POSSIBLE GENETIC DETERMINANTS OF GENTAMICIN RESISTANCE IN *LISTERIA MONOCYTOGENES*

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POSSIBLE GENETIC DETERMINANTS OF GENTAMICIN RESISTANCE IN *LISTERIA MONOCYTOGENES*

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ABSTRACT

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JAMIE NG MAY LING

Listeria monocytogenes is a Gram-positive foodborne pathogen capable of causing a foodborne infection known as listeriosis. There are two main types of listeriosis: non-invasive and invasive form which is often associated with a high mortality and hospitalisation rate among susceptible individuals. Gentamicin, used as an adjunct therapy with ampicillin, remains the treatment of choice for the life-threatening and invasive listeriosis. Nevertheless, there is little data on gentamicin resistance determinants in *L. monocytogenes.* The main objective of the study was to identify possible genetic determinants of gentamicin resistance in *L. monocytogenes*. In this study, a gentamicin-resistant mutant, B2b, was derived from *L. monocytogenes* ATCC 19115 by using the Luria-Delbrück experiment to determine the target of resistance in *L. monocytogenes*. Whole-genome sequencing was carried out to identify the mutation site of resistance. The mutant was also characterised using antimicrobial susceptibility testing and PCR. The gentamicin resistance in B2b was caused by a 10-bp deletion in *atpG2* which encodes a gamma subunit of the ATP synthase in *L. monocytogenes*. For biological validation by using reverse genetics, complementation and allelic exchange mutagenesis were carried out. Complementation of B2b with the wild-type *atpG2* reverted the resistant phenotype back to its sensitive state. When the same mutation was introduced into the wild-type ATCC 19115 via allelic

exchange, the development of gentamicin resistance was observed. The ATP level of B2b was significantly lower than the wild-type ATCC 19115, suggesting that the ATP production in B2b was potentially hampered by the *atpG2* mutation. Using *atpG2* PCR, various other mutations were identified in most of the gentamicin resistant mutants derived from ATCC 19115, indicating that *atpG2* mutations could be a major driving force of gentamicin resistance in *L. monocytogenes*. In addition, the mutation from B2b, when introduced into *L. ivanovii*, also caused gentamicin resistance in this *Listeria* species. In conclusion, *atpG2* mutations appear to be important determinants of gentamicin resistance not only in *L. monocytogenes* but possibly also in other *Listeria* species. These mutations could be a cause of treatment failure in *Listeria* infections treated with gentamicin. A better understanding of resistance mechanisms in *L. monocytogenes* is essential for the clinical management of potentially life-threatening foodborne infections caused by this organism. By adding new gene targets to routine molecular drug susceptibility tests, it will be possible to quickly identify strains that are resistant to gentamicin and choose the best course of treatment. Through the development of new drugs or drug combinations based on resistance mechanisms, it also can help to curb the global spread of gentamicin resistance.

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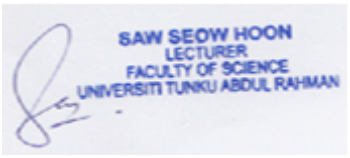

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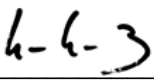

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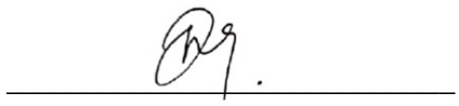
DECLARATION

I JAMIE NG MAY LING hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.



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LIST OF ABBREVIATIONS

AME	Aminoglycoside-modifying enzyme
AMR	Antimicrobial resistance
API	Analytical Profile Index
ATc	Anhydrotetracycline
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BC	Benzalkonium chloride
BHI	Brain-heart-infusion
CFU	Colony-forming unit
DNA	Deoxyribonucleic acid
Ery	Erythromycin
ESBL	Extended-spectrum beta-lactamase
EUCAST	European Committee on Antimicrobial Susceptibility Testing
gDNA	Genomic DNA
GM	Gentamicin
h	hour
IV	Intravenous
MAMA	Mismatch amplification mutation assay

MLVA	Multi locus variable number of tandem repeat analysis
MDR	Multidrug resistance
MHII	Mueller-Hinton II
MIC	Minimum inhibitory concentration
min	minute
mRNA	Messenger RNA
MU	million units
NTC	No-template control
PCR	Polymerase chain reaction
q	every (Latin: quaque)
RE	Restriction enzyme
RMTases	Ribosomal RNA methyltransferase
RND	Resistance-nodulation-cell division
rRNA	Ribosomal RNA
RTE	Ready-to-eat
SNP/InDel	Single nucleotide polymorphism/Insertion or Deletion
UV	Ultraviolet
VNTR	Variable-number-tandem-repeat
WGS	Whole-genome sequencing
wt	Wild type

CHAPTER 1

INTRODUCTION

1.1 Background

One of the biggest risks to public health in the 21st century is the emergence of bacterial antimicrobial resistance (AMR), which happens when changes in bacteria lead to a reduction in the efficacy of antimicrobial agents used for treatment or disinfection. In the past few decades, the number of multidrug resistant (MDR) pathogens has been rising due to the prolonged usage and improper handling of antimicrobials. Antimicrobial-resistant organisms could be transmitted through close contacts, environments, or food chains (Olaimat *et al.*, 2018). The spread of antimicrobial resistance among foodborne pathogens is a major public health concern especially for antibiotics that are commonly used in the treatment of foodborne illnesses.

Listeria monocytogenes is a foodborne pathogen capable of causing a foodborne infection known as listeriosis. Serious complications of invasive listeriosis in susceptible individuals, such as the newborn, elderly and immunocompromised patients, include septicaemia, meningitis, meningoencephalitis and in particular, perinatal infections which may result in abortion and stillbirth (Buchanan *et al.*, 2017). Listeriosis also contributes to an alarming mortality and hospitalisation rate of up to 30% and 92%, respectively, notably higher as compared to other

common foodborne illnesses (Swaminathan and Gerner-Smidt, 2007; Scallan *et al.*, 2011). Therefore, an effective antibiotic treatment is crucial to improve the outcome of this listerial infection.

The primary antibiotic for the treatment of listeriosis often involves the administration of a beta-lactam (e.g. ampicillin) alone or in conjunction with gentamicin (an aminoglycoside). However, most of the *L. monocytogenes* strains are tolerant to ampicillin. Due to the weak bactericidal activity of ampicillin, the killing of this bacteria can only take place at very high drug concentrations (32 times above the normal minimum inhibitory concentration [MIC]) (Hof, 2004). Generally, this shortcoming can be compensated by adding an aminoglycoside into the regimen (Hof, 2004). Various studies have successfully demonstrated the use of the combined ampicillin and gentamicin therapy to treat listeriosis (Moellering *et al.*, 1972; Scheld, 1983; Crum, 2002; Hof, 2003; Castellazzi, Marchisio and Bosis, 2018).

Gentamicin is one of the most widely used aminoglycosides in the treatment of life-threatening infections. This antibiotic acts by inhibiting the key steps in bacterial protein synthesis. It works in tandem with beta-lactams where the latter break down the bacterial cell wall and enable gentamicin to enter the bacterial cytoplasm and gain access to ribosomal targets. Once in the cytoplasm, gentamicin binds to the 16S rRNA of the 30S ribosomal subunit, interfering with the translation of mRNA and causing the formation of truncated or non-functional proteins which damage the membrane and other parts of the bacterial cell leading to rapid cell death (Beganovic *et al.*, 2018).

Resistance to aminoglycosides can be mediated by different mechanisms, including ribosomal modification through mutations and enzymatic actions, drug deactivation by aminoglycoside-modifying enzymes, decreased intracellular concentration of aminoglycosides as a result of alterations or modifications to the bacterial cell membrane, and active removal of aminoglycoside molecules from the bacterial cells via efflux pumps (Garneau-Tsodikova and Labby, 2016).

Mutations are one of the major pathways for the emergence of antibiotic resistance in bacteria (Woodford and Ellington, 2007). These mutations are frequently referred to as "target-gene mutations," in which the systems that the antibiotic targets are changed in a way that prevents the antibiotic from binding to its target, thus rendering the antibiotic less effective (Revitt-Mills and Robinson, 2020). In the laboratory, there are several approaches commonly used for the generation of mutants. The Luria-Delbrück experiment (Luria and Delbrück, 1943), originally designed to estimate bacterial mutation rates, is increasingly being employed in the modern era to select laboratory mutants and to study mechanisms of antibiotic resistance development (Ng *et al.*, 2018; Lee *et al.*, 2021) .

The isolation of antibiotic-resistant strains of *L. monocytogenes* from different sources, such as food, environment as well as human clinical samples, has increased in recent years, predominantly in antibiotics that are often used in the treatment of listeriosis (Olaimat *et al.*, 2018; Caruso *et al.*, 2020; Wiśniewski *et*

al., 2022). Nevertheless, resistance to gentamicin has rarely been documented. In this study, gentamicin-resistant mutants, derived *in vitro* using the Luria-Delbrück experiment from a previously susceptible strain, were selected and characterised to look for mutations associated with gentamicin resistance in *L. monocytogenes*. A better understanding of the resistance mechanism of gentamicin in *L. monocytogenes* would be helpful for the development of new drugs and diagnostic tools for the clinical management of potentially life-threatening foodborne infections caused by this organism.

1.2 Problem statements and hypothesis

Gentamicin resistance determinants in *L. monocytogenes* are not well-elucidated. In this study, it was hypothesized that novel resistance determinants may be involved in the emergence of gentamicin resistance in *L. monocytogenes*.

1.3 Objectives

The main aim of this study was to identify possible gentamicin resistance determinants in *L. monocytogenes*.

The specific objectives of this study were:

a) To decipher the mechanisms of gentamicin resistance in *L. monocytogenes* through the selection of gentamicin-resistant spontaneous mutants.

b) To identify the genetic determinants of gentamicin resistance in these mutants by sequencing analyses.

c) To validate the selected genetic determinant of gentamicin resistance in *L. monocytogenes* by reverse genetics.

d) To reconstruct the gentamicin resistance in another *Listeria* species.

CHAPTER 2

LITERATURE REVIEW

2.1 Foodborne pathogens

The global burden of foodborne diseases remains a significant problem in both developed as well as developing countries by affecting healthcare systems and contributing to the economic losses in sectors such as agriculture, tourism, food export and trade industries. Approximately, 600 million people, which is about 1 in every 10 people in the world, fell ill after consuming contaminated food with 420,000 deaths occurred annually (WHO, 2022). This alarming figure has resulted in an estimated loss of 33 million healthy life years. The South-East Asia (SEA) region holds the second highest number of foodborne diseases after the African region. Each year, more than 150 million cases and 175,000 deaths were reported in the SEA region (WHO, 2022). In Malaysia, the incidence of foodborne diseases is also on the rise, with 6,012 cases reported in 2016 as compared to 3,822 cases documented in 2010 and a mortality rate of 0.03 (Woh *et al.*, 2016). The high number of cases is partly attributed to the hot and humid climate, inadequate basic hygiene, and the consumption of raw food in traditional Malaysian cuisines, which are ideal conditions for the growth and transmission of foodborne pathogens (Abdul-Mutalib *et al.*, 2015; Ismail *et al.*, 2018).

Foodborne diseases are generally caused by the consumption of food contaminated with bacteria, viruses, fungi, parasites or toxins and chemical substances (Zhao *et al.*, 2014). In the United States, it is estimated that 31 major pathogens cause 9.4 million episodes of foodborne diseases annually, with viruses being the primary agents and bacterial infections often leading to hospitalizations and deaths (Scallan *et al.*, 2011). One of the most severe and life-threatening foodborne diseases is known as listeriosis. Invasive foodborne listeriosis is a very concerning bacterial infection which often leads to a high mortality rate (20-30%) among populations with underlying health conditions (Goulet *et al.*, 2012). In comparison, other common foodborne pathogens, such as *Salmonella* spp. and *Escherichia coli* O157:H7, have a much lower mortality rate of less than 1% (Scallan *et al.*, 2011). The high fatality rate caused by *L. monocytogenes* necessitates the need for a rapid and effective antibiotic treatment.

2.2 Genus *Listeria*

The genus *Listeria* consists of a group of Gram-positive, small rod-shaped, non-spore forming, and facultatively anaerobic bacteria of the family *Listeriaceae* (Orsi and Wiedmann, 2016). Generally, members of this genus are catalase-positive, oxidase-negative, and have a low GC genome content (<50%). They are commonly found to be motile at low temperatures (Luque-Sastre *et al.*, 2018). Other Gram-positive bacteria found to be phylogenetically related to *Listeria* include *Bacillus*, *Clostridium*, *Enterococcus*, *Streptococcus* and *Staphylococcus* (Feresu and Jones, 1988).

To date, there are up to 20 known species in the genus *Listeria* (Nwaiwu, 2020). These species can be further classified into two groups according to their phylogenetic relatedness with *L. monocytogenes*, the main pathogenic species of the genus. The two groups are known as the *"Listeria* sensu stricto" and "*Listeria* sensu lato" clades (Table 2.1) (Orsi and Wiedmann, 2016; Luque-Sastre *et al.*, 2018).

Table 2.1: Two groups of *Listeria* species.

Group	Species
Listeria sensu stricto	*L. monocytogenes, L. ivanovii, L. innocua, L. seeligeri, L. welshimeri, L. marthii*
Listeria sensu lato	*L. grayi, L. fleischmannii, L. floridensis, L. aquatica, L. newyorkensis, L. cornellensis, L. rocourtiae, L. weihenstephanensis, L. grandensis, L. riparia, L. booriae, L. costaricensis, L. goaensis, L. thailandensis*

Unlike the species in the "*Listeria* sensu lato" group, which are more commonly found and isolated from the natural environment or food-associated matrices, the "*Listeria* sensu stricto" group are able to colonise mammalian hosts and have been identified in the gastrointestinal tract of symptom-free animals, faeces and in food products of animal origin (Schardt *et al.*, 2017). Within the genus *Listeria*, two species are known to be pathogenic: *L. monocytogenes* and *L. ivanovii*. While *L. monocytogenes* is capable of causing diseases to both humans and animals, *L. ivanovii* primarily infects ruminants and less frequently in humans (Vázquez-Boland *et al.*, 2001).

2.2.1 *Listeria monocytogenes*

L. monocytogenes is an opportunistic foodborne pathogen which is widely distributed in nature. It can be found in a variety of environmental sources, such as soil, water, sewage, silage, vegetation, waste effluent and faeces of animals and humans (Freitag, Port and Miner, 2009). High risk foods which are prone to be contaminated by *L. monocytogenes* include ready-to-eat (RTE) vegetables, processed meat, uncooked poultry products, unpasteurized dairy products, smoked fish and raw seafood (Olaimat *et al.*, 2018). This pathogen, which is commonly found in the environment, is able to infiltrate the food chain and food-processing facilities (Buchanan *et al.*, 2017; Fharok, 2019; Chen *et al.*, 2021). As a result, governments and organisations responsible for ensuring the food safety in countries, such as the United States of America (USA), Austria, Australia, New Zealand, and Italy, have implemented a zero-tolerance policy for *L. monocytogenes* (i.e. absence of *L. monocytogenes* in a 25 g food sample) (Obaidat *et al.*, 2015).

The prevalence of this resilient organism in the environment is aided by its ability to adapt and withstand a wide range of external stresses. It can survive and grow at a temperature ranging from 0.5 ˚C to 45 ˚C, with an optimum temperature range between 30 to 37 ˚C (Low and Donachie, 1997). This is of particular concern, especially to the food industry, since it can replicate in refrigerated conditions and survive for long periods of time in frozen food products (Ramaswamy *et al.*, 2007). It can also tolerate a wide range of pH (pH 4.3 to 9.6) and high concentrations of salt (up to 20% w/v NaCl) (Zunabovic,

Domig and Kneifel, 2011). Another characteristic of *L. monocytogenes* is its ability to form biofilms on various contact surfaces, including stainless steel and plastic (Bremer, Monk and Osborne, 2001; Gandhi and Chikindas, 2007). The biofilms may pose a significant threat to public health as they are found to be more resistant to disinfectants and sanitizers than free-living bacterial cells (Lewis, 2001), causing their removal a major challenge.

Apart from its versatility in adapting to a broad range of extreme environmental conditions, *L. monocytogenes* is also a facultatively intracellular pathogen that can invade, survive and replicate within the host cells. After the intake of food contaminated with *L. monocytogenes*, the bacteria will colonise the gastrointestinal cells and attach to the surface receptors to translocate through the intestinal membrane via endocytosis. The presence of *L. monocytogenes* will then trigger the host defense mechanism, in which phagocytic cells (e.g. macrophages) will engulf the bacteria into their vacuoles. The bacteria mediate their escape from the membrane-bound vacuole by secreting listeriolysin O, a virulence factor encoded by the *hly*A gene, to degrade the vacuole in which they are entrapped. The bacteria then enter the host cytoplasm and rapidly divide and spread to adjacent cells by using the actin polymerization as a motility force (Tilney and Portnoy, 1989). Through this series of steps, this foodborne pathogen establishes an infection in humans with a combination of symptoms known as listeriosis.

2.3 Listeriosis

L. monocytogenes is known as the main causative agent of listeriosis, a foodborne disease that is mainly acquired through the consumption of food contaminated by infected animals or the environment (Hilliard *et al.*, 2018). The number of listeriosis cases varies among different countries and regions of the world with a rate of 0.1 - 10 cases per million people (WHO, 2018). Although relatively rare, the disease carries severe consequences for pregnant women, newborns, elderly people and immune-compromised individuals (Buchanan *et al.*, 2017). Infections in these groups are often associated with hospitalization and mortality rates of more than 92% and 20 to 30%, respectively, which are prominently higher than those foodborne diseases caused by other bacteria (Scallan *et al.*, 2011; Altuntas *et al.*, 2012; Du *et al.*, 2017).

Human listeriosis can manifest as a non-invasive or invasive form of the disease. Non-invasive infection is a mild febrile form of gastroenteritis which mainly affects healthy individuals. The usual symptoms include fever, vomiting, diarrhoea, abdominal pain, fatigue and myalgia. In most of these healthy individuals, this infection is self-limiting (Dalton *et al.*, 1997). This non-invasive infection normally lasts for 9 to 32 h after the consumption of tainted food with *L. monocytogenes* (Olaimat *et al.*, 2018). Invasive listeriosis is a more severe form of the disease in which infection usually spreads to the circulatory system and central nervous system (CNS) of susceptible individuals, resulting in septicaemia, meningitis or meningoencephalitis (Reda *et al.*, 2016). Cerebral listerial infections, such as rhombencephalitis, brain abscess, meningitis and

meningoencephalitis, are more commonly seen in elderly patients (>50 years old) (Brouwer *et al.*, 2006).

Pregnant women are about 20 times more likely to contract listeriosis than the general population (Southwick and Purich, 1996). During pregnancy, the hormonal changes in a pregnant woman such as the heightened production of the hormone progesterone reduced the overall body's immune system. The weakened defense system increases the risk of infections and illnesses during pregnancy. The pathogen, *Listeria* took advantage of this and invade the pregnant host causing invasive listeriosis infection (NSW, 2014). Infections during pregnancy can result in complications such as pre-term delivery, miscarriage, stillbirth, or neonatal infection (Mylonakis *et al.*, 2002). Neonatal infection can be acquired transplacentally or during passage in the birth canal. Clinical presentations of early onset neonatal listeriosis include bacteraemia, meningitis (usually a late-onset infection) and pneumonia (Jackson, Iwamoto and Swerdlow, 2010).

The diagnosis of invasive listerial infection is made based on clinical symptoms and by culturing the pathogen from a sterile site like blood, spinal fluid or amniotic fluid (Janakiraman, 2008). Stool or vaginal cultures were found to be less helpful in diagnosis as some women are just asymptomatic carriers (Southwick and Purich, 1996). The bacteria have been detected or isolated from the cervix, amniotic fluid, and placenta of pregnant women (Olaimat *et al.*, 2018). Gram stain is only useful in one third of the listerial infection cases since it is less sensitive in detecting intracellular organisms, such as *Listeria* (Silver, 1998).

The morphology of this organism also resembles that of other Gram-positive pathogens, such as the diplococcal shape of pneumococci and the diphtheroid shape of corynebacteria, and is therefore, easily misinterpreted (Janakiraman, 2008). As a result, direct microscopy cannot be used alone for diagnosis; it must be combined with microscopic and culture techniques, as well as biochemical, serological, or molecular methods to identify the isolate. Various other detection methods, which are more sensitive and rapid than culture, such as polymerase chain reaction (PCR) and real-time PCR, are also available for the diagnosis of listeriosis in humans.

Most of the listeriosis cases are sporadic with occasional large multi-state outbreaks that lead to hospitalisation and death. One of the largest reported outbreaks was in South Africa which took place in 2017-2019 and resulted in over 200 deaths and more than 1000 laboratory-confirmed positive cases. The neighbouring Sub-Saharan African countries were also affected by the outbreak which was due to meat products contaminated with *L. monocytogenes* imported from South Africa (Allam *et al.*, 2018; Smith *et al.*, 2019). Another notable listeriosis outbreak was linked to the consumption of rock melons (cantaloupe) from a farm in Australia. This outbreak resulted in 22 confirmed cases, 1 miscarriage and 7 deaths. Through whole-genome sequencing, the isolates from patients were linked to those 37 rock melons from the farm and its processing and packaging areas. A worldwide product recall was carried out because those contaminated batches of rock melons were also distributed internationally to eight other countries including Malaysia (Desai *et al.*, 2019). Although there have not been any reported outbreaks of foodborne listeriosis in Malaysia,

several prevalence studies have demonstrated the presence of *L. monocytogenes* in local foods (Jamali, Chai and Thong, 2013; Kuan *et al.*, 2017; Fharok, 2019; Wai *et al.*, 2020), indicating the potential spread and circulation of this foodborne pathogen in Malaysia.

2.4 Antibiotic treatments for listeriosis

Invasive *Listeria* infections are often associated with a high fatality rate and the general severity of invasive listeriosis warrants an immediate need of antibiotic treatments to control the disease. The most common and preferred antibiotic treatment for severe listeriosis is with a beta-lactam (ampicillin or penicillin) alone or in combination with an aminoglycoside (gentamicin) (Table 2.2). In general, listerial isolates are tolerant to beta-lactam antibiotics, with killing achieved only at extremely high concentrations and after a prolonged exposure to the drugs (Winslow *et al.*, 1983; Hof, 2004). Studies have shown that the addition of gentamicin to the beta-lactam treatment has a synergistic effect and is beneficial especially to higher-risk patients with listerial CNS infections and endocarditis (Mylonakis, Hohmann and Calderwood, 1998; Crum, 2002; Hof, 2004; Castellazzi, Marchisio and Bosis, 2018). The combination of these two antibiotics enables the bacterial cell wall to be broken down by ampicillin followed by the penetration of gentamicin, which is a strong bactericidal drug, into the cytoplasm of the bacteria (Hof, 2004; Beganovic *et al.*, 2018). The incorporation of gentamicin into the treatment regimen for listeriosis, however, had also been questioned by some authors. Some animal model studies had shown conflicting results on the effectiveness of

aminoglycosides for the treatment of listeriosis, as these antibiotics are unable to pass the blood-brain barrier (Temple and Nahata, 2000; Crum, 2002). Apart from that, due to the potential nephrotoxicity of gentamicin, this antibiotic has to be removed after 1 to 2 weeks of treatment, especially in elderly patients and patients treated alongside with other nephrotoxic drugs, such as cyclosporin A (Hof, 2004).

Table 2.2: Antibiotics treatment for different types of *Listeria* infections*.

Types of infection	Antibiotics treatment	Remarks
Meningitis	Ampicillin 2 gm IV q4-6h (or penicillin G 4 MU IV q4h) + gentamicin 1.7 mg/kg IV q8h x ≥ 3 weeks Alternatives: • TMP/SMX 3-5 mg/kg (trimethoprim) q6h IV x ≥ 3 weeks • Meropenem 2 g IV q8	Preferred for patients with normal renal function. Administration of gentamicin requires close monitoring of the renal function and may be stopped after 1-2 weeks when the condition of the patient improve significantly and/or the renal function starts to deteriorate.
Bacteremia (without meningitis)	Ampicillin 2 gm IV q4-6h (or penicillin G 4 MU IV q4h) + gentamicin 1.7 mg/kg IV q8h x 2 weeks	
Brain abscess, rhomboencephalitis or cerebritis	Ampicillin 2 gm IV q4-6h (or penicillin G 4 MU IV q4h) + gentamicin 1.7 mg/kg IV q8h x 4-6 weeks or longer	
Gastroenteritis	Antibiotic treatments are not required due to symptoms are mild and self-limiting. However, if diagnosed in susceptible patients, amoxicillin or TMP/SMX x 7d may be used.	

TMP/SMX: trimethoprim/sulfamethoxazole
*Adopted from (Shoham and Bartlett, 2018)

For the treatment of listerial meningitis in patients with beta-lactam allergies, trimethoprim in combination with sulfamethoxazole or meropenem alone is often recommended (Tunkel *et al.*, 2004). Other antibiotics that are also used to treat listeriosis include vancomycin, erythromycin, chloramphenicol, tetracycline, rifampicin and fluoroquinolones (Olaimat *et al.*, 2018). Non-meningeal infections are sometimes treated with vancomycin, while erythromycin is used for listeriosis during pregnancy (Alonso-Hernando *et al.*, 2012). In contrast, cephalosporins, such as ceftriaxone or cefotaxime, which are widely used in the empirical therapy for bacterial meningitis, are not effective against *L. monocytogenes*. Most of the *Listeria* isolates are naturally resistant to cephalosporins due to the limited number of proper penicillin binding proteins (PBP) available in their cytoplasmic membrane. Among the five PBPs found in the membrane of the bacterial cell, PBP3 is involved in the final step of peptidoglycan synthesis and the inhibition of this protein has lethal outcomes to the organism. Cephalosporins, unlike beta-lactams, penicillin and ampicillin, do not bind to the essential PBP3 with high affinity (Vicente *et al.*, 1990).

Development of febrile listerial gastroenteritis (mild listeriosis) among healthy individuals after the consumption of contaminated food will generally resolve within 2 days and at times even before the identification of the pathogen and source of infection. Therefore, these patients seldom require or receive antimicrobial treatments. The progression from gastroenteritis to a more severe invasive listeriosis is not common. The risk of developing invasive listeriosis, however, increases in elderly, pregnant, neonatal and immunocompromised patients. In this high-risk group of patients, treatment with amoxicillin or

TMP/SMX has been suggested (Ooi and Lorber, 2005). The antibiotic, TMP/SMX, has to be used with caution as it may result in serious side effects in pregnant women due to the interruption in the metabolism of folic acids. Therefore, it is only recommended to be used during early stages of pregnancy to reduce the risk of harmful effects to the foetus (Mardis, Conley and Kyle, 2012).

2.5 Aminoglycosides

Aminoglycosides are broad-spectrum antibiotics that are active against a wide range of Gram-negative and Gram-positive bacteria. They are one of the earliest clinically approved antibiotic classes and had been actively used in healthcare settings since the discovery of streptomycin in 1944. Over the years, many other members of aminoglycosides, such as neomycin, kanamycin, gentamicin, netilmicin, tobramycin, amikacin, arbekacin and plazomicin, were also introduced (Krause *et al.*, 2016). In general, the aminoglycosides consist of a main structure of amino sugars linked to a common dibasic aminocyclitol, 2-deoxystreptamine via glycosidic linkages (Mingeot-Leclercq, Glupczynski and Tulkens, 1999). They can be classified into four sub-groups of aminoglycosides based on the different aminocyclitol moieties (Table 2.3) (Magnet and Blanchard, 2005; Wachino and Arakawa, 2012). The chemical structure of representative antibiotic(s) from each sub-group is shown in Figure 2.1.

