EXPRESSION OF *marA* IN *SALMONELLA* TYPHIMURIUM

EXPOSED TO OXYTETRACYCLINE

IN VITRO AND

IN VIVO

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LIST OF ABBREVIATIONS

BSL	Biosafety level
CFU	Colony forming units
cDNA	Complementary DNA
DNA	Deoxyribonucleic acid
IM	Intramuscular
IV	Intravenous
M	Molar
Mar	Multiple antibiotic resistance
mg	Milligram
ml	Milliliter
μl	Microliter
mRNA	Mitochondrial RNA
NaOAc	Sodium acetate
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RT-qPCR	Reverse transcriptase real time polymerase chain reaction
SSC	Sodium chloride-sodium citrate
SDS	Sodium dodecyl sulfate
SQ	Subcutaneous

CHAPTER 1: INTRODUCTION

Approximately 1.4 million cases of human salmonellosis occur in the United

States each year. The most common causes of this disease are *Salmonella enterica*

serovars Typhimurium and Enteritidis. In humans, the primary source of infection is

contaminated animal food products. While this disease is primarily self-limiting, there

are many instances in which antibiotics are used for treatment; the current drug of choice

is ciprofloxacin (CDC, 2005). Recent outbreaks of multi-drug resistant *Salmonella*

infections in humans has led to an increasing concern about the use of antimicrobial

agents in food animals (Hsueh *et al*, 2004; Zansky *et al*, 2002).

The use of antimicrobial agents in food animals for growth promotion and

therapeutic purposes is believed to lead to emergence of resistant bacteria (Fox, 2001;

Furuya *et* al, 2006; Mlot, 2001). Of particular concern is the use of antimicrobial agents

in food animals leading to resistant bacterial infections in humans, especially those

caused by *Escherichia coli, Campylobacter* spp. and *Salmonella* spp. (Angulo *et al,* 2004;

Molbak, 2005). Consequently, use of antibiotics in food animals has been greatly

restricted, particularly the use of fluoroquinolones, such as ciprofloxacin. Indeed, current

strategies employed to reduce the emergence of resistance include a prohibition against

using the same antibacterial agent in both food animals and human patients. Thus,

resistance that develops against a particular antibacterial agent administered to a food

animal is believed to be unlikely to limit therapy of a human patient using another

antibacterial agent. For example, as ciprofloxacin is the treatment of choice for food-

borne salmonellosis in humans, it is not available for use in food animal medicine.

However, the assumption that resistance to one specific agent does not affect development of resistance to another drug may not be true. Recent discovery of a multiple antibiotic resistance (*mar*) locus chromosomally located in *Salmonella* species has complicated the issue. The *mar* locus responds to exposure to many classes of antimicrobial agents and disinfectants by increasing efflux protein expression and decreasing porin expression, leading to multi-drug resistance (Randall *et al*, 1997 and 2002; Sulavik *et al*, 1997). The increase in efflux protein expression has been found to increase resistance to fluoroquinolones (Chu *et al*, 2005; Goldman *et al*, 1996). This means that exposure to many different types of antimicrobials other than fluoroquinolones can lead to fluoroquinolone-resistant salmonellosis in humans. In fact, chloramphenicol and tetracycline have been found to induce the *mar* system in *E. coli* and increase resistance to nalidixic acid *in vitro* (Cohen *et al*, 1989). Nalidixic acid is a quinolone antibacterial agent closely related to the fluoroquinolones.

Discovery of the *mar* system in *Salmonella* may necessitate a change in the use of antimicrobial agents in food animals. Tetracyclines, for example, are commonly used in cattle, for both prophylactic and metaphylactic purposes. The assumption that use of tetracyclines in cattle will not promote emergence of fluoroquinolone resistance in human consumers may be incorrect. If exposure to oxytetracycline induces the expression of multi-drug resistance mechanisms, such as the *mar* system, the bacteria expressing these systems will survive exposure to moderate concentrations of oxytetracycline and are more likely to represent a higher proportion of the bacterial population contaminating beef products. These bacteria can then be transferred to human consumers, where they will be resistant to not only tetracyclines but also to other antibacterial agents, including

ciprofloxacin. Clearly, there is much research that needs to be conducted to more fully

understand the impact of the *mar* system *in vivo*, including the possibility that the use of

drugs like tetracyclines in beef cattle can lead to ciprofloxacin-resistant *Salmonella*

infections in humans.

CHAPTER 2: LITERATURE REVIEW

Multiple Drug Resistance (MDR)

Definition of multiple drug resistance (MDR)

Bacteria have many ways of resisting the effects of antibiotics or other types of chemicals commonly used to kill them. In the last ten years, research on bacterial resistance has found that many bacteria, both Gram-positive and Gram-negative, employ specific resistance mechanisms that work against many different classes of antibacterials by decreasing the concentration of drug within the cell (De *et al*, 2001; Hooper, 2002; Poole, 2001; Regelink *et al*, 1999). These mechanisms, termed multi-drug resistance mechanisms, provide bacteria with resistance to not just one class of antimicrobials, but to a wide range of different chemical classes. Emergence of these multi-drug resistance mechanisms has necessitated the development of new agents with novel mechanisms of action, which is becoming increasingly difficult to accomplish (Neu, 1992).

Overview of types of mechanisms of MDR

There are three main ways that bacteria become resistant to antibiotics: changes in the target site; destruction of the drug by enzymes; or decreasing/limiting drug concentration within the cell. Decreasing/limiting drug concentration within the cell is the main focus of this review and usually results from drug efflux mechanisms and porin down-regulation (Poole, 2002).

Target Alteration:

Many drugs used against bacteria require a specific binding site on or within the bacterium to cause death or inhibition of growth of the organism. A common mechanism of resistance involves a mutational changing of a particular binding site resulting in decreased or no binding affinity. This is seen commonly with rifampin, macrolides, and beta-lactam antibiotics (Neu, 1992; Poole, 2002).

Alteration by Enzymes:

The main mechanism of resistance to beta-lactam antibiotics is the production of beta-lactamase, an enzyme that cleaves the antibiotic rendering it inactive; this may be either outside the bacterium or within the periplasmic space. Resistance to aminoglycoside antibiotics is similar, but uses drug modification instead of enzymatic cleavage. An aminoglycoside must be transported across the inner cytoplasmic membrane of the gram-negative cell wall to reach the target site on the ribosome. The bacterium secretes enzymes that conjugate the aminoglycoside outside the cell, thus preventing the drug from being able to enter the cell (Neu, 1992).

Decreased Intracellular Drug Concentration Related to Efflux Pumps and Porin Expression:

Efflux proteins conferring resistance to multiple classes of drugs were originally identified in the mid 1980s as the P-glycoproteins, which are still studied today in drug - resistant tumors. Since then, many homologs of the P-glycoprotein system have been found in both Gram-positive and Gram-negative bacteria. Although most of these efflux

systems use either the proton motive force or ATP to expel antibacterial agents, it is still not clear that this is their intended function (Poole, 2001). Often, bacteria use the export pumps for purposes related more to the environment in which they normally live than to antibacterials. For example, it is thought that the efflux systems are often found in enteric bacteria such as *Salmonella* and *E. coli* because they are used to help pump out toxic compounds, such as bile acids, in that environment (Prouty *et al*, 2004). However, many studies have found that exposure to antimicrobials effects the gene expression of these efflux systems (Alonso *et al*, 2000; Kehrenberg *et al*, 2001; Lewis 2002).

Efflux systems are most commonly associated with tetracycline resistance, but recently have been found to provide resistance to many other antimicrobials (Kehrenberg *et al*, 2001; Poole, 2002) such as fluoroquinolones, aminoglycosides, macrolides, and beta-lactams, as well as antiseptics and disinfectants (Alonso *et al*, 2000; Putman *et al*, 2000). Hydrophobic agents that easily pass across the outer and inner membranes of Gram-negative bacteria are collected in the inner side of the cytoplasmic membrane and pumped out through an outer membrane channel. (See Figure 1)

Currently, there are four known classes of efflux pumps: the major facilitator superfamily, the small multidrug resistance family, the resistance-nodulation-cell division family, and the multidrug and toxic compound extrusion family (Putman *et al*, 2000).

The major facilitator superfamily (MFS) can be divided into two groups based on the number of transmembrane segments (TMS). The 12-TMS group has been identified in *Staphylococcus aureus*, *Bacillus subtilis*, *Lactococcus lactis*, *Escherichia coli*, *Cornyebacterium glutamicum*, many species of *Mycobacterium*, and may be involved in the fluoroquinolone resistance of *Streptococcus pneumoniae*. Although these 12-TMS

sequences are not identical in different bacteria, homology is shared between them. The other group of MFS is the 14-TMS group which has been shown to increase resistance to compounds that include, but are not limited to, quaternary ammonium compounds, diamines, intercalating dyes and possibly chlorhexidines, proving that multidrug resistance is not limited to systemic antibiotics, but also affects disinfectants and antiseptics. This set of transporters has been seen in *S. aureus*, *E. coli*, *B. subtilis*, *Mycobacterium* species and *Vibrio cholerae*. Again, these are not all exactly the same proteins, but they do show homology (Putman *et al*, 2000).

The small multidrug resistance family (SMR) of proteins are only 107 amino acids in length and appear to be formed in a tightly packed four-helix antiparallel bundle. The *smr* genes are located on an integron, and therefore are widely distributed, especially among gram-negative isolates. *Staphylococcus aureus* was the first bacterial species found to have this gene. *Escherichia coli*, *Klebsiella aerogenes*, *B. subtilis* and several species of *Mycobacterium* also appear to have *smr* genes (Putman *et al*, 2000).

The resistance-nodulation-cell division family (RND) has been associated with many species of Gram-negative bacteria. RND transporters interact with a membrane fusion protein and an outer membrane protein to allow drug transport across both the inner and outer cell membranes of Gram-negative bacteria. These RND pumps have been identified in *Pseudomonas aeruginosa*, *E. coli*, *Neisseria gonorrheoeae*, *Haemophilus influenzae*, *Burkholderia pseudomallei*, and *Stenotrophomonas maltophilia*. All of these pathogens have presented challenges in the therapy of human and animal health problems due to high incidences of resistance (Alonso *et al*, 2000; Putman *et al*, 2000).

The final family is the multidrug and toxic compound extrusion family (MATE). Thus far, this protein has been found only in *Vibrio parahaemolyticus* and *E. coli*, but has been shown to mediate resistance to dyes, fluoroquinolones, and aminoglycosides.

Gram-negative bacteria in general are more resistant to antimicrobials than the Gram-positive bacteria. This is mainly attributed to the Gram-negative complex outer membrane barrier, which is only selectively permeable. This membrane limits the access of chemicals to the inside of the cell, the main site of action for most commonly used antimicrobials (Ames, 1998). Gram-negative bacteria use water-filled (aqueous) channels, termed porins, in the outer membrane for acquisition of small nutrients, amino acids, some polar molecules and ions (De *et al*, 2001).

Porins were originally thought to exclude most drugs because of size restriction: however, more recent research has found that porins may be important in allowing drug access to the cell, particularly the hydrophilic agents that cannot diffuse across the outer membrane (Mallea *et al*, 1998; Nikaido, 1993). (See Figure 1) Several studies of Gram-negative bacteria *in vitro* have shown decreased porin expression in the presence of antibiotic, signifying that regulation of porin protein expression may be an important mechanism of resistance employed by these bacteria (Mallea *et al*, 1998; Regelink *et al*, 1999).

Studies have identified porins that allow passage of antibacterials in *Enterobacter* species, *Haemophilus* species, and, probably, *Mannheimia haemolytica*. The porins recognized have been designated Omp C and Omp F in *Enterobacteriaceae* species and Omp P2 in *Haemophilus* species. Recent studies have identified similar proteins in *M. haemolytica*.

In the presence of antibiotics, these experiments have indicated that resistant strains down-regulate porin proteins compared with those strains grown in the absence of antibiotics (De *et al*, 2001; Mallea *et al*, 1998). Down-regulation of the porin proteins appears to limit antibiotic access to the cell, resulting in resistance against many different classes of antibiotics. So far, porin regulation has been found to provide resistance to beta-lactams, chloramphenicols, and tetracyclines (De *et al*, 2001; Mallea *et al*, 1998). How exactly the porins are regulated and to what extent, still remains to be seen. Current research is focusing on learning more about porins and how they are controlled.

marRAB as a mediator of MDR

The multiple antibiotic resistance (*mar*) system is a chromosomally mediated form of multidrug resistance (Barbosa *et al*, 2000). The genes encoded by this system are responsible for up-regulating efflux protein systems and down-regulating porin protein expression along with a variety of other genes. This combination of increasing efflux and decreasing porin expression creates multi-drug resistance by decreasing intracellular concentration of both hydrophobic (efflux) and hydrophilic (porin) chemicals, decreasing the efficacy of a wide range of antimicrobial agents. There are five genes included in the *mar* system; *marO* encodes the operon, *marR* encodes the regulator, and *marA* is primarily responsible for the up and down-regulation of other proteins. The functions of *marB* and *marC* are still unclear. Under normal conditions, in the absence of any antimicrobials, the MarR protein represses *marO*, inhibiting the expression of *marA*, *B* and *C* (Alekshun *et al*, 2000; Cohen *et al*, 1993a; Martin *et al*, 2004). In the presence of certain antimicrobial agents, MarR becomes inhibited through binding to the agent. This

allows *marO* to initiate transcription and translation of *marA*, *B* and *C* (Martin *et al*, 1996). (See Figure 1).

The MarA protein is considered to be the master regulator (Alekshun *et al*, 1997; Gambino *et al*, 1993; Hachler *et al*, 1991; Jair *et al*, 1995). It is currently believed to up-regulate approximately 47 genes in some bacterial strains, including efflux components, as well as down-regulate approximately 15 genes including the porin proteins (Chollet *et al*, 2002; Cohen *et al*, 1988; Fralick, 1996). The *mar* system has been found in many different organisms, including *E. coli* O157:H7, *Enterobacter aerogenes,Campylobacter* spp., *Salmonella* spp. and others (Barbosa *et al*, 2000; Chollet *et al*, 2002 and 2004; Randall *et al*, 2003; Tavio *et al*, 2004; Yaron *et al*, 2003).

Compounds that are known to induce the mar system include salicylate, acetominophen, naphthoquinones, and sodium benzoate (Alekshun *et al*, 1999a; Cohen *et al*, 1993b; Randall *et al*, 2002; Seone *et al*, 1995). It is thought that certain antibiotics may also induce the system in certain bacteria, but not in others. For example, tetracycline is believed to induce *mar* in *E. coli*, but not in *Salmonella* (Randall *et al*, 2002). The expression of *marA* has been shown to increase resistance to a broad range of antibiotics, including tetracyclines, fluoroquinolones, and beta-lactams (Chu *et al*, 2005; Randall *et al*, 2002; White *et al*, 1997).

While the induction of the *mar* system is known to increase resistance to other compounds, the change in resistance is generally smaller than the resistance resulting from specific mutations or gene acquisitions. The current hypothesis is that *mar* allows bacteria an opportunity to mutate or acquire those genes that will confer a higher level of resistance and is therefore thought of as more of a stepping-stone to a high level of

resistance (Alekshun *et al*, 1997; Goldman *et al*, 1996). The relevance of this system *in vivo* is unknown at this time, but it is thought to be important in both human and veterinary medicine (Randall *et al*, 2001).

Relevance of MDR to Human and Animal Health

The development of resistant strains of bacteria is increasing the morbidity and mortality of bacterial diseases as well as the cost of both human and animal health care (Shlaes *et al*, 1997). Emergence of these pathogens is occurring at a frightening rate, and the development of new chemicals to combat these bacteria has not kept pace with the emergence of resistant strains. *Salmonella*, *Pseudomonas*, *Streptococcus*, *Enterobacter* and *Staphylococcus* strains are proving to be formidable enemies to hospitals and patients (Boyce, 2001; Russell, 2002). Infections caused by these bacteria, especially strains like methicillin-resistant *S. aureus*, vancomycin-resistant enterococci or fluoroquinolone-resistant *Salmonella,* are almost impossible to treat due to the lack of available drugs that are able to kill these pathogens (Boyce, 2001; Randall *et al*, 2004).

The largest problems are seen in humans and animals that may be immunocompromised, which explains why most problems occur in hospital situations. In these cases, normal flora or commensals become opportunistic pathogens and can be lethal. These are often the strains that are extremely resistant to a wide variety of drugs due to efflux expression or porin regulation (Boyce, 2001; FAAIR *et al*, 2002).

Relevance of Exposure to Selection/Expression of Resistance

Many studies have demonstrated that exposure to antimicrobials may actually be promoting development of resistance. It is understood that antibacterial agents do not initiate gene mutatation to resistant genotypes but select for those isolates that become resistant due to mutation or acquisition of resistance genes. *In vitro* studies demonstrate expression or down-regulation of certain multi-drug resistance mechanisms only in the presence of antibacterial agents (see marRAB) (De *et al*, 2001).

In the absence of antibacterial drugs, mechanisms such as the *marRAB* system may be a hindrance to growth and therefore are not always part of the expressed genome in these situations. Only in the presence of antibiotics do these mechanisms seem to allow the bacteria to survive better than those strains not expressing resistance.

These findings have led many to believe that the prophylactic use of broad-spectrum agents, especially in food animals, needs to be more tightly controlled (Levy, 1998). It appears that many of the resistant strains that cause severe problems in humans may be closely associated with, or even the same as, those strains found in healthy food animals that are receiving regular antibiotic treatment (Fox, 2001; Mlot, 2001). Although blame is often directed at food animal production and antibiotic use, many researchers are finding that any exposure to any antimicrobial at any time can lead to resistance. Therefore, the constant and inappropriate uses of these drugs in both food animals and in human medicine are providing opportunities for resistance to develop (Mlot, 2001).

Many steps have been taken by the FDA and the CDC to combat the emergence of antibacterial resistance, and specific committees have been set up to educate the public on the occurrence of resistance, the steps that can be taken to stop it, and why it is

important (Fox, 2001; Mlot, 2001; Shlaes *et al*, 1997). Currently, more research is being

done to determine how these mechanisms are regulated, and to develop new methods of

killing these resistant bacteria.

<center>*Salmonella* Typhimurium</center>

Overview of the Organism

　　　　Salmonella enterica serovar Typhimurium is a Gram-negative rod of the

Enterobacteriaceae family. It is a facultative intracellular organism that utilizes carrier

animals as its primary reservoir. *Salmonella* is primarily an enteric pathogen that is

capable of causing infection anywhere in the body once it crosses the gastrointestinal

epithelium (Radostits *et al*, 2000).

Pathogenesis of Infection

　　　　Many animals are carriers of *Salmonella* Typhimurium and show no clinical signs

of infection. However, once these animals are stressed they may start to show clinical

signs, but just importantly they shed the organism in the feces. Other animals that are

naïve to the organism ingest it from feces-contaminated food or water. Once in the

gastrointestinal tract, *Salmonella* uses fimbriae for attachment and invades the

gastrointestinal epithelium, causing vacuole formation in the epithelial cells. The

immune response results in large numbers of neutrophils infiltrating the *lamina propria*

and lumen surface. In addition, *Salmonella* can release a cytotoxin that inhibits protein

synthesis in eukaryotic cells, resulting in cell death. Endotoxin from the bacterial cell

wall as well as the host cell death result in severe inflammation of the lower small

<center>13</center>

intestine and colon resulting in secretory and/or hemorrhagic diarrhea (CDC, 2005; Radostits *et al*, 2000).

