AN ABSTRACT OF THE THESIS OF

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Title: Prevalence of the Multiple Antibiotic Resistance Operon (*marRAB*) in the Genus *Salmonella*

Abstract Approved: Scott S Cupp

The multiple antibiotic resistance operon (*marRAB* operon) is a member of the Multidrug resistance (Mdr) systems. Similar to other Mdr systems, this operon codes for resistance to structurally and functionally unrelated antibiotics. This operon has been shown to be conserved in the family Entrobacteriaceace. Within the genus *Salmonella*, a previous study (8) demonstrated *S. typhimurium* was positive for the *marRAB* operon while *S. arizonae* appeared not to contain the operon. Thus, the objective of this study was to determine the prevalence of the *marRAB* operon in genus *Salmonella*. Thirty different veterinary *Salmonella* isolates were examined using PCR, Southern blot, and dot blot analysis. PCR was performed on isolated chromosomal DNA from all thirty organisms using primers based on the *marRAB* operon of *S. typhimurium*. The predicted 2.2 kb band was amplified in 16 organisms, including *S. arizonae*. The cloned 2.2 kb PCR product of *Salmonella enteritidis* was subjected to DNA sequencing and shown to have approximately 99% sequence homology to the *marRAB* operon of *S. typhimurium*. Southern blot and dot blot analysis were performed on the PCR amplification products using the sequenced *marRAB* fragment as a probe. Southern blot data revealed that the probe hybridized with all PCR products appearing on the agarose gel. Dot blot analysis data indicated the probe hybridized with chromosomal DNA from all thirty organisms.

Induction studies were performed in the presence of low concentrations of either tetracycline or chloramphenicol to determine if the isolates have a functional *marRAB* operon. There was an overall increase in the resistance of almost all induced organisms to tetracycline, chloramphenicol, and ampicillin. Taken together, these data demonstrate conservation of the *marRAB* operon in the thirty *Salmonella* species examined.

Prevalence of the Multiple Antibiotic Resistance Operon (*marRAB*) in the Genus *Salmonella*

A Thesis

Submitted to

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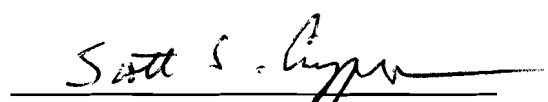
Kunonga, Ngoni Ishe



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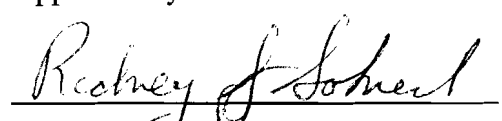

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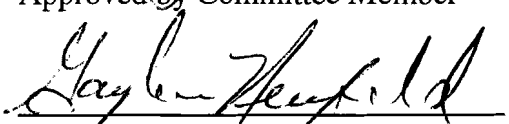

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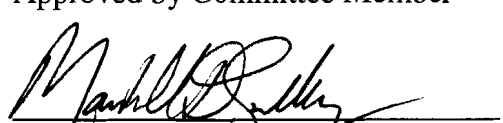

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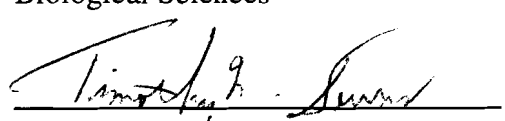

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PREFACE

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Introduction

I. Bacterial Antibiotic Resistance

Bacterial resistance to antibiotics is increasing faster than antibiotics can be successfully developed. To date, several thousand antibiotics have been developed with approximately one hundred being medically practical (11). Science has yet to determine whether the genes for resistance to antibiotics present in infectious bacteria have always been encoded in the genome of these organisms or are the result of random mutations (14).

Gram-negative bacteria have unlinked genes encoding resistance to various antibiotics that are plasmid-encoded. These plasmids, designated R-plasmids, are characterized as being self-transmissible and usually have the resistance gene located on a transposon (11,33). Some organisms, however, are able to confer resistance to multiple antibiotics which are structurally unrelated (14). For example, organisms resistant to chloramphenicol, ampicillin, and rifampin could have more than one unlinked gene residing either on a R-plasmid, chromosomal DNA, or both.

