ABSTRACT

Title of Dissertation: CHARACTERIZATION, DISTRIBUTION, AND EVOLUTIONARY ACQUISITION OF ANTIMICROBIAL RESISTANCE ELEMENTS AMONG DIVERSE *SALMONELLA* SUBSPECIES

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Insights into the evolution of antimicrobial resistance can be gleaned by examination of historical strains of *Salmonella* collected from a variety of locations, time periods, and sources. Here, the *Salmonella* Reference Collections were utilized to gain evidence into the distribution, prevalence, and reticulate nature of antimicrobial resistance from strains that represent the collective genetic diversity of *Salmonella*. Of the 141 strains examined, 25.5% were fully or intermediately resistant to one or more agents. Resistance to the older antimicrobials sulfisoxazole and streptomycin were more common than resistance to newer antimicrobials, with 15.6% and 14.2% of strains resistant, respectively. No strains presented with resistance to newer drugs--ciprofloxacin, cefoxitin, and ceftiofur specifically. Of particular importance was the identification of a correlation between strains isolated from food animal sources and the presence of resistance to streptomycin and kanamycin, as food animals have been implicated in the transfer of resistance elements through the food chain. Increased incidences of resistance were identified in serotypes Saintpaul, Heidelberg, and Typhimurium. Integrons, a major contributor to the horizontal transfer of resistance genes, were identified in 9.93% of strains and the most commonly harbored gene was *aadA1*, conferring resistance to streptomycin. Identification of exogenous genes responsible for the observed phenotypes revealed that 73.1% of resistant phenotypes could be accounted for by the presence of such an element. Analysis of horizontal gene transfer among the regulatory *mar, ram* and *sox* operon regions, which have been implicated in the development of multi-drug resistance via increased cellular efflux, revealed that recombination helped to maintain a handful of presumably beneficial alleles across subspecies I *S. enterica*. Diversification was particularly limited in the *sox* operon and in the global regulatory genes, as opposed to local regulators. Such lack of diversity speaks to the requirement for proper functioning of many processes in the cell. Retention of some clonality was seen in the closely-related SARA strains, with assortment of alleles more obvious across the more diverse SARB strains. Supported by evidence gathered here is the importance of

horizontal gene transfer in evolution of selective benefits harbored by bacterial pathogens, in particular, *S. enterica*.

CHARACTERIZATION, DISTRIBUTION, AND EVOLUTIONARY ACQUISITION OF ANTIMICROBIAL RESISTANCE ELEMENTS AMONG DIVERSE *SALMONELLA* SUBSPECIES

by

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List of Abbreviations

Ami: amikacin
Amp: ampicillin
AST: antimicrobial susceptibility testing
ATCC: American Type Culture Collection
Aug: amoxicillin-clavulanic acid
Axo: ceftriaxone
Chl: chloramphenicol
Cip: ciprofloxacin
CLSI: Clinical Laboratory Standards Institute
Cot: trimethoprim-sulfamethoxazole
DMSO: dimethyl sulfoxide
DNA: deoxyribonucleic acid
FDA: Food and Drug Administration
Fox: cefoxitin
GC: guanine-cytosine
gDNA: genomic DNA
Gen: gentamicin
HGT: horizontal gene transfer
Kan: kanamycin
Nal: nalidixic acid
NARMS: National Antimicrobial Resistance Monitoring System
MDR: multi-drug resistance
MIC: minimum inhibitory concentration
MLEE: multi-locus enzyme electrophoresis
MLST: multi-locus sequence typing
ORF: open reading frame
PCR: polymerase chain reaction
RND: resistance-nodulation-division
SAPE: streptavidin-phycoerythrin conjugate
SAR: *Salmonella* Reference
SEEC: *Salmonella-E. coli*
SGI: *Salmonella* Genomic Island
SGSC: *Salmonella* Genomic Stock Centre
SLV: single locus variant
Str: streptomycin
Tet: tetracycline
Tio: ceftiofur

1 Chapter 1: Introduction

1.1 *Salmonella*

Salmonella is a genus of Gram negative, rod-shaped bacteria often associated with foodborne illness in humans (Bell and Kyriakides, 2002; Scallan et al., 2011). The genus is comprised of two species, *S. bongori* and *S. enterica*, of which the latter is further subdivided into six subspecies: I, ssp. enterica; II, ssp. salamae; IIIa, ssp. arizonae; IIIb, ssp. diarizonae; IV, ssp. houtenae and VI, ssp. indica (Brenner et al., 2000). Serotyping, which utilizes variation of phase I (H1) and phase II (H2) flagellar and somatic lipopolysaccharide (O) antigens on the surface of bacterial cells, is commonly used to distinguish strains. Of the over 2500 recognized serotypes, more than 1500 are of subspecies I and associated with warm-blooded animals (Guibourdenche et al., 2010). *S. enterica* subspecies I strains are responsible for 99% of all reported human *Salmonella* infections in the United States (Centers for Disease Control, 2011).

In developed countries, gastroenteritis is the most common result of *Salmonella* infection, with symptoms including diarrhea, fever, and abdominal cramps. Most cases of salmonellosis are self-limiting and resolve in four to seven days (Centers for Disease Control). In certain cases, non-typhoidal *S. enterica* can spread outside of the gastrointestinal tract and cause invasive infection in the blood, joints, or bone (Acheson and Hohmann, 2001). Typhoidal infections, caused by human-restricted serotypes Typhi and Paratyphi A, are more common in less developed countries and cause potentially life-threatening enteric fever (Sanchez-Vargas, 2011). It is estimated that, each year, more than 1.2 million people are afflicted with non-typhoidal salmonellosis in the United States, resulting in over 23,000 cases of hospitalization and over 450 deaths. More than

one million of these illnesses are attributed to contaminated food or water, making *S. enterica* the leading cause of food-related hospitalization and death in the United States (Scallan et al., 2011). Surveillance data of infections provided by FoodNet, a collaborative effort between federal and state health laboratories, indicates that the incidence of *S. enterica* in the United States has not declined since reporting was initiated in 1996 (Centers for Disease Control, 2011). Globally, non-typhoidal enteric infections result in over 93 million cases of illness and an estimated 155,000 deaths per year. As a result of its broad host range and environmental niche occupation, many different foods have been found to be contaminated with *S. enterica*, including poultry, fruits, vegetables, eggs, and dairy products (Dunkley et al., 2009; Zhou et al., 2008; Eng et al., 2015).

Antimicrobial resistance first emerged in *S. enterica* in the mid-1960s in a strain of *S*. Typhimurium definitive type (DT) 29. The strain was associated with cattle in Great Britain and carried plasmid-encoded resistance to five antimicrobials: furazolidone, ampicillin, streptomycin, sulfonamides, and tetracycline. It disappeared as a major outbreak strain, only to be replaced by *S*. Typhimurium DT193 and DT204/204c around 1975. These strains were resistant to the same antimicrobials as *S*. Typhimurium DT29, with additional resistance to chloramphenicol, gentamycin, kanamycin, and trimethoprim. Outbreaks were primarily limited to veterinary cases in cattle and human contact with cattle in Europe (Threlfall et al., 2000). It was not until the early 1990s that the global dissemination of antimicrobial resistance in *S. enterica* became an important public human health issue (Butaye et al., 2006), when a strain of multi-drug resistant *S*. Typhimurium DT104 emerged. The ACSSuT phenotype (resistance to ampicillin,

chloramphenicol, streptomycin, sulfonamides, and tetracycline) displayed by DT104 continues to be identified in many *S.* Typhimurium strains, isolated from a variety of sources, including cattle, poultry, pigs, and humans. Despite a decrease in incidence of *S.* Typhimurium in more recent years (NARMS, 2014), multi-resistant strains of previously sensitive serotypes continue to be discovered (Palomo et al., 2013). The burden posed by antimicrobial resistance, due to the increased incidence of hospitalization from invasive non-typhoidal infections, is significant (Helms et al., 2002; Parsons et al., 2013; Varma et al., 2005). Patients at risk for an invasive infection, such as the very young, elderly, or immuno-compromised, are commonly given a fluoroquinolone antimicrobial but increasing incidences of resistance place limitations on the effectiveness of such treatment (Sanchez-Vargas, 2011; Crump et al., 2015). Additionally, typhoidal strains that are deemed susceptible to the drug ciprofloxacin but have an increased minimum inhibitory concentration (MIC) have been shown to result in enteric fever infections that require a longer course of treatment and more often result in treatment failure (Crump et al., 2008). *S. enterica* infections resistant to cephalosporins, a second commonly used class of drug particularly in children to whom a fluoroquinolone cannot be given, have been increasing in incidence globally since the late 1990s. The resistance determinant in these strains is usually attributed to the presence of an acquired Amp-C type β-lactamase gene. It is theorized that increased use of β-lactams in animals, particularly food animals, has played a role in the dissemination of this mobile genetic element (Miriagou et al., 2004). Increases in resistance are being seen in particular in strains acquired outside of the United States, primarily in serotypes Typhi and Paratyphi A acquired by travelers to Asia (Date et al., 2016).

While studies of the prevalence of antimicrobial resistance in historical collections is limited, there have been some insights into the evolutionary acquisition of phenotypic resistance as a result of the selective pressures created by antimicrobial usage. In England and Wales, the period from 1981 to 1988 was marked by a doubling of multi-resistant *S.* Typhimurium strains isolated from humans and a quadrupling of multi-resistant *S.* Typhimurium strains isolated from cattle. The cause of such increases were attributed to antimicrobial usage in cattle, reinforced by the finding that increases in multi-resistant strains from poultry, where the use of antimicrobials was not as intensive, did not show the same large increases over the seven year period (Threlfall et al., 1993). Other studies and retrospective reviews also point to the selective pressure exerted by antimicrobial use in food animal production as a primary source of multi-resistant *S. enterica*. A study by the Centers for Disease Control revealed that antimicrobial-resistant *S. enterica* outbreaks were more likely to be sourced back to a food animal than non-multi-resistant outbreaks (Holmberg et al., 1984). Additionally, there existed parallels between the drugs used in veterinary medicine and the types of resistance being seen in humans. The same parallels did not exist for drugs used in human medicine (Angulo, et al., 2000). A study of a historical collection of clinical *S. enterica* isolates from the United States, primarily from the top five serotypes implicated in foodborne infections, showed that resistance to streptomycin, sulfamethoxazole, tetracycline, and ampicillin increased over the course of several decades (Tadesse et al., 2016), before tapering off in the late 1990s. Resistance to newer antimicrobial agents such as ceftiofur, ceftriaxone, and ciprofloxacin, failed to be identified in any of the historical strains tested in that study. An older study comparing the MICs of a collection of pre-antibiotic era strains of

Escherichia coli to a collection of contemporary strains found that high-level resistance was primarily found in contemporary strains and almost non-existent in the pre-antibiotic era strains (Houndt and Ochman, 2000).

1.2 Resistance Mechanisms in *Salmonella*

Antimicrobial resistance can occur through four different mechanisms, mediated by different genetic elements in the bacterial cell. Breakdown of the drug can occur through acquisition of exogenous genes, which is commonly the case for aminoglycoside, beta-lactam, and chloramphenicol resistance (Michael et al., 2006; Alcaine et al., 2007). These exogenous genes are most often found on mobile genetic elements such as plasmids but can also be located on stably-integrated elements, like the *Salmonella* Genomic Island, discussed in more detail below. Drug target modification can occur through horizontal acquisition of a resistant form of the drug target or point mutations in the host genome. Resistance to sulfonamides, trimethoprim, and quinolones are commonly mediated through these genetic mechanisms (Spratt, 1994; Michael et al., 2006; Alcaine et al., 2007). Reduced uptake of the drug into the cell can also occur, most commonly mediated by porin expression changes (Alcaine et al., 2007). Multi-drug resistance that is not mediated by one or a combination of the mechanisms above is most likely to be mediated through active efflux from the cell (Alekshun and Levy, 1994; Nikaido, 1994). Active efflux can occur as a result of changes to regulatory gene functioning and the concomitant increase in expression of efflux pump genes, discussed in depth below.

1.2.1 Integrons

Integrons, first discovered in the 1980s, are composed of three components: an *int* gene coding for an integrase protein, a recombination site, and a promoter which is used to express integrated genes. The integrase gene is present at the 5', conserved region of the integron. The 3' conserved end of class 1 integrons contains a *qacEdelta/sul1* gene, which codes for resistance to sulfonamides. Genes are integrated through site-specific recombination at a site located just downstream from the *int* gene, which results in the variable region of the integron accumulating cassettes that are then expressed using the previously mentioned promoter. Integron classes, of which there are five known, are defined by the type of integrase gene harbored. Class 1 integrons harbor the largest variety of expressed resistance genes and are widely disseminated among Gram-negative bacteria. The other classes of integrons do not contain the wide variety of genes found in class 1 integrons. Integrons play an important role in antimicrobial resistance as a result of their ability to capture and express genetic material. Most integrons characterized to date contain either antimicrobial resistance or virulence genes. They can be present either as a part of the bacterial chromosome or on a plasmid. When present on a mobilizable or conjugative plasmid, the integron can be inter- and intra-species transferred to other bacteria, and it is believed that integrons pre-date the species divergence of some genera of bacteria (Mazel, 2006). Multiple gene cassettes can be inserted into a single integron, and in non-typhoidal *S. enterica*, integrons have been characterized that contain up to six different resistance genes (Krauland et al., 2009). In a study of *S. enterica* isolated from seafood, it was found that some integrons share much similarity to integrons isolated from *Vibrio cholera* seafood isolates in Asia, invoking the hypothesis that certain

integrons may have evolutionary ties to commercial seafood operations in this area of the world, where antimicrobial use in aquaculture is not restricted (Khan et al., 2009).

1.2.2 *Salmonella* Genomic Island

The *Salmonella* Genomic Island (SGI) was first characterized in *S.* Typhimurium DT104 and harbors the genes responsible for its multi-resistant phenotype. Since the initial discovery, many variations on the *S.* Typhimurium DT104 SGI have been identified in other *Salmonellae*. The SGI is a large, chromosomally-integrated element and contains 44 open reading frames and several other diverse genetic signatures. Fifteen of these open reading frames have no similarity to any other known genes. Of the open reading frames with known homology, some are related to plasmid genes coding for sex pili and DNA transfer proteins. The remaining open reading frames are primarily genes with antimicrobial resistance functions. The cryptic retronphage sequence, present at the 3' end of some SGI variants, seems to have originated with *Escherichia coli* phage Φ-R73 while another region, IS6100, was originally found in *Mycobacterium fortuitum* and *Klebsiella. oxytoca* (Boyd et al., 2000; Doublet et al., 2005). *Proteus mirabilis* was recently found to also contain previously characterized SGI variants (Boyd et al., 2008). While the unique evolutionary history of the SGI is not fully understood, the most likely explanation for the variation in gene and open reading frame arrangement and content is a combination of single insertion events and intra-island homologous recombination, which can cause regional deletions (Boyd et al., 2002). In all known instances, SGI is inserted between the *thdF* and *yidY* genes of the bacterial chromosome. The island is not able to self-mobilize but can be transferred if a plasmid coding for a DNA transfer mechanism is present (Doublet et al., 2005).

1.2.3 Efflux Pump Expression

An additional major mechanism causing multi-drug resistance in both natural and laboratory *S. enterica* strains is the active efflux of compounds, including antimicrobials, from the bacterial cell (Chen et al., 2007). *Salmonella* Typhimurium has nine efflux pump systems, with each system having varying degrees of substrate specificity. RND (resistance-nodulation-division) pumps have the broadest substrate specificity and can export, among other things, many classes of antimicrobials. The broad substrate specificity of these pumps makes them of particular clinical importance in the development of the multi-drug resistant infections (White et al., 2005). Of the five *Salmonella* RND efflux pump systems, all of them are dependent upon TolC, an inner membrane protein (Horiyama et al., 2010). Two other pump components, AcrA and AcrB have also been identified as important players in broad spectrum drug efflux. Over-production of these proteins lead to a significant decrease in susceptibility to eight different antimicrobials, and inactivation of *acrB* and *tolC* in a multi-drug resistant strain of *S.* Typhimurium DT104 resulted in a 16-fold drop in resistance to chloramphenicol, tetracycline, and florfenicol, despite the presence of an intact *Salmonella* Genomic Island (Baucheron et al., 2004).

Three regulatory operons (*mar*, *sox*, and *ram*) have been identified in *Salmonella* that regulate the expression of a large number of genes, including genes that code for efflux pump proteins and membrane porin proteins. Efflux pumps are known to contribute to the intrinsic resistance of Gram-negative bacteria to some antimicrobial compounds, and as described above, play a role in multi-drug resistance when they are

over-expressed. Such over-expression has been tied to mutations within the regulatory operons.

The *mar* and *sox* operons were first identified in *E. coli* and mutations in the regulatory genes *marR* and *soxR* were directly attributed to the development of multi-drug resistance. It was therefore hypothesized that similar mutations in the homologous *Salmonella* genes would also confer a multi-drug resistant phenotype, but constitutive mutations in these two operons were determined to have only a minimal role in multi-drug resistance. A third *Salmonella* operon, *ram*, is absent in *E. coli* but shares much homology with both the *E. coli* and *Salmonella mar* operons (van der Staaten et al., 2004a; Barbosa and Levy, 2000).

The *mar* operon contains four separate coding sequences: *marRABC* (Figure 1-1). *marRAB* are co-transcribed from one promoter while *marC* is transcribed from a separate promoter.

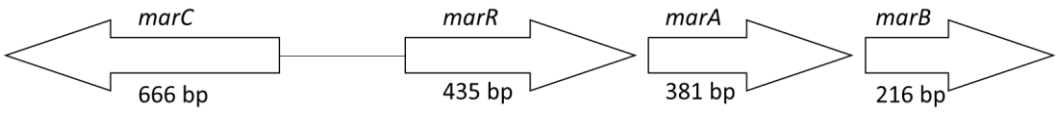


Figure 1-1. Diagram of the four genes present in the *mar* operon

Both promoters are contained within an intergenic region. The *mar* operon was initially identified in *Salmonella* Typhimurium after it was noted that there was a high level of sequence similarity to an operon previously characterized in *E. coli*. While the *marRA* region shares 91% sequence homology with its *E. coli* homolog, the *marB* gene is much more diverged, with only 42% sequence homology (Sulavik et al., 1997). The *marC* gene has no known function but is 91% homologous to *marC* from *E. coli* and shares additional homology with two hypothetical proteins in *E. coli*, as well as a

hypothetical protein in *Methanococcus jannaschii* (Alekshun and Levy, 1997). Despite the shared sequence identity, the functioning of the two operon regions seems to vary between bacterial species. Mutation in *marA* renders *E. coli* hypersensitive to tetracycline and chloramphenicol but has little effect on *Salmonella* sensitivity to these agents (Sulavik et al., 1997).

MarR negatively regulates the expression of *marRAB* through binding of the protein to a region just upstream of *marR* (Cohen et al., 1993; Martin and Rosner, 1995; Seoane and Levy, 1995). *marA* codes for a DNA-binding activator protein that competitively binds to the upstream region of *marR*, reducing MarR transcriptional repression (Martin et al., 1996). It is MarA that additionally aids in the regulation of many other genes, including those with virulence, efflux, and porin protein functions. MarA binds to a specific upstream region known as a marbox to positively activate transcription of these regulon genes (Gallegos et al., 1993; Sulavik et al., 1997).

A functional *marA* gene is required for inducible multiple antimicrobial resistance in *S.* Choleraesuis. When the gene is disrupted, exposure to low levels of salicylate fail to result in increases in MIC to tetracycline, chloramphenicol, nalidixic acid, and rifampin (Tibbets et al., 2005). It has been found that a decrease in *marA* expression, through deletion of the gene, is linked to a decrease in *acrB* expression, as well as a decrease in *soxS* expression (discussed below), along with a decrease in ciprofloxacin resistance (O'Regan et al., 2009).

It was recently discovered that MarB acts as a periplasmic protein, affecting the levels of MarA in the cell. When a stop codon was introduced into *marB*, MarA levels increased and complementation with the wildtype gene restored MarA to wildtype levels

(Vinue et al., 2013). While no known function has been identified for *marC*, its amino acid sequence indicates that it may be a membrane-bound transport protein and in one study, its presence was required for induction of fluoroquinolone resistance in *E. coli* (Goldman et al., 1996).

The *sox* operon, composed of two genes, *soxR* and *soxS* (Figure 1-2), is an important regulatory region, involved in response to oxidative and nitrosylative stress in the bacterial cell via a [2Fe-2S] cluster (Ding et al., 1996; Ding and Demple, 2000).

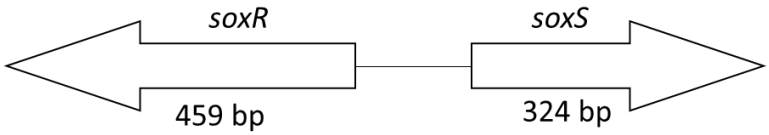


Figure 1-2. Diagram of the two genes present in the *sox* operon

Such stress activates SoxR, which then acts as a positive regulator of *soxS* expression. The SoxS protein then goes on to activate other genes responsible for protecting the cell from antimicrobial or oxidative attack. Among the genes activated by SoxS are those involved in down-regulation of porin expression and up-regulation of efflux pump expression (Chou et al., 1993; White et al., 1997). SoxS is responsible for the regulation of at least 15 genes in *S. enterica* (Pomposiello and Demple, 2000). Mutations in *soxR* can result in constitutive expression of *soxS*, increasing the positive regulatory effect that SoxR has on *soxS* expression. This in turn results in the development of ciprofloxacin resistance (Koutsolioutsou et al., 2001). It has also been shown that MarA and SoxS have overlapping regulatory functions and that in certain cases, the presence of both genes is required for proper regulatory functioning. MarA has been shown to enhance the affinity of SoxS for the promoter region of a minor porin protein gene *ompW* (Collao et al., 2013). The 459 bp *soxR* gene shares an intergenic region of 133 bp with the 324 bp *soxS*

gene (Kehrenberg et al., 2009). Despite its link to efflux pump expression in *E. coli*, only one study has been able to link constitutive mutations in *soxR* in *S. enterica* to the development of multi-drug resistance (Koutsolioutsou et al., 2001). Conversely, other studies have shown that an increase in SoxS is correlated with an increase in *acrB* expression and a decrease in *ompF* expression, similar to that seen with MarA (O'Regan et al., 2009).

The significant role of *ram* in the development of multi-drug resistance has only recently been elucidated, after it was determined that the *soxRS* locus was not the only regulatory operon involved in cellular protection against macrophages during host infection. The operon is composed of the two genes--582 bp *ramR* and 342 bp *ramA,* connected via a 288 bp intergenic region. This 288 bp intergenic region contains the promoters for both genes (Nikaido et al., 2008; Abouzeed et al. 2008) (Figure 1-3).

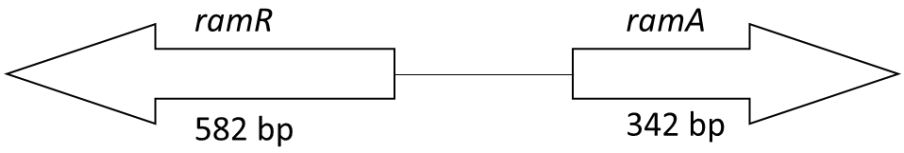


Figure 1-3. Diagram of the two genes present in the *ram* operon

In the absence of a functional *sox* locus, *ramA* is key to providing protection against oxidative agents (van der Straaten, 2004b). It was this discovery that led researchers to hypothesize that *ram* may also be involved in multi-drug resistance. The *ram* operon is 37% to 52% homologous to *marA* and *soxRS,* and RamA has the ability to bind to the marbox in *mar*-regulated genes in *E. coli* (van der Straaten et al., 2004a). The *ram* operon, while present in *Salmonella*, *Klebsiella,* and some *Enterobacter* species, is notably absent from *E. coli* (Chollet et al., 2004; George et al., 1995). RamA binds upstream of *acrAB* and *tolC,* and increased expression of *ramA,* via an inactivated RamR,

also up-regulates *acrAB* expression, providing evidence for RamA as a positive regulator of efflux pump expression. *ramR* acts as a local regulator of *ramA* expression. *ramA* is a global transcriptional activator and is a player in the expression of many other genes, as evidenced by the altered expression of over 220 genes when *ramA* was inactivated in serotype Typhimurium. Additionally, when *ramA* was over-expressed, more than 300 genes showed altered expression (Bailey et al, 2010).

Mutations in *ramR* can increase the expression of *ramA*, consequently over-inducing efflux pump expression. It has recently been noted that *ram* operon mutations have been implicated in the development of multi-drug resistance in *S. enterica* more frequently than mutations in *mar* and *sox* (Bailey *et al.*, 2010). Despite this evidence, seemingly contradictory findings have determined that deleting *ramA* in a set of clinical multi-drug resistant strains had little effect on the bacterial resistance profiles (van der Staaten, 2004a). Deletions in the promoter region of *ramA* or within *ramR* result in up-regulation of *ramA* (Zheng et al, 2009; Akiyama and Khan, 2011), which is associated with increased resistance to ciprofloxacin. In addition to the role of *ramA* in efflux pump expression, it was also found that inactivation of *ramA* affects the expression of genes associated with amino acid synthesis, glycolysis, ribosome synthesis, and decreased expression of genes involved in pathogenicity (Bailey et al, 2010).

As can be seen, the regulation of efflux-mediated multi-drug resistance is a complex issue and is most likely not regulated by one operon but potentially by all three of the operons discussed here, and much evidence speaks to the possibility of cross-talk between the activators and inducers of *mar*/Mar, *sox*/Sox, and *ram*/Ram.

1.3 Impact of Horizontal Gene Transfer

While the acquisition of exogenous antimicrobial resistance genes is one of the more obvious results of horizontal gene transfer in *Salmonella*, there exists evidence that horizontal gene transfer affects the bacterial cell through homologous recombination of genetic material. Homologous recombination among *S. enterica* strains was once thought be have a very limited impact on the population structure and the species was widely regarded as one of the few examples of a truly clonal bacteria. These assumptions were based mainly on the phylogenetic analysis of metabolic enzyme electrophoretic motility (MLEE) profiles and limited to a handful of serotypes (Reeves et al., 1989; Selander et al., 1990). Additional studies have shown that, in fact, recombination in *Salmonella* is more frequent and plays an important role in shaping the genetic relationships of closely related strains (Octavia and Lan, 2005; Brown et al., 2003; Didelot et al., 2011). Homologous recombination transfer events are difficult to identify, as the swapping of highly similar sequences between related organisms does not necessarily result in a discernible phylogenetic signal (Andam et al., 2011). In fact, it has been determined that for *S. enterica*, the frequency of horizontal gene transfer is increased between strains that are more closely-related (Brown et al., 2012; Brown et al., 2003; Didelot et al., 2011). This tendency to swap genes with close relatives, as opposed to those more diverged, is likely the result of similarities in replication, transcription, and translation mechanisms. Foreign DNA that is transferred to a bacterial cell would need to be recognized by the cellular functions in order to be successfully integrated and expressed. Additionally, niche separation places physical limitations on organisms that come into physical contact with one another (Andam et al., 2001; Didelot et al., 2011). It has also been noted that

bacterial strains with mutations in genes responsible for methyl-directed mismatch repair exist more frequently in environments with higher selective pressures, such as the food chain (Cebula et al., 2001). Such mutations increase the frequency with which homologous recombination occurs by relaxing the protective mechanisms that prevent integration of foreign DNA into the chromosome. These hyper-mutable phenotypes are estimated to be present in 1% of strains and can serve as a reservoir of novel mutations that may provide a fitness advantage under certain conditions or pressures (LeClerc et al., 1996, 1998). The identification of recombined alleles from a more homogenous population of bacteria requires comparison of gene trees, built from sequences of the locus in question, to a phylogeny built from whole-genome representative sequences. Discordance between the two phylogenies can be assumed to be the result of allelic shuffling between strains. It has been found that recombination between closely-related stains takes on a pattern of either assortive shuffling or, if the strains are from a more clonal lineage, a pattern of homogenization, wherein one or a few preferred alleles are harbored by strains of different serotypes (Brown et al., 2003, 2012). Importantly, it has been hypothesized that operons, clusters of genes with connected functionality, are the evolutionary product of horizontally transferred genetic material, since genes in close proximity to each other are more likely to be transferred to a donor as a single unit and would provide potential benefits to the donor only if all genes in the operon cluster were acquired (Lawrence and Roth, 1996).

1.4 SAR Collections

In the 1990s, three *Salmonella* reference collections were established from diverse serotypes of the genus *Salmonella*. The first collection, SARA, was established in 1991

by Beltran et al. and contains 72 *S. enterica* (Group I) isolates of five serotypes: *S.* Typhimurium, *S.* Saintpaul, *S.* Muenchen, *S.* Paratyphi B, and *S.* Heidelberg. The strains in this collection encompass 48 electrophoretic types, as determined by multi-locus enzyme electrophoresis (MLEE) of 24 chromosomally-coded, metabolic enzymes. They were selected for incorporation into this collection to represent the genomic variation of natural populations of *S.* Typhimurium and four other serotypes most closely related to it. Collectively, these five serotypes are known as "Typhimurium complex" strains (Beltran et al., 1991). The SARB collection was established in 1993 by E. F. Boyd et al. and contains 72 *S. enterica* isolates of 37 serotypes. Strains were selected for incorporation much the same way they were for SARA, based on multi-locus enzyme electrophoresis and are intended to represent the natural genetic variation of Group I *Salmonella* (Boyd et al., 1993). The SARC collection was established in 1996 by E. F. Boyd and is the most diverse of the three collections, containing representatives of the seven groups of the genus *Salmonella* (Boyd et al., 1996). The publicly available 16 strain set contains two strains from each Group but a larger, 96 strain set was also obtained from E.F. Boyd. All isolates in this larger collection were typed using MLEE and encompass 80 electrophoretic types. This last set represents the genetic variation in natural populations of the *Salmonella* genus.

1.5 Study Objectives

The aim of this study was to investigate the distribution, prevalence, and evolutionary transfer of antimicrobial resistance-conferring elements in *Salmonella* using three established *Salmonella* reference collections, which are considered to be a representation of the diversity of the *Salmonella* genus. Phenotypic characterization of

resistance profiles was conducted, along with genetic characterization of integron-associated genes. Further genetic characterization of genes harbored independent of integrons was conducted through utilization of a microarray chip containing all known resistance genes at the time of design. This information was mapped onto whole-genome representative phylogenetic data, to gain insight into the evolutionary mechanisms underpinning the acquisition of resistance-associated elements.

Additionally, three regulatory operons, which have been determined to be involved in efflux-mediated multi-drug resistance, were examined to determine the impact of horizontal gene transfer on the evolutionary history of the operon genes. A phylogenetic, tree-building approach was utilized, alongside tests of incongruence and incompatibility. Multi-locus sequence typing (MLST) data, taken as a representative of the whole chromosome, was used to build a phylogeny that was then compared to phylogenies built from genes that make up the individual operons. Incongruence between the MLST phylogeny and the gene trees would be indicative of the lateral transfer of DNA (Dykhuizen and Green, 1991). Secondly, strains from different genetic backgrounds will harbor identical alleles, as a result of allele swapping between strains that share overlapping niches. Recombination can also be assessed using measures of tree length incongruence (Brown et al., 2002) and analysis of network structures (Holmes et al., 1999). Genes that are impacted by horizontal gene transfer will often result in phylogenetic trees with poor bootstrap support as a result of reticulation in the evolutionary history, scrambling the inheritance of alleles, and this can be visualized with network graphs. Also of use were measures of pairwise site compatibility, obtained by comparing parsimoniously informative sites with each other to obtain a compatibility

matrix. Two sites were deemed incompatible when the substitutions at the sites require more than one step in a phylogeny to be accounted for (Jakobsen and Easteal, 1996). Here, this combination of methods was used to analyze the recombinatorial history of the three operon regions implicated in, among other functions, efflux-mediated multi-drug resistance.

2 Chapter 2: Materials and Methods

2.1 Reference Collections

The three SAR collections used in this study were obtained from the *Salmonella* Genetic Stock Centre at the University of Calgary, while the extended SARC collection was kindly provided by E.F. Boyd (University of Delaware). Any strains with documented discrepancies (Torpdahl and Ahrens, 2004; Porwollik et al.., 2004; Achtman et al.., 2012; Uzzau et al.., 1999) were eliminated. Additionally, some strains were duplicated across the SARA and SARB collections, which further reduced the strain number from the original 72 for each SARA and SARB. The extended SARC collection, because it was obtained from a long existing personal collection, was subjected to passes on differential xylose lysine deoxycholate agar and any strains that were visibly contaminated were eliminated from further study. The final subset of strains studied here consisted of 63 strains from SARA (Beltran et al., 1991), 50 strains from SARB (Boyd et al., 1993), 16 strains from SARC (Boyd et al., 1996), and 12 strains from the extended SARC. The SARA, SARB, and 16 strain subset of SARC are herein referred to by SGSC number while the remaining SARC strains from the extended collection are herein referred to by RKS number. The complete list of strains used can be found in Appendix Table 1.

2.2 Antimicrobial Susceptibility Testing

A phenotypic analysis of antimicrobial resistance was accomplished using a Sensititre broth micro-dilution system with CVM1-AGNF panels containing amikacin, ampicillin, amoxicillin/clavulanic acid, ceftriaxone, ceftiofur, chloramphenicol, ciprofloxacin, gentamicin, tetracycline, streptomycin, sulfisoxazole, nalidixic acid,

kanamycin, cefoxitin, and trimethoprim/sulfamethoxazole (Thermo Fisher Scientific, Trek Diagnostics, Cleveland, OH). Strains were grown overnight on 5% sheep's blood agar at 35°C for 16 hours. The agar plates were placed at 4°C until the inoculation of the antimicrobial plates. Colonies were selected from the blood agar plates and diluted into demineralized water. Turbidity was measured using a McFarland standard and the nephelometer included in the Sensititre AutoInoculator. 10 µL of this dilution was then added to 11 mL of pH adjusted Mueller-Hinton broth. This broth dilution was inoculated into Sensititre plates, using the AutoInoculator. Plates were incubated at 35°C for 16 hours before being read and interpreted using the Sensititre AutoReader and SWIN software system. Four widely used American Type Culture Collection (ATCC) control strains were used to validate all results: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212, and *Staphylococcus aureus* ATCC 29213. Established Clinical Laboratory Standards Institute guidelines and minimum inhibitory concentration breakpoints for broth micro-dilution were used to interpret results (CLSI, 2010). The MIC breakpoints used to determine cutoffs for resistance versus susceptibility are listed in Table 2-1. A streptomycin breakpoint is not provided by CLSI and as an alternative, the breakpoint of greater than or equal to 64 µg/mL used by the National Antimicrobial Resistance Monitoring System was utilized (NARMS, 2011).

Class	Drug	Interpretive Standard (μg/mL)		
		Susceptible	Intermediate	Resistant
Aminoglycosides	Amikacin	≤16	32	≥64
	Gentamicin	≤4	8	≥16
	Kanamycin	≤16	32	≥64
	Streptomycin	≤32	none	≥64
β-lactam/β-lactamase inhibitor	Amoxicillin-clavulanic acid	≤8/4	16/8	≥32/16
Cephems	Cefoxitin	≤8	16	≥32
	Ceftiofur	≤2	4	≥8
	Ceftriaxone	≤1	2	≥4
Folate Pathway Inhibitor	Trimethoprim-Sulfamethoxazole	≤2/38	none	≥4/76
	Sulfisoxazole	≤256	none	>256
Penicillin	Ampicillin	≤8	16	≥32
Phenicol	Chloramphenicol	≤8	16	≥32
Quinolones	Ciprofloxacin	≤1	2	≥4
	Nalidixic acid	≤16	none	≥32
Tetracycline	Tetracycline	≤4	8	≥16

Table 2-1. Minimum inhibitory concentration breakpoints for each drug studied, used to determine susceptible, intermediate, and resistant designations

2.2.1 Statistical Tests

Fisher's exact tests were executed on contingency tables of antimicrobial resistance data, using the XLSTAT statistical analysis package add-in (Addinsoft, France) for Microsoft Excel. Trends in resistance patterns over time were completed using the Mann-Kendall test, calculated using the MAKESENS Excel template (Salmi et al., 2002).

2.2.2 Hierarchical Clustering of Phenotypes

Hierarchical clustering of phenotypic antimicrobial resistance data, converted to binary characters (resistant=1, susceptible=0) was carried out using the XLSTAT statistical analysis package add-in (Addinsoft, France) for Microsoft Excel.

2.3 PCR and DNA Sequencing

2.3.1 Multi-locus Sequence Typing

An established seven-gene multi-locus sequence typing scheme (Kidgell et al., 2002) for *Salmonella* was utilized in order to establish a representation of the genomic background of each strain. The primer sequences used were obtained from http://mlst.warwick.ac.uk/mlst/dbs/Senterica and are listed below in Table 2-2. Genomic DNA was extracted using a Gentra Puregene Yeast/Bacteria Kit, from cultures grown on tryptic soy agar overnight at 37°C. The PCR conditions used for amplification were 1-10 ng DNA, 1 U Taq polymerase, 0.1 mM dNTPs, and 0.25 µM each primer in 1X buffer containing 1.5 mM $MgCl_2$ at a 50 µL total volume. Cycling conditions for all seven primer pairs were as follows: 95°C, 5 minutes; 30 cycles of 95°C for 1 minute, 55°C for 30 seconds, 72°C for 1 minute; 72°C for 5 minutes.

Primer Name		Primer Sequence (5'-3')
aroC	F	CCTGGCACCTCGCGCTATAC
	R	CCACACACGGATCGTGGCG
dnaN	F	ATGAAATTTACCGTTGAACGTGA
	R	AATTTCTCATTCGAGAGGATTGC
hemD	F	ATGAGTATTCTGATCACCCG
	R	ATCAGCGACCTTAATATCTTGCCA
hisD	F	GAAACGTTCCATTCCGCGCAGAC
	R	CTGAACGGTCATCCGTTTCTG
purE	F	ATGTCTTCCCGCAATAATCC
	R	TCATAGCGTCCCCCGCGGATC
sucA	F	AGCACCGAAGAGAAACGCTG
	R	GGTTGTTGATAACGATACGTAC
thrA	F	GTCACGGTGATCGATCCGGT
	R	CACGATATTGATATTAGCCCG

Table 2-2. Seven primer pairs used in multi-locus sequence typing

Purified PCR products were nucleotide cycle-sequenced in both directions using the Sanger dideoxy-chain termination method and the primers described above (MC Lab,

South San Francisco, CA). DNA Baser version 4 (Heracle BioSoft SRL) was used to assemble forward and reverse sequences into a single contig.

2.3.2 *Salmonella* Genomic Island, Integrase, and Integron Cassettes

Polymerase chain reaction was performed on all strains with primers specific to class 1 integrase and the conserved left and right junctions of the *Salmonella* Genomic Island. The primers used are listed in Table 2-3. The primers were previously designed and published (Boyd et al., 2000; Sandvang et al., 1998). Two *S.* Typhimurium DT104 strains were used as positive and negative controls, respectively: 96-5227 and S/921495 (Boyd et al., 2001). Any strains testing PCR-positive for *intI1* were further subjected to PCR to amplify the integron cassette. The PCR conditions used for amplification were 1-10 ng DNA, 1 U Taq polymerase, 0.1 mM dNTPs, and 0.25 µM each primer in 1X buffer containing 1.5 mM $MgCl_2$ at a 50 µL total volume. Cycling conditions were as follows: 95°C, 5 minutes; 30 cycles of 95°C for 1 minute, 55°C for 30 seconds, 72°C for 1 minute; 72°C for 5 minutes.

Primer Name	Target		Primer Sequence (5'-3')
intI1 F	class 1 integrase	F	CCT CCC GCA CGA TGA TC
intI1 R		R	TCC ACG CAT CGT CAG GC
int F	integron cassette	F	GGC ATC CAA GCA GCA AGC
int B		R	AAG CAG ACT TGA CCT GAT
U7-L12	SGI left junction	F	ACACCTTGAGCAGGGCAAAG
LJ-R1		R	AGTTCTAAAGGTTCGTAGTCG
C9-L2	SGI right junction, with retron	F	AGCAAGTGTGCGTAATTTGG
104-RJ		R	CTGACGAGCTGAAGCGAATTG
104-RJ	SGI right junction, without retron	F	CTGACGAGCTGAAGCGAATTG
104-D		R	ACCAGGCAAAACTACACAG

Table 2-3. Primer pairs used to amplify class 1 integrase gene, integron gene cassette, and left and right junctions of *Salmonella* Genomic Island

In order to identify the genes located within the integron cassette, purified PCR products were nucleotide cycle-sequenced in both directions using the Sanger dideoxy-chain termination method using the cassette primers described above (MC Lab, South San Francisco, CA). DNA Baser version 4 (Heracle BioSoft SRL) was used to assemble forward and reverse sequences into a single contig.

2.3.3 *mar*, *ram*, and *sox* Operons

Novel primers were designed in order to obtain sequences for the entirety of the three operons. *marRABC* was sequenced using five sets of overlapping primers, *ramRA* three sets, and *soxRS* two sets. The primer sequences and the respective anneal temperatures for each pair are listed in Table 2-4.

