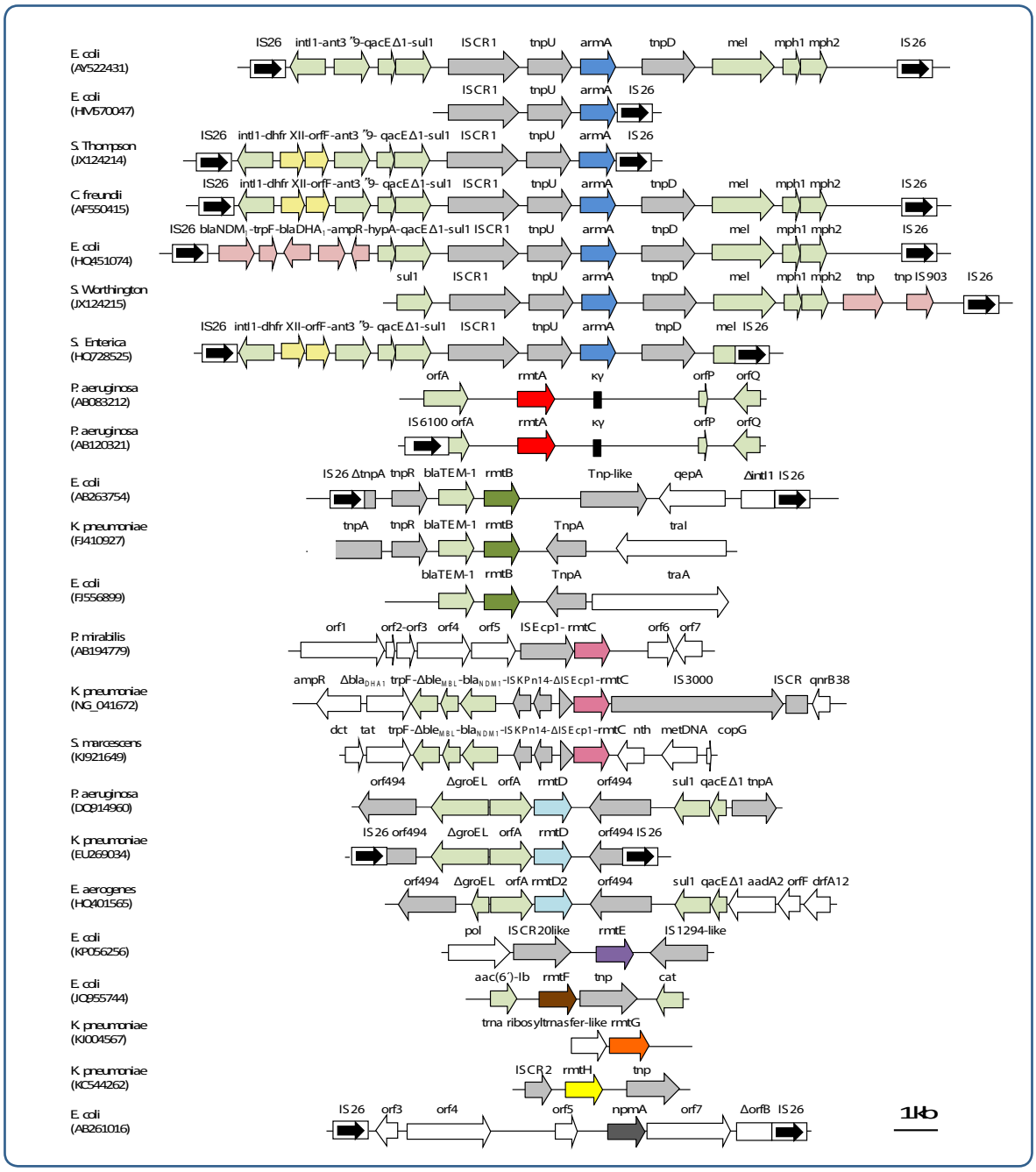


Figure 11. Diagram representing the genetic environment of the acquired 16S rRNA methyltransferases (Gutierrez et al., unpublished work)

This versatility also results in the selection of a genetic environment that is most suitable for an expression of the methyltransferases that confers high-level resistance while mitigating the biological cost incurred on the host. However, possibly the most important factor underlying the rapid dissemination of the 16S rRNA methyltransferases are the associated resistance determinants. A number of publications directly link the emergence of the acquired 16S methyltransferases with the global spread of ex-

tended spectrum β-lactamases including $bla_{\text{NDM-1}}$ *and* $bla_{\text{CTX-M}}$ (Doi *et al.*, 2016). Especially when the resistance determinant is found alongside other resistance genes on the same plasmid, any antibiotic treatment will co-select for both resistance genes, which in turn maintains the genes within the population.

3.3 Regulation associated with antibiotic resistance

Antibiotic resistance is, as repeatedly mentioned, a growing concern and, while there is a great emphasis on elucidating the mode of action of acquired resistance determinants and the surveillance of their dissemination, another very important field pertaining to antibiotic resistance is the study of their expressional regulation. This field is of great importance to elucidate the underlying factors of resistance gene dissemination. Resistance genes, especially those like the 16S rRNA methyltransferase encoding genes, may interfere with the 'natural order of things' within their host. Such interference generally incurs a fitness cost to the cell, which, in environments without selective pressure, can result in the out-competition of that strain and the subsequent loss of the resistance determinant (Wiesch *et al.*, 2010).

The complexity underlying regulation is immense and there are a plethora of strategies a bacterium may apply in order to fine-tune the expression of required genes. In this chapter, I will outline the various strategies employed by antibiotic producers and their intrinsic resistance mechanisms, as well as the emerging regulatory mechanisms of acquired resistance genes. But before discussing regulation with regards antibiotic resistance, I will present various aspects of global gene regulation and transcriptional induction.

3.3.1 Global transcriptional regulation

Depending on the environmental conditions and the current growth phase of the bacterium, the transcription levels from one moment to the next, and between different genes, vary significantly. For most genes a key factor in their transcriptional induction is the recognition and interaction of the gene's promoter region by the RNA polymerase (Browning *et al.*, 2004). The RNA polymerase is a ~400 kDa molecule that consists of 5 subunits (Figure 12).

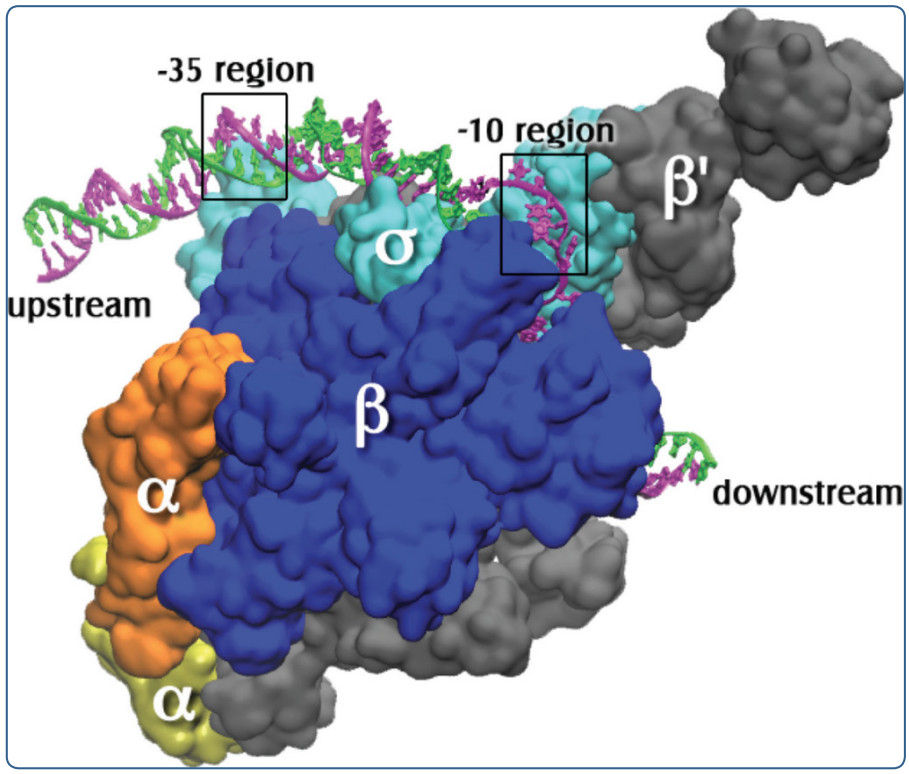


Figure 12. A representation of an RNA polymerase holoenzyme open complex. The core enzyme consists of a large β-subunit and a β'-subunit (blue and grey respectively), two α-subunits (orange and yellow), the small ω-subunit (not shown) and finally the sigma factor (turquoise) (Figure from Karpen et al., 2015).

The large β-subunit and the β'-subunit are assembled by binding the N-terminal domains of the α-subunits whereby they form a cleft that constitutes the active site. The fifth RNA polymerase component, the ω-subunit, is mainly a chaperone for the β'-subunit (Murakami *et al.*, 2003). However, this intact RNA polymerase alone is not able to recognize promoter regions without the presence of an additional protein known as a sigma factor, which is further explained in the next section (Figure 12). The interaction of the sigma factors with the core enzyme forms the complex known as the RNA polymerase holoenzyme, which is then able to interact with specific promoter regions (Gross *et al.*, 1998). This interaction ultimately unwinds the DNA in the region of the transcriptional start site (+1), and can commence the transcriptional cycles of transcription. This complex mechanism can be regulated at various phases, either at the level of RNA polymerase holoenzyme formation, promoter recognition by the RNA polymerase (more specifically, the sigma factor bound by the RNA polymerase), or at the level of the RNA polymerase activity (Browning *et al.*, 2016).

In recent years, the increasing importance of small regulatory RNAs has revealed another level of complexity within the regulatory network that underlies gene regulation. With regards to the objectives of this thesis, we will focus on the involvement of the sigma factors, as well as the specific promoter regions, in the induction of transcription.

3.3.1.1 Sigma factors

The specific association of sigma factors with the RNA polymerase is required not only for the initiation of transcription, but also for the selection of the genes to be transcribed. RNA polymerases are capable of interacting with a variety of sigma factors that are each responsible for the induction of specific groups of genes (Figure 13).

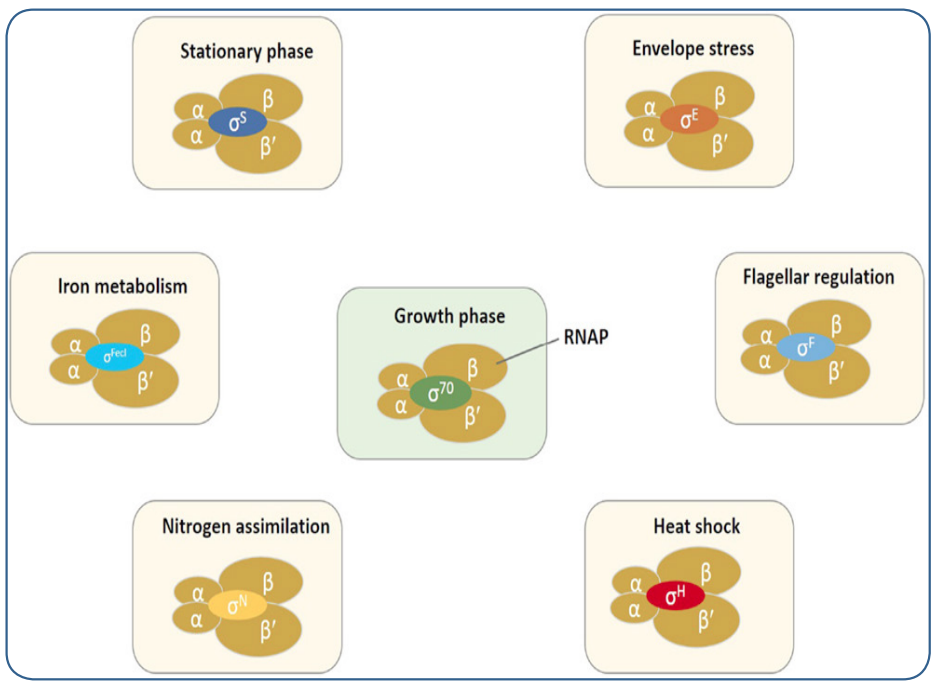


Figure 13. A representation of the complexes formed between RNA polymerases and the various sigma factors responsible for the variable transcription of genes (Figure from Tripathi et al., 2014)

The conventional model for the promoter recognition mediated by σ^{70} involves mainly the -35 box and -10 box (so called due to their respective distance from the transcriptional start site), as well as the discriminator sequence which are all recognized

by the sigma factor. In addition operator regions may also interact with the C-terminal domains of the α-subunits of the RNA polymerase holoenzyme (Browning *et al.*, 2004). All bacteria have primary housekeeping sigma factors (such as σ^{70} in *E. coli*), which are responsible for recognizing most promoter regions (Gross *et al.*, 1998). While the primary sigma factor recruits the RNA polymerase to most genes to be induced, most bacteria have a number of alternate sigma factors to dynamically modulate their transcriptome (Figure 13)(Gruber *et al.*, 2003).

Housekeeping σ^{70} factors are composed of four structural domains joined by flexible linkers that, when bound to the RNA polymerase, interact with the promoter elements (Murakami *et al.*, 2003). These sigma factors, also known as RpoD, are especially active during phases of rapid growth and preferentially induce the transcription of genes involved in protein synthesis (Ozaki *et al.*, 1991). These sigma factors are of vital importance, as there are a plethora of gene products associated with the assembly of the protein synthesis machinery that need to be induced dynamically to correspond to the growth conditions of the bacterium (Browning *et al.*, 2016). Other sigma factors such as RpoH, FliA, PvdS and RpoN, to name a few, are involved in vital processes within the cell, such as heat-shock, adhesion and flagellin biosynthesis, iron metabolism and virulence, and nitrogen metabolism respectively (Benvenisti et al,. 1995; Starnbach *et al.*, 1992; Cundliffe *et al.*, 1995; Ishimoto *et al.*, 1989). The fine-tuned orchestration of these factors governs the expression of a large number of genes within the cell responsible for vital processes (Browning *et al.*, 2004). Many of the main sigma factor promoters also compete with anti-sigma factors that are able to reduce sigma factor activity when their respective gene products are no longer required (Hughes *et al.*, 1998).

3.3.1.2 Sigma factor recognition of promoter regions initiates transcription

The precise constitution of the promoter region has a great effect on the activity of these essential sigma factors and the subsequent transcriptional levels. The core of the sigma factors can be thought of as a harmonic oscillator that is capable of expanding and contracting to interact with the promoter elements (Aoyama *et al.*, 1983). However, variations in the distance between the -35 box and the -10 box have a profound effect on the energy available for stabilization and the subsequent induction rate (Mandecki *et al.*, 1982; Aoyama *et al.*, 1983). The distance most commonly identified between sigma factor recognized promoter regions is 17 ± 1 bases, although initiation has been demonstrated with promoter regions separated by 15-20 bases (the spacings between the promoter elements are measured from the 3' end of the -35 box to the 5' end of the -10 box)(Figure 14)(Hawley *et al.*, 1983; Dombroski *et al.*, 1996).

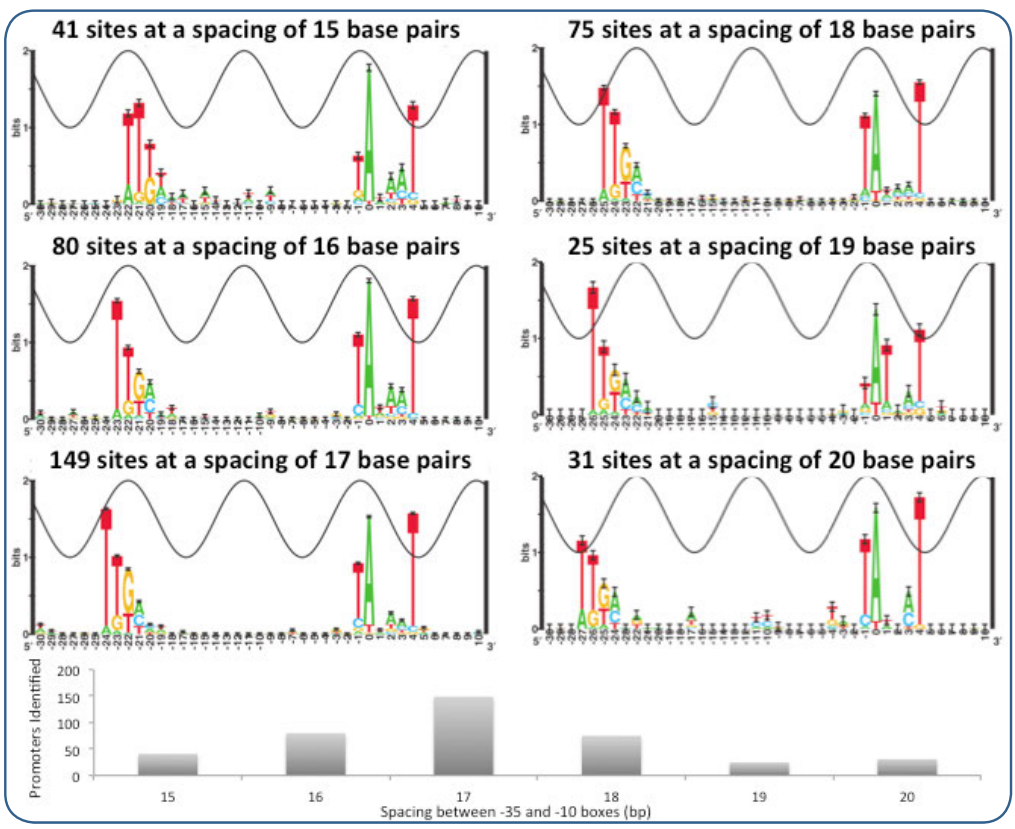


Figure 14. Representation of the promoter region conservation with regards to the spacing between the -35 and -10 boxes. It represents the number of putative sigma factors identified in the upstream region of 599 experimentally determined transcriptional start sites from the RegulonDb database (Salgado et al., 2001) (Figure adapted from Shultzaberger et al., 2007)

While the most common distance between two promoter regions appears to be 17 base pairs, it would not be accurate to describe this as the optimum distance for sigma factor recognition. In fact, this yet again, is a mechanism to fine tune the specific transcriptional levels required of each respective gene (Aoyama *et al.*, 1988). Interestingly, as seen in figure 14 above, the precise promoter conservation alters depending on the distance between the -35 and -10 promoter regions, although the overall trend remains quite constant. That being said, although the promoter recognition by sigma factors is variable, reports demonstrate that the interaction of the sigma factor with certain -10 boxes alone is sufficient to induce transcription (Kumar *et al.*, 1993; Barne *et al.*, 1997).

Furthermore, there are a number of additional elements that may influence sigma factor interactions with the respective promoter regions, such as transcription factors and repressors that are capable of binding to operators upstream of the promoter regions, where they either facilitate the recruitment of RNA polymerases or create a steric hindrance that inhibits the interaction with the RNA polymerase holoenzyme (Thieffry *et al.*, 1998). Such factors may function as part of a two component regulatory pathway that reacts to environmental conditions or with biosynthetic intermediates that precisely orchestrate the sequential initiation of required gene products (Browning *et al.*, 2016). The transcriptional regulation of vancomycin resistance, for example, is regulated by such a two component system (Arias *et al.*, 2000; Abadia et al,. 2002)

As previously mentioned, the regulatory mechanisms here outlined regarding the global regulation of transcription are just a selection of the variables mediating the prokaryotic transcriptome. As such, here I have introduced a small selection of mechanisms to illustrate the immense complexity of this process. Additionally, although I will mention a number of post-transcriptional mechanisms of regulation in the sections to follow, the transcriptional induction is just a relatively small aspect when it comes to the arsenal of regulatory tools available to the prokaryotic cell.

3.3.2 Regulation of intrinsic antibiotic resistance mechanisms

In order for the antibiotic producers to effectively protect themselves from the toxic activity of these potentially lethal secondary metabolites, it is fundamental that they express their intrinsic resistance genes in a timely fashion. Generally, there are two main strategies that can be employed to govern this timely expression. Firstly, in many antibiotic producers, the expression of the resistance gene is closely associated with the biosynthetic clusters that encode the antibiotic. The second expressional strategy is the induction by their cognate antibiotics or by intermediate molecules from their biosynthetic pathways (Mak *et al.*, 2014). There are some resistance genes, however, that are constitutively expressed, such as the *ermE* erythromycin resistance gene; although generally this is rare, and most of these genes are tightly regulated, most likely because the advantages of antibiotic resistance are typically only transient (Bibb et al,. 1985; Depardieu *et al.*, 2007).

With regards to the aminoglycosides, the Kgm and Kam family of intrinsic methyltransferases confer resistance to aminoglycosides by methylating G1405 and A1408 respectively (Beauclerk *et al.*, 1987). The genes of these intrinsic resistance determinants, such as *grmA*, *kmr* and *kamA,* are also commonly found within the biosynthetic clusters alongside the aminoglycosides gentamicin kanamycin and istamycin respectively (Magnet *et al.*, 2005).

3.3.2.1 Regulation of intrinsic resistance genes within the biosynthetic gene clusters of the antibiotic

It is stipulated that the regulatory mechanisms coupling the resistance gene directly to the production of the antibiotic are mechanistically diverse and have been associated with the origins of clinical antibiotic resistance and many of the resistance genes within these clusters have been shown to be expressed immediately before or alongside the antibiotic itself (Mak *et al.*, 2014). The regulatory mechanism underlying the induction of the

factors leading to the synthesis of the antibiotic are also well described. These regulatory mechanisms commonly known as cluster-situated regulators (CSRs) monitor a number of cellular aspects such as physiology, developmental states and environment (to name a few), to determine the appropriate quantity antibiotic to produce (Liu *et al.*, 2013).

One example of such a co-induction of resistance is the intrinsic streptomycin resistance gene *aphD*. This 6-phosphotransferase confers resistance to streptomycin in the producer *S. griseus* (Tohyama *et al.*, 1984; Distler *et al.*, 1987). The enzymatic modification produced by this enzyme renders the streptomycin inactive and permits the safe export of this potentially lethal secondary metabolite (Beyer *et al.*, 1996). The so-called A-factor initially binds to the Arp protein, which is a repressor belonging to the TetR-family of repressors (Onaka *et al.*, 1997; Cuthbertson *et al.*, 2013). The interaction between the A-factor and the repressor ArpA subsequently causes it to liberate the promoter region of *adpA,* which is a global regulator of sporulation and secondary metabolites such as the *str* cluster, which encodes streptomycin (Ohnishi *et al.*, 2005). The inducing activity of AdpA on the streptomycin pathway specific regulator *strR* also results in the expression of the downstream resistance gene product AphD (Figure 15) (Vujaklija *et al.*, 1991).

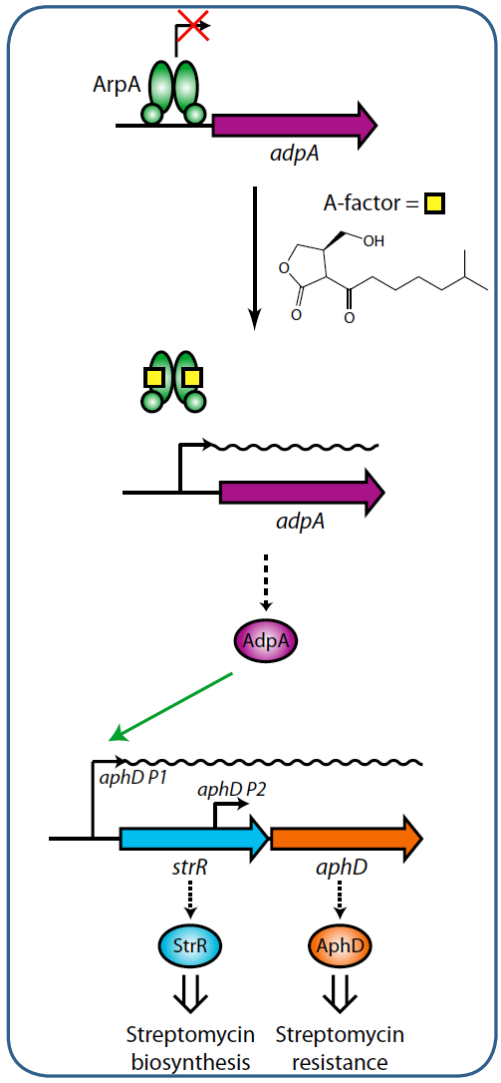


Figure 15. Figure outlining the regulatory cascade governing the expression of the streptomycin biosynthetic pathway (strR) alongside its respective resistance gene aphD (Mak et al., 2014)

This elegant mechanism ensures the presence of the streptomycin modyfing enzyme AphD before the antibiotic can accumulate in the cytoplasm and, as such, protects its host from the deleterious activity of this secondary metabolite. Another advantage of this cascade is that the A-factor is a diffusible signal, which permits the activation of this cascade of adjacent cells within the culture (Horinouchi, 2002).

3.3.2.2 Regulation of intrinsic resistance genes modulated by intracellular concentrations of the antibiotic

Depending on the specific antibiotic in question, the producing bacterium may draw an advantage from having a dynamically regulated resistance determinant that is not only expressed alongside the antibiotic, but also expressed proportionally according to the intracellular antibiotic concentrations.

Such a regulation of intrinsic resistance gene regulation was identified, among others, in *Streptomyces fradiae*. This bacterium produces the macrolide antibiotic tylosin, which acts on the ribosomal 50S subunit (Zalacain *et al.*, 1989). The biosynthetic gene cluster of this antibiotic harbors four tylosin resistance genes (*tlrA-D*) that protect the producer from the antibiotic (Liu *et al.*, 2000). These resistance genes consist of three rRNA methyltransferases and one efflux pump (*tlrC*) (Rosteck *et al.*, 1991). These four resistance genes constitute a type of fail-safe resistance mechanism for the host. TlrD, one of the rRNA methyltransferases, is constitutively expressed in small quantities, which ensures a certain degree of protection to the host by methylating and protecting the 23S rRNA whether the tylosin is being synthesized or not (Cundliffe *et al.*, 1991). The expression of the other resistance genes, however, is more complicated. It has been proposed that the expression of the resistance methyltransferase *tlrA* is regulated via a ribosome mediated transcriptional attenuation (Keleman *et al.*, 1994; Mak *et al.*, 2014). This mechanism relies on the presence of a small upstream ORF that is constitutively transcribed and translated. In the absence of the antibiotic, the ribosome rapidly translates a leader peptide sequence in the mRNA of *tlrA*, which results in a secondary structure in the mRNA that prevents the full length transcription of the *trlA* mRNA (Keleman *et al.*, 1994). This group then demonstrated that in the presence of the 'inducing' antibiotic, however, the ribosome briefly stalls in the leader peptide of the *tlrA* mRNA, which resolves the secondary structure responsible for prematurely terminating the transcription of the resistance methyltransferase. This 'stalling' is a result of nascent peptide interactions with the exit tunnel of the ribosome (Sunohara *et al.*, 2004). This in turn permits the complete transcription and subsequent translation

of the resistance determinant, but only in the presence of the antibiotic (i.e. only when actually needed). To further complicate this inducible intrinsic resistance conferred, the induction of *tlrA* requires the ribosome to have been previously methylated by the aforementioned *tlrD* (Liu *et al.*, 2002).

3.3.2.3 Regulation of intrinsic resistance genes in correspondence with the growth phase

Another interesting mechanism of resistance gene induction is found within *Streptomyces rimosus*, which produces oxytetracycline. This antibiotic acts on the 30S rRNA and blocks the access to aminoacyl-tRNAs required for translation (Petković *et al.*, 2006). Again, found within the biosynthetic cluster of this antibiotic, are the two intrinsic resistance genes *otrA* and *otrB* (also known as *tetA* and *tetB*) that provide self-defense via target protection and an efflux pump, respectively (Ohnuki *et al.*, 1985). Upstream of these resistance determinants, two promoter regions were identified, *otrAp1* and *oxySp1* (McDowall *et al.*, 1999). Based on the constitution of these promoter regions, when the cells are growing exponentially, the *otrA* intrinsic resistance gene is transcribed as a monocistronic mRNA following the transcriptional induction of the *otrAp1*. However, when the cells enter the stationary phase, where the expression of secondary metabolites increases, the activity of the *otrAp1* ceases, and the second promoter region, *oxySp1*, mediates the expression of the *otrA,* but this time it is co-transcribed along with two genes involved in the maturation of the oxytetracycline (McDowall *et al.*, 1999). Again, this mechanism ensures the expression of the resistance gene prior to the synthesis of the antibiotic, which will protect the cell from the deleterious effects of the antibiotic. Interestingly, the expression of the secondary metabolite and its various resistance genes has been linked to the activity of sigma factors, capable of modulating expression based on the growth phase of the cell (Mak *et al.*, 2014).

With the exception of a few more well characterized intrinsic resistance gene regulatory mechanisms, the data available regarding the co-expression of intrinsic resistance

determinants with their respective antibiotics is limited. In summary, these genes are generally mechanistically coupled to the synthesis of the antibiotic agent, which allows the producer to minimize any biological burden incurred by the resistance gene.

Due to the clinical relevance of the acquired resistance genes however, many more mechanisms have been described in detail, which I will outline in the following sections.

3.3.3 Regulation of acquired antibiotic resistance mechanisms

The regulation of acquired resistance genes differs substantially from that of the intrinsic resistance genes despite achieving the same goal (i.e. protection when required while mitigating the biological cost). One major difference, which I will describe towards the end of this section, is the fact that many (even most) of the acquired resistance genes are harbored within mobile genetic elements that are constantly evolving. Another major difference is that intrinsic resistance genes have co-evolved alongside the producer and optimized expressional profiles. Acquired resistance genes, however, tend to incur a more significant biological cost that is only mitigated after a period of compensation (Depardieu *et al.*, 2007). As mentioned at the beginning of this section pertaining to resistance gene regulation, conferring resistance to a bacterial host is only transiently advantageous (i.e. in the presence of the antibiotic). As such, it appears that acquired resistance genes, more often than not, are found alongside domains capable of regulating their expression.

As previously performed with the intrinsic resistance genes, I will focus on selected examples of both transcriptional and translational mechanisms of regulation commonly associated with clinically significant pathogens and antibiotics.

3.3.3.1 Transcriptional regulation of acquired antibiotic resistance genes (in class 1 integrons)

Although there are many examples of post-transcriptional mechanisms of resistance gene regulation, transcriptional mechanisms have the added advantage that biological resources are not devoted to the partial transcription/translation of resistance genes that are repressed. In this section I will highlight a form of transcriptional regulation that is closely associated with the mobile genetic elements the acquired resistance genes are so often identified in.

The transcription of cassette-associated resistance genes is often mediated by one or more 'strong' promoter regions and a number of 'weak' terminators (Collis *et al.*, 2002). Collis et al, demonstrated that both the position of the gene within the cassette as well as the position of the overall cassette had significant effects on the levels of resistance observed. In such a scenario, the resistance genes are expressed in relationship to their distance from the promoter region. As the 'weak' terminators moderate the number of RNA polymerases that reach the downstream gene products, this method can differentially express a number of resistance genes simultaneously (Depardieu *et al.*, 2007). Such an expression is often mediated by up to four integron-borne promoter regions of variable expressional capacities (Levesque *et al.*, 1994). A number of studies have focused on these particular promoter elements and their inductional efficacy versus the resistance levels they confer (Levesque *et al.*, 1994). These promoters have also frequently been associated with cassette-borne resistance genes that have lost their native promoter region. The expression of these genes is even more dependent on the order in which they appear within the cassette (Collis *et al.*, 2002).

Although there are a number of further transcriptional mechanisms associated with acquired resistance mechanisms, the above mentioned integron mediated regulation of resistance genes is a testament to the rapid evolutionary development facilitated by horizontal gene transfer.

3.3.3.2 Post-transcriptional regulation of resistance genes

Acquired resistance genes have been described under the regulation of a variety of post-transcriptional mechanisms governing their expressional profiles (Breidt et al., 1990). As with the transcriptional feedback mechanisms (coupling the transcriptional levels of the resistance gene to the expression of the antibiotic or biosynthetic intermediates), acquired resistance genes have repeatedly demonstrated the capacity to be induced by their respective antibiotic (Bailey *et al.*, 2008; He *et al.*, 2013). This section will highlight a number of key post-transcriptional regulatory mechanisms associated with antibiotic resistance determinants.

Riboswitch induction of resistance genes

Since the molecular characterization of riboswitches, this molecular gene 'switch' has found wide ranging applications in both the industrial biotechnologies and the medical field (Ketzer *et al.*, 2014). Riboswitches are small secondary mRNA structures found in the 5' untranslated region (UTR) that selectively express their respective gene products based on the presence of an inducing agent (Vitreschak *et al.*, 2004).

In the most common configuration of a molecular riboswitch, which has recently been identified to modulate the expression of aminoglycoside resistance genes, this mRNA structure sequesters the ribosomal binding site preventing the translation of the regulated gene (Figure 16)(He *et al.*, 2013; Jia *et al.*, 2013).

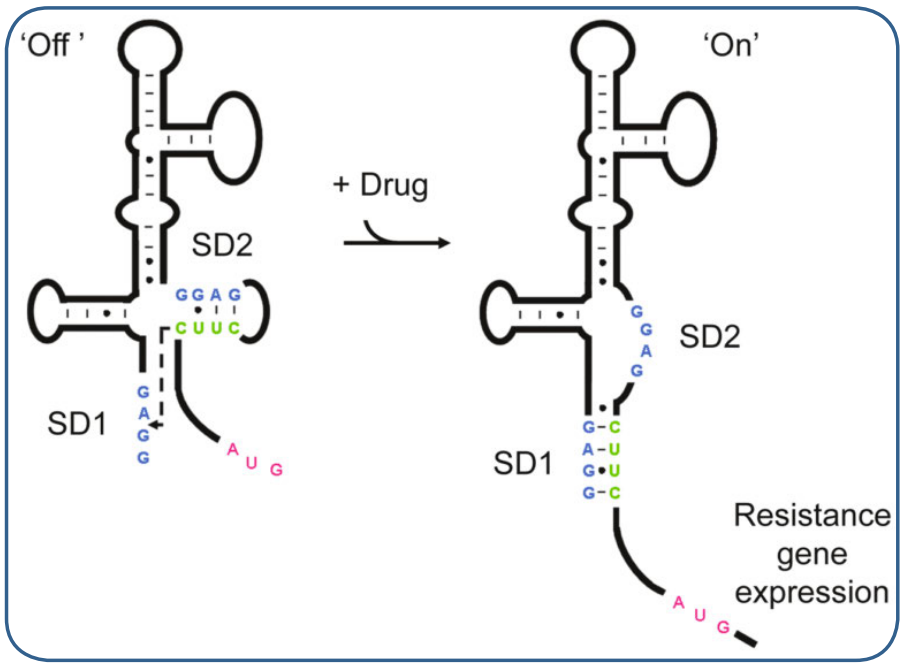


Figure 16. Representation of a molecular riboswitch that has been shown to regulate the expression of aminoglycoside resistance conferring adenyl- and acetyl- transferases. The presence of the inducing antibiotic causes a conformational change of the secondary structure, which liberates the ribosomal binding site resulting in the translation of the resistance gene(Figure from He et al., 2013)

This highly dynamic mechanism allows for the expressional levels of the resistance genes to be augmented by accumulating intracellular concentrations of the inducing antibiotic. By doing so, the host is sufficiently protected while minimizing the biological cost of producing the enzymes in the absence of antibiotic pressure (He *et al.*, 2013).

The riboswitch regulating the aminoglycoside modifying enzymes interacts directly with the inducing antibiotic. However, as the structurally diverse molecular 'switches' can potentially interact with a variety of molecules, it is possible factors specifically associated with the presence of the antibiotic can also interact with such riboswitches to induce the expression of the resistance gene (Vitreschak *et al.*, 2004).

Ribosome stalling mediated expression of resistance genes

Another regulatory mechanism that has been described for a number of resistance genes, especially ones acting on the ribosome, is ribosome stalling (Weisblum *et al.*, 1995; Lovett *et al.*, 1996; Bailey *et al.*, 2008; Ramu *et al.*, 2009). Such mechanisms rely on the programmed stalling of the ribosome as a result of amino acid interactions of the nascent peptide with ribosomal proteins of the exit tunnel. In the case of *ermC*, which is a macrolide resistance conferring 23S rRNA methyltransferase, in the absence of an inducing agent, hairpin loops that form the secondary structure within the 5' mRNA sequester the ribosomal binding site and prevent the subsequent translation of the resistance gene (Figure 17, A)(Bailey *et al.*, 2008).

