Plasmid-mediated high-level gentamicin resistance in bacteria for the turtle farm environment in Louisiana

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PLASMID-MEDIATED HIGH-LEVEL GENTAMICIN RESISTANCE IN BACTERIA FROM THE TURTLE FARM ENVIRONMENT IN LOUISIANA

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College
In partial fulfillment of the Requirements for the degree of Doctor of Philosophy

In

The Department of Biological Sciences

by

Maria Alejandra Diaz
B.S., Universidad Simon Bolivar, Venezuela 1993
December 2005
In memory of Ronald John Siebeling, Ph.D.
(1938-2002)
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v
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# TABLE OF CONTENTS

Dedication ...................................................................................................................................... ii  
Acknowledgements ...................................................................................................................... iii  
List of Tables .................................................................................................................................. x  
List of Figures .............................................................................................................................. xi  
Abstract ....................................................................................................................................... xiii  

Chapter 1: Introduction .................................................................................................................... 1  
1.1 Background - Gentamicin Resistance in Louisiana Pet Turtle Farms ....................................... 2  
1.2 Aminoglycoside Antibiotics ...................................................................................................... 5  
1.2.1 Clinical Use ...................................................................................................................... 6  
1.2.2 Mode of Action .............................................................................................................. 7  
1.2.3 The Ribosomal Target ................................................................................................. 9  
1.3 Resistance to Aminoglycosides .............................................................................................. 10  
1.3.1 Altered Target .............................................................................................................. 11  
1.3.1.1 Target-Site Mutations ....................................................................................... 11  
1.3.1.2 Enzymatic Modification of the Target Site ...................................................... 12  
1.3.2 Adaptive Resistance ..................................................................................................... 14  
1.3.3 Drug Impermeability: Reduced Uptake and Active Efflux of Antibiotics ...................... 14  
1.3.4 Enzymatic Modification of the Drug ............................................................................. 16  
1.3.4.1 Nomenclature of Aminoglycoside Modifying Enzymes ...................................... 17  
1.3.4.2 Aminoglycoside Acetyltransferases ..................................................................... 17  
1.4 High-Level Gentamicin Resistance (HLGR) ......................................................................... 20  
1.4.1 HLGR in Gram-Positive Organisms ............................................................................. 20  
1.4.2 Transfer of Gentamicin Resistance Genes in Gram-Positive Organisms ...................... 22  
1.5 Integrons .................................................................................................................................. 25  
1.5.1 Definition and Classes ................................................................................................. 25  
1.5.2 Class-1 Integrons: Structure ........................................................................................ 27  
1.5.3 Gene Expression ........................................................................................................... 30  
1.6 Conjugative Transposons in Gram-Negative Bacteria ........................................................... 32  
1.7 16S rRNA Methylases ............................................................................................................ 33  
1.8 Objectives ............................................................................................................................... 35  
1.9 Relevance of Study ................................................................................................................. 36  
1.10 References ............................................................................................................................ 36  

Chapter 2: Plasmid-Mediated High-Level Gentamicin Resistance Among Bacteria Isolated From Turtle Farms in Louisiana .................................................................................. 49  
2.1 Introduction ............................................................................................................................ 50  
2.2 Materials and Methods ......................................................................................................... 52  
2.2.1 Sample Recovery, Bacterial Isolation and Identification .............................................. 52  
2.2.2 Antibiotic Susceptibility Assays .................................................................................. 53  
2.2.3 R-Plasmid Isolation and Characterization ..................................................................... 56
2.2.4. Cloning and Identification of GEN-Resistance Gene(s) ...............................................58
2.2.5. Sequencing and Computer Analysis..............................................................................58
2.2.6. Screening for GEN Resistance Genes ...........................................................................59
2.3 Results ....................................................................................................................................61
   2.3.1. Gentamicin Resistance in Turtle Farms ........................................................................61
   2.3.2. R-plasmid Isolation .......................................................................................................64
   2.3.3. Identification of GEN Resistance Gene(s) ....................................................................64
   2.3.4. Distribution of GEN Resistance Genes in Bacterial Isolates from Turtle Farms ..........66
   2.3.5. Plasmid Heterogeneity...................................................................................................68
2.4 Discussion...............................................................................................................................68
2.5 References ..............................................................................................................................71

Chapter 3: Culture-Independent Identification of Bacteria Present in Gentamicin Solutions Used to Eradicate Salmonella spp. from Pet Turtles .................76
3.1 Introduction ............................................................................................................................77
3.2 Materials and Methods ...........................................................................................................78
   3.2.1. Sample Collection .........................................................................................................78
   3.2.2. DNA Extraction and Purification ..................................................................................79
   3.2.3. Amplification of 16S rRNA Genes from Bacterial Community DNA .........................79
   3.2.4. 16S rRNA Gene Library ................................................................................................80
   3.2.5. Amplification of 16S rRNA Genes ...............................................................................80
   3.2.6. ARDRA .........................................................................................................................81
   3.2.7. Partial Sequencing of Selected Clones ..........................................................................81
   3.2.8. 16S rRNA Gene Sequence Analysis .............................................................................81
3.3 Results and Discussion...........................................................................................................82
3.4 References ..............................................................................................................................90

Chapter 4: Integrons and Gene Cassettes in Bacteria from the Turtle Farm Environment ...........................................................................................................94
4.1 Introduction ............................................................................................................................95
4.2 Materials and Methods ...........................................................................................................97
   4.2.1. Bacterial Strains and Plasmids Used in This Study.......................................................97
   4.2.2. Antibiotic Susceptibility Assays....................................................................................97
   4.2.3. Screening for Genes Associated with Class-1 Integrons by PCR .................................97
   4.2.4. Sequencing and Computer Analysis............................................................................102
4.3 Results ..................................................................................................................................102
4.4 Discussion.............................................................................................................................104
4.5 References ............................................................................................................................109

Chapter 5: Overall Discussion and Conclusions ......................................................................114
5.1 References ............................................................................................................................137

Appendix A: Additional Data ......................................................................................................145

Appendix B: Letters of Permission ............................................................................................151

Vita ..............................................................................................................................................153
LIST OF TABLES

2.1. Bacterial Strains and Plasmids Used in This Study..............................................................54

2.2. Primers Used for PCR and Sequencing................................................................................60

2.3. Prevalence of Gentamicin Resistance Genes Among Bacterial Isolates From Turtle Farms ....................................................................................................................................62

2.4. R-Plasmids Isolated From Bacteria Recovered From Turtle Farms ....................................65

3.1. Organisms Identified by Partial Sequencing of 16S rRNA Genes.....................................85

3.2. Most Prevalent Genera Identified by Partial Sequencing of 16S rRNA Genes in Gentamicin Treatment Solutions From Five Different Locations in Louisiana in June, 2002...............................................................................................................................89

4.1. Bacterial Strains and Plasmids Used in This Study..............................................................98

4.2. Primers Used for PCR and Sequencing..............................................................................101

5.1. Conjugative Transfer of GEN Resistance Genes ...............................................................118

5.2. Aminoglycoside Modifying Enzymes Confering HLGR..................................................121

5.3. Ribosomal Methyltransferase Enzymes Confering HLAR...............................................122

5.4. General Characteristics of Integrons Class II, III and IV ..................................................128
LIST OF FIGURES

1.1. Distribution of Pet Turtle Farms in Louisiana.................................................................3
1.2. Chemical Structure of Gentamicin ..................................................................................7
1.3. Aminoglycoside Phosphotransferase Enzymes .............................................................19
1.4. Aminoglycoside Nucleotidytransferase Enzymes .........................................................19
1.5. Aminoglycoside Acetyltransferase Enzymes .................................................................19
1.6. General Structure of a Class-1 Integron ........................................................................28
2.1. HindIII and BamHI digestion profiles of R-plasmids ......................................................67
3.1. Selection of Appropriate Clones by M13 Amplification of Cloned 16S rRNA Gene Inserts..........................................................................................................................83
3.2. ARDRA ..........................................................................................................................84
3.3. Phylogenetic Tree of Organisms Identified in This Study ..............................................86
4.1. PCR Amplification Products of intI1 Gene.......................................................................103
4.2. PCR Amplification Products of Integron-Associated Genes intI1, aac(3)Ila, and aac(3)VIa .................................................................................................................................105
4.3. PCR Amplification Products Obtained With 5’CS-3’CS Primers ..................................106
5.1. Diagram of Transfer of GEN resistance ........................................................................118
5.2. PFGE of R-Plasmids .....................................................................................................119
5.3. General Structure of Complex Integrons ......................................................................135
A.1. Melt Curve Graph for Genes aac(3)Ila and rrn, Using SYBR-490 ..............................148
A.4. Melt Curve Graph for Gene aac(3)Ila, Using SYBR-490 ...........................................149
A.5. Real Time PCR Graph for Gene aac(3)Ila, Using SYBR-490 ....................................149
A.6. Melt Curve Graph for Gene *rrn*, Using SYBR-490 .......................................................... 150

A.7. Real Time PCR Graph for Gene *rrn*, Using SYBR-490 ................................................... 150
The sale of small turtles is banned from the US market due to concerns about their excretion of *Salmonella* spp. To produce a safe pet for the export market, the Louisiana pet turtle industry uses 1000 µg/ml gentamicin sulfate baths to eradicate *Salmonella* spp. from turtle eggs. In 1999, we analyzed bacterial samples recovered from turtle farms and found that strains of *Salmonella enterica* subsp. *arizonae* and other bacteria such as *Salmonella enterica* subsp. *enterica*, *Enterobacter cloacae*, *Citrobacter freundii*, *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia*, were resistant to high concentrations of gentamicin (>2000 µg/ml) and to other aminoglycosides. The goal of this study was to identify the gene(s) contributing to the high-level gentamicin resistance phenotype observed in bacteria from environmental samples with turtle farming activity, particularly the salmonellae, and to estimate their incidence in these bacteria, as well as to explore the molecular elements that contribute to the dissemination of gentamicin resistance in this context. R-plasmids from gentamicin resistant strains were transferred by conjugation and transformation to naïve *Escherichia coli* cells. Cloning and sequencing of the gentamicin resistance determinants on these plasmids revealed the presence of aminoglycoside acetyltransferase genes *aac(3)IIa* and *aac(3)VIIa*. Multiplex PCR assays showed that every gentamicin resistant isolate carried either one of these acetyltransferase genes. Pulsed-Field Gel Electrophoresis and restriction enzyme digestion analysis of R-plasmids carrying these genes revealed plasmids with sizes ranging from ca. 45 kb to 145 kb, with different restriction profiles, supporting their presence on mobile molecular elements. In effect, gene *aac(3)VIIa* was present as a gene cassette of a class 1 integron. Furthermore, evidence suggests that gene *aac(3)IIa* may be encoded as part of a complex class 1 integron. The presence of *aac(3)IIa* and *aac(3)VIIa* in mobile molecular elements explains the distribution of these genes in the different plasmids and organisms.
described in this study. Further studies are required to characterize these molecular elements, and to detect genes which may be co-selected in the presence of gentamicin. This work is one of the first to report dissemination of high-level gentamicin resistance in Gram-negative bacteria, particularly in environmental isolates of *Salmonella enterica* subsp. *arizonae*. 
CHAPTER 1: INTRODUCTION
1.1 Background - Gentamicin Resistance in Louisiana Pet Turtle Farms

The pet turtle industry began in the 1930s, with the sale of hatchlings in the United States pet market. In 1974, the sale of baby turtles was banned by the Food and Drug Administration (FDA) in the domestic market due to concerns about their excretion of the human pathogen *Salmonella* spp., etiological agent of Salmonellosis or “Salmonella food poisoning” (Siebeling, R.J., *et al*, 1975b; Cohen, M.L., *et al*, 1980; Michael-Marler, S., *et al*, 1983; Siebeling, R.J., *et al*, 1984; Izadjoo, M.J., *et al*, 1987; D’Aoust, J.Y., *et al*, 1990; Shane, S.M., *et al*, 1990; Ebani, V.V., *et al*, 2005). In spite of the domestic ban on the sale of small carapace turtles, the export market has continued to thrive. Thus, around 12 million hatchlings enter the export market each year to Western Europe, South America and Asia (Japan, China). The economic impact of the industry in the State of Louisiana is estimated around 30 million US dollars. The main geographical areas in Louisiana where turtle farming occurs are Pierre Part, Jonesville and Ponchatoula (*Figure 1.1*) (Siebeling, R.J., *et al*, 1975b; Siebeling, R.J., *et al*, 1975a; Michael-Marler, S., *et al*, 1983; Siebeling, R.J., *et al*, 1984).

In order to eradicate *Salmonella* spp. from turtle eggs, farmers follow a modified version of the protocol used in the turkey industry to treat eggs, the pressure-differential egg-dip procedure (Saif, Y.M. and Shelly, S.M., 1973; Greenfield, J., *et al*, 1975; Nivas, S.C., *et al*, 1975; Ekperigin, H.E. and McCapes, R.H., 1977; Ekperigin, H.E., *et al*, 1983). This modified, FDA-approved treatment is summarized as follows: 1) Every day from April through September, eggs are picked from fresh nests; at this point each egg harbors approximately 10-75 million bacteria (Siebeling, R.J., *et al*, 1984). 2) Eggs are immersed in a Clorox bath at 380 ppm (1 oz/gallon) for 10-15 min.; this sanitation step reduces bacterial counts to 10-50,000 per egg (Siebeling, R.J., *et al*, 1984). 3) Sanitized eggs are treated in baths of gentamicin sulfate (Garasol; Shering Corp., Bloomfield, N.J.), at 1000 ppm (1000 µg/ml), using the pressure-
differential method (Siebeling, R.J., *et al.*, 1975b; Michael-Marler, S., *et al.*, 1983; Siebeling, R.J., *et al.*, 1984). It is mandatory in the State of Louisiana to treat eggs with this antibiotic in order to eradicate *Salmonella* spp. Gentamicin sulfate was chosen for the treatment of turtle eggs since it presents various advantages at a commercial turtle farm; notably, it does not lose its antimicrobial activity when in solution or when exposed to freezing or auto-claving temperatures, allowing it to be reused after appropriate readjustments are made (Michael-Marler, S., *et al.*, 1983; Siebeling, R.J., *et al.*, 1984). 4) After a 60 day incubation period, turtles hatch, and before they can be exported, representative samples from each farm must be tested for

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**Figure 1.1: Distribution of Pet Turtle Farms in Louisiana**

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3
Salmonella spp. This test, called “Certification”, is done by independent FDA-certified laboratories; it involves the enrichment, isolation, and identification of Salmonella spp. through classic bacteriological and molecular assays (Siebeling, R.J., et al, 1975a; Michael-Marler, S., et al, 1983; Siebeling, R.J., et al, 1984).

S. enterica subsp. enterica serovar Enteritidis is the major cause of Salmonella food poisoning in the U.S., since it is a frequent contaminant of chicken eggs (Cohen, M.L., et al, 1980; Carattoli, A., 2003). Salmonella spp. is a mesophilic, Gram-negative, motile bacillus, bacteriologically characterized as a non-lactose fermentor, H₂S-positive, lysine-decarboxylase-positive microorganism. S. enterica subsp. arizonae, which commonly inhabits the cloacae of reptiles, can be readily differentiated bacteriologically from other salmonellae since it is a delayed lactose fermentor (Siebeling, R.J., et al, 1975a; Siebeling, R.J., et al, 1984; Ebani, V.V., et al, 2005).

It is well known that the use of any antibiotic presents a life cycle where resistance to the particular antibiotic will generally follow its implementation or use (Davies, J. and Smith, D.I., 1978; Levy, S.B., 1998; Mazel, D. and Davies, J., 1999; Levy, S.B., 2002). Gentamicin resistance in Salmonella spp. isolated from pet turtles was originally reported in Canada, in 1990, a study stating that turtles exported from Louisiana to Canada carried gentamicin-resistant Salmonella spp. (D'Aoust, J.Y., et al, 1990). Concerns of the emergence of gentamicin-resistant S. enterica subsp. arizonae, which could survive gentamicin treatment, had been previously reported in the turkey industry (Ekperigin, H.E., et al, 1983; Hirsh, D.C., et al, 1983). This gentamicin resistance problem was later addressed at L.S.U. in collaborative work between the laboratories of E.C. Achberger, and R.J. Siebeling (Jenson, M.A.B., 1992). This work falls under the umbrella of such collaboration (Diaz, M.A., et al, in press).
In July of 1999, 11 of 16 turtle farms in Pierre Part had shipment lots fail certification. Bacterial isolates were obtained for further studies from failed-lot turtle samples provided by the Central Analytical Laboratories, Belle Chase, LA. Identification by standard bacteriological assays and by API-20E (bioMérieux SA, Marcy l’Etoile, France) confirmed the presence of *S. enterica* subsp. *arizonae*. All isolates tested were resistant to concentrations of gentamicin >2000 µg/ml, which is higher than the concentration used to treat the eggs. Other bacteria, such as *Enterobacter cloacae*, *Citrobacter freundii*, *Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa*, among others, were also found to have this level of gentamicin resistance. We became interested in studying the high-level gentamicin resistance expressed by these bacteria since this resistance had not been reported in Gram-negative bacteria (Diaz, M.A., *et al*, 2000).

1.2 Aminoglycoside Antibiotics

Antibiotics are biochemical compounds produced by microorganisms which can inhibit the growth or kill other microorganisms (Levy, S.B., 1998). They act selectively on prokaryotic targets, which can be the bacterial cell wall, cell membrane function, nucleic acid synthesis, protein synthesis or other bacterial enzymatic pathways (Levy, S.B., 1998; Mazel, D. and Davies, J., 1999). Aminoglycoside antibiotics act by inhibiting prokaryotic protein synthesis (Davis, B.D., 1987; Wright, G.D., *et al*, 1998; Azucena, E. and Mobashery, S., 2001; Vakulenko, S.B. and Mobashery, S., 2003).

Aminoglycosides are produced by the Actinomycetes *Streptomyces* spp. (*i.e.* neomycin, kanamycin and tobramycin) and *Micromonospora* spp. (*i.e.* gentamicin and sisomicin). Gentamicin is produced by *Micromonospora purpurea*. There are also semisynthetic members of the family, which are created by chemical modification of the original molecules, *i.e.* amikacin and netilmicin (Davis, B.D., 1987; Wright, G.D., *et al*, 1998; Vakulenko, S.B. and Mobashery,
S., 2003). The first aminoglycoside, streptomycin, was discovered in 1944 by Waksman, from *Streptomyces griseus* (Waksman, S.A., 1958; Comroe, J.H., Jr., 1978).

Aminoglycosides consist of an aminocyclitol ring, 2-deoxystreptamine, or streptidine in the case of streptomycin, which is joined by glycosidic bonds to two or more aminosugars (Wright, G.D., *et al*, 1998). They are highly polar molecules of a relatively large size. The two major classes of 2-deoxystreptamine aminoglycosides are the 4,5-disubstituted neomycin, paramomycin, and ribostamycin, and the 4,6-disubstituted gentamicins and kanamycins. Gentamicin, as well as kanamycin and amikacin, belong to the 4,6-disubstituted class of the 2-deoxystreptamine aminoglycosides (*Figure 1.2*) (Davis, B.D., 1987; Wright, G.D., *et al*, 1998; Vakulenko, S.B. and Mobashery, S., 2003).

### 1.2.1. Clinical Use

Aminoglycosides are highly-potent, broad-spectrum, bactericidal antibiotics that can be used both on Gram-positive and Gram-negative bacteria (Moore, R.D., *et al*, 1987; Vakulenko, S.B. and Mobashery, S., 2003). Gram-negative aerobic bacilli are particularly sensitive to these antibiotics, and they are often the treatment of choice for infections from Gram-negative bacteria such as *Escherichia coli*, *Salmonella* spp., *Shigella* spp., *Enterobacter* spp., *Citrobacter* spp., *Acinetobacter* spp., *Proteus* spp., *Klebsiella* spp., *Serratia* spp., *Morganella* spp., and *Pseudomonas* spp (Vakulenko, S.B. and Mobashery, S., 2003). They are also used, generally in combination therapy with cell wall active agents, to treat infections caused by Gram-positive organisms such as *Staphylococcus aureus* and some streptococci (Vakulenko, S.B. and Mobashery, S., 2003), as well as infections due to intracellular bacteria (Maurin, M. and Raoult, D., 2001).

Aminoglycosides are relatively toxic to the eukaryotic host cell. Their therapeutic window is rather small, which means there's a small difference between the serum concentration
needed for bactericidal action and the concentration of the drug that is toxic to the host (Moore, R.D., et al, 1987; Wright, G.D., et al, 1998). At therapeutical concentrations, aminoglycosides may also affect eukaryotic cells by nonspecific binding to eukaryotic ribosomes. Their use can cause kidney and hearing damage, since these antibiotics can concentrate in these organs (Bennett, W.M., et al, 1982; Brion, N., et al, 1984). In spite of the resistance problems that have arisen since the early 1970’s and their relatively high toxicity on the host, they are still antibiotics of choice clinically, especially in combined therapeutic use with cell wall active β-lactam antibiotics (Kotra, L.P., et al, 2000; Vakulenko, S.B. and Mobashery, S., 2003).

### 1.2.2. Mode of Action

Aminoglycosides act by inhibiting prokaryotic protein synthesis (Davis, B.D., 1987; Azucena, E. and Mobashery, S., 2001). In order to take effect, they must first reach their target,

Once inside the cell, aminoglycosides inhibit prokaryotic protein synthesis by binding to the 30S ribosome subunit (Pfister, P., et al, 2003; Vakulenko, S.B. and Mobashery, S., 2003). This binding doesn't affect the formation of the initiation complex of protein synthesis, but rather, it interferes with the elongation of the nascent polypeptide. The process of proofreading is thus compromised, resulting in misreading of the genetic code or premature termination of the nascent chain (Konno, T., et al, 2004). This leads to a significant number of altered proteins. When inserted in the cell membrane, these abnormal proteins alter the membrane permeability, which allows an increased entry of the antibiotic into the cell. This step, in which more and more of the drug is able to enter the cell and bind to the ribosomal target, leads irreversibly to cell death. (Davis, B.D., 1987; Kotra, L.P., et al, 2000; Pfister, P., et al, 2003; Vakulenko, S.B. and Mobashery, S., 2003; Konno, T., et al, 2004).
1.2.3. The Ribosomal Target


The specificity and nature of aminoglycoside-ribosome contacts have been determined in detail with A-site mutants displaying various levels of resistance and with structural studies of the drug-target interaction (Moazed, D. and Noller, H.F., 1987; Pfister, P., et al, 2003). In this regard, A-site mutants displaying resistance to aminoglycoside antibiotics have been useful to determine the site of action of aminoglycoside antibiotics (Pfister, P., et al, 2003). Two critical regions have been identified for the binding of aminoglycosides at the A-site of the ribosome; these are an adenine residue at position 1408 (E. coli numbering) and the positioning of the U1406 and U1495 bases (Pfister, P., et al, 2003). Furthermore, the effects of particular A-site mutations vary according to the chemical characteristics of the drug molecule. For example, an A to G mutation in position 1408 impairs the insertion of 6'-NH3+ aminoglycosides inside the A-site helix, which makes these mutants display high-level resistance to these drugs (Pfister, P., et al, 2003). Moreover, chemical footprinting of A-site point mutants which display higher levels of resistance have also stressed the importance of this site in the binding and action of aminoglycosides (Carter, A.P., et al, 2000; Recht, M.I. and Puglisi, J.D., 2001).
The elucidation of the crystal structure of aminoglycoside antibiotics bound to the A-site of E. coli 16S ribosomal RNA has been useful in understanding the interaction of these drugs to their target site (Fourmy, D., et al, 1996; Carter, A.P., et al, 2000; Pfister, P., et al, 2003). Structural studies done with aminoglycosides such as paromomycin, tobramycin, and geneticin, bound to oligonucleotides which contain a minimal A-site have indicated a key role of position 1406 of the 16S rRNA, particularly in the binding of ring III of the aminoglycoside molecule (Moazed, D. and Noller, H.F., 1987; Fourmy, D., et al, 1996; Fourmy, D., et al, 1998a; Fourmy, D., et al, 1998b; Carter, A.P., et al, 2000; Pfister, P., et al, 2003). Thus, alterations of the binding sites of the ribosome or the aminoglycoside molecule that reduce the binding affinity of the drug to the ribosome lead to varied levels of resistance, according to the nature of these changes (Llano-Sotelo, B., et al, 2002; Pfister, P., et al, 2003; Vakulenko, S.B. and Mobashery, S., 2003).

1.3 Resistance to Aminoglycosides

Organisms can be intrinsically resistant to an antimicrobial or become resistant through mutations or by the acquisition of new genes (Levy, S.B., 1998; Mazel, D. and Davies, J., 1999; Rowe-Magnus, D.A. and Mazel, D., 1999). Bacteria can acquire resistance from an outside source through conjugation, transformation, and transduction (Mazel, D. and Davies, J., 1999). These mechanisms of genetic exchange allow bacteria to obtain favorable genes from other bacteria (Davies, J. and Smith, D.I., 1978; Levy, S.B., 1998). Other modes of horizontal gene transfer such as transposition, Gram-positive conjugative transposons, and the recently described conjugative transposons in Gram-negative organisms, as well as the integrons of some Gram-negative bacteria, allow bacteria to acquire new genes through intermolecular genetic exchange (Davies, J. and Smith, D.I., 1978; Stokes, H.W., et al, 2001; Pembroke, J.T., et al, 2002; Carattoli, A., 2003; Partridge, S.R. and Hall, R.M., 2003). These mobile DNA elements have played an important role in the evolution of microorganisms (Recchia, G.D. and Hall, R.M.,
Microorganisms can become resistant to antibiotics through a variety of mechanisms, which include the alteration of the drug’s target, reduced uptake or accumulation of the drug, and the enzymatic modification of the antibiotic (Davis, B.D., 1987; Levy, S.B., 1998; Mazel, D. and Davies, J., 1999).