Salmonella survives within macrophages by inhibiting phagolysosome fusion (Eriksson *et al*, 2003). It is this adaptation that allows the organism to invade the macrophages in the Peyer's patches of the intestine and use this site to gain access to other lymph nodes as well as the blood stream. Once in the blood stream, a severe septicemia develops that can lead to infection in almost any organ or site of the body, including the central nervous system (Radostits *et al*, 2000). Death is usually associated with endotoxemia.

In addition to colonization of carrier animals, *Salmonella* can be found in the environment. It is capable of replicating at temperatures between 8 and 45°C, can survive at a pH between 4 and 8 and in the presence or absence of oxygen. *Salmonella* is also extremely resistant to drying and therefore can live in the environment for several years (Radostits *et al*, 2000).

Importance as a Zoonotic Pathogen

In humans, salmonellosis is a disease caused by many different isolates of *Salmonella*, Typhimurium and Enteritidis being the most common serotypes. This disease is considered zoonotic because the primary route of infection is through contaminated food products, mainly improperly prepared eggs (Enteritidis) and meat (Typhimurium). In the United States, it is estimated that 1.4 million cases occur annually; of these cases, culture by the CDC confirms approximately 40,000 (CDC, 2005).

The human disease is characterized by fever, abdominal cramps and diarrhea that typically lasts 5-7 days. These symptoms alone do not necessarily require treatment, however, should the disease lead to dehydration or sepsis, treatment is required. Treatment includes fluids and antibiotics, specifically ampicillin, trimethoprim/sulfamethoxazole or ciprofloxacin. Approximately 600 people die each year in the United States due to salmonellosis, and these are usually the very young, old or immunocompromised patients. In 2% of patients, Reiter's syndrome can develop, which is characterized by pain in the joints, eye irritation and painful urination, and can lead to chronic arthritis (CDC, 2005).

Antibacterial Resistance in *Salmonella*

Salmonella Typhimurium phage type DT104 is perhaps the most recently notorious of the *Salmonella* strains. This phage type was originally identified in the early 1980s in the UK. DT104 is unique due to the chromosomal regulation of resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline. All of the multi-drug resistant strains isolated prior to this strain had plasmid-mediated resistance, believed to be acquired from other organisms. In addition, in the last 10 years, the susceptibility of DT104 to ciprofloxacin, a common antibiotic of choice for *Salmonella* infections in humans, has been declining. This resistance is chromosomally encoded and is believed to be a result of the use of fluoroquinolone antibiotics in food animals (Threlfall, 2002).

Phage type DT104 is not the only *Salmonella* organism exhibiting resistant phenotypes. In 2002, an outbreak of salmonellosis occurred in the United States due to a

strain of Salmonella Newport that was resistant to amoxicillin/clavulanate, ampicillin, cefoxitin, ceftiofur, cephalothin, chloramphenicol, streptomycin, sulfamethoxazol and tetracycline, with a few of the isolates resistant to kanamycin and ceftriazone (Zansky *et al*, 2002). Researchers in Germany identified 319 different strains of *Salmonella enterica* causing human infection throughout 2001. The German strains were resistant to beta-lactams, aminoglycosides, chloramphenicol, sulfonamides and tetracycline (Miko *et al*, 2005). In Taiwan, ciprofloxacin resistant strains of *Salmonella enterica* Typhimurium (non-DT104) and Cholerasuis were found that were transmitted from pigs to humans (Hsueh *et al*, 2004).

Currently, the largest concern relating to resistance of human salmonellosis is resistance to fluoroquinolones. Fluoroquinolone antibiotics are the treatment of choice for salmonelloses in humans, particularly the septic form of the disease. Resistance to fluoroquinolones is believed to result from the use of fluoroquinolones in food animals (Molbak, 2005; Furuya *et al*, 2006). The mechanisms for resistance to fluoroquinolones include mutations in genes *gyrA* and *parC*, encoding synthesis of the site of action, as well as efflux mechanisms (Chu *et al*, 2005; Olliver *et al*, 2004; Randall *et al*, 2004a and 2005). *Salmonella* Typhimurium has five different RND efflux systems, as well as four efflux systems belonging to the MFS, MATE and ABC families (Nishino, *et al*, 2005). In addition, *Salmonella* has a chromosomally located *mar* system that, as mentioned before, affects the expression both efflux and porin systems (Sulavik *et al*, 1997; Okusu *et al*, 1996). While it seems practical to stop the use of fluoroquinolones in food animals to decrease the incidence of resistant infections in humans, that alone may not be enough, as exposure to other compounds, including other antibiotics and disinfectants, may lead

to resistance to fluoroquinolones via the *mar* system (Cohen *et al*, 1989; Randall *et al*, 2004b). To assess the impact of antibiotic exposure on multi-drug resistance, the expression of genes encoding for resistance mechanisms must be well understood.

Gene Expression and Measurement

Methods for Assessing Gene Expression

Investigating bacterial responses to certain stressors, including antibacterial agents, cannot be accomplished without first understanding the degree to which these stressors affect gene expression. Differential gene expression may be influenced at several functional levels, including at the transcriptome. Methods employed to study gene transcription include the following:

Differential display using PCR

Generally, differential display using PCR is performed as follows: Two identical bacterial inocula are cultured in broth, one as a negative standard with minimal stress, the other with a stressor of interest, such as an antibiotic. Once the cultures have reached the mid-log phase of growth, the RNA is extracted and then reacted with a set of arbitrary or random primers to reverse transcribe the RNA. A second set of arbitrary primers is then added to make short segments of cDNA. These segments are then amplified by PCR and the products are separated on an agarose gel. The control and stressed group gel patterns are then compared and genes that are induced or repressed can be isolated for sequencing and identification (Handfield *et al*, 1999). An advantage of this technology is that it is

simple to perform, although somewhat time consuming. The most difficult aspect of this

experimental approach is the isolation of the bacterial RNA. Isolation of enough pure,

undegraded RNA from bacteria is not a trivial task, but once this is accomplished, the

methodology is both simple and effective.

Subtractive and differential hybridization

An alternative to differential display using PCR is subtractive and differential

hybridization. The techniques are very similar in that two cultures are grown, one control

and one stressed. The total RNA is again extracted from each sample; however, in this

case, a probe is used to bind and remove contaminant rRNA. The mRNA is then used to

synthesize cDNA by reverse transcriptase. PCR amplification of the cDNA fragments

using 5' adaptor oligonucleotides allows for biotin labeling of the control group genes at

the 5' end. The PCR products from both the control and stress groups are denatured and

then mixed together and allowed to hybridize. The hybridized genes are then passed over

a filter that binds the biotin labeled products and removes them, leaving only the

unlabeled segments. These unlabeled segments are denatured, mixed with the labeled

segments and allowed to hybridize again and passed over filter. This process is repeated

three times to ensure that the products recovered from the filtration process are unique

sequences found only in the stressed group of bacteria (Handfield *et al*, 1999). This

protocol identifies genes expressed under the stressed conditionsbut not those genes that

are repressed in stress. his protocol requires a high yield of good quality mRNA, which

as stated above is difficult to obtain. This protocol is highly specific in comparison to the

differential display, but requires further testing to investigate all changes in expression as it assesses only those genes that are induced under stress.

In vivo expression technology (IVET)

      In vivo expression technology (IVET) is used primarily to investigate genes expressed *in vivo*, versus those expressed only *in vitro*. DNA is cleaved into fragments using restriction endonuclease enzymes. These fragments, from the bacterial chromosome, are inserted into *E. coli* DH5αλ plasmid with selection for ampicillin resistance. These plasmids (suicide vectors) are then transformed into the bacterial strain of interest, which is then inoculated into the animal model. Bacteria are then recovered from the animal and plated on media with selective markers such as IPTG and Xgal (Handfield *et al*, 1999). Colonies can then be selected based on the expression of the gene *in vivo*. While this method can select for a large number of genes that may be associated with virulence, it is quite time consuming and expensive.

Northern blot

      Northern hybridization separates RNAs based on size using an agarose gel matrix. The relative abundance of certain RNAs can be measured based on the fluorescence once the gel is stained. To investigate a specific gene of interest, the RNA must be transferred to a solid matrix, such as a nylon membrane. The RNA can then be screened for certain sequences or genes of interest using labeled probes. Once the unbound probes are removed, those RNAs containing the sequence of interest can be identified and the abundance of that particular transcript determined (Sambrook *et al*, 2001).

This protocol has been used for many years and was originally described in 1977 (Alwine *et al*, 1977). There are several drawbacks to this type of analysis, including the isolation of large amounts of intact RNA needed for this type of experiment. In addition, the buffer for the agarose gels must contain formamide, a carcinogen, to inhibit the denaturation of the RNA during electrophoresis. The transfer of RNA from the gel to the solid matrix can be somewhat difficult depending on the size and abundance of RNA. While this method has been used extensively, it is time-consuming and technically difficult compared to the newer, similar method of microarray analysis (Sambrook *et al*, 2001).

Gfp reporter

Green fluorescent protein (gfp) is a detection tool that can be used two different ways to investigate gene expression. A *gfp:gene* construct can be made in a suicide vector and inserted into a bacterial chromosome. The bacteria can then be subjected to different stresses and, or cultured *in vivo* and the expression of that gene compared by measuring the level of fluorescence produced. While this approach appears straight-forward, it is actually quite difficult to create a *gfp:gene* construct with certain genes (Randall *et al*, 2001).

The second method for using *gfp* is to insert the *gfp* randomly into the bacterial chromosome. The fluorescence is then measured in and out of a host environment. Those bacteria that fluoresce *in vivo*, but not *in vitro* have the *gfp* inserted into a gene that is up regulated *in vivo*. In this way, *gfp* can be used to investigate many genes as opposed to the regulation of just one gene (Handfield *et al*, 1999).

Real time reverse transcriptase PCR

Real time PCR (qPCR) utilizes fluorescent molecules to measure the number of amplicons produced by each iteration of the PCR process, as opposed to just a final product. There are essentially two methods for fluorescence-labeling the PCR product: one is to use an intercalating agent, such as Sybrgreen$^®$, which binds any double stranded DNA product; the other is to use a labeled probe such as TaqMan® probes, molecular beacons or scorpions (Dharmaraj, 1998).

Sybrgreen® is very sensitive, but it binds to any double stranded product, such as primer-dimers. This method requires an extra step following completion of the PCR; a melting curve analysis. Generating a melting curve involves heating the product until the intercalating reagent is released. If one PCR product is present, all reactions will occur around one time point (i.e. one peak will be visible), but if non-specific binding or primer-dimers are present, they will melt at a different time/temperature creating more than one peak (Applied Biosystems, 2001).

TaqMan$^®$ technology is an example of a probe reaction in which primers and a probe are designed together. The probe has a quencher on one end and a fluorescent molecule on the other end. The probe initially binds the specific sequence; the primers then initiate elongation of the second strand, eventually cleaving the probe from the primary strand. The cleavage of the probe causes an increase in fluorescence, due to release from the quencher, that is measured at each step in the PCR cycle (Dharmaraj, 1998).

Real time PCR can be coupled with reverse transcriptase to detect and quantitate mRNA. Real time, reverse transcriptase PCR (RT-qPCR) is an extremely sensitive method that can be used to quantitate changes in gene expression. The sequence detection system first creates cDNA, which is then amplified by PCR, with detection of each amplicon. The resulting graph describing the production of fluorescence (related to DNA concentration) as a function of time forms a sigmoidal curve with the point of reference in the middle of the exponential phase of replication. A threshold (Ct) value is set once the fluorescence crosses this threshold and the cycle number is recorded and used for data analysis. The lower the Ct value, the higher the initial amount of mRNA template present, and the higher the level of expression of the gene of interest (Applied Biosystems, 2001; Dharmaraj, 1998).

There are two methods used to quantitate real time analysis; the standard curve method and the comparative Ct method. The standard curve method is constructed from RNA of known concentrations, which involves the assembly of cDNA plasmids for each gene of interest that are then reverse transcribed into the RNA standards and accurately quantitated. This curve is then used to determine the concentration for mRNA from the unknown sample. This method, while extremely accurate, is very time consuming, especially when there is more than one gene of interest (Applied Biosystems, 2001; Dharmaraj, 1998).

The second method of quantitation is the comparative Ct method. This compares the Ct values of the sample of interest with a control sample, such as a non-treated sample. The Ct values of both the sample of interest and the control must be normalized to a housekeeping gene, which is a gene that is expressed to the same extent in both

samples, and that has an amplification efficiency that is similar to that of the gene of interest. In bacteria, the housekeeping gene most commonly used is 16s RNA. To determine the amplification efficiency, the RNA is diluted to create different concentrations and the concentrations vs. Ct values are plotted for both the gene of interest and the housekeeping gene. The resulting plots should have similar slopes, indicating that the efficiency is similar for the two genes. The fold difference in expression is calculated using the equation 2^{-ddCt}, where ddCt is equal to dCt[sample]-dCt[reference]. In this equation, dCt[sample] is the Ct value for that sample, normalized to the housekeeping gene for that sample, and dCt[reference] is the Ct value for the reference normalized to the housekeeping gene for the reference (Applied Biosystems, 2001; Dharmaraj, 1998).

Microarray analysis

Microarray analysis usually involves genome wide expression analysis on a microscope slide. The array is created by selecting genes of interest and binding them to a microscope slide. There are several techniques for binding DNA to the slide, and several companies now offer commercially prepared slides with complete genomes for certain bacteria. The creation of the slide is crucial to the success of the experiment, and if done in the lab can be very time-consuming, depending on the number of genes of interest. For each gene, a PCR reaction must be performed to generate a large number of sequence copies. The PCR product must then be cleaned and placed in an appropriate buffer and an array printer is then used to transfer the PCR products to the slide (Hedge *et al*, 2000).

A large amount of RNA is required for this analysis; usually a minimum of 10-15 μg of each sample. The RNA is reverse transcribed to DNA with an amino-allyl labeled nucleotide (usually dUTP). This reaction is cleaned to remove any unused reactants and then the cDNA is labeled with a cyanine fluor, typically Cy3 for the control sample and Cy5 for the treatment sample. Following labeling, the reaction product is cleaned again and concentrated (Hedge *et al*, 2000; Stintzi, 2005).

Hybridization of the RNA to the array requires preparation of the slide with prehybridization buffer as well as resuspension of the labeled cDNA in the appropriate buffer for the slide. Once the labeled probe is applied to the array, it is allowed to hybridize for 16 to 20 hours. The slide is then cleaned using several different buffers containing different concentrations of phosphate buffered saline (PBS), sodium dodecyl sulfate (SDS) and sodium chloride-sodium citratein (SSC) to remove any unbound probes that would compromise analysis (Hedge *et al*, 2000).

Analysis of the slide involves scanning of the slide and image processing. There are several software packages that can assist in identifying spots and subtracting out the background for each spot individually, as background can vary throughout the slide. Normalization of the data can be achieved by adding a number of controls in increasing concentrations to both labeling reactions; the sum of these spots should be equal. Another normalization technique involves linear regression analysis of measured intensities of certain genes assumed to be equally expressed in the control and treated samples. There are other normalization procedures that have been incorporated into the software used to analyze the slide. Once the slide has been normalized, the genes that are

differentially expressed can be identified and analyzed for the fold difference in expression (Hedge *et al*, 2000).

Microarray can be used to investigate bacterial gene expression in many different stress conditions, including exposure to antibiotics. It can also be used to compare gene expression *in vivo* versus *in vitro* cultures. However, the large amount of RNA necessary for the assay makes *in vivo* collection difficult and time-consuming. In addition, the process of creating and hybridizing a sensitive array can be technically demanding (Hinton *et al*, 2004). Nevertheless, it is becoming an increasingly popular means of genome expression analysis and will likely become fairly common in bacterial studies.
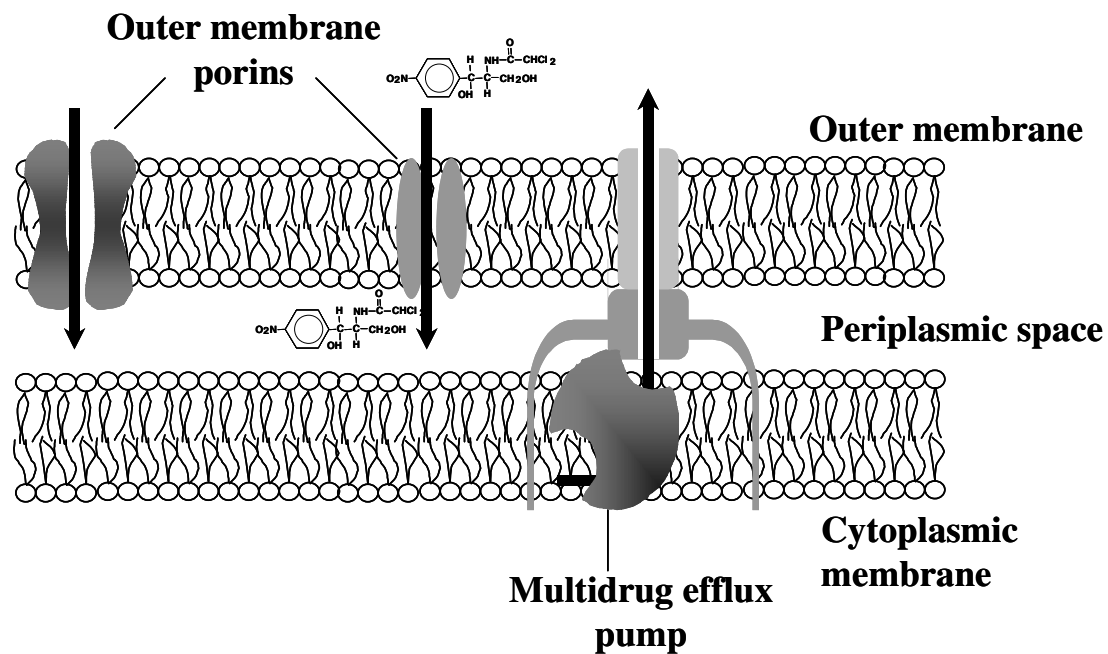
Figure 1. Diagram depicting both efflux pump and porin protein structures. Efflux pumps collect hydrophobic chemicals at the inner part of the cytoplasmic membrane and pump them out through an outer membrane channel. Porin proteins allow passage of hydrophilic molecules into the cell.
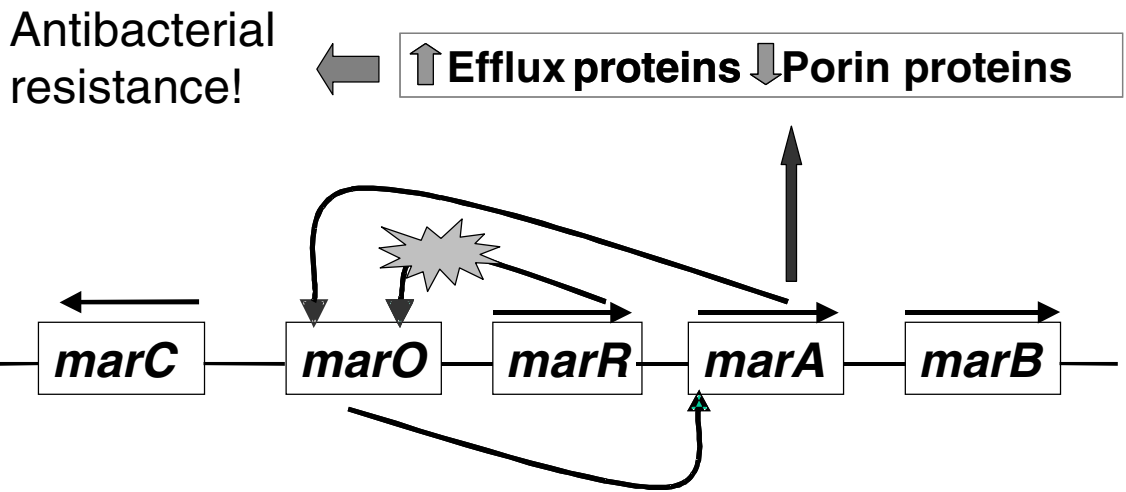
Figure 2. The *mar* regulon. Under normal conditions, MarR protein binds and inhibits *marO* from expression. In the presence of antibiotic, the MarR protein becomes bound and can no longer inhibit *marO*. MarO then initiates the transcription and translation of *marA, marB and marC*. MarA then initiates the transcription and translation of many other proteins including efflux and porin systems.