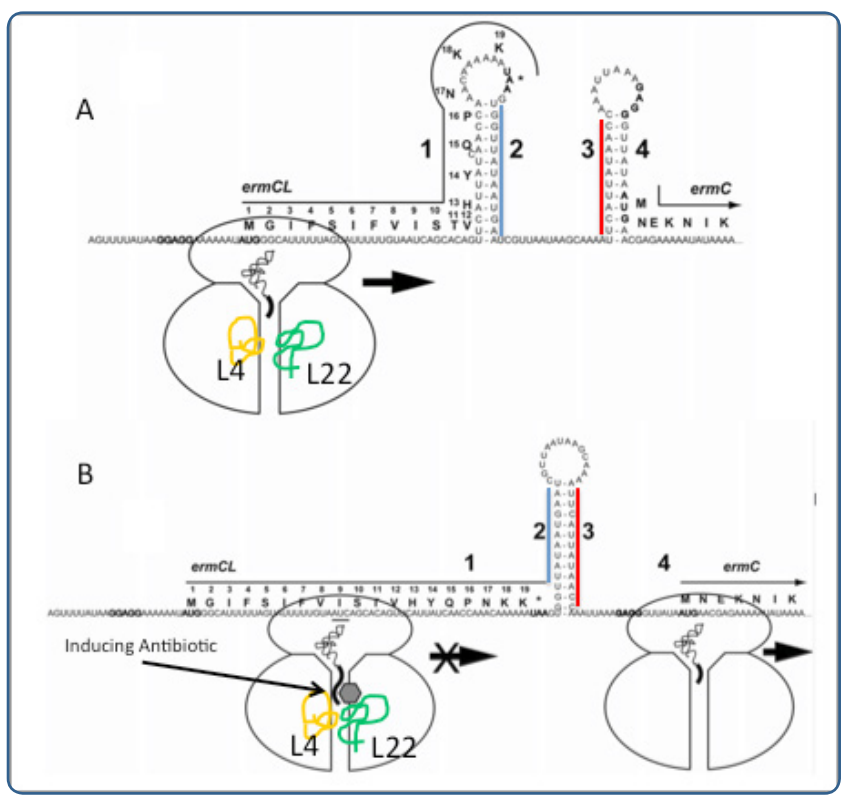


Figure 17. Representation of the secondary mRNA structures in the 5' UTR of the ermC resistance gene. A. Demonstrates the structural conformation in the absence of the inducing antibiotic, and B. demonstrates the conformational changes of ribosomal protein L4 and L22 upon the interaction of the inducing antibiotic with its ribosomal target site. This alteration leads to a brief stalling of the ribosome (due to the interactions of the ribosomal proteins with the nascent peptide), which resolves the secondary structures and thereby liberates the ribosomal binding site of the downstream resistance gene. (Figure adapted from Ramu et al., 2009)

In the case of *ermC*, which is a macrolide resistance conferring 23S rRNA methyltransferase, the presence of the antibiotic at its active site on the ribosome induces a conformational change, which in turn changes the position of ribosomal proteins L4 and L22 (Figure 17, B)(Bailey *et al.*, 2008; Ramu *et al.*, 2009). This conformational change alters the constitution of the ribosomal exit tunnel so that, specific amino acid sequences in the leader peptide (*ermCL*), which is constitutively transcribed and translated, briefly stall the ribosome at a very specific position (9th codon of *ermCL*)(Ramu *et al.*, 2009). The brief stall at this location results in a conformation change of the secondary structures, forming one large hairpin loop that ultimately liberates the ribosomal binding site of the downstream resistance gene, *ermC,* which in turn allows for the translation of the resistance gene in the presence of the inducing antibiotic (Bailey *et al.*, 2008).

Ramu *et al.*, also suggested that this regulatory mechanism could be responsible for the induction of a variety of genes conferring resistance to protein synthesis inhibitors (Ramu *et al.*, 2009). The chloramphenicol resistance genes (*cml* and *cat*), for example, are preceded by shorts ORFs that also appear to result in ribosome stalling in the presence of the inducing agent (i.e. chloramphenicol)(Lovett *et al.*, 1996). Furthermore, Ramu *et al.*, identified a putative leader peptide in a number of tetracycline resistance genes (*tetL*, *tetM* and *tetQ*) and in the 5' UTR of the aminoglycoside resistance conferring methyltransferase *armA* (Ramu *et al.*, 2009).

3.3.4 The impact of insertion sequences on the expression of resistance genes

The following example of acquired resistance gene expression, like the previous example, depends on mobile genetic elements. Beyond the fact that elements such as insertion sequences and transposons greatly facilitate the dissemination of antibiotic resistance, sequence components they harbor may exert a delicate control over the expressional profile of adjacent resistance genes (Chandler *et al.*, 2002).

Insertion sequence elements are transposable genetic elements generally between 0.8 and 2.5 kb that only harbor genes required for their translocation (Bennett *et al.*, 2004). Currently, over 1,000 IS elements have been identified in Gram-positive and Gram-negative bacteria (Siguier *et al.*, 2014). Insertion sequences are another significant driving force of horizontal gene transfer and are typically located on the chromosome or within plasmids, although for intercellular translocation they must be encoded on conjugative elements (Depardieu *et al.*, 2007).

Generally, there are two IS-mediated effects on antibiotic resistance gene expression. For genes encoding specific resistance mechanisms such as enzymes for target modification, antibiotic modification or efflux pumps, insertion sequences have been found to activate these genes via promoter region alteration (Depardieu *et al.*, 2007). On the other hand, insertion sequences have been shown to affect resistance gene expression by insertional inactivation of genes that have been associated with the modulation of resistance determinants such as repressors (e.g. *ampD*, *mexR*, *acrR* etc.)(Mahillon *et al.*, 1998).

The activation of genes via promoter region alteration is an interesting concept that has been associated with a number of resistance genes (Toleman *et al.*, 2006; Depardieu *et al.*, 2007; Berçot *et al.*, 2010). Essentially, insertion sequences harboring a -35 element or both the -35 and -10 promoter regions may insert upstream of genes that have a weak, incomplete or inactive promoter region, and thereby activate their expression (Depardieu *et al.*, 2007). Typically, promoter regions harbored on such insertion sequences correspond to the canonical consensus sequence TTGACA and TATAAT for the -35 box and the -10 box respectively, that are separated by 17 base pairs (Lisser *et al.*, 1993). These insertion sequences may activate both intrinsic and acquired resistance determinants. In *B. fragilis* it was discovered that most antibiotic resistance genes are expressed by means of promoter elements that are entirely borne on insertion sequences (Podglajen *et al.*, 1995). More recent studies have identified such insertion sequence borne promoters to regulate the expression of acquired resistance

genes such as the 16S rRNA methyltransferase *rmtC* as well as the extended spectrum b-lactamase gene $bla_{\text{TLA-1}}$ (Karah *et al.*, 2016; Berçot *et al.*, 2010).

As was the case with the integron mediated promoter elements, active promoter elements harbored on insertion sequences have the potential to rapidly evolve the expressional profile of resistance genes. Especially resistance genes that act in a concentration dependent manner such as modifying enzymes can, over time, develop increased expressional levels proportional to resistance levels (Depardieu *et al.*, 2007). While such high-levels of expression may not always be beneficial to the host, when there is selective antibiotic pressure, the up regulation of certain resistance genes by integron- or insertion sequence- harbored promoters may confer a selective advantage to the cell.

4 OBJECTIVES AND JUSTIFICATION

4 OBJECTIVES AND JUSTIFICATION

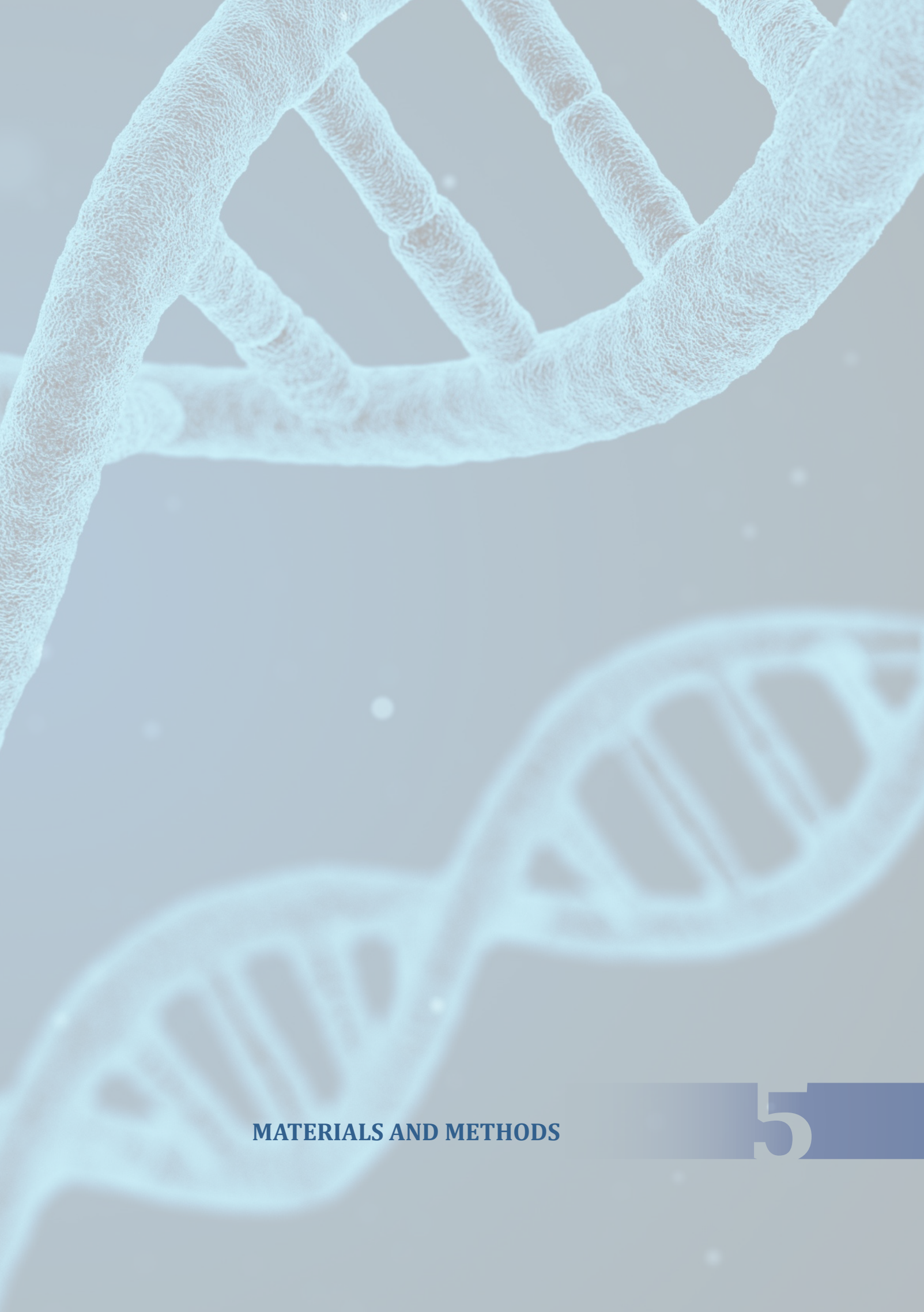
Currently antimicrobial resistance remains one of the most serious threats to modern medicine. As such, the selection and administration of antibiotics must be carefully considered, especially since the discovery of novel antimicrobial agents has reduced substantially. Agents that have previously been dismissed for unfavorable secondary activity are becoming increasingly valuable as the resistance prevalence towards the first choice antibiotic increases drastically. The aminoglycosides are a family of antibiotics that belong to this category. Shortly after their introduction to the market, problems with toxicity became apparent and they were no longer considered a drug of choice for physicians. However, increasingly worrisome resistance trends have forced physicians to reevaluate the application of aminoglycosides for life-threatening infections caused by Gram-negative pathogens.

However, among other resistance mechanisms, the 16S rRNA methyltransferases have emerged in Gram-negative pathogenic bacteria as an acquired resistance mechanism conferring high-level resistance to all clinically relevant aminoglycosides, even ones that have not yet been released to the market. Since their discovery in 2003, a total of 13 acquired methyltransferases have been identified including three variants. The rate at which these resistance determinant are spreading, combined with the broad resistance profile they confer to most clinically significant aminoglycosides, is jeopardizing the aminoglycosides as a viable last resort antibiotic.

As such the objectives of this study are the following:

1. Conduct a comprehensive *in silico* and *in vitro* study of the acquired 16S rRNA methyltransferases in relation with both house-keeping and intrinsic resistance conferrig methyltransferases to identify any regulatory mechanisms that may underlie the rapid dissemination and prevalence of these resistance determinants.

2. Evaluate any proposed regulatory hypotheses by conducting an in depth study of the most prevalent acquired 16S rRNA methyltransferase, *armA.*



5 MATERIALS AND METHODS

MATERIALS AND METHODS

5.1 In silico sequence management

A variety of programs were utilized to analyze sequence elements. CLC Workbench® (Qiagen Bioinformatics, USA) was used to perform a number of sequence analyses including the alignment of all reference sequences, the evaluation of sequence elements such as upstream ORFs and secondary structures as well as dendrograms to investigate the degree of relatedness among acquired and intrinsic aminoglycoside resistance methyltransferases. Dendrograms were typically performed with at least 1,000 replicates for the boot-strap values, and up to 10,000. Serial Cloner® (Serial Basics, France) was used to perform sequence annotations and to verify sequences obtained via Sanger Sequencing, which were compiled using the 4Peaks® software suite (Nucelobytes, Netherlands). Online sequence analysis tools such as β-prom (Softberry, USA) promoter prediction algorithms were used to identify upstream promoter elements.

5.2 Strains utilized

Unless otherwise stated, all the experiments in this project were carried out using the laboratory strain *E. coli* DH5α with the chromosomal genotype *fhuA2, lac(Δ)U169, phoA, glnV44, Φ80', lacZ(Δ)M15, gyrA96, recA1, relA1, endA1, thi-1* and *hsdR17* (Life Technologies, USA). Besides usually exhibiting a high transformation efficiency, this *E. coli* strain also harbors the alpha acceptor allele Δ*(lacZ)*M15 which is required for the blue/white screening of many *lacZ* based vectors. Unless otherwise stated, this strain was transformed via electroporation under the conditions described for small plasmids: 2.5 kV/cm, 25 μ F and 200 Ω (San Millan *et al.*, 2010).

5.3 Genetic constructions

The *armA* used to make all of the following constructions was amplified via High-Fidelity PCR from an *E. coli* strain bearing pMUR050 (Genbank accession number: AY522431.1). All constructions performed in this project were subcloned onto either the linearized pTOPO TA cloning vector with a pUC origin of replication derived from pBR322 (Thermo Fisher, USA) via amplification with *Taq* polymerase (Biotools, USA) using its un-templated adenine 3' overhang or, if the constructions were intended to be co-transformed with ColE1-like plasmids, constructions were cloned directly onto the cloning vector pACYC-184, derived from the non-ColE1 miniplasmid origin of replication p15A1 (Mo Bi Tech, Germany) via PCR amplification using oligonucleotides (Sigma-Aldrich, Spain) bearing restriction sites. Primers designed to be used with the In-Fusion HD cloning kit (Clontech, USA) were designed according to the manufacturers specifications. A full list of the oligonucleotides used to make the constructions used in this work can be found in table 2.

Table 2: Oligonucleotides used as part of this study, their sequence and reference

	Name	5'➔3'	Reference
Vector Primers	M13.F	GTAAAACGACGGCCAG	Invitrogen
	M13.R	CAGGAAACAGCTATGAC	Invitrogen
	pACYC.F	GAAATCGTCGTGGTATTCAC	This work
	pACYC.R	AACCAGACCGTTCAGCTGGA	This work
RACE	SMARTer RACE gsp1	CTCTTCCATTCCCTTCTCCTTTCCAGA	This work
	SMARTer RACE gsp1 + 15nt Vector Overlap	GATTACGCCAAGCTTCTCTTCCATTCCCTTCTCCTTTCCA	This work
lacZα Fusion	*armA:lacZα*.F + NdeI Site	TCGACCATATGTCGCCAGCCAGTGAATTGAG	This work
	armA:lacZα.R + AflII Site	TCGACTCTTAAGATTCTTATCCATTTGAAAGA	This work
Truncation Primers	*armA*(400nt UTR) + EcoR1 Site	AGCGTGGAATTCGAAATCCTGGCTGGCGTGAA	This work
	armA(195nt UTR) + EcoR1 Site	AGCGTGAATTCGAATCAATCAGGGGTGACGGCAG	This work
	armA(139nt UTR) + EcoR1 Site	AGCGTGAATTCCCGGAAAATTCTGATTGGAT	This work
	armA(116nt UTR) + EcoR1 Site	AGCGTGAATTCTTGTTCGGGGTGCGAAAACA	This work
	armA(35nt UTR) + EcoR1 Site	AGCGTGAATTCTTCCTATCAAATACTTAACT	This work
	armA.R + EcoR1 Site	AGCGTGAATTCATCCTCTATGTTGTCCAAGG	This work
3xFlag Tag	*armA*(400nt UTR) + BamH1 Site	TCGACGGATCCGAAATCCTGGCTGGCGTGAA	This work
	armA(Δ Stop Codon).R + BamH1 Site	GCATTGGATCCTTTCTGAAATCCACTAGTAA	This work
RT-qPCR	*armA*(RT-PCR).F	AGACGACGATAAAGTATAG	This work
	armA(RT-PCR).R	GTAATTCTCTTCCATTCC	This work
	dnaK(RT-PCR).F	AAACACATGAACATCAAA	This work
	dnaK(RT-PCR).R	TACCAAAGAACTCAGCAA	This work
	uidA(RT-PCR).F	GTCAATAATCAGGAAGTG	This work
	uidA(RT-PCR).R	AAAGAAATCATGGAAGTAA	This work

Table 3. Oligonucleotides designed as part of the -35 promoter region mutagenesis

		Name	5'➔3'	Reference
In Fusion HD Promoter Region Mutagenesis	-35 Nucleotide Substitutions	-35 tt ➔ gg position 2.F	GCGAATCAGGGGGGGACGGCAGAAATG	This work
		-35 aa ➔ cc position 2.R	ATTTCTGCCGTCCCCCCTGATTCGCG	This work
		-35 ga ➔ gg position 3.F	AGGGGTTGGCGGCAGAAATGGA	This work
		-35 ct ➔ cc position 3.R	TCTGCCGCCAACCCCTGATTCG	This work
		-35 cg ➔ gg position 4.F	GGGTTGAGGGCAGAAATGGACT	This work
		-35 gc ➔ cc position 4.R	TTTCTGCCCTCAACCCCTGATT	This work
		-35 gc ➔ gg position 5.F	GTTGACGGGAGAAATGGACTGG	This work
		-35 cg ➔ cc position 5.R	CATTTCTCCCGTCAACCCCTGA	This work
		-35 gg ➔ aa position 1.F	AATCAGGAATTGACGGCAGAAA	This work
		-35 cc ➔ tt position 1.R	CCGTCAATTCCTGATTCGCGTT	This work
		-35 tt ➔ aa position 2.F	TCAGGGGAAGACGGCAGAAATG	This work
		-35 aa ➔ tt position 2.R	TGCCGTCTTCCCCTGATTCGCG	This work
		-35 ga ➔ aa position 3.F	AGGGGTTAACGGCAGAAATGGA	This work
		-35 ct ➔ tt position 3.R	TCTGCCGTTAACCCCTGATTCG	This work
		-35 cg ➔ aa position 4.F	GGGTTGAAAGCAGAAATGGACT	This work
		-35 gc ➔ tt position 4.R	TTTCTGCTTTCAACCCCTGATT	This work
		-35 gc ➔ aa position 5.F	GTTGACGAAAGAAATGGACTGG	This work
		-35 cg ➔ tt position 5.R	CATTTCTTTCGTCAACCCCTGA	This work

Table 4. Oligonucleotides designed as part of the -10 promoter region mutagenesis

		Name	5'➔3'	Reference
In Fusion HD Promoter Region Mutagenesis	-35 Nucleotide Substitutions	-35 tt ➔ gg position 2.F	GCGAATCAGGGGGGGACGGCAGAAATG	This work
		-35 aa ➔ cc position 2.R	ATTTCTGCCGTCCCCCCTGATTCGCG	This work
		-35 ga ➔ gg position 3.F	AGGGGTTGGCGGCAGAAATGGA	This work
		-35 ct ➔ cc position 3.R	TCTGCCGCCAACCCCTGATTCG	This work
		-35 cg ➔ gg position 4.F	GGGTTGAGGGCAGAAATGGACT	This work
		-35 gc ➔ cc position 4.R	TTTCTGCCCTCAACCCCTGATT	This work
		-35 gc ➔ gg position 5.F	GTTGACGGGAGAAATGGACTGG	This work
		-35 cg ➔ cc position 5.R	CATTTCTCCCGTCAACCCCTGA	This work
		-35 gg ➔ aa position 1.F	AATCAGGAATTGACGGCAGAAA	This work
		-35 cc ➔ tt position 1.R	CCGTCAATTCCTGATTCGCGTT	This work
		-35 tt ➔ aa position 2.F	TCAGGGGAAGACGGCAGAAATG	This work
		-35 aa ➔ tt position 2.R	TGCCGTCTTCCCCTGATTCGCG	This work
		-35 ga ➔ aa position 3.F	AGGGGTTAACGGCAGAAATGGA	This work
		-35 ct ➔ tt position 3.R	TCTGCCGTTAACCCCTGATTCG	This work
		-35 cg ➔ aa position 4.F	GGGTTGAAAGCAGAAATGGACT	This work
		-35 gc ➔ tt position 4.R	TTTCTGCTTTCAACCCCTGATT	This work
		-35 gc ➔ aa position 5.F	GTTGACGAAAGAAATGGACTGG	This work
		-35 cg ➔ tt position 5.R	CATTTCTTTCGTCAACCCCTGA	This work

All constructions were cloned onto the pACYC vector in the forward orientation (with regards to the origin of replication) in the RT-PCR (reverse transcriptase polymerase chain reaction) confirmed absence of an active upstream promoter. Prior to sequencing, all constructions were carefully examined via PCR to confirm insert orientation and size. Constructions made using the In-Fusion HD cloning kit (Clontech, USA) were confirmed via outward PCRs. All constructions were analyzed via Sanger Sequencing techniques using flanking primers in both orientations.

Initial truncations were cloned onto the pTOPO-cloning vector using the *Taq* polymerase as described above. Further truncations were later performed using the pACYC-cloning vector. The various inserts were amplified using the high fidelity Phusion polymerase (Thermo Fisher, USA), and were then purified and digested using the EcoR1 restriction enzyme (Thermo Fisher, USA) as indicated by the manufacturer. Purified pACYC was also digested using the EcoR1 enzyme and subsequently dephosphorylated using calf intestinal alkaline phosphatase (CiP) (New England Biolabs, USA) to avoid self-ligation in the following steps. The insert and vector DNA concentrations were then obtained via electrophoresis or Nano Drop (Thermo Fisher, USA). After further purifications, the ligation reactions were designed to achieve a 12:1 insert to vector ratio and incubated overnight at 16°C with the T4 DNA ligase (New England Biolabs, USA). Following ligation, all constructions were electroporated into *E. coli* DH5α as described above.

5.3.1 lacZα reporter constructions

lacZα reporter constructions were performed as described by Alexander Mankin's group (Bailey *et al.*, 2008) who provided the pERMCα vector used to investigate the induction of the 23S rRNA erythromycin resistance methyltransferase *ermC*. The initial full length *armA* 5' UTR (400nt) including the first 4 codons of the *armA* gene were fused to codons 6-60 of the *lacZα* reporter gene using the NdeI (New England Bioloabs,

USA) and AflII (Takara, USA) restrictions sites of the reporter construct pERMCα. The construction was then subcloned onto the pTOPO-TA cloning vector prior to cloning onto the pACYC-184 vector via digestion enzymes.

5.3.2 Western Blot constructions

Constructions to be used for Western Blot protein quantification were performed using the commercial 3xFlagTag-cloning vector (Sigma-Aldrich, USA) as described by the manufacturer. Using the BamH1 restriction enzyme (New England Biolabs, USA), *armA* was cloned into the multiple cloning site, in frame with the 3xFlagTag, which consists of 3 tandem hydrophilic flag epitopes, 22 amino acids in length. The insert bearing the 3xFlagTag and the transcriptional terminator following the multiple cloning site was then amplified using oligonucleotides bearing the EcoR1 restriction site and later cloned onto the pACYC vector as all other constructions.

5.3.3 Promoter region mutagenesis

Finally, all site-specific mutations of the -35 and -10 promoter regions were performed using the In-Fusion HD cloning kit (Clontech, USA). Using the *armA(wt):Ft* constructions as a template, the plasmid was then linearized with an inverse PCR. As described by the manufacturer, primers were designed that include a 15bp overlap with each other at the 5' end. The desired nucleotide substitutions were located within this 15bp overlapping region. After a 15-minute incubation at 50°C, the constructions were then electroporated into DH5 α. After a 1-2 hour phenotypic expression shaking (100rpm) at 37°C, the transformants were selected using the plasmid mediated resistance to tetracycline. As always, the mutations were carefully confirmed via Sanger sequencing with both flanking primers.

5.4 Antimicrobial susceptibility testing

the minimal inhibitory concentration (MIC) for each of the constructions bearing *armA* was determined in at least 3 independent replicates and according to both the CLSI and EUCAST guidelines (CLSI, 2017; EUCAST, 2017). Both broth micro-dilutions in 96-well plates and disk diffusion testing were performed in Mueller-Hinton media (Oxoid, UK) with all appropriate constructions. The final values obtained in mg/L are the compiled average of the independent replicates. Additionally, antibiograms and MICs performed in the presence of sub-inhibitory concentrations of aminoglycosides were performed using 50% of the MIC as previously described (Zhanel *et al.*, 1992).

5.5 Bacterial growth conditions

All growth curves performed over the course of this project were performed in various volumes of Lysogeny Broth (LB) media (Conda Laboratories, Spain) depending on the subsequent application of the sample. Before any growth curve, the stability of the cloning vector was assessed over a period of 48 hours. The most frequently used vector pACYC-184 was shown to be 100% stable in the absence of antibiotic pressure. Bacterial growth was always performed at 37°C while shaking at 100rpm and assessed via spectrophotometry at λ600 using a Hitachi U-1900 spectrophotometer (Hitachi, Japan). Samples were diluted appropriately when the OD_{600} reached levels above 0.4-0.5. Measurements were taken, either every hour or in order to obtain the samples at a predetermined optical density (e.g. OD_{600} 0.2 & 0.4). Furthermore, all inoculations were performed from an overnight culture at an inoculation ratio of 1:100.

5.6 RNA extractions and reverse transcription

a number of the techniques utilized in the process require cDNA generated from RNA extractions. Prior to any RNA extractions performed, multiple growth curves were conducted to determine the precise colony forming units (CFU) at each time point. This

was used to calculate and standardize the recommended number of cells for the RNA extractions ($6x10^7$) at each time point. Both the RNA extraction and the reverse transcription were performed exactly as outlined by the manufacturer. RNA extractions were performed using Qiagen's RNeasy extraction kit (Qiagen, USA). As RNases are extremely abundant and stable in the environment, the working area, including pipettes, tip boxes and eppendorf racks were cleaned with RNase Zap (Thermo Fisher, USA) prior and during RNA extractions. Furthermore, this procedure was conducted with RNase free tips and eppendorfs to avoid RNase contamination. After every extraction performed, it is of upmost importance to perform a conventional PCR of the RNA to confirm the absence of any residual DNA. We performed this PCR using internal *armA* primers (Table 2). If this PCR indicated the presence of undigested DNA, the RNA was loaded back onto the micro-centrifuge column and treated with further courses of DNase treatments.

The RNA was then run on a standard electrophoresis gel to determine the quality of the RNA extraction based on ribosomal RNA degradation. After confirming the absence of residual DNA via PCR, the concentration of the RNA was determined using a Nanodrop. These concentrations were determined to generate cDNA from the same amount of RNA for each of the samples, especially if the samples were to be used for qPCR analysis. The reactions were then setup using the Superscript III reverse transcriptase (Invitrogen, USA) carefully following the instructions provided.

5.7 Rapid amplification of cDNA ends (RACE)

The rapid amplification of cDNA ends is an elegant approach to determine the full transcript lengths of any target gene. The SMARTer RACE kit (Clontech, USA) achieves this by extracting the total RNA from the samples followed by the joint action of the SMARTer II A universal oligonucleotide and the SMARTScribe reverse transcriptase. When the transcriptase acts on the 5' end of the RNA, its terminal activity adds a few

additional nucleotides to the 3' end of the first cDNA strand. The SMARTer II A Oligonucleotide contains a terminal stretch of modified bases that anneal to the extended cDNA tail, allowing the oligo to serve as a template for reverse transcription. SMARTScribe reverse transcription switches templates from the mRNA molecule to the SMARTer oligo, generating a complete cDNA copy of the original RNA with the additional SMARTer sequence at the end (Figure 18).

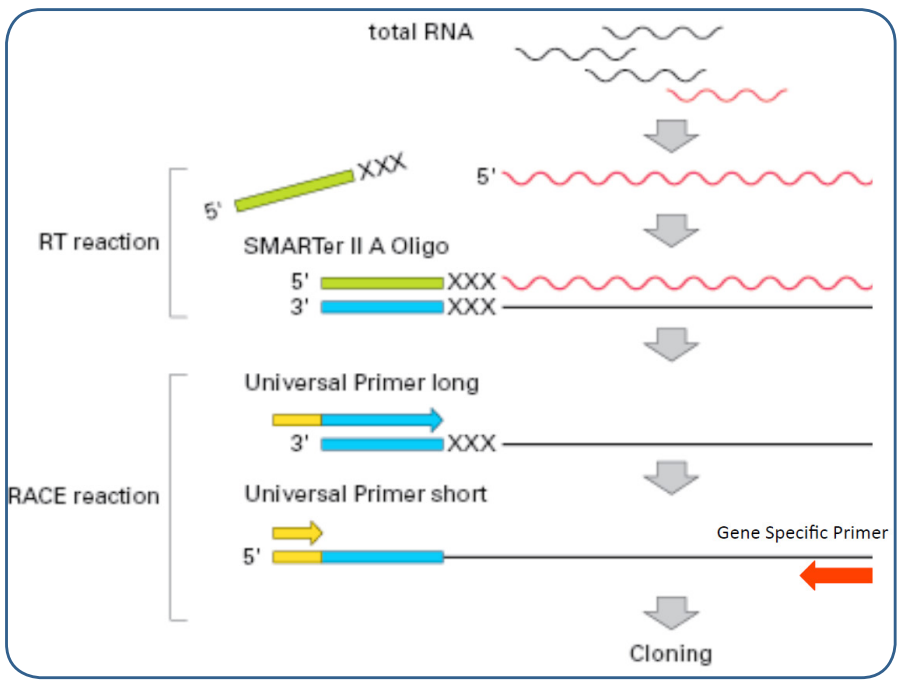


Figure 18. A representation of the SMARTer RACE activity with the SMARTer II A oligonucleotide tail (green) added to the total RNA prior to reverse transcription and the universal primer (yellow/blue) complementary to the previously added tail that can be used to amplify the 5' UTR via conventional PCR using the gene specific primer (red) (Figure adapted from SMARTer RACE manual, Clontech, USA).

Subsequently, the generated cDNA can be used directly in a standard PCR with gene-specific primers generating an amplicon from within the gene until the 5' end of the transcript. This procedure was carried out exactly as specified by the manufacturers applying all available controls at all times.

5.8 lacZα Screening

The *lacZα* blue/white screening was performed as outlined by Bailey *et al* (Bailey *et al.* 2008) utilizing either LB or MH plates as we found that BHI plates seem to completely inhibit the alpha-complementation of *lacZ*α. As our vector did not have an IPTG inducible promoter, our media did not require IPTG. Furthermore, rather than inoculating low percentage agar with the bacteria and applying this to media containing the x-gal (Sigma-Aldrich, USA) substrate, we chose to plate the bacteria directly on plates containing 40µg/ml of x-gal after performing a 0.5 McFarland from a fresh overnight inoculum. The effects of aminoglycosides on the expression of the *lacZ*α reporter gene were assessed using a range of gentamicin concentrations applied either via disks or within the plate itself. After plating, the strains were incubated at 37°C between 18 and 20 hours, after which the plates were incubated at 4°C for approximately 4 hours before taking the result of the screening. It is very important to standardize this screening method, based on the typical growth time of the bacteria, in our case *E. coli*.

5.9 Western Blot

Western Blots were carried out with an in-house protocol and self-made buffers compiled from literature (Biorad, USA). Please find the full protocol and buffer constitutions in the supplementary information. As previously described, all bacterial growth to be used for Western Blots was initiated with an inoculation ratio of 1:100 in LB from a fresh overnight culture. 1ml of the sample was then taken every hour or at the desired optical density, and pelleted by centrifugation at 13,400 rpm for 5 minutes. The pellet was then carefully air dried and suspended in 2-fold Laemli buffer containing fresh β-mercaptoethanol (Bio-Rad, USA) at a concentration of 1µl Laemli Buffer/0.01 Absorbance Units to standardize the cell count at various optical densities. After re-suspending, the samples were immediately frozen at -20°C for at least 24 hours.

Subsequently, the samples were de-thawed prior to denaturing the protein at 100°C for 5 minutes. Additionally, we prepared our own 50:50 mix of Magic Marker XP (Thermo Fisher, USA) and Benchmark (Thermo Fisher) Western Blot weight markers, as one can be seen on the membrane but not in the final image obtained, whereas the other reacts with the ECL reagents to be seen in the final image but not on the membrane. Unless otherwise specified, 2.5 μ l of the sample were then loaded onto a 4-20% Tris-Glycine gel (Invitrogen, USA) and run for 50-60 minutes at 200V. Once the electrophoresis was completed, we set up the Western Blot transfer to the nitrocellulose membrane (Bio-Rad, USA) overnight at 30V and 4°C.

The following day, the membranes were blocked by submerging the membranes in TBS with 1% Tween 20 (Panreac, USA) and 10% milk (Nestle, Switzerland) for 2 hours at room temperature while shaking at 100 rpm. Following 3-5 washes in fresh TBS-T the membranes were then exposed to the primary antibodies for 2.5 hours (shaking at 100rpm and room temperature), in this case anti-FlagTag (Sigma-Aldrich, USA) and the control anti-DnaK (Enzo, Spain) at concentrations of 1:500 and 1:2000 respectively in TBS-T as specified by the manufacturers.

The nitrocellulose membrane was simultaneously exposed to both primary antibodies in stomacher bags (Seward, UK) that were sealed around the membrane to achieve the highest possible exposure with relatively low volumes. Typically the antibodies were dissolved into 5-10mL of TBS-T/membrane, depending on the size of the membrane. After another 3-5 washes in TBS-T, the membranes were finally exposed to the secondary goat anti-mouse IgG horseradish peroxidase (HRP) conjugate (Life Technologies, USA) at a concentration of 1:5000 in TBS-T, as recommended by the manufacturer, for 1.5 hours (also shaking at 100rpm and room temperature), similarly to the primary antibodies although typically in about 10ml/membrane. The membranes were then thoroughly washed in TBS-T before rinsing them in distilled water prior to ECL reagent (Thermo Fisher, USA) exposure for approximately 5 minutes.

Using the Image Lab (Bio-Rad, USA) Western Blot exposure and quantification software, the membranes were then exposed to UV light for a total of 5 minutes while taking pictures of the membranes every 10 seconds. Quantifications of the signal strength/ protein quantity were then performed using the same Image Lab software suite. Unless otherwise stated, Western Blot images used were after a 60 second UV exposure, after which the software was then used to automatically identify lanes and bands. Minor adjustments were performed to ensure that the lanes captured the full signal strength of each image. Finally, using the DnaK signal as a reference (e.g. 1) the relative signal strength of ArmA could then be quantified. Most Western Blots were conducted and quantified at least 5 times. The results of this technique are presented as the average of all replicates.