1.3.1. Altered Target

There are two main modes through which bacteria can become resistant to aminoglycosides by modifications of the ribosome: 1) through mutations in ribosomal proteins or the rRNA, and 2) by enzymatic modification of the ribosome (Davis, B.D., 1987; Wright, G.D., et al, 1998; Vakulenko, S.B. and Mobashery, S., 2003).

1.3.1.1. Target-Site Mutations

As mentioned above, any mutation that affects the binding sites of the ribosome can reduce the binding affinity of the drug to the ribosome and lead to varied levels of resistance. The effects of such mutations depend on the nature of the mutation and on the chemistry of the drug (Fourmy, D., et al, 1996; Carter, A.P., et al, 2000; Llano-Sotelo, B., et al, 2002). For example, mutations that inhibit the binding of ring I of aminoglycosides confer high-level resistance to these drugs (Carter, A.P., et al, 2000; Recht, M.I. and Puglisi, J.D., 2001). Mutations U1406-to-A confer higher levels of resistance as well as a partial dominance of resistance to all 4,6 disubstituted 2-deoxystrepateamine aminoglycosides, by reducing the affinity of the antibiotic for the rRNA target (Recht, M.I. and Puglisi, J.D., 2001; Pfister, P., et al, 2003).

Mutational changes in the 16S rRNA, or on ribosomal proteins, have been reported to produce resistance mainly to streptomycin (Fernandez, R.O. and Anton, D.N., 1987; Melancon, P., et al, 1988). These mutations are of clinical importance, particularly in Mycobacterium
tuberculosis, since streptomycin is one of the drugs used for the treatment of tuberculosis (Gillespie, S.H., 2002; Vakulenko, S.B. and Mobashery, S., 2003).

1.3.1.2. Enzymatic Modification of the Target Site

The other mode of target site alteration leading to aminoglycoside resistance is through the enzymatic modification of the ribosome (Davis, B.D., 1987; Cundliffe, E., 1989; Wright, G.D., et al, 1998). It is not uncommon to find modifications of the ribosome in microorganisms producing antibiotics that target the ribosome, as a way of protecting themselves from the antibiotics they produce (Cundliffe, E., 1989; Yoshizawa, S., et al, 1998). An example of this can be found in some aminoglycoside-producing microorganisms where methylation of certain guanine residues of the 16S rRNA produce high-level resistance to aminoglycoside antibiotics (Cundliffe, E., 1989; Vakulenko, S.B. and Mobashery, S., 2003). Ribosomal modification has been well documented in the gentamicin producer Micromonospora purpurea, as well as other aminoglycoside-producers, such as Streptomyces tenjimariensis and Streptomyces tenebrarius (Piendl, W. and Bock, A., 1982; Piendl, W., et al, 1984; Cundliffe, E., 1989).

For years, several studies strived to identify the genes that confer high-levels of resistance in organisms that produce aminoglycosides (Cundliffe, E., 1989; Kojic, M., et al, 1992). Some of this research has focused on methylase enzymes which confer aminoglycoside resistance to aminoglycoside producing organisms (Thompson, J., et al, 1985). In one of these studies, J. Thompson et al (1985) cloned fragments from the gentamicin producer, Micromonospora purpurea into naïve E. coli cells and obtained a strain which was highly resistant to both gentamicin and to kanamycin. The cloned methylase activity acted on the 16S RNA 30S ribosomal subunits of E. coli, to produce the mentioned resistance phenotype. Later, in 1987, A. A. Beauclerk and E. Cundliffe observed the sites of action of Micromonospora purpurea’s rRNA methylases and determined that these enzymes made this aminoglycoside-producer resistant to
high levels of gentamicin by the conversion of residue G-1405 to 7-methylguanosine within the 
bacterial 16S ribosomal RNA (Beauclerk, A.A. and Cundliffe, E., 1987). The nature of such 
interactions has also been studied in detail, based on sequence analysis as well as structural 

Another group of researchers studied the aminoglycoside-resistance determinants that 
they cloned from *Micromonospora purpurea* and the sisomicin producer *Micromonospora rosea* 
(Kelemen, G.H., et al, 1991). They found that these resistance determinants showed a high 
degree of identity between them, both at the structural and the functional level. Their studies 
allowed them to identify the *grm* gene, which encodes a ribosomal RNA methyltransferase. 
Furthermore, M. Kojic and collaborators (Kojic, M., et al, 1992) cloned a sisomicin-gentamicin 
resistance methylase gene (*sgm*) from *Micromonospora zionensis* and found that its deduced 
amino acid sequence also showed similarity to Grm enzymes, as well as to the 16S rRNA 
methylase, KgmB.

The studies mentioned above show the effects that methylation of the ribosome can have 
on aminoglycoside efficiency. The importance of the background of the aminoglycoside 
producer strain for the effective action of gentamicin-resistance determinants has also been 
investigated (Kojic, M., et al, 1999). Consequently, *Micromonospora* spp. strains that produce 
aminoglycosides have been shown to express high-levels of resistance to their own products as 
well as to antibiotics that are structurally similar, that is, those with a 4,6-disubstituted 
deoxystreptamine aminocyclitol component (neomycin, kanamycin, or gentamicin). On the other 
hand, these strains also proved to be sensitive to aminoglycoside antibiotics in which the 
aminocyclitol ring had different types of substitutions, such as neomycin and apramycin (Kojic, 
M., et al, 1999). These studies, which illustrate the importance of the aminoglycoside producer 
background for the action of a particular resistance determinant, provide clues to the impact on
the phenotype that ribosomal modification for self-protection has in antibiotic producers (Kojic, M., et al, 1999).

Aminoglycoside resistance by enzymatic modification of the ribosome has recently become an important focus of study due to the discovery of enzymes that modify the 16SrRNA in bacteria other than antibiotic producers (Galimand, M., et al, 2003; Yokoyama, K., et al, 2003; Doi, Y., et al, 2004; Yamane, K., et al, 2004). This mode of aminoglycoside resistance by modifications of the target involving enzymatic methylation of the rRNA in non-antibiotic producers will be explained in a later section.

1.3.2. Adaptive Resistance

Adaptive resistance to aminoglycosides, which is a transient, reduced susceptibility to the drug, has been reported mainly in aerobic Gram-negative bacilli (Davis, B.D., 1987; Wright, G.D., et al, 1998). An example of these studies is one performed by Karlowski et al, 1996, where these researchers observed that stepwise increases in aminoglycoside concentrations allowed Pseudomonas aeruginosa to grow in concentrations of aminoglycosides that would normally kill the parent strains. However, adaptive changes are typically unstable, and are due to regulatory events rather than mutations or the acquisition of resistance genes, so they don’t persist once the selective pressure is removed (Karlowsky, J.A., et al, 1996). It is argued that such reduced susceptibility could be due to protein changes or alteration in regulation genes of the respiratory pathway, but the actual mechanism is poorly understood (Bryan, L.E. and Van Den Elzen, H.M., 1977; Karlowsky, J.A., et al, 1996).

1.3.3. Drug Impermeability: Reduced Uptake and Active Efflux of Antibiotics

In order to take effect, aminoglycosides must first penetrate the cell wall and membranes, and reach the bacterial ribosome (Bryan, L.E. and Van Den Elzen, H.M., 1977; Martin, N.L. and Beveridge, T.J., 1986; Davis, B.D., 1987). Some microorganisms have developed ways to inhibit
this important step of these drugs' mode of action. Aminoglycoside resistance due to reduced uptake of the drug is mainly seen in the pseudomonads and other non-fermentor Gram-negative bacilli (Bryan, L.E. and Van Den Elzen, H.M., 1977; Martin, N.L. and Beveridge, T.J., 1986). The mechanism of action of reduced drug uptake is generally due to membrane impermeabilization, which impairs the drug penetration into the cell (Bryan, L.E. and Van Den Elzen, H.M., 1977; Davis, B.D., 1987). This mode of resistance is clinically important since it is stable and it results in an intermediate susceptibility to all of the aminoglycosides (Miller, G.H., et al., 1997; Lister, P.D., 2002). The precise molecular mechanisms that produce the aminoglycoside impermeability type of resistance are yet unknown (Damper, P.D. and Epstein, W., 1981; Davis, B.D., 1987; Schlessinger, D., 1988).

Another mechanism bacteria use to reduce the accumulation of an antibiotic within the cell is through the active efflux, or pumping, of the drug out of the cell (Zgurskaya, H.I. and Nikaido, H., 2000). This mechanism is more frequently observed in non-fermentor Gram-negative bacilli (Martin, N.L. and Beveridge, T.J., 1986; Westbrock-Wadman, S., et al, 1999), but has been reported in other microorganisms as well, including Salmonella spp. and E. coli (Nikaido, H., et al, 1998; Rosenberg, E.Y., et al, 2000). Multidrug efflux pumps generally have low substrate specificity (Zgurskaya, H.I. and Nikaido, H., 2000). Furthermore, the presence of different efflux pumps may confer additive or multiplicative effects on drug resistance (Lee, A., et al, 2000). Multidrug efflux pumps confer Gram-negative bacteria useful survival strategies, which can follow the acquisition of foreign genes, as well as the activation or increased expression of innate efflux systems (Westbrock-Wadman, S., et al, 1999; Putman, M., et al, 2000; Zgurskaya, H.I. and Nikaido, H., 2000; Lister, P.D., 2002).

Efflux pump systems have been classified in different families. ATP-dependent multidrug transporters are those which use ATP as the energy source to drive the drug outside of
the cell. Secondary multidrug transporters mediate the efflux of antibiotics from the cell in a combined exchange with protons. The latter include 1) the major facilitator superfamily, 2) the small multidrug resistance superfamily, 3) the multidrug and toxic compound extrusion superfamily and 4) the resistance-nodulation-cell division family (Nikaido, H., et al, 1998; Zgurskaya, H.I. and Nikaido, H., 2000; Mao, W., et al, 2001; Lister, P.D., 2002).

*Pseudomonas aeruginosa*, which is an important nosocomial pathogen that often presents difficulties in therapy, is a non-fermentor Gram-negative bacillus which possesses intrinsic resistance to multiple drugs (Lister, P.D., 2002). Over 90% of aminoglycoside-resistant *P. aeruginosa* isolates from cystic fibrosis patients display the drug impermeability phenotype (Westbrock-Wadman, S., et al, 1999; Lister, P.D., 2002), highlighting the importance of this mechanism of resistance. *P. aeruginosa*’s natural resistance to aminoglycosides involves an active efflux system, or pumps, as shown by aminoglycoside accumulation assays in mutants. These involve energy-dependent systems that pump out aminoglycosides from the cell (Aires, J.R., et al, 1999). Some examples of efflux pumps which confer resistance to aminoglycosides include the MexXY-OprM efflux pump system found in *P. aeruginosa* (Mao, W., et al, 2001) and the AcrD pump reported in *E. coli* (Rosenberg, E.Y., et al, 2000).

1.3.4. Enzymatic Modification of the Drug

The most important mechanism of aminoglycoside resistance is the enzymatic modification of the antibiotic by Aminoglycoside Modifying Enzymes (AMEs), which results in a molecule with decreased binding affinity to the target site at the ribosome (Wright, G.D., et al, 1998; Vakulenko, S.B. and Mobashery, S., 2003). This mode of resistance involves the presence of genes that code for enzymes that modify these antibiotics. The family of AMEs is divided in three main classes according to their enzymatic activity: 1) the aminoglycoside phosphoryltransferases (APH) participate in the ATP-dependent phosphorylation of hydroxyl
groups in the drug; 2) the aminoglycoside nucleotidyltransferases (ANT) catalyze the ATP-dependent adenylation of certain hydroxyl groups; and 3) the aminoglycoside acetyltransferases (AAC) participate in the acetylCoA-dependent acetylation of specific aminoglycoside amino groups (Shaw, K.J., et al, 1993; Wright, G.D., et al, 1998; Azucena, E. and Mobashery, S., 2001; Vakulenko, S.B. and Mobashery, S., 2003). This last class of the AMEs constitutes the largest group in the family and is the group relevant to this study (Shaw, K.J., et al, 1993; van de Klundert, J.A. and Vliegenthart, J.S., 1993).

1.3.4.1. Nomenclature of Aminoglycoside Modifying Enzymes

AMEs display substrate specificity, which means that each enzyme will modify a specific chemical group, resulting in a particular phenotype (Shaw, K.J., et al, 1993; Wright, G.D., et al, 1998). Due to the large number of AMEs that have been described to date, a uniform nomenclature system was proposed by K.J. Shaw (Shaw, K.J., et al, 1993) to denominate these enzymes and avoid confusion. Briefly, the first three letters of the name should refer to the enzymatic action, followed by a number in parenthesis designating the group modified, then a Roman numeral, indicating the resistance profile, and lastly, a lower case letter indicating the individual gene or enzyme (Shaw, K.J., et al, 1993; van de Klundert, J.A. and Vliegenthart, J.S., 1993; Wright, G.D., et al, 1998; Vakulenko, S.B. and Mobashery, S., 2003). Figure 1.3, Figure 1.4 and Figure 1.5 indicate the chemical groups of an aminoglycoside molecule modified by different AMEs.

1.3.4.2. Aminoglycoside Acetyltransferases

Aminoglycoside acetyltransferases are the largest group in the family of the AMEs (Shaw, K.J., et al, 1993; Wright, G.D., et al, 1998). There are four classes of enzymes within this group, according to the amino group that is acetylated in the aminoglycoside molecule. These are AAC(1), AAC(3), AAC(2”), and AAC(6”) (Shaw, K.J., et al, 1993). These enzymes use the
acetyl group from an acetyl coenzyme-A donor molecule to modify the aminoglycoside molecule at the 1 and 3 positions of the 2-deoxystreptamine ring, and the 2” and 6” positions of the 6-aminohexose ring, respectively (Shaw, K.J., et al., 1993; Vakulenko, S.B. and Mobashery, S., 2003).

Several resistance patterns have been found within the class of AACs which modify the aminoglycoside molecule at position 3 of the 2-deoxystreptamine ring, or AAC(3). One of the resistance phenotypes of interest in this study corresponds to the AAC(3)-II resistance pattern (Diaz, M.A., et al., 2000; Diaz, M.A., et al., 2001). The phenotype of these enzymes involves resistance to gentamicin, tobramycin, sisomicin, netilmicin, and dibekacin (Shaw, K.J., et al., 1993; Miller, G.H., et al., 1997; Vakulenko, S.B. and Mobashery, S., 2003). Three different aac(3)-II genes have been identified to date, which encode enzymes AAC(3)-IIa, -IIb, and -IIc. Genes aac(3)-IIc and aac(3)-IIb have been reported to share 97% and 72% identity with the aac(3)-IIa gene, respectively (Vakulenko, S.B. and Mobashery, S., 2003). These enzymes had previously been named AAC(3)-III (Allmansberger, R., et al., 1985) and AAC(3)-V (Brau, B., et al., 1984; Rather, P.N., et al., 1992) until later analysis revealed them to be identical to the AAC(3)-II (Shaw, K.J., et al., 1993; Vakulenko, S.B. and Mobashery, S., 2003).

Clinically, AAC(3)-II enzymes are frequently found in Gram-negative bacteria, with a frequency that varies from 18% to 60% relative to other phenotypes (Miller, G.H., et al., 1997; Vakulenko, S.B. and Mobashery, S., 2003). The most frequent gene producing the AAC(3)-II phenotype is AAC(3)-IIa, which accounts for 85% of the bacteria presenting this phenotype (Miller, G.H., et al., 1997). This gene was originally identified in R-plasmids (Allmansberger, R., et al., 1985).

Three other AAC(3) resistance patterns, AAC(3)-III, AAC(3)-IV, and AAC(3)-VI, occur rarely among clinical isolates, and patterns AAC(3)-VII, -VIII, -IX, and -X have been discovered.
Figure 1.3. Aminoglycoside Phosphotransferase Enzymes

Figure 1.4. Aminoglycoside Nucleotidyltransferase Enzymes

Figure 1.5. Aminoglycoside Acetyltransferase Enzymes

1.4 High-Level Gentamicin Resistance (HLGR)

1.4.1. HLGR in Gram-Positive Organisms

The majority of aminoglycoside modifying enzymes present enough activities to result in effective resistance to aminoglycosides, but only certain phosphotransferase enzymes have been reported to produce high-levels of resistance to these antibiotics (Galim and, M., et al, 2003; Vakulenko, S.B. and Mobashery, S., 2003). To the best of our knowledge, there had been no reports in the literature of high-level resistance to gentamicin in Gram-negative bacteria before the start of this study in 1999 (Diaz, M.A., et al, 2000). There had been numerous reports on high-level gentamicin resistance in Gram-positive bacteria (Chow, J.W., 2000). Reports on the variety of structures involved in the transfer of these determinants in Gram-positive bacteria were also abundant (Ferretti, J.J., et al, 1986; Chow, J.W., 2000).

The enzyme AAC(6′)-APH(2″) was the first to be reported to cause the high-level gentamicin resistance phenotype (Shaw, K.J., et al, 1993; Chow, J.W., 2000). It confers high-level gentamicin resistance to Gram-positive organisms due to its 6′-N-acetyltransferase and 2″-O-phosphotransferase activities (Shaw, K.J., et al, 1993). The combined acetylation and phosphorylation of specific amino and hydroxyl groups by this enzyme gives it a broad substrate specificity in both enzymatic activities (Wright, G.D., et al, 1998; Kotra, L.P., et al, 2000).

High-level aminoglycoside resistance in enterococci is generally mediated by these aminoglycoside-modifying enzymes (Fluit, A.C., et al, 2003). This level of resistance eliminates the synergistic bactericidal effect that is normally obtained by the combined use of cell wall-active agents, such as β-lactams, with aminoglycosides (Chow, J.W., 2000), which is clinically relevant since this is often a treatment of choice for infections caused by these microorganisms (Miller, G.H., et al, 1997; Vakulenko, S.B. and Mobashery, S., 2003).

High-level gentamicin resistance, often referred to as HLGR, is found in almost all enterococcal species involved in human infection (Straut, M., et al, 1996; Chow, J.W., 2000; Fluit, A.C., et al, 2003). In fact, the majority of Enterococcus spp. tested to date have been shown to encode genes with homology to the gene aac(6’)-aph(2’), also found in the literature as the aac6-aph2 gene (Ferretti, J.J., et al, 1986).

The gene coding this bifunctional enzyme has been reported to be carried either by Tn4001-like structures or by Tn4001-truncated elements (Ferretti, J.J., et al, 1986) and to present insertion sequences IS256 and IS257 in its molecular environment. It has been detected in other HLGR strains of Gram-positive bacteria, such as Staphylococcus aureus, Streptococcus spp., Ent. faecalis and Ent. faecium (Ferretti, J.J., et al, 1986), and in the chromosome of Streptococcus mitis (Kaufhold, A. and Potgieter, E., 1993). Plasmid-borne gene aac(6’)-aph(2’”) has also been detected in other enterococcal species displaying HLGR, namely Ent. hirae, Ent. avium, and Ent. raffinosus (Sahm, D.F. and Gilmore, M.S., 1994; Straut, M., et al, 1996; Shiojima, M., et al, 1997). The genes encoding this bifunctional enzyme have been shown to be highly homologous in Enterococcus faecalis, Enterococcus faecium, and Streptococcus agalactiae (Ounissi, H., et al, 1990; Kaufhold, A., et al, 1992).
Recently, other genes have been reported to account for plasmid-encoded high-level gentamicin resistance in *Enterococcus faecium* (Chow, J.W., 2000; Vakulenko, S.B. and Mobashery, S., 2003). In 1998, S.F. Tsai *et al* identified a new high-level gentamicin resistance gene, *aph(2")-Id*, in *Enterococcus* spp. (Tsai, S.F., *et al*, 1998). The deduced amino acid sequence for this gene has a region which presents homology to the aminoglycoside-modifying enzyme APH(2")-Ic, which reportedly confers mid-levels resistance to gentamicin (Chow, J.W., *et al*, 1997); it also presents homology to the C-terminal domain of the bifunctional enzyme AAC(6')-APH(2") (Chow, J.W., *et al*, 1997; Chow, J.W., *et al*, 2001; Vakulenko, S.B. and Mobashery, S., 2003). Later, in 2000, S.J. Kao *et al* cloned a new high-level gentamicin resistance gene, *aph(2")-Ib*, from *Enterococcus faecium* strain SF11770, with homology to the enzymes AAC(6')-APH(2"), APH(2")-Ic, and APH(2")-Id (Kao, S.J., *et al*, 2000). Interestingly, gene *aph(2")-Ib* was recently detected in *E. coli* as well as in *Enterococcus faecium*, findings that suggest a horizontal transfer of these genes between Gram-positive and Gram-negative bacteria (Chow, J.W., *et al*, 2001). In conclusion, recently reported genes accounting for plasmid-encoded high-level gentamicin resistance in *Enterococcus faecium* include: *aph(2")-Ib* and *aph(2")-Id* (Tsai, S.F., *et al*, 1998; Chow, J.W., 2000; Kao, S.J., *et al*, 2000). It is relevant to mention that even though *aph(2")-Ib* has been reported in Gram-negative bacteria, there are no reports to date of any aminoglycoside modifying enzyme causing the HLGR phenotype in these bacteria (Galimand, M., *et al*, 2003; Yamane, K., *et al*, 2005).

1.4.2. **Transfer of Gentamicin Resistance Genes in Gram-Positive Organisms**

Various reports in Europe, the United States, and other places in the world have ascertained that HLGR is an important cause of nosocomial enterococcal infections (Murray, B.E., 1990; Fluit, A.C., *et al*, 2003). This may in part be due to the fact that HLGR determinants are present on mobile molecular elements which can facilitate their spread among Gram-positive
organisms (Ferretti, J.J., et al, 1986; Tolmasky, M.E., 2000; Fluit, A.C., et al, 2003). In this sense, several reports have revealed an ample variety of structures associated with the genetic variability and transfer of gentamicin resistance in Gram-positive organisms. For example, some studies have described the plasmid contents in clinical isolates of HLGR Enterococcus faecalis as highly heterogenous (Zervos, M.J., et al, 1986; Casetta, A., et al, 1998; Daikos, G.L., et al, 2003). These have found the gentamicin resistance determinants either on conjugative, or on nonconjugative, mobilizable plasmids. In addition, the bi-functional enzyme AAC(6’)-APH(2”) has been found to be encoded on both narrow and broad host range plasmids (Simjee, S. and Gill, M.J., 1997), as well as on conjugative transposons (Sahm, D.F. and Gilmore, M.S., 1994; Casetta, A., et al, 1998; Murray, B.E., 1998; Culebras, E. and Martinez, J.L., 1999; Simjee, S., et al, 1999).

Overall, studies on gentamicin resistance in enterococci report isolating plasmids from transconjugants with sizes ranging from 45 kb to larger than 80 kb. All these plasmids are reported to carry the gene aac(6’)-aph(2”) for HLGR. Nevertheless, the molecular environment or flanking regions of this gene is reported to vary significantly (Sahm, D.F. and Gilmore, M.S., 1994; Horvat, R.T., et al, 1998; Simjee, S., et al, 1999). As an example of this variability, one study reported this gene to be found as a Tn4001-like alpha element on the Ent. faecalis chromosome, as well as a Tn4001-truncated type I structure on a conjugative plasmid, and even on both (Casetta, A., et al, 1998). The heterogeneity observed in plasmid contents and in the molecular environments of this gene in clonally related enterococci argues against a clonal dissemination of gentamicin resistant strains (Culebras, E. and Martinez, J.L., 1999).

Studies done on Ent. faecalis have further characterized the plasmids carrying the HLGR determinants as pheromone-responsive conjugative plasmids (Shiojima, M., et al, 1997; Murray, B.E., 1998). These pheromone-responsive plasmids are characterized by giving a mating
response to small sex pheromones secreted by potential recipient cells. This event is involved in
the formation of mating aggregates and plasmid transfer (Salyers, A.A., et al, 1995; Shiojima,
M., et al, 1997; Tolmasky, M.E., 2000). Until recently, conjugative transposons had only been
reported in Gram-positive bacteria (Salyers, A.A., et al, 1995). However, recent studies
(Pembroke, J.T., et al, 2002) have described the presence of similar though unrelated structures
in Gram-negative bacteria. This subject has become a hot topic since and will be discussed in a
later section.

