INVESTIGATION OF A CRYPTIC AMINOGLYCOSIDE RESISTANCE GENE IN *SALMONELLA ENTERICA* SUBSPECIES *ENTERICA* SEROVAR TYPHIMURIUM

A Dissertation

by

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ABSTRACT

The gene *aac(6')-Iy* is a cryptic aminoglycoside acetyltransferase (AAC) that is present in almost all *Salmonella* species and subspecies. Despite its ubiquitous presence in *Salmonella*, it does not confer *Salmonella* with aminoglycoside resistance. The reason why *Salmonella* maintains this cryptic gene is unclear. This thesis aimed to describe the role of *aac(6')-Iy* in *S.* Typhimurium. Loss of *aac(6')-Iy* in *S.* Typhimurium did not confer a significant difference in resistance to gentamicin, kanamycin, and amikacin, confirming its cryptic phenotype as an antibiotic resistance gene. However, the gene is not entirely cryptic as it has expression under *in vitro* conditions. In addition, *aac(6')-Iy* is upregulated in intracellular *S.* Typhimurium during infection of both HeLa epithelial cells and RAW264.7 macrophages. These results suggested a role for the gene in intracellular survival, which was confirmed with infection assays in both HeLa epithelial cells and RAW264.7 macrophages. The pattern of survival in HeLa cells suggested a role for *aac(6')-Iy* in survival within the *Salmonella*-containing vacuole (SCV). The survival of an *aac(6')-Iy* mutant in both the cytosol and the SCV were measured to investigate whether *aac(6')-Iy* specifically affected survival within the SCV. Loss of *aac(6')-Iy* did not affect cytosolic survival within HeLa epithelial cells, but it did lead to a significant reduction in the percent of LAMP1-associated bacteria present in the SCV. This confirmed that *aac(6')-Iy* has a role in *S.* Typhimurium survival within the SCV. Loss of *aac(6')-Iy* also led to a significant competitive disadvantage in a murine model of systemic infection. Together these data demonstrate an alternative role for the antibiotic resistance gene in *aac(6')-Iy* in *Salmonella* survival and infection in the host. This is the first chromosomally encoded AAC shown to play a role in survival within epithelial cells and within the SCV.

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NOMENCLATURE

AAC	Aminoglycoside acetyltransferase
ATCC	American Type Culture Collection
CFU	Colony forming units
CI	Competitive index
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
DMEM	Dulbecco’s modified eagle medium
DPBS	Dulbecco’s phosphate buffered-saline
ESP	Early stationary phase
FBS	Fetal bovine serum
GM	Growth media
IPTG	Isopropyl β-d-1-thiogalactopyranosideand
LAMP1	Lysosome-associated membrane protein 1
LB	Lysogeny broth
LC	Liquid chromatography
NGS	Normal goat serum
NTS	Nontyphoidal serovar
MCS	Multiple cloning site
MHA	Mueller Hinton agar
MHB	Mueller Hinton broth
MIC	Minimum inhibitory concentration
MOI	Multiplicity of infection

MS	Mass spectrometry
OD	Optical density
ONPG	Ortho-nitrophenyl-β-galactoside
PCR	Polymerase Chain Reaction
PBS	Phosphate buffered saline
PI	Post-infection
PM	Phenotype microarray
PTS	Phosphotransferase
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SCV	*Salmonella*-containing vacuole
SDS	Sodium dodecyl sulfate
SSC	Saline-sodium citrate
STM	*Salmonella* Typhimurium
SPI	*Salmonella* Pathogenicity Island
TS	Typhoidal serovar
UV	Ultraviolet
WT	Wild-type
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

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CHAPTER I

INTRODUCTION

Background

The *aac(6')-Iy* gene is a cryptic aminoglycoside resistance gene first noted when a massive 60-kb deletion occurred in *Salmonella enterica serovar* Enteritidis (Magnet *et al*., 1999). This deletion put the promoter for *nmpC* upstream of the *aac(6')-Iy* gene, leading to constitutive expression and aminoglycoside resistance in the isolate (Magnet *et al*., 1999). This was an unusual case; however, as aminoglycoside resistance was now conferred by a chromosomally encoded acetyltransferase and not by an external source of DNA. Without an external source of DNA, *Salmonella* is usually not aminoglycoside resistant. The gene is found in most *Salmonella*, and it is always in the same genomic environment by a group of putative metabolic genes termed the *sgc* cluster (Magnet *et al*., 1999; Figure 1). The reason behind the maintenance of *aac(6')-Iy* in *Salmonella* and the role of *aac(6')-Iy* and the *sgc* cluster remain to be discovered.

Specific Aims

The long-term goal of this project is to understand the role of the *aac(6')-Iy* and *sgc* genes in *S.* Typhimurium survival and virulence. The hypothesis is that *aac(6')-Iy* has an alternate role than aminoglycoside resistance and is required for *S.* Typhimurium survival and proliferation in macrophages. We will examine this hypothesis with these specific aims.

AIM I. Determine if deletion of *aac(6')-Iy* reduces the minimum inhibitory concentration of aminoglycoside antimicrobial drugs in *S.* Typhimurium. This aim will be carried out with wild-type (WT) S. Typhimurium, an isogenic *aac(6')-Iy* deletion mutant and complemented mutant, and resistance will be measured via an E-test. Previous research demonstrated the ability of AAC(6')-Iy to acetylate aminoglycosides, however *S.* Typhimurium does not demonstrate an aminoglycoside resistant phenotype. We will measure the minimum inhibitory concentrations of the aminoglycosides gentamicin and amikacin for WT *S.* Typhimurium compared to an *aac(6')-Iy* deletion mutant, in order to demonstrate whether *aac(6')-Iy* provides any aminoglycoside resistance in *in vitro* conditions. The hypothesis is that *aac(6')-Iy* does not provide any aminoglycoside resistance under *in vitro* conditions.

AIM II. Determine transcriptional regulation of *aac(6')-Iy* and the *sgc* cluster. This aim will be carried out via *lacZ* transcriptional fusions to the *sgc* and *aac(6')-Iy* genes, northern blot analysis, and real time PCR. It is expected that expression will be low, and *aac(6')-Iy* expression should be nonexistent or low when the bacteria are grown aerobically in lysogeny broth (LB) but will be increased under SPI-2 inducing conditions. Whether the *sgc* genes are transcribed in one transcriptional unit or not has also not been determined. Northern blots and real time PCR will

demonstrate whether there is a separation in transcription between *sgcQ* and *sgcA*, as well as the strength of expression.

AIM III. Determine if *aac(6')-Iy* and the *sgc* genes play a role in *S.* Typhimurium survival within eukaryotic cells. This aim will be carried out via gentamicin protection assays in epithelial cells (HeLa) and macrophages (RAW264.7 cells). Survival of the bacteria 24 hours post-infection will be measured via colony counts on plates. It is expected that the *aac(6')-Iy* and *sgc* mutants will have decreased survival in RAW264.7 cells, but not HeLa cells, due to their increased expression in conditions experienced in a macrophage and putative regulation by SPI-2 regulator SlyA.

CHAPTER II

LITERATURE REVIEW

Introduction to *Salmonella*

Salmonella is a Gram-negative, facultative intracellular bacillus that is transmitted via the fecal-oral route. This genus includes bacteria that cause both typhoid/paratyphoid fever and a non-typhoidal self-limiting gastroenteritis. Bacteria in these two groups are commonly referred to as typhoidal *Salmonella* (TS) or nontyphoidal *Salmonella* (NTS), respectively.

Typhoidal *Salmonella* consist of the disease typhoid fever and paratyphoid fever. Typhoid fever causes a multi-systemic disease characterized by fever, abdominal pain, and sepsis (Crump *et al*., 2015). It is associated with contaminated food, unhygienic conditions and unclean water, and as a result, it typically affects those living in impoverished conditions. Paratyphoid fevers cause a similar febrile illness. According to the Centers for Disease Control and Prevention (CDC), the TS group affects 16-26 million people per year (11-21 million for typhoid fever; 5 million for paratyphoid fever) (CDC 2020). According to the World Health Organization (WHO), typhoid fever kills up to 161,000 people per year (WHO 2021).

Nontyphoidal *Salmonella* (NTS) causes a foodborne illness with gastroenteritis and fever. Most cases recover without treatment; however, *Salmonella* can cause severe or life-threatening illnesses in the elderly, children, or immunocompromised. Nontyphoidal *Salmonella* is one of the top four causes of diarrhea around the world (WHO 2021). It affects 153 million people per year with 57,000 deaths (CDC 2019). The burden of NTS is heavy in sub-Saharan Africa, due to underdevelopment and a high incidence of the human immunodeficiency virus (HIV) (Gordon *et al*., 2008). Within this region, NTS is one of the top causes of sepsis in humans (Morpeth *et al*.,

2010). Within the United States, NTS infections account for 1.35 million infections and 420 deaths per year (CDC 2021). Although not as severe as sub-Saharan Africa, NTS infections come at a high economic cost. In 2014, NTS infections cost the United States almost $3.7 billion dollars in medical costs and productivity loss (Economic Research Service (ERS) 2014).

These statistics demonstrate the importance of *Salmonella* research. This dissertation will focus on characterizing an antibiotic resistance gene in *Salmonella*. This chapter will provide a background on *Salmonella*, leading into more specific details on the wild-type *Salmonella* used in this research: *Salmonella enterica* subspecies *enterica* serovar Typhimurium (*S.* Typhimurium).

Salmonella Nomenclature and History

Despite having "salmon" in the name, *Salmonella* is not named after the fish, but after a veterinary pathologist Daniel Elmer Salmon. A scientist in Salmon's group Theobald Smith had isolated *Salmonella enterica* subspecies *enterica* serovar Choleraesuis from pigs around the 1880s. Salmon's group was not the first group to isolate *Salmonella*. Karl Joseph Eberth and Rudolf Virchow had seen bacilli within typhoid patient tissue in 1880. In 1884, George Theodor Gaffky grew *Salmonella* in pure culture. It was Joseph Leon Lignières who proposed that the pathogen be named after Salmon in 1885.

Salmonella nomenclature has evolved several times since its discovery in the late 1800s (Brenner *et al*., 2000; Eng *et al*., 2015). Currently, the genus *Salmonella* has two species: *Salmonella bongori* and *Salmonella enterica*. Within the species *enterica* are six subspecies: *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV), and *indica* (VI). *Salmonella bongori* was formerly subspecies V but was updated to species status in 2005

(Tindall *et al*., 2005). Members of subspecies *enterica* (I) are named after the place of discovery, disease it causes, and/or animal it infects (Ryan *et al*., 2017). Non-subspecies *enterica* (I) serovars are named after their antigenic formula based on the Kauffmann-White scheme (Ryan *et al*., 2017). Subspecies *enterica* (I) contains most mammalian *Salmonella* infections, with serovar Typhimurium and Enteritidis often contributing to most NTS infections and Typhi and Paratyphi A, B, and C constituting the typhoidal serovars (Jajere 2019; Ferrari *et al*., 2019).

While the actual bacterium was not grown in pure culture until the 1880s, *Salmonella* is a historical disease (Khosla 2008; Galán 2016). In 430 B.C., a disease whose symptoms matched typhoid fever ravaged Athens (Galán 2016; Thucydides 1965). This was known as the "plague of Athens," and killed around 75,000-100,000 people, almost ¼ of the population (Littman 2009). Typhoid was only suspect until DNA sequencing of teeth from burial sites showed a match to *Salmonella* Typhi, supporting that typhoid fever was the plague of Athens (Papagrigorakis *et al*., 2006). In 1519, when the Spanish arrived in Mexico, the Aztec population numbered around 25 million people. However, outbreaks occurred in Mexico, known as *cocoliztli*, killing 7-18 million people. The identity of the *cocoliztli* was debated as well, until DNA sequencing of teeth from burial sites in Mexico provided strong support that *Salmonella* Paratyphi C, one of the agents of paratyphoid fever, was at least one of the *cocoliztli* (Vågene *et al*., 2018). Paratyphi C, though rare today, may have affected a wide scope of Europe thousands of years ago. A screen of almost 3,000 ancient human skeletons across western Eurasia led to the reconstruction of 8 *Salmonella* genomes all matching closely to Paratyphi C (Key *et al*., 2020). These few examples show the scope of *Salmonella*'s effect on human history.

Treatment and Multidrug Resistance

Currently, fluoroquinolones, third generation cephalosporins, and the macrolide azithromycin are the antibiotics of choice for non-typhoidal *Salmonella* infections in humans. Antibiotics are reserved only for life-threatening illnesses and are not recommended for normal treatment of non-typhoidal *Salmonella* infections, due to an association between antibiotic treatment and increased *Salmonella* fecal shedding and symptoms in patients (Murase *et al*., 2000; Barbara *et al*., 2001). Although antibiotics are discouraged for *Salmonella* treatment, fluoroquinolone, and third-generation cephalosporin-resistant non-typhoidal *Salmonella* isolates have been discovered, prompting concern (Cuypers *et al*., 2018; Saito *et al*., 2017). Fluoroquinolones themselves have limitations for *Salmonella* treatment as they cannot be used in children or pregnant women.

For typhoid fever, similar to NTS infections, fluoroquinolones, third generation cephalosporins, and azithromycin are also used as first-line antibiotics (Milligan *et al*., 2018). Unlike NTS, antibiotic treatment is recommended as it reduces disease severity and length (Kariuki 2015). There are two vaccines available for typhoid fever, one taken orally and one injected (Milligan *et al*., 2018). While both vaccines increase immunity to typhoid, both lose efficacy over time and require boosters (CDC 2020).

In the United States, the CDC estimates the occurrence of 100,000 non-typhoidal drug-resistant *Salmonella* infections per year and 3,800 typhoidal drug-resistant infections caused by serovar Typhi per year. Surveillance on antimicrobial resistance to the drugs ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline (ACSSuT) has shown a decrease in human isolates over the years (NARMS 2017). However, resistance to ampicillin, streptomycin, sulfonamides, and tetracycline rose around 20% from 2014 to 2015 in an NTS

strain from human isolates (CDC 2018). Multidrug resistance (MDR) is becoming a concern in subspecies *enterica* strains, due to significant associations of MDR isolates with more severe clinical disease outcome (Krueger *et al*., 2014).

As with NTS, *Salmonella* Typhi antibiotic resistance is an issue. In regions of the world where Typhi is endemic, over ⅓ of isolates are multidrug resistant (MDR) (Kariuki 2015). The CDC reports that *S.* Typhi infections resistant to the popular fluoroquinolone drug, ciprofloxacin, was at 74% in 2017 (CDC 2019). Reports of MDR *S.* Typhi isolates started in the 1960s with resistance to the original first-line antibiotics documented; and it was only after a study in Vietnam found MDR in around 90% of typhoid cases that ciprofloxacin was advocated as a treatment option (Dyson *et al*., 2019). One notable MDR isolate is the H58 haplotype, which spread throughout Asia and Africa (Wong *et al*., 2016). In 2017, an MDR isolate resistant to the original first-line antibiotics, fluoroquinolones, and third-generation cephalosporins was found in Pakistan (Klemm *et al*., 2018). These isolates were classified as Extensively Drug Resistant (XDR) and found to be the H58 haplotype (Dyson *et al*., 2019, Klemm *et al*., 2018). With the recent emergence of XDR Typhi strains, there is reasonable concern over the future of typhoid treatment options (Levine & Simon 2018).

Introduction to serovar Typhimurium

Salmonella enterica subspecies *enterica* serovar Typhimurium (*S.* Typhimurium) is one of the most common *Salmonella* strains in the United States. *S.* Typhimurium earned its name from its ability to cause a typhoid-like fever in mice, hence *typhi* for "typhoid" and *murium* for "mouse." *S.* Typhimurium is not host-adapted, but can survive in a broad range of hosts, including humans, poultry, cattle, mice, and pigs. *S.* Typhimurium can also survive in

contaminated flour and vegetables. As a result, it is common to see *S.* Typhimurium outbreaks associated with both animal and non-animal products.

S. Typhimurium strains can be differentiated based on phage type. Phage typing is based on the ability of different phages to lyse the bacteria (Rabsch 2007). Several phage types for *S.* Typhimurium are associated with host preference, disease severity, or MDR phenotype. Phage type DT104 is a notable example due to its MDR phenotype, its disease severity in humans and animals, and its rapid spread globally (Poppe *et al*., 1998; Leekitcharoenphon *et al*., 2016). There are several phage typing systems for *S.* Typhimurium: the Lilleengen type (LT) strains of *Salmonella*, including the representative *S.* Typhimurium strain LT2; the Felix/Callow system with their 12 phage types; and the Anderson definitive phage types (DTs), which includes DT104 (Lilleengen 1984; Callow 1959; Anderson *et al*., 1977). While phage type can be used to differentiate strains, there is redundancy in nomenclature. For instance, the widely used laboratory *S.* Typhimurium strain 14028s has the same phage type as LT2 (Jarvik *et al*., 2010; Lilleengen 1984). However, phage typing is epidemiologically useful, as it is used to trace the source of outbreaks for *S.* Typhimurium (Baggesen *et al*., 2010).

Salmonella Pathogenesis

When *Salmonella* enters the digestive tract, it first must survive the harsh environment of the stomach and reach the small intestine. Once in the small intestine, *Salmonella* senses several environmental signals that activate invasion genes and prepare the bacteria to invade the intestinal epithelial cells. These signals include pH, osmolarity, oxygen, and short and long chain fatty acids (Galán & Curtiss 1990; Lee & Falkow 1990; Lee *et al*., 1992; Bajaj *et al*., 1996). Following signal

recognition, *Salmonella* upregulates genes found mainly on *Salmonella* pathogenicity island-1 (SPI-1) to invade the intestinal cells and gain access to the host.

Pathogenicity islands in *Salmonella* are groups of genes acquired via horizontal transfer that aid in virulence and survival within the host. *S.* Typhimurium, to date, has six known islands. *Salmonella* pathogenicity island-1 (SPI-1) and -2 (SPI-2) code for their own type III secretion systems (T3SS), which inject effector proteins into the host cell, promoting uptake, invasion, and survival within the host's epithelial cells and macrophages (Galan & Curtiss R 3rd. 1989; Lostroh & Lee, 2001; Zhou *et al*., 2001; Galan *et al.*, 2001; Waterman, Holden, 2003).

SPI-1 encodes proteins necessary for successful invasion of epithelial cells and triggering of inflammation in the intestines (Coombes *et al.,* 2005; Hapfelmeier *et al.,* 2004**)**. The SPI-1 T3SS can pierce host cell membranes and inject bacterial effector proteins into the cell (Kubori *et al.*, 1998; Sukhan *et al*., 2001; Lou *et al*., 2019). The effector proteins cause cytoskeletal actin rearrangement and the engulfment of the bacteria into the host cell (Zhou *et al*., 1999; Lou *et al*., 2019). Control of expression in this island is coordinated by the regulatory protein HilA (Bajaj *et al.*, 1995; Bajaj *et al*., 1996). SPI-1 is also affected by other conditions, such as pH and osmolarity (Galán & Curtiss 1990; Lee & Falkow 1990; Lee *et al*., 1992; Bajaj *et al.*, 1996).

When *Salmonella* invades the intestinal cells, it begins to form a niche within the host cell termed the *Salmonella*-containing vacuole (SCV) in host cells (Takeuchi 1967; Takeuchi & Sprinz 1967; Kihlstrom & Latkovic 1978; Finlay & Falkow 1989; Steele-Mortimer 2008; **Figure 1**). At this stage, *Salmonella* pathogenicity island-2 (SPI-2) genes are upregulated to form another T3SS needle complex that will inject effector proteins into the host cell and aid in establishment of the SCV and long-term survival of the bacteria within the host (Jennings *et al*., 2017). Within epithelial cells, there can exist two populations of *Salmonella*: ones within the

SCV and others that have escaped out into the cytosol of the host cell (**Figure 1**; Knodler *et al*., 2014). The cytosolic population hyper-replicates and, at later stages of infection, account for half of the total *Salmonella* population within epithelial cells (Knodler *et al*., 2014).

Once within epithelial cells, *Salmonella* can escape into the intestinal *lamina propria* where it can be subsequently phagocytized by macrophages. Within the macrophage, *Salmonella* must survive against the respiratory burst, antimicrobial peptides, acidic environment, iron-binding proteins, and metal ion toxicity (Cederlund *et al*., 2011; Rathman *et al*., 1996; Di *et al*., 2006; Fritsche *et al*., 2007; Nairz *et al*., 2008; Achard *et al*., 2012; Kapetanovic *et al*., 2016). *Salmonella* subverts the macrophage's attacks by preventing the phagolysosome fusion, creating another SCV within the macrophage (**Figure 1**; Buchmeier *et al*., 1991). It is within the SCV that *Salmonella* defends itself against the macrophage's attacks. Unlike epithelial cells, *Salmonella* is unable to create a cytosolic population due to the presence of caspase-1 and -11 that inhibit replication (Thurston *et al*., 2016). *Salmonella* can invade intestinal cells and subvert macrophage attacks to create a niche within the two cell types termed the *Salmonella*-containing vacuole (SCV). This mechanism allows *Salmonella* to live long-term within susceptible hosts.

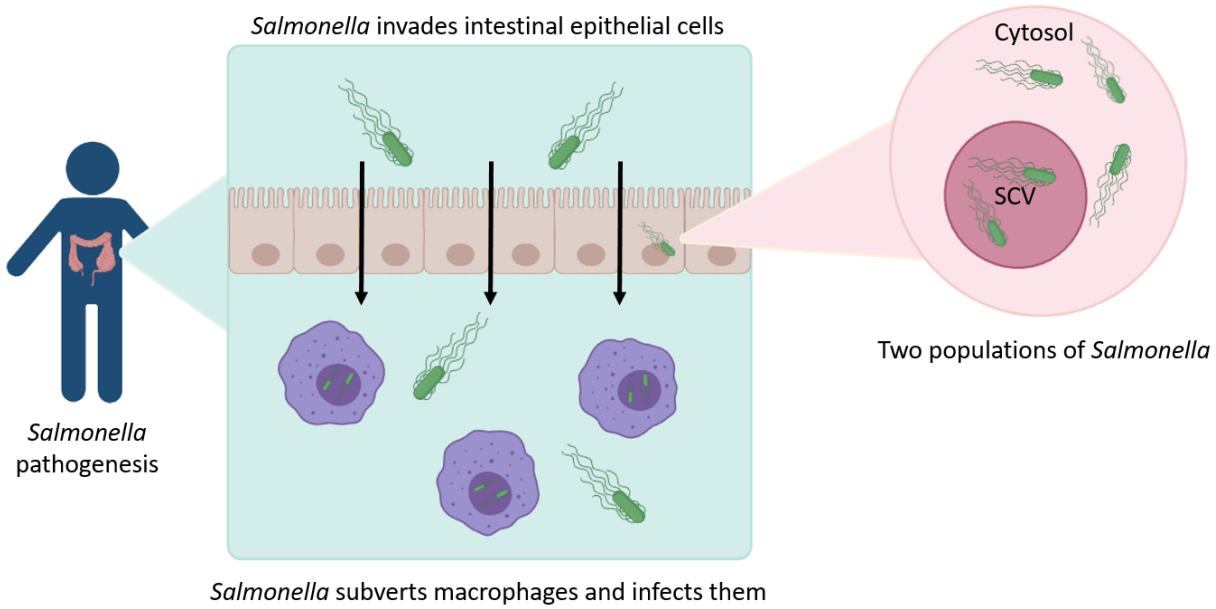


Figure 1. Diagram of *Salmonella* invasion in the intestines. Figure created with BioRender.com.

Introduction to aminoglycoside antibiotics

Aminoglycosides were one of the first classes of antibiotics discovered. The first aminoglycoside was streptomycin, which was discovered and isolated from *Streptomyces griseus* (Schatz et al., 1944). Like streptomycin, most early aminoglycosides came from soil bacteria. The order of development of notable aminoglycosides is as follows: streptomycin, neomycin, kanamycin, gentamicin, tobramycin, and amikacin. These aminoglycoside antibiotics were widely used until the discovery of less toxic antibiotics. Used systemically, aminoglycosides can have side effects for the host including ototoxicity and nephrotoxicity. Aminoglycosides are now used primarily for topical therapy or treatment of resistant bacteria.

Aminoglycosides are inositol derivatives consisting of a hydroxyl group, amino groups, and an amino sugar, with the hydroxyl and amino groups playing a role in its functional activity. Their mechanism of action leads to improper protein translation by interacting with the 16S

rRNA of the 30S subunit of the ribosome (Moazed & Noller 1987). Specifically, aminoglycosides bind the A-site of the ribosome and cause mistranslation of the mRNA, by allowing the wrong tRNA to bind to the site (**Figure 2**; Ogle et al., 2002). This mechanism is bactericidal, possibly due to inhibition of protein translation or the effect of mistranslated membrane proteins causing membrane destabilization (Wallace & Davis 1973; Davis et al., 1986). Aminoglycoside uptake into bacterial cells requires an aerobic environment and they cannot penetrate eukaryotic cells. Hence, aminoglycosides are not used to treat infections caused by obligate anaerobic and intracellular pathogens.

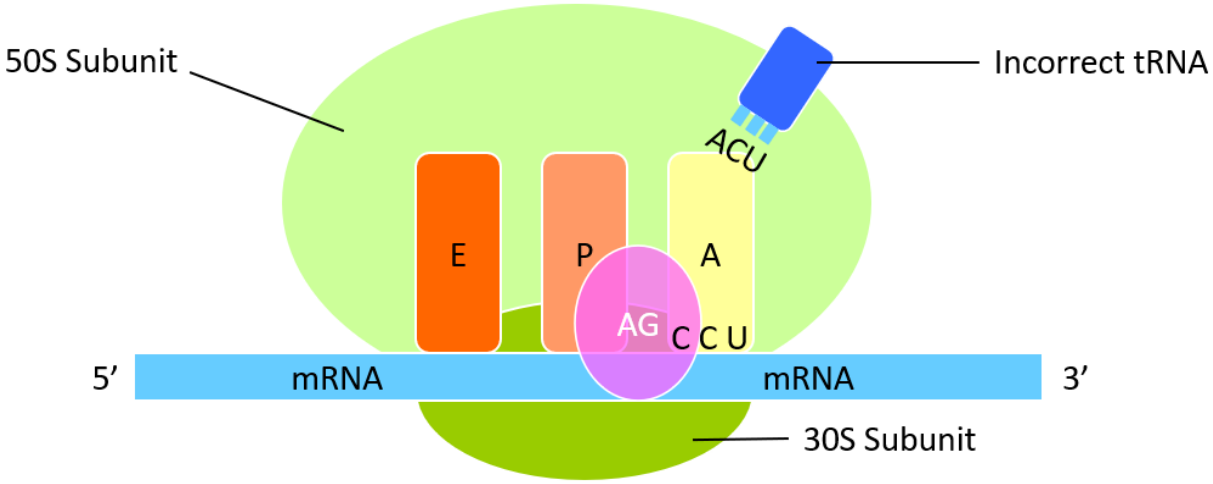


Figure 2. Diagram of aminoglycoside binding to ribosome. Aminoglycosides bind the 30S subunit of the ribosome and cause mistranslation of the mRNA, resulting in faulty proteins. Aminoglycoside = AG.

Aminoglycosides can produce similar mRNA:tRNA mismatch in eukaryotic ribosomes. Aminoglycosides with 6' hydroxyl groups induce missense errors in translation (Wilhelm et al., 1978). Similar to their effects on bacterial ribosomes, such aminoglycosides bind to the A-site within the eukaryotic ribosome, specifically the h44 decoding region, which is involved with

decoding of the mRNA and correct pairing of tRNA in accordance with the codon (Fan-Minogue & Bedwell, 2008; Hobbie et al., 2007). Aminoglycoside binding increases misreads in the A-site, leading to incorrect tRNA:mRNA pairing (Prokhorova et al., 2017).

Aminoglycosides have ototoxic and nephrotoxic effects in humans. Ototoxic effects are attributed to its effect on the eukaryotic mitochondrial ribosome, in which the aminoglycoside induces protein mistranslation similar to its effect on cytosolic ribosomes (Hobbie et al., 2008; Matt et al., 2012; Shulman et al., 2014). This leads to creation of reactive oxygen species and oxidative damage to the cells (Hobbie et al., 2008; Matt et al., 2012; Shulman et al., 2014). Around 20% of patients develop hearing loss when treated with aminoglycosides (Frymark et al., 2010). The mechanism of nephrotoxicity is not fully understood; however, the current explanation is that aminoglycosides accumulate in the renal cortex, leading to kidney damage (Vandewalle et al., 1981). This damage is reversible if aminoglycoside treatment is discontinued (WHO 2010).

Aminoglycosides are approved by the Food & Drug Administration for animal use in the United States although the American Association of Bovine Practitioners (AABP) has supported a voluntary ban on the use of aminoglycosides in cattle since 1994 (AABP 1994). Aminoglycosides may be used for treatment of infection in cattle, swine, poultry, and companion animals (Gehring et al., 2005). Unfortunately, aminoglycosides contributed to most of the antimicrobial drug contamination of meat products in 2018 (USDA 2018). A market analysis of the United States' aminoglycoside market showed and predicted an increase in aminoglycoside sales for all uses, veterinary, medical, and environmental, from 2012 to 2022 (Grand View Research 2016). As a result, the possibility of increased aminoglycoside resistance among foodborne pathogens remains a concern.

Aminoglycoside Resistance

Mutations in the ribosomal proteins, rRNA, and aminoglycoside uptake mechanisms provide limited protection. As mentioned before, aminoglycosides bind the 30S ribosomal subunit and interfere with translation. Mutations in the ribosomal proteins can confer protection by interfering with aminoglycoside binding (Garneau-Tsodikova & Labby 2016). 16S rRNA mutations provide protection for the bacteria against either aminoglycosides with a streptamine ring or aminoglycosides with a 2-deoxystreptamine ring (Recht & Puglisi 2001). There can also be mutations that affect aminoglycoside uptake (Ahmad *et al*., 1980). As uptake requires a certain membrane potential, these mutations modify the electron transport chain to interfere with antibiotic uptake (Ibacache-Quiroga *et al*., 2018). A mutation in *fusAI*, one of the elongation factors involved in translation, conferred resistance against 2-deoxystreptamine aminoglycosides (Bolard *et al*., 2018).

As with most antibiotics, bacteria have acquired resistance genes to aminoglycosides. Most aminoglycoside resistance genes are on mobile elements, such as plasmids, making the spread of aminoglycoside resistant bacteria concerning (Davies & Wright 1997). There are three groups that aminoglycoside resistance genes fall into: efflux pumps, 16S rRNA methyltransferases, or aminoglycoside modifying enzymes.

Efflux pumps have been implicated in multiple bacteria's aminoglycoside resistance. Efflux pumps work by exporting the antibiotic out of the bacteria, thereby preventing the antibiotic from binding its target. The problematic drug resistant *Pseudomonas aeruginosa* has low aminoglycoside resistance due to its RND-type efflux pump MexXY-OprM system (Aires *et al*., 1999; Mine *et al*., 1999). *Escherichia coli* has a highly similar efflux pump AcrD that is involved in efflux of several compounds, including aminoglycosides (Rosenberg *et al*., 2000).

Acinetobacter baumannii also has an ortholog of MexXY termed Ade that is responsible for aminoglycoside efflux (Magnet *et al*., 2001). *Burkholderia cenopacia*, which is intrinsically resistant to multiple antibiotics, has an RND-type transporter AmrAB-OprA efflux system that is specific for both aminoglycosides and macrolides (Moore *et al*., 1999). *Stenotrophomonas maltophilia*, a growing nosocomial-acquired pathogen, has an efflux pump SmeYZ that is induced with ribosomal damage (Adegoke *et al*., 2017; Calvopiña *et al*., 2020). Efflux pumps can be mobile via plasmids or transposons and are difficult to counteract due to their ability to efflux a wide range of antibiotics (Sun *et al*., 2014).