Table 2.3: Four sub-groups of aminoglycosides.

Type of sub-group	Example of antibiotic(s) in the sub-group
No deoxystreptamine	streptomycin*
A mono-substituted deoxystreptamine ring	apramycin
4,5-di-substituted deoxystreptamine ring	neomycin, ribostamycin
4,6-di-substituted deoxystreptamine ring	gentamicin, amikacin, kanamycin, tobramycin, and plazomicin

*Consists of a streptidine ring to which two or more amino-modified sugars are bound via glycosidic linkages

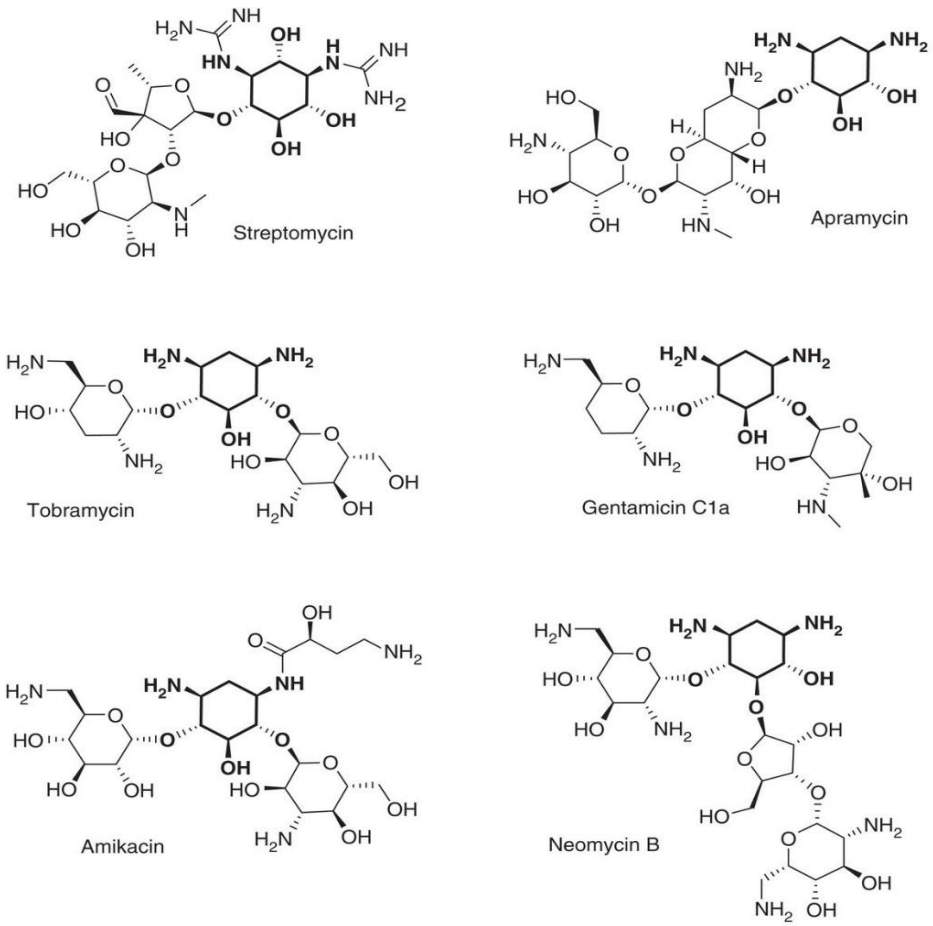

Figure 2.1: Chemical structures of the representative antibiotics from each sub-group of aminoglycosides. The streptidine or deoxystreptamine rings are drawn in bold. Adopted from (Krause *et al.*, 2016).

The main target of aminoglycosides is the bacterial small ribosomal subunit (30S) which is comprised of the 16S rRNA. The antibiotics of this class bind with high affinity to the 30S subunit and cause conformational changes in the tRNA acceptor aminoacyl-site (A-site) found in the 30S subunit to inhibit the protein synthesis. This action results in the misreading of the codon during the translocation step, permitting the incorrect amino acids to assemble into a mistranslated polypeptide that may cause damage to the membrane or other parts of the bacterial cell (Mingeot-Leclercq, Glupczynski and Tulkens, 1999; Kotra, Haddad and Mobashery, 2000; Ramirez and Tolmasky, 2010). Some of the members in this class of antibiotics can also interfere with protein synthesis (I) by inhibiting the elongation process or initiation pathway (Davis, 1987; Kotra, Haddad and Mobashery, 2000; Wilson, 2014). The binding mechanism and downstream effects vary among the different chemical structures of aminoglycosides. Nevertheless, all antibiotics of this class are known to exert rapid bactericidal activity (Davis, 1987; Mingeot-Leclercq, Glupczynski and Tulkens, 1999).

Apart from their use in monotherapy, aminoglycosides are also often used concomitantly with antibiotics from a different class in treatment for a broad range of infections (Avent *et al.*, 2011; Jackson, Chen and Buising, 2013). Combination therapy is usually indicated for the treatment of those patients with severe infections in order to reduce mortality and improve patient prognosis (Tamma, Cosgrove and Maragakis, 2012). Some of the benefits of using a mixture of antibiotics for treatment are (1) to widen the therapy coverage with two or more antimicrobial agents of different properties and mechanisms of

action to make sure that the aetiological agent is at least effectively covered by one active antibiotic in the regimen, (2) to improve the clinical outcome–by taking advantage of the synergistic effect observed *in vitro* between two different antimicrobial agents, or (3) to help delay the development of antibiotic resistance (Le *et al.*, 2011; Pankuch *et al.*, 2011). Clinically, aminoglycosides are frequently used in conjunction with beta-lactams for the treatment of sepsis and some other hospital infections with high fatality or for the empirical therapy when there are concerns that the etiological agent of the disease may be a multi-drug resistant pathogen (Dellinger *et al.*, 2013).

2.5.1 Gentamicin

Gentamicin is one of the few aminoglycosides that is synthesized naturally by *Micromonospora purpurea*, a Gram-positive bacterium found in the environment. Unlike the other aminoglycosides, such as streptomycin, kanamycin or neomycin, that are produced from the genus *Streptomyces*, gentamicin and other related antibiotics (verdamicin, netilmicin, mutamicin) have the names ending with 'micin' instead of 'mycin'. The change in the naming suffix is to differentiate their biological backgrounds from those antibiotics that were derived from *Streptomyces* (Serio *et al.*, 2018). Belonging to the family of aminoglycosides, the primary mechanism of action for gentamicin is the inhibition of bacterial protein synthesis. Unlike other antibiotics that possess the same mechanism of protein synthesis inhibition, such as tetracyclines, clindamycin, chloramphenicol, macrolides, that are bacteriostatic, the aminoglycosides (including gentamicin) are bactericidal

(Davis, 1987; Mingeot-Leclercq, Glupczynski and Tulkens, 1999; Serio *et al.*, 2018).

The entry of gentamicin into bacterial cells happen in three stages; the first step increases the permeability of the membrane of the cells while the subsequent second and third steps are energy-dependent processes in the cell cytoplasm. The polycationic gentamicin molecules will first attach themselves to the negatively charged bacterial cell membrane. This process takes place in the phospholipids and lipopolysaccharides (LPS) of Gram-negative bacteria and in the phospholipids and teichoic acids of Gram-positive bacteria. The binding between the antibiotic molecules and components in the bacterial cell membrane results in the displacement of magnesium ions (Davis, 1987). These ions are essential for the cross linkage and stabilization of the outer cell membrane structure. The removal of these ions disrupts the membrane of the bacterial cell, leading to an increase in the cell permeability and thus, initiating the gentamicin uptake pathway (Hancock, 1984; Ramirez and Tolmasky, 2010). The gentamicin molecules then travel into the cytoplasm of the bacterial cell via energy-dependent processes which are electron-transport mediated. Upon gaining access to the cytoplasm, the antibiotic molecules inhibit the protein synthesis and the resulting mistranslated proteins lead to the damage of cellular components such as the cell membrane (Davis, 1987). An enhanced permeability in the cell membrane further facilitates the uptake of gentamicin molecules into the bacterial cytoplasm, resulting in a rapid inhibition of protein synthesis, mistranslation and accelerate killing of the bacterial cells (Ramirez and Tolmasky, 2010).

Gentamicin is used to treat clinically severe bacterial infections, such as sepsis, urinary tract infections, endocarditis, meningitis and pneumonia, due to their rapid killing effect. Because of its poor absorption in the gastrointestinal tract, gentamicin is administered intravenously, intramuscularly or topically (Ramirez and Tolmasky, 2010). Despite its therapeutic usefulness, however, gentamicin is administered with caution as it is associated with side effects like ototoxicity and nephrotoxicity. Different strategies had been taken to reduce these aminoglycoside-induced toxicities which include shorter treatment duration and once-daily intravenous dosing (Avent *et al.*, 2011). The administration of once-daily dosing results in a higher peak concentration of the drug and a longer inter-dosing interval. This allows for a more rapid elimination of the pathogen and a longer time for the kidneys to recover in between doses. Although the serum concentration of the antibiotic declines during the dosing intervals, the post-antibiotic effect of the antibiotic ensures the continuous killing of the bacterial cells during the dosing intervals (Stankowicz, Ibrahim and Brown, 2015).

Gentamicin, one of the most popular antibiotics in the aminoglycoside family, is widely used due to its long history and effectiveness against multiple Gram-negative and some Gram-positive bacteria, including emerging MDR pathogens like carbapenem-resistant Enterobacteriaceae (CRE) (Gonzalez-Padilla *et al.*, 2015). Apart from human infections, gentamicin is also useful against zoonotic infections such as plague (caused by *Yersinia pestis*) and tularemia (caused by *Francisella tularensis*). Traditionally, streptomycin was used for the treatment of these infections. However, gentamicin is now the preferred drug due to its

broader availability and similar efficacy to streptomycin (Snowden and Stovall, 2011).

The mechanism of aminoglycoside uptake requires oxygen and an active electron transport system. Therefore, the use of aminoglycosides, including gentamicin, is not effective against obligate anaerobic organisms such as *Bacteroides fragilis* and *Clostridium perfringens*. Facultative anaerobes, grown under low oxygen conditions, were found to be less susceptible to these antibiotics (Bryan, Kowand and Van den Elzen, 1979). As a result, some facultative anaerobes transitioned into the anaerobic phase to evade aminoglycoside treatment. A study by Knudsen *et al.* (2016) demonstrated that when *L. monocytogenes* was exposed to the sublethal concentration of four different antibiotics (including gentamicin), a switch from aerobic to anaerobic mechanisms in the organism was observed to prevent the production of reactive oxygen species that may result in cell death. The switch to anaerobic metabolism was also shown to cause changes in the *Listeria* cell phenotypes linked to antibiotic tolerance at a higher lethal concentration.

The broad-spectrum activity of gentamicin is improved through the synergy with other antimicrobial drugs of different classes. These interactions show that the combined effect of two drugs is better than the sum of their individual effects. The added benefit of synergy when gentamicin is co-administered with a beta-lactam antibiotic is widely used for the treatment of some complex nosocomial infections, dose optimisation and reduction of adverse side effects of the drugs (Krause *et al.*, 2016). The synergism occurs when the beta-lactam drug causes

damage to the cell membrane of bacteria to allow a better diffusion of gentamicin into the bacterial cytoplasm. Pharmacodynamic synergism was also observed when the high serum concentration of gentamicin helped to reduce the bacterial load in the bloodstream so that the beta-lactam antibiotic could then work more effectively in removing the remaining bacterial cells. However, not all antibiotics have an increased activity when they are combined with gentamicin. Antagonism was observed when gentamicin was used together with antimicrobial agents such as tetracyclines, macrolides and chloramphenicol (D'Alessandri, McNeely and Kluge, 1976; Giguère, Prescott and Dowling, 2013).

2.6 Mechanisms of aminoglycosides resistance

Aminoglycoside resistance is mediated by different mechanisms, such as mutations and enzymatic modifications of the ribosome, deactivation of the drugs by aminoglycoside-modifying enzymes (AMEs), reduced intracellular concentration of aminoglycosides due to changes or modifications in the bacterial cell membrane and active removal of aminoglycoside molecules out of the bacterial cells via efflux pumps. These mechanisms of aminoglycoside resistance are depicted in Figure 2.2.

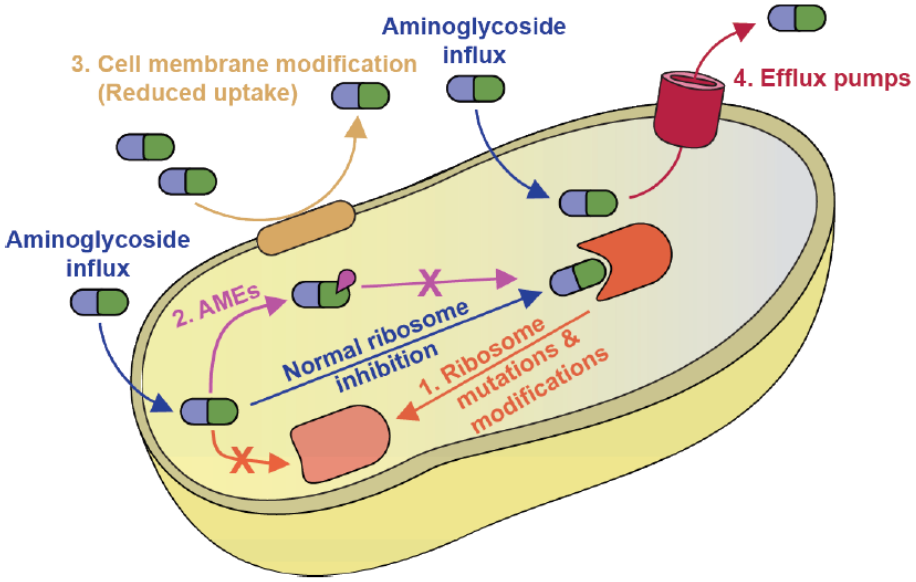

Figure 2.2: Different mechanisms contributing to the development of aminoglycoside resistance. Adopted from (Garneau-Tsodikova and Labby, 2016).

The conventional mode of antibiotic resistance usually occurs through changes in the key target of the antibiotic. Aminoglycosides inhibit protein synthesis by binding to the A-site located in the 16S rRNA of the 30S bacterial ribosomal subunit. This mechanism of action can be interrupted due to mutations or enzymatic modifications of the ribosome (Garneau-Tsodikova and Labby, 2016). The target-based mutations, however, are not commonly seen in aminoglycoside resistance because most bacterial species have several copies of rRNA encoding genes with the exception of *Mycobacterium* and *Borrelia* spp. These two bacterial genera only carry a single copy of the 16S rRNA gene or ribosomal operon which increases the likelihood of aminoglycoside resistance due to ribosomal mutations. Studies have shown that mutations in *rrs* and *rpsL* genes which encode the 16S rRNA and ribosomal protein S12 are causes of aminoglycoside resistance in *M. tuberculosis* clinical isolates (Springer *et al.*, 2001; Maus, Plikaytis and Shinnick, 2005). Similarly, the same mutations in the

ribosome were also observed in *B. burgdorferi* which conferred a high-level of resistance to spectinomycin and other aminoglycosides (Criswell *et al.*, 2006).

Apart from mutations, enzymatic modifications of the target site in the ribosomes can also confer aminoglycoside resistance. One notable example is the 16S rRNA methyltransferases (16S-RMTases) which add methyl groups to the specific rRNA nucleotide residues, thereby reducing the affinity of aminoglycosides for their ribosomal target. This phenomenon often results in a high-level and widespread aminoglycoside resistance (Wachino and Arakawa, 2012). RMTases are generally acquired by mobile genetic elements like plasmids containing the RMTases gene. The RMTases are divided into two general groups, classified based on the specific modification of nucleotide residues. The enzymes for methylation at the N7 position of nucleotide G1405 are more common, with RmtA being the first enzyme isolated from a *Pseudomonas aeruginosa* clinical strain in 1997 (Yokoyama *et al.*, 2003). This was followed by the discovery of more enzymes, including ArmA, RmtB1, RmtB2, RmtC, RmtD1, RmtD2, RmtE, RmtF, RmtG and RmtH (Krause *et al.*, 2016). These enzymes confer resistance to 4,6-disubstituted aminoglycosides such as gentamicin, amikacin, kanamycin and tobramycin. The second group of the 16S-RMTases focuses on the methylation at the N1 position of A1408. The enzyme in this group, NpmA, confers resistance to 4,6-disubstituted, 4,5-disubstituted (e.g. neomycin) and monosubstituted (e.g. apramycin) aminoglycosides (Mingeot-Leclercq, Glupczynski and Tulkens, 1999; Garneau-Tsodikova and Labby, 2016). However, NpmA is seldom found in clinical isolates.

The most widespread mechanism of resistance to aminoglycosides is the inactivation of the antibiotics by the AMEs. These enzymes, which modify the structure of aminoglycosides via enzymatic reactions, are divided into three groups: aminoglycoside acetyltransferases (AACs), aminoglycoside phosphotransferases (APHs) and aminoglycoside adenyltransferases (ANTs). The AME genes are found to be encoded on the same plasmid as the 16S-RMTases (Garneau-Tsodikova and Labby, 2016; López Díaz *et al.*, 2017). The AACs are the largest AME subgroup which acetylates the amino groups found on aminoglycosides. Some common members in the AAC family include AAC(6')-1, AAC(3)-IIa and AAC(3)-I which are actively involved in the modification of aminoglycosides, such as tobramycin, netilmicin, amikacin and gentamicin (Shaw *et al.*, 1993). The AAC(6')-APH(2") bifunctional enzyme is responsible for conferring a high-level of gentamicin resistance in *Staphylococcus*, *Enterococcus*, and *Streptococcus agalactiae* clinical isolates (Kaufhold *et al.*, 1992).

The second largest AME subgroup is APH enzymes which catalyse the transfer of a phosphate group from ATP to the hydroxyl substituents present on the aminoglycosides. Among the APH enzymes, the most clinically relevant member is the APH(3') subfamily which confers resistance to kanamycin and neomycin and is found diversely in both Gram-negative and Gram-positive organisms (Ramirez and Tolmasky, 2010). The last AME subgroup is the ANT enzymes which transfer an adenosine monophosphate (AMP) group from ATP to a hydroxyl group on the aminoglycoside molecule. Although not as prevalent

as the AAC and APH enzymes, ANT(2”) is also a significant contributor to aminoglycoside resistance in Enterobacteriaceae and *P. aeruginosa* (Holbrook and Garneau-Tsodikova, 2018).

Besides mutations and enzymatic modifications, the mechanisms of resistance to aminoglycosides had also been demonstrated via efflux systems. Some major efflux pumps belong to the resistance nodulation division (RND) family, a tripartite efflux superfamily that is often associated with aminoglycoside resistance in clinically relevant pathogens, such as Enterobacteriaceae, *P. aeruginosa* and *Acinetobacter* spp. Alterations in RND protein-encoding genes (e.g. MexY from *P. aeruginosa*) resulted in an increase in susceptibility of the pathogen to aminoglycosides. These findings suggested that the RND efflux proteins may play a role in the resistance to aminoglycosides (Westbrock-Wadman *et al.*, 1999; Magnet and Blanchard, 2005). The RND efflux systems, when overexpressed, contribute to clinical aminoglycoside resistance, particularly in cystic fibrosis patients infected with *P. aeruginosa* (Poole, 2011).

Another instrumental factor that causes the resistance to aminoglycosides is modifications or changes in the bacterial cell membrane which subsequently reduce the concentration of intracellular aminoglycosides. Porins are channels found on the outer membrane and are actively involved in the uptake of several antibiotics, such as beta-lactams, fluoroquinolones, tetracycline and chloramphenicol into the bacterial cell. Resistance to these antibiotics was observed when there were functional changes to the bacterial porins. However, limited data is available to support that porins are involved in the resistance to

aminoglycosides. Studies conducted *in vitro* have linked the transient kanamycin resistance to the reduced expression of the OmpF porin found in *E. coli* and a complete knockout of *ompF* gene led to the resistance to both gentamicin and kanamycin in an *E. coli* mutant (Fei *et al.*, 2012). However, these results are inconclusive as some other studies have shown that the uptake of aminoglycosides is still possible even in porin-deficient mutants (Hancock, 1984; Serio *et al.*, 2018).

2.6.1 Mechanisms of gentamicin resistance in *L. monocytogenes*

The recovery of resistant strains of *L. monocytogenes* has increased in recent years due to the widespread use of antibiotics in the treatment of listeriosis (Olaimat *et al.*, 2018). Nevertheless, mechanisms of gentamicin resistance in *L. monocytogenes* are still not well-elucidated. Based on the limited studies in this area of research, gentamicin resistance genes found in this pathogen appeared to be acquired from other microorganisms via horizontal gene transfer (Baquero *et al.*, 2020). For instance, the gentamicin modification bifunctional enzymes, *aac6'-aph2*, are found on the plasmid pIP501 which belongs to the Inc18 plasmid family. This wide-host-range plasmid can be transmitted from *Streptococcus* to *Listeria* and re-transferrable back to *Streptococcus* (Vicente, Baquero and Pérez-diaz, 1988; Kohler, Vaishampayan and Grohmann, 2018).

Apart from that, adaptive gentamicin resistance had also been reported. A decrease in gentamicin susceptibility of *L. monocytogenes* was observed after an exposure to benzalkonium chloride (BC), which is one of the most widely used

disinfectants in the food processing industry. The susceptibility of the antibiotic was, however, restored in the presence of an efflux inhibitor, reserpine, suggesting that the mechanism of gentamicin resistance in those BC-adapted strains might be associated with efflux pumps (Rakic-Martinez *et al.*, 2011). Interestingly, reduced gentamicin susceptibility was also found in *L. monocytogenes* clinical strains (with the ST6 genotype) which were isolated from patients suffering with meningitis. The plasmid pLMST6 carrying the efflux transporter *emrC* was found in these isolates and was also reported to be linked to the increase in ST6 listerial meningitis cases in Netherlands (Kremer *et al.*, 2017).

The mechanisms described above are acquired genetic determinants of gentamicin resistance. However, little is known about the intrinsic mechanisms of reduced gentamicin susceptibility in *Listeria*.

2.7 ATP synthase and its contribution to aminoglycoside resistance

ATP synthase is an enzyme made up of two components, an integral membrane portion (F_0) and a catalytic portion (F_1). F_1 consists of the α_3, β_3, γ, δ, and ε subunits, which function to synthesize or hydrolyze ATP while F_0 is formed by the a, b, and c subunits, which act as a channel for the translocation of protons. The *atpG2* gene encodes the gamma subunit of this synthase in *L. monocytogenes* and is involved in the production of ATP from ADP when a proton gradient is present across the membrane. Apparently, the gamma chain is

believed to be important in the regulation of ATPase activity and the flow of protons through the F_0 complex (Nakanishi-Matsui, Sekiya and Futai, 2016).

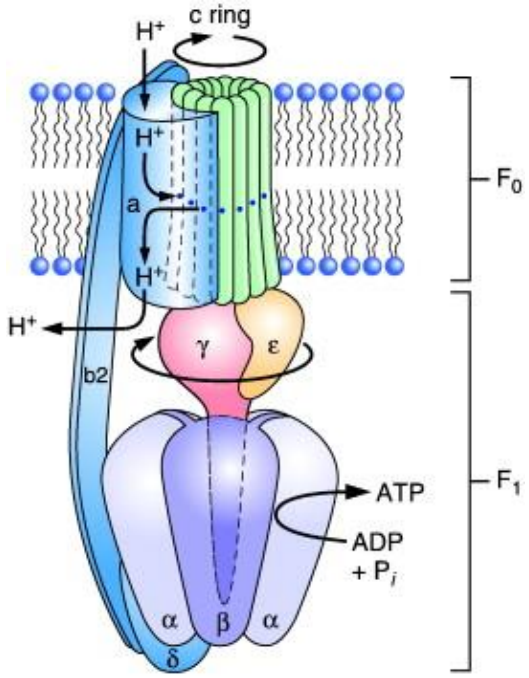

Figure 2.3: The components of a bacterial ATP synthase. Adopted from (Hicks *et al.*, 2010).

Although ATP synthase is commonly found in all bacteria, the role played by this important enzyme differs across bacterial genera and species (Balemans *et al.*, 2012). The function of ATP synthase includes generating high cellular energy to support the growth of mycobacterial cells (Cox and Cook, 2007; Haagsma *et al.*, 2010) or to sustain the proton motive force produced in *Chlorobium limicola* by photosynthesis or respiration (Xie *et al.*, 1993). Apart from that, ATP synthase has been linked to pH homeostasis, which allows foodborne pathogens, such as *L. monocytogenes* and *S. enterica* serovar Typhimurium, to survive in low pH environments (Foster and Hall, 1991; Cotter, Gahan and Hill, 2000).

Interestingly, mutations in genes encoding the ATP synthase have previously been associated with aminoglycoside resistance in bacteria (Miller *et al.*, 1980; Taber *et al.*, 1987; Magnet and Blanchard, 2005). A study by Humbert and Altendorf (1989) showed that a mutated gamma subunit of the ATP synthase was associated with resistance to aminoglycosides (neomycin, gentamicin, and streptomycin) in *E. coli*. This mutation was present in the form of a 2-bp insertion, which led to a truncated gamma subunit. Typically, the uptake of aminoglycosides occurs at a higher membrane potential. Therefore, when the gamma subunit of the ATP synthase is mutated, the influx of protons into the membrane may become unregulated, leading to a decrease in the membrane potential. This would then prevent the uptake of the antibiotic, resulting in the development of resistance (Mates *et al.*, 1982; Ramirez and Tolmasky, 2010).

CHAPTER 3

METHODOLOGY

3.1 Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 3.1. *Listeria* isolates were routinely cultured at 37 ˚C on brain-heart infusion (BHI) agar or in BHI broth (Oxoid). Antibiotics or other chemicals were added to the culture media when necessary. Culture media were prepared according to what were described in Appendix A. The two main plasmids used in this study were obtained from the Addgene repository: pMSP3545 was a gift from Gary Dunny (Addgene plasmid #46888; http://n2t.net/addgene:46888; RRID: Addgene_46888) and pHoss1 was a gift from Attila Karsi (Addgene plasmid #63158; http://n2t.net/addgene:63158; RRID: Addgene_63158). All the bacterial strains and plasmids were kept in BHI broth supplemented with 15% glycerol and stored at -80 ˚C for further characterisation.

3.2 Mutant selection and determination of mutation frequency

The method used to select gentamicin-resistant mutants was adapted from the Luria-Delbrück experiment which generates spontaneous mutants (Luria and Delbrück, 1943). Briefly, *L. monocytogenes* ATCC 19115 (American Type Culture Collection [ATCC]), with a starting culture of 10^3 CFU/mL, was

inoculated in the BHI broth and incubated to a cell density of approximately 10^9 CFU/mL. Two parallel cultures (B1 and B2 series), grown independently, were plated on BHI agar supplemented with 10 mg/L gentamicin (Sigma-Aldrich) and incubated at 37 ˚C for 24-48 h (Figure 3.1). The gentamicin concentration was set at a concentration 4-fold higher than the MIC of ATCC 19115 (2.5 mg/L). The mutation frequency was expressed as the ratio of the number of mutant colonies to the total viable count (Vickers, O'Neill and Chopra, 2007).