Primer Name		Primer Sequnce (5'-3')	Anneal Temp (°C)
mar1	F	AGA CAA ACG CTA ATT TCA GAC CAT GC	57
	R	ATT CTC TAT CTG GCG GAA CGC TAT GG	
mar2	F	GTG ATC CGG TAT TTG TGT GGC G	57
	R	GCG TAC TGG TGA AGC TAA CGC	
mar3	F	TAA TTC CTG ATG CAG GTC TTG CCC	57
	R	GCC ATT TCG CCA GTG TGC AAG TTA	
mar4	F	TGA ATT GGC CGA TGC CAC GAT TTG	57
	R	AAA CGC AAT ATT GGC CGT CGG TTC	
mar5	F	ATG AGT CGC CGG AAG CGA AA	57
	R	AAA TGG CCA GTG ACG CTG GAA GAA	
ram1	F	CGT GCA GTG TTT GAC CGT CCA TTA	48
	R	CGC AGG TGT TGC AGA AGG AAC ATT	
ram2	F	GCT CAT CTT TGG TCG CGA AAT AGC	48
	R	CCG CAC ATT TAC GGC AAC AGC AAT	
ram3	F	ATT GCT GTT GCC GTA AAT GTG CGG TG	48
	R	ATG TCA TTC GCT TTA TCT GGC GGC	
soxR	F	AGC GGT TGG TCG ATA TGT TC	56
	R	GCT GCG AAC GAG ACT GAT TT	
soxS	F	ATT CAT CGC CTG GCT ACA AC	56
	R	GCC GTT GGT TAC CGC TAT TA	

Table 2-4. Primer pairs used for amplification and DNA sequencing of segments of the three operon genes, listed with the anneal temperature used in PCR cycling

PCR cycling conditions and DNA sequencing were as described above for multi-locus sequence typing, with the exception of anneal temperature. DNA Baser version 4 (Heracle BioSoft SRL) was used to assemble overlapping operon sequences into a single contig.

2.4 Microarray Analysis of Resistance Genes

The FDA-SEEC microarray chip utilized here was developed and validated previously and commercially printed by Affymetrix with probes complimentary to unique regions of the selected genes, including antimicrobial resistance genes (Jackson et al., 2011). The FDA-SEEC array, in addition to 747 antimicrobial resistance elements, contains probes specific to four bacterial genera genomes: *Escherichia coli*, *Shigella* spp., *S. enterica*, and *Vibrio cholera*. The study here aimed to utilize the array for its ability to identify exogenous antimicrobial resistance genes. Any resistant phenotypes that are not the result of imported genes, such as quinolone resistance, which is commonly determined by point mutations in DNA replication mechanism genes, cannot be accounted for with this methodology. Probe target matches to antimicrobial resistance genes, compared against the database ResFinder (Center for Genomic Epidemiology, Denmark) (Zankari et al., 2012), are listed in Table A-8.

Genomic DNA (gDNA) was isolated using the DNeasy® Blood and Tissue Kit (Qiagen) following the manufacturer's protocol for Gram-negative bacteria, with the inclusion of a 10 minute incubation period at 65°C after the addition of Buffer A. After purification, the gDNA was concentrated using Centricon YM-30 columns. The final concentration was then determined using a NanoDrop® spectrophotometer. gDNA was

digested using DNase I at a concentration of 0.020 U/µl in 1X All-Phor-One buffer. For each digestion reaction, 10 µg of DNA was used in a final reaction volume of 40 µL. The samples were incubated at room temperature for 1 minute and then placed at 99°C for 15 minutes to stop the reaction. The digested gDNA was biotin labeled in a reaction containing 1X terminal deoxynucleotidyl transferase buffer, 37 µM biotin-11-ddATP, and 1.1 U/µL terminal transferase in a final volume of 54 µL. This reaction was then incubated at 37°C for 3 to 5 hours.

Hybridization of the digested and labeled gDNA to an FDA-SEEC array Affymetrix GeneChip® was carried as described in the GeneChip® Expression Analysis Technical Manual. Briefly, a 146 µL volume of hybridization buffer containing 1X hybridization buffer, 50 µM B2 control oligo, 0.1 mg/mL salmon sperm DNA, 0.5 mg/ml BSA, and 7.8% DMSO. The samples were vortexed and then centrifuged at 13,000 rpm for 30 seconds. This was followed by a 1 minute incubation at 98°C. Samples were once again centrifuged at 13,000 rpm for 5 minutes. The solution was then injected into a GeneChip® and the chips were incubated in a hybridization oven with 60 rpm rotation at 45°C for 16 hours.

After hybridizing, the hybridization solution was removed and replaced with 130 µL of Wash Buffer A. The chip was stored at 4°C until the washing step. Wash and stain solutions were prepared fresh the same day. The Affymetrix FS-450 fluidics station was also primed ahead of time with Wash Buffers A and B, using the protocol provided on the fluidics station. The chips were then placed into the fluidics station, with appropriate volumes of SAPE and antibody solutions in vials. The Mini_prok2v1 fluidics script was

then used to carry out the remainder of the staining and washing procedure. Afterwards, the chips were read with the GeneChip® Scanner 3000 7G running GCOS v1.4 software.

2.4.1 Probe Sets and Present/Absence Calls

Each gene represented on the array was detected using a combination of probe pairs--11 perfect match and 11 mismatch oligos. The mismatch oligos contain one base at the 13th position of the 25-mer that does not match with the sequence in question while the perfect match oligos contain 25-mers that are completely specific to the region in question. Present/absent calls of genes are determined using an algorithm based on a discrimination score, R. R is equal to the difference between the perfect match signal and the mismatch signal, divided by the sum of the perfect match signal and the mismatch signal.

2.5 Gene Characteristics and Evolution

2.5.1 MLST Phylogeny and Population Structure

Multiple-sequence alignment of MLST gene segments and operon genes were conducted using BioEdit version 7.2.5 (Hall, 1999). The MLST sequences obtained for each strain were given a unique numerical designation for each allele and for the combination of alleles, referred to as a sequence type, based on the publically available MLST database for *Salmonella enterica* (http://mlst.warwick.ac.uk/mlst/dbs/Senterica). These designations are listed in Table A-2. Aligned MLST nucleotide matrices were subjected to phylogenetic analysis using MEGA5. Phylogenetic trees were produced in MEGA5 using the maximum likelihood method. Model tests were run on each data set prior to tree building to best determine the appropriate model for the given sequence set. The initial tree for the heuristic search was obtained by applying the neighbor-joining

method. Support values were obtained by bootstrap iterations of 1000. The tree with the highest log likelihood was selected for further analyses (Tamura et al., 2011).

Population structure analyses on MLST sequences were performed using Structure version 2.3.4, using a Bayesian clustering method (Pritchard et al., 2000; Falush et al., 2003). This program uses allele frequency to identify admixture among a population, which results in members of a population deriving the overall genetic makeup from one or more subpopulations. This allows for a more intricate assessment of strain relatedness than a strictly phylogenetic approach would allow. Ten replicate runs were performed for each assumed number of populations, k=2 through k=10. The admixture model with correlated allele frequencies was used. For each independent run, 50,000 burn-in generations were followed by 100,000 generations. The estimated natural log likelihood was used to determine the k value that best fit the data.

Allelic profiles and single- and double- locus variant groups were generated using MLSTest version 1.0.1.23 (Tomasini et al., 2013). A grouping of strains was deemed a single-locus variant when they share six out of seven identical alleles. Similarly, double-locus variants shared five out of seven alleles.

2.5.2 Operon Gene Characterization and Compatibility

Sequence characterization statistics were obtained using DNAsp version 5 (Librado and Rozas, 2009). These statistics included nucleotide diversity values, GC content, polymorphic and parsimonious site identification, synonymous and non-synonymous mutation identification, and allelic diversity values (Bonetto et al., 2006).

MLSTest was used to conduct incongruence length difference (ILD) tests, using the BioNJ method with 1000 permutations (Zelwer and Daubin, 2004). The ILD test is a

statistical measure of congruence between data sets, measured by combining two data matrices into one larger data set and then generating two random submatrices that are the same size as the original sets. Tree length differences between the original data set and the randomly generated data set are statistically analyzed and a p value is generated, which is then used to determine whether or not to reject a null hypothesis of congruence. Intra- and inter-gene compatibility matrices were generated with version 5.2 of DAMBE (Data Analysis in Molecular Biology and Evolution). Parsimoniously informative sites were pairwise compared with each other and deemed compatible if they could be accounted for once in a phylogeny. The compatibility matrix data were then subjected to posterior analysis to obtain percentage values for each gene-to-gene and within-gene comparison (Xia and Xie, 2001).

MEGA5 (Tamura et al., 2001) was used to conduct a codon-based Z-test of purifying selection, averaged over all sequence pairs. Comparison of non-synonymous mutation to synonymous mutation was used to assess the impact of evolutionary pressure on a given sequence (neutral evolutionary pressure versus purifying selective pressure). Neutral evolutionary pressure would result in approximately equal non-synonymous and synonymous mutations while a purifying selective pressure would reduce the number of non-synonymous changes, due to the likelihood that an amino acid change would have a negative impact on protein functioning. The null hypothesis of strict-neutrality ($dN = dS$) and an alternative hypothesis ($dN < dS$) were used, dN and dS being the per-site numbers of non-synonymous and synonymous mutations, respectively. Values of p less than 0.05 were considered significant. The variance of the difference was computed using the bootstrap method with 1000 replicates. Analyses were conducted using the Nei-Gojobori

method (Nei and Gojobori, 1986). dN/dS ratio values for each operon gene were obtained using START2 version 0.9.0 (Jolley et al., 2001).

2.5.3 Reticulation in Operon Genes

NeighborNet (Bryant and Moulton, 2004) splits trees, used to visualize the reticulate nature of operon sequences, were produced using SplitsTree version 4.14.3 (Huson and Bryant, 2006; Huson, 1998). Character sets remained uncorrected. The Jaccard coefficient with unweighted pair-group average was used. Tanglegrams, used to compare phylogenies built from different sets of sequences through connected taxa, were created using Dendroscope version 3.5.7 (Huson and Scornavacca, 2012).

3 Chapter 3: Strain Metadata

3.1 Strain Source, Date, and Location of Isolation

The set of strains used here were collected over several decades. Eleven percent of strains (n=15) were collected in the 1960s or earlier, 10% of strains (n=14) were collected in the 1970s, 35% of strains (n=63) were collected in the 1980s and the remaining 45% of strains (n=49) have an unknown collection date. The collections, however, were assembled in the early 1990s, so all strains are at least as old as the respective collection.

The strains used here were also collected from a variety of sources. Forty-two percent of the strains (n=59) have a human or clinical source, 30% of the strains (n=42) had no source listed, 11% of the strains (n=15) were isolated from food animals, 9% of the strains (n=13) were isolated from companion animals, and the remaining were either isolated from food, a wild animal, an environmental source (including water or sewage) or had an unclear source listed.

In addition to source and dates of isolation, the strains also varied by the location from which they were collected. Forty-three percent of strains (n=61) were collected in the United States and Mexico, 35% of the strains (n=49) were collected in Europe, and the remaining (n=25) were collected in either the Middle East, Africa, Europe, Asia, Pacific, were a laboratory strain, or had an unclear or unknown location of collection. Three strains listed as being isolated in Georgia were considered to have an unclear location because it was not indicated if this was the country or the state, as was one listed as being isolated from the Canal Zone.

Of the strains collected from a human source, over one-third had unknown dates of collection (n=22), of a total of 60 collected. Of the 44 strains with unknown sources, an even larger percentage had unknown collection dates as well (n=27). Of the 56 strains collected in the United States, 18 (32%) had an unknown date of collection. Of the 48 strains collected in Europe, 12 (25%) had an unknown date of collection. Table 3-1 contains metadata, broken into number of strains, for each date and source or location.

Source	1953	1954	1958	1964	1965	1966	1967	1968	1972	1974	1976	1977	1978	1979	1981	1982	1983	1984	1985	1986	1987	1988	unknown	Total
Human	1		1		2			1	1	1	4			1	5	2		1	3	1	3	11	22	60
Wild Animal									1												1			2
Food Animal							1									1				5	7	1		15
Companion Animal						1					1	1							1	3	6			13
Environmental					2					1							1			1				5
Food											2													2
Unknown, other, or unclear		1		2	1		1	1					1						1	1		8	27	44
Location																								
US					1	1	2	1	1		1							1	5	10	15		18	56
Mexico, Central and South America														1								2	8	11
Africa				1											1	1					1		1	5
Middle East, Asia, and Pacific		1			2						1						1			1			4	10
Europe			1		2					2	5	1			4	2					1	18	12	48
Laboratory																							4	4
Unknown or unclear	1			1				1	1				1										2	7
Total	1	1	1	2	5	1	2	2	2	2	7	1	1	1	5	3	1	1	5	11	17	20	49	

Table 3-1. Strain metadata, by dates of collection and source or location

4 Chapter 4: Antimicrobial Phenotypes and Genotypes

4.1 Antimicrobial Susceptibility Testing

4.1.1 Resistance and Susceptibility

Antimicrobial susceptibility testing of the 141 strains revealed that 25.5% (n=36) were fully resistant or intermediately resistant to one or more of the 15 drugs in the Sensititre panel (Table S-2). Nearly 75% (n=105) were pan-susceptible to all drugs tested. Eight strains (5.7%) tested positive for intermediate resistance to at least one agent. One of these eight strains (strain SGSC 2213) was intermediately resistant to two different drugs, amikacin and amoxicillin/clavulanic acid. No strains were fully or intermediately resistant to cefoxitin, ceftiofur, ceftriaxone, or ciprofloxacin (Figure 4-1). Resistance to ampicillin, commonly used to treat salmonellosis in the 1980s (Smith et al., 1984), was found in 12 strains with no strain showing intermediate resistance to this drug. Resistance to chloramphenicol and trimethoprim/sulfamethoxazole, other drugs of choice for clinical treatment in prior decades (Rowe at al., 1997), were found in eight and five strains, respectively. One additional strain was intermediately resistant to chloramphenicol. Twelve strains were resistant to tetracycline, an antimicrobial commonly used in food animals (Landers et al., 2012).

The most common antimicrobial resistance phenotypes observed were sulfisoxazole and streptomycin resistance, with 15.6% and 14.2% of strains, respectively. Ampicillin and tetracycline resistance phenotypes were the next most common, representing 8.5% of all strains (n=12, each). Of strains resistant to one or more antimicrobials (24.8%, n=35), 62.8% and 57.1%, respectively, were resistant to sulfisoxazole and streptomycin. The least common resistant phenotypes were to

trimethoprim/clavulanic acid and nalidixic acid, found in 8.6% and 5.7% of strains, respectively. Of the 35 strains exhibiting full resistance to at least one drug, ten were resistant to four or more agents (28.6%), and seven (20%) were resistant to five or more antimicrobial agents. The majority of strains resistant to one or more drugs (51.4%, n=18) were resistant to two or fewer antimicrobials, while 71% were resistant to three or fewer.

The phenotypic resistance profiles of the 35 strains resistant to one or more antimicrobial agents were grouped according to the particular drug patterns. Twenty-five different patterns existed, with singular resistance to streptomycin being the most common (n=5). The next most common patterns, with three strains each, were singular resistance to sulfisoxazole and sulfisoxazole with trimethoprim/sulfamethoxazole resistance. Two additional strains, each, were resistant to the combination of streptomycin, sulfisoxazole, tetracycline and ampicillin, chloramphenicol, kanamycin, streptomycin, sulfisoxazole, tetracycline.

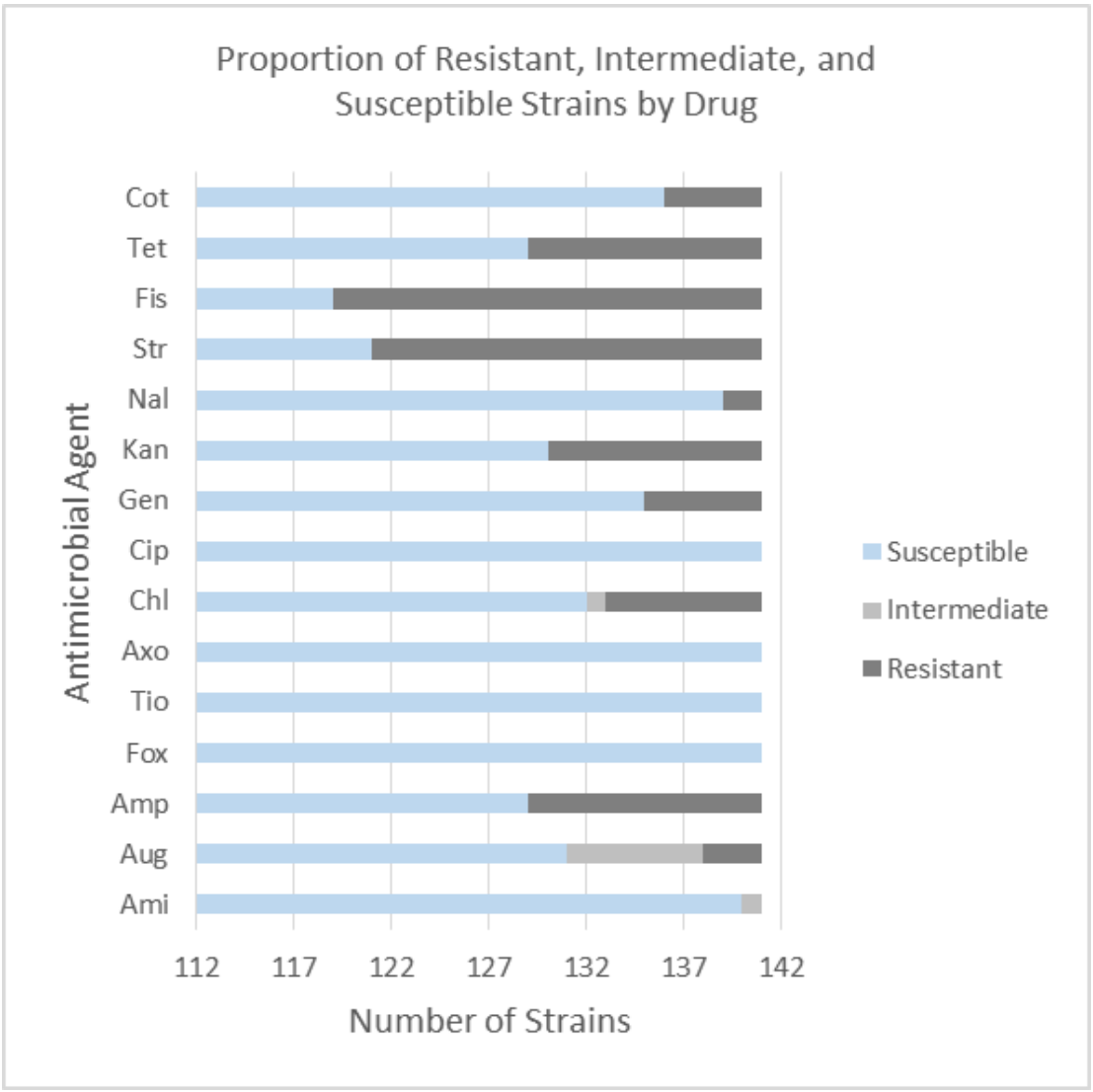


Figure 4-1. Bar graph displaying the number of resistant, intermediate, and susceptible strains, by drug tested. Cot: trimethoprim/sulfamethoxazole; Tet: tetracycline; Fis: sulfisoxazole; Str: streptomycin; Nal: nalidixic acid; Kan: kanamycin; Gen: gentamycin; Cip: ciprofloxacin; Chl: chloramphenicol; Axo: ceftriaxone; Tio: ceftiofur; Fox: cefoxitin; Amp: ampicillin; Aug: amoxicillin/clavulanic acid; Ami: amikacin.

4.1.2 Distribution of Minimum Inhibitory Concentrations

For amoxicillin/clavulanic acid, ampicillin, ceftriaxone, ciprofloxacin, kanamycin, tetracycline, and trimethoprim/sulfamethoxazole, the majority of the strains tested were inhibited by the smallest dose of drug tested. As CLSI does not have an established breakpoint, the NARMS breakpoint of 64 μg/mL was instead used. In certain

cases, a breakpoint of greater than or equal to 32 μg/mL has been determined to be a more appropriate measure of resistance (Tyson et al., 2016) but because the panel used here did not contain concentrations less than 32 μg/mL, it was not possible to distinguish lower values of MIC. Strains for the remaining agents--amikacin, cefoxitin, ceftiofur, chloramphenicol, gentamicin, nalidixic acid, and sulfisoxazole--were not inhibited by the smallest dose tested but remained susceptible. Table 4-1 lists the percentage of strains found to have an MIC at each breakpoint.

Antimicrobial Agent	Range tested (µg/mL)	0.02	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512
Amikacin	0.5-64						2	18	64	13	2	0	1	0			
Amoxicillin/clavulanic acid	1/0.5-32/16							76	16	0	1	5	2				
Ampicillin	1-32							81	11	0	0	0	9				
Cefoxitin	0.5-32						1	16	52	28	4	0	0				
Ceftiofur	0.12-8				3	3	14	76	4	0	0						
Ceftriaxone	0.25-64					100	0	0	0	0	0	0	0	0			
Chloramphenicol	2-32								4	45	45	1	6				
Ciprofloxacin	0.015-4	79	20	0	1	1	0	0	0	0							
Gentamicin	0.25-16					13	66	16	1	0	0	4					
Kanamycin	8-64										91	1	0	8			
Nalidixic acid	0.5-32						1	2	24	71	1	0	1				
Streptomycin	32-64												86	14			
Sulfisoxazole	16-256											13	33	37	1	0	16
Tetracycline	4-32									91	0	1	8				
Trimethoprim/sulfamethoxazole	0.12/2.38-4/76				92	4	0	0	0	4							

Table 4-1. Percentage of strains found to have a minimum inhibitory concentration value at each measured concentration, by antimicrobial agent. Total number of strains tested was 141. Black boxes indicate concentrations that were not tested. Gray boxes indicate the resistant breakpoint for each drug. Bold vertical lines indicate the intermediate resistance breakpoint, where applicable.

It has previously been reported that strains from natural populations can show increases in the average MIC over the course of decades (Houndt and Ochman, 2000). In order to determine if this was the case for the strains tested here, an unpaired t-test was used to compare the average MIC of susceptible strains from the 1980s to the remaining strains collected before 1980. Using a significant p value of less than 0.05, it was determined that there was a statistical difference between MICs for streptomycin and sulfisoxazole. Additionally, an MIC increase that broached significance ($p < 0.1$) over time was noted for amoxicillin/clavulanic acid. No statistical difference was detected for any of the other drugs tested. In order to further examine changes in MICs over time, the Mann-Kendall test was used to identify the presence of upward trends in the concentrations at which strains were inhibited by each drug. Those strains for which date of isolation information was available were included in the analysis. When strains with MICs that deemed them resistant to a particular drug were included in the calculations, a positive upward trend over time was identified for ampicillin, kanamycin, streptomycin, sulfisoxazole, tetracycline, and trimethoprim/sulfamethoxazole, a trend also noted by others for feral enteric bacteria populations over time (Houndt and Ochman, 2000). When resistant strains were excluded from the analysis, a positive upward trend was identified for sulfisoxazole, indicating that even though the MICs were still low enough to render the strains sensitive to the drug, a subtle, yet meaningful, increase was identified in the lowest level of drug required to inhibit growth.

4.1.3 Association with Source, Location, Date, and SAR Collections

Table 4-2 contains dates of isolation, source, and location metadata for each strain resistant to one or more antimicrobials.

Strain no.	Serotype, Subspecies, or Species	Date	Source	Location	Ami	Amp	Aug	Gen	Kan	Str	Fis	Cot	Chl	Tet	Nal	No. Resistant Phenotypes
2997	ssp. *salamae*	1965	Human	California							■					1
2241	Paratyphi B	1974	Water	Scotland									▒			0
2230	Paratyphi B	1976	Food	Middle East						■						1
3045	*S. bongori*	1977	Lizard	United Kingdom						■						1
2239	Paratyphi B	1981	Human	France		■			■							2
2470	Dublin	1982	Bovine	France						■						1
3025	ssp. *houtenae*	1986	Vacuum cleaner	Guam							■					1
3086	ssp. *houtenae*	1986	Human	Illinois							■					1
2184	Typhimurium	1986	Rabbit	Indiana		■	▒				■					2
2467	Derby	1986	Swine	Minnesota						■	■			■		3
2469	Dublin	1986	Cattle	Idaho		■	▒			■	■			■		4
2468	Derby	1986	Turkey	Pennsylvania				■	■	■	■			■		5
2244	Muenchen	1986	Cow	Kentucky		■	▒		■	■	■		■	■		6
3068	ssp. *diarizonae*	1987	Human	Oregon						■						1
2189	Typhimurium	1987	Parrot	California							■	■				2
2190	Typhimurium	1987	Opposum	California						■	■					2
2217	Heidelberg	1987	Turkey	Colorado					■	■				■		3
2516	Senftenberg	1987	Chicken	Maryland				■		■	■					3
2218	Heidelberg	1987	Turkey	Arizona				■	■	■	■					4
2245	Muenchen	1987	Chicken	Florida		■	■		■	■	■					5
2225	Paratyphi B	1988	Cow	France											■	1
2479	Haifa	1988	-	Scotland							■	■				2
2482	Indiana	1988	-	Scotland							■	■				2
2206	Saintpaul	1988	Human	France		■				■	■			■		3
2517	Stanley	1988	-	Scotland						■	■			■		3
2207	Saintpaul	1988	Human	France		■	▒			■	■	■	■			4
2208	Saintpaul	1988	Human	France		■	▒		■	■	■		■	■		6
2528	Wien	1988	Human	France		■	■	■	■	■	■	■	■	■		7
2187	Typhimurium	-	-	Norway										■		1
2197	Typhimurium	-	-	Yugoslavia						■						1
2215	Heidelberg	-	-	Brazil		■	▒									1
2214	Heidelberg	-	-	Israel									■	■		2
2185	Typhimurium	-	-	Mongolia						■	■				■	3
2219	Heidelberg	-	Human	North Carolina							■		■	■		3
2213	Heidelberg	-	Human	Mexico	▒	■	▒	■	■	■	■		■	■		7
2494	Newport	-	Human	Mexico		■	■	■	■	■	■		■			7
No. Exhibiting Full Resistance					0	12	3	6	11	20	22	5	8	12	2	

Table 4-2. Strains exhibiting intermediate or full resistance to one or more antimicrobials, in order of ascending date of isolation. Black boxes indicate full resistance while gray boxes indicate intermediate resistance. Metadata identifying serotype, source, location and date of isolation are included.

Of the strains with known isolation dates, strain RKS 2997 was the oldest in the strain set used here to be resistant to at least one drug. This strain, subspecies salamae, was isolated in 1965 from a human source in California and was resistant to sulfisoxazole. This was the only strain from the 1960s that tested positive for any resistance. The remaining 12 strains isolated in the 1960s were pan-susceptible to the antimicrobials tested here.

Moreover, strains SGSC 2230 and RKS 3045 were the only two, out of a total 14 isolated in the 1970s, resistant to any agent. Both were streptomycin resistant but susceptible to all other agents. Twenty-four strains collected during the 1980s were resistant to one or more drugs, out of a total of 63 (38.1%). The remaining 39 strains from the 1980s were pan-susceptible. Of the 49 strains with unknown collection dates, nine were resistant to one or more drugs (18.4%). All strains resistant to three or more agents with a known source were isolated from either humans or food animals. All strains with known collection dates that were resistant to two or more agents were isolated in the 1980s, while all strains with any resistant phenotype that were isolated before the 1980s were resistant to only one agent.

For the 97 strains for which source data was available, Fisher's exact test was used to determine whether there existed a statistical correlation between the type of source of the strains (human, food animal, companion animal, environmental, wild animal, or food) and the presence of one or more resistant phenotypes. Strains from a food animal source were more likely to be resistant to one or more drugs ($p < 0.05$). Strains from a food animal source were also more likely to be resistant to three or more drugs ($p < 0.05$), but the correlation did not hold when strains resistant to four or more drugs were considered ($p > 0.1$). No other statistical correlations were found for resistance and other source type. For the 135 strains for which location of isolation data existed, Fisher's exact test was used to determine the presence or absence of a statistical correlation to the number of resistant phenotypes. No correlation for strains that were resistant to one or more drugs was found, nor was any correlation for strains resistant to four or more drugs found.

For the 92 strains for which date of collection information existed, an examination was made to ascertain correlation between period of collection (1950s, 1960s, 1970s, 1980-1985, and 1986-1989) and the presence of resistance to one or more drugs. A strong correlation was found between an isolation date from 1986 to 1989 and resistance to one or more agents ($p < 0.01$). The correlation between a date of isolation between 1986 to 1989 holds when strains resistant to three or more antimicrobials were considered ($p < 0.05$) No statistical support for a correlation between SAR collection (A, B or C) and the presence of resistance to one or more antimicrobials or resistance to four or more antimicrobials was found ($p > 0.1$).

4.1.4 Strains Grouped by Structure MLST

To gain a clearer picture of how resistance was distributed among strains and across the SARA and SARB collections, resistance patterns were analyzed against subpopulations determinations made using MLST data (Figure 4-2), analyzed with the program Structure (Pritchard at al., 2000). This program allows for a more detailed representation of the ancestral makeup of individuals from a population than is provided by the distance-based maximum likelihood phylogeny. Subpopulations present among a larger population were identified through Bayesian clustering of allele frequencies and each strain received an assignment to one or more subpopulations. Multiple subpopulation assignment was indicative of admixture, or recombination, between individuals. Due to an inability to obtain complete MLST sequences for all strains from the SARC collection using the primers listed here, they were eliminated from further analyses. Using both the phylogeny built from MLST data and the Structure subpopulation assignments, resistant phenotypes were plotted against the genetic data

representative of each strain in the *S. enterica* strains studied here. A total of eight subpopulations were identified and are indicated by group numbers in Figure 4-2.

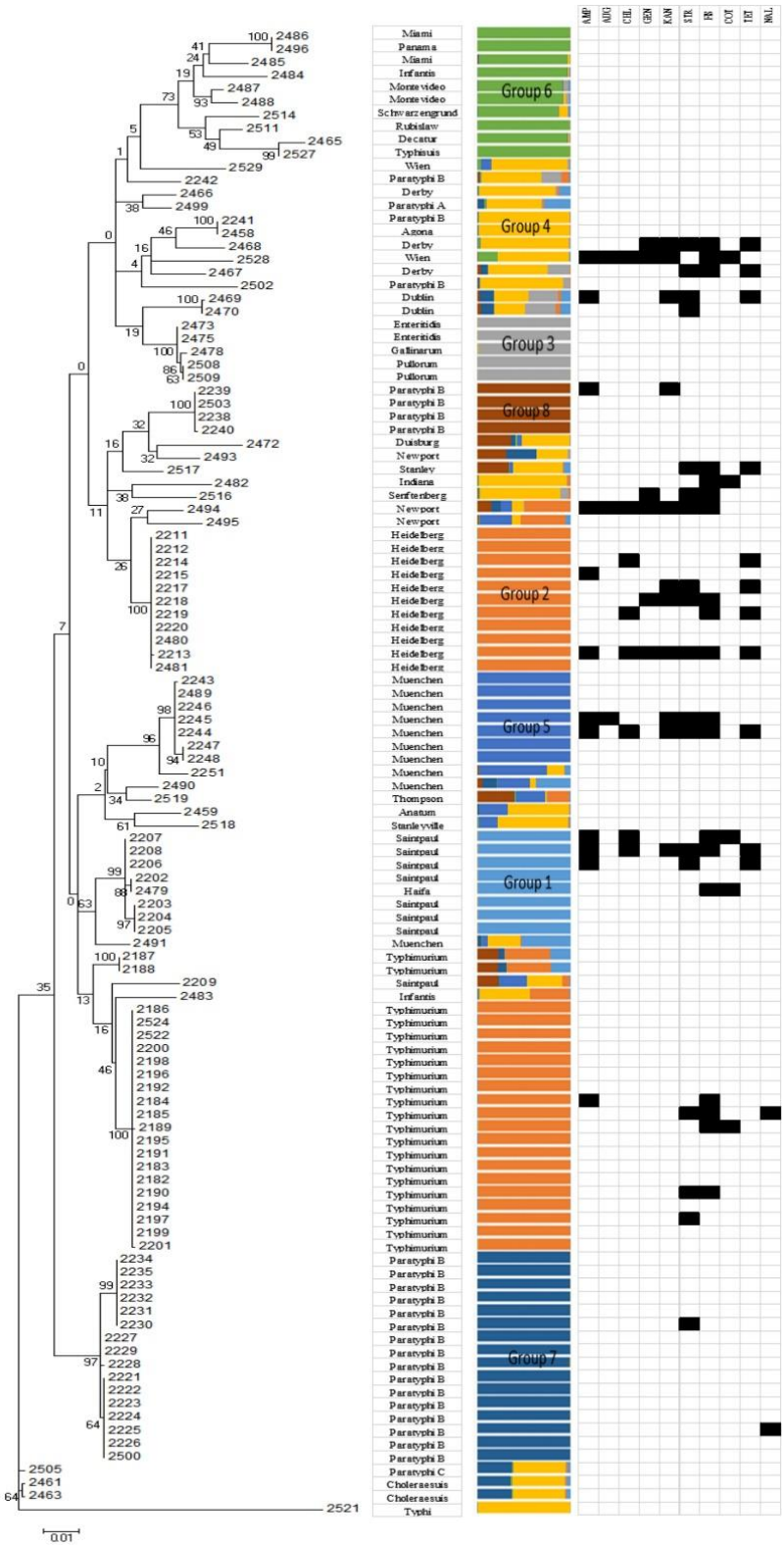


Figure 4-2. Maximum likelihood phylogeny of SARA and SARB, aligned with Structure subpopulation designations, group assignments based on Structure subpopulations, and an antibiogram table. Phylogeny was built using concatenated MLST data for each strain. Structure output for k=8 populations for SARA and SARB strains is shown as colored rectangles, with the eight subpopulations

indicated as groups and each population assigned a unique color (Group 1, light blue; Group 2, orange; Group 3, gray; Group 4, yellow; Group 5, medium blue; Group 6, green; Group 7, dark blue; Group 8, red). Strains derived from multiple subpopulations are aligned next to rectangles containing two or more colors, with color bar size proportional to percentage of subpopulation. Black boxes in the antibiogram indicate presence of resistance, by antimicrobial agent. Single locus variants are outlined in orange boxes on the phylogenetic tree.

Each group was assigned a unique color. Strains derived from multiple subpopulations were aligned next to rectangles containing two or more colors, with color bar size proportional to the percentage of subpopulation membership. Strains with multiple group memberships were assigned to the group from which the highest percentage of its makeup was derived. Strains from Groups 2 (orange) and 4 (yellow) failed to cluster together on the phylogenic tree, with strains from each group falling into different clades. This may be the result of conflicting phylogenetic signals in the MLST data, resulting in an inability to determine the precise placement of all strains that make up these two groups.

The highest number of individual antimicrobial resistance phenotypes, per strain, was found in Group 1, which was composed primarily of Saintpaul strains. A total of 15 resistant phenotypes were present in Group 1, distributed among 10 strains (7.1% of all strains), representing 15.6% of the total number of antimicrobial resistant phenotypes identified. Forty percent of the strains in Group 1 were resistant to one or more drugs, the highest percentage among the eight groups. Twenty percent were resistant to four or more drugs, second only to Group 5, for the percentage of multi-drug resistant strains making up the group. For ampicillin, sulfisoxazole, trimethoprim/sulfamethoxazole, chloramphenicol, and tetracycline, the highest percentage of strains exhibiting resistance were from Group 1. In Group 2, composed of a mix of Typhimurium, Heidelberg and Newport strains, 38.2% were resistant to one or more agents. This group accounted for 39.6% of all antimicrobial resistance phenotypes identified while representing 24.1% of

the total strains. Of the strains in Group 2, at least one was resistant to each drug for which a resistant phenotype was identified in the total population. No resistant phenotypes were identified in Group 3, made up of Gallinarum, Enteriditis and Pullorum strains. Thirty-two percent of Group 4 strains, a mix of serotypes which included Wien and Derby, were resistant to one or more drugs while 12.0% were resistant to four or more. The total number of antimicrobial resistant phenotypes accounts to 29.2% of those identified over all groups. Group 5, made up of eight serotype Muenchen strains, contained the highest percentage of multi-drug resistant strains (25%). The two multi-drug resistant strains were resistant to five and six antimicrobials, respectively, with both exhibiting resistance to ampicillin, kanamycin, streptomycin, and sulfisoxazole. This group accounted for 11.5% of all resistant phenotypes while representing 5.7% of strains, the second highest phenotype-to-strain ratio of the eight groups. Group 6, composed of 15 strains of a variety of serotypes, including Montevideo and Miami, exhibited no antimicrobial resistant phenotypes. Two strains (12.5% of the total strains) in Group 7, comprised of solely serotype Paratyphi B, were resistant to one agent each. Resistant phenotypes in this group accounted for only 2.1% of the total number of resistant phenotypes. The single resistant strain in Group 8 (a second set of primarily serotype Paratyphi B) was resistant to two drugs and accounted for 2.1% of all resistant phenotypes. Multi-drug resistant strains were found in Groups 1, 2, 4, and 5. Of the three strains resistant to seven drugs, two of them were from Group 2, with the remaining strain from Group 4. 92 out the 96 antimicrobial resistant phenotypes were found in strains from Group 1, 2, 4 or 5. The total number of strains represented by these four groups was 77 (54.6% of the total number). Using Fisher's exact test to determine the

presence of a correlation between group membership and resistance to one or more antimicrobials, it was determined that strains from Group 2 were proportionally less pan-susceptible, and strains from Groups 3 and 6 were proportionally less resistant to one or more agents ($p < 0.05$). Strains from Groups 3, 6, 7, and 8 were proportionally less resistant to four or more agents while strains from Group 5 were proportionally less resistant to three or fewer agents ($p < 0.05$).

4.1.5 Antimicrobial Resistance Phenotype Associations and Clustering

Fisher's exact test was used to identify associations between multi-drug resistance and individual drugs. When multi-drug resistant strains were considered to be those resistant to four or more drugs, a statistical correlation was noted between the presence of multi-drug resistance and individual resistance to ampicillin, amoxicillin/clavulanic acid, chloramphenicol, gentamicin and kanamycin ($p < 0.05$). Gentamicin resistance was only seen in strains resistant to a total of three or more antimicrobials, and in all but one case, was accompanied by resistance to streptomycin. Amoxicillin/clavulanic acid resistance was only seen in strains resistant to a total of five or more drugs.

In order to better visualize the clustering of antimicrobial resistant phenotypic patterns and compare these to the source and location of strains, hierarchical clustering was carried out on a matrix of phenotypic data, converted to binary characters, with resistance being equal to 1 and susceptible being equal to 0. Five major clusters were identified (Fig. 4-3).

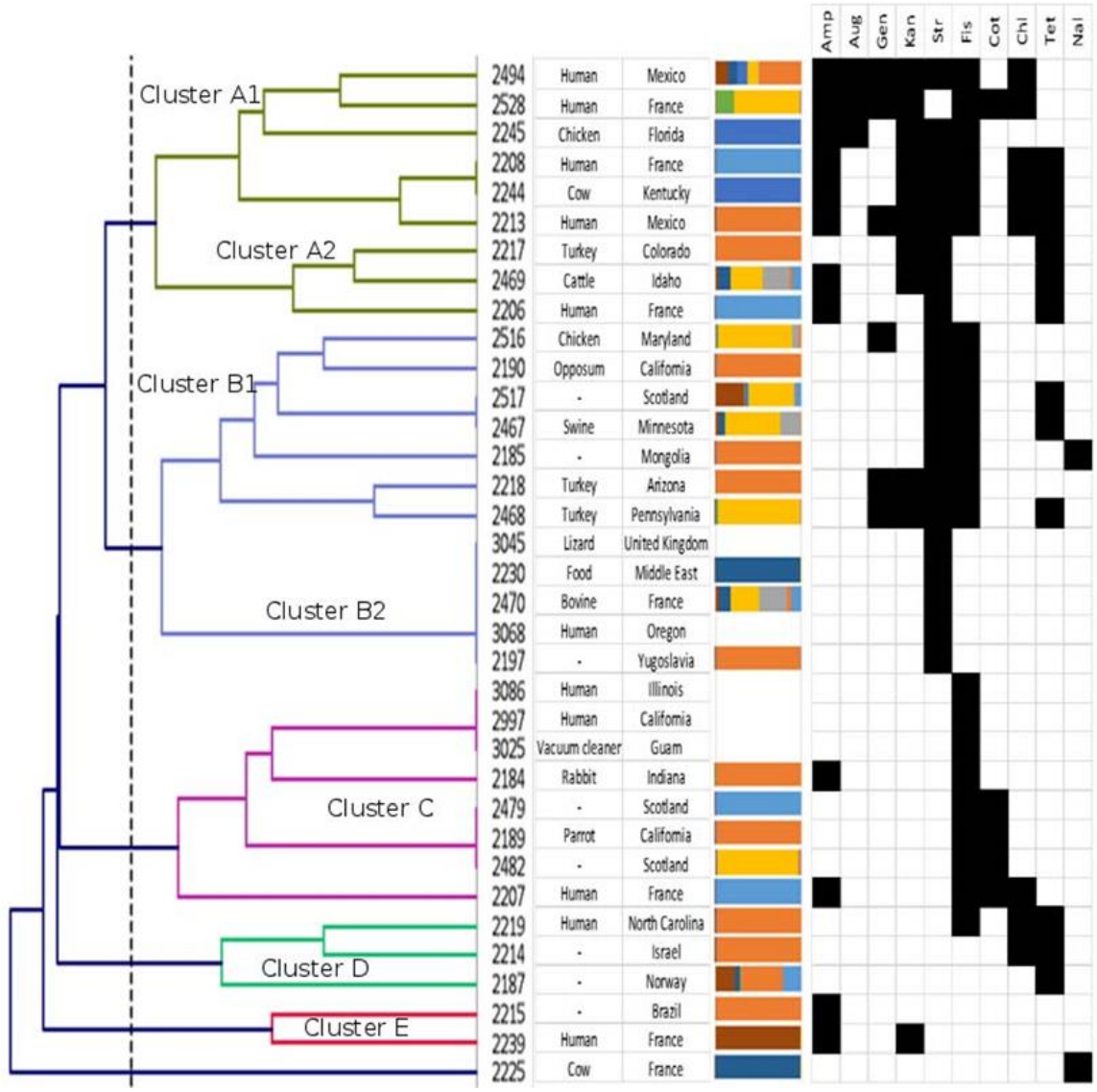


Figure 4-3. Hierarchical clustering dendrogram, produced using phenotypic resistance data, converted to binary characters (resistance=1, susceptible=0). Taxa are aligned next to source and location of isolation metadata, population Structure determinations, and an antibiogram indicating phenotypic resistance, by drug (black squares indicate resistance and white squares indicate susceptibility). The dotted line depicts the clustering dissimilarity cutoff value, determined by the clustering algorithm.