16S rRNA methyltransferases (RMT) are often found on plasmids and pose an issue due to their worldwide spread. RMTs work by modifying a specific rRNA nucleotide, blocking the aminoglycoside's action on the ribosome. RMTs can confer resistance to either 4,6-di-substituted aminoglycosides or to both 4,6- and 4,5-di-substituted aminoglycosides, depending on which nucleotide they interact with. The first finding of an RMT was a plasmid borne RMT termed RmtA in *Pseudomonas aeruginosa* (Yokoyama *et al*., 2003). It was highly suggested that *rmtA* originated from an aminoglycoside producer (Yokoyama *et al*., 2003). After this, more plasmid-borne RMTs were discovered in aminoglycoside-resistant isolates. To date, the clinically isolated plasmid borne RMTs are *rmtA, rmtB,* and *armA, rmtC, rmtD, rmtE, rmtF, rmtG, rmtH,* and *npmA* (Yokoyama *et al*., 2003; Galimand *et al*., 2003; Doi *et al*., 2004; Wachino *et al*., 2006; Doi *et al*., 2007; Davis *et al*., 2010; Galimand *et al*., 2012; Bueno *et al*., 2013; O'Hara *et al*., 2013; Wachino *et al*., 2007; Doi *et al*., 2016). The RMT NpmA found in *E. coli* is particularly concerning as it provides the duo resistance to 4,5 and 4,6-di-substituted aminoglycosides (Wachino *et al*., 2007). Aminoglycoside-resistance conferring RMTs are prevalent among

Enterobacteriaceae and pose a concern as aminoglycosides are a last resort antibiotic for multidrug resistant strains (Doi *et al*., 2016).

Enzymatic inactivation of aminoglycosides via aminoglycoside modifying enzymes (AMEs) is the most common method of aminoglycoside resistance. Like RMTs, AMEs are thought to originate from aminoglycoside-producing bacteria and are often found on plasmids (Benveniste *et al*., 1973; Shaw *et al*., 1993). AMEs are grouped into three families: nucleotidyltransferases (ANTs), aminoglycoside phosphotransferases (APHs), and aminoglycoside acetyltransferases (AACs). AMEs work by decreasing the affinity of the aminoglycoside for the ribosome via modification of the antibiotic (Llano-Sotelo *et al*., 2002). This mechanism is carried out by adenylation via ANTs, phosphorylation via APHs, or acetylation via AACs. AMEs are further subgrouped based on the position they modify, marked by a number in parentheses, and their resistance profile, marked by a Roman numeral (Ramirez & Tolmasky 2010). For example, AAC(6')-Iy is an acetyltransferase that modifies the 6' position on the aminoglycoside and falls into the type I resistance profile. The lowercase letter at the end of the name is to further separate different AMEs that have identical modification and resistance profiles (Ramirez & Tolmasky 2010). This section will focus on enzymes within the AAC family.

Aminoglycoside Acetyltransferases (AACs)

AACs comprise the majority of AMEs and are found in both Gram-positive and Gram-negative organisms. AAC(6')-IV (also known as AAC(6')-Ib) was the first AME and AAC discovered in *E. coli* in 1965 (Okamoto & Suzuki 1965). Similar to the RMTs, they are thought to originate from aminoglycoside producers, such as actinomycetes (Benveniste & Davies 1973).

All AACs fall into the GCN5-related *N*-acetyltransferase superfamily and use acetyl-CoA to acetylate amine groups on the aminoglycoside (Vetting *et al*., 2005). As mentioned before, this acetylation results in reduced affinity of the aminoglycoside to the ribosome (Llano-Sotelo *et al*., 2002).

Classes of AACs

AACs fall into different classes, depending on the position of the amine they acetylate on the aminoglycoside. The classes are AAC(1), AAC(3), AAC(2'), and AAC(6'). The AAC(1) class is quite small and has only been found in *E. coli*, *Campylobacter* spp., and actinomycetes (Hedges & Shannon 1984; Lovering *et al*., 1987; Gomez-Luis *et al*., 1999; Sunada *et al*., 1999). AAC(1) enzymes acetylate the amino group on position 1 of the 2-deoxystreptamine ring on the aminoglycoside (**Figure 3**). The first AAC(1) was discovered in 1984 in *E. coli* from animal isolates (Hedges & Shannon 1984). The authors were unable to identify what type of acetyltransferase it was, only noting that it could not be an AAC(3) or AAC(6'). In 1987, the enzyme was characterized and named AAC(1) (Lovering *et al*., 1987). This chromosomally encoded AAC mono-acetylates apramycin, butirosin, lividomycin, and paromomycin, and duo-acetylates ribostamycin and neomycin (Lovering *et al*., 1987). A soil actinomycete isolate with a novel resistance profile had an AAC(1) that differs from the AAC(1) in *E. coli*. While this AAC(1) was able to acetylate paromomycin, it did not confer paromomycin resistance to the isolate (Sunada *et al*., 1999). Additionally, the actinomycete AAC(1) did not acetylate apramycin as fast as the *E. coli* AAC(1) (Sunada *et al*., 1999). Beyond *E. coli* and actinomycete, there may be an AAC(1) in *Campylobacter* spp., although the data has not been published (Gomez-Luis *et al*., 1999).

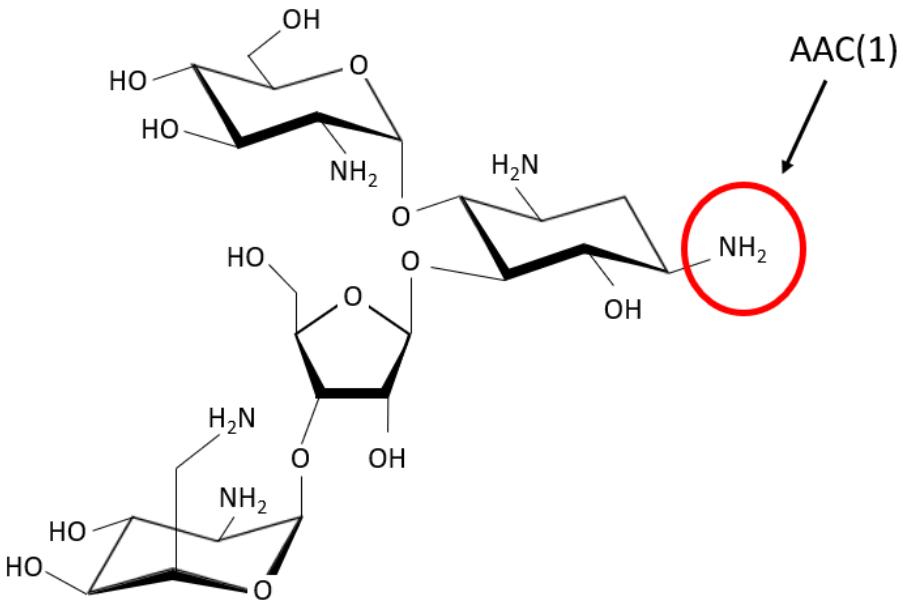


Figure 3. Chemical structure of paromomycin. The AAC(1) enzymes acetylate the primary amino group on the 2-deoxystreptamine ring (position marked by red circle).

The AAC(2') class has two subclasses, and as the name informs, these enzymes acetylate the 2' amine of the aminoglycoside (**Figure 4**). AAC(2')-I genes can confer resistance to dibekacin, kanamycin B, 6'-*N*-ethylnetilmicin, gentamicin, netilmicin, and tobramycin, while AAC(2')-II genes confer resistance to only kasugamycin (Aínsa *et al*., 1996; Yoshii *et al*., 2012; Pawlowski *et al*., 2016). The AAC(2') enzymes discovered so far are AAC(2')-Ia in *Providencia stuartii*, AAC(2')-Ib in *Acinetobacter baumannii* and *Mycobacterium fortuitum*, AAC(2')-Ic in *Mycobacterium tuberculosis* and *Mycobacterium bovis*, AAC(2')-Id in *Mycobacterium smegmatis*, AAC(2')-Ie in *Mycobacterium leprae* (putative), AAC(2')-IIa in *Burkholderia glumae* and *Acidovorax avenae*, and AAC(2')-IIb in *Paenibacillus* sp. LC231 (Chevereau *et al*., 1974; Lin *et al*., 2015; Aínsa *et al*., 1996; Aínsa *et al*., 1997; Yoshii *et al*., 2012; Pawlowski *et*

al., 2016). AAC(2')-Ia, -Ib, -Ic, and -Id will be discussed in detail later on in this review. AAC(2')-Ie is a putative AAC(2') that was found in the genome of *Mycobacterium leprae*, and shares around 78% nucleotide identity to *M. tuberculosis* AAC(2')-Ic (Aínsa *et al*., 1997). AAC(2')-IIa and -IIb were discovered in agricultural or environmental bacteria. AAC(2')-IIa was found in kasugamycin resistant isolates of the rice pathogens *B. glumae* and *A. avenae* (Yoshii *et al*., 2012). AAC(2')-IIa confers resistance to kasugamycin, but not to neomycin, kanamycin, tobramycin, and gentamicin (Yoshii *et al*., 2012). In kasugamycin-resistant isolates of *B. glumae*, AAC(2')-IIa is encoded on the chromosome on an integrated IncP island, suggesting the gene arrived via horizontal transfer (Yoshii *et al*., 2012). AAC(2')-IIb was discovered in a cave-dwelling *Paenibacillus* sp. LC231 as part of an antibiotic resistance survey (Pawlowski *et al*., 2016). This enzyme is chromosomally encoded and was credited for only kasugamycin resistance in the isolate (Pawlowski *et al*., 2016). An interesting note is the prevalence of AAC(2') enzymes found on the chromosome of bacteria, rather than on extrachromosomal DNA elements. While AAC(2')-IIa's presence on an island suggests it is on a mobile element, the majority of AAC(2') genes are ubiquitous in their respective bacteria and, when discovered, were hypothesized to serve other cellular functions beyond antibiotic resistance.

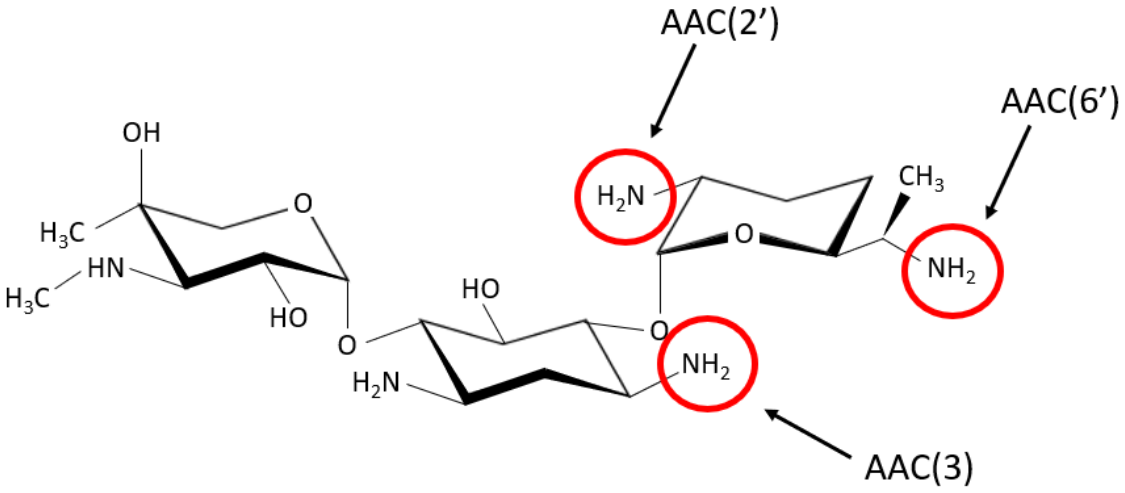


Figure 4. Chemical structure of gentamicin. The amino groups where AAC(2'), AAC(3'), and AAC(6') acetylate on the aminoglycoside are shown with a red circle.

AAC(3) is mainly found on plasmids or integrons, and consists of ten subclasses. The subclasses are: Ia, Ib, Ic, Id, Ie, IIa, IIb, IIc, IId, IIe, IIIa, IIIb, IIIc, IVa, VIa, VIIa, VIIIa, IXa, Xa, and XI. Subclass V was removed, as it turned out to be identical to AAC(3)-II (Shaw *et al*., 1993). The AAC(3) enzymes acetylate the amino group on position 3 of the aminoglycoside (**Figure 4**). The AAC(3)-I genes confer resistance to astromicin/fortimicin, gentamicin, and sisomicin. AAC(3)-Ia (*S. marcescens, E. coli, Acinetobacter baumannii, Klebsiella pneumoniae, Klebsiella oxytoca, P. aeruginosa, S.* Typhimurium, *Proteus mirabilis*), AAC(3)-Ib (*P. aeruginosa*), AAC(3)-Ic (*P. aeruginosa*), AAC(3)-Id (*S. enterica, P. mirabilis, Vibrio fluvialis*), and AAC(3)-Ie (*E. coli, S. enterica, P. mirabilis, P. aeruginosa*) are mainly found on plasmids or other mobile elements in their bacteria (Witchitz 1972; Umezawa *et al*., 1973; LeGoffic & Moreau 1973; Brzezinska *et al*., 1972; Wohlleben *et al*., 1989; Javier Teran *et al*., 1991;

Schwocho *et al*., 1995; Doublet *et al*., 2004; Levings *et al*., 2005; Gionechetti *et al*., 2008; Ho *et al*., 2010).

The AAC(3)-II genes confer resistance to dibekacin, 2'-*N*-ethylnetilmicin, 6'-*N*-ethylnetilmicin, gentamicin, netilmicin, sisomicin, kanamycin, and tobramycin (Shaw *et al*., 1993). AAC(3)-IIa/AAC(3)-Va (*K. pneumoniae, Enterobacter cloacae, Actinobacillus pleuropneumoniae*, *S.* Typhimurium, *Citrobacter freundii*, *P. aeruginosa*), AAC(3)-IIb/AAC(3)-Vb (*E. coli, A. faecalis*, and *S. marcescens*), AAC(3)-IIc (*E. coli, P. aeruginosa*, *S.* Typhimurium serovar Virchow, *S. marcescens*), AAC(3)-IId (*E. coli*), and AAC(3)-IIe (*E. coli*) have mainly been found on plasmids (van de Klundert & Vliegenthart 1993; Allmansberger *et al*., 1985; Mugnier *et al*., 1996; Shaw *et al*., 1993; Rather *et al*., 1992; Dahmen *et al*., 2010; Oteo *et al*., 2006; Dubois *et al*., 2008; Wilson & Hall 2010; Javier Teran *et al*., 1991; Ho *et al*., 2010). However, AAC(3)-IIc was integrated into the genome of *S. enterica* serovar Virchow (Wilson & Hall 2010).

The AAC(3)-III genes have only been discovered in *P. aeruginosa*, and confer resistance to gentamicin, sisomicin, tobramycin, kanamycin, neomycin, and paromomycin (Vliegenthart *et al*., 1991; Norris *et al*., 2010; Shahid & Malik 2005). AAC(3)-IVa (*E. coli*, *Campylobacter jejuni*, *Pseudomonas stutzeri*) and AAC(3)-VIa (*Enterobacter cloacae, S. enterica, E. coli*) were discovered on plasmids and confer resistance to gentamicin, netilmicin, tobramycin, kanamycin, and neomycin (Brau *et al*., 1984; Heuer *et al*., 2002; Call *et al*., 2010; Rather *et al*., 1993). AAC(3)-VIIa (*Streptomyces rimosus*), AAC(3)-VIIIa (*Streptomyces fradiae*), AAC(3)-IXa (*Micromonospora chalcea*), and AAC(3)-Xa (*Streptomyces griseus*) have been found chromosomally encoded in actinomycetes (Lopez-Cabrera *et al*., 1989; Salauze *et al*., 1991; Ishikawa *et al*., 2000). AAC(3)-Xa also acetylates the 3" amino group of arbekacin and

amikacin, making it the first AAC(3") enzyme (Ishikawa *et al*., 2000). A newly discovered AAC(3)-XI was discovered in *Corynebacterium striatum* in 2015 (Galimand *et al*., 2015). This subclass provides resistance to gentamicin and tobramycin (Galimand *et al*., 2015).

The next class AAC(6') is the largest class of AACs that used to have only two subclasses AAC(6')-I and AAC(6')-II. Both subclasses can acetylate tobramycin, netilmicin, and 2'-*N*-ethylnetilmicin (Shaw *et al*., 1993). The difference between the two subclasses is that AAC(6')-I can acetylate amikacin and gentamicin C1a and C2, while AAC(6')-II cannot acetylate amikacin, but can acetylate all gentamicin forms (Shaw *et al*., 1993; Rather *et al*., 1992). Recently, a novel subclass of AAC(6') was discovered in *Burkholderia spp*. termed AAC(6')-III (Zhang *et al*., 2020). This acetyltransferase is chromosomally encoded, lacks mobile elements, and is conserved in *Burkholderia* and confers resistance to tobramycin, but not amikacin or gentamicin (Zhang *et al*., 2020). Currently, AAC(6')-III has not been found in other bacteria.

Due to both the prevalence of and small variations within AAC(6') enzymes, their nomenclature can be confusing and there is no consensus on the proper way to distinguish these enzymes (Ramirez & Tolmasky 2010). These factors also contribute to confusion in naming some AAC(6') genes, with instances occurring that two different enzymes had the same name (Vanhoof *et al*., 1998; Casin *et al*., 2003; Lambert *et al*., 1994b). Researchers wanting to publish data about a novel AAC(6') should take precautions to ensure their gene/protein does not match with any other AACs and that they have chosen a unique name.

Notable AAC(6') enzymes include AAC(6')-Ib and AAC(6')-Ie. AAC(6')-Ib is a highly mobile gene that is widespread among *Enterobacteriaceae*. Ramirez *et al*. covers this gene extensively in their review (Ramirez *et al*., 2013). The *aac(6')-Ib* gene variant *aac(6')-Ib-cr*

provides resistance to amikacin, the two forms of gentamicin, and notably, fluoroquinolones. This leads some to believe *aac(6')-Ib-cr* is a unique class of AAC, however since it evolved from *aac(6')-Ib*, it is named as an AAC(6') (Robicsek *et al*., 2006). Another widespread acetyltransferase, AAC(6')-Ie is a unique enzyme in that it also functions as the APH(2")-Ia enzyme. AAC(6')-Ie/APH(2")-Ia are encoded on the same gene and are the same polypeptide (Culebras & Martinez 1999). This bifunctional protein is an important source of aminoglycoside resistance in *Staphylococcus* and *Enterococcus* species, providing resistance to almost every aminoglycoside (Culebras & Martinez 1999; Daigle *et al*., 1999). There are other fusion AAC proteins, such as ANT(3")-Ii/AAC(6')-IId (*S. marcescens*), AAC(6')-30/AAC(6')-Ib' (*P. aeruginosa*), and AAC(3)-Ib/AAC(6')-Ib" (*P. aeruginosa*)(Centron & Roy 2002; Mendes *et al*., 2004; Dubois *et al*., 2002). There is no consensus on how or why these bifunctional proteins exist; either they are ancestral genes from which other aminoglycoside resistance genes arose or they are cassette fusions (Centron & Roy 2002; Naas *et al*., 1999).

Epidemiological Significance

Aminoglycoside resistance is worldwide. There have been many studies evaluating the prevalence of aminoglycoside resistance genes in certain sample populations over the last 5 years. In China, a study looked at aminoglycoside resistance genes in *Klebsiella pneumoniae* isolates from hospitals. Exactly 50% of their isolates contained *aac* genes, specifically 49/162 carried *aac(3)-II* and 32/162 carried *aac(6')-Ib* (Liang *et al*., 2015). As mentioned before, the *aac(6')-Ib-cr* gene variant is problematic due to its ability to confer fluoroquinolone resistance. A screen of quinolone-resistant clinical isolates of *A. baumannii*, *P. aeruginosa*, and *S. maltophilia* from China showed that 60% of pandrug resistant and 22% of multidrug resistance

isolates had *aac(6')-Ib-cr* (Ming *et al*., 2020). In Egypt, the two most prevalent AME genes from clinical isolates of Gram-negative bacteria were *aac(3')-IIa* (40%) and *aac(6')-Ib* (30%)(Abo-State *et al*., 2018). In Iran, 42.6% of clinical isolates of *Enteroccocus* had *aac(6')-Ie-aph(2')-I* genes (Amini *et al*., 2018).

Antibiotic resistance is a multi-faceted problem, with resistance developing in human medicine and the animal and agricultural industry (McEwen & Collignon 2018). AACs are a contributing factor to aminoglycoside resistance. In Korea, sequencing of the gut microbiome in swine and cattle revealed a high abundance of *aac(6')* in swine (97.22%) and a low abundance in cattle (12.2%) (Lim *et al*., 2020). In India, screening of quinolone-resistant *E. coli* in goats after a 5-day marbofloxacin administration revealed 4/8 resistant isolates carried the *aac(6')-Ib-cr* gene (Bhardwaj *et al*., 2020). A metagenomic study of treated water in a wastewater plant in Singapore revealed the presence of *aac(6')-I* and *aac(6')-II* genes, along with other resistance genes (Ng *et al*., 2019).

In the United States (U.S.), there are several agencies that monitor the spread of antibiotic resistance. The National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS) works with the U.S. Centers for Disease Control (CDC), the U.S. Food and Drug Administration (FDA), and the U.S. Department of Agriculture (USDA), as well as health departments to monitor the spread of antibiotic resistance among enteric pathogens. The SENTRY database created by the JMI laboratories tracks antibiotic resistance worldwide via submissions to their portal (Pfaller *et al*., 1998). Their interactive heat map allows the user to explore susceptibility to a variety of antibiotics. Countries like Mexico, Poland, and Russia have 45.23%, 24.24%, and 40.97% susceptibility in their *Enterobacteriaceae* isolates to tobramycin.

Although aminoglycosides are not often used for human medicine, resistance still poses a concern, because aminoglycosides are often used as last-resort antibiotics for serious infections.

Chromosomal Aminoglycoside Acetyltransferases

Most sources of aminoglycoside resistance genes are via mobile elements. However, some non-antibiotic producing bacteria contain chromosomal AACs. One of the first discoveries of a chromosomally encoded gene was due to an outbreak of a netilmicin-resistant *S. marcescens* in a neonatal unit (Champion *et al*., 1988). A search for the culpable resistance gene led to the finding of a chromosomally-encoded *aac(6')-Ic* gene in the *S. marcescens* isolate (Champion *et al*., 1988). This *aac(6')-Ic* is found in all *S. marcescens,* regardless of whether aminoglycoside resistance is present (Shaw *et al*., 1992). Under normal circumstances, *aac(6')-Ic* is poorly expressed, but exposure to aminoglycosides results in mutations that lead to higher expression, resulting in aminoglycoside resistance (Shaw *et al*., 1992). A palindromic sequence at its promoter suggests an operator that could be binding to and repressing transcription. It was hypothesized that mutations in either this putative operator or the promoter region could cause the increased expression (Shaw *et al*., 1992). While it was suggested that the gene could serve a role in primary metabolism, there have been no further studies to confirm this (Shaw *et al*., 1992).

Oftentimes chromosomal AACs can acetylate a broad range of aminoglycosides, however with poor substrate specificity or turnover. The chromosomally-encoded, species-specific AAC(2')-Ii from *Enterococcus faecium* is one example of an AAC that lacks aminoglycoside specificity (Costa *et al*., 1993; Wright & Ladak 1997). AMEs often display a positive correlation with their MIC phenotypic values and their kinetic rate at sub-level

aminoglycoside concentrations (Radika & Northrop 1984). This prevents cell death, as the enzyme works efficiently when aminoglycoside concentrations are not yet lethal (Radika & Northrop 1984). However, the MIC phenotype of AAC(2')-Ii is only increased with its kinetic rate at saturated aminoglycoside concentrations (Wright & Ladak 1997). At saturated aminoglycoside concentrations, the cell is on the verge of being killed by aminoglycosides, so an efficient aminoglycoside resistance enzyme should not be in the early phase of increasing its expression at this late stage. This, and the low level specificity of AAC(2')-Ii, has prompted a suggestion that AAC(2')-Ii may serve a physiological role rather than an antimicrobial resistance role for aminoglycosides (Wright & Ladak 1997). A low substrate turnover rate with aminoglycosides was also observed with AAC(6')-Ig in *Acinetobacter haemolyticus* (Stogios *et al*., 2016). While able to acetylate a broad range of aminoglycosides, AAC(6')-Ig had significantly less substrate turnover rates compared to other AAC(6') enzymes (Stogios *et al*., 2016). This species-specific chromosomal AAC has a large active site cleft that is only partially occupied when acetylating aminoglycosides, suggesting the enzyme's true role could be acetylation of a larger substrate (Rudant *et al*., 1997; Stogios *et al*., 2016). This partial occupancy was also found with AAC(6')-Iy of *S.* Typhimurium and AAC(6')-Ih of *Acinetobacter baumannii* (Stogios *et al*., 2016). Indeed, the dimeric structure of AAC(6')-Ig is very similar to AAC(6')-Iy and -Ih (Stogios *et al*., 2016). While AAC(6')-Iy is chromosomal, AAC(6')-Ih is found only on plasmids in *Acinetobacter* spp. (Stogios *et al*., 2016). However, analysis of 11 *Acinetobacter*-specific AAC(6') enzymes showed sequence conservation in the unoccupied spaces of the cleft, suggesting a similar role among these enzymes beyond antibiotic resistance (Stogios *et al*., 2016). This analysis included several species-specific chromosomal AAC(6')

enzymes found in the *Acinetobacter* genus, such as AAC(6')-Ih in *Acinetobacter* sp. 6 and AAC(6')-Ij in *Acinetobacter* sp. 13 (Rudant *et al*., 1994; Lambert *et al*., 1994).

Due to their ubiquitous nature and low-level expression, chromosomally encoded AACs might serve physiological roles in bacteria, rather than exist solely for antibiotic resistance. The first study to show another role beyond antibiotic resistance for a chromosomally-encoded AAC was with *aac(2')-Ia* (Payie *et al*., 1996). AAC(2')-Ia was first discovered in *Providencia stuartii* in 1974 and is found only in this species (Chevereau *et al*., 1974). Like *aac(6')-Ic*, *aac(2')-Ia* has low level expression, resulting in aminoglycoside susceptibility (Chevereau *et al*., 1974; Yamaguchi *et al*., 1974; Macinga & Rather 1999). The gene is regulated by several **a**minoglycoside **a**cetyltransferase **r**egulator or *aar* genes, which has been extensively covered in a review (Rather *et al*., 1993; Macinga & Rather 1999). High expression of *aac(2')-Ia* is due to mutations in the *aar* genes and results in resistance to 6'-*N*-ethylmicin, gentamicin, tobramycin, and netilmicin (Macinga & Rather 1999). In 1995, analysis of peptidoglycan *O*-acetylation in *P. stuartii* found that over- or under-expression of the *aac(2')-Ia* gene led to more or less *O*-acetylation, respectively (Payie *et al*., 1996). *O*-acetylation of peptidoglycan increases its resistance to lysozymes/muramidases that hydrolyze peptidoglycan and break it down (Rather *et al*., 1993). Further research showed that aminoglycosides are poor substrates for AAC(2')-Ia (Franklin & Clarke 2001). This suggests AAC(2')-Ia main purpose could be *O*-acetylation of peptidoglycan rather than antibiotic resistance.

Like AAC(2')-Ia, another chromosomal AAC was found to serve a role in peptidoglycan metabolism (Ainsa *et al*., 1997). The gene *aac(2')-Id* is found in all strains of *Mycobacterium smegmatis* (Ainsa *et al*., 1997). Deletion of the gene in *M. smegmatis* resulted in increased susceptibility to lysozymes. As mentioned before, *O*-acetylation of peptidoglycan plays a role in

lysozyme resistance. Analysis of peptidoglycan acetylation revealed that loss of *aac(2')-Id* resulted in less peptidoglycan acetylation (Ainsa *et al*., 1997). The gene *aac(2')-Id* was discovered as a result of hybridization experiments in search of homologues to another chromosomally ubiquitous AAC, *aac(2')-Ib* in *Mycobacterium fortuitum* (Ainsa *et al*., 1997). This search also led to the discovery of *aac(2')-Ic* in *Mycobacterium tuberculosis*.

The chromosomal gene *aac(2')-Ic* in *M. tuberculosis* is present in all strains and has low level expression (Ainsa *et al*., 1997). While able to acetylate aminoglycosides, it does not provide good resistance (Barrett *et al*., 2008). While its true physiological purpose is unknown, a structural analysis of the protein revealed it could be involved in mycothiol biosynthesis (Vetting *et al*., 2002). Other *Mycobacterium* spp., such as *M. abscessus* and *M. leprae* , carry chromosomal *aac* genes as well (Rominski *et al*., 2017; Luthra *et al*., 2018; Ainsa *et al*., 1997). However, the most well-known of these chromosomal AACs is the Eis (Enhanced intracellular survival) protein also found in *M. tuberculosis*.

Eis was discovered as a result of a screening for *M. tuberculosis* genes required for intracellular survival within macrophages (Wei *et al*., 2000). The study constructed a plasmid library and cloned them into *M. smegmatis*, which does not survive well within macrophages. When the plasmid containing *eis* was cloned into *M. smegmatis*, survival increased within the macrophage by over 2-fold. This gene is found in pathogenic strains of *Mycobacterium* and is absent in nonpathogenic species (Wei *et al*., 2000). It encodes for an acetyltransferase capable of acetylating both aminoglycosides, arylalkylamines, human histone H3, and the nucleoid-associated protein HU (Duan *et al*., 2016; Pan *et al*., 2018; Ghosh *et al*., 2016; Green *et al*., 2018). Eis does not have a traditional AAC name, as it modifies the 3" amine and the γ-amine of different aminoglycosides, making it a novel AAC (Houghton *et al*., 2013a). Eis can acetylate

amikacin, capreomycin, netilmicin, hygromycin, kanamycin, neamine, neomycin B, paromomycin, ribostamycin, sisomicin, and tobramycin (Chen *et al*., 2011; Houghton *et al*., 2013b; Reeves *et al*., 2013). Depending on the species of *Mycobacterium*, Eis can acetylate the arylalkylamines: histamine, octopamine, and tryamine (Pan *et al*., 2018). Mutations in the *eis* promoter region are screened for in *M. tuberculosis* isolates, due to their ability to increase expression of *eis*, and therefore aminoglycoside resistance (Chakravorty *et al*., 2015; Pholwat *et al*., 2016; Kambli *et al*., 2016).

Expression of *eis* is regulated by different factors involved in cellular survival. The SigA sigma factor, also known as *rpoV*, in *M. tuberculosis* promotes bacterial intracellular growth in human monocytes and in mice (Wu *et al*., 2009). It regulates both housekeeping genes and genes involved in pathogenesis (Collins *et al*., 1995; Steyn *et al*., 2002; Wu *et al*., 2009). Upregulation of *sigA* results in increased intracellular survival in a macrophage, possibly as a result of upregulation of *sigA* targets (Wu *et al*., 2009). SigA binds to the promoter region of *eis*, and over-expression of *sigA* - either in a plasmid or naturally in a wild-type strain - results in upregulation of *eis* (Roberts *et al*., 2004; Wu *et al*., 2009). As mentioned before, upregulation of *sigA* results in an increased survival within monocytes (Wu *et al*., 2009). This increase in survival is likely due to upregulation of *eis*, as deletion of *eis* in a *sigA*-upregulated wild-type strain resulted in decreased survival within monocytes (Wu *et al*., 2009). The response in bacteria increases survival under starvation conditions (Primm *et al*., 2000). The stringent response regulator Rel_{MTB} negatively regulates *eis* in such conditions. Consistent with its ability to confer antibiotic resistance, *eis* is also part of the *whiB7* regulon (Morris *et al*., 2005; Sowajassatakul *et al*., 2018). The transcriptional regulator *whiB7* is upregulated when the bacteria are exposed to antibiotics and fatty acids, and activates genes involved in antibiotic and

fatty acid resistance (Morris *et al*., 2005). This regulation suggests that the bacteria make full use of Eis in intracellular survival and antibiotic resistance. Despite these regulations, deletion of *eis* has no effect on *M. tuberculosis* growth in human monocytes or mice (Samuel *et al*., 2007). However, deletion of *eis* has a profound effect on the human immune response to *M. tuberculosis*.