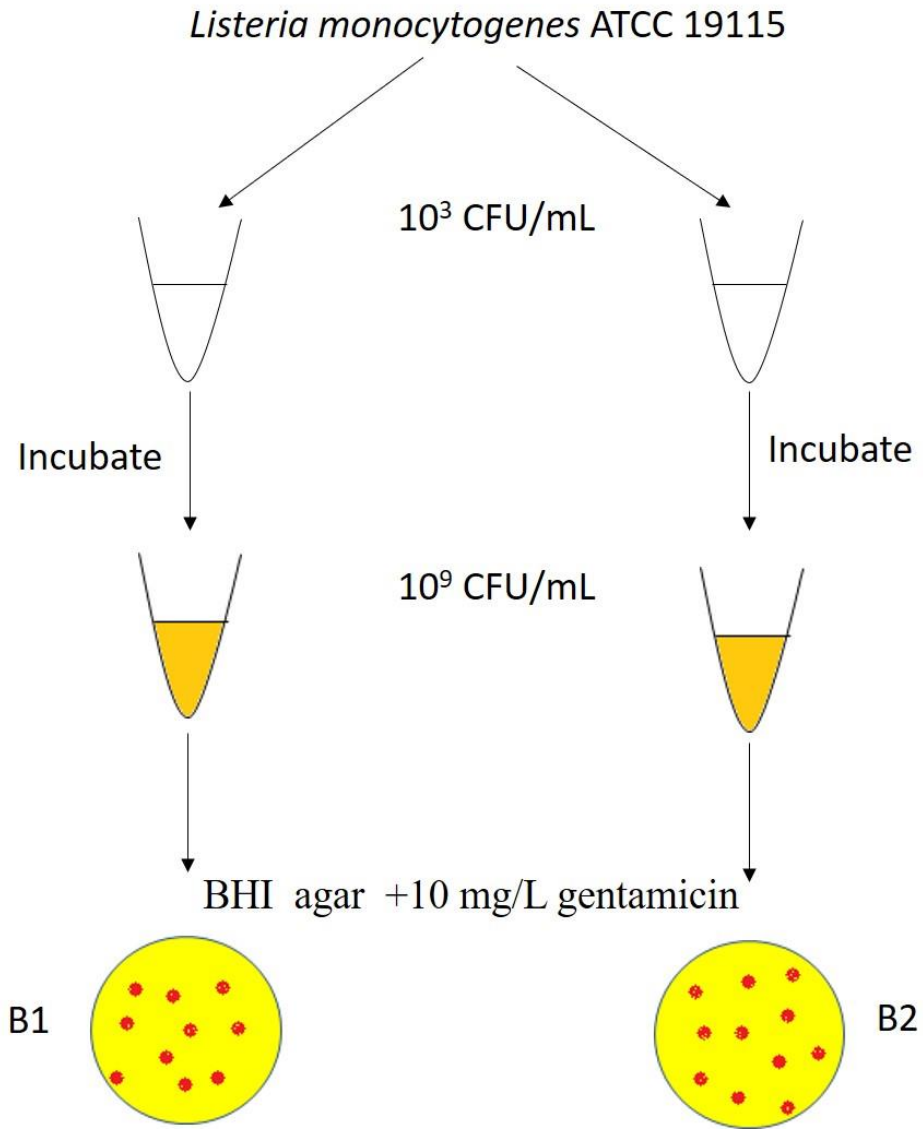

Figure 3.1: Workflow of the Luria-Delbrück experiment. The B1 and B2 series of mutants arose independently of each other.

Table 3.1: Bacterial strains and plasmids used in this study.

Bacterial strain(s) or plasmid	Description	Source / Reference
Bacterial strain(s)		
ATCC 19115	Gentamicin-susceptible, parental, and wild-type *L. monocytogenes* strain	ATCC
B2b B1b - B1l B2c - B2l	Gentamicin-resistant mutants derived from ATCC 19115	This study
ATCC 19115-pMSP3545	ATCC 19115 transformed with the empty pMSP3545 plasmid	This study
ATCC 19115- pMSP3545-*atpG2*^{mut}	ATCC 19115 transformed with pMSP3545 carrying the mutant *atpG2* gene	This study
ATCC 19115- pMSP3545-*atpG2*^{wt}	ATCC 19115 transformed with pMSP3545 carrying the wild-type *atpG2* gene	This study
ATCC 19115-pHoss1-*atpG2*^{mut}	ATCC 19115 transformed with pHoss1 carrying the mutant *atpG2* gene	This study
E. coli ESBL 184-379	A positive control for the *aac (3')-IIa* gentamicin resistance gene	This study
K. pneumoniae ESBL UVA 16-3	A positive control for the *armA* gentamicin resistance gene	This study
E. faecium NKS 31-3	A positive control for the *aac (6')-aph (2")* gentamicin resistance gene	This study
E. coli NEB5α	Competent cells	NEB
S. aureus ATCC 29213	A positive control strain for antimicrobial susceptibility testing	ATCC
LM Q01	*L. monocytogenes* strain isolated from food	This study

Table 3.1: Bacterial strains and plasmids used in this study (continued).

Bacterial strain(s) or plasmid	Description	Source / Reference
Bacterial strain(s)		
LM A17, LM 0221A, LM 12214A, LM 23719A, LM 12115A, LM 5914A, LM 27717A	Clinical strains of *L. monocytogenes* isolated from cervical swab, blood, tissue, cerebrospinal fluid and ear swab	This study
ATCC 19119	Gentamicin-susceptible, parental, and wild-type *L. ivanovii* strain	ATCC
ATCC 19115-AE-B2b	*L. monocytogenes* ATCC 19115 with the B2b mutation in the *atpG2* gene, introduced using the allelic exchange	This study
ATCC 19119-AE-B2b	*L. ivanovii* ATCC 19119 with the B2b mutation in the *atpG2* gene, introduced using the allelic exchange	This study
Plasmid		
pMSP3545	A nisin-inducible expression vector for Gram-positive bacteria	(Bryan *et al.*, 2000)
pHoss1	A vector used for allelic exchange in Gram-positive bacteria	(Abdelhamed, Lawrence and Karsi, 2015)
pMSP3545-*atpG2*^{wt}	pMSP3545 carrying the wild-type *atpG2* gene from *L. monocytogenes*	This study
pHoss1-*atpG2*^{mut}	pHoss1 carrying the mutant *atpG2* gene from *L. monocytogenes* B2b	This study
pHoss1-Li-*atpG2*^{wt}	pHoss1 carrying the wild-type *atpG2* gene from *L. ivanovii*	This study
pHoss1-Li-*atpG2*^{mut}	pHoss1 carrying the mutant *atpG2* gene from *L. ivanovii*, generated by site-directed mutagenesis	This study

3.3 Antimicrobial susceptibility testing

The antibiotic resistance/susceptibility patterns of the *Listeria* isolates were screened using Stokes disk diffusion and broth microdilution.

3.3.1 Stokes disk diffusion

The Stokes disk diffusion method (Phillips *et al.*, 1991) was used for the rapid comparison of gentamicin susceptibility between the mutant and the control strain of *L. monocytogenes*. In this test, each bacterial strain was adjusted to 0.5 McFarland. The control strain was spread on one half of a cation-adjusted Mueller-Hinton agar plate (Isolab) while the test strain was spread over the other half of the plate. A gentamicin 10 µg disk (Oxoid) was placed at the middle between the two halves of the plate. The inoculated plates were incubated at 35 °C with 5% CO_2 for 24 h. The zones of inhibition were measured. As clinical breakpoints of gentamicin have not been described for *L. monocytogenes*, the inhibition zone diameters were interpreted using the EUCAST breakpoints (susceptible ≥18 mm, resistant <18 mm) (EUCAST, 2022) for *S. aureus*, a fellow Gram-positive bacterium. The same method was also used to detect if the mutant was cross-resistant to other antibiotics, including ampicillin, chloramphenicol, ciprofloxacin, erythromycin, trimethoprim-sulfamethoxazole, tetracycline, vancomycin and other aminoglycosides (amikacin, kanamycin and neomycin).

3.3.2 Broth microdilution

MICs were determined using broth microdilution (Balouiri, Sadiki and Ibnsouda, 2016). Serial two-fold dilutions of an antibiotic were prepared in Mueller-Hinton II (MHII) broth (Becton Dickinson) and pipetted into a 96-well microtiter plate (NEST). To each well, the test organism was added to a final concentration of 5×10^5 CFU/mL. The plate was then sealed and incubated at 35 ˚C for 20 h. The MIC was determined as the lowest concentration of antibiotic that prevented any visible growth of the bacterial strains. A viable control, which was the test strain in the antibiotic-free broth, was set up in every assay. Each test condition was tested in at least two biological replicates, with each biological replicate being tested in technical duplicates.

3.4 Biochemical test

Biochemical profiles of the selected mutant, B2b, and its wild type, ATCC 19115, and *L. ivanovii* ATCC 19119 were determined by the Analytical Profile Index (API) for *Listeria* system (BioMérieux, Marcy-l'Etoile, France). The API test strip consists of the following 10 tests: the arylamidase activity (the DIM test), hydrolysis of esculin, α-mannosidase activity, and acid production from D-arabitol, D-xylose, L-rhamnose, Methyl alpha-D-glucopyranoside, D-ribose, glucose-1-phosphate, and D-tagatose. Freshly grown bacterial culture was emulsified in an ampoule containing 2 mL of sterile API suspension medium; the turbidity of the inoculated medium was adjusted to 1 McFarland with sterile distilled water. About 3 mL of distilled water was poured into the supplied tray

to create a humid atmosphere. The test strip was removed from its individual packaging and placed in the tray. The bacterial suspension was pipetted into the test strip consisting of 10 reaction wells (100 µL for DIM test and 50 µL for the other tests). The tray was then covered with a lid and incubated for 18 to 24 h at 37 ˚C in aerobic conditions. After incubation, a single drop of ZYM B (supplied by the manufacturer) was added to the first well (DIM test) and allowed to react for 3 min at room temperature. The test strip results were then ready to be read and the colour changes were interpreted as per the manufacturer's guidelines (Table 3.2).

Table 3.2: API biochemical tests interpretation guidelines for *Listeria*.

Tests	Reactions/Enzymes	Results	
		Negative	**Positive**
DIM	Arylamidase enzyme activity	Pale orange /Pink-beige /Grey-beige	Orange
ESC	Hydrolysis (esculin)	Pale yellow	Black
αMAN	α-Mannosidase	Colourless	Yellow
DARL	Acidification (D-arabitol)	Red/Orange-red	Yellow /Yellow-orange
XYL	Acidification (xylose)		
RHA	Acidification (rhamnose)		
MDG	Acidification (methyl-α-D-glucopyranoside)		
RIB	Acidification (ribose)		
G1P	Acidification (glucose-1-phosphate)		
TAG	Acidification (tagatose)		

3.5 Molecular Analyses

3.5.1 End-point PCR

Total DNA was extracted using Quick-DNA Fungal/Bacterial Miniprep (Zymo Research) according to the manufacturer's instructions. The PCR mix contained GoTaq Green Mastermix (Promega) (1×), primers (forward and reverse, 0.2 µM each), and PCR-grade water. The DNA was added to a final amount of 10 ng. Amplification was carried out using Veriti Thermal Cycler (Thermo Scientific). In every run, at least one no-template control was included. PCR products were resolved on 1% gel pre-casted with 1× RedSafe Nucleic Acid Staining Solution (Intron Biotechnology) and visualised under ultraviolet ray at 312 nm on UVIPURE transilluminator (Uvitec). All the primers were synthesised by Integrated DNA Technologies. The sequence of the primers, target gene, product length and annealing temperature of each PCR assay were summarised in Table 3.3. When necessary, the PCR amplicons and primers were sent to Apical Scientific (Malaysia) for Sanger sequencing.

3.5.2 Multi locus variable-number-tandem-repeat analysis (MLVA) PCR

Genotyping of B2b and ATCC 19115 was carried out using PCR-based multi locus variable-number-tandem-repeat analysis (MLVA) (Lindstedt *et al.*, 2008) with the primers listed in Table 3.3. The PCR amplicons were sequenced using Sanger technology (Apical Scientific, Malaysia). For each of the recommended loci, the copy number was determined by using Pattern Locator

(PATLOC) (Mŕazek and Xie, 2006), which is a sequence motif recognition software that could identify the VNTR sequences in the uploaded PCR sequence.

3.5.3 Mismatch amplication mutation assay (MAMA) PCR

MAMA is a PCR-based technique widely used for the discrimination of mutations (Zirnstein *et al.*, 1999). Unlike the conventional end-point PCR, this technique differs in the mismatch primer at the 3'-end which prevents the Taq DNA polymerase from performing the extension during amplification (Figure 3.2). In this study, MAMA PCR was used for the detection of a 10-bp deletion in the *atpG2* gene carried by the mutants. The forward primer was designed in a manner where the 3' end partially falls within the deleted sequence. This prevents the amplification of the sequence of the mutants with the right mutation. The sequences of MAMA PCR primers are described in Table 3.3.

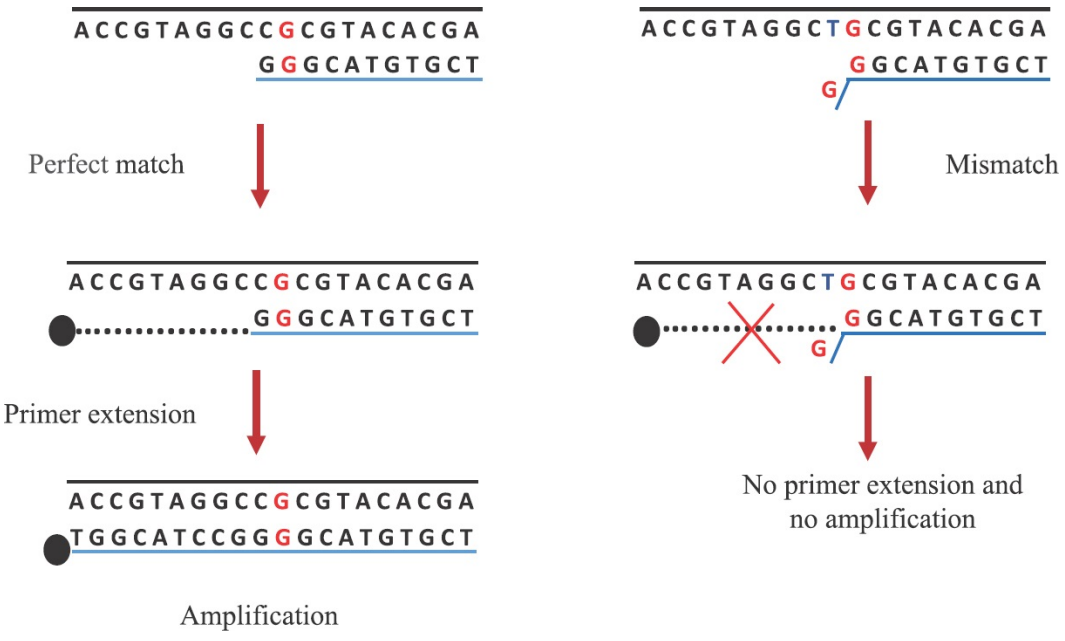

Figure 3.2: Mismatch at the 3' end of the primer prevents an amplification in MAMA PCR. Adopted from (Deekshit *et al.*, 2019).

3.6 Genome sequencing

The total DNA of the B2b mutant was extracted using ZR Fungal/Bacterial DNA Miniprep (Zymo Research). The purity, concentration and integrity of the DNA sample were assessed using Nanodrop (Thermo Scientific) and gel electrophoresis. The sample was then submitted to Novogene for a PCR-free library preparation using NEBNext Ultra II DNA Library Prep Kit (New England Biolabs). For the library construction, the DNA was randomly sheared into short fragments of 350 bp. These fragments were then end-repaired, undergone phosphorylation and addition of polyA tails and further ligated with Illumina adapters. The fragments with the adapters were PCR-amplified, size-selected and purified. The constructed library was then assessed with Qubit, real-time PCR and Bioanalyzer. The quantified library of B2b was sequenced by NovaSeq 6000 (Illumina) using the 2×150 bp method. After that, the reads were processed using CASAVA (Hosseini *et al.*, 2010). Clean paired-end reads were mapped to the genome of the reference strain (ATCC 19115) using BWA (Cock *et al.*, 2010). The average sequencing depth was 266×. The SNP/InDel detection was performed using GATK (Depristo *et al.*, 2011) and annotated using ANNOVAR (Wang, Li and Hakonarson, 2010).

Table 3.3: PCR primers.

Primer	Sequence (5' – 3')	Target	Product length (bp)	Annealing temperature (˚C)	Purpose	Reference
LM_16S F LM_16S R	ACGCAAGGAATCTTATTCACGG CCTCTCAAAACTGAACAAATAGAGA	*16S rRNA*	1661	59	End point PCR to detect mutations in *16S rDNA* region for B2b	This study
LMV1 F LMV1 R	CGTATTGTGCGCCAGAAGTA MAMCAACRCAACAACAAACAG	VNTR locus V1	396	58	MLVA	(Lindstedt *et al.*, 2008)
LMV2 F LMV2 R	TAGATGCGGTTGAGRTAGAYR CTGGMTYMATWGGATTTACTKGAT	VNTR locus V2	491	55	MLVA	(Lindstedt *et al.*, 2008)
LMV6 F LMV6 R	AAAAGCCCCRATTGGATA CTCGCTGTTTTCTGWTTTCTTAGG	VNTR locus V6	232	58	MLVA	(Lindstedt *et al.*, 2008)
LMV7 F LMV7 R	TCMAAAATCAAGCACAAATCACTG TAGCAAGCAWAYGCCTGTCCAKA	VNTR locus V7	449	57	MLVA	(Lindstedt *et al.*, 2008)
LMV9 F LMV9 R	AACGGTKRCKGATTTACTTC CTTGGYGTCGAGGCATTTA	VNTR locus V9	530	52	MLVA	(Lindstedt *et al.*, 2008)
AAC (3')-IIa F AAC (3')-IIa R	CGGAAGGCAATAACGG TCACGATGTCCTGCG	*aac (3')-IIa*	757	52	Screening of GMR gene	This study
ArmA F ArmA R	ATTCTGCCTATCCTAATTGG ACCTATACTTTATCGTCGTC	*armA*	315	52	Screening of GMR gene	This study
AAC (6')-APH (2") F AAC (6')-APH (2") R	ACAGAGCCTTGGGAAGATGAA CCTCGTGTAATTCATGTTCTGGC	*aac (6')-aph (2")*	349	59	Screening of GMR gene	This study

MLVA: Multi-locus variable-number-tandem-repeat analysis
GMR: Gentamicin resistance

Table 3.3: PCR primers (continued).

Primer	Sequence (5' – 3')	Target	Product length (bp)	Annealing temperature (˚C)	Purpose	Reference
LM_atpG2F LM_atpG2R	TATCCATGGGTTTGGCATCTTTAATC GATATT TACTGCAGCTATTCTAGTGCGGCTG Forward primer RE (NcoI-HF) Reverse primer RE (PstI-HF)	*atpG2*	863 (ATCC 19115) 853 (B2b)	Partial (56) Full (67)	Amplify *atpG2* gene of ATCC 19115 and B2b	This study
LM_gdh F LM_gdh R	TATGCATGCGGATGGCACAAACATC CAC TATCTAGATTAAATAATACCTTGAG AAATCATTGT Forward primer RE (SphI) Reverse primer RE (XbaI)	*gdh*	1376	58	WGS verification	This study
pMSP3545_F pMSP3545_R	ATAACGCGAGCATAATAAACGGC TGGCTATCAATCAAAGCAACACG	pMSP3545	247	60	Cloning and transformation	This study
pHoss1_F pHoss1_R	GTCGTCATCTACCTGCCTGG CCTGGAGCTGGTATATAAGTCCCT	pHoss1	295	60	Allelic exchange	This study
LM_ext atpG2-F LM_ext atpG2-R	GCGAAACTTGAAGCAGCATT TCCTCCTCACTTACCTTCCC	*atpG2*	1025	58	Screening of clinical and environmental isolates, and other mutants of LM	This study

RE: Restriction enzyme
WGS: Whole-genome sequencing
LM: *L. monocytogenes*
LI: *L. ivanovii*

Table 3.3: PCR primers (continued).

Primer	Sequence (5' – 3')	Target	Product length (bp)	Annealing temperature (˚C)	Purpose	Reference
LI_ext atpG2-F LI_ext atpG2-R	GCGAAACTTGAAGCAGCATT TCCTCCTCACTTACCTTCCC	*atpG2*	1030	58	Screening of reconstructed LI mutants via allelic exchange	This study
LI_IPCR_SDM-F LI_IPCR_SDM-R	TGACTTATCACTACAATATAATCG CGGATGCATTATCTGTC		2999	53	To introduce 10-bp deletion in the LI *atpg2* gene	This study
MAMA_PCR-F MAMA_PCR-R	ACAGACAATGCATCCGATTT CTCCTCACTTACCTTCCCA		167	60	To screen for colonies with 10-bp deletion mutation in *atpG2* gene	This study
LM_AE atpG2-F LM_AE atpG2-R	TTAGTCGACATAAATATCTGGATGAT GTACC TAACCATGGAGTAGCTAGGGTTGGTT Forward primer RE (SalI-HF) Reverse primer RE (NcoI-HF)		2000	Partial (56) Full (65)	To knockout the 10-bp from LM *atpG2* for allelic exchange	This study

RE: Restriction enzyme
LM: *L. monocytogenes*
LI: *L. ivanovii*

3.7 Cloning and transformation

3.7.1 Molecular cloning and transformation into *E. coli*

The target gene was amplified using Q5 High-Fidelity 2× Master Mix (NEB). The PCR amplicons were then cleaned up using QIAquick PCR Purification Kit (Qiagen). The primers used are described in Table 3.3. The purified amplicon was cloned into the plasmid using restriction enzymes and T4 DNA ligase (NEB). The ligated mixture was purified using DNA Clean and Concentrator-5 (Zymo Research). The transformation of recombinant plasmids into NEB 5-alpha competent *E. coli* cells was carried out using heat shock at 42 ˚C for 30 s. After a recovery in the SOC broth (NEB), the culture was spread onto a BHI agar (Oxoid) supplemented with the appropriate antibiotics and incubated at 37 ˚C overnight.

The transformed colonies were screened using colony PCR. Using DNA-spin Plasmid Purification Kit (iNtRON Biotechnology), the cloned plasmids were extracted from the BHI broth cultures (containing 100 mg/L erythromycin) of the positive colonies. Sanger sequencing was performed to confirm that no unwanted mutations were introduced into the insert.

3.7.2 Preparation of *Listeria* electro-competent cells and electroporation

Listeria electro-competent cells were prepared as described previously by (Park and Stewart, 1990). In brief, an overnight culture was diluted with freshly prepared BHI broth supplemented with 0.5 M sucrose (1:100), and allowed to grow at 37 ˚C with shaking until an OD_{600} of ~ 0.2 was achieved. The culture was then treated with penicillin G (10 µg/mL) and incubated for another 2 h. The culture was incubated on ice for 10 min followed by a centrifugation at 8,000 × g and 4 °C for another 10 min. The harvested cells were washed three times with cold, sterile washing solution consisting of 1 mM HEPES (pH 7) and 0.5 M sucrose. The cells were resuspended in 200 µL of the ice-cold washing solution and then frozen on dry ice for 10 min. The electrocompetent cells were then stored in a -80 ˚C ultra-deep freezer for future use.

One µg of the recombinant plasmid was added to the electro-competent cells. Electroporation was carried out using Eporator (Eppendorf) at 1,000 V. After the cells were electroporated, BHI broth supplemented with 0.5 M sucrose was added immediately and the culture was incubated statically at 37 ˚C for 1 h. The culture was then plated on a BHI agar supplemented with 10 mg/L erythromycin. Recombinant plasmids were detected in selected colonies using colony PCR.

3.8 Biological validation by reverse genetics

3.8.1 Complementation

After the identification of the mutation in the *atpG2* gene of B2b using WGS (Section 3.6), the wild-type *atpG2* gene was cloned (Section 3.7.1) into the expression plasmid, pMSP3545 (Figure 3.3). The recombinant plasmid was then electroporated into B2b. To induce the expression of the cloned, wild-type *atpG2* gene, the transformant was transferred to BHI broth supplemented with 25 ng/ml of nisin (Alfa Aesar) for an overnight incubation. The following morning, Stokes disk diffusion (Section 3.3.1) was carried out to determine the gentamicin susceptibility of the induced transformant. B2b transformed with the empty pMSP3545 plasmid served as the empty-plasmid control. If the mutation was indeed a resistance determinant, complementation with the wild-type gene should revert the resistance phenotype of the B2b mutant back to the susceptible phenotype.

3.8.2 Allelic exchange mutagenesis

A *Listeria* colony transformed with the recombinant pHoss1 plasmid (Figure 3.4) which carried the mutated *atpG2* gene was streaked on a BHI agar supplemented with 10 mg/L erythromycin and incubated at a plasmid-replication-nonpermissive temperature of 42 ˚C for 2 days. This process was repeated twice to allow the plasmid to integrate into the chromosome of the host cell and initiate the homologous recombination event. A single colony was then

passed twice in BHI broth (without erythromycin) at 30 ˚C for 24 h before spreading on BHI agar containing anhydrotetracycline and gentamicin. Colonies were screened by the MAMA PCR (Section 3.5.3). For those MAMA PCR-negative colonies (i.e. with mutation), the whole coding sequence of *atpG2* was amplified and sequenced to confirm the presence of the mutation of interest. The workflow and principle of the experiment are further illustrated in Figure 3.5.

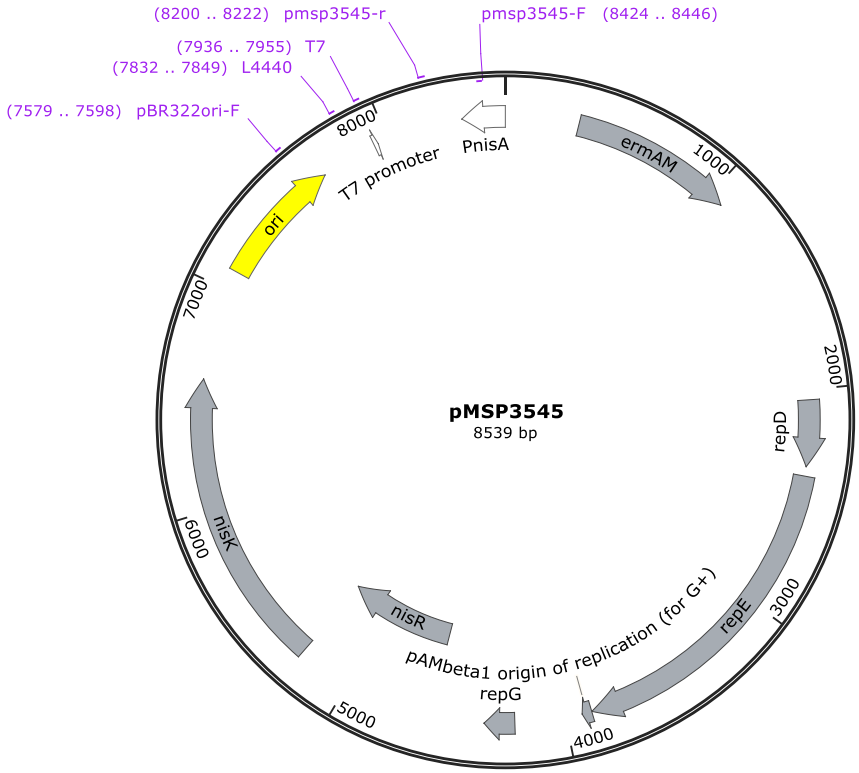

Figure 3.3: Plasmid map of pMSP3545. Adopted from (Bryan *et al.*, 2000). An inducible expression vector for Gram-positive bacteria which contains the nisin-inducible P*nis*A promoter, the pAMB1 replicon for expression in gram-positive bacteria and genes encoding NisR and NisK, the two-component signalling mechanism for activating transcription from P*nis*A in the presence of nisin.