5.10 RT-qPCR

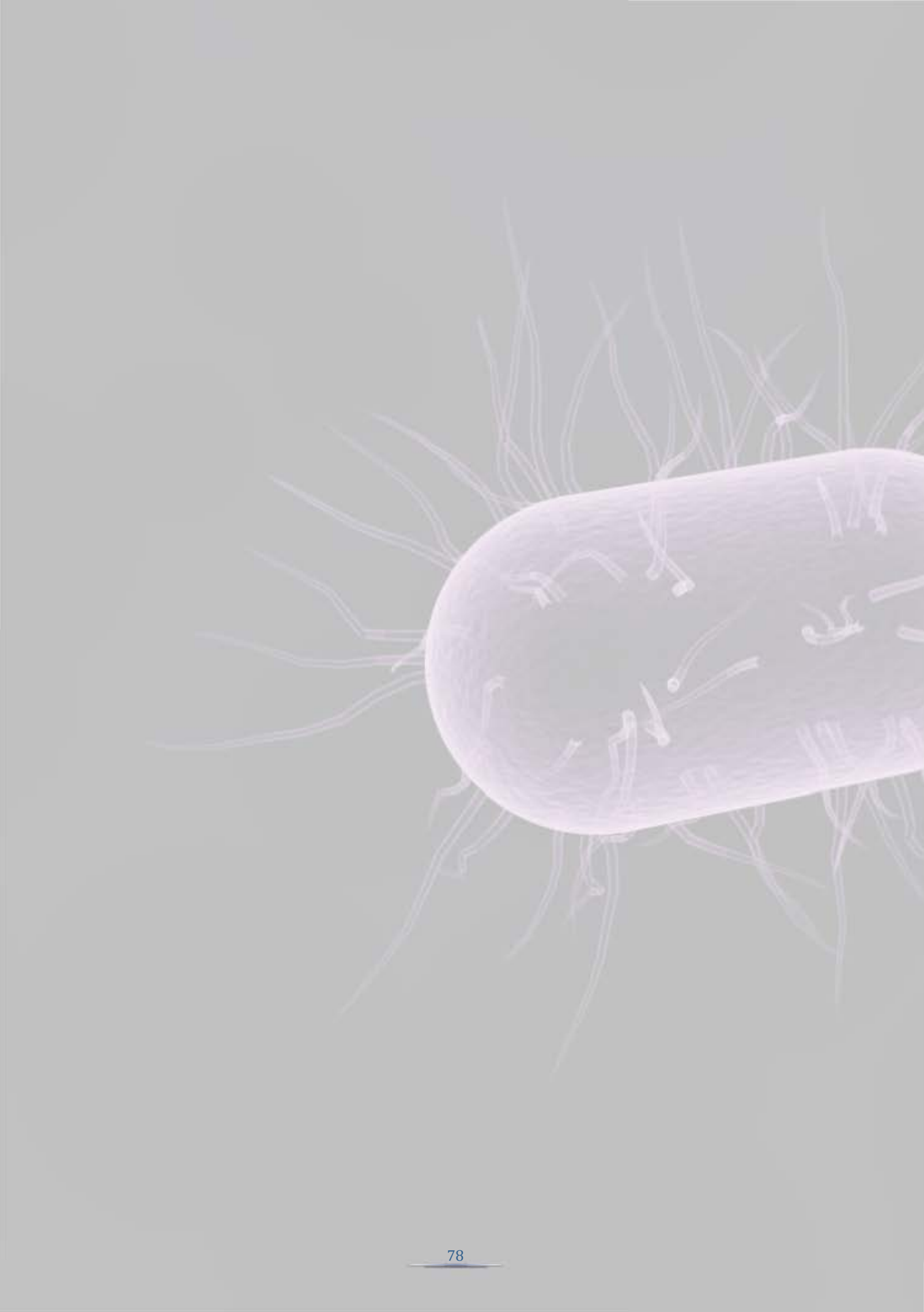
To investigate the growth phase dependent expression of *armA,* we performed RT-qPCRs of samples taken every hour over a time span of 16 hours. As the RNA extractions should be performed with approximately $6x10^7$ cells, prior to the actual growth curve a number of growth curves were conducted with the samples to determine the amount of colony forming units (CFU) at each time point and optical density. Using this we extracted the volume required to acquire $6x10^7$ cells at each time point. Although the RT-PCRs were conducted with a monocopy reference gene (*uidA*), extracting the RNA from the same number of cells at each time makes the results much more reliable. In order to take the plasmid copy number into account, the curves were simultaneously performed in duplicate, extracting the RNA from one and the DNA from the other. Additionally, at each time point 1mL aliquots were taken for simultaneous Western Blot analysis. At each time point, the appropriate volume was pelleted down via centrifugation for 5 minutes at 6,000 rpm and then stored at -80°C until the RNA and DNA extractions were performed as outline above.

The qPCRs were performed in quadruplicate with a My iQ Single Color Real-Time PCR Detection System (Bio-Rad laboratories), using the iQ SYBR Green Supermix (Bio-Rad Laboratories). qPCR conditions were calibrated to obtain the optimal amplification conditions for each gene (oligonucleotides utilized can be found in table 2). This includes determining the optimal primer concentrations, temperature and threshold for a high reaction efficiency (Table 5).

Table 5. RT-qPCR conditions utilized (oligonucleotides presented in table 2)

	Concentration	Temperature	Amplicon Size
armA.F	4µM	48.5°C	212nt
armA.R	4µM		
uidA.F	4µM	51.3°C	201nt
uidA.R	4µM		

It is important to point out that qPCRs of plasmid borne genes have been shown to require prior restriction enzyme digestion (Providenti *et al.*, 2006), which was performed using the PstL restriction enzyme (TaKaRa, USA) as directed by the manufacturer. As briefly mentioned above, the qPCR was conducted using the monocopy chromosomal control gene, *uidA*. All the primers designed generated an amplicon of 200nt. All conditions were carefully calibrated to achieve the optimal amplification of each of these genes prior to the first RT-qPCR. Additionally, we performed an efficiency control in each qPCR to be performed, using a five-fold dilution series of an independent PstI digested DNA extraction of the sample. Finally, RT-qPCRs with an efficiency lower than 97% (or over 105%) – as determined by the internal control dilutions - and an r^2 lower than .99 were discarded and repeated.



6 RESULTS

RESULTS

6.1 *In silico* analysis of the 16S rRNA methyltransferases and their 5' upstream regions

6.1.1 Coding region conservation of the 16S rRNA methyltransferases

6.1.1.1 CDS conservation of the acquired aminoglycoside resistance conferring methyltransferases

Our investigation of the regulatory mechanisms underlying the expression of the acquired resistance conferring 16S rRNA methyltransferases started with a comprehensive *in silico* analysis. Initially, amino acid sequence alignments were performed to assess the degree of relatedness among the acquired 16S rRNA methyltransferases (Figure 19).

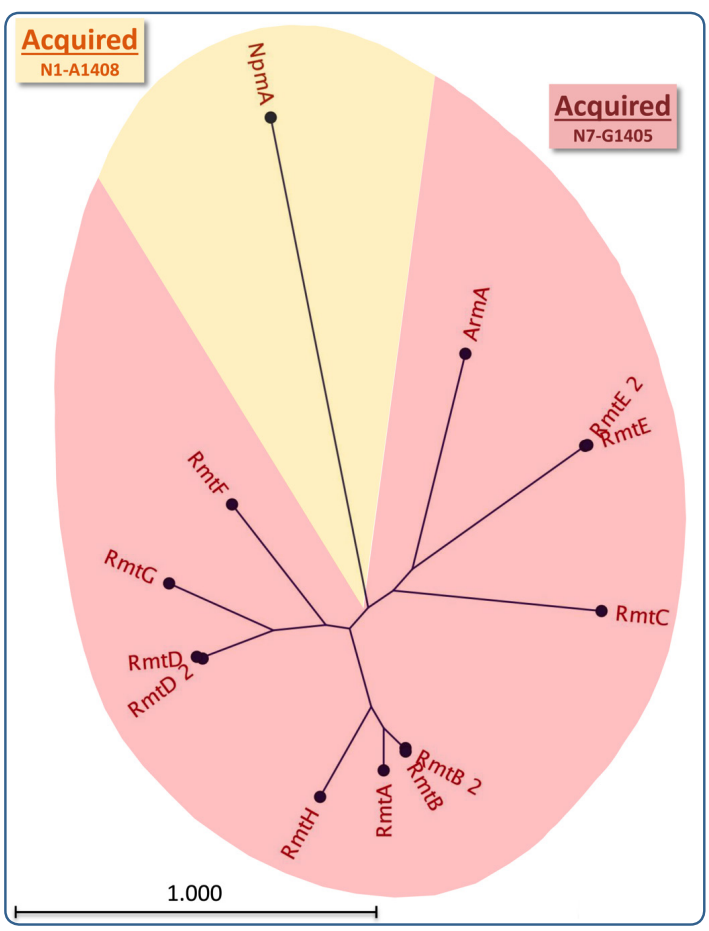


Figure 19. Radial dendrogram of the acquired G1405 and A1408 methyltransferases. Bootstrap analysis conducted with 10,000 replicates.

Amino acid sequence based dendrograms demonstrate high degrees of conservation among methyltransferase variants such as RmtD:RmtD 2 and RmtE:RmtE 2, which is not surprising as, for the 16S rRNA methyltransferases, a single amino acid change is sufficient for the gene to be classified as a new methyltransferase variant. RmtA and RmtB exhibit the highest degree of relatedness among the other unique methyltransferases (81% amino acid identity). RmtH and RmtB (and consequently RmtA) also share a significant amino acid identity (64% and 63% amino acid identity, respectively). Finally, RmtG and RmtD 2 share an amino acid identity of 59% and 57% with RmtD, respectively. While there appear to be three clusters of the acquired 16S rRNA methyltransferases (i. RmtA, RmtB, RmtB 2 RmtH; ii. RmtE, RmtE 2; iii. RmtD, RmtD 2, RmtG) the other acquired methyltransferases do not appear to exhibit a high degree of conservation. Generally, this suggests that the acquired 16S rRNA methyltransferases originated from distinct ancestral origins via convergent evolution. The only A1408 methyltransferase, NpmA, is made up of a unique amino acid sequence that is unlike any of the G1405 methyltransferases.

6.1.1.2 Acquired vs. intrinsic aminoglycoside resistance conferring methyltransferases CDS conservation

To further investigate any possible relationships between the acquired and intrinsic resistance conferring methyltransferases, which have been described as a putative origin of several acquired methyltransferases (Liou *et al.*, 2006), we constructed a further dendrogram including both groups of methyltransferases, some of which have been listed in table 1 of the introduction (Figure 20).

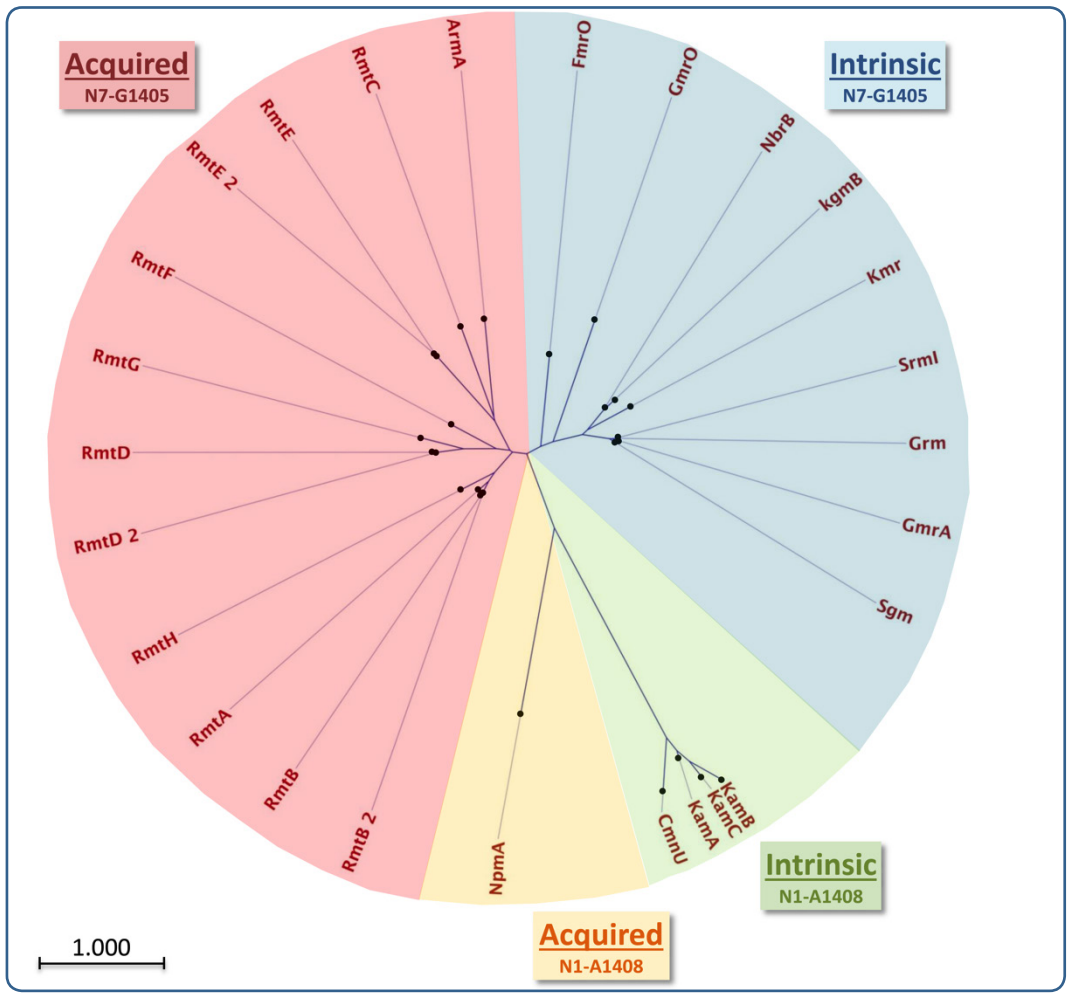


Figure 20. Radial dendrogram of the acquired resistance methyltransferases belonging to the acquired G1405 methylating Arm (red) and A1408 methylated Pam (yellow) family of methyltransferases contrasted with intrinsic resistance conferring methyltransferases belonging to the G1405 methylating Kgm (blue) and A1408 methylating Kam (green) family of methyltransferases. Bootstrap analysis conducted with 10,000 replicates.

Interestingly, the intrinsic methyltransferases do not exhibit a significant degree of relatedness to any of the acquired G1405 methylating enzymes. In fact, the methylases included within this dendrogram appear to segregate based on their origin (e.g. acquired vs. intrinsic) and function (e.g. G1405 or A1408 methylases). As such, NpmA and the methyltransferases belonging to Kam family differ significantly from the G1405 methylating enzymes.

6.1.1.3 The conservation between the resistance conferring and house-keeping 16S rRNA methyltransferases

We then decided to increase the scope of this comparative study to include non-resistance-conferring, intrinsic methyltransferases (or house-keeping methyltransferases) to identify a possible origin of the acquired methyltransferases (Figure 21).

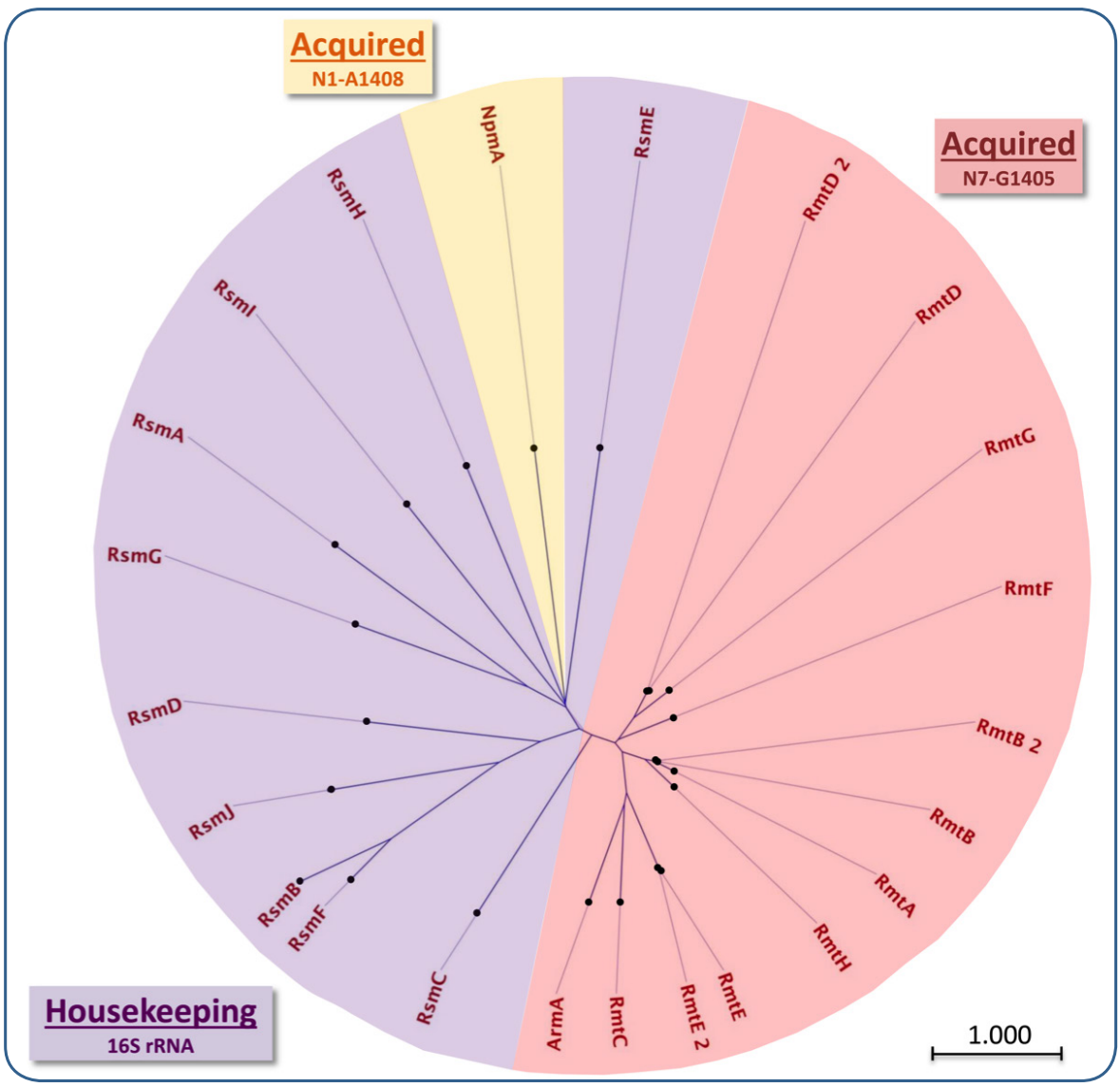


Figure 21. Radial dendrogram presenting the acquired G1405 methylating Arm family (red), the acquired A1408 methylating Pam family (yellow) with the ribosomal small subunit methyltransferases of the Rsm family (pink). Bootstrap analysis conducted with 10,000 replicates.

Based on the amino acid alignments performed, the acquired 16S rRNA methyltransferases do not share a very high degree of conservation with 16S rRNA methyl-

transferases from the Rsm family of methyltransferases. That being said, some housekeeping methyltransferases (e.g. RsmC) appear to be more closely related to acquired methyltransferases (such as ArmA) than to other members of the Rsm family. RsmC, which methylates position N2 of G1207, shares a 71.31% amino acid identity with the acquired methyltransferase ArmA. As previously observed, these methyltransferases also form clusters based on their target residue and origin as is to be expected. Interestingly NpmA appears to be more similar to members of the Rsm family of housekeeping methyltransferases than with the Arm family.

Finally, we constructed a last dendrogram, presenting all the here analyzed methyltransferases, again to ascertain any possible trends that could indicate a possible origin of the methyltransferases (Figure 22).

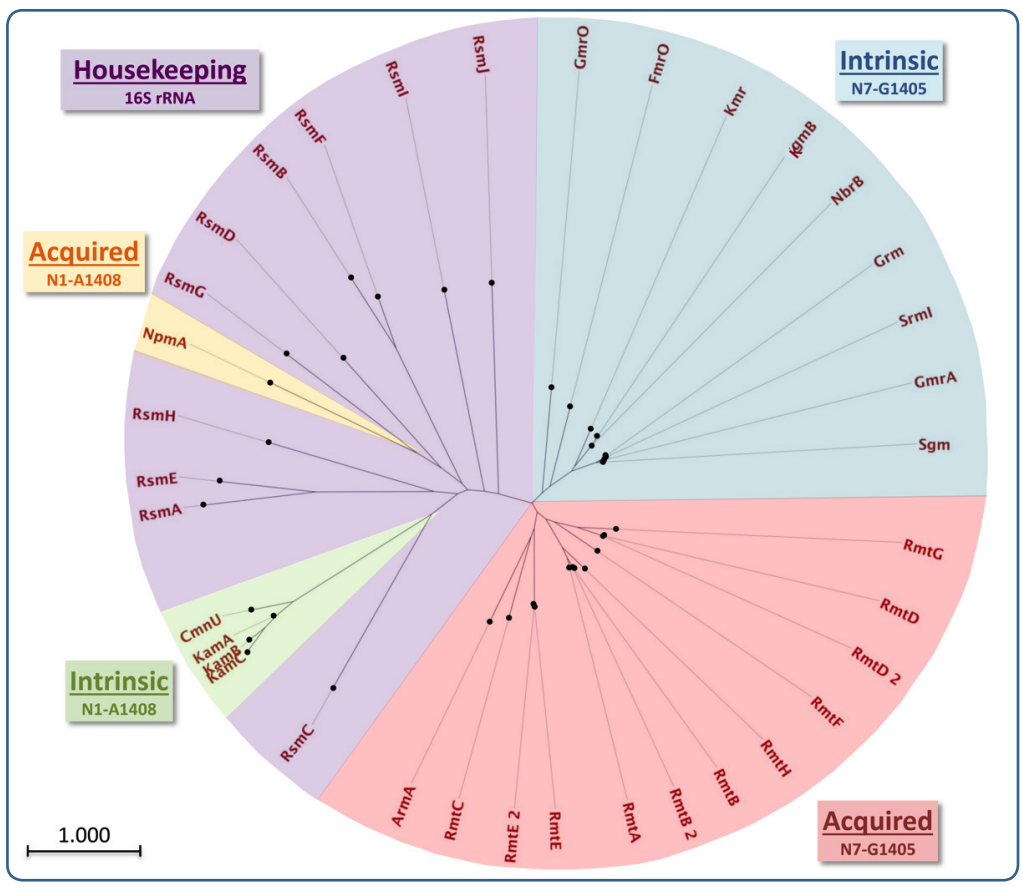


Figure 22. Further dendrogram presenting all previously analyzed methyltransferase groups belonging to the acquired resistance methyltransferases (Arm and Pam), the intrinsic resistance conferring methyltransferases (Kgm and Kam) and finally the intrinsic 16S rRNA housekeeping methyltransferases (Rsm). Bootstrap analysis conducted with 10,000 replicates.

While the above presented figure indicates that the Arm family of methyltransferases is more closely related to the Rsm family than to the Kgm family, the degrees of similarity are unfortunately too low to identify a conclusive origin. Generally amino acid conservations range between conserved areas and non-conserved areas. As such, the conclusion again, is that based on their amino acid sequences, the methyltransferases appear to have several ancestral distinct origins.

6.1.2 The plasticity of the 16S rRNA methyltransferase 5' upstream regions

As literature has demonstrated the significance of the 5' untranslated region in gene regulation (Breidt *et al*., 1990; Bailey *et al*., 2008; He *et al*., 2013), comprehensive evaluations of the 5' upstream regions of the acquired and intrinsic resistance confirming methyltransferases in relation to their downstream coding regions were performed.

6.1.2.1 Acquired 16S rRNA methyltransferase 5' upstream region conservation

Before presenting the results of the 5' upstream region alignments, it should be pointed out that, for a number of genes, it was difficult or impossible to find sequences alongside an extended upstream region. *rmtB 2*, for example, only has two sequences uploaded on Genbank, neither of which contains an untranslated region. As such, *rmtB 2* has been excluded from the remainder of this study. For the acquired methyltransferases, full-length upstream region were generally available, however, the chromosomally encoded intrinsic methyltransferases rarely contained sequences of the genetic environment. We found that these methylases had far fewer annotated sequences uploaded to Genbank. Based on these limitations, all 5' upstream region alignments were performed with a region of 400nt upstream from the coding region. Furthermore, as previously shown, the genetic environment of the 16S rRNA methyltransferases is highly variable. As such, choosing to analyze 400 nt allows us to capture an upstream region

that is most conserved alongside the various genes. Sequences used were assessed to be the most representative upstream regions of the respective methyltransferases.

In the previous section we aligned the amino acid sequences of the various 16S methyltransferases. We then analyzed the nucleic acid sequence conservation of the 5' upstream regions (Table 6).

Table 6. Nucleotide conservation of 400 nt upstream of the acquired methyltransferase coding regions. Conservations of more than 50% have been marked bold.

armA	rmtA	rmtB	rmtC	rmt D	rmtD 2	rmtE	rmtE 2	rmtF	rmtG	rmtH	npmA	
	25%	28.25%	30%	28.75%	29%	**73.25%**	23.18%	22.25%	31.5%	26.25%	26%	armA
		22.25%	19.5%	26.75%	27%	23.25%	21.89%	26.75%	27.5%	23.75%	30%	rmtA
			25.25%	25.5%	21.5%	31%	20.6%	18%	27.25%	28.75	27.5%	rmtB
				27.75	29.75%	31.75%	24.46%	15.5%	26.25%	26%	15.25%	rmtC
					97%	22%	22.1%	27.25%	**63.75%**	24.25%	29.25%	rmtD
						20.25%	22.1%	26.25%	**64%**	24.5%	34%	rmtD 2
							32.19%	20.25	24.25%	30%	21.75%	rmtE
								25.11%	33.69%	19.53%	24.25%	rmtE 2
									29.5%	26.5%	26.25%	rmtF
										21.25%	25%	rmtG
											25.25%	rmtH
												npmA

Interestingly, this nucleotide sequence alignment demonstrated that 5' upstream regions of *armA* and *rmtE* appear to share notable degree of conservation (73.25%). Further investigation revealed that *armA* and *rmtE* share a 243nt upstream element, which will be discussed in depth in the following sections. While *rmtD* and its variant *rmtD 2* have maintained a highly similar 5' upstream region (97%), it appears that *rmtE 2* has acquired a novel 5' upstream region compared to *rmtE*. Further investigation revealed an ISCR20 insertion sequence upstream of the *rmtE* coding region. The original upstream region was still intact upstream of this insertion sequence. Additionally, the upstream regions of *rmtG* and *rmtD* (and consequently *rmtD* 2), show a surprisingly high conservation (63.75%).

The dendrogram generated based on these alignments further highlights the aforementioned associations (Figure 23).

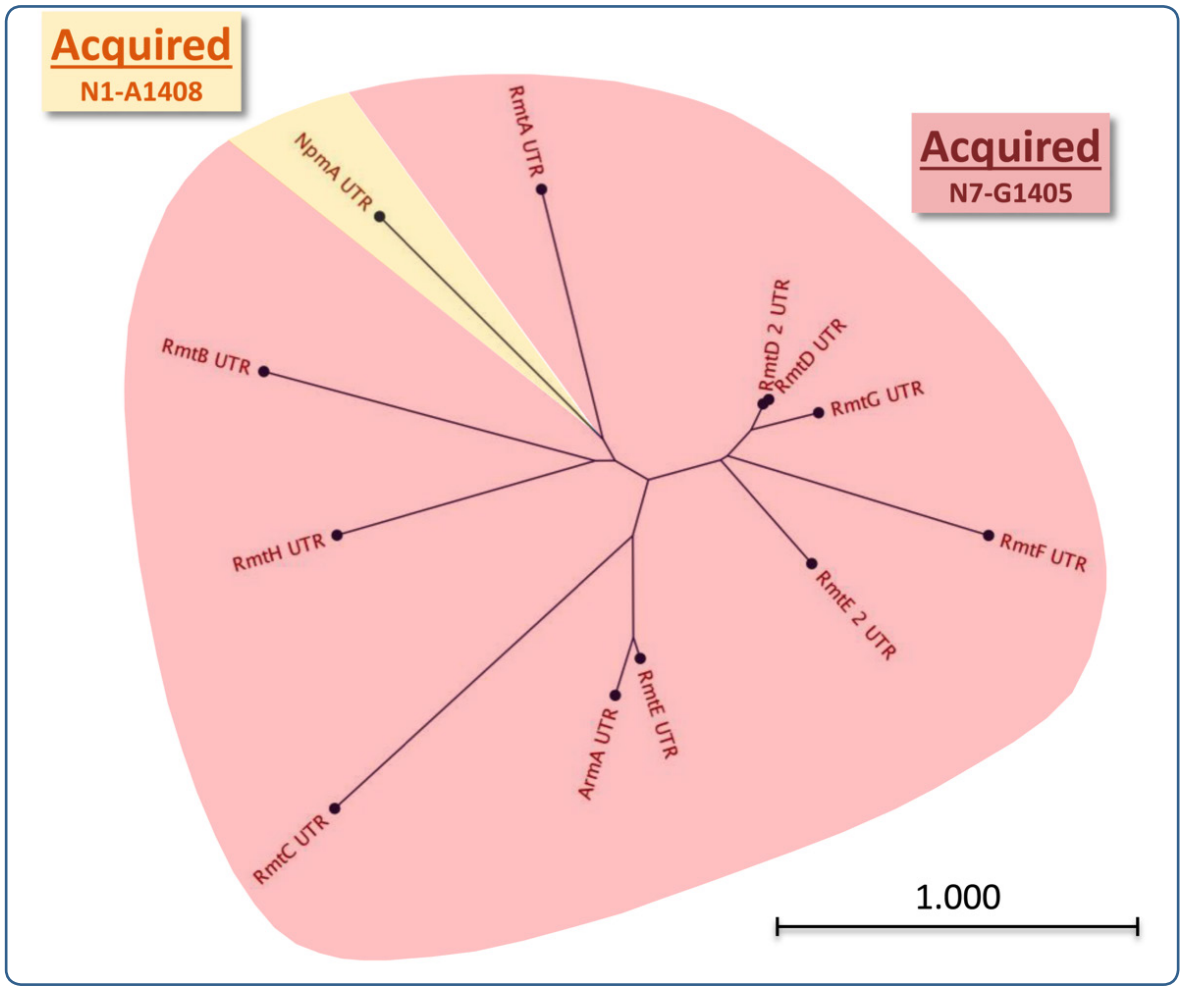


Figure 23. Dendrogram generated from alignments of the acquired methyltransferase upstream region (400 nt). Based on sequence availability, rmtB 2 was excluded from this analysis. Bootstrap analysis conducted with 10,000 replicates.

Figure 23 differs from previously presented trees of the coding regions, which has interesting implications regarding the conservation versus acquisition of novel upstream regions. To highlight this, Table 7 below is a table containing both the coding regions and the 5' upstream regions of the acquired methyltransferases.

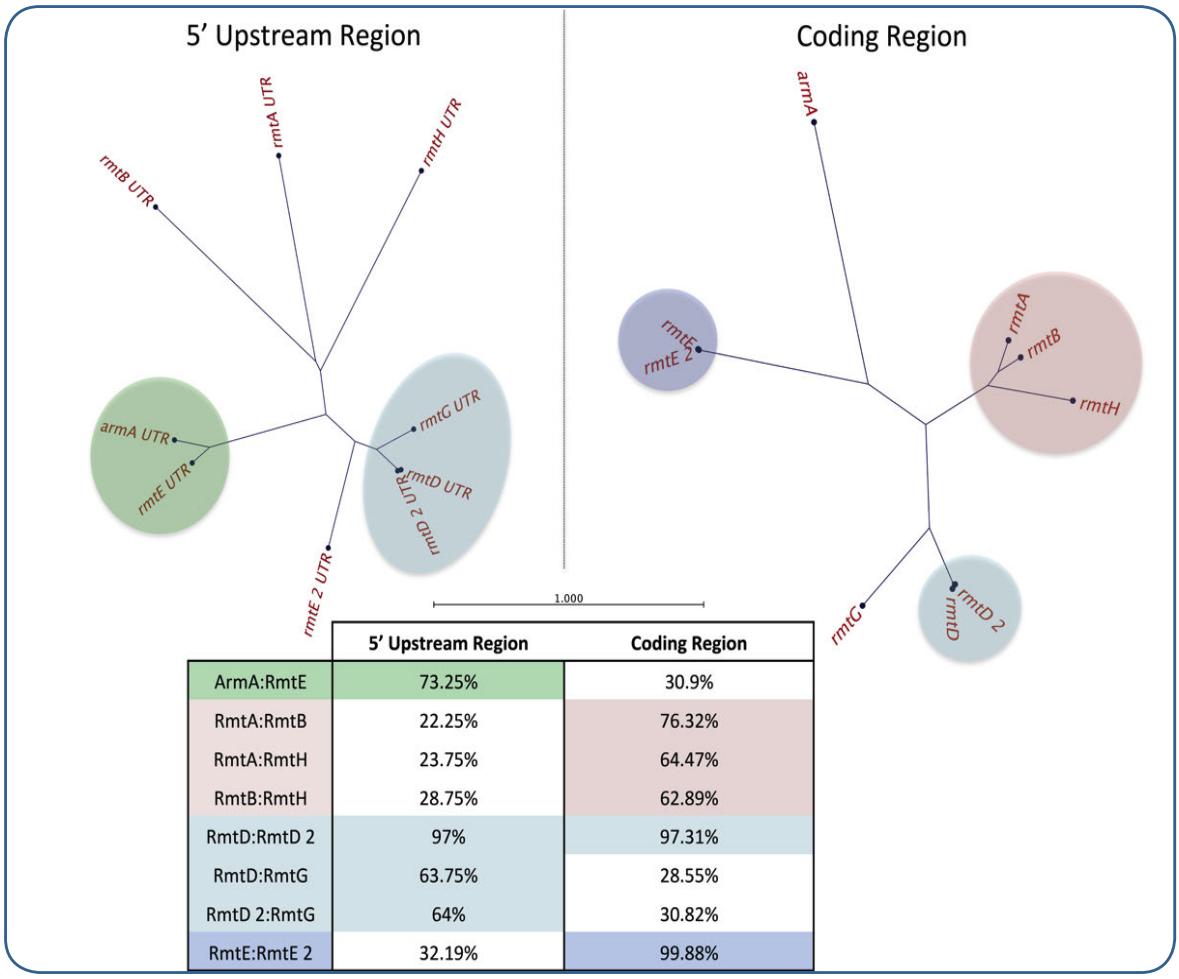
Table 7. Nucleotide conservation levels of the 400 nt upstream region of the acquired methyltransferases (red) vs. nucleotide conservations of the methyltransferase coding regions (blue).

5' UPSTREAM REGIONS

CODING REGION	armA	rmtA	rmtB	rmtC	rmtD	rmtD 2	rmtE	rmtE 2	rmtF	rmtG	rmtH	npmA	5' UPSTREAM REGIONS
armA		25%	28.25%	30%	28.75%	29%	**73.25%**	23.18%	22.25%	31.5%	26.25%	26%	armA
rmtA	26.49%		**22.25%**	19.5%	26.75%	27%	23.25%	21.89%	26.75%	27.5%	**23.75%**	30%	rmtA
rmtB	25.58%	**76.32%**		25.25%	25.5%	21.5%	31%	20.6%	18%	27.25%	**28.75%**	27.5%	rmtB
rmtC	29.79%	24.23%	21.99%		27.75	29.75%	31.75%	24.46%	15.5%	26.25%	26%	15.25%	rmtC
rmtD	28.04%	36.9%	23.41%	22.58%		**97%**	22%	22.1%	27.25%	**63.75%**	24.25%	29.25%	rmtD
rmtD 2	28.17%	36.68%	22.75%	22.18%	**97.31%**		20.25%	22.1%	26.25%	**64%**	24.5%	34%	rmtD 2
rmtE	30.9%	33.09%	33.45%	26.36%	24.09%	23.84%		**32.19%**	20.25	24.25%	30%	21.75%	rmtE
rmtE 2	30.9%	33.09%	33.45%	26.12%	24.09%	23.84%	**99.88%**		25.11%	33.69%	19.53%	24.25%	rmtE 2
rmtF	20%	29.36%	39.62%	26.83%	48.46%	48.33%	27.86%	29.56%		29.5%	26.5%	26.25%	rmtF
rmtG	22.52%	35.6%	34.47%	20.45%	**28.55%**	**30.82%**	31.27%	31.27%	26.42%		21.25%	25%	rmtG
rmtH	25.58%	**64.47%**	**62.89%**	24.59%	37.11%	36.84%	22.38%	22.38%	41.28%	25.79%		25.25%	rmtH
npmA	23.77%	23.81%	22.88%	22.7%	29.3%	29.17%	24.09%	24.09%	24.23%	25.53%	23.55%		npmA
	armA	rmtA	rmtB	rmtC	rmtD	rmtD 2	rmtE	rmtE 2	rmtF	rmtG	rmtH	npmA	

CODING REGION

In table 7, it is interesting that, although ArmA and RmtE have low levels of nucleotide conservation (30.9%), their 5' upstream regions exhibit a high degree of relatedness. Similarly, RmtG and RmtD (and consequently RmtD 2) also harbor a low nucleotide sequence conservation (28.55% and 30.82% respectively), while their upstream regions are quite conserved (63.75% and 64% respectively). Conversely, other methyltransferases such as RmtA, RmtB and RmtH share high levels of amino acid conservation whereas their 5' upstream regions have drastically changed over time. For clarity, figure 24 below presents the aforementioned methyltransferases that exhibit 5' upstream region conservation and/or coding region conservation.