Cluster A was made up of nine strains primarily resistant to chloramphenicol, ampicillin and kanamycin. All amoxicillin/clavulanic acid resistant strains were present in Cluster A. All strains displaying full or intermediate resistance to amoxicillin/clavulanic acid were also resistant to ampicillin. Positive correlations between ampicillin and amoxicillin/clavulanic acid; ampicillin and chloramphenicol; and

ampicillin and kanamycin resistances were detected using Fisher's exact test ($p < 0.05$). A weakly significant statistical correlation was detected between the presence of chloramphenicol and kanamycin resistance ($p < 0.1$). No strain in this cluster was resistant to less than three antimicrobial agents. Cluster B was comprised of 12 strains, all resistant to streptomycin, with two strains demonstrating co-resistance to gentamicin and kanamycin, for which there was a statistically significant positive correlation ($p < 0.05$). The eight strains in Cluster C were characterized by resistance to sulfisoxazole, with the number of total resistance phenotypes ranging from one to four. Cluster D was characterized by resistance to tetracycline, with two out of the three members of this cluster also resistant to chloramphenicol. There existed a weakly significant correlation between these two phenotypes ($p < 0.1$). The two strains in Cluster E were both resistant to ampicillin, with one of these also resistant to kanamycin, for which a correlation was previously mentioned. Strain 2225, resistant to only nalidixic acid, remained un-clustered. Of the five clusters identified, four contained at least one strain from a human source while food animal-associated strains were only found in Clusters A and B. Phenotype clustering did not correlate to group membership or location of isolation, with strains from different continents represented in each clade.

Among the strains resistant to one or more antimicrobials, those exhibiting ampicillin resistance were most likely to be associated with a human source ($p < 0.05$), with five of the seven ampicillin-resistant, human-isolated strains contained within Cluster A. Also more likely to be associated with a human source were those resistant to chloramphenicol ($p < 0.05$). Four out of six chloramphenicol-resistant human isolates were found in Cluster A. These correlations echo the phenotypic drug-to-drug

correlations mentioned above, as well as drug-to-MDR phenotype correlations. Strains resistant to one or more antimicrobials and from a food animal source were more likely to be resistant to streptomycin or kanamycin ($p < 0.05$). Out of the ten food-animal associated strains, nine were resistant to streptomycin while six were resistant to kanamycin. Streptomycin and kanamycin resistance grouped with Clusters A and B, and all but one food animal isolated strain clustered similarly.

NeighborNet analysis of the binary phenotype data revealed further the reticulate nature of antimicrobial resistance patterns (Figure 4-4). Many parallel paths between strains were indicative of the conflicting phylogenetic signal produced by presumably the horizontal transfer of resistance determinants between taxa. Strains that clustered together under hierarchical analysis, in some cases, failed to cluster together on the splits tree, indicating that similarity of phenotype did not necessarily correlate to reticulation groupings.

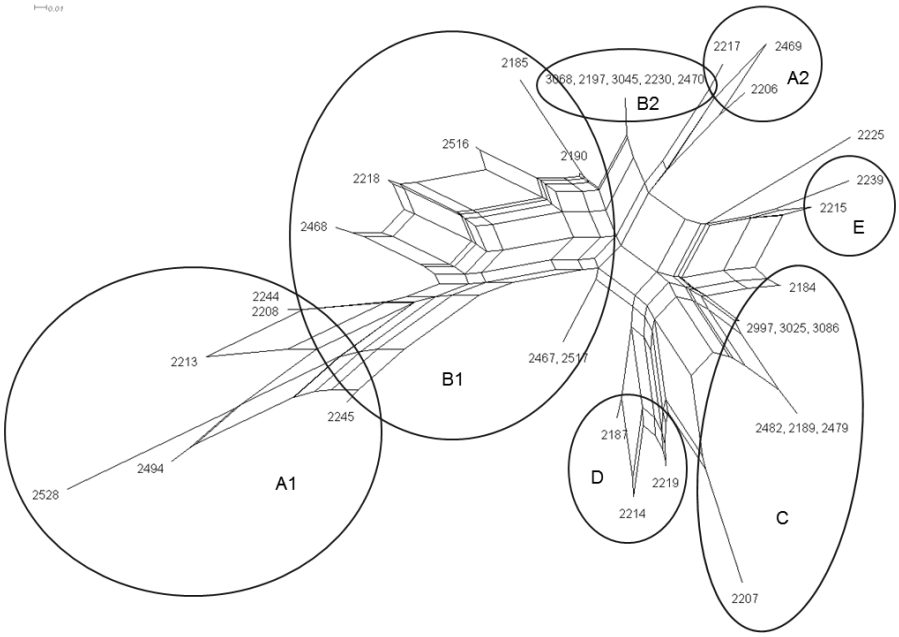


Figure 4-4. NeighborNet graph of binary resistance profiles

Splits tree diagram was produced using binary resistance data for each strain exhibiting resistance to one or more drugs. Cluster groupings, as determined above, are outlined with black circles and the corresponding letter designations.

4.2 Integrase, Integron Cassettes and *Salmonella* Genomic Island

4.2.1 Class 1 Integrase

Using the primers and conditions described in Materials and Methods, *intI1* was found to be present in 9.93% of strains (n=14). Nine strains from the SARA collection tested PCR-positive, as well as five strains from the SARB collection. No strains from SARC tested positive for a class 1 integrase gene. The *intI1* gene was only found in strains resistant to two or more antimicrobials. Table 4-3 lists the percent of resistant phenotypes in strains with and without a class 1 integrase gene.

	intI-positive (n=14)	*intI-negative* (n=127)	Fisher's p-value
Gentamicin	35.71%	4.76%	**0.028**
Kanamycin	50.00%	19.05%	*0.060*
Streptomycin	64.29%	52.38%	0.365
Amoxicillin-clavulanic acid	14.29%	4.76%	0.348
Trimethoprim-Sulfamethoxazole	28.57%	4.76%	*0.071*
Sulfisoxazole	100.00%	38.10%	**0.000**
Ampicillin	42.86%	28.57%	0.477
Chloramphenicol	42.86%	9.52%	**0.030**
Nalidixic acid	0.00%	9.52%	0.353
Tetracycline	42.86%	28.57%	0.304
Resistant to three or more	78.57%	28.57%	**0.006**
Resistant to four or more	57.14%	9.52%	**0.006**
Resistant to five or more	42.86%	4.76%	**0.010**

Table 4-3. Percent of resistant phenotypes in strains with and without class 1 integrase Significant p values (< 0.05) are highlighted in bold while weakly significant p values (< 0.10) are italicized.

Of the strains that were PCR-positive for a class 1 integrase gene, 100% (n=14) were resistant to sulfisoxazole, 50% (n=7) were resistant to kanamycin, 64.29% (n=9) were resistant to streptomycin, and 42.86% (n=6) were resistant to ampicillin,

chloramphenicol, and tetracycline. Resistance to sulfisoxazole was presumably due to the presence of *sul1* on the 3' conserved end of the class 1 integron. The presence of *intI1* statistically correlated to the phenotypic resistance of two or more drugs, four or more drugs, and five or more drugs ($p < 0.05$). Of the strains resistant to four or more agents, only two strains (2469 and 2494) did not contain *intI1*. 2494 was PCR-negative for a class 1 integrase gene but was resistant to a total of seven agents tested. Interestingly, this strain tested positive for the *qacEdelta*/*sul1* region in the microarray testing (see below). It is possible that the strain contained integron cassettes but underwent a deletion event of *intI1* at some point after acquisition of the cassettes. Of the 14 strains testing positive for *intI1*, 78.57% (n=11) were resistant to three or more drugs, 57.14% (n=8) were resistant to four of more drugs and 42.86% (n=6) were resistant to five or more.

The presence of a class 1 integrase gene was found to have a positive correlation to phenotypic resistance to chloramphenicol, gentamicin, and sulfisoxazole. While not all sulfisoxazole resistant strains harbored a class 1 integrase gene (38.10% of *intI1*-negative strains were sulfisoxazole resistant), there was a statistical correlation ($p < 0.05$) between the presence of sulfisoxazole resistance and *intI1*. Only one strain that was gentamicin resistant failed to test PCR-positive for a class 1 integrase, but as mentioned above, this strain did test positive through microarray for the 3' conserved end of an integron cassette, indicating that it may have at one point harbored a complete cassette. Two additional drugs, kanamycin and trimethoprim/sulfamethoxazole, approached significance ($p < 0.1$) when tested for a correlation to the presence of *intI1*. No correlation was found between the presence of *intI1* and the source of the strain.

4.2.2 Integron Cassettes

aadA1 was the most commonly identified integron cassette gene, found in 11 out of the 14 strains. Four strains had cassettes that contained a *dhfr* gene, responsible for trimethoprim resistance. Two strains contained *oxa-1*, a beta-lactamase gene, responsible for ampicillin resistance. Strain 2528 contained an erythromycin resistance gene not found in any other strains tested (Table 4-4).

Strain no.	Serotype	Integron Cassette (5'-3')	BLAST Match	Ami	Amp	Aug	Gen	Kan	Str	Fis	Cot	Chl	Tet	Nal	No. Resistant Phenotypes
2189	Typhimurium	*dhfr1 aadA1*	LN794247 Typhimurium pSBLT												2
2190	Typhimurium	*aadA1*	CP012931 Heidelberg pN13-01290_23												2
2479	Haifa	*dfrA16*	*E. coli* pEC448_OXA163 CP015078												2
2219	Heidelberg	*aadA1*	CP016585 Heidelberg pSH14-009_99												3
2516	Senftenberg	*aadA1*	CP016585 Heidelberg pSH14-009_99												3
2517	Stanley	*aadA1*	CP016585 Heidelberg pSH14-009_99												3
2207	Saintpaul	*dhfr1 aadA1*	LN794247 Typhimurium pSBLT												4
2218	Heidelberg	*aadB aadA2*	Bovismorbificans strain HP507391												4
2245	Muenchen	*oxa-1 aadA1*	Typhimurium strain 18-425 JN003856												5
2468	Derby	*aadA1*	CP016585 Heidelberg pSH14-009_99												5
2208	Saintpaul	*aadA1*	CP012931 Heidelberg pN13-01290_23												6
2244	Muenchen	*oxa-1 aadA1*	Typhimurium strain 18-425 JN003856												6
2213	Heidelberg	*aac(6')33 aadA1*	novel												7
2528	Wien	*dfrA5 ereA2*	AY827837 Wien												7
Total No. Exhibiting Full Resistance				0	6	2	5	7	9	14	4	6	6	0	

Table 4-4. Strains which contained a class 1 integrase gene and the genes present in the corresponding integron cassette. Black boxes indicate full resistance and gray boxes indicate intermediate resistance.

Strain 2189, despite carrying an *aadA1* gene in an integron cassettes, did not present with phenotypic resistance to streptomycin, one of the two drugs for which it encodes resistance. Spectinomocin, the other drug, was not among the drugs in the panel that were tested. The strain did however exhibit resistance to trimethoprim/sulfamethoxazole, resistance to which is encoded by *dhfr1*, the other gene in the integron cassette. Strain 2207 also carried *aadA1* and similarly did not exhibit phenotypic resistance to streptomycin but did exhibit resistance to trimethoprim/sulfamethoxazole. It is possible that the streptomycin MIC breakpoint used here, which is an unofficial breakpoint used by the NARMS program, does not properly account for varying levels of resistance. It has been found that a 32 μg/mL breakpoint is more appropriate, as it increases the correlation between genotypic resistance and phenotypic resistance in *S. enterica* and *E. coli* strains (Tyson et al., 2016). As the range of streptomycin tested here did not include concentrations less than 32 μg/mL, it is possible that the strains carrying these resistance genes have MICs that would distinguish them from strains not carrying any streptomycin resistance genes.

Strains 2190, 2208, 2468, 2516, and 2517 all carried a lone *aadA1* gene that was a BLAST match for a gene sequenced from Heidelberg strains. All five of these strains had phenotypic resistance to streptomycin. Strain 2219 carried a lone *aadA1* gene but tested as susceptible to streptomycin. Strain 2218 carried *aadA2* and *aadB*, which confer resistance to streptomycin and gentamicin/kanamycin, respectively. All of these phenotypes were detected in the strain. Strains 2244 and 2245 both contained $bla_{\text{oxa-1}}$ genes, followed by *aadA1*. $bla_{\text{oxa-1}}$ confers resistance to ampicillin, which was phenotypically detected in both strains. Strain 2479 carries a single gene cassette, *dfrA16*,

which was responsible for the phenotypic resistance to trimethoprim, one of the drugs in the trimethoprim/sulfamethoxazole combination. Another strain carried a different trimethoprim resistance gene, *dfrA5*. This gene was found in combination with *ereA2* in strain 2528. *ereA2* confers resistance to erythromycin, which was not in the panel of drugs tested here.

Using the cassette primers described here, it was not possible to obtain a PCR product for sequencing for strain 2213. As a side note, it was later determined through whole genome sequencing of this strain in a project not discussed in detail here that the integron contained a novel cassette, the 5' end of which contained a hypothetical protein, followed by *aac*(6')-33 and *aadA1* (Kroft et al., 2013). It is possible that the hypothetical protein at the 5' end of the integron prevented successful PCR amplification of the integron cassette.

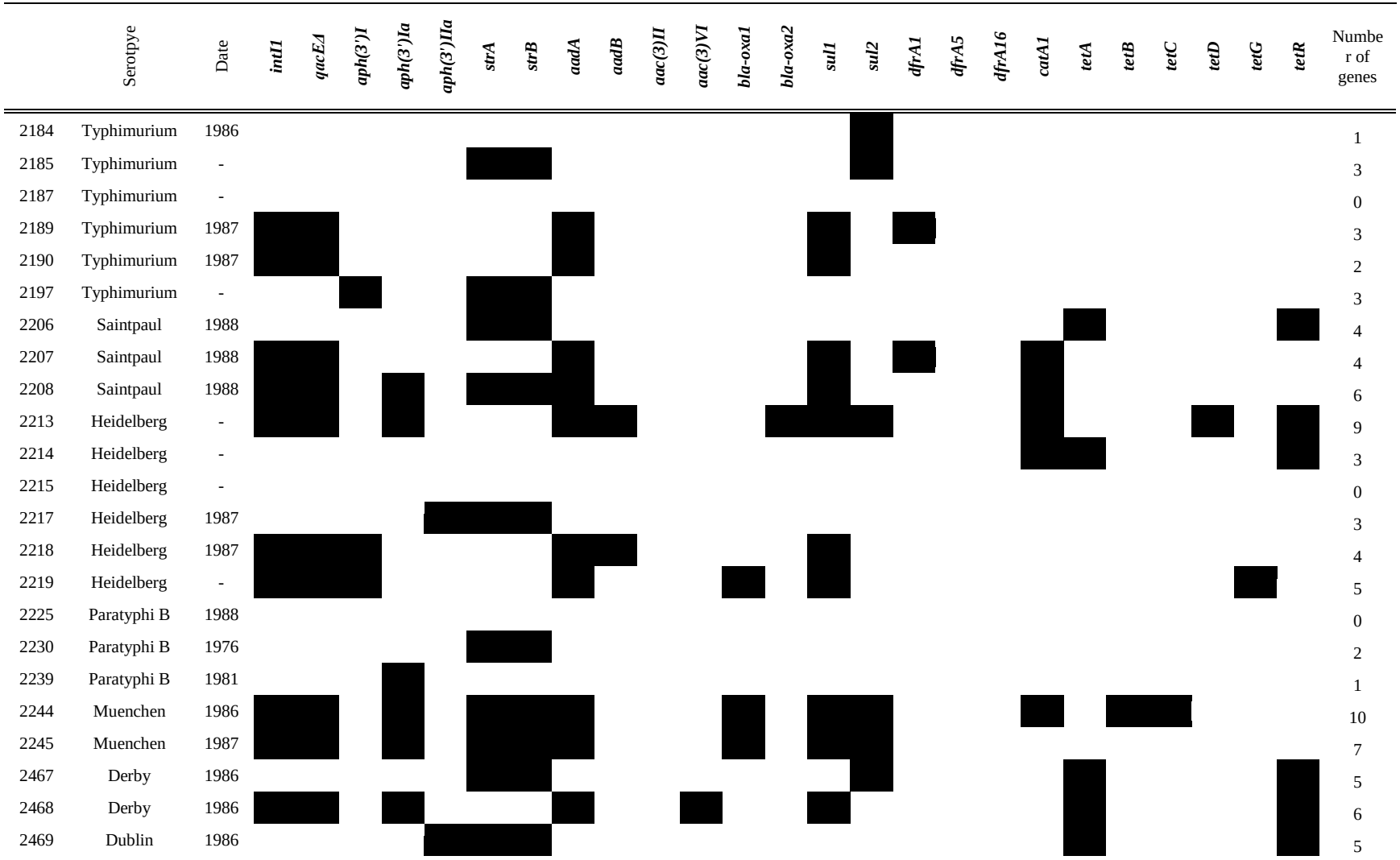
4.2.3 *Salmonella* Genomic Island

Using the primers listed in the Materials and Methods section, all 141 strains tested negative for the presence of the *Salmonella* Genomic Island.

4.3 Antimicrobial Resistance Genes Detected by Microarray

Microarray analysis was carried out on the strains which presented with phenotypic resistance to one or more antimicrobials. Among the 34 strains analyzed using the microarray chip, eight were determined to not be carrying any resistance genes (Table 4-7). The remaining 26 strains harbored at least one resistance gene. Twenty-three different resistance genes were identified, providing resistance to five classes of antimicrobials: aminoglycosides, beta-lactams, sulfonamides, chloramphenicol, and tetracycline. Also noted was the presence of elements associated with integrons: *intI1* and

qacEdelta. All strains that tested positive previously via PCR for *intI1* also tested positive through microarray analysis. As mentioned previously, strain 2494 did not harbor *intI1* but did harbor the 3' conserved end of an integron cassette, along with the corresponding *sul1* gene.


Serotpye
Date
intI1
qacEΔ1
aph(3')I
aph(3')Ia
aph(3')IIa
strA
strB
aadA
aadB
aac(3)II
aac(3)VI
bla-oxa1
bla-oxa2
sul1
sul2
dfrA1
dfrA5
dfrA16
catA1
tetA
tetB
tetC
tetD
tetG
tetR
Number of genes
2184 Typhimurium 1986 1
2185 Typhimurium - 3
2187 Typhimurium - 0
2189 Typhimurium 1987 3
2190 Typhimurium 1987 2
2197 Typhimurium - 3
2206 Saintpaul 1988 4
2207 Saintpaul 1988 4
2208 Saintpaul 1988 6
2213 Heidelberg - 9
2214 Heidelberg - 3
2215 Heidelberg - 0
2217 Heidelberg 1987 3
2218 Heidelberg 1987 4
2219 Heidelberg - 5
2225 Paratyphi B 1988 0
2230 Paratyphi B 1976 2
2239 Paratyphi B 1981 1
2244 Muenchen 1986 10
2245 Muenchen 1987 7
2467 Derby 1986 5
2468 Derby 1986 6
2469 Dublin 1986 5

2470	Dublin	1982																										0
2479	Haifa	1988	■	■						■						■				■								3
2482	Indiana	1988															■											1
2494	Newport	-		■		■					■				■	■					■							5
2516	Senftenberg	1987	■	■						■			■			■												3
2517	Stanley	1988	■	■						■						■						■					■	4
2528	Wien	1988	■	■		■				■		■				■			■									5
2997	ssp. salamae	1965																												0
3045	*S. bongori*	1977																										0
3068	ssp. diarizonae	1987																										0
3086	ssp. houtenae	1986																										0

Table 4-5. Antimicrobial resistance genes identified using microarray. Black boxes indicate the presence of the given gene.

4.3.1 Aminoglycosides

Among the 34 strains that were subjected to microarray analysis, nine different aminoglycoside genes were detected. *aph(3')-IIa* was detected in two strains, 2217 and 2469. This gene was responsible for resistance to kanamycin, among other drugs that were not tested here. Also responsible for kanamycin resistance was *aph(3')I*, detected in strains 2197, 2218, 2219, and 2189. Despite testing positive by microarray for this gene, strains 2197 and 2219 were not phenotypically resistant to kanamycin. The most common aminoglycoside gene found was *ant(3')Ia*, also known as *aadA*. This gene was identified above, having been found to be part of integron cassettes in several strains. Under microarray analysis, it was detected in strains 2189, 2190, 2207, 2208, 2213, 2218, 2219, 2244, 2245, 2468, 2479, 2516, 2517 and 2528. It provided resistance to streptomycin. Several phenotypic discrepancies were also noted with respect to *aadA*. Despite testing genotypically-positive for resistance, five strains remained phenotypically susceptible to streptomycin: 2189, 2207, 2219, 2479, and 2528. Again, it is possible that the breakpoint for streptomycin utilized here is not an accurate epidemiological cutoff and that these strains are in fact resistant at a 32 μg/mL cutoff. *aac(3)-VI* was detected in strains 2468 and 2516. This gene conferred resistance to gentamicin and low level resistance to kanamycin. In strains 2213, 2218, and 2494, gene *aadB* (also known as *ant(2")-Ia*) was found, providing resistance to gentamicin and kanamycin. Strain 2528 was the only strain in which the gene *aac(3)-II* was found, responsible for gentamicin resistance. Two linked genes, conferring resistance to streptomycin, known as *strA* and *strB* (or *aph(6)-Ia* and *aph(6)-Id*, respectively) were found in strains 2185, 2197, 2206, 2208, 2217, 2230, 2244, 2245, 2467, and 2469.

4.3.2 Beta-lactam

Two genes, responsible for resistance to ampicillin, were detected in five strains. Strains 2219, 2244, and 2245 contain $bla_{oxa\text{-}1}$ and strains 2213 and 2492 contain $bla_{oxa\text{-}2}$. Strain 2219 has no phenotypic resistance to ampicillin but the remaining four strains did test as resistant to ampicillin.

4.3.3 Folate Pathway Inhibitor

Five genes that provide resistance to folate pathway inhibitors were detected in 18 strains. The most common, *sul1*, was detected in 15 strains (2189, 2190, 2207, 2208, 2213, 2218, 2219, 2244, 2245, 2468, 2479, 2494, 2516, 2517, and 2528). The *sul2* gene was detected in seven strains (2184, 2185, 2213, 2244, 2245, 2467, and 2482). Both of these genes provide resistance to sulfisoxazole and, in part, resistance to trimethoprim/sulfamethoxazole. Three other genes, *dfrA1, dfrA16,* and *dfrA5* were detected that confer resistance to trimethoprim, the other component of the trimethoprim/sulfamethoxazole combination drug. Strains 2189 and 2207 contained a *dfrA1* gene while strains 2479 and 2528 contained *dfrA16* and *dfrA5*, respectively. All strains containing a *sul* gene were resistant to sulfisoxazole and all strains containing and *sul* and *dfr* genes together were resistant to trimethoprim/sulfamethoxazole.

4.3.4 Phenicol

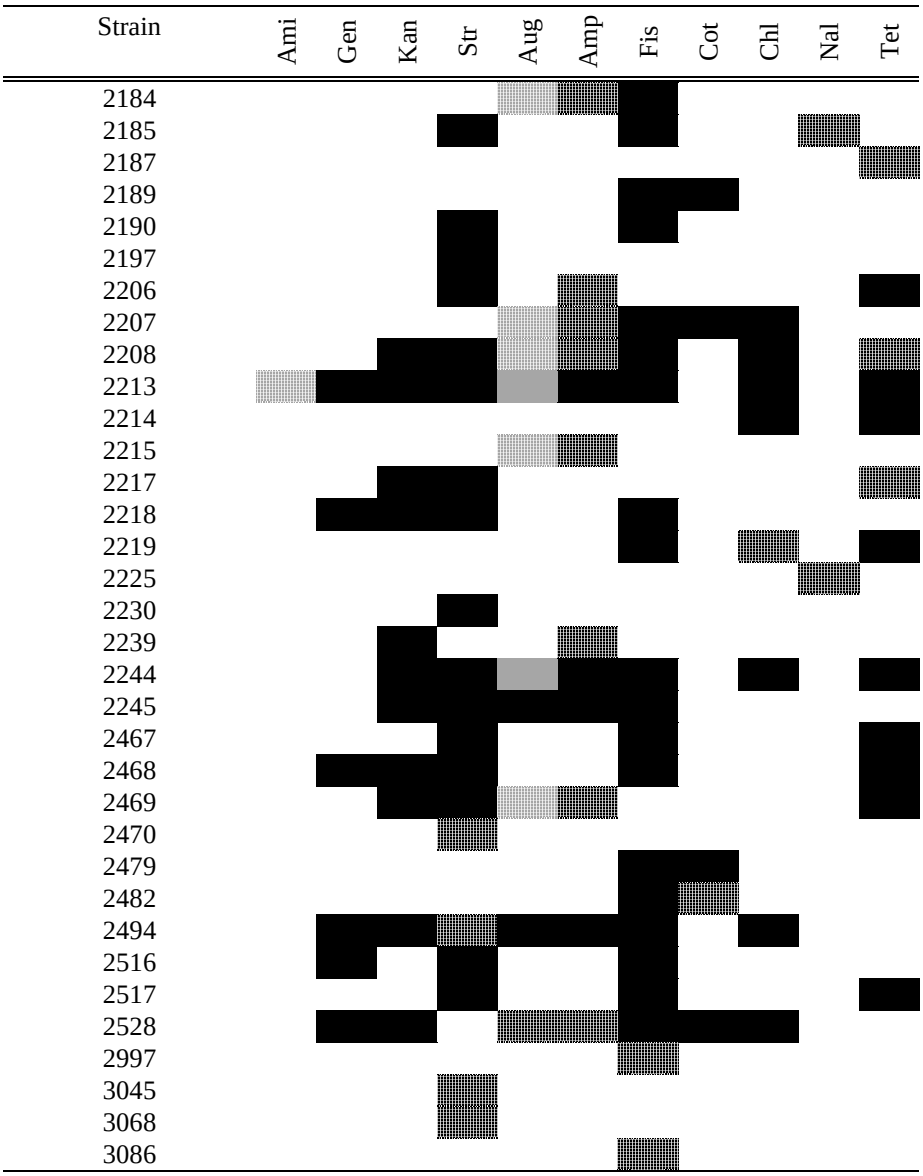
One gene responsible for chloramphenicol resistance was detected in six strains. These six strains (2207, 2208, 2213, 2214, 2244, and 2494) were all phenotypically resistant to chloramphenicol.

4.3.5 Tetracycline

Five genes, responsible for tetracycline resistance utilizing a tetracycline-specific efflux pump, were identified. Also present in all but one case was a corresponding *tetR* regulator gene. Strains 2206, 2214, 2467, 2468, 2469 and 2517 contained a *tetA* gene. One strain, 2213, contained a *tetD* gene. Strain 2244 contained both a *tetB* and *tetC* gene. Strain 2219 contained a *tetG* gene but no *tetR*, which would explain the lack of phenotypic resistance seen in this strain. All other strains exhibited phenotypic resistance to tetracycline.

4.4 Unexplained Phenotypes

As mentioned above, some strains remained susceptible to drugs to which they were genotypically-resistant. The opposite effect was also seen. Some strains tested as resistant to certain drugs but no genetic element was identified through microarray that would explain the phenotypes observed. Gentamicin and kanamycin were the only two drugs for which a gentamicin- or kanamycin-resistant phenotype was fully explained in all strains by the presence of a corresponding gene (Table 4-6).



	Ami	Gen	Kan	Str	Aug	Amp	Fis	Cot	Chl	Nal	Tet
No. Intermediate and Resistant Phenotypes	1	6	11	20	10	12	21	5	8	2	12
No. Unexplained by Microarray Results	1	0	0	4	6	8	2	1	1	2	3
Percent Unexplained Phenotypes	100%	0%	0%	20%	60%	67%	10%	20%	13%	100%	25%

Table 4-6. Phenotypes unexplained by microarray results Black solid boxes indicate the presence of resistance that has an identified genetic component. Gray solid boxes indicate intermediate resistance that has an identified genetic component. Hashed boxes indicate resistance for which no genetic component was identified.

In all other cases, there was at least one instance of the phenotype not being explained.

Both strains resistant to nalidixic acid have unexplained phenotypes. Because nalidixic

acid resistance is commonly mediated by point mutations in DNA topoisomerase and DNA gyrase genes and not by exogenous genes, the microarray method would not have identified a genetic component responsible for the phenotypic resistance. The majority of ampicillin and amoxicillin/clavulanic acid phenotypes had no detected corresponding gene. Only twelve strains out of the 35 tested by microarray had resistance profiles that were completely explained by the presence of exogenous genes.

4.5 Role of Regulatory Operon Mutations In Antimicrobial Resistance

In light of the presence of genetically unexplained phenotypes is discussed above, examination of mutations in the *mar*, *ram*, and *sox* operons, which play a role in regulation of efflux pump expression, was undertaken to determine whether any strains contained point mutations that might have affected the phenotypic resistance observed. However, it was unlikely that any of the strains here were over-expressing efflux pumps, due to the lack of ciprofloxacin resistance seen. Resistance to ciprofloxacin is primarily the result of active efflux in the cell (Baucheron et al., 2002; Giraud et al., 2000), so any strain exhibiting enhanced efflux due to an operon mutation would also present with ciprofloxacin resistance. Examination of the predicted amino acid mutations in the *mar*, *ram*, and *sox* operons revealed that only one strain that exhibited multi-drug resistance (strain 2244) had a change in amino acid that was unique to the strain in question, and this strain had all phenotypes accounted for by exogenous genes. Additional examination of the intergenic regions also failed to reveal any mutations unique to strains exhibiting unexplained resistant phenotypes. Therefore, it was unlikely that any resistant phenotypes observed here were the result of mutations in these three regulatory operon sequences.

4.6 Discussion

Antimicrobial resistance among isolates from the SAR collections, strains which were selected as genetic representatives of the genus, demonstrate the extent to which resistance was distributed. Strains from a variety of backgrounds, including genetic, geographic, and temporal, were found to display varying levels of resistance. Here, the reticulate nature of resistance was illustrated. Identical resistant phenotypes were distributed among strains that showed high levels of genetic diversity. This was especially evidenced by resistance to older drugs like sulfisoxazole and streptomycin. Resistance to these two drugs was not restricted to strains of the subspecies *enterica,* with one *S. bongori* strain displaying resistance to streptomycin. Combinations of resistant phenotypes that did not lend themselves to being resolved in a linear, bifurcating fashion were also seen, which was not surprising, considering the horizontal nature of the transfer of many antimicrobial resistance genes (Tosini et al., 1998; Autunes el at, 2005; Guerra et al., 2002). Despite the seemingly random assortment of phenotypes across genetically diverse strains, correlations were able to be drawn between certain drug phenotypes and the presence of multi-drug resistance, as defined by resistance to four or more drugs.

As had been previously reported, historical *Salmonella* strains here were most typically resistant to older antimicrobials that have a history of clinical or agricultural use. A previous study of over 2000 contemporary *Salmonella* strains from clinical sources were studied similarly to determine the change in phenotypes over time. As was found here, the majority of the strains were resistant to streptomycin, sulfisoxazole, tetracycline, and ampicillin and at similar percentages. This same study also failed to identify any strains resistant to the newer antimicrobials ceftiofur, ceftriaxone, and

ciprofloxacin. Considering that the strains studied here came from a multitude of sources, it is important to note that the prevalence of resistance was not significantly different from strains that were collected from only clinical sources, where the selective pressure on the organism is presumably much greater (Tadesse et al., 2016). Resistance to streptomycin, sulfisoxazole, and tetracycline in *S. enterica* was discovered decades ago and because of the use of these drugs in both food animals and humans, it is not surprising that the phenotypes would present in the strains studied here (Bissett et al., 1974). While the majority of the strains analyzed were susceptible to every drug tested, approximately one-quarter showed either intermediate or full resistance to one or more drugs. No strain (for which a date of isolation was available) was pre-dated by the introduction of clinical use for the given drug. Almost all of the resistance seen in these strains were to drugs that were clinically introduced prior to 1970, with the exception being the three strains that were resistant to amoxicillin/clavulanic acid, the combination of which was used clinically starting in 1981 in the United Kingdom (Geddes et al., 2007). The two drugs for which resistance was most commonly seen, streptomycin and sulfisoxazole, are two of the oldest antimicrobials used, having been introduced in 1946 and 1936, respectively (Lewis, 2013). Ampicillin, chloramphenicol, and trimethoprim/sulfamethoxazole were commonly used to treat infections in the 1980s but are no longer used because of the development of resistance to these drugs. Increases in this resistance pattern were noted for human isolates collected from 1987 to 1994 previously (Su et al., 2004). Two strains studied here exhibit this phenotype, both isolated from humans in 1988.

When comparisons were made to antimicrobial resistance phenotypes and dates of isolation, it was determined that not only were strains isolated from the 1980s more likely to be resistant to one or more drugs, as compared to strains isolated in earlier decades, there was a statistically significant temporal change in MIC for several drugs. This increase in MIC over time was seen for ampicillin, kanamycin, streptomycin, sulfisoxazole, tetracycline, and trimethoprim/sulfamethoxazole, which correlated well to findings from other studies of enteric bacteria. Increasing trends in resistance to ampicillin, sulfisoxazole, and tetracycline were found from the period 1950 to 2002 in *E. coli* (Tadesse et al., 2012). In a study of human isolates, resistance to one or more antimicrobials was identified more often in strains from the period 1984 to 1985 than 1979 to 1980 (16% versus 24%, respectively) (MacDonald et al., 1987). Here, an upward trend in MIC was also seen for strains susceptible to sulfisoxazole, an indication that despite the overall classification as susceptible, strains were tolerant to increasing concentrations of the drug, eventually rendering them resistant should the trend continue. This phenomenon, called MIC creep, has been identified in other species of bacteria, including *Streptococcus pneumoniae* and *Staphylococcus aureus*, to penicillin and vancomycin, respectively (Fernández et al., 2011; Steinkraus et al., 2007).

The only statistical correlation between the presence of resistance to three or more agents and a particular type of source was that of strains from a food animal source. Of the 35 strains resistant to one or more drugs, 28.6% (n=10) were from a food animal source. Eight of these ten strains were resistant to at least three drugs. This echoes previous findings that showed that two-thirds of multi-resistant *Salmonella* outbreaks from 1971 to 1983 were attributable to a food animal source (Holmberg et al., 1984).

Similarly, higher levels of resistance in *E. coli* from animal sources, as compared to human sources, has been noted (Tadesse at al., 2012). This correlation has public health implications, as resistant *Salmonella* in food animals has been attributed to the development of resistant infections in humans, and additionally, food animals can act as reservoirs of resistance gene determinants, which are then shed and transferred to other animals or to the environment (Angulo et al., 2000; Silbergeld et al., 2008; Aarestrup, 2005). In the collections used here, which were not selected based on clinical or veterinary significance, the prominence of food-animal associated resistance is important because it speaks to the potential evolutionary origins of antimicrobial resistance in *Salmonella*.

Increases in MDR have been seen in Typhimurium and Heidelberg strains, in a comparison of pre-1960 strains to post-1989 strains. Typhimurium was most often more resistant to more agents than other serotypes. During this period, the number of Typhimurium strains resistant to one or more antimicrobials also increased (Tadesse et al., 2016). In this study here, the group containing Typhimurium and Heidelberg strains was found to be negatively correlated to pan-susceptibility. Increases in multi-drug resistance for Typhimurium have been attributed to the rise of Typhimurium DT104, which commonly harbors resistance to five antimicrobial agents--ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline (Helms et al., 2005). No strains in this study exhibited this specific combination of phenotypes, indicating that perhaps the tendency for this grouping of strains to be less pan-susceptible exists independently of the characteristic DT104 resistance.

While a correlation between sulfisoxazole and multi-drug resistance was not found here (sulfisoxazole exists in a significant number of strains in the absence of MDR), all but one multi-drug resistant strain was resistant to sulfisoxazole. This was most likely attributable to the sulfisoxazole phenotype being maintained through its presence in class 1 integrons, which are stable in the bacterial chromosome or plasmid outside of an evolutionary pressure. Integrons have been well-documented agents in the dissemination of antimicrobial resistance, through their ability to capture and retain resistance genes (Mazel, 2006; Butaye, 2006). They have been found in not only pathogenic bacteria but in environmental strains of non-pathogenic bacteria. In fact, it has been argued that the origins of the class 1 integrons were soil microbes, and it was the intersection of niches, along with selective pressure exerted by antibiotic use that led to the development of integrons becoming a critical mechanism for antimicrobial gene capture in the food chain (Gillings et al., 2008). Here it was found that integrons were only present in strains resistant to two or more antimicrobials and that the percent of strains resistant to a particular drug was higher when the strain harbored an integron, even when the resistance element was not present in the integron cassette. This was the case for chloramphenicol resistance, which shows a statistical correlation to the presence of *intI1*, despite no chloramphenicol resistance gene being found in any integron cassettes. Of the 14 strains that tested positive for *intI1*, 12 of them came from a human or food animal source. The remaining two strains (2189 and 2190) were isolated from a parrot and an opossum, indicating that the reach of integrons is not limited to the food chain, and their dissemination is not strictly tied to the presence of a strong selective pressure, like the clinical and agricultural use of antimicrobials.

The presence of several antimicrobial resistance genes in strains from food animal and human sources points to their establishment in the food chain well before they became a public health concern. The coexisting genes *strA* and *strB* were first genetically characterized from an *E. coli* strain in 1989 (Schloz et al., 1989), years after strains studied here were isolated. Tens strains were found to harbor these two genes, the earliest one being a Paratyphi B strain from a food source in 1976. They were also found in strains from the 1980s and from a variety of sources, including humans, swine, poultry, and bovine. *aac(3)II* and *aac(3')VI* were first characterized from bovine isolates in France in 1984 (Chaslus-Dancla et al.,1987; Hedges and Shannon, 1984; Frye and Jackson, 2013). Here, these genes were identified in a chicken strain from 1987, a turkey strain from 1986, and a human strain from 1988. The first *sul* gene was initially sequenced in 1988 (Sundstrom et al., 1988), after it was already well-established in strains from even wider range of sources. The *sul1*gene here was found in strains from humans, turkey, bovine, poultry, opossum, and parrot sources. Also of note was the presence of two *tet* genes, *tetB* and *tetC*, in strain 2244 alongside the presence of *tetG*. Though becoming more commonly isolated from food animals, due to the widespread practice of feeding tetracycline to animals (Frye et al., 2011), it is uncommon to find multiple *tet* genes in the same strain. Also uncommon is the presence of *tetG* outside of the *Salmonella* Genomic Island (Boyd et al**.,** 2001; Frech and Schwarz, 2000), as is the case for strain 2219 (Michael et al., 2006). This gene was first isolated from *Vibrio anguillarum* in 1981 (Aoki et al., 1987).

Comparison of identified genes to exhibited phenotypes demonstrated that not all phenotypic resistance was accounted for by exogenous genes. Of the 108 intermediate

and resistant phenotypes, 26.9% existed without a corresponding gene having been identified through integron sequencing or microarray analysis. It is possible that point mutations exist in the target genes; mutations in the quinolone resistance determining region of *gyrA* are a common cause of nalidixic acid resistance (Cloeckaert and Chaslus-Dancla, 2001; Strahilevitz et al., 2009). Such point mutations could not have been identified using the specific microarray used here. Besides nalidixic acid, ampicillin and amoxicillin/clavulanic acid resistance were the most commonly identified phenotypes without a genotypic counterpart. It is possible that the strains contain genes that were not represented on the microarray.

Two strains here exhibiting high-level resistance, 2208 and 2528, have three and two unexplained phenotypes each—strain 2208 to amoxicillin/clavulanic acid (intermediate), ampicillin, and tetracycline and strain 2528 to amoxicillin/clavulanic acid and ampicillin. It was possible that there existed increased efflux in the cell, due to mutations in a variety of genes. Below, mutations in the *mar*, *ram*, and *sox* regulatory operon genes were analyzed but no predicted amino acid change was unique to these two strains. Amino acid mutations in the *ram* operon unique to individual strains that presented with phenotypic resistance were identified for strains 2214, 2244, 2469, 2470, and 2516. However, it is unlikely that any of these mutations were the cause of the resistance patterns seen. Strain 2244 was multi-drug resistant but all of its phenotypes were accounted for with exogenous genes. Strain 2469 was resistant to four agents, with only its resistance to ampicillin and amoxicillin/clavulanic acid being unexplained, and it shared the predicted amino acid change with other, non-resistant strains. The remaining strains are not multi-drug resistant, and any efflux changes would be seen in an increase

in resistance to additional drugs, specifically ciprofloxacin. For strains that did not exhibit multi-drug resistance, such as the four strains from the SARC collection that were resistant to either streptomycin or sulfisoxazole, it was possible that point mutations in the target genes had inhibited the drug's ability to bind to the target protein. It was also possible that the microarray probes in the Affymetrix chip did not hybridize to all of the resistance genes present in the individual strains. Any point mutations in a resistance gene could alter the affinity of the probe for the target, reducing the observed signal. A separate whole genome sequencing study that focused on sequencing of two of the SARA strains, 2213 and 2244, revealed the presence of genes that were not detected by microarray. For strain 2213, four genes (*aac(6')-Iy*, *aadA5*, *aac(6')-33*, and bla_{TEM}) were identified through *in silico* analysis of whole genome sequencing data that were not identified through microarray. For strain 2244, one gene, *aac(6')-Iaa*, was identified through *in silico* analysis but failed to be detected by microarray (Kroft et al., 2013). It is possible that other genes in other strains went undetected as well.