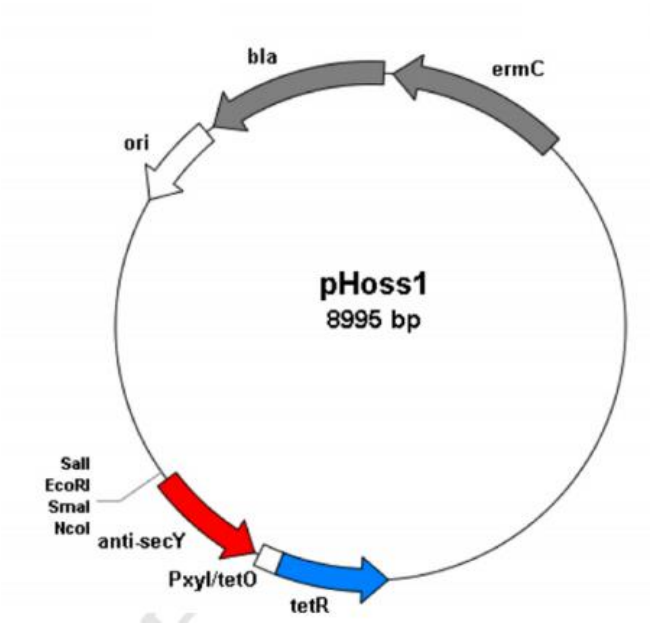

Figure 3.4: Plasmid map of pHoss1. Adopted from (Abdelhamed, Lawrence and Karsi, 2015). This is a suicide plasmid for Gram-positive bacteria. This plasmid contains a heat-sensitive origin of replication, a selectable marker (*ermC* which confers resistance to erythromycin) and a counter-selectable marker (the *secY* antisense cassette driven by an inducible *Pxyl*/*tetO* promoter which confers susceptibility to anhydrotetracycline).

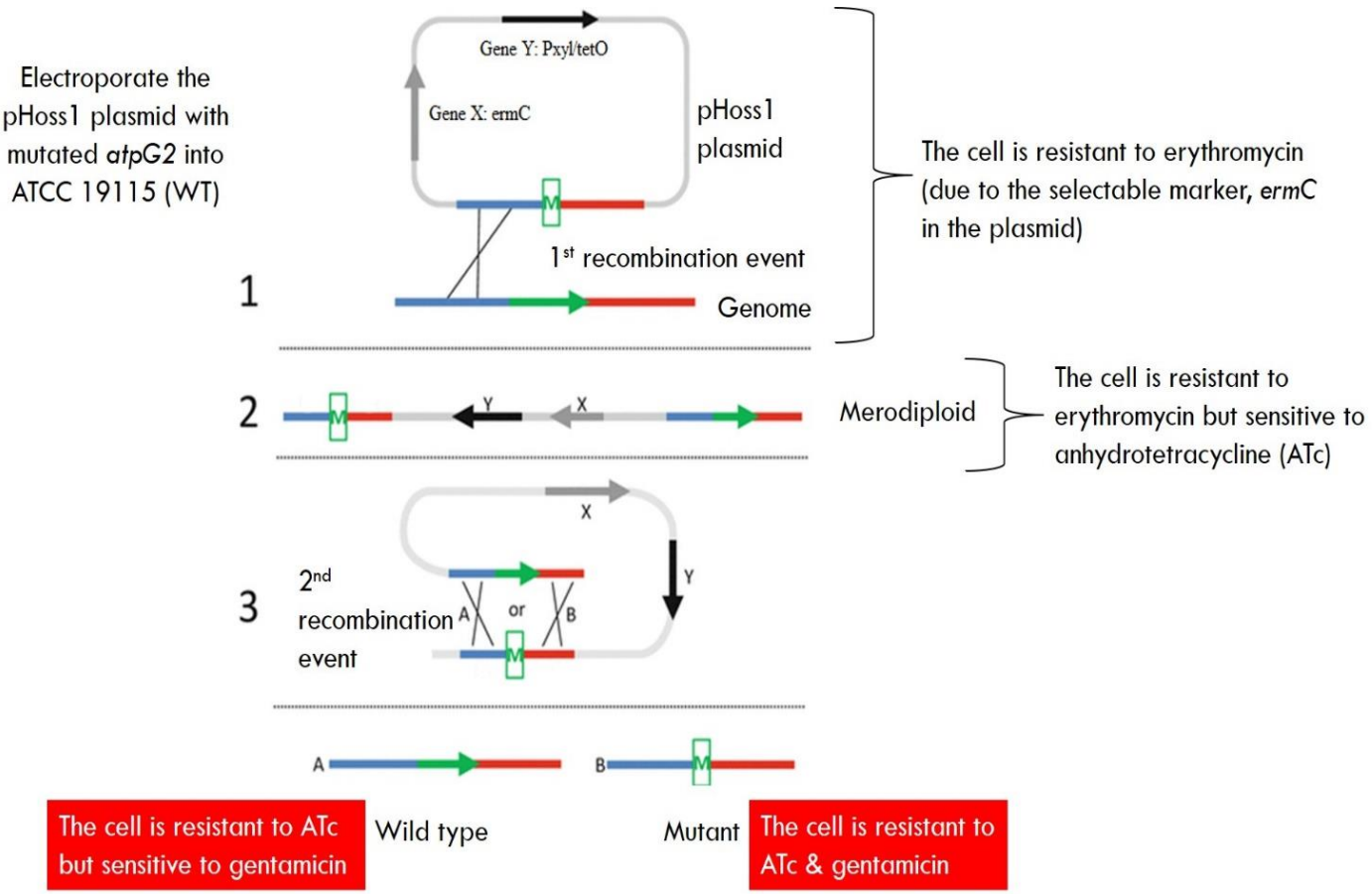

Figure 3.5: Workflow and principle of the allelic exchange experiment. WT: Wild type

3.8.3 Site-directed mutagenesis

The *atpG2* gene sequence of *L. ivanovii* is slightly different from that of *L. monocytogenes* (Appendix B). Therefore, after cloning the *L. ivanovii atpG2* gene into the pHoss1 plasmid, site-directed mutagenesis (Toyobo) was used to introduce the 10-bp deletion orthologous to the one found in B2b (Figure 3.6). The inverse PCR of the recombinant plasmid (pHoss1 carrying the *atpG2* gene of *L. ivanovii* ATCC 19119) was carried out using a pair of primers designed specifically to introduce the 10-bp deletion (Table 3.3 and Figure 3.7B). After PCR, the template plasmid (with methylation as it was purified from *E. coli*) was removed using *Dpn*I, a restriction enzyme which specifically removes the methylated DNA. The unmethylated PCR amplicons would then be self-ligated through the enzymatic actions of the T4 polynucleotide kinase and ligase. These self-ligated PCR products were then purified and transformed into *E. coli* competent cells (Section 3.7.1). The propagated and purified plasmid was then used as the suicide plasmid to introduce the desired mutation into *L. ivanovii* ATCC 19119 (Section 3.8.2).

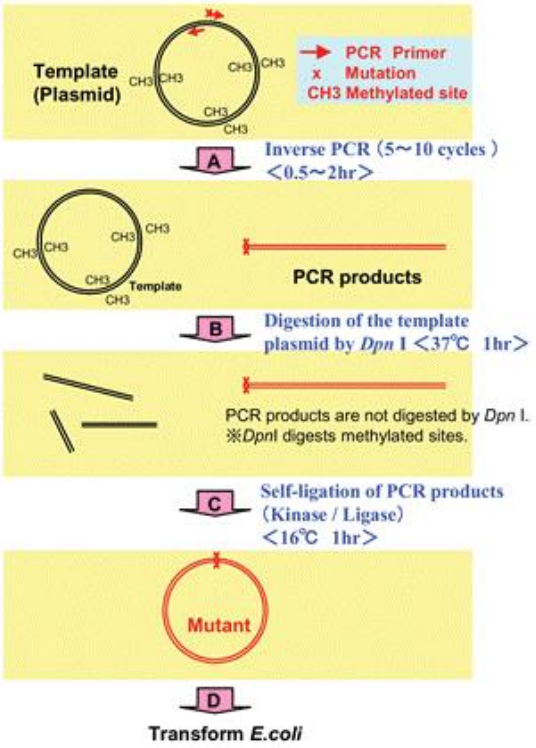

Figure 3.6: Workflow of site-directed mutagenesis. Adopted from (Toyobo, 2004)**.**

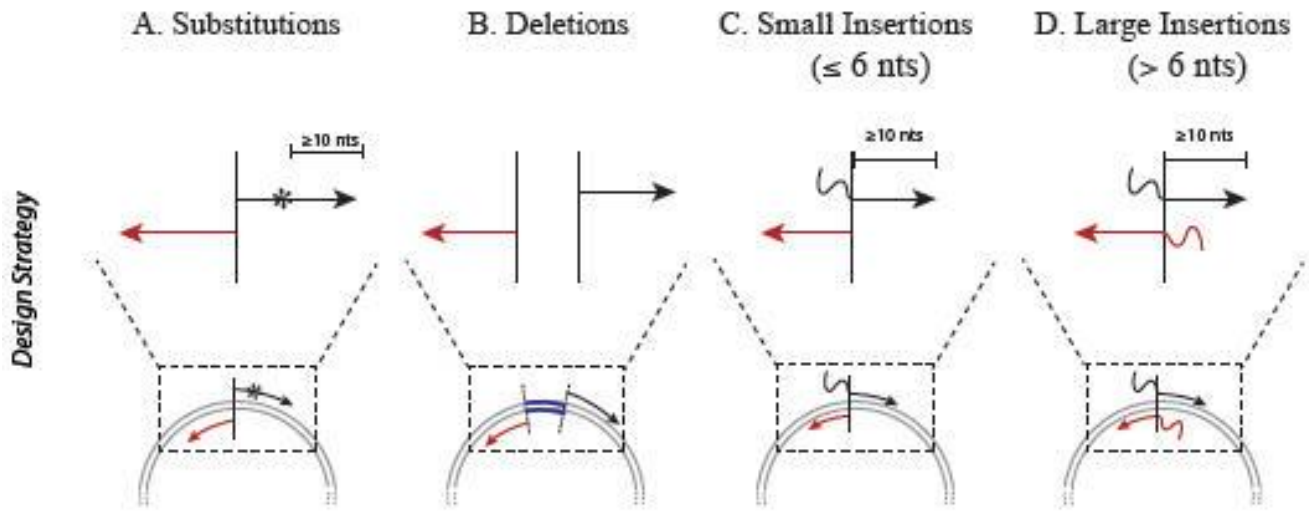

Figure 3.7: Inverse PCR applicable for the introduction of mutations, such as substitutions, deletions and insertions, into a plasmid. Adopted from (Toyobo, 2004). The primer design (B. Deletions) was used in this study to generate the 10-bp deletion in the target plasmid.

3.9 Qualitative and quantitative catalase test

3.9.1 Qualitative catalase test

Equal volumes of the catalase reagent (Thermo Scientific) were dropped onto a clean glass slide. Fixed volumes of standardised *Listeria* suspension were then added onto the reagent. The formation of bubbles was observed.

3.9.2 Quantitative catalase test

Standardised inocula were plated onto agar deeps (prepared in 15-ml tubes with graduation marks). After a 24 h incubation at 37 ˚C, the catalase reagent was added to the agar deeps. The resulted columns of bubbles were measured in mL. Parallel cultures on the agar deeps were prepared. After incubation, the growth on the deep was re-suspended in saline and the suspension was measured using McFarland densitometer. To obtain the standardized catalase activity, the volume of bubbles was normalized by the McFarland value.

3.10 Fitness cost

To compare the fitness cost between *L. monocytogenes* ATCC 19115 and B2b in broth, each isolate was inoculated into 30 mL of BHI broth to a final concentration of 5 x 10^5 CFU/mL. Both cultures were allowed to shake at 37 ˚C and their McFarland readings were taken every 2 h for up to 24 h.

3.11 ATP chemiluminescence assay

ATP levels were measured using ATP Chemiluminescence Assay Kit (Elabscience), following the protocol as recommended by the manufacturer. Both the *L. monocytogenes* ATCC 19115 and B2b were cultured in BHI broth and allowed to grow at 37 ˚C with shaking. The cells were harvested at log phase when both cultures reached 2.5 McFarland. The pellet that formed after a centrifugation was washed twice with the phosphate-buffered saline (PBS). The extraction buffer was then added to the pellet and the mixture was boiled for 10 min. The boiled lysates were centrifuged and the supernatants were transferred to a 96-well black microplate. An equal volume of enzyme working solution was then added into the wells with samples and the serially diluted standards. After mixing, both the standards and samples were measured by Tecan Spark Chemiluminescence analyser. Under the catalysation of the luciferase enzyme, ATP reacted with the substrate luciferin and emitted chemiluminescence (Figure 3.8). The chemiluminescence intensity was proportional to the concentration of ATP within a detection range recommended by the manufacturer.

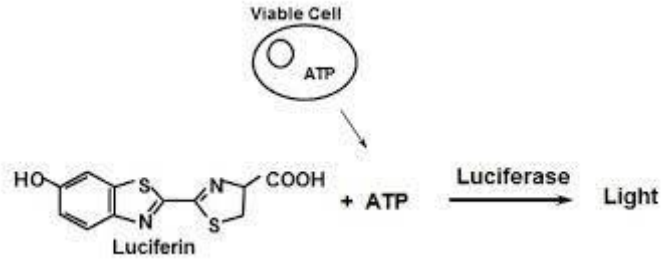

Figure 3.8: Catalytic reaction between the substrate luciferin and the ATP in bacterial cells leads to the emission of chemiluminescence.

3.12 pH assays

L. monocytogenes ATCC 19115 and B2b were cultured in BHI broth and allowed to grow at 37 ˚C with shaking. The overnight cultures were then adjusted to 2 McFarland. The cultures (10 µL) were then spotted in triplicates on BHI agar at pH 5 and pH 7. The plates were incubated overnight at 37 ˚C.

3.13 Efflux inhibitor assay

For the efflux inhibition study, broth microdilution was used (Section 3.3.2). The efflux inhibitor reserpine was added into the gentamicin-supplemented MHII broth to a final concentration of 10 mg/L, a recommended concentration previously described for *L. monocytogenes* (Godreuil *et al.*, 2003; Guérin *et al.*, 2014).

3.14 Statistical analyses

For all quantitative experiments, biological triplicates were carried out. The results were presented as mean (± standard deviation) and the two experimental groups (the mutant vs wild type) were compared using the unpaired Student's t-test with the p-value set at 0.05 as the minimal level of significance. The analyses were performed using GraphPad Prism 5.

CHAPTER 4

RESULTS

4.1 Mutant selection

Through the Luria-Delbrück experiment, which involved plating saturated ATCC 19115 cultures onto gentamicin-containing agar plates, 75 and 96 mutant colonies were selected from the B1 and B2 culture plates, respectively. B1 and B2 mutants were selected independently of each other (Figure 3.1). Additional parallel cultures were used for the viable count (3.3×10^9 CFU/mL, enumerated through serial dilution) on agars without the antibiotic. These mutants were developed at a frequency of $5.2 \pm 0.92 \times 10^{-8}$. Table 4.1 summarises the results of the Stokes disk diffusion. Ten to 11 mutants selected from the B1 and B2 series of experiments were subjected to Stokes disk diffusion. These mutants have an inhibition zone size of at least 17 to 20 mm smaller than that of the wild-type ATCC 19115 strain.

Table 4.1: Inhibition zone sizes of the B1 and B2 series of mutants selected using the Luria-Delbrück experiment.

Mutant	Inhibition zone size (mm) [1]
B1b	11
B1c	9
B1d	10
B1e	10
B1f	10
B1g	10
B1h	10
B1i	11
B1j	9
B1k	10
B2b	8
B2c	9
B2d	9
B2e	9

[1] The zone size of the wild-type ATCC 19115 was 28 mm.

Table 4.1: Inhibition zone sizes of the B1 and B2 series of mutants selected using the Luria-Delbrück experiment (continued).

Mutant	Inhibition zone size (mm) [1]
B2e	9
B2f	11
B2g	9
B2h	9
B2i	10
B2j	9
B2k	10
B2l	10

[1] The zone size of the wild-type ATCC 19115 was 28 mm.

4.2 Preliminary characterisations of the gentamicin-resistant mutant B2b

4.2.1 Antimicrobial susceptibility testing of B2b

The most resistant mutant, B2b (with the smallest inhibition zone of 8 mm) (Table 4.1 and Figure 4.1), was selected for further characterisations. This mutant was deemed resistant to gentamicin by the EUCAST guideline. The B2b mutant, when subjected to broth microdilution (see Section 3.3.2), was found to have a MIC of 40 mg/L.

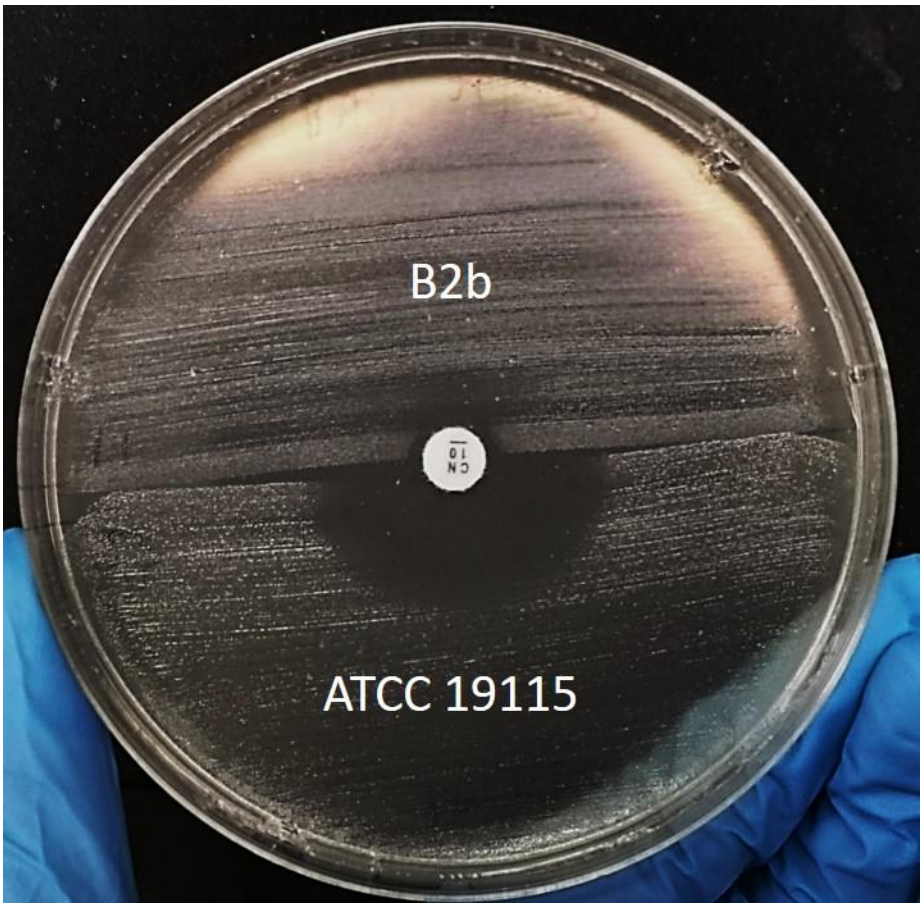

Figure 4.1: Stokes disk diffusion of the gentamicin-resistant mutant, B2b (top) vs the parental strain, ATCC 19115 (bottom).

4.2.2 Cross-resistance of B2b with other antibiotics

Table 4.2 shows the results seen when B2b was tested for the development of cross resistance, against other antibiotics: ampicillin, chloramphenicol, ciprofloxacin, erythromycin, trimethoprim-sulfamethoxazole, tetracycline, vancomycin and other aminoglycosides (amikacin, kanamycin and neomycin). B2b was found to be cross-resistant to other aminoglycosides (amikacin, kanamycin and neomycin) but not with other classes of antibiotics tested.

Table 4.2: Results of antimicrobial susceptibility testing of *L. monocytogenes* B2b and ATCC 19115.

Antibiotic	Inhibition zone diameter (mm)	
	B2b	**ATCC 19115**
Ampicillin	22	23
Chloramphenicol	29	28
Ciprofloxacin	24	22
Erythromycin	32	32
Tetracycline	32	33
Trimethoprim-sulfamethoxazole	29	29
Vancomycin	22	19
Gentamicin	8	28
Amikacin	8	18
Kanamycin	9	23
Neomycin	9	20

4.2.3 Analytical Profile Index (API) biochemical test

Figure 4.2 and Table 4.3 summarise the biochemical profiles of *L. monocytogenes* B2b and ATCC 19115. No changes in the biochemical profiles were detected in both strains. There were, however, changes in the biochemical test results between these two isolates (B2b and ATCC 19115) and *L. ivanovii* ATCC 19119.

Table 4.3: Test results of API biochemical tests of *L. monocytogenes* ATCC 19115 and B2b, and *L. ivanovii* ATCC 19119.

Test	*L. monocytogenes* ATCC 19115	*L. monocytogenes* B2b	*L. ivanovii* ATCC 19119
DIM*	-	-	+
Esculin hydrolysis	+	+	+
α-mannosidase	+	+	-
D-arabitol	+	+	+
D-xylose	-	-	+
L-rhamnose	+	+	-
methyl-αD-glucopyranoside	+	+	+
D-ribose	-	-	+
Glucose-1-phosphate	-	-	+
D-tagatose	-	-	-

*DIM test: to detect the presence or absence of arylamidase
+: positive reaction
-: negative reaction

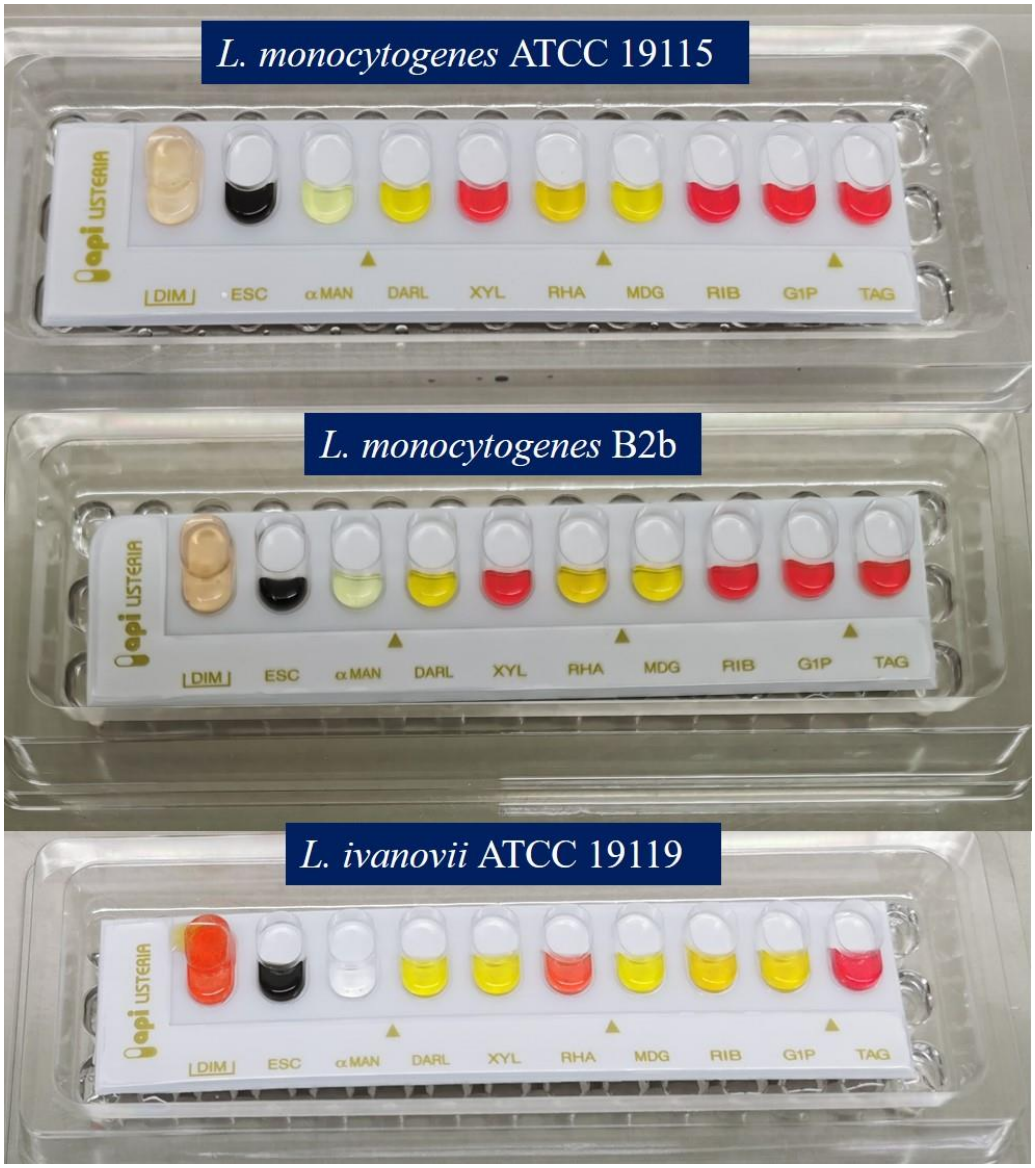

Figure 4.2: API biochemical tests of *L. monocytogenes* ATCC 19115 and B2b, and *L. ivanovii* ATCC 19119 after 24 h of incubation.

4.2.4 Multi-locus variable-number-tandem-repeat analysis (MLVA)

Table 4.4 shows the results observed from the genotyping of B2b and ATCC 19115. To confirm their clonal relationship, B2b and ATCC 19115 were subjected to genotyping using MLVA (Lindstedt *et al.*, 2008). The results showed that B2b had the same copy numbers (genotypes) as ATCC 19115 across all five recommended loci.

Table 4.4: MLVA of *L. monocytogenes* B2b and ATCC 19115.

Locus	Copy number[1]		VNTR sequence	Expected amplicon size (bp)
	ATCC 19115	B2b		
V1	16	16	GTATTT	396
V2	22	22	GTAGATCCG	491
V6	3	3	AGTACCACCAACACC	232
V7	1	1	TAAAACCTA	449
V9	4	4	AGAAAAACC	530

[1] Due to small VNTR sizes (6-15 bases), standard gel electrophoresis might not be able to identify the copy-number changes. Therefore, the amplicons were sequenced using Sanger technology. These sequences were then used to determine the copy numbers of each VNTR locus of ATCC 19115 and B2b. VNTR: variable-number tandem repeat.