Eis affects cytokine secretion in the immune response to *M. tuberculosis*. Examination of infected host cells revealed Eis is secreted into the host cytoplasm during infection (Samuel *et al*., 2007). TNF-α and IL-10 play significant roles in the immune response to *M. tuberculosis*, maintaining a balance between the T_H1 and T_H2 responses (Flynn *et al*., 1995; Gong *et al*., 1996). Addition of Eis into human monocyte culture altered levels of TNF-α and IL-10 in a dose-dependent manner, with TNF-α decreasing and IL-10 increasing when the concentration of Eis was at 5 and 10 μg/mL. (Samuel *et al*., 2007). To further investigate the role of Eis in cytokine secretion, infection of human monocytes was performed and compared in an *eis* deletion mutant, wild-type, and complemented strain; the deletion mutant showed a significant increase in TNF-α production and a significant decrease in IL-10 (Samuel *et al*., 2007). This corroborated that the Eis protein decreases TNF-α and increases IL-10 secretion in *M. tuberculosis* infections. In another study, macrophages infected with a deletion mutant of *eis* displayed greater levels of the pro-inflammatory cytokines TNF-alpha and IL-6 compared to the control strain with *eis* (Shin *et al*., 2010). Eis also increases IL-10 and IFN-γ production and decreases TNF-α and IL-4 production in T-cells (Lella & Sharma 2007). The mechanism behind this effect is the Eis inhibition of the ERK1/2 and JAK pathways, which are used to increase TNF-α and IL-4 production and decrease IL-10 and IFN-γ production. Eis can also acetylate the human histone H3, which binds to the IL-10 promoter region (Duan *et al*., 2016). Eis' ability to affect cytokine

production may ruin the balance between a proper T_H1 and T_H2 response in tuberculosis infections (Lella & Sharma 2007).

In addition, Eis also plays a role in autophagy, reactive oxygen species (ROS) generation, inflammation, and cell death in tuberculosis infections. It most likely exerts its effect through acetylation of the dual-specificity protein phosphatase 16 (DUSP16)/mitogen-activated protein kinase phosphatase-7 (MKP-7)(Kim *et al*., 2012). Autophagy is the process of removing organelles from the cell and helps overcome the *M. tuberculosis*-induced blockage of membrane trafficking (Shin *et al*., 2010). Deletion of *eis* resulted in increased amounts of autophagic vacuoles within infected macrophages, along with increased production of proinflammatory cytokines (Shin *et al*., 2010). This was due to an increased amount of NOX-derived ROS production in the macrophage infected with the *eis* deletion strain (Shin *et al*., 2010). Deletion of *eis* also resulted in increased cell death, as a result of increases in caspase-independent cell death (CICD) and JNK-dependent regulation of ROS signaling (Shin *et al*., 2010). This trend of increased autophagy, inflammation, and cell death with an *eis* mutant held true in mouse studies as well (Shin *et al*., 2010), suggesting that Eis inhibits autophagy, ROS production, and cell death in normal tuberculosis infections.

Introduction to AAC(6')-Iy

The gene *aac(6')-Iy* is a cryptic aminoglycoside resistance gene first noted when a large, 60-kb deletion occurred in a clinical infection attributed to S. Enteritidis (Magnet *et al*., 1999). This deletion removed the native promoter of the *aac(6')-Iy* gene and put the promoter for the gene *nmpC* upstream of the *aac(6')-Iy* gene, leading to increased expression and aminoglycoside resistance in the isolate (Magnet *et al*., 1999). This was an unusual case, as *Salmonella* normally

acquires aminoglycoside resistance through external sources of DNA, and this aminoglycoside resistance was conferred by a chromosomally encoded gene. However, despite having a chromosomally encoded aminoglycoside resistance gene, wild-type *Salmonella* does not show resistance to concentrations of aminoglycosides that would be expected in the serum of patients treated with clinically appropriate doses of aminoglycosides.

The gene *aac(6')-Iy* was acquired early in the evolution of *Salmonella*, as the gene is found in *Salmonella bongori* (Magnet *et al*., 1999). It was reported to be in all *Salmonella* subspecies, except *Salmonella enterica* subspecies *arizonae* (Magnet *et al.*, 1999; **Figure 5**). The *aac(6')-Iy* gene is consistently in the same genomic environment in *Salmonella* (Magnet *et al*., 1999; **Figure 5**). Magnet *et al.* conducted a search for the presence of this gene in closely related *Escherichia coli* and *Citrobacter freundii* and showed the absence of *aac(6')-Iy* in their genomes, suggesting that the *aac(6')-Iy* is specific for *Salmonella* (Magnet *et al*., 1999). In *Salmonella, aac(6')-Iy* is found adjacent to the *sgc* operon which includes 7 genes that encode putative members of a carbon metabolism locus. The *sgc* operon was first discovered in *E. coli* (Reizer *et al*., 1994). Since then, it has never been characterized, although its genes are related to galactitol, lactose, and cellobiose utilization genes (Reizer *et al.*, 1994).

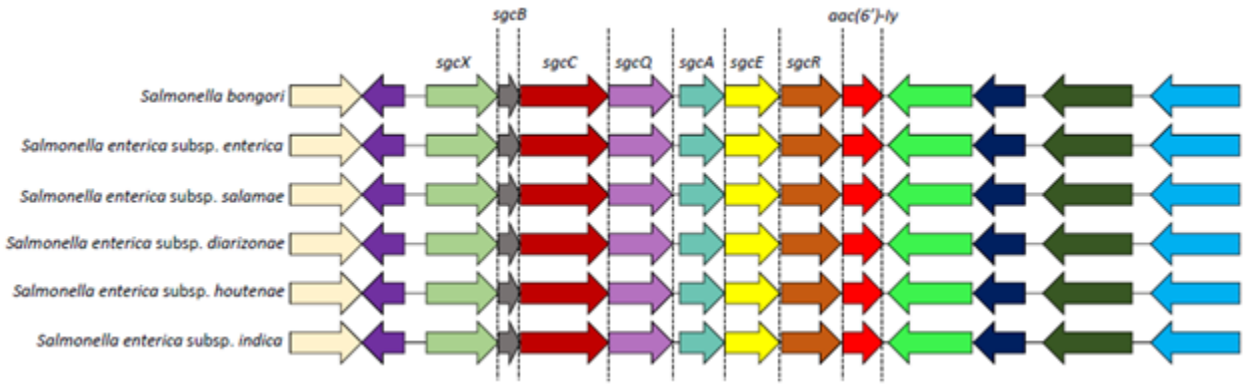


Figure 5. Genomic presence and environment of *aac(6')-Iy*. PATRIC was used to analyze the genomic presence and environment of *aac(6')-Iy* in *Salmonella* (Davis *et al*., 2020). Subspecies *arizonae* does not have the *sgc* and *aac(6')-Iy* genes. Note that *Salmonella bongori* also contains the *aac(6')-Iy* gene (86% identity), suggesting the gene's long-term presence in *Salmonella*.

What is AAC(6')-Iy?

The protein encoded in *aac(6')-Iy* is an N-acetyltransferase that acetylates aminoglycosides at the 6' amine position, putting the enzyme in the large AAC(6') family (Magnet *et al*., 1999). While able to acetylate neomycin, kanamycin, tobramycin, amikacin, dibekacin, sisomicin, netilmicin, gentamicin, 2'-*N*-ethylnetilmicin, and ribostamycin, the gene does not normally confer resistance in *Salmonella* (Magnet *et al*., 1999). Structural analysis of the protein in its dimer formation found that it is most similar to *Saccharomyces cerevisiae* Hpa2 histone acetyltransferase (Vetting *et al*., 2004). This similarity led to finding that AAC(6')-Iy rapidly acetylates both calf thymus histone III-S and the human histone H3 proteins (Vetting *et al*., 2004). Despite this, whether it does acetylate host cell histones *in vivo* is unknown. Other proteins in the same superfamily as AAC(6')-Iy can also acetylate eukaryotic histones, such as *Enteroccocus faecium*'s AAC(6')-Ii and tabtoxin resistance protein (He *et al*., 2003).

Regulation of the *aac(6')-Iy* gene is unknown. Its expression may depend on the *sgc* promoter, due to a lack of an upstream transcription termination signal (Magnet *et al*., 1999). The presence of a trans-sRNA upstream of the gene within the *sgc* cluster could indicate translational regulation, as well. This sRNA, *ryjB*, lies at the 5' end of *sgc*A (STM1615), which putatively binds to its own RNA transcript and can repress translation. If *aac(6')-Iy* is expressed under the same promoter as the *sgc* cluster, then *ryjB* binding to the mRNA produced could inhibit translation of the genes following *sgc*A (STM1615), including *aac(6')-Iy*. It is unknown whether *aac(6')-Iy* expressed under *in vitro* conditions can provide the cell some level of aminoglycoside resistance. Salipante and Hall demonstrated that *aac(6')-Iy*, termed aac(6')-Iaa due to small differences in nucleotide sequence, reached the limit of its evolutionary potential, and the chances of developing a beneficial mutation that would confer better aminoglycoside resistance was low (Salipante & Hall 2003).

According to SalComD23580 from the Hinton Lab, *aac(6')-Iy*'s expression increases more than 2-fold in response to NaCl shock, anaerobic shock, peroxide shock and nitric oxide shock in SPI-2 inducing media, and within macrophages (Kröger *et al*., 2013; Srikumar *et al*., 2015). These data hint at expression in conditions experienced within the macrophage. Although AAC(6')-Iy is able to acetylate aminoglycosides, aminoglycosides do not penetrate eukaryotic cells and their transport into bacterial cells are inhibited by high osmolarity, low pH, and anaerobic conditions (Mingeot-Leclercq *et al*., 1999; Damper & Epstein 1981; Xiong *et al*., 1996). As a result, some conditions in which *aac(6')-Iy* expression is induced are conditions in which aminoglycoside antibiotics would not work as effectively. The true role of *aac(6')-Iy* in *Salmonella* has, therefore, not been elucidated.

Genomic environment of aac(6')-Iy

The *aac(6')-Iy* gene is downstream and partially overlaps a group of genes termed the *sgc* cluster (**Figure 6**). This cluster was first discovered in *E. coli* (Reizer *et al*., 1994), and its functions were never investigated in either *E. coli* or *Salmonella*. The genes in this putative operon are related to genes involved in carbohydrate metabolism. There are 8 genes in this putative operon, with *aac(6')-Iy* constituting the last gene of the operon. While the *sgc* genes are found in *E. coli*, the *aac(6')-Iy* gene is not found in *E. coli*. The presence of this cluster of genes in most *Salmonella* strains and across related bacteria, and their putative functions suggest a physiological role in *S.* Typhimurium.

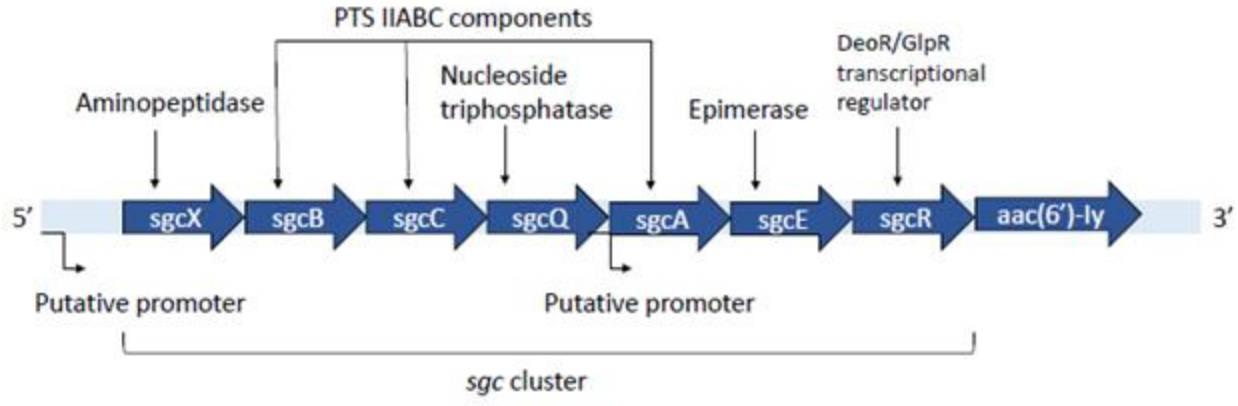


Figure 6. Diagram of the *sgc* cluster with predicted protein functions.

The first gene in this putative operon is termed *sgc*X (STM1612) and encodes for a putative aminopeptidase or endoglucanase. The second gene is termed *sgc*B (STM1613) and encodes for a putative PTS IIB component. The third gene is *sgc*C (STM1614) and encodes for a putative PTS IIC component. The fourth gene is *sgc*Q (STM1615) and encodes for a putative nucleoside triphosphatase. The fifth gene is *sgc*A (STM1616) and encodes for a putative PTS IIA component. The sixth gene is *sgc*E (STM1617) and encodes for a putative epimerase. The seventh gene is *sgc*R (STM1618) and encodes for a putative DeoR/GlpR transcriptional regulator

and may act to inhibit transcription of the operon. The eighth gene is *aac(6')-Iy* (STM1619) and encodes for an acetyltransferase that can acetylate aminoglycoside and eukaryotic histone proteins.

Similar to *aac(6')-Iy*, the *sgc* cluster is found in all *Salmonella* species, except *Salmonella arizonae* (Magnet *et al*., 1999; **Figure 5**). Analysis software predict a KpLE2 phage-like element in the *sgc* genes, suggesting a horizontal transfer event in the ancestry of *Salmonella*. The *sgc* genes are also found in other Gram-negative bacteria, such as *E. coli*. However, the position does not remain consistent in closely related bacteria. In *E. coli* K-12, the *sgc* operon lies on a different position of the chromosome compared to *S.* Typhi CT18 (Bishop *et al*., 2005).

Genetic regulation of the cluster remains largely unstudied. In *E. coli*, there is a consensus sequence for sigma-70, encoded by *rpoD* upstream of *sgc*X (Shimada *et al*., 2013). Since sigma-70 controls various genes related to growth, this further suggests a role for the *sgc* cluster in growth of the organism. Curran *et al*. demonstrated that SlyA, a transcriptional regulator required for virulence, binds in the promoter region of *sgc*X, and over-expression of *slyA* leads to downregulation of the *sgc* genes (Curran *et al*., 2017). There is also a trans-sRNA termed *ryjB* in the 5' end of *sgc*A (STM1615), which may regulate translation of the downstream genes in the cluster. This sRNA is expressed in *in vitro* conditions, in both LB and M9 media (Kawano *et al*., 2005). In addition, the long intergenic region between *sgc*Q and *sgc*A might include a promoter, however this has not been empirically proven.

Environmental regulation of the cluster hints at a role for the operon in the macrophage. According to SalComD23580 from the Hinton Lab, in *Salmonella*, *sgc*R (STM1618) has increased expression at peroxide shock and nitric oxide shock in SPI-2 inducing conditions, early

stationary phase, and with anaerobic shock (Kröger *et al*., 2013; Srikumar *et al*., 2015). The other genes in the cluster experience upregulation in a macrophage environment (Kröger *et al*., 2013; Srikumar *et al*., 2015). In *Escherichia coli*, the sRNA *ryjB* is upregulated at high Mg2+ concentrations (Raghavan *et al*., 2011). However, actual survival of *Salmonella* mutants with and without the *sgc* genes in macrophage-like conditions and in macrophage survival assays has not been tested.

Transposon studies have revealed a role for some of the individual *sgc* genes in animal models of infection. Chaudhuri *et al*., using the method of transposon-directed insertion-site sequencing (TraDIS), created a transposon library and screened genes that resulted in reduced fitness of *S*. Typhimurium in chickens, pigs, and cattle (Chaudhuri *et al*., 2013). In their data, *sgc*B (STM1613), *sgc*Q (STM1615), and *sgc*R (STM1618) mutants had reduced fitness in a cattle model.

Only three chromosomal AACs were shown to have a physiological function in their bacteria (*P. stuartii* (AAC(2')-Ia, *M. smegmatis* AAC(2')-Id, and pathogenic *Mycobacterium* Eis). Evidence for a physiological role for AAC(6')-Iy lies in its ability to acetylate eukaryotic histones, its proximity to a putative metabolic operon, and bioinformatics data showing upregulation within the macrophage (Vetting *et al*., 2004; Magnet *et al*., 1999; Kröger *et al*., 2013; Srikumar *et al*., 2015). The next chapters will investigate whether AAC(6')-Iy serves a role beyond antibiotic resistance in *Salmonella*.

CHAPTER III

INVESTIGATION OF *AAC(6')-IY*

Introduction

The clinical case report describing the 60-kb deletion in *Salmonella enterica* serovar Enteritidis that resulted in loss of native control suggests that, if *aac(6')-Iy* is sufficiently expressed, it provides resistance to concentrations of aminoglycosides used clinically. Protein purification research demonstrated that *aac(6')-Iy* is an *N*-acetyltransferase that can acetylate aminoglycosides and eukaryotic histone proteins (Vetting *et al*., 2004). In addition, previous research on chromosomal AACs showed that AAC(2')-Ia and AAC(2')-Id could acetylate peptidoglycan, and loss of the genes resulted in a decreased resistance to lysozyme (Payie *et al*., 1995; Ainsa *et al*., 1997). It is also unknown whether AAC(6')-Iy could play a role in lysozyme resistance. The role of *aac(6')-Iy* in aminoglycoside resistance and lysozyme resistance is unclear for *S.* Typhimurium.

Knowledge of the transcriptional regulation of *aac(6')-Iy* and conditions in which it could be upregulated are also limited. The promoter for *aac(6')-Iy* has not been discovered. There are two putative promoters driving expression of the *sgc* and *aac(6')-Iy* genes, one upstream of *sgcX*, the putative aminopeptidase, and the other in the intergenic region between *sgcQ*, the putative nucleoside triphosphatase, and *sgcA*, the putative PTS IIA component. In addition, there is an sRNA within the *sgc* cluster that may also regulate expression of the *sgc* and *aac(6')-Iy* genes. The last gene in the *sgc* cluster *sgcR* encodes for a putative transcriptional regulator and may also regulate expression of these genes. RNA-seq data demonstrated increased expression of *aac(6')-Iy* to NaCl shock, anaerobic shock, peroxide shock and nitric oxide shock in SPI-2 inducing

media, and within the macrophage (Kröger *et al*., 2013; Srikumar *et al*., 2015). This data suggests that *aac(6')-Iy* is upregulated in environments found within host cells.

Once in the intestines, *Salmonella* invades the intestinal epithelial cells and forms the SCV (Takeuchi 1967; Takeuchi & Sprinz 1967; Kihlstrom & Latkovic 1978; Finlay & Falkow 1989; Steele-Mortimer 2008). Within epithelial cells, *Salmonella* exists as two populations: one within the SCV and one within the cytosol (Knodler *et al*., 2014). There are multiple effectors responsible for creation and maintenance of the SCV with the *sifA* gene being essential for maintenance of the SCV membrane (Beuzón *et al*., 2000). Loss of *sifA* results in loss of the vacuolar membrane and escape into the cytosol (Beuzón *et al*., 2000). Once in the cytosol, *Salmonella* is then able to leave the intestinal epithelial cells via an extrusion mechanism and enter the intestinal lumen (Knodler *et al*., 2010). Within the *lamina propria*, macrophages can encounter and phagocytize *Salmonella.* In the macrophage, *Salmonella* subverts the phagolysosome fusion and establishes another SCV (Steele-Mortimer 2008). In macrophages, *Salmonella* can only exist in the SCV due to the presence of caspase-1 and -11 in the cytosol (Thurston *et al*., 2016).

There are several methods to look at *Salmonella* survival within the cytosol and the SCV. A popular method to analyze cytosolic survival is the chloroquine resistance assay. Chloroquine accumulates within endosomes and kills bacteria present within the SCV, but not in the cytosol (Knodler *et al*., 2014). This method compares the cytosolic CFU/mL obtained from infected host cells treated with chloroquine with total CFU/mL obtained from untreated infected host cells. One can also take advantage of a *sifA* mutant leading to loss of the SCV membrane to obtain a cytosolic population of bacteria within the host cell. To analyze SCV survival, immunofluorescence microscopy and live microscopy have also been used to evaluate the population of *Salmonella* within host cells and their intracellular location. LAMP1 is often used as a marker for the SCV

and % LAMP1-positive bacteria have been used to estimate the amount of bacteria present in the SCV (Knodler *et al*., 2010).

This chapter looks to answer the three aims for investigation of *aac(6')-Iy*: 1) determine if deletion of *aac(6')-Iy* reduces the minimum inhibitory concentration of aminoglycoside antimicrobial drugs in *S.* Typhimurium, 2) determine transcriptional regulation of *aac(6')-Iy*, and 3) determine if *aac(6')-Iy* plays a role in *S.* Typhimurium survival within eukaryotic cells.

Materials and Methods

Bacterial strains, plasmids, and growth conditions

A *Salmonella enterica* serovar Typhimurium derivative of strain ATCC 14028, IR715, that is resistant to nalidixic acid was used as wild-type (WT) (Stojiljkvoci & Bäumler *et al*., 1995). *Salmonella* strains were grown in lysogeny broth (LB) with aeration at 37ºC, unless indicated otherwise. A deletion mutant of *aac(6')-Iy* was obtained from Dr. Helene Andrews-Polymenis' lab that was constructed via the Datsenko & Wanner method (Datsenko & Wanner 2000). A *Salmonella* strain containing pKD46 was used to construct a deletion mutant of the *sgc* genes using the Datsenko & Wanner method (Datsenko & Wanner 2000). A low-copy plasmid pWSK29 was used to complement *aac(6')-Iy* in *trans* (Wang & Kushner 1991). The plasmids pCP20 and pCE36 were used for construction of chromosomal *lacZ* transcriptional fusions (Cherepanov & Wackernagel 1995; Ellermeier *et al*., 2002). When necessary, antibiotics were used in the following concentrations: chloramphenicol 30 µg/mL, nalidixic acid 50 µg/mL, and carbenicillin 100 µg/mL.

Cell culture conditions

HeLa (ATCC® CCL-2™) and RAW 264.7 cells (ATCC® TIB-71™) were grown at 37°C in a 5% CO_2 incubator in growth media (GM) consisting of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS).

Primers used in this study

Primers were obtained from Millipore Sigma and re-suspended in nuclease-free water (Millipore Sigma, USA).

Table 1. Primers, probes, and oligonucleotides used for Chapter III

Primer	Sequence (5' to 3')	Purpose
aac(6')-Iy 5'	ACGTTATCAGCAGGCGGGAT	Confirmation of Deletion
aac(6')-Iy 3'	ATCTGCCCCAGTAATATAAA	
C1	TTATACCAAGGCGACAAGG	
C2	GATCTTCCGTCACAGGTAGG	
Lac	GACCATTTTCAATCCGCA	Confirmation of *lacZ*
Km	TTTCTAGAGCTGTTAAAAGGACA	
aac-HindIII-F	TAAGCAAAGCTTATGAGCCAACAGCGCCCCGA	Complementation
aac-BamHI-R	TGCTTACTCGAGTCAACAACGCTTTCGGTA	
M13(-21)-F	TGTAAAACGACGGCCAGT	
M13(-40)-R	GTCATAGCTGTTTCCTG	
aac(6')-Iy-RT-F	CGCCCGTGGTTTTCCTT	qRT-PCR
aac(6')-Iy-RT-R	CCGCGTTGACGGAATGA	
aac(6')-Iy Probe	AAGGTATTTTTGTTCTCCC	
16S RT-F	CAACGCGAAGAACCTTAC	
16S RT-R	CCCAACATTTCACAACAC	
16S Probe	TTGACATCCACAGAAGAATCCAGAGA	
aac(6')-Iy DNA Probe F1	GTATTTTTGTTCTCCCCTCA	Northern Blot
aac(6')-Iy DNA Probe R2	TCAACAACGCTTTCGGTAGA	
aac-XhoI-F (RNA Probe)	TAAGCACTCGAGATGGACATCAGGCAAATGAAC	
aac-HindIII-R (RNA Probe)	GAATTGAAGCTTTCAACAACGCTTTCGGTAGA	

Construction of strains used in this study

A deletion of *aac(6')-Iy* marked with chloramphenicol resistance was obtained from Dr. Andrews-Polymenis' lab as mentioned before. In order to move the mutation to the desired WT strain, phage P22 was used to transduce the mutation into WT. The deletion mutant was confirmed via PCR and Southern blot.

The low-copy plasmid pWSK29 was used for complementation of *aac(6')-Iy* by inserting the gene into the multiple cloning site (MCS). Putative plasmids were screened by blue/white screening on LB agar plates supplemented with carbenicillin, isopropyl β-d-1-thiogalactopyranosideand (IPTG), and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal). White colonies were screened and checked for the presence of *aac(6')-Iy* in the MCS of pWSK29 via PCR and Sanger sequencing.

lacZ fusions were created using the pCP20 plasmid and the pCE36 plasmid. Briefly, the temperature-sensitive plasmid pCP20 was electroporated into either the *aac(6')-Iy* or *sgc* deletion mutants. Then, the plasmid pCE36 containing *lacZ* was electroporated into the deletion mutants containing pCP20, which flipped out the *lacZ* gene from pCE36 into the area of the deletion mutant, creating a chromosomal *lacZ* fusion. Fusions were confirmed with PCR.

In order to obtain fluorescent bacteria for immunofluorescence microscopy, the pFPV-mCherry plasmid (Addgene, USA) was transformed into the WT and *aac(6')-Iy* strains (Drecktrah *et al*., 2008).

E-test to measure minimum inhibitory concentration

An Epsilometer test (E-test® strips; bioMérieux, USA) was used to determine the minimum inhibitory concentration (MIC) of the aminoglycoside for the WT and the *aac(6')-Iy*

deletion mutant. Briefly, three to five colonies of a strain were picked from a freshly grown plate and suspended in 1X PBS. The turbidity was adjusted to be equivalent with a 0.5 McFarland standard. This suspension was inoculated onto a Mueller-Hinton plate using a sterile cotton swab. Next, a commercially available E-test strip of either gentamicin or amikacin was placed onto the plate. The plate was incubated for 18-20 hours at 37ºC. After incubation, the MIC was determined by matching the zone of inhibition to the number on the E-test strip.

Minimum inhibitory concentration in microbroth dilution

The MIC of WT and the *aac(6')-Iy* mutant were determined by microbroth dilution. Briefly, fresh colonies were picked from a plate and resuspended in sterile saline to a concentration equivalent with a 0.5 McFarland standard. This culture was further diluted 1:150 in Mueller Hinton broth and used for inoculation. Aliquots of 100 µL of MHB only were placed into all wells of the 96-well plate. Aliquots of 100 µL of 2X the highest concentration of antibiotic desired was added to the first row and mixed via pipetting 10X. A dilution series was created by removing 100 µL from the wells of the first row and mixed with the wells of the next row. This was continued for each row until the last row was reached, where 100 µL was removed after mixing. The diluted bacterial culture was then added to the wells of the plate. The plate was covered with the Breathe-Easy® sealing membrane (Millipore Sigma, USA), and the plate was incubated at 37ºC overnight with no shaking. The MIC was determined by determining the lowest concentration where there was no bacterial growth in the plate 24 hours after inoculation.

Lysozyme Sensitivity Disk Diffusion Assay

The ability of the bacteria to inhibit the activity of lysozyme on the cultures was determined using the lysozyme disk diffusion assay. Briefly, one colony was picked from a plate and grow overnight. The overnight culture was diluted 1:100 and grown to OD600 = 0.4. A 100 µL aliquot of this culture was added to 4 mL 0.75% soft mueller hinton agar (MHA) held at 50°C, vortexed, and poured onto plates containing 15 mL solidified 1.5% MHA. The plates were left to dry for 20-30 minutes. Whatman filter paper disks (70 mm) that had been autoclaved for 30 minutes at 121ºC were used as disks in this assay. A 5 µL aliquot of 100 µg/mL lysozyme was pipetted onto the paper disks, and the disks were placed onto the agar plates. There were three replicates per plate and the experiment was repeated three times. The plates were incubated at 37ºC overnight and the diameter of the zone of inhibition was measured using a ruler.

Beta-galactosidase assays

Analysis of *sgc* and *aac(6')-Iy* expression was performed using beta-galactosidase assays. Briefly, cultures were diluted 1:100 and grown until the desired growth phase. The OD_{600} of the cells was read and recorded at each time-point. 500 µL of cell culture was added to 500 µL of Z-buffer. Then, cells were lysed with chloroform and 0.1% SDS. An aliquot of 200 µL of ortho-nitrophenyl-β-galactoside (ONPG) was added and incubated at 28ºC until a yellow color developed. The reaction was stopped by adding 500 µL of 1 M Na_2CO_3. The cells were centrifuged for 1 minute at 10,000 *g* and the OD_{420} of the supernatant was read. Miller units were calculated using the formula: $(1000*OD_{420}) / (\text{time in minutes}*\text{volume of cells added in mL}*OD_{600})$.

Bacterial survival in HeLa cells assay

Bacterial survival in HeLa cells were performed as described before with some modifications (Steele-Mortimer 2018). Briefly, HeLa cells were seeded on cell culture plates and incubated at 37°C with 5% CO_2 overnight. The seeded HeLa cells were infected at an multiplicity of infection (MOI) of 100 and incubated at 37°C with 5% CO_2 for 10 minutes. The wells were washed twice with 1X DPBS, fresh GM added, and incubated for an additional 20 minutes. After 20 minutes, the media was replaced with GM containing 50 µg/mL gentamicin and incubated for 40 min. Media was then replaced with GM containing 5 µg/mL gentamicin and incubated for the remainder of the experiment. At 1.5-, 8-, and 16-hours post-infection, wells were washed once with 1X PBS and 1% Triton-X was added to lyse cells at 4°C for 10 minutes. Bacteria were diluted and spot plated onto LB plates with antibiotics to calculate CFU/mL. An *invA* deletion mutant defective in invasion of HeLa cells was used as a control. Chloroquine resistance assays were performed with similar steps to the gentamicin protection assay, with an additional step of replacing the media with GM containing 400 µM chloroquine (Sigma) 1 hour before lysis. % cytosolic CFU/mL was calculated by dividing the cytosolic CFU/mL from the treated wells to the total CFU/mL from the untreated wells and multiplying by 100.

Bacterial survival in RAW264.7 macrophages assay

Bacterial survival in RAW264.7 cells was performed using a similar gentamicin protection assay. Briefly, 1 x 10^5 cells/well RAW264.7 cells were seeded in a 24-well plate 24 hours before infection. The same day, strains used for the study were cultured in LB and incubated for 18 hours. After 18 hours, the samples were opsonized with 10% mouse serum (in PBS) for 30 minutes at 37°C, as previously described (Lathrop *et al*., 2019). The samples were further diluted in GM to

achieve the appropriate MOI. The wells were washed once with 1X PBS and infected at an MOI 10. Infection was synchronized by centrifugation at 1000 *x* g for 10 minutes. The plates were incubated for 30 minutes at 37°C, washed twice with PBS, and then incubated for 1 hour in 100 μg/mL gentamicin. After 1 hour, media was replaced with maintenance media containing 10 μg/mL gentamicin for the remainder of the experiment. At 24 hours post-infection, samples were washed, lysed, and spot plated as described before. The fold change in CFU/mL was calculated by dividing the CFU/mL at 24 hours by the CFU/mL at 2 hours.

RNA Extraction

RAW264.7 and HeLa cells were seeded in 6-well plates at concentration of 3 x 10^6 cells/well or 5 x 10^4 cells/well, respectively. Infections were carried out as described previously. At 4- and 6-hours post-infection for RAW264.7 cells and 8- and 16-hours post-infection for HeLa cells, wells were washed once with 1X PBS and samples were collected using the TRIzol reagent (ThermoFisher, USA). RNA was extracted using a combination of TRIzol and the Qiagen RNeasy mini kit (ThermoFisher, USA; Qiagen, USA). Briefly, chloroform was added to samples in TRIzol and centrifuged. The aqueous phase containing RNA was mixed with 70% ethanol and added to a Qiagen RNeasy column (Qiagen, USA). From there, extraction was carried out following the manufacturer's protocol. DNase I (Life Technologies Corporation, USA) was used for DNA digestion during column extraction.