	5' Upstream Region	Coding Region
ArmA:RmtE	73.25%	30.9%
RmtA:RmtB	22.25%	76.32%
RmtA:RmtH	23.75%	64.47%
RmtB:RmtH	28.75%	62.89%
RmtD:RmtD 2	97%	97.31%
RmtD:RmtG	63.75%	28.55%
RmtD 2:RmtG	64%	30.82%
RmtE:RmtE 2	32.19%	99.88%

Figure 24. Dendrogram of the acquired methyltransferases demonstrating the plasticity of the upstream region (400 nt) in contrast to the coding regions. High levels of conservation (>50%) have been marked with corresponding colors in both the table and the dendrogram Bootstrap analysis conducted with 10,000 replicates.

The alignments presented demonstrate an interesting trend pertaining to the conservation of the 5' upstream region among the acquired methylases. It appears that the 5' region of the different methylases generally have low levels of conservation; nevertheless all the methyltransferases are capable of producing unusually high levels of aminoglycoside resistance under the regulation of each particular 5' upstream region. The identified regions that maintain a high level of similarity within the 5' upstream region may bear regulatory features that favor a stringent expressional pattern resulting in the high level resistance conferred. That being said, as the publications pertaining to these genes generally do not determine MIC over a concentration of 128 or 256 mg/L it is hard to ascertain whether or not the various methyltransferases and their 5' upstream regionsconfer the maximum resistance possible.

6.1.2.2 Acquired vs. intrinsic resistance conferring methyltransferases 5' upstream region conservation

As previously conducted with the coding regions of the methyltransferases, our next step was to determine whether or not intrinsic resistance conferring methyltransferases shared a degree of conservation with elements found upstream of each other and/or the acquired methyltransferases (Figure 25). As was the case with *rmtB 2* in the previous section, there were no available sequences of the *kamC* 5' upstream regions and, as such, it has been excluded from further analyses. Furthermore, unfortunately, based on sequence availability, we were unable to include the upstream regions of the housekeeping methyltransferases within the following assays.

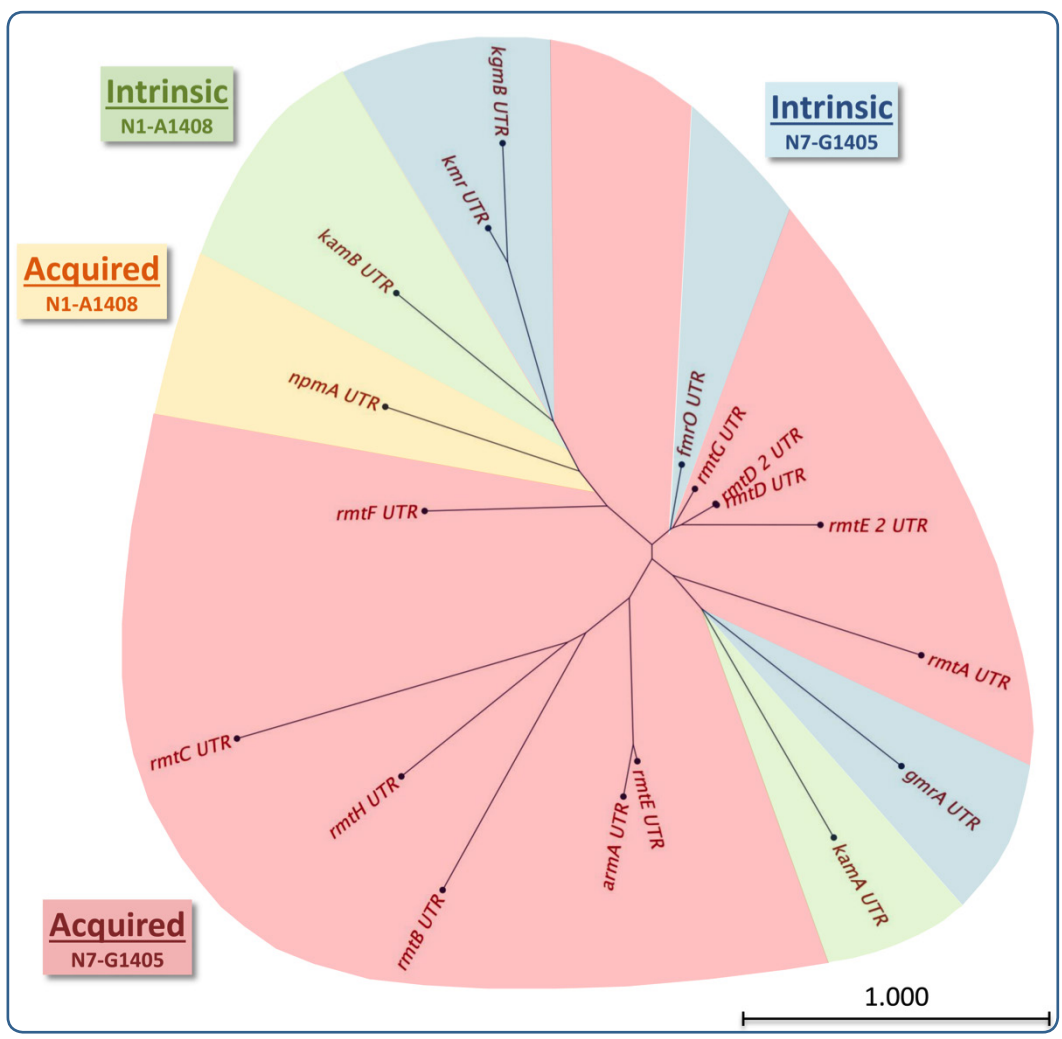


Figure 25. Dendrogram of the 400 nt upstream region of the acquired 16S rRNA methylases including the 400 nt upstream region of the intrinsic resistance conferring methyltransferases. Bootstrap analysis conducted with 10,000 replicates.

Figure 25 demonstrates substantial differences to amino acid alignments of acquired and intrinsic resistance methyltransferases (figure 20), generally there does not appear to be a significant degree of relatedness among the 5' regions of the intrinsic and acquired methyltransferases. Unlike the amino acid alignments presented in section, the methyltransferases do not appear to form clusters based on origin or function with regards to their 5' region identity. That being said, the *rmtG* upstream region shares 67% identity with the 5' region of the intrinsic resistance methyltransferase *fmrO.*

6.1.3 Acquired 16S methyltransferases promoter predictions

6.1.3.1 Degree of -35 and -10 box conservation among the acquired 16S methyltransferases

With the exception of certain cases, the *in silico* analysis of the methyltransferase coding regions and 5' upstream region demonstrated a high degree of plasticity in the upstream region of the acquired methyltransferases. To further investigate any common features regarding the regulation of their expression, we then assessed whether or not upstream elements such as promoters harbor a higher degree of conservation.

Utilizing the online promoter prediction algorithms, the 5' upstream regions of the acquired 16S rRNA methyltransferases were processed for putative promoter elements. Figure 26 below portrays the position of the -35 and -10 promoter elements within the 400nt 5' upstream region analyzed.

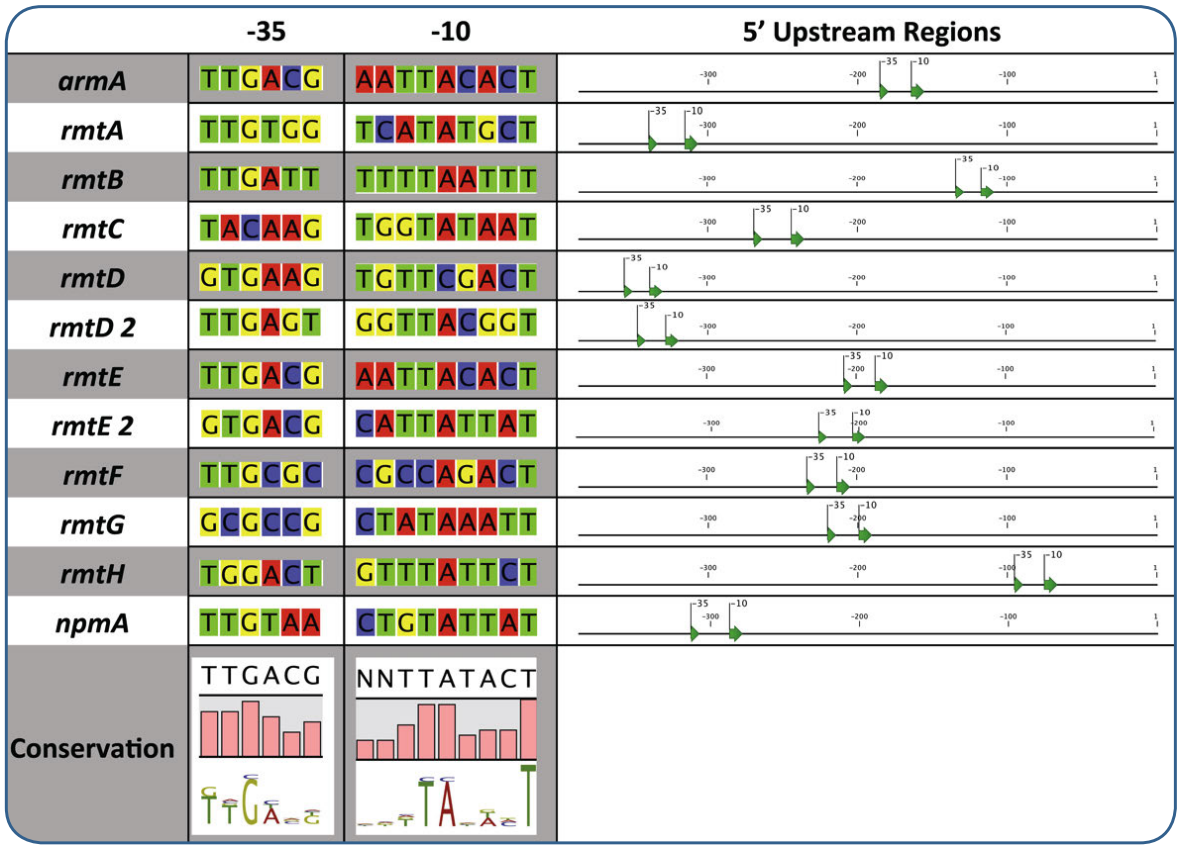


Figure 26. Alignments of the putative -35 and -10 promoter regions of the acquired 16S rRNA methyltransferases including a diagram of the promoter distance from the coding region of the methyltransferases (right). Promoter regions were analyzed within a region of 400nt upstream of the CDS.

The nucleotide conservation of all promoters analyzed revealed a general conservation of a -35 TTGACG motif and a -10 TTATACT motif. Interestingly, *armA* and *rmtE* (that harbored upstream regions that shared 73.25% nucleotide sequence conservation) both harbor this exact promoter sequence. This promoter sequence shares a high-level conservation with promoters that have been experimentally confirmed to interact with the σ^{70} factor, which will be further discussed in the following sections. The distance of the promoters from the start codons ranged between 67 and 330 nucleotides, measuring from 3' of the -10 box, where the average distance of the promoter elements from the coding region was approximately 214 nucleotides. While the general conservation within the 5' upstream region among the 16S rRNA methyltransferases was limited, it appears that the promoter motifs harbor a more significant degree of conservation.

6.2 The characterization of the *armA* 5' UTR

6.2.1 armA reference sequence conservation

The remainder of this study will focus on the most disseminated 16S rRNA methyltransferase, ArmA. In addition to being the most prevalent acquired methyltransferase, ArmA is also harbored by the broadest host-range compared to the other acquired methyltransferases. As such, it has been proposed to harbor a regulatory mechanism able to adapt to the large variations in ribosome count throughout the growth phases of the host bacteria (Ramu *et al*., 2009).

To isolate the most representative 5' upstream region of *armA*, all available reference sequences of *armA* were downloaded from Genbank and aligned to evaluate upstream conservation (Figure 27).

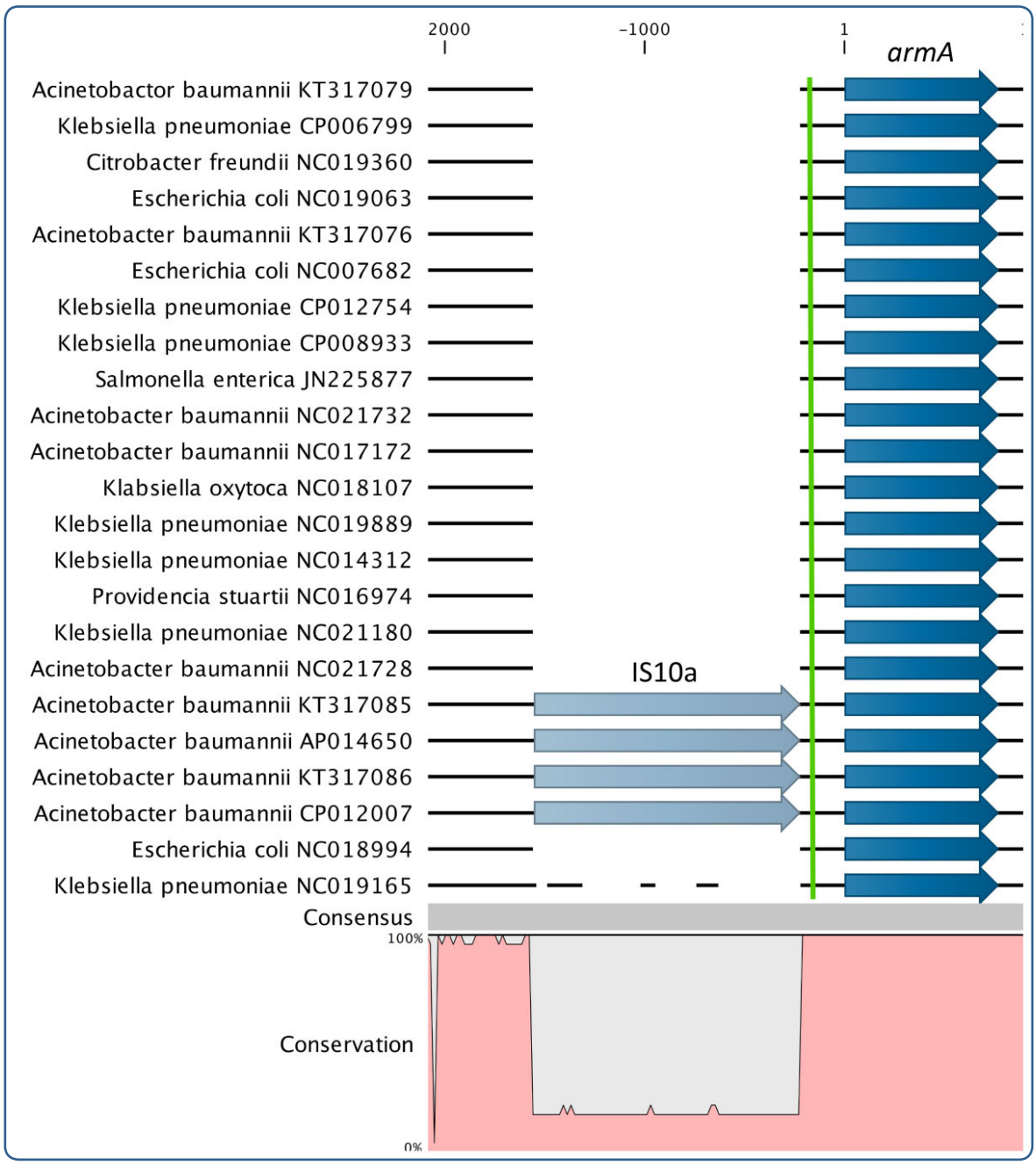


Figure 27. Alignments of all available reference sequences of armA from Genbank. Accession numbers and bacterial species are included on the left. The putative promoter regions identified are denoted by a green line within the highly conserved region.

As *armA* has thus far almost exclusively been identified within the transposon Tn1548, it is not surprising that the upstream region is highly conserved. Generally, non-coding regions acquire more single nucleotide mutations than coding regions; however, we identified a 221 nt region immediately upstream of *armA* that was highly conserved in 100% of the available sequences of *armA*. This region contains the aforementioned

putative -35 and -10 element (between 186 nt and 156 nt from the start codon) that is 100% conserved throughout all sequences of *armA* (Figure 26). 4 of the 23 sequences analyzed (17.4%) harbored a 1,335 nt insertion sequence 221 nt upstream of the ATG start codon of *armA*. Using an online database (ISfinder), this insertion sequence was identified as the insertion sequence IS10a, which belongs to the IS4 family. Interestingly, this insertion sequence interrupts an open reading frame in the 5' upstream region of *armA* that has previously been described as a putative leader peptide involved in the regulation of *armA* (Ramu *et al*., 2009). Based on the publications describing sequences harboring this insertion sequence, resistance levels do not appear to be affected by the insertion sequence, suggesting that the translation of this leading peptide is not required for the functional expression of *armA*.

6.2.2. *In vitro* identification of the *armA* 5' Untranslated Region

In order to accurately dissect the untranslated region of this 16S rRNA methyltransferase it was necessary to identify the full-length of the 5' UTR. This was conducted in the presence and absence of aminoglycoside pressure, via the rapid amplification of cDNA ends (RACE). After treating the synthesized cDNA with a 5' terminal universal adaptor capable of interacting with the complementary SMARTer RACE oligonucleotide, it was possible to amplify the full 5' UTR via PCR from within the gene using a gene specific primer. The resulting amplicon was then subcloned and sent to sequencing. Both in the presence and absence of antibiotic pressure the *armA(wt)* 5' UTR was determined to be 139 nucleotides upstream of the *armA* start codon via Sanger Sequencing (Figure 28).

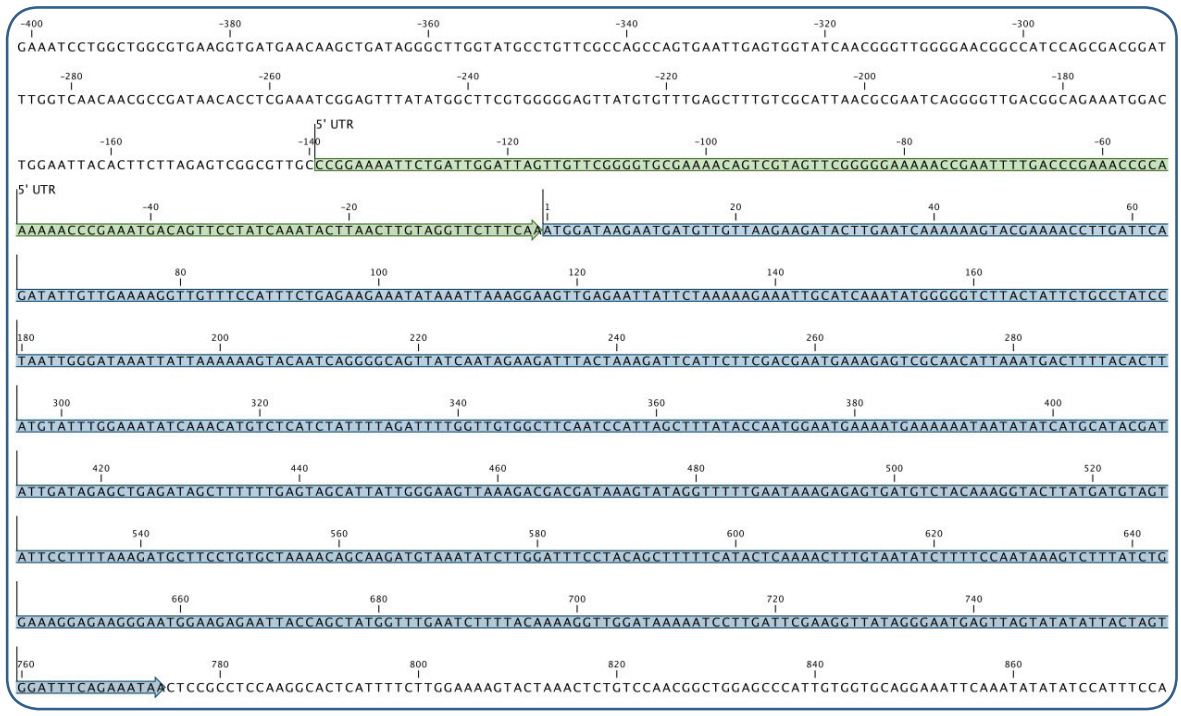


Figure 28. Sequence map containing the armA coding region and orientation (blue arrow) as well as the RACE confirmed 5' UTR of armA. The identified 5' UTR of armA is precisely 139 nt from the armA coding sequence.

The repeatedly obtained transcript length of 139 nt corresponds to the high-level 5' UTR sequence conservation described in the previous section (Figure 27). In 100% of the *armA* sequences aligned the here-identified 5' UTR is conserved. The results of the 5' UTR RACE were then confirmed by extracting RNA from *E. coli* DH5α bearing a *WT* plasmid that harbors *armA*. RT-PCR analysis then confirmed this transcript using various primers upstream and downstream from the predicted transcriptional start.

The RT-PCR performed of RNA extractions after 2, 4, 6 and 8 hours of growth confirmed the absence of a transcript upstream of the here-predicted 139 nt 5' UTR. While RT-PCRs conducted of a 139 nt or less of the 5' UTR were positive, all RT-PCR using primers upstream of this region were negative at all time points analyzed (Figure 29).

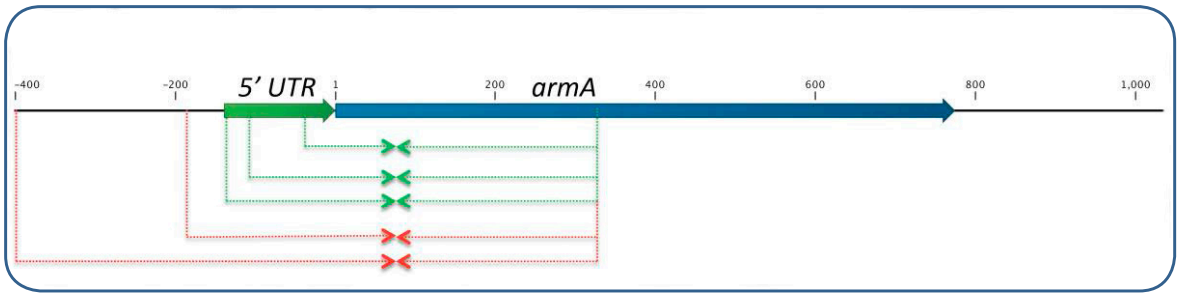


Figure 29. Diagram of the RT-PCRs conducted using various primers from within the 5' UTR (green) of armA (blue) to investigate the presence of any active promoter regions upstream of the RACE predicted 5' UTR. Using primers from within the predicted 5' UTR resulted in an amplicon suggesting the presence of this 5' UTR within the mRNA, while RT-PCRS performed with any primers upstream (red) of the predicted 5' UTR did not amplify the cDNA, suggesting no upstream promoter regions.

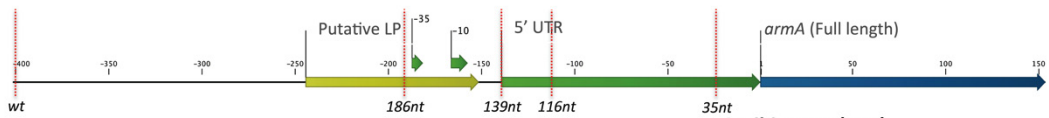
These assays were conducted in various conditions to ensure a single transcriptional start site. Based on the plethora of possible regulatory mechanisms, it was important to confirm not only the length of the 5' UTR but also to rule out multiple upstream transcriptional start sites. These results demonstrate that both in the presence and absence of aminoglycosides, throughout the growth phases of DH5α harboring pMUR050 bearing *armA*, the transcriptional start site of *armA* is always 139 nt upstream of the *armA* start codon.

With regards to the *armA* 3' UTR, we decided not to performed the 3' UTR RACE identification because an *in silico* investigation revealed a very clear Rho-independent transcriptional terminator loop starting 20 nt from the 3' extreme of *armA*. As such, we predict that the full transcript length of *armA* is 946 nt, which is made up of a 139 nt 5' UTR and a 774 nt coding region followed by a 33 nt 3' UTR.

6.2.3 Phenotypic effects upon the truncation of the *armA* 5' UTR

To identify regulatory elements within this untranslated region, our next step was to perform various truncations of the 5' UTR to determine the phenotypic impact on the *armA* resistance profile. These constructions were cloned onto the non-ColE1 cloning

vector pACYC-184. Subsequently RT-PCR analysis confirmed the absence of any active upstream promoter region. As described in materials and methods, these constructs were cloned via PCR amplification with a high fidelity polymerase, after which they were carefully confirmed via sequencing. As the resistance profile conferred by the various constructions is an informative method of assessing expressional levels, we performed MICs and antibiograms of five truncations to identify regions of the 5' UTR required for the expression of this resistance determinant (Figure 30).



Length of 5' UTR	MIC (mg/L)	Antibiogram (mm)		
		Gentamicin	Kanamycin	Amikacin
400nt (*wt*)	1024	6	6	6
186nt	512	6	6	6
139nt	<0.25	**26**	**28**	**27**
116nt	<0.25	**25**	**29**	**28**
35nt	<0.25	**24**	**28**	**26**
DH5α Control	<0.25	26	28	27

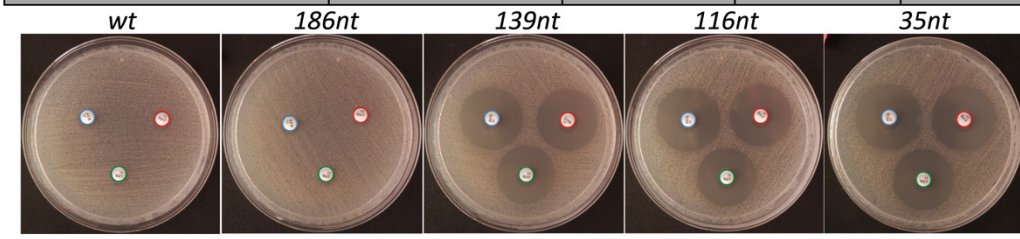


Figure 30. The antibiotic resistance profile conferred by active ArmA with truncated 5' upstream regions. MICs were determined with gentamicin according to the CLSI and EUCAST guidelines. Numbers reflect the measured halo size with the susceptible phenotype marked in bold. Antibiograms in the lower portion of the figure were conducted with selected 4,6 2-DOS aminoglycosides amikacin 30 µg (blue) kanamycin 30 µg (red) and 10 µg gentamicin (green). All antibiograms were conducted according to the CLSI and EUCAST guidelines (CLSI, 2017; EUCAST, 2017).

The results obtained from this truncational assay once again support the findings of the RACE predicted 5' UTR. These results demonstrate that between -400 nt and -139 nt from the *armA* start codon there is a regulatory domain absolutely required for the expression of *armA*. Upon the truncation of this domain, the levels of resistance conferred drop to that of the susceptible DH5*α* control. As the construction bearing the

186 nt 5' upstream region has a truncated putative leader peptide, it appears that the full length leading peptide is not required for the expression of *armA*. That being said, there is a one fold reduction in the MIC between the *WT* and the 186 nt construction, which will be further investigated in the following chapter. This seems to support the prediction of an *in silico* promoter region immediately upstream of the transcriptional start site. Both the minimum inhibitory concentrations and the antibiograms were performed in the presence and absence of subinhibitory levels of aminoglycosides. However, under no conditions did low levels of aminoglycosides affect the resistance profile exhibited by the constructions.

To further investigate this expressional pattern, we then decided to directly quantify the amount of ArmA translated via Western Blot. As outlined in the materials and methods, we initially tagged *armA* bearing the full 400 nt upstream region with a C-terminal domain Triple FlagTag. Truncations of this tagged construction then allowed us to directly assess translational levels of *armA* under the regulation of various lengths of the 5' upstream region (Figure 31).

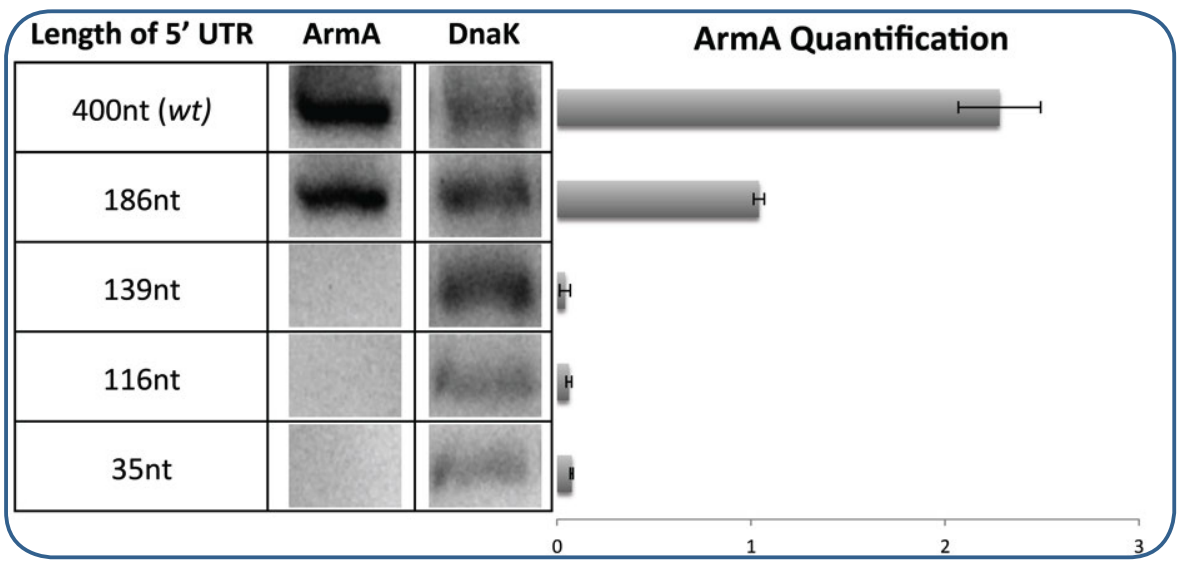


Figure 31. Western blot analysis of armA variants with truncated 5' upstream region. Western blots conducted at an optical density (OD_{600}) of 0.2. Expressional quantification was conducted using the DnaK Western Blot reference protein.

Using Western Blots to directly quantify the translational levels of *armA* at various time points throughout the growth phases of *E. coli* bearing these constructions, once again confirmed the presence of a domain necessary for the accurate expression of *armA* encoded between -400 nt and -139 nt upstream of the *armA* coding region. Although the Western Blot quantification indicates that the construction bearing the 186 nt upstream region has a lower level of expression, it still produces a substantial amount of ArmA, again suggesting that the leading peptide is not absolutely required for the expression of *armA.*

6.2.4 Evaluation of the *armA* expressional profile

To further elucidate the expressional profile of this resistance determinant, we designed a *lacZα* reporter construction using the pERMCα vector. By fusing 400 nucleotides of the *armA* 5' upstream region bearing the first 4 codons of *armA* to codons 6-60 of the *lacZα* reporter gene we were able to identify any environmental factors that influence the expression of this resistance methyltransferase (Figure 32).

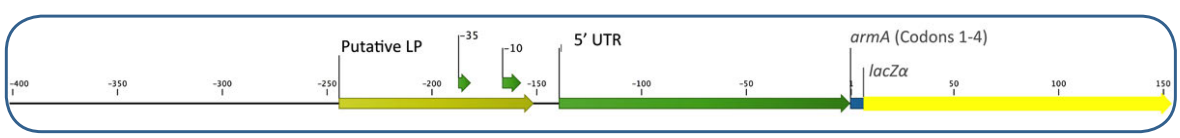


Figure 32. Genetic map of the armA upstream region of 400 nt harboring the putative leader peptide (dark yellow), the putative promoter region (green arrows) and the first four codons of armA (blue) fused to the lacZα reported gene (yellow).

The first result of this expressional assay demonstrated that the expression of the *armA* resistance gene appears to be constitutive (Figure 33).

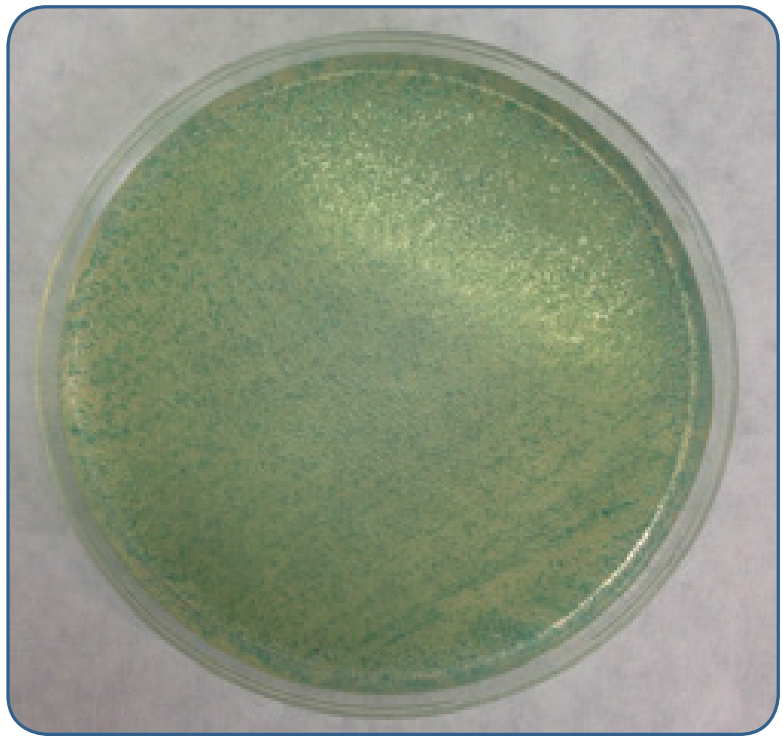


Figure 33. Plate of the armA:lacZα reporter construct on Mueller-Hinton agar with x-gal in the absence of antibiotics or any known inducing agents. Bacteria were plated after obtaining a 0.5 McFarland turbidity inoculum.

Both in the presence and absence of aminoglycosides, there was a constitutive expression of the *lacZα* gene product under the regulation of the full-length *armA* 5' UTR.

To further investigate the results of the truncational assay outlined in the previous section, we fused each truncation to the *lacZα* reporter gene (Figure 34).

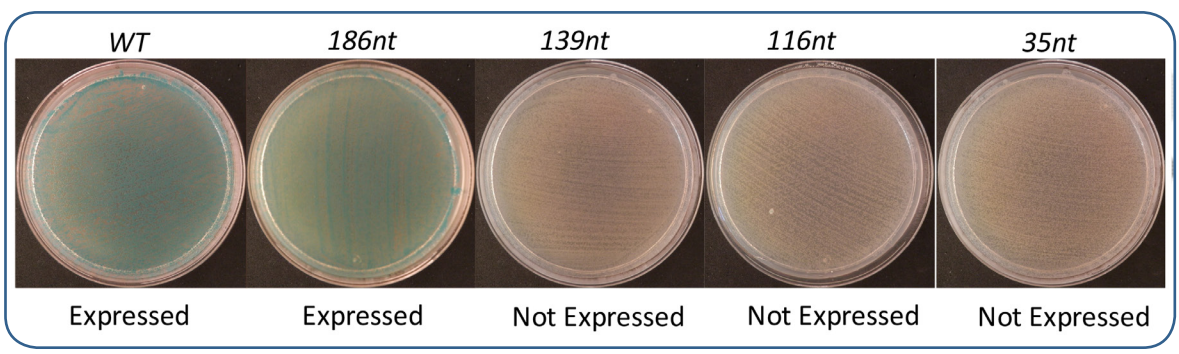


Figure 34. armA variants with truncated 5' upstream regions plated on Mueller Hinton agar with x-gal. Both the WT and 186 nt UTR variant indicate the expression of the lacZα reporter gene under the regulation of the respective upstream regions (blue plates).

The results of the 5' upstream region truncations fused to the *lacZα* reporter construct aligned perfectly with our previous results regarding essential domains of the 5' UTR. As previously identified, the region between -400 and -139 nt upstream of the *armA* coding region harbors domains absolutely required for the expression of the 16S rRNA methyltransferase *armA*.