CHAPTER 3: HYPOTHESIS AND EXPERIMENTAL OBJECTIVES

The goal of this research was to evaluate the contribution of the *mar* regulon to survival of *Salmonella enterica* serovar Typhimurium, in the absence of antibiotics or in the presence of subtherapeutic or therapeutic levels of oxytetracyline. The underlying hypothesis upon which this goal was based is that the prevalence of bacteria that are antibiotic resistant due to the expression of multidrug resistance systems increases when cattle are administered prophylactic antibiotics and that the wide substrate specificity of these systems confers resistance not only to antibiotics used prophylactically but also to antibiotics used therapeutically in cattle and human consumers of contaminated beef products. To achieve this goal, experiments were organized into two phases:

Phase I

Previous studies have reported that expression of the *mar* system in *E. coli* is affected by many different substrates, including tetracycline, but that expression of the *mar* system in *Salmonella* may not be induced by tetracycline (Randall *et al*, 2002). In the first phase, *in vitro* experiments were conducted to determine whether exposure of *Salmonella* Typhimurium to oxytetracycline induced expression of the *mar* regulon. In addition, the genome of *Salmonella* Typhimurium was screened to identify other genes that might play a role in promoting survival of the bacterium in the presence of oxytetracycline. Specific experimental objectives were to:

(1) develop methods for studying the expression of *marA* in the presence and absence of oxytetracycline;

(2) identify and measure any incubation time-dependent changes in expression of *marA* in response to different concentrations of oxytetracycline; and

(3) perform a microarray analysis of the *Salmonella* Typhimurium genome to identify any genes other than *marA* involved in survival of the bacterium in the presence of oxytetracycline.

Phase II

The second phase was designed to investigate the effect of oxytetracycline on *marA* expression in *Salmonella* Typhimurium *in vivo*, using a bovine subcutaneous tissue chamber model. The study was conducted in cattle because contaminated beef is the most common source of human infection. The specific objectives of this phase were to:

(1)   design an effective experimental protocol for infection of tissue chambers to allow infected tissue fluid collection and analysis of the samples using RNA isolation and real-time PCR.

(2)   use the RT-qPCR protocol designed in Phase I to identify any changes in expression of *marA in vivo* in response to parental administration of oxytetracycline at different doses; and

(3)   based on the results of the microarray analysis in Phase I, investigate the roles of additional, selected genes that may facilitate survival of *Salmonella* Typhimurium *in vivo*, using RT-qPCR.

CHAPTER 4: EXPRESSION OF *marA* IN *SALMONELLA ENTERICA* SEROVAR

TYPHIMURIUM EXPOSED TO OXYTETRACYCLINE *IN VITRO*

Introduction

Infections caused by *Salmonella* spp., particularly multi-drug resistant strains, are a recurring challenge to public health (Threlfall, 2002; Zansky *et al*, 2002). Of particular concern is the transmission of resistant *Salmonella* from food animals to human consumers of animal food products (Hsueh *et al*, 2004; Angulo *et al*, 2004; Miko *et al*, 2005). Use of antibiotics in food producing animals is suspected to promote the emergence of multi-drug resistant organisms that can lead to potentially life threatening infections in humans (Angulo *et al*, 2004; Mlot, 2001; Fox, 2001). Recent human outbreaks have been linked to *Salmonella* Newport and Typhimurium DT104 (Hsueh *et al*, 2004; Miko *et al*, 2005).

Salmonella enterica serovar Typhimurium is one of several Gram-negative bacteria that employ the chromosomally located *mar* regulon as a mechanism of multi-drug resistance (Randall *et al*, 2001b and 2002). The *mar* regulon consists of 5 genes: *marR*, the repressor; *marO*, the operator; *marA*, the master regulator; and *marB* and *marC*, the functions of which have not been clearly determined. (Figure 2) Under normal conditions (the absence of antibiotics or chemicals), *marR* represses *marO* by binding to it and prohibiting transcription. In the presence of certain chemicals, *marR* becomes bound to the chemical and cannot bind *marO*. This leads to transcription and translation of *marA, B* and *C*. The MarA protein is responsible for up-regulation of approximately 50 genes and the down-regulation of approximately 15-20 genes. The genes affected

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include the *acrAB* efflux system (up-regulated) and the Omp porin proteins (down-regulated) (Randall *et al*, 2002; Alekshun *et al*, 1997, 1999; Seoane *et al*, 1995b). Both efflux pumps and porins are non-specific and allow transmembrane passage of a wide variety of antibacterial agents belonging to different chemical classes. Coordinated regulation of efflux pumps and porin expression restricts the intracellular accumulation of antibacterial agents, which must be in sufficiently high concentrations at intracellular sites of action to be effective. Thus, increased expression of *marA* creates multiple antibiotic resistant phenotypes (Gambino *et al*, 1993; Nishino *et al*, 2006; Eaves *et al*, 2004; Sulavik *et al*, 1997; Cohen *et al*, 1988).

Previous studies reported that the *marA* gene in *E. coli* is sensitive to tetracyclines, which are commonly used in the therapy and prophylaxis of cattle diseases. Current strategies designed to retard the emergence of resistance include avoiding use of the same antibacterial agents in food animals and humans because development of resistance against one agent is thought not to affect development of resistance against the other. However, if exposure to one antibacterial agent promotes emergence of multi-drug resistance, this assumption may not be correct. Therefore, the objectives of this study were to identify and measure any incubation time-dependent changes in expression of *marA* in response to different concentrations of oxytetracycline, and perform a microarray analysis of the *Salmonella* Typhimurium genome to identify any genes other than *marA* that may be important in allowing the bacterium to survive in the presence of oxytetracycline.

Materials and Methods

Effect of oxytetracycline exposure on expression of *marA* in *Salmonella* Typhimurium

Previous studies conducted by Randall and coworkers (2002) indicated that expression of *marA* in *E. coli* is induced by exposure to oxytetracycline, but the responsiveness of *marA* in *Salmonella* Typhimurium had yet to be confirmed. Therefore, a series of gene expression studies were conducted to investigate the effect of exposure to oxytetracycline on expression of *Salmonella* Typhimurium *marA*: Initially, bacteria were exposed to oxytetracycline concentrations at or below the MIC value, and the effect on gene expression was compared to that produced by a positive control, salicylate. Thereafter, the range of oxytetracycline concentrations was expanded to include concentrations greater than the MIC. Finally, the effect of duration of exposure to oxytetracycline (incubation time) on gene expression was studied.

Effect of oxytetracycline at concentrations ≤ MIC versus salicylate

Taqman® primers and probes (Roche Molecular Systems, Inc., Pleasanton, CA) were selected for the *marA* gene and 16s RNA (internal control) using Primer Express® software (Applied Biosystems Inc., Foster City, CA). *Salmonella enterica* serovar Typhimurium LT2 (ATCC 700720) was streaked on LB Miller (DIFCO, Becton Dickinson Microbiology Systems, Sparks, MD) agar plates with increasing concentrations of oxytetracycline (0, 0.01, 0.05, 0.5, 1, and 2 μg/ml) or salicylate (0, 1,10, 20, 50 and 100 μg/ml). The concentration range selected for oxytetracycline was based on the MIC for the bacterial isolate (2 μg/ml), which was determined using a standard

dilution method in LB Miller broth. Inoculated plates were incubated at 37°C for 20

hours and individual colonies were then selected from the plates and transferred to LB

Miller broth (DIFCO, Becton Dickinson Microbiology Systems, Sparks, MD) containing

corresponding concentrations of oxytetracycline (0, 0.01, 0.05, 0.5, 1, and 2 μg/ml) or

salicylate (0, 1, 10, 20, 50 and 100 μg/ml). Cultures were grown in broth to mid-log

phase and samples were collected for RNA extraction.

Effect of oxytetracycline at concentrations ≥ MIC

Further studies of the effect of oxytetracycline on expression of *marA* investigated

the responsiveness of *Salmonella* Typhimurium to a wider range of oxytetracycline

concentrations, including a concentration higher than the MIC. The experimental

methods employed for culture of bacteria in oxytetracycline were identical to those

described above, except that initial culture on LB Miller plates occurred in the absence of

oxytetracycline. Individual colonies cultured on solid media were selected and

inoculated into LB Miller broth containing 0, 1, 2 and 4 μg/ml of oxytetracycline and

incubated at 37°C. Using a reference standard curve constructed by plotting the

spectrophotometric absorbance of serial dilutions of bacteria in LB Miller broth against

the concentrations of bacteria determined by spot plate counts, the mid-log growth phases

of the broth cultures were identified and samples were collected for extraction of RNA

and determination of oxytetracycline concentrations by bioassay.

Effect of incubation time on oxytetracycline-induced expression of marA

For these experiments, bacterial colonies isolated on LB Miller plates were inoculated into LB Miller broth containing 2 μg/ml oxytetracycline or 100 μg/ml salicylate. Using the absorbance-CFU reference curve, samples were collected at early-, mid- and late-log phases of growth. Samples were subjected to the following analyses: RNA was extracted for gene expression analysis, oxytetracycline was determined by bioassay, and MIC values were determined for a variety of antibacterial agents and compared with those of the inoculation strain.

Assay of oxytetracycline concentration

Samples collected for determination of oxytetracycline were centrifuged and the supernatants were subjected to microbiological assay. Briefly, *Bacillus cereus* (ATCC 11778) was cultured for 4 hours in Nutrient agar. A 12" x 12" glass plate was prepared by soaking in 5% hypochlorite solution for 30 minutes, rinsing in deionized water and air drying. It was then sterilized by wiping with 70% propanol and placed in an oven at 65°C for 15 minutes. The plate was then placed on an adjustable stage to ensure it was level. Approximately 600 ml of Nutrient agar was inoculated with 0.5 ml of the *B. cereus* culture and then poured into the leveled glass plate and allowed to solidify. Wells were suctioned in the agar using a grid pattern. Duplicate aliquots (150 μl) of samples or concentration standards (0, 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 15, 25 μg/ml) were pipetted into wells, the plate was refrigerated at 4°C for 1 hour and then incubated at 37°C overnight. Following incubation, a concentration standard curve was established by plotting

diameters of the zones of inhibition against known concentrations of oxytetracycline.

Concentrations of the samples were determined.

Extraction of RNA and measurement of *marA* expression

RNA extraction was performed using a modified hot phenol method (Naikare *et al*, 2006; Stintzi *et al*, 2003). Briefly, 10 ml of broth sample was mixed immediately after collection with 1 ml ice-cold stop solution consisting of 10% buffered phenol in ethanol. The mixture was centrifuged at 4°C for 15 minutes at 3500g. The supernatant was removed and the pellet was resuspended in 800 μl lysozyme solution (0.5 mg/ml lysozyme in TE 10,1 pH 8) before addition of 80 μl 10% SDS. The solution was placed in a 64°C water bath for 1-2 minutes, 88 μl of 1M sodium acetate was added, and the sample was mixed. An equal volume of water-saturated phenol was then added, and the sample was again placed in the 64°C water bath for 8 minutes. The samples were quickly transferred to ice and then centrifuged for 30 minutes at 19,000g and 4°C. The aqueous layer was then transferred to a tube containing an equal volume of chloroform, mixed and then centrifuged for 15 minutes at 19,000g and 4°C. Again, the aqueous layer was collected and mixed with 1/10 volume 3M sodium acetate, EDTA to 1 mM, and 2.5x volumes 99% cold ethanol. The samples were then allowed to precipitate at -80°C for at least 24 hours. Following precipitation, the samples were centrifuged for 1 hour at 19,000g and 4°C to pellet the RNA. The supernatant was removed and the pellet washed three times with 1 ml 80% ethanol, and then placed in a speed vacuum for 20 minutes to dry. Samples were then resuspended in 100 μl RNase free water, DNase treated and cleaned on a Qiagen RNeasy® column. RNA was then checked for DNA by PCR,

integrity was confirmed by agarose gel electrophoresis and extracted RNA was quantitated using RiboGreen® RNA quantification reagent (Molecular Probes, Eugene, Oregon).

Expression of *marA* was assessed by RT-qPCR using appropriate primers and probes. Eurogentec® one-step RT-qPCR MasterMix Plus kits were used following the supplied protocol. All the RT-qPCR reactions were performed on an ABI Prism 7700 Sequence detector. A comparative analysis was done assuming the 16s gene (normalizing gene) was maximally expressed under all conditions. The ddCt method was used for data analysis to determine the fold differences in levels of expression (Applied Biosystems, 2001). Standard deviations were used to determine statistical significance between fold differences.

Microarray analysis to identify other genes affected by exposure to oxytetracycline.

Increased synthesis of MarA protein can be expected to affect the expression of several other genes, including those involved in synthesis of efflux and porin proteins (Alekshun *et al*, 1997). In order to study those genes regulated by *marA*, as well as any other genes that may be affected by oxytetracycline exposure, expression of the entire genome was assessed by microarray analysis. A specific oligonucleotide-based microarray (Combimatrix Custom Array®) was designed to evaluate expression of approximately 99% (approximately 12,000 genes) of the *Salmonella* Typhimurium genome. At least 40 probes per transcript were employed to interrogate the genes generally recognized to be involved in antibiotic resistance, including the *mar* regulon, and those encoding for multi-drug efflux pumps and outer membrane porins.

Gene expression of mid-log phase bacteria cultured in 2 μg/ml oxytetracycline were compared to bacteria grown in LB Miller broth with no oxytetracycline, using a previously described hybridization protocol (Ducey *et al*, 2005). Briefly, RNA was extracted using the modified hot-phenol method described above. The RNA concentration was determined using Ribogreen® (Molecular Probes) and the absence of DNA was confirmed by PCR. Using equivalent amounts (16 μg) of RNA from each sample, the RNA was reverse transcribed to cDNA in the presence of aminoallyl nucleotides. The RNA was mixed with 8 μl of 5x 1st strand buffer, 2 μl of DTT (0.1M), 10 μg random primer and DEPC water to 34.35 μl, and then incubated sequentially at 65°C for 5 minutes and 42°C for 5 minutes. Thereafter, the nucleotide and reverse transcriptase reagents were mixed (1 μl dGTP, dATP and dCTP at 20 mM, 1.3 μl of dTTP at 5 mM, 1.35 μl of aminoallyl-dUTP at 10 mM and 2 μl of Superscript® II (Invitrogen)) and 5.55μl was added to the RNA mixture and allowed to incubate at 42°C for 2 hours. The reaction was then stopped by base hydrolysis of the RNA using 4 μl of 50 mM EDTA and 2 μl of 10N NaOH and incubated at 65°C for 20 minutes. This reaction was then neutralized with 4 μl of 5M acetic acid. To remove the free amine, the reactants and products were filtered through a Microcon® 30 filter with 450 μl of water. The filtrate was centrifuged at 9800g for 8 minutes, repeated 4 times. The Microcon® 30 filter was then inverted in a new tube and the sample collected by centrifuging at 16,000g for 1 minute. It was then dried down in a speed vacuum to 9 μl.

At this time, the aminoallyl labeled cDNA was coupled to the Cy dye. Each sample was resuspended in 10 μl of 0.1 μl of Na-carbonate. Monofunctional NHS-ester Cy3 and Cy5 solutions were made by resuspending one vial of dye in 65 μl water-free

DMSO. Ten microliters of Cy3 solution was added to the control (no oxytetracycline) and 10 μl of Cy5 solution was added to the treated sample (2 μg/ml oxytetracycline). These were then incubated for 1 hour at room temperature in the dark. Following incubation, 35 μl of 100 mM NaOAc (pH 5.2) was added and uncoupled dye was removed using a Qiagen® PCR purification kit. Buffer PB (500 μl) was added to the mixture and all applied to the column and centrifuged. The column was then washed with 750 μl of PE buffer 4 times. The sample was then eluted with 40 μl of water at pH 7.4.

To prepare the samples for hybridization, the now Cy - labeled probes were dried down in a speed vacuum, resuspended in 23.25 μl of water and mixed together. The following were added individually and mixed by pipetting between each addition: 2.5 μl 10 μg/ml salmon sperm DNA, 25 μl formamide, 25 μl 20x SSC and 1 μl 10% SDS. This solution was then denatured at 99°C for 2 minutes, cooled to 42°C, then hybridized to the slide.

The Combimatrix Custom Array® slides were washed with 100 μl of water at 65°C for 10 minutes. They were then prepared with a prehybridization solution containing 250 μl of 20x SSC, 250 μl formamide, 10 μl of 10% SDS, 10 mg BSA and water to 1 ml and incubated for 30 minutes at 42°C. Once the prehybridization was complete, the solution was removed and the hybridization solution containing the labeled probes was added to the slide and the slide incubated at 42°C overnight (~16 hours). The hybridization solution was then removed and the slide washed with 2x SSC and 0.1% SDS for 5 minutes at 42°C. The next wash was with 0.1x SSC and 0.1% SDS for 10 minutes at room temperature. This was followed by 5 washes with 0.1x SSC for 1

minute each and two final washes with 2x PBS for 5 minutes each. The imaging solution was added following the final wash and the array scanned using a Perkin Elmer Scan Array® microarray scanner. The slides were scanned at 532 nm (Cy3) and 635 nm (Cy5) wavelengths at 5μm resolution and 70, 80 and 90% gain. GenePix Pro 3.0.5 software (Axon Instruments, Foster City, California) was used to evaluate the fluorescence intensity of each spot. Spot registration was optimized manually and exported to OriginPro 7 software (OriginLab Corporation, Northampton, Massachusetts). The spots were then filtered, and those with bad signals excluded. The background was subtracted and the fluorescence intensity in each wavelength $\log_2$ transformed and normalized using MIDAS software (The Institute for Genomic Research; http://www.tigr.org/software/). To analyze the microarray data, the significant analysis of microarray (SAM) algorithm was used. This Microsoft Excel software add in is available at http://www-stat.stanford.edu/~tibs/SAM/.

Results

Exposure to salicylate resulted in a concentration-dependent increase in the level of expression of *marA* (Figure 3). The growth curves from this experiment indicated that increasing concentrations of salicylate did not effect the growth of *Salmonella*, as the mid-log phase was reached at ~4 hours in all concentrations (Figure 4). When exposed to oxytetracycline, concentrations lower than the MIC produced no statistically significant difference in the level of expression of *marA*. However, at the MIC of 2 μg/ml, a large, statistically significant difference in expression was observed (Figure 5).