To obtain the controls for comparing expression of *aac(6')-Iy* in a macrophage, overnight bacterial cultures were diluted 1:1000 in 25 mL LB and outgrown to early stationary phase (ESP) or OD600 around 2.0. 60% methanol was added to 1 mL of culture to fix transcription and the culture pelleted. Supernatant was removed and the pellet stored at -80C for further extraction. For

comparing expression in epithelial cells, bacteria grown to late log phase (3.5 hours) was collected and stored similarly. RNA extraction was carried out using the Qiagen RNeasy mini kit (Qiagen, USA).

Northern blot

Northern blots were performed using RNA isolated from WT grown under the conditions described above. Probes were designed for the 3' ends of the *aac(6')-Iy* gene. Both digoxigenin (DIG)-labeled DNA and RNA probes were used. DIG-labeled DNA probes designed to the 3' ends of the *aac(6')-Iy* gene were created by using the DIG-labeled nucleotide kit (Roche, USA) using the primers aac(6')-Iy DNA Probe F1and aac(6')-Iy DNA Probe R2 (**Table 1**) for amplification of the probe sequence. DIG-labeled RNA probes were created by cloning *aac(6')-Iy* into pWSK29 under the control of the T7 promoter and using the DIG RNA labeling kit (SP6/T7; Roche, USA) to create DIG-labeled *aac(6')-Iy* RNA probe (**Table 1**). A 1 µg aliquot of RNA from cultures grown to ESP (OD600 = 2.0) were run on a denaturing formaldehyde gel and then blotted onto a positively charged Nylon membrane, either via upward capillary transfer or downward alkaline capillary transfer. The blot was UV cross-linked and allowed to dry at 37ºC in a hybridization bag. DIG Easy Hyb™ hybridization buffer (Roche, USA) was added to the bag, sealed, and then incubated in a water bath at 45ºC for 30 minutes. The buffer was removed and the probe in DIG Easy Hyb™ hybridization buffer was then added to the bag and incubated in a water bath at 45ºC overnight. The probe was removed, and the blot stringently washed in both 2X saline-sodium citrate (SSC) with 0.1% sodium dodecyl sulfate (SDS) and 0.5X SSC with 0.1% SDS. The DIG Wash and Block Buffer Set (Roche, USA) was used for washing and detection. The blot was rinsed in 1X maleic acid then blocked for 30 minutes. After blocking, the blot was incubated with anti-

digoxigenin-Ab (diluted 1:10,000 in 1X blocking solution) followed by washing and detection. The blot was analyzed using the BioRad Chemidoc MP Gel Documentation System (BioRad, USA).

Quantitative real time PCR analysis

RNA samples were converted to complementary DNA (cDNA) using TaqMan™ Reverse Transcription Reagents (ThermoFisher Scientific, USA). Quantitative PCR (qPCR) was conducted using primers and probes listed in **Table 1** and the TaqMan Fast Advanced Master Mix (ThermoFisher Scientific, USA) on the Applied Biosystems 7500 Fast Real-Time PCR System (ThermoFisher Scientific, USA).

Immunofluorescence microscopy experiments

HeLa cells were seeded on Nunc™ Lab-Tek™ 8-well Chamber Slide System (Thermo Scientific, USA). The cells were infected with the WT and *aac(6')-Iy* strains containing pFPV-mCherry at an MOI 50 and gentamicin protection assays were carried out as described previously. At 1.5-, 8-, and 16-hours post-infection, cells were washed once with 1X DPBS and fixed with 2.5% paraformaldehyde (pre-warmed to 37°C) for 10 minutes. The cells were washed three times with 1X PBS for 10 minutes each and then blocked and permeabilized with 500 µL PBS containing 10% (v/v) normal goat serum (NGS) and 0.1% (w/v) saponin (SS-PBS) for 15 minutes. The buffer was quickly replaced with 125 µL of mouse monoclonal anti-LAMP-1 antibody in SS-PBS (1:1000, clone H4A3, Developmental Studies Hybridoma Bank, USA) and incubated for 45 minutes. The cells were washed three times again and then 125 µL of Alexa Fluor 488-conjugated goat anti-mouse IgG in SS-PBS (1:800, Life Technologies, USA) were added to the wells and

incubated for 45 minutes. The cells were washed three times again and then 1 drop of ProLong™ Diamond Antifade Mountant with DAPI (Invitrogen, USA) was added to the wells and stored at 4°C until imaging. Images were taken on an Olympus Fluoview FV3000 confocal laser scanning microscope with a PlanApo N SC2, BFP1 60X objective lens (Olympus Corporation, Japan). Fiji/ImageJ was used to re-construct images and count total bacteria and LAMP-associated bacteria (Schindelin *et al*., 2012; Fiji/ImageJ, USA).

Animal Experiments

Bacteria were grown overnight in 10 mL LB with appropriate antibiotics and then pelleted and re-suspended in PBS. A 1:1 mixture of WT:*aac(6')-Iy* mutant culture was used to infect mice and for plating to determine inoculum CFU/mL.

C57BL/6 mice were obtained from The Jackson Laboratory (USA) and maintained at the Texas A&M University Medical Research Engineering Building (MREB). Mice were maintained at 72°F and had a 12 hour day/night cycle. 10-week old mice were infected orally with 100 µL of the 1:1 culture of WT:*aac(6')-Iy* and infection was allowed to proceed for 3 days. Mice were euthanized humanely 3 days PI and the mesenteric lymph nodes, Peyer's patches, spleen, liver, and cecum were removed, weighed, and homogenized in 3 mL PBS, serially diluted, and plated on LB containing antibiotics to enumerate bacteria in each organ.

Statistical analyses

All statistical analyses (Student's *t*-test, ANOVA, Wilcoxon each pair, etc.) were performed using JMP Pro (SAS Institute Inc., USA).

Results

The gene aac(6')-Iy does not play a role in aminoglycoside resistance

The minimum inhibitory concentration (MIC) of the aminoglycoside antibiotics gentamicin, amikacin, and kanamycin were measured against the WT, *aac(6')-Iy* deletion mutant, and the complemented *aac(6')-Iy* strain using both the E-test and microbroth dilution methods. Loss of *aac(6')-Iy* did not lead to a significant change in *S.* Typhimurium resistance to gentamicin, amikacin, and kanamycin compared to WT (**Figure 7**). The MIC for amikacin was 3 µg/mL for WT, 2.7 µg/mL for *aac(6')-Iy*, and 7 µg/mL for the complemented *aac(6')-Iy*. For gentamicin, the MIC was 2.3 µg/mL for both WT and complemented *aac(6')-Iy* and 2.7 µg/mL for *aac(6')-Iy*. For kanamycin, the MIC was 1.9 µg/mL for WT, 1.3 µg/mL for *aac(6')-Iy*, and 2.3 µg/mL for the complemented *aac(6')-Iy*. There was an increase of 2 µg/mL amikacin resistance for the complemented *aac(6')-Iy* compared to WT.

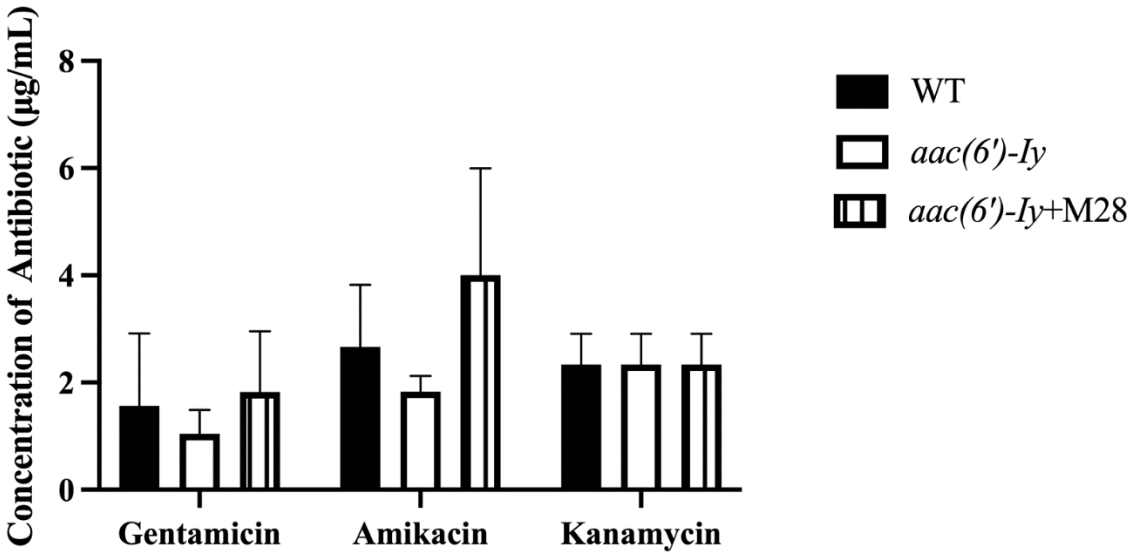


Figure 7. Aminoglycoside susceptibility. MIC results for WT, an *aac(6')-Iy* deletion mutant (Δ*aac*), and a complemented *aac(6')-Iy* mutant for amikacin, gentamicin, and kanamycin demonstrate that loss of *aac(6')-Iy* had no effect on MIC to all aminoglycosides, but complementation caused an increase in resistance to amikacin. Data was collected from 3 independent experiments and analyzed with ANOVA followed by multiple *t*-test comparisons.

The gene aac(6')-Iy does not affect lysozyme resistance.

A disk diffusion assay was performed to measure the diameter of inhibition of lysozyme to WT, *aac(6')-Iy*, and the complemented *aac(6')-Iy*. There was no significant change in lysozyme resistance in an *aac(6')-Iy* deletion mutant or complemented *aac(6')-Iy* strain compared to WT (**Figure 8)**. The diameter of inhibition (mm) remained 10 mm for WT, *aac(6')-Iy*, and complemented *aac(6')-Iy*.

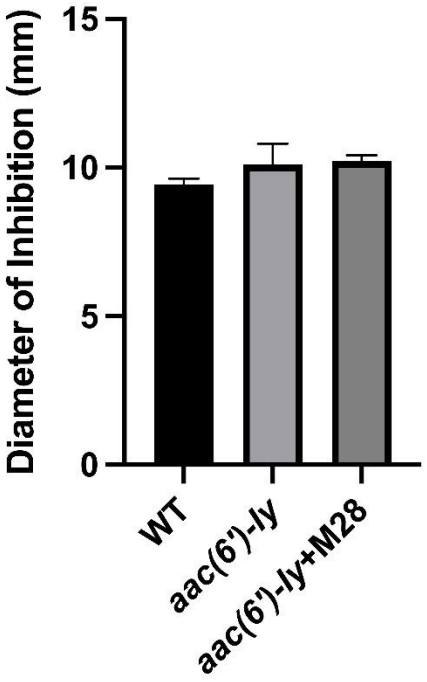


Figure 8. The gene *aac(6')-Iy* does not play a role in lysozyme resistance. The diameter of inhibition was 10 mm for WT, *aac(6')-Iy*, and the complemented *aac(6')-Iy* (*aac(6')-Iy* + M28). Shown are the means and standard deviation from 3 independent experiments. Data were analyzed using ANOVA followed by multiple *t*-test comparisons.

The gene aac(6')-Iy has expression under in vitro conditions

Beta-galactosidase assays were performed to measure expression of *aac(6')-Iy::lacZ* and *sgc::lacZ* fusions in LB. At late-log phase of growth, there was significantly more *aac(6')-Iy* expression compared to *sgc* expression (p-value<0.001; **Figure 9A**). Beta-galactosidase assays of the *aac(6')-Iy::lacZ* fusion in LB vs. SPI-2 inducing broth showed that expression of *aac(6')-Iy* was unchanged between the two broths (**Figure 9B**).

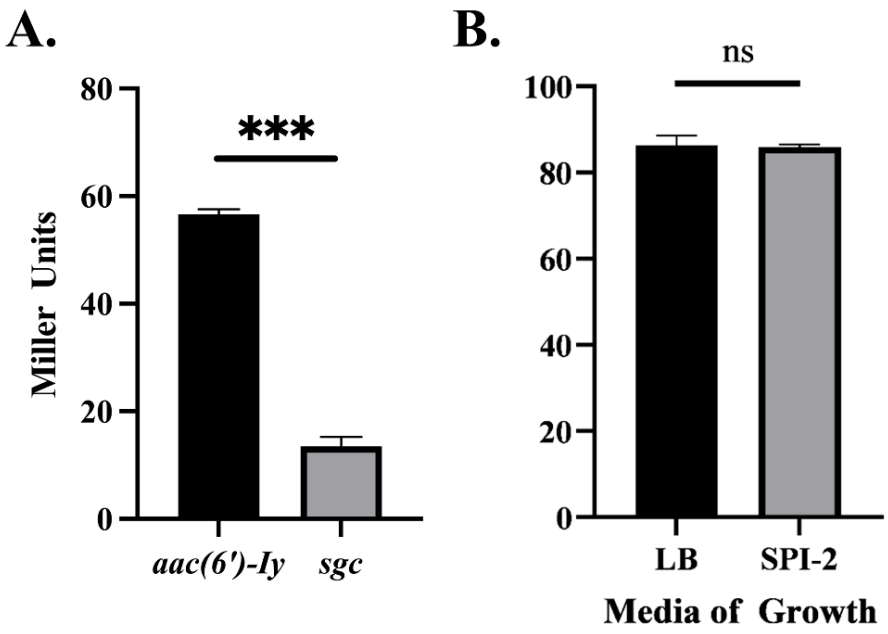


Figure 9. Beta-galactosidase assays of an *aac(6')-Iy::lacZ* fusion and an *sgc::lacZ* fusion. **A)** Beta-galactosidase assays were performed on late-log cultures of *aac(6')-Iy::lacZ* and *sgc::lacZ* fusion mutants grown in LB. There was a significant difference in expression between *aac(6')-*

Iy::lacZ and *sgc::lacZ*, with *aac(6')-Iy::lacZ* showing a higher level of expression. This suggests *aac(6')-Iy* might be under different transcriptional regulation compared to the *sgc* genes. Data was analyzed with Student's *t*-test (n = 3 in triplicate). *** represents p-value<0.001. **B)** Expression of *aac(6')-Iy::lacZ* did not differ in late-log growth in both LB and SPI-2 inducing media. Shown are the means and standard deviation from 3 independent experiments. Data were analyzed using ANOVA followed by multiple *t*-test comparisons.

Northern blot unable to detect aac(6')-Iy transcripts

A northern blot was performed using DIG-labeled DNA probes to *aac(6')-Iy* and WT RNA collected at early stationary phase (OD600 = 1.0). The northern blot could not detect the presence of an *aac(6')-Iy* transcript. DIG-labeled RNA probes were created and used to increase sensitivity, but it did not detect an *aac(6')-Iy* transcript.

Upregulation of aac(6')-Iy in host cells

qRT-PCR was performed on RNA collected from infected host cells RAW264.7 macrophages and HeLa epithelial cells at 4- and 6-hours post-infection (PI) for RAW264.7 cells and 8- and 16-hours PI for HeLa cells. Expression of *aac(6')-Iy* in these times post-infection was compared to RNA collected from *in vitro* grown cultures in LB. The gene *aac(6')-Iy* is upregulated greater than 10-fold in RAW264.7 macrophages at 4- and 6-hours PI and greater than 5-fold in HeLa epithelia cells at 8- and 16-hours PI (**Figure 10**).

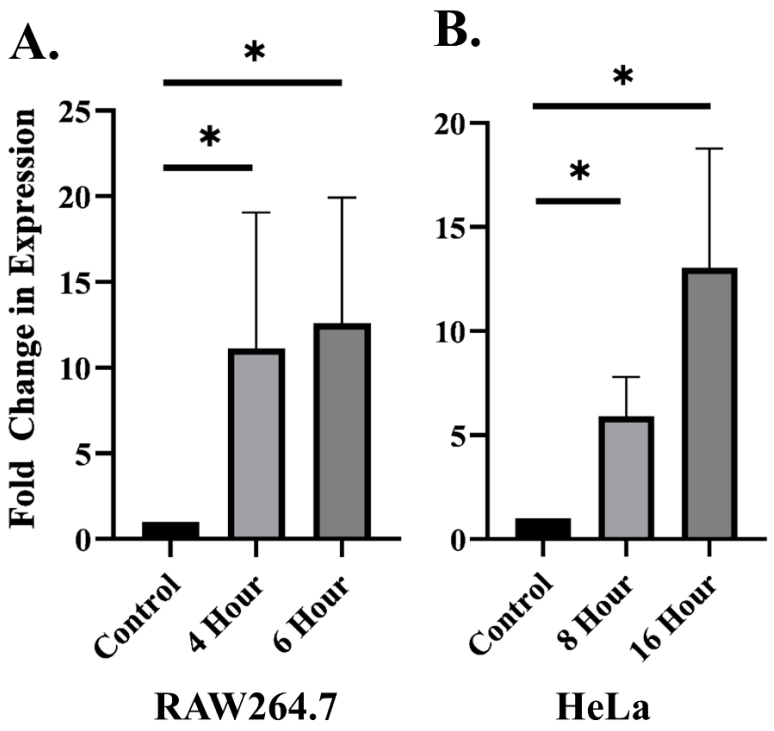


Figure 10. Upregulation of *aac(6')-Iy* in host cells. Quantitative RT-PCR was performed to measure expression of the gene *aac(6')-Iy* in RAW264.7 macrophages (A) and HeLa epithelial cells (B) post-infection. RNA was collected from *in vitro* grown WT bacteria at ESP (OD600 ~ 2.0), as well as infected RAW264.7 macrophages at 4 and 6 hours PI (A) and infected HeLa cells at 8 and 16 hours PI (B). The 16S ribosomal RNA (rRNA) gene was used as the reference gene and the RNA collected from early stationary phase (ESP) bacteria was used as the endogenous control. Shown are the mean fold changes and standard deviation from 3 independent experiments. Fold change was calculated as $2^{-\Delta\Delta Ct}$. * represents fold change>2.

The gene aac(6')-Iy plays a role in intra-macrophage survival

The fold change in the CFU/ml following infection of RAW264.7 macrophages for 2 hours and 24 hours showed that the *aac(6')-Iy* deletion mutant had a significant reduced fold change

within the macrophage compared to both WT and a complemented *aac(6')-Iy* mutant (**Figure 11**). The complemented *aac(6')-Iy* (*aac(6')-Iy*+M28) restored survival.

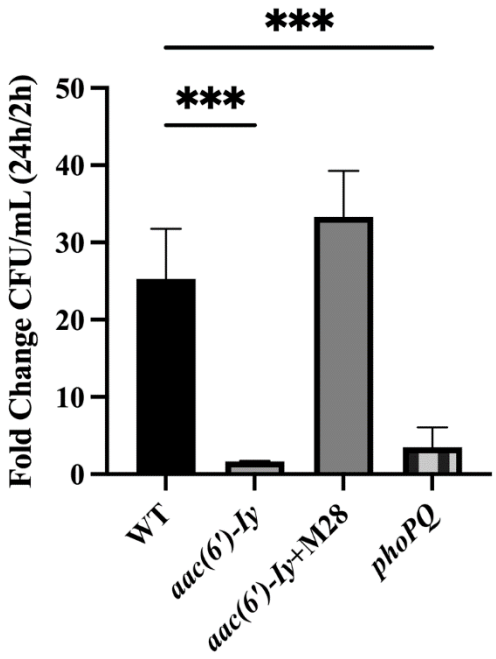


Figure 11. The gene *aac(6')-Iy* is important for intra-macrophage survival. Loss of *aac(6')-Iy* resulted in a significant decrease in survival within RAW264.7 cells. Complementation of *aac(6')-Iy* (*aac(6')-Iy* + M28) restored survival. The fold change in CFU/mL was calculated by dividing the 24-hour CFU/mL by the 2-hour CFU/mL. The deletion mutant *phoPQ* was used as control. Shown are the means and standard deviation from 3 independent experiments. Data were analyzed using ANOVA followed by multiple *t*-test comparisons. *** represents p-value < 0.001.

The gene aac(6')-Iy plays a role in intra-epithelial cell survival

A gentamicin protection assay was carried out in HeLa epithelial cells and the fold change in CFU/mL at 1.5 hours vs. 8 or 16 hours PI was calculated. Loss of *aac(6')-Iy* did not result in an

invasion defect (**Figure 12**), nor a survival defect at 8 hours post-infection (**Figure 13A**). However, loss of *aac(6')-Iy* resulted in a survival defect 16 hours post-infection in HeLa cells (**Figure 13B**).

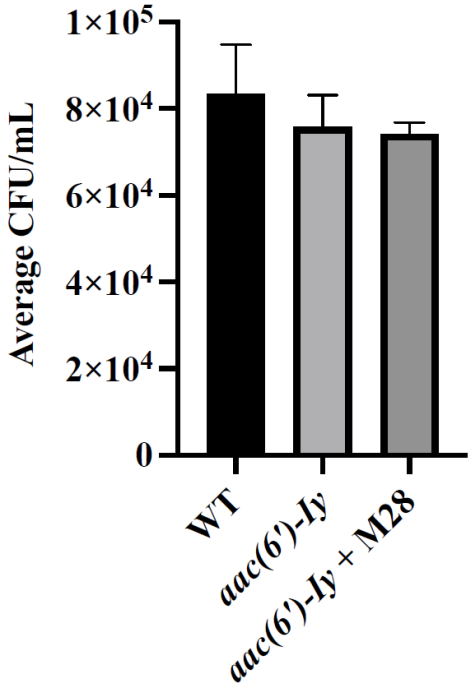


Figure 12. Loss of *aac(6')-Iy* had no effect on invasion of epithelial cells. HeLa epithelial cells were infected with cultures grown to late-log phase and invasion was measured by lysing the cells 1.5 hours post-infection and the bacteria plated. There was no significant difference in CFU/mL between WT and *aac(6')-Iy* or between WT and the complemented *aac(6')-Iy* strain (*aac(6')-Iy*+M28). The deletion mutant *invA* was used as control. Shown are the means and standard deviation from 3 independent experiments. Data were analyzed using ANOVA followed by multiple *t*-test comparisons.

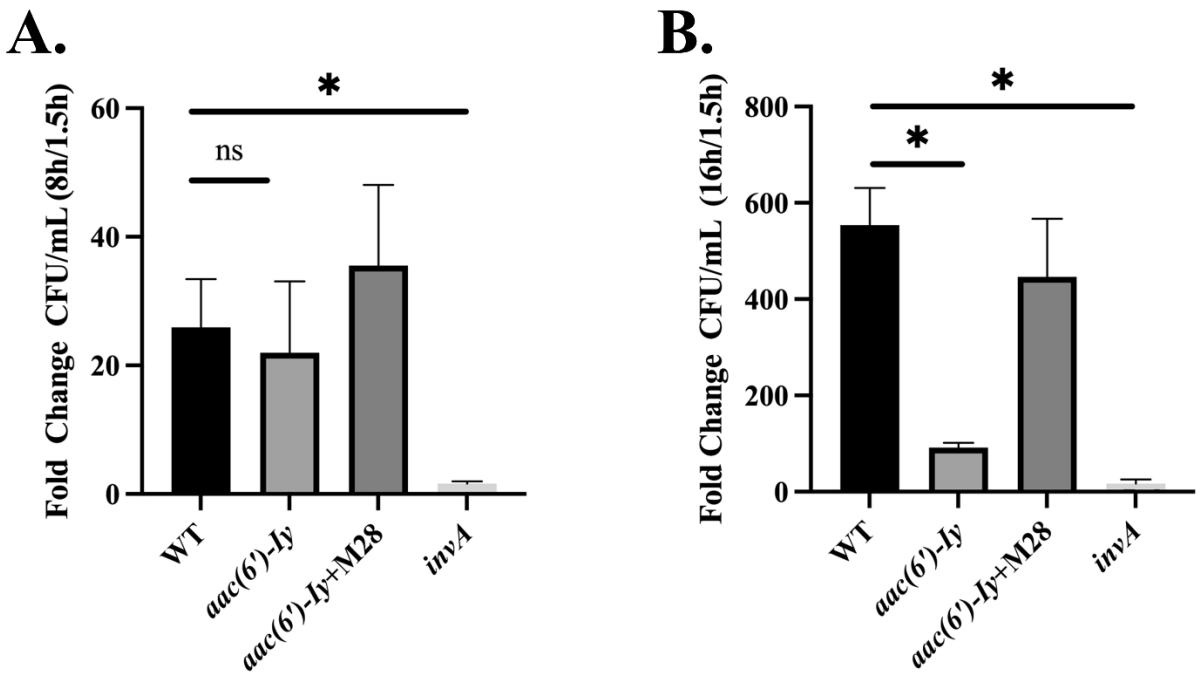


Figure 13. The gene *aac(6')-Iy* is important for survival in epithelial cells. HeLa cells were infected with WT, *aac(6')-Iy*, and *aac(6')-Iy* + M28 (complemented *aac(6')-Iy*) at an MOI 100. At 8 hours (A) and 16 hours (B) post-infection, cells were lysed and bacteria plated to determine CFU/mL. The fold change in CFU/mL was calculated by dividing CFU/mL at 8 or 16 hours by the CFU/mL at 1.5 hours post-infection. The deletion mutant *invA* was used as a control. Shown are the means and standard deviation from 3 independent experiments. Data were analyzed using ANOVA followed by multiple *t*-test comparisons. * represents *p*-value <0.05.

The gene aac(6')-Iy does not affect cytosolic survival within HeLa cells

HeLa epithelial cells were infected with WT, *aac(6')-Iy*, *sifA*, and a double mutant of *aac(6')-Iy sifA*. At 16 hours post-infection, there was no significant difference in normalized CFU/mL between a *sifA* mutant and the double mutant of *aac(6')-Iy sifA* (**Figure 14A**.

A chloroquine resistance assay was performed to further corroborate that *aac(6')-Iy* does not play a role in cytosolic survival. At 16 hours PI, there was no significant difference in % cytosolic bacteria between WT, *aac(6')-Iy*, and the complemented *aac(6')-Iy* sample (**Figure 14B**).

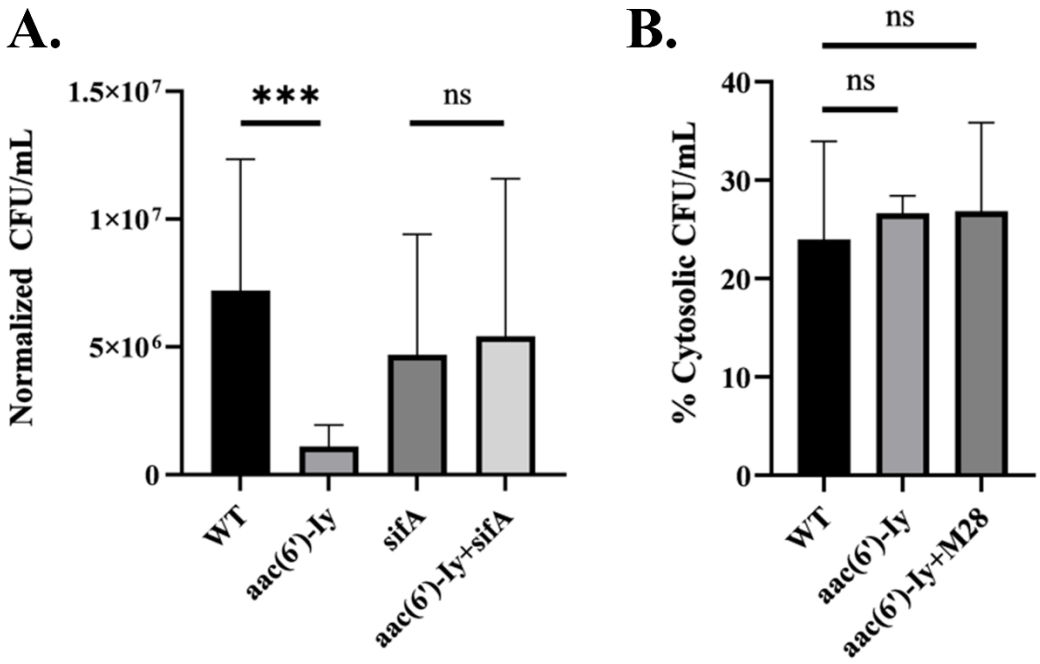
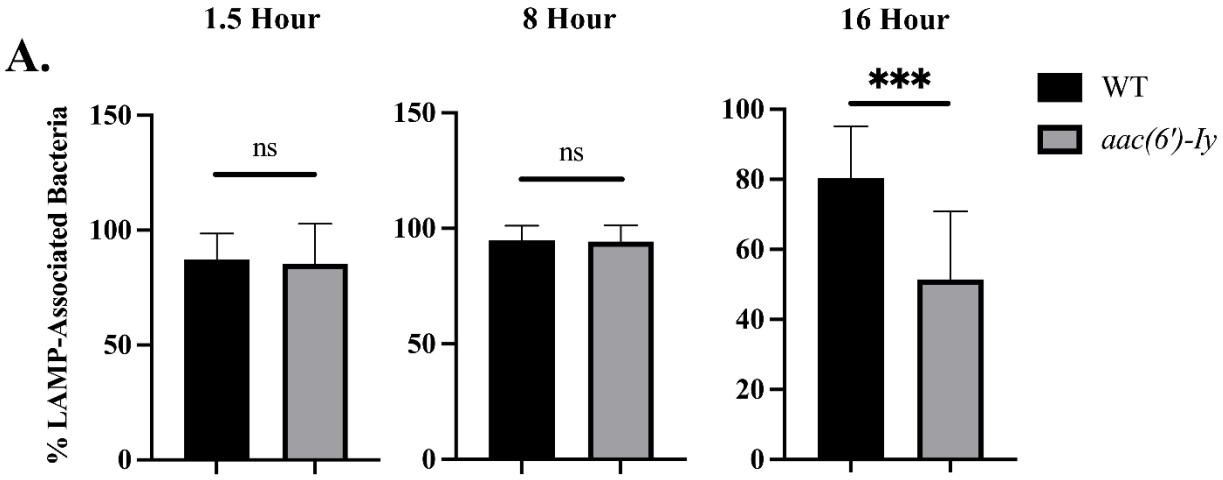


Figure 14. Cytosolic *aac(6')-Iy* mutants do not have a survival defect in HeLa cells. **A)** HeLa cells were infected at an MOI 100 with WT, *aac(6')-Iy*, and *aac(6')-Iy*+M28. The % cytosolic CFU/mL was calculated by dividing the average CFU/mL from the chloroquine-treated wells with the average CFU/mL from the untreated wells and multiplying by 100. Shown are the means and standard deviation from 3 independent experiments. Data were analyzed using ANOVA followed by multiple *t*-test comparisons. **B)** HeLa cells were infected at an MOI 100 with WT, *aac(6')-Iy*, *sifA*, and an *aac(6')-Iy sifA* double mutant. At 16 hours post-infection, cells were lysed and bacteria spot-plated to determine CFU/mL. Shown are the means and standard deviation from 3 independent experiments. Data were analyzed using ANOVA followed by multiple *t*-test comparisons. *** represents p-value<0.05.

Loss of aac(6')-Iy results in less LAMP-associated bacteria

Lysosome-associated membrane protein 1 (LAMP1) is a protein found on the SCV membrane and serves as a marker for the SCV (Steele-Mortimer *et al*., 1999; Knodler *et al*., 2010). Immunofluorescence was performed to look at the percentage of LAMP1-associated bacteria at 1.5-, 8-, and 16-hours PI for both WT and *aac(6')-Iy*. At 1.5 and 8 hours PI, there was not a significant difference in % LAMP1-associated bacteria between WT and *aac(6')-Iy* (**Figure 15A**). However, at 16 hours PI, the *aac(6')-Iy* mutant had significantly less % LAMP1-associated bacteria compared to WT (p-value<0.0001).