Mechanisms of antibiotic resistance are diverse. For example, an unlinked gene can encode a protein which degrades or enzymatically alters an antibiotic (9,38). Alternatively, some mechanisms of antibiotic resistance are accomplished by simply overexpressing the protein that is targeted by the antibiotic. Regulating cell permeability, however, is one of the main mechanisms utilized by gram-negative bacteria to confer resistance to antibiotics. This is achieved in part by reducing the number of outer membrane porins that are expressed. Outer membrane porins (Omp) are partly

responsible for the influx of antibiotics into a bacteria (4,33). However, very little is known about how the various porins are involved in antibiotic influx. In *Escherichia coli*, OmpF and OmpC are the most abundant porins with OmpF being largely responsible for antibiotic influx at low temperature (7,13).

Chromosomal-borne antibiotic resistance genes, collectively known as Multidrug resistance (Mdr) genes, have been recently identified and characterized (14). These genes encode resistance to structurally and functionally unrelated antibiotics. The gene products of the Mdr genes regulate antibiotic levels in a bacteria non-specifically by regulating the influx and efflux of antibiotics via membrane transporters (14).

Mdr genes are found associated with various enteric organisms. For example, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Serratia marcesens, Neisseria gonorrhoeae, Pseudomonas aeruginosa,* and *Proteus vulgaris* have an Mdr system which is possibly organized in the form of an operon (12,14,17,22,26,32). These systems, when induced, encode resistance to structurally unrelated antibiotics. The mechanism of action of these systems involves regulating cell permeability through efflux pumps, influx of antibiotics, or a combination of both.

II. Multiple Antibiotic Resistance Operon (*marRAB* operon)

E. coli has various Mdr systems that confer resistance to a wide variety of compounds such as antibiotics, dyes, and detergents (6,14,35). One of the Mdr systems of *E. coli* is known as the multiple antibiotic resistance operon (*marRAB* operon) and was first described in 1983 (15,16). In this initial study, *E. coli* was incubated with subinhibitory concentrations of either tetracycline or chloramphenicol. This resulted in

the organism becoming resistant to various structurally unrelated antibiotics, such as tetracycline, chloramphenicol, β-lactams, puromycin, and nalidixic acid for 50-200 generations after removal of the inducing antibiotic. The mechanism of this resistance was temperature dependent and induction by tetracycline and chloramphenicol was 1.5 times higher at 30^0C than at 37^0C. Analysis of the resistant cells demonstrated that chloramphenicol acetyltransferase or β-lactamase were not present. The region of the chromosome involved in this resistance was found at 34 minutes in the genome. This operon was later shown to be present in *Salmonella tyhpimurium* (14,41).

A. Structure and function

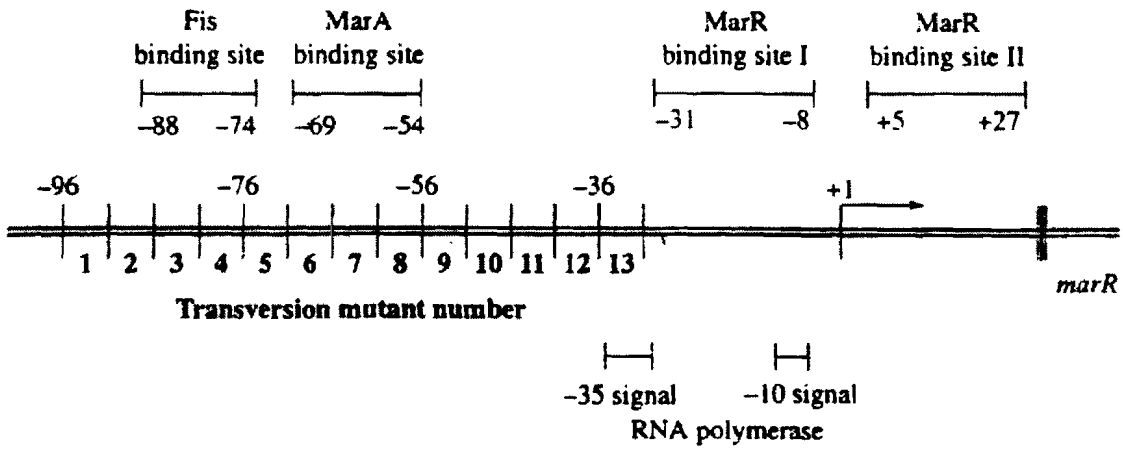
The *marRAB* operon is composed of four structural genes, designated *marR*, *marA*, *marB*, and *marC* (1). An associated regulatory region known as *marO* functions as both an operator and promoter (5). Figure 1 illustrates the organization of the *marRAB* operon. When the operon is activated, all four structural genes are transcribed with *marR*, *marA* and *marB* being transcribed downstream as one 1.4 kb mRNA transcript (1,27). Upstream of *marO*, *marC* is transcribed as a 1.0 kb transcript (5,27,29).