The next set of experiments were designed to examine more closely the expression of *marA* in relation to a wider range of initial oxytetracycline concentrations and to correlate these results with any changes in drug concentration occurring as a function of incubation time. Growth curves of *Salmonella* cultured in 0, 1, 2, and 4 μg/ml of oxytetracycline in LB broth indicated significant concentration – dependent slowing in growth (Figure 6). However, even though growth rate was decreased at the 2 and 4 μg/ml concentrations, the bacteria still remained viable and entered an exponential phase of growth after a prolonged initial stationary phase. The 4 μg/ml samples (twice the MIC of 2 μg/ml) reached mid-log phase at around 52 hours. The corresponding bioassay results indicated that oxytetracycline had decreased (probably as a result of degradation) to a concentration of ~1.6 μg/ml at this time (Figure 7). The 2 μg/ml samples reached mid-log phase at about 40 hours with a corresponding oxytetracycline concentration of 0.6 μg/ml. Interestingly, the highest level of *marA* expression occurred at 2 μg/ml oxytetracycline, and not at the higher 4 μg/ml concentration, even though the latter had decreased to a level below the MIC by the time the mid-log sample was collected (Figure 8).

Experiments conducted to assess the expression of *marA* as a function of incubation time confirmed that growth of the bacterium is closely correlated with oxytetracycline concentration and that the microorganism does not enter an exponential growth phase before the concentration of drug degrades to a level below MIC (Figure 9). Analysis of *marA* expression by RT-qPCR indicated that the level of expression was highest at mid-log phase (~36 hours) when *Salmonella* Typhimurium was incubated in an initial oxytetracycline concentration of 2 μg/ml oxytetracycline versus the relative lack of

gene expression in mid-log phase (~4 hours) bacteria incubated without oxytetracycline (Figure 10). The increased expression of *marA* in samples exposed to oxytetracycline was not only correlated with increased resistance to oxytetracycline, as confirmed by the higher MIC (8 μg/ml) of the bacterium after incubation versus the pre-incubation inoculum (2 μg/ml), but the MIC values for several other chemically unrelated antibacterial agents (chloramphenicol, ampicillin and gentamicin) were also higher in the incubated samples (Table 1).

Incubation conditions that produced the highest level of *marA* expression were selected for microarray analysis: Bacteria were cultured in 2 μg/ml oxytetracycline and samples were collected at mid-log phase of growth and RNA was extracted. The RNA isolated at that time point was compared with RNA isolated from samples grown in LB broth with no oxytetracycline. The goal of the microarray was to identify genes affected by up-regulation of the MarA protein as well as unrelated genes affected by oxytetracycline exposure. In contrast to the real-time PCR experiments, microarray analysis indicated no significant change in the *mar* regulon or the efflux genes and porin genes usually associated with *mar* regulon function. However, approximately 72 other genes were up-regulated, but only a few of them are considered to be antibacterial resistance genes (Table 2). Many of the genes that were up-regulated encode for ribosomal subunit proteins. Approximately 125 genes were down-regulated. While many of these genes encode for putative outer and inner membrane proteins, none of these currently are recognized as porin proteins (Table 3). Many of the proteins that were down regulated are involved in flagella production and assembly.

Discussion

Many chemicals have been found to induce the *mar* system in *Salmonella*, including salicylate (primary inducer), chloramphenicol, dinitrophenol, paraquat, benzoate and bile acids (Randall *et al*, 2001b, 2002; Prouty *et al*, 2004). It has been hypothesized that the *mar* system evolved to aid in the survival of enteric bacteria in the hostile environment of the gastrointestinal tract, because expression of the system confers resistance to bile acids (Prouty *et al*, 2004). While tetracycline has been found to induce the *mar* system in *E. coli*, it has not been implicated in the induction of the system in *Salmonella* (Randall *et al*, 2002). The present research demonstrated that the *mar* regulon is in fact induced in *Salmonella* Typhimurium exposed to oxytetracycline, but not to the same extent as in response to salicylate.

Concurrent induction of *marA* expression, and increase in the MIC value for oxytetracycline, provides strong circumstantial evidence that *marA* expression is related to antibacterial resistance. Indeed, concurrent increases in the MIC values for several other chemically unrelated antibacterial agents is consistent with the induction of multi-drug resistance mechanisms mediated by efflux pumps and porin proteins. In the cases of chloramphenicol and gentamicin, the two-fold increases in MIC values were relatively modest and were consistent with the functions of efflux pumps and porin proteins. However, the MIC of ampicillin increased eight-fold, suggesting the involvement of additional resistance mechanisms. It is probable that expression of the *mar* system may promote acquisition of additional resistance capabilities (through gene mutations or

transfer of resistance genes) by allowing higher numbers of bacteria to survive and replicate (Randall *et al*, 2002, Chu *et al*, 2005). As demonstrated in the present study, this is most likely to occur at concentrations similar to the MIC value, which would result from dosage regimens that are not high enough to ensure elimination of the bacteria.

The observation that bacteria inoculated into broth containing oxytetracycline at the MIC level not only express *marA* but also grow exponentially after a prolonged stationary phase has very important implications for *in vitro* sensitivity testing. Considering that such testing utilizes an 18-hour incubation period, which is of shorter duration than the stationary phase observed in the present study, a sensitivity determination based on the absence of growth at the MIC level may overestimate susceptibility to an antibacterial agent. Indeed, the results of the present study suggest that the MIC value may vary, depending on the duration of exposure to the antibacterial agent and the expression status of resistance genes that may be induced by exposure to the agent.

As stated before, approximately 125 genes were down regulated in the presence of oxytetracycline, and approximately 72 genes were up regulated. Genes that were noticeably absent from these lists include the *mar* genes, *marR, marO, marA, marB* and *marC*. Also missing were the genes that encode for the *acrAB* efflux system and the Omp porin proteins that are believed to be regulated by the *mar* regulon (Alekshun *et al*, 1999; Cohen *et al*, 1988). There are several possible explanations for this finding: The sensitivity of the RT-qPCR system is much higher than that of the microarray system, which may be one reason the changes seen with PCR are not seen with the microarray. Another possibility is that the RNA was not extracted in large enough quantity or good

enough quality to achieve the sensitivity needed to observe those changes. The genes that were up regulated include several ribosomal subunit proteins, which may be related to resistance to oxytetracycline. Oxytetracycline exerts its antibacterial effect by inhibiting the binding of aminoacyl tRNA to the A site on the 30S ribosomal subunit, thus inhibiting bacterial protein synthesis. Other proteins of interest include several outer membrane proteins of unknown function that were up regulated: The putative S-adenosylmethionine-dependant methyltansferase (STM3109) is involved in macrolide resistance. Putative outer membrane protein (STM1819) is actually a starvation inducible outer membrane lipoprotein that has been noted in previous experiments to be down-regulated in the presence of antibiotic and possibly in response to *mar*, but was up-regulated in the present experiment (Price *et al*, 2000). The major cold shock protein (STM3649) up regulated here has been found to be involved in the stress response in certain bacterial species (Katzif *et al*, 2003; Mangalappalli-Illathu *et al*, 2006). The magnesium transport protein, MgtC (STM3764), is required for intramacrophage replication as it allows for increased uptake of magnesium (Rang *et al*, 2007). Tetracyclines bind magnesium to gain entry into the cell and for binding to the ribosomal subunit, which may lead to low magnesium availability for bacterial cellular needs. Thus, up-regulation of this gene would promote uptake of magnesium for cellular processes and facilitate bacterial survival (Chopra *et al*, 2001). A few other proteins of interest include the putative SAM-dependent methyltrasfease (STM4500) that is involved in tellurite resistance and the starvation inducible inner membrane protein, PsiE (STM4226), both of which were up regulated in the microarray.

A number of the down-regulated genes are also of particular interest: Several are putative outer membrane proteins and transport proteins of unknown function. The majority of the down-regulated genes are involved in flagella production and assembly. The decrease in production of the flagellar proteins has previously been reported as a response to antimicrobials (Bader *et al*, 2003). Perhaps the flagellar proteins are down regulated as a means to conserve amino acids in a stress environment, or perhaps they are a liability because they facilitate host recognition of the bacterial cell.

The interest in oxytetracycline stems from the fear that the prophylactic and metaphylactic use of antimicrobials in food animals may result in resistant infections in humans (Angulo *et al*, 2004; Mlot, 2001; Fox, 2001). Oxytetracycline is an approved and commonly used antibacterial in food animals, both prophylactically and metaphylactically. Label indications include bacterial pneumonia (shipping fever), pink eye, as well as any other susceptible infection (Liquimycin®LA200, Pfizer). The implications of oxytetracycline causing *mar* induction are significant because it raises the possibility that prophylactic use of oxytetracycline in food animals may result in resistance not only to oxytetracycline but also to other antibacterial agents that are used commonly to treat human infections, such as fluoroquinolones. While this research does not indicate that oxytetracycline – induced *mar* expression led to increased resistance to fluoroquinolones, based on the MIC data and the microarray data, resistance to several other important antibacterial agents was induced and there is still need for concern that drugs labeled for use in food animals may lead to resistant infections in humans.
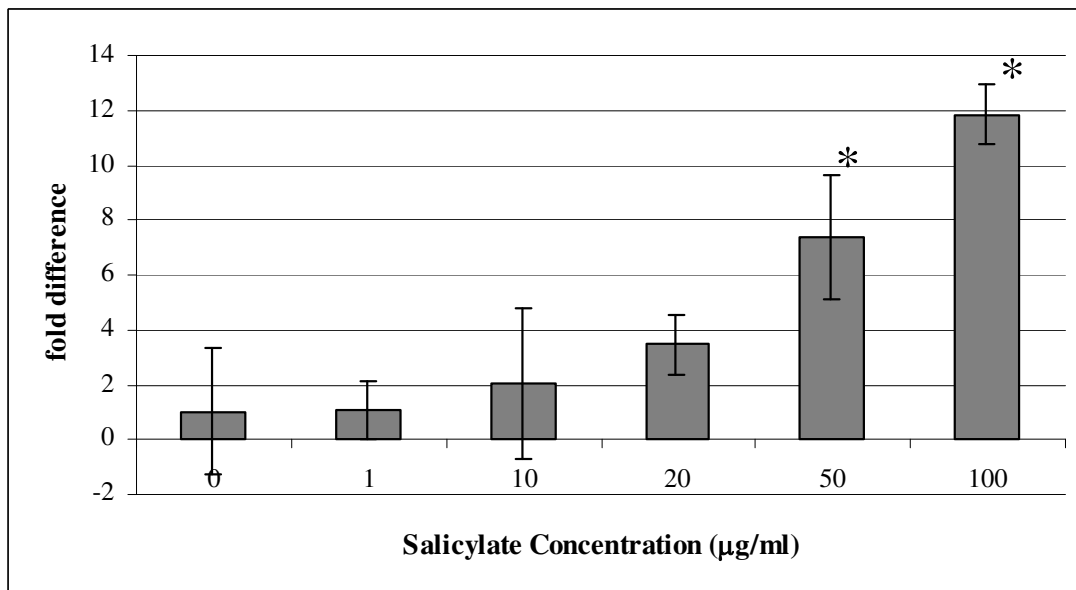
Figure 3. RT-qPCR results from initial salicylate experiments done in duplicate. Each
column represents the mean fold difference plus and minus the standard
deviation for each sample. This graph indicates an increase in *marA*
expression with exposure to increasing concentrations of salicylate.
Statistically significant changes are seen at the higher concentrations of 50
and 100 μg/ml (*).
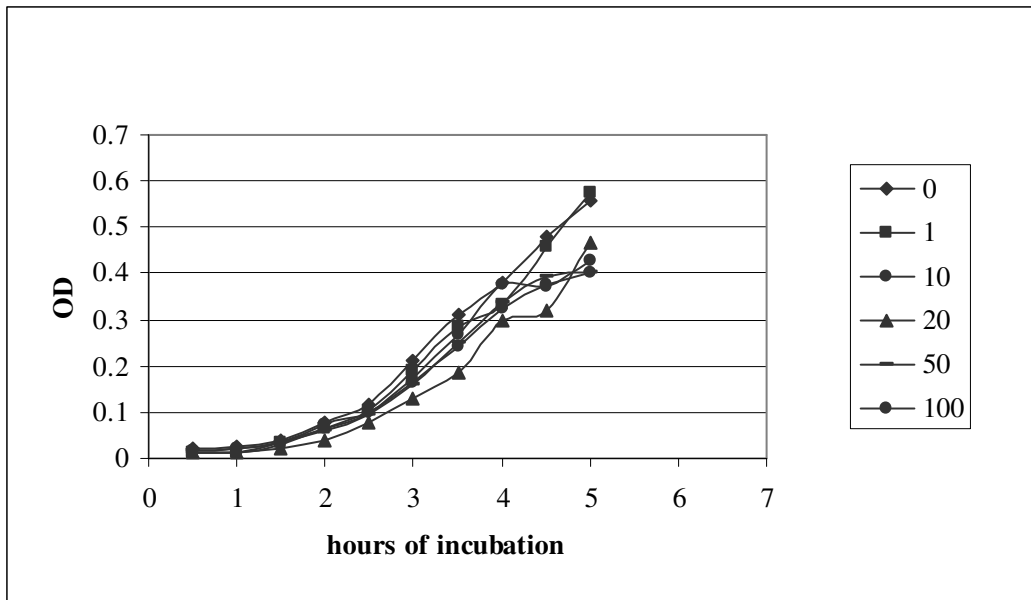
Figure 4. This growth curve indicates the change in OD600 of *Salmonella* Typhimurium over time in increasing concentrations of salicylate (μg/ml). These results represent one of two growth curves done in the presence of salicylate. The increasing concentrations of salicylate do not affect the growth rate of *Salmonella.*
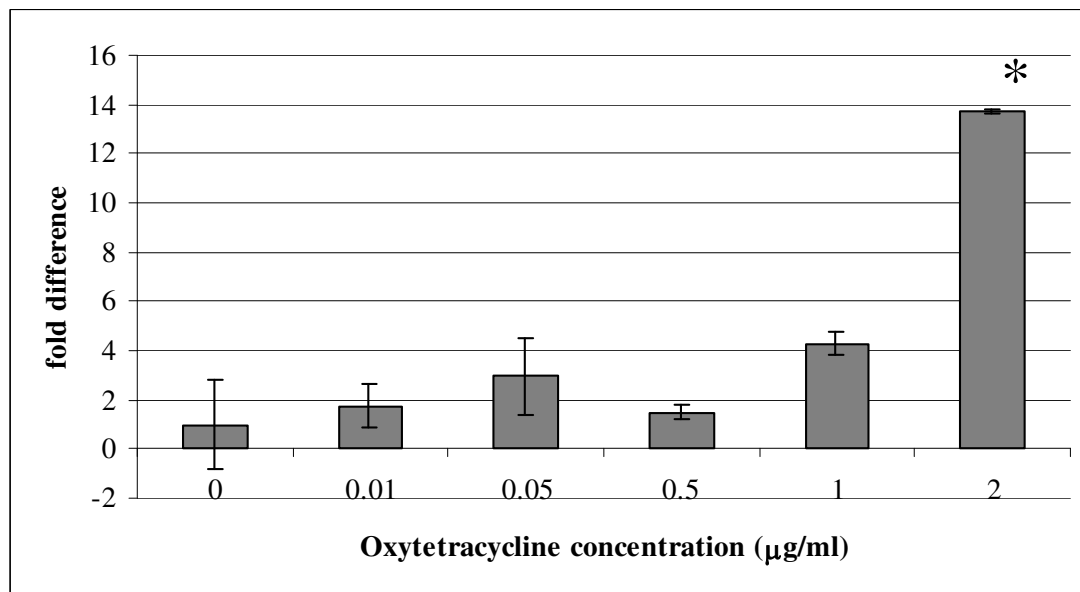
Figure 5. RT-qPCR results from oxytetracycline experiments in *Salmonella* Typhimurium done in triplicate. This graph indicates a statistically significant increase in expression of *marA* at 2 μg/ml (*). Columns represent the mean fold difference plus and minus the standard deviation.
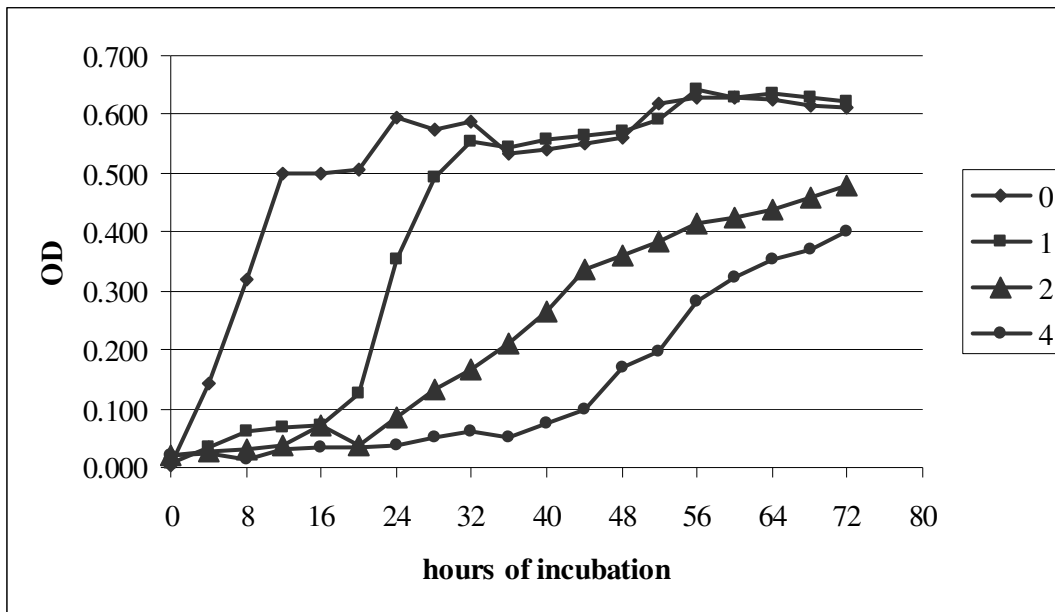
Figure 6. Graph of the growth curve (OD 600) of *Salmonella* in increasing
    concentrations of oxytetracycline (0, 1, 2, and 4 µg/ml). This represents one of
    three replicates. In all replicates, the 4 µg/ml was significantly slower in
    growth, but did grow given enough time.
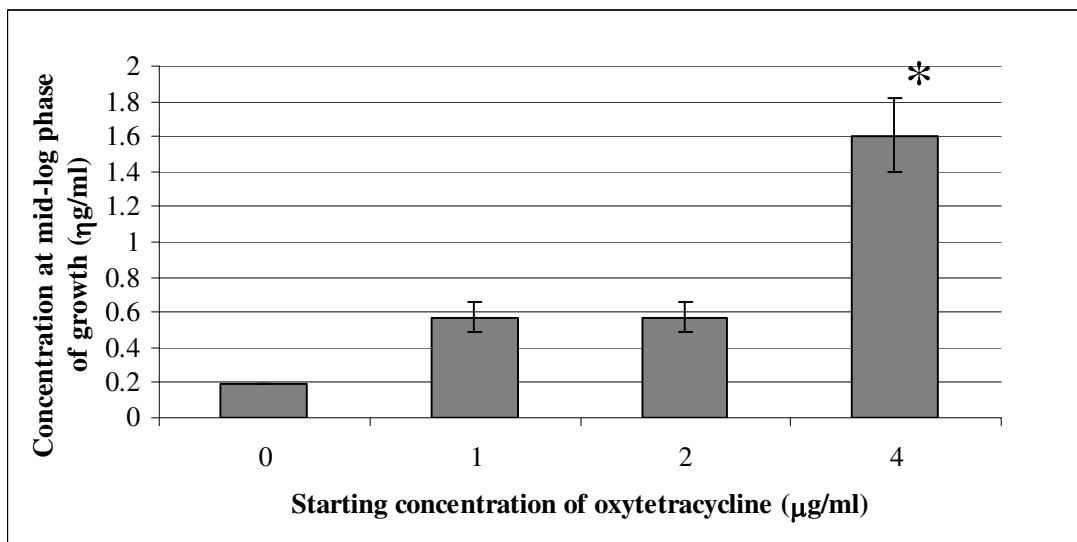
Figure 7. Graph showing the initial concentration of oxytetracycline versus the concentration at mid-log phase of growth of *Salmonella* as determined by bioassay. Each column represents the mean plus and minus standard deviation of the concentration of oxytetracycline (µg/ml) at mid-log phase of growth done in triplicate. Those samples started at 1 and 2 µg/ml reached mid-log phase at just above 0.5 µg/ml. The sample started at 4 µg/ml reached mid-log phase at around 1.5 µg/ml, a statistically significant difference(*).
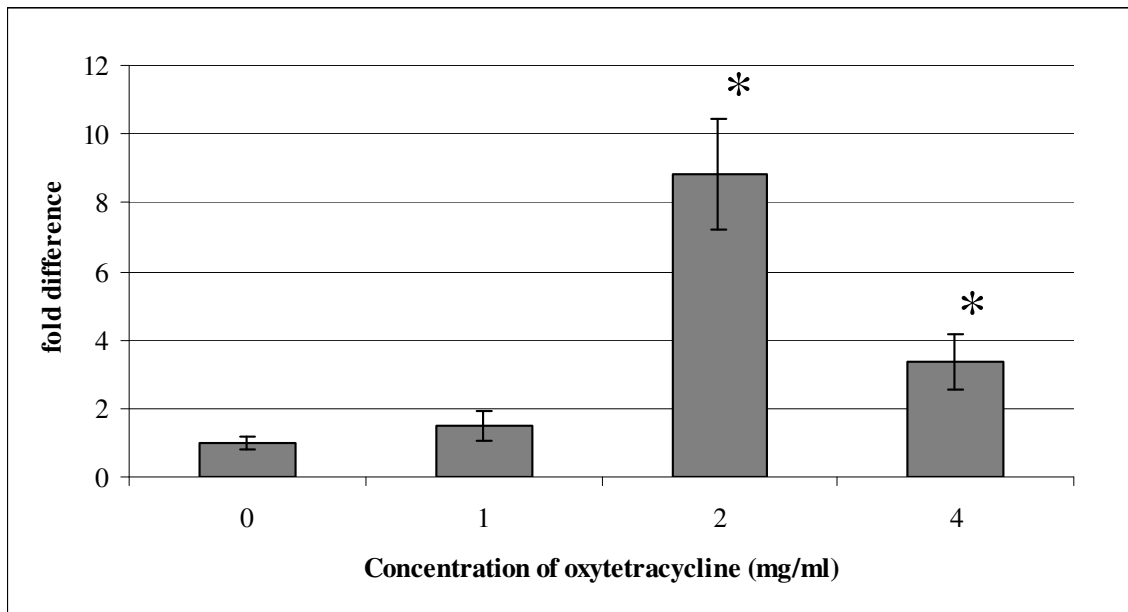
Figure 8. Graph depicting the RT-qPCR results of samples taken at mid-log phase in 0,
1, 2, and 4 μg/ml oxytetracycline. Each column represents the mean plus and
minus the standard deviation of the fold difference for *marA* expression for
that sample. Three biological replicates were done and two RT-qPCR
replicates for each biological sample were analyzed. This graph is a
compilation of all replicates, biological and RT-qPCR. A statistically
significant difference in expression can be seen at 2 and 4 μg/ml, but 2μg/ml
has a much higher fold difference in expression than 4μg/ml (*).
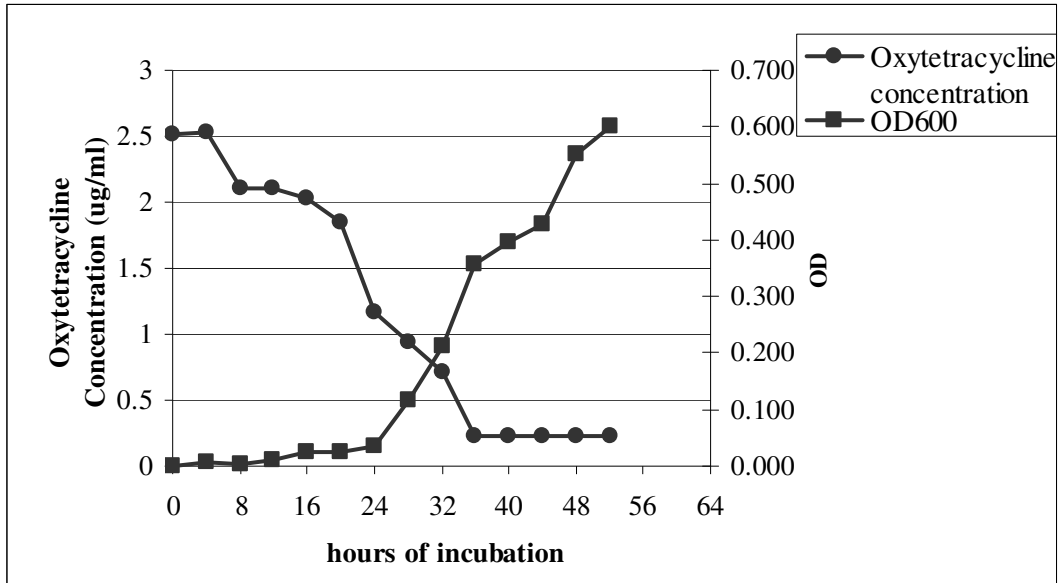
Figure 9. Graph of oxytetracycline concentration and OD600 of *Salmonella* versus time
indicating that growth of *Salmonella* does not actually start until the
concentration of oxytetracycline falls below 1 μg/ml.
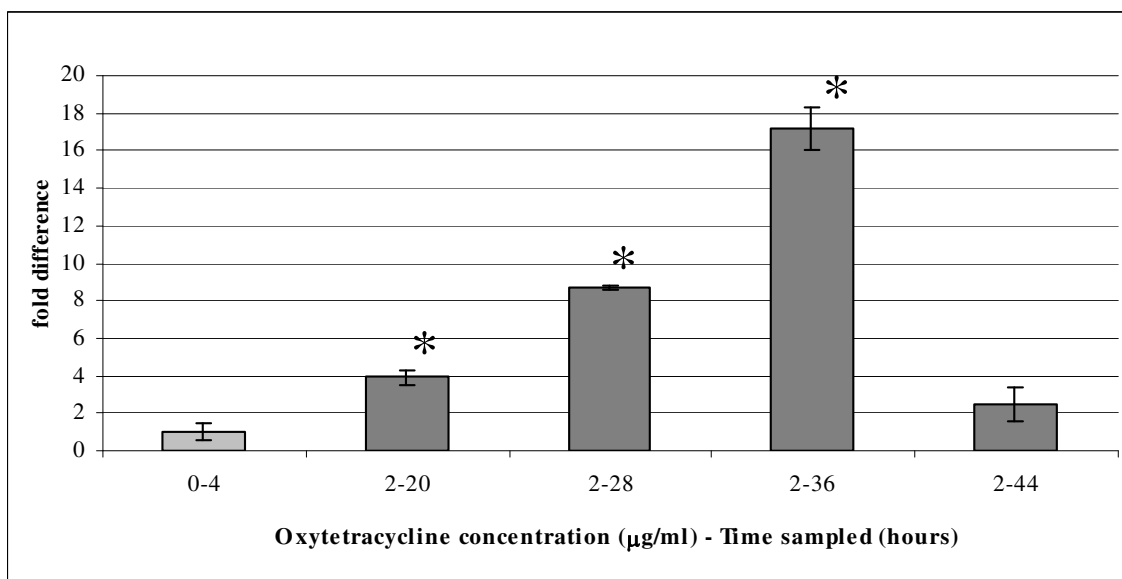
Figure 10. Graph of the level of expression of *marA* in 2 μg/ml oxytetracycline at early, mid and late log phase of the growth curve versus expression in LB without oxytetracycline. Columns are mean difference plus and minus the standard deviation for that sample. This data is from one of three biological experiments with two RT-qPCR analyses done for each biological replicate. Statistically significant differences can be seen at 20, 28 and 36 hours with the highest fold difference at 36 hours, mid-log phase (*).