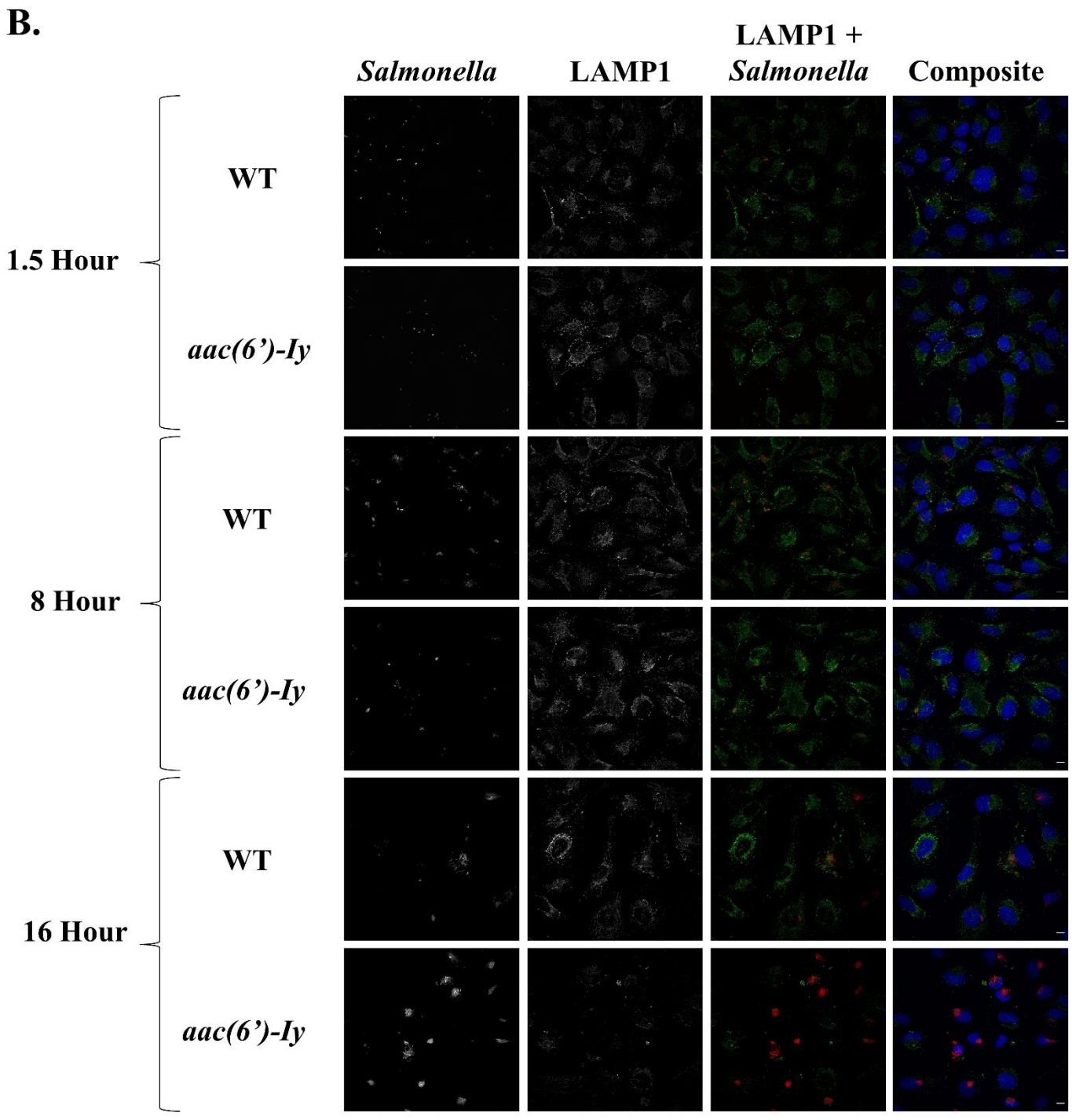


Figure 15. The gene *aac(6')-Iy* contributes to survival within the SCV. Immunofluorescence of HeLa cells infected with either WT or *aac(6')-Iy* containing the pFPV-mCherry plasmid at 1.5-, 8-, and 16-hours PI. **A)** The % LAMP1-associated bacteria were compared between WT and

aac(6')-Iy. There was significantly less % LAMP1-associated bacteria at 16-hours PI (p-value<0.0001). Bacteria were counted from n=50 cells in 3 independent experiments. Data were analyzed with the Student's *t*-test **B)** Representative images of infected HeLa cells at 1.5, 8, and 16 hours PI used for data analysis. Scale bars are 10 µm. DAPI was used to stain DNA. The LAMP1 + *Salmonella* images show *Salmonella* in red and LAMP1 in green. The composite images show *Salmonella* in red, LAMP1 in green, and DNA in blue.

The gene aac(6')-Iy confers a competitive advantage in C57BL/6 mice

The $\log_{10}$ competitive index ($\log_{10}$ CI) was calculated from CFU/g obtained from the liver, cecum, spleen, mesenteric lymph nodes, and spleen of 6 C57BL/6 mice that were infected with a 1:1 mixture of WT and *aac(6')-Iy*. Compared to the inoculum, the overall $\log_{10}$ CI was greater than 0 in all organs (**Figure 16**). The Student's *t*-test showed that the ratio in the cecum was significant, suggesting loss of *aac(6')-Iy* leads to a significant competitive disadvantage in the cecum of the mouse (p-value<0.05)

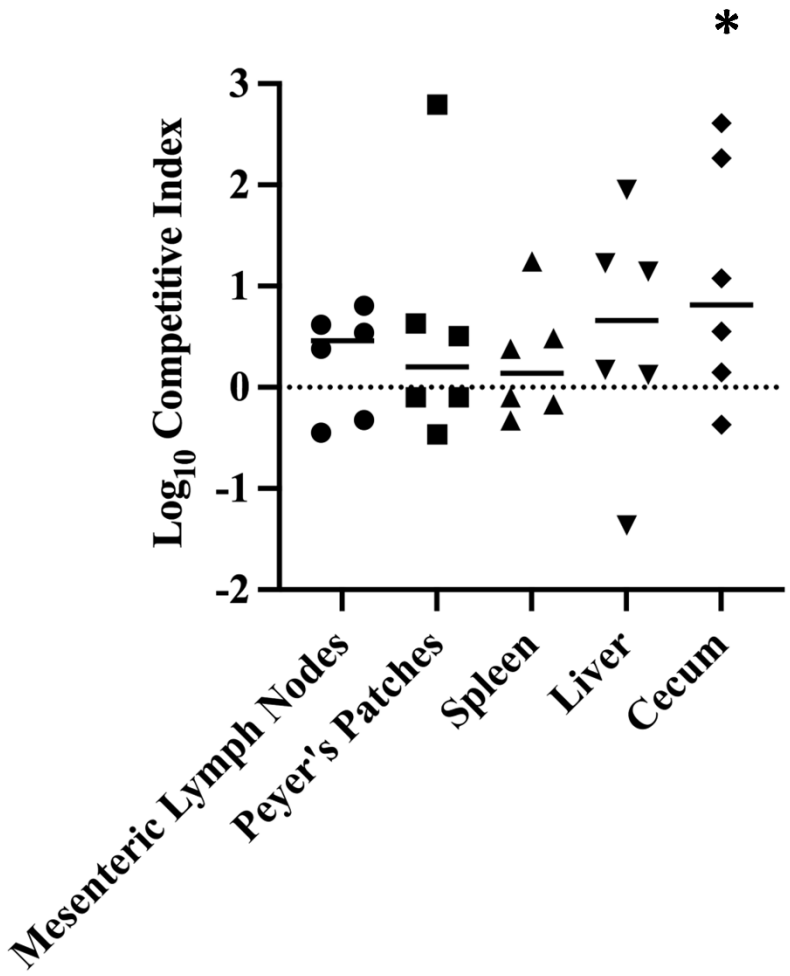


Figure 16. The gene *aac(6')-Iy* contributes to *Salmonella* infection of the host. Loss of *aac(6')-Iy* leads to a significant competitive disadvantage in the cecum in a systemic model of infection. The rest of the organs had a $\log_{10}$ CI>0, indicating that WT had a greater competitive advantage in these organs, but it was not significant. CI were calculated by dividing the output ratio of WT/*aac(6')-Iy* by the input ratio of WT/*aac(6')-Iy*. The $\log_{10}$ CI was calculated by log transforming the CI. Data were analyzed using Student's *t*-test. * means p-value<0.05.

Discussion

Purified AAC(6')-Iy protein is able to acetylate aminoglycosides (Vetting *et al*., 2004). Despite the presence of *aac(6')-Iy* in *S.* Typhimurium, there is no inherent clinical aminoglycoside resistance (Magnet et al. 2001). Deletion of *aac(6')-Iy* did not result in a decrease in resistance to kanamycin, amikacin, or gentamicin at wild-type levels of expression (Figure 7). However, complementation of *aac(6')-Iy* resulted in higher levels of resistance to amikacin (Figure 7). This may be due to placement of the *aac(6')-Iy* gene under the control of a constitutively expressed *lac* promoter in pWSK29, thereby increasing expression of *aac(6')-Iy* in the complemented strain. This supports previous findings that if *aac(6')-Iy* is increased in expression, it confers aminoglycoside resistance to the bacteria (Magnet *et al*., 2001). This is consistent with evidence that at wild-type levels of expression, *aac(6')-Iy* does not confer aminoglycoside resistance, supporting its status as a cryptic aminoglycoside resistance gene. Its role in *S.* Typhimurium is still unknown.

One possible role for *aac(6')-Iy* was acetylation of peptidoglycan conferring lysozyme resistance. AAC(2')-Ia from *Providencia stuartii* and AAC(2')-Id from *Mycobacterium smegmatis O*-acetylate peptidoglycan and increase lysozyme resistance for the bacteria (Payie *et al*., 1995; Ainsa *et al*., 1997). Here, loss of *aac(6')-Iy* and complementation did not affect lysozyme resistance in *Salmonella* (Figure 8). From this phenotype, it can be conferred that AAC(6')-Iy may not *O*-acetylate peptidoglycan like AAC(2')-Ia or AAC(2')-Id. It is likely AAC(6')-Iy acetylates another target in *Salmonella*, but it is unknown what that target is.

The gene *aac(6')-Iy* is termed cryptic because its presence does not confer aminoglycoside resistance. This was attributed to the gene having little to no level expression under *in vitro* conditions used to measure the minimum inhibitory concentrations of antibiotics in

clinical laboratories. A beta-galactosidase assay was performed to look at expression of an *aac(6')-Iy*::*lacZ* fusion, as well compare expression of *aac(6')-Iy* with an *sgc::lacZ* fusion (Figure 9A). The gene *aac(6')-Iy* had a moderate level of expression that was unexpected for a cryptic gene. Surprisingly, expression of an *aac(6')-Iy*::lacZ was significantly higher compared to expression of an *sgc::lacZ* fusion at late-log phase of growth. This suggests differential regulation on the *aac(6')-Iy* gene and possibly supports that *aac(6')-Iy* might be regulated independently from the *sgc* genes.

Comparison of *aac(6')-Iy* expression in LB and SPI-2 inducing broth showed no difference at the late-log phase of growth (Figure 9B). This was surprising as *aac(6')-Iy* expression is upregulated in macrophages (Srikumar *et al*., 2015). It is possible that *aac(6')-Iy* is upregulated under oxidative stress instead of nutrient conditions, as *aac(6')-Iy* is upregulated when the bacteria are exposed to hydrogen peroxide, nitric oxide, and high osmolarity (Srikumar *et al*., 2015).

A northern blot was performed to confirm the size of the *aac(6')-Iy* transcript and to attempt to deduce where its promoter might be located. Unfortunately, the northern blot was unable to detect *aac(6')-Iy* transcript with either DIG-labeled DNA and RNA probes. This is possibly due to the low expression and the size of the *aac(6')-Iy* transcript. While *aac(6')-Iy* is not completely cryptic, it still has a low level of expression that could make detection difficult. This same issue was encountered in a previous study on *aac(6')-Iy* (Magnet *et al*., 1999). However, from the same study, a northern blot detected *aac(6')-Iy* in a mutant strain of *Salmonella* which had a 60 kb deletion within the genome that placed the *nmpC* promoter and gene upstream of *sgcER* and *aac(6')-Iy* (Magnet *et al*., 1999). The size of the *aac(6')-Iy* transcript from this mutant was about 2,700 base pairs, suggesting that *aac(6')-Iy* is encoded with some of the *sgc* genes (Magnet *et al*.,

1999). If *aac(6')-Iy* is transcribed with the *sgc* genes, it is possible that the northern blot failed because the transcript was too large to transfer to the blot. In order to increase transfer, a downward alkaline capillary transfer was used. This method increased the transfer of large transcripts, but the *aac(6')-Iy* was not detected. As a result, the size of the *aac(6')-Iy* transcript could not be determined.

Previous RNA-seq data showed that *aac(6')-Iy* is upregulated in intracellular(-like) conditions, such as in the macrophage (Kröger *et al*., 2013; Srikumar *et al*., 2015). In order to confirm this data, qRT-PCR was used to look at fold change of *aac(6')-Iy* expression in RAW264.7 macrophages 4- and 6-hours post-infection (Figure 10A). The results found that *aac(6')-Iy* is upregulated in macrophages and suggests a possible role for the gene in macrophage infection and/or survival. In order to rule out whether *aac(6')-Iy* only plays a role in macrophage infection and/or survival, the fold change of *aac(6')-Iy* expression in infected HeLa epithelial cells 8- and 16-hours post-infection was also determined. The gene *aac(6')-Iy* is also upregulated >5-fold in HeLa epithelial cells at 8- and 16-hours post-infection (Figure 10B). This suggests that *aac(6')-Iy* plays a role in *Salmonella* infection and/or survival in both epithelial cells and macrophage cells.

In order to investigate whether *aac(6')-Iy* plays a role in intracellular survival, a gentamicin protection assay was performed in RAW264.7 macrophages (Figure 11). In RAW264.7 macrophages, loss of *aac(6')-Iy* led to a significant survival defect 24 hours PI, suggesting *aac(6')-Iy* plays a significant role in intra-macrophage survival. This is not the first chromosomal AAC to contribute to do so. The Eis (**E**nhanced **I**ntracellular **S**urvival) protein in *M. tuberculosis* is a well-characterized AAC that is found within pathogenic strains of *Mycobacterium* (Wei *et al*., 2000). It affects cytokine secretion, autophagy, reactive oxygen species (ROS) generation, inflammation,

and cell death in tuberculosis infections (Samuel *et al*., 2007; Shin *et al*., 2010). Along with Eis, AAC(6')-Iy is the second chromosomal AAC shown to play a role in survival within macrophages.

In order to determine whether *aac(6')-Iy* only played a role in intra-macrophage survival, the invasion and survival of an *aac(6')-Iy* deletion mutant was investigated in a gentamicin protection assay in HeLa epithelial cells. Loss of *aac(6')-Iy* had no effect on invasion of epithelial cells (Figure 12) but led to a significant survival defect 16 hours PI, but not at 8 hours PI (Figure 13). This pattern of survival was similar to a SPI-2 deletion mutant and is attributed to a defect in vacuolar survival (Malik-Kale *et al*., 2012). This is the first chromosomal AAC that has been shown to have a role in survival within epithelial cells (Figure 12).

Salmonella can survive in both the cytosol and vacuole in epithelial cells. In macrophages, *Salmonella* only survives within the vacuole, as the cytosol interferes with growth with caspase-I and –II (Thurston *et al*., 2016). The survival defect within RAW264.7 macrophages and the similar pattern of survival within HeLa epithelial cells suggests that *aac(6')-Iy* may play a specific role in intra-vacuolar survival. To rule out whether *aac(6')-Iy* contributes to cytosolic survival, a *sifA* mutant was utilized as well as the chloroquine resistance assay (Figure 14). A *sifA* mutant leads to loss of vacuolar membrane integrity and escape of bacteria to the cytosol (Beuzón *et al*., 2000). If *aac(6')-Iy* plays no role in cytosolic survival, a double mutant of *aac(6')-Iy sifA* would have no reduction in survival compared to a *sifA* mutant. After 16 hours PI, there was no difference in the normalized CFU/mL of the *aac(6')-Iy sifa* double mutant compared to the *sifA* mutant (Figure 14B). The chloroquine resistance assay further confirmed that loss of *aac(6')-Iy* did not result in a defect in cytosolic survival. Chloroquine accumulates within endosomes in eukaryotic cells and kills the bacteria within the SCV, but not the cytosol (Klein *et al*., 2016; Finlay & Falkow 1988). At 16 hours PI, there was no difference in the % cytosolic CFU/mL between WT, *aac(6')-Iy*, or

complemented *aac(6')-Iy* (*aac(6')-Iy*+M28 in Figure 14A). This suggests the reduction in survival within epithelial cells is not cell-wide but is associated with a reduction in survival within the SCV.

Immunofluorescence microscopy was used to determine whether loss of *aac(6')-Iy* would result in a decrease in LAMP1-associated bacteria. Similar to the gentamicin protection assays, at 1.5 and 8 hours PI, there was not a significant difference between WT and *aac(6')-Iy* (Figure 15). However, at 16 hours PI, there was a significant reduction in % LAMP1-associated bacteria in the *aac(6')-Iy*. This result, along with the *sifA* and chloroquine resistance assay data, support the hypothesis that the survival defect seen at 16 hours PI in HeLa epithelial cells is due to a survival defect in the SCV. The role of *aac(6')-Iy* in survival within the SCV is still unknown.

A competitive index assay was performed to determine whether loss of *aac(6')-Iy* led to a difference in competitive advantage in C57BL/6 mice in a systemic model of infection. Loss of *aac(6')-Iy* led to a competitive disadvantage ($\log_{10}$ CI>0) in the liver, cecum, spleen, and Peyer's Patches, but not the mesenteric lymph nodes. There was a significant competitive disadvantage in the cecum. The role of *aac(6')-Iy* in infection of the cecum is unclear, however it is possible that *aac(6')-Iy* could play a role in the other organs as well, but the effect could not be seen as mice were sacrificed early. This data suggests an *in vivo* role of *aac(6')-Iy* in *Salmonella* infection of the cecum and possibly survival within a host. Besides *aac(6')-Iy*, only the *eis* gene in *M. tuberculosis* has shown a significant competitive disadvantage in mice (Wei *et al*., 2000). More experiments should be performed to confirm whether loss of *aac(6')-Iy* is important for infection of a host.

The gene *aac(6')-Iy* is the second chromosomal AAC shown to play a role in bacterial survival within host cells as well as in an animal model of infection. Its mechanism for how it contributes to bacterial survival, specifically within the SCV, is unknown. However, this

Salmonella-specific gene is important for the bacteria's ability to survive within the host and should be researched further to discover its function in the bacteria.

CHAPTER IV

INVESTIGATION OF THE *SGC* CLUSTER IN *S.* TYPHIMURIUM

Introduction

Originally hypothesized to serve a role in carbohydrate metabolism, the *sgc* cluster consists of seven genes and was originally discovered in *E. coli* (Reizer *et al*., 1994). The seven genes putatively encode an aminopeptidase or endoglucanase, PTS IIB component, PTS IIC component, nucleoside triphosphatase, PTS IIA component, epimerase, and a DeoR/GlpR transcriptional regulator, respectively. The functions of each gene and their target carbohydrate is unknown.

The genetic regulation of the *sgc* genes is largely unstudied. There are several possible regulators for the *sgc* genes in *Salmonella*: SlyA, the sRNA *ryjB* found within the *sgc* genes, and *sgcR* which might regulate expression of the genes (Shimada *et al*., 2013; Curran *et al*., 2017; Kawano *et al*., 2005). SlyA is a transcriptional regulator that contributes to *Salmonella* survival against oxidative stress and is important for intra-macrophage survival (Buchmeier *et al*., 1997). According to SalcomD23580, the *sgc* genes are upregulated in different environmental conditions such as oxidative stress, early stationary phase, anaerobic shock, and within the macrophage (Kröger *et al*., 2013; Srikumar *et al*., 2015). This suggests a role for the *sgc* genes in intra-macrophage survival.

Materials & Methods

Bacterial strains, plasmids, and growth conditions

A *Salmonella enterica* serovar Typhimurium derivative of strain ATCC 14028, IR715, that is resistant to nalidixic acid was used as wild-type (WT; Stojiljkvoci & Bäumler 1995). *Salmonella* strains were grown in LB with aeration at 37ºC, unless indicated otherwise. When necessary, antibiotics were used in the following concentrations: chloramphenicol 30 µg/mL, nalidixic acid 50 µg/mL, and kanamycin 100 µg/mL.

Cell culture conditions

RAW 264.7 cells (ATCC® TIB-71™) were grown at 37°C in a 5% CO_2 incubator in growth media (GM) consisting of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS).

Construction of strains used in this study

A deletion of the *sgc* genes was made using the Wanner method (Datsenko & Wanner 2000). As mentioned before, the low-copy plasmid pWSK29 was used to construct a complemented strain. The In-Fusion® cloning kit was used to clone the *sgc* genes into pWSK29 (Takara Bio, USA).

Primers used in this study

Primers were obtained from Millipore Sigma and re-suspended in nuclease free water (Millipore Sigma; USA) and are listed in **Table 2**.

Table 2. Primers used for Chapter IV

Primer	Sequence (5' to 3')
sgcX+P1	ACGCTCAACGACTGATTGTTTTATCACCACGGAGACGCCAGTGTAGGCTGGAGCTGCTTC
sgcR+P2	CAGTGATCCAGATGGGTTCTGTTCATTTGCCTGCATATGAATATCCTCCTTAG
sgcX 5'	TTATACCAAGGCGACAAGG
sgcR 3'	GATCTTCCGTCACAGGTAGG
sgc-pWSK29-F	CGGGCCCCCCCTCGATTAACGCGCTCCTGATGAGG
sgc-pWSK29-R	TACCGTCGACCTCGAATGACCTTTTCTGTGCAGGAAACG

Phenotype Microarray Plates

A phenotype microarray was performed using the OmniLog® reader and Phenotype MicroArray (PM) plates from Biolog (Biolog, USA). Briefly, the samples were grown overnight on LB plates. Following the manufacturer's protocol, the plate was inoculated and suspended in IF-0. The turbidity was adjusted to 42%T. 15 mL of the 42%T suspension was added to 75 mL IF-0+tetrazolium dye and mixed. This new suspension was used to inoculate PM plates 1 and 2 per sample. PM 1 and PM 2A plates were loaded into the OmniLog® instrument and readings were taken every 15 minutes (Biolog, USA).

Bacterial survival in RAW264.7 macrophages assay

Bacterial survival in RAW264.7 cells was performed using a 96-well gentamicin protection assay, as described before (Wu *et al*., 2014). Briefly, RAW264.7 cells were seeded in 4 wells per strain in a 96-well cell culture plate. The same day, samples were cultured in 5 mL of LB with appropriate antibiotics and grown overnight. The next day, cultures were diluted 1:50 and outgrown 4 hours. The wells were then washed once with 1X DPBS and then infected with media

containing *Salmonella* strains at an MOI of 20. Plates were centrifuged at 1,000 *x* g for 10 minutes and then incubated at 37°C with 5% CO2 for 30 minutes. Plates were washed twice with 1X DPBS and then GM containing 100 μg/mL gentamicin was added to the wells and the plates incubated for 1 hour. After 1 hour, the media was replaced with GM containing 10 μg/mL gentamicin for the remainder of the experiment. At 2- and 24-hours PI, 3 of the 4 wells were lysed with 1% Triton X-100 and the CFU/mL enumerated via plating. One well was left to estimate the cells/mL remaining. The CFU/cell was calculated by dividing the CFU/mL by the cells/mL.

Statistical analysis

All statistical analyses were performed with JMP Pro (SAS Institute Inc., USA).

Results

The sgc cluster is not used for metabolism under extracellular conditions

A Biolog Phenotype MicroArray was performed on a *sgc* deletion mutant compared to WT. A chart of the average height of each curve was created and the difference between WT and *sgc* was used to determine whether there was a significant difference (**Figure 17** and **Table 3**). Numbers over 50 represented a significant difference in growth. After accounting for errors in the experiment (PM1 plate; H8 2,3-butanediol had a technical error which resulted in a significant difference), it was found that there was no significant difference in growth in 190 different carbon sources (**Figure 17** and **Table 3**). Besides 2,3-butanediol, the largest average height difference was found in D-glucuronic acid at an average height difference of approximately 40.

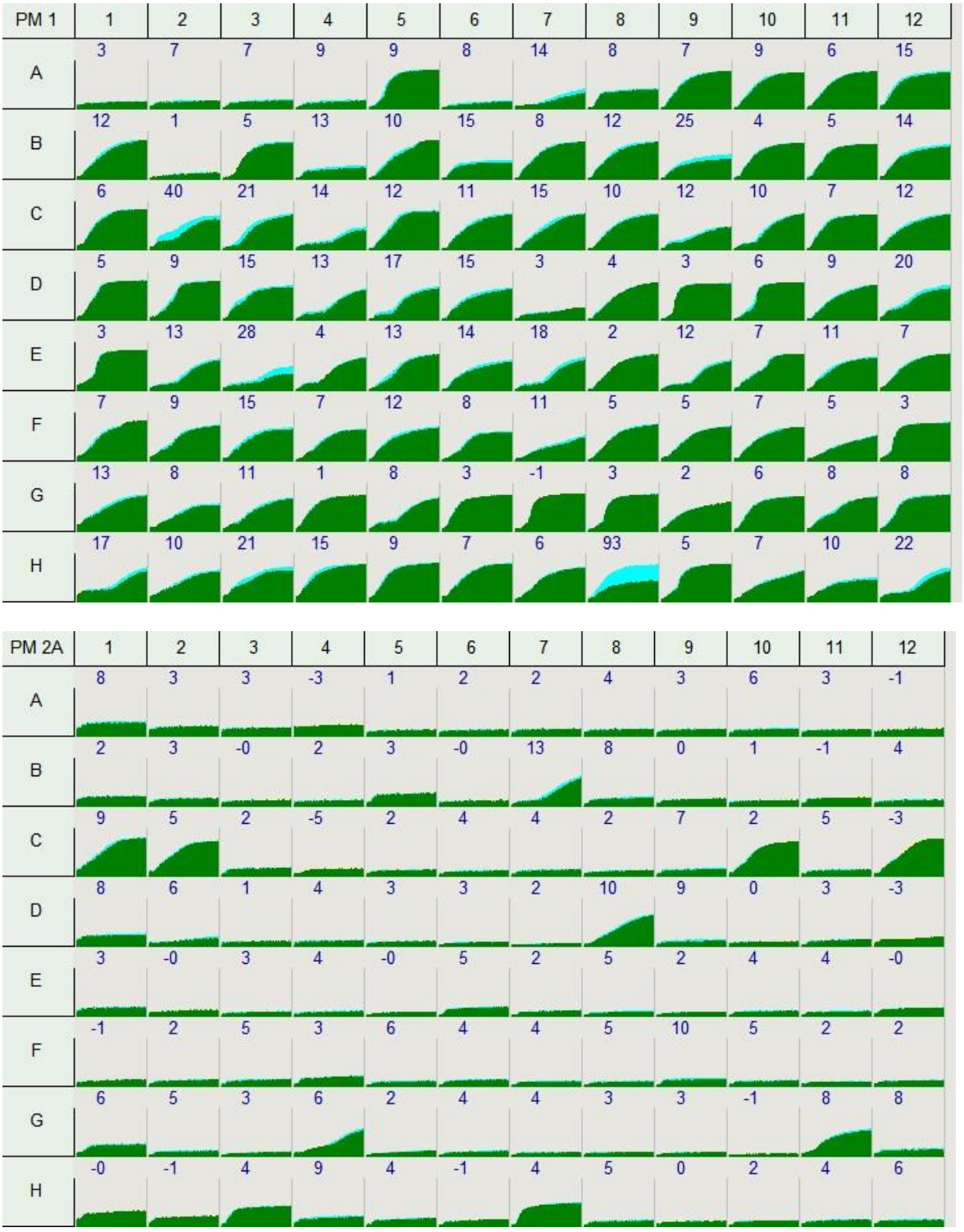


Figure 17. Growth comparison charts of WT (green) compared to an *sgc* mutant (light blue). The average height difference between WT and the *sgc* mutant was used to look at whether loss of *sgc* affected metabolism of certain carbon sources in the PM 1 and PM 2A plates. An average

height difference greater than 50 was considered significant. The graph was generated using the OmniLog® system (Biolog, USA). Data was collected from 2 independent experiments.

Table 3. Carbon sources in PM 1 and PM 2A plates and the average height difference between the WT and *sgc* mutant.