To investigate whether or not the presence of aminoglycosides is involved with the expressional levels of the *armA:lacZα* reporter construct, we then added gentamicin in the form of 10 μg disks to the screening (Figure 35).

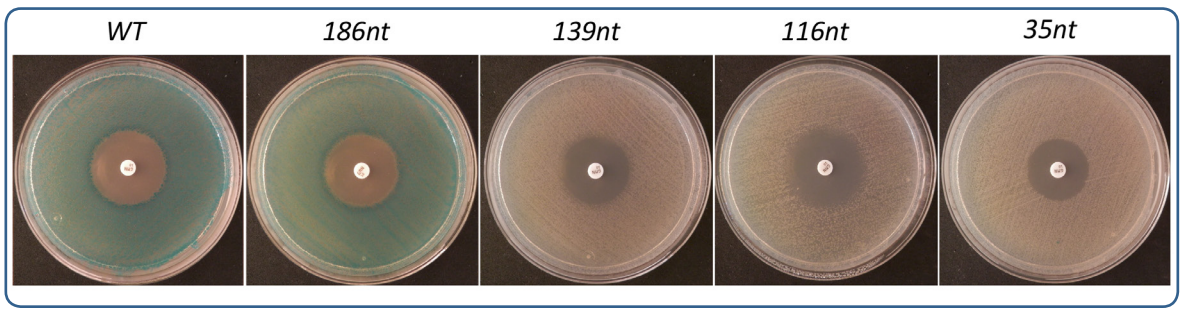


Figure 35. armA variants with truncated 5' upstream regions plated on Mueller Hinton agar with x-gal in the presence of gentamicin. Bacteria plated after making a 0.5 McFarland turbidity inoculum.

At first glance in Figure 35, in the *WT* as well as in the strain harboring a 186 nt UTR it does appear that there is a region of augmented *lacZα* expression in the presence of the aminoglycoside. We believe this is attributed to the colony size within the subinhibitory range of the antibiotic disk. To ascertain whether or not this is case, we performed a number of Western Blots in the presence and absence of various concentrations of gentamicin (Figure 36).

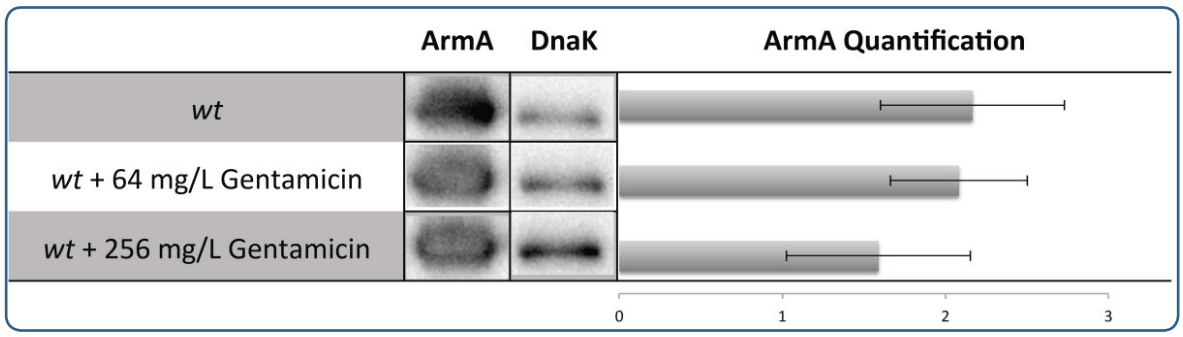


Figure 36. Western Blot quantification of WT armA in the absence and presence of various concentrations of gentamicin. Quantification performed using DnaK as a reference (Y-axis).

These Western Blots suggest that antibiotics do not augment the expression of active *armA* under the regulation of its full 5' UTR. Although not shown, further Western Blots performed in the presence and absence of various 4,6 2-DOS aminoglycosides did not reveal any effect on the expressional levels of *armA*.

Finally, to assess whether or not a feedback mechanism modulates the expressional levels of *armA*, we co-transformed *E. coli* DH5 α with the *armA:lacZα* reporter construct alongside active *armA* and *rmtC* in *trans* (Figure 37).

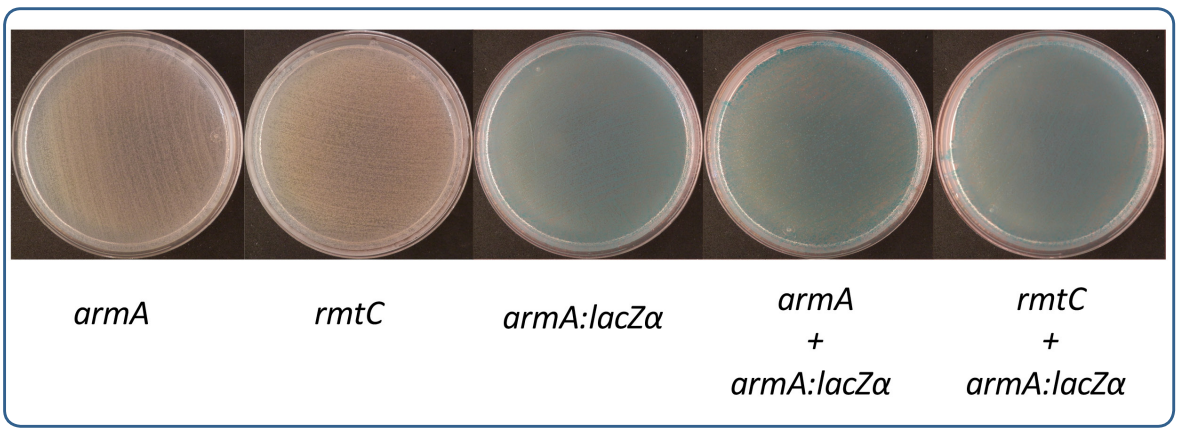


Figure 37. Cotransformations of the armA:lacZα reporter construct with active armA borne on the pTOPO cloning vector and rmtC on the pBAD cloning vector demonstrated no reduction in the expression of the reporter gene in the presence of the methyltransferase armA or the methyl group at postion G1405.

The co-transformations were maintained using selective antibiotic concentrations and the presence of both plasmids was confirmed via PCR. This assay was conducted in the presence and absence of two different acquired 16S rRNA methyltransferases. This was done to assess whether, either the presence of active methyltransferase or the presence of the methyl group on G1405 had an impact on the expressional levels of *armA*. However, neither the presence of *armA* on a high copy number plasmid, nor the presence of *rmtC* in *trans* appears to affect the expressional levels of *armA.*

6.3 Growth phase dependent expression of *armA* mediated by σ^{70} transcription factor promoter region

6.3.1 Evaluation of the predicted σ^{70} promoter region of *armA*

Based on the data obtained to this point, the expression of *armA* does not appear to be affected by post-transcriptional regulatory activities. Both the phenotypic and translational assays confirm the complete lack of ArmA upon truncation of the region containing the promoter (Figures 30 and 31). Before experimentally investigating this promoter, we carefully analyzed it *in silico* and compared it to previously identified promoters.

Online promoter prediction algorithms revealed that promoters of this sequence have known interactions with the σ^{70} factor RpoD. The RpoD family of sigma factors preferentially induces the transcription of genes associated with fast growth, such as ribosomal operons and a number of other factors involved in protein synthesis.

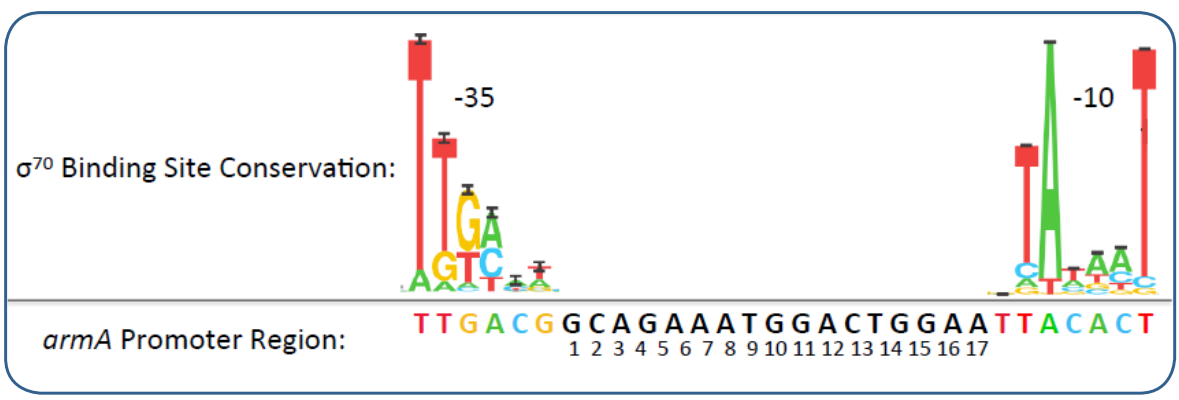


Figure 38. Conservation of the experimentally confirmed RpoD recognized promoter region (Figure adapted from Shultzaberger et al., 2007).

Figure 38 contrasts the promoter region identified within the region required for the expression of *armA* with the most conserved nucleotides of promoters experimentally identified to interact with σ^{70} factor RpoD. In addition to nucleotide conservation, a key modulator of activity for this type of transcription factor is the distance of the -35 and -10 box of the promoter region, where the most common distance between the

two elements identified was 17 nucleotides. This factor is capable of fine-tuning the transcriptional levels of the gene product, and as the distance between the -35 and -10 boxes of the *armA* promoter region is precisely this most common distance, it could support the unusually high MIC produced by this resistance determinant. Based on literature dissecting promoter regions, the nucleotide conservation demonstrated above is sufficient to initiate transcription via this housekeeping sigma factor. Interestingly, the RACE and RT-PCR confirmed transcriptional start site is 17 nucleotides from the 3' end of the -10 box. Typically, the -10 box is between 8 and 14 nucleotides from the transcriptional start site, although larger spacings have previously been described (Shulzaberger et al., 2007).

6.3.2 Single Nucleotide resolution of promoter elements required for the fine-tuned expression of *armA*

6.3.2.1 Initial mutagenesis of highly conserved nucleotides

To assess whether or not this putative promoter is responsible for the expression of *armA* we performed mutations of conserved nucleotides within the promoter region. To achieve this we initially synthesized 3 mutants of the 400 nt *armA* variant bearing the 3xFlagTag to subsequently assess the translational levels alongside the resistance profile. The first of them had the two most conserved nucleotides of the -35 box (TT➔GG) substituted with a double guanine. The second mutant generated also had two base-pairs substituted with a double 'GG', but within the -10 we chose to mutate one highly conserved nucleotide and an adjacent less conserved nucleotide (TT➔GG) to assess whether or not the expressional levels expected correspond with the σ^{70} promoter region hypothesis. The third mutant generated as part of this initial mutagenesis contained 'GG' substitutions in both the -35 and the -10 box (Figure 39). Subsequently, we assessed the expression of *armA* via both the resistance profile generated and the translational levels.

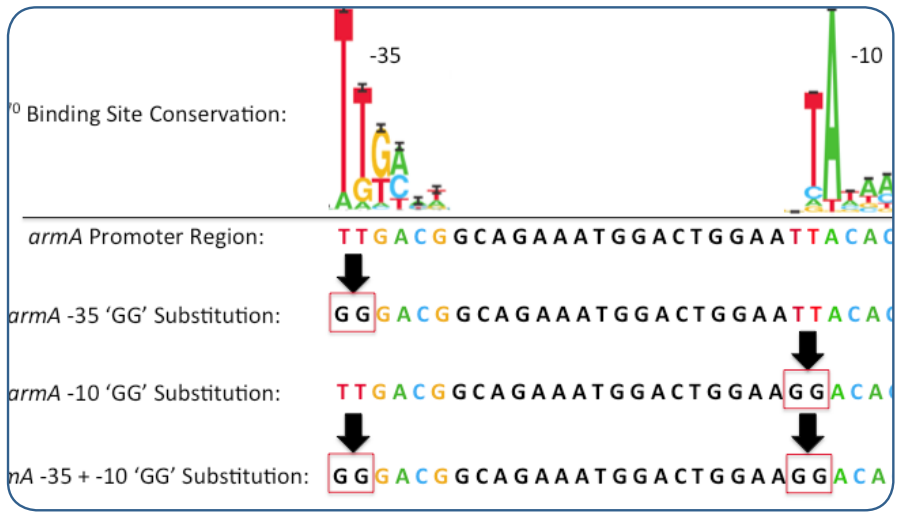


Figure 39. Diagram of the initial promoter region substitutions performed in contrast to the RpoD recognized promoter region conservation. 'GG' substitutions performed (framed red) of the -35 box, the -10 box and both the -35 and the -10 box (Figure adapted from Shultzaberger et al., 2007).

These constructions, generated via an outward PCR and the In-Fusion HD cloning kit, were then carefully examined via Sanger sequencing, after which their resistance profile and translational levels were examined (Figure 40).

Construction	MIC (mg/L)	Antibiogram (mm)		
		Gentamicin	Kanamycin	Amikacin
wt	1024	6	6	6
-35 GG substitution	<0.25	**27**	**27**	**26**
-10 GG Substitution	16	**26**	**28**	**26**
-35 & -10 GG substitution	<0.25	**27**	**28**	**27**
DH5α Control	<0.25	26	28	27

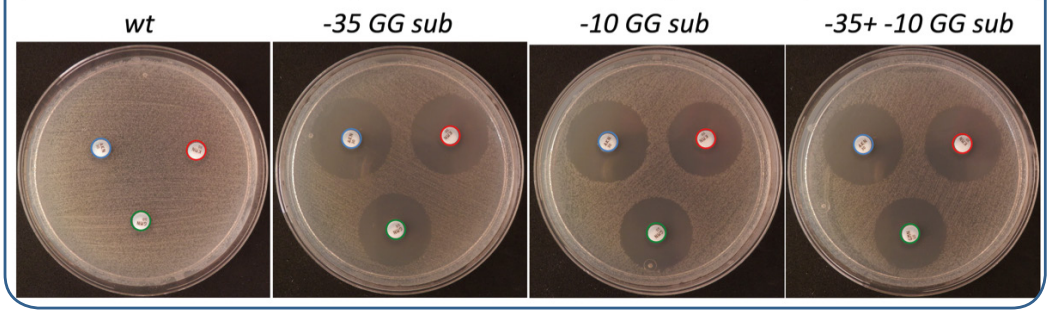


Figure 40. Antibiotic resistance profile conferred by armA(wt) and armA with initial GG substitutions of the -35 box, the -10 box and both elements (-35 & -10 boxes). MICs were determined with gentamicin according to the CLSI and EUCAST guidelines. Numbers reflect the measured halo size with the susceptible phenotype marked in bold. Antibiograms in the lower portion of the figure were conducted with selected 4,6 2-DOS aminoglycosides amikacin 30 µg (blue) kanamycin 30 µg (red) and 10 µg gentamicin (green). All antibiograms were conducted according to the CLSI and EUCAST guidelines (CLSI, 2017; EUCAST, 2017).

The results pertaining to the resistance profile of the initial -35 and/or -10 box mutants aligns precisely with the hypothesis of a promoter region recognized by a σ^{70} factor. Interestingly, the -10 box, where the substituted nucleotides are one highly conserved and one less conserved, exhibits low levels of *armA* expression. This is to be expected as only one of the most conserved nucleotides was mutated, suggesting that the presence of the adjacent adenine is sufficient to induce very low levels of *armA* transcription. To investigate the effects this has on the translational levels of *armA* we then conducted Western Blot assays (Figure 41).

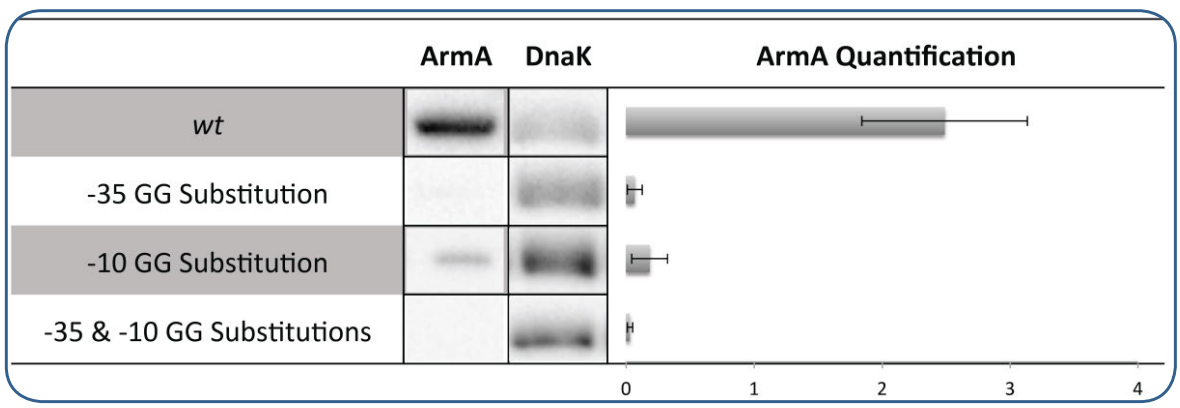


Figure 41. Western Blot expressional analysis of the initial GG substitutions performed in the -35 and -10 boxes of the predicted promoter region. Western Blot quantification carried out using DnaK as a reference as previously described.

The Western Blot translational levels align very accurately with the results obtained regarding the resistance profile of each of these mutants. While there is a drastic reduction in the translated ArmA in each of the mutants, the -10 box GG substitution (of one highly conserved and one less conserved nucleotide) displays levels of translation that are higher than in those constructions where the most conserved nucleotides are substituted.

6.3.2.2 Comprehensive -35 and -10 GG substitution assay

The initial results obtained from the mutagenesis of the promoter region strongly suggest the presence and activity of a promoter known to interact with the σ^{70} factor. To further investigate crucial elements within the promoter region we performed a number of further mutants of both the -35 and the -10 promoter regions (Figure 42). To obtain a complete understanding regarding the various promoter elements we decided to substitute nucleotides in pairs with both double guanine and double adenine mutations as previously described by Miura *et al* (Miura *et al*., 2015).

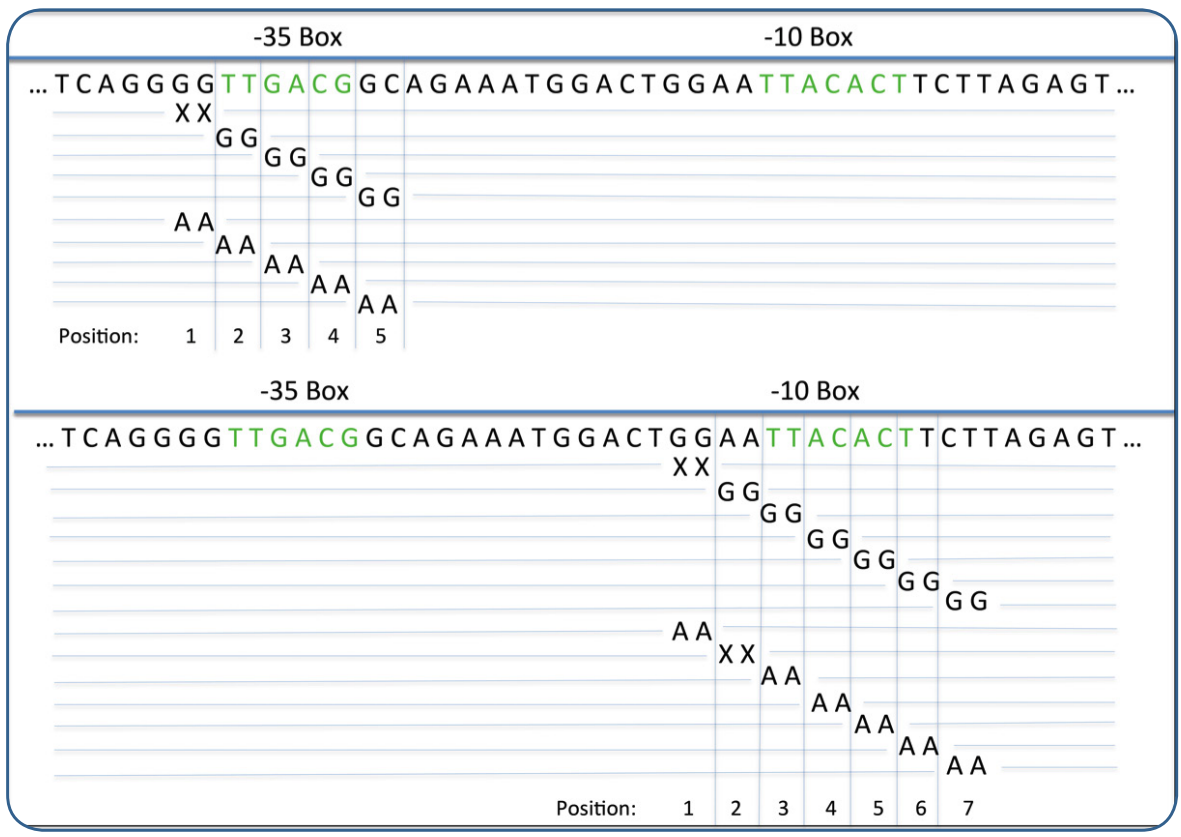


Figure 42. Figure demonstrating the various substitutions performed to ascertain nucleotides required for the expression of armA at a single nucleotide resolution.

This was necessary to not only identify the specific nucleotides most required for the appropriate transcriptional induction of *armA*, but also to identify the extremes of the promoter regions. To this point, every mutation of these regions has resulted in a significant reduction of MIC. Despite previous results indicating the appropriate

expression of *armA* even upon the truncation of the leading peptide, these mutants again confirm that the drastic reduction of aminoglycoside resistance is a result of the promoter region substitutions and not due to amino acid changes within the putative leader peptide. Again, after carefully examining the constructions via Sanger sequencing, we investigated the effects of the mutagenesis on the resistance profile and translational levels.

-35 box substitutions

Figure 43 below presents the effects of the double base pair substitutions on both the resistance profile and the translational levels of each 'GG' substitution performed throughout the -35 box of the *armA* promoter.

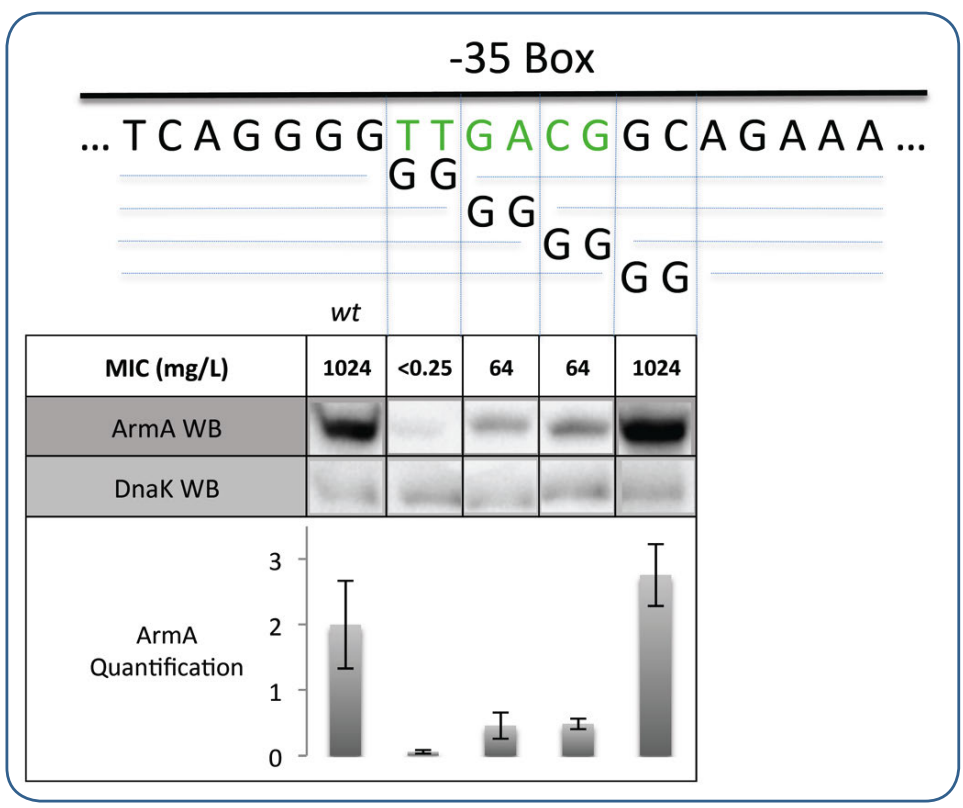


Figure 43. Resistance levels and Western Blot expressional analysis of GG substitutions throughout the -35 box. Gentamicin MICs were determined according the CLSI and EUCAST guidelines (CLSI, 2017; EUCAST 2017). Western Blots were performed in triplicate and quantified using DnaK levels as a reference. Western Blots presented are representative of the expressional trends observed and quantifications were performed using all replicates.

The GG substitutions performed at every position of the -35 promoter region clearly identify key nucleotides involved in the expression of *armA*. Modifying nucleotides at position 2 of the -35 box (TT➔GG) had the most pronounced effect on both the resistance levels conferred and the Western Blot translational levels. Substitutions of positions 3 and 4 significantly reduced both this resistance levels as well as the translational levels of *armA*. Based on the RpoD promoter region conservation, these results support the involvement of the RpoD housekeeping sigma factor in the translational induction of *armA*.

As mentioned above, substitutions were performed with both double guanine and double adenine nucleotide pairs. Figure 44 below presents the results obtained from the double adenine substitutions at every position of the -35 box.

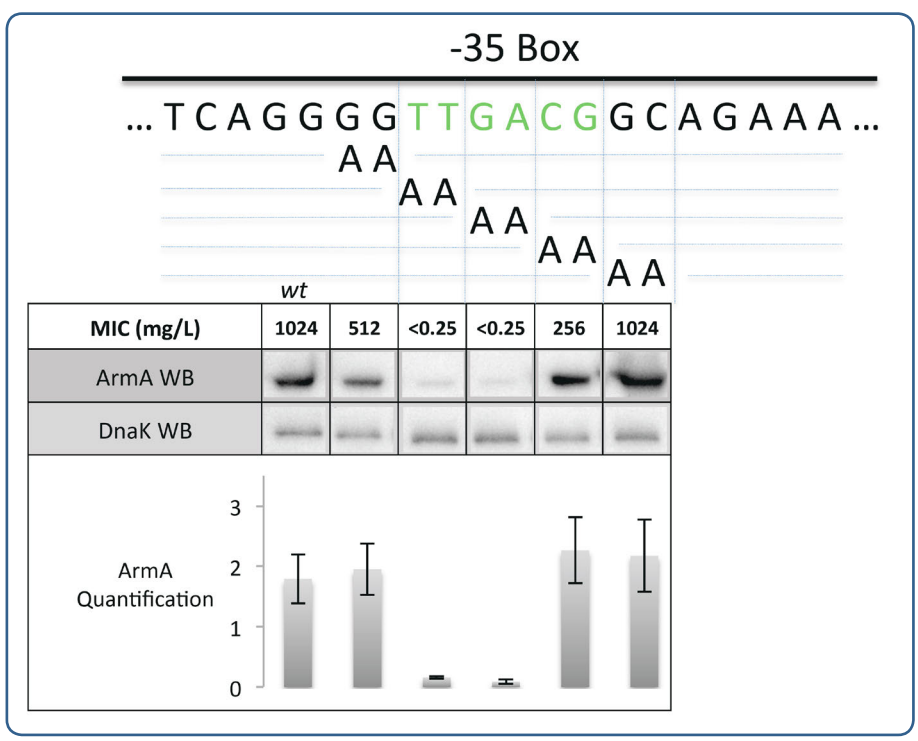


Figure 44. Resistance levels and Western Blot expressional analysis of 'AA' substitutions throughout the -35 box. Gentamicin MICs were determined according the CLSI and EUCAST guidelines (CLSI, 2017; EUCAST 2017). Western Blots were performed in triplicate and quantified using DnaK levels as a reference. Western Blots presented are representative of the expressional trends observed and quantifications were performed using all replicates.

The results obtained for the double adenine substitutions revealed further information required for the transcriptional induction of *armA*. Again, substitution of the two most conserved nucleotides results in a drastic reduction of MIC and Western Blot expressional levels. Interestingly, 'AA' substitutions at position 3 (GA➔AA) reduces the expressional levels of *armA* more than the previous substitution from (GA➔GG). This indicates that in addition to the most conserved thymines at position 2, the guanine of position 3 is absolutely required for the transcriptional induction of the resistance gene.

Both 'GG' and 'AA' substitutions at position #1 and #5, outside of the conserved promoter region, have little to no effect on both the resistance profile and translational levels of ArmA. This further supports that the phenotypic effects observed in the initial mutagenesis assay are a result of disturbing the promoter region rather than altering the putative peptide, which thus far has not demonstrated any involvement with the expression of *armA*. In fact, it appears that the entire promoter region, including generally unconserved nucleotides of sigma factor recognized promoters, is required to generate WT levels of *armA* expression.

-10 Box substitutions

Following the same model as previously described for the -35 box, the -10 box was substituted at all positions with both 'GG' and 'AA' substitutions. Subsequently, the resistance profile and translational assays were assessed via MIC and Western Blot respectively. Figure 45 below, presents the results obtained regarding the expressional levels of *armA* after substituting its -10 box with double guanine nucleotides.

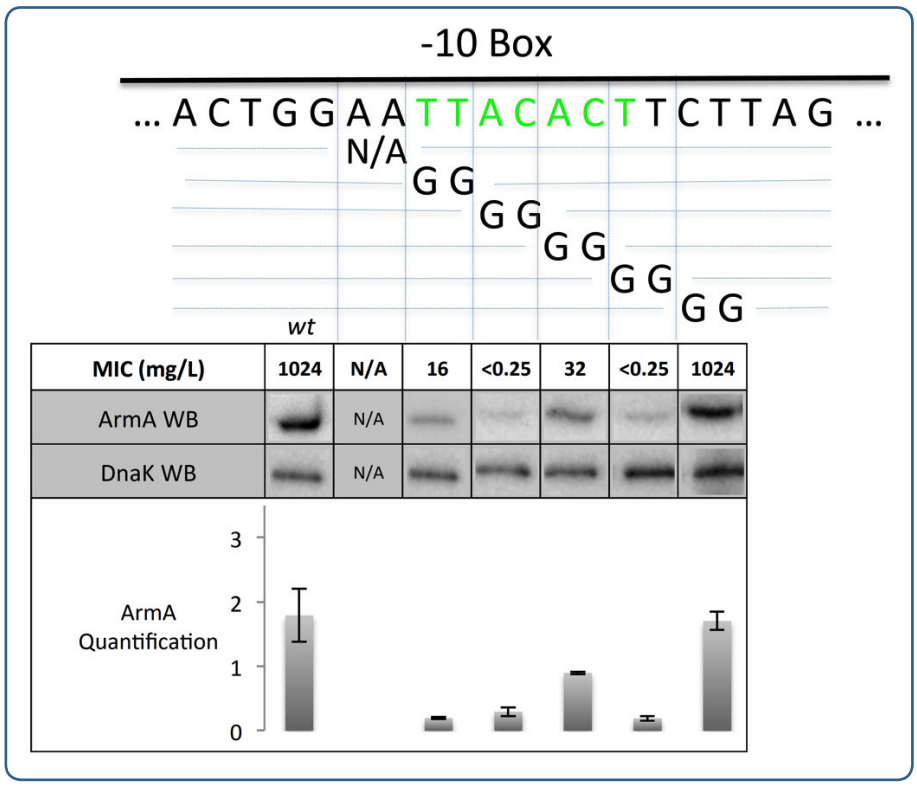


Figure 45. Resistance levels and Western Blot expressional analysis of 'GG' substitutions throughout the -10 box. We were unable to obtain the 'GG' substitutions of position 1 (N/A). Gentamicin MICs were determined according the CLSI and EUCAST guidelines (CLSI, 2017; EUCAST 2017). Western Blots were performed in triplicate and quantified using DnaK levels as a reference. Western Blots presented are representative of the expressional trends observed and quantifications were performed using all replicates.

As observed with the substitutions of the -35 box, this study clearly reveals nucleotides required to produce WT levels of *armA* expression. While substitutions of the less conserved double thymine group of position 2 permit low levels of *armA* expression, substitution of the adenine in position 3 (the most conserved nucleotide of the RpoD recognized -10 box) results in a complete lack of MIC conferred. Futhermore 'GG' substitution of position 4, which is generally unconserved in RpoD recognized promoters, also permits low levels of *armA* expression. While, substitutions of the first thymine of position 5 (the second most conserved nucleotide of the RpoD recognized -10 box) also results in a complete inhibition of *armA* expression. As previously observed, substitutions beyond the 5' and 3' extremes of the promoter regions do not affect the resistance level nor the expressional levels of *armA*.

Figure 46 below, presents the results of the -10 'AA' substitutions as previously described. In this case, however, we have introduced one further position, to evaluate the involvement of an extended -10 region.

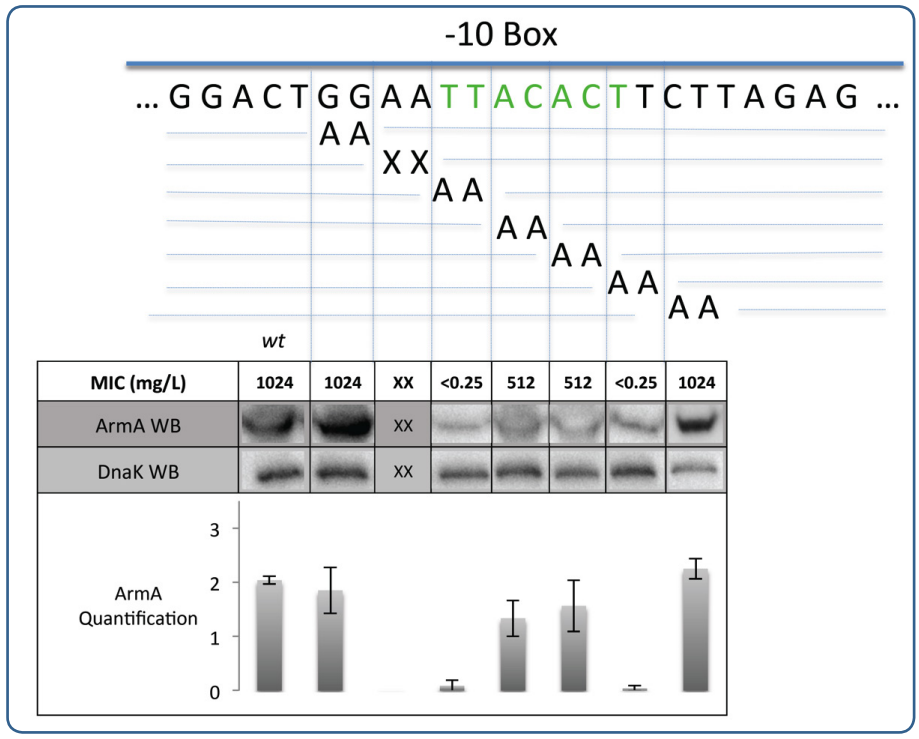


Figure 46. Resistance levels and Western Blot expressional analysis of 'AA' substitutions throughout the -10 box. The XX pertains to nucleotides that were not substituted as the original sequence harbors those nucleotides. Gentamicin MICs were determined according the CLSI and EUCAST guidelines (CLSI, 2017; EUCAST 2017). Western Blots were performed in triplicate and quantified using DnaK levels as a reference. Western Blots presented are representative of the expressional trends observed and quantifications were performed using all replicates.

The results obtained from the AA substitutions of Figure 46, confirmed the involvement of both the double thymine group at position 3 (previously position 2). Furthermore, it confirms the importance of the adenine group in position 4 (previously 3), because in this substitution series this nucleotide is not affected and the MIC and Western Blot levels are close to the WT phenotype. Positions 4 and 5 (previously 3 and 4 respectively) each already harbor an adenine, and as such, only the cytosine in mutated at each postion, which again permit *armA* expressional levels to reach almost that of the WT. Final, as also observed with the 'GG' substitutions (Figure 45), the substitution of the thymine at the 3' extreme of the -10 box results in expressional levels close to the

susceptible DH5 α control. Again mutagenesis of nucleotides adjacent to the promoter regions did not affect expressional levels of *armA*.