i) MarC and MarB

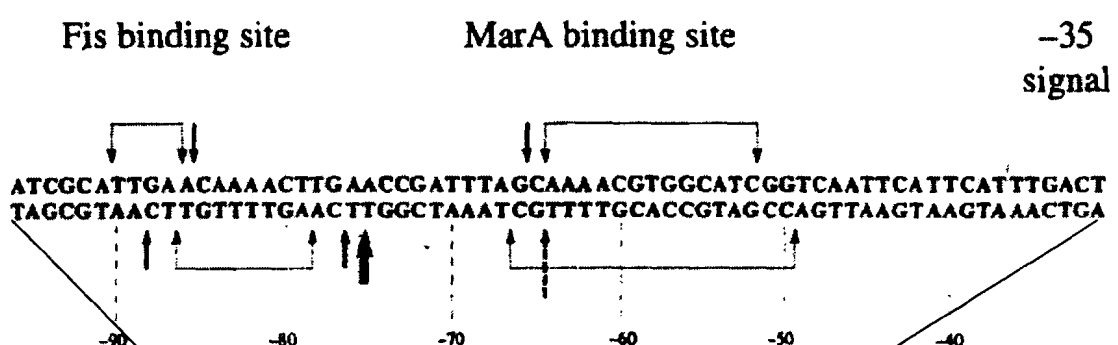
Little is known about the functions of MarC and MarB. Both have been shown are needed for full phenotypic expression of the operon (5,27). Mutation studies have shown organisms containing only *marA* and *marB* are two to three fold less resistant to multiple antibiotics than cells containing *marC, marA*, and *marB* (18). The amino acid sequence for MarC of *E. coli* is 91% identical to that of *S. typhimurium* (41). The MarA

Fig. 1. Organization of the *marRAB* operon (30). (A) *mar* promoter region; (B) detailed illustration of *marO*; (C) *marRAB* operon.

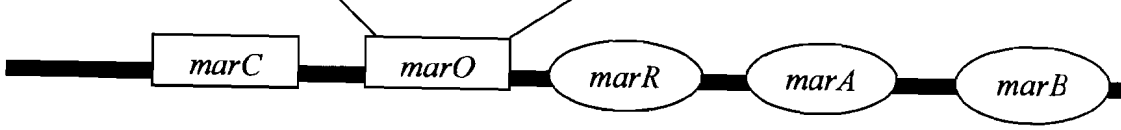
A


Fis
binding site
MarA
binding site
MarR
binding site I
MarR
binding site II
−88 −74
−69 −54
−31 −8
+5 +27
−96
−76
−56
−36
+1
1 2 3 4 5 6 7 8 9 10 11 12 13
marR
Transversion mutant number
−35 signal
−10 signal
RNA polymerase

B


Fis binding site
MarA binding site
−35
signal
ATCGCATTGAACAAAACTTGAACCGATTTAGCAAAACGTGGCATCGGTCAATTCATTCATTTGACT
TAGCGTAACTTGTTTTGAACTTGGCTAAATCGTTTTGCACCGTAGCCAGTTAAGTAAGTAAACTGA
-90
-80
-70
-60
-50
-40

C


marC
marO
marR
marA
marB

and MarB proteins of *E. coli* are 86% and 42% identical to MarA and MarB of *S. typhimurium*, respectively (41).