HLGR genes can also be transferred among different genera of Gram-positive organisms
(Sahm, D.F. and Gilmore, M.S., 1994). One example of this is a study which identifies the same
antimicrobial resistance genes in enterococci and staphylococci on the enterococcal transposon
Tn5384. The study supports the evolution of a composite transposon through cointegration of
enterococcal and staphylococcal plasmids, though this genetic transfer between enterococci and
staphylococci is still not well defined nor understood. Additionally, the molecular structures
mentioned so far also play a key role in the dissemination of resistant strains between different
regions of the world (Fluit, A.C., et al, 2003; Hallgren, A., et al, 2003). This dissemination is
illustrated by a study in the UK (Simjee, S., et al, 1999) that detected a Tn5281-like element
carrying the \textit{aac(6')-aph(2'')} gene (with and without an associated 70 kb plasmid) in clinical
isolates of \textit{Enterococcus faecium}, which had been originally detected in a \textit{Enterococcus faecalis}

In general, studies conducted on the origin and spread of the \textit{aac(6')-aph(2'')} gene
suggest that the origin of this gene is not clonal, but it is rather due to the combination of events
such as plasmid conjugation, together with multiple insertion events, which have facilitated the
rapid spread of this aminoglycoside resistance gene among Gram-positive bacteria (Simjee, S.
and Gill, M.J., 1997; Culebras, E. and Martinez, J.L., 1999).
1.5 Integrons

1.5.1. Definition and Classes

Integrons are gene-capture molecular elements that contain an integrase gene, named \( intI \), and an arrangement of gene cassettes which are expressed from a common promoter (Stokes, H.W. and Hall, R.M., 1989; Collis, C.M. and Hall, R.M., 1995). They were first described by H.W. Stokes and R.M. Hall (Stokes, H.W. and Hall, R.M., 1989) and since then, the literature abounds with reports of these molecular elements, which are often found on the plasmids and/or the chromosomes of Gram-negative bacteria (Hall, R.M. and Stokes, H.W., 1993; Recchia, G.D. and Hall, R.M., 1997; Fluit, A.C. and Schmitz, F.J., 1999; Hall, R.M., \textit{et al}, 1999; Rowe-Magnus, D.A. and Mazel, D., 1999).

Gene cassettes are defined as small mobile elements generally composed of a single gene, plus a site known as the 59-base element \( (59be) \) or \( attC \) (Stokes, H.W. and Hall, R.M., 1989; Hall, R.M. and Collis, C.M., 1995). Gene cassettes can be inserted or excised from the integron through site-specific recombination, an event that is catalized by the integrase \( IntI \) (Stokes, H.W. and Hall, R.M., 1989; Collis, C.M. and Hall, R.M., 1992b; Hall, R.M. and Collis, C.M., 1995; Stokes, H.W., \textit{et al}, 1997; Collis, C.M., \textit{et al}, 2001).

The majority of gene cassettes described to date code for antibiotic resistance (Fluit, A.C. and Schmitz, F.J., 1999; Fluit, A.C. and Schmitz, F.J., 2004). Integrons which encode these resistance genes are known as Multiple Resistance Integrons or MRIs. More than 70 different antibiotic resistance gene cassettes have been reported in the MRIs to date, and they include resistance genes to most of the existing antibiotic families (Recchia, G.D. and Hall, R.M., 1995; Fluit, A.C. and Schmitz, F.J., 1999; Mazel, D. and Davies, J., 1999).

Integrons are divided into different classes, according to the \( IntI \) integrase and the general gene arrangement that they encode. Before 2001, only four classes of integrons had been
identified, mainly from clinical isolates. These were integrons class-1 (Stokes, H.W. and Hall, R.M., 1989), class-2, class-3 (Collis, C.M., et al, 2002a) and class-4 (Mazel, D., et al, 1998).


In addition to the four classes of integrons originally reported (Stokes, H.W. and Hall, R.M., 1989; Mazel, D., et al, 1998; Collis, C.M., et al, 2002a), new integron classes have been discovered in recent studies (Nield, B.S., et al, 2001; Stokes, H.W., et al, 2001). This Australian group designed degenerate primers from conserved sequences in the genes coding for the known integrases and used them to screen for new putative integrases in environmental samples. This
same screening strategy proved helpful in a later study conducted to detect new gene cassettes from the environment (Stokes, H.W., et al, 2001).

1.5.2. Class-1 Integrons: Structure

Class-1 integrons are the most prevalent integrons among clinical isolates (Hall, R.M. and Stokes, H.W., 1993; Fluit, A.C. and Schmitz, F.J., 1999). They are characterized by the presence of two conserved sequences, known as the 5’CS and 3’CS, which flank a variable region (Figure 1.6) (Bennett, P.M., 1999). The 5’-conserved region contains the integrase gene intI1 and a gene cassette attachment site, attI. The 3’-conserved region is characterized by the presence of a sulfonamide resistance gene, sul1, a quaternary ammonium compound resistance gene, qacEΔ1, and open reading frames orf5 and orf6, with unknown functions. The variable region of class-1 integrons can code for one or more gene cassettes (Stokes, H.W. and Hall, R.M., 1989; Hall, R.M. and Stokes, H.W., 1993).

Gene cassettes are excised from integrons as covalently-closed circles (Collis, C.M. and Hall, R.M., 1992b; Collis, C.M. and Hall, R.M., 1992a). They are generally inserted at a specific site in the integron, known as the attI site (Collis, C.M. and Hall, R.M., 1992b; Collis, C.M., et al, 1993; Stokes, H.W., et al, 1997). This site, together with the 59be site, are the substrates for the site-specific recombinase IntI (Hall, R.M. and Collis, C.M., 1995; Collis, C.M., et al, 1998; Collis, C.M., et al, 2001). The majority of gene cassettes are found integrated at the attI site of integrons, but some gene cassettes have also been described on secondary sites, or 2°rs (Stokes, H.W. and Hall, R.M., 1989; Recchia, G.D., et al, 1994; Hall, R.M. and Collis, C.M., 1995; Recchia, G.D. and Hall, R.M., 1995; Collis, C.M., et al, 2001). IntI integrases bind preferentially to the adjacent attI site for the recombination reaction with a 59be site (Collis, C.M., et al, 2002b).
As mentioned above, gene cassettes possess a 59be which, together with the attI site, serves as the substrate for the integron integrase (Collis, C.M., et al, 1993; Hall, R.M. and Collis, C.M., 1995; Stokes, H.W., et al, 1997). The 59be or attC sites of different gene cassettes can vary considerably in their length and in sequence. Thus, they can measure anywhere from 57 to 141 base pairs (Hall, R.M., et al, 1991; Collis, C.M., et al, 1993; Hall, R.M. and Collis, C.M., 1995).

59-base elements are unique for each gene cassette. There are regions at the ends of these sites, measuring approximately 25 bp, which display a certain degree of similarity and which are imperfect inverted repeats of each other (Hall, R.M., et al, 1991; Collis, C.M., et al, 1993; Hall,

As opposed to attC sites, integron attI sites are not closely related to each other, and they do not share the features described in the previous paragraphs for attC (Hall, R.M., et al, 1991; Partridge, S.R., et al, 2000). The attI site only has one site structure, with two directly repeated integrase-binding domains located at the left side (Collis, C.M. and Hall, R.M., 1995; Hall, R.M. and Collis, C.M., 1995; Collis, C.M., et al, 2001).

As mentioned above, the sequences which flank gene cassettes can vary a great deal between the different integron classes (Hall, R.M., et al, 1994; Stokes, H.W., et al, 1997; Stokes, H.W., et al, 2001). However, identical cassettes have been found to be encoded in integrons of different classes, which indicates that the gene cassette pool is probably shared between them (Fluit, A.C. and Schmitz, F.J., 1999; Rowe-Magnus, D.A. and Mazel, D., 1999; Rowe-Magnus, D.A. and Mazel, D., 2001; Fluit, A.C. and Schmitz, F.J., 2004). The fact that gene cassettes may possibly be exchanged between the different integron classes advocates that they may have played an important role in bacterial evolution (Rowe-Magnus, D.A. and Mazel, D., 1999; Rowe-Magnus, D.A., et al, 2001; Rowe-Magnus, D.A. and Mazel, D., 2001; Rowe-Magnus, D.A., et al, 2002). In effect, the flexibility that integrons confer to bacteria allows them to adapt rapidly to unpredictable environmental challenges by allowing bacteria to acquire favorable genes that can result in an improved fitness (Hall, R.M., et al, 1999; Rowe-Magnus, D.A. and Mazel, D., 1999; Rowe-Magnus, D.A. and Mazel, D., 2001; Fluit, A.C. and Schmitz, F.J., 2004).
Likewise, any gene that proves to be useless may be eliminated via a similar mechanism (Hall, R.M., et al, 1999; Fluit, A.C. and Schmitz, F.J., 2004). On the other hand, the majority of the cassettes in the SIs are not expressed. Even so, they are believed to be maintained in the absence of selective pressure as a genetic resource for the evolution of new genes (Rowe-Magnus, D.A. and Mazel, D., 1999; Rowe-Magnus, D.A., et al, 2001; Rowe-Magnus, D.A. and Mazel, D., 2001; Rowe-Magnus, D.A., et al, 2002).

The origins of the gene cassettes are not known, yet it is suggested that cassettes from multiple resistance integrons may be relatively ancient structures (Recchia, G.D. and Hall, R.M., 1997). Moreover, it is suspected that MRIs evolved from super integrons through the recruitment of super-integron gene cassettes (Rowe-Magnus, D.A., et al, 2002; Hall, R.M. and Stokes, H.W., 2004). This is illustrative of how super-integrons have had an important impact on the bacterial evolution of antibiotic resistance through the recruitment of super-integron gene cassettes (Rowe-Magnus, D.A., et al, 2002; Hall, R.M. and Stokes, H.W., 2004).

1.5.3. Gene Expression

Integrons can be regarded as natural bacterial expression vectors (Collis, C.M. and Hall, R.M., 1995; Hall, R.M. and Collis, C.M., 1995). There is a considerable degree of variability in the level of expression of the genes encoded as gene cassettes. This variability of expression can be accounted for by the intrinsic characteristics of both the gene cassettes and the integron (Levesque, C., et al, 1994; Collis, C.M. and Hall, R.M., 1995; Hall, R.M. and Collis, C.M., 1995; Recchia, G.D. and Hall, R.M., 1995).

The expression of gene cassettes has been intensely studied at the transcriptional level (Collis, C.M. and Hall, R.M., 1995; Hall, R.M. and Collis, C.M., 1995; Ploy, M.C., et al, 2000). Gene cassettes are inserted in integrons in the same orientation with respect to their coding sequence. Their initiation codons are located close to their 5’ end (Hall, R.M. and Collis, C.M.,
1995). With a few exceptions, gene cassettes do not contain a promoter of their own. They are instead expressed from a common promoter, P\textsubscript{ant} or P\textsubscript{1}, or rarely from another promoter, P\textsubscript{2} (Levesque, C., et al, 1994; Collis, C.M. and Hall, R.M., 1995; Stokes, H.W., et al, 2001). The P\textsubscript{1} promoter sequence has strong sequence similarity to the \textit{Escherichia coli} $\sigma^{70}$ promoter consensus sequence. Both P\textsubscript{1} and P\textsubscript{2} promoters are located in the 5’-conserved segment (5’-CS) of class 1 integrons. P\textsubscript{ant} is in a region in the 5’-CS called the 59-conserved segment, located at 59 bases from the gene cassettes; it is 200 bp upstream from the cassette-encoded genes inserted at the \textit{attI} site (Levesque, C., et al, 1994; Collis, C.M. and Hall, R.M., 1995).

Several factors can affect the expression of the gene cassettes in an integron (Ploy, M.C., et al, 2000). Multiple studies on the promoter sequences have confirmed that variations in the sequence of P\textsubscript{ant} can affect the level of expression of a given gene by more than 20-fold (Levesque, C., et al, 1994; Collis, C.M. and Hall, R.M., 1995). Thus, mutations and natural variants of P\textsubscript{ant} have been shown to have an effect on the expression of gene cassettes, as measured by the level of antibiotic resistance conferred by the cassettes encoded downstream of such promoters (Levesque, C., et al, 1994; Ploy, M.C., et al, 2000). Another factor that can significantly affect the expression of gene cassettes is the order in which the cassette is inserted in the integron. Thus, the level of resistance conferred is higher for the resistance genes that are located in the first place, \textit{i.e.} the first cassette. Likewise, it is reduced when there are preceding cassettes (Levesque, C., et al, 1994; Collis, C.M. and Hall, R.M., 1995; Ploy, M.C., et al, 2000). In effect, when various cassette configurations are present, several RNA transcripts can be detected, with different lengths. From the length of the most abundant transcripts, it has become evident that they terminate near the 59\textit{be} end of individual cassettes, which suggests that the 59\textit{be} may also act as transcription terminators (Levesque, C., et al, 1994; Collis, C.M. and Hall, R.M., 1995).
1.6 Conjugative Transposons in Gram-Negative Bacteria

Conjugative transposons are DNA elements that can excise themselves to form a covalently closed circular intermediate (Salyers, A.A., et al, 1995). Subsequently, this intermediate can reintegrate itself within the same organism, or it can be transferred by conjugation to a recipient cell and then integrate in the recipient’s genome (Salyers, A.A., et al, 1995; Pembroke, J.T., et al, 2002; Partridge, S.R. and Hall, R.M., 2003). These integrative molecular elements were first found in Gram-positive bacteria such as the streptococci and enterococci (Tolmasky, M.E., 2000), and were barely mentioned before 1996 in connection with Gram-negative bacteria (Pembroke, J.T., et al, 2002). Conjugative transposon-like elements were later detected in Gram-negative bacteria, such as Bacteroides spp. (Salyers, A.A., et al, 1995; Pembroke, J.T., et al, 2002), and though characterized to a lesser extent, in the Enterobacteriaceae. Conjugative transposon-like elements have been reported in Salmonella spp., Proteus spp., in the SXT element in Vibrio spp. and in the element known as R391 (Salyers, A.A., et al, 1995; Pembroke, J.T., et al, 2002; Partridge, S.R. and Hall, R.M., 2003).

Gram-negative conjugative transposons differ considerably from those of Gram-positive organisms (Salyers, A.A., et al, 1995; Pembroke, J.T., et al, 2002; Partridge, S.R. and Hall, R.M., 2003). Some distinguishing features are that they generally are larger than Gram-positive conjugative transposons and they carry genes which are derived from diverse sources. In fact, they resemble what would be an intermediate between a phage and a plasmid, or “phagemid”. However, this denomination has been avoided to prevent confusing them with the genetically engineered elements (Pembroke, J.T., et al, 2002).

Gram-negative conjugative transposons have a broad host range, which gives them a key role in the dissemination of antibiotic resistance genes among these bacteria (Salyers, A.A., et al, 1995; Pembroke, J.T., et al, 2002; Partridge, S.R. and Hall, R.M., 2003). Many conjugative
Transposons are also able to mobilize plasmids, so the resistance genes do not need to be coded on the conjugative transposon themselves in order to be transferred (Salyers, A.A., et al, 1995).

STX is a conjugative integrating element derived from *Vibrio cholerae* (Beaber, J.W., *et al*, 2002b; Pembroke, J.T., *et al*, 2002). Genomic and functional research done on this integrating antibiotic resistance gene transfer element has provided insight on the structure of conjugative transposons. Another such structure, known as R391, has also provided a basis to define the genetic structure of these resistance gene mobilization determinants (Beaber, J.W., *et al*, 2002a; Pembroke, J.T., *et al*, 2002). Interestingly, conjugative transposons are thought to be related to enteric pathogenicity islands in the *Enterobacteriaceae* (Pembroke, J.T., *et al*, 2002).

### 1.7 16S rRNA Methylases

As mentioned before, one of the modes of acquisition of aminoglycoside resistance through alteration of the target site involves the enzymatic methylation of the 16S rRNA (Carter, A.P., *et al*, 2000; Pfister, P., *et al*, 2003; Vakulenko, S.B. and Mobashery, S., 2003). This mode of ribosomal protection, involving the production of 16S rRNA methylase enzymes, was previously detected and reported only in aminoglycoside-producing actinomycetes (Thompson, J., *et al*, 1985; Cundliffe, E., 1989). Recently, however, this mode of resistance has also been reported in Gram-negative organisms such as *Pseudomonas aeruginosa* (Yokoyama, K., *et al*, 2003; Yamane, K., *et al*, 2004), *Serratia marcescens* (Doi, Y., *et al*, 2004), and, more relevant to this study, members of the *Enterobacteriaceae* (Galimand, M., *et al*, 2003; Yamane, K., *et al*, 2005). This type of modification is clinically important, since it confers high-level resistance to a broad range of aminoglycosides, whereas aminoglycoside-modifying enzymes are substrate-specific, so they don’t confer resistance to different types of aminoglycosides (Shaw, K.J., *et al*, 1993; Miller, G.H., *et al*, 1997; Galimand, M., *et al*, 2003; Vakulenko, S.B. and Mobashery, S., 2003).
So far the discussion has centered on the mechanisms of Gram-positive organisms and aminoglycoside producers to resist high-levels of these antibiotics. In effect, until recently there had been no reports, to the best of our knowledge, of the acquisition of genes conferring high-levels of resistance to aminoglycosides in Gram-negative organisms (Galimand, M., et al, 2003). In the first stages of our studies, we had hypothesized that we would find a second gene which could act in synergy with the already identified \( \text{aac}(3)\text{II} \) gene product to confer high-level gentamicin resistance to several members of the \( \text{Enterobacteriaceae} \) isolated in our laboratories and which displayed the HLGR phenotype (Diaz, M.A., et al, 2000).

Recently, however, a group of investigators from the Pasteur Institut in Paris, published a study that has reoriented our research. In brief, M. Galimand (Galimand, M., et al, 2003) found that the high-level aminoglycoside resistance in a clinical strain of \( \text{Klebsiella pneumoniae} \) was due to both the presence of the \( \text{aac3-II} \) gene as well as to a new aminoglycoside resistance methylase gene: \( \text{armA} \). The enzyme encoded by the latter, ArmA, reportedly shares 37-47% similarity with the 16S rRNA m(7)G methyltransferases from various actinomycetes (Galimand, M., et al, 2003). In this study, these investigators demonstrated that methylation of 16S rRNA can confer high-level resistance to a broad-range of aminoglycosides in Gram-negative organisms, including the pathogen they studied. In addition, the same clinical strain of \( \text{Klebsiella pneumoniae} \) also had genes \( \text{bla}_{\text{TEM1}}, \text{bla}_{\text{CTX-M}}, \text{dfrA12}, \text{sul1} \) and \( \text{ant}(3")9 \), which conferred resistance to beta-lactams, trimethoprim, sulfonamides and streptomycin-spectinomycin, respectively. Interestingly, the gene \( \text{armA} \) has a low GC content, making it improbable that this gene originated from aminoglycoside-producing actinomycetes (Galimand, M., et al, 2003).

Concurrently in Japan, a group of researchers (Yokoyama, K., et al, 2003) cloned and reported an aminoglycoside-resistance gene, \( \text{rmtA} \), from a clinical isolate of \( \text{Pseudomonas aeruginosa} \) AR-2. The enzyme encoded by this gene, RmtA, confers high-level resistance to
various aminoglycosides, with a MIC>1024 mg/L (Yokoyama, K., *et al*, 2003). The enzyme was shown to be a methylase as it was able to incorporate radiolabeled methyl groups into the 30S ribosome. As opposed to ArmA, this newly identified enzyme showed both sequence and functional similarities to the 16S rRNA methylases of aminoglycoside-producing actinomycetes (Yokoyama, K., *et al*, 2003; Yamane, K., *et al*, 2004; Yamane, K., *et al*, 2005). Also, the G+C content of the *rmtA* gene is about 55%, which is similar to aminoglycoside-producing organisms. Thus, these studies support a possible horizontal gene transfer from aminoglycoside-producing microorganisms to *P. aeruginosa* (Yokoyama, K., *et al*, 2003; Yamane, K., *et al*, 2004; Yamane, K., *et al*, 2005). In fact, it has been proposed that gene *rmtA* may have been transferred by a mobile genetic element such as Tn5041 (Yamane, K., *et al*, 2004; Yamane, K., *et al*, 2005).

Recently, another 16S rRNA methylase was isolated in Japan (Doi, Y., *et al*, 2004). This plasmid-borne methylase was isolated from *Serratia marcescens* and confers high-level resistance to aminoglycosides, including the kanamycins and gentamicins. The enzyme shares 82% identity with RmtA, so it was named RmtB (Yokoyama, K., *et al*, 2003; Doi, Y., *et al*, 2004; Yamane, K., *et al*, 2005). Its origin is also suspected to be from aminoglycoside producing organisms (Doi, Y., *et al*, 2004; Yamane, K., *et al*, 2005). Interestingly, ribosomal methylase genes have also been detected, encoded as gene cassettes, in a culture-independent gene cassette screening study (Nield, B.S., *et al*, 2004).

### 1.8 Objectives

The objectives of this study were to identify the gene or genes that contribute to high-level gentamicin resistance in bacteria isolated from environmental samples with turtle farming activity in Louisiana, and to determine their incidence in these bacteria, particularly *Salmonella* spp. Additionally, to explore the molecular elements that contribute to the dissemination of gentamicin resistance among bacteria isolated at the turtle farms.
1.9 Relevance of Study

The work presented in this thesis is among the first to report high-level gentamicin resistance in Gram-negative bacteria. It constitutes one of the first reports of aminoglycoside resistance genes \( aac(3)\text{IIa} \) and \( aac(3)\text{VIa} \) in environmental isolates of \( Salmonella enterica \) subsp. \( arizonae \). Additionally, this study illustrates the potential of transfer of antibiotic resistance genes, particularly gentamicin resistance, among bacteria in an environment with turtle farming activity, a matter of particular concern as it involves the zoonotic pathogen \( Salmonella \) spp.

This research provides epidemiological data describing the resistance to aminoglycosides currently used both in the turtle farms and in the clinical setting. The data presented highlights the current inefficacy of the antibiotic gentamicin to eradicate \( Salmonella \) spp. from turtle eggs. We hope to persuade the relevant authorities in Louisiana to revise the mandate to use gentamicin for turtle egg treatment and to stress the need to develop new treatments to eradicate \( Salmonella \) from turtle hatchlings.

1.10 References


CHAPTER 2:

PLASMID-MEDIATED HIGH-LEVEL GENTAMICIN RESISTANCE AMONG BACTERIA ISOLATED FROM TURTLE FARMS IN LOUISIANA *

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2.1 Introduction

eggs presents various advantages at a commercial turtle farm level, since it does not lose its antimicrobial activity when in solution or when exposed to freezing or autoclaving temperatures (Michael-Marler, S., et al, 1983; Siebeling, R.J., et al, 1984).

Gentamicin belongs to the aminoglycoside family of antibiotics. The two major classes of 2-deoxystreptamine aminoglycosides are the 4,5-disubstituted molecules, which include neomycin, paramomycin, and ribostamycin, and the 4,6-disubstituted molecules, which include gentamicin, kanamycin and amikacin (Shaw, K.J., et al, 1993; Wright, G.D., et al, 1998; Vakulenko, S.B. and Mobashery, S., 2003). Aminoglycosides are highly-potent, broad-spectrum, bactericidal antibiotics that are often used in combination therapy with cell wall active agents to treat infections caused by Gram-positive organisms such as Staphylococcus aureus and streptococci (Miller, G.H., et al, 1997; Wright, G.D., et al, 1998; Vakulenko, S.B. and Mobashery, S., 2003) as well as intracellular bacteria (Maurin, M. and Raoult, D., 2001; Vakulenko, S.B. and Mobashery, S., 2003). Gram-negative aerobic bacilli are also sensitive to these antibiotics, which makes them a treatment of choice for these infections (Wright, G.D., et al, 1998; Vakulenko, S.B. and Mobashery, S., 2003). Aminoglycoside antibiotics act by inhibiting prokaryotic protein synthesis (Davis, B.D., 1987; Mazel, D. and Davies, J., 1999). They induce misreading of the genetic code by binding to the 16S ribosomal RNA A-site in bacteria (Moazed, D. and Noller, H.F., 1987; Carter, A.P., et al, 2001). The most important mechanism of aminoglycoside resistance is the enzymatic modification of the antibiotic by aminoglycoside modifying enzymes, which are divided according to their enzymatic activity into the aminoglycoside phosphoryltransferases (APHs), the aminoglycoside nucleotidyltransferases (ANTs) and the aminoglycoside acetyltransferases (AACs) (Davis, B.D., 1987; Shaw, K.J., et al, 1993; Wright, G.D., et al, 1998; Vakulenko, S.B. and Mobashery, S., 2003). In spite of the resistance problems that have arisen since the early 1970s and their
relatively high toxicity to the host, they are still antibiotics of clinical choice, especially in combined therapeutic use with β-lactams (Miller, G.H., et al, 1997; Wright, G.D., et al, 1998; Vakulenko, S.B. and Mobashery, S., 2003).

Antibiotic use constitutes a selective pressure which can ultimately result in the emergence of resistant strains; thus, bacteria resistant to a particular antibiotic are often detected following its implementation or use (Davies, J. and Smith, D.I., 1978; Miller, G.H., et al, 1997; Levy, S.B., 1998; Mazel, D. and Davies, J., 1999). Gentamicin resistance in Salmonella spp. isolated from pet turtles was originally reported in Canada, in a 1990 study stating that turtles imported from Louisiana carried gentamicin-resistant Salmonella spp. (D'Aoust, J.Y., et al, 1990). Concerns of the emergence of gentamicin-resistant Salmonella enterica subsp. arizonae which could survive gentamicin treatment had been previously reported in the turkey farm industry (Ekperigin, H.E., et al, 1983; Hirsh, D.C., et al, 1983). In July of 1999, several Louisiana pet turtle export shipment lots failed certification. We received bacterial isolates from failed-lot turtle samples from the certification laboratory, and we detected strains that were resistant to concentrations of gentamicin >2000 µg/ml. The goal of the present study was to identify the gene or genes which contribute to the high-level gentamicin resistance phenotype observed in enteric bacteria, particularly Salmonella spp., from environmental samples with turtle farming activity in Louisiana, and to determine the incidence of such genes in the turtle farm environment.