Bacteria have several types of mechanisms available to them through which they can acquire and retain elements conferring resistance to antimicrobials. Soil bacteria have been identified as the source for many mobile elements, and this speaks to the importance of horizontal gene transfer in the development of resistance in the food chain and in clinically relevant strains of pathogenic bacteria, like *S. enterica*. But as shown here, some resistance seemed to be more sporadic in nature and without an identifiable exogenous gene. Regardless, the recombinatory nature of antimicrobial resistance remains to be an important mechanism by which bacteria from different genetic and environmental backgrounds can acquire resistance.

5 Chapter 5: Evolutionary Analyses of Regulatory Operon Regions

In order to assess the impact that homologous recombination has had on the evolution of three regulatory operon regions, a combination of cladistics analysis, incongruence length difference testing and network structure analysis were used. Homologous recombination can often be difficult to detect in highly similar sequences, so a combination of methods aids in the elucidation of evolutionary history. Visualization of topological discordance, or incongruence, was achieved through tree comparison to whole-genome representative MLST data, whereby two phylogenies were compared for topological congruence and displacement of taxa. Taxa that clustered differently in gene trees, as compared to prior-agreement MLST trees were assumed to have been affected by recombination at the gene or genes in question, resulting in placement on the gene tree that did not agree with the MLST tree. This method, however, requires that taxa affected by recombination have a phylogenetic signal that differentiate them from those taxa most related. In cases where recombination does not result in a discernable change, tanglegram analysis would fail to yield an accurate picture of those strains affected. For this reason, additional visualization is required. Network structuring, which allows for visualization of reticulation, is useful for displaying the multiple possible pathways to reach a given sequence. Recombination often results in conflicting phylogenetic signals that cannot be resolved completely with a bifurcating tree model, and network structuring helps to resolve this visualization problem by effectively displaying multiple possible trees in one diagram. Additionally, clustering comparison was utilized, whereby groupings of strains were compared between the whole-genome representative MLST data and the gene or operon being analyzed for recombination. Such comparison of groupings is useful for the

visualization of homogenization and assortive effects that recombination can produce. Homogenization results in strains from different clonal groupings or MLST clusters harboring identical or highly similar alleles. Assortive recombination results in a scrambling affect, whereby unrelated alleles are distributed in a more haphazard manner across an MLST phylogeny. Both of these effects have been noted in *S. enterica* strains and there exists a correlation between the genetic distance between strains and the type of pattern that recombination takes on. In more highly-related strains, homogenization is primarily seen, while assortive patterns are seen in strains making up Group I, subspecies *enterica* (Brown et al., 2012).

Analysis of the evolutionary pressure on a gene region can be useful for determining the extent of negative selective pressure exerted upon a gene or operon. In cases where the selective pressure on a gene is effectively neutral, mutations leading to amino acid substitutions will occur approximately as often as mutations causing no amino acid change. In the case of regulatory operons, which have well-established cellular functions that are critical for survival of the bacteria, it can be assumed that they are under strict negative selective pressure which has the effect of purging novel amino acid mutations from a population. Analysis of such pressure can be measured using a codon-based Z-test, which determines whether there exists a statistical difference between synonymous mutation frequency and non-synonymous mutation frequency.

Due to the limited number of strains from SARC for which operon sequencing was successful, evolutionary analyses of the regulatory operon regions were conducted on only those from SARA and SARB. Several Paratyphi B strains were also excluded,

due to an inability to obtain complete *mar* sequences. The resulting subset contained 104 strains.

5.1 Housekeeping Gene Phylogenies

5.1.1 SARA

The 56 SARA collection strains used here, members of what is referred to as the Typhimurium complex, were made up of strains of five serotypes: Typhimurium, Saintpaul, Muenchen, Heidelberg, and Paratyphi B. Under MLST phylogenetic analysis, the strains separated into seven clades, with two Typhimurium strains forming their own grouping and three strains of Paratyphi B also grouping outside the remaining Paratyphi B strains. One Saintpaul (strain 2209) and two Paratyphi B (strains 2241 and 2242) failed to cluster with the other strains. Of the 56 total strains, 50 were members of a single-locus variant group and none were members of a double-locus variant group. Such groups are considered to be clonal complexes.

5.1.2 SARB

The 48 strains of SARB used here were members of subspecies I *Salmonella*, a more diverse set of strains than SARA. The strains came from a variety of serotypes and the phylogeny built from MLST data separated the strains into nine clades, with two strains remaining ungrouped (2500 and 2521). Six strains belonged to three single-locus variant pairs: 2469 and 2470, 2465 and 2527, and 2522 and 2524. Nine strains were members of three double-locus groups: 2473, 2475, 2478, 2508, and 2509; 2461 and 2463; 2487 and 2488.

5.2 Evolutionary Pressures on *mar* Operon

In order to examine the impact of negative evolutionary pressure on the *mar* operon, nucleotide and allelic diversity were examined, as well as the ratio of synonymous to non-synonymous mutations in each of the operon genes. Of the 104 *marRABC* sequences obtained from the SARA and SARB collections, 53 were unique. The 56 SARA strains harbored 19 unique alleles while the 48 SARB strains harbored 40 unique alleles. The 666 bp *marC* gene was made up of the most alleles across both collections, with 33 different sequences, and thus had the most allelic diversity. The 435 bp *marR* gene was made up of 18 different alleles. The 381 bp *marA* gene was made up of 13 alleles and the 216 bp *marB* gene, 15 alleles. Allelic diversity, the probability of selecting two different alleles from the pool of total alleles, was highest for *marC*, with a value of 0.934. *marB* had the lowest allelic diversity, with a value of 0.679. While there were 15 different *marB* alleles across both SAR collections, almost three-quarters of the strains had one of two alleles (n=77). One single allele was present in over half of the strains (n=54). This was in contrast to *marC*, of which no single allele was present in more than 18.3% of strains (n=19). The allelic diversities for *marA* and *marR* fell between, with values of 0.772 and 0.807, respectively. Despite the differences in allelic diversity, all four genes had very similar nucleotide diversity values, all falling within 0.0008 of each other. Under the codon-based Z-test for selective pressure, *marB* was the only gene that for which the hypothesis of neutral evolution could not be rejected. Of the total 14 mutations in *marB*, six of them resulted in predicted amino acid sequence changes. In contrast, the *marR*, *marA*, and *marC* genes all produced p values that rejected the hypothesis of neutral evolution, in favor of a hypothesis of negative selective

pressure. *marC* had the lowest dN/dS ration, with a value of 0.0310, followed closely by that of *marR* (dN/dS=0.0395) and *marA* (dN/dS=0.0558). The dN/dS ration was used as an indicator of the type of selective pressure on a gene or DNA region. dN was the average number of non-synonymous substitutions per non-synonymous site while dS was the average number of synonymous substitutions per synonymous site. A dN/dS value below 1 was indicative of negative selective pressure exerted upon a gene, due to the purging of novel amino acid mutation from a population in question. Interestingly, of the three non-synonymous substitutions in *marR*, all were present in the SARA strains but none were present in the SARB strains—the predicted amino acid sequence for *marR* in the SARB strains was completely conserved while three sites in the SARA strain were not conserved.

5.2.1 SARA

5.2.1.1 Topological Incongruence and Network Structuring for marRABC

Alignment of the MLST maximum likelihood phylogenetic tree, a representative of the whole-genome phylogeny, alongside the maximum likelihood tree built from *marRABC* sequences showed limited displacement of certain strains (Figure 5-1). The tanglegram below was used to display the placement of identical taxa, connected with lines, in each phylogeny.

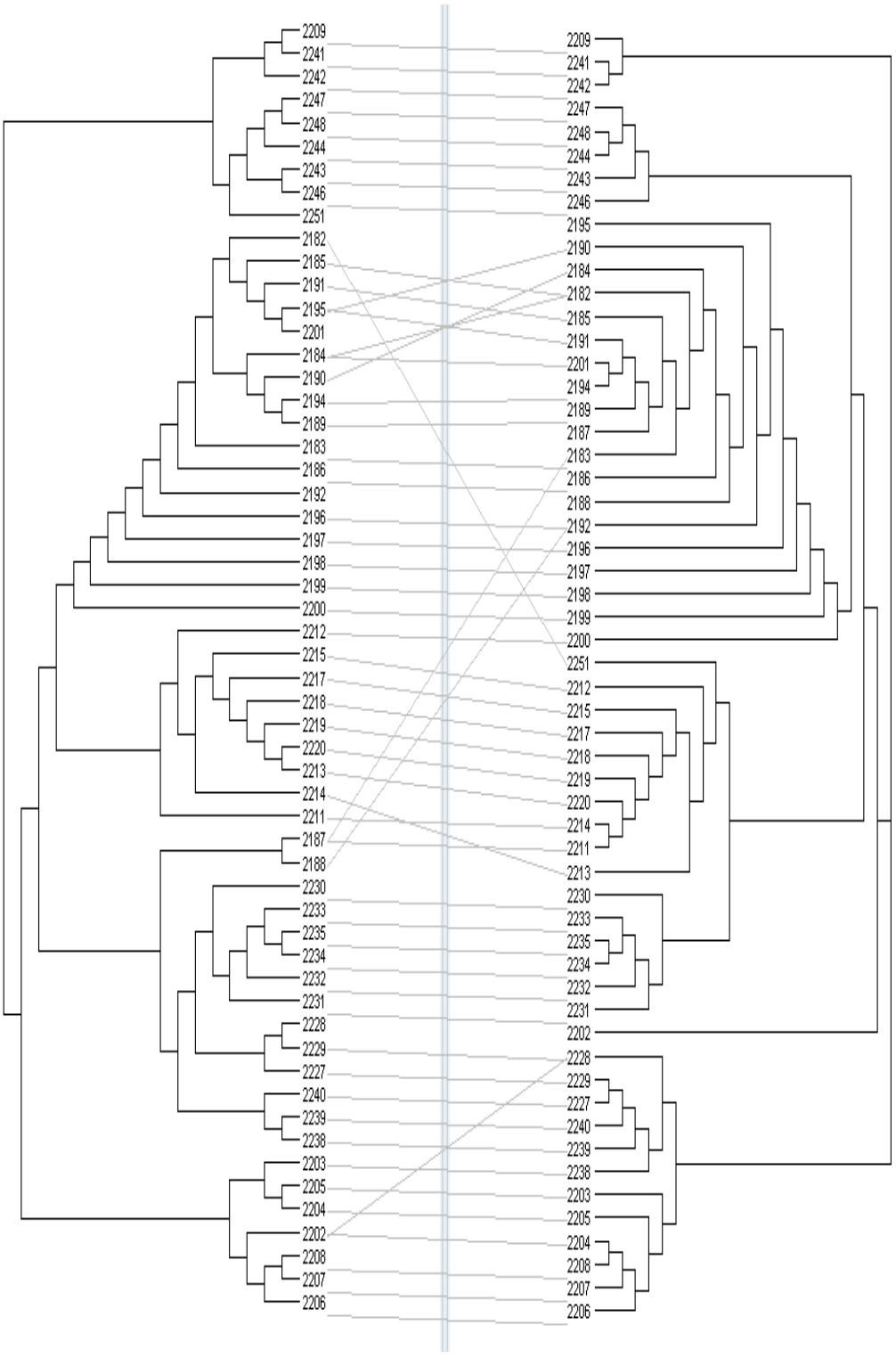


Figure 5-1. Tanglegram showing the MLST maximum likelihood phylogeny (left) alongside the *marRABC* phylogeny (right). Lines were used to connect identical taxa.

In particular, strains 2202, 2187, 2188, and 2251 clustered differently under MLST analysis than they did under *mar* analysis. Typhimurium strains 2187 and 2188, under MLST analysis, clustered in a branch removed from Paratyphi B strains but under *marRABC* analysis, were most similar to the other Typhimurium strains. Strain Saintpaul 2202, which clustered with other Saintpaul strains under MLST analysis, instead clustered with Paratyphi B strains in the tree built from *marRABC* data. Similarly, Muenchen 2251 was most similar to other Muenchen strains under MLST but failed to retain such clustering under analysis of *marRABC* sequences, instead clustering with Heidelberg strains.

Splits tree analysis of the concatenated *marRABC* sequences (Figure 5-2), used to display reticulation when conflicting phylogenetic signals exist in the data, showed significant network structuring, with no one strain or group of strains contributing to the overall network, indicative of shuffling of alleles within the operon. Parallel lines in the splits tree displayed the different evolutionary paths between the sequences in question.

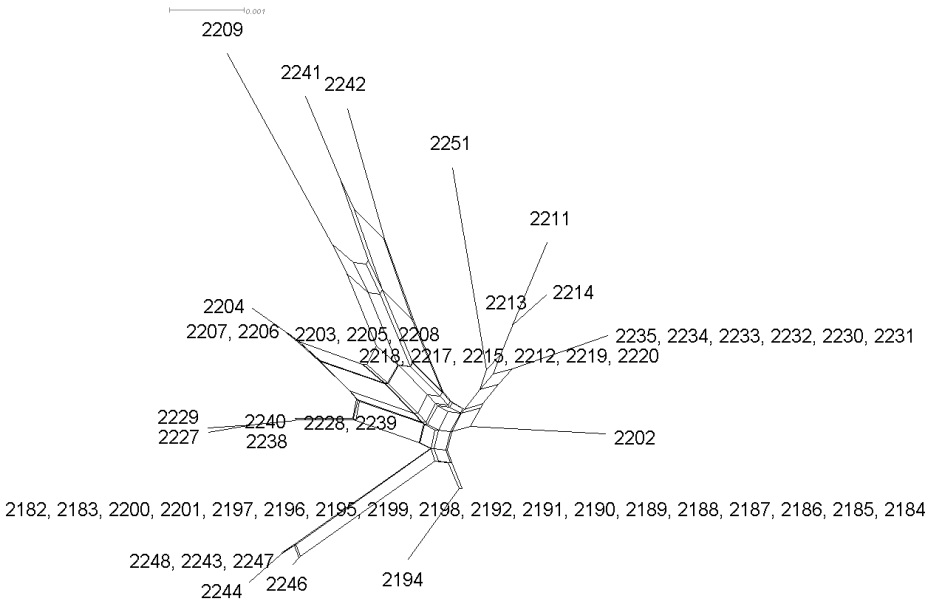


Figure 5-2. NeighborNet splits tree of *marRABC* sequences from SARA strains.

ILD tests between the individual *mar* genes showed congruence between all combinations of *mar* genes, with the exception of *marC* combined with *marA*. Removal of *marC* from the concatenated sequences resulted in a splits diagram with noticeably less network structuring (Figure 5-3), possibly indicating that *marC* shuffling was a significant contributor to the overall lack of bifurcating structuring of the operon.

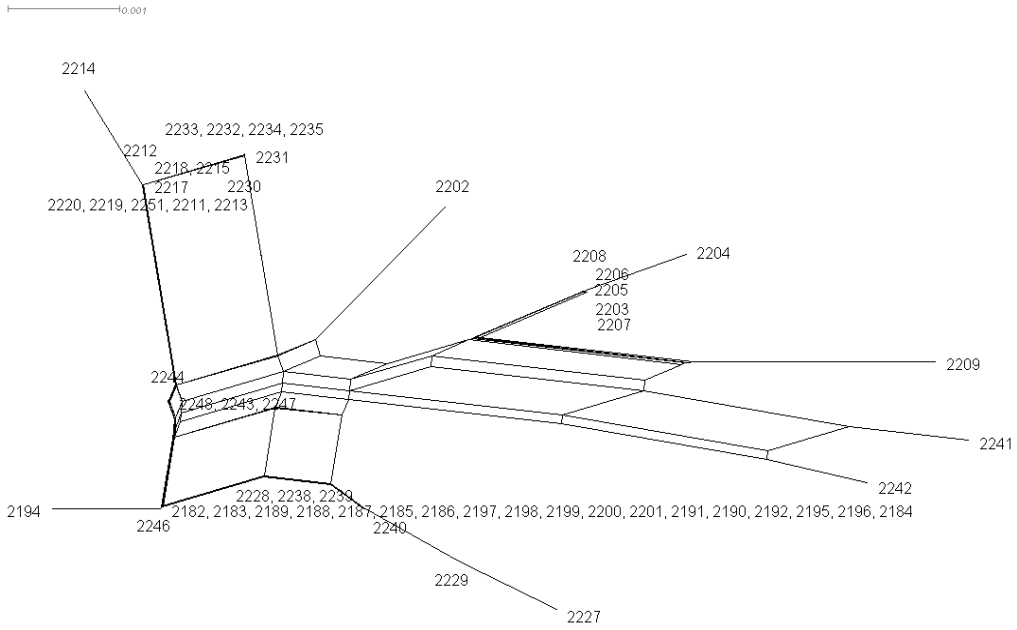


Figure 5-3. NeighborNet splits tree of *marRAB* sequences from SARA strains (*marC* excluded)

Despite the inability to reject the hypothesis of congruence when individual *mar* genes were compared to each other, the *marRAB* concatenated sequences did reject the hypothesis of congruence, when compared to MLST sequences. Additionally, each *mar* gene, when compared to MLST data individually, failed to reject the hypothesis of congruence. Failure to reject the hypothesis of congruence could, however, be limited by

nucleotide diversity in the operon, which could signify a homogenizing affect across alleles from different strains.

5.2.1.2 Homogenization with Limited Diversification of marR

In order to investigate the impact of recombination on the *marR* gene, the MLST phylogeny was compared to a phylogeny built from the *marR* gene. The *marR* gene separated into five clades, indicated by unique colors for each clade in Figure 5-4. The majority of strains (n=33), converged into a single clade (indicated as allele types 1 and 2, with light blue boxes). These 33 strains comprised three serotypes. All Typhimurium, Muenchen, and Heidelberg strains had *marR* alleles that fell into this single clade, despite these strains belonging to four separate clades in the MLST tree. This included the two Typhimurium strains, 2187 and 2188, which formed their own clade under MLST analysis (yellow boxes in MLST column). With the exception of strain 2202, the remaining strains had alleles that fell into one of three clades. Strain 2202 had an allele that formed its own clade, indicated with a grey box. Despite an inability to cluster strains 2209, 2241, and 2242 under MLST phylogenetic analysis, they formed a single clade, along with the remaining Saintpaul strains, in the *marR* tree (medium blue boxes).

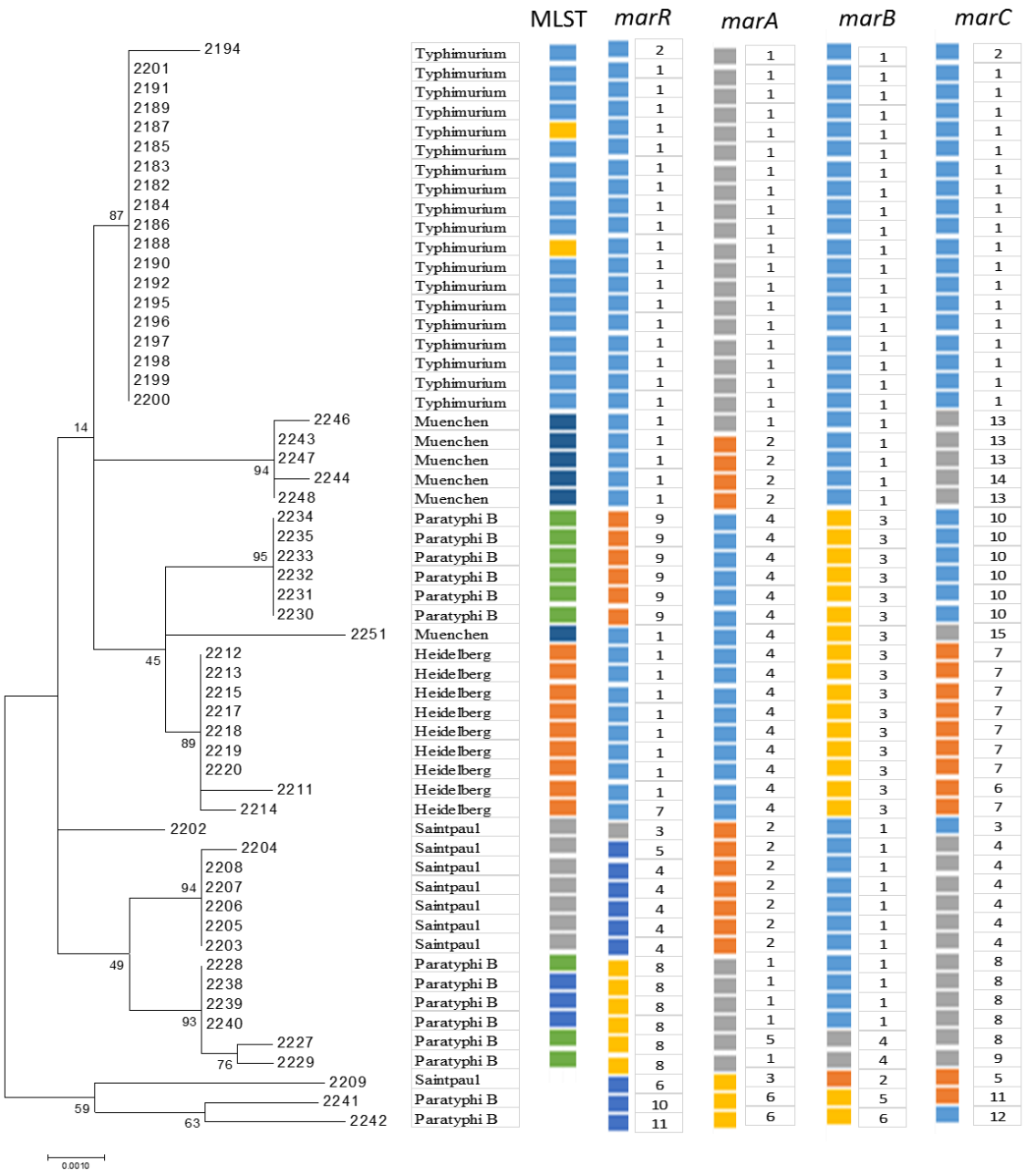


Figure 5-4. Maximum likelihood phylogeny built from *marRABC* sequences of SARA strains (left), aligned next to serotypes, MLST clade groupings, and clade groupings for each individual *mar* gene. Bootstrap values are presented as percentages. Colored boxes are used to represent clade designations for MLST, *marR*, *marA*, *marB*, and *marC*. Each clade was assigned a unique color and each bar column color set is independent of each other. Numerical allele designations are listed next to each colored box, and each set of allele numbers is also independent of each other. White boxes indicate an inability to assign a clade designation for MLST data.

Incongruence length difference testing indicated congruence between MLST sequences and *marR* sequences. Incompatibility scores between parsimonious sites of the

marR gene revealed that 40% of these sites were pairwise incompatible with each other, indicating that there may have been repeated crossover events that inserted mutations in a manner than interfered with the bifurcating phylogeny.

When parsimonious *marR* sites were compared pairwise to the other parsimonious sites of the remaining three genes in the operon, 30% of sites are incompatible, with the highest incompatibility seen for sites from the *marA* gene (45%). Sites in *marR* were 22.9% incompatible with those in *marC* and 26.7% incompatible with those in *marB*. Taken together, these data were indicative of some recombination but not enough to obscure the entirety of the *marR* phylogeny, when it was compared to that of MLST sequences.

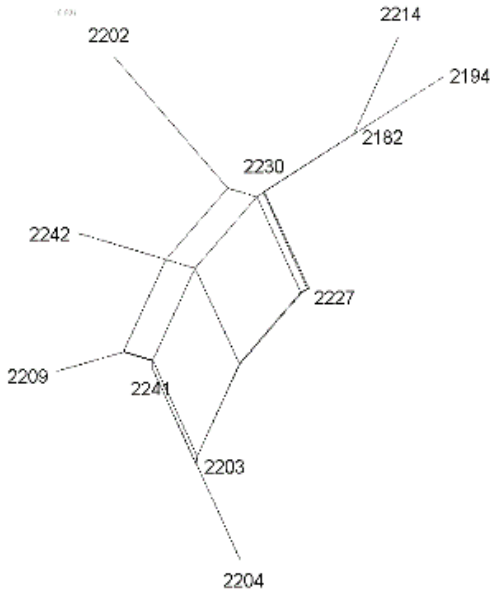


Figure 5-5. NeighborNet splits tree of unique *marR* alleles. One representative strain was selected for each unique allele.

Splits tree diagraming of the eleven unique *marR* alleles from the SARA collection revealed some network structuring that was not resolved when any one single strain was removed. The lack of a strictly bifurcating tree-like phylogeny also speaks to the possible impact of recombination on this alleles among strains from the Typhimurium complex.

5.2.1.3 Homogenization of marA With Mixing of Alleles Between Serotypes

Among SARA strains, there existed only six different *marA* alleles, and two of these alleles were present in 73.2% of strains (n=41). Three alleles were present in 52 strains, with only two strains having alleles that were unique. These two unique alleles (indicated by numbers 3 and 5) clustered with alleles 6 and 1, respectively (Figure 5-4). The *marA* alleles, collectively, separated into four clades. Unlike the *marR* locus, which had some diversification along clonal lines with respect to Muenchen and Paratyphi B strains, a greater level of homogenization is seen in *marA*. For instance, all Saintpaul and Muenchen strains (with the exception of strains 2246 and 2251) shared the same *marA* allele, listed as allele 2 in Figure 5-4. Typhimurium strains all harbored the same *marA* allele, which was also shared with a clade of Paratyphi B strains (allele 1). Paratyphi B strains that clustered together under MLST analysis (indicated with green boxes under MLST on Figure 5-4) harbored three different *marA* alleles. Heidelberg strains all harbored the same *marA* allele, one that was shared with Muenchen strain 2251 and six Paratyphi B strains (allele 4).

Despite the homogenization described above, incongruence length difference testing indicated congruence between MLST data and that from *marA*. This could have been the result of the limited nucleotide diversity seen in *marA*. In contrast, incompatibility analysis of parsimonious sites showed that 33.3% of sites within *marA* are incompatible with each other, indicative of shuffling of nucleotide polymorphisms in a manner that was inconsistent with bifurcating phylogenies. Additionally, *marA* parsimoniously informative sites were pairwise incompatible with 41.7% of the remaining three genes in the operon. *marA* sites were most compatible with sites in the

marB gene, with an incompatibility score of 25%, compared to the higher scores of 46.4% and 45% for *marC* and *marR*, respectively.

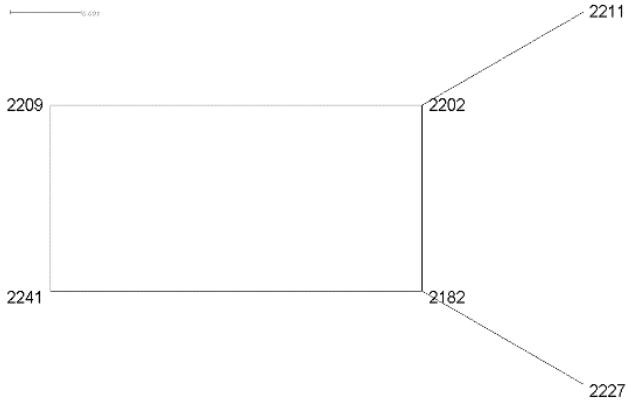


Figure 5-6. NeighborNet splits tree of unique *marA* alleles. One representative strain was selected for each unique allele.

Splits tree analysis of *marA* from SARA strains showed almost no network structure, which was limited by the small number of alleles. Removal of 2209 or 2241 from the split network resulted in a bifurcating tree.

5.2.1.4 Homogenization of marB With Limited Diversity

While all the genes from the *mar* operon had similar levels of nucleotide diversity, the *marB* gene had the lowest among strains from the SARA collection. *marB* also interestingly was not under significant negative evolutionary pressure, as measured by a codon-based Z-test. The limited diversity was responsible for 51 out of the 56 strains from SARA having one of two alleles. Six alleles were found to be present in all strains, with four of those present in just five strains. Saintpaul strain 2209 and Paratyphi B strains 2241 and 2242 had unique alleles while Paratyphi B stains 2227 and 2229 shared another allele. The most common allele (listed as allele 1 in Figure 5-4) was found in 35 strains, including all Typhimurium strains, four Muenchen strains, seven Saintpaul strains, and four Paratyphi B strains. The second most common allele was contained

within all Heidelberg strains, one Muenchen strain, and six Paratyphi B strains (allele 3 in Figure 5-4).

As was the case for *marR* and *marA*, despite homogenization of alleles across multiple serotypes, the incongruence length difference test indicated congruence between MLST data and *marB* sequences. Incompatibility between parsimonious sites within the *marB* gene was 0% and *marB* sites were 22.9% incompatible with parsimonious sites in the remaining three *mar* operon genes, the lowest value of the four comparisons made. The *marB* gene was least compatible with *marC*, with 38.1% of parsimonious sites being incompatible. *marB* was the smallest of the *mar* genes and the low incompatibility scores could have been the result of an overall lack of parsimoniously informative sites.

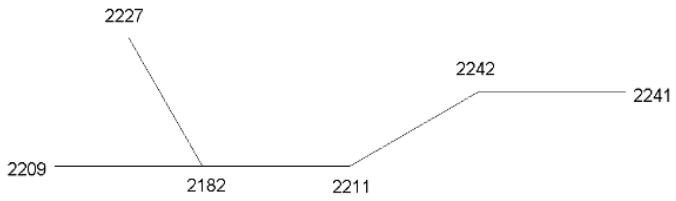


Figure 5-7. NeighborNet splits tree of unique *marB* alleles. One representative strain was selected for each unique allele.

A splits tree built from the six unique alleles showed a tree-like structure, with no networks. Network structuring may be limited by the lack of diversity of alleles and the lack of diversity of the nucleotides present in the *marB* gene, as well as the limited length of the gene.

5.2.1.5 Homogenization of marC

The *marC* gene was the most diverse among the *mar* operon genes, both in terms of allelic diversity and nucleotide diversity. Negative selective pressure appeared to have

greatly impacted the gene, according to its dN/dS ration. Only two sites out of a total 22 polymorphic sites contained mutations that resulted in a predicted amino acid sequence change. Among the SARA strains, *marC* was made up of 15 different alleles, which was more than that found in *marR*, *marA*, or *marB*. Despite the relatively high allelic diversity, upon phylogenetic analysis, the 15 alleles separated into only three clades. When aligned with MLST clades, there was limited retention of clonality among the alleles. For example, the first clade of *marC* alleles (indicated by blue boxes in Figure 5-4) were possessed by strains from four different MLST clades. *marC* alleles in gray boxes, the second of three clades for this gene, were possessed by strains from four different MLST clades. The third clade of *marC* genes, indicated by orange boxes, was possessed by all Heidelberg strains and two strains that failed to cluster under MLST analysis (Saintpaul strain 2209 and Paratyphi B strain 2241).

The incongruence length difference test supported the hypothesis of congruence, when compared to MLST data. The gene appeared to have undergone little intra-gene shuffling, as measured by incompatibility of pairwise parsimoniously informative sites. Only 4.8% of sites within the *marC* gene were incompatible with each other. The *marR* and *marA* genes, in comparison, had much higher intra-gene incompatibility values.

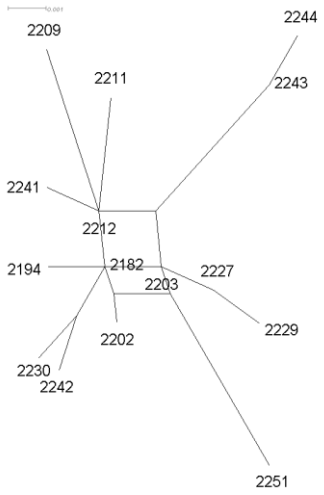


Figure 5-8. NeighborNet splits tree of unique *marC* alleles from SARA strains. One representative strain was selected for each unique allele.

When *marC* sequences were used to build a splits tree diagram, it was possible to see an amount of network structuring, possibly indicative of the insertion of mutations in a manner that interfered with a bifurcating tree-like structure.

5.2.2 SARB

5.2.2.1 Topological Incongruence and Network Structuring of marRABC

Splits tree analysis of the *marRABC* operon region showed network structuring of the 40 unique operon allele types, with no single strain or group of strains contributing to the overall structure (Figure 5-9).

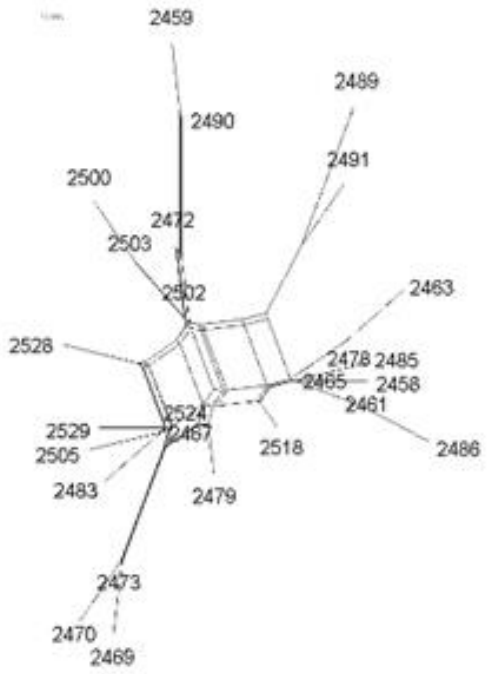


Figure 5-9. NeighborNet splits tree of 40 unique *marRABC* sequences from SARB strains

Alignment of the MLST phylogeny and that built from *marRABC* strains showed displacement of several strains, indicative of incongruence between the two sets of sequences. Notably, strains 2461, 2463, 2522, 2467, 2516, and 2491 clustered differently on the *marRABC* tree than they did on the MLST tree (Figure 5-10).

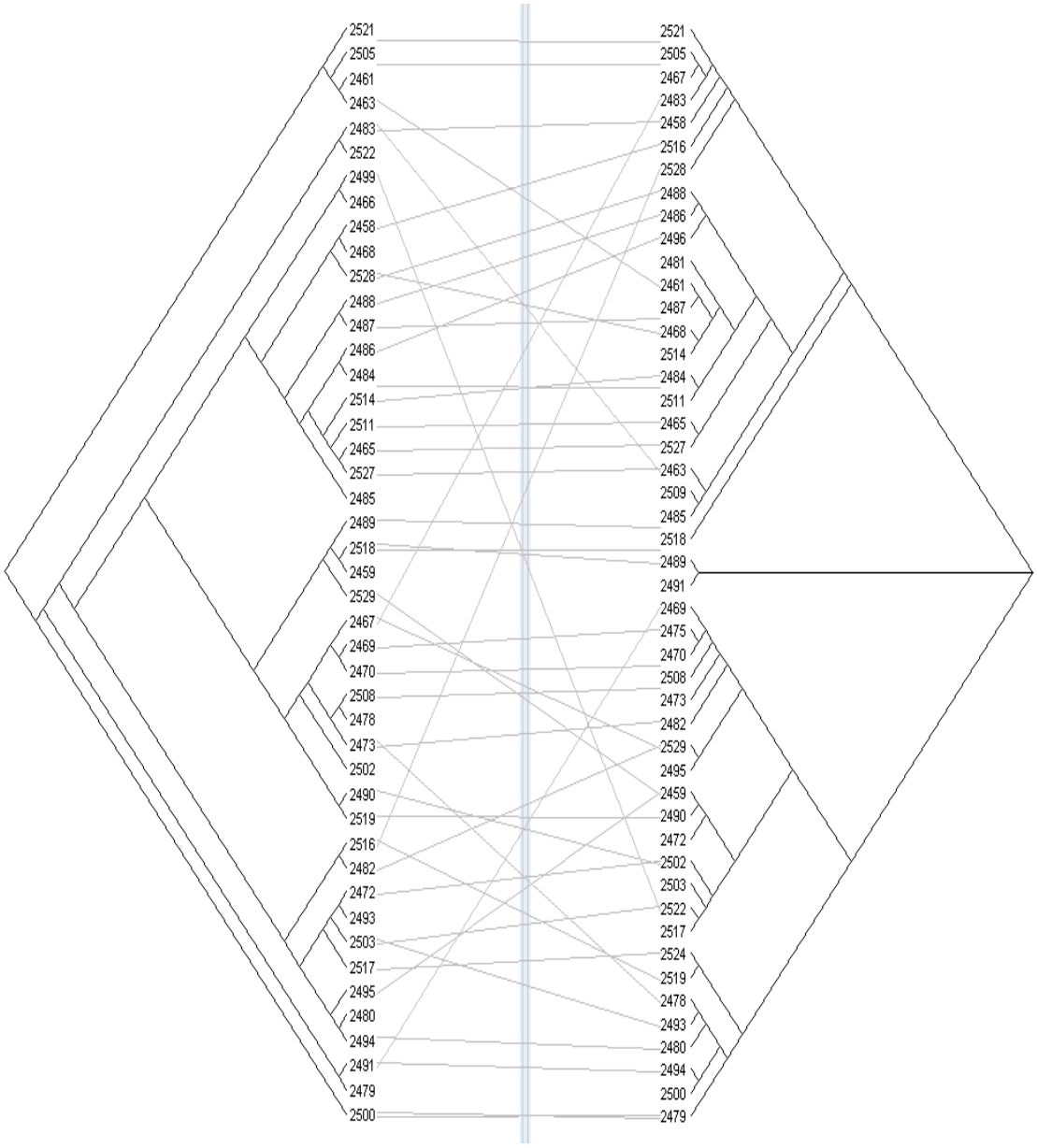


Figure 5-10. Tanglegram of MLST maximum likelihood phylogeny (left) connected to *marRABC* phylogeny (right) of SARB strains.

Additionally, as can be seen in Figure 5-11, MLST clades were distributed across much of the *marRABC* tree, with only a handful of strains retaining similar clustering—of the nine strains indicated by gray MLST boxes, five remained together on the *mar* tree (strains 2473, 2508, 2469, 2470, and 2475) and of the nine strains indicated by light blue

MLST boxes, seven (strains 2484, 2511, 2496, 2486, 2488, 2465, and 2527) remained clustered on the *mar* tree.

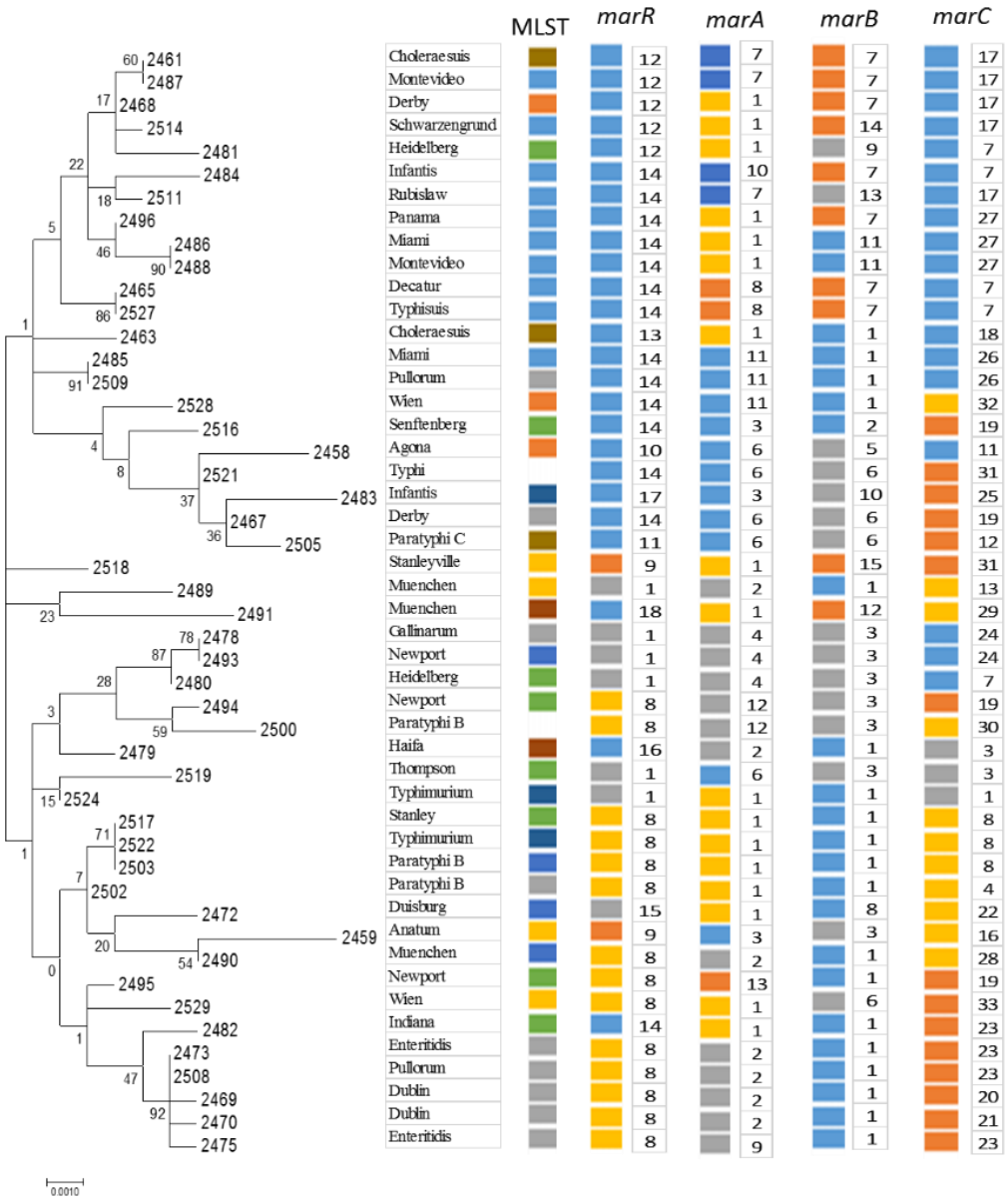


Figure 5-11. Maximum likelihood phylogeny built from *marRABC* sequences of SARB strains (left). Bootstrap support values are presented as percentages. Serotypes are listed next to each taxa. Colored boxes are used to represent clade designations for MLST, *marR*, *marA*, *marB*, and *marC*. Each clade was assigned a unique color and each bar column color set is independent of each other. Numerical allele designations are listed next to each colored box. White boxes indicate an inability to assign a clade designation for MLST data.