Table 1. MIC values for *Salmonella* Typhimurium wild type versus oxytetracycline induced. This data represents one of three replicates and all had similar results. The most significant changes are seen in oxytetracycline, chloramphenicol, ampicillin and gentamicin.

Antibiotic	Wild Type	Mar-Induced
Oxytetracycline	2	8
Erythromycin	>16	>16
Ciprofloxacin	<0.125	<0.125
Chloramphenicol	4	8
Ampicillin	2	16
Gentamicin	4	8

Table 2. Genes up-regulated in the presence of oxytetracycline as determined by microarray analysis. Those in bold are involved in antibiotic resistance.

UP

ribosomal subunit protein S20
30S ribosomal subunit protein S2
protein chain elongation factor EF-Ts
putative RHS-like protein
protohaeme IX farnesyltransferase
cytochrome o ubiquinol oxidase subunit I
Sec-independent protein secretion pathway component
7,8-diaminopelargonic acid synthetase
protein chain initiation factor IF-1
putative inner membrane protein
50S ribosomal subunit protein L32
putative outer membrane protein
putative outer membrane lipoprotein
50S ribosomal subunit protein L20
putative peptide transport protein
putative outer membrane protein
50S ribosomal subunit protein L25
NADH dehydrogenase transcriptional repressor
50S ribosomal subunit protein L19
tRNA (guanine-7-)-methyltransferase
16S rRNA processing protein
30S ribosomal subunit protein S16
putative cytoplasmic protein
putative S-adenosylmethionine-dependent methyltransferase
putative cytoplasmic protein
50S ribosomal subunit protein L27
30S ribosomal subunit protein S9
possible dehydrogenase
factor-for-inversion stimulation protein
50S ribosomal subunit protein L17
30S ribosomal subunit protein S11
30S ribosomal subunit protein S13
preprotein translocase
50S ribosomal subunit protein L15
30S ribosomal subunit protein S5

50S ribosomal subunit protein L18
50S ribosomal subunit protein L6
50S ribosomal subunit protein L5
30S ribosomal subunit protein S17
50S ribosomal subunit protein L29
30S ribosomal subunit protein S3
50S ribosomal subunit protein L22
30S ribosomal subunit protein S19
50S ribosomal subunit protein L2
50S ribosomal subunit protein L23
50S ribosomal subunit protein L4
50S ribosomal subunit protein L3
30S ribosomal subunit protein S10
30S ribosomal subunit protein S7
30S ribosomal subunit protein S12
dehydroquinate synthase
putative ribonucleoprotein related-protein
major cold shock protein
50S ribosomal subunit protein L33
Mg2+ transport protein
acetolactate synthase I large subunit
small heat shock protein
50S ribosomal subunit protein L34
50S ribosomal subunit protein L31
preprotein translocase
50 S ribosomal subunit protein L11
50S ribosomal subunit protein L1
putative inner membrane protein
30S ribosomal subunit protein S6
primosomal replication protein N
30S ribosomal subunit protein S18
50S ribosomal subunit protein L9
putative SAM-dependent methyltransferase
putative cytoplasmic protein

Table 3. Genes down-regulated in the presence of oxytetracycline as determined by microarray analysis. Those in bold are involved in antibiotic resistance.

DOWN

putative cytoplasmic protein
putrescine/ornithine antiporter
ornithine decarboxylase isozyme
stress response DNA-binding protein
putative minor tail protein
anaerobic dimethyl sulfoxide reductase subunit A
anaerobic dimethyl sulfoxide reductase subunit B
pyruvate formate lyase I
putative FlgK/FlgL export chaperone
anti-FliA factor
flagella basal body P-ring formation protein precursor
flagellar basal-body rod protein
cell-proximal portion of basal-body rod
basal-body rod modification protein
flagellar hook protein
cell-proximal portion of basal-body rod
flagellar basal-body rod protein
flagellar L-ring protein precursor
putative flagellar basal body protein
flagellar biosynthetic protein
flagellar hook-associated protein 1
flagellar hook-associated protein 3
MltA-interacting protein A
putative periplasmic protein
putative outer membrane protein
transcriptional regulator
putative periplasmic transport protein
methyl-accepting chemotaxis protein III
mannose-specific enzyme IIAB
mannose-specific enzyme IIC
mannose-specific enzyme IID
hypothetical protein
chemotactic response protein
chemotaxis regulator
methyl esterase
methyl accepting chemotaxis protein II
chemotaxis docking protein
chemotaxis sensory histidine protein kinase
chemotaxis protein
flagellar motor proton conductor component
putative periplasmic binding transport protein
putative FliA-regulator
sigma 28
lysine-N-methylase
flagellin
flagellar hook-associated protein 2
flagellar protein
possible FliD export chaperone
putative flagellar hook-basal body protein
flagellar motor switch protein
flagellar assembly protein
flagellar protein
flagellar hook-length control protein
flagellar biosynthetic protein
flagellar motor switch protein
flagellar biosynthetic protein
polyhedral body protein
periplasmic glycerophosphodiester phosphodiesterase
putative chemotaxis signal transduction protein
lysine/cadaverine transport protein

lysine decarboxylase 1
secreted effector protein
transcriptional regulator
invasion regulatory protein
putative cytoplasmic protein
needle complex export protein
needle complex assembly protein
needle complex inner membrane lipoprotein
needle complex minor subunit
needle complex major subunit
needle complex inner membrane protein
invasion protein regulatory protein
invasion protein transcriptional activator
invasion protein precursor
protein tyrosine phosphatase/GTPase activating protein
secretion chaparone
putative acyl carrier protein
secreted effector protein
translocation machinery component
translocation machinery component
translocation machinery component
secretion chaperone
needle complex export protein
needle complex export protein
needle complex export protein
needle length control protein
needle complex assembly protein
needle complex secretion ATPase
secretion chaperone
needle complex export protein
invasion protein
outer membrane secretin precursor
invasion regulatory protein
needle complex outer membrane lipoprotein precursor
glycine cleavage complex protein H
fructose-bisphosphate aldolase
periplasmic L-asparaginase II
putative methyl-accepting chemotaxis protein
putative cytoplasmic protein
putative methyl-accepting chemotaxis protein
aerotaxis sensor receptor
L-serine deaminase
pyruvate formate-lyase 4/2-ketobutyrate formate-lyase
propionate kinase/acetate kinase II
L-threonine/L-serine permease
threonine dehydratase
phosphoenolpyruvate carboxykinase
methyl-accepting transmembrane citrate/phenol chemoreceptor
hypothetical protein
ketodeoxygluconokinase
putative cytoplasmic protein
putative fructose-1,6-bisphosphate aldolase"
triosephosphate isomerase
putative methyl-accepting chemotaxis protein
putative ABC exporter outer membrane component
putative inner membrane protein
putative cytoplasmic protein
putative regulatory protein
putative DNA-binding protein
methyl-accepting chemotaxis protein I

CHAPTER 5: THE *IN VIVO* EFFECT OF OXYTETRACYCLINE ON EXPRESSION
OF THE *SALMONELLA* TYPHIMURIUM GENES, *marA*, *cspA*, *mgtC*, AND *slp*, IN
CATTLE.

Introduction

Multi-drug resistant bacteria, including non-typhoidal salmonelloses, are
responsible for increased morbidity and mortality of infections in humans and animals
(Threlfall, 2002; Molbak, 2005; Furuya *et al*, 2006). Of particular concern are the use of
antibacterial agents in food animals and the possibility that such use may cause the
emergence of resistant food-borne infections in humans (Angulo *et al*, 2004; Mlot, 2001;
Fox, 2001; Gomez *et al*, 1997). The current, FDA-sanctioned, approach to minimizing
this risk is to restrict the use of antimicrobial agents in food animals to those agents not
commonly used in humans, thus preserving the susceptibility of human pathogens to
other antibacterial agents used exclusively in humans (Molbak, 2005; Furuya *et al*, 2006).
However, the recent discovery of multidrug resistant mechanisms, such as those encoded
by the *mar* regulon, has raised concerns that this approach may not be prudent. As
demonstrated by the experiments described in Chapter 4, the *mar* system responds to the
presence of certain antimicrobial agents and chemicals by up-regulating synthesis of
MarA protein, which in turn up-regulates genes encoding for efflux systems and down-
regulates genes encoding for porin proteins, thus creating multi-drug resistance in
response to exposure to a single chemical (Alekshun *et al*, 1999a, 1997, Randall *et al*,
2002). Therefore, it is possible that exposure to antibacterial agents routinely used in
food animals, and not in humans, could lead to multi-drug resistance against a variety of

chemically unrelated antibacterial agents, including those used only in humans. For example, it may be possible, considering the involvement of the *mar* system, to cause resistance to fluoroquinolones (restricted use in food animals) by exposure to oxytetracycline (commonly used in food animals).

Quinolones represent a mechanistic group of antibacterial agents considered to be one of only a few available drug classes appropriate for use against resistant infections, including human salmonellosis. However, several recent outbreaks of salmonelloses in humans have led to the discovery that while ciprofloxacin and nalidixic acid are recognized to be drugs of choice for treating resistant *Salmonella* infections, they are no longer effective in all cases, due to resistance. Fluoroquinolones are not available for widespread prophylactic use in food animals in the United States, and yet resistant, food-borne outbreaks have still occurred (Molbak, 2005; Hsueh *et al*, 2004; Zansky *et al*, 2002; Miko *et al*, 2005), thus suggesting that fluoroquinolone resistance in humans may have been caused by use of different antibacterial agents in food animals and emergence of multi-drug resistance.

The experiments described in Chapter 4 clearly demonstrated that exposure of *Salmonella* Typhimurium to oxytetracycline induced the expression of the *marA* gene and concurrently caused the development of multi-drug resistance to oxytetracycline and several other chemically unrelated antibacterial agents. However, the role of the *mar* regulon has not been studied *in vivo* and it is possible that exposure to oxytetracycline may also affect the expression of *marA*-independent mechanisms that confer antibacterial resistance. Therefore the goal of this study was to investigate the *in vivo* effect of

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oxytetracycline, at low and high doses, on *marA* expression as well as the expression of any other genes that may be involved in oxytetracycline-induced multi-drug resistance.

Selection of genes, other than *marA*, that may be involved in oxytetracycline-induced multi-drug resistance was guided by the results of the *in vitro* microarray experiment described in Chapter 4. This experiment compared *Salmonella* Typhimurium cultured in the absence of antibacterial agents with bacteria grown in broth with 2 ug/ml oxytetracycline. The genes selected, *cspA*, *mgtC*, and *slp*, encode for a cold shock protein, a magnesium transport protein and a starvation-induced outer membrane protein, respectively.