4.2.5 PCR screening of other gentamicin resistance genes

Figures 4.3, 4.4, and 4.5 show the outcome of B2b when it was screened for previously reported genetic determinants of gentamicin resistance. One such genetic determinant is mutations in the *16S rRNA* gene, which encodes the molecular target of aminoglycosides (Kotra, Haddad and Mobashery, 2000). No mutations were detected in the *16S rRNA* genes of both B2b and ATCC 19115.

When B2b and ATCC 19115 were subjected to PCR screening of some commonly found gentamicin-resistance genes, such as those encoding aminoglycoside N-acetyltransferase AAC (3')-IIa, 16S rRNA methylase ArmA, aminoglycoside acetyltransferase and phosphotransferase bifunctional enzyme AAC (6')-APH (2"), none of these genes were detected in B2b and ATCC 19115

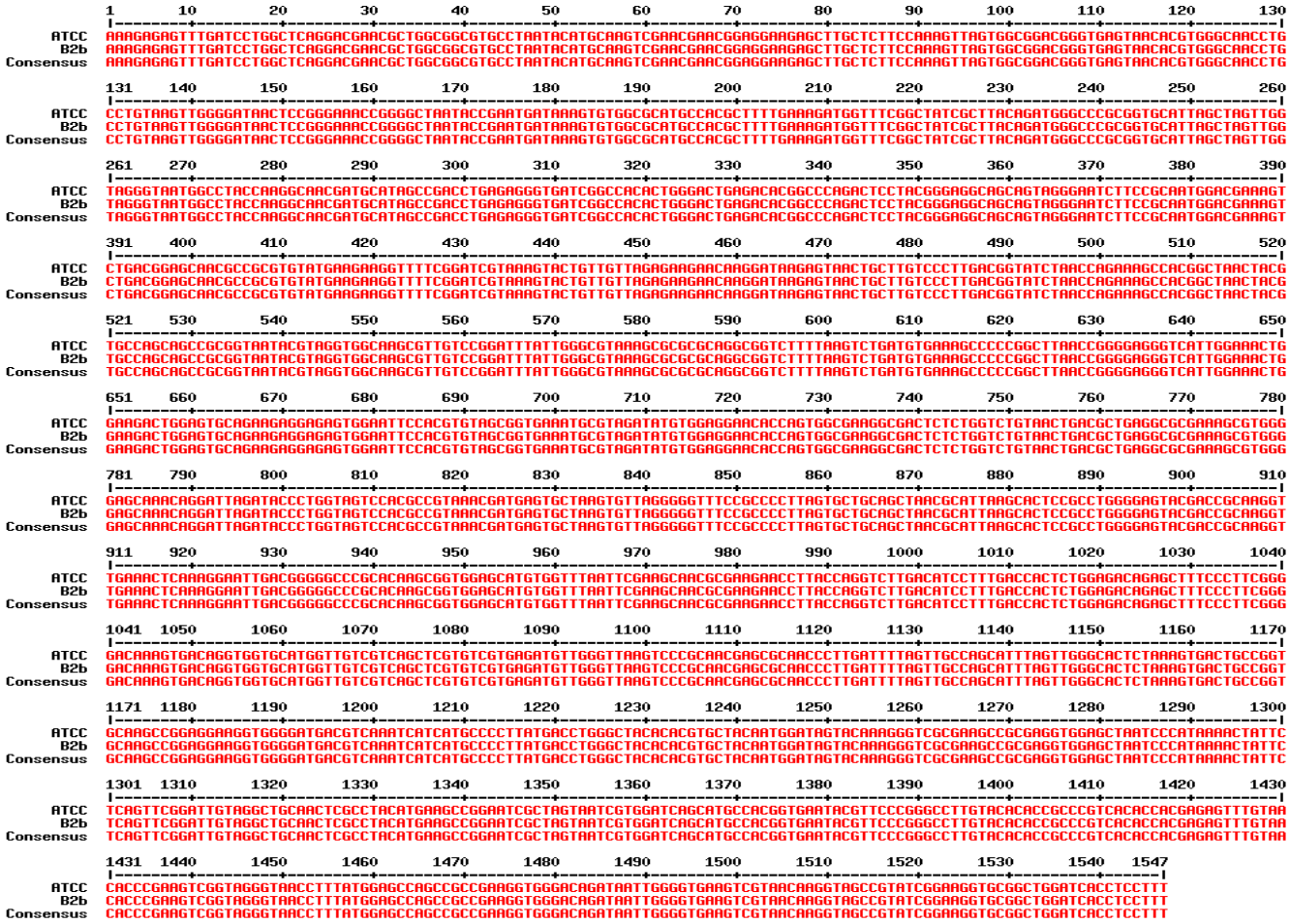

Figure 4.3: *16S rRNA* gene sequence alignment of *L. monocytogenes* B2b and ATCC 19115. Red-coloured text indicates identical sequences

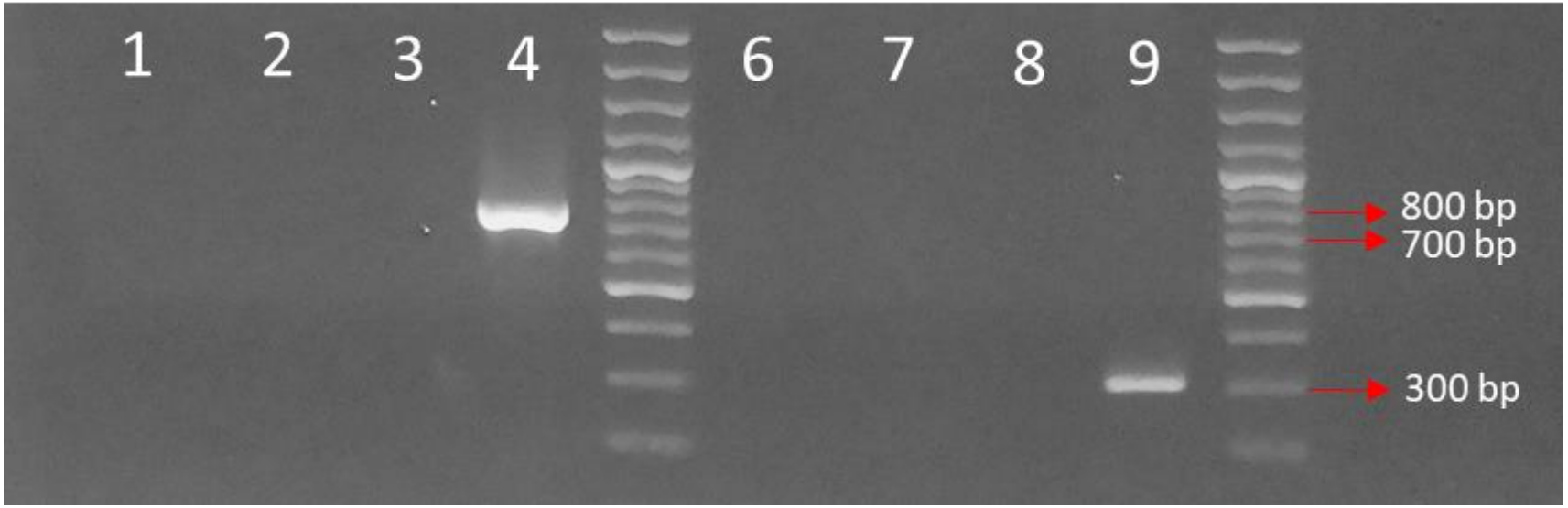

Figure 4.4: PCR screening of the genes encoding AAC (3')-IIa and ArmA.
The *aac (3')-IIa* gene PCR (expected size: 757 bp). Lane 1: NTC; lane 2: ATCC 19115; lane 3: B2b; lane 4: Positive control *E. coli* ESBL 184-379 isolated from a patient in 2015.
The *armA* gene PCR (expected size: 315 bp). Lane 6: NTC; lane 7: ATCC 19115; lane 8: B2b; lane 9: Positive control *K. pneumoniae* ESBL UVA 16-3 isolated from a patient in 2017.
Marker: 100-bp DNA ladder.

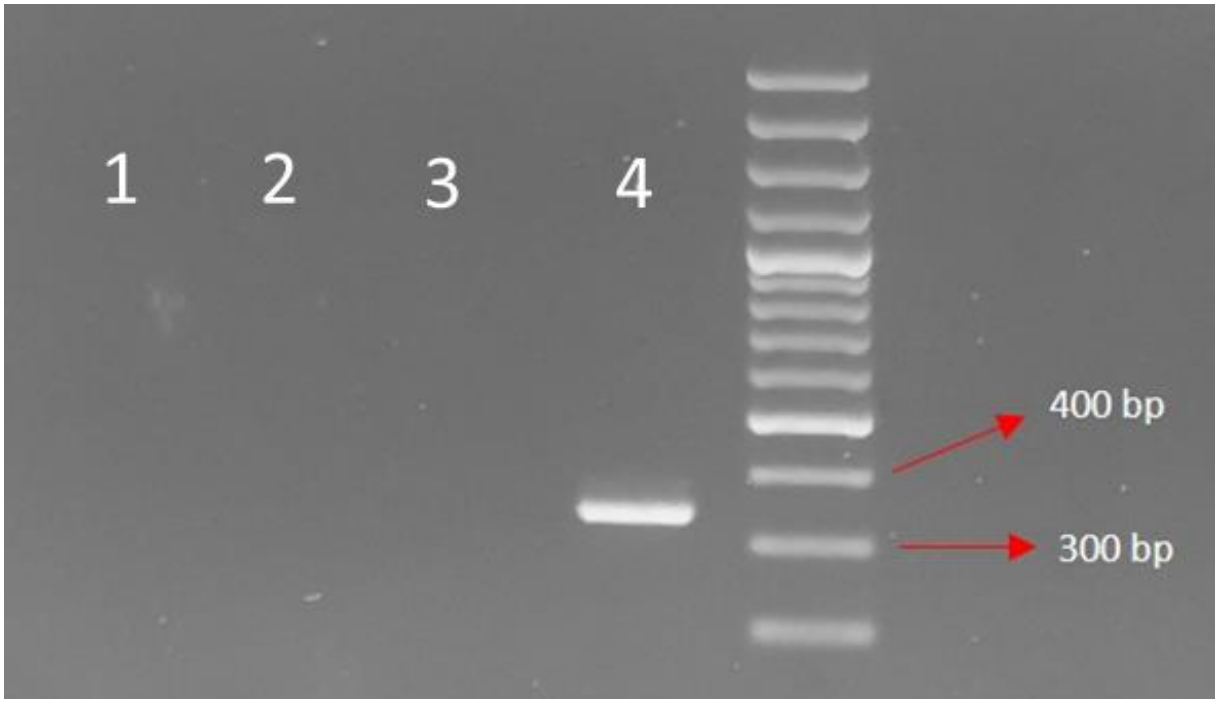

Figure 4.5: PCR screening of the gene encoding AAC (6')-APH (2").
The *aac (6')-aph (2")* gene PCR (expected size: 349 bp). Lane 1: NTC; lane 2: ATCC 19115; lane 3: B2b; lane 4: Positive control *E. faecium* NKS 31-3 isolated from a patient in 2017.
Marker: 100-bp DNA ladder.

4.2.6 Efflux inhibition assay

Table 4.5 depicts the testing for possible efflux involvement in the increase of resistance in B2B. An efflux pump inhibitor, reserpine, was added to the gentamicin broth microdilution assay. No changes in the MIC were observed for both ATCC 19115 and B2b, with and without the addition of reserpine.

Table 4.5: Efflux inhibition assay of ATCC 19115 and B2b.

Test strain	Gentamicin MIC (mg/L)
ATCC 19115	2.5
ATCC 19115 added with reserpine	2.5
B2b	40
B2b added with reserpine	40

4.3 Whole-genome sequencing and biological validation by reverse genetics

4.3.1 Whole-genome sequencing

The whole-genome sequencing of B2b was performed using the Illumina NovaSeq 6000 sequencing platform, and the result showed that there were 6,821,368 raw reads and 99.87% were effective reads after filtration. When the B2b reads were mapped with the reference genome (ATCC 19115) at an average depth of 266×, two mutations were observed in the following genes: *gdh* and *atpG2*. Using PCR, the same *gdh* mutation was also found in the laboratory ATCC 19115 and thus, the effect of this mutation on the function of the *gdh* gene was not further explored as it was unlikely to be the mutation associated with the gentamicin resistance (Figure 4.6). Further PCR verification showed that the 10-bp deletion in *atpG2* was found specifically in the B2b mutant (absent in ATCC 19115) (Figure 4.7). Raw sequencing reads of B2b were deposited in European Nucleotide Achieve (ENA) (accession number: PRJEB53473) (Figure 4.8).

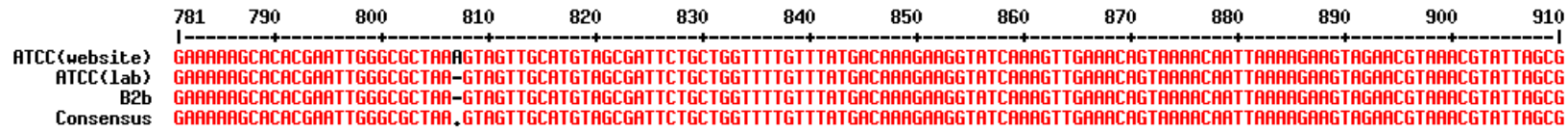

Figure 4.6: Multiple sequence alignment of partial *gdh* sequences from ATCC 19115 (downloaded from the ATCC website and amplified from the laboratory strain) and B2b. Sequencing reads of B2b were mapped to the ATCC 19115 genome downloaded from the ATCC website. The *gdh* gene sequence of the ATCC 19115 laboratory strain was different from the one downloaded at position 807. As a result, B2b, a mutant derived from the laboratory strain of ATCC 19115, also carried the same mutation in this gene.

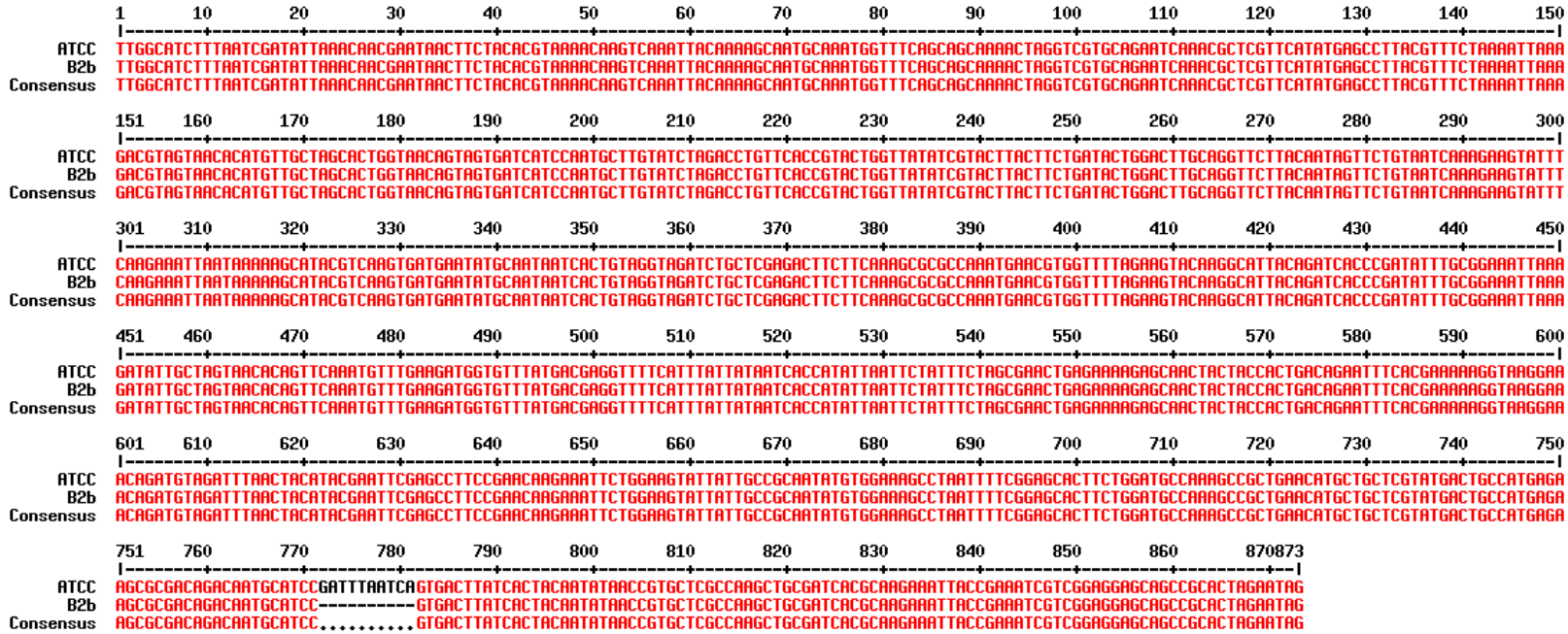

Figure 4.7: The 10-bp deletion in *atpG2* found in the B2b mutant, as compared to the *atpG2* of the wild-type ATCC 19115.

Figure 4.8: The raw sequencing data deposited in the European Nucleotide Archive (Accession number: PRJEB53473).

4.3.2 Biological validation by reverse genetics

The results from the complementation experiments indicated that, when the wild-type *atpG2* gene was cloned and transformed into the B2b mutant, the resistance phenotype was reverted back to its susceptible state (Figure 4.9). For further verification, the 10-bp deletion of *atpG*2, found in B2b, was introduced via allelic exchange into the wild-type ATCC 19115 strain. As expected, these reconstructed mutants from the allelic exchange experiment were resistant to gentamicin (Figure 4.10).

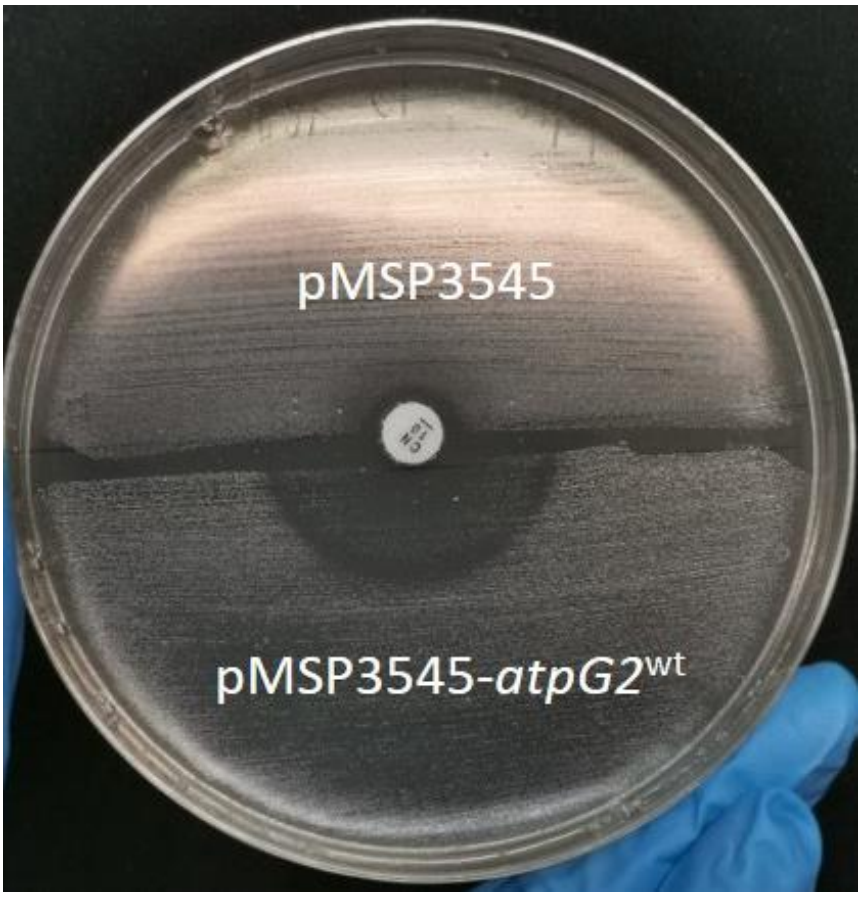

Figure 4.9: Stokes disk diffusion of B2b transformed with pMSP3545 as the empty plasmid control (top) vs B2b complemented with pMSP3545-*atpG2*^{wt} (bottom).

Figure 4.10: Stokes disk diffusion of reconstructed mutant of ATCC 19115 with the B2b mutation via allelic exchange (top) vs wild-type *L. monocytogenes* ATCC 19115 (bottom).

4.4 Further characterisations of B2b

4.4.1 Catalase test

Figure 4.11 showed the results from the investigation of the catalase activities in the B2b mutant and the wild-type ATCC 19115 strain. It has previously been shown that ATP can imitate catalase activities through the decomposition of H_2O_2 (Shi *et al.*, 2019). As the gamma subunit of ATP synthase is involved in ATP synthesis, the catalase activities of the B2b mutant and the wild-type ATCC 19115 strain were therefore investigated. Interestingly, B2b demonstrated a significantly lower catalase activity than ATCC 19115 (p-value = 0.02).

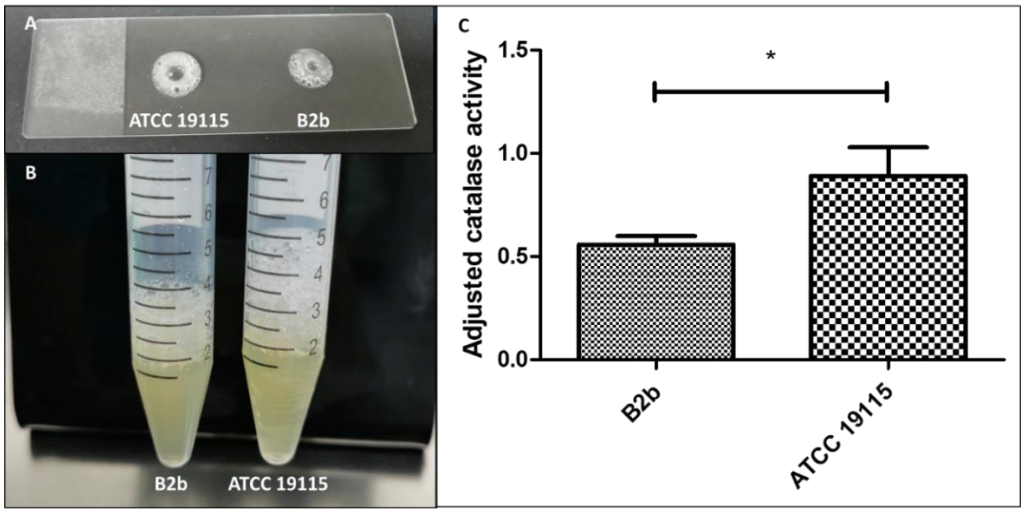

Figure 4.11: Catalase tests of B2b and ATCC 19115. (A) Qualitative catalase test. Equal volumes of the catalase reagent were dropped on a glass slide before equal volumes of standardised suspension of both strains were added using a multi-channel pipette. **(B) Quantitative catalase test.** The column of bubbles was lower in B2b as compared to that in ATCC 19115. **(C) Adjusted catalase activities.** The result in (B) was adjusted by the McFarland value of the 24 h bacterial growth (on a separate agar deep) resuspended in saline. After the volume of bubbles was adjusted by the number of bacterial cells (expressed in McFarland), a direct comparison between the two strains could be made. *p-value <0.05.

4.4.2 ATP chemiluminescence assay

The luciferase chemiluminescence assay results were shown in Figure 4.12. The ATP levels of B2b and ATCC 19115 were quantified to determine if the *atpG2* mutation in B2b could hamper the ATP production. The ATP level was found to be lower in B2b (p-value <0.001, fold difference = -1.6) than in ATCC 19115.

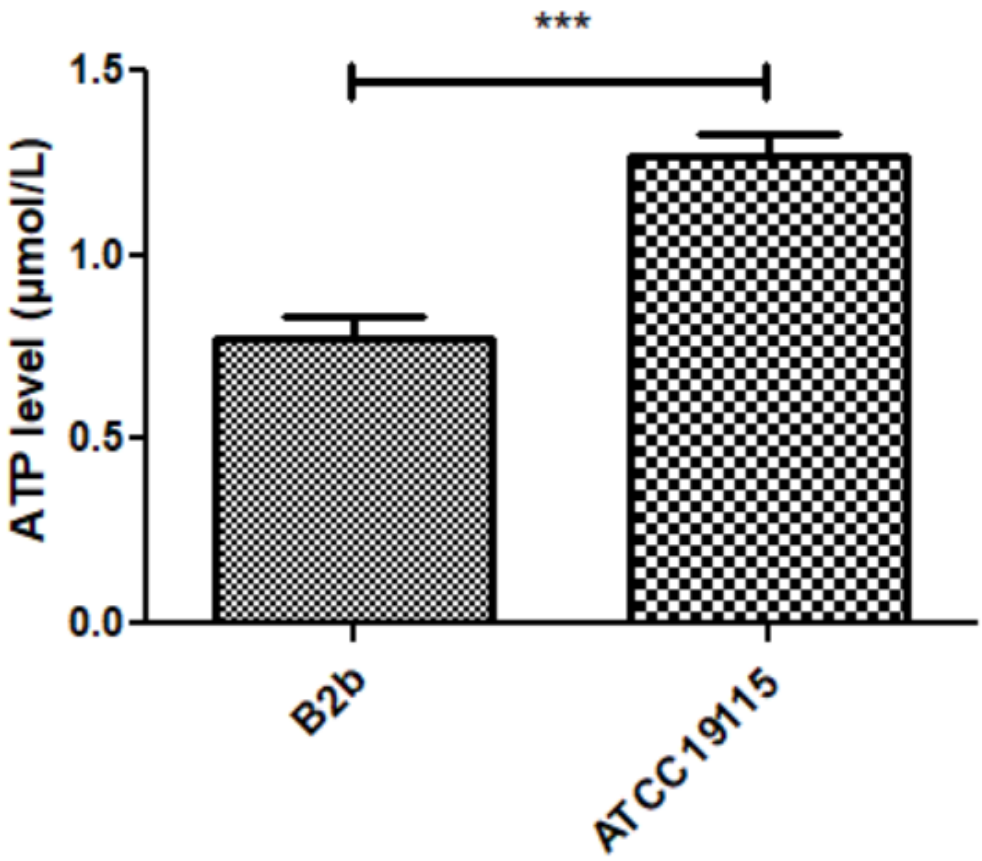

Figure 4.12: ATP levels of B2b and ATCC 19115. ***p-value <0.001.

4.4.3 pH assay

It was also noted that, B2b, possibly with a defect in the ATP synthase due to the *atpG2* mutation, did not grow as well as the wild-type ATCC 19115 on the medium with a lower pH (pH 5) (Figure 4.13). In addition, gentamicin inhibition zone sizes of B2b and ATCC 19115 were found to be decreased at pH 5 as compared to their zone sizes at pH 7 (Figure 4.14).