Incongruence length difference tests comparing the MLST sequences to *marRABC* sequences from SARB strains rejected the hypothesis of congruence. When the four *mar* genes were compared pairwise to each other, the hypothesis of congruence failed to be rejected for *marR:marA*, *marR:marB*, and *marB:marA* but was rejected when *marC* was compared to each of the other three genes.

5.2.2.2 Assortment with Homogenization of marR Across Genetically Diverse Taxa

Of the four *mar* operon genes, *marR* from the SARB collections had the least nucleotide diversity and the second lowest allelic diversity. Unlike the *marR* genes from SARA, there were no predicted amino acid substitutions in the gene, and *marR* was the only gene among the eight operon genes studied here for which this was the case. The 48 *marR* alleles from the SARB strains examined here separated into four distinct clades, as compared to the nine clades that become apparent under MLST analysis. The most frequently found *marR* alleles were alleles numbered 8 and 14, making up the majority of strains clustering into the blue and yellow clades, respectively (Figure 5-11). Together, these two alleles comprised 28 out of the 56 total SARB strains. None of the four clades of *marR* alleles clustered completely together on the *marRABC* tree, indicative of shuffling between taxa of different genetic backgrounds. The majority of alleles clustered in light blue remained together but three other taxa with similar *marR* alleles (numbered 14, 16 and 18)) were distributed at different locations on the *marRABC* tree. The remaining three *marR* clades (indicated with orange, yellow, and grey boxes) retained even less clustering. One single allele, present in two strains (Stanleyville strain 2518 and

Anatum strain 2459) formed its own *marR* clade but were not located within the same *marRABC* clade.

Homogenization of *marR* across niches was seen, with strains that were either host-adapted or -restricted harboring alleles from strains that were host generalists. For example, Typhi strain 2521, a human-restricted serotype, had an allele identical to Typhisuis strain 2527, a swine-restricted serotype. This was also notable because strain 2521 was one of two strains that failed to cluster into a distinct clade under MLST phylogenetic analysis. The second strain, Paratyphi B 2500, harbored a *marR* allele identical to, among others, a Dublin strain and two other Paratyphi B strains.

Splits tree analysis of the 12 unique *marR* alleles from SARB strains showed limited network structuring (Figure 5-12), with several alleles being derived from the allele harbored by strain 2465. Incongruence length difference testing between MLST sequences and *marR* sequences rejected the hypothesis of congruence, which supported the allele swapping described above.

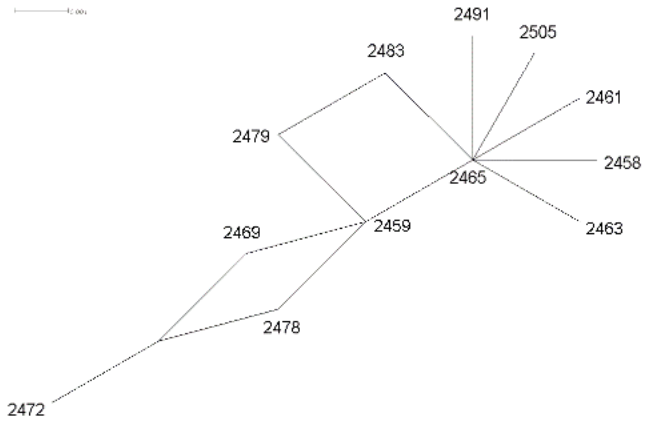


Figure 5-12. NeighborNet splits tree of unique *marR* sequences from SARB strains. One representative strain was selected for each unique allele.

It is possible to see here that in contrast to the overall genetic diversity of the SARB strains, which together represent the genetic diversity *Salmonella enterica*, the *marR* allele has retained a significant amount of homogeneity with some assortment of alleles across taxa.

5.2.2.3 Assortment of marA Across Genetically Diverse Taxa

The *marA* gene from SARB strains was made up of 12 alleles, with 35.4% of strains containing the same allele, referred to here as allele 1. These 12 alleles were divided into five clades, with two clades containing just two alleles each. None of the five clades clustered completely together on the *marRABC* tree. Two Cholerasuis strains, 2461 and 2463, under MLST analysis were double-locus variants and thus members of a clonal complex. Despite this, they carried *marA* alleles that clustered separately. Strains 2473, 2475, and 2478, also members of a double-locus variant group, harbored three different *marA* alleles, though these alleles were members of the same *marA* clade.

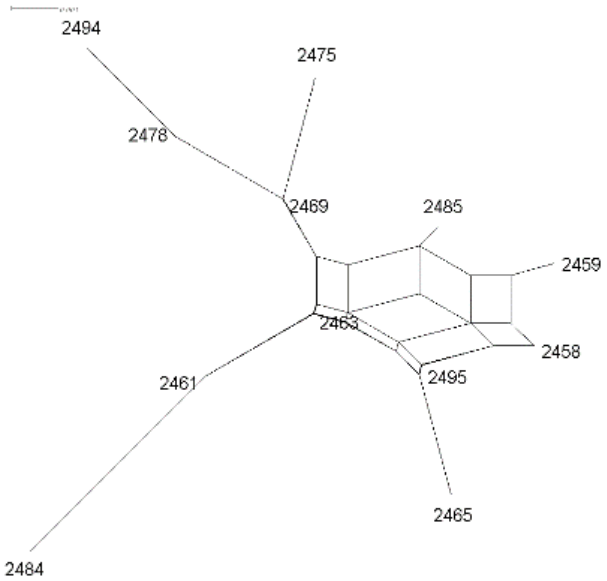


Figure 5-13. NeighborNet splits tree of unique *marA* sequences from SARB strains. One representative strain was selected for each unique allele.

Network structuring of the unique *marA* alleles revealed some reticulate evolution in the history of the gene (Figure 5-13). ILD tests comparing MLST sequences to *marA* sequences rejected the hypothesis of congruence, supporting the evidence gleaned from phylogenetic comparisons of the two sets of data. Additionally, 20% of parsimonious sites are incompatible with one another and 41.7% of *marA* sites were incompatible with sites in the remaining three operon genes.

5.2.2.4 Assortment of marB Across Genetically Diverse Taxa

The *marB* locus from SARB strains was comprised of 14 alleles, divided into three clades. 39.6% of strains harbored one single *marB* allele. This locus had the highest nucleotide diversity of the four genes in the operon, with a value of 0.0064 average substitutions per site. Unlike the other three genes, *marB* had a high ratio of non-synonymous to synonymous substitutions, and under Z-test analysis of neutrality, could not reject the hypothesis of neutral evolution. The three clades comprising the *marB* alleles were dispersed across the *marRABC* tree, with no one clade clustering completely together. Two double-locus variant groups had phylogenies that were disrupted under *marB* analysis. Gallinarum strain 2478 harbored a *marB* allele from a clade different than that of the rest of the strains making up the double-locus variant group. This was also the case for Cholerasuis strains 2461 and 2463. Each of these strains harbored *marB* alleles from different *marB* clades.

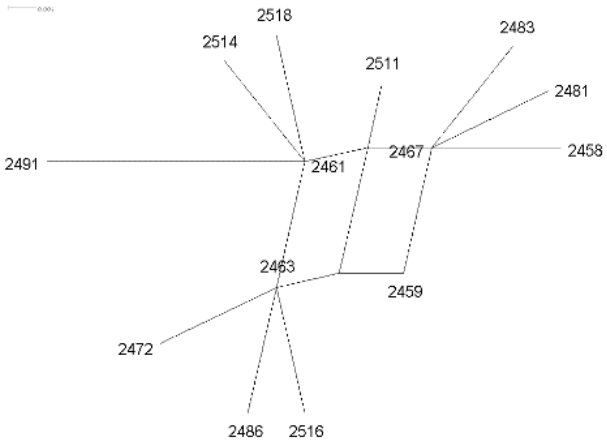


Figure 5-14. NeighborNet splits tree of unique *marB* sequences from SARB strains. One representative strain was selected for each unique allele.

NeighborNet analysis shows minimal network structuring of the *marB* allele (Figure 5-14), which became completely resolved when the allele harbored by strain 2459 was removed from the analysis. The *marB* allele was the only one of the four *mar* operon genes that was deemed to be congruent with the MLST sequences under ILD analysis. It was also congruent with *marA* and *marR*.

5.2.2.5 Assortment and Diversification of marC Alleles

The *marC* gene was the only one of the *mar* genes that did not have a dominant allele or alleles harbored by a majority of strains. The two most commonly identified alleles were present in five strains each, whereby the other *mar* genes were composed of one or two alleles that were found in a third or more of the population. Additionally, the number of *marC* alleles identified was 26, more than another gene in the *mar* operon. Despite this, it retained a moderate amount of nucleotide diversity and appeared to be under a negative selective pressure, an interesting finding considering that the function of the gene has not been identified. The 26 unique alleles of *marC* separated into four clades and these clades were dispersed across the *marRABC* tree. No one clade of *marC*

clustered completely together, with the exception of the alleles 1 and 3 (gray boxes in Figure 5-11). The three strains containing these two alleles remained clustered together on the *marRABC* tree.

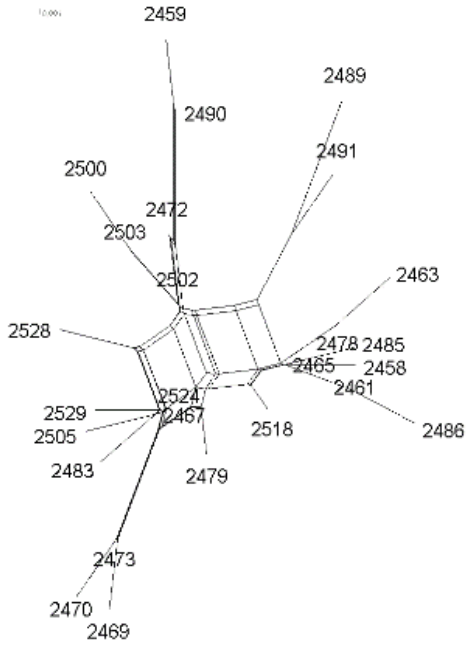


Figure 5-15. NeighborNet splits tree of unique *marC* sequences from SARB strains. One representative strain was selected for each unique allele.

The reticulate nature of the *marC* gene was revealed through a NeighborNet splits tree of the 26 unique alleles present in strains from the SARB collection (Figure 5-15). Network structuring, indicative of conflicting phylogenetic signals in the sequence set, was not resolved by removal of one or a handful of strains. In support of these findings, ILD scores indicated that *marC* not only failed to be congruent with the MLST data but failed to be congruent with the other three genes in the operon as well. Additionally, 36.4% of parsimoniously informative sites were incompatible with those in the remaining three genes.

5.3 Evolutionary Pressures on *ram* Operon

Of the 104 SARA and SARB strains for which *ramRA* sequences were obtained, 50 were unique. The 582 bp *ramR* gene had 27 different alleles while the 342 bp *ramA* gene had 14 different alleles. Only four of the *ramA* alleles were represented by SARA strains. The most common *ramR* allele was found in 25.7% of SARA and SARB strains, collectively. In contrast, over 60% of *ramA* sequences were represented by only two alleles.

ramR had 30 total polymorphic sites, with 20 of these being parsimoniously informative. *ramA* had 13 polymorphic sites, with seven of them being parsimoniously informative. The *ramA* and *ramR* genes had similar levels of nucleotide diversity, with 0.0051 and 0.0068 average substitutions per site, respectively. The *ramR* strains from SARA contained no non-synonymous substitutions, while there existed four non-synonymous substitutions, across 24 polymorphic sites, in strains from the SARB collection. The codon-based Z-test indicated that *ramR* was under negative selective pressure, an indication that the number of non-synonymous mutations was statistically lower than the number of synonymous mutations. Such negative selective pressure results in the purging of novel amino acid mutations from a population, in preference of conserved sequences that have already been well-established as functional cellular proteins. In contrast to *ramR*, the *ramA* gene appeared to be under less negative pressure, as sequences from SARA strains failed to reject the hypothesis of neutral selective pressure. Of the four polymorphic sites in *ramA* alleles from SARA strains, two of them resulted in predicted amino acid mutations. Sequences from SARB however, did reject

the test hypothesis of neutral selective pressure, with only three sites having amino acid polymorphisms, in the total of 12 polymorphic nucleotide sites.

5.3.1 SARA

5.3.1.1 Topological Incongruence and Network Structuring of ramRA

In order to assess topological incongruence between MLST and *ramRA* phylogenies, a tanglegram was constructed from the maximum likelihood trees for each group of sequences, whereby identical taxa were connected in order to display displacement between clades (Figure 5-16). Made visible was the displacement of strain Saintpaul 2209 from its grouping under MLST with Paratyphi B and Muenchen strains, to a clade made up of Heidelberg strains. It was possible that this strain had undergone recombination at the *ram* locus with a Heidelberg strain at some point in the evolutionary history of the strain, seeing as it harbors a divergent form of the allele that was conserved across all Heidelberg strains studied here. Also displaced from their MLST groupings were strains 2208 (Saintpaul) and 2188 (Typhimurium). Under MLST analysis, strain 2208 clustered with other Saintpaul strains but in the *ramRA* phylogeny, contained an allele identical to the one harbored by the majority of Typhimurium strains. Strain 2188, which shared an identical MLST sequence type with strain 2187, was displaced to the Typhimurium clade under *ramRA* analysis.

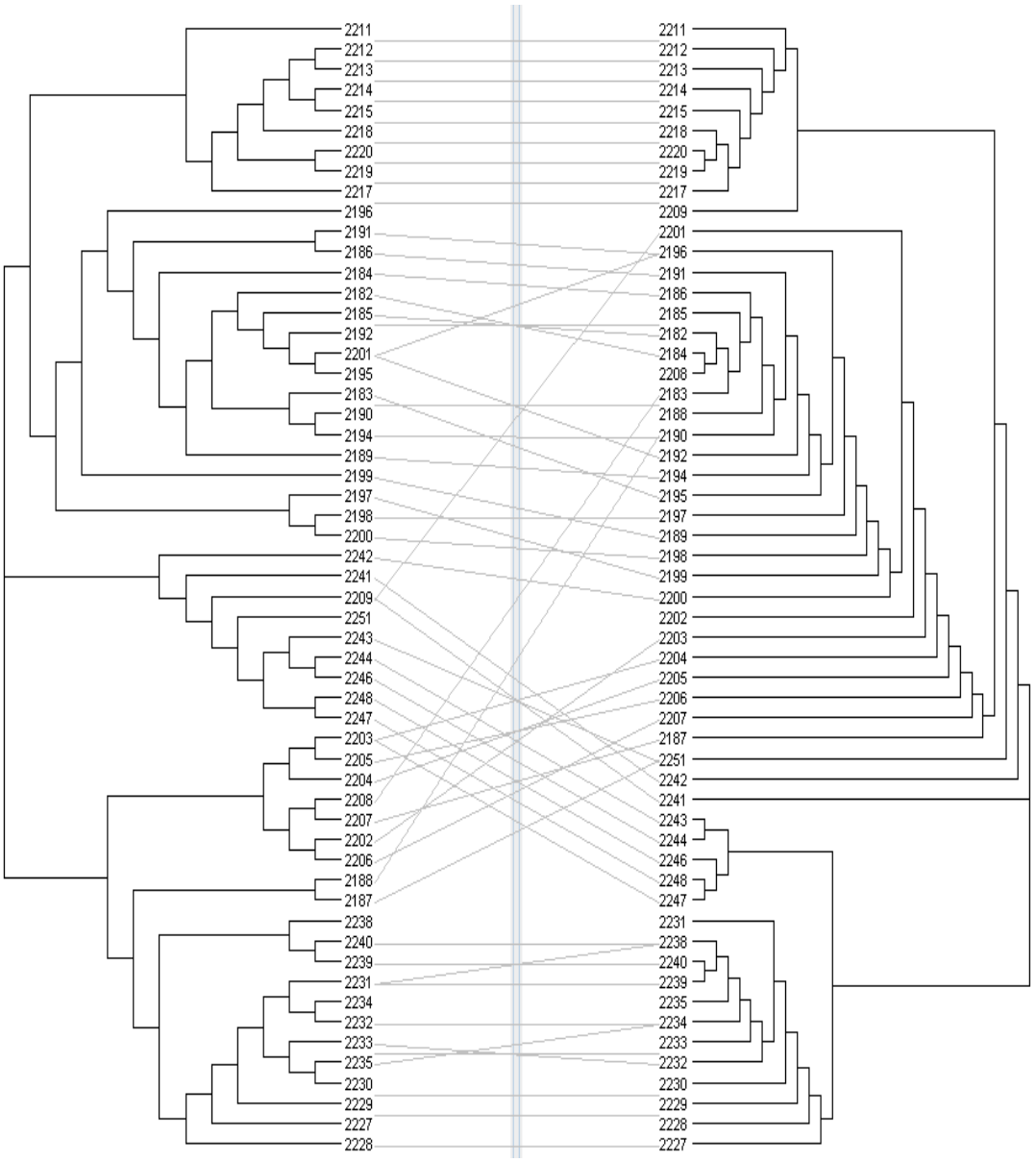


Figure 5-16. Tanglegram showing the SARA MLST maximum likelihood phylogeny (left) alongside the *ramRA* phylogeny (right). Lines are used to connect identical taxa.

A NeighborNet splits tree, used to visualize reticulation among a set of sequences, was built using *ramRA* from the SARA strains (Figure 5-17). Network structure in the *ramRA* splits graph was minimal and became completely resolved when strains 2209, 2241, and 2251 were removed.

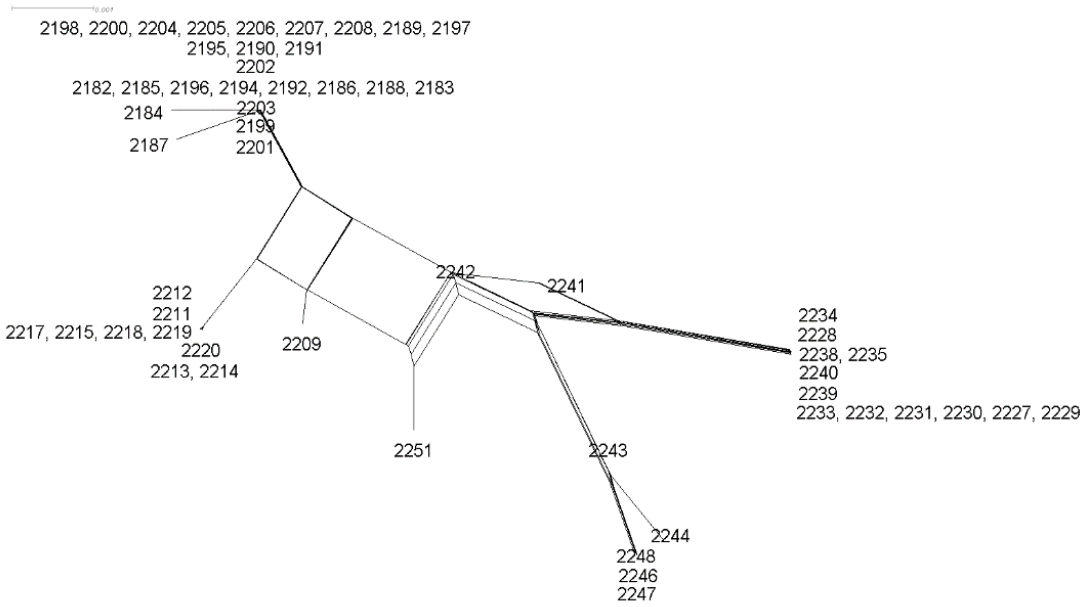


Figure 5-17. NeighborNet splits tree of *ramRA* sequences from SARA

When the incongruence length difference test was used to compare MLST sequence data to *ramRA* from SARA strains, the hypothesis of congruence failed to be rejected, indicating that there was limited incongruence. Taken together, we can see that *ramRA* incongruence was limited to a handful of strains, and when these strains were eliminated from analysis, congruence improves. However, the limited sequence diversity of *ramRA* sequences could give a false impression of the relative impact of homologous recombination on the locus. Any homogenization across taxa would obscure reticulation and congruence scores. To gain a better understanding of the impact that horizontal gene transfer has had on the locus, a cladistics-based analysis was undertaken.

5.3.1.2 ramR Showed Some Homogenization Across Serotypes

The *ramR* gene from SARA was comprised of 10 alleles, with just three of them (identified as alleles 1, 5, and 6) making up 80.4% of the population. None of the alleles

contained a predicted amino acid polymorphism. In order to investigate the impact of recombination on the *ramR* gene, a tree built from *ramR* sequences was compared to the concatenated MLST phylogeny (Figure 5-18). From this alignment, it was possible to see that while *ramR* sequences separated into six clades, the *ramA* sequences separated into only two clades. All Typhimurium and Saintpaul strains in this clade, with the exception of Typhimurium strain 2184 and 2187, shared the same *ramR* allele. As mentioned previously, Typhimurium and Saintpaul strains are members of two different clonal complexes. Strains 2184 and 2187 have two different but related alleles at this locus. Despite the MLST grouping that separated Typhimurium strains 2187 and 2188 from the remaining Typhimurium strains, clustering was retained for *ramR*. The lack of diversification between Saintpaul strains and Typhimurium strains, which clustered independently under MLST analysis, could be indicative of possible recombination, resulting in a homogenizing effect for these two serotypes. A similar lack of diversification was seen for the cluster of Paratyphi B strains (excluding strains 2242 and 2241), which, in the MLST phylogeny, form separate clades and different single-locus variant groups. Here, we can see that these two groups of Paratyphi B shared the same *ramR* allele. The Heidelberg strains all had the same *ramR* allele, as do all but one Muenchen strain. This strain, 2251, harbored an allele that was more similar to that of the Heidelberg strains. Saintpaul strain 2209, which remained ungrouped in the MLST phylogeny, clustered with the Heidelberg strains.

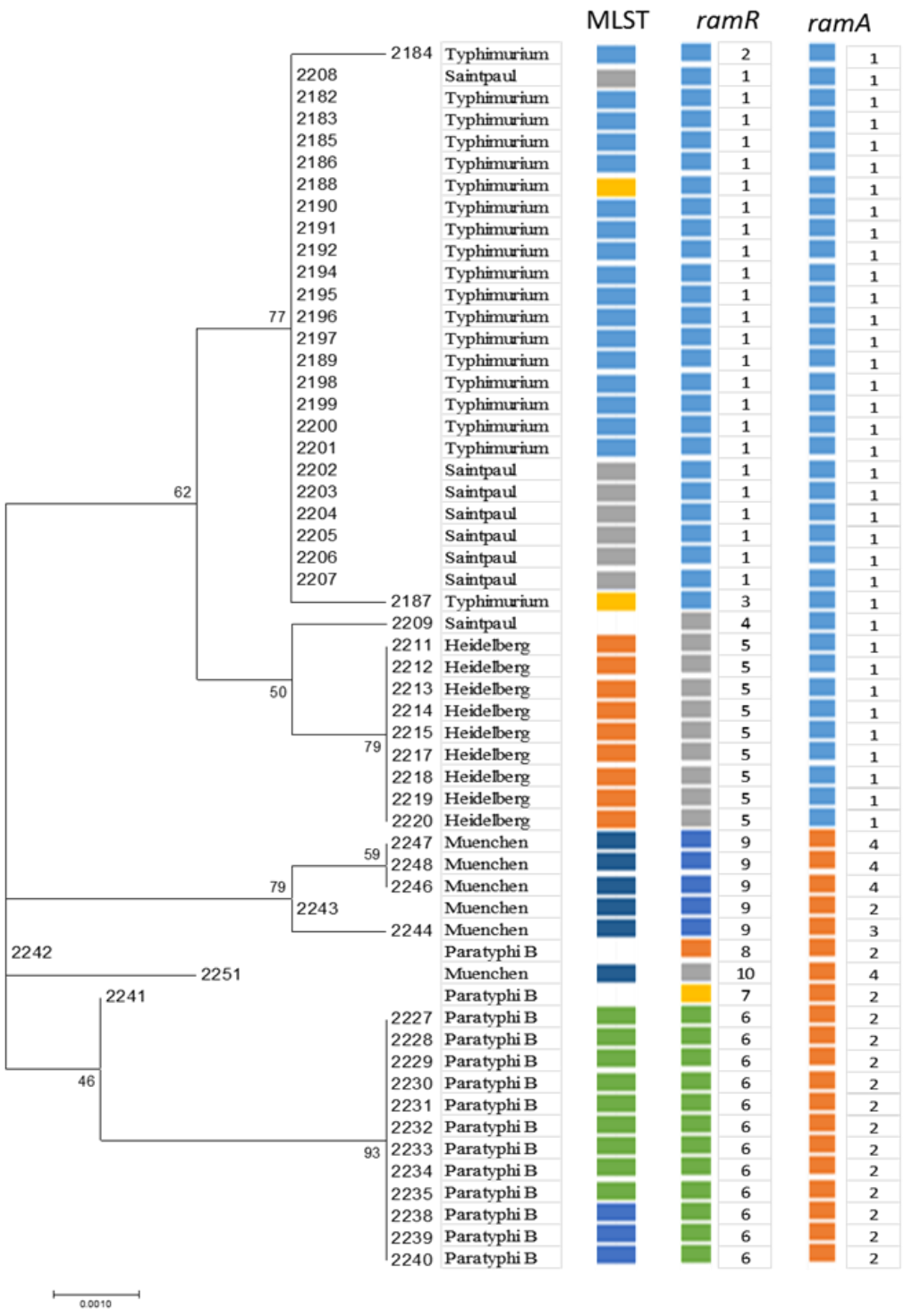


Figure 5-18. Maximum likelihood phylogeny built from *ramRA* sequences of SARA strains. Bootstrap values are presented as percentages. Serotypes are listed next to each taxa. Colored boxes are used to represent clade designations for MLST, *ramR* and *ramA*. Each clade was assigned a unique color and each bar column color set is independent of each other. Numerical allele designations are listed next to each colored box. White boxes indicate an inability to assign a clade designation for MLST data.

Incongruence length difference testing, a statistical method used to determine congruence between two sets of sequence data, showed congruence between *ramR* and *ramA*, as well as congruence between the concatenated MLST sequences and *ramR*, indicating that there was limited recombination between the strains of SARA. In support of this were intragenic incompatibility scores. *ramR* and *ramA*, respectively, have scores of 5.6% and 0.0%, indicating that there have been few repeated mutations affecting the nucleotide sequences of each gene. However, failure to reject the hypothesis of congruence would be limited by the overall sequence similarity between SARA strains.

Splits tree analysis of the *ramR* from SARA strains showed minimal network structure, which was completely resolved when allele numbers 4, 7, and 8 (corresponding to strains 2241, 2242, and 2209, respectively) were removed from the network (Figure 5-19). This was also indicative of limited recombination that can be attributed to a handful of strains in the Typhimurium complex population, in addition to the limited homogenizing that was seen between Typhimurium and Saintpaul strains and the limited divergence that would be expected between the two clonal complexes of Paratyphi B (Figure 5-19). Network structuring was also limited by the relatively small number of *ramR* alleles.

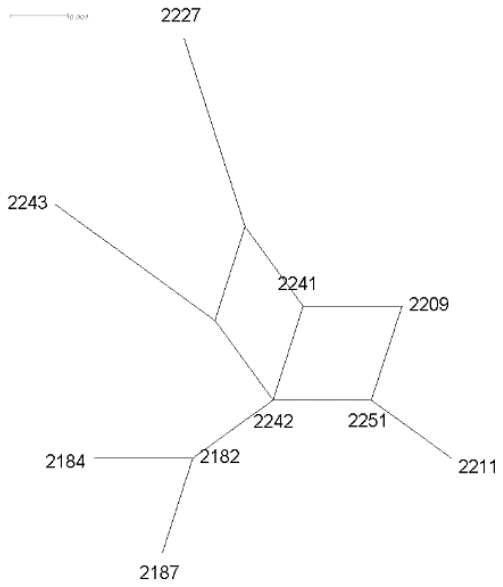


Figure 5-19. NeighborNet splits tree built using unique *ramR* sequences from SARA

5.3.1.3 ramA Homogenization Between Strains

As was done for *ramR*, a *ramA* tree was aligned with the phylogenetic tree built from concatenated MLST data. The SARA strains shared only four alleles among the 56 strains studied here. Under cladistics analysis, one allele formed a cluster by itself and this allele (indicated here as *ramA* allele 1) was present in the majority of strains (n=36, 64%). There were clear delineations for homogenization of *ramA*, with all Saintpaul, Typhimurium, and Heidelberg strains harboring a single, undifferentiated allele. The remaining 20 strains had three *ramA* alleles that clustered together under phylogenetic analysis. All Paratyphi B strains harbored the same allele. The Muenchen strains harbored three different yet related alleles. It is obvious that *Salmonella* strains from the SARA collection have a specific set of *ramA* sequences that have been selectively retained. Interestingly though, of the four nucleotide polymorphisms present in these alleles, two of them were non-synonymous, resulting in predicted amino acid mutations. While there was limited nucleotide diversity among *ramA* in SARA, there was an amount

of amino acid diversity, contributing to the inability described above to reject a hypothesis of neutral evolutionary pressure on the gene. Splits tree analysis of the four alleles show a star-like pattern, with no network structuring (Figure 5-20). However, any reticulation in the history of the gene would be obscured by the homogenization of nucleotide sequences and the small number of alleles.

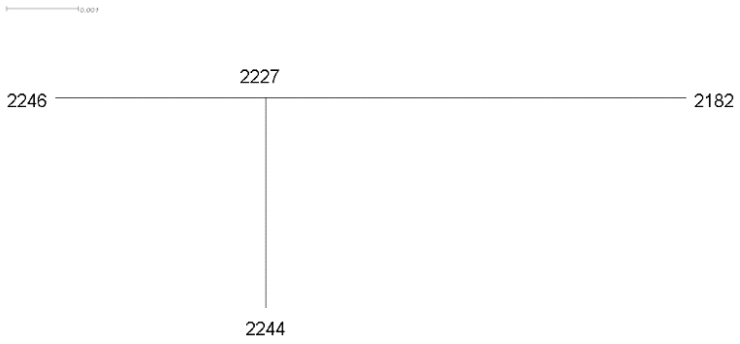


Figure 5-20. NeighborNet splits tree built using unique *ramA* alleles from SARA

5.3.2 SARB

5.3.2.1 Topological Incongruence and Network Structuring of ramRA

A phylogeny built using MLST data from the SARB strains was aligned next to a tree built from *ramRA* for the same set of strains to allow for visualization of topological incongruence (Figure 5-21). Many strains were displaced from their respective clades on the MLST tree into different clades in the *ramRA* tree. The MLST tree, which was here used to represent the whole genome phylogeny, showed discordance with the operon tree, indicative of evolutionary shuffling that is not supported by a bifurcating tree alone. The ILD test also rejected the hypothesis of congruence for MLST and *ramRA* sequences.

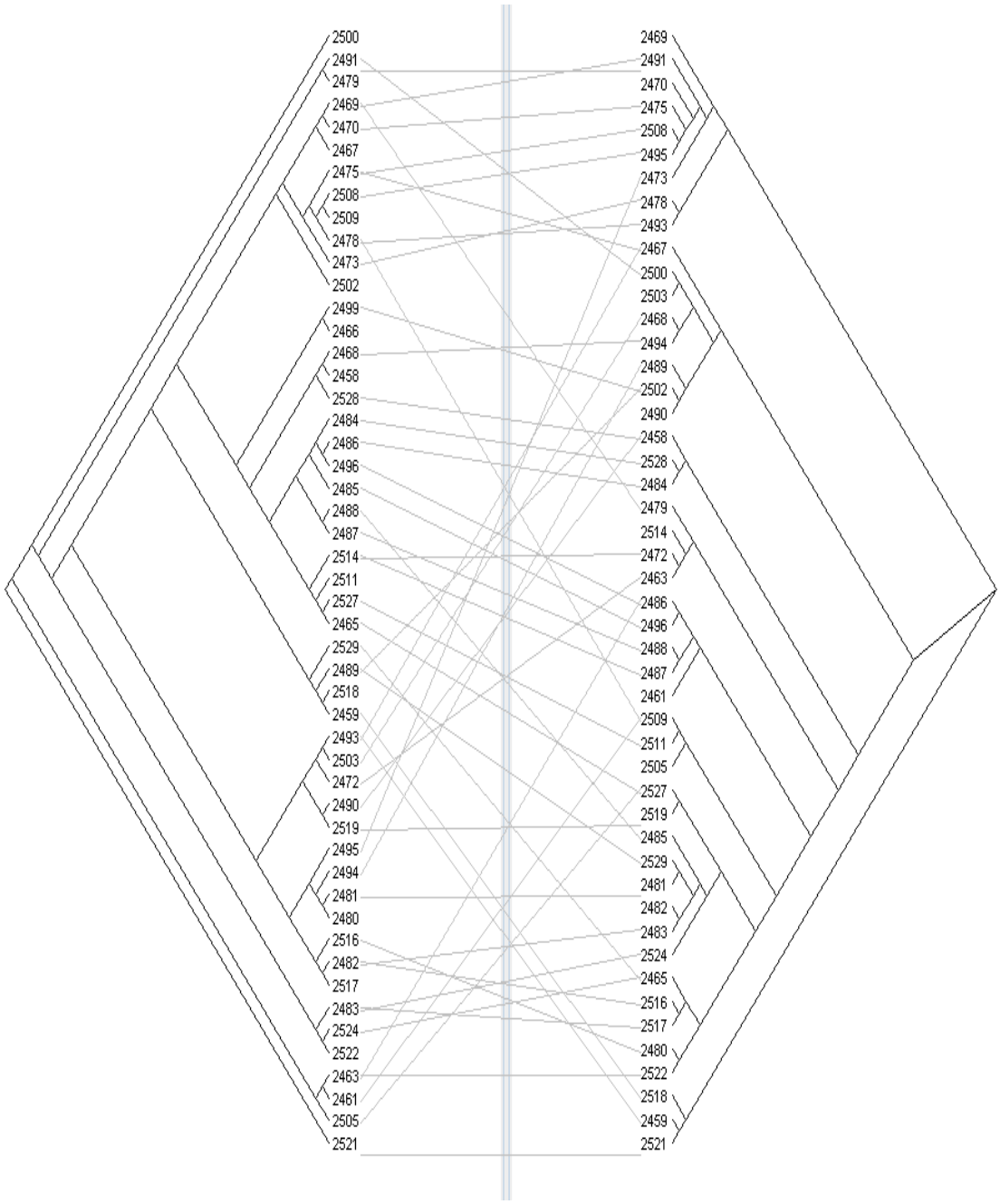


Figure 5-21. Tanglegram displaying MLST maximum likelihood phylogeny (left), with identical taxa connected to the maximum likelihood *ramRA* phylogeny (right)

When the *ramRA* sequences were used to build a splits network, the reticulate nature of the *ramRA* operon was visible further (Figure 5-22). The splits tree revealed many parallel paths and lack of a strictly star-like network, indicative of the many possible evolutionary steps take to reach a given sequence.

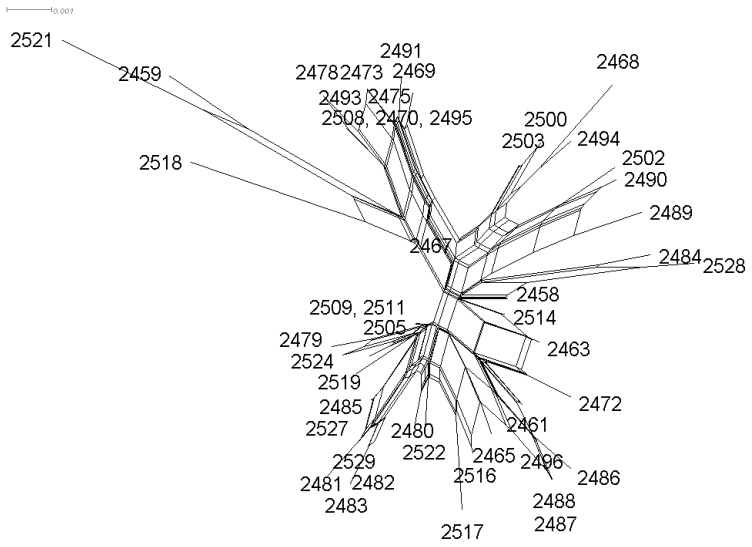


Figure 5-22. NeighborNet splits tree built using *ramRA* sequences from SARB strains

5.3.2.2 Evidence For ramR Allele Shuffling, Including Among Niches

Among the 48 strains analyzed that belong to the SARB collection, 25 different *ramR* alleles were identified. These *ramR* alleles separated into six clades, with all but three strains falling into four of these clades (Figure 5-23). The majority of strains (n=41) have an allele that was a member of one of three clades. There was little congruence across the MLST and *ramR* data sets, with identical or similar alleles being distributed across diverse *S. enterica* strains. Excluding strains 2518, 2459, and 2521, each of the four remaining *ramR* clades was comprised of strains from at least four different MLST clades. This was evidence for extensive allelic shuffling between strains from a variety of genetic and environmental backgrounds. For instance, Gallinarum strain 2478 (poultry-restricted) had the same *ramR* allele as a Newport strain 2493. Choleraesuis strain 2461 (swine-adapted) shared *ramR* alleles with Montevideo, Miami, and Panama strains but not with the other Choleraesuis strain 2463, which had a unique allele not found in any

other strain studied here. Paratyphi C strain 2505 (human-restricted) shared *ramR* alleles with a Rubislaw and a Pullorum strain. Two Paratyphi B strains (2500 and 2503) shared the same *ramR* allele, despite clustering separately under MLST analysis. Additionally, strains sharing the same evolutionary history, as defined by MLST clonal complexes, had different *ramR* alleles. Such was the case for strains 2522 and 2524. Strains from the other two single-locus variant groups retained the same *ramR* allele.

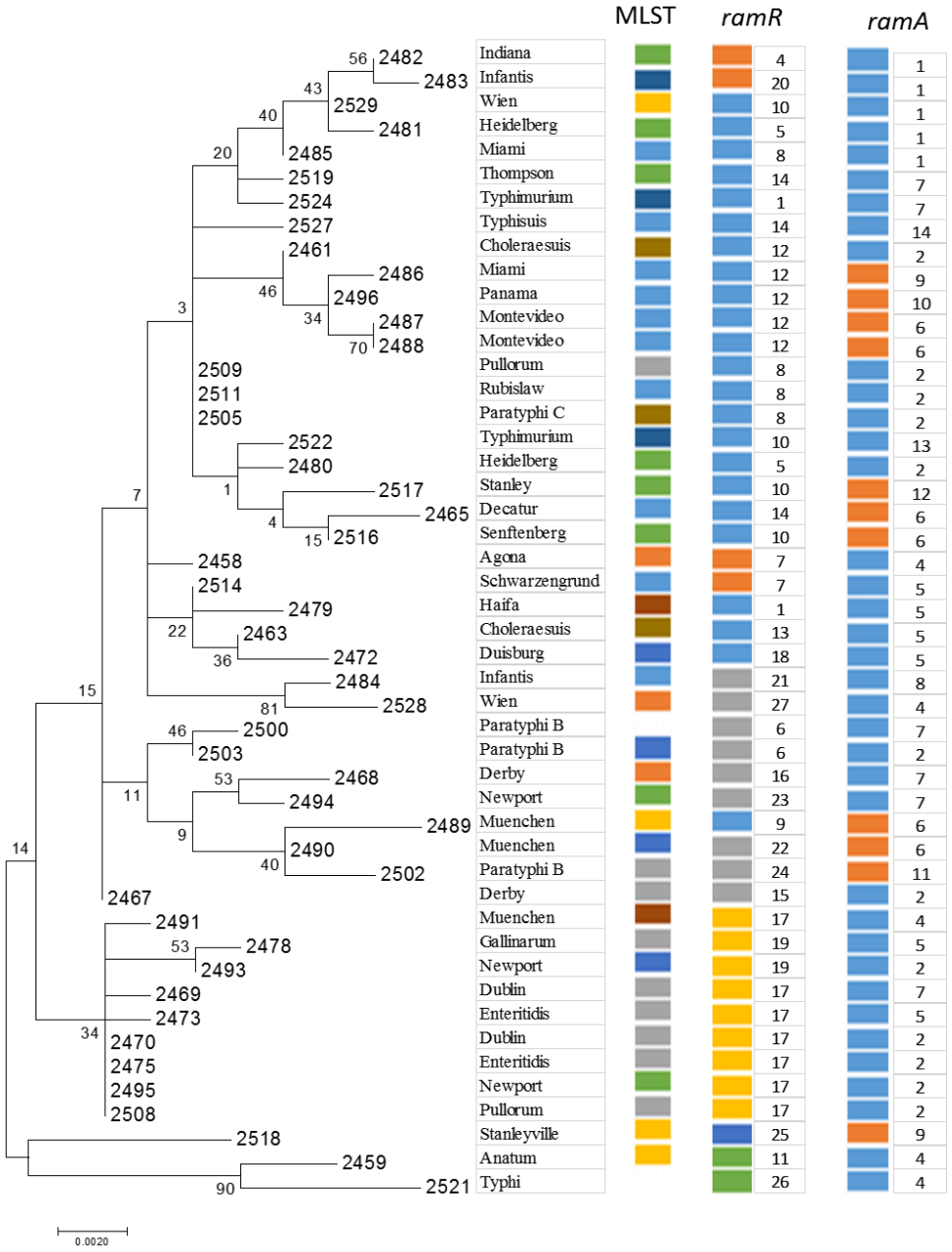


Figure 5-23. Maximum likelihood phylogeny built from *ramRA* sequences of SARB strains. Bootstrap values are presented as percentages. Serotypes are listed next to each taxa. Colored boxes are used to represent clade designations for MLST, *ramR* and *ramA*. Each clade was assigned a unique color and each bar column color set is independent of each other. Numerical allele designations are listed next to each colored box. White boxes indicate an inability to assign a clade designation for MLST data.