ii) MarR

The first structural gene to be transcribed downstream of the operator/promoter region is *marR*. MarR of *E. coli* is 144 amino acids long and functions as a negative transcriptional regulator of the *marRAB* operon. This protein represses not only its own transcription, but also *marA*, *marB*, and *marC*. Repression of the *marRAB* operon occurs when MarR binds to two positions on the operator region known as site I and site II (Fig 1). Site I and Site II are 80% identical (31). When MarR binds, a 21 base pair region is covered up on each site, overlapping the -35 and -10 positions (30,31). Actual repression of the operon occurs when MarR binds at site I whereas occupation of site II by MarR has no effect (30,39). MarR and MarA bind at different positions on *marO*; MarA at the marbox and MarR at site I and II. The marbox is a DNA segment that is part of the promoter region (*marO)*. It is located at the -69 to -54 position of *marO* (30). When MarA binds to the marbox, transcriptional activation of *marRAB* operon occurs. Although MarR and MarA bind at different positions on *marO*, evidence suggests they compete with each other for binding to their respective positions (39).

iii) MarA

MarA is a transcriptional activator that induces its own transcription and that of *marR*, *marB*, and *marC* (27,28,41,42). In *E. coli,* the marbox is bound by MarA (41). An accessory transcriptional activator known as Fis, enhances the binding of MarA to the

marbox (39). MarA has a DNA binding motif that binds to the promoter of the *marRAB* operon. This motif is also an ambidextrous transcriptional activator that binds to various promoters, such as *micF, soxRS* and *fumC* (23,24). The *micF* promoter regulates the expression of OmpF (4,14). At 30^0C, OmpF is more abundant than OmpC. When *E. coli* is induced with either tetracycline or chloramphenicol at 30^0C, the expression of MarA increases (4,14,21). MarA is a transcriptional activator of *micF* mRNA; *micF* mRNA is an antisense post-transcriptional regulator of *ompF*. When *micF* mRNA is expressed, it binds to *ompF* mRNA which in turn reduces the expression of *ompF* when either tetracycline or chloramphenicol are present.

B. Structural and functional homology of MarA to other proteins

MarA is a member of Xyls/AraC, a family of transcriptional activators characterized by a helix-turn-helix DNA binding motif. This family is composed of three subgroups with over 27 members (20). Relative to other members of the Xyls/AraC family, MarA is more homologous to SoxS (a protein that activates resistance to antibiotics and oxidative agents), Rob (no known function), and TetD (no known function) (2,5,19,20,25,36,43). The homology of the helix-turn-helix DNA binding motif of MarA to that of other proteins in this class makes it an ambidextrous transcriptional activator of various promoters.

III. Objective

In this study, the prevalence of the *marRAB* operon in the genera *Salmonella* was determined using the polymerase chain reaction, dot blot analysis, Southern blot analysis,

and antibiotic resistance patterns. A prior study (8) showed the *marRAB* operon was prevalent in the family Enterobacteriaceace; however, the same study also demonstrated that it may or may not be present within the genus *Salmonella.* These inconclusive data prompted the current investigation.

Materials and Methods

I. Bacterial Strains, Plasmids, and Growth Conditions

Thirty *Salmonella* species (Table 1) were routinely propagated in Brain Heart Infusion (BHI) media or on agar plates at 37°C. *Escherichia coli* DH5-α, a routine cloning strain of *E. coli*, was grown on Luria-Bertani (LB) media at 37°C. For long-term storage, bacterial cultures were stored in BHI -20% glycerol at -70°C. Cells containing the plasmid pT7Blue-3 (Novagen, Inc., Madison, WI) were selected using 100 μg/ml ampicillin. The chromogenic substrate X-gal (5-bromo-4-chloro-3-indoly-β-D-galactoside) was used at 20 μg/μl. A complete listing of media and solutions used in this study is depicted in Table 2.