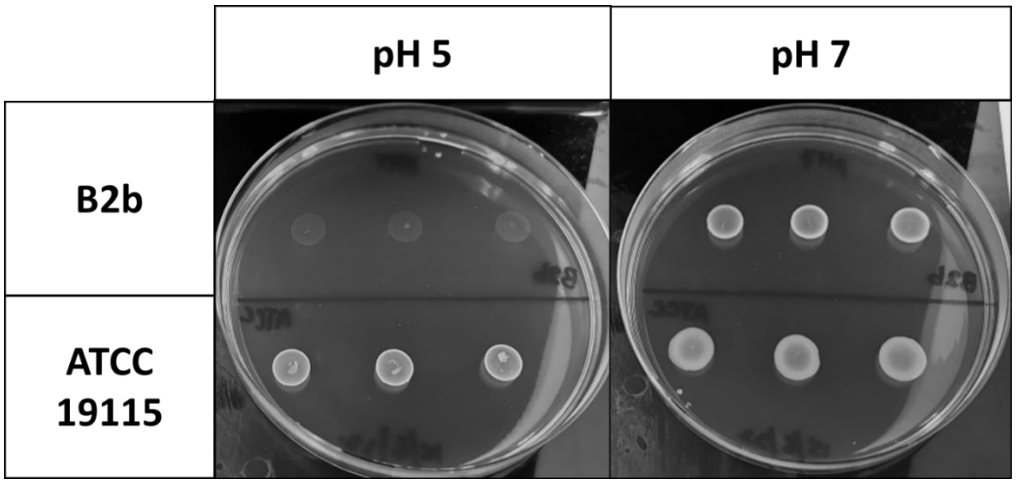

Figure 4.13: The overnight growth of B2b and ATCC 19115 on agar at different pH. The experiment at pH 7 served as the viable control to demonstrate that both strains were viable at the time of the experiment.

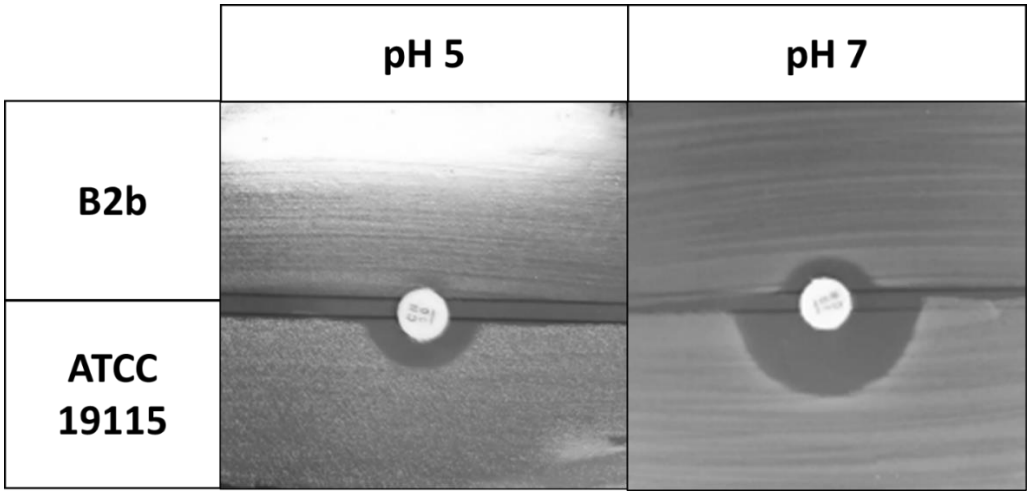

Figure 4.14: Gentamicin susceptibility of B2b and ATCC 19115 at pH 5 and 7. Zone diameters of both strains were smaller at pH 5 than 7. The ATCC 19115 zone size at pH 5 was approaching the B2b zone size at pH 7.

4.4.4 Growth rate

Figure 4.15 depicted the fitness cost of the gentamicin resistance, as determined by the assessment of the growth rates of B2b and ATCC 19115. B2b was found to have a slower growth rate and smaller colony size in comparison with ATCC 19115 after a 24 h incubation, indicating that the replication of B2b was impeded.

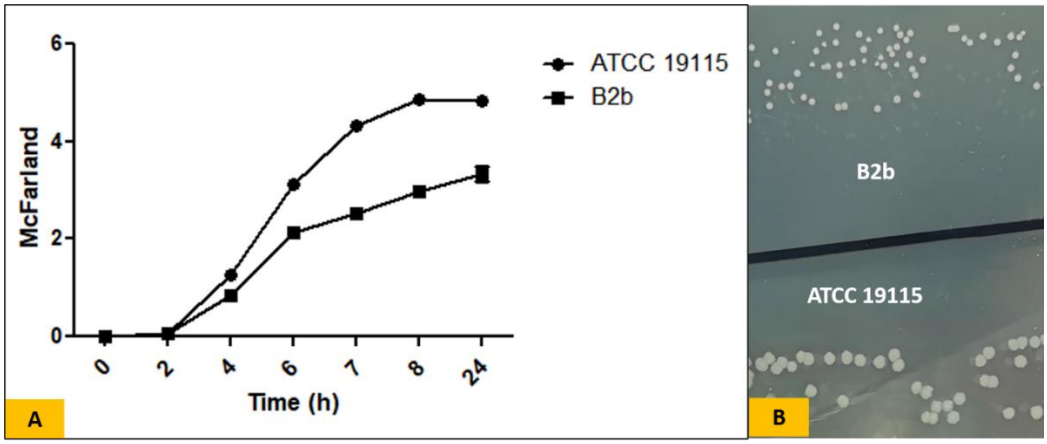

Figure 4.15: Growth rate of B2b and ATCC 19115. (A) Growth kinetics in broth. Each data point was presented as mean ± standard deviation. (B) Colonies of B2b (above) and ATCC 19115 (bottom) on a solid medium after a 24 h incubation.

4.5 Introduction of the *atpG2* mutation into *L. ivanovii*

Figure 4.16 showed the results of the investigation on whether the B2b mutation could cause gentamicin resistance in another *Listeria* species, where the mutation was introduced into the corresponding region of the *L. ivanovii* ATCC 19119 genome via allelic exchange. The presence of the 10-bp deletion in *atpG2* in the genomes of the recovered mutants of *L. ivanovii* was confirmed

with PCR and Sanger sequencing (Appendix C). Stokes disk diffusion showed that these *L. ivanovii* mutants were resistant to gentamicin.

Figure 4.16: Stokes disk diffusion of the allelic exchange mutant of *L. ivanovii* ATCC 19119 which carried the mutated *atpG2* gene with the 10-bp deletion orthologous to the deletion found in B2b (top) vs the wild-type *L. ivanovii* ATCC 19119 (bottom).

4.6 Screening of *atpG2* mutations in clinical and environmental isolates as well as other mutants

4.6.1 Screening of *atpG2* mutations in clinical and environmental isolates

Table 4.6 showed the results of 8 clinical or environmental *L. monocytogenes* isolates which were screened for the gentamicin resistance phenotype and the presence of *atpG2* mutations using Stokes disk diffusion and PCR-Sanger sequencing, respectively. Seven of these isolates were neither gentamicin-resistant (zone diameter: 23-28 mm) nor carrying any non-synonymous mutations. Only one isolate (12214A) was found to carry a point mutation in the DNA sequence that led to an amino acid substitution at position

135 (leucine to valine). However, the results indicated that the isolate was not resistant to gentamicin.

4.6.2 Screening of *atpG2* diversities in clinical and environmental isolates from a public database

Table 4.7 showed the output of 350 *atpG2* gene sequences downloaded from the complete genomes of *L. monocytogenes* in Genbank (between the years of 2002 and 2022). These isolates obtained globally were from human, animal, environment and food samples. Some genetic diversities in the *atpG2* sequences of these strains (96.45-100% similarity compared to the ATCC 19115 sequence) were observed. However, results indicated that none of them carried the 10-bp deletion found in the mutant B2b (Appendix D).

4.6.3 Screening of *atpG2* mutations in other selected spontaneous mutants

Table 4.8 showed the output of the screening of B1 and B2 mutants for *atpG2* mutations using PCR-Sanger sequencing. Other than B2b, most (85 % or 17/20) of the other selected spontaneous mutants from B1 and B2 series were also found to have mutations in the *atpG2* gene.

Table 4.6: DNA and amino acid sequence of mutation in *atpG2* of the clinical and environmental isolates.

Isolate	Origin of isolate	Inhibition zone size (mm)*	Mutation in *atpG2* (DNA sequence)	Mutation in *atpG2* (amino acid sequence)
LM Q01	Raw chicken meat, 2012	26	No mutation	No mutation
LM A17	Cervical swab, 2017	25	No mutation	No mutation
LM 0221A	Blood, 2021	23	No mutation	No mutation
LM 12214A	Cerebrospinal fluid, 2014	25	Point mutation at position 403 T → G	Amino acid substitution at position 135 L → V (leucine to valine)
LM 23719A	Tissue, 2019	28	No mutation	No mutation
LM 12115A	Blood, 2015	27	No mutation	No mutation
LM 5914A	Blood, 2014	27	No mutation	No mutation
LM 27717A	Ear swab, 2017	25	No mutation	No mutation

*The zone size of the parental strain, ATCC 19115 was 28 mm.

Table 4.7: Genetic diversity of *L. monocytogenes atpG2* genes downloaded from Genbank.

Genome accession(s)	Identity (%) *
CP054846.1, CP054040.1, CP053630.1, CP053628.1, CP053632.1, CP053478.1, CP053357.1, CP043177.2, CP044432.2, CP006596.2, CP044430.2, CP012021.2, CP023862.1, CP014252.2, CP016213.2, CP014250.2, CP007600.2, CP006046.4, CP045749.1, CP045751.1, CP045748.1, CP045745.1, CP045746.1, CP045747.1, CP032671.1, CP030810.1, CP030834.1, CP030809.1, CP030808.1, CP030807.1, CP030806.1, CP030805.1, CP030804.1, CP030803.1, CP041213.1, CP040988.1, CP041014.1, CP039751.1, CP031141.1, CP033612.1, CP031674.1, CP035187.1, CP031476.1, CP030101.1, CP011398.2, CP025219.1, CP025220.1, CP025565.1, CP028333.1, LT985475.1, LT985474.1, CP026043.1, CP023321.1, CP015508.1, CP023050.1, CP023052.1, CP016629.1, CP007169.1, LR698978.1, CP007167.1, CP007526.1, CP007525.1, CP007462.1, CP007461.1, CP007460.1, CP007459.1, CP008821.1, CP022020.1, CP015593.1, CP020022.1, CP019625.1, CP019624.1, CP019622.1, CP019620.1, CP019619.1, CP019616.1, CP019615.1, CP013289.1, CP013288.1, CP013285.1, CP006047.2, CP007686.1, CP011004.1, CP010346.1, CP009897.1, CP101619.1, CP087264.1, CP064373.1, CP092060.1, CP092059.1, CP006874.1, CP007210.1, CP006594.1, CP006600.1, CP006599.1, CP006598.1, CP006597.1, CP006592.1, CP076626.1, CP076375.1, CP076127.1, CP075871.1, CP050025.1, CP050024.1, CP050023.1, CP071154.1, LR999861.1, LR999860.1, CP018148.2, CP018149.2, CP046478.1, CP068979.1, CP068600.1, CP068601.1, CP067362.1, HF558398.1, FR733642.2, FR720325.1, FR733646.1, FR733645.1, FR733644.1, FR733643.1, CP003414.1, CP063382.1, CP063383.1, CP062129.1, CP062124.1, CP060526.1, GU067768.1, FM242711.1, AE017262.2	100.00

*Sequences downloaded from Genbank were compared with the atpG2 sequence of *L. monocytogenes* ATCC 19115.

Table 4.7: Genetic diversity of *L. monocytogenes atpG2* genes downloaded from Genbank (continued).

Genome accession(s)	Identity (%) *
CP020774.1	99.89
CP032672.1, CP076126.1	99.77
CP028183.1, CP098507.1, HG813249.1, CP076644.1, CP075878.1	98.74
CP076669.1, CP065028.1	98.51
CP054039.1, CP033738.1, CP069380.1	98.28
CP048401.1, CP032673.1, CP032670.1, CP009242.1, CP002816.1, FM211688.1, CP001175.1	98.17
CP013287.1, CP013286.1, FR733651.1	97.82
CP054042.1, CP045970.1, CP032669.1, CP007583.1, CP029175.1, CP038642.1, CP025221.1, CP025222.1, CP008773.1, CP008772.1, CP008771.1, CP008770.1, CP008769.1, CP008768.1, CP008767.1, CP008766.1, CP008765.1, CP008703.1, CP007527.1, CP008836.1, CP007021.1, CP007020.1, CP007019.1, CP007018.1, CP007017.1, CP007011.1, CP007010.1, CP007009.1, CP007008.1, CP007007.1, CP008837.1, CP007538.1, CP020833.1, CP020832.1, CP020831.1, CP019618.1, CP019617.1, CP019170.1, CP019167.1, CP019165.1, CP019164.1, CP018685.1, CP013919.1, CP013724.1, CP009258.1, CP001602.2, HG813247.1, CP006940.1, CP006862.1, CP006861.1, CP006860.1, CP006859.1, CP006858.1, CP075873.1, CP075872.1, CP075877.1, CP075874.1, CP050030.1, CP050029.1, CP050028.1, CP064843.1, CP063240.1, CP063381.1, CP002001.1, CP001604.1	97.48

*Sequences downloaded from Genbank were compared with the *atpG2* sequence of *L. monocytogenes* ATCC 19115.

Table 4.7: Genetic diversity of *L. monocytogenes atpG2* genes downloaded from Genbank (continued).

Genome accession(s)	Identity (%) *
CP048400.1, CP044429.1, CP032668.1, CP041211.1, CP033737.1, LR134400.1, LR134397.1, CP029372.1, CP028412.1, CP028413.1, CP028411.1, CP028410.1, CP028408.1, CP028409.1, CP028405.1, CP028406.1, CP028407.1, CP028404.1, CP028403.1, CP028402.1, CP028400.1, CP028401.1, CP028396.1, CP028394.1, CP028397.1, CP028395.1, CP028399.1, CP028398.1, CP028393.1, CP028392.1, CP025560.1, LT906436.1, CP007171.1, CP007170.1, CP020828.1, CP019623.1, CP011345.1, CP014790.1, CP014261.1, CP013722.1, CP007689.1, CP007688.1, CP007685.1, CP007684.1, CP092058.1, CP092056.1, CP092061.1, CP090054.1, CP090052.1, CP007160.1, CP076625.1, HG421741.1, CP050027.1, CP050129.1, CP050026.1, CP068392.1, FR733647.1, CP068150.1, CP062126.1, CP060435.1, CP060434.1, CP060433.1, CP060432.1, CP060431.1, CP060430.1, CP060429.1, CP058256.1, CP002002.1	97.37
CP046362.1, CP046361.1, CP045969.1, CP030837.1, CP021174.1, CP027029.1, LT985476.1, CP007200.1, CP007199.1, CP007198.1, CP007197.1, CP020830.1, CP020827.1, CP019614.1, CP013723.1, CP007687.1, CP011397.1, CP093220.1, CP092057.1, CP006593.1, CP006591.1, CP076125.1, CP076051.1, CP075876.1, CP075875.1, CP068599.1, FR733650.1, CP002004.1	97.25
CP023861.1, CP045972.1, CP030813.1, CP030836.1, CP030835.1, CP030870.1, CP030812.1, CP030811.1, CP025568.1, CP025567.1, CP021325.1, CP025443.1, CP025442.1, CP025440.1, CP025438.1, CP025259.1, CP025082.1, CP023752.1, CP023754.1, CP007196.1, CP007195.1, CP007194.1, CP061814.1, CP074104.1, CP025201.1, CP068977.1, FR733649.1, FR733648.1, CP002003.1, AL591983.1	97.14
CP054041.1, CP062127.1	96.68
CP090057.1, HE999705.1, HE999704.1	96.45

*Sequences downloaded from Genbank were compared with the *atpG2* sequence of *L. monocytogenes* ATCC 19115.

Table 4.8: Characteristics of 21 gentamicin-resistant mutants selected using the Luria-Delbrück experiment.

Mutant	Inhibition zone size (mm) [1]	Mutation in *atpG2* (gene sequence) [2]	Mutation in AtpG2 (amino acid sequence)
B1b	11	Substitution at position 367 (c → t)	Truncated protein with a premature stop codon at position 123
B1c	9		
B1d	10		
B1h	10		
B1i	11		
B1j	9		
B1e	10	Deletion (atgttgct) at position 164-171 (8 bp)	Frame shift at position 55 followed by a premature stop codon at position 58
B1f	10		
B1g	10	Insertion (attt) between positions 507 and 508	Frame shift at position 172 followed by a premature stop codon at position 174
B1k	10	Substitution at position 689 (t → a)	Amino acid substitution at position 230 I →N (isoleucine to asparagine)

[1] The zone size of the wild-type ATCC 19115 was 28 mm.

[2] The *atpG2* mutation in B2b was identified using WGS. *atpG2* mutations of the remaining mutants were screened with PCR.

Table 4.8: Characteristics of 21 gentamicin-resistant mutants selected using the Luria-Delbrück experiment (continued).

Mutant	Inhibition zone size (mm) [1]	Mutation in *atpG2* (gene sequence) [2]	Mutation in AtpG2 (amino acid sequence)
B2b	8	Deletion (gatttaatca) at position 772-781 (10 bp)	Frame shift at position 258
B2c	9	Substitution at position 367 (c → t)	Truncated protein with a premature stop codon at position 123
B2e	9		
B2g	9		
B2h	9		
B2k	10		
B2f	11	Substitution at position 388 (c → t)	Truncated protein with a premature stop codon at position 130
B2j	9		
B2d	9	No mutation	No mutation
B2i	10		
B2l	10		

[1] The zone size of the wild-type ATCC 19115 was 28 mm.

[2] The *atpG2* mutation in B2b was identified using WGS. *atpG2* mutations of the remaining mutants were screened with PCR.

CHAPTER 5

DISCUSSION

5.1 Mutant selection

In the present study, a total of 21 spontaneous mutants (Table 4.1) were selected from the wild-type *L. monocytogenes* ATCC 19115 using the Luria-Delbrück experiment. The isolation and characterisation of these mutants could help to elucidate potential mechanisms of gentamicin resistance in *L. monocytogenes*. They were recovered at a frequency of $5.2 \pm 0.92 \times 10^{-8}$ which, in accordance with the definition by Baquero *et al.* (2004), was considered as a weakly hypermutable frequency. This finding is in line with previous observations that gentamicin resistances were rare among *L. monocytogenes* strains (Baquero *et al.*, 2020). Similar mutation frequencies for single-step resistance had also been reported in *L. monocytogenes* exposed to rifampicin and trimethoprim (Morse *et al.*, 1999; Korsak and Krawczyk-Balska, 2017). Gentamicin, rifampicin, and trimethoprim are some of the antibiotics that can be used for the treatment of listeriosis. The development of *in vitro* mutants that are resistant to these antibiotics may indicate the potential emergence of resistant clinical strains that can compromise the effectiveness of drug therapies (Martinez and Baquero, 2000; Haeseker *et al.*, 2013).

5.2 Characterisations of B2b

5.2.1 Antimicrobial susceptibility testing

The 21 selected spontaneous mutants, with inhibition zone sizes ranging from 8 to 11 mm, were deemed resistant to gentamicin by the EUCAST guideline (sensitive ⩾18 mm, resistant <18 mm) (Figure 4.1). The inhibition zone sizes of these mutants were not prominently different from each other (differed by 3 or less mm). Nonetheless, due to budgetary constraints, only B2b, which had an inhibition zone size of 8 mm, was selected for further characterisations. B2b (gentamicin MIC: 40 mg/L, Table 4.5), was found to have a 16-fold increase in the gentamicin MIC as compared to the wild-type strain (2.5 mg/L), indicating that B2b exhibited a relatively low-level of gentamicin resistance as compared with the high-level gentamicin resistance (MIC >2000 mg/L) mediated by the aminoglycoside-modifying enzyme AAC(6')-APH(2") which was reported in *Enterococcus* spp. (Leclercq *et al.*, 1992; Sparo, Delpech and Allende, 2018). Apart from gentamicin, B2b was found to be cross-resistant to other aminoglycosides, including amikacin, kanamycin and neomycin (Table 4.2). This implies that the molecular determinant carried by this mutant might play an important role in resistances to different classes of aminoglycosides.

5.2.2 Biochemical tests and genotyping

Both ATCC 19115 and B2b demonstrated no differences in terms of their biochemical profiles (Table 4.3). Both strains were found to not have any

enzymatic activities of arylamidase. However, they were able to hydrolyse esculin, and acidify D-arabitol, L-rhamnose and methyl-αD-glucopyranoside. The α-mannosidase was also found to be present in both strains. Their identical biochemical profiles suggest that the molecular determinant in B2b might not be involved in the metabolic pathways of these carbon sources in *L. monocytogenes*. Meanwhile, the enzymatic activities of arylamidase, esculin hydrolysis, and D-arabitol, D-xylose, methyl-αD-glucopyranoside, D-ribose and glucose-1-phosphate acidification were observed in *L. ivanovii* ATCC 19119. These profiles corresponded exactly to the previously reported biochemical profiles of *L. monocytogenes* and *L. ivanovii* (Allerberger, 2003; Yehia, Ibraheim and Hassanein, 2016).

Using PCR and Sanger sequencing, the MLVA genotyping results showed that B2b had the same genotypes as ATCC 19115 across all the five recommended loci tested (Table 4.4). This, along with the results from the biochemical tests, suggests a clonal relationship between them, implying that B2b was unlikely to be an outcome of laboratory contamination but a true descendent of the wild-type *L. monocytogenes* ATCC 19115 through the experimental evolution.

5.2.3 Screening of previously reported resistance determinants

Upon the addition of reserpine, a commonly used efflux pump inhibitor in *L. monocytogenes* studies (Mata, Baquero and Pérez-Díaz, 2000; Godreuil *et al.*, 2003; Guérin *et al.*, 2014), no changes were observed in the gentamicin MICs of ATCC 19115 and B2b (Table 4.5). A previous study showed that the gentamicin

susceptibility of BC-adapted *L. monocytogenes* strains was restored after the addition of reserpine and proposed that the gentamicin resistance observed in these BC-adapted strains may be related to the outcome of efflux activities (Rakic-Martinez *et al.*, 2011). However, the efflux inhibition assay of the current study did not support this finding and indicated that reserpine-sensitive efflux proteins were unlikely to be responsible for the gentamicin resistance observed in the mutant B2b.

Through PCR screening, no mutations were detected in *16S rDNA* (encoding the molecular target of aminoglycosides, Figure 4.3) of B2b. In addition, other commonly reported gentamicin-resistance genes, such as those encoding AAC (3')-IIa, ArmA and AAC (6')-APH (2"), were also not found in both B2b and ATCC 19115 (Figure 4.4 and Figure 4.5). These results suggested that a novel *L. monocytogenes* determinant might be involved in the gentamicin resistance observed in B2b. The absence of the gene encoding AAC (6')-APH (2") also seemed to explain why B2b did not develop a high-level resistance against gentamicin (i.e. MIC >2000 mg/L), as reported previously in *Enterococcus* spp. (Leclercq *et al.*, 1992; Sparo, Delpech and Allende, 2018). Seeing that the phenotypic and molecular assays used in this study could not identify its resistance determinant, WGS of B2b was warranted to determine the potential mutation involved in the gentamicin resistance.

5.2.4 Whole-genome sequencing and biological validation by reverse genetics

Although two mutations were revealed by the WGS analysis (Figure 4.6), only the 10-bp deletion in the *atpG2* gene was found specifically in the B2b mutant (Figure 4.7), indicating that the mutation in this gene was very likely to be the cause of the gentamicin resistance.

To confirm the potential causal role of this mutation in the gentamicin resistance in B2b, biological validation by using reverse genetics were carried out via the complementation assay and allelic exchange mutagenesis. Through the complementation analysis, when the wild-type *atpG2* gene was introduced into the B2b mutant, the gentamicin susceptibility phenotype was restored (Figure 4.9). On the other hand, the reconstructed mutants, produced through the allelic exchange mutagenesis, were also found to be resistant to gentamicin (Figure 4.10). These consistent findings substantiate that the mutation in *atpG2* is the most probable cause of the gentamicin resistance observed in B2b.

5.2.5 Catalase and ATP chemiluminescence assays

The function of *atpG2* gene in *L. monocytogenes* is to encode the gamma subunit of the ATP synthase which is involved in the production of ATP. A catalase test was carried out because ATP was previously reported to have the ability to decompose hydrogen peroxide (Shi *et al.*, 2019). As expected, B2b, which harboured a nonsense mutation in the *atpG2* gene, was found to have a lower catalase activity than ATCC 19115 (Figure 4.11). However, one major limitation of this assay was that, it has to be assumed that the intrinsic catalase activities of B2b and ATCC 19115 were at a similar level. Dissimilar intrinsic

catalase activities would have interfered with the estimation of the ATP levels in the bacterial strains. However, in the present study, it was not possible to rule out this possibility. Thus, a direct measurement of ATP levels in B2b and ATCC 19115 was warranted by using the ATP chemiluminescence assay. This assay showed a significant reduction of ATP level in B2b (Figure 4.12) as compared to ATCC 19115. This implicates that the ATP synthesis was hampered in the B2b mutant due to the mutation in the *atpG2* gene.

5.2.6 pH assays

The growth of B2b was negatively affected when growing on a medium with a lower pH as compared to the wild-type strain (Figure 4.13). This observation is congruent with previous findings that ATP synthase maybe linked to pH homeostasis and thus, allowing foodborne pathogens, such as *L. monocytogenes* and *S. enterica* serovar Typhimurium, to survive in low pH environments (Foster and Hall, 1991; Cotter, Gahan and Hill, 2000). Therefore, it is plausible that B2b might have a reduced level of virulence as the *atpG2* mutation might severely hamper the survival of this foodborne pathogen in the acidic environment, such as in the stomach.

5.3 Potential mechanism of gentamicin resistance in B2b

Aminoglycosides, such as gentamicin, enter into bacterial cells through three key steps, by first increasing the permeability of the bacterial membrane followed by two energy-dependent processes (Ramirez and Tolmasky, 2010).

The uptake of aminoglycosides usually takes place at a higher membrane potential (Mates *et al.*, 1982). The mutated gamma subunit of the ATP synthase might lead to a decrease in the membrane potential of B2b, thus preventing the uptake of the antibiotic and resulting in the development of resistance.

It has previously been reported that the rate of ATP synthesis in bacteria can increase exponentially by increasing their membrane potential (Dimroth, Kaim and Matthey, 2000). Since the uptake of gentamicin occurs at a higher membrane potential (and thus the sensitivity), the ATP production should also be higher in wild-type ATCC 19115 (gentamicin sensitive) than B2b. In line with this hypothesis, the ATP level of ATCC 19115 was higher than the level of B2b (Figure 4.12), substantiating that the *atpG2* mutation might have caused a reduction in the membrane potential, leading to a decreased uptake of gentamicin in B2b.

To further confirm the association between the membrane potential and gentamicin uptake, both B2b and ATCC 19115 were subjected to gentamicin susceptibility testing at different pH. An acidic pH has been shown to be able to reduce the membrane potential and the uptake of gentamicin, causing the development of resistance in *S. aureus* (Mates *et al.*, 1982). Consistent with the finding by Mates *et al.* (1982), gentamicin inhibition zone sizes of B2b and ATCC 19115 decreased at pH 5 as compared to their zone sizes at pH 7 (Figure 4.14). This reiterates the importance of the bacterial membrane potential to the cellular uptake of gentamicin.