Additionally, there was evidence for intra-gene shuffling, with 24.2% of sites within *ramR* being incompatible with other sites in the gene. The splits tree showed network structuring, with no single strain or handful of strains contributing to the overall network (Figure 5-24).

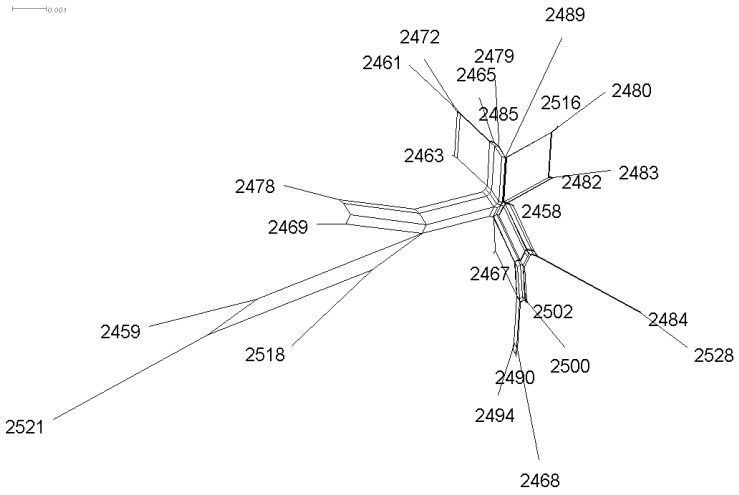


Figure 5-24. NeighborNet splits tree of unique *ramR* alleles from SARB strains

5.3.2.3 Homogenization of ramA Within Subspecies I Salmonella

The *ramA* alleles from SARB strains were collectively less diverse than those from *ramR*, and among the 48 strains, only 13 different alleles were identified. Six of these 13 alleles were present in 71.4% of strains (n= 40). The 13 total alleles separated into two clades, with alleles 6, 9, 10, 11, and 12 clustering separately from the remaining eight alleles. When aligned along the MLST tree, it was possible to see that clustering of the two clades of alleles was not maintained, with allele 6 in particular, distributed

throughout the *ramRA* tree. Allele 2, another common *ramA* allele was also distributed across strains that do not cluster together on the *ramRA* tree.

The splits tree showed star-like network structuring with no reticulate network (Figure 5-25). Intra-gene incompatibility was 0.0%--all parsimoniously informative sites within *ramA* were compatible with each other. Visualization of reticulation would be limited by the lack of nucleotide diversity seen at this locus.

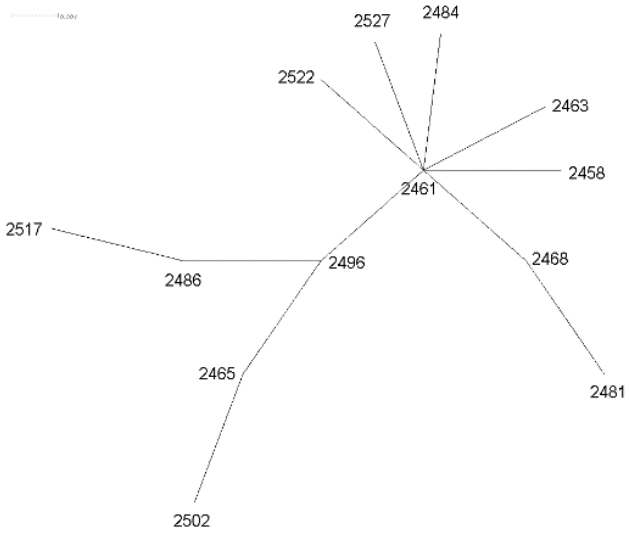


Figure 5-25. NeighborNet splits tree built using unique *ramA* alleles from SARB strains

5.3.2.4 Evidence for Intra-Operon Shuffling Among ramRA

The tanglegram below shows two maximum likelihood trees, built from the sequences of *ramR* and *ramA* (Figure 5-26). Identical taxa were then connected with lines, demonstrating the lack of topological congruence among the two genes. Not only was the overall topology of the trees different, but many taxa failed to cluster in the same manner.

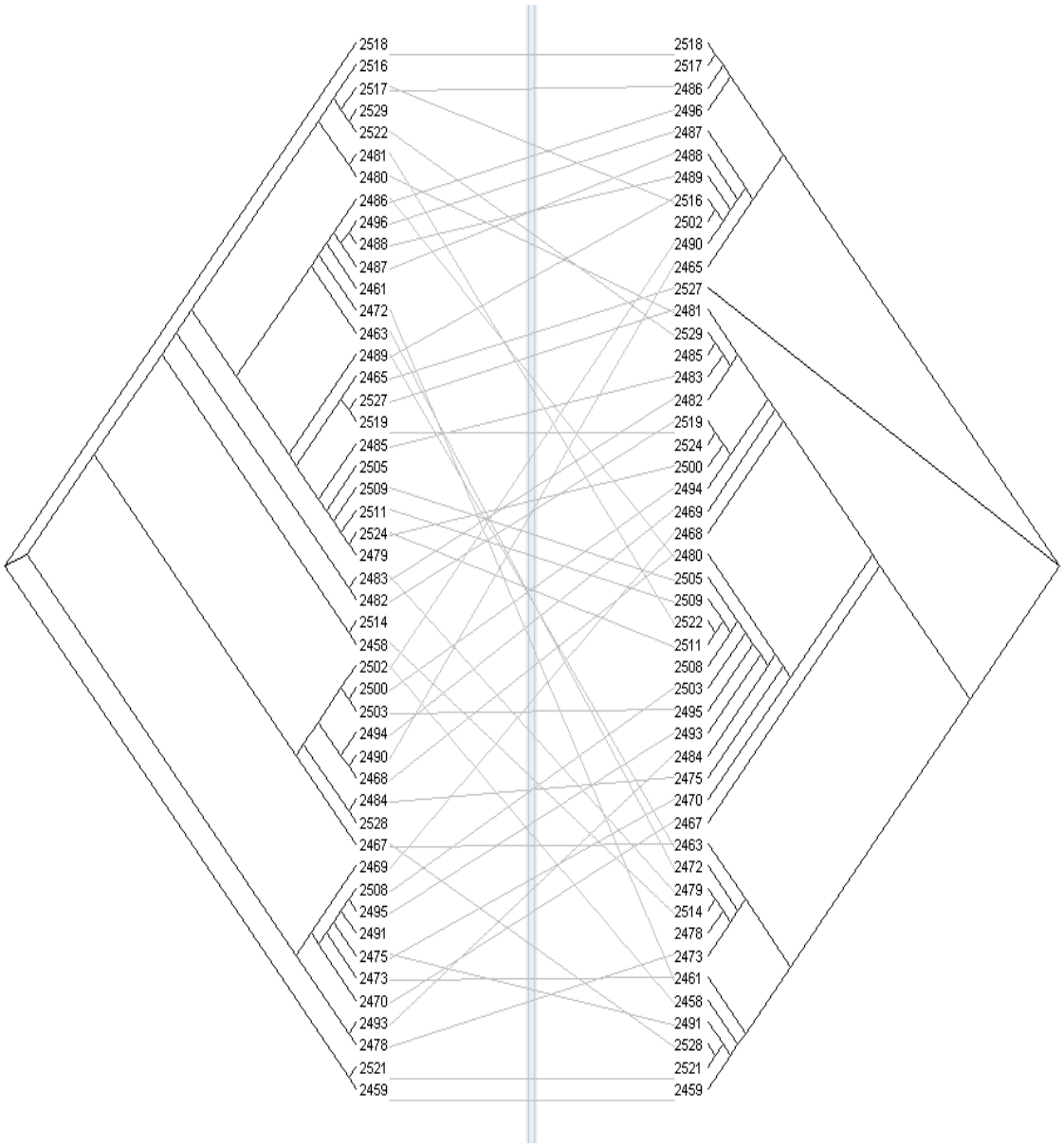


Figure 5-26. Tanglegram showing *ramR* maximum likelihood phylogeny connected to *ramA* maximum likelihood phylogeny.

When parsimoniously informative sites were compared to each other, it was determined that inter-gene incompatibility is 47.6%. That is, almost half of parsimoniously informative sites had mutations that cannot be accounted for with less than two steps on a bifurcating tree. High incompatibility scores can be indicative of allele swapping and recombination, which in this case, impacted the two genes in different ways. In support

of this was the ILD score, which rejected the hypothesis of congruence, when *ramR* and *ramA* were compared to one another. Additionally, the ILD test was used to compare the inter-genic region, containing the promoters for each gene, to the genes individually. In both cases, the inter-genic region was incongruent with the coding region.

5.4 Evolutionary Pressure on *sox* Operon

Of the 104 SARA and SARB strains for which *soxRS* sequences were obtained, 23 were unique. The 459 bp *soxR* gene had 19 different alleles while the 324 bp *soxS* gene had nine different alleles, and only two of these were represented by SARA strains. These nine alleles distributed across 104 strains contributed to a very low allelic diversity of 0.266. The allelic diversity is defined as the probability of randomly selecting two different alleles from the pool of total alleles. In comparison, *soxR* had an allelic diversity of 0.87. Over 90% of the strains had one of two *soxS* alleles and the remaining seven *soxS* alleles were spread across less than 10% of strains. *soxR* had 14 total polymorphic sites, with 11 of these being parsimoniously informative. *soxS* had 10 polymorphic sites, with 4 of them being parsimoniously informative. As echoed by the allelic diversity, the total nucleotide diversity of *soxS* was significantly lower than that of *soxR*, a difference of about 3.6-fold. Despite its relatively higher level of diversity, *soxR* contained no non-synonymous substitutions (dN/dS=0.00) and as a result, the predicted amino acid sequence for this gene were conserved across all strains. The p value of the Z-test statistic indicated that it was, as can be presumed from the lack of non-synonymous mutations, under negative selective pressure. In contrast, despite its much lower nucleotide diversity, *soxS* had three non-synonymous mutations in its 10 polymorphic sites. The p value for the Z-test statistic indicated that it was also under a negative selective pressure, when all

sequences are analyzed collectively. However, when analyzed by SAR collection, the test hypothesis of neutrality failed to be rejected for SARA strains while the analysis of SARB sequences did reject the test hypothesis of neutrality. This inability to reject the hypothesis of neutrality was likely due to the overall lack of mutations in *soxS* from SARA strains.

5.4.1 SARA

5.4.1.1 Limited Topological Incongruence and Network Structuring

Alignment of the concatenated MLST tree with the *soxRS* tree for SARA strains further supported the hypothesis of low levels of clonality among the operon (Figure 5-27). With the exception of four strains, clustering was conserved along the two trees. As was mentioned earlier, strains 2187 and 2188 were displaced from their own cluster under MLST analysis and instead clustered with the remaining Typhimurium and Saintpaul strains. Strains 2241 and 2242 also clustered differently on the MLST and *soxRS* trees. On the MLST phylogeny, 2241 and 2242, both Paratyphi B, clustered with other Paratyphi B strains but in the *soxRS* tree, they clustered with a group largely composed of Typhimurium strains. Congruence was otherwise maintained for the remaining strains.

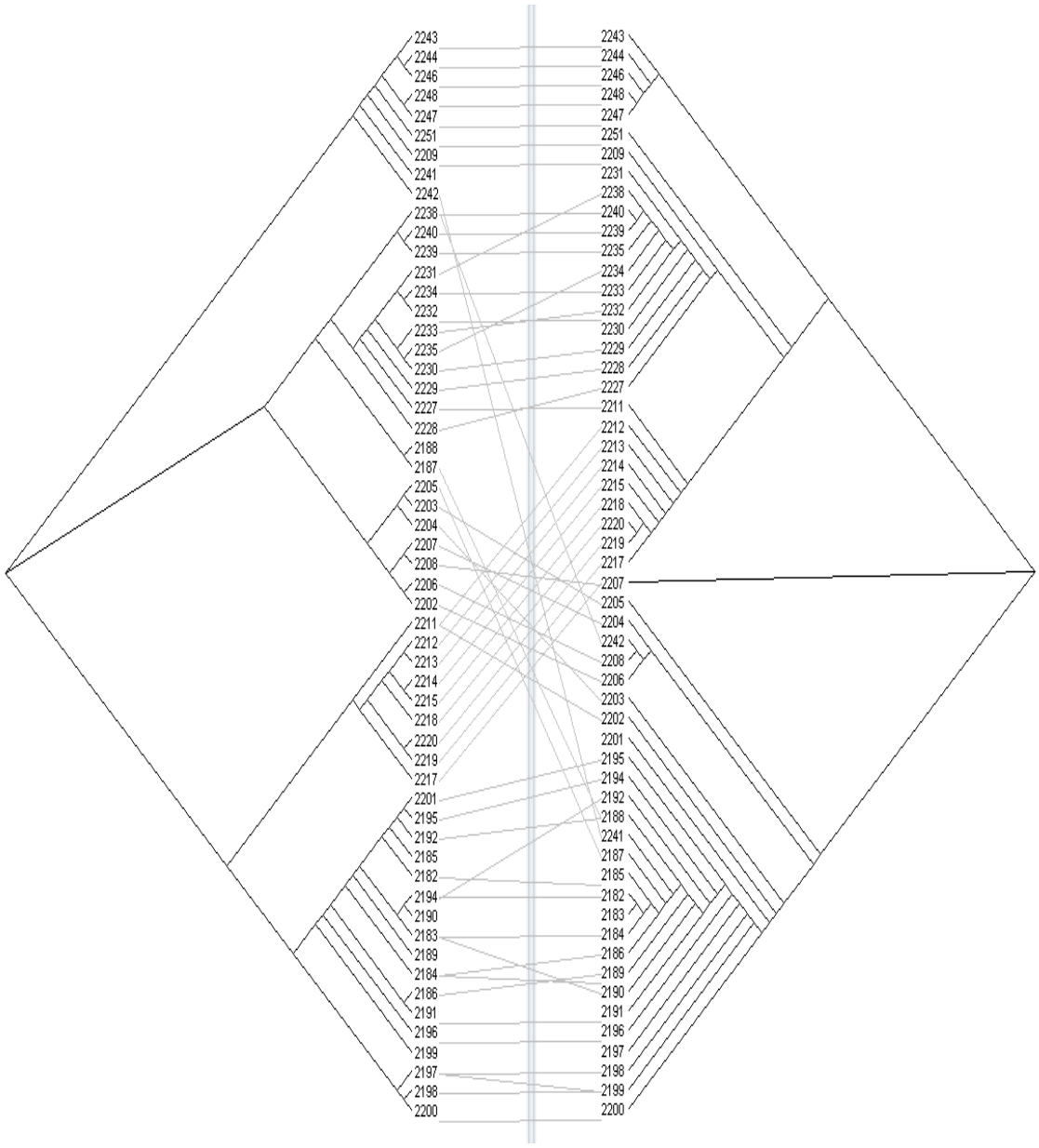


Figure 5-27. Tanglegram showing MLST maximum likelihood phylogeny (left) connected to *soxRS* maximum likelihood phylogeny (right)

The four major groupings of *soxR* were echoed in the splits tree of *soxRS* (Figure 5-28). Network structure in the *soxR* splits graph was minimal and became completely resolved when the Muenchen allele was removed. This is possible evidence for intra-genic recombination in that allele.

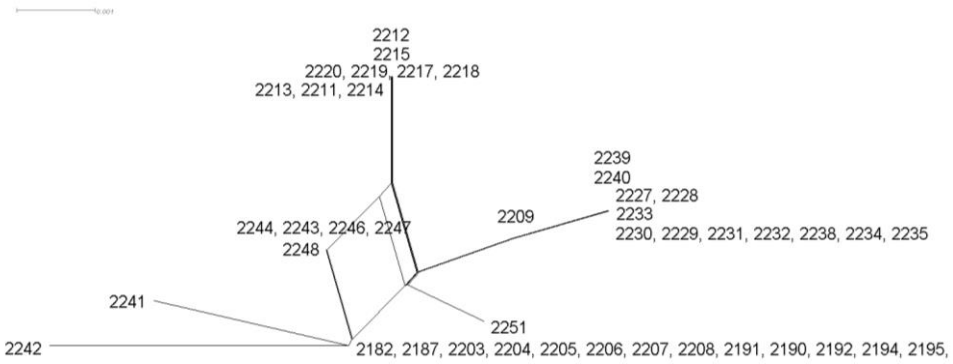


Figure 5-28. NeighborNet splits tree built using *soxRS* sequences from SARA strains

5.4.1.2 soxR Lacks Diversification in Typhimurium Complex Strains

In order to investigate the impact of recombination on the *soxR* gene, a tree built from *soxR* sequences was compared to the concatenated MLST tree (Figure5-29). From this alignment, it was possible to see that all Typhimurium strains and all but one Saintpaul strain shared the same *soxR* allele.

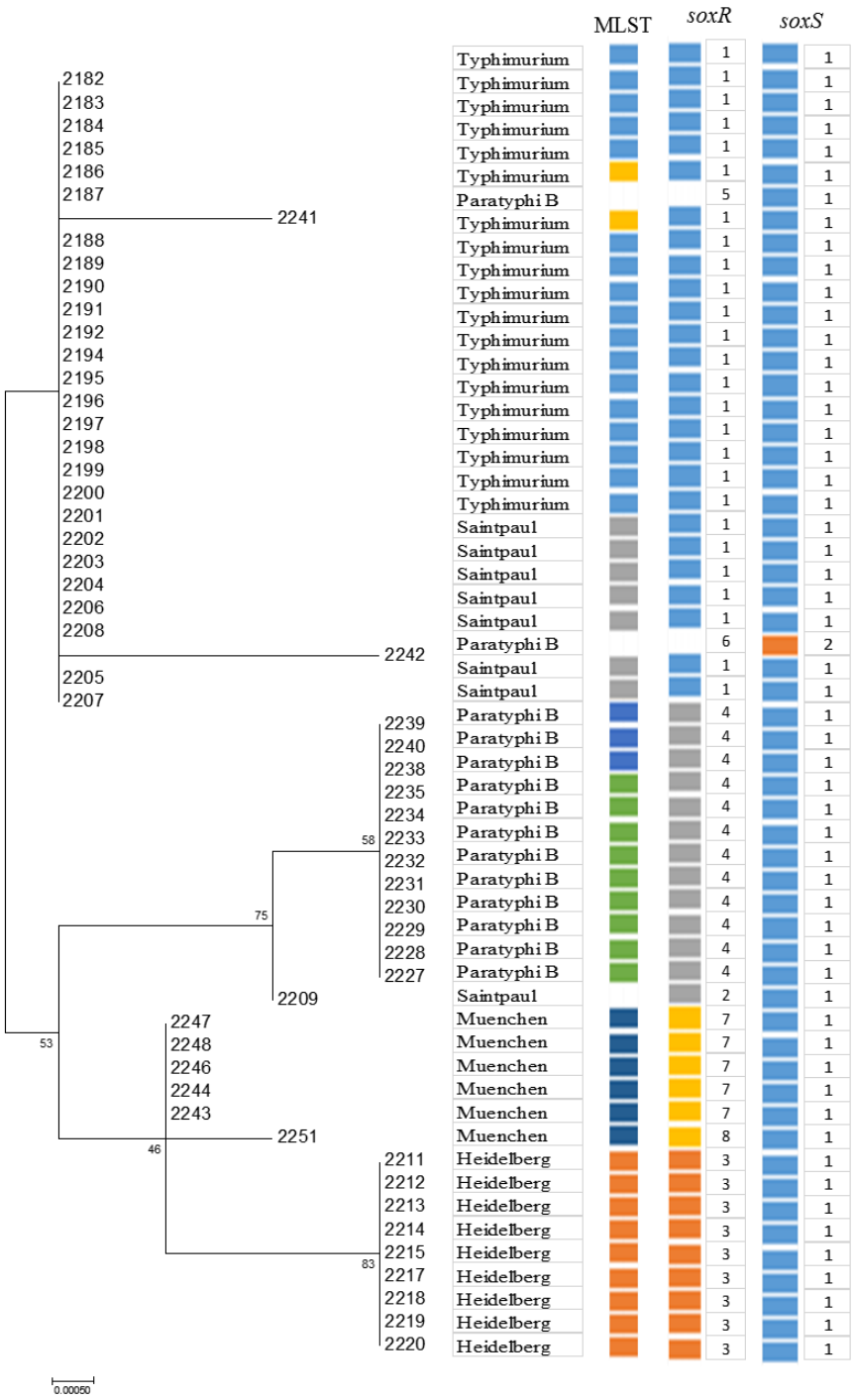


Figure 5-29. Maximum likelihood phylogeny built from *soxRS* sequences of SARA strains. Bootstrap values are presented as percentages. Serotypes are listed next to each taxa. Colored boxes are used to represent clade designations for MLST, *soxR* and *soxS*. Each clade was assigned a unique color and each bar column color set is independent of each other. Numerical allele designations are listed next to each colored box. White boxes indicate an inability to assign a clade designation for MLST data.

This includes two Typhimurium strains (2187 and 2188) that failed to cluster with the remaining Typhimurium strains in the MLST phylogeny and formed their own independent cluster. The two clades of Paratyphi B, separated in the MLST phylogeny shared the same *soxR* allele. The Muenchen and Heidelberg strains retained separation of *soxR* clades. Here we can see that strains belonging to two different serotype groups shared the same *soxR* allele—Typhimurium strains and Saintpaul strains. The same was true for Paratyphi B strains. Under MLST analysis, they formed different groupings, while both being made up of solely Paratyphi B strains, separated into two different single-locus variant groupings. And despite being members of two different clonal complexes, they retained the same *soxR* allele. Retention of clonality could be seen in the Heidelberg strains, which all clustered together in the MLST tree and all had the same unique *soxR* allele. Muenchen strains, with the exception of 2251, also all had the same unique *soxR* allele. The *soxR* allele of strain 2251 was the same as the allele for the other five Muenchen strains, with an additional polymorphism at nucleotide 249, resulting in a silent A to G mutation.

Network structuring, as revealed through a splits tree of the eight *soxR* alleles (Figure 5-30), showed that there was limited reticulate evolution at play between the alleles. Removal of the allele harbored by strain 2243 resulted in a completely star-like graph. However, network structuring could be limited by the lack of nucleotide diversity seen in *soxR* from SARA strains.

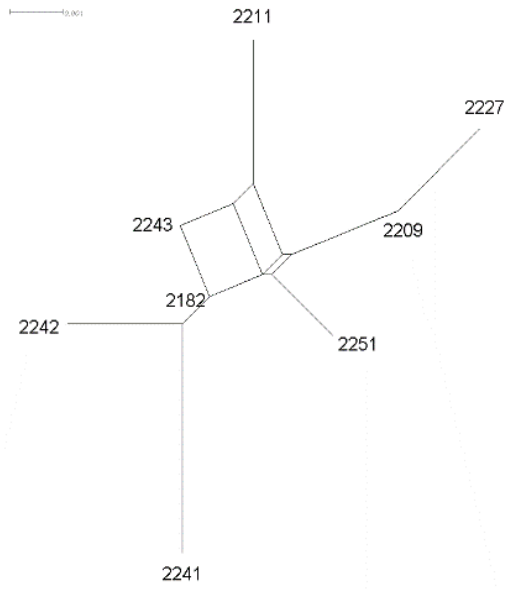


Figure 5-30. NeighborNet splits tree built using unique *soxR* sequences from SARA strains.

Pairwise site compatibility information indicates that 30% of *soxR* parsimoniously informative sites were incompatible with each other, indicative of repeated mutations that obscure the phylogenetic signal.

5.4.1.3 soxS Homogenization Between Typhimurium Complex Strains

As was done for *soxR*, a *soxS* tree was aligned with the phylogenetic tree built from concatenated MLST data. Interestingly, all SARA strains except 2242 shared a single *soxS* allele. Strains that fell into clades under MLST analysis and under analysis of *soxR* failed to separate, indicating that there were limited preferred alleles for *soxS*. Strain 2242, the only strain with a different *soxS* allele, had a C to T silent transition mutation at nucleotide 198 and a T to C silent transition mutation at nucleotide 312 that differentiated it from the other *soxS* alleles present in SARA strains. Such significant lack of diversity of the *soxS* gene among five different serotypes of *S. enterica* indicated that not only was this allele preferred, but that allele swapping over time has cleansed the population of much variation that we would expect to see if recombination were not at play.

5.4.2 SARB

5.4.2.1 Topological Incongruence and Network Structuring

Splits tree analysis shows that the *soxRS* genes from SARB strains had an amount of reticulate evolution, with no one allele type contributing exclusively to this (Figure 5-31).

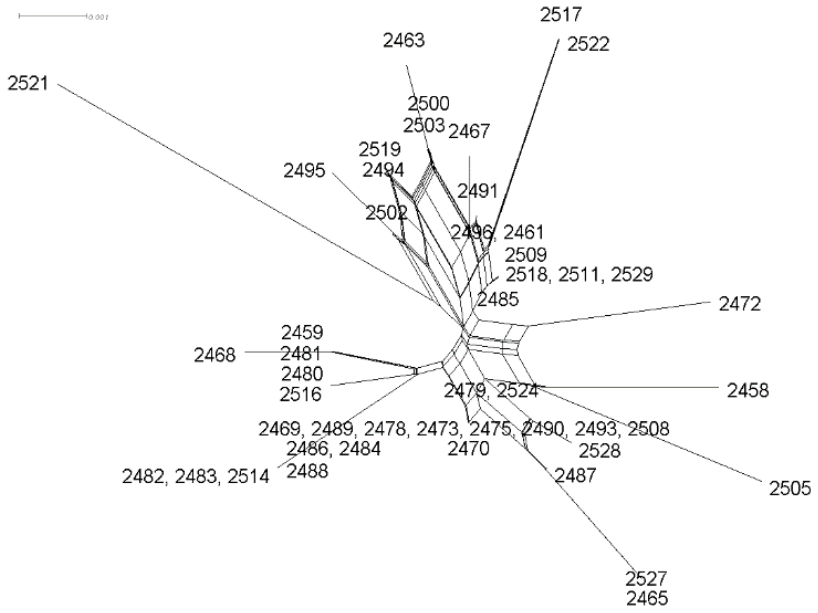


Figure 5-31. NeighborNet splits tree built using *soxRS* sequences from SARB strains

Topological incongruence visualized with a tanglegram was also consistent with the swapping of alleles between taxa (Figure 5-32), with many strains failing to align when the MLST and *soxRS* trees were placed next to each other. An incongruence length difference test also rejected the hypothesis of congruence, when the MLST data is compared to *soxRS* data for SARB strains.

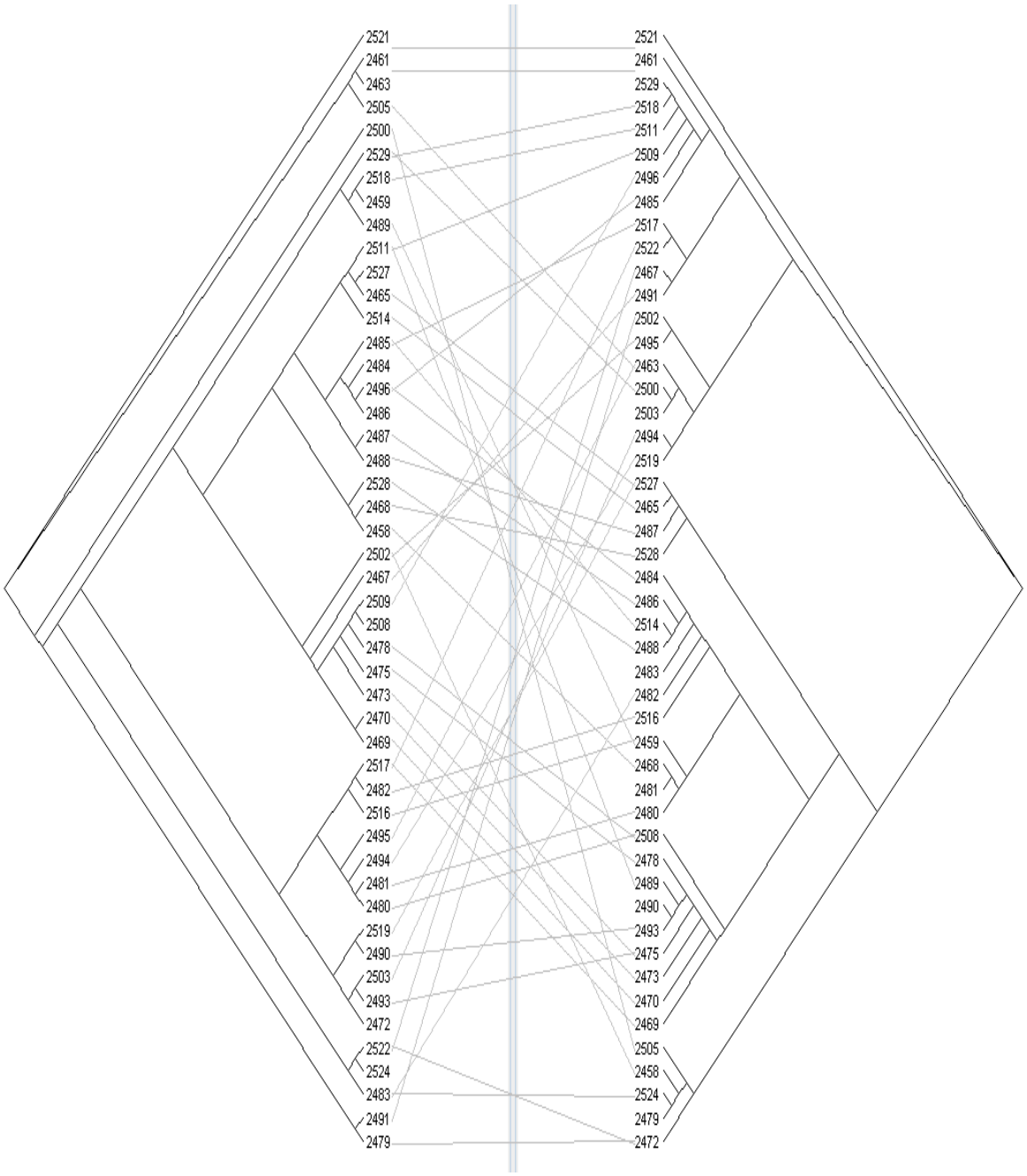


Figure 5-32. Tanglegram displaying MLST phylogeny (left) connected to *soxRS* phylogeny (right) of SARB strains

5.4.2.2 Assortment of soxR Within Subspecies I Salmonella

Of the 48 strains analyzed that belong to the SARB collection, the *soxR* alleles separated into four clades, as compared to the nine clade groupings for MLST data

(Figure 5-33). There was little congruence across the two data sets, with identical or similar alleles being distributed across diverse *S. enterica* strains. Each of the four *soxR* clades was comprised of strains from at least three different MLST clades. This was indicative of extensive allelic shuffling, over the history of the subspecies, between strains from different genetic backgrounds. For instance, strains 2461 and 2509, serotypes Cholerasuis and Pullorum, respectively, are both host-adapted to different species of mammal—swine and poultry. Despite this, they both shared the same *soxR* and *soxS* alleles, indicating some level of niche overlap which has led to the strains having identical sequences at this loci. Of the six strains belonging to three single-locus variant groups in SARB (2469 and 2470, 2465 and 2527, 2522 and 2524), two pairs retained the same set of *soxR* alleles. The third pair however, strain 2522 ad 2524, had two different *soxR* alleles. Despite being members of a clonal complex, the two strains had experienced divergence of their *sox* operon.

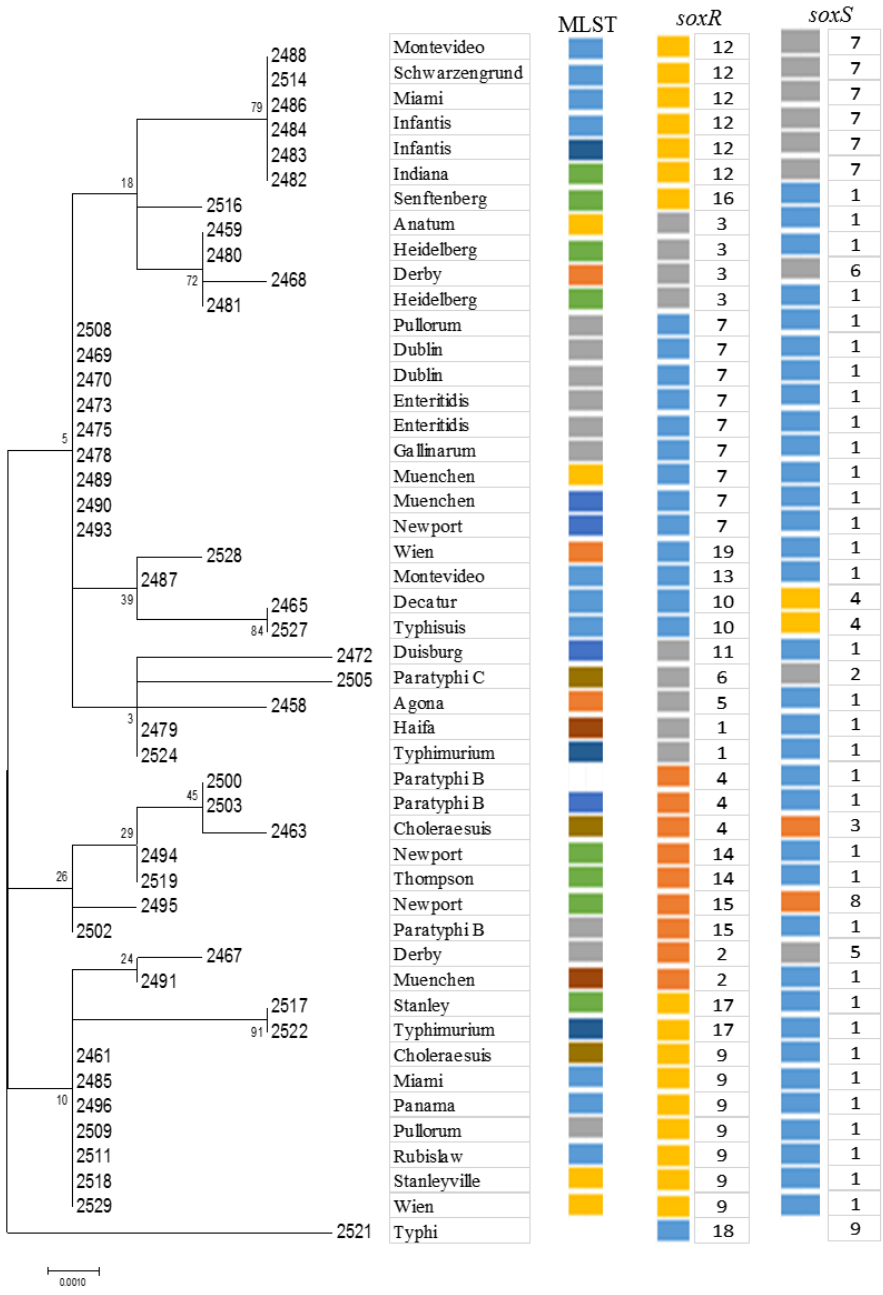


Figure 5-33. Maximum likelihood phylogeny built from *soxRS* sequences of SARB strains. Bootstrap values are presented as percentages. Serotypes are listed next to each taxa. Colored boxes are used to represent clade designations for MLST, *soxR* and *soxS*. Each clade was assigned a unique color and each bar column color set is independent of each other. Numerical allele designations are listed next to each colored box. White boxes indicate an inability to assign a clade designation for MLST data.

Splits tree analysis showed that the *soxR* genes from SARB strains had an amount of reticulate evolution (Figure 5-34), with no one allele type contributing exclusively to this, as was the case for SARA *soxR* sequences.

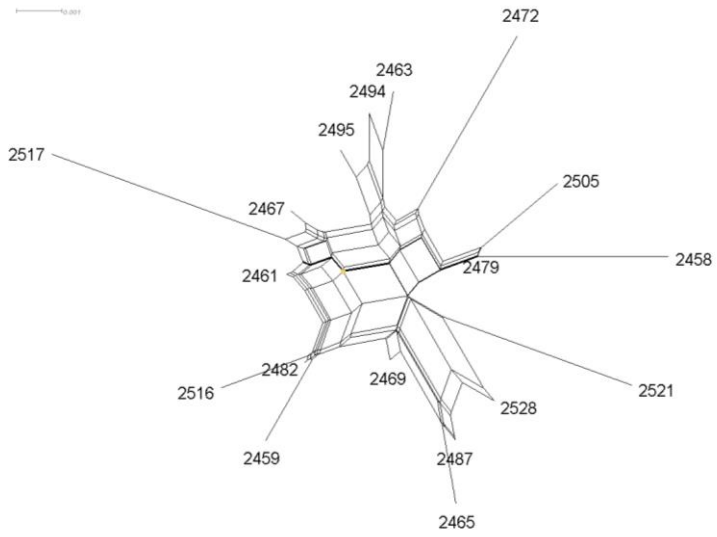


Figure 5-34. NeighborNet splits tree built using unique *soxR* sequences from SARB strains

Incompatibility within the SARB *soxR* gene was 36.1%, indicative of repeated mutations that obscured phylogenetic signals.

5.4.2.3 Homogenization of soxS Within Subspecies I Salmonella

As was seen in SARA, there was a limited amount of genetic diversity among *soxS* alleles, resulting in a homogenizing effect for this gene. Though *soxS* alleles among SARB strains were more diverse than SARA *soxS* alleles, the majority of the strains had retained conserved alleles. The majority of the strains (n=34, 73%) share a single *soxS* allele.

The splits tree graph is star-like (Figure 5-35), with no network structure, indicating that the phylogeny for *soxS* could be reproduced in a single bifurcating tree.

However, the limited diversity of *soxS* alleles could have obscured any reticulate evolutionary signals, and that was likely the case for this gene.

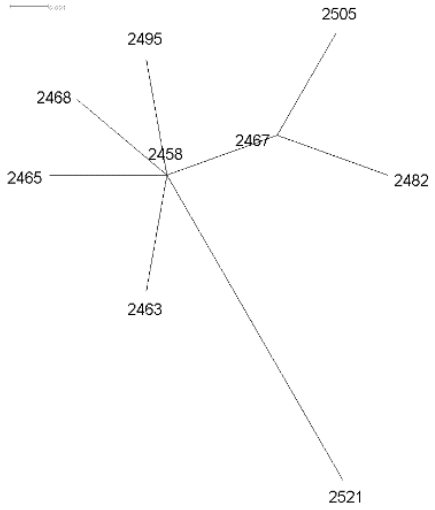


Figure 5-35. NeighborNet splits tree built using unique *soxS* sequences from SARB strains

Despite the lack of reticulation seen in the splits tree, there still existed evidence for recombination which had the effect of spreading a small number of closely-related alleles of *soxS* throughout subspecies I strains of *Salmonella*.

5.5 Discussion

Horizontal gene transfer, as it applies to the operon genes examined here, appeared to play a significant role in shaping the evolutionary history of the *mar*, *ram*, and *sox* genes, a finding that was consistent with previous studies of this strain set (Brown et al., 2003; Brown et al., 2012). Among the SARA collection, a group of closely-related "Typhimurium complex" strains, displacement of a handful of strains upon tree-building comparison to the whole-genome representative MLST phylogeny was seen. While cursory examination might indicate that recombination had been limited to just strains showing clustering variations, examination of gene clades from the strain set showed significant homogenization, wherein strains from one or more serotypes retained identical

alleles. Of the seven operon genes examined from the SARA collection, each showed evidence of significant homogenizing recombination. A truly clonal population would show complete linkage disequilibrium of alleles, but in this case, it was observed that alleles crossed not only serotype boundaries but also clonal complex boundaries, as defined by MLST loci. This was particularly evident for *soxS*, which shows almost complete convergence into a single, identical allele. Of the five serotypes and seven MLST clades, only one strain showed any variation in *soxS* allele sequence.

Examination of the impact of intra-operon recombination through cladistics analysis and incongruence testing of the SARA strains indicated that horizontal transfer did not necessarily affect whole operons but acted on genes individually. In the case of the *sox* operon, the nearly complete convergence for *soxS* into one clade is not echoed by similar convergence in *soxR*. Instead, congruence was observed across just two serotypes—Saintpaul and Typhimurium strains. One Saintpaul strain did not cluster with the remaining strains of its serotype but had a unique allele that was most similar to the alleles harbored by Paratyphi B strains. If horizontal transfer of the operon was impacting the operon as a whole, similar convergence across the *soxR* region of the operon would be seen. Instead, *soxR* remains more clonal, with the exception of the previously mentioned convergence. Congruence tests indicated that *soxR*, *soxS*, and the inter-genic region were congruent but this finding was likely the result of the limited nucleotide diversity in the *soxS* operon.