PM 1 Plate		PM 2 Plate	
Chemical	Average Height Difference	Chemical	Average Height Difference
A1 Negative Control	2.724	A1 Negative Control	7.906
A2 Negative Control	6.625	A2 Negative Control	2.786
A3 Negative Control	7.271	A3 Negative Control	2.672
A4 α-Cyclodextrin	8.625	A4 Tween 20	-3.25
A5 Dextrin	8.677	A5 Tween 40	0.703
A6 Glycogen	8.25	A6 Tween 80	2.078
A7 Maltitol	14.286	A7 Gelatin	2.422
A8 Maltotriose	8.271	A8 L-Alaninamide	3.557
A9 D-Maltose	7.26	A9 L-Alanine	2.802
A10 D-Trehalose	8.906	A10 D-Alanine	6.161
A11 D-Cellobiose	6.292	A11 L-Arginine	2.984
A12 β-Gentiobiose	14.734	A12 L-Asparagine	-1.005
B1 D-Glucose-6- Phosphate	12	B1 L-Aspartic Acid	2.234
B2 α-D-Glucose-1- Phosphate	1.448	B2 D-Aspartic Acid	3.083
B3 L-Glucose	4.984	B3 L-Glutamic Acid	-0.104
B4 α-D-Glucose	13.135	B4 D-Glutamic Acid	1.818
B5 α-D-Glucose	9.714	B5 L-Glutamine	2.922
B6 α-D-Glucose	14.708	B6 Glycine	-0.104
B7 3-O-Methyl-DGlucose	8.052	B7 L-Histidine	13.448
B8 α-Methyl-DGlucoside	11.563	B8 L-Homoserine	8.286

B9 β-Methyl-DGlucoside	24.833	B9 Hydroxy-LProline	0.484
B10 D-Salicin	3.823	B10 L-Isoleucine	0.943
B11 D-Sorbitol	5.052	B11 L-Leucine	-1.401
B12 N-Acetyl-DGlucosamine	14.26	B12 L-Lysine	3.922
C1 D-Glucosaminic Acid	6.318	C1 L-Methionine	9.469
C2 D-Glucuronic Acid	39.729	C2 L-Ornithine	5.469
C3 Chondroitin-6- Sulfate	20.536	C3 L-Phenylalanine	2.177
C4 Mannan	13.703	C4 L-Proline	-4.771
C5 D-Mannose	11.734	C5 L-Serine	2.099
C6 α-Methyl-DMannoside	11.229	C6 D-Serine	4.052
C7 D-Mannitol	15.365	C7 L-Threonine	3.682
C8 N-Acetyl-β-DMannosamine	10.344	C8 D-Threonine	1.896
C9 D-Melezitose	12.422	C9 L-Tryptophan	6.901
C10 Sucrose	9.974	C10 L-Tyrosine	2.464
C11 Palatinose	7.01	C11 L-Valine	4.677
C12 D-Turanose	11.609	C12 Ala-Ala	-3.281
D1 D-Tagatose	5.005	D1 Ala-Arg	7.615
D2 L-Sorbose	9.073	D2 Ala-Asn	5.896
D3 L-Rhamnose	15.495	D3 Ala-Asp	1.417
D4 L-Fucose	12.703	D4 Ala-Glu	4.396
D5 D-Fucose	17.042	D5 Ala-Gln	2.839
D6 D-Fructose-6- Phosphate	15.281	D6 Ala-Gly	3.333
D7 D-Fructose	3.432	D7 Ala-His	1.589
D8 Stachyose	3.552	D8 Ala-Ile	10.219
D9 D-Raffinose	2.964	D9 Ala-Leu	9.281
D10 D-Lactitol	6.292	D10 Ala-Lys	0.354
D11 Lactulose	8.781	D11 Ala-Met	2.521
D12 α-D-Lactose	20.12	D12 Ala-Phe	-3.021
E1 Melibionic Acid	2.823	E1 Ala-Pro	3.099
E2 D-Melibiose	13.099	E2 Ala-Ser	-0.339
E3 D-Galactose	28.177	E3 Ala-Thr	2.625
E4 α-Methyl-DGalactoside	4.005	E4 Ala-Trp	4.094
E5 β-Methyl-DGalactoside	13.219	E5 Ala-Tyr	-0.052
E6 N-AcetylNeuraminic Acid	13.786	E6 Ala-Val	4.62
E7 Pectin	18.359	E7 Arg-Ala (b)	1.516
E8 Sedoheptulosan	2.453	E8 Arg-Arg (b)	5.276
E9 Thymidine	12.047	E9 Arg-Asp	1.911
E10 Uridine	6.891	E10 Arg-Gln	4.271
E11 Adenosine	11.01	E11 Arg-Glu	4.38

E12 Inosine	6.844	E12 Arg-Ile (b)	-0.38
F1 Adonitol	7.443	F1 Arg-Leu (b)	-0.885
F2 L- Arabinose	9.146	F2 Arg-Lys (b)	2.068
F3 D-Arabinose	15.063	F3 Arg-Met (b)	4.865
F4 β-Methyl-DXylopyranoside	6.766	F4 Arg-Phe (b)	2.786
F5 Xylitol	11.656	F5 Arg-Ser (b)	6.411
F6 Myo-Inositol	8.417	F6 Arg-Trp	3.646
F7 Meso-Erythritol	10.802	F7 Arg-Tyr (b)	4.318
F8 Propylene glycol	5.427	F8 Arg-Val (b)	4.661
F9 Ethanolamine	4.505	F9 Asn-Glu	10.26
F10 D,L-α-GlycerolPhosphate	6.917	F10 Asn-Val	5.453
F11 Glycerol	5.094	F11 Asp-Ala	2.469
F12 Citric Acid	3	F12 Asp-Asp	2.417
G1 Tricarballylic Acid	13.375	G1 Asp-Glu	5.641
G2 D,L-Lactic Acid	8.38	G2 Asp-Gln	5.13
G3 Methyl D-lactate	10.781	G3 Asp-Gly	3
G4 Methyl pyruvate	0.708	G4 Asp-Leu	6.432
G5 Pyruvic Acid	7.859	G5 Asp-Lys	2.469
G6 α-Keto-Glutaric Acid	2.854	G6 Asp-Phe	3.62
G7 Succinamic Acid	-0.781	G7 Asp-Trp	4.474
G8 Succinic Acid	2.583	G8 Asp-Val	2.625
G9 Mono-Methyl Succinate	2.198	G9 Glu-Ala	3.031
G10 L-Malic Acid	6.484	G10 Glu-Asp	-0.948
G11 D-Malic Acid	7.948	G11 Glu-Glu	8.307
G12 Meso-Tartaric Acid	8.432	G12 Glu-Gly	8.24
H1 Acetoacetic Acid (α)	17.276	H1 Glu-Ser	-0.401
H2 γ-Amino-NButyric Acid	10.453	H2 Glu-Trp	-0.766
H3 α-Keto-Butyric Acid	20.526	H3 Glu-Tyr	3.599
H4 α-HydroxyButyric Acid	14.828	H4 Glu-Val	8.755
H5 D,L-β-HydroxyButyric Acid	8.63	H5 Gln-Glu	4.443
H6 Glycolic Acid	6.891	H6 Gln-Gln	-0.536
H7 Butyric Acid	5.979	H7 Gln-Gly	4.224
H8 2,3-Butanediol	92.651	H8 Gly-Ala	5.26
H9 3-Hydroxy-2- Butanone	4.917	H9 Gly-Arg	0.448
H10 Propionic Acid	7.401	H10 Gly-Asn	1.828
H11 Acetic Acid	10.38	H11 Gly-Asp	3.74
H12 Hexanoic Acid	22.406	H12 α-D-Glucose	6.125

The sgc cluster plays a role in intra-macrophage survival

Similar to loss of *aac(6')-Iy*, loss of the *sgc* cluster led to reduction in survival in RAW264.7 macrophages 24 hours post-infection (**Figure 18**).

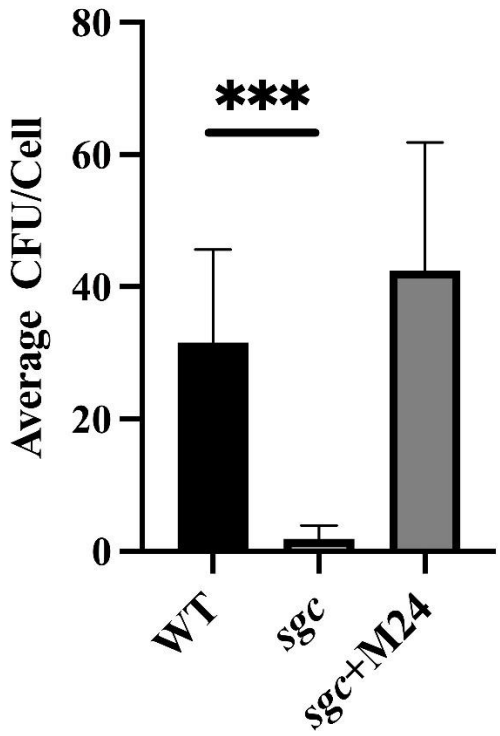


Figure 18. Loss of *sgc* leads to decreased *Salmonella* survival in RAW264.7 macrophages. Complementation (*sgc*+M24) restored survival. Shown are the means and standard deviation from 3 independent experiments. Data were analyzed using ANOVA followed by multiple *t*-test comparisons. *** represents p-value<0.05.

Discussion

In order to investigate the growth of a *sgc* deletion mutant with different carbon sources, the Biolog Phenotype Microarray plates and Omnilog® reader were used. Loss of *sgc* had no effect on growth in 190 different carbon sources compared to WT. There was a significant difference in growth in pyruvic acid (PM1 plate; H8 well), however that was due to an error in one experiment, and the difference could not be justified. There was almost a significant difference in carbon utilization of D-glucuronic acid (C2 on PM 1) between WT and the *sgc* deletion mutant. There were several limitations to this experiment. First, only two independent experiments were performed. Additional replicates would have helped confirm true differences between the two samples. Second, there was no complemented strain of the *sgc* mutant used to further confirm that the differences were accurate. Further experiments would need to be done to accurately compare growth of *sgc* in carbon sources compared to WT.

Similar to *aac(6')-Iy*, an *sgc* deletion mutant had a significant survival defect in RAW264.7 macrophages 24 hours post-infection. Complementation restored survival. This suggests the *sgc* genes play a role in intra-macrophage survival, however it is unknown whether it plays a role in epithelial cell survival like *aac(6')-Iy*. Given that the *sgc* genes are similar to metabolism genes, it's possible the *sgc* cluster is involved in metabolism required for survival in macrophages (Reizer *et al*., 1999). Given the proximity of the *sgc* genes to *aac(6')-Iy*, the *sgc* genes may play similar roles in bacterial survival within the host as *aac(6')-Iy* does. Like *aac(6')-Iy*, the exact function of the *sgc* genes is also unknown.

CHAPTER V

CONCLUSION

The gene *aac(6')-Iy* was first discovered and classified as an antibiotic resistance gene due to its ability to confer aminoglycoside resistance (Magnet *et al*., 1999). However, it was termed cryptic because in almost all *Salmonella* species and subspecies, it does not confer aminoglycoside resistance (Magnet *et al*., 1999). The status of this gene as a chromosomal AAC was particularly intriguing as most currently known chromosomal AACs either serve or are hypothesized to serve physiological roles. Even more-so, *aac(6')-Iy* is found at the end of a putative metabolic operon termed the *sgc* cluster, which further provided support that AAC(6')-Iy's primary role may be physiological. The question of whether *aac(6')-Iy* serves a physiological role in *S.* Typhimurium was investigated with the following aims.

AIM I. **Determine if deletion of *aac(6')-Iy* reduces the minimum inhibitory concentration of aminoglycoside antimicrobial drugs in *S.* Typhimurium.**

We looked at whether *aac(6')-Iy* contributed to aminoglycoside resistance under *in vitro* conditions. The MIC of the aminoglycosides kanamycin, gentamicin, and amikacin were determined for the WT, the *aac(6')-Iy* mutant, and the complemented strain *aac(6')-Iy*+M28. Loss of *aac(6')-Iy* did not affect the MIC for all 3 aminoglycosides, confirming the cryptic phenotype of *aac(6')-Iy*. However, restoration of *aac(6')-Iy* by introduction of a plasmid containing *aac(6')-Iy* under the control of the *lacZ* promoter increased the MIC of amikacin against this strain. This confirmed that *aac(6')-Iy* is able to confer aminoglycoside resistance if expression is increased. We next asked the question of whether *aac(6')-Iy* may play a role in

peptidoglycan acetylation leading to increased lysozyme resistance. Loss of *aac(6')-Iy* and complementation had no effect on lysozyme resistance, suggesting that AAC(6')-Iy does not acetylate peptidoglycan like AAC(2')-Ia and AAC(2')-Id (Payie *et al*., 1995; Ainsa *et al*., 1997). This aim confirmed the cryptic aminoglycoside resistance phenotype of *aac(6')-Iy* and ruled out a role for this gene in lysozyme resistance.

AIM II. **Determine transcriptional regulation of *aac(6')-Iy* and the *sgc* cluster.**

In this aim, we evaluated expression and regulation of *aac(6')-Iy*. The expression of *aac(6')-Iy* under *in vitro* conditions was measured to determine whether its cryptic resistance phenotype translated into cryptic expression. Beta-galactosidase assays showed that *aac(6')-Iy* has a moderate level of expression and was expressed significantly higher than the *sgc* cluster. This suggested both that *aac(6')-Iy* may be regulated independently from the *sgc* genes and was not entirely cryptic. We next evaluated conditions that might upregulate *aac(6')-Iy*. Expression of *aac(6')-Iy* was unchanged between LB and SPI-2 inducing broth. The SPI-2 inducing broth was used in order to determine whether *aac(6')-Iy* might have shared regulation with SPI-2. This result was surprising as qRT-PCR analysis of infected RAW264.7 macrophages revealed that *aac(6')-Iy* is upregulated >10-fold at 4 and 6 hours PI. This suggests *aac(6')-Iy* is regulated by conditions found in the macrophage, but not in SPI-2 inducing broth. Previous RNA-seq data showed upregulation of *aac(6')-Iy* under high osmolarity, hydrogen peroxide shock, and nitric oxide shock, so it is possible that *aac(6')-Iy* is upregulated in stressful conditions and not the specific nutrient environment found within the macrophage (Kröger *et al*., 2013; Srikumar *et al*., 2015). Quantitative RT-PCR analysis of infected HeLa cells showed that *aac(6')-Iy* is upregulated >6-fold 8 and 16 hours PI. This suggested a role for *aac(6')-Iy* in intracellular

survival. Interestingly, aminoglycosides cannot penetrate eukaryotic cells and are not effective in intracellular environmental conditions (Mingeot-Leclercq *et al*., 1999; Damper & Epstein 1981; Xiong *et al*., 1996). This further suggested that the primary role for *aac(6')-Iy* is physiological rather than antibiotic resistance.

AIM III. **Determine if *aac(6')-Iy* and the *sgc* genes play a role in *S.* Typhimurium survival within eukaryotic cells.**

We addressed the contribution of *aac(6')-Iy* to bacterial survival in both RAW264.7 macrophages and HeLa epithelial cells. Loss of *aac(6')-Iy* resulted in a significant decrease in fold change CFU/mL in both RAW264.7 macrophages 24 hours PI and in HeLa epithelial cells 16 hours PI. Loss of *aac(6')-Iy* did not affect the fold change in CFU/mL in HeLa epithelial cells at 8 hours PI. This pattern of survival resembled that of a *SPI-2* deletion mutant, where a survival defect due to decreased in survival in the SCV was present 16 hours PI, but not at 8 hours PI in HeLa cells (Malik-Kale *et al*., 2012). This evidence suggested that the gene *aac(6')-Iy* may play a role in SCV survival. This hypothesis was further supported by the fact that within macrophages *Salmonella* survives only in the SCV, due to the presence of caspases in the cytosol that inhibit replication (Holden *et al*., 2016).

To determine whether *aac(6')-Iy* played a role in SCV survival, we first ruled out whether it played a role in cytosolic survival. Cytosolic survival was determined using a *sifA* mutant and a chloroquine resistance assay. SifA is essential for maintenance of the SCV membrane integrity and loss of *sifA* results in loss of the SCV and escape of bacteria into the cytosol (Beuzón *et al*., 2000). HeLa cells were infected with either a *sifA* or a double mutant of *aac(6')-Iy* and *sifA*. The CFU/mL were normalized and compared between the two mutants at 16

hr PI. There was no difference in normalized CFU/mL between the *sifA* mutant and the *aac(6')-Iy sifA* double mutant, suggesting that *aac(6')-Iy* does not affect survival within the cytosol. We confirmed these results using a chloroquine resistance assay. Chloroquine accumulates within endosomes and kills bacteria present within the SCV, but not in the cytosol (Knodler *et al*., 2014). We measured the % cytosolic population (measured by dividing the cytosolic CFU/mL by the total CFU/mL x 100) and found that the % cytosolic population was unaffected by loss of *aac(6')-Iy* and complementation of *aac(6')-Iy*. This confirmed that loss of *aac(6')-Iy* does not affect survival within the cytosol.

We next addressed whether *aac(6')-Iy* affected SCV survival by measuring the % LAMP1-associated bacteria using immunofluorescence microscopy. LAMP1 is a marker for the SCV and LAMP1-associated bacteria were presumed to be within the SCV (Steele-Mortimer *et al*., 1999; Knodler *et al*., 2010). Loss of *aac(6')-Iy* had no effect on the % LAMP1-associated bacteria at 1.5 and 8 hours PI in HeLa cells, which corroborated the infection assay results. At 16 hours PI, loss of *aac(6')-Iy* resulted in a significant decrease in % LAMP1-associated bacteria, meaning loss of *aac(6')-Iy* resulted in significantly fewer bacteria present in the SCV. This confirmed that the gene *aac(6')-Iy* contributes to *Salmonella* survival within the SCV.

The preceding results demonstrated that *aac(6')-Iy* plays a role in *Salmonella* survival within host cells *in vitro*, but the *in vivo* role of *aac(6')-Iy* in *Salmonella* infection in hosts needed to be determined. C57BL/6 mice were infected with a 1:1 mixture of WT and the *aac(6')-Iy* deletion mutant in a systemic model of infection. Loss of *aac(6')-Iy* resulted in a significant competitive disadvantage for *Salmonella* in the mice in the cecum. This finding demonstrates that *aac(6')-Iy* serves a role for *Salmonella* in both host cell survival and infection within the host, specifically in the cecum.

This *aac(6')-Iy* gene is the first chromosomal AAC shown to play a role in epithelial cell survival and the second chromosomal AAC shown to play a role in macrophage survival and infection in mice. This study showed the primary role of *aac(6')-Iy* was not antibiotic resistance, but *Salmonella* survival within host cells and during infection of the host. These findings are helpful for explaining why *Salmonella* maintained this "cryptic" gene and its presence in all *Salmonella* species and subspecies, excluding subsp. *arizonae*. One major question unanswered by this study is the exact function of *aac(6')-Iy* within *Salmonella*. One possible role is acetylation of eukaryotic histone proteins (Vetting *et al*., 2004). A purified AAC(6')-Iy protein is able to acetylate both the calf thymus histone III-S and the human histone H3 protein (Vetting *et al*., 2004). The findings from this study support further research into whether AAC(6')-Iy may acetylate histone proteins within host cells.

In addition to elucidating the role of *aac(6')-Iy*, the role of the *sgc* genes was also analyzed in this study. We initially searched for a target carbohydrate for the proteins encoded by the *sgc* genes and to determine whether this putative operon could play a role in intra-macrophage survival as well. Loss of *sgc* did not significantly affect metabolism of ~200 carbon sources under *in vitro* conditions. This could be due to low levels of expression of the *sgc* genes or limitations of the experiment. Infection assays of RAW264.7 macrophages revealed that loss of the *sgc* genes led to a survival defect 24 hours PI. This suggests a role for the *sgc* genes in intra-macrophage survival. More research should be performed to elucidate the function of the *sgc* genes, their target carbohydrate, and whether they serve similar roles to host cell survival as *aac(6')-Iy*.

We initiated this study knowing that *aac(6')-Iy* was a cryptic aminoglycoside resistance gene present in almost all *Salmonella* species and subspecies. The physiologic benefit for

Salmonella to maintain this cryptic gene was unclear, so we began our investigation to identify the role this gene might play in *S.* Typhimurium. We found that *aac(6')-Iy* contributed significantly to *S.* Typhimurium survival within host cells. Specifically, *aac(6')-Iy* contributes to survival within the SCV of host cells. Furthermore, *aac(6')-Iy* also confers a competitive advantage in systemic infection of a host. This study discovered a role for *aac(6')-Iy* in *S.* Typhimurium, but not its mechanism. Further experiments are needed to discover the mechanism by which AAC(6')-Iy contributes to *Salmonella* survival within and infection of the host.

CHAPTER VI

FUTURE WORK

This study presented findings on an *aac(6')-Iy* deletion mutant showing reduced intracellular survival and a competitive disadvantage in the cecum of C57BL/6 mice. The target of *aac(6')-Iy* was not elucidated with this study. Future work is needed to determine the function of *aac(6')-Iy* in *Salmonella*. This chapter will discuss future directions for *aac(6')-Iy* research.

As mentioned in the introduction, the AAC(6')-Iy protein in its dimer formation is similar to the *Saccharomyces cerevisiae* Hpa2 histone acetyltransferase (Vetting et al., 2004). AAC(6')-Iy is able to acetylate both the calf thymus histone III-S and the human histone H3 proteins (Vetting et al., 2004). This is not the only chromosomal AAC able to acetylate eukaryotic histones. The Eis protein's acetylation of the human histone H3 protein contributes to *M. tuberculosis* virulence (Duan *et al*., 2016). This acetylation contributes to alteration of IL-10 production of host cells, leading to an increase in the anti-inflammatory cytokine IL-10 (Duan *et al*., 2016). If AAC(6')-Iy is able to acetylate the human histone H3 protein, it is possible cytokine production could be altered similar to the Eis protein. Enzyme-linked immunosorbent assays (ELISAs) could be performed on supernatants collected from infected host cells to determine whether loss of *aac(6')-Iy* alters the production of IL-10. Alternatively, addition of a purified AAC(6')-Iy protein into macrophage cell culture can also show whether there is a dose-dependent effect on cytokine production, similar to the work done by Samuel *et al*., 2007.

The target of AAC(6')-Iy is unknown in *Salmonella* or in host cells. One possible route to narrow down the target for AAC(6')-Iy could be an acetylome assay. In this assay, protein can be extracted from infected host cells containing either the WT, *aac(6')-Iy* deletion mutant, or the

complemented *aac(6')-Iy* strain. The peptides can then be digested and immunoprecipitated with anti-acetyl-lysine antibodies, as AAC(6')-Iy was shown to perform lysine acetylation on the histone proteins (Vetting *et al*., 2004). LC-MS could then be performed to identify the lysine-acetylated proteins and compare the results between the WT, deletion mutant, and the complemented mutant. This assay would help in finding putative targets of AAC(6')-Iy for further research.

The *sgc* cluster genes also do not have a described function. Early data suggests the *sgc* cluster plays a role in intra-macrophage survival, however it is unknown whether it plays a role in intra-epithelial cell survival or the specific intra-vacuolar survival that *aac(6')-Iy* contributes to. The specific *sgc* genes' functions are also unknown. Protein purification of each *sgc* gene could help to elucidate their possible functions. In addition, whether the *sgc* genes are transcribed as one transcriptional unit or in separate units is also unknown. Research done on *aac(6')-Iy* suggest at least some of the *sgc* genes are transcribed together (Magnet *et al*., 1999). The hypothesized target for the *sgc* genes is a pentose or pentitol sugar (Reizer *et al*., 1999). The phenotype microarray data did not find a significant difference in carbon utilization between a WT and *sgc* deletion mutant under *in vitro* conditions. It is possible that the *sgc* genes are used for metabolism under intracellular conditions and play a role in *Salmonella* survival within host cells, similar to *aac(6')-Iy*.

There is much that is unknown about *aac(6')-Iy* and the *sgc* cluster, but their importance for *Salmonella* has been elucidated with this study. More work must be done to confirm and discover the role of these genes in *Salmonella*.

REFERENCES

1. Magnet S, Courvalin P, Lambert T. Activation of the cryptic *aac(6')-Iy* aminoglycoside resistance gene of *Salmonella* by a chromosomal deletion generating a transcriptional fusion. *J Bacteriol*. 1999;181(21):6650–6655.

2. Crump JA, Sjölund-Karlsson M, Gordon MA, Parry CM. Epidemiology, Clinical Presentation, Laboratory Diagnosis, Antimicrobial Resistance, and Antimicrobial Management of Invasive *Salmonella* Infections. *Clin Microbiol Rev*. 2015;28(4):901–937. doi:10.1128/CMR.00002-15.

3. Centers for Disease Control & Prevention (2020). "Typhoid Fever and Paratyphoid Fever Questions & Answers." Retrieved August 26, 2021, from https://www.cdc.gov/typhoid-fever/sources.html.

4. World Health Organization (2021). "Immunization, Vaccines and Biologicals: Typhoid." Retrieved August 26, 2021, from https://www.who.int/teams/immunization-vaccines-and-biologicals/diseases/typhoid.

5. World Health Organization (2021). "Salmonella (non-typhoidal)." Retrieved August 26, 2021, from https://www.who.int/news-room/fact-sheets/detail/salmonella-(non-typhoidal).

6. Centers for Disease Control & Prevention (2019). "Salmonellosis (Nontyphoidal)." Retrieved August 26, 2021, from https://wwwnc.cdc.gov/travel/yellowbook/2020/travel-related-infectious-diseases/salmonellosis-nontyphoidal.

7. Gordon MA, Graham SM, Walsh AL, Wilson L, Phiri A, Molyneux E, Zijlstra EE, Heyderman RS, Hart CA, Molyneux ME. Epidemics of invasive *Salmonella* enterica

serovar enteritidis and S. enterica serovar typhimurium infection associated with multidrug resistance among adults and children in Malawi. *Clin Infect Dis*. 2008;46(7):963-9.

8. Morpeth SC, Ramadhani HO, Crump JA. Invasive non-Typhi *Salmonella* disease in Africa. *Clin Infect Dis*. 2009;49(4):606–611. doi:10.1086/603553.
9. Centers for Disease Control & Prevention (2021). "Salmonella Homepage." Retrieved August 26, 2021, from https://www.cdc.gov/Salmonella/index.html.
10. Economic Research Service (ERS), U.S. Department of Agriculture (USDA). Cost Estimates of Foodborne Illnesses. http://ers.usda.gov/data-products/cost-estimates-of-foodborne-illnesses.aspx.
11. Brenner FW, Villar RG, Angulo FJ, Tauxe R, Swaminathan B. *Salmonella* nomenclature. *J Clin Microbiol*. 2000;38(7):2465–2467.
12. Eng S-K, Pusparajah P, Mutalib N-SA, Ser H-L, Chan K-G, Lee L-H. *Salmonella*: A review on pathogenesis, epidemiology and antibiotic resistance. *Frontiers in Life Science*. 2015;8(3):284-293. doi:10.1080/21553769.2015.1051243.
13. Tindall BJ, Grimont PA, Garrity GM, Euzeby JP. Nomenclature and taxonomy of the genus *Salmonella*. *Int J Syst Evol Microbiol*. 2005;55:521-524.
14. Ryan MP, O'Dwyer J, Adley CC. Evaluation of the Complex Nomenclature of the Clinically and Veterinary Significant Pathogen *Salmonella*. *Biomed Res Int*. 2017;2017:3782182.
15. Jajere SM. A review of *Salmonella enterica* with particular focus on the pathogenicity and virulence factors, host specificity and antimicrobial resistance including

multidrug resistance. *Vet World*. 2019;12(4):504–521. doi:10.14202/vetworld.2019.504-521.

16. Ferrari RG, Rosario DKA, Cunha-Neto A, Mano SB, Figueiredo EES, Conte-Junior CA. Worldwide Epidemiology of *Salmonella* Serovars in Animal-Based Foods: a Meta-analysis. *Appl Environ Microbiol*. 2019;85(14):e00591-19. Published 2019 Jul 1. doi:10.1128/AEM.00591-19.
17. Khosla SN. *Typhoid Fever Its Cause, Transmission, and Prevention*. New Delhi: Atlantic Publ.; 2008.
18. Galán JE. Typhoid toxin provides a window into typhoid fever and the biology of *Salmonella* Typhi. *Proc Natl Acad Sci U S A*. 2016;113(23):6338–6344. doi:10.1073/pnas.1606335113.
19. Thucydides. The History of the Peloponnesian War. revised Ed Library of Alexandria; Athens Greece: 1965.
20. Littman RJ. The plague of Athens: epidemiology and paleopathology. *Mt Sinai J Med*. 2009;76(5):456-67.
21. Papagrigorakis MJ, Yapijakis C, Synodinos PN, Baziotopoulou-Valavani E. DNA examination of ancient dental pulp incriminates typhoid fever as a probable cause of the Plague of Athens. *Int J Infect Dis*. 2006;10(3):206-14.
22. Vågene ÅJ, Herbig A, Campana MG, Robles García NM, Warinner C, Sabin S, Spyrou MA, Andrades Valtueña A, Huson D, Tuross N, Bos KI, Krause J. *Salmonella enterica* genomes from victims of a major sixteenth-century epidemic in Mexico. *Nat Ecol Evol*. 2018;2(3):520-528.

23. Key FM, Posth C, Esquivel-Gomez LR, Hübler R, Spyrou MA, Neumann GU, Furtwängler A, Sabin S, Burri M, Wissgott A, Lankapalli AK, Vågene ÅJ, Meyer M, Nagel S, Tukhbatova R, Khokhlov A, Chizhevsky A, Hansen S, Belinsky AB, Kalmykov A, Kantorovich AR, Maslov VE, Stockhammer PW, Vai S, Zavattaro M, Riga A, Caramelli D, Skeates R, Beckett J, Gradoli MG, Steuri N, Hafner A, Ramstein M, Siebke I, Lösch S, Erdal YS, Alikhan NF, Zhou Z, Achtman M, Bos K, Reinhold S, Haak W, Kühnert D, Herbig A, Krause J. Emergence of human-adapted *Salmonella* enterica is linked to the Neolithization process. *Nat Ecol Evol*. 2020;4(3):324-333.

24. Murase T, Yamada M, Muto T, Matsushima A, Yamai S. Fecal excretion of *Salmonella* enterica serovar typhimurium following a food-borne outbreak. *J Clin Microbiol*. 2000;38(9):3495–3497.

25. Barbara G, Stanghellini V, Berti-Ceroni C, *et al*. Role of antibiotic therapy on long-term germ excretion in faeces and digestive symptoms after *Salmonella* infection. *Aliment Pharmacol Ther*. 2000;14(9):1127-31.

26. Cuypers WL, Jacobs J, Wong V, Klemm EJ, Deborggraeve S, Van Puyvelde S. Fluoroquinolone resistance in *Salmonella*: insights by whole-genome sequencing. *Microb Genom*. 2018;4(7):e000195.

27. Saito S, Koori Y, Ohsaki Y, *et al*. Third-generation cephalosporin-resistant non-typhoidal *Salmonella* isolated from human feces in Japan. *Jpn J Infect Dis*. 2018;70(3):301-304.

28. Milligan R, Paul M, Richardson M, Neuberger A. Vaccines for preventing typhoid fever. *Cochrane Database Syst Rev*. 2018;5(5):CD001261. Published 2018 May 31. doi:10.1002/14651858.CD001261.pub4

29. Kariuki S, Gordon MA, Feasey N, Parry CM. Antimicrobial resistance and management of invasive *Salmonella* disease. *Vaccine*. 2015;33 Suppl 3(0 3):C21–C29. doi:10.1016/j.vaccine.2015.03.102.

30. Centers for Disease Control & Prevention (2020). "Typhoid Fever and Paratyphoid Fever Vaccination." Retrieved August 26, 2021, from https://www.cdc.gov/typhoid-fever/typhoid-vaccination.html.

31. Centers for Disease Control and Prevention (CDC). National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS): Human Isolates Surveillance Report for 2015 (Final Report). Atlanta, Georgia: U.S. Department of Health and Human Services, CDC, 2017.

32. Antibiotic / Antimicrobial Resistance (AR / AMR). Centers for Disease Control and Prevention. https://www.cdc.gov/drugresistance/biggest_threats.html#non. Published November 26, 2018.

33. Krueger AL, Greene SA, Barzilay EJ, *et al*. Clinical outcomes of nalidixic acid, ceftriaxone, and multidrug-resistant nontyphoidal *Salmonella* infections compared with pansusceptible infections in FoodNet sites, 2006-2008. *Foodborne Pathog Dis*. 2014;11(5):335-41.

34. Centers for Disease Control and Prevention (2019). "Drug-Resistant Salmonella Serotype Typhi." Retrieved August 26, 2021, from https://www.cdc.gov/drugresistance/pdf/threats-report/salmonella-typhi-508.pdf.

35. Dyson ZA, Klemm EJ, Palmer S, Dougan G. Antibiotic resistance and typhoid. *Clin Infect Dis*. 2019;68(2):S165-S170.

36. Wong VK, Baker S, Pickard DJ, Parkhill J, Page AJ, Feasey NA, Kingsley RA, Thomson NR, Keane JA, Weill FX, Edwards DJ, Hawkey J, Harris SR, Mather AE, Cain AK, Hadfield J, Hart PJ, Thieu NT, Klemm EJ, Glinos DA, Breiman RF, Watson CH, Kariuki S, Gordon MA, Heyderman RS, Okoro C, Jacobs J, Lunguya O, Edmunds WJ, Msefula C, Chabalgoity JA, Kama M, Jenkins K, Dutta S, Marks F, Campos J, Thompson C, Obaro S, MacLennan CA, Dolecek C, Keddy KH, Smith AM, Parry CM, Karkey A, Mulholland EK, Campbell JI, Dongol S, Basnyat B, Dufour M, Bandaranayake D, Naseri TT, Singh SP, Hatta M, Newton P, Onsare RS, Isaia L, Dance D, Davong V, Thwaites G, Wijedoru L, Crump JA, De Pinna E, Nair S, Nilles EJ, Thanh DP, Turner P, Soeng S, Valcanis M, Powling J, Dimovski K, Hogg G, Farrar J, Holt KE, Dougan G. Phylogeographical analysis of the dominant multidrug-resistant H58 clade of *Salmonella* Typhi identifies inter- and intracontinental transmission events. *Nat Genet*. 2015;47(6):632-9.

37. Klemm EJ, Shakoor S, Page AJ, Qamar FN, Judge K, Saeed DK, Wong VK, Dallman TJ, Nair S, Baker S, Shaheen G, Qureshi S, Yousafzai MT, Saleem MK, Hasan Z, Dougan G, Hasan R. Emergence of an Extensively Drug-Resistant *Salmonella enterica* Serovar Typhi Clone Harboring a Promiscuous Plasmid Encoding Resistance to Fluoroquinolones and Third-Generation Cephalosporins. *mBio*. 2018;9(1):e00105-18.

38. Levine MM & Simon R. The Gathering Storm: Is Untreatable Typhoid Fever on the Way? *mBio*. 2018;9:e00482-18.

39. Rabsch W. *Salmonella* typhimurium phage typing for pathogens. *Methods Mol Biol*. 2007;394:177-211.

40. Poppe C, Smart N, Khakhria R, Johnson W, Spika J, Prescott J. *Salmonella* typhimurium DT104: a virulent and drug-resistant pathogen. *Can Vet J*. 1998;39(9):559–565.