The cold shock protein, CspA, has been found to increase resistance in several bacteria, including *Staphylococcus aureus* and *Salmonella enterica* serovar Enteritidis (Katzif *et al*, 2003; Mangalappalli-Illathu *et al*, 2006). The magnesium transport protein, MgtC, is up regulated to support intracellular growth of bacteria, and in conditions of low magnesium concentration (Rang *et al*, 2007). Oxytetracycline chelates magnesium to gain entry into the bacterial cell as well as to bind to the ribosomal subunit. Magnesium bound to oxytetracycline would not be available to the bacterium, unless *mgtC* is up-regulated (Chopra *et al*, 2001). The starvation-induced outer membrane protein, Slp, has been found to down-regulate in the presence of antibiotic and possibly in response to *mar* expression (Price *et al*, 2000). However, our experiments demonstrated an increase in the expression of this protein in response to oxytetracycline *in vitro*.

The *in vivo* model selected for this study was a bovine soft-tissue infection model, established by inoculation of tissue chambers implanted subcutaneously in cattle (Clarke *et al*, 1989, 1996). These chambers form an abscess like environment, where immune

cells and drugs can enter and exit, but the infectious agent, in this case *Salmonella*, is primarily confined to the chamber. Implantation of several chambers in each animal allowed collection of multiple samples over a period of several days, thus minimizing the number of animals needed. Furthermore, *Salmonella* bacteria could be harvested without being contaminated with other enteric bacterial organisms.

Materials and Methods

Animals

Nine cross-bred calves were obtained from local sources. Shortly after arrival, calves were confirmed to be healthy by physical examination and housed in outdoor pens. They were fed free choice prairie grass hay and a commercial grain ration containing 14% protein. Health was monitored daily by assessing rumen fill, attitude, respiratory function, and rectal temperature. The study protocol was approved by the Oklahoma State University Institutional Animal Care Use Committee and the Biosafety Committee. At the conclusion of each experiment, cattle were euthanized by pentobarbital overdose, after initial sedation with IM xylazine. Chambers were removed and incinerated, and cattle were disposed of according to approved biosafety protocols.

Implantation of tissue chambers

Tissue chambers were constructed of Delrin®, a non-reactive thermoplastic (Delrin®, EI du Pont de Nemours & Co, Wilmington, DE), and the top of each chamber was covered with a medical-grade, silicon-dacron elastomer (Technical Products,

Decatur, GA) to allow percutaneous collection of samples. Chambers measured 4.6 cm

internal diameter, 5.2 cm outer diameter, and 1.5 cm in depth. Chambers were

assembled, autoclaved and surgically implanted, as previously described (Clarke *et al*,

1989, 1996). Each calf had 2 tissue chambers implanted subcutaneously in each

paralumbar fossa for a total of 4 chambers per calf. Implanted chambers were allowed to

heal for several months and then checked for sterility by culturing an aspirate of chamber

fluid aerobically and anaerobically; all chambers determined to be infected were removed

prior to inoculation.

Preparation of inocula

Chambers were inoculated with an ATCC strain of *Salmonella enterica* serovar

Typhimurium LT2 (ATCC 700720). The minimum inhibitory concentration for

oxytetracycline against this isolate was determined to be 2.0 µg/ml. *Salmonella*

Typhimurium isolates were initially streaked for purity and then cultured for 5 hours

(mid-log phase) in LB Miller broth. Thereafter, the culture was centrifuged at 9800g for

15 minutes to pellet the cells, the supernatant was removed and the cells were

resuspended in phosphate buffered saline (PBS) to the concentration of bacteria required

for each experiment.

Experimental design

Cattle were moved to an indoor BSL-2 facility 2 days prior to inoculation to allow

time acclimatization. Chambers were sampled prior to inoculation to confirm sterility.

Chambers were then inoculated with *Salmonella* Typhimurium and tissue chamber fluid

was collected periodically, according to the protocol for each experiment. Samples were analyzed for determination of bacterial concentration (CFU/ml) and oxytetracycline activity (in treated cattle), and RNA was extracted for RT-qPCR analysis of gene expression.

The first experiment involved two calves (#52 and #33). The *Salmonella* Typhimurium inoculum was diluted in PBS to a concentration of 6.25 x 10^6 CFU/ml and 1 ml was injected into each of the chambers. Chambers were sampled immediately following inoculation, and then at 5 and 24 hours post inoculation. Starting 24 hours after inoculation, one calf (#52) was administered 2 IV doses (20 mg/kg) of oxytetracycline (LA200®, Pfizer), 24 hours apart. The other calf (#33) received one IM dose of oxytetracycline (10 mg/kg) 24 hours after chamber inoculation. Samples were collected every 12 hours for 10 days and analyzed for bacterial and oxytetracycline concentration. Samples for RNA extraction were collected immediately prior to oxytetracycline treatment and at 2, 5 and 10 days post-treatment.

In the second experiment, all chambers in 2 calves (#34 and #67) were inoculated with a higher concentration of bacteria, 2.75 x 10^{10} CFU/ml in 1 ml, to provide more mRNA for extraction and analysis. Samples for determination of bacterial concentration were collected at 5 and 24 hours post inoculation. At 24 hours post inoculation, cattle were treated with oxytetracycline: one calf (#67) received 20 mg/kg, IV, twice, 24 hours apart; and the other calf (#34) received one IM dose of 10 mg/kg. Samples were then collected on days 1, 2, 3, 4, 5, 7, 11 and 17 post-treatment and analyzed for bacterial and oxytetracycline concentration. Samples for RNA extraction were collected before administration of oxytetracycline and on days 1, 4, 7, 11 and 17, post treatment.

The third experiment was conducted using five calves (#78, #3, #18, #20, and #11). Each chamber in all calves was inoculated with 1 ml containing ~6 x 10^9 CFU of *Salmonella* Typhimurium. Samples were collected immediately post inoculation and at 1, 2, 3, 4, 5, 6, 7, 8, and 9 days after inoculation for determination of bacterial concentration. Twenty-four hours after inoculation, chambers were sampled for RNA extraction. Cattle were then treated with oxytetracycline: Two cattle (#78 and #3) received one IM injection of 10 mg/kg; two cattle (#18 and #20) received two IV doses of 20 mg/kg, 24 hours apart and one animal (#11) served as a negative control and received no oxytetracycline. Samples collected at 1, 2, 3, 4, 5, 6, 7, and 8 days after the initial treatment day were analyzed for the concentration of oxytetracycline. Samples were also collected at 1, 2, 3, 4, 6, and 8 days after initial treatment for bacterial RNA extraction.

Sample analysis

Samples (0.5 ml) for determination of CFU/ml were immediately placed on ice and analyzed using spot plate counts on LB Miller agar plates, after serial dilution in PBS.

Oxytetracycline concentrations in samples (0.5 ml) were determined by bioassay, using the methods described in Chapter 4.

RNA extraction in preparation for analysis of gene expression proved to be very challenging due to the low numbers of bacteria within the chambers, particularly in animals receiving higher doses of oxytetracycline, and the interference of eukaryotic cells. Several protocols were tested on *in vitro* cultures to determine the most effective method of extraction. Protocols tested included the Qiagen RNeasy® (Valencia, CA) and

Ambion Ribopure® (Austin, TX) kits, a hot phenol protocol designed for intracellular pathogens and a modified hot phenol method used in the *in vitro* experiments described in Chapter 4, respectively. Initially, *Salmonella* was grown *in vitro* to a concentration of ~1x10^8 CFU/ml and diluted to 10^7, 10^6, 10^5, 10^4, and 10^3 CFU/ml. These dilutions were then extracted using all of the protocols listed above. Based on these results, the hot phenol and the modified hot phenol methods were selected for further testing using tissue chamber fluid infected with *Salmonella* Typhimurium. Ultimately, the modified hot phenol method (Naikare *et al*, 2006; Stintzi *et al*, 2003) was determined to yield the best results and was selected for extraction of *in vivo* samples (10 ml) collected from tissue chambers.

Assessment of gene expression

The RT-qPCR primer and probe sets were designed using Primer Express® software. Eurogentec® one-step RT-qPCR MasterMix Plus kits were used, following the supplied protocol. All the RT-qPCR reactions were performed on an ABI Prism 7700 Sequence detector®. A comparative analysis was used, assuming the 16s gene (normalizing gene) was maximally expressed in all conditions. The ddCt method was used for data analysis to determine the fold differences in level of expression (Applied Biosystems, 2001). Standard deviations were used to determine statistical significance between fold differences.

The primary purpose of these experiments was to study the effect oxytetracycline on *marA* expression. Therefore, in the first and second cattle experiments, only *marA* was studied due to the limited amount of RNA collected and extracted from the samples.

Fortunately, the third experiment yielded much larger quantities of RNA due to optimization of the experimental protocol. This success allowed investigation of several other genes of interest, which were selected for further study based on the results of the *in vitro* microarray data described in Chapter 4. These genes encode for the cold shock protein (STM 3649), the magnesium transport protein (STM 3764), and the starvation outer membrane protein (STM 1819).

Results

The first experiment served as a pilot study to optimize methods and procedures. Figure 11 describes the changes in chamber fluid CFU/ml for *Salmonella* Typhimurium in both the IV- and IM-treated cattle. While the concentration of bacteria in the oxytetracycline IV-treated calf decreased dramatically to approximately 100 CFU/ml, the infection was not sterilized and bacterial numbers eventually rebounded to a final concentration very similar to that seen in the IM-treated calf. The bacterial numbers produced by the inoculation employed in this first experiment were insufficient to support expression analysis of more than one gene, thus necessitating a change in protocol in subsequent experiments. Bioassay of oxytetracycline activity (Figure 12) indicated a large difference in oxytetracycline concentrations between the IV- and IM-treated calves. These results explain the sudden decrease in the number of bacterial cells at approximately the same time as the second spike in oxytetracycline concentration after administration of the 20 mg/kg dose. The IM treatment produced a much lower peak concentration of approximately 1 ug/ml, which was lower than the MIC for the

inoculation isolate (2 µg/ml). The IV treated calf had a peak concentration of approximately 6 µg/ml. The RT-qPCR results indicated a slight increase in expression of *marA* in the IM-treated animal at 24 hours post-treatment, followed by decreasing expression during the rest of the sampling period. Intravenous administration of the higher 20 mg/kg dose caused a slight increase in *marA* expression at 24 hours post-treatment, but the expression of *marA* was much higher at 10 days post-treatment (Figure 13). This pattern of expression mirrored the changes in oxytetracycline concentration as a function of time.

The second experiment yielded similar results to those of the first. Numbers of bacteria in tissue chambers decreased significantly in both the IV- and IM-treated animals (Figure 14). The CFU/ml counts in the IV-treated animal appeared to rebound a little faster, but by the end of the experiment the bacterial numbers in IV- and IM-treated tissue chambers were similar. In addition, the oxytetracycline bioassay results were also similar. The large peak in concentration for the IV calf was slightly higher than that for the IV calf in the first experiment, reaching a peak concentration of 6.7µg/ml. The IM-treated calf had a peak concentration of approximately 0.5µg/ml, about half that observed in the first experiment (Figure 15). Samples collected for analysis of gene expression were used to optimize methods for RNA extraction. These efforts enabled development and selection of procedures for analysis of multiple genes in the third experiment, but unfortunately it was not possible to perform RT-qPCR analysis of gene expression in this experiment.

In the third experiment, two animals received the low IM dose, two animals received the high IV dose and one animal served as an untreated control. Bacterial

counts in tissue chamber fluid decreased in all cattle, including in the non-treated control animal, thus demonstrating the effect of host defenses in the absence of antibacterial agent (Figure 16). However, all treated cattle still had lower numbers of bacteria than the non-treated control, and those cattle treated with the higher IV dose of oxytetracycline had lower bacterial counts than those treated with the lower IM dose. Although bacterial counts decreased, none of the infections were sterilized. The concentration of oxytetracycline was significantly higher in the IV-treated cattle than in the IM-treated cattle, as expected, although there was more variation than expected between the two IV-treated cattle (approximately 16 μg/ml and 7 μg/ml). Neither of the IM-treated cattle ever had a concentration above 2 μg/ml (the MIC). In both the IV- and IM-treated cattle, the concentration of oxytetracycline continued to decrease gradually through the duration of the experiment, and even reached undetectable levels in 3 of the 4 treated cattle (Figure 17).

For this third experiment, 20 ml of infected tissue chamber fluid sample was extracted from each of the chambers for RNA analysis. The expression of *marA* was significantly increased in both the IV- and IM-treated cattle, but the fold difference in expression was much higher in the IV-treated cattle. In both the IM- and IV-treated cattle, one set of samples indicated an increase in expression early in the experiment followed by a decline, while the other set did not increase until later in the experiment. This appears to be a variation associated with the *marA* gene, as the other genes studied were more consistent between the two animals for each treatment (Figures 18 and 19). Figures 20 and 21 describe the expression of the cold shock protein, which is increased in the IV-treated animals and more so than in the IM-treated animals, with the exception of

the spike in expression in #78 on day 10. The fold difference in expression of this gene

was not nearly as high as that observed in the *marA* data. The magnesium transport

protein also had increased expression in the treated cattle (Figures 22 and 23). In this

case, the one IM–treated animal, #78, had the highest level of expression. With the

exception of this animal, the IV-treated animals had higher levels of expression, but again

not as high as the *marA* gene. The starvation outer membrane protein was expressed the

most in the animal that did not receive any oxytetracycline (Figures 24 and 25); none of

the other animals had any significant increase in expression.

Discussion

Oxytetracycline is not the treatment of choice for salmonelloses in humans and its

use as a prophylactic and therapeutic agent in cattle is believed, therefore, to pose little

risk with regard to promoting development of resistance of human infections to

therapeutic drugs, such as the fluoroquinolones. However, the results of the *in vitro*

experiments described in Chapter 4 indicated that exposure of *Salmonella* Typhimurium

to oxytetracycline at concentrations close to the MIC value caused the expression of

marA to increase, and that this response was correlated with increased resistance to

oxytetracycline as well as several other chemically unrelated antibacterial agents. This

observation was consistent with the hypothesis that oxytetracycline can induce expression

of the *mar* regulon, which regulates expression of a number of multi-drug resistant

mechanisms, including efflux pumps and outer membrane porin proteins, and that

activity of these systems can confer resistance to antibacterial agents unrelated to the

original inducer. In an effort to test this hypothesis further, it was necessary to confirm

that exposure to oxytetracycline *in vivo* also caused expression of the *marA* gene. In

addition, it was recognized that other genes may also be involved, as suggested by the *in*

vitro microarray data, which implicated *cspA*, *mgtC*, and *slp*.

The *in vitro* studies described in Chapter 4 indicated that *marA* expression was

correlated with oxytetracycline concentration, and that the highest expression occurred at

concentrations that were 25 – 50% of the MIC. This result was potentially important

because it suggested that the tissue concentrations produced by prophylactic and

therapeutic dosage regimens may be relevant to the likelihood of resistant strains

emerging. Therefore, different doses of oxytetracycline were employed in the *in vivo*

experiments to investigate the effect of drug dose and resultant tissue concentrations. In

this respect, the tissue chamber model is ideally suited to the study objectives because it

provides a means of measuring the concentration of antibacterial agent in the same *in*

vivo location occupied by the bacterial pathogen. Close examination of the

oxytetracycline concentration-time plots (Figure 17) and the *marA* expression data reveal

similar correlations between drug concentration and gene expression: Significantly

increased gene expression was observed only in oxytetracycline-treated animals.

Furthermore, animals receiving the lower IM dose demonstrated expression levels that

were much lower than those produced by the higher IV dose. However, it was apparent

also that excessively high concentration had a negative impact on expression, as indicated

by the difference between responses induced by the IV dose in the two experimental

animals in the third experiment. Significant expression in calf number 18 occurred only

after the very high peak drug concentration had declined, whereas the response in calf

number 20, which had a lower peak oxytetracycline concentration, occurred more quickly. Interestingly, *marA* expression was increased substantially in the presence of oxytetracycline that was several-fold higher than the MIC value, thus indicating a higher resistance of the isolate *in vivo* than was observed *in vitro*. Specifically, *marA* was up-regulated when the oxytetracycline concentration was between 6 and 8 μg/ml, but at concentrations above this *marA* was expressed at the same level as it was in the absence of antibacterial agent. At concentrations below the 6 – 8 μg/ml level, the expression of *marA* was quite variable between animals. It can be assumed from previous studies (Gambino *et al*, 1993) as well as the *in vitro* MIC data reported in Chapter 4, that tissue chamber bacteria surviving oxytetracycline at concentrations higher than the MIC are probably also multi-drug resistant.

The gene (*cspA*) that encodes for the cold shock protein is believed to be involved in adaptation of *Salmonella* to different stressors, including exposure to antibacterial agents (Katzif *et al*, 2003; Mangalappalli-Illathu *et al*, 2006). Expression analysis of this gene *in vivo* indicated that even in the animal receiving no treatment (#11), the expression of *cspA* was altered, although not to the same degree as in the treated animals. The IV treated animals once again had a higher fold change in expression than the IM animals, reflecting the higher concentration of oxytetracycline and its effect on the bacterial cells. With the exception of one animal that received the lower IM dose, there appeared to be an optimal concentration necessary for *cspA* expression, as confirmed by the relative responses in the two animals receiving the high IV dose: the calf with the lower tissue concentration had higher levels of expression that the calf with the higher tissue concentrations.

The magnesium transport protein (*mgtC*) data indicates an increase in expression in both the IM- and IV-treated animals early in the experiment, and a subsequent decrease as the concentration of oxytetracycline declines. The expression response was greatest in the IM treated cattle, again suggesting a drug concentration dependency whereby the optimal concentration is neither too high nor too low. Apparently, the *mgtC* gene is sensitive to lower concentrations of oxytetracycline, which may be related to the role of MgtC in promoting intracellular survival of *Salmonella* (Rang *et al*, 2007) and the relative protection from effective drug concentration that the intracellular locations provide.

The starvation outer membrane protein (*slp*) is normally down regulated in the presence of antibiotic (Price *et al*, 2000). However, in the *in vitro* microarray study, this protein was up-regulated in the presence of oxytetracycline. In contrast, the data for the *in vivo* study are more consistent with previously published reports in that there was no evidence of increased gene expression, but gene expression was significantly higher in non-treated animals versus those that received oxytetracycline.