The *ramRA* operon also showed evidence of intra-operon shuffling, as the convergence patterns for *ramR* and *ramA* are not the same. Saintpaul and Typhimurium strains harbored *ramR* alleles which clustered together under cladistics analysis while

ramA showed convergence of two groups of serotypes—Saintpaul, Typhimurium, and Heidelberg into one group and Muenchen and Paratyphi B into another group. Congruence testing supported this finding, with *ramA* being incongruent with the inter-genic region and to a lesser extent, *ramR*.

marRABC intra-operon interchange among SARA strains was apparent upon similar examination, with each of the four genes showing homogenization between groups of different serotypes. In one instance, a single *marB* allele was comprised of four serotypes—Typhimurium, Muenchen, Saintpaul, and Paratyphi B. Convergence of *marR* showed inclusion of Typhimurium strains with Muenchen and Saintpaul strains, while grouping differently under cladistics analysis of *marA*. Incongruence testing supported this conclusion of intra-operon allele exchange, whereby *marC* was incongruent with *marA* and to a lesser extent *marB*, and *marA* congruence with the inter-genic region approached incongruence.

While recombination in SARA took on characteristics of homogenization, we observed a different pattern in SARB. Instead of a homogenizing affect, recombination in the form of allele shuffling had scrambled the operon phylogenies. These findings were consistent with previous work (Brown et al., 2003; Brown et al., 2012), whereby SARB strains showed assorting of genes, as opposed to homogenization across clonal groups. In certain cases, some homogenization was still seen, with genetically divergent strains sharing the same allele. The *soxS* gene displayed the least amount of assortment, instead retaining little allelic diversity, even at the subspecies level. Of the 48 SARB strains, 34 had identical alleles. The *marRAB* genes also retained a level of visible homogenization, with over one-third of strains harboring one or two different alleles. The assortive effect

of recombination was most obvious in the *ramRA* operon. Taken with the evidence that the operons analyzed here are under negative selective pressure, with presumably the global regulators having more restrictions on nucleotide and amino acid composition, it would then make sense that homogenization would be more apparent throughout the global regulator phylogenies while assortment would be more apparent throughout local regulator phylogenies.

As was seen with the SARA strains, shuffling of alleles was not limited to entire operon segments but affected operon genes individually. This was supported by incongruence scores, which indicated that not only were *ramR* and *ramA* incongruent with each other but they were both incongruent with the inter-genic region that contains promoters for both genes. Incongruence testing of *marRABC* indicated that *marC* was incongruent with *marR*, *marA*, *marB*, and the inter-genic promoter region. The inter-genic region was also incongruent with *marR*, *marA*, and *marB*. The remaining three pairwise comparisons indicated congruence for *marR* and *marA*, *marR* and *marB*, and *marB* and *marA*. As was seen for the SARA strain operon sequences, all *sox* comparisons indicated congruence but this was likely the result of the limited nucleotide diversity in *soxS*.

In spite of this evidence of extensive recombination among operon alleles, it appeared that assortive recombination did not affect strains equally. Four strains, which shared the same MLST clade, retain identical sequences for every operon gene examined here, with the exception of *marC*. In that case, the *marC* alleles harbored by these strains clustered in the same clade, however. Had they been affected by recombination, it would be likely that the alleles would be shuffled in a manner that did not preserved allelic

congruence across all three operons. Such a tendency for variation in recombination frequencies has been noted previously (Didelot et al., 2011).

In all cases, the local repressor gene (*marR* and *ramR*) or activator (*soxR*) had more nucleotide diversity than the global activator gene. The global regulatory genes are influenced by more cellular factors than the local regulatory genes, which have one primary role in the cell. More balance is required for the global activators, since any potential change in amino acid sequence could have a cascade of implications. It then makes sense that less variation and more homogenization was observed among these global regulatory genes. In particular, a predominance of a handful of alleles was seen, with the majority of strains harboring one or two alleles. Such conservation among the nucleotide sequences of *marR*, *marA*, and *marB* echoed the limited allelic diversity seen for *ramRA* and *soxRS* among SARA strains. This speaks to the nature of the operon as a regulatory region and the strict evolutionary constraints on variation. That being said, not every gene in the operons appeared to be under similar levels of evolutionary pressure. *marC*, despite also being under a negative evolutionary pressure, had accumulated much more allelic variation than its counterparts while retaining similar levels of nucleotide diversity, possibly indicating that there were fewer limitations on allelic preference for this gene.

As is the case for the *soxRS* operon, we saw that the local repressor, *ramR* had accumulated more nucleotide diversity than the global activator, *ramA*. This was in contrast to the lack of amino acid diversity, as evidenced by complete conservation of amino acid sequences in the strains from the SARA collection. Among the SARB strains, all sites are conserved, with the exception of four sites in each *ramR* and *ramA* but the

majority of strains retained a single amino acid sequence. The limited variations in amino acid sequence speak to the regulatory nature of the proteins, as any changes must be tightly controlled to preserve proper functioning as a regulatory operon. This high level of control did not however, mean that the operon had not undergone recombination. As can be seen by the incongruence between SARB MLST sequences and both *ramR* and *ramA* sequences, that the evolutionary history of these two genes has involved shuffling of alleles, whereby a preferred allele was swapped with a strain of a different genetic history. Additionally, as seen in SARB strains, the two genes in the operon had become incongruent with one another, indicating that allele swapping had occurred that affected one gene but not the other over the course of the evolutionary history of the operon.

The regulatory nature of the *sox* operon requires that its evolution be strictly controlled, as any strain that develops deleterious mutations will be eliminated from the population. From the data presented above, it was observed that *soxS* diversity among strains had been tightly controlled, with strains from different genetic and environmental backgrounds harboring identical or similar alleles. In contrast to the very low nucleotide diversity seen in the *soxS* gene from SARA, there were a handful of non-synonymous mutations (three amino acid changes in four strains) in SARB strains, perhaps indicating that the gene had acquired amino acid changes that optimized its functioning in a specific host or environmental setting. This was unlike the *soxR* gene, which had a greater level of nucleotide diversity but retained no non-synonymous mutations—its amino acid sequence was completely conserved across all strains studied here. Taken with the evidence that supports allelic swapping and recombination between *soxRS* alleles, it was

possible to see that recombination resulting in the homogenization of allelic diversity has played an important part in the evolution of the *soxRS* operon in *S. enterica*.

The successful integration and utilization of DNA transferred through recombination is influenced by many factors, including the traditional Darwinian selection of alleles that produce a desirable phenotype. Adding complexity to the process are cellular functions that impact which DNA sequences are able to become first, integrated, and then expressed efficiently. It has been noted that recombination between more closely related strains occurs more frequently (Brown et al., 2003). Closely-related strains will likely have similar restriction-modification (R-M) systems, which prevent introduced DNA from being degraded or limit the size of recombined segments; a relationship exists between segment size and genetic-relatedness, with larger segments being more easily transferred between strains that are more similar (Milkman, 1997; Bullas et al., 1980). It has been found that *S. enterica* undergoes recombination between alleles responsible for the restriction-modification system, creating molecular similarity that then allows for increased ease of recombination between the closely-related subspecies I strains (Brown et al., 2012). The panmictic structure of R-M genes has been attributed to the ability of *S. enterica* to freely recombine DNA amongst each other, as evidenced here by the conservation of regulatory alleles, with significant lack of linkage disequilibrium, particularly as it applies to the global regulators *soxS*, *marA*, and *ramA*. Compatibility of transfer RNA systems also impact the efficiency with which newly integrated DNA is translated. Genes that contain codons that match the pre-existing tRNA pool will be translated faster, leading to a higher cellular growth rate. It has been shown that genes that are highly-expressed are under a higher tRNA selective pressure

and organisms with similar tRNA systems will be more likely to horizontally transfer genes (Tuller, 2011). Defects in the mismatch repair system, which exist in natural populations of *S. enterica*, relax some of the barriers to homologous recombination, leading to increased transfer of genetic material (Cebula and LeClerc, 1997; Brown et al., 2001). Such defects also increase the rate of point mutation in a cell, leading to the rise of novel, beneficial genotypes that can then be transferred throughout a population of bacteria under selective pressure (LeClerc et al., 1996). This would have the effect of creating a gene-tree phylogeny that is spotted with unique alleles. However, in instances where allelic preference is strong, homologous recombination can play a role in rescuing deleterious mutations from these members that are mutating and recombining at a higher frequency, resulting in homogenization throughout a population.

The observation that the local regulatory genes studied here retained greater levels of clonality than the global regulators, which appeared much more homogenized, could be due to conservation of tRNA pools and the inherent codon bias that results. A variety of conditions can result in the activation of transcription of the global regulator genes but they are primarily stress-related, such as oxidative or chemical attack. Under this increased selective pressure, rapid translation of the global regulatory protein would be crucial to survival of the cell. Codons that are well-represented in the cellular tRNA pool and available for immediate use during translation would increase the speed at which the global regulator proteins are produced. It may be for this reason that the global regulatory genes are more conserved and are represented by fewer alleles across both the SARA and SARB collections, as compared to the local regulators. Alleles that enable the most rapid and efficient translation of the global regulatory protein under stress conditions would

lead to increased survival and over-representation of the preferred allele or alleles in the surviving population once the stressor is removed. The translation of local regulatory genes would be less impacted by existing tRNA reserves, as such translation occurs during periods of non-stress and therefore the genes would be able to accumulate more allelic diversity without having a negative impact on survivability. It was noted here that the *marC* gene, while appearing under significant selective pressure, as evidence by very limited non-synonymous substitutions, had accumulated a greater level of allelic diversity than its counterparts in the *mar* operon. While amino acid conservation seems critical to proper functioning, conservation of codon usage seems less critical. While the exact functioning of the gene is not known, if it is not utilized during times of cellular stress or is translated less frequently, demand on existing tRNA pools would be decreased, allowing for utilization of less common codons.

The tendency for the local regulator genes to retain an amount of clonality in the SARA strains, with some homogenization, as compared to the highly homogenized global regulator genes, may speak to the clonal expansion of strains that have become highly adapted to multiple environments. Such adaptation would increase the fitness of a population and increase invasion into a variety of niches. The large population size would then increase the overall allelic frequency, creating a pool of fit clones, available as donors of optimized alleles. In instances of deleterious mutation, such as those occurring in individuals with mutator phenotypes due to defects in the methyl-directed mismatch repair system, these optimized alleles would be readily transferred to the mutator cells, rescuing their mutated, less fit alleles and the identical allelic profile of a population would be maintained. Possibly as a result of niche optimization or relaxed amino acid

sequence requirements, as compared to the global regulators, the local regulators retain greater levels of clonality, with instances of homogenization.

Ecological structuring plays a role in horizontal gene transfer, as populations that reside in the same or overlapping hosts will be more likely to come into contact with one another. Using the metadata from the strains analyzed here however, no clear ecological or geographic partitioning between related alleles was observed. Such analysis was limited by several factors. First, not all of the strains utilized here had known sources of isolation. There was also unequal distribution of sources between serotypes. For instance, all Saintpaul strains were isolated from a human source, giving the impression that there may have been some structuring involved with this serotype but it is not possible to determine that without being able to compare non-human isolated Saintpaul strains to those used here. The majority of stains from the SARB collections that had source information available were also from a human source, despite being from a more broad genetic background than the SARA strains. Additionally, human isolated strains likely originated from another source, as the majority of *S. enterica* infections are foodborne. With the metadata available, it was not possible to determine the original source of the human-isolated stains. Lack of ecological structuring was particularly evident with Typhimurium strains, whereby strains from several sources were found to have identical alleles. Sources in this instance included humans, rabbit, horse, parrot, dog, and opossum. This may speak to the interplay between the environment, humans, and agricultural settings, allowing for transmission of a particular lineage of bacteria to multiple hosts, particularly when a serotype or subtype is well-adapted to carriage by a wide variety of host species. While all strains used in this study did have location of isolation data

available, there were no clear geographic delineations between operon clade groupings. This was likely the result of a couple of factors. First, the strains were isolated over the course of several decades and it is impossible to know from where strains originated, as strains can spread across the globe quite easily, due to the ease with which humans travel and the international nature of food production (Butaye et al., 2006; Davis et al., 2002). Second, in the strains utilized here, there was an over-representation of strains isolated from the United States and Europe, making it difficult to accurately determine whether there was, in fact, a global dissemination of operon alleles among strains from a variety of regions.

Salmonella enterica was once regarded as a clonal species of bacteria (Selander et al., 1996; Reeves et al., 1989), but several studies have since illuminated the important role that recombination has played in the genetic history of the organism (Brown et al., 2002; 2003, Kotewicz et al., 2002). Here, we present more evidence of the role that homologous recombination has on regulatory operon regions. Analysis of 104 strains from two well-characterized collections of *Salmonella enterica* subspecies *enterica* revealed the different effects that recombination has had on these regulatory regions. Strains from the more genetically-related SARA collection showed significant evidence of homogenization and disruption of linkage disequilibrium, whereby strains from different serotypes and clonal complexes harbored identical alleles. This was especially evident for the global regulatory gene *soxS*, which showed almost no genetic variation across the 56 strains from the SARA collection that were studied. Recombination in SARB collection strains showed evidence of some homogenization across genetically-diverse taxa, also in the form of disruption of linkage disequilibrium but also showed

evidence of allele shuffling and assortment. Operon genes from the SARB collection showed more genetic diversity than those from SARA and genes showed evidence of scrambling, when compared to whole-genome representative phylogenies. Also present was evidence of intra-operon shuffling, indicating that operons did not undergo homologous recombination as intact units and instead underwent shuffling at the gene level, which has previously been attributed to the repeated rescuing of deleterious mutations (Brown et al., 2012). Taken together, we can see that recombination played a significant role in maintaining a preferred set of operon alleles among strains of *Salmonella enterica*, which speaks to the importance of these alleles as regulatory elements that are tightly controlled at the evolutionary level.

6 Conclusions and Future Work

The antimicrobial resistance patterns and associations observed in this study re-iterate what has become known in recent decades regarding the dissemination and emergence of antimicrobial resistance in *Salmonella*. The tendency for resistance determinates to be horizontally transferred was evidenced by the reticulation of phenotypes and lack of correlation between geographic locations of isolation and specific drug resistance patterns. Despite this, the importance of the interplay between environmental, ecological, and genetic factors influencing the acquisition and maintenance of antimicrobial resistance in bacteria is a topic that is not completely understood. While horizontal transfer of resistance elements and the development of point mutations are the genetic driving forces behind such evolution, there exist factors which influence the ability of a strain to acquire and maintain a resistant phenotype. Antimicrobial resistance has varying levels of fitness costs on the host cell, depending on

many factors, including the genetic background of the strain, the mechanism of resistance, the number of drugs to which the bacterium is resistant, and the specific MIC conferred (Vogwill and MacLean, 2015; Melnyk at al., 2014). Plasmid-mediated resistance has been shown to result in lower fitness costs to the cell than chromosomal mutations and additionally, compensatory mutations in the chromosome can ameliorate plasmid cost, which are then passed on vertically, creating a clonal population which is more adapted to plasmid carriage (Vogwill and MacLean, 2015). During the process of adjusting for reduced fitness, cellular compensatory mutations occur more frequently than phenotypic resistance reversal (Andersson and Hughes, 2011). A similar balancing act plays out with respect to point mutations in drug target genes, whereby reduced fitness can be overcome by mutations at other locations on the chromosome (Martinez and Baquero, 2000). Important in the acquisition of beneficial mutations is recombination with other strains. Structured environments, whereby bacteria are fixed to a particular surface and therefore become compartmentalized, allow for more genetic variability in a population as a result of lessening of competition between cells. Under these circumstances, a greater number of total alleles can be maintained, creating diversity that can be utilized in the instance of changing selective pressure (Martinez and Baquero, 2000). In support of this, it has been found that asymptomatic *S. enterica* strains in swine are a reservoir of genetic diversity, including those with multi-drug resistant phenotypes (Perron et al., 2008b). Similarly, hyper-mutators, cells which present with defects in the methyl-directed mismatch repair system, can produce novel mutations and increased recombination frequencies, resulting in an increased ability to survive antimicrobial challenge (Levy et al., 2004; LeClerc et al., 1996). Increased mutation rates can also

result from sub-lethal exposure to a variety of antimicrobials, through modulation of SOS response machinery in the cell (Chopra et al., 2003; Rodriguez-Rojas et al., 2013).

The knowledge that the three operon regions—*mar*, *ram*, and *sox*--were affected by repeated events of homologous recombination has implications for the development of antimicrobial resistance. Limited nucleotide diversity across closely-related "Typhimurium complex" strains and less closely-related subspecies I strains speaks to the potential for rescuing of deleterious alleles from a population. Such purging of diversity maintains preferred alleles and ensures the proper functioning of not only the regulatory operons themselves but of the cascade of regulation that follows from the action of the global operators. Conversely, under a strong selective pressure, such as that exerted by application of an antimicrobial agent or agents, mutation could become beneficial and the acquisition of novel alleles would give a population a selective advantage. And as has previously been discovered, there exists in a population at any given time, individuals who generate novel alleles at a higher rate (LeClerc et al., 1996). Under circumstances, for instance, where increased efflux in the cell would render a population resistant to a range of antimicrobials, recombination could allow a beneficial mutation to be acquired by previously sensitive individuals, thereby creating a new niche occupation. And as we have seen over the last few decades, *Salmonella* has been able to expand well beyond its previous niches, in terms of both host occupation and geographic dissemination. Homologous recombination has a great adaptive value and combined with other mechanisms of horizontal gene transfer, gives bacterial populations many options for coping with the strong selective pressures exerted by antimicrobials in the environment and the food chain.

In other genera of bacteria, the existence of "high-risk" clones has been reported, which have an increased capacity to occupy a variety of niches. These clones, as a result of the same mechanisms that bestow them with increased persistence, also have a tendency to be more virulent and present with antimicrobial resistance (Baquero et al., 2013). It is possible that similar "high-risk" clones exist in *S. enterica*, as evidenced by the international, clonal dissemination of many antimicrobial resistant serotypes, including Typhimurium, Kentucky, Schwarzengrund, and Newport (Le Hello et al., 2011; Threlfall, 2000; Aarestrup et al., 2007; Butaye et al., 2006). Studies have indicated that swine-isolated strains of particular genetic lineages within *S. enterica* are more likely to evolve multi-drug resistance than others (Perron et al., 2008a). Such multi-level interaction between selective pressures, bacterial genetic background, and mechanisms of resistance calls for an increased understanding of the multiple genetic factors that influence a bacterial population's ability to acquire, maintain, and spread antimicrobial resistance. To this end, future work will focus on determining the genetic mechanisms responsible for the observed phenotypes through whole-genome sequencing of the strains utilized here. Whole-genome sequencing will allow for a more complete genetic picture of not only the resistance mechanisms, both those located on mobile elements and within the chromosome, but the genetic background of the strains harboring resistance, resulting in a better understanding of how different lineages of *Salmonella* are able to evolutionarily adapt to the acquisition and retention of phenotypic antimicrobial resistance. Additionally, these historical isolates can also serve to aid in measures of rapid, short-term evolutionary changes, by providing a reference point for comparison to newer isolates that may have accumulated genetic changes as a result of increased

selective pressures exerted by altered niche environments and antimicrobial usage in animals and humans.

7 Appendix

Table A-1: Strain[a] Metadata

SGSC	SARA	RKS No.	Serotype	Source	Locality	Date
2182	2	4939	Typhimurium	-	Laboratory	-
2183	3	145	Typhimurium	Horse	Rhode Island	1987
2184	4	183	Typhimurium	Rabbit	Indiana	1986
2185	5	810	Typhimurium	-	Mongolia	-
2186	6	345	Typhimurium	Human	Ohio	-
2187	7	821	Typhimurium	-	Norway	-
2188	8	811	Typhimurium	-	Finland	-
2189	9	203	Typhimurium	Parrot	California	1987
2190	10	154	Typhimurium	Opposum	California	1987
2191	11	829	Typhimurium	-	Thailand	-
2192	12	147	Typhimurium	Horse	Louisiana	1987
2194	14	842	Typhimurium	-	Panama	-
2195	15	149	Typhimurium	Dog	Texas	1987
2196	16	350	Typhimurium	Human	North	-
2197	17	1164	Typhimurium	-	Yugoslavia	-
2198	18	151	Typhimurium	Horse	Iowa	1987
2199	19	93	Typhimurium	Human	Mexico	-
2200	20	839	Typhimurium	-	France	-
2201	21	4535	Typhimurium	Heron	Oregon	-
2202	22	1688	Saintpaul	Human	Massachusetts	-
2203	23	1689	Saintpaul	Human	Pennsylvania	-
2204	24	1690	Saintpaul	Human	Texas	-
2205	25	1380	Saintpaul	-	France	-
2206	26	3748	Saintpaul	Human	France	1988
2207	27	3755	Saintpaul	Human	France	1988
2208	28	3763	Saintpaul	Human	France	1988
2209	29	1686	Saintpaul	Human	Florida	-
2211	31	560	Heidelberg	Swine	Maryland	1987
2212	32	562	Heidelberg	Dog	Texas	1986
2213	33	576	Heidelberg	Human	Mexico	-
2214	34	1364	Heidelberg	-	Israel	-
2215	35	1389	Heidelberg	-	Brazil	-
2217	37	543	Heidelberg	Turkey	Colorado	1987
2218	38	540	Heidelberg	Turkey	Arizona	1987
2219	39	646	Heidelberg	Human	North	-
2220	40	1347	Heidelberg	-	United States	-
2221	41	3222	Paratyphi B	Human	France	1976
2222	42	3279	Paratyphi B	Human	Scotland	1974
2223	43	3305	Paratyphi B	Human	Africa	1982
2224	44	3265	Paratyphi B	Human	Middle East	<1965

2225	45	3596	Paratyphi B	Cow	France	1988
2226	46	3294	Paratyphi B	Human	Europe	1981
2227	47	3249	Paratyphi B	Sewage	Scotland	1983
2228	48	3237	Paratyphi B	Human	Scotland	1982
2229	49	3267	Paratyphi B	Sewage	United	<1965
2230	50	3202	Paratyphi B	Food	Middle East	1976
2231	51	3193	Paratyphi B	Human	France	1976
2232	52	3614	Paratyphi B	Cow	France	1987
2233	53	3605	Paratyphi B	Human	France	1988
2234	54	3597	Paratyphi B	Human	France	1988
2235	55	3211	Paratyphi B	Human	France	1981
2238	58	3218	Paratyphi B	Human	France	1981
2239	59	3219	Paratyphi B	Human	France	1981
2240	60	3192	Paratyphi B	Food	France	1976
2241	61	3277	Paratyphi B	Water	Scotland	1974
2242	62	3215	Paratyphi B	Human	Africa	1981
2243	63	4283	Muenchen	Human	France	1988
2244	64	4129	Muenchen	Cow	Kentucky	1986
2245	65	4135	Muenchen	Chicken	Florida	1987
2246	66	4277	Muenchen	Human	Massachusetts	-
2247	67	4317	Muenchen	Human	Mexico	-
2248	68	4292	Muenchen	Human	France	1988
2251	71	4272	Muenchen	Human	North	-
SGSC	SARB	RKS No.	Serotype	Source	Locality	Date
2458	1	1701	Agona	-	Peru	-
2459	2	2403	Anatum	Human	Washington	-
2461	4	1280	Choleraesuis	Swine	Minnesota	1986
2463	6	3169	Choleraesuis	-	Thailand	1954
2465	8	4647	Decatur	-	France	-
2466	9	246	Derby	Avian	Oklahoma	1986
2467	10	241	Derby	Swine	Minnesota	1986
2468	11	243	Derby	Turkey	Pennsylvania	1986
2469	12	1518	Dublin	Cattle	Idaho	1986
2470	13	4717	Dublin	Bovine	France	1982
2472	15	4239	Duisburg	-	Scotland	1988
2473	16	53	Enteritidis	-	Rhode Island	-
2475	18	69	Enteritidis	-	Connecticut	-
2478	21	2962	Gallinarum	Human	Connecticut	1972
2479	22	4241	Haifa	-	Scotland	1988
2480	23	539	Heidelberg	Chicken	Pennsylvania	1987
2481	24	1391	Heidelberg	-	Thailand	-
2482	25	4250	Indiana	-	Scotland	1988
2483	26	1490	Infantis	Human	North	-
2484	27	1452	Infantis	-	Senegal	-

2485	28	2833	Miami	Human	Georgia	1953
2486	29	4381	Miami	Human	French	1979
2487	30	1762	Montevideo	Human	Georgia	-
2488	31	1740	Montevideo	Human	Florida	-
2489	32	3121	Muenchen	-	Laboratory	-
2490	33	4288	Muenchen	Human	France	1988
2491	34	4300	Muenchen	Human	France	1988
2493	36	2016	Newport	Human	North	-
2494	37	1915	Newport	Human	Mexico	-
2495	38	1956	Newport	Snake	Massachusetts	1987
2496	39	1793	Panama	-	Italy	-
2499	42	4993	Paratyphi A	-	Laboratory	-
2500	43	3222	Paratyphi B	Human	France	1976
2502	45	3201	Paratyphi B	Human	France	1976
2503	46	3274	Paratyphi B	Water	United	<1965
2505	48	4587	Paratyphi C	-	France	-
2508	51	2266	Pullorum	-	Germany	-
2509	52	2246	Pullorum	-	Germany	-
2511	54	4938	Rubislaw	-	Laboratory	-
2514	57	4261	Schwarzengrund	-	Scotland	1988
2516	59	2358	Senftenberg	Chicken	Maryland	1987
2517	60	4264	Stanley	-	Scotland	1988
2518	61	4267	Stanleyville	-	Scotland	1988
2519	62	1767	Thompson	Human	Florida	-
2521	64	3320	Typhi	-	Dakar	1988
2522	65	284	Typhimurium	Human	Mexico	-
2524	67	837	Typhimurium	-	France	-
2527	70	3133	Typhisuis	Swine	Iowa	1967
2528	71	4000	Wien	Human	France	1988
2529	72	3998	Wien	Human	France	1988
SGSC	SARC	RKS No.	Species,	Source	Locality	Date
3029	1	s4194	Typhimurium	Human	England	1958
3036	2	s3333	Typhi	-	Dakar	1988
3039	3	s2985	ssp. *salamae*	Human	Massachusetts	1985
3047	4	s2993	ssp. *salamae*	-	-	1964
3061	5	s2980	ssp. *arizonae*	Corn	Oregon	1986
3063	6	s2983	ssp. *arizonae*	Human	California	1985
3068	7	s2978	ssp. *diarizonae*	Human	Oregon	1987
3069	8	s2979	ssp. *diarizonae*	Human	California	1984
3074	9	s3015	ssp. *houtenea*	Animal	Canal Zone	1968
3086	10	s3027	ssp. *houtenea*	Human	Illinois	1986
3100	11	s3041	*S. bongori*	Frog	-	1972
3103	12	s3044	*S. bongori*	Parakeet	United States	1976
3116	13	s2995	ssp. *indica*	-	India	1965

3118	14	s3057	ssp. *indica*	-	-	1978
3120	15	s3013	ssp. VII	-	Tonga	1964
3121	16	s3014	ssp. VII	Human	Florida	1968
-	54	**2997**	ssp. *salamae*	Human	California	1965
-	68	**3023**	ssp. *houtenea*	Human	Nebraska	1985
-	49b	**3002**	ssp. *salamae*	Reptile	Iowa	1985
-	46	**2999**	ssp. *salamae*	-	Connecticut	1967
-	69	**3019**	ssp. *houtenea*	-	Michigan	1985
-	76	**3031**	ssp. *houtenea*	Human	South	1987
-	65	**3025**	ssp. *houtenea*	Vacuum	Guam	1986
-	5	**3045**	*S. bongori*	Lizard	United	1977
-	48b	**2998**	ssp. *salamae*	Tortoise	Iowa	1966
-	23	**1762**	Montevideo	Human	Georgia	-
-	4	**3051**	*S. bongori*	Human	Sudan	1987
-		**837**	Typhimurium	-	France	-

[a]Metadata for *Salmonella* Reference Collection strain information for 141 strains. *S. enterica* ssp. *enterica* strains are referred to by serotype and all others by subspecies or species. Numbers in bold indicate strain designations used to refer to each strain in this study, for those without an SGSC number.

Table A-2. Multi-locus sequence type designations for 139 strains

		aroC	*dnaN*	*hemD*	*hisD*	*purE*	*sucA*	*thrA*	ST
SARA	2182	10	7	12	9	5	9	2	19
	2183	10	7	12	9	5	9	2	19
	2184	10	7	12	9	5	9	2	19
	2185	10	7	12	9	5	9	2	19
	2186	10	7	12	9	5	9	2	19
	2187	18	14	12	9	5	18	21	36
	2188	18	14	12	9	5	18	21	36
	2189	10	7	50	9	5	9	2	98
	2190	10	7	12	9	5	9	2	19
	2191	10	7	12	9	5	9	2	19
	2192	10	7	12	9	5	9	2	19
	2194	10	7	12	9	5	9	2	19
	2195	10	7	12	9	5	9	2	19
	2196	10	7	12	9	5	9	2	19
	2197	10	7	12	9	5	9	2	19
	2198	10	7	12	9	5	9	2	19
	2199	10	7	12	9	5	9	2	19
	2200	10	7	12	9	5	9	2	19
	2201	10	7	12	9	5	9	46	99
	2202	5	14	21	9	6	12	17	49
	2203	5	21	18	9	6	12	17	50
	2204	5	21	18	9	6	12	17	50
	2205	5	21	18	9	6	12	17	50
	2206	5	14	18	9	6	12	17	27
	2207	5	14	18	9	6	12	17	27
	2208	5	14	18	9	6	12	17	27
	2209	3	36	43	38	16	42	38	95
	2211	2	7	9	9	5	9	12	15
	2212	2	7	9	9	5	9	12	15
	2213	2	7	9	9	434	9	12	1516
	2214	2	7	9	9	5	9	12	15
	2215	2	7	9	9	5	9	12	15
	2217	2	7	9	9	5	9	12	15
	2218	2	7	9	9	5	9	12	15
	2219	2	7	9	9	5	9	12	15
	2220	2	7	9	9	5	9	12	15
	2221	2	14	24	14	37	19	8	86
	2222	2	14	24	14	37	19	8	86
	2223	2	14	24	14	37	19	8	86
	2224	2	14	24	14	37	19	8	86
	2225	2	14	24	14	37	19	8	86
	2226	2	14	24	14	37	19	8	86
	2227	2	14	24	14	2	19	8	43
	2228	2	14	24	14	49	19	8	149
	2229	2	14	24	14	2	19	8	43
	2230	2	2	24	14	2	19	8	110
	2231	2	2	24	14	2	19	8	110
	2232	2	2	24	14	2	19	8	110
	2233	2	2	24	14	2	19	8	110
	2234	2	2	24	14	2	19	8	110
	2235	2	2	24	14	2	19	8	110
	2238	20	4	23	14	16	19	18	42
	2239	20	4	23	14	16	19	18	42
	2240	20	4	23	14	16	19	189	734
	2241	3	3	7	4	3	3	7	13
	2242	47	45	47	47	39	9	43	89
	2243	41	42	43	12	9	12	53	111
	2244	41	42	43	12	9	12	2	82
	2245	41	42	43	12	9	12	2	82
	2246	41	42	43	12	9	12	2	82
	2247	41	42	43	58	9	12	2	112
	2248	41	42	43	58	9	12	2	112
	2251	9	9	6	12	9	12	2	18
SARB	2458	3	3	7	4	3	3	7	13

	2459	10	14	15	31	25	20	33	64
	2461	34	31	35	14	26	6	8	66
	2463	36	31	35	14	26	34	8	68
	2465	38	34	38	35	28	35	22	—
	2466	39	35	8	36	29	9	36	71
	2467	19	20	3	20	5	22	22	40
	2468	40	36	3	37	29	9	36	72
	2469	5	2	3	6	5	5	10	10
	2470	5	2	3	38	5	5	10	73
	2472	14	37	39	33	30	19	37	75
	2473	5	2	3	7	6	6	11	11
	2475	5	2	3	7	6	6	11	11
	2478	5	2	42	7	31	6	11	78
	2479	5	14	21	9	6	12	17	49
	2480	2	7	9	9	5	9	12	15
	2481	2	7	9	9	5	9	12	15
	2482	8	8	11	11	5	11	15	17
	2483	17	18	22	17	5	21	19	32
	2484	11	11	17	40	32	9	40	79
	2485	42	40	17	41	33	36	41	80
	2486	22	11	25	21	10	23	23	48
	2487	43	41	16	13	34	13	4	4
	2488	43	41	16	42	35	13	4	81
	2489	41	42	43	12	9	12	2	82
	2490	41	9	21	12	8	37	17	83
	2491	44	14	44	14	15	38	17	84
	2493	16	43	45	43	36	39	42	5
	2494	2	2	15	14	15	20	12	31
	2495	10	7	21	12	15	12	12	46
	2496	22	11	25	21	10	23	23	48
	2499	45	4	8	44	27	9	8	85
	2500	2	14	24	14	37	19	8	86
	2502	46	44	46	46	38	18	34	88
	2503	20	4	23	14	16	19	18	42
	2505	48	31	35	14	26	40	44	90
	2508	5	2	3	7	31	41	11	92
	2509	5	2	3	7	31	41	11	92
	2511	42	46	48	48	40	35	4	94
	2514	43	47	49	49	41	15	3	96
	2516	7	6	8	8	7	8	13	14
	2517	16	16	26	18	8	12	18	51
	2518	51	48	43	50	42	43	45	97
	2519	14	13	18	12	14	18	1	26
	2521	1	1	2	1	1	1	9	3
	2522	10	7	12	9	5	9	2	19
	2524	10	7	12	9	5	9	2	19
	2527	38	34	38	35	28	35	4	70
	2528	52	3	40	51	44	45	3	101
	2529	53	49	51	52	45	46	48	102
SARC	837	10	7	12	9	5	9	2	19
	1762	43	41	16	13	34	13	4	4
	2997	335	176	27	22	18	85	91	—
	2998	284	176	187	380	18	371	24	—
	2999	25	176	77	123	18	91	91	3403
	3002	25	176	27	214	18	181	169	581
	3019	30	28	31	27	467	29	476	1869
	3023	30	28	31	27	467	29	30	433
	3025	128	119	100	27	22	129	30	958
	3029	10	7	12	9	5	9	19	—
	3031	483	28	355	454	493	29	30	2053
	3036	10	7	12	9	5	9	19	—
	3039	405	176	27	214	18	474	249	—
	3045	132	28	33	137	126	245	30	—
	3047	288	176	27	327	250	24	91	—
	3051	132	285	216	134	24	133	398	975
	3061	65	25	29	24	20	50	497	131
	3063	56	25	28	83	207	26	78	2402

3068	145	26	30	144	21	145	28	430
3074	30	28	31	151	22	29	30	2309
3086	31	28	32	28	23	29	81	596
3100	32	29	33	29	24	30	30	—
3103	32	149	34	30	24	31	50	—
3116	55	149	458	30	24	546	50	—
3120	380	482	276	458	240	360	392	2265
3121	380	482	276	511	240	360	208	2868

Table A-3: Minimum Inhibitory Concentrations[a] for Each Drug Tested, By Strain

	Ami	Aug	Amp	Fox	Tio	Axo	Chl	Cip	Gen	Kan	Nal	Str	Fis	Tet	Cot
2182	= 2	≤ 1/0.5	≤ 1	= 2	= 0.5	≤ 0.25	= 4	≤ 0.015	= 0.5	≤ 8	= 2	≤ 32	32	≤ 4	≤ 0.12
2183	= 1	≤ 1/0.5	≤ 1	= 2	= 1	≤ 0.25	= 4	≤ 0.015	≤ 0.25	≤ 8	= 4	≤ 32	= 32	≤ 4	≤ 0.12
2184	= 2	= 16/8	> 32	= 2	= 1	≤ 0.25	= 8	≤ 0.015	= 0.5	≤ 8	= 4	≤ 32	> 256	≤ 4	≤ 0.12
2185	= 2	≤ 1/0.5	≤ 1	= 4	= 1	≤ 0.25	= 4	= 0.12	= 0.5	≤ 8	> 32	> 64	> 256	≤ 4	≤ 0.12
2186	= 2	≤ 1/0.5	≤ 1	= 1	= 1	≤ 0.25	= 8	≤ 0.015	= 0.5	≤ 8	= 4	≤ 32	= 64	≤ 4	≤ 0.12
2187	= 2	≤ 1/0.5	≤ 1	= 2	= 0.5	≤ 0.25	= 4	≤ 0.015	= 0.5	≤ 8	= 4	≤ 32	= 32	> 32	≤ 0.12
2188	= 2	≤ 1/0.5	≤ 1	= 2	= 1	≤ 0.25	= 8	≤ 0.015	≤ 0.25	≤ 8	= 4	≤ 32	= 32	≤ 4	≤ 0.12
2189	= 2	≤ 1/0.5	≤ 1	= 4	= 1	≤ 0.25	= 8	≤ 0.015	= 0.5	≤ 8	= 2	≤ 32	> 256	≤ 4	> 4
2190	= 2	= 2	= 2	= 4	= 1	≤ 0.25	= 8	= 0.03	= 0.5	≤ 8	= 4	> 64	> 256	≤ 4	= 0.25
2191	= 4	≤ 1/0.5	≤ 1	= 4	= 1	≤ 0.25	= 8	≤ 0.015	= 0.5	≤ 8	= 4	≤ 32	= 32	≤ 4	≤ 0.12
2192	= 2	≤ 1/0.5	≤ 1	= 2	= 1	≤ 0.25	= 4	= 0.03	= 0.5	≤ 8	= 2	≤ 32	= 64	≤ 4	≤ 0.12
2194	= 2	≤ 1/0.5	≤ 1	= 2	= 1	≤ 0.25	= 4	= 0.03	= 0.5	≤ 8	= 4	≤ 32	= 32	≤ 4	≤ 0.12
2195	= 2	≤ 1/0.5	≤ 1	= 1	= 0.25	≤ 0.25	= 4	≤ 0.015	= 0.5	≤ 8	= 4	≤ 32	= 64	≤ 4	≤ 0.12
2196	= 2	≤ 1/0.5	≤ 1	= 2	= 1	≤ 0.25	= 8	= 0.03	= 0.5	≤ 8	= 4	≤ 32	= 32	≤ 4	≤ 0.12
2197	= 4	≤ 1/0.5	= 2	= 4	= 1	≤ 0.25	= 8	≤ 0.015	= 0.5	≤ 8	= 4	> 64	= 32	≤ 4	≤ 0.12
2198	= 1	≤ 1/0.5	≤ 1	= 2	= 1	≤ 0.25	= 8	≤ 0.015	= 0.5	≤ 8	= 4	≤ 32	= 32	≤ 4	≤ 0.12
2199	= 2	≤ 1/0.5	≤ 1	= 2	= 1	≤ 0.25	= 4	≤ 0.015	= 0.5	≤ 8	= 4	≤ 32	= 32	≤ 4	≤ 0.12
2200	= 2	≤ 1/0.5	= 2	= 2	= 1	≤ 0.25	= 8	= 0.03	= 0.5	≤ 8	= 4	≤ 32	= 32	≤ 4	≤ 0.12
2201	= 2	≤ 1/0.5	≤ 1	= 2	= 1	≤ 0.25	= 8	≤ 0.015	= 0.5	≤ 8	= 4	≤ 32	= 32	≤ 4	≤ 0.12
2202	= 2	≤ 1/0.5	≤ 1	= 1	= 1	≤ 0.25	= 4	≤ 0.015	= 0.5	≤ 8	= 4	≤ 32	= 64	≤ 4	≤ 0.12
2203	= 2	≤ 1/0.5	≤ 1	= 1	= 0.25	≤ 0.25	= 4	≤ 0.015	= 0.5	≤ 8	= 4	≤ 32	= 64	≤ 4	≤ 0.12
2204	= 2	≤ 1/0.5	≤ 1	= 2	= 1	≤ 0.25	= 4	≤ 0.015	= 0.5	≤ 8	= 4	≤ 32	= 64	≤ 4	≤ 0.12
2205	= 2	≤ 1/0.5	≤ 1	= 2	= 0.5	≤ 0.25	= 4	≤ 0.015	0.5	≤ 8	= 4	≤ 32	= 32	≤ 4	≤ 0.12
2206	= 2	= 8	> 32	= 2	= 1	≤ 0.25	= 8	≤ 0.015	≤ 0.25	≤ 8	= 2	> 64	= 32	> 32	≤ 0.12
2207	= 1	= 16/8	> 32	= 1	= 1	≤ 0.25	> 32	≤ 0.015	≤ 0.25	≤ 8	= 2	≤ 32	> 256	≤ 4	> 4
2208	= 4	= 16/8	> 32	= 4	= 1	≤ 0.25	> 32	≤ 0.015	= 1	> 64	= 4	> 64	> 256	> 32	= 0.25
2209	= 2	≤ 1/0.5	≤ 1	= 1	= 1	≤ 0.25	= 8	≤ 0.015	= 0.5	≤ 8	= 4	≤ 32	= 32	≤ 4	≤ 0.12
2211	= 2	≤ 1/0.5	≤ 1	= 2	= 1	≤ 0.25	= 8	≤ 0.015	= 0.5	≤ 8	= 4	≤ 32	= 32	≤ 4	≤ 0.12
2212	= 2	≤ 1/0.5	≤ 1	= 1	= 1	≤ 0.25	= 4	≤ 0.015	= 0.5	≤ 8	= 2	≤ 32	≤ 16	≤ 4	≤ 0.12
2213	= 32	= 16/8	> 32	= 2	= 1	≤ 0.25	> 32	≤ 0.015	> 16	> 64	= 4	> 64	> 256	> 32	= 0.25
2214	= 2	= 2	≤ 1	= 4	= 1	≤ 0.25	> 32	≤ 0.015	= 1	≤ 8	= 4	≤ 32	= 32	> 32	≤ 0.12
2215	= 2	= 16/8	> 32	= 1	= 1	≤ 0.25	= 8	≤ 0.015	= 0.5	≤ 8	= 4	≤ 32	= 32	≤ 4	≤ 0.12