2.2 Materials and Methods

2.2.1. Sample Recovery, Bacterial Isolation and Identification

The main geographical areas in Louisiana where turtle farming is conducted are Pierre Part, Jonesville and Ponchatoula (Michael-Marler, S., et al, 1983; Siebeling, R.J., et al, 1984; Izadjoo, M.J., et al, 1987). Samples were taken from various commercial pet turtle farms in
Louisiana during the egg-laying season (April through September), from 1999 to 2002. Samples were individually taken at different steps of the egg sanitation process, since each step involved different exposures to gentamicin (GEN): 1) turtle hatchlings which had failed the Certification assay (i.e., they were excreting *Salmonella* spp.) and which were provided by Central Analytical Laboratories, Belle Chase, LA (Siebeling, R.J., *et al*., 1975a; Michael-Marler, S., *et al*., 1983); 2) eggs, which were collected directly from dirt nests and which had not yet been treated with GEN; 3) the 1000 µg/ml GEN solution used to treat eggs and 4) water from pet turtle farm ponds. Each sample was taken separately and only one isolate of each genus and species was recovered per sample, to avoid double inclusion of the same isolate. Samples were enriched for *Salmonella* spp. using selenite cistine broth (Difco, Detroit, MI), and isolated on bismuth sulfite (Difco) plates as previously described (Siebeling, R.J., *et al*., 1975a; Siebeling, R.J., *et al*., 1984). Gram-negative bacteria were routinely grown at 37°C using McConkey (Difco) plates. Isolates were identified using API-20E or API-NE (bioMérieux SA, Marcy l'Etoile, France). Conjugation assays were done using Heart Infusion (HI) media (Difco). Laboratory strains were grown using Luria-Bertani (LB) media (Sambrook, J., *et al*., 1989). All GEN-resistant strains were propagated in media supplemented with 100 µg/ml GEN. A summary of all laboratory bacterial strains and plasmids used in this study is found in Table 2.1.

### 2.2.2. Antibiotic Susceptibility Assays

The minimal inhibitory concentration (MIC) assays were performed in microdilution plates, using Mueller-Hinton (MH) broth (Difco), following the procedure recommended by the National Committee for Clinical Laboratory Standards (NCCLS) (NCCLS, 1999), using the following aminoglycoside antibiotics: GEN, kanamycin (KAN), streptomycin (STR), neomycin (NEO), tobramycin (TOB), amikacin (AMK), netilmicin (NET) and sisomicin (SIS) (Sigma, St.
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<th>Strain or plasmid</th>
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<td><em>E. coli</em> TOP10</td>
<td>Electrocompetent or chemically competent. F&lt;sup&gt;mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 deoR araD139 (ara-leu)7697 galU galK rpsL (Str&lt;sup&gt;r&lt;/sup&gt;) endA1 nupG GEN&lt;sup&gt;s&lt;/sup&gt; (MIC: &lt;3.9 µg/ml)</td>
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<td><em>E. coli</em> strain BM14</td>
<td>AZI; GEN&lt;sup&gt;s&lt;/sup&gt; (MIC: &lt;3.9 µg/ml)</td>
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<td><strong>Environmental strains</strong></td>
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<td></td>
</tr>
<tr>
<td><em>S. enterica</em> subsp. <em>enterica</em>, strain S5</td>
<td>GEN KAN TOB; GEN MIC: &gt;2000 µg/ml</td>
<td>turtle nest eggs&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>S. enterica</em> subsp. <em>arizonae</em>, strain A40</td>
<td>GEN KAN TOB; GEN MIC: &gt;2000 µg/ml</td>
<td>infected turtles&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>S. enterica</em> subsp. <em>arizonae</em>, strain PH5</td>
<td>GEN KAN TOB; GEN MIC: &gt;2000 µg/ml</td>
<td>turtle pond water&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>E. cloacae</em>, strain E19</td>
<td>GEN KAN TOB; GEN MIC: &gt;2000 µg/ml</td>
<td>infected turtles&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>C. freundii</em>, strain C14</td>
<td>GEN KAN TOB; GEN MIC: &gt;2000 µg/ml</td>
<td>turtle nest eggs&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pGEM&lt;sup&gt;®&lt;/sup&gt;-7Zf</td>
<td>cloning and sequencing vector; AMP</td>
<td>Promega</td>
</tr>
<tr>
<td>pCR2.1-TOPO</td>
<td>cloning and sequencing vector; KAN</td>
<td>Invitrogen</td>
</tr>
</tbody>
</table>

(table cont’d)
<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSal5</td>
<td>R-plasmid isolated from <em>S. enterica</em> subsp. <em>enterica</em>, strain S5</td>
<td>this study</td>
</tr>
<tr>
<td>pAri40</td>
<td>R-plasmid isolated from <em>S. enterica arizonae</em>, strain A40</td>
<td>this study</td>
</tr>
<tr>
<td>pAriPH5</td>
<td>R-plasmid isolated from <em>S. enterica</em> subsp. <em>arizonae</em>, strain PH5</td>
<td>this study</td>
</tr>
<tr>
<td>pEnt19</td>
<td>R-plasmid isolated from <em>E. cloacae</em>, strain E19</td>
<td>this study</td>
</tr>
<tr>
<td>pCit14</td>
<td>R-plasmid isolated from <em>C. freundii</em>, strain C14</td>
<td>this study</td>
</tr>
<tr>
<td>pGSal5h</td>
<td>pGEM(^\text{®})-7Zf derivative containing a 5.5 kb HindIII fragment from pSal5</td>
<td>this study</td>
</tr>
<tr>
<td>pGEnt19b</td>
<td>pGEM(^\text{®})-7Zf derivative containing a 5.0 kb BamHI fragment from pEnt19</td>
<td>this study</td>
</tr>
</tbody>
</table>

\(^a\) isolated from nest egg homogenate (26, 37, 38) at the Louisiana turtle farms, July 1999; \(^b\) supplied by Central Analytical Laboratory, Belle Chase, LA, July 1999, \(^c\) isolated from turtle breeding pond water in Louisiana, June 2000. Abbreviations: AMP, ampicillin; AZI, sodium azide; GEN, gentamicin; GEN\(^s\), gentamicin sensitive; KAN, kanamycin; TOB, tobramycin.
Louis, MO). In all cases, the starting antibiotic concentration tested was 2000 µg/ml serially diluted two-fold to 3.9 µg/ml.

The Kirby-Bauer disk-diffusion assay was performed using MH agar plates according to NCCLS standards (NCCLS, 1999); using the following antibiotics: GEN (10 µg), KAN (30 µg), TOB (10 µg), AMK (30 µg), ampicillin (AMP) (10 µg), tetracycline (TET) (30 µg), chloramphenicol (CHL) (30 µg), polymixin B (PMB) (300 units), cephalothin (CEF) (30 µg), cefotaxime (CTX) (30 µg), trimethoprim (TMP) (5 µg) and nitrofurantoin (NIT) (300 µg) (Difco). Inhibition zone breakpoints were interpreted according to information supplied by the manufacturer and to NCCLS standards (NCCLS, 1999).

2.2.3. R-Plasmid Isolation and Characterization

Conjugation was done to detect plasmids carrying GEN-resistance determinants. In each bacterial mating experiment, a GEN-resistant organism was used as the donor. Representative isolates of each genus and species (chosen based on similar resistance phenotypes) were used as donors and included

- *S. enterica* subsp. *enterica* serovar Enteritidis,
- *S. enterica* subsp. *arizonae*,
- *E. cloacae*,
- *C. freundii*,
- *P. aeruginosa*,
- *Acinetobacter baumannii*,
- *Stenotrophomonas maltophilia*,
- *Proteus vulgaris* and
- *Providencia stuartii*.

*E. coli* strain BM14, resistant to sodium azide (AZI) was used as the recipient (Cloeckaert, A., *et al.*, 2000). Bacterial mating was done by mixing 1 ml of a log-phase culture of GEN-resistant donor cells with 4 ml of log-phase recipient AZI-resistant *E. coli* BM14 cells and incubating at 37°C for 16 hours, with no shaking. Transconjugant AZI-resistant, GEN-resistant *E. coli* colonies were selected on HI plates supplemented with 100 µg/ml GEN and 500 µg/ml AZI.

R-plasmids from GEN-resistant bacteria were purified using QIAfilter plasmid kits (Qiagen, Valencia, CA), following instructions provided by the manufacturer for low copy
number plasmids. Plasmids were eluted in molecular grade water at 65°C and stored at -20°C. To minimize the possibility of working with strains carrying more than one plasmid (mobilizable plasmids that might co-transfer during conjugation) and to better assess the resistance phenotypes (AZI-resistant transconjugant *E. coli* have a slower growth rate), plasmids isolated from transconjugants were used to transform naïve OneShot TOP10 *E. coli* cells (Invitrogen, Carlsbad, CA), using a Bio-Rad Gene Pulser II electroporator (BioRad, Hercules, CA), following recommendations from the manufacturer. Briefly, 50 µl electrocompetent cells were transferred to a chilled 0.1 cm cuvette with 1-2 µl of diluted plasmid DNA. Pulses were given at 2.0 kV, 25 µF and 100-200 Ω for 5 msec. Transformant GEN-resistant *E. coli* cells were selected on LB plates supplemented with 100 µg/ml GEN.

To compare their restriction fragment profiles, R-plasmids were isolated as described above from environmental GEN-resistant isolates, transconjugant *E. coli*, and transformant *E. coli* cells. DNA concentration of uncut plasmids was estimated by comparison to Supercoiled Plasmid DNA ladder (Bayou Biolabs, Harahan, LA), after 0.5% Agarose I (Sigma) in 0.5x TBE (BioRad) gel electrophoresis migration. R-plasmids were digested separately with EcoRI, HindIII or BamHI (New England Biolabs, Beverly, MA), following recommendations from the manufacturer for each restriction enzyme. Restriction enzyme digestion profiles were assessed using 0.5% agarose III (Amresco, Solon, OH) gel electrophoresis in TAE buffer (Sambrook, J., *et al*, 1989). The 1 kb DNA ladder and λ DNA-Hind III Digest (New England BioLabs) were used as molecular size standards. Image analysis was done by UV transillumination of ethidium bromide-stained gels using the Quantity One imaging software (BioRad).

The sizes of the large R-plasmids were estimated using Pulse Field Gel Electrophoresis. Briefly, 0.8% Pulse Field Electrophoresis Agarose (Sigma) plugs were made with R-plasmids isolated as described above. Agarose plugs were loaded onto a 90 ml, 1% agarose (Sigma) gel
in 0.5x TBE buffer and run in 0.5x TBE buffer using a CHEF–DR II system (BioRad). Electrophoresis was run for 22 h at 6 V/cm, 120° and 14°C with switch times ramping 1-25 seconds. The Midrange I PFG Marker (New England BioLabs) and the BAC-Tracker Supercoiled DNA Ladder (Epicentre Biotechnologies, Madison, WI) were used as a molecular size standards. Image analysis was done after staining with ethidium bromide, using Quantity One imaging software.

2.2.4. Cloning and Identification of GEN-Resistance Gene(s)

A vast number of genes are known to confer GEN resistance in Gram-negative bacteria (Shaw, K.J., et al, 1993; Wright, G.D., et al, 1998; Vakulenko, S.B. and Mobashery, S., 2003). To determine which gene(s) confer GEN resistance in the isolates of this study, these were cloned and sequenced as follows: R-plasmids isolated as described above were digested with EcoRI, HindIII or BamHI, according to the manufacturer’s recommendations. Restriction fragments were purified using QIAquick Spin columns (Qiagen) and ligated into previously digested pGEM®-7Zf (Promega, Piscataway, NJ). Ligation reactions were used to transform E. coli TOP10 chemically competent cells (Invitrogen) following the protocol provided by the manufacturer. Recombinant clones carrying GEN-resistance determinants were selected on LB plates supplemented with 100 µg/ml GEN and 50 µg/ml AMP. Recombinant plasmids were purified with QIAprep Spin columns (Qiagen) and inserts were sequenced using the vector’s internal sequencing primers.

2.2.5. Sequencing and Computer Analysis

Sequencing was done with fluorescent dye-labeled dideoxynucleotides with an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequence analysis was done using VectorNTI (Invitrogen), BLAST (Altschul, S.F., et al, 1997) and GeneBank

2.2.6. Screening for GEN Resistance Genes

Multiplex PCR assays were designed to screen environmental isolates for the GEN-resistance genes that were identified through cloning and sequencing of GEN resistance determinants. Multiplex PCR reactions included primers to detect resistance genes \textit{aac(3)IIa} and \textit{aac(3)VIa}, as well as a fragment of the 16S rRNA gene as an internal control for each reaction (Vliegenthart, J.S., \textit{et al}, 1990). Primers were designed using Vector NTI (Invitrogen). PCR screening of genes \textit{intI} and \textit{sulI}, commonly associated with class 1 integrons (Stokes, H.W. and Hall, R.M., 1989; White, P.A., \textit{et al}, 2001), and of \textit{armA}, \textit{rmtA} and \textit{rmtB}, the only genes reported to confer high level aminoglycoside resistance in Gram-negative bacteria (Yamane, K., \textit{et al}, 2005), was done in separate reactions using published primers (Schwocho, L.R., \textit{et al}, 1995; Sunde, M. and Sorum, H., 1999; Galimand, M., \textit{et al}, 2003; Yokoyama, K., \textit{et al}, 2003; Doi, Y., \textit{et al}, 2004; Yamane, K., \textit{et al}, 2005). All primers used are summarized in Table 2.2. To prepare DNA for PCR, pelleted cells from 1 ml of overnight cultures were resuspended in 100 µl dH₂O and lysed through two 5 min. freeze-boil cycles. 2 µl of the supernatants were used as templates in 50 µl PCR reactions. PCR was done using the FailSafe™ PCR System with buffer “G” (Epicentre Biotechnologies) according to instructions provided by the manufacturer. Thermocycler conditions were set as follows: [1X (96°C, 5 min); 35X (96°C, 30 sec; 58°C, 30 sec; 70°C, 60 sec); (70°C, 7 min)]. To amplify the \textit{armA} gene, PCR was done using the same conditions stated above, but various annealing temperatures (40°C, 42.2°C, 44.1°C, 46.9°C, 48.9°C, 50.2°C and 51°C) were assayed in different reactions. PCR products were visualized by agarose gel electrophoresis using 2% Agarose I in 0.5x TBE buffer and staining with ethidium bromide. Some PCR products were sequenced to confirm
<table>
<thead>
<tr>
<th>PCR target</th>
<th>Primer</th>
<th>Amplicon size (bp)</th>
<th>Sequence (5' → 3')</th>
<th>Accession No. (Reference)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>aac(3)-Ila</em></td>
<td>aacIIaF</td>
<td>900</td>
<td>GGG AAT TCA GAG GAG ATA TCG CGA TGC ATA CG</td>
<td>X13543 (*)</td>
</tr>
<tr>
<td></td>
<td>aacIIaR</td>
<td></td>
<td>CAT TGT CGA CGG CCT CTA ACC</td>
<td></td>
</tr>
<tr>
<td><em>aac(3)-VIa</em></td>
<td>aacVIaF</td>
<td>465</td>
<td>CGC TCA GGC GAT ATG GTG AT</td>
<td>L22613 (*)</td>
</tr>
<tr>
<td></td>
<td>aacVIaR</td>
<td></td>
<td>CAT AAT GGA CGG CGG TGA CT</td>
<td></td>
</tr>
<tr>
<td><em>armA</em></td>
<td>metF</td>
<td>777</td>
<td>CAA ATG GAT AAG AAT GAT GAT GAT</td>
<td>AY220558 (18)</td>
</tr>
<tr>
<td></td>
<td>metR</td>
<td></td>
<td>TTA TTT CTG AAA TCC ACT</td>
<td></td>
</tr>
<tr>
<td><em>rmtA</em></td>
<td>RMTA-F</td>
<td>634</td>
<td>CTA GCG TCC ATC CTT TCC TC</td>
<td>AB083212 (46)</td>
</tr>
<tr>
<td></td>
<td>RMTA-R</td>
<td></td>
<td>TTT GCT TCC ATG CCC TTG CC</td>
<td></td>
</tr>
<tr>
<td><em>rmtB</em></td>
<td>MBH-F</td>
<td>771</td>
<td>GGA ATT CCA TAT GAA CAT CAA CG A TGC CCT</td>
<td>AB103506 (13)</td>
</tr>
<tr>
<td></td>
<td>MBH-R</td>
<td></td>
<td>CCG CTC GAG TCC ATT CTT TTT TAT CAA G TA</td>
<td></td>
</tr>
<tr>
<td><em>intI1</em></td>
<td>intIF</td>
<td>845</td>
<td>CTA CCT CTC ACT AGT GAG GGG CGG</td>
<td>X15852 (34,40)</td>
</tr>
<tr>
<td></td>
<td>intIR</td>
<td></td>
<td>GGG CAG CAG CGA ATG CGA G GC</td>
<td></td>
</tr>
<tr>
<td><em>sulI1</em></td>
<td>sulF1</td>
<td>840</td>
<td>ATG GTG ACG GTG TTT GCC AT</td>
<td>U12441 (18,40)</td>
</tr>
<tr>
<td></td>
<td>sulR</td>
<td></td>
<td>CTA GGC ATG ATC TAA CCC TC</td>
<td></td>
</tr>
<tr>
<td><em>rrn</em></td>
<td>rrlA</td>
<td>330</td>
<td>TAA CAC ATG CAA GT C GA A CG</td>
<td>(43)</td>
</tr>
<tr>
<td></td>
<td>rrl2B</td>
<td></td>
<td>CCC ATT GTG CAA TAT TCC CC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M13F (-20)</td>
<td>variable</td>
<td>GTA AAA CGA CGG CCA G</td>
<td>(Invitrogen)</td>
</tr>
<tr>
<td></td>
<td>M13R</td>
<td></td>
<td>CAG GAA ACA GCT ATG AC</td>
<td></td>
</tr>
</tbody>
</table>

* E.C. Achberger, personal communication
amplification of the appropriate genes and to compare with published sequences in the databases. For sequencing, PCR products were purified using QIAquick Spin columns and ligated into pCR2.1-TOPO using the TOPO-TA cloning kit (Invitrogen), as suggested by the manufacturer. Sequencing of appropriate clones was done using the internal vector sequencing primers, as described above.

2.3 Results

2.3.1. Gentamicin Resistance in Turtle Farms

Bacterial genera and species recovered from the turtle farm setting are summarized in Table 2.3, and they include *S. enterica* subsp. *enterica*, *S. enterica* subsp. *arizonae*, *E. cloacae*, *C. freundii*, *P. aeruginosa*, *A. baumannii*, *S. maltophilia*, *A. faecalis*, *P. vulgaris*, and *P. stuartii*. The antibiotic resistance phenotypes of the organisms tested show that in addition to resistance to GEN, these organisms exhibited resistance to other aminoglycosides such as KAN and TOB, but not to AMK. Some organisms were also resistant to AMP (Table 2.3). Organisms such as *P. aeruginosa* and *S. maltophilia* were resistant to the majority of the antibiotics assayed.

As summarized in Table 2.3, the majority of GEN-resistant bacteria displayed high-level resistance to GEN (MIC>2000 µg/ml). GEN-sensitive bacteria were primarily recovered from untreated eggs and breeding ponds and presented GEN MICs from <3.9-7.8 µg/ml (Table 2.3), whereas the majority of isolates recovered from contaminated turtles and the GEN dip solutions showed elevated resistance to GEN. Nevertheless, GEN-resistant isolates recovered from untreated eggs and breeding ponds also expressed high-level of resistance. *E. coli* strain BM14, *E. coli* OneShot TOP10, and *E. coli* ATCC 25922, used as GEN-sensitive controls, had GEN MICs <3.9-7.8 µg/ml (Table 2.1).
Table 2.3. Prevalence of Gentamicin Resistance Genes Among Bacterial Isolates From Turtle Farms

<table>
<thead>
<tr>
<th>Sample&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Identified organism</th>
<th>Number collected</th>
<th>GEN&lt;sup&gt;r&lt;/sup&gt; n (%)</th>
<th>Resistance phenotype&lt;sup&gt;b&lt;/sup&gt;</th>
<th>GEN MIC (µg/ml)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>% Incidence&lt;sup&gt;d&lt;/sup&gt;acon(3)IIa</th>
<th>acon(3)VIa</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pond water</strong></td>
<td><em>Citrobacter freundii</em></td>
<td>43</td>
<td>7 (16)</td>
<td>GEN KAN TOB</td>
<td>&gt;2000</td>
<td>43</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td><em>Enterobacter cloacae</em></td>
<td>20</td>
<td>3 (15)</td>
<td>GEN KAN TOB</td>
<td>&gt;2000</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td><em>Pseudomonas aeruginosa</em></td>
<td>4</td>
<td>2 (50)</td>
<td>GEN KAN TOB AMP (CHL) (CTX) NIT (TET) TMP</td>
<td>&gt;2000</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td><em>S. enterica subsp. arizonae</em></td>
<td>38</td>
<td>4 (11)</td>
<td>GEN KAN TOB</td>
<td>&gt;2000</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td><em>Stenotrophomonas maltophilia</em></td>
<td>7</td>
<td>3 (43)</td>
<td>GEN KAN TOB AMP (CHL) (CTX) NIT (TET) TMP</td>
<td>&gt;2000</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td><strong>Nest eggs</strong></td>
<td><em>Citrobacter freundii</em></td>
<td>49</td>
<td>6 (12)</td>
<td>GEN KAN TOB</td>
<td>&gt;2000</td>
<td>33</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td><em>Enterobacter cloacae</em></td>
<td>44</td>
<td>2 (5)</td>
<td>GEN KAN TOB</td>
<td>&gt;2000</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td><em>Escherichia coli</em></td>
<td>3</td>
<td>1 (33)</td>
<td>GEN KAN TOB</td>
<td>&gt;2000</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td><em>Proteus vulgaris</em></td>
<td>1</td>
<td>1 (100)</td>
<td>GEN AMP NIT PMB (TMP)</td>
<td>500</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td><em>Pseudomonas aeruginosa</em></td>
<td>12</td>
<td>5 (42)</td>
<td>GEN KAN TOB AMP (CHL) (CTX) NIT (TET) TMP</td>
<td>&gt;2000</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td><em>S. enterica subsp. enterica</em></td>
<td>4</td>
<td>2 (50)</td>
<td>GEN KAN TOB</td>
<td>&gt;2000</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td><em>S. enterica subsp. arizonae</em></td>
<td>53</td>
<td>11 (21)</td>
<td>GEN KAN TOB</td>
<td>&gt;2000</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td><strong>GEN soln.</strong></td>
<td><em>Acinetobacter baumannii</em></td>
<td>1</td>
<td>1 (100)</td>
<td>GEN KAN TOB AMP TMP</td>
<td>&gt;2000</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td><em>Alcaligenes xylosoxidans</em></td>
<td>2</td>
<td>2 (100)</td>
<td>GEN KAN TOB AMP (CHL) CTX NIT TET TMP</td>
<td>&gt;2000</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td><em>Pseudomonas aeruginosa</em></td>
<td>3</td>
<td>3 (100)</td>
<td>GEN KAN TOB AMP (CHL) CTX NIT TET TMP</td>
<td>&gt;2000</td>
<td>100</td>
<td>-</td>
</tr>
</tbody>
</table>

(table cont’d)
<table>
<thead>
<tr>
<th>Bacterial Species</th>
<th>Isolate Code</th>
<th>GEN Resistance</th>
<th>Antibiotic Sensitivity</th>
<th>MIC (µg/ml)</th>
<th>O Additional Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pseudomonas fluorescens</em></td>
<td>2</td>
<td>(100)</td>
<td>GEN KAN TOB AMP (CHL)</td>
<td>&gt;2000</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(CTX) NIT (TET) TMP</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Turtles</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Alcaligenes faecalis</em></td>
<td>1</td>
<td>(100)</td>
<td>GEN KAN TOB AMP TMP</td>
<td>&gt;2000</td>
<td>100</td>
</tr>
<tr>
<td><em>Citrobacter freundii</em></td>
<td>4</td>
<td>(100)</td>
<td>GEN KAN TOB</td>
<td>&gt;2000</td>
<td>-</td>
</tr>
<tr>
<td><em>Enterobacter cloacae</em></td>
<td>3</td>
<td>(100)</td>
<td>GEN KAN TOB</td>
<td>&gt;2000</td>
<td>-</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>1</td>
<td>(100)</td>
<td>GEN KAN TOB</td>
<td>&gt;2000</td>
<td>-</td>
</tr>
<tr>
<td><em>Proteus vulgaris</em></td>
<td>2</td>
<td>(100)</td>
<td>GEN AMP NIT PMB (TMP)</td>
<td>500</td>
<td>100</td>
</tr>
<tr>
<td><em>Providencia stuartii</em></td>
<td>3</td>
<td>(100)</td>
<td>GEN AMP NIT PMB (TMP)</td>
<td>500</td>
<td>100</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>8</td>
<td>(63)</td>
<td>GEN KAN TOB AMP (CHL)</td>
<td>&gt;2000</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(CTX) NIT (TET) TMP</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>S. enterica subsp. arizonae</em></td>
<td>28</td>
<td>(100)</td>
<td>GEN KAN TOB</td>
<td>&gt;2000</td>
<td>-</td>
</tr>
</tbody>
</table>

*a* Samples were taken from nest egg homogenate, turtle breeding pond water and GEN soln., as previously described (26, 37, 38), at turtle farms in Louisiana, 1999-2002. Infected turtle isolates were supplied by Central Analytical Laboratory, Belle Chase, LA, July 1999. 

*b* Resistance phenotype and *c* MIC value displayed by >90% of GEN resistant isolates, for each genus and species. GEN sensitive isolates presented GEN MIC <3.9-7.8 µg/ml. 