41. Leekitcharoenphon P, Hendriksen RS, Le Hello S, Weill FX, Baggesen DL, Jun SR, Ussery DW, Lund O, Crook DW, Wilson DJ, Aarestrup FM. Global Genomic Epidemiology of *Salmonella* enterica Serovar Typhimurium DT104. *Appl Environ Microbiol*. 2016;82(8):2516–2526. Published 2016 Apr 4. doi:10.1128/AEM.03821-15.

42. Lilleengen K. Typing *Salmonella* by means of bacteriophage. *Acta Pathol Microbiol Scand Suppl*. 1948;77:11-125.

43. Callow BR. A new phage-typing scheme for *Salmonella* typhi-murium. *J Hyg (Lond)*. 1959;57(3):346–359. doi:10.1017/s0022172400020209

44. Anderson ES, Ward LR, Saxe MJ, de Sa JD. Bacteriophage-typing designations of *Salmonella* typhimurium. *J Hyg (Lond)*. 1977;78(2):297–300. doi:10.1017/s0022172400056187

45. Jarvik T, Smillie C, Groisman EA, Ochman H. Short-term signatures of evolutionary change in the *Salmonella* enterica serovar typhimurium 14028 genome. *J Bacteriol*. 2010;192(2):560–567. doi:10.1128/JB.01233-09.

46. Baggesen DL, Sørensen G, Nielsen EM, Wegener HC. Phage typing of *Salmonella* Typhimurium - is it still a useful tool for surveillance and outbreak investigation? *Euro Surveill*. 2010;15(4):19471.

47. Galán JE, Curtiss R 3rd. Expression of Salmonella typhimurium genes required for invasion is regulated by changes in DNA supercoiling. *Infect Immun*. 1990;58(6):1879-1885. doi:10.1128/iai.58.6.1879-1885.1990.

48. Lee CA, Falkow S. The ability of Salmonella to enter mammalian cells is affected by bacterial growth state. *Proc Natl Acad Sci U S A*. 1990;87(11):4304-4308. doi:10.1073/pnas.87.11.4304.

49. Ikuta T, Ausenda S, Cappellini MD. Mechanism for fetal globin gene expression: role of the soluble guanylate cyclase-cGMP-dependent protein kinase pathway. *Proc Natl Acad Sci U S A*. 2001;98(4):1847-1852. doi:10.1073/pnas.041599798.

50. Bajaj V, Lucas RL, Hwang C, Lee CA. Co-ordinate regulation of Salmonella typhimurium invasion genes by environmental and regulatory factors is mediated by control of hilA expression. *Mol Microbiol*. 1996;22(4):703-14.

51. Galán JE, Curtiss R 3rd. Cloning and molecular characterization of genes whose products allow Salmonella typhimurium to penetrate tissue culture cells. *Proc Natl Acad Sci U S A*. 1989;86(16):6383-6387. doi:10.1073/pnas.86.16.6383

52. Lostroh CP & Lee CA. The Salmonella pathogenicity island-1 type III secretion system. *Microbes Infect*. 2001;3(14-15):1281-91.

53. Zhou D, Chen LM, Hernandez L, Shears SB, Galán JE. A Salmonella inositol polyphosphatase acts in conjunction with other bacterial effectors to promote host cell actin cytoskeleton rearrangements and bacterial internalization. *Mol Microbiol*. 2001;39(2):248-59.

54. Galán JE. Salmonella interactions with host cells: type III secretion at work. *Annu Rev Cell Dev Biol*. 2001;17:53-86.

55. Waterman SR & Holden DW. Functions and effectors of the Salmonella pathogenicity island 2 type III secretion system. *Cell Microbiol*. 2003;5(8):501-11.

56. Coombes BK, Wickham ME, Lowden MJ, Brown NF, Finlay BB. Negative regulation of Salmonella pathogenicity island 2 is required for contextual control of virulence during typhoid. *Proc Natl Acad Sci U S A*. 2005;102(48):17460-17465. doi:10.1073/pnas.0505401102.

57. Hapfelmeier S, Ehrbar K, Stecher B, Barthel M, Kremer M, Hardt WD. Role of the Salmonella pathogenicity island 1 effector proteins SipA, SopB, SopE, and SopE2 in Salmonella enterica subspecies 1 serovar Typhimurium colitis in streptomycin-pretreated mice. *Infect Immun*. 2004;72(2):795-809. doi:10.1128/IAI.72.2.795-809.2004.

58. Kubori T, Matsushima Y, Nakamura D, Uralil J, Lara-Tejero M, Sukhan A, Galán JE, Aizawa SI. Supramolecular structure of the Salmonella typhimurium type III protein secretion system. *Science*. 1998;280(5363):602-5.

59. Sukhan A, Kubori T, Wilson J, Galán JE. Genetic analysis of assembly of the Salmonella enterica serovar Typhimurium type III secretion-associated needle complex. *J Bacteriol*. 2001;183(4):1159-1167. doi:10.1128/JB.183.4.1159-1167.2001.

60. Lou L, Zhang P, Piao R, Wang Y. *Salmonella* Pathogenicity Island 1 (SPI-1) and Its Complex Regulatory Network. *Front Cell Infect Microbiol*. 2019;9:270. Published 2019 Jul 31. doi:10.3389/fcimb.2019.00270.

61. Zhou D, Mooseker MS, Galán JE. An invasion-associated Salmonella protein modulates the actin-bundling activity of plastin. *Proc Natl Acad Sci U S A*. 1999;96(18):10176-10181. doi:10.1073/pnas.96.18.10176.

62. Bajaj V, Hwang C, Lee CA. hilA is a novel ompR/toxR family member that activates the expression of Salmonella typhimurium invasion genes. *Mol Microbiol*. 1995;18(4):715-27.

63. Takeuchi A. Electron microscope studies of experimental Salmonella infection. I. Penetration into the intestinal epithelium by Salmonella typhimurium. *Am J Pathol*. 1967;50(1):109-136.

64. Takeuchi A & Sprinz H. Electron-Microscope Studies of Experimental Salmonella Infection in the Preconditioned Guinea Pig: II. Response of the Intestinal Mucosa to the Invasion by Salmonella typhimurium. *Am J Pathol*. 1967;51(1):137-161.

65. Kihlström E & Latkovic S. Ultrastructural studies on the interaction between Salmonella typhimurium 395 M and HeLa cells. *Infect Immun*. 1978;22(3):804-809. doi:10.1128/iai.22.3.804-809.1978.

66. Finlay B & Falkow S. Salmonella as an intracellular parasite. *Mol Microbiol*. 1989;3(12):1833-1841.

67. Steele-Mortimer O. The Salmonella-containing vacuole: moving with the times. *Curr Opin Microbiol*. 2008;11(1):38-45. doi:10.1016/j.mib.2008.01.002.

68. Jennings E, Thurston TLM, Holden DW. Salmonella SPI-2 Type III Secretion System Effectors: Molecular Mechanisms And Physiological Consequences. *Cell Host Microbe*. 2017;22(2):217-231.

69. Knodler LA, Nair V, Steele-Mortimer O. Quantitative assessment of cytosolic Salmonella in epithelial cells. *PLoS One*. 2014;9(1):e84681. Published 2014 Jan 6. doi:10.1371/journal.pone.0084681.

70. Cederlund A, Gudmundsson GH, Agerberth B. Antimicrobial peptides important in innate immunity. *FEBS J*. 2011;278(20):3942-51.

71. Rathman M, Sjaastad MD, Falkow S. Acidification of phagosomes containing Salmonella typhimurium in murine macrophages. *Infect Immun*. 1996;64(7):2765-2773. doi:10.1128/iai.64.7.2765-2773.1996.

72. Di A, Brown ME, Deriy LV, Li C, Szeto FL, Chen Y, Huang P, Tong J, Naren AP, Bindokas V, Palfrey HC, Nelson DJ. CFTR regulates phagosome acidification in macrophages and alters bactericidal activity. *Nat Cell Biol*. 2006;8(9):933-44.

73. Fritsche G, Nairz M, Theurl I, Mair S, Bellmann-Weiler R, Barton HC, Weiss G. Modulation of macrophage iron transport by Nramp1 (Slc11a1). *Immunobiology*. 2007;212(9-10):751-7.

74. Nairz M, Theurl I, Ludwiczek S, Theurl M, Mair, SM, Fritsche G, Weiss G. The co-ordinated regulation of iron homeostasis in murine macrophages limits the availability of iron for intracellular Salmonella typhimurium. *Cell Microbiol*. 2007;9(9):2126-40.

75. Achard MES, Stafford SL, Bokil NJ, Chartres J, Bernhardt PV, Schembri MA, Sweet MJ, McEwan AG. Copper redistribution in murine macrophages in response to Salmonella infection. *Biochem J*. 2012;444(1):51-7.

76. Kapetanovic R, Bokil NJ, Achard MES, Ong CY, Peters KM, Stocks CJ, Phan M, Monteleone M, Schroder K, Irvine KM, Saunders BM, Walker MJ, Stacey KJ, McEwan AG, Schembri MA, Sweet MJ. Salmonella employs multiple mechanisms to subvert the TLR-inducible zinc-mediated antimicrobial response of human macrophages. *FASEB J*. 2016;30(5):1901-12.

77. Buchmeier NA, Heffron F. Inhibition of macrophage phagosome-lysosome fusion by Salmonella typhimurium. *Infect Immun*. 1991;59(7):2232-2238. doi:10.1128/iai.59.7.2232-2238.1991.

78. Thurston TLM, Matthews SA, Jennings E, Alix E, Shao F, Shenoy AR, Birrell MA, Holden DW. Growth inhibition of cytosolic Salmonella by caspase-1 and caspase-11 precedes host cell death. *Nat Commun*. 2016;13292(2016).

79. Schatz A, Bugle E, Waksman SA. Streptomycin, a Substance Exhibiting Antibiotic Activity Against Gram-Positive and Gram-Negative Bacteria. 1944. *Clin Orthop Relat Res*. 2005;(437):3-6.

80. Moazed D, Noller HF. Interaction of antibiotics with functional sites in 16S ribosomal RNA. *Nature*. 1987;327(6121):389-394.

81. Ogle JM, Murphy FV, Tarry MJ, Ramakrishnan V. Selection of tRNA by the Ribosome Requires a Transition from an Open to a Closed Form. *Cell*. 2002;111(5):721-732.

82. Wallace BJ, Davis BD. Cyclic blockade of initiation sites by streptomycin-damaged ribosomes in Escherichia coli: an explanation for dominance of sensitivity. *J Mol Biol*. 1973;75(2):377-390.

83. Davis BD, Chen LL, Tai PC. Misread protein creates membrane channels: an essential step in the bactericidal action of aminoglycosides. *Proc Natl Acad Sci U S A*. 1986;83(16):6164-6168.

84. Wilhelm JM, Jessop JJ, Pettitt SE. Aminoglycoside antibiotics and eukaryotic protein synthesis: stimulation of errors in the translation of natural messengers in extracts of cultured human cells. *Biochemistry*. 1978;17(7):1149-1153.

85. Fan-Minogue H, Bedwell DM. Eukaryotic ribosomal RNA determinants of aminoglycoside resistance and their role in translational fidelity. *RNA*. 2008;14(1):148-157.

86. Hobbie SN, Kalapala SK, Akshay S, *et al*. Engineering the rRNA decoding site of eukaryotic cytosolic ribosomes in bacteria. *Nucleic Acids Res*. 2007;35(18):6086-6093.

87. Prokhorova I, Altman RB, Djumagulov M, *et al*. Aminoglycoside interactions and impacts on the eukaryotic ribosome. *Proc Natl Acad Sci U S A*. 2017;114(51):E10899-E10908.

88. Hobbie SN, Akshay S, Kalapala SK, Bruell CM, Shcherbakov D, Böttger EC. Genetic analysis of interactions with eukaryotic rRNA identify the mitoribosome as target in aminoglycoside ototoxicity. *Proc Natl Acad Sci U S A*. 2008;105(52):20888–20893.

89. Matt T, Ng CL, Lang K, *et al*. Dissociation of antibacterial activity and aminoglycoside ototoxicity in the 4-monosubstituted 2-deoxystreptamine apramycin. *Proc Natl Acad Sci U S A*. 2012;109(27):10984-10989.

90. Shulman E, Belakhov V, Wei G, *et al*. Designer Aminoglycosides That Selectively Inhibit Cytoplasmic Rather than Mitochondrial Ribosomes Show Decreased Ototoxicity: A STRATEGY FOR THE TREATMENT OF GENETIC DISEASES. *J Biol Chem*. 2014;289(4):2318-2330.

91. Frymark T, Leech H, Mullen R, *et al*. Evidence-Based Systematic Review: Drug-Induced Hearing Loss—Gentamicin. *ASHA's National Center for Evidence-Based Practice in Communication Disorders*. 2010.

92. Vandewalle A, Farman N, Morin J-P, *et al*. Gentamicin incorporation along the nephron: Autoradiographic study on isolated tubules. *Kidney Int*. 1981;19(4):529-539.

93. Gentamicin (Review). World Health Organization. http://www.who.int/selection_medicines/committees/subcommittee/2/gentamicin/en/. Published December 11, 2010. Accessed April 23, 2018.

94. American Association of Bovine Practitioners. Aminoglycoside use in cattle. Accessed June 3, 2019.

95. Gehring R, Haskell SR, Payne MA, *et al*. Aminoglycoside residues in food of animal origin. *J Am Vet Med Assoc*. 2005;227(1):63-6.

96. USDA Food Safety Inspection Services Web site. National Residue program data: the "red book." Available at https://www.fsis.usda.gov/wps/wcm/connect/d6baddf7-0352-4a0e-a86d-32ba2d46 13ba/2018-red-book.pdf?MOD=AJPERES. Accessed May 9, 2019.

97. Grand View Research. (2016, April). *Aminoglycosides Market Analysis By Drug (Neomycin, Tobramycin, Gentamicin, Amikacin, Paromomycin, Streptomycin, Kanamycin), By Mode of Administration (Feed, Injectables (Parenteral), Intra-mammary, Topical, Oral), By Application (Veterinary, Skin, Respiratory, UTI & Pelvic Disease, Neonatal Sepsis) And Segment Forecasts To 2022*. Retrieved from https://www.grandviewresearch.com/industry-analysis/aminoglycoside- market.

98. Garneau-Tsodikova S, Labby KJ. Mechanisms of Resistance to Aminoglycoside Antibiotics: Overview and Perspectives. *Medchemcomm*. 2016;7(1):11-27. doi:10.1039/C5MD00344J.

99. Recht MI, Puglisi JD. Aminoglycoside resistance with homogeneous and heterogeneous populations of antibiotic-resistant ribosomes. *Antimicrob Agents Chemother*. 2001;45(9):2414-2419. doi:10.1128/aac.45.9.2414-2419.2001.

100. Ahmad MH, Rechenmacher A, Böck A. Interaction between aminoglycoside uptake and ribosomal resistance mutations. *Antimicrob Agents Chemother*. 1980;18(5):798-806. doi:10.1128/aac.18.5.798.

101. Ibacache-Quiroga C, Oliveros JC, Couce A, Blázquez J. Parallel Evolution of High-Level Aminoglycoside Resistance in *Escherichia coli* Under Low and High Mutation Supply Rates. *Front Microbiol*. 2018;9:427. Published 2018 Mar 19. doi:10.3389/fmicb.2018.00427.

102. Bolard A, Plésiat P, Jeannot K. Mutations in Gene *fusA1* as a Novel Mechanism of Aminoglycoside Resistance in Clinical Strains of Pseudomonas aeruginosa. *Antimicrob Agents Chemother*. 2018;62(2):e01835-17. Published 2018 Jan 25. doi:10.1128/AAC.01835-17.

103. Davies J & Wright GD. Bacterial resistance to aminoglycoside antibiotics. *Trends Microbiol*. 1997;5(6):234-40.

104. Aires JR, Köhler T, Nikaido H, Plésiat P. Involvement of an active efflux system in the natural resistance of Pseudomonas aeruginosa to aminoglycosides. *Antimicrob Agents Chemother*. 1999;43(11):2624–2628.

105. Mine T, Morita Y, Kataoka A, Mizushima T, Tsuchiya T. Expression in Escherichia coli of a new multidrug efflux pump, MexXY, from Pseudomonas aeruginosa. *Antimicrob Agents Chemother*. 1999;43(2):415–417.

106. Rosenberg EY, Ma D, Nikaido H. AcrD of Escherichia coli is an aminoglycoside efflux pump. *J Bacteriol*. 2000;182(6):1754–1756. doi:10.1128/jb.182.6.1754-1756.2000.

107. Magnet S, Courvalin P, Lambert T. Resistance-nodulation-cell division-type efflux pump involved in aminoglycoside resistance in Acinetobacter baumannii strain BM4454. *Antimicrob Agents Chemother*. 2001;45(12):3375–3380. doi:10.1128/AAC.45.12.3375-3380.2001.

108. Moore RA, DeShazer D, Reckseidler S, Weissman A, Woods DE. Efflux-mediated aminoglycoside and macrolide resistance in Burkholderia pseudomallei. *Antimicrob Agents Chemother*. 1999;43(3):465–470.

109. Adegoke AA, Stenström TA, Okoh AI. *Stenotrophomonas maltophilia* as an Emerging Ubiquitous Pathogen: Looking Beyond Contemporary Antibiotic Therapy. *Front Microbiol*. 2017;8:2276. Published 2017 Nov 30. doi:10.3389/fmicb.2017.02276.

110. Calvopiña K, Dulyayangkul P, Avison MB. Mutations in Ribosomal Protein RplA or Treatment with Ribosomal Acting Antibiotics Activates Production of Aminoglycoside Efflux Pump SmeYZ in Stenotrophomonas maltophilia. *Antimicrob Agents Chemother*. 2020;64(2):e01524-19. doi: 10.1128/AAC.01524-19.

111. Sun J, Deng Z, Yan A. Bacterial multidrug efflux pumps: mechanisms, physiology and pharmacological exploitations. *Biochem Biophys Res Commun*. 2014;453(2):254-67.

112. Yokoyama K, Doi Y, Yamane K, Kurokawa H, Shibata N, Shibayama K, Yagi T, Kato H, Arakawa Y. Acquisition of 16S rRNA methylase gene in Pseudomonas aeruginosa. *Lancet*. 2003;362(9399):1888-93.

113. Galimand M, Courvalin P, Lambert T. Plasmid-mediated high-level resistance to aminoglycosides in Enterobacteriaceae due to 16S rRNA methylation. *Antimicrob Agents Chemother*. 2003;47(8):2565–2571. doi:10.1128/aac.47.8.2565-2571.2003.

114. Doi Y, Yokoyama K, Yamane K, Wachino J, Shibata N, Yagi T, Shibayama K, Kato H, Arakawa Y. Plasmid-mediated 16S rRNA methylase in Serratia marcescens conferring high-level resistance to aminoglycosides. *Antimicrob Agents Chemother*. 2004;48(2):491–496. doi:10.1128/aac.48.2.491-496.2004.

115. Wachino J, Yamane K, Shibayama K, Kurokawa H, Shibata N, Suzuki S, Doi Y, Kimura K, Ike Y, Arakawa Y. Novel plasmid-mediated 16S rRNA methylase, RmtC, found in a proteus mirabilis isolate demonstrating extraordinary high-level resistance against various aminoglycosides. *Antimicrob Agents Chemother*. 2006;50(1):178–184. doi:10.1128/AAC.50.1.178-184.2006.

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

117. Davis MA, Baker KN, Orfe LH, Shah DH, Besser TE, Call DR. Discovery of a gene conferring multiple-aminoglycoside resistance in Escherichia coli. *Antimicrob Agents Chemother*. 2010;54(6):2666–2669. doi:10.1128/AAC.01743-09.

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

119. Bueno MF, Francisco GR, O'Hara JA, de Oliveira Garcia D, Doi Y. Coproduction of 16S rRNA methyltransferase RmtD or RmtG with KPC-2 and CTX-M group extended-spectrum β-lactamases in Klebsiella pneumoniae [published correction appears in Antimicrob Agents Chemother. 2016 Jan;60(1):714]. *Antimicrob Agents Chemother*. 2013;57(5):2397–2400. doi:10.1128/AAC.02108-12.

120. O'Hara JA, McGann P, Snesrud EC, Clifford RJ, Waterman PE, Lesho EP, Doi Y. Novel 16S rRNA methyltransferase RmtH produced by Klebsiella pneumoniae associated with war-related trauma. *Antimicrob Agents Chemother*. 2013;57(5):2413–2416. doi:10.1128/AAC.00266-13.

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

122. Doi Y, Wachino JI, Arakawa Y. Aminoglycoside Resistance: The Emergence of Acquired 16S Ribosomal RNA Methyltransferases. *Infect Dis Clin North Am*. 2016;30(2):523–537. doi:10.1016/j.idc.2016.02.011.

123. Benveniste R, Davies J. Aminoglycoside antibiotic-inactivating enzymes in actinomycetes similar to those present in clinical isolates of antibiotic-resistant bacteria. *Proc Natl Acad Sci U S A*. 1973;70(8):2276–2280. doi:10.1073/pnas.70.8.2276.

124. Shaw KJ, Rather PN, Hare RS, Miller GH. Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. *Microbiol Rev.* 1993;57(1):138–163.

125. Llano-Sotelo B1, Azucena EF Jr, Kotra LP, Mobashery S, Chow CS. Aminoglycosides modified by resistance enzymes display diminished binding to the bacterial ribosomal aminoacyl-tRNA site. *Chem Biol*. 2002;9(4):455-63.

126. Ramirez MS, Tolmasky ME. Aminoglycoside modifying enzymes. *Drug Resist Updat*. 2010;13(6):151–171. doi:10.1016/j.drup.2010.08.003.

127. Okamoto S & Suzuki Y. Chloramphenicol-, dihydrostreptomycin-, and kanamycin-inactivating enzymes from multiple drug-resistant *Escherichia coli* carrying episome 'R'. *Nature*. 1965;208(5017):1301-3.

128. Benveniste R, Davies J. Aminoglycoside antibiotic-inactivating enzymes in actinomycetes similar to those present in clinical isolates of antibiotic-resistant bacteria. *Proc Natl Acad Sci U S A.* 1973;70(8):2276–2280. doi:10.1073/pnas.70.8.2276.

129. Vetting MW, S de Carvalho LP, Yu M, Hegde SS, Magnet S, Roderick SL, Blanchard JS. Structure and functions of the GNAT superfamily of acetyltransferases. *Arch Biochem Biophys*. 2005;433(1):212-26.

130. Hedges RW & Shannon KP. Resistance to apramycin in *Escherichia coli* isolated from animals: detection of a novel aminoglycoside-modifying enzyme. *J Gen Microbiol*. 1984;130(3):473-82.

131. Lovering AM, White LO, Reeves DS. AAC(1): a new aminoglycoside-acetylating enzyme modifying the Cl aminogroup of apramycin. *J Antimicrob Chemother*. 1987;20(6):803-13.

132. Gomez-Luis, R.; Vergara, Y.; Lopez, L.; Castillo, J.; Rubio, M. (1999). 1-N-Aminoglycoside acetyltransferase [AAC(1)] in clinical isolates of *Campylobacter* spp.. Interscience Conference on Antimicrobial. Agents and Chemotherapy; San Francisco, CA.

133. Sunada A, Nakajima M, Ikeda Y, Kondo S, Hotta K. Enzymatic 1-N-acetylation of paromomycin by an actinomycete strain #8 with multiple aminoglycoside resistance and paromomycin sensitivity. *J Antibiot (Tokyo)*. 1999;52(9):809-14.

134. Aínsa JA, Martin C, Gicquel B, Gomez-Lus R. Characterization of the chromosomal aminoglycoside 2'-N-acetyltransferase gene from *Mycobacterium fortuitum*. *Antimicrob Agents Chemother*. 1996;40(10):2350–2355.

135. Yoshii A, Moriyama H, Fukuhara T. The novel kasugamycin 2'-*N*-acetyltransferase gene aac(2')-IIa, carried by the IncP island, confers kasugamycin resistance to rice-pathogenic bacteria. *Appl Environ Microbiol*. 2012;78(16):5555–5564. doi:10.1128/AEM.01155-12.

136. Pawlowski AC, Wang W, Koteva K, Barton HA, McArthur AG, Wright GD. A diverse intrinsic antibiotic resistome from a cave bacterium. *Nat Commun*. 2016;7:13803. Published 2016 Dec 8. doi:10.1038/ncomms13803.

137. Chevereau M, Daniels PJ, Davies J, LeGoffic F. Aminoglycoside resistance in bacteria medicated by gentamicin acetyltransferase II, an enzyme modifying the 2'-amino group of aminoglycoside antibiotics. *Biochemistry*. 1974;13(3):598-603.

138. Lin T, Tang CG, Li QH, Ji J, Ge HY, Zhang XY, Sun HP. Identification of aac(2')-I type b aminoglycoside-modifying enzyme genes in resistant *Acinetobacter baumannii*. *Genet Mol Res*. 2015;14(1):1828-35.

139. Aínsa JA, Pérez E, Pelicic V, Berthet FX, Gicquel B, Martín C. Aminoglycoside 2'-*N*-acetyltransferase genes are universally present in mycobacteria: characterization of the aac(2')-Ic gene from *Mycobacterium tuberculosis* and the aac(2')-Id gene from *Mycobacterium smegmatis*. *Mol Microbiol*. 1997;24(2):431-41.

140. Witchitz JL. Plasmid-mediated gentamicin resistance not associated with kanamycin resistance in Enterobacteriaceae. *J Antibiot (Tokyo)*. 1972;25(10):622-4.

141. Umezawa H, Yagisawa M, Matsuhashi Y, Naganawa H, Yamamoto H. Letter: Gentamicin acetyltransferase in *Escherichia coli* carrying R factor. *J Antibiot (Tokyo)*. 1973;26(10):612-4.

142. Taitt CR, Leski TA, Stockelman MG, Craft DW, Zurawski DV, Kirkup BC, Vora GJ. Antimicrobial resistance determinants in Acinetobacter baumannii isolates taken from military treatment facilities. *Antimicrob Agents Chemother*. 2014;58(2):767-81. doi: 10.1128/AAC.01897-13. Epub 2013 Nov 18. PMID: 24247131; PMCID: PMC3910874.

143. Le Goffic F & Moreau N. Purification by affinity chromatography of an enzyme involved in gentamicin inactivation. *FEBS Lett*. 1973;29(3):289-291.

144. Brzezinska M, Benveniste R, Davies J, Daniels PJ, Weinstein J. Gentamicin resistance in strains of *Pseudomonas aeruginosa* mediated by enzymatic *N*-acetylation of the deoxystreptamine moiety. *Biochemistry*. 1972;11(5):761-5.

145. Wohlleben W, Arnold W, Bissonnette L, Pelletier A, Tanguay A, Roy PH, Gamboa GC, Barry GF, Aubert E, Davies J, *et al*. On the evolution of Tn21-like multiresistance transposons: sequence analysis of the gene (*aacC1*) for gentamicin acetyltransferase-3-I (AAC(3)-I), another member of the Tn21-based expression cassette. *Mol Gen Genet*. 1989;17(2-3):202-8.

146. Javier Terán F, Alvarez M, Suárez JE, Mendoza MC. Characterization of two aminoglycoside-(3)-*N*-acetyltransferase genes and assay as epidemiological probes. *J Antimicrob Chemother*. 1991;28(3):333-46.

147. Schwocho LR, Schaffner CP, Miller GH, Hare RS, Shaw KJ. Cloning and characterization of a 3-N-aminoglycoside acetyltransferase gene, aac(3)-Ib, from Pseudomonas aeruginosa. *Antimicrob Agents Chemother*. 1995 Aug;39(8):1790-6. doi: 10.1128/aac.39.8.1790.

148. Doublet B, Weill FX, Fabre L, Chaslus-Dancla E, Cloeckaert A. Variant Salmonella genomic island 1 antibiotic resistance gene cluster containing a novel 3'-N-aminoglycoside acetyltransferase gene cassette, aac(3)-Id, in Salmonella enterica serovar newport. *Antimicrob Agents Chemother*. 2004 Oct;48(10):3806-12. doi: 10.1128/AAC.48.10.3806-3812.2004. PMID: 15388438; PMCID: PMC521890.

149. Levings RS, Partridge SR, Lightfoot D, Hall RM, Djordjevic SP. New integron-associated gene cassette encoding a 3-N-aminoglycoside acetyltransferase. *Antimicrob Agents Chemother*. 2005 Mar;49(3):1238-41. doi: 10.1128/AAC.49.3.1238-1241.2005.

150. Gionechetti F, Zucca P, Gombac F, Monti-Bragadin C, Lagatolla C, Tonin E, Edalucci E, Vitali LA, Dolzani L. Characterization of antimicrobial resistance and class 1

integrons in *Enterobacteriaceae* isolated from Mediterranean herring gulls (*Larus Cachinnans*). *Microb Drug Resist*. 2008;14(2):93-9.

151. Ho P, Wong RC, Lo SW, Chow K, Wong SS, Que T. Genetic identity of aminoglycoside-resistance genes in *Escherichia coli* isolates from human and animal sources. *J Med Microbiol*. 2010;59(Pt6):702-707.

152. van de Klundert JA, Vliegenthart JS. Nomenclature of aminoglycoside resistance genes: a comment. *Antimicrob Agents Chemother*. 1993 Apr;37(4):927-8. doi: 10.1128/aac.37.4.927.

153. Allmansberger R, Bräu B, Piepersberg W. Genes for gentamicin-(3)-*N*-acetyl-transferases III and IV. II. Nucleotide sequences of three AAC(3)-III genes and evolutionary aspects. *Mol Gen Genet*. 1985;198(3):514-20.

154. Mugnier P, Dubrous P, Casin I, Arlet G, Collatz E. A TEM-derived extended-spectrum beta-lactamase in Pseudomonas aeruginosa. *Antimicrob Agents Chemother*. 1996 Nov;40(11):2488-93.

155. Rather PN, Mierzwa R, Hare RS, Miller GH, Shaw KJ. Cloning and DNA sequence analysis of an aac(3)-Vb gene from Serratia marcescens. *Antimicrob Agents Chemother*. 1992 Oct;36(10):2222-7. doi: 10.1128/aac.36.10.2222.

156. Dahmen S, Bettaieb D, Mansour W, Boujaafar N, Bouallègue O, Arlet G. Characterization and Molecular Epidemiology of Extended-Spectrum Beta-Lactamases in Clinical Isolates of Enterobacteriaceae in a Tunisian University Hospital. *Microb Drug Resist*. 2010;16(2):163-70.

157. Oteo J, Navarro C, Cercenado E, Delgado-Iribarren A, Wilhelmi I, Orden B, García C, Miguelañez S, Pérez-Vázquez M, García-Cobos S, Aracil B, Bautista V, Campos J.

Spread of Escherichia coli strains with high-level cefotaxime and ceftazidime resistance between the community, long-term care facilities, and hospital institutions. *J Clin Microbiol*. 2006 Jul;44(7):2359-66. doi: 10.1128/JCM.00447-06.

158. Dubois V, Arpin C, Dupart V, Scavelli A, Coulange L, André C, Fischer I, Grobost F, Brochet J, Lagrange I, Dutilh B, Jullin J, Noury P, Larribet G, Quentin C. Beta-lactam and Aminoglycoside Resistance Rates and Mechanisms Among Pseudomonas Aeruginosa in French General Practice (Community and Private Healthcare Centres). *J Antimicrob Chemother*. 2008;62(2):316-23.

159. Wilson NL, Hall RM. Unusual class 1 integron configuration found in Salmonella genomic island 2 from Salmonella enterica serovar Emek. *Antimicrob Agents Chemother*. 2010 Jan;54(1):513-6. doi: 10.1128/AAC.00895-09.

160. Vliegenthart JS, Ketelaar-van Gaalen PA, van de Klundert JA. Nucleotide sequence of the aacC3 gene, a gentamicin resistance determinant encoding aminoglycoside-(3)-N-acetyltransferase III expressed in Pseudomonas aeruginosa but not in Escherichia coli. *Antimicrob Agents Chemother*. 1991 May;35(5):892-7. doi: 10.1128/aac.35.5.892.