Both -35 and -10 promoter regions harbor conserved elements that correspond with conserved promoter regions of the sigma factor RpoD. Mutagenesis of non-conserved nucleotides of this promoter region resulted in a drastic reduction of *armA* expressed although low levels of expression were recorded. Substituting the most conserved nucleotides of the promoter region completely inhibited *armA* expression resulting in the resistance phenotype of the susceptible Dh5 α control. This study also demonstrated, that while some -10 boxes alone are capable of inducing transcription, the expression of *armA* relies on both promoter regions being intact to reach WT expressional levels. Furthermore, substitutions adjacent to the promoter regions had no affect on the expression of *armA*. As these substitutions confer amino acid changes in every occasion the previously described putative leader peptide does not appear to be involved for the expression of WT levels of *armA*.

6.3.3 Growth phase dependent expression of *armA*

All the previously obtained results demonstrate that the expression of *armA* is solely regulated by the above identified promoter region. As this promoter region was identified as a region that has been shown to interact with the σ^{70} factor RpoD, we decided to investigate the expressional profile of *armA* throughout various growth curves. As previously mentioned, the RpoD sigma factors are especially active in periods of rapid cellular growth and is strongly associated with the induction of genes involved in protein synthesis. Therefore, we decided to investigate both the transcriptional and translational levels of *armA* throughout growth. All growth curves were performed in the absence of antibiotics, as we have previously demonstrated that the presence of antibiotics has no effect on the expression of *armA*. Prior to this study the stability of the *armA* bearing vector was shown to be 100% over a period of 48 hours.

6.3.3.1 Translational levels of ArmA over a 12 hour growth period

Primarily, we conducted a Western blot translational assay of both 3xFlagTagged constructions bearing its promoter region within the pACYC-184 cloning vector. All growth curves were conducted at least in triplicates and an average expressional value is presented in the figures below (Figure 47).

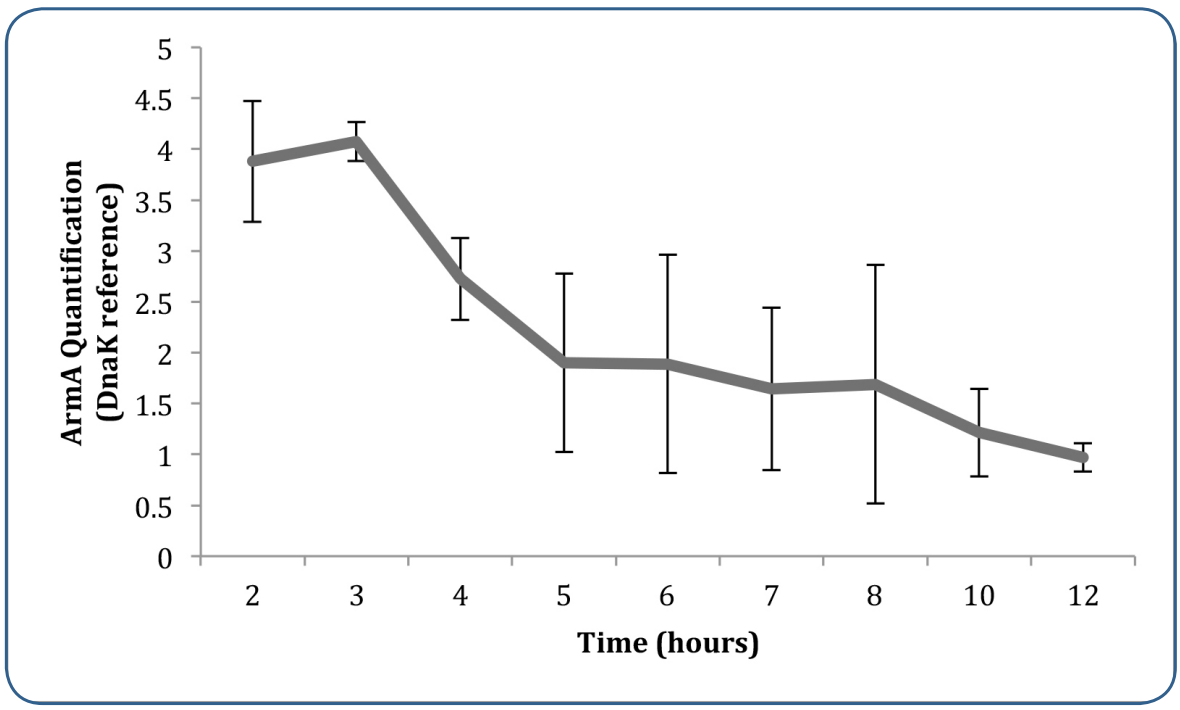


Figure 47. Western Blot expressional levels of armA over a 12 hour growth period. As with all previous Western Blots the levels of armA were quantified using DnaK as a reference. All growth curves and Western Blots were performed repeatedly and the average is presented.

The translational assays conducted over a 12-hour growth curve presented above demonstrate an interesting expressional profile of the 16S rRNA methyltransferase ArmA. *armA* alongside its functional promoter exhibits significantly higher expression between 2-5 hours after inoculation. This time period corresponds with the early exponential growth phase. As the exponential phase of bacterial growth is highly dependent on the upregulated transcription of genes involved in the protein synthesis machinery, it would appear that the expressional profile of *armA* corresponds with that to be expected of a gene induced by the sigma factor RpoD. Such a mechanism would result in expressional levels of the resistance gene that accurately match the expressional levels of its target site within the host.

To further investigate the association of this expressional peak with the identified promoter region, we performed growth curves using the initial 'GG' substitutions performed. The *armA* expressional profile of constructions harboring a 'GG' substitution of the most conserved nucleotides of the -35 box and/or 'GG' substitutions of one highly conserved nucleotide and one less conserved nucleotide of the -10 box were then also analyzed over a growth period of 12 hours (Figure 48).

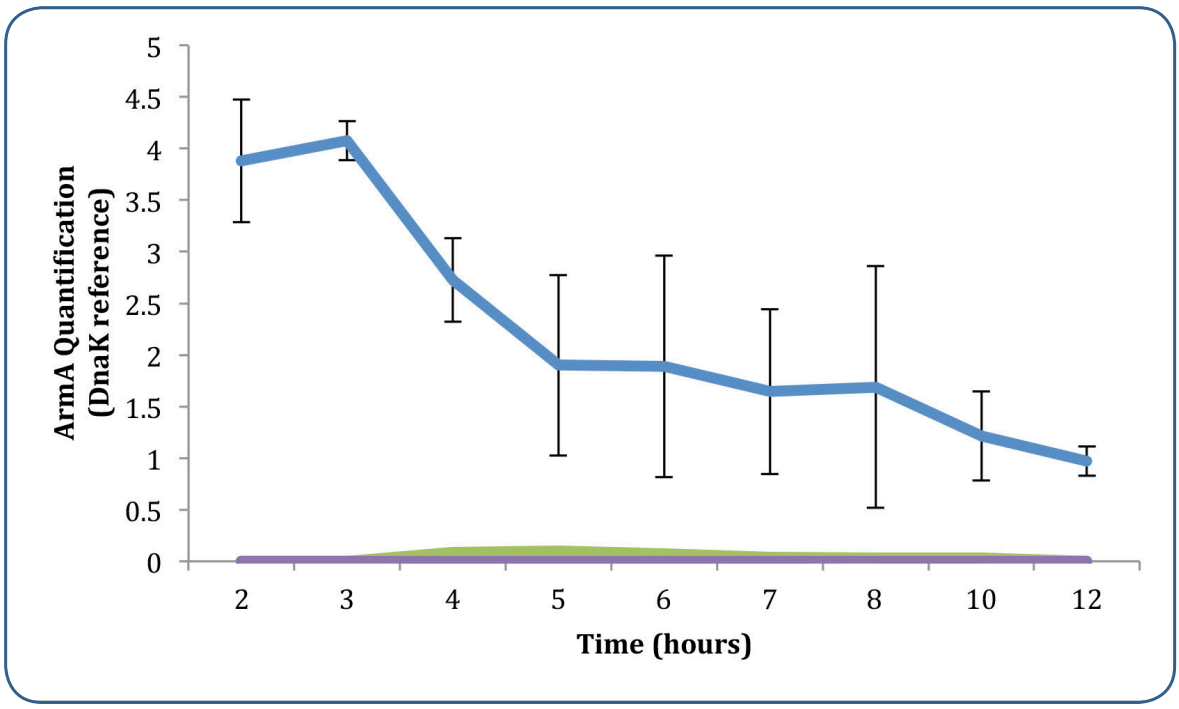


Figure 48. Western Blot expressional levels of armA(wt) (blue) and the initial 'GG' substitutions of the -35 (purple), the -10 (green) and the -35 and -10 double mutant (red – levels too low to be visualized in the figure). As with all previous Western Blots the levels of armA were quantified using DnaK as a reference.

While the very low levels of expression, of the *armA* construction harboring a 'GG' substitution within the -10 box, correspond to previous results, the expressional levels of all of the initial promoter region mutants are virtually 0. To further investigate the associated between the promoter region and the expressional peak observed, these constructions were cloned in the presence of an upstream promoter region. The results of this preliminary study will be discussed in the discussion.

6.3.3.2 Transcriptional levels of *armA* over a 12 hour growth period

To ensure that our constructions are a reflection of the *WT* expressional pattern of *armA*, we then performed RT-qPCRs of this resistance determinant in its *WT* environment on the plasmid pMUR050. To quantify the levels of *armA* transcription we used the chromosomal reference gene *uidA*. The growth curves performed were conducted under exactly the same conditions as the growth curves used to investigate the translational profile in the previous section (Figure 49).

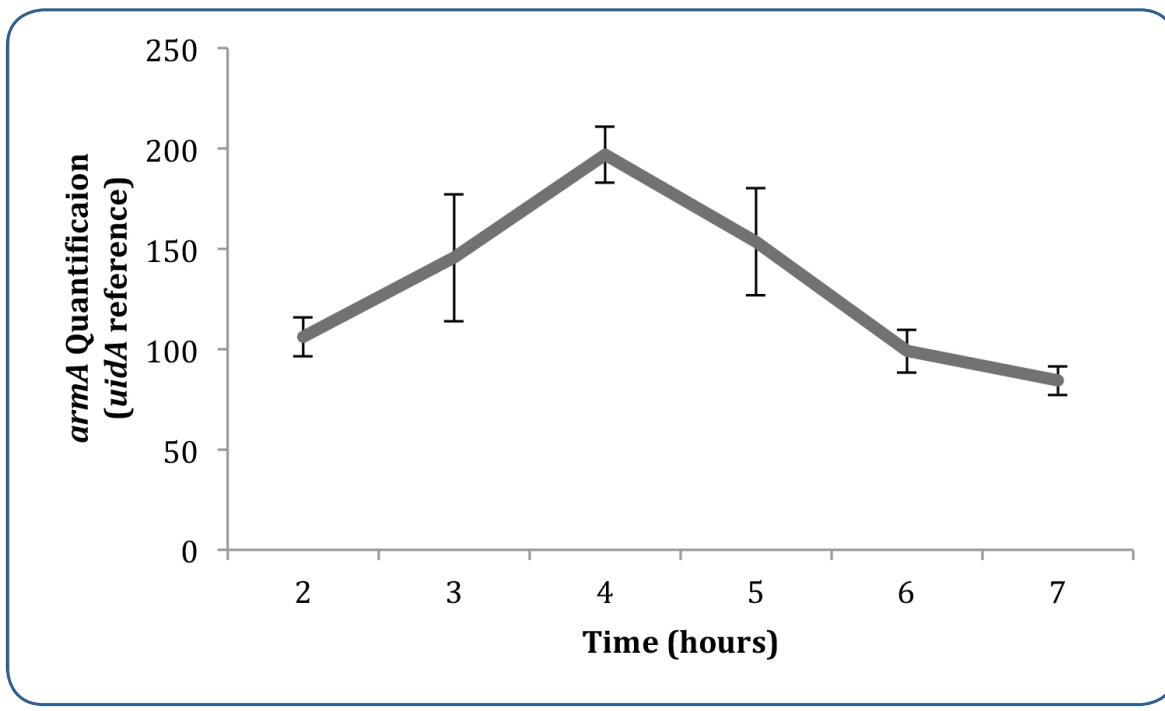
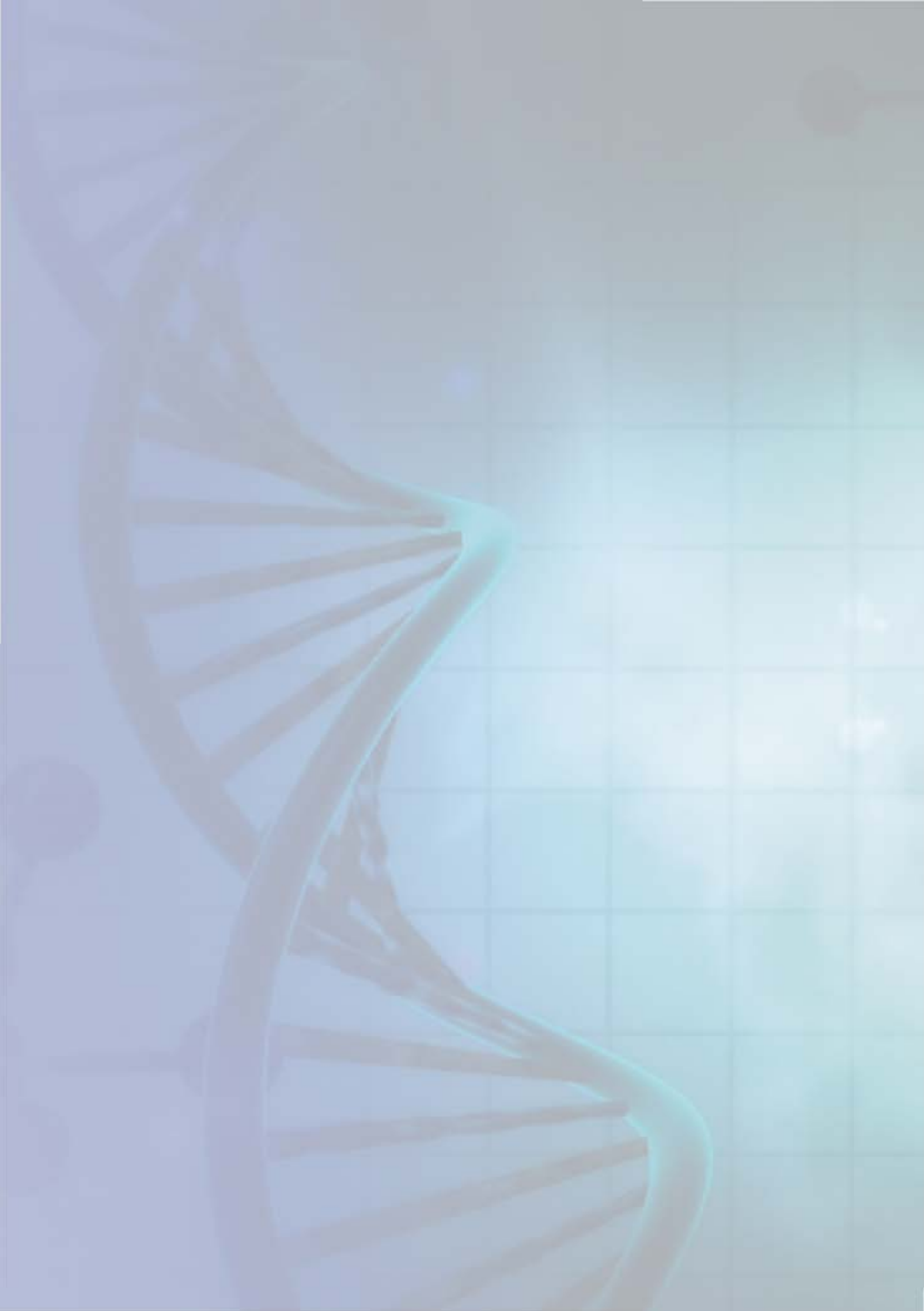


Figure 49. Preliminary RT-qPCR analysis of the transcriptional levels of armA throughout the early exponential phase of growth, using the chromosomal and constitutive uidA gene as a reference. Simultaneous DNA extractions were used to account for the plasmid copy number. Finally, the quantifications were performed using the published formula (San Millan et al., 2014)

The results obtained pertaining to the transcriptional levels of *armA* again demonstrate increased expressional levels between 2-5 hours after inoculation. The transcriptional levels of *armA* double in this period as compared to the *uidA* reference gene. This observed increase again corresponds to the early exponential phase of growth, thus supporting the hypothesis of the recognition of the *rpoD* transcription factor by the identified promoter region. That being said, surprisingly, the RT-qPCR expressional

analysis revealed lower transcriptional levels in the early growth phase than observed in the Western Blot analysis. We believe that this discrepancy is the result of performing extractions in the very early phases of growth. Furthermore, these RT-qPCRs are a preliminary study of the transcriptional levels, further studies of the *armA* transcriptional profile will be described in the discussion.



DISCUSSION

DISCUSSION

7.1 The origin of the 16S rRNA methyltransferases and the evolution of their 5' upstream regions

7.1.1 The coding region conservation of the acquired 16S rRNA methyltransferases

This study commenced with a comprehensive *in silico* investigation of the coding regions of the acquired 16S methyltransferases. The aim of this portion of the study was to identify conserved regions of this resistance determinant that could possibly provide indications of a putative ancestral methyltransferase capable of shedding light on the origins of the resistance determinant and any 'original' regulatory mechanism associated with its expression.

Our results strongly suggest that the acquired 16S rRNA methyltransferases convergently evolved from several different ancestral enzymes (Figure 20). In terms of high-level conservation among the acquired methyltransferases, RmtB and its variant RmtB2, RmtD and its variant RmtD2, and finally RmtE and its variant RmtE2 display by far the highest level of identity, which is to be expected. For the aminoglycoside resistance conferring methyltransferases a single amino acid change is sufficient for the gene to be considered a variant. There are, however, limited examples of acquired methyltransferases that exhibit a degree of conservation, which may indicate a common ancestor for those genes. For example, RmtA and RmtB exhibit the highest degree of amino acid conservation at approximately 81%. This suggests that these two methyltransferases, did at some point, share a common ancestor. To address this further it is interesting to look at the epidemiology of the two genes. Although RmtA was discovered prior to RmtB, RmtB is far more disseminated and described than RmtA. While it is possible that RmtA is a derivative of RmtB and, by chance, it was discovered first, it is also possible that the variations accumulated by RmtB have resulted in a significant

selective advantage of this novel resistance gene. To investigate such a relationship, more information and experimental evidence regarding factors such as biological burden (e.g. fitness cost) of each gene, is required. Furthermore, the more recently identified methyltransferase RmtH shares a relatively high degree of conservation (64.47%) with RmtA (and consequently RmtB – 62.89% - and its variant RmtB 2). These findings would support a putative common ancestor for this cluster of acquired 16S rRNA methyltransferases (RmtA, RmtB (and RmtB 2) and RmtH). However, some of these methyltransferases have not been described very often and further data regarding their resistance profile and more importantly their biological cost would facilitate studies such as this one.

When analyzing such trends within publications there is a slight problem with the acquired 16S rRNA methyltransferases, which is that, generally, studies investigating resistance levels in isolated bacteria do not typically determine the actual MIC conferred by the methyltransferase (unless the publication specifically focuses on these resistance determinants). Generally, aminoglycoside MICs over 128-256 mg/L already strongly indicate the presence of a 16S rRNA methyltransferase, although such MICs can also be the result of numerous modifying enzymes. With regards to putative regulatory domains underlying these resistance levels, it would be of interest to obtain the actual MICs of all acquired 16S rRNA methyltransferases as well as data pertaining to the biological burden they incur on the host.

In summary, this portion of the study demonstrated a high degree of plasticity among the coding regions of the acquired resistance determinants suggesting that they convergently evolved from a number of ancestral genes. To gain further insights into the precise relationship of these genes, further *in vitro* studies with a broader selection of methyltransferases would be required.

7.1.2 Investigating the origin of the acquired methyltransferases

We then decided to expand the analysis to include a number of key intrinsic, resistance conferring, 16S rRNA methyltransferases. As outlined in the introduction, for the producers of antibiotics that act on the prokaryotic ribosome, such as aminoglycosides, intrinsic resistance conferred via target modification is an advantageous strategy, due to the high-level resistance that is achieved. In these producers, such a resistance mechanism has frequently been found within the cluster responsible for the biosynthesis of the antibiotics themselves (Cundliffe *et al*., 2010). The advantages of co-transcribing the resistance gene alongside the antibiotic are two fold. Primarily, the goal of the bacterium is to produce the methyltransferase simultaneously or prior to the production of the antibiotic. By managing the expression of the resistance determinant in such a way, the bacterium can ensure resistance from the auto-toxic effects of the antibiotic. Secondly, depending on the growth phase of the bacterium, the secondary metabolite, that is, the antibiotic, is unlikely to be constitutively expressed and, as such, the constitutive expression of the protective resistance mechanism is not required. Although the transcription and translation of such methyltransferases have been shown to not incur a significant biological burden to the cell, modifications of a machinery as highly conserved as the prokaryotic ribosome has been shown to incur some reduction in host fitness (Lioy *et al.*, 2014).

The above-mentioned organization of intrinsic 16S rRNA methyltransferases is the underlying reason for our *in silico* investigation of the methyltransferase coding regions. The identification of an intrinsic methyltransferase that bears high levels of conservation with any acquired methyltransferase would allow us to investigate the original environment and regulatory framework of the methyltransferases. That being said, the dendrograms generated pertaining to these conservations revealed that, despite NpmA forming a segregated cluster with other N1-A1408 methyltransferases such as KamA-C as well as CmnU, it appears that amongst that cluster the amino acid conservation remains low and that these methyltransferases are also likely to have

evolved convergently. FmrO also shares a degree of conservation with some of the N7-G1405 methyltransferases, although again, no clear ancestral linkage could be made. In fact, the dendrograms demonstrated clusters among the analyzed methyltransferases that clearly correspond with origin (e.g. intrinsic vs. acquired) and function (e.g. target residue). Our results suggest that the ancestral origin of the acquired resistance methyltransferases is not one of the intrinsic resistance conferring methyltransferases described. That being said, based on the GC content of the intrinsic methyltransferases (64-72%) originating from their Gram positive aminoglycoside producers compared to the GC content of the acquired methyltransferases (30-59%), it is possible that the resistance genes have had to change significantly to compensate for their new genetic environment in Gram negative hosts (Liou *et al*., 2006). Liou *et al* went on to demonstrate that the acquired resistance methyltransferase *armA* is capable of functioning in the low GC content Gram positive bacterium *B. subtilis*.

However, based on the results obtained until this point, it appears that, perhaps, these acquired resistance conferring methyltransferases may have originated from RNA methyltransferases other than those responsible for conferring aminoglycoside resistance to the producers. As such, we expanded our analysis yet again to include a select number of housekeeping 16S rRNA methyltransferases. While there are a vast amount of methyltransferases within the bacterial cell, based on the structure of their target molecule, we have limited this study to housekeeping methyltransferases that act on the 16S rRNA. The analyzed housekeeping methyltransferases belong to the ribosomal small subunit methyltransferase (Rsm) family and methylate a variety of residues within the 16S rRNA. The resulting CDS conservation corresponded with previously observed trends of the methyltransferases. Again, clusters were formed based on the origin and function of the gene in question without any significant degrees of conservation among the various groups formed. The aminoacid sequence of ArmA did show a relatively high amino acid conservation with the housekeeping methyltransferase RsmC (71.37%). However, a number of the highly conserved domains within the

methyltransferase regions were disrupted. As such it is insufficient to suggest it as a ancestral origin of ArmA.

In conclusion, the coding regions of the various 16S rRNA methyltransferases did not indicate the direct linkage between the acquired, intrinsic and housekeeping methyltransferases analyzed. While there are clearly many more methyltransferases that could be included within this study, based on the scope of our study and the correctly annotated sequences available, we were unable to identify any strong correlations.

7.1.3 The maintenance vs. evolution of the 5' upstream regions of acquired methyltransferases

Over the past decades, the literature pertaining to gene regulation has increasingly focused on the involvement of the 5' UTR in the accurate transcription and translation of gene products (Breidt *et al.*, 1990; Bailey *et al.*, 2008; Ramu *et al.*, 2009; He *et al.*, 2013). Furthermore, it has become clear that gene products associated with targets that drastically vary in quantity over growth phases, such as the protein synthesis machinery, oftentimes harbor a dynamic mechanism of regulation. While such mechanisms can be transcriptionally and/or translationally mediated, many domains responsible for such an auto-regulation rely on elements harbored within the 5' upstream region of the gene in question. This information, along with the published *in silico* prediction of a regulatory domain upstream of the *arm*A methyltransferase, led us to focus on this region early on in the study (Ramu *et al*., 2009).

The *in silico* dissection of this 5' region was conducted identically to the analyses of the coding regions. Initially, our study focused on the acquired methyltransferases and then we broadened the analyses to include intrinsic methyltransferases with associated non-coding regions on Genbank. Based on the aforementioned limitations regarding the availability of annotated sequences and their 5' regions, we analyzed a region of

400nt upstream of the start codon. Although there are select examples of longer UTRs, the majority of 5' UTR harbored regulatory domains are within 200-300 nucleotides of the coding region (Ramu et al., 2009). Furthermore, a number of both the intrinsic and acquired methyltransferases had a very small number of sequences uploaded. RmtB 2, for example, only had two sequences uploaded to Genbank, neither of which harbored a genetic environment. That being said, we proceeded to analyze the 5' upstream regions of this group of methyltransferases with the data available to us.

Initial alignments of this 400nt selection upstream of the CDS revealed a variety of interesting trends. Primarily, that the clusters generated among the methyltransferases have altered significantly. For example, NpmA, the only acquired N1-A1408 methyltransferase, is no longer segregated from the rest of the methyltransferases as was observed within the coding region alignments. The second very interesting observation was the acquisition of novel 5' regions among some methyltransferase variants (RmtE vs. RmtE 2) and the maintenance of others (RmtD and RmtD 2). Interestingly, although RmtE and its variant RmtE2 exhibit a 99.88% amino acid sequence conservation, the conservation of the 5' upstream region is 32.19%, suggesting the acquisition of a novel upstream environment. Again, as the number of uploaded sequences and publications of RmtE and its variant are very limited, it is difficult to accurately interpret these results. It is possible that RmtE has such a low prevalence because its current genetic environment results in an over expression of the gene, which confers too high of a biological burden to the host (e.g. augmented levels of SAM sequestration). Over the course of our study, we did realize that bacteria carrying 16S rRNA methyltransferases on high copy number plasmids appear to grow at a slower rate, most likely due to such an increased fitness burden. Conversely, it is very possible that, upon moving from one host species to another, *rmtE* acquired an insertion sequence to compensate its biological cost to the new host (Depardieu *et al.*, 2007). However, as mentioned, without further sequences and experimental evidence pertaining to fitness cost and resistance profile, it is difficult to extrapolate more information from this phenomenon.

However, the aforementioned case of RmtE and its variant, is not the only observed case where the coding region displays high degrees of conservation whereas the 5' upstream region appears to be entirely unique. RmtA and RmtB share a 76.32% nucleotide conservation within the coding region, however their 5' regions share less than 23% identity. This phenomenon was also observed with RmtH and RmtA (and consequently RmtB), where in both cases the coding region conservation was elevated whereas the 5' region conservation was <30%. One thing that is clearly demonstrated by this incongruence of 5' region and the CDS is that the 5' upstream regions of the acquired methyltransferases exhibit a high degree of plasticity. Such a genetic plasticity would be a very elegant mechanism for bacteria to develop regulatory domains capable of expressing appropriate quantities of the resistance determinant.

However, despite not finding a universally conserved domain in the upstream region of these methyltransferases (which would not have been expected in genes derived from a variety of ancestors) there were a number of elements that appeared conserved among certain methyltransferases that do not share high levels of CDS conservation. One such example is the relatively high level conservation between the *rmtG* and *rmtD* 5' upstream region (and consequently *rmtD2*) – 63.75% (and 64%). Although these upstream regions harbor scattered fractions of conserved regions, closer analysis does not reveal any clear origin of this sequence. The high degree of conservation observed between the 5' regions of *armA* and *rmtE* however lead to more interesting findings. These two sequences are the only two 5' upstream regions that harbor a highly conserved region upstream of the CDS. We identified a 243nt sequence that was 100% conserved between these two methyltransferases.

Initially, comparisons of this common sequence with online databases revealed only sequences upstream of either *armA* or *rmtE* based on the number of uploaded sequences. However, further investigations using low similarity BLAST parameters revealed an ~80% conservation to a sequence previously identified upstream of a β-lactamase

gene. In this publication this region was identified as a portion of an ISCR20 element (Berçot *et al*., 2010), but the experimental evidence was limited and degree of conservation, perhaps, too low to draw further conclusions. That being said, there are cases of 16S rRNA methyltransferases, such as RmtC, that are expressed by means of insertion sequence elements harboring promoter regions that have been identified to induce the expression of downstream gene products. As outlined in the introduction, insertion sequence borne promoters have been shown to modulate the expression of adjacent resistance genes (Depardieu *et al*., 2007). This evidence does suggest high levels of variability in the upstream region of the acquired 16S rRNA methyltransferases, as the 243nt most likely originate from an insertion sequence that has been interrupted by further insertion sequences. Or, perhaps, both methyltransferases were initially mobilized by the the same insertion sequence which was later deleted.

7.1.4 The conservation of 5' upstream regions with regards to select intrinsic resistance conferring methyltransferases

The results obtained in this section are very interesting with regards to the prior results when comparing the CDS of the acquired methyltransferases to the intrinsic or housekeeping methyltransferases. When comparing dendrograms of the CDS, the various proteins analyzed clustered form based on their origin and function. If the 5' elements of these proteins share a long evolutionary history with the coding region, then arguably they would be expected to also segregate into clusters based on the function and origin of their respective coding region. This was not the case for the analyzed 16S rRNA methyltransferases. The 5' regions of the Kam and Pam family of methyltransferases are no longer clearly distinguished from the N7-G1405 methyltransferases and, in general, the intrinsic Kgm methyltransferases appear to be far less segregated than previously observed with regards to their coding regions.

However, this analysis revealed a single case in which an intrinsic methyltransferase shared a significant identity with one of the acquired regions. The upstream region of the intrinsic resistance conferring methyltransferase *fmrO* shares a 67% nucleotide sequence conservation with the upstream region of the acquired methyltransferase *rmtG.* And as was the case with the alignment between the *rmtD* and *rmtG*, the conservation of *rmtG* with *fmrO* consists of scattered portions of conserved nucleotides, however, the overall nucleotide identity is insufficient to reveal any ancestral origin of this region.

Despite only finding a limited number of conserved regions or domains within the 5' upstream region, whether novel or maintained, they mediate an expressional profile that leads to the high-level aminoglycoside resistance that is typical of the 16S rRNA methyltransferases. While the similarities between *fmrO* and certain acquired resistance methyltransferases could be an indication of a native 5' upstream region, high degrees of sequence conservation would not really be expected based on their different genetic environment. While the intrinsic resistance methyltransferases are generally harbored within chromosomal clusters alongside the antibiotic or intermediate components the acquired methyltransferases are on mobile genetic elements that have varied greatly over time. With regards to the GC content previously mentioned, the possibility does remain that the acquired methyltransferases did originate from intrinsic resistance methyltransferases of Gram positive producers but they have had to evolve greatly to function within Gram positive bacteria, which could explain the diffuse pattern observed among the acquired resistance methyltransferase alignments.

7.1.5 Coding region promoter elements identified

To this point, no overwhelming conservations have been identified within the regions upstream of the analyzed 16S rRNA methyltransferases. The final aspect of the upstream region analyzed as part of the *in silico* investigation were the putative promoter elements. As such, using the Softberry promoter prediction algorithms (Soft-

berry, USA), we proceeded to predict any putative promoters in the regions upstream of the methyltransferase coding regions. These predictions revealed that all acquired 16S methyltransferases harbor a -35 and -10 box within the 400nt analyzed. These alignments were the first aspect of these resistance conferring methyltransferases that exhibited a high degree of conservation among all acquired methyltransferases. The promoter region of prokaryotes typically consists of a 'TTGACA' -35 box and a 'TATAAT' -10 element. The acquired methyltransferase promoter conservations differed by only one nucleotide from the most conserved -35 and -10 elements of prokaryotic promoters. These predictions performed with β-prom did reveal further information though; in a number of the methylases, it predicted a promoter region known to interact with the σ^{70} factor RpoD, which will be further discussed in the following sections.

Interestingly, the promoter prediction algorithms were unable to identify most promoter regions within the available 400nt upstream region of the intrinsic resistance conferring 16S rRNA methyltransferases. This is most likely due to the mechanism by which these intrinsic resistance conferring genes are regulated. As most commonly they are found within biosynthetic clusters alongside the antibiotic it is possible that their respective promoter region is harbored within the 5' region of genes (related to the biosynthesis of the antibiotic or the antibiotic itself) that they are co-transcribed with. One intrinsic resistance conferring methyltransferase, *fmr0*, was predicted to harbor an RpoD recognized promoter region, however, it did not share sequence conservation with the conserved promoter region of the acquired 16S methyltransferases.

At an average distance of 221nt from the resistance gene coding region, the methyltransferase 5' upstream regions correspond in length with untranslated regions demonstrated to harbor a number of regulatory mechanisms. Based on these lengths, we performed an extensive study of putative secondary structures within these regions; however, no secondary structure elements or patterns were identified that correspond with known post-transcriptional regulatory mechanisms.

7.2 The *armA* 5' untranslated region

Using the sequence alignments performed with all available sequences of *armA,* we were able to demonstrate a very high level of conservation within at least 221nt from the *armA* coding region. The conservation observed within this region again suggested the presence of a putative regulatory domain harbored within the 5' UTR. Interestingly, 17.4% of the sequences analyzed harbored an IS10a insertion sequence immediately before this 221nt region (which contains the predicted promoter region). The publications regarding those strains appeared to indicate *WT* levels of resistance conferred by ArmA. This is interesting because this insertion sequence truncates the 5' portion of the leading peptide previously identified as a putative leader peptide involved in a regulatory mechanism mediated by ribosome stalling (Ramu *et al*., 2009). This information led us to question the involvement of this putative leader peptide.

Furthermore, the high degree of conservation allowed us to pinpoint a region of interest for further experimental evaluations of the 5' UTR. As *armA* has only ever been found within Tn1548 (until very recently), a certain degree of conservation is to be expected. However, generally non-coding regions acquire point mutations at a higher rate than coding regions, and it was interesting to find such a high degree of conservation in all *armA* sequences available online. Such a high degree of conservation is typically indicative of a biological role played by that region. When comparing the level of conservation within this highly conserved region to the upstream region, it was evident that there were significantly more point mutations and alterations. Now that we had identified a highly conserved region upstream of *armA,* we were able to design our experimental approach to characterize the untranslated region governing the expression of *armA.*

7.2.1 Defining the extremes of the *armA* transcript

The identification of the precise transcript of *armA* was a crucial first step for the experimental dissection of this promoter region. The 139nt 5' UTR region demonstrated via the RACE was, as mentioned, confirmed multiple times under conditions in the presence and absence of antibiotics. This analysis was performed with *armA* in its *WT* genetic environment harbored on pMUR050. As defining the 5' UTR is of upmost importance to our study, we found it necessary to confirm these results via RT-PCR analysis. The RT-PCR conducted with RNA extractions at 2, 4, 6 and 8 hours were performed to absolutely confirm the absence of any transcript longer than that identified via RACE. The 139nt 5' UTR not only aligns with the highly conserved region identified when assessing the degree of conservation in the upstream region of *armA*, but it also corresponds with the predicted -35 and -10 promoter region. Additionally, this result provides further evidence as to the involvement of the previously identified putative leader peptide. Under no conditions does this region appear to be co-transcribed alongside *armA*, which again suggests that this element is not required for the appropriate expression of *armA*. In fact, this putative leader peptide is most likely a small portion of a transposase gene that has been truncated and altered over time. In the future, it would be of value to insert a frame shift mutation within this putative ORF to conclusively confirm whether or not it influences the expression of *armA*.

7.2.2 Identification of regulatory domains within the 5' UTR

Now that we had identified the extremes of the *armA* transcript, we decided to investigate the phenotypic effects observed upon the truncation of key elements within the 5' UTR. As outlined in the materials and methods, the following constructions were all cloned onto the pAYCY-184 cloning vector. Prior to any further experiments, we performed RNA extractions to confirm the absence of any upstream, vector-borne, promoter regions that may influence the transcription rates of *armA*. This analysis conclusively demonstrated the absence of a vector-borne upstream promoter region, and,

as such, we were able to proceed with the experiments regarding the 5' UTR elements.