5.4 Fitness cost

As ATP is involved in the cellular respiration of bacteria, a defect in the ATP production could potentially slow down their growth rates. As expected, B2b was found to have a slower growth rate when growing in non-selective culture environments, indicating that the replication of B2b was impeded (Figure 4.15). This also suggests that the *atpG2* mutation might be conferring a fitness cost to the mutant. However, costly resistances are rarely observed among clinical isolates (Woodford and Ellington, 2007), suggesting the potential development of compensatory mutations or other feedback mechanisms. This may constitute an interesting angle for future studies on B2b.

5.5 Introduction of the *atpG2* mutation into *L. ivanovii*

Through allelic exchange mutagenesis, mutants of *L. ivanovii*, which harboured the 10-bp deletion at the region orthologous to the one in B2b, were recovered. Stokes disk diffusion showed that these *L. ivanovii* mutants were resistant to gentamicin (Figure 4.16), suggesting that the *atpG2* mutation found in *L. monocytogenes* B2b could also contribute to gentamicin resistance in another *Listeria* species. Apart from *L. monocytogenes*, *L. ivanovii* is another *Listeria* species known to cause human diseases. Although more common in the ruminants, *L. ivanovii* has been reported to cause gastroenteritis and bacteraemia in humans (Guillet *et al.*, 2010). The *atpG2* gene sequence of *L. ivanovii* is slightly different from that of *L. monocytogenes* (Appendix B), which might affect how the mutation impacted the function of the ATP synthase. This could

potentially explain why the orthologous mutation of B2b (B2b zone size: 8 mm, ATCC 19115 zone size: 28 mm, Figure 4.1) did not result in a similar magnitude of increase in gentamicin resistance in *L. ivanovii* (ATCC 19119-AE-B2b zone size: 14 mm, ATCC 19119 zone size: 25 mm, Figure 4.16).

5.6 Screening of *atpG2* mutations in other isolates

5.6.1 Clinical or environmental isolates

Eight *L. monocytogenes* isolates from clinical or environmental origins were screened for the gentamicin resistance and the presence of *atpG2* mutations. However, 7/8 of these isolates were neither gentamicin-resistant nor carrying any non-synonymous mutations (Table 4.6). Only one isolate (12214A) was found to carry a point mutation in the DNA sequence that led to an amino acid substitution at position 135 (leucine to valine) (Table 4.6). This change, however, did not lead to gentamicin resistance (23 mm, interpreted as sensitive based on the breakpoint described in Section Stokes disk diffusion). It is possible that the mutation found in 12214A did not change the membrane potential. This is consistent with a previous finding that not all mutations in *atpG* can lead to a reduction in aminoglycoside susceptibility (Aalap *et al.*, 2014).

In addition, through bioinformatic analysis, a varying degree of diversity (96.45 to 100 %) (Table 4.7) was observed among the 350 *atpG2* gene sequences downloaded from the public database Genbank (Appendix C). The 10-bp deletion carried by the mutant B2b, however, was not found in these sequences.

Unfortunately, gentamicin susceptibility/resistance patterns of these strains were not made available by the authors. Therefore, it was not possible for the current study to correlate the genetic diversity of *atpG2* with gentamicin susceptibility or resistance in these isolates.

5.6.2 Spontaneous mutants

Interestingly, besides B2b, most (85 % or 17/20) of the other selected spontaneous mutants from B1 and B2 series also have mutations in the *atpG2* gene (Table 4.8). These mutations were present in the form of substitution, deletion or insertion at different loci of the *atpG2* gene. As the B1 and B2 series of mutants arose independently of each other (Figure 3.1), the finding of *atpG2* mutations in most of the B1 (100 % or 10/10) and B2 (73 % or 8/11, including B2b) mutants appears to suggest that *atpG2* mutations could potentially be a major gentamicin-resistance determinant in *L. monocytogenes*. This should warrant future studies by expanding the number of replicates (of independent cultures) in the Luria-Delbrück experiment. In addition, it may also be interesting to investigate whether the *atpG2* mutations would still be a major resistance determinant when the gentamicin concentration is increased in the selection process. For the remaining three gentamicin-resistant mutants (B2d, B2i and B2l), in which mutations were not detected in the *atpG2* gene, further investigations are needed. One interesting follow-up would be to identify the promoter sequence of *atpG2* in these mutants, which may provide useful hints on whether the gentamicin resistances found in these mutants were due to altered gene expression levels of *atpG2*.

CHAPTER 6

CONCLUSION

In recent years, *L. monocytogenes* isolated from a variety of sources, including food, the environment, and human clinical samples, have become increasingly resistant to antibiotics, especially those often used to treat listeriosis (Olaimat *et al.*, 2018). Although gentamicin is one of the main antibiotics used for treatment, very few studies have been conducted to elucidate the molecular determinants of gentamicin resistance in *L. monocytogenes*. This study was carried out to identify possible genetic determinants that may be involved in the emergence of gentamicin resistance in this foodborne pathogen.

6.1 Potential novel genetic determinants of gentamicin resistance in *Listeria*

In summary, the findings from this study showed various mutations in the *atpG2* gene to be the cause of gentamicin resistance in *L. monocytogenes* exposed to the antibiotic in *in vitro* cultures. While the role of the gamma subunit of ATP synthase in conferring aminoglycoside resistance had been documented in *E. coli* (Humbert and Altendorf, 1989), it has never been reported in *Listeria*. This study also demonstrated that one of the mutations, when introduced by allelic exchange into another pathogenic species of *Listeria* (*L. ivanovii*), could also lead to gentamicin resistance.

Clinically, gentamicin resistance has rarely been reported in *L. monocytogenes*. However, the findings of the present study indicate that the development of gentamicin resistance is possible in this pathogen and *atpG2* mutations could be a cause of treatment failure in *Listeria* infections treated with gentamicin.

In addition, this study has resulted in a better understanding of mechanisms associated with antibiotic resistance in *Listeria*. A better understanding of resistance mechanisms in *L. monocytogenes* is essential for the clinical management of potentially life-threatening foodborne infections caused by this organism. By adding new gene targets to routine molecular drug susceptibility tests, it will be possible to quickly identify strains that are resistant to gentamicin and choose the best course of treatment. Through the development of new drugs or drug combinations based on resistance mechanisms, it can also help to curb the global spread of gentamicin resistance.

6.2 Limitations and future studies

Although the findings of this study had shown that mutations in *atpG2* could lead to gentamicin resistance in laboratory-selected mutants of *L. monocytogenes*, neither gentamicin resistance nor resistance-associated *atpG2* mutations were detected in the clinical isolates. Future endeavours should be made to collect and study more *Listeria* clinical isolates, especially those recovered from patients treated with gentamicin, which could harbour gentamicin resistance determinants that are clinically relevant.

Apart from that, it would also be interesting to explore the other subunits of the ATP synthase and their roles in aminoglycoside resistance in *Listeria*. Based on the findings of this study, it seems plausible that any loss-of-function mutations in genes encoding the components of ATP synthase that regulate the membrane potential would be able to contribute to the development of gentamicin resistance in *L. monocytogenes*. Besides that, to further verify the importance of the bacterial membrane potential to the cellular uptake of gentamicin, the B2b mutant and wild-type can be treated with a proton motive force inhibitor, such as carbonyl cyanide-m-chlorophenylhydrazone or ammonia sulphate, prior to the gentamicin susceptibility testing.

In addition, mutations in *atpG2* might also affect the ability of *L. monocytogenes* in establishing an infection. Therefore, for future studies, it would be interesting to investigate (1) how the *atpG2* mutations could impact the virulence of these mutants in animal studies and (2) if compensatory mutations could be developed in these mutants to offset the detrimental effects (e.g. fitness cost) of the gentamicin-resistance mutations.

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APPENDIX A

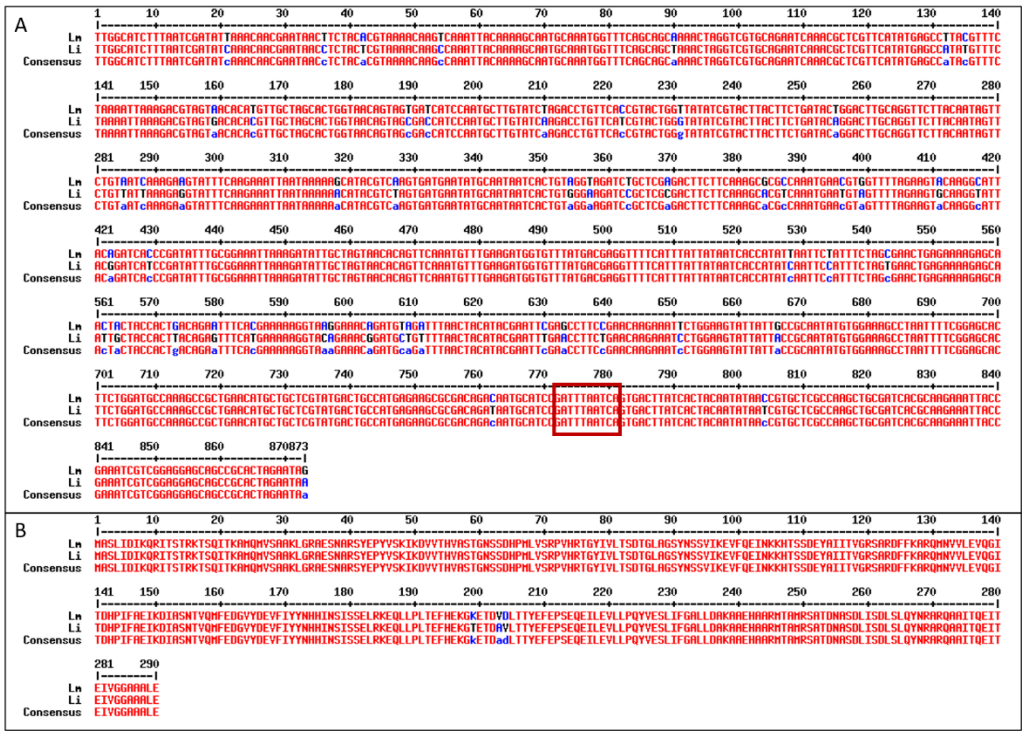
Preparation of culture media

Medium	Recipe (per L)	Remarks
Cation-adjusted Mueller-Hinton II agar	N/A	Pre-poured culture media
Cation-adjusted Mueller-Hinton II broth	22 g of powder	N/A
BHI agar	47 g of powder	N/A
BHI broth	37 g of powder	N/A
Listeria electro-competent cell growth medium (BHI with 0.5 M sucrose)	37 g of BHI broth powder 171.2 g of 1 M sucrose	N/A

The agar and broth were prepared using dehydrated culture media (in powder form). Distilled water was added to a final volume of 1 L and the media were sterilised by autoclaving at 121°C for 15 min. N/A: Not applicable.

APPENDIX B

The *atpG2* gene and amino acid sequences of *L. ivanovii* ATCC 19119 as compared to that of *L. monocytogenes* ATCC 19115

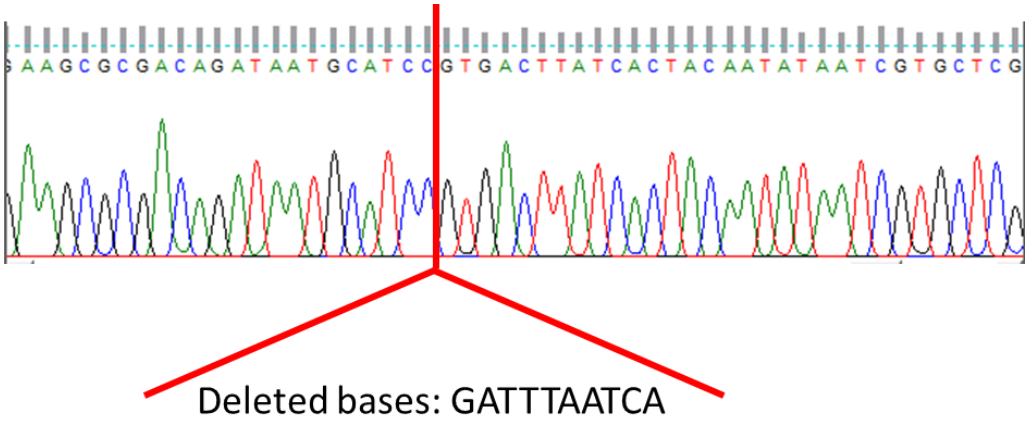


A. Difference between the *atpg2* gene sequences of *L. monocytogenes* ATCC 19115 (Lm) and *L. ivanovii* ATCC 19119 (Li).
B. Difference between the Atpg2 amino acid sequences of *L. monocytogenes* ATCC 19115 (Lm) and *L. ivanovii* ATCC 19119 (Li).

The red rectangle indicates the region of the 10-bp deletion found in *L. monocytogenes* B2b.

APPENDIX C

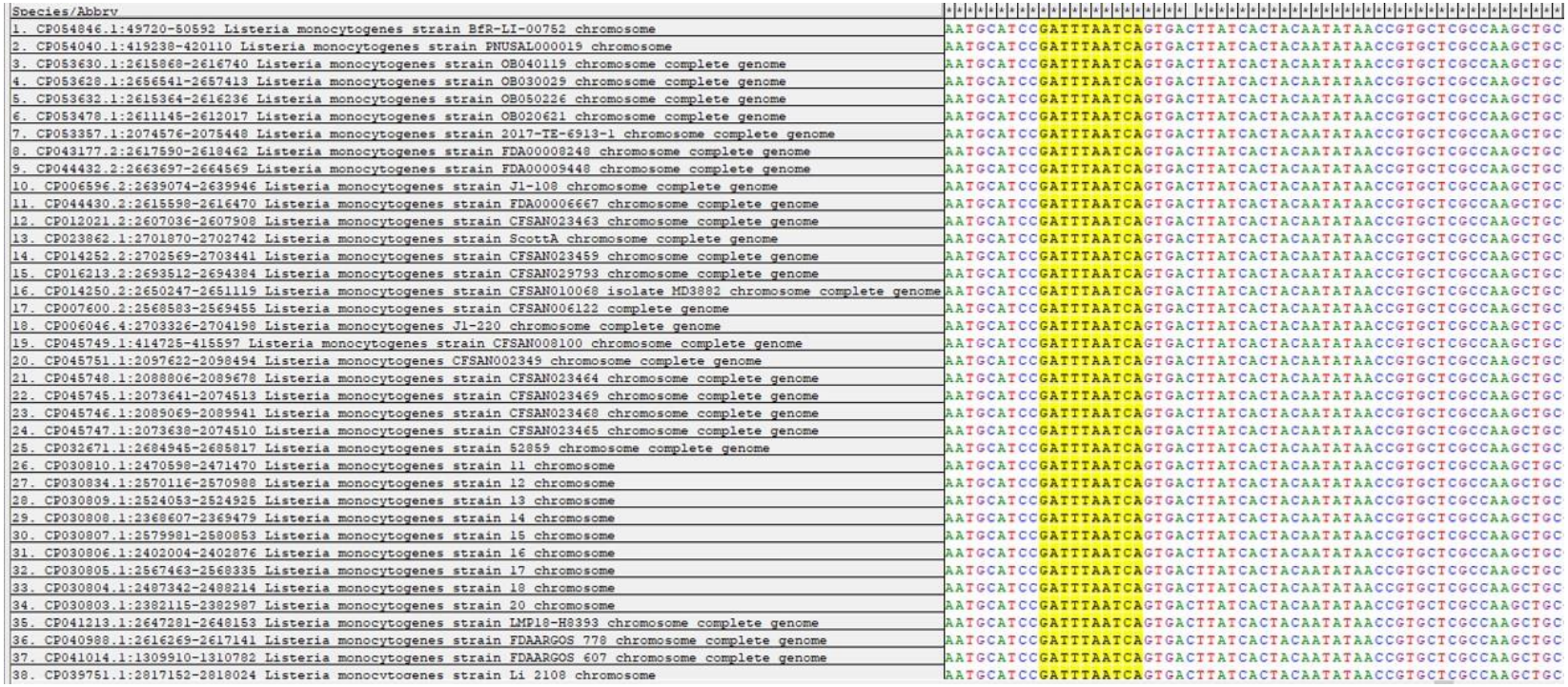
The presence of the 10-bp deletion in the *atpG2* gene of the recovered *L. ivanovii* mutants was confirmed with PCR and Sanger sequencing



The vertical red line indicates the region of the 10-bp deletion.

APPENDIX D

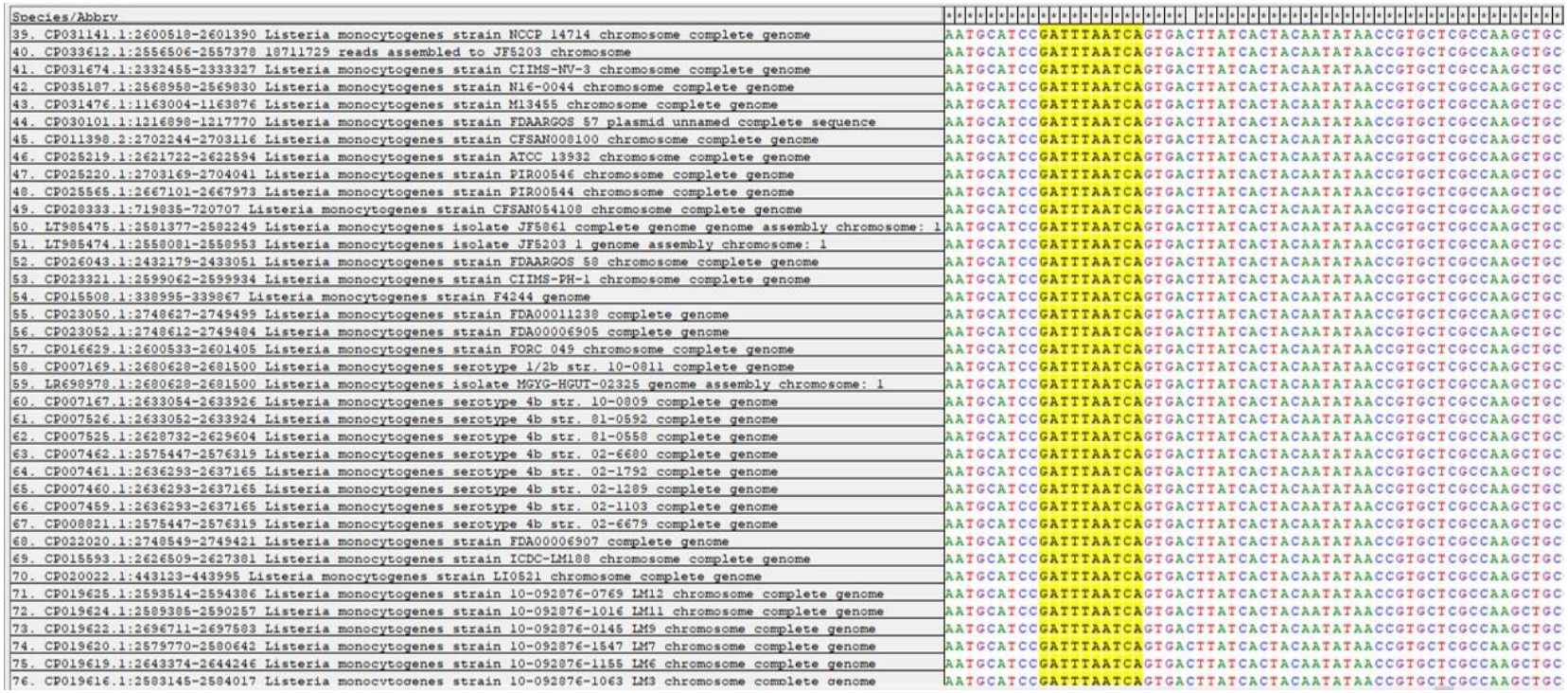
Multiple sequence alignment of 350 *atpG2* gene sequences downloaded from the public database Genbank



The highlighted sequences indicate the 10 bp which were deleted in *L. monocytogenes* B2b.

APPENDIX D

Multiple sequence alignment of 350 *atpG2* gene sequences downloaded from the public database Genbank (continued)

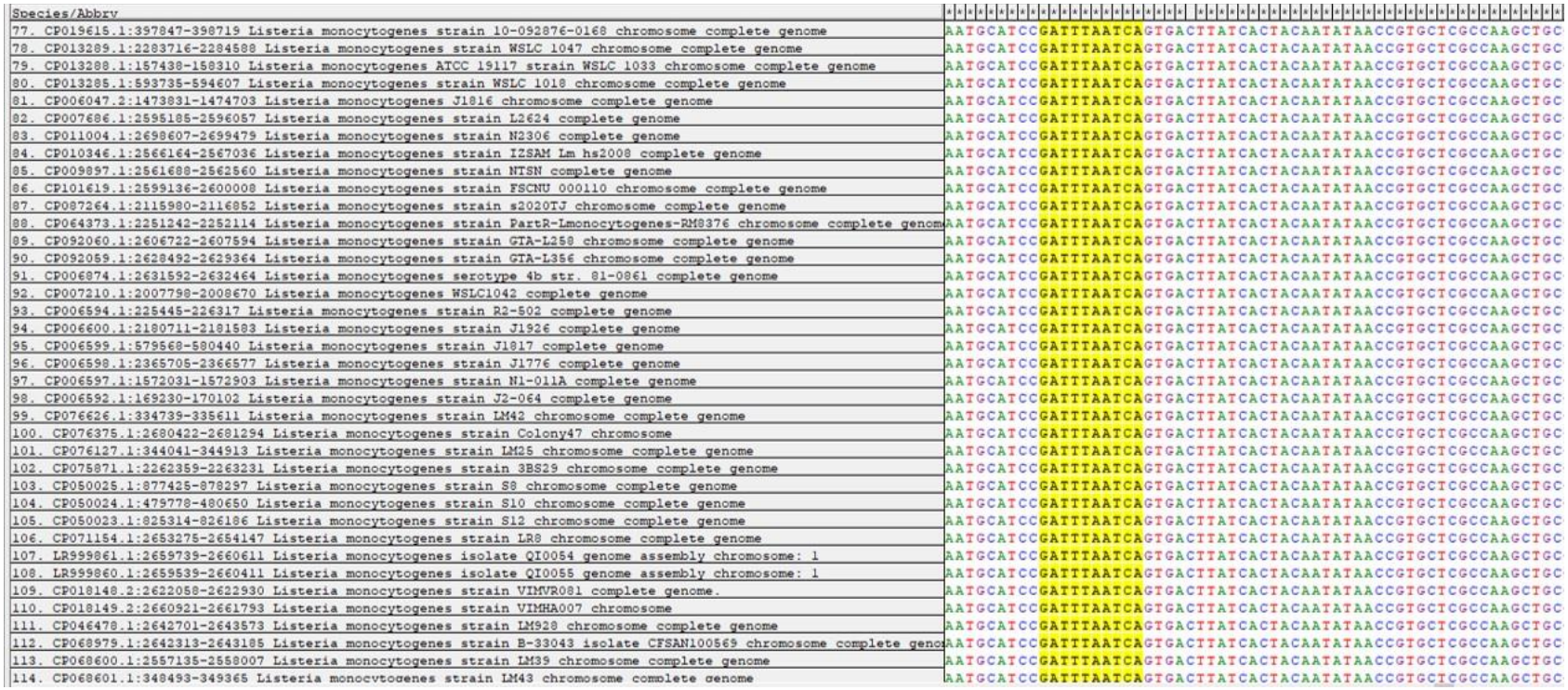


Species/Abbrv	
39. CP031141.1:2600518-2601390 Listeria monocytogenes strain NCCP 14714 chromosome complete genome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
40. CP033612.1:2556506-2557378 18711729 reads assembled to JF5203 chromosome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
41. CP031674.1:2332455-2333327 Listeria monocytogenes strain CIIMS-NV-3 chromosome complete genome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
42. CP035187.1:2568958-2569830 Listeria monocytogenes strain N16-0044 chromosome complete genome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
43. CP031476.1:1163004-1163876 Listeria monocytogenes strain M13455 chromosome complete genome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
44. CP030101.1:1216898-1217770 Listeria monocytogenes strain FDAARGOS 57 plasmid unnamed complete sequence	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
45. CP011398.2:2702244-2703116 Listeria monocytogenes strain CFSAN008100 chromosome complete genome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
46. CP025219.1:2621722-2622594 Listeria monocytogenes strain ATCC 13932 chromosome complete genome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
47. CP025220.1:2703169-2704041 Listeria monocytogenes strain PIR00546 chromosome complete genome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
48. CP025565.1:2667101-2667973 Listeria monocytogenes strain PIR00544 chromosome complete genome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
49. CP028333.1:719835-720707 Listeria monocytogenes strain CFSAN054108 chromosome complete genome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
50. LT985475.1:2581377-2582249 Listeria monocytogenes isolate JF5861 complete genome genome assembly chromosome: 1	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
51. LT985474.1:2558081-2558953 Listeria monocytogenes isolate JF5203_1 genome assembly chromosome: 1	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
52. CP026043.1:2432179-2433051 Listeria monocytogenes strain FDAARGOS 58 chromosome complete genome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
53. CP023321.1:2599062-2599934 Listeria monocytogenes strain CIIMS-PH-1 chromosome complete genome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
54. CP015508.1:338995-339867 Listeria monocytogenes strain F4244 genome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
55. CP023050.1:2748627-2749499 Listeria monocytogenes strain FDA00011238 complete genome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
56. CP023052.1:2748612-2749484 Listeria monocytogenes strain FDA00006905 complete genome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
57. CP016629.1:2600533-2601405 Listeria monocytogenes strain FORC_049 chromosome complete genome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
58. CP007169.1:2680628-2681500 Listeria monocytogenes serotype 1/2b str. 10-0811 complete genome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
59. LR698978.1:2680628-2681500 Listeria monocytogenes isolate MGYG-HGUT-02325 genome assembly chromosome: 1	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
60. CP007167.1:2633054-2633926 Listeria monocytogenes serotype 4b str. 10-0809 complete genome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
61. CP007526.1:2633052-2633924 Listeria monocytogenes serotype 4b str. 81-0592 complete genome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
62. CP007525.1:2628732-2629604 Listeria monocytogenes serotype 4b str. 81-0558 complete genome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
63. CP007462.1:2575447-2576319 Listeria monocytogenes serotype 4b str. 02-6680 complete genome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
64. CP007461.1:2636293-2637165 Listeria monocytogenes serotype 4b str. 02-1792 complete genome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
65. CP007460.1:2636293-2637165 Listeria monocytogenes serotype 4b str. 02-1289 complete genome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
66. CP007459.1:2636293-2637165 Listeria monocytogenes serotype 4b str. 02-1103 complete genome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
67. CP008821.1:2575447-2576319 Listeria monocytogenes serotype 4b str. 02-6679 complete genome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
68. CP022020.1:2748549-2749421 Listeria monocytogenes strain FDA00006907 complete genome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
69. CP015593.1:2626509-2627381 Listeria monocytogenes strain ICDC-LM188 chromosome complete genome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
70. CP020022.1:443123-443995 Listeria monocytogenes strain LI0521 chromosome complete genome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
71. CP019625.1:2593514-2594386 Listeria monocytogenes strain 10-092876-0769 LM12 chromosome complete genome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
72. CP019624.1:2589385-2590257 Listeria monocytogenes strain 10-092876-1016 LM11 chromosome complete genome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
73. CP019622.1:2696711-2697583 Listeria monocytogenes strain 10-092876-0145 LM9 chromosome complete genome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
74. CP019620.1:2579770-2580642 Listeria monocytogenes strain 10-092876-1547 LM7 chromosome complete genome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
75. CP019619.1:2643374-2644246 Listeria monocytogenes strain 10-092876-1155 LM6 chromosome complete genome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
76. CP019616.1:2583145-2584017 Listeria monocytogenes strain 10-092876-1063 LM3 chromosome complete genome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC

The highlighted sequences indicate the 10 bp which were deleted in *L. monocytogenes* B2b.