161. Norris A, Ozen C, Serpersu EH. Thermodynamics and Kinetics of Association of Antibiotics With the Aminoglycoside Acetyltransferase (3)-IIIb, a Resistance-Causing Enzyme. *Biochemistry*. 2010;49(19):4027-35.

162. Shahid M & Malik A. Resistance due to aminoglycoside modifying enzymes in *Pseudomonas aeruginosa* isolates from burns patients. *Indian J Med Res*. 2005;122(4):324-9.

163. Bräu B, Pilz U, Piepersberg W. Genes for gentamicin-(3)-N-acetyltransferases III and IV: I. Nucleotide Sequence of the AAC(3)-IV Gene and Possible Involvement of an IS140 Element in Its Expression. *Mol Gen Genet*. 1984;193(1):179-87.

164. Heuer H, Krögerrecklenfort E, Wellington EMH, Egan S, van Elsas JD, van Overbeek L, Collard J-M, Guillaume G, Karagouni AD, Nikolakopoulou TL, Smalla K. Gentamicin resistance genes in environmental bacteria: prevalence and transfer. *FEMS Microbiol Ecol*. 2002;42(2):289-302.

165. Call DR, Singer RS, Meng D, Broschat SL, Orfe LH, Anderson JM, Herndon DR, Kappmeyer LS, Daniels JB, Besser TE. blaCMY-2-positive IncA/C plasmids from Escherichia coli and Salmonella enterica are a distinct component of a larger lineage of plasmids. *Antimicrob Agents Chemother*. 2010 Feb;54(2):590-6. doi: 10.1128/AAC.00055-09.

166. Rather PN, Mann PA, Mierzwa R, Hare RS, Miller GH, Shaw KJ. Analysis of the aac(3)-VIa gene encoding a novel 3-N-acetyltransferase. *Antimicrob Agents Chemother*. 1993 Oct;37(10):2074-9. doi: 10.1128/aac.37.10.2074.

167. López-Cabrera M, Pérez-González JA, Heinzel P, Piepersberg W, Jiménez A. Isolation and nucleotide sequencing of an aminocyclitol acetyltransferase gene from Streptomyces rimosus forma paromomycinus. *J Bacteriol*. 1989 Jan;171(1):321-8. doi: 10.1128/jb.171.1.321-328.1989.

168. Salauze D, Perez-Gonzalez JA, Piepersberg W, Davies J. Characterization of aminoglycoside acetyltransferase-encoding genes of neomycin-producing *Micromonospora chalcea* and *Streptomyces fradiae*. *Gene*. 1991;101(1):143-8.

169. Ishikawa J, Sunada A, Oyama R, Hotta K. Identification and characterization of the point mutation which affects the transcription level of the chromosomal 3-N-acetyltransferase gene of Streptomyces griseus SS-1198. *Antimicrob Agents Chemother*. 2000 Feb;44(2):437-40. doi: 10.1128/aac.44.2.437-440.2000.

170. Galimand M, Fishovitz J, Lambert T, Barbe V, Zajicek J, Mobashery S, Courvalin P. AAC(3)-XI, a new aminoglycoside 3-N-acetyltransferase from Corynebacterium striatum. *Antimicrob Agents Chemother*. 2015 Sep;59(9):5647-53. doi: 10.1128/AAC.01203-15.

171. Rather PN, Munayyer H, Mann PA, Hare RS, Miller GH, Shaw KJ. Genetic analysis of bacterial acetyltransferases: identification of amino acids determining the specificities of the aminoglycoside 6'-N-acetyltransferase Ib and IIa proteins. *J Bacteriol*. 1992 May;174(10):3196-203. doi: 10.1128/jb.174.10.3196-3203.1992.

172. Zhang G, Li J, Ai G, He J, Wang C, Feng J. A new intrinsic aminoglycoside 6′-N-acetyltransferase subclass, AAC(6′)-III, in *Burkholderia pseudomallei, Burkholderia mallei* and *Burkholderia oklahomensis*. *J Antimicrob Chemother*. 2020;75(5):1352-1353.

173. Ramirez MS, Tolmasky ME. Aminoglycoside modifying enzymes. *Drug Resist Updat*. 2010;13(6):151-171. doi:10.1016/j.drup.2010.08.003.

174. Vanhoof R, Hannecart-Pokorni H, Content J. Nomenclature of genes encoding aminoglycoside-modifying enzymes. *Antimicrob Agents Chemother*. 1998;42(2):438.

175. Casin I, Hanau-Berçot B, Podglajen I, Vahaboglu H, Collatz E. Salmonella enterica serovar Typhimurium bla(PER-1)-carrying plasmid pSTI1 encodes an extended-

spectrum aminoglycoside 6'-N-acetyltransferase of type Ib. *Antimicrob Agents Chemother*. 2003 Feb;47(2):697-703. doi: 10.1128/aac.47.2.697-703.2003.

176. Lambert T, Ploy MC, Courvalin P. A Spontaneous Point Mutation in the aac(6')-Ib' Gene Results in Altered Substrate Specificity of Aminoglycoside 6'-N-acetyltransferase of a *Pseudomonas Fluorescens* Strain. *FEMS Microbiol Lett*. 1994;115(2-3):297-304.

177. Ramirez MS, Nikolaidis N, Tolmasky ME. Rise and dissemination of aminoglycoside resistance: the aac(6')-Ib paradigm. *Front Microbiol*. 2013 May 17;4:121. doi: 10.3389/fmicb.2013.00121.

178. Robicsek A, Strahilevitz J, Jacoby GA, Macielag M, Abbanat D, Park CH, Bush K, Hooper DC. Fluoroquinolone-modifying enzyme: a new adaptation of a common aminoglycoside acetyltransferase. *Nat Med*. 2006;12(1):83-8.

179. Culebras E & Martínez JL. Aminoglycoside Resistance Mediated by the Bifunctional Enzyme 6'-N-aminoglycoside acetyltransferase-2''-O-aminoglycoside Phosphotransferase. *Front Biosci*. 1999;4:D1-8.

180. Daigle DM, Hughes DW, Wright GD. Prodigious Substrate Specificity of AAC(6')-APH(2''), an Aminoglycoside Antibiotic Resistance Determinant in *Enterococci* and *Staphylococci*. *Chem Biol*. 1999;6(2):99-110.

181. Centrón D, Roy PH. Presence of a group II intron in a multiresistant Serratia marcescens strain that harbors three integrons and a novel gene fusion. *Antimicrob Agents Chemother*. 2002 May;46(5):1402-9. doi: 10.1128/aac.46.5.1402-1409.2002.

182. Mendes RE, Toleman MA, Ribeiro J, Sader HS, Jones RN, Walsh TR. Integron carrying a novel metallo-beta-lactamase gene, blaIMP-16, and a fused form of

aminoglycoside-resistant gene aac(6')-30/aac(6')-Ib': report from the SENTRY Antimicrobial Surveillance Program. *Antimicrob Agents Chemother*. 2004 Dec;48(12):4693-702. doi: 10.1128/AAC.48.12.4693-4702.2004.

183. Dubois V, Poirel L, Marie C, Arpin C, Nordmann P, Quentin C. Molecular characterization of a novel class 1 integron containing bla(GES-1) and a fused product of aac3-Ib/aac6'-Ib' gene cassettes in Pseudomonas aeruginosa. *Antimicrob Agents Chemother*. 2002 Mar;46(3):638-45. doi: 10.1128/aac.46.3.638-645.2002.

184. Centrón D, Roy PH. Presence of a group II intron in a multiresistant *Serratia marcescens* strain that harbors three integrons and a novel gene fusion. *Antimicrob Agents Chemother*. 2002 May;46(5):1402-9. doi: 10.1128/aac.46.5.1402-1409.2002.

185. Naas T, Poirel L, Nordmann P. Molecular Characterisation of In51, a Class 1 Integron Containing a Novel Aminoglycoside Adenylyltransferase Gene Cassette, aadA6, in *Pseudomonas Aeruginosa. Biochim Biophys Acta*. 1999;1489(2-3):445-51.

186. Liang C, Xing B, Yang X, Fu Y, Feng Y, Zhang Y. Molecular epidemiology of aminoglycosides resistance on *Klebsiella pneumonia* in a hospital in China. *Int J Clin Exp Med.* 2015 Jan 15;8(1):1381-5.

187. Ming DS, Chen QQ, Chen XT. Detection of 5 Kinds of Genes Related to Plasmid-Mediated Quinolone Resistance in Four Species of Nonfermenting Bacteria with 2 Drug Resistant Phenotypes. *Can J Infect Dis Med Microbiol*. 2020 Apr 9;2020:3948719. doi: 10.1155/2020/3948719.

188. Abo-State MAM, Saleh YE, Ghareeb HM. Prevalence and sequence of aminoglycosides modifying enzymes genes among *E. coli* and *Klebsiella* species isolated from Egyptian hospitals. *J Radiat Res Appl Sc*. 2018;11(4):408-415.

189. Amini F, Krimpour HA, Ghaderi M, Vaziri S, Ferdowsi S, Azizi M, Amini S. Prevalence of aminoglycoside resistance genes in *Enterococcus* strains in Kermanshah, Iran. *Iran J Med Sci*. 2018;43(5):487-493.

190. McEwen SA & Collignon PJ. Antimicrobial resistance: a one health perspective. *Microbiol Spectr*. 2018;6(2).

191. Lim S-K, Kim D, Moon D-C, Cho Y, Rho M. Antibiotic resistomes discovered in the gut microbiomes of Korean swine and cattle. *GigaScience*. 2020;9(5):giaa043.

192. Bhardwaj P, Kaur G, Rampal S. Impact of marbofloxacin administration on the emergence of marbofloxacin-resistant *E. coli* in faecal flora of goats and elucidation of molecular basis of resistance. *J Glob Antimicrob Resist*. 2020;21:116-123.

193. Ng C, Tan B, Jiang XT, Gu X, Chen H, Schmitz BW, Haller L, Charles FR, Zhang T, Gin K. Metagenomic and Resistome Analysis of a Full-Scale Municipal Wastewater Treatment Plant in Singapore Containing Membrane Bioreactors. *Front Microbiol*. 2019 Feb 18;10:172. doi: 10.3389/fmicb.2019.00172.

194. Pfaller MA, Jones RN, Doern GV, Kugler K. Bacterial pathogens isolated from patients with bloodstream infection: frequencies of occurrence and antimicrobial susceptibility patterns from the SENTRY antimicrobial surveillance program (United States and Canada, 1997). *Antimicrob Agents Chemother*. 1998 Jul;42(7):1762-70.

195. Champion HM, Bennett PM, Lewis DA, Reeves DS. Cloning and characterization of an AAC(6') gene from *Serratia marcescens*. *J of Antimicrob Chemother*. 1988;22(5):587-596.

196. Shaw KJ, Rather PN, Sabatelli FJ, Mann P, Munayyer H, Mierzwa R, Petrikkos GL, Hare RS, Miller GH, Bennett P, Downey P. Characterization of the chromosomal

aac(6')-Ic gene from Serratia marcescens. *Antimicrob Agents Chemother*. 1992 Jul;36(7):1447-55. doi: 10.1128/aac.36.7.1447.

197. Costa Y, Galimand M, Leclercq R, Duval J, Courvalin P. Characterization of the chromosomal aac(6')-Ii gene specific for Enterococcus faecium. *Antimicrob Agents Chemother*. 1993 Sep;37(9):1896-903. doi: 10.1128/aac.37.9.1896.

198. Wright GD & Ladak P. Overexpression and characterization of the chromosomal aminoglycoside 6'-*N*-acetlytransferase from *Enteroccocus faecium*. *Antimicrob Agents Chemother*. 1997;41(5):956-60.

199. Radika K & Northrop DB. The kinetic mechanism of kanamycin acetyltransferase derived from the use of alternative antibiotics and coenzymes. *J Biol Chem*. 1984;259(20):12543-6.

200. Stogios PJ, Kuhn ML, Evdokimova E, Law M, Courvalin P, Savchenko A. Structural and Biochemical Characterization of Acinetobacter Spp. Aminoglycoside Acetyltransferases Highlights Functional and Evolutionary Variation Among Antibiotic Resistance Enzymes. *ACS Infect Dis*. 2017;3(2):132-143.

201. Rudant E, Courvalin P, Lambert T. Loss of intrinsic aminoglycoside resistance in *Acinetobacter haemolyticus* as a result of three distinct types of alterations in the aac(6')-Ig gene, including insertion of IS17. *Antimicrob Agents Chemother*. 1997;41(12):2646-51.

202. Lambert T, Gerbaud G, Courvalin P. Characterization of the chromosomal aac(6')-Ij gene of Acinetobacter sp. 13 and the aac(6')-Ih plasmid gene of Acinetobacter baumannii. *Antimicrob Agents Chemother*. 1994 Sep;38(9):1883-9. doi: 10.1128/aac.38.9.1883.

203. Payie KG, Strating H, Clarke AJ. The role of *O*-acetylation in the metabolism of peptidoglycan in *Providencia stuartii*. *Microb Drug Resist*. 1996;2(1):135-40.

204. Chevereau M, Daniels PJ, Davies J, LeGoffic F. Aminoglycoside resistance in bacteria medicated by gentamicin acetyltransferase II, an enzyme modifying the 2'-amino group of aminoglycoside antibiotics. *Biochemistry*. 1974;13(3):598-603.

205. Yamaguchi M, Mitsuhashi S, Kobayashi F, Zenda H. A 2'-*N*-acetylating enzyme of aminoglycosides. *J Antibiot (Tokyo)*. 1974;27(7):507-15.

206. Rather PN, Orosz E, Shaw KJ, Hare R, Miller G. Characterization and transcriptional regulation of the 2'-N-acetyltransferase gene from Providencia stuartii. *J Bacteriol*. 1993;175(20):6492-6498. doi:10.1128/jb.175.20.6492-6498.1993.

207. Macinga DR & Rather PN. The chromosomal 2'-*N*-acetyltransferase of *Providencia stuartii*: physiological functions and genetic regulation. *Front Biosci*. 1999;4:D132-40.

208. Rather PN, Orosz E, Shaw KJ, Hare R, Miller G. Characterization and transcriptional regulation of the 2'-N-acetyltransferase gene from Providencia stuartii. *J Bacteriol*. 1993 Oct;175(20):6492-8. doi: 10.1128/jb.175.20.6492-6498.1993.

209. Franklin K, Clarke AJ. Overexpression and characterization of the chromosomal aminoglycoside 2'-N-acetyltransferase of Providencia stuartii. *Antimicrob Agents Chemother*. 2001 Aug;45(8):2238-44. doi: 10.1128/AAC.45.8.2238-2244.2001.

210. Aínsa JA, Pérez E, Pelicic V, Berthet FX, Gicquel B, Martín C. Aminoglycoside 2'-N-acetyltransferase Genes Are Universally Present in Mycobacteria: Characterization of the *aac(2')-Ic* Gene From *Mycobacterium Tuberculosis* and the *aac(2')-Id* Gene From *Mycobacterium Smegmatis*. *Mol Microbiol*. 1997;24(2):431-41.

211. Barrett OJ, Pushechnikov A, Wu M, Disney MD. Studying aminoglycoside modification by the acetyltransferase class of resistance-causing enzymes via microarray. *Carbohydr Res*. 2008;343(17):2924-31.

212. Vetting MW, Hegde SS, Javid-Majd F, Blanchard JS, Roderick SL. Aminoglycoside 2'-*N*-acetyltransferase from *Mycobacterium tuberculosis* in complex with coenzyme A and aminoglycoside substrates. *Nat Struct Biol*. 2002;9(9):653-8.

213. Rominski A, Selchow P, Becker K, Brülle JK, Molin MD, Sander P. Elucidation of *Mycobacterium abscessus* aminoglycoside and capreomycin resistance by targeted deletion of three putative resistance genes. *J Antimicrob Chemother*. 2017;72(8):2191-2200.

214. Luthra S, Rominski A, Sander P. The Role of Antibiotic-Target-Modifying and Antibiotic-Modifying Enzymes in *Mycobacterium abscessus* Drug Resistance. *Front Microbiol*. 2018 Sep 12;9:2179. doi: 10.3389/fmicb.2018.02179.

215. Wei J, Dahl JL, Moulder JW, Roberts EA, O'Gaora P, Young DB, Friedman RL. Identification of a Mycobacterium tuberculosis gene that enhances mycobacterial survival in macrophages. *J Bacteriol*. 2000 Jan;182(2):377-84. doi: 10.1128/jb.182.2.377-384.2000.

216. Duan L, Yi M, Chen J, Li S, Chen W. *Mycobacterium tuberculosis* EIS gene inhibits macrophage autophagy through up-regulation of IL-10 by increasing the acetylation of histone H3. *Biochem Biophys Res Commun*. 2016;473(4):1229-1234.

217. Pan Q, Zhao F-L, Ye B-C. Eis, a novel family of arylalkylamine *N*-acetyltransferase (EC 2.3.1.87). *Sci Rep*. 2018;8:2435.

218. Ghosh S, Padmanabhan B, Anand C, Nagaraja V. Lysine acetylation of the *Mycobacterium tuberculosis* HU protein modulates its DNA binding and genome organization. *Mol Microbiol*. 2016;100(4):577-88.

219. Green KD, Chen W, Garneau-Tsodikova S. Identification and characterization of inhibitors of the aminoglycoside resistance acetyltransferase Eis from Mycobacterium tuberculosis. *ChemMedChem*. 2012 Jan 2;7(1):73-7. doi: 10.1002/cmdc.201100332.

220. Houghton JL, Biswas T, Chen W, Tsodikov OV, Garneau-Tsodikova S. Chemical and structural insights into the regioversatility of the aminoglycoside acetyltransferase Eis. *Chembiochem*. 2013 Nov 4;14(16):2127-35. doi: 10.1002/cbic.201300359.

221. Chen W, Green KD, Garneau-Tsodikova S. Cosubstrate tolerance of the aminoglycoside resistance enzyme Eis from Mycobacterium tuberculosis. *Antimicrob Agents Chemother*. 2012 Nov;56(11):5831-8. doi: 10.1128/AAC.00932-12.

222. Houghton JL, Green KD, Pricer RE, Mayhoub AS, Garneau-Tsodikova S. Unexpected N-acetylation of capreomycin by mycobacterial Eis enzymes. *J Antimicrob Chemother*. 2013 Apr;68(4):800-5. doi: 10.1093/jac/dks497.

223. Reeves AZ, Campbell PJ, Sultana R, et al. Aminoglycoside cross-resistance in Mycobacterium tuberculosis due to mutations in the 5' untranslated region of whiB7. *Antimicrob Agents Chemother*. 2013;57(4):1857-1865. doi:10.1128/AAC.02191-12.

224. Pan Q, Zhao FL, Ye BC. Eis, a novel family of arylalkylamine N-acetyltransferase (EC 2.3.1.87). *Sci Rep*. 2018;8(1):2435. Published 2018 Feb 5. doi:10.1038/s41598-018-20802-6.

225. Chakravorty S, Lee JS, Cho EJ, Roh SS, Smith LE, Lee J, Kim CT, Via LE, Cho SN, Barry CE 3rd, Alland D. Genotypic susceptibility testing of Mycobacterium tuberculosis isolates for amikacin and kanamycin resistance by use of a rapid sloppy molecular beacon-based assay identifies more cases of low-level drug resistance than phenotypic Lowenstein-Jensen testing. *J Clin Microbiol*. 2015 Jan;53(1):43-51. doi: 10.1128/JCM.02059-14.

226. Pholwat S, Stroup S, Heysell S, Ogarkov O, Zhdanova S, Ramakrishnan G, Houpt E. eis Promoter C14G and C15G Mutations Do Not Confer Kanamycin Resistance in Mycobacterium tuberculosis. *Antimicrob Agents Chemother*. 2016 Nov 21;60(12):7522-7523. doi: 10.1128/AAC.01775-16.

227. Kambli P, Ajbani K, Nikam C, Sadani M, Shetty A, Udwadia Z, Georghiou SB, Rodwell TC, Catanzaro A, Rodrigues C. Correlating rrs and eis promoter mutations in clinical isolates of Mycobacterium tuberculosis with phenotypic susceptibility levels to the second-line injectables. *Int J Mycobacteriol*. 2016 Mar;5(1):1-6. doi: 10.1016/j.ijmyco.2015.09.001.

228. Wu S, Barnes PF, Samten B, Pang X, Rodrigue S, Ghanny S, Soteropoulos P, Gaudreau L, Howard ST. Activation of the eis gene in a W-Beijing strain of Mycobacterium tuberculosis correlates with increased SigA levels and enhanced intracellular growth. *Microbiology*. 2009 Apr;155(Pt 4):1272-1281. doi: 10.1099/mic.0.024638-0.

229. Collins DM, Kawakami RP, de Lisle GW, Pascopella L, Bloom BR, Jacobs WR Jr. Mutation of the principal sigma factor causes loss of virulence in a strain of the Mycobacterium tuberculosis complex. *Proc Natl Acad Sci U S A*. 1995 Aug 15;92(17):8036-40. doi: 10.1073/pnas.92.17.8036.

230. Steyn AJ, Collins DM, Hondalus MK, Jacobs WR Jr, Kawakami RP, Bloom BR. Mycobacterium tuberculosis WhiB3 interacts with RpoV to affect host survival but is dispensable for in vivo growth. *Proc Natl Acad Sci U S A.* 2002 Mar 5;99(5):3147-52. doi: 10.1073/pnas.052705399.

231. Roberts EA, Clark A, McBeth S, Friedman RL. Molecular characterization of the eis promoter of Mycobacterium tuberculosis. *J Bacteriol*. 2004 Aug;186(16):5410-7. doi: 10.1128/JB.186.16.5410-5417.2004.

232. Primm TP, Andersen SJ, Mizrahi V, Avarbock D, Rubin H, Barry CE 3rd. The stringent response of Mycobacterium tuberculosis is required for long-term survival. *J Bacteriol*. 2000 Sep;182(17):4889-98. doi: 10.1128/jb.182.17.4889-4898.2000.

233. Morris RP, Nguyen L, Gatfield J, Visconti K, Nguyen K, Schnappinger D, Ehrt S, Liu Y, Heifets L, Pieters J, Schoolnik G, Thompson CJ. Ancestral antibiotic resistance in Mycobacterium tuberculosis. *Proc Natl Acad Sci U S A.* 2005 Aug 23;102(34):12200-5. doi: 10.1073/pnas.0505446102.

234. Sowajassatakul A, Prammananan T, Chaiprasert A, Phunpruch S. Overexpression of eis without a mutation in promoter region of amikacin- and kanamycin-resistant Mycobacterium tuberculosis clinical strain. *Ann Clin Microbiol Antimicrob*. 2018 Jul 16;17(1):33. doi: 10.1186/s12941-018-0285-6.

235. Samuel LP, Song C-H, Wei J, Roberts EA, Dahl JL, Barry CE, Jo E-K, Friedman RL. Expression, production and release of the Eis protein by *Mycobacterium tuberculosis* during infection of macrophages and its effect on cytokine secretion. *Microbiology*. 2007;153(Pt2):529-540.

236. Flynn JL, Goldstein MM, Chan J, Triebold KJ, Pfeffer K, Lowenstein CJ, Schreiber R, Mak TW, Bloom BR. Tumor necrosis factor-alpha is required in the protective immune response against Mycobacterium tuberculosis in mice. *Immunity*. 1995;2(6):561-72.

237. Gong JH, Zhang M, Modlin RL, et al. Interleukin-10 downregulates Mycobacterium tuberculosis-induced Th1 responses and CTLA-4 expression. *Infect Immun*. 1996;64(3):913-918. doi:10.1128/iai.64.3.913-918.1996.

238. Shin DM, Jeon BY, Lee HM, Jin HS, Yuk JM, Song CH, Lee SH, Lee ZW, Cho SN, Kim JM, Friedman RL, Jo EK. *Mycobacterium tuberculosis* eis regulates autophagy, inflammation, and cell death through redox-dependent signaling. *PLoS Pathog*. 2010 Dec 16;6(12):e1001230. doi: 10.1371/journal.ppat.1001230.

239. Lella RK & Sharma C. Eis (Enhanced Intracellular Survival) protein of *Mycobacterium tuberculosis* disturbs the cross regulation of T-cells. *J Biol Chem*. 2007;282(26):18671-5.

240. Kim KH, An DR, Song J, Yoon JY, Kim HS, Yoon HJ, Im HN, Kim J, Kim DJ, Lee SJ, Kim KH, Lee HM, Kim HJ, Jo EK, Lee JY, Suh SW. Mycobacterium tuberculosis Eis protein initiates suppression of host immune responses by acetylation of DUSP16/MKP-7. *Proc Natl Acad Sci U S A*. 2012 May 15;109(20):7729-34. doi: 10.1073/pnas.1120251109.

241. Reizer J, Michotey V, Reizer A, Saier MH Jr. Novel phosphotransferase system genes revealed by bacterial genome analysis: unique, putative fructose- and glucoside-specific systems. *Protein Sci*. 1994;3(3):440–450. doi:10.1002/pro.5560030309.

242. Davis JJ, Wattam AR, Aziz RK, Brettin T, Butler R, Butler RM, Chlenski P, Conrad N, Dickerman A, Dietrich EM, Gabbard JL, Gerdes S, Guard A, Kenyon RW, Machi D, Mao C, Murphy-Olson D, Nguyen M, Nordberg EK, Olsen GJ, Olson RD, Overbeek JC, Overbeek R, Parrello B, Pusch GD, Shukla M, Thomas C, VanOeffelen M, Vonstein V, Warren AS, Xia F, Xie D, Yoo H, Stevens R. The PATRIC Bioinformatics Resource Center: expanding data and analysis capabilities. *Nucleic Acids Res*. 2020 Jan 8;48(D1):D606-D612. doi: 10.1093/nar/gkz943.

243. Vetting MW, Magnet S, Nieves E, Roderick SL, Blanchard JS. A bacterial acetyltransferase capable of regioselective *N*-acetylation of antibiotics and histones. *Chem Biol*. 2004;11:565–573.

244. He H, Ding Y, Bartlam M, *et al*. Crystal structure of tabtoxin resistance protein complexed with acetyl coenzyme A reveals the mechanism for beta-lactam acetylation. *J Mol Biol*. 2003;325(5):1019-30.

245. Salipante SJ & Hall BG. Determining the Limits of the Evolutionary Potential of an Antibiotic Resistance Gene. *Mol Biol Evol*. 2003;20(4):653-659.

246. Kröger C, Colgan A, Srikumar S, *et al*. An Infection-Relevant Transcriptomic Compendium for *Salmonella enterica* Serovar Typhimurium. *Cell Host Microbe*. 2013;14(6):683-695.

247. Srikumar S, Kröger C, Hébrard M, *et al*. RNA-seq Brings New Insights to the Intra-Macrophage Transcriptome of *Salmonella* Typhimurium. *PLoS Pathog*. 2015;11(11):e1005262.

248. Mingeot-Leclercq MP, Glupczynski Y, Tulkens PM. Aminoglycosides: activity and resistance. *Antimicrob Agents Chemother*. 1999;43(4):727–737.

249. Damper PD, Epstein W. Role of the membrane potential in bacterial resistance to aminoglycoside antibiotics. *Antimicrob Agents Chemother*. 1981;20(6):803–808.

250. Xiong YQ, Caillon J, Drugeon H, Potel G, Baron D. Influence of pH on adaptive resistance of Pseudomonas aeruginosa to aminoglycosides and their postantibiotic effects. *Antimicrob Agents Chemother*. 1996;40(1):35–39.

251. Bishop AL, Baker S, Jenks S, *et al*. Analysis of the hypervariable region of the *Salmonella* enterica genome associated with tRNA(leuX). *J Bacteriol*. 2005;187(7):2469-82.

252. Shimada T, Yamazaki Y, Tanaka K, & Ishihama A. The Whole Set of Constitutive Promoters Recognized by RNA Polymerase RpoD Holoenzyme of *Escherichia coli*. *PLoS One*. 2013;9(3): e90447.

253. Curran TD, Abacha F, Hibberd SP, Rolfe MD, Lacey MM, Green J. Identification of new members of the Escherichia coli K-12 MG1655 SlyA regulon. *Microbiology*. 2017;163(3):400-409.

254. Kawano M, Reynolds AA, Miranda-Rios J, Storz G. Detection of 5'- and 3'-UTR-derived small RNAs and cis-encoded antisense RNAs in Escherichia coli. *Nucleic Acids Res*. 2005;33(3):1040-50.

255. Raghavan R, Groisman EA, Ochman H. Genome-wide detection of novel regulatory RNAs in *E. coli*. *Genome Res*. 2011;21(9):1487-97.

256. Chaudhuri RR, Morgan E, Peters SE, *et al*. Comprehensive assignment of roles for *Salmonella* typhimurium genes in intestinal colonization of food-producing animals. *PLoS Genet*. 2013;9(4):e1003456.

257. Beuzón CR, Méresse S, Unsworth KE, *et al*. Salmonella maintains the integrity of its intracellular vacuole through the action of SifA [published correction appears in EMBO J 2000 Aug 1;19(15):4191]. *EMBO J*. 2000;19(13):3235-3249. doi:10.1093/emboj/19.13.3235.

258. Knodler LA, Vallance BA, Celli J, et al. Dissemination of invasive Salmonella via bacterial-induced extrusion of mucosal epithelia. *Proc Natl Acad Sci U S A*. 2010;107(41):17733-17738. doi:10.1073/pnas.1006098107.

259. Stojiljkovic I, Bäumler AJ, Heffron F. Ethanolamine utilization in Salmonella typhimurium: nucleotide sequence, protein expression, and mutational analysis of the cchA cchB eutE eutJ eutG eutH gene cluster. *J Bacteriol*. 1995;177(5):1357-1366. doi:10.1128/jb.177.5.1357-1366.1995.

260. Datsenko KA, Wanner BL. One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. *Proc Natl Acad Sci U S A*. 2000;97(12):6640-6645. doi:10.1073/pnas.120163297.

261. Wang RF & Kushner SR. Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in Escherichia coli. *Gene*. 1991;100:195-9.

262. Cherepanov PP & Wackernagel W. Gene disruption in Escherichia coli: TcR and KmR cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. *Gene*. 1995;158(1):9-14.

263. Ellermeier CD, Janakiraman A, Slauch JM. Construction of targeted single copy lac fusions using lambda Red and FLP-mediated site-specific recombination in bacteria. *Gene*. 2002;290(1-2):153-61.

264. Drecktrah D, Levine-Wilkinson S, Dam T, et al. Dynamic behavior of Salmonella-induced membrane tubules in epithelial cells. *Traffic*. 2008;9(12):2117-2129. doi:10.1111/j.1600-0854.2008.00830.x.

265. Steele-Mortimer O. Infection of epithelial cells with Salmonella enterica. *Methods Mol Biol*. 2008;431:201-11.

266. Lathrop SK, Cooper KG, Binder KA, et al. Salmonella Typhimurium Infection of Human Monocyte-Derived Macrophages. *Curr Protoc Microbiol*. 2018;50(1):e56. doi:10.1002/cpmc.56.

267. Schindelin J, Arganda-Carreras I, Frise E, et al. Fiji: an open-source platform for biological-image analysis. *Nat Methods*. 2012;9(7):676-682. Published 2012 Jun 28. doi:10.1038/nmeth.2019.

268. Steele-Mortimer O, Méresse S, Gorvel JP, Toh BH, Finlay BB. Biogenesis of Salmonella typhimurium-containing vacuoles in epithelial cells involves interactions with the early endocytic pathway. *Cell Microbiol*. 1999;1(1):33-49.

269. Buchmeier N, Bossie S, Chen CY, Fang FC, Guiney DG, Libby SJ. SlyA, a transcriptional regulator of Salmonella typhimurium, is required for resistance to oxidative stress and is expressed in the intracellular environment of macrophages. *Infect Immun*. 1997;65(9):3725-3730. doi:10.1128/iai.65.9.3725-3730.1997.

270. Wu J, Pugh R, Laughlin RC, et al. High-throughput assay to phenotype Salmonella enterica Typhimurium association, invasion, and replication in macrophages. *J Vis Exp*. 2014;(90):e51759. Published 2014 Aug 11. doi:10.3791/51759.