*d* % GEN resistant strains carrying *aac(3)IIa* or *aac(3)VIa*. Abbreviations: AMP, ampicillin; CHL, chloramphenicol; CTX, cefotaxime; GEN, gentamicin; GENr, GEN resistance; GEN soln., GEN egg dip solution (1000 µg/ml); KAN, kanamycin; NIT, nitrofurantoin; PMB, polymyxin B; TET, tetracycline; TMP, trimethoprim; TOB, tobramycin; antibiotics in parenthesis denote intermediate resistance.
2.3.2. R-plasmid Isolation

When checked for plasmids, environmental GEN-resistant isolates showed one or more plasmids. Nevertheless, transconjugant *E. coli* resistant to GEN and AZI were obtained only from the strains of *S. enterica* subsp. *enterica*, *S. enterica* subsp. *arizonae*, *E. cloacae* and *C. freundii*. Transconjugants' GEN MICs were all >2000 µg/ml. Likewise, the antibiograms were similar between donor organisms and their *E. coli* transconjugants. Furthermore, OneShot TOP10 *E. coli* cells transformed with R-plasmids isolated from these transconjugants acquired the same resistance phenotypes as the transconjugant from which the R-plasmid had been isolated. The resulting phenotypes and genotypes of these transformant *E. coli* are summarized in Table 2.4.

2.3.3. Identification of GEN Resistance Gene(s)

R-plasmid pSal5 was isolated from GEN-resistant *E. coli* obtained after transformation with plasmids from *S. enterica* (strain S5) (Table 4). Recombinant plasmid pGSal5h was obtained from a 5.5 kb HindIII restriction fragment of pSal5 that was ligated into pGEM®-7Zf. Sequence analysis of this GEN-resistant clone revealed the presence of the aminoglycoside resistance gene *aac(3)IIa*, which was 98% identical to that in GenBank database accession no. X13543 (Brau, B., *et al*, 1984; Allmansberger, R., *et al*, 1985; Vakulenko, S.B. and Mobashery, S., 2003).

Preliminary screening to detect gene *aac(3)IIa* in other environmental isolates revealed that some GEN-resistant organisms did not carry *aac(3)IIa*. Thus, R-plasmid pEnt19, originally from *E. cloacae* (strain E19) (Table 2.4), was used for cloning and sequencing since this plasmid conferred GEN resistance, but did not carry *aac(3)IIa*. Recombinant plasmid pGEnt19b was obtained from a 5 kb BamHI fragment of R-plasmid pEnt19 ligated into
<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Size (kb)</th>
<th>RFLP type</th>
<th>Resistance phenotype&lt;sup&gt;a&lt;/sup&gt;</th>
<th>GEN MIC (µg/ml)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>GEN resistance and integron-associated genes&lt;sup&gt;b&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>pSal5</td>
<td>ca. 45</td>
<td>A</td>
<td>GEN KAN TOB</td>
<td>&gt;2000</td>
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</tr>
<tr>
<td>pAri40</td>
<td>ca. 145</td>
<td>B</td>
<td>GEN KAN TOB</td>
<td>&gt;2000</td>
<td>-</td>
</tr>
<tr>
<td>pAriPH5</td>
<td>ca. 45</td>
<td>B</td>
<td>GEN KAN TOB</td>
<td>&gt;2000</td>
<td>-</td>
</tr>
<tr>
<td>pEnt19</td>
<td>ca. 115</td>
<td>C</td>
<td>GEN KAN TOB</td>
<td>&gt;2000</td>
<td>-</td>
</tr>
<tr>
<td>pCit14</td>
<td>ca. 45</td>
<td>D</td>
<td>GEN KAN TOB</td>
<td>&gt;2000</td>
<td>+</td>
</tr>
</tbody>
</table>

<sup>a</sup> determined using transformant TOP10 *E. coli* cells; <sup>b</sup> as determined by PCR and/or sequencing
pGEM®-7Zf. Sequence analysis of pGEnt19b revealed the presence of aminoglycoside acetyltransferase gene \textit{aac(3)VIa}, which was 99% identical to that in GenBank database accession no. L22613 (Rather, P.N., \textit{et al}, 1993). Sequencing and PCR additionally revealed integron class 1 associated genes \textit{intI1} and \textit{sul1} (\textbf{Table 2.4}), indicating the presence of \textit{aac(3)VIa} as a gene cassette of a class-1 integron.

Genes \textit{aac(3)IIa} or \textit{aac(3)VIa} have not been reported in the literature to confer high-level GEN resistance (Brau, B., \textit{et al}, 1984; Allmansberger, R., \textit{et al}, 1985; Rather, P.N., \textit{et al}, 1993; Vakulenko, S.B. and Mobashery, S., 2003). We thus attempted to amplify by PCR the 16S rRNA methylase genes recently reported in the literature which do confer a high-level aminoglycoside resistance phenotype in Gram-negative bacteria, namely \textit{armA} (Galimand, M., \textit{et al}, 2003), \textit{rmtA} (Yokoyama, K., \textit{et al}, 2003; Yamane, K., \textit{et al}, 2004) and \textit{rmtB} (Doi, Y., \textit{et al}, 2004). Nevertheless, these genes were not detected in the isolates tested (\textbf{Table 2.4}).

\textbf{2.3.4. Distribution of GEN Resistance Genes in Bacterial Isolates From Turtle Farms}

Multiplex PCR assays designed to screen for the presence of resistance genes \textit{aac(3)IIa} and \textit{aac(3)VIa} on bacterial isolates from turtle farms (N=336), revealed that every GEN-resistant organism tested carried either one of these acetyltransferase genes (Table 3). Gene \textit{aac(3)IIa} was present in 37% of the GEN-resistant isolates tested (n=101); it was detected mainly in non-fermentors but was also found in \textit{S. enterica} subsp. \textit{enterica} and \textit{C. freundii}. Gene \textit{aac(3)VIa} accounted for 94% of GEN resistance in \textit{Salmonella} spp. (n=35). Neither gene was detected in GEN-sensitive organisms, including GEN-sensitive controls previously described. \textit{intI1} was detected by PCR in all GEN-resistant bacteria carrying \textit{aac(3)VIa}, but not in organisms carrying \textit{aac(3)IIa}. 

66
Figure 2.1. HindIII and BamHI digestion profiles of R-plasmids pSal5 (lanes 1 & 4), pAri40 (lanes 2 & 5) and pEnt19 (lanes 3 & 6). λ-DNA-Hind III digest, used as a molecular standard, is indicated on the left. Arrowheads indicate bands carrying genes aac(3)IIa (►) and aac(3)Vla (◄).
2.3.5. Plasmid Heterogeneity

R-plasmids pSal5, pAri40, and pEnt19 showed different HindIII and BamHI RFLP profiles (Figure 2.1). Multiplex PCR of resistance genes aac(3)IIa and aac(3)VIa (Table 2.4) plus hybridization of these genes with digested R-plasmids (Figure 2.1) showed that pSal5 carried aac(3)IIa, and that pAri40 and pEnt19 carried aac(3)VIa, confirming that these genes are plasmid-borne. The mentioned R-plasmids differed in size as well, as visualized after PFGE and ethidium bromide staining. These results are summarized in Table 2.4.

2.4 Discussion

Turtles and other reptiles are known to excrete S. enterica subsp. arizonae and other salmonellae (Cohen, M.L., et al, 1980; D'Aoust, J.Y., et al, 1990; Ebani, V.V., et al, 2005). The GEN resistance that has emerged in the last decade presents a threat to the efficacy of the GEN sulfate treatment used by the turtle industry to eradicate Salmonella spp. In this study, we found two genes which are carried by bacteria recovered from environmental samples with turtle farming activity which confer high-level GEN resistance. These genes are aac(3)IIa and aac(3)VIa, which code for aminoglycoside acetyltransferase enzymes (Brau, B., et al, 1984; Allmansberger, R., et al, 1985; Rather, P.N., et al, 1993). Gene aac(3)IIa presents the AAC(3)II aminoglycoside resistance pattern phenotype, which involves resistance to GEN, TOB, SIS, NET, and DBK (Brau, B., et al, 1984; Allmansberger, R., et al, 1985; Shaw, K.J., et al, 1993; Vakulenko, S.B. and Mobashery, S., 2003). These genes are reported to be detected in Gram-negative bacteria in the clinical setting with a relatively higher frequency (18-60%) with respect to other aminoglycoside resistance patterns (Shaw, K.J., et al, 1993; Miller, G.H., et al, 1997; Wright, G.D., et al, 1998; Vakulenko, S.B. and Mobashery, S., 2003). Gene aac(3)IIa, originally identified in R-plasmids (Allmansberger, R., et al, 1985), reportedly accounts for 85% of the AAC(3)-II phenotype (Miller, G.H., et al, 1997; Wright, G.D., et al, 1998;

The majority of aminoglycoside modifying enzymes can provide effective resistance to aminoglycosides, but only certain phosphotransferase enzymes detected in Gram-positive bacteria have been reported to produce high-levels of resistance to these antibiotics (Ferretti, J.J., et al, 1986; Shaw, K.J., et al, 1993; Wright, G.D., et al, 1998; Chow, J.W., 2000; Vakulenko, S.B. and Mobashery, S., 2003). When we started this study there had been no reports, to the best of our knowledge, of the acquisition of genes conferring high-level aminoglycoside resistance in environmental Gram-negative bacteria (M.A. Diaz, E.C. Achberger, V.R. Srinivasan and R.J. Siebeling, Abstr. 100th Amer. Soc. Microbiol. Gen. Meeting, abstr. Z-4, 2000). Recently however, Galimand et al (Galimand, M., et al, 2003) found that the high-level aminoglycoside resistance phenotype in a clinical strain of Klebsiella pneumoniae was due to the presence of the aminoglycoside resistance gene armA. This strain of K. pneumoniae also presented genes aac(3)II, blaTEM1, blaCTX-M, dfrA12, sul1 and ant(3")9, conferring resistance to aminoglycosides, β-lactams, trimethoprim, sulfonamides and streptomycin-spectinomycin, respectively (Galimand, M., et al, 2003). In this study, we did not detect gene armA by PCR in any of the isolates screened, in spite of detecting certain shared elements in some of the strains, such as genes aac(3)IIa and sul1 (Table 4). We also screened for two other high-level aminoglycoside resistance genes: rmtA, cloned and identified from a
clinical isolate of \textit{P. aeruginosa} AR-2 in Japan (Yokoyama, K., \textit{et al}, 2003) and plasmid-borne 16S rRNA methylase \textit{rmtB}, isolated from \textit{Serratia marcescens} (Doi, Y., \textit{et al}, 2004). Nevertheless, we did not detect by PCR any other resistance gene that could act in synergy with the already identified \textit{aac(3)IIa} or \textit{aac(3)Vla} gene products or by itself, to give the high-level GEN resistance phenotype observed in the bacteria we isolated in the turtle farms.

The current study is the first to report the genes which confer GEN resistance to bacteria in the turtle farm environment, namely \textit{aac(3)IIa} and \textit{aac(3)Vla}. PCR and sequencing experiments confirmed that these resistance genes are present on mobile genetic elements that can facilitate their horizontal transfer among bacteria in the environment (Allmansberger, R., \textit{et al}, 1985; Rather, P.N., \textit{et al}, 1993; Fluit, A.C. and Schmitz, F.J., 1999; White, P.A., \textit{et al}, 2001; Heuer, H., \textit{et al}, 2002; Carattoli, A., 2003). Both genes are found on R-plasmids of the \textit{Enterobacteriacea} (Brau, B., \textit{et al}, 1984; Allmansberger, R., \textit{et al}, 1985; Rather, P.N., \textit{et al}, 1993). Until recently (Galimand, M., \textit{et al}, 2003), only gene \textit{aac(3)Vla} had been reported as a gene cassette within a class 1 integron (Rather, P.N., \textit{et al}, 1993; Wright, G.D., \textit{et al}, 1998; White, P.A., \textit{et al}, 2001; Yamane, K., \textit{et al}, 2005). Recent studies, however, have also detected the \textit{aac(3)II} gene surrounded by integron elements (Galimand, M., \textit{et al}, 2003). In fact, the molecular environment of \textit{armA} gene has been depicted as a composite or complex integron environment and is found downstream of orf513 coding for a putative recombinase or transposase (Partridge, S.R. and Hall, R.M., 2003). We are currently conducting research to examine the molecular environment of the genes found.

GEN-resistant isolates were more prevalent in samples where exposure to GEN was greater, but were also detected in turtle pond water and nest eggs, consonant with the notion that antibiotic use can favor the selection of resistant strains (Miller, G.H., \textit{et al}, 1997; Levy, S.B., 1998). The presence of these genes on mobile molecular elements can facilitate their
transfer and spread among different bacteria in the turtle farm environment (Fluit, A.C. and Schmitz, F.J., 1999; Heuer, H., et al, 2002; Carattoli, A., 2003). However, additional studies are required to determine if the interspecies dissemination of these genes occurred at the turtle farms. This study illustrates the potential of transfer of antibiotic resistance genes, particularly GEN resistance, between different genera in the turtle farms, a matter of particular public health concern as it involves the human pathogen *Salmonella* spp. The epidemiological data presented stresses the need to develop an alternate method for the eradication of *Salmonella* spp. from turtle eggs. This is the first study to describe the genes involved in GEN resistance in *S. enterica* subsp. *arizonae*. It is important to monitor the presence of these resistance genes and molecular transfer elements in bacteria like the salmonellae, since these cause zoonosis and represent possible resistance reservoirs and can thus be important in the development of multiresistance in bacteria (Boyd, D., et al, 2002; Carattoli, A., 2003; Ebani, V.V., et al, 2005), as well as in its transfer between animals and humans.

2.5 References


CHAPTER 3:

CULTURE-INDEPENDENT IDENTIFICATION OF BACTERIA PRESENT IN GENTAMICIN SOLUTIONS USED TO ERADICATE _SALMONELLA_ SPP. FROM PET TURTLES
3.1 Introduction

The sale of small turtles, *Pseudemys (Trachemys) scripta elegans*, has been an important economic activity in the State of Louisiana since the 1930s (Siebeling, R.J., *et al*., 1975b; Cohen, M.L., *et al*., 1980). In spite of the domestic ban imposed on the sale of small carapace turtles due to salmonellosis outbreaks that were traced back to these pets, the export market has continued to thrive (Michael-Marler, S., *et al*., 1983; Siebeling, R.J., *et al*., 1984; Izadjoo, M.J., *et al*., 1987). However, in order for baby turtles to be certified for export to Europe, Asia and South America, *Salmonella* spp. must be eradicated from the turtles (Siebeling, R.J., *et al*., 1975a; Michael-Marler, S., *et al*., 1983; Siebeling, R.J., *et al*., 1984). For this, turtle farms follow a modified version of the FDA-approved pressure-differential gentamicin treatment protocol used in the turkey farm industry (Saif, Y.M. and Shelly, S.M., 1973; Greenfield, J., *et al*., 1975; Ekperigin, H.E., *et al*., 1983; Hirsh, D.C., *et al*., 1983). In brief, turtle eggs are treated in a solution of 1000 ppm (µg/ml) gentamicin by the pressure differential method to ensure penetration of the antibiotic into the eggs (Siebeling, R.J., *et al*., 1975b; Michael-Marler, S., *et al*., 1983; Siebeling, R.J., *et al*., 1984). The use of the gentamicin sulfate solution (Garasol; Shering Corp., Bloomfield, N.J.) was chosen for its practical advantage since it has a stable shelf life and can be boiled and filtered, and it retains its bactericidal activity, allowing the solution to be stored and reused after appropriate adjustments (Siebeling, R.J., *et al*., 1975b; Michael-Marler, S., *et al*., 1983; Siebeling, R.J., *et al*., 1984).

Gentamicin belongs to the family of the aminoglycosides, which are highly-potent, broad-spectrum bactericidal antibiotics, that are commonly used clinically in combination with β-lactams for the treatment of Gram-positive organisms such as *Staphylococcus aureus* and the streptococci as well as aerobic Gram-negative infections (Shaw, K.J., *et al*., 1993; Wright, G.D., *et al*., 1998; Vakulenko, S.B. and Mobashery, S., 2003). Gentamicin is a 4,6-disubstituted 2-
deoxystreptamine aminoglycoside (Wright, G.D., et al, 1998; Vakulenko, S.B. and Mobashery, S., 2003). Aminoglycoside antibiotics act by inhibiting prokaryotic protein synthesis (Davis, B.D., 1987; Mazel, D. and Davies, J., 1999). They induce misreading of the genetic code by binding to the 16S rRNA A-site in the bacterial ribosome (Moazed, D. and Noller, H.F., 1987). Microorganisms can be intrinsically resistant or become resistant to aminoglycosides through a variety of mechanisms, which include: 1) the alteration of the drug’s target through mutational or enzymatic modifications of the ribosome; 2) the reduced uptake or accumulation of the drug, generally by means of active efflux pumps; and 3) most frequently, the enzymatic modification of the antibiotic molecule by aminoglycoside modifying enzymes (Shaw, K.J., et al, 1993; Wright, G.D., et al, 1998; Vakulenko, S.B. and Mobashery, S., 2003).

In recent years, using a bacteriological culturing approach, we have detected gentamicin resistant organisms in commercial turtle farms in Louisiana (Diaz, M.A., et al, in press). However, the assessment of the diversity of a microbial community can be largely underestimated by these classic culturing techniques (Ward, D.M., et al, 1998; Hengstmann, U., et al, 1999), and organisms which may contribute to maintaining the gentamicin resistance gene pool in the turtle farms could be missed by conventional antibiotic resistance assays. Therefore with this study we intended to identify, using a culture-independent approach, organisms present in the 1000 µg/ml gentamicin sulfate treatment solutions used to eradicate Salmonella spp. from turtle eggs.

3.2 Materials and Methods

3.2.1. Sample Collection

Gentamicin (GEN) treatment solutions were collected in June, 2002 from pet turtle farms in five different locations in Louisiana. Samples were collected observing sterile conditions. The GEN dip solutions had been used and stored at the farms as usual, for 3-4 days
to treat approximately 20,000 turtle eggs. The dip solutions had an original concentration of 1000 µg/ml GEN, which is recalibrated to this concentration to prior to reuse (Siebeling, R.J., et al., 1984). They were kept at 4°C at the farms, then transported and stored for one year in our laboratory at the same temperature.

3.2.2. DNA Extraction and Purification

Ten ml of each unfiltered GEN dip solution were centrifuged to pellet any bacterial cells present in the treatment solution after the customary year of storage at 4°C. The pellet was resuspended in 300 µl saline-EDTA (Sambrook, J., et al., 1989). Cells were lysed by adding 15 µl of lysozyme (BioRad, Hercules, CA) mixing and incubating for 30 min. at 37°C. This was followed by adding 5 µl of Proteinase K (BioRad) and 10 µl SDS (10% w/v) (BioRad) and incubating at 60°C for 10 min. and finally, performing 3 freeze–thaw cycles (10 min. at -80°C followed by 10 min. at 60°C). 300 µl of the supernatants were stored at -20°C for further analysis. The presence of DNA from the lysates was verified on 0.5% agarose I (Sigma, St. Louis, MO) gels in 0.5x TBE buffer (BioRad) after staining with ethidium bromide. Image analysis was done using the Quantity One imaging software (BioRad). Total community DNA was purified using a Prep-A-Gene purification kit (BioRad), according to the manufacturer’s instructions.

3.2.3. Amplification of 16S rRNA Genes From Bacterial Community DNA

In order to amplify the 16S rRNA genes of the bacterial community in each sample, PCR reactions were performed on total community DNA using the universal eubacterial primers: 27f (5’-GAG TTT GAT CCT GGC TCA G-3’) and 1525r (5’-AGA AAG GAG GTG ATC CAG CC-3’) (Rainey, F.A., et al., 1996). These primers bind to highly conserved sequences of the bacterial 16S rRNA gene (Brosius, J., et al., 1978) and produce amplicons of 1.5 kb. Amplification of the 16S rRNA genes was done using Taq polymerase. PCR reactions
were carried out with a Perkin Elmer PCR system 2400 thermocycler (Applied Biosystems, Foster City, CA). Briefly, 6 min. of a hot start step at 98°C were followed by 35 cycles with 30 sec. denaturing at 94°C, 30 sec. primer annealing at 52°C and 60 sec. elongation at 72°C; then a final elongation step of 7 min. at 72°C. PCR products were checked by agarose gel electrophoresis using 2% agarose I in 0.5x TBE buffer (BioRad) and staining with ethidium bromide.

### 3.2.4. 16S rRNA Gene Library

In order to construct a library containing representative clones of the 16S rRNA genes of all the bacteria present in each sample (Ward, D.M., et al, 1998; Hengstmann, U., et al, 1999; Amann, R. and Ludwig, W., 2000), the amplification products obtained with the universal eubacterial primers were ligated into pCR 2.1-TOPO (Invitrogen, Carlsbad, CA) using the TOPO-TA cloning kit (Invitrogen), as indicated by the manufacturer. Recombinant plasmids were transformed into chemically competent TOP10 *Escherichia coli* cells (Invitrogen) and transformants were selected using Luria-Bertani (LB) (Sambrook, J., et al, 1989) plates supplemented with 50 µg/ml kanamycin (KAN), as recommended by the manufacturer. Transformants that grew on LB-KAN plates were picked and grown in LB-KAN media for plasmid isolation.

### 3.2.5. Amplification of 16S rRNA Genes

To screen for clones with appropriate insert size, DNA inserts from each of these clones were amplified using M13 universal primers (5’- GTA AAA CGA CGG CCA G – 3’ and 5’-CAG GAA ACA GCT ATG AC– 3’). Amplification of the 16S rRNA genes was done using Taq polymerase. PCR reactions were carried out using a Perkin Elmer PCR system 2400 thermocycler, with an initial heating step of 3 min. at 94°C, followed by 25 cycles with 30 sec. denaturing at 94°C, 30 sec. primer annealing at 52°C and 60 sec. elongation at 72°C; then a
final elongation step of 9 min. at 72°C. PCR products were separated on 2% agarose I gels, using the 100 bp DNA ladder (Invitrogen) as molecular standards, and visualized after staining with ethidium bromide.

3.2.6. ARDRA

16S rRNA PCR products were digested using TaqI for 1 h. at 65°C. Restriction fragments were separated in 3.0% GenepureSieve GQA agarose gels (ISC BioExpress) in TBE (BioRad). Agarose gels were tempered at 55-60°C for 25 min. before pouring, then cooled for 30 min. at room temperature and 30 min. at 5°C, before use. Different restriction fragments were analyzed by comparison to DNA Molecular Weight Marker VIII (Roche Diagnostics, Basel, Switzerland) using the Bionumerics ARDRA software (Applied Maths, St.-Martens-Latem, Belgium).

3.2.7. Partial Sequencing of Selected Clones

Recombinant plasmids with appropriate sized inserts (1.5 kb) were purified from transformant E. coli to use for sequencing, using QIAprep Spin columns (Qiagen, Valencia, CA) as recommended by the manufacturer. Clones were sequenced using the 27f primer. Automated sequencing was done with fluorescent dye-labeled dideoxynucleotides using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

3.2.8. 16S rRNA Gene Sequence Analysis

Phylogenetic and molecular evolutionary analyses were performed using the MEGA software, version 3.1 (Kumar, S., et al., 2004). Phylogenetic trees were generated using the neighbor-joining method (Saitou, N. and Nei, M., 1987) and evolutionary distance matrices were calculated using the Jukes-Cantor algorithm (Jukes, T.H. and Cantor, C., 1969).

3.3 Results and Discussion

Five 16S rRNA gene libraries were constructed from the GEN dip solutions recovered at the turtle farms in June 2002, in Louisiana. Figure 3.1 shows some of the PCR products obtained from the amplification of cloned 16S rRNA genes. Approximately 550 clones were screened for the appropriate insert size of 1.5 kb; around 300 of these had the correct insert size and were thus selected for further identification analysis. ARDRA analysis was used as a guideline to select representative clones to sequence and revealed different TaqI restriction digestion patterns, as illustrated in Figure 3.2.

Sequences obtained from partial sequencing of the cloned 16S rRNA inserts lead to the identification of both culturable and uncultured organisms from the samples studied. Organisms (and accession numbers of the closest nucleotide sequence hits obtained through BLAST) identified by partial sequencing of the cloned 16S rRNA genes are summarized in Table 3.1.