APPENDIX D

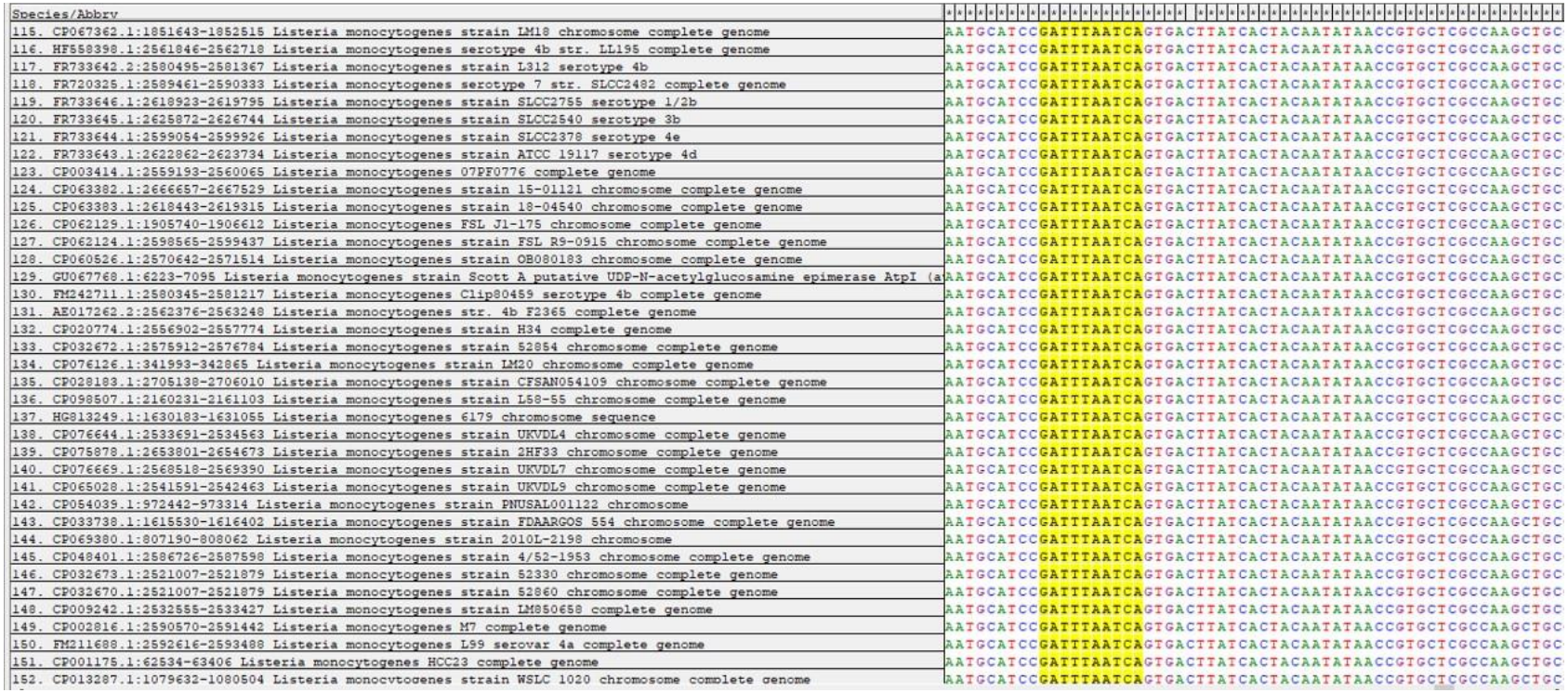
Multiple sequence alignment of 350 *atpG2* gene sequences downloaded from the public database Genbank (continued)



The highlighted sequences indicate the 10 bp which were deleted in *L. monocytogenes* B2b.

APPENDIX D

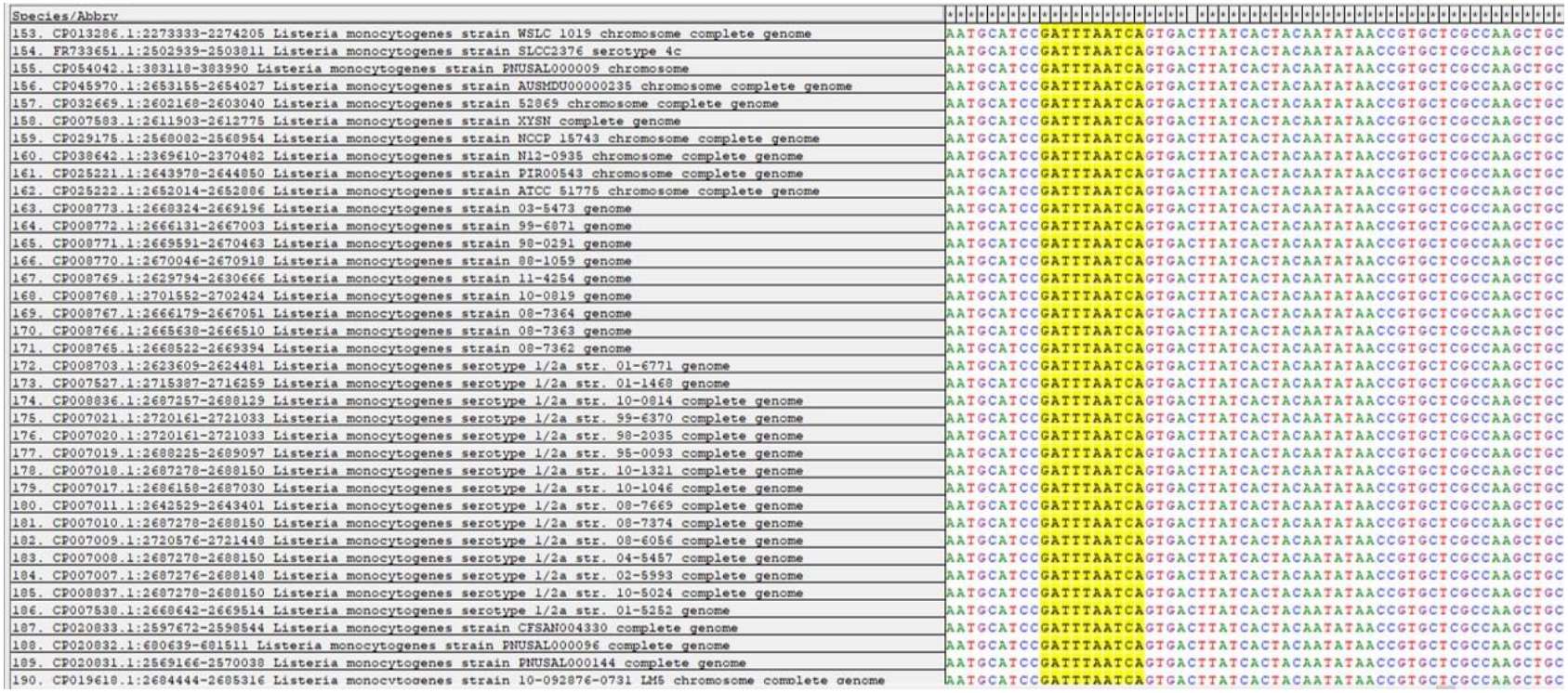
Multiple sequence alignment of 350 *atpG2* gene sequences downloaded from the public database Genbank (continued)



The highlighted sequences indicate the 10 bp which were deleted in *L. monocytogenes* B2b.

APPENDIX D

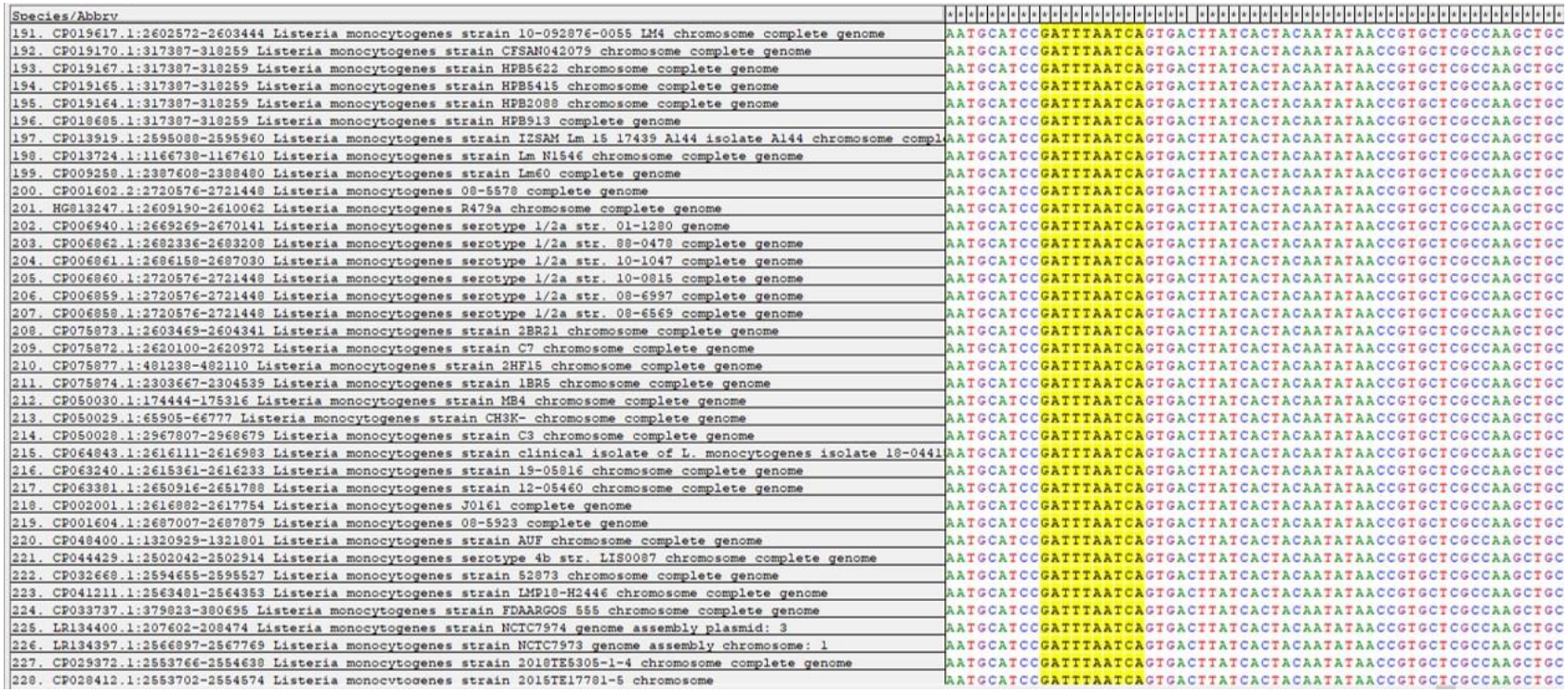
Multiple sequence alignment of 350 *atpG2* gene sequences downloaded from the public database Genbank (continued)



The highlighted sequences indicate the 10 bp which were deleted in *L. monocytogenes* B2b.

APPENDIX D

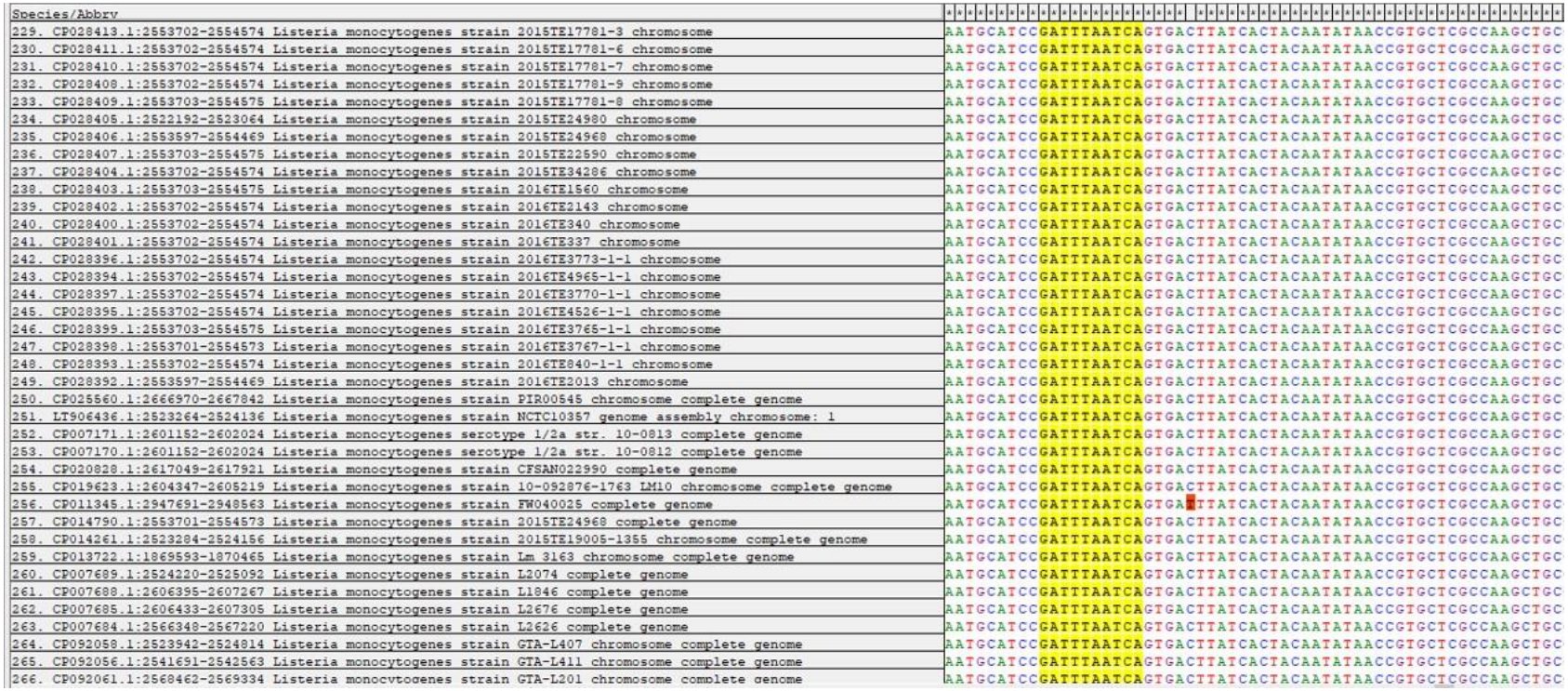
Multiple sequence alignment of 350 *atpG2* gene sequences downloaded from the public database Genbank (continued)



The highlighted sequences indicate the 10 bp which were deleted in *L. monocytogenes* B2b.

APPENDIX D

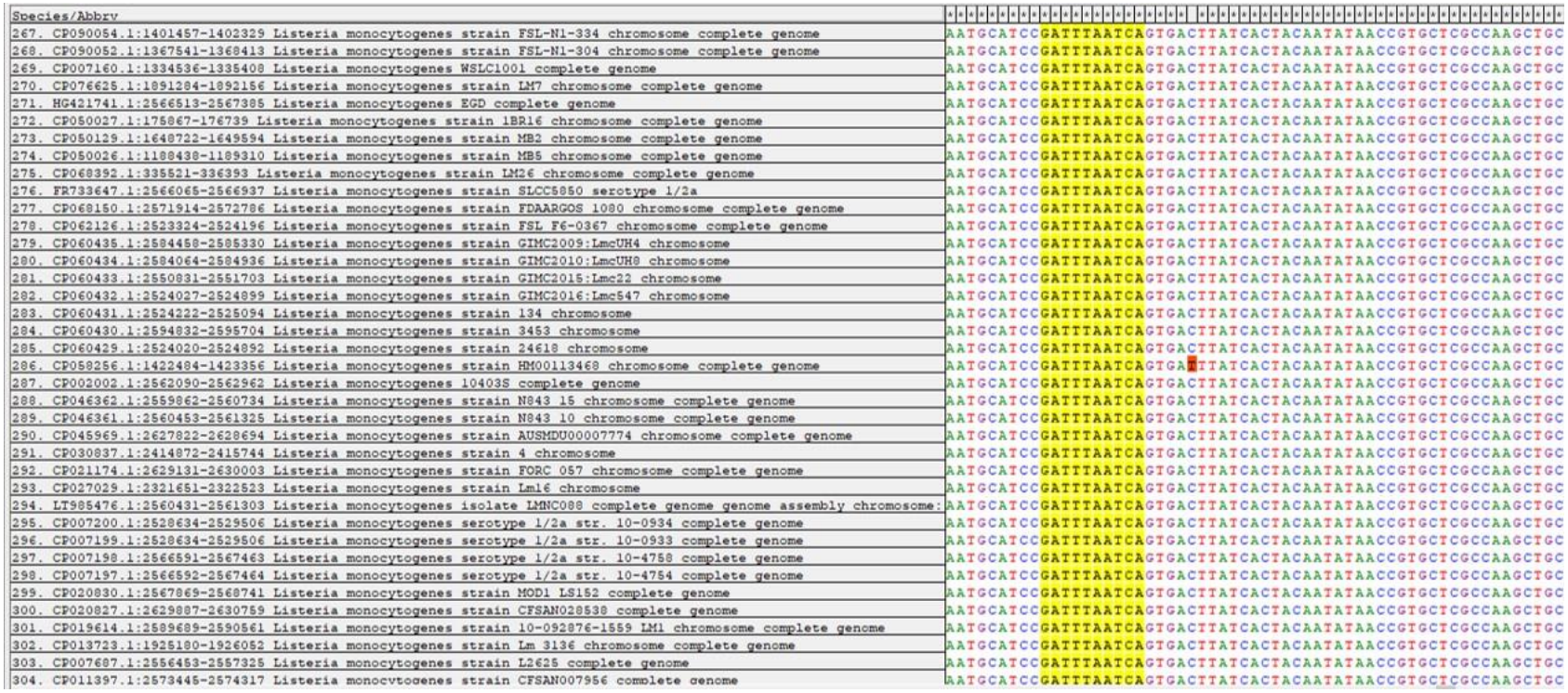
Multiple sequence alignment of 350 *atpG2* gene sequences downloaded from the public database Genbank (continued)



The highlighted sequences indicate the 10 bp which were deleted in *L. monocytogenes* B2b.

APPENDIX D

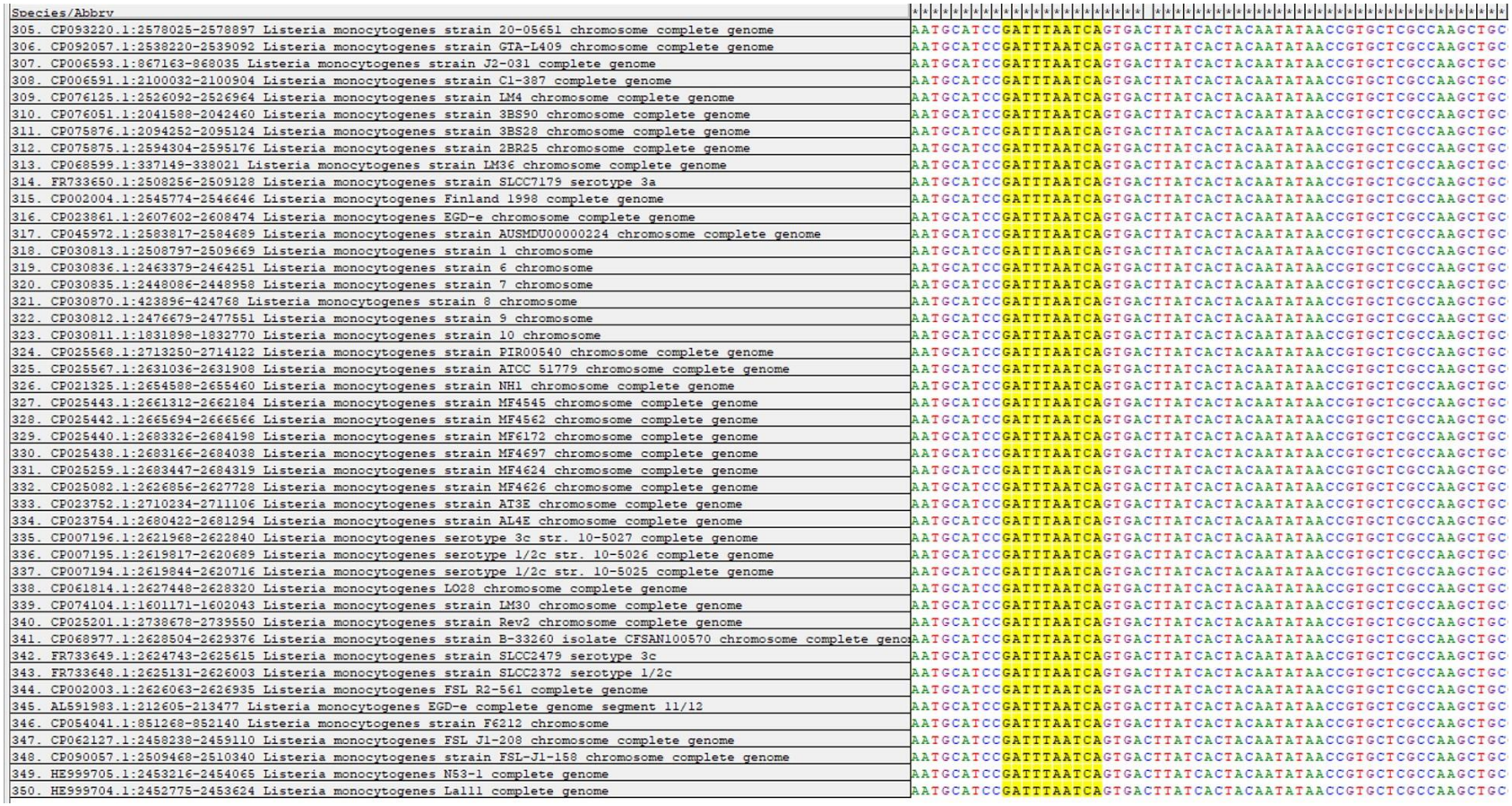
Multiple sequence alignment of 350 *atpG2* gene sequences downloaded from the public database Genbank (continued)



The highlighted sequences indicate the 10 bp which were deleted in *L. monocytogenes* B2b.

APPENDIX D

Multiple sequence alignment of 350 *atpG2* gene sequences downloaded from the public database Genbank (continued)



Species/Abbrv	
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306. CP092057.1:2538220-2539092 Listeria monocytogenes strain GTA-L409 chromosome complete genome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
307. CP006593.1:867163-868035 Listeria monocytogenes strain J2-031 complete genome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
308. CP006591.1:2100032-2100904 Listeria monocytogenes strain C1-387 complete genome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
309. CP076125.1:2526092-2526964 Listeria monocytogenes strain LM4 chromosome complete genome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
310. CP076051.1:2041588-2042460 Listeria monocytogenes strain 3BS90 chromosome complete genome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
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312. CP075875.1:2594304-2595176 Listeria monocytogenes strain 2BR25 chromosome complete genome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
313. CP068599.1:337149-338021 Listeria monocytogenes strain LM36 chromosome complete genome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
314. FR733650.1:2508256-2509128 Listeria monocytogenes strain SLCC7179 serotype 3a	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
315. CP002004.1:2545774-2546646 Listeria monocytogenes Finland 1998 complete genome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
316. CP023861.1:2607602-2608474 Listeria monocytogenes EGD-e chromosome complete genome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
317. CP045972.1:2583817-2584689 Listeria monocytogenes strain AUSMDU00000224 chromosome complete genome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
318. CP030813.1:2508797-2509669 Listeria monocytogenes strain 1 chromosome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
319. CP030836.1:2463379-2464251 Listeria monocytogenes strain 6 chromosome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
320. CP030835.1:2448086-2448958 Listeria monocytogenes strain 7 chromosome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
321. CP030870.1:423896-424768 Listeria monocytogenes strain 8 chromosome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
322. CP030812.1:2476679-2477551 Listeria monocytogenes strain 9 chromosome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
323. CP030811.1:1831898-1832770 Listeria monocytogenes strain 10 chromosome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
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326. CP021325.1:2654588-2655460 Listeria monocytogenes strain NH1 chromosome complete genome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
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329. CP025440.1:2683326-2684198 Listeria monocytogenes strain MF6172 chromosome complete genome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
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333. CP023752.1:2710234-2711106 Listeria monocytogenes strain AT3E chromosome complete genome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
334. CP023754.1:2680422-2681294 Listeria monocytogenes strain AL4E chromosome complete genome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
335. CP007196.1:2621968-2622840 Listeria monocytogenes serotype 3c str. 10-5027 complete genome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
336. CP007195.1:2619817-2620689 Listeria monocytogenes serotype 1/2c str. 10-5026 complete genome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
337. CP007194.1:2619844-2620716 Listeria monocytogenes serotype 1/2c str. 10-5025 complete genome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
338. CP061814.1:2627448-2628320 Listeria monocytogenes LO28 chromosome complete genome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
339. CP074104.1:1601171-1602043 Listeria monocytogenes strain LM30 chromosome complete genome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
340. CP025201.1:2738678-2739550 Listeria monocytogenes strain Rev2 chromosome complete genome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
341. CP068977.1:2628504-2629376 Listeria monocytogenes strain B-33260 isolate CFSAN100570 chromosome complete geno	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
342. FR733649.1:2624743-2625615 Listeria monocytogenes strain SLCC2479 serotype 3c	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
343. FR733648.1:2625131-2626003 Listeria monocytogenes strain SLCC2372 serotype 1/2c	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
344. CP002003.1:2626063-2626935 Listeria monocytogenes FSL R2-561 complete genome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
345. AL591983.1:212605-213477 Listeria monocytogenes EGD-e complete genome segment 11/12	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
346. CP054041.1:851268-852140 Listeria monocytogenes strain F6212 chromosome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
347. CP062127.1:2458238-2459110 Listeria monocytogenes FSL J1-208 chromosome complete genome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
348. CP090057.1:2509468-2510340 Listeria monocytogenes strain FSL-J1-158 chromosome complete genome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
349. HE999705.1:2453216-2454065 Listeria monocytogenes N53-1 complete genome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC
350. HE999704.1:2452775-2453624 Listeria monocytogenes La111 complete genome	AATGCATCCGATTTAATCAGTGACTTATCACTACAATATAACCGTGCTCGCCAAGCTGC

The highlighted sequences indicate the 10 bp which were deleted in *L. monocytogenes* B2b.

PUBLICATION AND SYMPOSIUM PARTICIPATION

The manuscript generated based on the findings of this study has been accepted for publication in the Journal of Medical Microbiology. In addition, a part of this study's data was presented during the NAIST-UM-USM-UTAR Joint Symposium Bioscience and Biotechnology on 1st of September, 2022.

Publication reference:

Ng, J. M. L. et al., 2022. Mutations in *atpG2* may confer resistance to gentamicin in *Listeria monocytogenes*, *Journal of Medical Microbiology*, 71(12) pp.1-9.