The results obtained from this truncational assay were very informative regarding the significance of the identified upstream elements. Both the resistance profile and the Western blot analysis demonstrate a small reduction in the MIC and translational levels, respectively, between the construction bearing the *WT* 5' upstream region. and the construction bearing the 186nt 5' upstream region. While this could be indicative of a role played by the leading peptide, we believe this is based on the novel genetic environment immediately upstream of the promoter region after cloning onto the pACYC-184 vector. Based on environmental methyltransferases that confer high levels of resistance despite having this leader peptide truncated, in addition to later experiments where we induce amino acid changes within this ORF, it is unlikely that it plays a significant role in the expression of *armA*.

Furthermore, this truncational study very clearly identified a region between -139 and -400 nucleotides that is absolutely required for the expression of *armA*. The truncation of the region bearing the putative promoter region leads to a complete reversion of aminoglycoside resistance to the levels of susceptible bacteria. The results obtained lead us to believe that the cause of this is the removal of the promoter region rather than the truncation of the putative leader peptide, as the construction bearing the promoter region, yet missing a significant portion of the leading peptide, still produces high levels of aminoglycoside resistance. The resistance profiles of the constructions bearing a 5' upstream region of 139nt or less demonstrated the absence of any further promoter regions within this area.

7.2.3 Environmental factors that influence *armA* expression

The next step of our study was to gain insights regarding environmental factors that may influence the expression of *armA*. As literature demonstrates, there are a number of auto-regulatory feedback mechanisms that may affect the expression of a resistance determinant (Bailey *et al*., 2008; He *et al.,* 2013). This was especially relevant to *armA* for several reasons. Primarily, *armA* acts on the protein synthesis machinery, which drastically varies in quantity per cell based on the growth phase of the bacterium. As such, to adequately protect the cell, the expression of the methyltransferase should be proportional to the number of ribosomes present at any given moment. Secondly, such a regulatory mechanism is even more likely when considering the ramifications of modifying a macromolecule as highly conserved as the prokaryotic ribosome. Literature has demonstrated that, while neither the transcription nor translation of *armA* confer a significant biological burden to the cell, its action on the ribosome does reduce bacterial fitness (Lioy *et al.*, 2014). This, again, constitutes a scenario in which it would be very beneficial to the host to only express the resistance determinant in conditions where it is absolutely required (i.e. in the presence of the antibiotic). Resistance conferring methyltransferases, such as *ermC* (a 23S rRNA methyltransferase that confers macrolide resistance) have been clearly demonstrated to regulate their expression based on the presence of an inducing antibiotic (Bailey *et al*., 2008). However, this type of regulatory activity is not the only post-transcriptionally mediated mechanism. For a number of aminoglycoside modifying enzymes, riboswitches have been identified that only permit the expression of the resistance gene in the presence of the inducing antibiotic (He *et al*., 2013). Furthermore, taking a step away from resistance conferring gene-products, ribosomal co-factors such as intrinsic methyltransferases and ribosomal proteins have been shown to be, very frequently, regulated in a dynamic manner based on ribosome count (e.g. molecular mimicry)(Green *et al*., 1997).

To determine the expressional profile of armA and to assess whether or not its expression is affected by environmental factors such as the presence of an inducing anti-

biotic, in this case aminoglycosides, or perhaps by the presence of excess methyltransferase, we then constructed a *lacZα* reporter fusion. Initially, we fused the full (400nt) 5' upstream region of *armA* to *lacZα,* as previously described by Bailey *et al* (Bailey *et al*., 2008). All assays were again performed in the confirmed absence of any upstream promoter regions that may influence the transcriptional levels of *armA*. Initially, this reporter construct under the regulation of the full-length 5' upstream region of *armA* was then plated on x-gal to investigate the natural levels of expression in the absence of any known inducing agents. These early results demonstrated that the expression of *lacZα* under the regulation of the *armA* 5' upstream region is constitutive in both the presence and absence of an inducing antibiotic, which in this case would be the aminoglycosides. X-gal plates containing various subinhibitory concentrations of aminoglycosides demonstrated no visual changes in the transcriptional levels of the reporter gene. Furthermore, the placement of a aminoglycoside bearing disk on an x-gal plate did not produce any increase in *lacZα* expression in the regions containing subinhibitory concentrations of aminoglycosides, which would be the case if the transcriptional levels were affected by the presence of aminoglycosides. This is a very important result when investigating the expressional regulation of this resistance methyltransferase.

This result, however, is not enough to suggest the absence of a post-transcriptional mechanism of regulation. Rather than requiring a transcriptional induction, the expression of *armA* could very well be modulated by an inhibitory feedback mechanism. Such regulatory mechanisms are common to gene products involved with protein synthesis. Ribosomal proteins such as (but not limited to) S15, L20 and L35 have been shown to interact with their own 5' UTR if their primary target site has already been acted on, as part of a regulatory mechanism known as molecular mimicry (Guiller et al., 2002; Ehresmann et al., 2004; Guiller et al., 2005). In such cases, the superfluous ribosomal proteins recognize portions of their own mRNA, which mimics their ribosomal target sites to prevent the translational of further, unnecessary, proteins.

To determine whether or not this is the case, we then electroporated the various *lacZα* reporter constructs alongside active methyltransferases *armA* as well as *rmtC* (borne on cloning vectors pTOPO and pBAD respectively). We chose to perform this assay with two different methyltransferases to determine if the effects observed by the presence of active methyltransferases, were the result of the methyltransferases themselves or methylation of residue G1405 of the ribosome. An hypothesis at the time was that, either, superfluous amounts of ArmA are inhibiting the expression of further methyltransferases (by interacting with elements within the 5' UTR) or perhaps, the methylation itself is blocking the translation of further methyltransferase in a ribosome stalling type mechanism. Nevertheless, neither the presence of active ArmA nor the presence of active RmtC demonstrated any effects on the expression of our *lacZα* reporter construct. These results do indicate that, despite the highly conserved, relatively long 5' UTR of *armA*, there do not appear to be any post-transcriptional regulatory effects on the expression of *armA*. Rather, they seem to indicate that constitutive expression of the resistance determinant mediate by the previously identified promoter region.

Although, the RpoD sigma factor induces the transcription of a large number of genes, finding this highly conserved promoter region upstream of *armA* would permit expressional levels to match that of its target-site (i.e. the ribosome). While not being one of the post-transcriptional mechanisms previously described, this would be an elegant form of ensuring sufficient levels of the resistance gene in the cell. A high degree of genetic plasticity in the upstream region of *armA* would allow for a type of trial and error process in which the resistance genes gradually obtain an optimal promoter region.

7.3 Identification of -35 and -10 box nucleotides necessary for the transcriptional induction of *armA*

7.3.1 Promoter region conservation with RpoD recognized promoters

Despite suggestions from literature pertaining to the auto-regulatory capacity of the *armA* 5' UTR, our studies to this point, have revealed an expression that appears constitutive both in the absence and presence of antibiotic pressure and excess gene product. As such, our attention shifted to dissect the promoter region elements required for the accurate expression of *armA*. As previously mentioned, the promoter region identified by the online prediction service was highly conserved with a region known to interact with the σ^{70} factor RpoD. This association is based on high sequence conservation and the precise distance between the -35 and the -10 elements. Literature review demonstrates that this common transcription factor is especially active in conditions of rapid growth (i.e. the exponential phase). This association is based on the fact that the RpoD family of sigma factors is responsible for the induction of a wide range of genes directly associated with ribosomal proteins and the protein synthesis machinery. It has been shown that in *E. coli* the ribosome count in the cell may vary from over 70,000 in fast growing cells to under 20,000 in slow growth conditions (Bremer *et al.*, 2008). For protein synthesis to function correctly, this large variation must be complemented by a precise expression of all the cofactors required to accurately assemble a functioning ribosome. One such mechanism of the cell is to utilize specific groups of transcription factors that induce large clusters of genes associated with required functions.

While some resistance genes might gain selective advantage by being under the stringent control of either an inducing or inhibiting agent, it appears that, based on the high sequence conservation with RpoD recognized promoter regions, *armA* may be co-induced alongside a wide variety of genes responsible for protein synthesis. Such a regulatory mechanism would be an elegant form for an acquired resistance gene to mitigate the biological cost conferred to the cell while ensuring the expression of sufficient

methyltransferase in the cytoplasm to act on all ribosomes within the cell. While target modification is generally associated with very high levels of resistance, a co-induction of the resistance methyltransferase alongside components of its target site may also contribute to the high levels of resistance observed.

7.3.2 Site-directed mutagenesis of most conserved -35 and -10 elements

Initial site-directed mutagenesis was designed to experimentally confirm the involvement of the identified promoter region in the expression of *armA*. Three separate mutants ('GG' substitutions) were initially created targeting the two most conserved nucleotides of the -35 box and two nucleotides of the -10 box (one highly conserved and another less conserved) as well as a final construction harboring both mutations. This was performed as such to not only confirm the involvement of the *in silico* predicted promoter region, but also to assess the degree to which the nucleotide conservation affects the expression of the resistance gene. The results obtained strongly indicate the involvement of this active promoter in the expression of *armA*. Furthermore, the effects of the mutagenesis were more significant when substituting two highly conserved nucleotides of the -35 than they were when mutating one highly conserved and one less conserved nucleotide of the -10 box. With this, we demonstrate that the highly conserved promoter elements of an RpoD recognized region are specifically involved with the expression of *armA*.

Interestingly, the mutagenesis of the -35 region completely inhibited the expression of the downstream gene. Oftentimes, the -10 promoter region alone is capable of recruiting the RNA polymerase to commence transcription. In the case of the *armA* promoter, both elements appear to be required for the expression of the resistance determinant. This further supports the involvement of the RpoD sigma factor as the promoter sites on which they act maintain a high degree of conservation in both the -35 and -10 box of *armA*.

7.3.3 Dissection of the promoter elements required for the expression of *armA*

The results obtained from the first mutants generated, conclusively demonstrate the activity of this promoter region in the expression of *armA*. Our next objective was to further analyze both the specific nucleotides involved in the induction of transcription as well as the 5' and 3' extremes of the -35 and -10 box.

Interestingly, nucleotide substitutions performed adjacent to the conserved -35 and -10 box exhibited no reduction in the MIC and expression levels as assessed by Western Blot. This demonstrates two things: i. the *armA* promoter region conservation, again, aligns with that of the RpoD sigma factor recognized regions; and ii. the effects of the mutagenesis on the expression of *armA* are the result of altering nucleotides within the promoter region. This second point was important to address because this promoter region is harbored within the putative leader peptide upstream of *armA*. While the substitutions alter the promoter region sequence, they also significantly alter the putative amino acid sequence of the leader peptide. However, in total 6 regions adjacent to the promoter sites were substituted and none resulted in any reduction of neither the resistance profile nor the translational levels. Conversely, the substitution of any nucleotide that was highly conserved with the nucleotide sequence of an RpoD recognized region lead to the almost complete lack of expression. Interestingly, substitutions of much less conserved nucleotides also significantly reduced the expression of *armA*, although not completely. This may suggest that the specific promoter region responsible for the expression of *armA* is closely associated with the high-level MIC achieved. Based on the low level of conservation of these nucleotides, we would have expected to witness less of an effect of the resistance profile and the translational levels. In all results of the promoter regions the MIC and Western Blot expressional levels appeared to be proportional.

The extensive mutagenic dissection of this promoter region reveals that every nucleotide of the promoter elements plays a role in the perfect expressional level of *armA*.

While some nucleotides are absolutely required to express *armA*, the substitution of others permits the expression of low levels of *armA*, although nowhere near the levels reached by the intact promoter region.

7.3.4 Growth phase dependent expression of *armA*

Expressional levels of *armA* were consistently shown to be up to 4 times higher throughout the early growth phases between 2-5 hours than during the stationary phase. These time points correspond to the exponential phase observed via spectrophotometry. After the exponential phase, while there is ArmA present, it is in a smaller but consistent quantity. Arguably, the levels of ArmA observed after the exponential phase could be the residual methyltransferase in the cytoplasm despite the lack of newly transcribed/translated protein. While this does correspond to patterns that would be expected of RpoD induction, these results do not conclusively determine whether the peak in protein levels in the cytoplasm is mediated transcriptionally or post-transcriptionally.

Preliminary growth curves conducted of the initial constructions with 'GG' substitutions of the more or less conserved elements of the promoter region are an interesting way of implicating this particular promoter region with the expressional peak observed. Unfortunately, based on the extremely low levels of expression of the mutants, it is difficult draw such conclusions. To address this, we have cloned the various constructions harboring mutated promoter regions in the opposite orientation on the pACYC vector. RT-qPCR analysis demonstrates, that in this orientation, there is a constitutive upstream promoter. These constructions permit baseline levels of expression that allow us to evaluate the expressional peak witness early in the exponential phase. Furthermore, these baseline expressions serve as a control for the expressional peak witnessed in *armA* harboring its native promoter. As the expressional pattern of *armA* with an upstream promoter do not exhibit any form of expressional peak, this allows

us to conclude that the expressional peak in in fact a result of the promoter region and not plasmid copy number related.

As such, the final experiments performed were to assess the transcriptional levels of *armA* throughout growth via RT-qPCR. This study revealed a similar pattern to the previously observed in the Western Blot quantified ArmA growth curves. There is a notably higher transcription of *armA* observed precisely between 2-5 hours, which again corresponds to the exponential growth phase observed via spectrophotometry. Furthermore this aligns with an expressional pattern to be expected from an RpoD induced gene. Additionally, this demonstrates that the levels of transcription and translation of *armA* appear to be proportional, suggesting the absence of post-transcriptional regulatory events. It is, however, unfortunately not sufficient to conclusively demonstrate the association of the RpoD with the expression of *armA*. Additional controls at a known concentration would be necessary to confirm this expressional pattern. To our knowledge, *uidA* is constitutively expressed throughout growth; however, global transcriptional up regulation during the exponential phase may also lead to an expressional peak of *uidA*. Such a trend, would, in fact, strengthen our results as even with regards to the *uidA* expression during the exponential phase we observe a peak in the expression of *armA*. This qPCR was able to further demonstrate that the effects witnessed were a result of the transcriptional induction of *armA* rather than a phenomenon associated with the plasmid copy number. qPCRs performed of DNA exractions using *uidA* as a control demonstrated a relatively constant plasmid count over the growth period. To further elucidate such an association one would have to perform *in vitro* transcription assays in the presence and absence of this specific sigma factor.

Nevertheless, the expression of *armA* does appear to be associated with conditions of rapid growth, which is, of course, mediated by the number of active ribosomes in the cell. This study was performed using the most prevalent 16S rRNA methyltransferase and, perhaps, this particular mechanism of induction underlies its epidemiological

success. The co-induction of *armA* with the protein synthesis machinery and all of its cofactors would be an elegant way to achieve maximum levels of resistance while mitigating the biological cost. Furthermore, this promoter region is the representation of the most conserved promoter sequence among all 16S rRNA methyltransferases. That being said, not all of the promoter regions assessed were predicted to be RpoD family promoter sites. However, based on the levels of promoter region conservation, perhaps the 16S methyltransferases are slowly being selected alongside this specific promoter region. Our results show that any small modification of the promoter region has drastic effects on the resulting resistance profile, which has serious ramifications on the selective advantage conferred by the resistance gene. Whether by horizontal gene transfer (e.g. insertion sequences) or single point mutations, resistance genes may obtain, or develop, promoter regions capable of increased resistance levels with reduced biological cost. Several ISCR elements have been identified to be associated with the 16S rRNA methyltransferases and many other resistance genes (Reference). Of these, a number have been shown to harbor outward facing promoters to selectively regulate the expression of adjacent (resistance) genes (Toleman *et al*., 2006; Depardieu *et al*., 2007; Berçot *et al*., 2010). This variability within the promoter region greatly facilitates the acquisition and subsequent maintenance of a promoter region that, as previously mentioned, maximizes the resistance level conferred while minimizing the biological burden on the host.

In environments with high levels of antibiotic use (e.g. hospitals, farms, etc.) resistance genes harboring a promoter that facilitates extremely high level resistance will confer a significant advantage to the host. Conversely, small modifications, in the case of *armA*, of nucleotides that are not highly conserved as part of the transcription factor binding region may render the bacterial host sensitive to antibiotics in the environment.

7.4 Future work

There are a number of studies that would permit this research line to reveal more details about both the origin of the acquired 16S rRNA methyltransferases as well as their expressional profile. It would be extremely interesting to carry out in depth fitness assays of all 16S rRNA methyltransferases including intrinsic ones. While Liou *et al*, have demonstrated that the fitness cost is a result of the methylation of residue G1405 itself rather than the transcription/translation of the resistance gene, it would be interesting to know if such a biological burden also occurs within the producers. Furthermore, a large scale *in vitro* study with a larger database of prokaryotic RNA methyltransferases would surely provide candidates for ancestral origins of the acquired 16S rRNA methyltransferases. Additionally, the availability of all extended 5' upstream regions would permit further studies of elements within this region.

With regards to the proposed growth dependent expression of *armA*, it would be very interesting to perform *in vitro* transcription assays to assess whether or not, the RpoD sigma factor alone is capable of inducing the transcription of *armA*, or if further factors are involved.

Finally, our next steps are to investigate, the effects of both the aminoglycosides and the acquired 16S rRNA methyltransferases on the fidelity of the prokaryotic ribosome. Using a very novel technique known as ribosome profiling, we intend to elucidate the precise impact that both the antibiotic and this resistance determinant have to the translational rates and efficiency of the ribosome.


∞/0.17/D
N PLAN
20X/0.40

8 CONCLUSIONS

CONCLUSIONS

The conclusions of this doctoral thesis are as follows:

- **FIRST.** The *in silico* analysis of the acquired, intrinsic and housekeeping 16S rRNA methyltransferases revealed that the acquired 16S rRNA methyltransferases convergently evolved from various ancestral origins.

- **PRIMERA.** El análisis *in silico* de las metiltransferasas del ARNr 16S endógenas, intrínsecas y adquiridas indica que estas últimas, las metiltransferasas adquiridas, evolucionaron de forma convergente a partir de varios orígenes ancestrales.

- **SECOND.** Among all the acquired 16S rRNA methyltransferases, there is an overall conservation of the -35 (TTGACG) and -10 (TTACACT) promoter sequence.

- SEGUNDA. Existe una conservación generalizada de las secuencias -35 (TTGACG) y -10 (TTACACT) del promotor de las metiltransferasas del ARNr 16S adquiridas.

- **THIRD.** Using ArmA, the most prevalent 16S rRNA acquired methyltransferase, as a model system, we have identified a 5' untranslated region of 139 nucleotides that is highly conserved among all available sequences.

- TERCERA. Usando como modelo ArmA, la metiltransferasa del ARNr 16S adquirida más prevalente, hemos identificado una región no codificante de 139 nucleótidos altamente conservada entre todas las secuencias disponibles.

- **FOURTH.** Despite having been previously identified as a region harboring post-transcriptional mechanisms of regulation, neither the presence of antibiotics nor active methyltransferase had any consequences on the intracellular levels of ArmA.

- **CUARTA.** A pesar de haberse identificado previamente mecanismos de regulación post-transcripcionales en esa región, la presencia de antibióticos o de metiltransferasa activa no modifican los niveles intracelulares de ArmA.

- **FIFTH.** We have demonstrated the involvement of a promoter region responsible for the expression of *armA*, which shows a high degree of conservation with promoter regions known to interact with the sigma factor RpoD.

- **QUINTA.** Hemos demostrado la existencia de una región promotora responsable de la expresión de *armA*, la cual presenta un alto grado de conservación con regiones promotoras que interactúan con el factor sigma RpoD.

- **SIXTH.** A single nucleotide resolution of this promoter region demonstrates that substitution of its most conserved nucleotides completely inhibits the expression of *armA*. Even mutations of non-conserved nucleotides result in a drastic reduction of the high-level resistance conferred, suggesting that this specific promoter sequence is required to achieve the full resistance phenotype.

- **SEXTA.** A través de una caracterización nucleótido a nucleótido de esta región promotora hemos comprobado que la sustitución de los nucleótidos más conservados de dicha región inhibe completamente la expresión de *armA*. Yendo más allá, mutaciones de los nucleótidos no conservados suponen una drástica reducción de los altos niveles de resistencia conferidos por ArmA, sugiriendo que la secuencia específica de este promotor es necesaria para alcanzar el máximo fenotipo de resistencia.

- **SEVENTH.** The analysis of the *armA* expressional profile throughout bacterial growth demonstrates an increased activity of ArmA in the early exponential phase in-keeping with the transcriptional induction of the σ^{70} factor RpoD.

- **SÉPTIMA.** El análisis del perfil de expresión de *armA* a lo largo del crecimiento bacteriano ha evidenciado una mayor actividad de ArmA en los comienzos de la fase exponencial, acorde con la inducción de la transcripción del factor σ^{70} RpoD.

9

BIBLIOGRAPHY

9 BIBLIOGRAPHY

Abadía, L. P., Courvalin, P., & Perichon, B. (2002). vanE Gene Cluster of Vancomycin-Resistant Enterococcus faecalis BM4405. *Journal of Bacteriology*. https://doi.org/10.1128/JB.184.23.6457-6464.2002

Abraham, E. P., & Chain, E. (1940). An enzyme from bacteria able to destroy penicillin. 1940. *Reviews of Infectious Diseases*. https://doi.org/10.1038/146837a0

Al Sheikh, Y. A., Marie, M. A. M., John, J., Krishnappa, L. G., & Dabwab, K. H. M. (2014). Prevalence of 16S rRNA methylase genes among β -lactamase-producing Enterobacteriaceae clinical isolates in Saudi Arabia. *The Libyan Journal of Medicine*. https://doi.org/10.3402/ljm.v9.24432

Aoyama, T., & Takanami, M. (1988). Supercoiling response of E. coli promoters with different spacer lengths. *Biochimica et Biophysica Acta*, *949*(3), 311–317.

Aoyama, T., Takanami, M., Ohtsuka, E., Taniyama, Y., Marumoto, R., Sato, H., & Ikehara, M. (1983). Essential structure of E. coli promoter: effect of spacer length between the two consensus sequences on promoter function. *Nucleic Acids Research*.

Arias, C. A., Courvalin, P., & Reynolds, P. E. (2000). vanC cluster of vancomycin-resistant Enterococcus gallinarum BM4174. *Antimicrobial Agents and Chemotherapy*, *44*(6), 1660–1666.

Bailey, M., Chettiath, T., & Mankin, A. S. (2008). Induction of erm(C) expression by noninducing antibiotics. *Antimicrobial Agents and Chemotherapy*, *52*(3), 866–874. https://doi.org/10.1128/AAC.01266-07

Barlow, M. (2009). What antimicrobial resistance has taught us about horizontal gene transfer. *Methods in Molecular Biology (Clifton, N.J.)*, *532*, 397–411. https://doi.org/10.1007/978-1-60327-853-9_23

Barne, K. A., Bown, J. A., Busby, S. J., & Minchin, S. D. (1997). Region 2.5 of the Escherichia coli RNA polymerase sigma70 subunit is responsible for the recognition of the "extended-10" motif at promoters. *The EMBO Journal*. https://doi.org/10.1093/emboj/16.13.4034

Beauclerk, A. A., & Cundliffe, E. (1987). Sites of action of two ribosomal RNA methylases responsible for resistance to aminoglycosides. *Journal of Molecular Biology*, *193*(4), 661–671.

Bennett, P. M. (2004). Genome plasticity: insertion sequence elements, transposons and integrons, and DNA rearrangement. *Methods in Molecular Biology (Clifton, N.J.)*, *266*, 71–113. https://doi.org/10.1385/1-59259-763-7:071

Benveniste, R., & Daviest, J. (1973). Aminoglycoside Antibiotic-Inactivating Enzymes in Actinomycetes Similar to Those Present in Clinical Isolates of Antibiotic-Resistant Bacteria, *70*(8), 2276–2280.

Benvenisti, L., Koby, S., Rutman, A., Giladi, H., Yura, T., & Oppenheim, A. B. (1995). Cloning and primary sequence of the rpoH gene from pseudomonas aeruginosa. *Gene*, *155*(1), 73–76. https://doi.org/http://doi.org/10.1016/0378-1119(94)00829-H

Berçot, B., Poirel, L., & Nordmann, P. (2008). Plasmid-mediated 16S rRNA methylases among extended-spectrum β -lactamase-producing Enterobacteriaceae isolates. *Antimicrobial Agents and Chemotherapy*, *52*(12), 4526–4527. https://doi.org/10.1128/AAC.00882-08

Berçot, B., Poirel, L., Silva-Sanchez, J., & Nordmann, P. (2010). Association of the Extended-Spectrum β -Lactamase Gene bla(TLA-1) with a Novel ISCR Element, ISCR20 . *Antimicrobial Agents and Chemotherapy*. https://doi.org/10.1128/AAC.00075-10

Beyer, S., Distler, J., & Piepersberg, W. (1996). The str gene cluster for the biosynthesis of 5'-hydroxystreptomycin in Streptomyces glaucescens GLA.0 (ETH 22794): new operons and evidence for pathway-specific regulation by StrR. *Molecular & General Genetics : MGG*, *250*(6), 775–784.

Bibb, M. J., Janssen, G. R., & Ward, J. M. (1985). Cloning and analysis of the promoter region of the erythromycin resistance gene (ermE) of Streptomyces erythraeus. *Gene*, *38*(1–3), 215–226.

Böttger, E. C., Springer, B., Prammananan, T., Kidan, Y., & Sander, P. (2001). Structural basis for selectivity and toxicity of ribosomal antibiotics. *EMBO Reports*, *2*(4), 318–323. https://doi.org/10.1093/embo-reports/kve062

Breidt, F., & Dubnau, D. (1990). Identification of cis-acting sequences required for translational autoregulation of the ermC methylase. *Journal of Bacteriology*, *172*(7), 3661–3668.

Bremer, H., & Dennis, P. P. (2008). Modulation of Chemical Composition and Other Parameters of the Cell at Different Exponential Growth Rates. *EcoSal Plus*, *3*(1). https://doi.org/10.1128/ecosal.5.2.3

Browning, D. F., & Busby, S. J. (2004). The regulation of bacterial transcription initiation. *Nature Reviews. Microbiology*, *2*(1), 57–65. https://doi.org/10.1038/nrmicro787

Browning, D. F., & Busby, S. J. W. (2016). Local and global regulation of transcription initiation in bacteria. *Nature Reviews. Microbiology*. England. https://doi.org/10.1038/nrmicro.2016.103

Bryan, L. E., & Kwan, S. (1983). Roles of Ribosomal Binding , Membrane Potential , and Electron Transport in Bacterial Uptake of Streptomycin and Gentamicin, *23*(6), 835–845.

Bud, R. (2007). *Penicillin: Triumph and Tragedy*. Oxford University Press. Retrieved from https://books.google.es/books?id=At0nwXNJXHcC

Bueno, M. F. C., Francisco, G. R., De Oliveira Garcia, D., & Doi, Y. (2016). Complete sequences of multidrug resistance plasmids bearing rmtD1 and rmtD2 16S rRNA methyltransferase genes. *Antimicrobial Agents and Chemotherapy*, *60*(3), 1928–1931. https://doi.org/10.1128/AAC.02562-15

Bueno, M. F. C., Francisco, G. R., O'Hara, J. A., de Oliveira Garcia, D., & Doi, Y. (2013). Coproduction of 16S rRNA methyltransferase RmtD or RmtG with KPC-2 and CTX-M group extended-spectrum beta-lactamases in Klebsiella pneumoniae. *Antimicrobial Agents and Chemotherapy*, *57*(5), 2397–2400. https://doi.org/10.1128/AAC.02108-12

Bugg, T. D. H., Wright, G. D., Dutka-Malen, S., Arthur, M., Courvalin, P., & Walsh, C. T. (1991). Molecular basis for vancomycin resistance in Enterococcus faecium BM4147: biosynthesis of a depsipeptide peptidoglycan precursor by vancomycin resistance proteins VanH and VanA. *Biochemistry*, *30*(43), 10408–10415. https://doi.org/10.1021/bi00107a007

Busse, H. J., Wostmann, C., & Bakker, E. P. (1992). The bactericidal action of streptomycin: membrane permeabilization caused by the insertion of mistranslated proteins into the cytoplasmic membrane of Escherichia coli and subsequent caging of the antibiotic inside the cells due to degradation of these pr. *Journal of General Microbiology*, *138*(3), 551–561. https://doi.org/10.1099/00221287-138-3-551

Carattoli, A., Bertini, A., Villa, L., Falbo, V., Hopkins, K. L., & Threlfall, E. J. (2005). Identification of plasmids by PCR-based replicon typing. *Journal of Microbiological Methods*, *63*(3), 219–228. https://doi.org/10.1016/j.mimet.2005.03.018

Carattoli, A., Villa, L., Poirel, L., Bonnin, R. A., & Nordmann, P. (2012). Evolution of IncA/C blaCMY-(2)-carrying plasmids by acquisition of the blaNDM-(1) carbapenemase gene. *Antimicrobial Agents and Chemotherapy*, *56*(2), 783–786. https://doi.org/10.1128/AAC.05116-11

Cate, J. H., Yusupov, M. M., Yusupova, G. Z., Earnest, T. N., & Noller, H. F. (1999). X-ray Crystal Structures of 70S Ribosome Functional Complexes. *Science*, *285*(5436), 2095–2104. https://doi.org/10.1126/science.285.5436.2095

Chandler, M., & Mahillon, J. (2002). Insertion Sequences Revisited. In *Mobile DNA II* (pp. 305–366). American Society of Microbiology. Retrieved from http://www.asmscience.org/content/book/10.1128/9781555817954.chap15

Collis, C. M., Kim, M.-J., Stokes, H. W., & Hall, R. M. (2002). Integron-encoded IntI integrases preferentially recognize the adjacent cognate attI site in recombination with a 59-be site. *Molecular Microbiology*, *46*(5), 1415–1427.

Cooksey, R. C., Morlock, G. P., McQueen, A., Glickman, S. E., & Crawford, J. T. (1996). Characterization of streptomycin resistance mechanisms among Mycobacterium tuberculosis isolates from patients in New York City. *Antimicrobial Agents and Chemotherapy*, *40*(5), 1186–1188.

Courvalin, P. (1994). Transfer of antibiotic resistance genes between gram-positive and gram-negative bacteria. *Antimicrobial Agents and Chemotherapy*.

Cundliffe, E. (1989). How antibiotic-producing organisms avoid suicide. *Annual Review of Microbiology*, *43*, 207–233. https://doi.org/10.1146/annurev.mi.43.100189.001231

Cundliffe, E., & Demain, A. L. (2010). Avoidance of suicide in antibiotic-producing microbes. *Journal of Industrial Microbiology & Biotechnology*, *37*(7), 643–672. https://doi.org/10.1007/s10295-010-0721-x

Cundliffe, E., & Zalacain, M. (1991). Cloning of tlrD, a fourth resistance gene, from the tylosin producer, Streptomyces fradiae. *Gene*, *97*(1), 137–142.

Cunliffe, H. E., Merriman, T. R., & Lamont, I. L. (1995, May). Cloning and characterization of pvdS, a gene required for pyoverdine synthesis in Pseudomonas aeruginosa: PvdS is probably an alternative sigma factor. *Journal of Bacteriology*.

Davies, J. E. (1997). Origins, acquisition and dissemination of antibiotic resistance determinants. *Ciba Foundation Symposium*, *207*, 15–35.

Depardieu, F., Podglajen, I., Leclercq, R., Collatz, E., & Courvalin, P. (2007). Modes and Modulations of Antibiotic Resistance Gene Expression. *Clinical Microbiology Reviews*. https://doi.org/10.1128/CMR.00015-06

Department of Commerce and Business administration. (1906). *Mortality Statistics*. Retrieved from https://www.cdc.gov/nchs/data/vsushistorical/mortstatsh_1906.pdf

Distler, J., Ebert, A., Mansouri, K., Pissowotzki, K., Stockmann, M., & Piepersberg, W. (1987). Gene cluster for streptomycin biosynthesis in Streptomyces griseus: nucleotide sequence of three genes and analysis of transcriptional activity. *Nucleic Acids Research*, *15*(19), 8041–8056.

Doi, Y., Adams-Haduch, J. M., & Paterson, D. L. (2008). Genetic environment of 16S rRNA methylase gene rmtD. *Antimicrobial Agents and Chemotherapy*, *52*(6), 2270–2272. https://doi.org/10.1128/AAC.00037-08

Doi, Y., de Oliveira Garcia, D., Adams, J., & Paterson, D. L. (2007). Coproduction of novel 16S rRNA methylase RmtD and metallo-beta-lactamase SPM-1 in a panresistant Pseudomonas aeruginosa isolate from Brazil. *Antimicrobial Agents and Chemotherapy*, *51*(3), 852–856. https://doi.org/10.1128/AAC.01345-06

Doi, Y., Wachino, J., & Arakawa, Y. (2016). Aminoglycoside Resistance: The Emergence of Acquired 16S Ribosomal {RNA} Methyltransferases. *Infectious Disease Clinics of North America*, *30*(2), 523–537. https://doi.org/http://doi.org/10.1016/j.idc.2016.02.011

Doi, Y., Yokoyama, K., Yamane, K., Wachino, J., Shibata, N., Yagi, T., … Arakawa, Y. (2004). Plasmid-Mediated 16S rRNA Methylase in. *Society*, *48*(2), 491–496. https://doi.org/10.1128/AAC.48.2.491

Dombroski, A. J., Johnson, B. D., Lonetto, M., & Gross, C. A. (1996). The sigma subunit of Escherichia coli RNA polymerase senses promoter spacing. *Proceedings of the National Academy of Sciences of the United States of America*.

Edson, R. S., & Terrell, C. L. (1999). The Aminoglycosides. *Mayo Clinic Proceedings*, *74*(5), 519–528. https://doi.org/http://doi.org/10.4065/74.5.519

Ehresmann, C., Ehresmann, B., Ennifar, E., Dumas, P., Garber, M., Mathy, N., … Serganov, A. (2004). Molecular mimicry in translational regulation: the case of ribosomal protein S15. *RNA Biology*, *1*(1), 66–73.

Eliopoulos, G. M. (1989). Synergism and antagonism. *Infectious Disease Clinics of North America*, *3*(3), 399–406.

European Medicines Agency. (2010). *Annual report* (Vol. 44). Retrieved from http://www.ema.europa.eu/docs/en_GB/document_library/Annual_report/2010/05/WC500090712.pdf

Fair, R. J., & Tor, Y. (2014). Perspectives in Medicinal Chemistry Antibiotics and Bacterial Resistance in the 21st Century. *Perspectives in Medicinal Chemistry*, 25–64. https://doi.org/10.4137/PMC.S14459.Received

Feldman, M. B., Terry, D. S., Altman, R. B., & Blanchard, S. C. (2010). Aminoglycoside activity observed on single pre-translocation ribosome complexes. *Nat Chem Biol*, *6*(1), 54–62. Retrieved from http://dx.doi.org/10.1038/nchembio.274

Finken, M., Kirschner, P., Meier, A., Wrede, A., & Bottger, E. C. (1993). Molecular basis of streptomycin resistance in Mycobacterium tuberculosis: alterations of the ribosomal protein S12 gene and point mutations within a functional 16S ribosomal RNA pseudoknot. *Molecular Microbiology*, *9*(6), 1239–1246.

Fleming, A. (1929). On the Antibacterial Action of Cultures of a Penicillium, with Special Reference to their Use in the Isolation of B. influenzæ. *British Journal of Experimental Pathology*.

Forge, A., & Schacht, J. (2000). Aminoglycoside Antibiotics, *506*, 3–22.

Forrest, R. D. (1982). Early history of wound treatment. *Journal of the Royal Society of Medicine*.

Fourmy, D., Recht, M. I., Blanchard, S. C., & Puglisi, J. D. (1996). Structure of the A site of Escherichia coli 16S ribosomal RNA complexed with an aminoglycoside antibiotic. *Science*, *274*(5291), 1367 LP-1371. Retrieved from http://science.sciencemag.org/content/274/5291/1367.abstract

Freeman, C. D., Nicolau, D. P., Belliveau, P. P., & Nightingale, C. H. (1997). Once-daily dosing of aminoglycosides: review and recommendations for clinical practice. *The Journal of Antimicrobial Chemotherapy*, *39*(6), 677–686.

Galimand, M., Courvalin, P., & Lambert, T. (2003). Plasmid-mediated high-level resistance to aminoglycosides in Enterobacteriaceae due to 16S rRNA methylation. *Antimicrobial Agents and Chemotherapy*, *47*(8), 2565–2571.