To perform phylogenetic and molecular evolutionary analyses, sequences were aligned and edited using VectorNTI (Invitrogen) and BioEdit (Hall, T.A., 1999), as described above. For this, a representative clone was selected from each group of identical sequences and designated as an operational taxonomic unit (OTU) (Table 1). Sequence data corresponding approximately to bases numbers 100 to 500 (E. coli numbering) (Brosius, J., et al., 1978) was used to generate phylogenetic trees and distance matrices as presented in Figure 3.3. In this study we found a more diverse group of organisms to survive GEN treatment than
Figure 3.1. Selection of Appropriate Clones by M13 Amplification of Cloned 16S rRNA Gene Inserts. Lanes 1-21 (gels A and B) show M13 PCR products obtained from different clones. 100 bp DNA ladder (Invitrogen) was used as molecular size standard (M). Clones with an appropriate size insert of 1.5 kb were further analyzed. Clones from gel A, lanes 4, 5, 18, 19, 21, and gel B, lanes 2, 5, 6, 7, 17, 20, were excluded from the study due to their improper size.
Figure 3.2: ARDRA. Lanes 2-12 & 14-23 show some of the restriction digests of the 16S rRNA PCR products obtained using restriction enzyme Taq1. Five different restriction patterns are evident: A (lanes 1, 11, 16, 19), B (lanes 2, 3, 5, 6, 12), C (lanes 4, 7, 10, 14, 15, 20-23), D (lane 8), and E (lanes 17, 18). M: DNA Molecular Weight Marker VIII (Roche Diagnostics)
Table 3.1. Organisms Identified by Partial Sequencing of 16S rRNA Genes

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<tr>
<td>Pseudomonas sp. 48XA</td>
<td>DQ103513</td>
<td>100</td>
<td>18</td>
<td>clone JVB1</td>
</tr>
<tr>
<td>Pseudomonas sp. WT OTU2</td>
<td>AY965247</td>
<td>99</td>
<td>4</td>
<td>clone PPA2</td>
</tr>
<tr>
<td>Uncultured bacterium clone LS27</td>
<td>AY559425</td>
<td>99</td>
<td>2</td>
<td>clone JVB6</td>
</tr>
<tr>
<td>Uncultured bacterium clone Tc65</td>
<td>AF445125</td>
<td>95</td>
<td>2</td>
<td>clone JVA5</td>
</tr>
<tr>
<td>Uncultured bacterium clone TM09 16S</td>
<td>AY838517</td>
<td>99</td>
<td>8</td>
<td>clone PPA5</td>
</tr>
<tr>
<td>Uncultured bacterium DSSD57</td>
<td>AY328755</td>
<td>99</td>
<td>28</td>
<td>clone PPB3</td>
</tr>
<tr>
<td>Uncultured beta proteobacterium clone ccslm36</td>
<td>AY133096</td>
<td>98</td>
<td>4</td>
<td>clone PPC1</td>
</tr>
<tr>
<td>Uncultured Planctomycetales bacterium clone M10Ba43</td>
<td>AY360633</td>
<td>97</td>
<td>2</td>
<td>clone JVA4</td>
</tr>
<tr>
<td>Xenophilus azovorans</td>
<td>AF285414</td>
<td>99</td>
<td>2</td>
<td>clone JVA3</td>
</tr>
</tbody>
</table>

1 Total number of clones with indicated sequence identities. 2 Operational Taxonomic Units (OTUs) used to construct phylogenetic trees
Figure 3.3. Phylogenetic Tree of Organisms Identified in This Study
originally anticipated. In fact, using a culture-dependent approach we had only identified *Alcaligenes* spp. and *Pseudomonas* spp. of the organisms listed in Table 3.1. (Diaz, M.A., *et al*, in press). In effect, the assessment of microbial diversity is largely underestimated by classic culture methods (Ward, D.M., *et al*, 1998; Hengstmann, U., *et al*, 1999). In conventional culturing methods, specific growth conditions favor the growth of bacteria that are better adapted to the given culture conditions, which do not necessarily reflect the natural environment. Consequently, microorganisms identified by these methods do not illustrate the microbial community significantly (Ward, D.M., *et al*, 1998; Hengstmann, U., *et al*, 1999; Amann, R. and Ludwig, W., 2000). In fact, the fraction of microbial diversity which has been obtained from culture methods has been estimated to be less than one per cent of the total microbial diversity (Amann, R.I., *et al*, 1995; Amann, R. and Ludwig, W., 2000).

Table 3.2 summarizes the percentages of organisms identified in each sample. All species found with the studies performed had been previously reported. On the other hand, previously reported uncultured bacteria were identified in all of the samples tested. In effect, these previously reported uncultured bacteria had been detected in environmental samples. Though it is not possible to infer an ecological role or phenotype for the organisms identified based on the phylogenetic position of their 16S rRNA gene sequence, the information provided should prove useful as a qualitative tool for the study of these bacterial communities (Ward, D.M., *et al*, 1998; Hengstmann, U., *et al*, 1999). Furthermore, this type of study could aid in the detection of possible antibiotic producers or other organisms which help maintain the antibiotic resistance pool and yet are missed by classical bacteriological drug-resistance testing.

The most common genera encountered in the GEN treatment solutions were *Comamonas* spp. (34%), *Pseudomonas* spp. (12%), and *Methylphilus* spp. (11%) as can be seen in Table 3.2. *Comamonas* spp. was found in every location studied. It is important to
mention, though, that the culture-independent approach to the identification of bacteria in a community, based on 16S rRNA gene sequencing, is known to have important limitations as a quantitative tool (Rainey, F.A., et al, 1996; Hengstmann, U., et al, 1999). One such limitation is the bias obtained from the proportions of the 16S rRNA sequence types that are recovered (Hengstmann, U., et al, 1999). In addition, it is also not possible with the present approach to determine the resistance mechanisms which allow the identified non-culturable bacteria to survive such high levels of GEN. However, in a previous study, we found two genes which are carried by bacteria recovered from environmental samples with turtle farming activity, which exhibit high-level GEN resistance (Diaz, M.A., et al, in press). These are $aac(3)\text{II}a$ and $aac(3)\text{VI}a$, which code for aminoglycoside acetyl-transferase enzymes. Only gene $aac(3)\text{II}a$ was detected in pseudomonads and other non-fermentor Gram-negative bacilli in the studies performed (Diaz, M.A., et al, in press). We do not discard the possibility of the dissemination of these genes amongst some of the organisms detected in this study. Other resistance mechanisms often observed in the identified bacteria include adaptive resistance and reduced uptake of the antibiotic (Karlowsky, J.A., et al, 1996; Putman, M., et al, 2000). In addition, recent studies performed elsewhere have reported plasmid-mediated genes which confer high-level aminoglycoside resistance in Klebsiella pneumoniae (Galimand, M., et al, 2003), Pseudomonas aeruginosa (Yokoyama, K., et al, 2003) Serratia marcescens (Doi, Y., et al, 2004) and other microorganisms (Yamane, K., et al, 2005). Further studies would be needed to determine the resistance mechanisms which allow these bacteria to survive such high-levels of GEN.

In this study, we used a culture-independent approach for the identification of bacteria that can survive or thrive in GEN treatment solutions as it provides a less biased technique for
### Table 3.2. Most Prevalent Genera Identified by Partial Sequencing of 16S rRNA Genes in GEN Treatment Solutions From Five Different Locations in Louisiana in June, 2002.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Sample Site</th>
<th>JVA (n=60)</th>
<th>JVB (n=76)</th>
<th>PPA (n=56)</th>
<th>PPB (n=76)</th>
<th>PPC (n=30)</th>
<th>Total % (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Comamonas</em> spp.</td>
<td></td>
<td>63.3 (38)</td>
<td>7.9 (6)</td>
<td>39.3 (22)</td>
<td>13.2 (10)</td>
<td>86.7 (26)</td>
<td>34 (102)</td>
</tr>
<tr>
<td><em>Pseudomonas</em> spp.</td>
<td></td>
<td>23.7 (18)</td>
<td>35.7 (20)</td>
<td></td>
<td></td>
<td></td>
<td>13 (38)</td>
</tr>
<tr>
<td><em>Methylophilus</em> spp.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>11 (34)</td>
</tr>
<tr>
<td><em>Alcaligenes</em> spp.</td>
<td></td>
<td>31.6 (24)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>8 (24)</td>
</tr>
<tr>
<td><em>Collimonas</em> spp.</td>
<td></td>
<td>21.1 (16)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5 (16)</td>
</tr>
<tr>
<td><em>Flexibacter</em> spp.</td>
<td></td>
<td>20.0 (12)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4 (12)</td>
</tr>
<tr>
<td>other</td>
<td></td>
<td>10.0 (6)</td>
<td>13.2 (10)</td>
<td>10.7 (6)</td>
<td>5.3 (4)</td>
<td></td>
<td>9 (26)</td>
</tr>
<tr>
<td>Uncultured bacteria clones</td>
<td></td>
<td>6.7 (4)</td>
<td>2.6 (2)</td>
<td>14.3 (8)</td>
<td>36.8 (28)</td>
<td>13.3 (4)</td>
<td>15 (46)</td>
</tr>
</tbody>
</table>
the qualitative study of bacterial communities (Ward, D.M., *et al*, 1998; Hengstmann, U., *et al*, 1999; Amann, R. and Ludwig, W., 2000; Heuer, H., *et al*, 2002). With this approach, we identified bacteria that are able to survive high concentrations of GEN (1000 µg/ml) for one year at 4°C, which are the conditions at which the antibiotic baths are stored, and may possibly serve as antibiotic resistance reservoirs but yet would escape detection by classic antibiotic resistance monitoring.

### 3.4 References


CHAPTER 4:
INTEGRONS AND GENE CASSETTES IN BACTERIA FROM
THE TURTLE FARM ENVIRONMENT
4.1 Introduction

Gentamicin is an antibiotic which belongs to the family of the aminoglycosides, which are broad-spectrum antibiotics commonly used in combination with β-lactams for the treatment of aerobic Gram-negative infections (Miller, G.H., et al., 1997; Vakulenko, S.B. and Mobashery, S., 2003). In recent studies, bacteria which were highly resistant to gentamicin were recovered from various sources on commercial turtle farms in Louisiana (D'Aoust, J.Y., et al., 1990; Diaz, M.A., et al., 2000). This lead to investigation into resistance determinants as well as the molecular environment of the resistance genes identified (Diaz, M.A., et al., 2001; Diaz, M.A., et al., 2002b; Diaz, M.A., et al., 2002a). Plasmid-encoded aminoglycoside acetyltransferase genes \( aac(3)\text{IIa} \) and \( aac(3)\text{VIa} \) were found to be the most prevalent in gentamicin resistant bacteria recovered from turtle farms (Diaz, M.A., et al., 2000; Diaz, M.A., et al., in press). Furthermore, the gene \( aac(3)\text{VIa} \) was found within a class 1 integron (Stokes, H.W. and Hall, R.M., 1989; Diaz, M.A., et al., 2001; Diaz, M.A., et al., 2002b).

Integrons, which were first described by Stokes and Hall in 1989, are gene-capture molecular elements that contain an integrase gene, \( intI \), and an arrangement of gene cassettes which are expressed from a common promoter, \( P_{\text{ant}} \) (Stokes, H.W. and Hall, R.M., 1989; Collis, C.M., et al., 1998; Ploy, M.C., et al., 2000). These molecular elements have since been detected on plasmids and in the chromosomes of Gram-negative bacteria (Hall, R.M. and Stokes, H.W., 1993; Fluit, A.C. and Schmitz, F.J., 1999). Class-1 integrons, the most prevalent among clinical isolates (Fluit, A.C. and Schmitz, F.J., 1999; White, P.A., et al., 2001; Fluit, A.C. and Schmitz, F.J., 2004), are characterized by two conserved regions (5’-CS and 3’-CS) flanking a variable region (Hall, R.M. and Collis, C.M., 1995). The 5’ conserved region includes the integrase gene \( intI1 \) and a gene cassette attachment site, \( attI \) (Collis, C.M., et al., 1993; Hall, R.M. and Collis, C.M., 1995; Partridge, S.R., et al., 2000). The 3’ conserved region is characterized by the


The goal of the present study was to characterize the class-1 integrons in gentamicin-resistant isolates recovered from pet turtle farms and to investigate any genes which may be co-selected in the presence of gentamicin.

4.2 Materials and Methods

4.2.1. Bacterial Strains and Plasmids Used in This Study

Bacterial strains used in this study were isolated previously (Diaz, M.A., et al, in press) from environmental samples with turtle farming activity and are summarized in Table 4.1. Gram-negative bacteria were routinely grown at 37°C using McConkey (Difco, Detroit, MI) plates. Laboratory strains were grown using Luria-Bertani (LB) media (Sambrook, J., et al, 1989). All GEN-resistant strains were propagated in media supplemented with 100 µg/ml GEN. A summary of all laboratory bacterial strains and plasmids used in this study is found in Table 4.1.

4.2.2. Antibiotic Susceptibility Assays

The Kirby-Bauer disk-diffusion assay was done using MH agar plates according to NCCLS standards (NCCLS, 1999) using the following antibiotics: GEN (10 µg), KAN (30 µg), TOB (10 µg), AMK (30 µg), ampicillin (AMP) (10 µg), tetracycline (TET) (30 µg), chloramphenicol (CHL) (30 µg), polymixin B (PMB) (300 units), cephalothin (CEF) (30 µg), cefotaxime (CTX) (30 µg), trimethoprim (TMP) (5 µg), and nitrofurantoin (NIT) (300 µg) (Difco). Inhibition zone breakpoints were interpreted according to information supplied by the manufacturer and to NCCLS standards (NCCLS, 1999).

4.2.3. Screening for Genes Associated with Class-1 Integrons by PCR

In order to screen for the presence of putative integrases and gene cassettes which could contribute to the spread and increased level of gentamicin resistance, we screened for putative
Table 4.1. Bacterial Strains and Plasmids Used in This Study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Source or Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacterial strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> TOP10</td>
<td>Electrocompetent or chemically competent. F (mcrA \Delta(mrr-hsdRMS-mcrBC)) (\Phi80lac\Delta M15 \Delta lacX74) (recA1) (deoR) (araD139 (ara-leu)7697) (galU) (galK) (rpsL) (Str(^5)) (endA1) (nupG) GEN(^5) (MIC: &lt;3.9 µg/ml)</td>
<td>Invitrogen</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> strain PS7</td>
<td>GEN MIC: &gt;2000 µg/ml</td>
<td>(12)</td>
</tr>
<tr>
<td><em>Stenotrophomonas maltophilia</em> strain St16</td>
<td>GEN MIC: &gt;2000 µg/ml</td>
<td>(12)</td>
</tr>
<tr>
<td><em>Salmonella enterica</em> subsp. enterica strain S5</td>
<td>GEN KAN TOB; GEN MIC: &gt;2000 µg/ml</td>
<td>(12)</td>
</tr>
<tr>
<td><em>Salmonella enterica</em> subsp. arizonae strain PH5</td>
<td>GEN KAN TOB; GEN MIC: &gt;2000 µg/ml</td>
<td>(12)</td>
</tr>
<tr>
<td><em>Enterobacter cloacae</em> strain E19</td>
<td>GEN KAN TOB; GEN MIC: &gt;2000 µg/ml</td>
<td>(12)</td>
</tr>
<tr>
<td><em>Citrobacter freundii</em> strain C14</td>
<td>GEN KAN TOB; GEN MIC: &gt;2000 µg/ml</td>
<td>(12)</td>
</tr>
</tbody>
</table>

(table cont’d)
<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCR2.1-TOPO</td>
<td>cloning and sequencing vector; KAN</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pCSt16</td>
<td>pCR2.1-TOPO derivative with 1 kb insert from 5’CS-3’CS PCR obtained from</td>
<td>this study</td>
</tr>
<tr>
<td></td>
<td><em>Stenotrophomonas maltophilia</em> strain St16</td>
<td></td>
</tr>
<tr>
<td>pCSeS5</td>
<td>pCR2.1-TOPO derivative with 1 kb insert from 5’CS-3’CS PCR obtained from</td>
<td>this study</td>
</tr>
<tr>
<td></td>
<td><em>Salmonella enterica</em> subsp. <em>enterica</em> strain S5</td>
<td></td>
</tr>
<tr>
<td>pCSaPH5</td>
<td>pCR2.1-TOPO derivative with 1 kb insert from 5’CS-3’CS PCR obtained from</td>
<td>this study</td>
</tr>
<tr>
<td></td>
<td><em>Salmonella enterica</em> subsp. <em>arizonae</em> strain PH5</td>
<td></td>
</tr>
<tr>
<td>pCEcE19</td>
<td>pCR2.1-TOPO derivative with 1 kb insert from 5’CS-3’CS PCR obtained from</td>
<td>this study</td>
</tr>
<tr>
<td></td>
<td><em>Enterobacter cloacae</em> strain E19</td>
<td></td>
</tr>
<tr>
<td>pCCfC14</td>
<td>pCR2.1-TOPO derivative with 1 kb insert from 5’CS-3’CS PCR obtained from</td>
<td>this study</td>
</tr>
<tr>
<td></td>
<td><em>Citrobacter freundii</em> strain C14</td>
<td></td>
</tr>
<tr>
<td>pCPaPs7</td>
<td>pCR2.1-TOPO derivative with 2.5 kb insert from 5’CS-3’CS PCR obtained from</td>
<td>this study</td>
</tr>
<tr>
<td></td>
<td><em>Pseudomonas aeruginosa</em> strain Ps7</td>
<td></td>
</tr>
</tbody>
</table>
integrase genes and gene cassettes in the turtle pond environment using degenerate primers previously published (Nield, B.S., et al., 2001; Stokes, H.W., et al., 2001). Degenerate primers used to detect these putative genes and PCR conditions are summarized in Table 4.2. Different size PCR products were purified from agarose gels using QIAquick Spin kits (Qiagen, Valencia, CA) and cloned using Invitrogen’s TOPO-TA cloning kit (Invitrogen, Carlsbad, CA), as recommended by the manufacturer. Recombinant plasmids were transformed into chemically competent TOP10 *Escherichia coli* cells (Invitrogen), and transformants were selected on LB-KAN plates (50 µg/ml). DNA inserts from these clones were amplified using the M13 universal primers.

In addition, PCR assays were designed to screen environmental isolates for genes commonly associated with class 1 integrons, using primers published elsewhere (Levesque, C., et al., 1995; Schwocho, L.R., et al., 1995; Sandvang, D., et al., 1997; Galimand, M., et al., 2003) and summarized in Table 4.2. PCR screening of genes *intI*, *sul1* (Stokes, H.W. and Hall, R.M., 1989; Schwocho, L.R., et al., 1995), and of *armA*, reported to confer high-level aminoglycoside resistance in Gram-negative bacteria and to be associated with complex class-1 integrons (Galimand, M., et al., 2003; Galimand, M., et al., 2005), was done in separate reactions. PCR assays were also performed to detect complex integron-associated *orf513*, coding a putative recombinase. Other primer sets used to amplify these integron regions include: 1) 5'CS and 3'CS; 2) 5'CS and *orf513*-R and 3) *orf513*-F and *sulR*.

To prepare DNA for PCR, pelleted cells from 1 ml of overnight cultures were resuspended in 100 µl dH$_2$O and lysed through two 5 min. freeze-boil cycles. Two µl of the supernatants were used as templates in 50 µl PCR reactions. PCR was done using the FailSafe™ PCR System, buffer “G”, or buffer “F” (Epicentre Biotechnologies, Madison, WI),
<table>
<thead>
<tr>
<th>PCR target</th>
<th>Primer</th>
<th>Amplicon size (bp)</th>
<th>Sequence (5’ → 3’)</th>
<th>Accession No. (Reference)</th>
</tr>
</thead>
<tbody>
<tr>
<td>intI1</td>
<td>intIF</td>
<td>845</td>
<td>CTA CCT CTC ACT AGT GAG GGG CGG GGG CAG CAG CGA AGT CGA GGC</td>
<td>X15852 (44, 47)</td>
</tr>
<tr>
<td>intI1</td>
<td>intIR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>intI1</td>
<td>int1F</td>
<td>568</td>
<td>ACA TGT GAT GCC GAC GCA CGA</td>
<td>M95287 (17, 47)</td>
</tr>
<tr>
<td>intI1, intI2, intI3</td>
<td>hep35</td>
<td>491</td>
<td>TGC GGG TYA ARG ATB TKG ATT T CAR CAC ATG CTG GTA RAT</td>
<td>(54)</td>
</tr>
<tr>
<td>cassette array</td>
<td>hep74</td>
<td>variable</td>
<td>CGG GAT CCC GGA CGG CAT GCA CGA TTT GTA GAT GCC ATC GCA AGT ACG AG</td>
<td>(54)</td>
</tr>
<tr>
<td>cassette array</td>
<td>hep51</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>intIN / array</td>
<td>HS286</td>
<td>variable</td>
<td>GGG ATC CTC SGC TKG ARC GAM TTG TTA GVC</td>
<td>(30, 48)</td>
</tr>
<tr>
<td>intIN / array</td>
<td>HS287</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>intIN / array</td>
<td>HS298</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cassette array</td>
<td>5’CS</td>
<td>variable</td>
<td>GGC ATC CAA GCA GCA AGC</td>
<td>M73819 (24)</td>
</tr>
<tr>
<td>cassette array</td>
<td>3’S</td>
<td></td>
<td>AAG CAG ACT TGA CCT GAT</td>
<td></td>
</tr>
<tr>
<td>orf513</td>
<td>orf513F</td>
<td></td>
<td>CTC GCT ACA TCC AAC AAG AA TGT CAC GCC AGT TAG TAT CG</td>
<td>(31)</td>
</tr>
<tr>
<td>orf513</td>
<td>orf513R</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aac(3)II</td>
<td>aac3F</td>
<td>860</td>
<td>ATG CAT ACG CGG AAG GCA AT CTA ACC TGA AGG CTC GCA A</td>
<td>L22613 (17)</td>
</tr>
<tr>
<td>aac(3)II</td>
<td>aac3R</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aac(3)-IIa</td>
<td>aacIIaF</td>
<td>900</td>
<td>GGG AAT TCA GAG GAG ATA TCG CGA TGC ATA CG CAT TGT CGA CGG CCT CAT ACC</td>
<td>X13543 (12)</td>
</tr>
<tr>
<td>aac(3)-IIa</td>
<td>aacIIaR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aac(3)-VIa</td>
<td>aacVlaF</td>
<td>465</td>
<td>CGC TCA GGC GAT ATG GTG AT CAT ATT GGA GCG CGG TGA CT</td>
<td>L22613 (12)</td>
</tr>
<tr>
<td>aac(3)-VIa</td>
<td>aacVlaR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sul1</td>
<td>sulF1</td>
<td>840</td>
<td>ATG GTG ACG GTG TGC GCC AT CTA GCC ATG ATC TAA CCC TC</td>
<td>U12441 (17, 47)</td>
</tr>
<tr>
<td>sul1</td>
<td>sulR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M13F (-20)</td>
<td>variable</td>
<td></td>
<td>GTA AAA CGA CGG CCA G</td>
<td>(Invitrogen)</td>
</tr>
<tr>
<td>M13R</td>
<td></td>
<td></td>
<td>CAG GAA ACA GCT ATG AC</td>
<td></td>
</tr>
</tbody>
</table>
according to instructions provided by the manufacturer. Thermocycler conditions were set as follows: [1X (96°C, 5 min); 35X (96°C, 30 sec; 55°C, 30 sec; 70°C, 60 sec); (70°C, 7 min)].

PCR products were visualized by agarose gel electrophoresis using 2% Agarose I in 0.5x TBE buffer and staining with ethidium bromide. Some of the PCR products obtained were sequenced in order to confirm amplification of the appropriate genes and to compare with published sequences in the databases. For this, PCR products were purified using QIAquick Spin columns (Qiagen) and ligated into pCR2.1-TOPO using the TOPO-TA cloning kit (Invitrogen), as indicated by the manufacturer. Sequencing of appropriate clones was done using the internal vector sequencing primers, as described above.

4.2.4. Sequencing and Computer Analysis

Sequencing was done using the M13 universal primers with fluorescent dye-labeled dideoxynucleotides with an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequence analysis was done using VectorNTI (Invitrogen), BLAST (2) and GeneBank (National Center for Biotechnology Information, available through http://www.ncbi.nlm.nih.gov/BLAST/).

4.3 Results

The goal of this study was to search for integron classes carrying gentamicin resistance genes which could also contribute to the co-selection of other resistance genes under gentamicin selective pressure. PCR amplification of gene intI1 using primers intIF and intIR (Schwocho, L.R., et al., 1995) allowed the detection of class 1 integrase in E. coli-pAriPH5, E. coli-pEntE19, but not in Pseudomonas aeruginosa, Stenotrophomonas maltophilia, or E. coli-pSalS5 (Figure 4.1). Since aac(3)IIa was encoded in the latter organisms, and this gene was reported to be associated with gene armA, as well as orf513, and intI1 (Galimand, M., et al., 2003; Galimand, M., et al., 2005), these genes were screened for with the primers published by
Figure 4.1. PCR Amplification Products of intI1 Gene. PCR reaction was carried out using intIF and intIR primers. Lane 1, *Ps. aeruginosa*; lane 2, *E. coli*-pSalS5; lane 3, *E. coli*-pAriPH5; lane 4, *E. coli*-pEntE1; lane M, 100 bp molecular size marker.
these researchers (Galimand, M., et al, 2003; Partridge, S.R. and Hall, R.M., 2003). Gene armA was not detected in any of the isolates assayed in this study (Chapter 2). In addition, screening for complex-integron associated orf513 has not been detected in the isolates tested thus far. However, when screening for integrase class 1 with this primer set, it was possible to detect intI1 in isolates carrying aac(3)IIa, as well as those carrying aac(3)VIa. Figure 4.2 summarizes these findings.

Primers hep35 and hep36 anneal to conserved regions of integrase genes intI1, intI2, and intI3 (White, P.A., et al, 2001). Primers hep74 and hep51 were designed to amplify class 2 integron cassette regions by annealing to attI2 and to orfX, which is downstream of the cassette region within Tn7 (White, P.A., et al, 2001). Moreover, primers HS286, HS287 and HS298 have been used to detect integrase genes and cassettes from environmental DNA with success (Nield, B.S., et al, 2001; Stokes, H.W., et al, 2001). Numerous amplicons with appropriate sizes (greater than 1 kb) were obtained from over 200 isolates tested, and cloned into pCR2.1-TOPO using the TOPO-TA cloning kit, but sequencing of these clones did not lead to the detection of integrase genes other than intI1.

PCR performed using primer set 5’CS and 3’CS (Levesque, C., et al, 1995) produced amplicons with sizes which could include gene cassettes. Interestingly, different sizes of amplicons were obtained from isolates which are known to carry the same gene (Figure 4.3). Preliminary sequencing has confirmed that these are in fact integron elements, or cassettes (data not shown). Sequencing of these clones is currently underway.