This preliminary *in vivo* study of *marA*, *cspA*, *mgtC* and *slp*, indicates that not only are the bacteria responding to the host environment, but also to the concentration of oxytetracycline, as expected. The up-regulation of *marA* is of particular concern because this protein alone is responsible for conferring multi-drug resistance by the activation and inhibition of so many other proteins (Gambino *et al*, 1993, Alekshun *et al*, 1997), including those involved in efflux pumps and porins. While the present study did not directly address the possibility of oxytetracycline exposure in food animals causing the emergence of multi-drug resistant *Salmonella* Typhimurium infections in humans, it is

clear that the up-regulation of *marA* and differential expression of *cspA*, *mgtC* and *slp*

provides strong mechanistic evidence that this is possible. Furthermore, the results

confirming the correlation between oxytetracycline concentration and gene expression

indicate that emergence of resistance may be related to drug dose, thus raising the

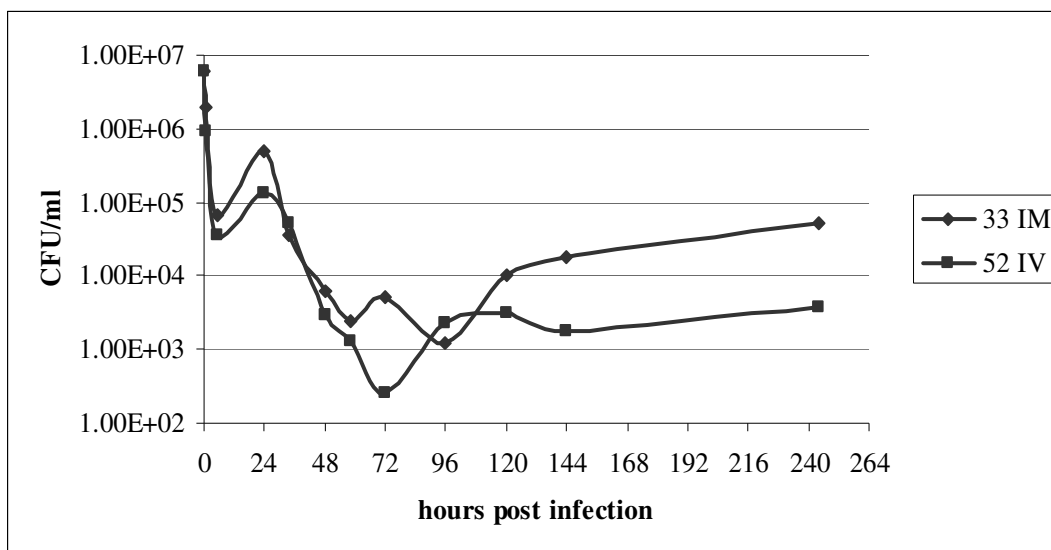probability that doses could be optimized to minimize emergence of resistance.

Figure 11. Growth curve depicting the changes in CFU concentration of *Salmonella* in the tissue chamber for the first cattle experiment. The oxytetracycline IV treated animal has a much lower bacterial cell count at 72 hours post infection, but rebounds and does not ever reach zero.
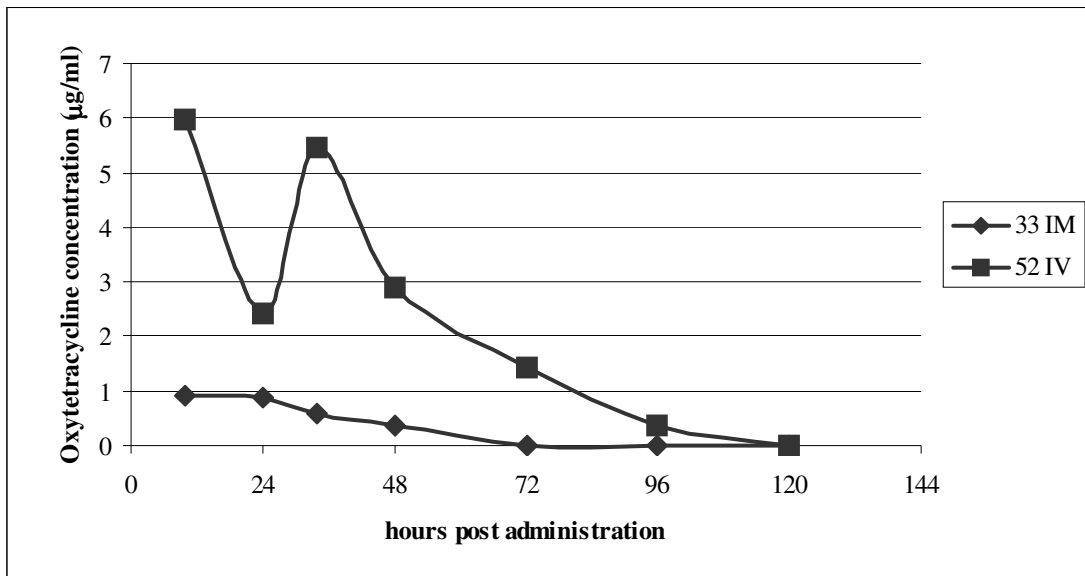
Figure 12. Graph depicting the bioassay results for the first cattle experiment. The IV
treated animal had a peak concentration of 6 μg/ml after the initial dose, and a
second peak at 5.5 μg/ml. Following the second peak the concentration
dropped to zero by 120 hours. The IM treated calf had an initial concentration
around 1 μg/ml that then dropped to zero by 96 hours.
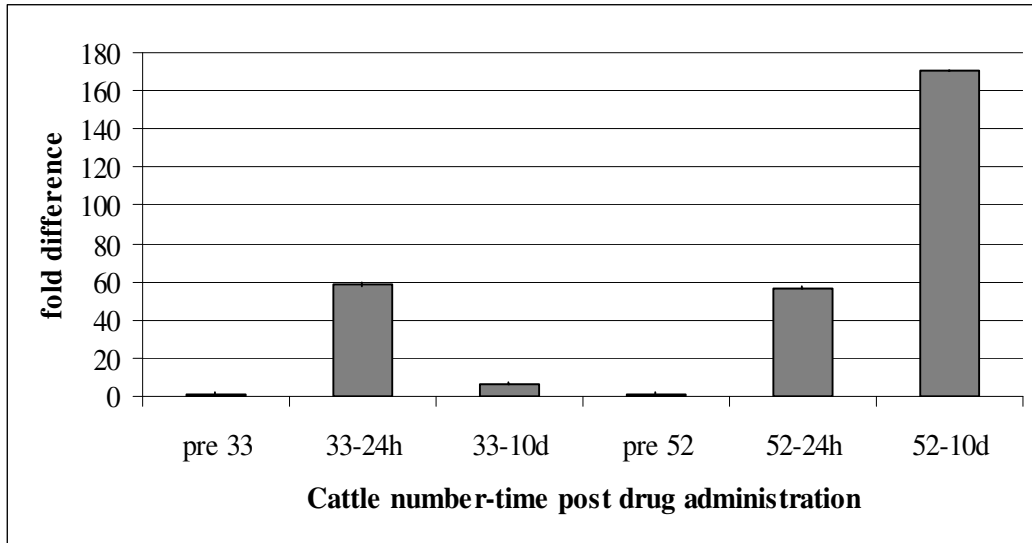
Figure 13. Graph depicting the RT-qPCR results for the first cattle experiment. Calf number 33 received one IM dose of oxytetracycline and had a slight increase in expression of *marA* at 24 hours post-drug administration and then it declined again by 10 days. Calf number 52 received two IV doses of oxytetracycline. The expression of *marA* was slightly elevated at 24 hours post-treatment and had an even greater increase in expression at 10 days post treatment.
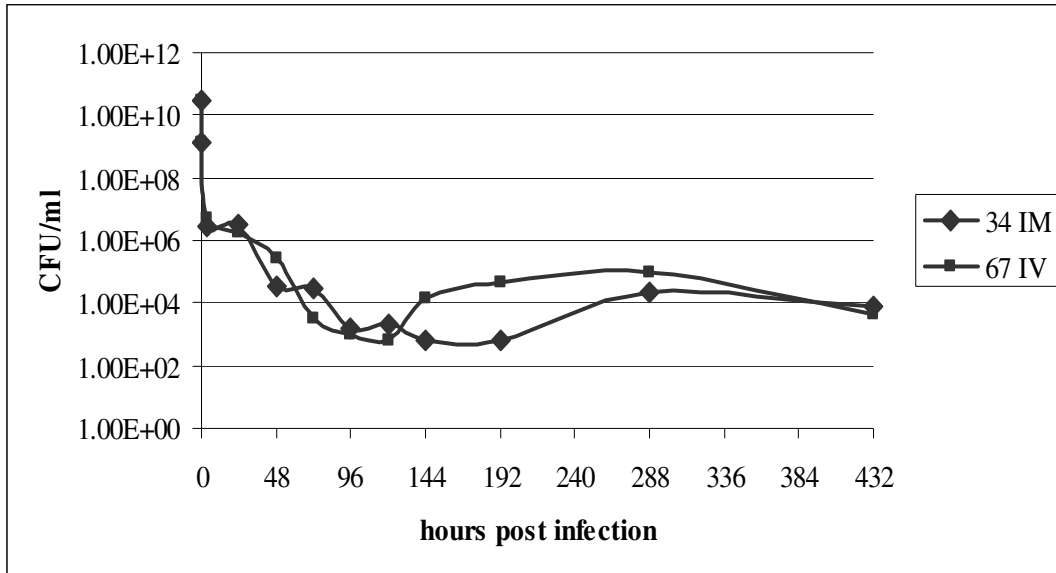
Figure 14. Growth curve depicting bacterial cell numbers for *Salmonella* for the second
cattle experiment. This experiment started with a higher inoculum and
therefore did not reach as low of a nadir as the first experiment. Again, the
number of bacterial cells did not reach zero.
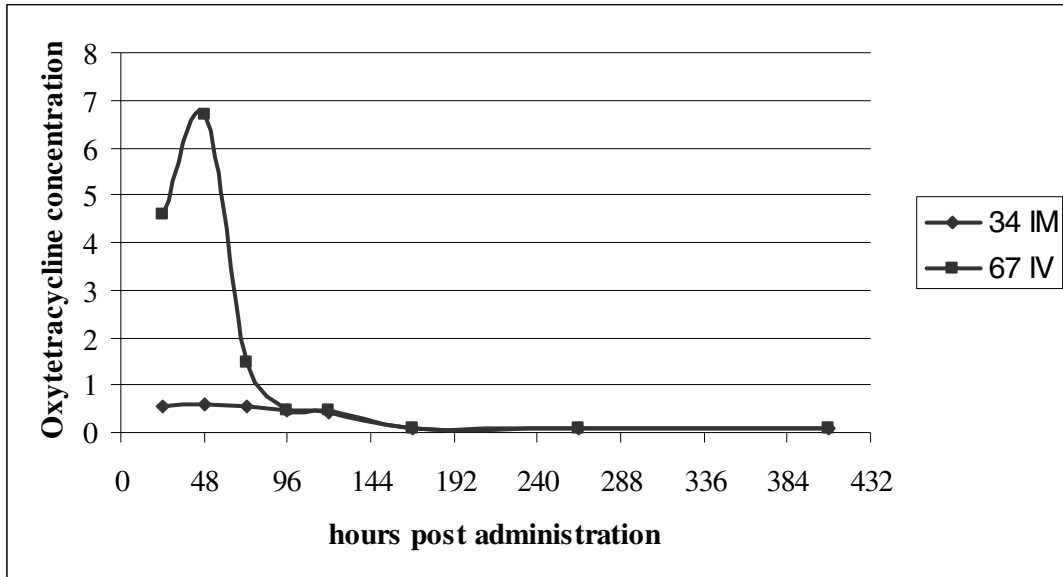
Figure 15. Graph depicting oxytetracycline bioassay results for the second cattle
experiment. Number 67 had two IV treatments of oxytetracycline 24 hours
apart, the peak for this calf was almost 7 μg/ml of oxytetracycline following
the second treatment. The concentration then drops sharply to zero by 168
hours. The IM treated calf (#34) had an initial concentration of less than 1
μg/ml and it stayed about this concentration until it dropped to zero at 168
hours.
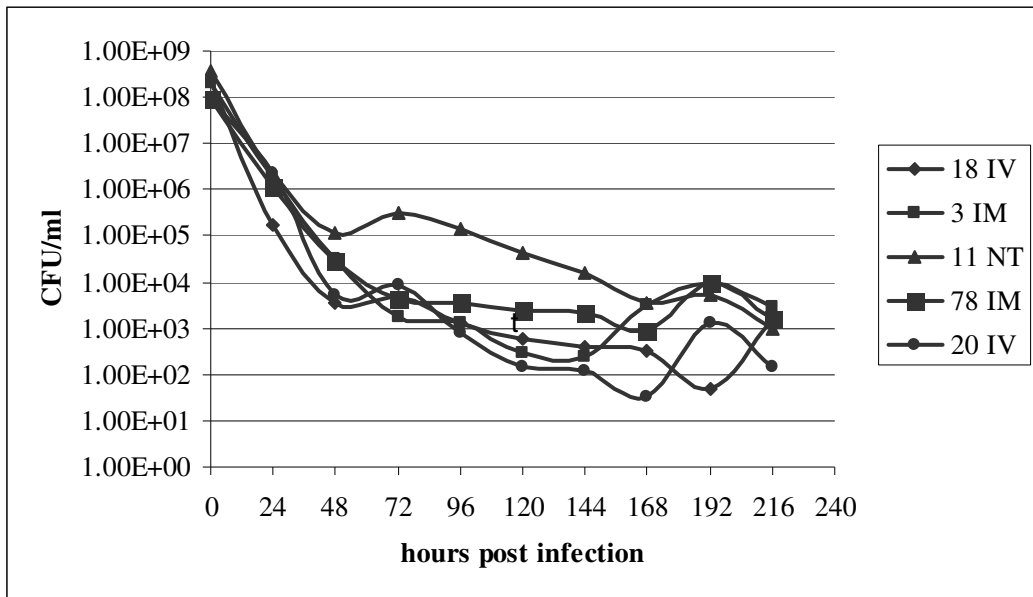
Figure 16. Growth curve depicting bacterial cell numbers for *Salmonella* in the third
cattle experiment. In all animals the bacterial cell counts dropped, including
calf #11 which received no treatment (NT). The IV oxytetracycline - treated
cattle (#18 and #20) had the lowest bacterial cell counts, but by 216 hours the
bacterial cell counts for all cattle had reached about the same level of
1.00E+03.
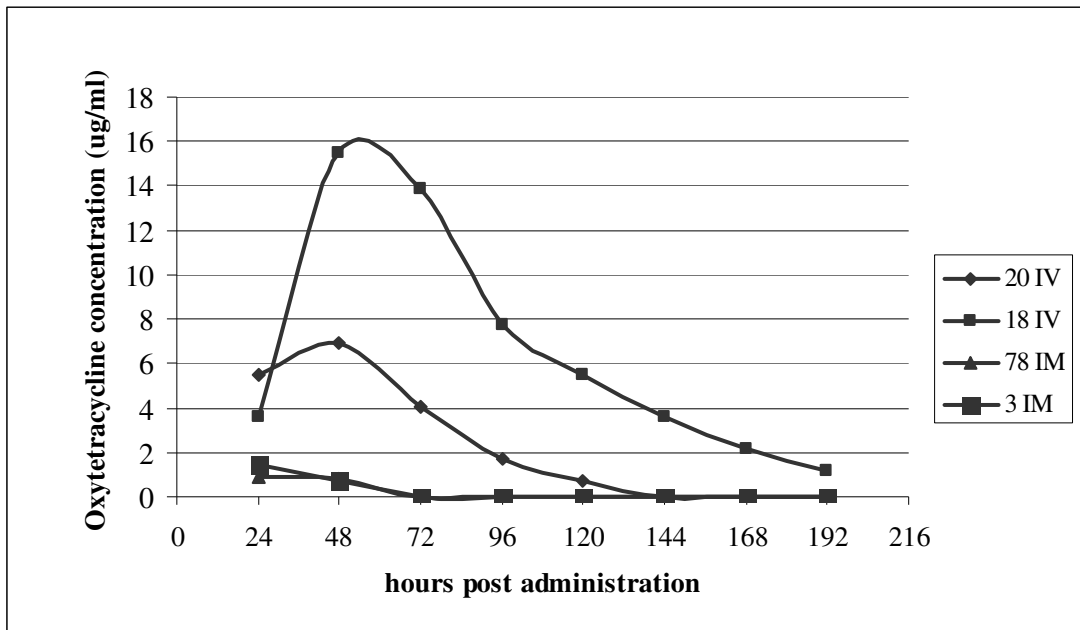
Figure 17. Graph depicting the bioassay results for the third cattle experiment. Calf #20
        had a similar pattern to the previously IV treated cattle, starting around 7
        μg/ml and declining to zero by 168 hours. The IM treated cattle also had
        similar patterns to those seen in the previous experiments. Calf #18 had a
        peak concentration of almost 16 μg/ml, a very high concentration that did not
        reach zero by 192 hours.
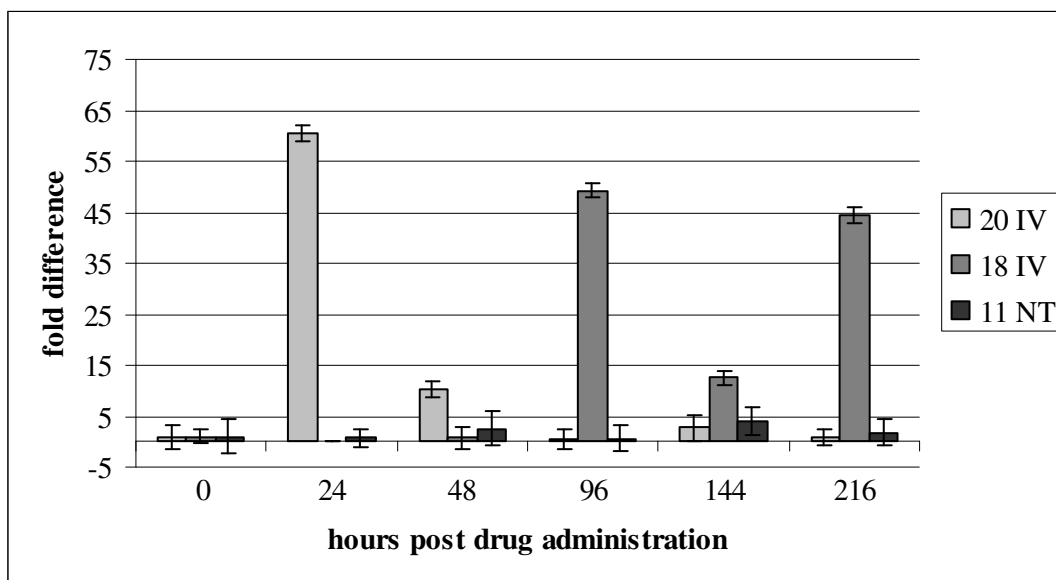
Figure 18. Expression of *marA* gene in IV oxytetracycline - treated cattle. Calf #20 started with a high level of expression of *marA* that gradually decreased as the concentration decreased. Calf #18 had a low concentration that peaked at around 96 hours, decreased at 144 hours and then increased again at 216 hours. The no-treatment control (NT) had no significant change in expression. The columns represent the mean fold difference plus and minus the standard deviation based on the RT-qPCR analyses done in duplicate.
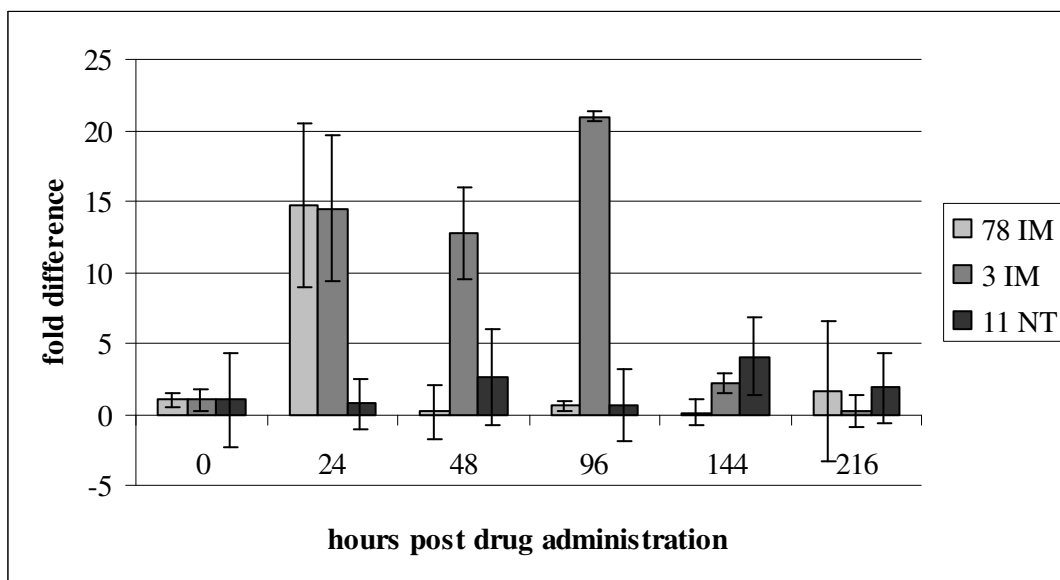
Figure 19. Expression of *marA* in oxytetracycline IM treated cattle. Both of the IM
treated cattle had an increase in expression of *marA* at 24 hours post treatment
compared to the animal receiving no treatment (#11 NT). However, at 48 and
96 hours, #3 continued to have increased expression while #78 had returned to
the same as #11. Columns represent mean fold difference plus and minus the
standard deviation based on the RT-qPCR analyses done in duplicate.
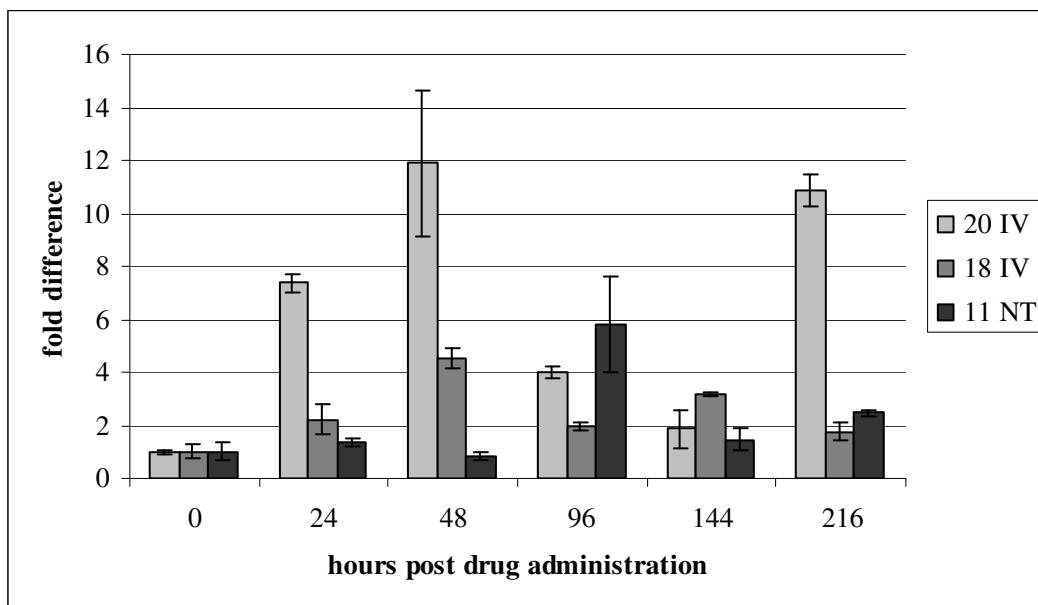
Figure 20. Expression of STM 3469 the cold shock protein in IV oxytetracycline - treated cattle. Calf #20 had a much larger increase in expression that #18 for the IV treated animals even though #18 had much higher concentration of oxytetracycline. Calf #11 (no treatment) had a significant increase in expression at 96 hours. Columns represent mean fold difference plus and minus the standard deviation based on the RT-qPCR analyses done in duplicate.
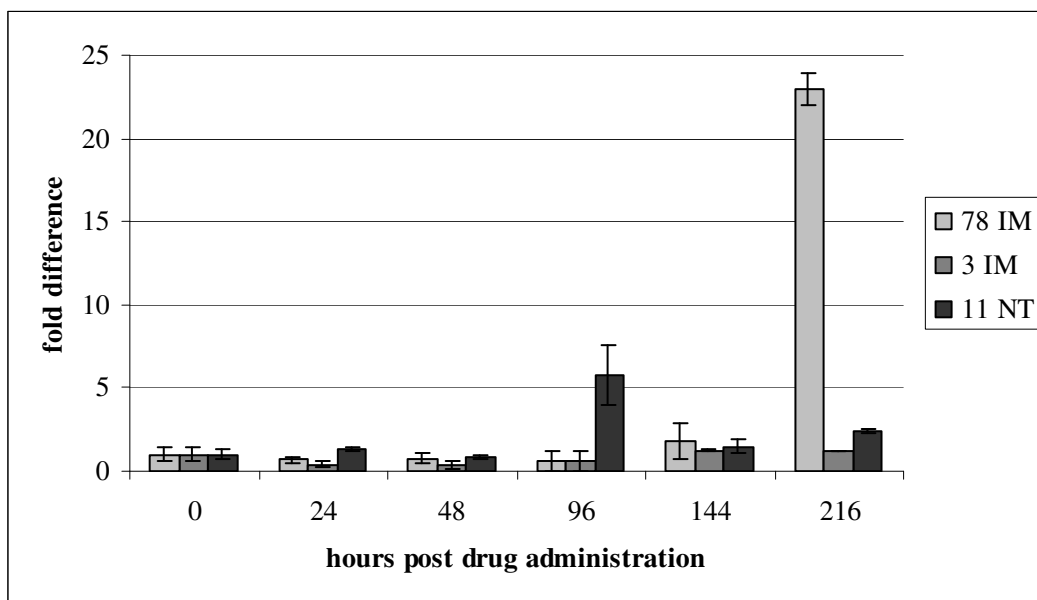
Figure 21. Expression of cold shock protein (STM 3469) in IM oxytetracycline - treated cattle. Calf #3 had no increase in expression of the cold shock protein throughout the experiment. Calf #78 had an increase in expression only at 216 hours. Columns represent mean fold difference plus and minus the standard deviation based on the RT-qPCR analyses done in duplicate.
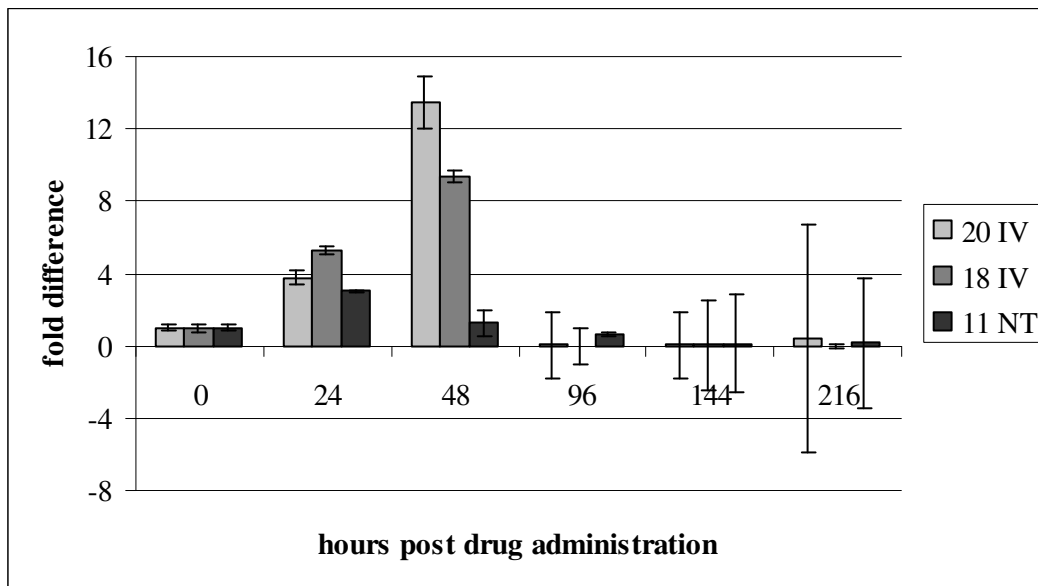
Figure 22. Expression of STM 3764 Magnesium transport protein in IV oxytetracycline -
treated cattle. Calf #20 and calf #18 had increases in expression of the
magnesium transport protein at 24 and 48 hours compared to the calf
receiving no treatment. After 48 hours, no difference was seen. Columns
represent mean fold difference plus and minus the standard deviation based on
the RT-qPCR analyses done in duplicate**.**
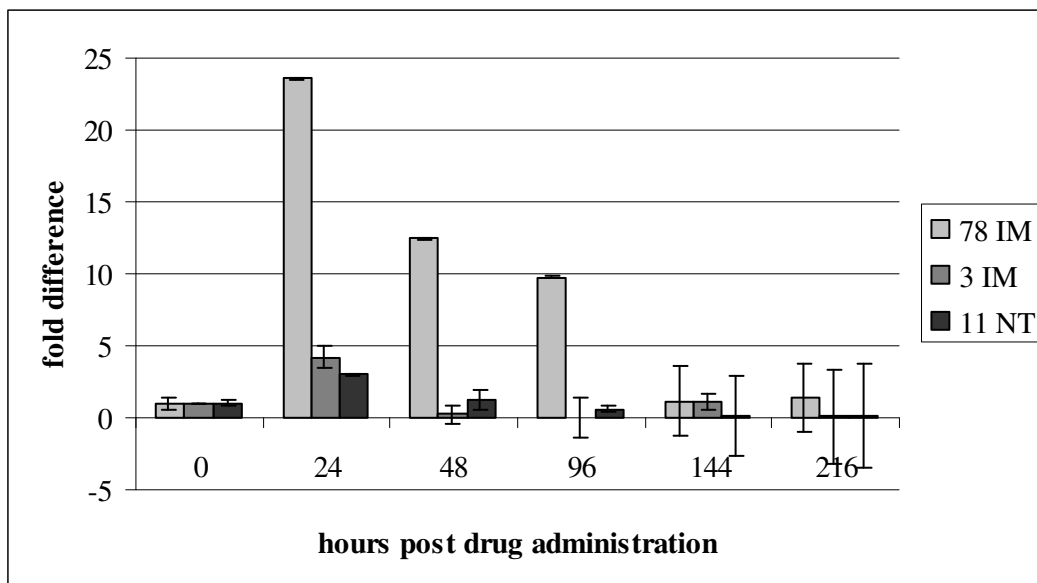
Figure 23. Expression of magnesium transport protein (STM 3764) in IM
oxytetracycline - treated cattle. Calf #78 had a significant increase at 24 hours
that gradually decreased throughout the experiment. Calf #3 had a slight
increase at 24 hours, but not to the same degree as #78. Columns represent
mean fold difference plus and minus the standard deviation based on the RT-
qPCR analyses done in duplicate.
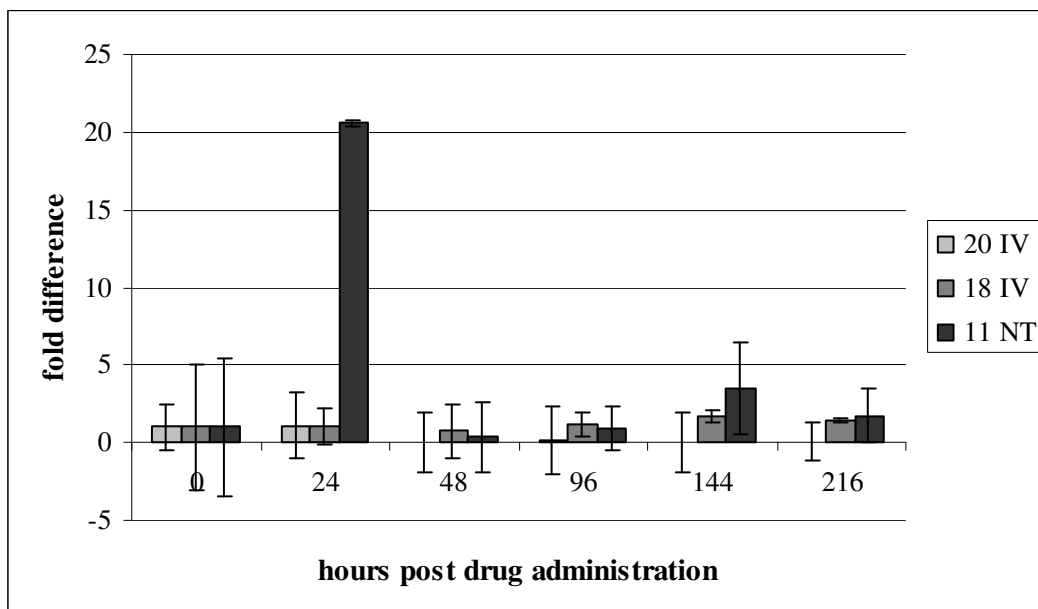
Figure 24. Expression of STM 1819 Starvation Outer Membrane Protein in IV
oxytetracycline - treated animals. The highest change in expression for this
protein was seen in the #11 calf that received no treatment. This spike was
seen at 24 hours post treatment for the treated calves. Columns represent mean
fold difference plus and minus the standard deviation based on the RT-qPCR
analyses done in duplicate.
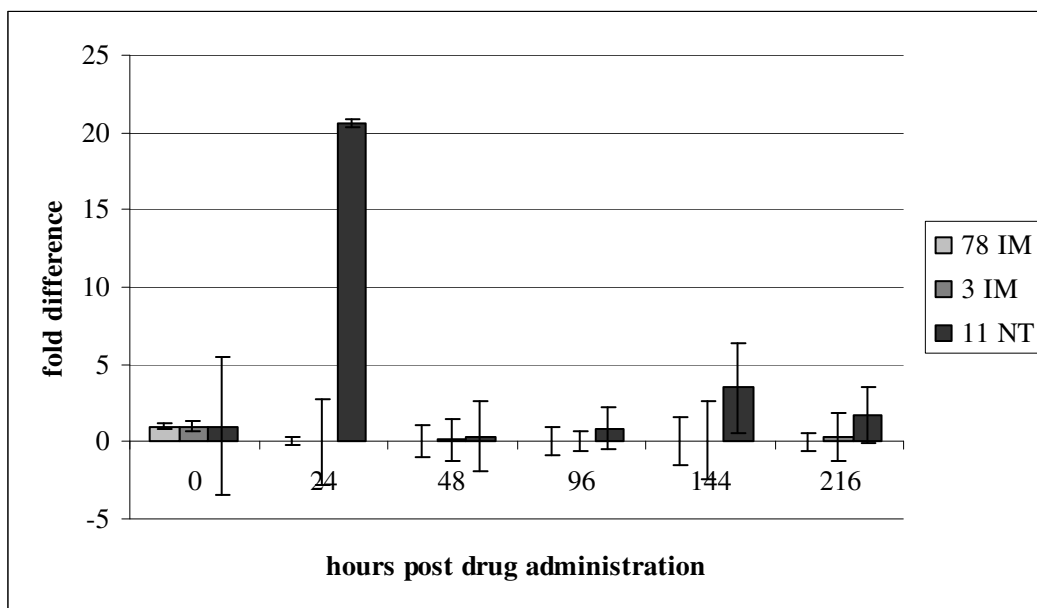
Figure 25. Expression of starvation outer membrane protein (STM 1819) in IM
oxytetracycline-treated cattle. The highest level of expression is again seen in
the animal that received no treatment at all, #11, at 24 hours. Columns
represent mean fold difference plus and minus the standard deviation based on
the RT-qPCR analyses done in duplicate.