Galimand, M., Courvalin, P., & Lambert, T. (2012). RmtF, a new member of the aminoglycoside resistance 16S rRNA N7 G1405 methyltransferase family. *Antimicrobial Agents and Chemotherapy*, *56*(7), 3960–3962. https://doi.org/10.1128/AAC.00660-12

Galimand, M., Sabtcheva, S., & Lambert, T. (2005). Worldwide Disseminated. *Society*, *49*(7), 2949–2953. https://doi.org/10.1128/AAC.49.7.2949

Garneau-Tsodikova, S., & Labby, K. J. (2016). Mechanisms of Resistance to Aminoglycoside Antibiotics: Overview and Perspectives. *MedChemComm*, *7*(1), 11–27. https://doi.org/10.1039/C5MD00344J

Gołębiewski, M., Kern-Zdanowicz, I., Zienkiewicz, M., Adamczyk, M., Żyliǹska, J., Baraniak, A., … Cegłowski, P. (2007). Complete Nucleotide Sequence of the pCTX-M3 Plasmid and Its Involvement in Spread of the Extended-Spectrum β-Lactamase Gene bla(CTX-M-3) . *Antimicrobial Agents and Chemotherapy*. https://doi.org/10.1128/AAC.00457-07

González-Zorn, B., Teshager, T., Casas, M., Porrero, M. C., Moreno, M. A., Courvalin, P., & Domínguez, L. (2005). armA and Aminoglycoside Resistance in Escherichia coli. *Emerging Infectious Diseases*, *11*(6), 954–956. https://doi.org/10.3201/eid1106.040553

Granier, S. A., Hidalgo, L., Millan, A. S., Escudero, J. A., Gutierrez, B., Brisabois, A., & Gonzalez-Zorn, B. (2011). ArmA methyltransferase in a monophasic Salmonella enterica isolate from food. *Antimicrobial Agents and Chemotherapy*, *55*(11), 5262–5266. https://doi.org/10.1128/AAC.00308-11

Green and, R., & Noller, H. F. (1997). Ribosomes and Translation. *Annual Review of Biochemistry*, *66*(1), 679–716. https://doi.org/10.1146/annurev.biochem.66.1.679

Gross, C. A., Chan, C., Dombroski, A., Gruber, T., Sharp, M., Tupy, J., & Young, B. (1998). The functional and regulatory roles of sigma factors in transcription. *Cold Spring Harbor Symposia on Quantitative Biology*, *63*, 141–155.

Gruber, T. M., & Gross, C. A. (2003). Multiple sigma subunits and the partitioning of bacterial transcription space. *Annual Review of Microbiology*, *57*, 441–466. https://doi.org/10.1146/annurev.micro.57.030502.090913

Guillier, M., Allemand, F., Dardel, F., Royer, C. A., Springer, M., & Chiaruttini, C. (2005). Double molecular mimicry in Escherichia coli: Binding of ribosomal protein L20 to its two sites in mRNA is similar to its binding to 23S rRNA. *Molecular Microbiology*, *56*(6), 1441–1456. https://doi.org/10.1111/j.1365-2958.2005.04644.x

Guillier, M., Allemand, F., Raibaud, S., Dardel, F., Springer, M., & Chiaruttini, C. (2002). Translational feedback regulation of the gene for L35 in Escherichia coli requires binding of ribosomal protein L20 to two sites in its leader mRNA: a possible case of ribosomal RNA-messenger RNA molecular mimicry. *RNA (New York, N.Y.)*, *8*(7), 878–89. https://doi.org/10.1017/S1355838202029084

Gutell, R. R. (1994). Collection of small subunit (16S- and 16S-like) ribosomal RNA structures: 1994. *Nucleic Acids Research*, *22*(17), 3502–3507.

Gutierrez, B., Escudero, J. A., San Millan, A., Hidalgo, L., Carrilero, L., Ovejero, C. M., … Gonzalez-Zorn, B. (2012). Fitness cost and interference of Arm/Rmt aminoglycoside resistance with the RsmF housekeeping methyltransferases. *Antimicrobial Agents and Chemotherapy*, *56*(5), 2335–2341. https://doi.org/10.1128/AAC.06066-11

Hawley, D. K., & McClure, W. R. (1983). Compilation and analysis of Escherichia coli promoter DNA sequences. *Nucleic Acids Research*.

Hayashi, S. F., Norcia, L. J., Seibel, S. B., & Silvia, A. M. (1997). Structure-activity relationships of hygromycin A and its analogs: protein synthesis inhibition activity in a cell free system. *The Journal of Antibiotics*, *50*(6), 514–521.

He, W., Zhang, X., Zhang, J., Jia, X., Zhang, J., Sun, W., … Murchie, A. I. H. (2013). Riboswitch control of induction of aminoglycoside resistance acetyl and adenyl-transferases. *RNA Biology*, *10*(8), 1266–1273. https://doi.org/10.4161/rna.25757

Heilman, D. H. (1945). Cytotoxicity of streptomycin and streptothricin. *Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine (New York, N.Y.)*, *60*, 365–367.

Hettig, R. A., & Adcock, J. D. (1946). Studies on the Toxicity of Streptomycin for Man: A Preliminary Report. *Science (New York, N.Y.)*, *103*(2673), 355–357. https://doi.org/10.1126/science.103.2673.355

Holmes, D. J., & Cundliffe, E. (1991). Analysis of a ribosomal RNA methylase gene from Streptomyces tenebrarius which confers resistance to gentamicin. *Molecular & General Genetics : MGG*, *229*(2), 229–237.

Holmes, D. J., Drocourt, D., Tiraby, G., & Cundliffe, E. (1991). Cloning of an aminoglycoside-resistance-encoding gene, kamC, from Saccharopolyspora hirsuta: comparison with kamB from Streptomyces tenebrarius. *Gene*, *102*(1), 19–26.

Hopkins, K. L., Escudero, J. A., Hidalgo, L., & Gonzalez-Zorn, B. (2010). 16S rRNA methyltransferase RmtC in Salmonella enterica serovar Virchow. *Emerging Infectious Diseases*, *16*(4), 712–715. https://doi.org/10.3201/eid1604.090736

Horinouchi, S. (2002). A microbial hormone, A-factor, as a master switch for morphological differentiation and secondary metabolism in Streptomyces griseus. *Frontiers in Bioscience : A Journal and Virtual Library*, *7*, d2045-57.

Hu, F., Munoz-Price, L. S., DePascale, D., Rivera, J. I., & Doi, Y. (2014). Klebsiella pneumoniae Sequence Type 11 Isolate Producing RmtG 16S rRNA Methyltransferase from a Patient in Miami, Florida. *Antimicrobial Agents and Chemotherapy*. 1752 N St., N.W., Washington, DC. https://doi.org/10.1128/AAC.02632-14

Hughes, K. T., & Mathee, K. (1998). The anti-sigma factors. *Annual Review of Microbiology*, *52*, 231–286. https://doi.org/10.1146/annurev.micro.52.1.231

Husain, N., Obranic, S., Koscinski, L., Seetharaman, J., Babic, F., Bujnicki, J. M., … Sivaraman, J. (2011). Structural basis for the methylation of A1408 in 16S rRNA by a panaminoglycoside resistance methyltransferase NpmA from a clinical isolate and analysis of the NpmA interactions with the 30S ribosomal subunit. *Nucleic Acids Research*, *39*(5), 1903–1918. https://doi.org/10.1093/nar/gkq1033

Ioannis, B., Samonis, G., Vardakas, K. Z., Chrysanthopoulou, S., & Falagas, M. E. (2005). Effect of Aminoglycoside and β-Lactam Combination Therapy versus β-Lactam Monotherapy on the Emergence of Antimicrobial Resistance: A Meta-analysis of Randomized, Controlled Trials. *Clinical Infectious Diseases*, *41*(2), 149. https://doi.org/10.1086/430912

Jana, M. S. (2006). Molecular understanding of aminoglycoside action and resistance, 140–150. https://doi.org/10.1007/s00253-005-0279-0

Jenner, L., Demeshkina, N., Yusupova, G., & Yusupov, M. (2010). Structural rearrangements of the ribosome at the tRNA proofreading step. *Nature Structural & Molecular Biology*, *17*(9), 1072–1078. https://doi.org/10.1038/nsmb.1880

Jia, X., Zhang, J., Sun, W., He, W., Jiang, H., Chen, D., & Murchie, A. I. H. (2013). Riboswitch control of aminoglycoside antibiotic resistance. *Cell*, *152*(1–2), 68–81. https://doi.org/10.1016/j.cell.2012.12.019

Jiang, H., Talaska, A. E., Schacht, J., & Sha, S.-H. (2007). Oxidative imbalance in the aging inner ear. *Neurobiology of Aging*, *28*(10), 1605–1612. https://doi.org/http://doi.org/10.1016/j.neurobiolaging.2006.06.025

Jin, J. S., Kwon, K. T., Moon, D. C., & Lee, J. C. (2009). Emergence of 16S rRNA methylase rmtA in colistin-only-sensitive Pseudomonas aeruginosa in South Korea. *International Journal of Antimicrobial Agents*. Netherlands. https://doi.org/10.1016/j.ijantimicag.2008.10.024

Kadurugamuwa, J. L., Clarke, A. J., & Beveridge, T. J. (1993). Surface action of gentamicin on Pseudomonas aeruginosa. *Journal of Bacteriology*.

Karah, N., Dwibedi, K., Sjöström, K., Edquist, P., Johansson, A., Wai, N., & Uhlin, E. (2016). Novel Aminoglycoside Resistance Transposons and Transposon- Derived Circular Forms Detected in Carbapenem-Resistant Acinetobacter baumannii Clinical Isolates, *60*(3), 1801–1818. https://doi.org/10.1128/AAC.02143-15.Address

Karlowsky, J. A., Hoban, D. J., Zelenitsky, S. A., & Zhanel, G. G. (1997). Altered denA and anr gene expression in aminoglycoside adaptive resistance in Pseudomonas aeruginosa. *The Journal of Antimicrobial Chemotherapy*, *40*(3), 371–376.

Karpen, M. E., & deHaseth, P. L. (2015). Base flipping in open complex formation at bacterial promoters. *Biomolecules*, *5*(2), 668–678. https://doi.org/10.3390/biom5020668

Kelemen, G. H., Zalacain, M., Culebras, E., Seno, E. T., & Cundliffe, E. (1994). Transcriptional attenuation control of the tylosin-resistance gene tlrA in Streptomyces fradiae. *Molecular Microbiology*, *14*(4), 833–842.

Ketzer, P., Kaufmann, J. K., Engelhardt, S., Bossow, S., von Kalle, C., Hartig, J. S., … Nettelbeck, D. M. (2014). Artificial riboswitches for gene expression and replication control of DNA and RNA viruses. *Proceedings of the National Academy of Sciences* , *111*(5), E554–E562. Retrieved from http://www.pnas.org/content/111/5/E554.abstract

Kotra, L. P., Haddad, J., & Mobashery, S. (2000). Aminoglycosides: Perspectives on mechanisms of action and resistance and strategies to counter resistance. *Antimicrobial Agents and Chemotherapy*, *44*(12), 3249–3256. https://doi.org/10.1128/AAC.44.12.3249-3256.2000

Kudo, F., & Eguchi, T. (2016). Aminoglycoside Antibiotics: New Insights into the Biosynthetic Machinery of Old Drugs. *Chemical Record*, *16*(1), 4–18. https://doi.org/10.1002/tcr.201500210

Kumar, A., Malloch, R. A., Fujita, N., Smillie, D. A., Ishihama, A., & Hayward, R. S. (1993). The minus 35-recognition region of Escherichia coli sigma 70 is inessential for initiation of transcription at an "extended minus 10" promoter. *Journal of Molecular Biology*, *232*(2), 406–418. https://doi.org/10.1006/jmbi.1993.1400

Lee, C.-S., Li, J.-J., & Doi, Y. (2015). Complete sequence of conjugative IncA/C plasmid encoding CMY-2 beta-lactamase and RmtE 16S rRNA methyltransferase. *Antimicrobial Agents and Chemotherapy*. United States. https://doi.org/10.1128/AAC.00852-15

Lévesque, C., Brassard, S., Lapointe, J., & Roy, P. H. (1994). Diversity and relative strength of tandem promoters for the antibiotic-resistance genes of several integron. *Gene*, *142*(1), 49–54. https://doi.org/http://doi.org/10.1016/0378-1119(94)90353-0

Levitan, A. A. (1967). In Vitro Antibacterial Activity of Kasugamycin. *Applied Microbiology*.

Liou, G. F., Yoshizawa, S., Courvalin, P., & Galimand, M. (2006). Aminoglycoside resistance by ArmA-mediated ribosomal 16S methylation in human bacterial pathogens. *Journal of Molecular Biology*, *359*(2), 358–364. https://doi.org/10.1016/j.jmb.2006.03.038

Lioy, V. S., Goussard, S., Guerineau, V., Yoon, E., Courvalin, P., Galimand, M., & Grillot-Courvalin, C. (2014). Aminoglycoside resistance 16S rRNA methyltransferases block endogenous methylation, affect translation efficiency and fitness of the host. *RNA (New York, N.Y.)*, *20*(3), 0–10. https://doi.org/10.1261/rna.042572.113

Lisser, S., & Margalit, H. (1993). Compilation of E. coli mRNA promoter sequences. *Nucleic Acids Research*, *21*(7), 1507–1516.

Liu, G., Chater, K. F., Chandra, G., Niu, G., & Tan, H. (2013). Molecular regulation of antibiotic biosynthesis in streptomyces. *Microbiology and Molecular Biology Reviews : MMBR*, *77*(1), 112–143. https://doi.org/10.1128/MMBR.00054-12

Liu, M., & Douthwaite, S. (2002). Resistance to the macrolide antibiotic tylosin is conferred by single methylations at 23S rRNA nucleotides G748 and A2058 acting in synergy. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(23), 14658–14663. https://doi.org/10.1073/pnas.232580599

Liu, M., Kirpekar, F., Van Wezel, G. P., & Douthwaite, S. (2000). The tylosin resistance gene tlrB of Streptomyces fradiae encodes a methyltransferase that targets G748 in 23S rRNA. *Molecular Microbiology*, *37*(4), 811–820.

Livermore, D. M., Mushtaq, S., Warner, M., Zhang, J.-C., Maharjan, S., Doumith, M., & Woodford, N. (2011). Activity of aminoglycosides, including ACHN-490, against carbapenem-resistant Enterobacteriaceae isolates. *The Journal of Antimicrobial Chemotherapy*, *66*(1), 48–53. https://doi.org/10.1093/jac/dkq408

Lovett, P. S., & Rogers, E. J. (1996). Ribosome regulation by the nascent peptide. *Microbiological Reviews*, *60*(2), 366–385.

Magnet, S., & Blanchard, J. S. (2005). Molecular insights into aminoglycoside action and resistance. *Chemical Reviews*, *105*(2), 477–497. https://doi.org/10.1021/cr0301088

Mahillon, J., & Chandler, M. (1998). Insertion sequences. *Microbiology and Molecular Biology Reviews : MMBR*, *62*(3), 725–774.

Mak, S., Xu, Y., & Nodwell, J. R. (2014). The expression of antibiotic resistance genes in antibiotic-producing bacteria. *Molecular Microbiology*, *93*(3), 391–402. https://doi.org/10.1111/mmi.12689

Mandecki, W., & Reznikoff, W. S. (1982). A lac promoter with a changed distance between -10 and -35 regions. *Nucleic Acids Research*, *10*(3), 903–912.

Martínez, J. L. (2008). Antibiotics and Antibiotic Resistance Genes in Natural Environments. *Science*, *321*(5887), 365 LP-367. Retrieved from http://science.sciencemag.org/content/321/5887/365.abstract

McDowall, K. J., Thamchaipenet, A., & Hunter, I. S. (1999). Phosphate control of oxytetracycline production by Streptomyces rimosus is at the level of transcription from promoters overlapped by tandem repeats similar to those of the DNA-binding sites of the OmpR family. *Journal of Bacteriology*, *181*(10), 3025–3032.

Melançon, P., Tapprich, W. E., & Brakier-Gingras, L. (1992). Single-base mutations at position 2661 of Escherichia coli 23S rRNA increase efficiency of translational proofreading. *Journal of Bacteriology*.

Melnikov, S., Ben-Shem, A., Garreau de Loubresse, N., Jenner, L., Yusupova, G., & Yusupov, M. (2012). One core, two shells: bacterial and eukaryotic ribosomes. *Nature Structural & Molecular Biology*, *19*(6), 560–7. https://doi.org/10.1038/nsmb.2313

Mingeot-Leclercq, M. P., Glupczynski, Y., & Tulkens, P. M. (1999). Aminoglycosides: activity and resistance. *Antimicrobial Agents and Chemotherapy*, *43*(4), 727–737.

Miura, C., Komatsu, K., Maejima, K., Nijo, T., Kitazawa, Y., Tomomitsu, T., … Namba, S. (2015). Functional characterization of the principal sigma factor RpoD of phytoplasmas via an in vitro transcription assay. *Scientific Reports*, *5*(July), 11893. https://doi.org/10.1038/srep11893

Moazed, D., & Noller, H. F. (1987). Interaction of antibiotics with functional sites in 16S ribosomal RNA. *Nature*, *327*(6121), 389–394.

Murakami, K. S., & Darst, S. A. (2003). Bacterial RNA polymerases: the wholo story. *Current Opinion in Structural Biology*, *13*(1), 31–39.

Nikaido, H. (1994). Prevention of drug access to bacterial targets: permeability barriers and active efflux. *Science (New York, N.Y.)*, *264*(5157), 382–388.

O'Hara, J. A., McGann, P., Snesrud, E. C., Clifford, R. J., Waterman, P. E., Lesho, E. P., & Doi, Y. (2013, May). Novel 16S rRNA Methyltransferase RmtH Produced by Klebsiella pneumoniae Associated with War-Related Trauma. *Antimicrobial Agents and Chemotherapy*. 1752 N St., N.W., Washington, DC. https://doi.org/10.1128/AAC.00266-13

O'Neill, J. (2014). Antimicrobial Resistance : Tackling a crisis for the health and wealth of nations. *Review on Antimicrobial Resistance*, (December), 1–16. https://doi.org/10.1038/510015a

Ohnishi, Y., Yamazaki, H., Kato, J.-Y., Tomono, A., & Horinouchi, S. (2005). AdpA, a central transcriptional regulator in the A-factor regulatory cascade that leads to morphological development and secondary metabolism in Streptomyces griseus. *Bioscience, Biotechnology, and Biochemistry*, *69*(3), 431–439. https://doi.org/10.1271/bbb.69.431

Ohnuki, T., Katoh, T., Imanaka, T., & Aiba, S. (1985). Molecular cloning of tetracycline resistance genes from Streptomyces rimosus in Streptomyces griseus and characterization of the cloned genes. *Journal of Bacteriology*, *161*(3), 1010–1016.

Onaka, H., & Horinouchi, S. (1997). DNA-binding activity of the A-factor receptor protein and its recognition DNA sequences. *Molecular Microbiology*, *24*(5), 991–1000.

Ozaki, M., Wada, A., Fujita, N., & Ishihama, A. (1991). Growth phase-dependent modification of RNA polymerase in Escherichia coli. *Molecular & General Genetics : MGG*, *230*(1–2), 17–23.

Park, S. S. R., Park, J. J. W., Ban, Y. H. Y., Sohng, J. K., & Yoon, Y. J. (2013). 2-Deoxystreptamine-containing aminoglycoside antibiotics: Recent advances in the characterization and manipulation of their biosynthetic pathways. *Natural Product Reports*, *30*(1), 11–20. https://doi.org/10.1039/c2np20092a

Perichon, B., Courvalin, P., & Galimand, M. (2007). Transferable resistance to aminoglycosides by methylation of G1405 in 16S rRNA and to hydrophilic fluoroquinolones by QepA-mediated efflux in Escherichia coli. *Antimicrobial Agents and Chemotherapy*, *51*(7), 2464–2469. https://doi.org/10.1128/AAC.00143-07

Petković, H., Cullum, J., Hranueli, D., Hunter, I. S., Perić-Concha, N., Pigac, J., … Long, P. F. (2006). Genetics of Streptomyces rimosus, the Oxytetracycline Producer. *Microbiology and Molecular Biology Reviews*. https://doi.org/10.1128/MMBR.00004-06

Podglajen, I., Breuil, J., Casin, I., & Collatz, E. (1995). Genotypic identification of two groups within the species Bacteroides fragilis by ribotyping and by analysis of PCR-generated fragment patterns and insertion sequence content. *Journal of Bacteriology* , *177*(18), 5270–5275. Retrieved from http://jb.asm.org/content/177/18/5270.abstract

Poehlsgaard, J., & Douthwaite, S. (2005). The bacterial ribosome as a target for antibiotics. *Nat Rev Micro*, *3*(11), 870–881. Retrieved from http://dx.doi.org/10.1038/nrmicro1265

Poirel, L., Dortet, L., Bernabeu, S., & Nordmann, P. (2011). Genetic Features of bla(NDM-1)-Positive Enterobacteriaceae. *Antimicrobial Agents and Chemotherapy*. 1752 N St., N.W., Washington, DC. https://doi.org/10.1128/AAC.00585-11

Poole, K. (2005). Efflux-mediated antimicrobial resistance. *The Journal of Antimicrobial Chemotherapy*, *56*(1), 20–51. https://doi.org/10.1093/jac/dki171

Providenti, M. A., O'Brien, J. M., Ewing, R. J., Paterson, E. S., & Smith, M. L. (2006). The copy-number of plasmids and other genetic elements can be determined by SYBR-Green-based quantitative real-time PCR. *Journal of Microbiological Methods*, *65*(3), 476–487. https://doi.org/10.1016/j.mimet.2005.09.007

Putman, M., van Veen, H. W., & Konings, W. N. (2000). Molecular properties of bacterial multidrug transporters. *Microbiology and Molecular Biology Reviews : MMBR*, *64*(4), 672–693.

Rahman, M., Prasad, K. N., Pathak, A., Pati, B. K., Singh, A., Ovejero, C. M., … Gonzalez-Zorn, B. (2015). RmtC and RmtF 16S rRNA methyltransferase in NDM-1-producing Pseudomonas aeruginosa. *Emerging Infectious Diseases*, *21*(11), 2059–2062. https://doi.org/10.3201/eid2111.150271

Ramirez, M. S., & Tolmasky, M. E. (2010, December). Aminoglycoside Modifying Enzymes. *Drug Resistance Updates : Reviews and Commentaries in Antimicrobial and Anticancer Chemotherapy*. https://doi.org/10.1016/j.drup.2010.08.003

Ramu, H., Mankin, A., & Vazquez-Laslop, N. (2009). Programmed drug-dependent ribosome stalling. *Molecular Microbiology*, *71*(4), 811–824. https://doi.org/10.1111/j.1365-2958.2008.06576.x

Recht, M. I., Douthwaite, S., & Puglisi, J. D. (1999). Basis for prokaryotic specificity of action of aminoglycoside antibiotics, *18*(11), 3133–3138.

Recht, M. I., Fourmy, D., Blanchard, S. C., Dahlquist, K. D., & Puglisi, J. D. (1996). RNA Sequence Determinants for Aminoglycoside Binding to an A-site rRNA Model Oligonucleotide. *Journal of Molecular Biology*, *262*(4), 421–436. https://doi.org/http://dx.doi.org/10.1006/jmbi.1996.0526

Rice, L. B. (2008). Federal Funding for the Study of Antimicrobial Resistance in Nosocomial Pathogens: No ESKAPE. *The Journal of Infectious Diseases*, *197*(8), 1079. https://doi.org/10.1086/533452

Rosteck, P. R. J., Reynolds, P. A., & Hershberger, C. L. (1991). Homology between proteins controlling Streptomyces fradiae tylosin resistance and ATP-binding transport. *Gene*, *102*(1), 27–32.

Salgado, H., Santos-Zavaleta, A., Gama-Castro, S., Millán-Zárate, D., Díaz-Peredo, E., Sánchez-Solano, F., … Collado-Vides, J. (2001). RegulonDB (version 3.2): transcriptional regulation and operon organization in Escherichia coli K-12. *Nucleic Acids Research*. Oxford, UK.

San Millan, A., Heilbron, K., & MacLean, R. C. (2014). Positive epistasis between co-infecting plasmids promotes plasmid survival in bacterial populations. *The ISME Journal*, *8*(3), 601–12. https://doi.org/10.1038/ismej.2013.182

Schwalbe, R. S., Stapleton, J. T., & Gilligan, P. H. (1987). Emergence of vancomycin resistance in coagulase-negative staphylococci. *The New England Journal of Medicine*, *316*(15), 927–931. https://doi.org/10.1056/NEJM198704093161507

Shaw, K. J., Rather, P. N., Hare, R. S., & Miller, G. H. (1993). Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. *Microbiological Reviews*, *57*(1), 138–163.

Shlaes, D. (2010). *Antibiotics - The perfect Storm*.

Shultzaberger, R. K., Chen, Z., Lewis, K. A., & Schneider, T. D. (2007). Anatomy of Escherichia coli Σ^{70} promoters. *Nucleic Acids Research*, *35*(3), 771–788. https://doi.org/10.1093/nar/gkl956

Siguier, P., Gourbeyre, E., & Chandler, M. (2014). Bacterial insertion sequences: Their genomic impact and diversity. *FEMS Microbiology Reviews*, *38*(5), 865–891. https://doi.org/10.1111/1574-6976.12067

Spellberg, B., Blaser, M., Guidos, R. J., Boucher, H. W., Bradley, J. S., Eisenstein, B. I., … Gilbert, D. N. (2011). Combating antimicrobial resistance: policy recommendations to save lives. *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America*, *52 Suppl 5*, S397-428. https://doi.org/10.1093/cid/cir153

Spellberg, B., Guidos, R., Gilbert, D., Bradley, J., Boucher, H. W., Scheld, W. M., … Edwards, J. J. (2008). The epidemic of antibiotic-resistant infections: a call to action for the medical community from the Infectious Diseases Society of America. *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America*, *46*(2), 155–164. https://doi.org/10.1086/524891

Starnbach, M. N., & Lory, S. (1992). The fliA (rpoF) gene of Pseudomonas aeruginosa encodes an alternative sigma factor required for flagellin synthesis. *Molecular Microbiology*, *6*(4), 459–469.

Sunohara, T., Jojima, K., Yamamoto, Y., Inada, T., & Aiba, H. (2004). Nascent-peptide-mediated ribosome stalling at a stop codon induces mRNA cleavage resulting in nonstop mRNA that is recognized by tmRNA. *RNA (New York, N.Y.)*, *10*(3), 378–386.

Taber, H. W., Mueller, J. P., Miller, P. F., & Arrow, A. S. (1987). Bacterial uptake of aminoglycoside antibiotics. *Microbiological Reviews*.

Takano, M., Okuda, M., Yasuhara, M., & Hori, R. (1994). Cellular toxicity of aminoglycoside antibiotics in G418-sensitive and -resistant LLC-PK1 cells. *Pharmaceutical Research*, *11*(5), 609–615.

Thieffry, D., Salgado, H., Huerta, A. M., & Collado-Vides, J. (1998). Prediction of transcriptional regulatory sites in the complete genome sequence of Escherichia coli K-12. *Bioinformatics*, *14*(5), 391. https://doi.org/10.1093/bioinformatics/14.5.391

Tijet, N., Andres, P., Chung, C., Lucero, C., Low, D. E., Galas, M., … Amilaga, A. D. V. (2011). rmtD2, a new allele of a 16S rRNA methylase gene, has been present in Enterobacteriaceae isolates from Argentina for more than a decade. *Antimicrobial Agents and Chemotherapy*, *55*(2), 904–909. https://doi.org/10.1128/AAC.00962-10

Tischendorf, G. W., Zeichhardt, H., & Stöffler, G. (1975). Architecture of the Escherichia coli ribosome as determined by immune electron microscopy. *Proceedings of the National Academy of Sciences of the United States of America*.

Tohyama, H., Shigyo, T., & Okami, Y. (1984). Cloning of streptomycin resistance gene from a streptomycin producing streptomycete. *The Journal of Antibiotics*, *37*(12), 1736–1737.

Toleman, M. A., & Walsh, T. R. (2011). Combinatorial events of insertion sequences and ICE in Gram-negative bacteria. *FEMS Microbiology Reviews*, *35*(5), 912–935. https://doi.org/10.1111/j.1574-6976.2011.00294.x

Toleman, M. a, Bennett, P. M., & Walsh, T. R. (2006). ISCR elements: novel gene-capturing systems of the 21st century? *Microbiology and Molecular Biology Reviews : MMBR*, *70*(2), 296–316. https://doi.org/10.1128/MMBR.00048-05

Tolmasky, M. E. (2007). Aminoglycoside-Modifying Enzymes: Characteristics, Localization, and Dissemination. In *Enzyme-Mediated Resistance to Antibiotics* (pp. 35–52). American Society of Microbiology. Retrieved from http://www.asmscience.org/content/book/10.1128/9781555815615.ch04

Tripathi, L., Zhang, Y., & Lin, Z. (2014). Bacterial sigma factors as targets for engineered or synthetic transcriptional control. *Frontiers in Bioengineering and Biotechnology*, *2*(September), 33. https://doi.org/10.3389/fbioe.2014.00033

Tsai, A., Uemura, S., Johansson, M., Puglisi, E. V., Marshall, R. A., Aitken, C. E., … Puglisi, J. D. (2013). The Impact of Aminoglycosides on the Dynamics of Translation Elongation. *Cell Reports*, *3*(2), 497–508. https://doi.org/10.1016/j.celrep.2013.01.027

Vaara, M. (1992). Agents that increase the permeability of the outer membrane. *Microbiological Reviews*.

Ventola, C. L. (2015). The Antibiotic Resistance Crisis Part 1 : Causes and Threats, *40*(4), 277–283.

Vicens, Q., & Westhof, E. (2002). Crystal structure of a complex between the aminoglycoside tobramycin and an oligonucleotide containing the ribosomal decoding a site. *Chemistry and Biology*, *9*(6), 747–755. https://doi.org/10.1016/S1074-5521(02)00153-9

Vining, L. C. (1990). Functions of secondary metabolites. *Annual Review of Microbiology*, *44*, 395–427. https://doi.org/10.1146/annurev.mi.44.100190.002143

Vitreschak, A. G., Rodionov, D. A., Mironov, A. A., & Gelfand, M. S. (2004). Riboswitches: the oldest mechanism for the regulation of gene expression? *Trends in Genetics : TIG*, *20*(1), 44–50. https://doi.org/10.1016/j.tig.2003.11.008

Vujaklija, D., Ueda, K., Hong, S. K., Beppu, T., & Horinouchi, S. (1991). Identification of an A-factor-dependent promoter in the streptomycin biosynthetic gene cluster of Streptomyces griseus. *Molecular & General Genetics : MGG*, *229*(1), 119–128.

Wachino, J.-I., Yamane, K., Shibayama, K., Kurokawa, H., Shibata, N., Suzuki, S., … Arakawa, Y. (2006). Novel plasmid-mediated 16S rRNA methylase, RmtC, found in a proteus mirabilis isolate demonstrating extraordinary high-level resistance against various aminoglycosides. *Antimicrobial Agents and Chemotherapy*, *50*(1), 178–184. https://doi.org/10.1128/AAC.50.1.178-184.2006

Wachino, J., & Arakawa, Y. (2012). Exogenously acquired 16S rRNA methyltransferases found in aminoglycoside-resistant pathogenic Gram-negative bacteria: An update. *Drug Resistance Updates*, *15*(3), 133–148. https://doi.org/http://dx.doi.org/10.1016/j.drup.2012.05.001

Wachino, J. I., Shibayama, K., Kimura, K., Yamane, K., Suzuki, S., & Arakawa, Y. (2010). RmtC introduces G1405 methylation in 16S rRNA and confers high-level aminoglycoside resistance on Gram-positive microorganisms. *FEMS Microbiology Letters*, *311*(1), 56–60. https://doi.org/10.1111/j.1574-6968.2010.02068.x

Wachino, J., Shibayama, K., Kurokawa, H., Kimura, K., Yamane, K., Suzuki, S., … Arakawa, Y. (2007). Novel plasmid-mediated 16S rRNA m1A1408 methyltransferase, NpmA, found in a clinically isolated Escherichia coli strain resistant to structurally diverse aminoglycosides. *Antimicrobial Agents and Chemotherapy*, *51*(12), 4401–4409. https://doi.org/10.1128/AAC.00926-07

Wainwright, M., & Swan, H. T. (1986). C.G. Paine and the earliest surviving clinical records of penicillin therapy. *Medical History*.

Waksman, S. A. (1944). Antibiotic Substances, Production by Microörganisms—Nature and Mode of Action . *American Journal of Public Health and the Nations Health*.

Waksman, S. A., Lechevalier, H. A., & Harris, D. A. (1949). Neomycin-Production and antibiotic properties. *Journal of Clinical Investigation*.

Waksman, S. A., Reilly, H. C., & Schatz, A. (1945). Strain Specificity and Production of Antibiotic Substances: V. Strain Resistance of Bacteria to Antibiotic Substances, Especially to Streptomycin. *Proceedings of the National Academy of Sciences of the United States of America*.

Weinstein, M. J., Wagman, G. H., Oden, E. M., & Marquez, J. A. (1967). Biological activity of the antibiotic components of the gentamicin complex. *Journal of Bacteriology*.

Weisblum, B. (1995). Erythromycin resistance by ribosome modification. *Antimicrobial Agents and Chemotherapy*, *39*(3), 577–585. https://doi.org/10.1128/AAC.39.3.577

WHO. (2007). Critically Important Antimicrobials for Human Medicine : Critically Important Antimicrobials for Human Medicine :, (May), 29–31.

Wiesch, P. S. Zur, Engelstadter, J., & Bonhoeffer, S. (2010). Compensation of fitness costs and reversibility of antibiotic resistance mutations. *Antimicrobial Agents and Chemotherapy*, *54*(5), 2085–2095. https://doi.org/10.1128/AAC.01460-09

Woodcock, J., Moazed, D., Cannon, M., Davies, J., & Noller, H. F. (1991). Interaction of antibiotics with A- and P-site-specific bases in 16S ribosomal RNA. *The EMBO Journal*.

World Health Organisation (WHO). (2014). GHANA: Factsheets of Health Statistics 2014. *2014*, (1), 1–5.

Wright, G. D., & Ladak, P. (1997). Overexpression and characterization of the chromosomal aminoglycoside 6'-N-acetyltransferase from Enterococcus faecium. *Antimicrobial Agents and Chemotherapy*.

Wu, H. Y., Miller, G. H., Guzman Blanco, M., Hare, R. S., & Shaw, K. J. (1997). Cloning and characterization of an aminoglycoside 6'-N- acetyltransferase gene from Citrobacter freundii which confers an altered resistance profile. *Antimicrobial Agents and Chemotherapy*, *41*(11), 2439–2447.

Xie, J., Talaska, A. E., & Schacht, J. (2011). New developments in aminoglycoside therapy and ototoxicity. *Hearing Research*. https://doi.org/10.1016/j.heares.2011.05.008

Xiong, Y., Caillon, J., Drugeon, H., Potel, G., & Clinique, A. (1996). Influence of pH on Adaptive Resistance of Pseudomonas aeruginosa to Aminoglycosides and Their Postantibiotic Effects, *40*(1), 35–39.

Yamane, K., Doi, Y., Yokoyama, K., Yagi, T., Kurokawa, H., Shibata, N., … Arakawa, Y. (2004). Genetic environments of the rmtA gene in Pseudomonas aeruginosa clinical isolates. *Antimicrobial Agents and Chemotherapy*, *48*(6), 2069–2074. https://doi.org/10.1128/AAC.48.6.2069-2074.2004

Yamane, K., Wachino, J., Suzuki, S., Shibata, N., Kato, H., Shibayama, K., … Arakawa, Y. (2007). 16S rRNA methylase-producing, gram-negative pathogens, Japan. *Emerging Infectious Diseases*, *13*(4), 642–646. https://doi.org/10.3201/eid1304.060501

Yokoyama, K., Doi, Y., Yamane, K., Kurokawa, H., Shibata, N., Shibayama, K., … Arakawa, Y. (2003). Acquisition of 16S rRNA methylase gene in Pseudomonas aeruginosa. *Lancet (London, England)*, *362*(9399), 1888–1893. https://doi.org/10.1016/S0140-6736(03)14959-8

Yoshizawa, S., Fourmy, D., & Puglisi, J. D. (1998). Structural origins of gentamicin antibiotic action, *17*(22), 6437–6448.

Zalacain, M., & Cundliffe, E. (1989). Methylation of 23S rRNA caused by tlrA (ermSF), a tylosin resistance determinant from Streptomyces fradiae. *Journal of Bacteriology*, *171*(8), 4254–4260.