2217	= 2	≤ 1/0.5	≤ 1	= 2	= 1	≤ 0.25	8	≤0.015	1	> 64	= 4	> 64	≤ 16	> 32	≤ 0.12
2218	= 2	≤ 1/0.5	≤ 1	= 2	= 1	≤ 0.25	= 8	≤ 0.015	> 16	> 64	= 4	> 64	> 256	≤ 4	≤ 0.12
2219	= 2	≤ 1/0.5	≤ 1	= 1	= 1	≤ 0.25	> 32	≤ 0.015	= 0.5	≤ 8	= 2	≤ 32	> 256	= 16	≤ 0.12
2220	= 2	≤ 1/0.5	≤ 1	= 1	= 0.5	≤ 0.25	= 4	≤ 0.015	0.5	≤ 8	= 4	≤ 32	32	≤ 4	≤ 0.12
2221	= 2	≤ 1/0.5	≤ 1	= 2	= 0.5	≤ 0.25	= 4	≤ 0.015	= 1	≤ 8	= 2	≤ 32	= 64	≤ 4	≤ 0.12
2222	= 4	≤ 1/0.5	= 2	= 4	= 1	≤ 0.25	= 8	= 0.03	= 1	≤ 8	= 8	≤ 32	= 64	≤ 4	≤ 0.12
2223	= 2	≤ 1/0.5	≤ 1	= 1	= 0.5	≤ 0.25	= 4	≤ 0.015	= 0.5	≤ 8	= 2	≤ 32	≤ 16	≤ 4	≤ 0.12
2224	= 2	≤ 1/0.5	≤ 1	= 1	≤ 0.12	≤ 0.25	≤ 2	≤ 0.015	= 0.5	≤ 8	= 1	≤ 32	= 64	≤ 4	≤ 0.12
2225	= 8	= 2	= 2	= 4	= 1	≤ 0.25	= 8	= 0.25	= 1	≤ 8	> 32	≤ 32	= 64	≤ 4	≤ 0.12
2226	= 2	≤ 1/0.5	≤ 1	= 2	= 0.5	≤ 0.25	= 4	≤ 0.015	= 0.5	≤ 8	= 4	≤ 32	= 32	≤ 4	≤ 0.12
2227	= 2	≤ 1/0.5	≤ 1	= 2	= 1	≤ 0.25	= 4	≤ 0.015	= 0.5	≤ 8	= 4	≤ 32	= 64	≤ 4	≤ 0.12
2228	= 2	≤ 1/0.5	= 2	= 4	= 2	≤ 0.25	= 8	= 0.03	= 0.5	≤ 8	= 4	≤ 32	= 128	≤ 4	≤ 0.12
2229	= 4	≤ 1/0.5	≤ 1	= 4	= 1	≤ 0.25	= 4	≤ 0.015	= 1	≤ 8	= 2	≤ 32	= 64	≤ 4	≤ 0.12
2230	= 4	≤ 1/0.5	≤ 1	= 2	= 1	≤ 0.25	= 8	= 0.03	= 1	≤ 8	= 4	> 64	= 64	≤ 4	≤ 0.12
2231	= 2	≤ 1/0.5	= 2	= 4	= 1	≤ 0.25	= 8	= 0.03	= 0.5	≤ 8	= 4	≤ 32	= 128	≤ 4	≤ 0.12
2232	= 1	≤ 1/0.5	≤ 1	= 1	= 0.25	≤ 0.25	= 4	≤ 0.015	= 0.5	≤ 8	= 2	≤ 32	= 64	≤ 4	≤ 0.12
2233	= 4	≤ 1/0.5	≤ 1	= 2	= 1	≤ 0.25	= 4	= 0.03	= 0.5	≤ 8	= 2	≤ 32	= 64	≤ 4	≤ 0.12
2234	= 2	≤ 1/0.5	≤ 1	= 2	= 1	≤ 0.25	= 8	≤ 0.015	= 0.5	≤ 8	= 4	≤ 32	= 64	≤ 4	≤ 0.12
2235	= 4	≤ 1/0.5	= 2	= 4	= 1	≤ 0.25	= 8	= 0.03	= 0.5	≤ 8	= 4	≤ 32	= 64	≤ 4	≤ 0.12
2238	= 2	≤ 1/0.5	≤ 1	= 2	= 1	≤ 0.25	= 4	≤ 0.015	= 0.5	≤ 8	= 4	≤ 32	= 32	≤ 4	≤ 0.12
2239	= 2	= 8	> 32	= 2	= 1	≤ 0.25	= 4	= 0.03	= 0.5	> 64	= 4	≤ 32	= 32	≤ 4	≤ 0.12
2240	= 1	≤ 1/0.5	≤ 1	= 2	= 1	≤ 0.25	= 4	≤ 0.015	≤ 0.25	≤ 8	= 4	≤ 32	= 64	≤ 4	≤ 0.12
2241	= 1	≤ 1/0.5	≤ 1	= 2	= 1	≤ 0.25	= 16	= 0.03	≤ 0.25	≤ 8	= 4	≤ 32	= 64	≤ 4	≤ 0.12
2242	= 2	≤ 1/0.5	≤ 1	= 2	= 1	≤ 0.25	= 4	≤ 0.015	= 0.5	≤ 8	= 2	≤ 32	= 64	≤ 4	≤ 0.12
2243	= 2	≤ 1/0.5	≤ 1	= 2	= 1	≤ 0.25	= 4	= 0.03	= 0.5	≤ 8	= 4	≤ 32	= 64	≤ 4	≤ 0.12
2244	= 4	= 16/8	> 32	= 4	= 2	≤ 0.25	> 32	≤ 0.015	= 0.5	> 64	= 4	> 64	> 256	> 32	≤ 0.12
2245	= 4	> 32/16	> 32	= 2	= 2	≤ 0.25	= 4	≤ 0.015	= 1	> 64	= 2	> 64	> 256	≤ 4	= 0.25
2246	= 2	≤ 1/0.5	≤ 1	= 2	= 0.5	≤ 0.25	= 4	≤ 0.015	0.5	≤ 8	= 4	≤ 32	= 64	≤ 4	≤ 0.12
2247	= 2	≤ 1/0.5	≤ 1	= 2	= 1	≤ 0.25	= 4	≤ 0.015	= 1	≤ 8	= 4	≤ 32	= 32	≤ 4	≤ 0.12
2248	= 1	≤ 1/0.5	≤ 1	= 2	= 1	≤ 0.25	= 4	≤ 0.015	= 0.5	≤ 8	= 4	≤ 32	= 64	≤ 4	≤ 0.12
2251	= 2	≤ 1/0.5	≤ 1	= 1	= 1	≤ 0.25	= 4	≤ 0.015	= 0.5	≤ 8	= 4	≤ 32	= 32	≤ 4	≤ 0.12
2458	= 2	≤ 1/0.5	≤ 1	= 4	= 1	≤ 0.25	= 8	≤ 0.015	= 0.5	≤ 8	= 4	≤ 32	= 64	≤ 4	≤ 0.12
2459	= 1	≤ 1/0.5	≤ 1	= 4	= 1	≤ 0.25	= 8	≤ 0.015	= 0.5	≤ 8	= 4	≤ 32	= 32	≤ 4	≤ 0.12
2461	= 2	≤ 1/0.5	≤ 1	= 2	= 1	≤ 0.25	= 8	≤ 0.015	= 0.5	≤ 8	= 4	≤ 32	= 64	≤ 4	≤ 0.12

2463	= 2	≤ 1/0.5	≤ 1	= 1	= 1	≤ 0.25	= 4	≤ 0.015	= 0.5	≤ 8	= 4	≤ 32	≤ 16	≤ 4	≤ 0.12
2465	= 1	≤ 1/0.5	≤ 1	= 1	≤ 0.12	≤ 0.25	≤ 2	≤ 0.015	≤ 0.25	≤ 8	≤ 0.5	≤ 32	≤ 16	≤ 4	≤ 0.12
2466	= 8	≤ 1/0.5	≤ 1	= 4	= 1	≤ 0.25	= 8	≤ 0.015	= 2	≤ 8	= 4	≤ 32	= 32	≤ 4	≤ 0.12
2467	= 1	= 2	≤ 1	= 4	= 1	≤ 0.25	= 8	≤ 0.015	= 0.5	≤ 8	= 2	> 64	> 256	> 32	≤ 0.12
2468	= 4	≤ 1/0.5	≤ 1	= 4	= 1	≤ 0.25	= 8	≤ 0.015	> 16	> 64	= 4	> 64	> 256	> 32	≤ 0.12
2469	= 2	= 16/8	> 32	= 8	= 1	≤ 0.25	= 8	= 0.03	= 0.5	> 64	= 4	> 64	= 64	> 32	≤ 0.12
2470	= 8	= 2	≤ 1	= 1	≤ 0.12	≤ 0.25	≤ 2	≤ 0.015	= 1	≤ 8	= 4	> 64	≤ 16	≤ 4	≤ 0.12
2472	= 2	= 2	≤ 1	= 2	= 1	≤ 0.25	= 8	≤ 0.015	= 1	≤ 8	= 4	≤ 32	= 64	≤ 4	≤ 0.12
2473	= 1	≤ 1/0.5	≤ 1	= 2	= 1	≤ 0.25	= 4	≤ 0.015	≤ 0.25	≤ 8	= 4	≤ 32	= 32	≤ 4	≤ 0.12
2475	= 2	≤ 1/0.5	≤ 1	= 2	= 1	≤ 0.25	= 4	≤ 0.015	= 0.5	≤ 8	= 4	≤ 32	≤ 16	≤ 4	≤ 0.12
2478	= 2	≤ 1/0.5	≤ 1	= 2	= 1	≤ 0.25	= 4	≤ 0.015	= 0.5	≤ 8	= 4	≤ 32	= 32	≤ 4	≤ 0.12
2479	= 2	≤ 1/0.5	≤ 1	= 1	= 1	≤ 0.25	= 4	≤ 0.015	= 0.5	≤ 8	= 4	≤ 32	> 256	≤ 4	> 4
2480	= 2	≤ 1/0.5	≤ 1	= 1	= 1	≤ 0.25	= 8	≤ 0.015	= 0.5	≤ 8	= 4	≤ 32	= 32	≤ 4	≤ 0.12
2481	= 4	≤ 1/0.5	≤ 1	= 2	= 1	≤ 0.25	= 8	≤ 0.015	= 0.5	≤ 8	= 4	≤ 32	= 64	≤ 4	≤ 0.12
2482	= 4	≤ 1/0.5	≤ 1	= 2	= 1	≤ 0.25	= 8	= 0.03	= 1	≤ 8	= 4	≤ 32	> 256	≤ 4	> 4
2483	= 2	≤ 1/0.5	≤ 1	= 4	= 1	≤ 0.25	= 8	≤ 0.015	= 0.5	≤ 8	= 2	≤ 32	= 32	≤ 4	≤ 0.12
2484	= 4	≤ 1/0.5	≤ 1	= 2	= 1	≤ 0.25	= 8	≤ 0.015	= 1	≤ 8	= 4	≤ 32	= 32	≤ 4	≤ 0.12
2485	= 2	= 2	= 2	= 4	= 1	≤ 0.25	= 8	= 0.03	= 0.5	≤ 8	= 4	≤ 32	= 32	≤ 4	≤ 0.12
2486	= 1	≤ 1/0.5	≤ 1	= 2	= 0.5	≤ 0.25	= 4	≤ 0.015	0.5	≤ 8	= 2	≤ 32	= 32	≤ 4	≤ 0.12
2487	= 2	≤ 1/0.5	≤ 1	= 2	= 1	≤ 0.25	= 8	≤ 0.015	= 0.5	≤ 8	= 4	≤ 32	= 64	≤ 4	≤ 0.12
2488	= 2	≤ 1/0.5	≤ 1	= 2	= 1	≤ 0.25	= 8	≤ 0.015	= 0.5	≤ 8	= 4	≤ 32	= 32	≤ 4	≤ 0.12
2489	= 2	≤ 1/0.5	≤ 1	= 8	= 1	≤ 0.25	= 4	≤ 0.015	≤ 0.25	≤ 8	= 4	≤ 32	= 32	≤ 4	≤ 0.12
2490	= 2	= 2	≤ 1	= 2	= 1	≤ 0.25	= 8	≤ 0.015	= 0.5	≤ 8	= 2	≤ 32	= 32	≤ 4	≤ 0.12
2491	= 2	≤ 1/0.5	≤ 1	= 2	= 1	≤ 0.25	= 4	≤ 0.015	= 0.5	≤ 8	= 4	≤ 32	= 64	≤ 4	≤ 0.12
2493	= 2	≤ 1/0.5	≤ 1	= 4	= 1	≤ 0.25	= 4	≤ 0.015	= 0.5	≤ 8	= 4	≤ 32	= 64	≤ 4	≤ 0.12
2494	= 4	> 32/16	> 32	= 4	= 2	≤ 0.25	> 32	≤ 0.015	> 16	> 64	= 2	> 64	> 256	≤ 4	≤ 0.12
2495	= 1	≤ 1/0.5	≤ 1	= 1	= 1	≤ 0.25	= 4	≤ 0.015	= 0.5	≤ 8	= 2	≤ 32	≤ 16	≤ 4	≤ 0.12
2496	= 2	≤ 1/0.5	≤ 1	= 2	= 0.5	≤ 0.25	= 4	≤ 0.015	= 0.5	≤ 8	= 2	≤ 32	= 64	≤ 4	≤ 0.12
2499	≤ 0.5	= 2	≤ 1	= 4	= 1	≤ 0.25	= 4	≤ 0.015	≤ 0.25	≤ 8	= 2	≤ 32	≤ 16	≤ 4	≤ 0.12
2500	= 4	≤ 1/0.5	≤ 1	= 2	= 1	≤ 0.25	= 8	≤ 0.015	= 1	≤ 8	= 4	≤ 32	= 64	≤ 4	≤ 0.12
2502	= 2	≤ 1/0.5	≤ 1	= 2	= 1	≤ 0.25	= 8	≤ 0.015	= 1	≤ 8	= 4	≤ 32	= 32	≤ 4	≤ 0.12
2503	= 2	≤ 1/0.5	≤ 1	= 2	= 1	≤ 0.25	= 8	= 0.03	= 0.5	≤ 8	= 4	≤ 32	≤ 16	≤ 4	≤ 0.12
2505	= 2	≤ 1/0.5	≤ 1	= 4	= 1	≤ 0.25	= 8	≤ 0.015	= 0.5	≤ 8	= 4	≤ 32	= 64	≤ 4	≤ 0.12
2508	≤ 0.5	≤ 1/0.5	≤ 1	= 4	= 0.5	≤ 0.25	= 4	≤ 0.015	≤ 0.25	≤ 8	= 2	≤ 32	≤ 16	≤ 4	≤ 0.12

2509	≤ 0.5	≤ 1/0.5	≤ 1	≤ 0.5	≤ 0.12	≤ 0.25	≤ 2	≤ 0.015	≤ 0.25	≤ 8	= 1	≤ 32	≤ 16	≤ 4	≤ 0.12
2511	= 2	≤ 1/0.5	≤ 1	= 2	= 1	≤ 0.25	= 8	≤ 0.015	= 1	≤ 8	= 4	≤ 32	= 32	≤ 4	≤ 0.12
2514	= 4	= 2	≤ 1	= 2	= 1	≤ 0.25	= 8	≤ 0.015	= 1	≤ 8	= 4	≤ 32	= 32	≤ 4	≤ 0.12
2516	= 2	≤ 1/0.5	≤ 1	= 4	= 1	≤ 0.25	= 8	= 0.03	> 16	= 16	= 4	> 64	> 256	≤ 4	≤ 0.12
2517	= 2	= 2	≤ 1	= 2	= 1	≤ 0.25	= 4	≤ 0.015	= 0.5	≤ 8	= 4	> 64	> 256	> 32	≤ 0.12
2518	= 2	≤ 1/0.5	≤ 1	= 2	= 1	≤ 0.25	= 8	≤ 0.015	= 0.5	≤ 8	= 4	≤ 32	= 64	≤ 4	≤ 0.12
2519	= 2	= 2	≤ 1	= 2	= 1	≤ 0.25	= 8	≤ 0.015	= 0.5	≤ 8	= 4	≤ 32	= 32	≤ 4	≤ 0.12
2521	= 1	≤ 1/0.5	≤ 1	= 2	= 0.5	≤ 0.25	= 4	≤ 0.015	≤ 0.25	≤ 8	= 2	≤ 32	= 64	≤ 4	≤ 0.12
2522	= 2	≤ 1/0.5	≤ 1	= 2	= 0.5	≤ 0.25	= 4	≤ 0.015	= 0.5	≤ 8	= 4	≤ 32	= 64	≤ 4	= 0.25
2524	= 2	≤ 1/0.5	= 2	= 4	= 1	≤ 0.25	= 4	≤ 0.015	= 1	≤ 8	= 2	≤ 32	= 64	≤ 4	≤ 0.12
2527	= 4	≤ 1/0.5	≤ 1	= 2	= 1	≤ 0.25	= 4	≤ 0.015	= 0.5	≤ 8	= 2	≤ 32	= 32	≤ 4	≤ 0.12
2528	= 2	>32/16	> 32	= 2	= 1	≤ 0.25	> 32	≤ 0.015	> 16	> 64	= 2	≤ 32	> 256	≤ 4	> 4
2529	= 2	≤ 1/0.5	≤ 1	= 4	= 1	≤ 0.25	= 8	≤ 0.015	0.5	≤ 8	= 4	≤ 32	= 64	≤ 4	≤ 0.12
3029	= 2	= 2	≤ 1	= 2	= 1	≤ 0.25	= 8	≤ 0.015	= 1	≤ 8	= 4	≤ 32	= 32	≤ 4	≤ 0.12
3036	= 2	= 2	= 2	= 4	= 1	≤ 0.25	= 8	≤ 0.015	= 1	≤ 8	= 4	≤ 32	= 64	≤ 4	≤ 0.12
3039	= 2	= 2	≤ 1	= 4	= 1	≤ 0.25	= 8	≤ 0.015	= 0.5	≤ 8	= 2	≤ 32	= 32	≤ 4	≤ 0.12
3047	= 2	= 2	≤ 1	= 2	= 1	≤ 0.25	= 8	≤ 0.015	= 0.5	≤ 8	= 4	≤ 32	≤ 16	≤ 4	≤ 0.12
3061	= 1	≤ 1/0.5	≤ 1	= 2	= 1	≤ 0.25	= 8	≤ 0.015	= 0.5	≤ 8	= 4	≤ 32	= 64	≤ 4	≤ 0.12
3063	= 1	≤ 1/0.5	≤ 1	= 2	= 0.5	≤ 0.25	= 4	≤ 0.015	≤ 0.25	≤ 8	= 2	≤ 32	≤ 16	≤ 4	≤ 0.12
3068	= 4	= 2	= 2	= 8	= 2	≤ 0.25	= 4	≤ 0.015	= 1	≤ 8	= 4	> 64	= 64	≤ 4	≤ 0.12
3069	= 2	≤ 1/0.5	≤ 1	= 1	= 0.5	≤ 0.25	= 4	≤ 0.015	= 0.5	≤ 8	= 2	≤ 32	= 32	≤ 4	≤ 0.12
3074	= 1	≤ 1/0.5	≤ 1	= 1	= 0.25	≤ 0.25	≤ 2	≤ 0.015	= 0.5	≤ 8	= 1	≤ 32	≤ 16	≤ 4	≤ 0.12
3086	= 2	= 2	= 2	= 4	= 2	≤ 0.25	= 8	= 0.03	= 0.5	≤ 8	= 4	≤ 32	> 256	≤ 4	≤ 0.12
3100	= 1	≤ 1/0.5	≤ 1	= 2	= 1	≤ 0.25	= 4	≤ 0.015	= 0.5	≤ 8	= 4	≤ 32	≤ 16	≤ 4	≤ 0.12
3103	= 2	= 2	≤ 1	= 4	= 1	≤ 0.25	= 8	= 0.03	= 0.5	≤ 8	= 4	≤ 32	= 64	≤ 4	≤ 0.12
3116	= 2	= 2	≤ 1	= 2	= 1	≤ 0.25	= 4	≤ 0.015	= 0.5	≤ 8	= 2	≤ 32	= 64	≤ 4	≤ 0.12
3118	= 1	≤ 1/0.5	≤ 1	= 1	= 0.5	≤ 0.25	≤ 2	≤ 0.015	= 0.5	≤ 8	= 2	≤ 32	≤ 16	≤ 4	≤ 0.12
3120	= 2	≤ 1/0.5	≤ 1	= 8	= 1	≤ 0.25	= 4	≤ 0.015	= 0.5	≤ 8	= 4	≤ 32	≤ 16	≤ 4	≤ 0.12
3121	= 2	≤ 1/0.5	≤ 1	= 4	= 1	≤ 0.25	= 4	≤ 0.015	= 0.5	≤ 8	= 4	≤ 32	≤ 16	≤ 4	≤ 0.12
837	= 1	≤ 1/0.5	≤ 1	= 2	= 0.5	≤ 0.25	= 4	= 0.03	≤ 0.25	≤ 8	= 4	≤ 32	= 64	≤ 4	≤ 0.12
1762	= 2	≤ 1/0.5	≤ 1	= 2	= 0.5	≤ 0.25	= 4	≤ 0.015	= 0.5	≤ 8	= 4	≤ 32	= 64	≤ 4	≤ 0.12
2997	= 2	≤ 1/0.5	≤ 1	= 4	= 1	≤ 0.25	= 8	= 0.03	= 0.5	≤ 8	= 4	≤ 32	> 256	≤ 4	= 0.25
2998	= 2	≤ 1/0.5	≤ 1	= 4	= 1	≤ 0.25	= 4	≤ 0.015	= 0.5	≤ 8	= 4	≤ 32	= 64	≤ 4	≤ 0.12
2999	= 1	≤ 1/0.5	≤ 1	= 4	= 0.5	≤ 0.25	= 8	= 0.03	≤ 0.25	≤ 8	= 2	≤ 32	= 64	≤ 4	≤ 0.12

3002	= 1	= 2	= 2	= 8	= 1	≤ 0.25	= 8	= 0.03	= 0.5	≤ 8	= 4	≤ 32	= 64	≤ 4	≤ 0.12
3019	= 1	≤ 1/0.5	≤ 1	= 2	= 0.5	≤ 0.25	= 4	≤ 0.015	= 0.5	≤ 8	= 2	≤ 32	= 32	≤ 4	≤ 0.12
3023	= 2	≤ 1/0.5	≤ 1	= 2	= 1	≤ 0.25	= 8	= 0.03	≤ 0.25	≤ 8	= 4	≤ 32	= 64	≤ 4	≤ 0.12
3025	= 2	≤ 1/0.5	≤ 1	= 2	= 1	≤ 0.25	= 4	≤ 0.015	= 0.5	≤ 8	= 4	≤ 32	> 256	≤ 4	≤ 0.12
3031	= 1	≤ 1/0.5	≤ 1	= 2	= 1	≤ 0.25	= 4	= 0.03	≤ 0.25	≤ 8	= 4	≤ 32	= 64	≤ 4	≤ 0.12
3045	= 2	= 2	= 2	= 4	= 1	≤ 0.25	= 4	≤ 0.015	= 0.5	≤ 8	= 4	> 64	= 32	≤ 4	≤ 0.12
3051	= 1	≤ 1/0.5	≤ 1	= 4	= 1	≤ 0.25	= 8	= 0.03	≤ 0.25	≤ 8	= 4	≤ 32	= 64	≤ 4	≤ 0.12

[a]MICs in solid-lined boxes were deemed resistant while those in dash-lined boxes were intermediately resistant.

Table A-4: Operon Gene Characteristics

	No. Alleles	Allelic Diversity[a]	No. polymorphic sites (No. parsimonious sites)[b]	No. synonymous mutations (No. non-synonymous mutations)[c]	G+C Content[d]	Nucleotide Diversity[e]	dN/dS[f]	Z-test p value[g]
	MLST (3336 bp)							
SARA	21	0.897	105 (83)	93 (14)	59.1%	0.0078	0.0407	**0.000**
SARB	43	0.996	229 (140)	195 (37)	59.1%	0.0126	0.0281	**0.000**
	marR (435 bp)							
SARA	11	0.652	10 (5)	7 (3)	48.1%	0.0037	0.0654	**0.035**
SARB	12	0.816	10 (5)	10 (0)	48.1%	0.0039	0.0000	**0.010**
	marA (381 bp)							
SARA	6	0.691	5 (4)	5 (0)	46.8%	0.0031	0.0000	**0.042**
SARB	12	0.84	10 (7)	7 (3)	46.7%	0.0048	0.0609	**0.013**
	marB (216 bp)							
SARA	6	0.535	5 (3)	4(1)	50.3%	0.0030	0.0564	0.086
SARB	14	0.805	12 (3)	7 (6)	50.4%	0.0064	0.1592	0.056
	marC (666 bp)							
SARA	15	0.853	22 (8)	21 (2)	53.3%	0.0038	0.0266	**0.001**
SARB	26	0.963	25 (13)	22 (4)	53.2%	0.0052	0.0261	**0.000**
	ramR (582 bp)							
SARA	10	0.748	11 (9)	11 (0)	52.8%	0.0052	0.0000	**0.002**
SARB	25	0.955	28 (19)	24 (4)	53.0%	0.0079	0.0464	**0.000**
	ramA (342 bp)							
SARA	4	0.519	4 (3)	2 (2)	50.2%	0.0032	0.3005	0.188
SARB	13	0.883	12 (7)	9 (3)	50.3%	0.0056	0.1384	**0.020**
	soxR (459 bp)							
SARA	8	0.716	8 (5)	8 (0)	54.7%	0.0043	0.0000	**0.009**
SARB	18	0.923	14 (9)	15 (0)	54.8%	0.0056	0.0000	**0.002**
	soxS (324 bp)							
SARA	2	0.036	2 (0)	2 (0)	51.5%	0.0002	0.0000	0.062
SARB	9	0.488	10 (3)	7 (3)	51.4%	0.0027	0.1356	**0.035**

[a]The chance of selecting two different alleles at random from the pool of total alleles.
[b]Polymorphic sites are those containing two or more different nucleotides. Parsimonious sites contain two or more nucleotides and occur with a frequency of two or more.
[c]Synonymous mutations do not result in a predicted amino acid change. Non-synonymous mutations do result in a predicted amino acid change.
[d]Percent of guanine and cytosine nucleotides.
[e]Average number of per site nucleotide differences.
[f]Ratio of the number of non-synonymous substitutions to the number of synonymous substitutions.

[g]The probability of rejecting a null hypothesis of neutrality in favor of an alternative hypothesis of purifying selection, as measured by a codon-based Z-test. Values in bold are considered significant ($p < 0.05$

Table A-5: Operon Allele Types[a] by Strain

	marA	*marB*	*marC*	*marR*	*ramA*	*ramR*	*soxR*	*soxS*
2182	1	1	1	1	1	1	1	1
2183	1	1	1	1	1	1	1	1
2184	1	1	1	1	1	2	1	1
2185	1	1	1	1	1	1	1	1
2186	1	1	1	1	1	1	1	1
2187	1	1	1	1	1	3	1	1
2188	1	1	1	1	1	1	1	1
2189	1	1	1	1	1	1	1	1
2190	1	1	1	1	1	1	1	1
2191	1	1	1	1	1	1	1	1
2192	1	1	1	1	1	1	1	1
2194	1	1	2	2	1	1	1	1
2195	1	1	1	1	1	1	1	1
2196	1	1	1	1	1	1	1	1
2197	1	1	1	1	1	1	1	1
2198	1	1	1	1	1	1	1	1
2199	1	1	1	1	1	1	1	1
2200	1	1	1	1	1	1	1	1
2201	1	1	1	1	1	1	1	1
2202	2	1	3	3	1	1	1	1
2203	2	1	4	4	1	1	1	1
2204	2	1	4	5	1	1	1	1
2205	2	1	4	4	1	1	1	1
2206	2	1	4	4	1	1	1	1
2207	2	1	4	4	1	1	1	1
2208	2	1	4	4	1	1	1	1
2209	3	2	5	6	1	4	2	1
2211	4	3	6	1	1	5	3	1
2212	4	3	7	1	1	5	3	1
2213	4	3	7	1	1	5	3	1
2214	4	3	7	7	1	5	3	1
2215	4	3	7	1	1	5	3	1
2217	4	3	7	1	1	5	3	1
2218	4	3	7	1	1	5	3	1
2219	4	3	7	1	1	5	3	1
2220	4	3	7	1	1	5	3	1
2227	5	4	8	8	2	6	4	1
2228	1	1	8	8	2	6	4	1
2229	1	4	9	8	2	6	4	1

2230	4	3	10	9	2	6	4	1
2231	4	3	10	9	2	6	4	1
2232	4	3	10	9	2	6	4	1
2233	4	3	10	9	2	6	4	1
2234	4	3	10	9	2	6	4	1
2235	4	3	10	9	2	6	4	1
2238	1	1	8	8	2	6	4	1
2239	1	1	8	8	2	6	4	1
2240	1	1	8	8	2	6	4	1
2241	6	5	11	10	2	7	5	1
2242	6	6	12	11	2	8	6	2
2243	2	1	13	1	2	9	7	1
2244	2	1	14	1	3	9	7	1
2246	1	1	13	1	4	9	7	1
2247	2	1	13	1	4	9	7	1
2248	2	1	13	1	4	9	7	1
2251	4	3	15	1	4	10	8	1
2458	6	5	11	10	4	7	5	1
2459	3	3	16	9	4	11	3	1
2461	7	7	17	12	2	12	9	1
2463	1	1	18	13	5	13	4	3
2465	8	7	7	14	6	14	10	4
2467	6	6	19	14	2	15	2	5
2468	1	7	17	12	7	16	3	6
2469	2	1	20	8	7	17	7	1
2470	2	1	21	8	2	17	7	1
2472	1	8	22	15	5	18	11	1
2473	2	1	23	8	5	17	7	1
2475	9	1	23	8	2	17	7	1
2478	4	3	24	1	5	19	7	1
2479	2	1	3	16	5	1	1	1
2480	4	3	7	1	2	5	3	1
2481	1	9	7	12	1	5	3	1
2482	1	1	23	14	1	4	12	7
2483	3	10	25	17	1	20	12	7
2484	10	7	7	14	8	21	12	7
2485	11	1	26	14	1	8	9	1
2486	1	11	27	14	9	12	12	7
2487	7	7	17	12	6	12	13	1
2488	1	11	27	14	6	12	12	7
2489	2	1	13	1	6	9	7	1

2490	2	1	28	8	6	22	7	1
2491	1	12	29	18	4	17	2	1
2493	4	3	24	1	2	19	7	1
2494	12	3	19	8	7	23	14	1
2495	13	1	19	8	2	17	15	8
2496	1	7	27	14	10	12	9	1
2500	12	3	30	8	7	6	4	1
2502	1	1	4	8	11	24	15	1
2503	1	1	8	8	2	6	4	1
2505	6	6	12	11	2	8	6	2
2508	2	1	23	8	2	17	7	1
2509	11	1	26	14	2	8	9	1
2511	7	13	17	14	2	8	9	1
2514	1	14	17	12	5	7	12	7
2516	3	2	19	14	6	10	16	1
2517	1	1	8	8	12	10	17	1
2518	1	15	31	9	9	25	9	1
2519	6	3	3	1	7	14	14	1
2521	6	6	31	14	4	26	18	9
2522	1	1	8	8	13	10	17	1
2524	1	1	1	1	7	1	1	1
2527	8	7	7	14	14	14	10	4
2528	11	1	32	14	4	27	19	1
2529	1	6	33	8	1	10	9	1

[a]Alleles for each of the seven genes analyzed were assigned a unique number. Duplicate profiles were eliminated from analyses where their inclusion was unnecessary, resulting in a 72 strain subset, highlighted in gray. Allele types that included an amino acid change from reference strain 2182 were outlined with a box.

Table A-6: Pairwise Incongruence Length Difference Test p Values[a]

SARA

	marC	intergenic	*marR*	*marA*	*marB*
marC					
intergenic	0.866				
marR	1.000	1.000			
marA	0.001	0.087	1.000		
marB	0.068	0.332	1.000	0.184	
MLST	0.255	0.803	0.188	0.170	0.496

	ramR	intergenic	*ramA*
ramR			
intergenic	0.124		
ramA	0.072	0.027	
MLST	0.288	0.698	0.129

	soxR	intergenic	*soxS*
soxR			
intergenic	1.000		
soxS	1.000	1.000	
MLST	0.545	1.000	0.973

SARB

	marC	intergenic	*marR*	*marA*	*marB*
marC					
intergenic	0.001				
marR	0.001	0.020			
marA	0.001	0.001	0.920		
marB	0.006	0.006	0.914	0.113	
MLST	0.001	0.001	0.025	0.001	0.216

	ramR	intergenic	*ramA*
ramR			
intergenic	0.005		
ramA	0.001	0.002	
MLST	0.001	0.007	0.001

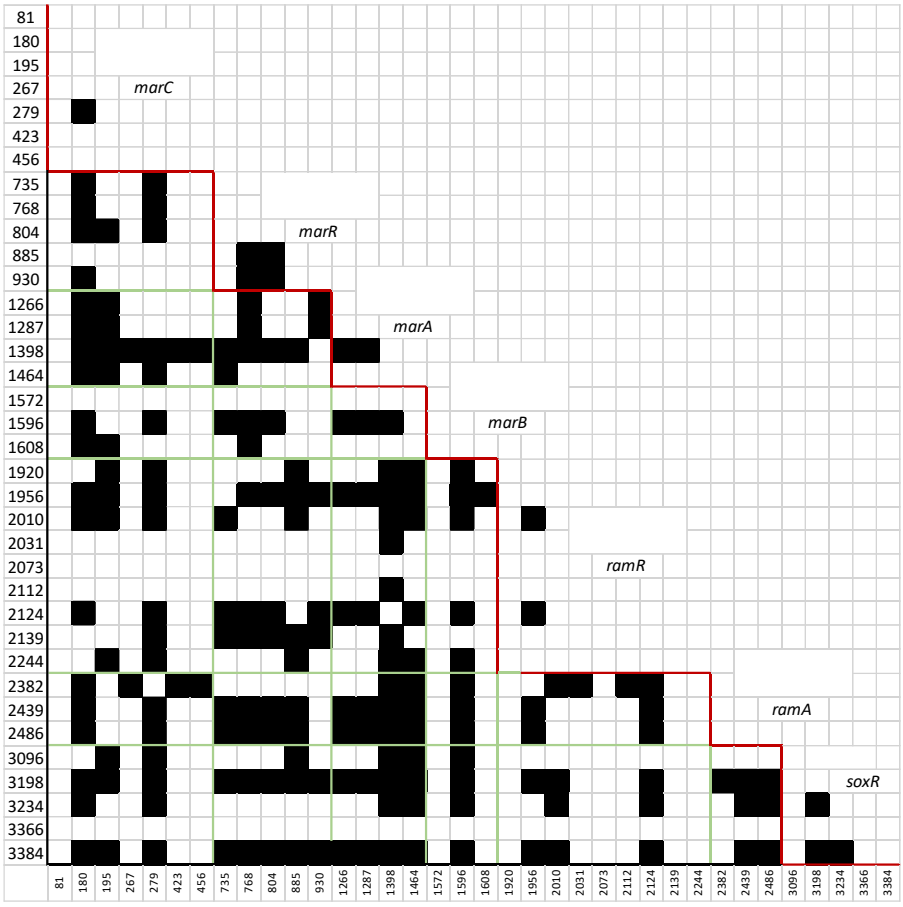
	soxR	intergenic	*soxS*
soxR			
intergenic	1.000		
soxS	0.996	1.000	

MLST	0.001	1.000	0.829

[a]p values in dark yellow indicate rejection of the null hypothesis of congruence while those in light yellow approach significance

Table A-7: Incompatibility Matrices[a]

SARA



SARB

[a]Pairwise incompatibility of parsimoniously informative nucleotide sites for the operon genes sequenced, for SARA and SARB strains. Numbers on the left and bottom indicate nucleotide sites from concatenated gene sequences of the seven operon gene set. Black boxes indicate incompatibility while white boxes indicate lack of incompatibility.

Table A-8: Microarray Probe Target Matches

Resistance Gene	Resistance Phenotype	Accession no.
aph(3')-Ia	Aminoglycoside	V00359
blaOXA-14	Beta-lactam	L38523
tetA(P)	Tetracycline	HQ399624
blaFOX-1	Beta-lactam	X77455
blaACC-1	Beta-lactam	AM939420
tet(Y)	Tetracycline	EF495198
tet(L)	Tetracycline	X60828
tet(D)	Tetracycline	X65876
tet(Z)	Tetracycline	AF121000
ere(B)	Macrolide	X03988
tcr	Tetracycline	D38215
tet(Q)	Tetracycline	X58717
tet(W)	Tetracycline	AJ427422
tet(T)	Tetracycline	L42544
tet(S)	Tetracycline	DQ377340
tetB(P)	Tetracycline	NC_010937
aac(6')Ib-cr	Fluoroquinolone and aminoglycoside	EF210035
dfrB2	Trimethoprim	DQ839391
aac(6')Ib-cr	Fluoroquinolone and aminoglycoside	EF210035
sul1	Sulphonamide	AY224185
aac(6')-Ia	Aminoglycoside	M18967
erm(B)	Macrolide	AF368302
ant(4')-IIa	Aminoglycoside	M98270
ARR-2	Rifampicin	HQ141279
tet(L)	Tetracycline	X60828
dfrA16	Trimethoprim	AF077008
aph(3')-Ic	Aminoglycoside	X62115
aac(6')-Ic	Aminoglycoside	M94066
dfrA13	Trimethoprim	Z50802
tet(B)	Tetracycline	AJ277653
dfrA18	Trimethoprim	AJ310778
aadA4	Aminoglycoside	Z50802
aac(6')-IIa	Aminoglycoside	M29695
blaEBR-1	Beta-lactam	AF416700
aac(6')-IIb	Aminoglycoside	L06163
dfrA10	Trimethoprim	L06418
catA1	Phenicol	V00622
cat	Phenicol	M35190
blaCTX-M-14	Beta-lactam	AF252622
aac(2')-Ia	Aminoglycoside	L06156

blaMUS-1	Beta-lactam	AF441286
cat(pC221)	Phenicol	X02529
catQ	Phenicol	M55620
catB8	Phenicol	AF227506
blaOXA-24	Beta-lactam	AJ239129
erm(C)	Macrolide	V01278
blaOXA-61	Beta-lactam	AY587956
tet(Y)	Tetracycline	EF495198
aph(3')-VIIa	Aminoglycoside	M29953
blaTUS-1	Beta-lactam	AF441287
blaLCR-1	Beta-lactam	X56809
QnrA1	Quinolone	AY070235
blaBES-1	Beta-lactam	AF234999
cphA1	Beta-lactam	X57102
blaB-3	Beta-lactam	AF189299
aac(3)-IIIa	Aminoglycoside	X55652
mph(B)	Macrolide	D85892
blaIND-1	Beta-lactam	AF099139
blaCGB-1	Beta-lactam	EF672680
blaOXA-18	Beta-lactam	EU503121
strB	Aminoglycoside	M96392
aph(3')-VIa	Aminoglycoside	X07753
blaOXA-7	Beta-lactam	X75562
blaKPC-2	Beta-lactam	AY034847
blaGES-1	Beta-lactam	HQ170511
strA	Aminoglycoside	M96392
blaJOHN-1	Beta-lactam	AY028464
aph(3'')-Ia	Aminoglycoside	M16482
mph(A)	Macrolide	D16251
aac(3)-IVa	Aminoglycoside	X01385
blaCARB-5	Beta-lactam	AF135373
cepA	Beta-lactam	L13472
aph(3')-Vb	Aminoglycoside	M22126
blaTLA-1	Beta-lactam	AF148067
blaGIM-1	Beta-lactam	JF414726
aph(3')-IV	Aminoglycoside	X03364
aac(3)-VIIIa	Aminoglycoside	M55426
cmlA1	Phenicol	M64556
aph(3')-Va	Aminoglycoside	K00432
blaCME-1	Beta-lactam	AJ006275
blaBRO-1	Beta-lactam	Z54180
blaSFO-1	Beta-lactam	AB003148
aac(3)-IIIc	Aminoglycoside	L06161

aph(3')-Ib	Aminoglycoside	M20305
aph(6)-Ia	Aminoglycoside	AY971801
blaSME-1	Beta-lactam	Z28968
blaSPM-1	Beta-lactam	AY341249
aac(3)-IIb	Aminoglycoside	M97172
aac(3)-VIIa	Aminoglycoside	M22999
blaPER-2	Beta-lactam	X93314
aac(3)-IXa	Aminoglycoside	M55427
aph(6)-Ib	Aminoglycoside	X05648
aac(3)-VIa	Aminoglycoside	M88012
blaCTX-M-8	Beta-lactam	AF189721
aac(3)-Xa	Aminoglycoside	AB028210
blaOXY-2-7	Beta-lactam	Z49084
cfxA	Beta-lactam	U38243
aph(4)-Ia	Aminoglycoside	V01499
ere(A)	Macrolide	AY183453
aph(4)-Ib	Aminoglycoside	X03615
blaCMY-65	Beta-lactam	JF780936
blaCMY-1	Beta-lactam	X92508
tet(H)	Tetracycline	U00792
QnrB42	Quinolone	JN680743
tet(X)	Tetracycline	AB097942
blaL1	Beta-lactam	EF126059
blaA	Beta-lactam	AY954728

Antimicrobial probe BLAST matches, listed next to the type of resistance conferred and the BLAST accession number, determined utilizing ResFinder (Center for Genomic Epidemiology), using a 20% minimum length value and 90% minimum match identity.

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