CHAPTER 6: SUMMARY AND CONCLUSIONS

The growing concern of antimicrobial resistance has led to changes in the regulations for antibacterial drug use in both human and animal medicine. The current strategy for preventing the spread of resistance includes utilizing antibacterial agents with spectra of activity that narrowly target the specific etiological pathogens as opposed to using broad-spectrum drugs, as well as limiting the use of antibacterial agents in food animal medicine to those not used in human medicine. This approach is predicated on the understanding that exposure to one antibacterial agent will not lead to emergence of resistance to other mechanistic classes of agents. However, this strategy does not take into account the involvement of multi-drug resistance mechanisms, such as the *mar* regulon, that may be induced by exposure to one agent and then confer phenotypic resistance to many different chemical classes of antibacterial agents.

The discovery of the *mar* system in *Salmonella* brought into question the wisdom of current regulatory measures (Sulavik *et al*, 1997). *Salmonella* is a common food-borne pathogen of humans that frequently requires the use of antibiotic for treatment (CDC 2005). Recent outbreaks of multi-drug resistant *Salmonella* infections have placed the use of antimicrobials in food animals under even more scrutiny (Hseuh *et al*, 2004; Zansky *et al*, 2002). Isolates resistant to the fluoroquinolones are of particular concern as this mechanistic group is considered the last line of defense for treatment of *Salmonella* in humans (Molbak, 2005).

In summary, the results of the present study indicated that expression of *marA* in *Salmonella* Typhimurium could be induced by oxytetracycline, in contrast to the

conclusions of a previously published report (Randall *et al*, 2002). Furthermore, exposure of *Salmonella* Typhimurium to oxytetracycline produces a concentration-dependent response whereby expression is maximized when concentrations are neither too high nor too low. Depending on whether such exposure occurs in an *in vitro* or *in vivo* environment, maximal expression generally occurs at concentrations 0.25 – 0.5 times or 3 – 4 times the MIC value, respectively. This observation suggests that dosage regimens for oxytetracycline in cattle could be optimized to minimize expression of the *mar* regulon and any resultant emergence of resistance. Finally, oxytetracycline-induced increase in *marA* expression was correlated with increased resistance of oxytetracycline and several other mechanistically unrelated antibacterial agents *in vitro* and survival of the bacterium *in vivo*, thus presenting strong circumstantial evidence in support of the underlying project hypothesis; that the prevalence of bacteria that are antibiotic resistant due to the expression of multidrug resistance systems increases when cattle are administered prophylactic antibiotics and that the wide substrate specificity of these systems confers resistance not only to antibiotics used prophylactically but also to antibiotics used therapeutically in cattle and human consumers of contaminated beef products. Although the results of the *in vitro* study failed to identify development of fluoroquinolone resistance in response to oxytetracycline exposure, previously published research (Cohen *et al*, 1989) reported that use of tetracycline antibiotics did lead to fluoroquinolone resistance in *E. coli*, so there is still a possibility that this could occur in *Salmonella* under different experimental conditions.

Obviously, further studies are needed to confirm that oxytetracycline-induced expression of *marA* is directly responsible for development of multi-drug resistance, and

that this resistance can be transferred to consumers of beef products. The factors relevant to this possibility clearly are very complex, as is illustrated by the possible involvement of the three other genes (*cspA*, *mgtC* and *slp*) identified in the *in vitro* study and investigated in the *in vivo* study.

An unexpected finding of the current research project is that *in vitro* incubation of *Salmonella* Typhimurium in liquid media containing oxytetracycline at or higher than the MIC inhibits growth for at least 18 hours, but that with longer incubation the bacterium will replicate exponentially, and that this delayed growth is correlated with an increase in resistance. This observation has important implications with respect to the interpretation of *in vitro* sensitivity data (these usually are derived from 18-hour cultures) and may explain why bacterial isolates classified as being intermediately susceptible may not respond favorably to therapeutic doses designed only to achieve tissue concentrations that barely exceed the MIC.

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