II. Isolation of DNA

A. Chromosomal DNA isolation

Chromosomal DNA was isolated using a modification of an established procedure (3,37). Organisms were inoculated into 6 ml of BHI and incubated for 18 hr at 37°C with shaking at 250 rpm in a New Brunswick Series 25 Incubator (Edison, NJ). Cells were harvested by centrifugation at 4,000 rpm for 5 min in a Sorvall model GLC-1 General Laboratory Centrifuge (Newton, CT). The supernatant was discarded and the cells resuspended in 4 ml of Tris-ethylenediaminetetraacetic acid (TE) buffer. Cells were lysed upon addition of 200 μl of 20% sodium dodecyl sulfate (SDS) and subsequent

Table 1. *Salmonella* species used in this study

Organism #	Organism	Stereotype	Source
1	*Salmonella agona*	B	University of Iowa
2	*Salmonella anatum*	E1	Kansas State University Veterinary Medicine
3	*Salmonella arizonae*[a]		Kansas State university Veterinary Medicine
4	*Salmonella bardo*	C3	University of Iowa
5	*Salmonella brandenburg*	B	Kansas State University Veterinary Medicine
6	*Salmonella choleraesuis*	C1	Kansas State University Veterinary Medicine
7	*Salmonella derby*	B	Kansas State University Veterinary Medicine
8	*Salmonella enteritidis*	D1	Kansas State University Veterinary Medicine
9	*Salmonella gallinarum*	D1	Kansas State University Veterinary Medicine
10	*Salmonella give*	E1	Kansas State University Veterinary Medicine
11	*Salmonella hamburg*	H1	University of Iowa
12	*Salmonella hartford*	C1	University of Iowa
13	*Salmonella heidelberg*	B	University of Iowa
14	*Salmonella kentucky*	C3	Kansas State University Veterinary Medicine
15	*Salmonella mbandaka*	C1	Kansas State University Veterinary Medicine
16	*Salmonella meleagridis*	E1	University of Iowa
17	*Salmonella montevideo*	C1	Kansas State University Veterinary Medicine
18	*Salmonella muenster*	E1	Kansas State University Veterinary Medicine
19	*Salmonella newport*	C2	Kansas State University Veterinary Medicine
20	*Salmonella oranienburg*	C1	Kansas State University Veterinary Medicine
21	*Salmonella paratyphi*	A	Kansas State University Veterinary Medicine
22	*Salmonella pullorum*	D1	Kansas State University Veterinary Medicine
23	*Salmonella reading*	B	Kansas State University Veterinary Medicine
24	*Salmonella rubislaw*	F	Kansas State University Veterinary Medicine
25	*Salmonella schottmuelleri*	B	Kansas State University Veterinary Medicine
26	*Salmonella stanley*	B	University of Iowa
27	*Salmonella thompson*	C1	Kansas State University Veterinary Medicine
28	*Salmonella typhimurium*	B1	Kansas State University Veterinary Medicine
29	*Salmonella uganda*	E1	Kansas State University Veterinary Medicine
30	*Salmonella urbana*	N	University of Iowa

[a]Belongs to the subgenus III (10)

incubation at 60°C for 15 min. Incubation at 37°C for 20 min in the presence of 10 µl of RNase (10 mg/ml) followed by incubation at 60°C for 2 hr with 30 µl of proteinase K (10 mg/ml) was performed. Phenol extraction was carried out by adding 4 ml of phenol, gently mixing by inversion, and centrifugation at 4,000 rpm for 5 min. The upper layer (aqueous phase) was transferred to a fresh 15 ml conical tube and an additional phenol extraction and chloroform/isoamyl alcohol (24:1) extraction was performed as described above. DNA was precipitated by adding 15 ml of ice cold 100% ethanol. The DNA was transferred to a 1.5 ml microcentrifuge tube and dried in a ISS 110 Savant speedvac for two minutes. Once dried, the DNA was resuspended in 200 µl of TE and concentration and purity were determined as described below.

B. Isolation of plasmid DNA

Plasmid DNA was isolated using a modified protocol (3,37). Briefly, individual colonies were incubated in 3 ml of LB broth containing the appropriate antibiotic for 16 hr at 37°C with shaking at 250 rpm. Cells were harvested by centrifugation for 30 sec at 13,000 x g and resuspended in 200 µl of Solution I. Cells were lysed upon the addition of 200 µl of Solution II and subsequent incubation for 10 min at room temperature. Protein-chromosomal DNA complexes were precipitated by adding 150 µl of Solution III. After incubation on ice for 10 min, centrifugation was performed for 5 min at 13,000 x g. The supernatant was added to 240 µl of TE and 10 µl of 1 mg/ml RNase. After 15 min of incubation at room temperature, 500 µl of 1.6 M NaCl-13% Polyethylene Glycol 8000.