4.4 Discussion

The goal of the present study was to characterize the integrons in gentamicin resistant isolates recovered from the pet turtle farm environment, since these molecular elements can
Figure 4.2. PCR Amplification Products of Integron-Associated Genes *intI1*, *aac(3)IIa*, and *aac(3)Vla*. Lanes 1 and 5, amplification of *aac(3)II* using primers aacIIF and aacIIR (Galimand, M., *et al.*, 2003); lanes 2 and 6, multiplex PCR amplification of genes *aac(3)IIa* and *aac(3)Vla* using primers aacIIaF, aacIIaR, aacVlaF and aacVlaR (Vliegenthart, J.S., *et al.*, 1990; E.C. Achberger, personal communication); lanes 3 and 7, amplification of *intI1* using primers int1F and int1R (Galimand, M., *et al.*, 2003); lanes 4 and 8, amplification of *intI1* using primers int1F and int1R (Schwocho, L.R., *et al.*, 1995); lanes M, 100 bp molecular size marker.
Figure 4.3. PCR Amplification Products of 5’CS-3’CS Primers. Lane 1, *Pseudomonas aeruginosa* strain Ps7; lane 2, *Stenotrophomonas maltophilia* strain St16; lane 3, *Salmonella enterica* subsp. *enterica* strain S5; lane 4, *Salmonella enterica* subsp. *arizonae* strain PH5; lane 5, *Enterobacter cloacae* strain E19; lane 6, *Citrobacter freundii* strain C14.
contribute not only to the spread of gentamicin resistance but also to that of other resistance genes of public health importance, which may be co-selected in the presence of gentamicin.

Four different classes of integrons had been identified before 2001, encoding different integrase genes and overall gene arrangements; these were integrons class-1 (Stokes, H.W. and Hall, R.M., 1989), class-2, class-3 (Collis, C.M., et al, 2002) and class-4 (Mazel, D., et al, 1998). In a previous study, we had detected the presence of the gene \( aac(3)\text{VI}a \) associated with the integron class-1 integrase gene. However, isolates carrying \( aac(3)\text{II}a \) had been considered \( \text{intI1} \)-negative when assayed with the primer set described (Schwacho, L.R., et al, 1995; Diaz, M.A., et al, in press), which was consistent with reports at the time (Brau, B., et al, 1984; Allmansberger, R., et al, 1985; Rather, P.N., et al, 1992; Rather, P.N., et al, 1993; Diaz, M.A., et al, 2000; Diaz, M.A., et al, 2001). On the other hand, we had detected the presence of \( aac(3)\text{II}a \) in different plasmids and diverse bacterial genera of organism, so we hypothesized that the gene was encoded within a mobile molecular element, such as another integron besides class-1, possibly a superintegron as described in \( \text{Xanthomonas} \) spp., \( \text{Shewanella} \) spp., and \( \text{Pseudomonas} \) spp. (Rowe-Magnus, D.A., et al, 1999; Rowe-Magnus, D.A. and Mazel, D., 1999; Vaisvila, R., et al, 2001). For this reason we screened for the presence of putative integrase (Nield, B.S., et al, 2001) and gene cassette genes (Stokes, H.W., et al, 2001), using degenerate primers designed elsewhere to detect these elements from environmental DNA. None of the PCR amplicons obtained with these primers rendered any gene which could be associated to an integron element, other than the ones already identified (Diaz, M.A., et al, 2002b; Diaz, M.A., et al, 2002a).

Preliminary sequencing of the different integron molecular environments in this study did not lead to the detection of any mutation which could have an effect on the expression of the gentamicin resistance gene cassettes. Gene \( aac(3)\text{VI}a \) was found to be in the first position in
the gene cassette array, near the integrase gene. On the other hand, even though we did not
detect intI1 gene associated with the gene aac(3)Ia in our preliminary work, we did detect
other integron elements in bacteria carrying this gene (Diaz, M.A., et al, 2002b; Diaz, M.A., et
al, 2002a). Interestingly, recent studies have also depicted the aac(3)Ia gene to be surrounded
by integron elements (Galimand, M., et al, 2003; Galimand, M., et al, 2005). In fact, the
molecular environment of the armA gene has been depicted as a composite integron
environment, characterized by the presence of a 5'-CS and a 3'-CS interspanning a variable
region which includes a sul1 gene and a gene known as orf513 (Partridge, S.R. and Hall, R.M.,
2003; Galimand, M., et al, 2005). This same type of arrangement has later been described in
other organisms and it is thought to involve a mobile integrative structure known as a
latter are DNA elements that can excise themselves to form a covalently closed circular
intermediate (Salyers, A.A., et al, 1995); this intermediate can reintegrate itself within the same
organism or, it can be transferred by conjugation to a recipient cell and then integrate in the
recipient’s genome (Salyers, A.A., et al, 1995; Pembroke, J.T., et al, 2002; Partridge, S.R. and
Hall, R.M., 2003). These integrative molecular elements were first found in Gram-positive
bacteria such as the streptococci and enterococci (Tolmasky, M.E., 2000) and were barely
mentioned before 1996 in connection with Gram-negative bacteria (Pembroke, J.T., et al,
2002). Conjugative transposon-like elements have later been detected in Gram-negative
bacteria such as Bacteroides spp. (Salyers, A.A., et al, 1995; Pembroke, J.T., et al, 2002) and
though characterized in a lesser extent, in the Enterobacteriaceae. They have been reported in
Salmonella spp., Proteus spp., in the R391 element and in the SXT element in Vibrio spp.
The variable region of class-1 integrons can code for one or more gene cassettes (Stokes, H.W. and Hall, R.M., 1989; Hall, R.M. and Stokes, H.W., 1993). In this study, we used published conserved sequence primers (Levesque, C., et al, 1995; Sandvang, D., et al, 1997) to screen for the presence of different gene arrays in the isolates under study. Interestingly, this not only lead to the detection of the expected gene cassette amplicons from organisms known to be carrying gene \( aac(3)\text{VI}_{a} \) within a class-1 integron, but we also obtained amplicons for organisms which carried gene \( aac(3)\text{II}_{a} \) and which were recently detected to carry \( \text{intII} \). Therefore, it is possible that gene \( aac(3)\text{II}_{a} \) is carried by a complex integron and thus was not detected as such with the primer pair used to detect the classic structure of integron class-1 (Galimand, M., et al, 2003). Sequencing of amplicons obtained with 5’CS-3’CS primers will shed light in this regard (Levesque, C., et al, 1995; Sunde, M. and Sorum, H., 1999). Transfer of \( aac(3)\text{II}_{a} \) may involve a complex integron class-1. The presence of \( aac(3)\text{II}_{a} \) and \( aac(3)\text{VI}_{a} \) in mobile molecular elements explains the distribution of these genes in the different bacterial genera described in this study (Holmes, A.J., et al, 2003). Further studies of their molecular environment are required in order to detect and characterize these mobile molecular elements, and to detect genes which may be co-selected in the presence of gentamicin.

4.5 References


CHAPTER 5:
OVERALL DISCUSSION AND CONCLUSIONS
Salmonella enterica subsp. enterica serovar Enteritidis is the major cause of Salmonella spp. food poisoning in the U.S., since it is a frequent contaminant of chicken eggs (Cohen, M.L., et al., 1980; Carattoli, A., 2003). S. enterica subsp. arizonae commonly inhabits the cloaca of reptiles without causing disease in these animals (Siebeling, R.J., et al., 1975a; Siebeling, R.J., et al., 1984; Ebani, V.V., et al., 2005). Turtle farming has been an important economic activity in the state of Louisiana since the 1930s, contributing approximately 30 million dollars to the State’s economy (Siebeling, R.J., et al., 1975b). The tracing of several salmonellosis outbreaks to pet turtles in the 1970s lead to a domestic ban on the sale of small carapace turtles in 1975 by the FDA (Siebeling, R.J., et al., 1975a; Cohen, M.L., et al., 1980; Shane, S.M., et al., 1990). The estimated 300,000 cases of human salmonellosis that could be traced to pet turtles (Siebeling, R.J., et al., 1975a; Cohen, M.L., et al., 1980), reportedly accounted for an approximate 14% of Salmonella spp. infections per year (Cohen, M.L., et al., 1980). The domestic ban on the sale of baby turtles did not affect the export market significantly, which continued to prosper, with 12 million hatchlings being exported from Louisiana to Western Europe, South America, and Asia every year, accounting for about 90% of the pet turtle world market (Michael-Marler, S., et al., 1983; Siebeling, R.J., et al., 1984; Izadjoo, M.J., et al., 1987). However, to guarantee a safe pet for the market, turtle export is subject to prior testing and certifying hatchlings as Salmonella-spp.-free by independent FDA-approved laboratories, which perform routine bacteriological and molecular assays such as PCR, before certifying hatchlings for export (Siebeling, R.J., et al., 1984).

Antibiotic use constitutes a selective pressure that favors the growth of bacterial strains which are resistant, or have acquired resistance, to the particular antibiotic (Davies, J. and Smith, D.I., 1978; Levy, S.B., 1998; Mazel, D. and Davies, J., 1999; Levy, S.B., 2002). Thus, the development of gentamicin resistance has been of concern in the agricultural setting,
particularly in the turkey farm industry, where this antibiotic has been used to eradicate *Salmonella* spp. from turkey eggs (Ekperigin, H.E., *et al*, 1983; Hirsh, D.C., *et al*, 1983). The concern of the emergence of gentamicin-resistant *Salmonella* spp. has also been voiced for pet turtles being exported from Louisiana to other countries (D'Aoust, J.Y., *et al*, 1990). This study constitutes a report of the molecular causes of the gentamicin resistance problematic in the turtle farms.

Bacteria that were identified by standard bacteriological assays from the samples taken at the turtle farms include organisms such as *Salmonella enterica* subsp. *enterica*, *Salmonella enterica* subsp. *arizonae*, *Enterobacter cloacae*, *Citrobacter freundii*, *Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa*, among others (Chapter 2). These organisms were found to express high-level resistance to gentamicin. This is of particular concern given that aminoglycosides are reportedly antibiotics of choice for the treatment of infections from Gram-negative bacteria such as *Escherichia coli*, *Salmonella* spp., *Shigella* spp., *Enterobacter* spp., *Citrobacter* spp., *Acinetobacter* spp., *Proteus* spp., *Klebsiella* spp., *Serratia* spp., *Morganella* spp., and *Pseudomonas* spp. (Vakulenko, S.B. and Mobashery, S., 2003).

study, the majority of the organisms tested for plasmids showed the presence of one or more plasmids. Some of these plasmids were shown to be conjugative plasmids, as determined by bacterial mating experiments (Chapter 2) in which plasmids were transferred from environmental isolates to a laboratory AZI resistant *E. coli* strain BM14. Transconjugant organisms were not obtained from all environmental donor strains assayed, as is summarized in **Table 5.1**. It is possible that no transconjugants were obtained for donor strains such as *Pseudomonas* spp., *Stenetrophomonas* spp., and *Acinetobacter* spp., and other non-fermenting organisms, since they are more distant phylogenetically to the recipient *E. coli* strain used in these experiments, than the organisms for which mating experiments were successful, namely, *Salmonella* spp., *Enterobacter* spp., and *Citrobacter* spp. Consequently, numerous barriers for conjugation could have taken effect in the former. A scheme illustrating the assays performed for the transfer of GEN resistance is depicted in **Figure 5.1**. From these experiments it was evidenced that the conjugative R-plasmids isolated from all transconjugants and transformants obtained in this study have relatively large sizes, ranging from ca. 45 to 145 kbp, as can be seen in **Figure 5.2**.

Microorganisms can be intrinsically resistant to an antibiotic, or become resistant to the drug through mutations of the antibiotic target site, or by the acquisition of new resistance genes (Levy, S.B., 1998; Mazel, D. and Davies, J., 1999; Rowe-Magnus, D.A. and Mazel, D., 1999). Bacteria can become resistant to antibiotics through a variety of mechanisms, which include the modification of the antibiotic target, reduced accumulation of the drug within the cell, and the enzymatic modification of the antibiotic molecule (Davis, B.D., 1987; Levy, S.B., 1998; Mazel, D. and Davies, J., 1999). The two main modes through which bacteria can become resistant to aminoglycosides by modifications of the ribosome are through mutations in
Table 5.1. Conjugative Transfer of GEN Resistance Genes

<table>
<thead>
<tr>
<th>Donor</th>
<th>Recipient</th>
<th>Gm\textsuperscript{R} Azi\textsuperscript{R} Transconjugants</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>Stenotrophomonas maltophilia</em></td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>Acinetobacter baumanii</em></td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>Proteus vulgaris</em></td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>Providencia stuartii</em></td>
<td><em>E. coli BM14 Azi\textsuperscript{R}</em></td>
<td>-</td>
</tr>
<tr>
<td><em>Salmonella. enterica subsp. enterica</em></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td><em>Salmonella enterica subsp. arizonae</em></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td><em>Enterobacter cloacae</em></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><em>Citrobacter freundii</em></td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

Figure 5.1. Diagram of Transfer of GEN resistance
Figure 5.2. PFGE of R-plasmids isolated from transconjugants obtained after conjugation with *Salmonella enterica* subsp. *enterica* serovar Enteritidis, *Salmonella enterica* subsp. *arizonae* strains PH5 and A40, *Enterobacter cloacae* and *Citrobacter freundii*. Lane M, PFG molecular size marker; lane 1, plasmid pSalS5; lane 2, plasmid pAriPH5; lane 3, plasmid pAriA40; lane 4, plasmid pEnt19; lane 5, plasmid pCit14.
ribosomal proteins or the rRNA and by the enzymatic modification of the ribosome (Davis, B.D., 1987; Wright, G.D., et al, 1998; Vakulenko, S.B. and Mobashery, S., 2003). Any mutation that affects the binding sites of the ribosome can reduce the binding affinity of the drug to the ribosome and lead to varied levels of resistance. The effects of such mutations depend on the nature of the mutation and on the chemistry of the drug (Fourmy, D., et al, 1996; Carter, A.P., et al, 2000; Llano-Sotelo, B., et al, 2002). Nevertheless, it is unlikely that the organisms investigated in the present study expressed such high-levels of resistance to gentamicin due to mutations in the target site, the ribosome, since these levels of resistance were transferable by conjugation in at least some organisms, such as Salmonella spp., Enterobacter spp., and Citrobacter spp., as shown in Chapter 2.

The other mode of target site alteration leading to aminoglycoside resistance is through the enzymatic modification of the ribosome (Davis, B.D., 1987; Cundliffe, E., 1989; Wright, G.D., et al, 1998). This is a common mechanism used by aminoglycoside producers to protect themselves from the antibiotics they produce (Cundliffe, E., 1989; Yoshizawa, S., et al, 1998). Aminoglycoside resistance by enzymatic modification of the ribosome has recently been detected in bacteria other than antibiotic producers (Galimand, M., et al, 2003; Yokoyama, K., et al, 2003; Doi, Y., et al, 2004; Yamane, K., et al, 2004). Usually the activities of most aminoglycoside modifying enzymes result in effective resistance to aminoglycoside antibiotics, but only a few phosphotransferase enzymes have been reported to produce high-levels of resistance to these antibiotics, namely the bifunctional enzyme aph(2")-aac(6’) (Ferretti, J.J., et al, 1986), and phosphotransferases aph(2")Ib, aph(2")Ic and aph(2")Id (Galimand, M., et al, 2003; Vakulenko, S.B. and Mobashery, S., 2003). Table 5.2 summarizes the main features of these enzymes, which confer the HLGR phenotype in Gram-positive organisms. Table 5.3,
<table>
<thead>
<tr>
<th>Gene</th>
<th>Characteristics</th>
<th>Identified in</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>aph(2&quot;)-aac(6')</td>
<td>- HLGR</td>
<td>Staph. aureus</td>
<td>Ferretti et al (1986)</td>
</tr>
<tr>
<td></td>
<td>- Tn4001</td>
<td>streptococci</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- IS256</td>
<td>enterococci</td>
<td></td>
</tr>
<tr>
<td>aph(2&quot;)-Ib</td>
<td>- HLGR</td>
<td>Ent. faecium</td>
<td>Kao et al (2000)</td>
</tr>
<tr>
<td>-Ic</td>
<td>- plasmid-encoded</td>
<td>enterococci</td>
<td>Chow et al (1997)</td>
</tr>
<tr>
<td>-Id</td>
<td></td>
<td></td>
<td>Tsai et al (1998)</td>
</tr>
<tr>
<td>Gene</td>
<td>Characteristics</td>
<td>Identified in</td>
<td>Reference</td>
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<tr>
<td>------</td>
<td>-----------------</td>
<td>---------------</td>
<td>-----------</td>
</tr>
<tr>
<td>armA</td>
<td>- HLAR&lt;br&gt;- plasmid-encoded&lt;br&gt;- 37-47% similarity AG producers&lt;br&gt;- low GC content&lt;br&gt;- \textit{aac3-II dhfrXII, sulI, ant3''9, bla}<em>{TEM1} and \textit{bla}</em>{CTX-M}</td>
<td>\textit{Klebsiella pneumoniae} clinical strain</td>
<td>Galimand \textit{et al} (2003)</td>
</tr>
<tr>
<td>rmtA</td>
<td>- HLAR&lt;br&gt;- plasmid-encoded&lt;br&gt;- MIC&gt;1024 mg/L&lt;br&gt;- 55% GC content&lt;br&gt;- Tn5041</td>
<td>\textit{Pseudomonas aeruginosa} clinical strain</td>
<td>Yokoyama \textit{et al} (2003)</td>
</tr>
<tr>
<td>rmtB</td>
<td>- HLAR&lt;br&gt;- 82% identity with RmtA&lt;br&gt;- plasmid-encoded</td>
<td>\textit{Serratia marcescens} clinical strain</td>
<td>Doi \textit{et al} (2004)</td>
</tr>
</tbody>
</table>
on the other hand, summarizes the characteristics of genes conferring the HLAR phenotype in Gram-negative organisms.

To the best of our knowledge, there had been no reports in the literature of high-level resistance to gentamicin in Gram-negative bacteria before this study was started in 1999 (Diaz, M.A., et al, 2000; Diaz, M.A., et al, in press). On the other hand, there had been an abundance of reports on high-level gentamicin resistance for Gram-positive bacteria (Chow, J.W., 2000).

To be effective, aminoglycosides must first penetrate the cell wall and membranes and reach their target, the bacterial ribosome (Bryan, L.E. and Van Den Elzen, H.M., 1977; Martin, N.L. and Beveridge, T.J., 1986; Davis, B.D., 1987). Some microorganisms can inhibit this important step of the aminoglycoside mode of action, particularly the pseudomonads and other non-fermentor Gram-negative bacilli (Bryan, L.E. and Van Den Elzen, H.M., 1977; Martin, N.L. and Beveridge, T.J., 1986). This mechanism of reduced uptake of the drug is generally due to membrane impermeabilization, which impairs drug penetration into the cell (Bryan, L.E. and Van Den Elzen, H.M., 1977; Davis, B.D., 1987) and results in an intermediate susceptibility to all of the aminoglycosides (Miller, G.H., et al, 1997; Lister, P.D., 2002). In addition, adaptive resistance to aminoglycosides is often reported for aerobic Gram-negative bacilli, such as Pseudomonas spp. (Davis, B.D., 1987; Wright, G.D., et al, 1998). This resistance is thought to be due to regulatory events rather than mutations or the acquisition of resistance genes, so it does not persist once the selective pressure is removed (Karlowsky, J.A., et al, 1996). We do not discount the possibility that this mechanism may contribute to the multiple drug resistance phenotypes displayed by the non-fermenting organisms identified in this study (Chapters 2 and 3). This could act in synergy with the expression of the resistance gene aac(3)IIa detected in the majority of these organisms.
Another mechanism of reduced accumulation of an antibiotic within the bacterial cell is through the active efflux or pumping of the drug out of the cell (Zgurskaya, H.I. and Nikaido, H., 2000), which is more frequently observed in non-fermentor Gram-negative bacilli (Martin, N.L. and Beveridge, T.J., 1986; Westbrock-Wadman, S., et al, 1999), but has also been reported in *S.* *coli* (Nikaido, H., *et al*, 1998; Rosenberg, E.Y., *et al*, 2000). Multidrug efflux pumps can be acquired as foreign genes, or alternatively, the reduced accumulation of an antibiotic can be due to the activation or the increased expression of an innate efflux system (Westbrock-Wadman, S., *et al*, 1999; Putman, M., *et al*, 2000; Zgurskaya, H.I. and Nikaido, H., 2000; Lister, P.D., 2002). Although multidrug efflux pumps generally have low substrate specificities (Zgurskaya, H.I. and Nikaido, H., 2000), some studies have reported efflux pumps that pump aminoglycosides out of the cell (Aires, J.R., *et al*, 1999; Rosenberg, E.Y., *et al*, 2000; Mao, W., *et al*, 2001). Furthermore, the presence of different efflux pumps may confer additive or multiplicative effects on drug resistance (Lee, A., *et al*, 2000).

In this study, it is improbable that the high-level gentamicin resistance observed in bacteria other than non-fermentors is due to the activation of innate efflux pumps, since this phenotype was transferable through conjugation and transformation, suggesting the genes are plasmid-borne. It is possible that this mechanism can account for the multiple antibiotic resistance phenotypes observed in the non-fermenting organisms, but further studies are necessary to ascertain such information. While in this study we did not detect (Chapter 2) a plasmid-encoded efflux pump which could be transferred among the organisms in the turtle farm environment, this possibility is not entirely discarded and should be tested in the future using aminoglycoside accumulation assays.
By far the most important mechanism of aminoglycoside resistance is the enzymatic modification of the drug by aminoglycoside modifying enzymes, resulting in a molecule with decreased binding affinity to the ribosome (Wright, G.D., et al., 1998; Vakulenko, S.B. and Mobashery, S., 2003). The largest group of these enzymes, the aminoglycoside acetyltransferases, participate in the acetylCoA-dependent acetylation of specific aminoglycoside amino groups (Shaw, K.J., et al., 1993; Wright, G.D., et al., 1998; Azucena, E. and Mobashery, S., 2001; Vakulenko, S.B. and Mobashery, S., 2003). Aminoglycoside modifying enzymes display substrate specificity, which means that each enzyme will modify a specific chemical group, resulting in a particular phenotype (Shaw, K.J., et al., 1993; Wright, G.D., et al., 1998).

According to the nomenclature proposed by K.J. Shaw et al., (Shaw, K.J., et al., 1993), AAC(3)IIa is an acetyltransferase enzyme which participates in the addition of an acetyl group to the amino group at position 3 of the 2-deoxystreptamine ring of the aminoglycoside molecule, giving resistance profile “II” by gene a (Shaw, K.J., et al., 1993; van de Klundert, J.A. and Vliegenthart, J.S., 1993; Wright, G.D., et al., 1998; Vakulenko, S.B. and Mobashery, S., 2003). Enzyme AAC(3)VIa uses the acetyl group from an acetyl coenzyme-A donor molecule to modify the aminoglycoside molecule at the position 3 of the 2-deoxystreptamine ring, and confers resistance profile VI, by gene a (Shaw, K.J., et al., 1993; Vakulenko, S.B. and Mobashery, S., 2003).

aminoglycoside modifying enzyme presents substrate specificity, the antibiotic resistance phenotype alone cannot be used in a study to determine the gene confering this resistance since there may be cumulative effects from multiple enzymes (Shaw, K.J., et al., 1993; Miller, G.H., et al., 1997). Thus, in this study, the problem was approached by cloning and sequencing of the genes which conferred gentamicin resistance to bacteria isolated at the turtle farms and which were transferred by conjugation and transformation to naïve E.coli. As discussed in Chapter 2, these experiments lead to the identification of genes aac(3)IIa and aac(3)Vla (Diaz, M.A., et al, in press).

The gene aac(3)IIa was originally identified in R-plasmids in Enterobacteraceae (Allmansberger, R., et al, 1985), as well as aac(3)VI, originally cloned from a conjugative plasmid of Enterobacter cloacae. The enzymes encoded by these genes reportedly share 50% amino acid identity (Rather, P.N., et al, 1993; Vakulenko, S.B. and Mobashery, S., 2003). In effect, all gentamicin resistant organisms tested in this study were positive for either one of the two aac(3) genes detected in the cloning and sequencing of gentamicin resistance determinants. These genes were detected in quite diverse organisms from the turtle farms, which were also different from the organisms originally reported on (Brau, B., et al, 1984; Allmansberger, R., et al., 1985; Rather, P.N., et al, 1993). Furthermore, each of these genes were found to be encoded on different plasmids, as shown in Chapter 2, all of which supports their dissemination through mobile molecular elements.

In this regard, it is relevant to state that when we began this study, only gene aac(3)Vla had been reported to be encoded as a gene cassette in a class-1 integron (Brau, B., et al, 1984; Allmansberger, R., et al, 1985; Rather, P.N., et al, 1992; Rather, P.N., et al, 1993; Diaz, M.A., et al, 2000; Diaz, M.A., et al, 2001). Integrons are gene-capture molecular elements that contain an integrase gene, named intI, and an arrangement of gene cassettes which are

Four different classes of integrons had been identified before 2001, according to the IntI integrase and the general gene arrangement that they encode, namely, class-1 (Stokes, H.W. and Hall, R.M., 1989), class-2, class-3 (Collis, C.M., et al, 2002) and class-4 (Mazel, D., et al, 1998). The main distinguishing features of integron classes, other than class-1, are summarized in Table 5.4. In this study, we detected the presence of the integrase gene of integron class-1 associated with the gene aac(3)VIa. In effect, all isolates carrying this gene were also positive by PCR to intI1 (Chapter 2). However, isolates carrying aac(3)IIa were not intI1-positive by this assay, with the primer set described in Chapter 2.

### Table 5.4. General Characteristics of Integrons Class II, III and IV

<table>
<thead>
<tr>
<th>Class II</th>
<th>Class III</th>
<th>Class IV</th>
<th>Other classes</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Included in the family of Tn7 transposons</td>
<td>- Only one described to date</td>
<td>- Super-integron (SI) or ‘integron island’</td>
<td>- Only the integrases detected, from environmental DNA</td>
</tr>
<tr>
<td>- Presence of Tn7 transposition genes</td>
<td>- Presence of Tn7 transposition genes</td>
<td>- First described in <em>Vibrio cholerae</em></td>
<td></td>
</tr>
<tr>
<td>- a defective integrase gene <em>intI2</em>, located at 5’CS</td>
<td>- Integrase gene <em>intI3</em> has 61% homology with <em>intI1</em></td>
<td>- Integrase gene <em>intI4</em></td>
<td><em>intI5, intI6, intI7 …</em></td>
</tr>
<tr>
<td>- 3 integrated gene cassettes: <em>dhfrI-sat-aadA1</em></td>
<td>- contains gene cassette <em>blaIMP</em></td>
<td>- ~ 100 gene cassettes</td>
<td></td>
</tr>
<tr>
<td>- No <em>sulI</em> gene</td>
<td>- Presence of a part of gene <em>aacA4</em></td>
<td>- gene cassettes encoded may have virulence functions (not antibiotic resistance)</td>
<td></td>
</tr>
<tr>
<td>- <em>att</em> site located between gene <em>intI2</em> and 1st resistance gene, as in class I integrons.</td>
<td>- Atypical recombination sites around gene cassette</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
encoded in integrons of different classes, which indicates that the gene cassette pool may be shared between them (Fluit, A.C. and Schmitz, F.J., 1999; Rowe-Magnus, D.A. and Mazel, D., 1999; Rowe-Magnus, D.A. and Mazel, D., 2001; Fluit, A.C. and Schmitz, F.J., 2004).

In this study, we detected the presence of \textit{aac(3)Ila} in different plasmids and diverse bacterial genera of organism, so it was hypothesized that the gene was encoded within a mobile molecular element such as another integron besides class-1, possibly even a superintegron as described in \textit{Xanthomonas} spp., and \textit{Pseudomonas} spp. (Rowe-Magnus, D.A., \textit{et al}, 1999; Rowe-Magnus, D.A. and Mazel, D., 1999; Vaisvila, R., \textit{et al}, 2001). For this reason we screened for the presence of putative integrase (Nield, B.S., \textit{et al}, 2001) and gene cassette (Stokes, H.W., \textit{et al}, 2001) genes, using degenerate primers designed to detect these elements in DNA from environmental bacteria (Nield, B.S., \textit{et al}, 2001; Stokes, H.W., \textit{et al}, 2001). These assays, though numerous and quite extensive (Diaz, M.A., \textit{et al}, 2002b; Diaz, M.A., \textit{et al}, 2002a), did not lead to detection of an integrase or gene cassette from the isolates studied. Thus, this screening strategy which proved useful in detecting new integrases and gene cassettes from the environment in Australia (Stokes, H.W., \textit{et al}, 2001), was quite unfruitful in this study, as well as others (J. Davies, personal communication). Nevertheless, this approach and others which involve the molecular screening for antibiotic resistance genes is important and should be pursued, as many of the organisms that help maintain antibiotic resistance gene pools are not necessarily cultivable, and thus escape detection through classic antimicrobial testing, as seen in Chapter 3.

Class-1 integrons are the most prevalent integrons among clinical isolates (Hall, R.M. and Stokes, H.W., 1993; Fluit, A.C. and Schmitz, F.J., 1999). Their basic structure is characterized by the presence of two conserved sequences, known as the 5'-CS and 3'-CS, which flank a variable region. The 5'-conserved region contains the integrase gene \textit{intI1} and a
gene cassette attachment site, *attI*. The 3'-conserved region is characterized by the presence of a sulfonamide resistance gene, *sul1*, a quaternary ammonium compound resistance gene, *qacEΔ1* and open reading frames orf5 and orf6, with unknown functions (Stokes, H.W. and Hall, R.M., 1989; Hall, R.M. and Stokes, H.W., 1993). These conserved regions have proven useful to design primers that allow the detection of gene cassette arrangements encoded within the variable region of class-1 integrons (Levesque, C., *et al*, 1995; Sandvang, D., *et al*, 1997). This variable region of class-1 integrons can code for one or more gene cassettes (Stokes, H.W. and Hall, R.M., 1989; Hall, R.M. and Stokes, H.W., 1993). In this study, we used these published 5’CS-3’CS primers (Sandvang, D., *et al*, 1997) to screen for the presence of different gene arrays in the isolates under study. This lead to the detection of the expected gene cassettes from organisms known to be carrying gene *aac(3)VIa* within a class-1 integron, but interestingly, we also obtained amplicons for organisms which had been negative for *intI1* (Chapter 2) and which carried gene *aac(3)IIa*.

Gene cassettes can be exchanged between the different integron classes, which supports the notion that they may have played an important role in bacterial evolution (Rowe-Magnus, D.A. and Mazel, D., 1999; Rowe-Magnus, D.A., *et al*, 2001; Rowe-Magnus, D.A. and Mazel, D., 2001; Rowe-Magnus, D.A., *et al*, 2002; Holmes, A.J., *et al*, 2003). In effect, the genetic flexibility that integrons confer bacteria allows them to adapt rapidly to environmental challenges by allowing bacteria to acquire favorable genes that can result in an improved fitness as well as eliminate those that prove useless (Hall, R.M., *et al*, 1999; Rowe-Magnus, D.A. and Mazel, D., 1999; Rowe-Magnus, D.A. and Mazel, D., 2001; Fluit, A.C. and Schmitz, F.J., 2004). On the other hand, even though the majority of the cassettes in the SIs are not expressed, they are believed to be maintained in the absence of selective pressure as a genetic resource for the evolution of new genes (Rowe-Magnus, D.A. and Mazel, D., 1999; Rowe-

There is great variability in the level of expression of genes encoded as gene cassettes. This can be due to intrinsic characteristics of both the gene cassettes and the integron (Levesque, C., *et al.*, 1994; Collis, C.M. and Hall, R.M., 1995; Hall, R.M. and Collis, C.M., 1995; Recchia, G.D. and Hall, R.M., 1995). Gene cassettes are inserted in integrons in the same orientation to their coding sequence, with their initiation codons located near their 5’ end (Hall, R.M. and Collis, C.M., 1995). Generally, gene cassettes do not code a promoter of their own, but are rather expressed from a common promoter, $P_{\text{ant}}$ or $P_1$, or rarely from $P_2$ (Levesque, C., *et al.*, 1994; Collis, C.M. and Hall, R.M., 1995; Stokes, H.W., *et al.*, 2001). Both $P_1$ and $P_2$ promoters are located in the 5’-conserved segment (5’-CS) of class 1 integrons. $P_1$ is located in a region 200 bp upstream from the genes-cassettes inserted at the $attI_1$ site (Levesque, C., *et al.*, 1994; Collis, C.M. and Hall, R.M., 1995).

Several factors can affect the expression of the gene cassettes in an integron (Ploy, M.C., *et al.*, 2000). The $P_1$ promoter sequence has strong sequence similarity to the *E. coli* promoter consensus sequence. Studies on integron promoter sequences support that variations in the sequence of $P_1$ can significantly affect the level of expression of a given gene (Levesque, C., *et al.*, 1994; Collis, C.M. and Hall, R.M., 1995). In this study, we did not detect any mutation which could have an effect on the expression of the gentamicin resistance gene cassettes.

Another factor which can significantly affect the expression of gene cassettes is the order in which the cassette is inserted in the integron. Thus, the level of resistance conferred is higher for the resistance genes that are located in the first place, *i.e.* the first cassette. Likewise, it is reduced when there are preceding cassettes (Levesque, C., *et al.*, 1994; Collis, C.M. and
Hall, R.M., 1995; Ploy, M.C., et al., 2000). In this sense, it is not surprising to have found in this study gene \( aac(3)VIa \) to be in the first position in the array of gene cassettes, near the integrase gene.

As stated before, when we began this study, only the gene \( aac(3)VIa \) had been reported as a gene cassette within a class-1 integron. In our preliminary work, we had detected integron elements in bacteria carrying the \( aac(3)IIa \) gene as well (Diaz, M.A., et al., 2002b; Diaz, M.A., et al., 2002a; Diaz, M.A., et al., in press). However, recent studies by others have also depicted the \( aac(3)II \) gene to be surrounded by integron elements (Galimand, M., et al., 2003). In fact, the molecular environment of the \( armA \) gene has been depicted as a composite or complex class 1 integron environment (Partridge, S.R. and Hall, R.M., 2003). Complex class 1 integrons, as the classical class 1 integrons, possess a 5’-conserved sequence (5’-CS) encoding an integrase gene and a gene cassette attachment site, \( attI \). The variable sequence can encode one or more resistance gene cassettes. Then, instead of a single 3’-conserved sequence, there are two 3’-conserved sequences: 3’-CS1 and 3’-CS2, both of which encode quaternary ammonium compound gene qacE\( \Delta1 \) and \( sulI \). The 3’-CSs are spanning a conserved region, CR, and a unique region. The CR typically encodes orf513, encoding a putative recombinase, and a potential recombination site. The unique region generally encodes resistance genes, which are not encoded as gene cassettes. The general structure of complex integrons is presented in Figure 5.3 (Partridge, S.R. and Hall, R.M., 2003; Doublet, B., 2004).

This type of arrangement has been described in other organisms, involving a mobile integrative structure known as a conjugative transposon (Salyers, A.A., et al., 1995; Partridge, S.R. and Hall, R.M., 2003), which could further contribute to its dissemination. Conjugative transposons are DNA elements that can excise themselves to form a covalently closed circular intermediate (Salyers, A.A., et al., 1995). This intermediate can then reintegrate itself within the
same organism or, it can be transferred by conjugation to a recipient cell and then integrate in
the recipient’s genome (Salyers, A.A., et al, 1995; Pembroke, J.T., et al, 2002; Partridge, S.R.
and Hall, R.M., 2003). First found in Gram-positive bacteria such as the streptococci and
enterococci (Tolmasky, M.E., 2000) and barely mentioned before 1996 in Gram-negative
bacteria (Pembroke, J.T., et al, 2002), conjugative transposon-like elements have later been
detected in Gram-negative bacteria such as Bacteroides spp. (Salyers, A.A., et al, 1995;
Pembroke, J.T., et al, 2002) and though characterized in a lesser extent, in the
Enterobacteriaceae. They have been reported in Salmonella spp., Proteus spp., in the SXT
element in Vibrio spp. and in the element known as R391 (Salyers, A.A., et al, 1995;

Because the aac(3)IIa gene product was not reported to confer high-level gentamicin
resistance, we hypothesized in the first stages of our studies that we would find a second gene
which could act in synergy with the already identified genes to confer high-level gentamicin
resistance to several members of the Enterobacteriaceae that we had isolated in our
laboratories and which displayed the HLGR phenotype (Diaz, M.A., et al, 2000; Diaz, M.A., et
al, in press). We redirected our study following a recent report that high-level aminoglycoside
resistance in a clinical strain of Klebsiella pneumoniae was due to the presence of armA, a new
aminoglycoside resistance methylase gene with only 37-47% similarity to the 16S rRNA
methyltransferases from actinomycetes (Galimand, M., et al, 2003). This strain also presented
genes aac(3)II, blaTEM1, blaCTX-M, dfrA12, sul1 and ant(3")9, conferring resistance to
aminoglycosides, β-lactams, trimethoprim, sulfonamides and streptomycin-spectinomycin,
respectively. Other genes conferring high-level aminoglycoside resistance in Gram-negative
bacteria have been found in a Japanese clinical isolate of Pseudomonas aeruginosa AR-2
(Yokoyama, K., et al, 2003) and Serratia marcescens (Doi, Y., et al, 2004), namely, methylase
enzymes \textit{rmtA} and \textit{rmtB} (Doi, Y., \textit{et al}, 2004; Yamane, K., \textit{et al}, 2005). The GC content of these genes and functional analysis of these enzymes support a possible horizontal gene transfer from aminoglycoside-producing microorganisms (Yokoyama, K., \textit{et al}, 2003; Doi, Y., \textit{et al}, 2004; Yamane, K., \textit{et al}, 2005). In this study, in spite of quite numerous PCR, cloning and sequencing assays performed with the aim of identifying any of these methylase enzymes in the isolates obtained from the turtle farm environment, we did not obtain any amplicon which, upon its sequencing, confirmed the presence of any of these methylase-encoding genes in our isolates.

Interestingly, when we screened for other genes associated with the reported methylase genes (Galimand, M., \textit{et al}, 2003; Galimand, M., \textit{et al}, 2005), we obtained amplification of the \textit{intI1} gene for isolates containing gene \textit{aac(3)IIa}. As mentioned before, this is in contradiction with what we had obtained using a different primer set, mentioned in Chapter 2. It is then possible that gene \textit{aac(3)IIa} is carried by a complex integron and thus was not detected as such with the primer pair used to detect the classic structure of integron class-I. The general structure of complex integrons is illustrated in Figure 5.3 (Partridge, S.R. and Hall, R.M., 2003; Doublet, B., 2004). Sequencing of regions encoding gene cassettes will shed light in this regard (Chapter 4) (Levesque, C., \textit{et al}, 1995; Sandvang, D., \textit{et al}, 1997; Sunde, M. and Sorum, H., 1999; Nield, B.S., \textit{et al}, 2004). Interestingly, recent investigations have found integron-encoded ribosomal methylase genes (Nield, B.S., \textit{et al}, 2004).

In conclusion, there are two genes which contribute to the high-level gentamicin resistance in bacteria isolated from sites with turtle farming activity in Louisiana. These genes are \textit{aac(3)IIa} and \textit{aac(3)Vla}, which code for aminoglycoside acetyl-transferase enzymes. These confer resistance to various aminoglycosides and were detected on different conjugative plasmids. The resistance genes detected in bacteria recovered from environmental samples with
Integrons:

- **attI**: cassette (circular form)
- **attC**: conserved sequence
- **intI**: integrase
- **3' CS**: unique region (resistance genes)

Complex Integrons:

- **intI**: unique region
- **qacEΔ**: gene cassettes
- **sulI**: 3'-CS1
- **orf513**: CR
- **qacEΔI**: unique region
- **sulI**: 3'-CS2

Figure 5.3. General Structure of Complex Integrons
turtle farming activity are coded on class-1 integrons. The molecular environment differed amongst the diverse isolates, which supports their presence on molecular gene exchange elements. Thus, transfer of these genes may involve a complex class-1 integron. Further studies of their molecular environment are required in order to detect and characterize such mobile structures.

This work constitutes one of the first reports of high-level gentamicin resistance in Gram-negative bacteria. It is one of the first to report aminoglycoside resistance genes \textit{aac(3)\textsubscript{IIa}} and \textit{aac(3)\textsubscript{VIa}} in environmental isolates of \textit{Salmonella enterica} subsp. \textit{arizonae}. Additionally, this study illustrates the potential of transfer of antibiotic resistance genes, particularly gentamicin resistance, among bacteria in an environment with turtle farming activity, a matter of particular concern as it involves the human pathogen \textit{Salmonella} spp. This research provides epidemiological data of resistance to aminoglycosides currently used both in the turtle farms and in the clinical setting. The data presented highlights the current inefficacy of the antibiotic gentamicin to eradicate \textit{Salmonella} spp. from turtle eggs. We hope to persuade the relevant authorities in Louisiana to revise the mandate of using gentamicin for turtle egg treatment, and to stress the need to develop new treatments to eradicate \textit{Salmonella} spp. from turtle hatchlings.

This work does not undermine the value of antibiotic use. It does, however, intend to emphasize the importance of monitoring the inevitable emergence of antibiotic resistance, so its appropriate implementation can be redirected accordingly. It is a process in flux, far from being static, which involves continuous vigilance, and surveillance. Ultimately, one of the solutions with the highest impact on antibiotic resistance is the adequate education of people who use them, at any level.
5.1 References


APPENDIX A:

ADDITIONAL DATA
Real-time PCR, also referred to as Q-PCR, is a variation of the PCR assay in which the exact amount of PCR amplification products can be monitored as it is being made. This is in part due to the properties of the fluorescent molecules used to track these products. One of these molecules, SyberGreen, binds to DNA with much higher affinity for double-stranded than single-stranded DNA. In addition, when bound to double-stranded DNA, SyberGreen fluoresces, which is detected by the spectrophotometer built into the thermocycler, thus monitoring the formation of PCR product as it is being made, or in real time.

In this study, real-time PCR was standardized to detect gene \( \text{aac}(3)\text{Ila} \). Primers designed within conserved sequences of the \( \text{rrn} \) gene were used to amplify part of the ribosomal gene as a control or reference (Chapter 2). The primers used to amplify gene \( \text{aac}(3)\text{Ila} \) were designed using the Beacon Designer 2.0 software (Biosoft International, Palo Alto, CA). Genomic DNA used for all reactions was obtained from a strain of \( \text{Salmonella enterica} \) subsp. \( \text{enterica} \) which was positive by conventional PCR and sequencing to gene \( \text{aac}(3)\text{Ila} \) (Chapter 2).

The figures included in this Appendix show the suitability of all primers designed to amplify genes \( \text{aac}(3)\text{Ila} \) and \( \text{rrn} \). SybrGreen (SYBR-490) was used as the fluorescein molecule to track the real-time progression of all Q-PCR reactions. Figure A.1., Figure A.4., and Figure A.6. show the melting curve graphs for genes \( \text{aac}(3)\text{Ila} \) and \( \text{rrn} \) using SYBR-490. It is evident from these figures that the primer sets designed produce only one amplicon in each case. In effect, melting points for \( \text{aac}(3)\text{Ila} \) gene amplicons are all at \( 86^\circ \text{C} \) and those for \( \text{rrn} \) gene amplicons are all at \( 88^\circ \text{C} \). Two much smaller curves, in blue and red, which peak around \( 82.5^\circ \text{C} \), correspond to the primer controls in which no DNA was added, and evidence the suitability of primers designed for real-time PCR.
Figure A.5. and Figure A.7. present results from real-time PCR graphs for genes \textit{aac(3)IIa} and \textit{rrn}, as obtained by using SYBR-490. These graphs show the logarithmic plot of real-time PCR through all cycles. The CT values can be seen intersecting the orange horizontal line, which was set at a point where all PCR reactions were linear. From these graphs, it is evident that different DNA concentrations intersect the horizontal line after different amounts of cycles, as is expected. Thus, the more diluted the sample, the more cycles it takes to reach a certain product quantity. In addition, all replicas intersect the horizontal line at the same number of cycles. Furthermore, primer controls, where no DNA was added, can be seen to the far right in red and blue, and show that no significant amplification is detected throughout PCR cycles. Thus, this is an accurate measurement of the amount of \textit{aac(3)IIa} and \textit{rrn} amplicons in a particular sample.

Standard curves were done with 1:10 serial dilutions of Genomic DNA. Figure A.2. and Figure A.3. show the standard curves obtained when plotting SYBR-490 values with the dilutions of the DNA assayed, throughout the progression of PCR cycles. The high correlation coefficients obtained in both curves indicate that both primer pairs designed can be appropriately used for the quantitation of gene \textit{aac(3)IIa} from unknown samples. Because the amount of gene \textit{rrn} does not vary within a particular bacterial strain, differences in \textit{rrn} quantities will account for any variation due to experimental manipulation or error. Thus, it can be used as a reference to compare against variations in \textit{aac(3)IIa} amounts. With this methodology, variations in the number of \textit{aac(3)IIa}, following exposure to different treatments, could be measured. Furthermore, the expression of this gene could also be monitored, using these same primers and conditions, but using total bacterial cDNA as template DNA. This Appendix includes data from experiments unfinished as of yet and which will be published as a communication of a technique standardization.
Figure A.1. Melt Curve Graph for Genes *aac(3)IIa* and *rrn*, Using SYBR-490

Figure A.2. Standard Curve Graph for Gene *aac(3)IIa*, Using SYBR-490

Figure A.3. Standard Curve Graph for Gene *rrn*, Using SYBR-490
Figure A.4. Melt Curve Graph for Gene \textit{aac(3)IIa}, Using SYBR-490

Figure A.5. Real Time PCR Graph for Gene \textit{aac(3)IIa}, Using SYBR-490
Figure A.6. Melt Curve Graph for Gene *rrn*, Using SYBR-490

Figure A.7. Real Time PCR Graph for Gene *rrn*, Using SYBR-490
APPENDIX B:
LETTER OF PERMISSION
November 10, 2005

Journals Department
American Society for Microbiology
1752 N St., NW
Washington, DC 20036
Fax: (202) 942-9355

To Whom It May Concern:

I am writing to request permission to incorporate the manuscript entitled “Plasmid-Mediated High-Level Gentamicin Resistance Among Enterics Isolated from Pet Turtles in Louisiana” (AEM01471-05) of which I am first author, into my doctoral dissertation. The manuscript, coauthored by R. K Cooper, A. Cloeckaert and R.J. Siebeling, has been accepted for publication in the Public Health section of Applied and Environmental Microbiology (AEM), and is scheduled for publication in vol. 76, issue 1, in January 2006. A copy of the signed copyright transfer agreement is included with this letter. I will include the entirety of the manuscript as a separate chapter of my dissertation, entitled “Plasmid-Mediated High-Level Gentamicin Resistance in Bacteria from the Turtle Farm Environment in Louisiana”, with only minor formatting changes.

Please contact me at the above address for any additional information.

Cordially,

Maria Alejandra Diaz
VITA

María Alejandra was born in Maracaibo, Venezuela, to Javier Antonio Díaz and Alejandra Páez. She grew up with her siblings Javier Enrique and María Patricia, and later also Juan Carlos and Daniel Alberto. Her parents are currently retired professors from La Universidad del Zulia, Venezuela. Growing up surrounded by the world of academia exposed her early on to science and to other cultures. María Alejandra first visited the LSU campus when she was only one year old, as both of her parents came to this University to work on their Master’s of Science degrees. She attended Colegio San Vicente de Paúl for her elementary education in Maracaibo. As a child, she traveled often with her family through the Colombian and Venezuelan Andes, where she developed a deep love for contemplating nature.

As a pre-teen, she traveled to Irvine, California, and then to Tempe, Arizona, as her parents worked on their doctorate degrees. She then returned with her family to her home school to complete her high-school studies, where she performed exceptionally well in science and language-related courses. At 15, she won the 2nd place –out of 40,000 students nationwide- in a national math contest, CENAMEC. In her senior year of high-school she was elected Valedictorian by her classmates and teachers. Upon high-school graduation from Colegio San Vicente de Paúl, she left her natal city to continue her studies in Caracas. There, she pursued a degree in biological sciences at Universidad Simón Bolívar, “the University of Excellence”, where besides obtaining a solid and complete undergraduate education, she also discovered the beauty of her country in the fieldtrips to the Caribbean, the rain forest, the Amazon and the Venezuelan plains, which happily were required by the curriculum of her major.

Upon receiving her bachelor’s degree, she accepted a job offer at IBM, which allowed her to participate in the improvement of the scientific education of her country. To complement her “hard science” education, she completed all required coursework for a master’s of science
in psychology. Later, she participated in a contest that rewarded her with a fully paid scholarship for a master’s degree in educational computing and technologies at the University of Hartford, Connecticut. After this, she was offered a position as a research assistant at her Alma Mater where she participated in research on the biochemistry and immunology of the intracellular microorganism \textit{Anaplasma marginale}. During this time, she was awarded with two full scholarships for two consecutive years by the French Embassy, to France. The first one allowed her to attend a graduate course in bioinformatics at the \textit{Institut Pasteur}, in Paris. The following year, she did an internship in collaborative research at the \textit{Institut National de la Recherche Agronomique}, in Tours.

Maria Alejandra was recruited to pursue her doctorate degree in microbiology by her late mentor, Dr. Ronald J. Siebeling. Above all in her doctorate, she is grateful she had the opportunity to learn from such an exceptional Professor, “El Magnífico”, as he teasingly signed. She attended the General Meetings of the American Society for Microbiology yearly, in what were Dr. Siebeling’s laboratory’s legendary trips. There, she was delighted to meet many “celebrities” of the antibiotic resistance world, such as Levy, Davies, Courvalin, Tolmaski, and Fluit. Maria Alejandra held a Teaching Assistant position concurrent with her studies, which allowed her to enjoy teaching both senior-level and basic-level courses in microbiology. Thus, she has taught the laboratories of general microbiology, immunology-serology, and pathogenic microbiology. In addition, she was recipient of the Graduate School Tuition Award. Upon the unfortunate and unexpected death of her advisor, Maria Alejandra finished her research under the guidance and support of Dr. Richard K. Cooper. Maria Alejandra presented and discussed her work yearly at the American Society for Microbiology General Meetings. The material presented in each of the chapters of this dissertation will be submitted for publication.
Maria Alejandra values most in life the bonds of love: romantic, family, and friendship. She feels profound satisfaction and pride with the places her professional and intellectual activities have taken her. Maria Alejandra defines herself as a Citizen of the World: her various sojourns and bonds with people in different countries have given her a sense of belonging to a greater entity that goes beyond frontiers, languages and religions. Her vocation to science is intimately linked to her belief that the world can constantly be made a better place for everyone. Maria Alejandra will be awarded her doctorate degree at the Fall Commencement ceremony of Louisiana State University.