Table 2. Media and Solutions used in this study

Media and Reagents	Composition
BHI broth[a]	37g BHI bring to 1 L with ddH_2O
BHI-20% Glycerol	37 g BHI 0.2 L Glycerol bring to 1 L with ddH_2O
LB broth[a]	10 g Tryptone 5 g Yeast Extract 10 g NaCl bring to 1 L with ddH_2O
2X LB	20 g Tryptone 10 g Yeast Extract 1 g NaCl bring to 1 L with ddH_2O
$CaCl_2$-Glycerol	0.8 L 1 M $CaCl_2$ 0.20 L Glycerol
LB/Amp/X-gal [a]	10 g Tryptone 5 g Yeast Extract 10 g NaCl 100 μg/ml Amp 20 μg/ml X-gal bring to 1 L with ddH_2O
Sodium dodecyl sulfate (SDS)	20 g SDS bring to 100 ml with ddH_2O
RNAse	1 mg/ml
Proteinase K	1 mg/ml
Tris-EDTA (TE)	10 mM Tris pH 8.0 1 mM EDTA pH 8.0
50X Tris- Acetic Acid EDTA (TAE)	242 g Tris 57.1 ml Glacial Acetic Acid 4 ml 0.5M EDTA bring to 1 L with ddH_2O
1X TAE	20 ml 50X TAE bring to 1 L with ddH_2O

[a] 20 g of agar was added to make agar plates

Table 2. Continued

Media and Reagents	Composition
Solution I	50 mM Sucrose 25 mM Tris pH 8.0 10 mM EDTA
Solution II	0.2N NaOH 1% SDS
Solution III	60 ml 5 M Potassium acetate 11.5 ml Acetic acid ddH_2O to 100 ml
5M Potassium acetate	49 g Potassium acetate ddH_2O to 100 ml
1.6 M NaCl-13% PEG	9.35 g NaCl 13 g PEG (8000) ddH_2O to 100 ml
Tris-EDTA (TE)	10 mM Tris pH 8.0 1 mM EDTA pH 8.0
3 M Sodium Acetate	40.8 g Sodium acetate ddH_2O to 100 ml
Chloroform/isoamyl alcohol (24:1)	96 ml Chloroform 4 ml Isoamyl alcohol
Ethidium Bromide (EtBr)[b]	0.1 g EtBr ddH_2O to 10 ml

[b] Wrap in foil (EtBr is light sensitive)

Table 2. Continued

Media and Reagents	Composition
Genius buffer #1 (pH 7.5)	0.1 M Tris 0.15 M NaCl
Genius wash buffer	Genius Buffer #1 3% Tween 20
Genius detection buffer pH 9.5 prior to addition of $MgCl_2$. Check pH after adding $MgCl_2$	0.1 M Tris 50 mM $MgCl_2$ 0.1 M NaCl
5X Blocking reagent[c]	10 g Blocking reagent Genius buffer #1 to 100 ml
1 M Na_2HPO_4	14.2 g 1 M Na_2HPO_4 ddH_2O to 100 ml
1 M NaH_2PO_4	13.8 g 1 M NaH_2PO_4 ddH_2O to 100 ml
1 M Sodium phosphate buffer (pH 7.0)	Titrate 60 ml of 1M $Na_2H(PO_4)$ with 1 M $NaH_2(PO_4)$ to get pH 7.0
Blocking solution	40 ml 5× Blocking reagent Genius buffer #1 to 200 ml
High SDS buffer or Hybridization Sol. (40 ml)	10 ml 20X SSC 2.8 g SDS 8 ml 5X Blocking reagent (pH 7) 2 ml 1 M Sodium phosphate buffer 20 ml Foramide
Base Solution 0.4 M NaOH, 0.6M NaCl	16.0 g NaOH 35.3 g NaCl bring to 1 L with ddH_2O
Acid Solution 0.25 M HCl	20 ml concentrated HCl 980 ml H_2O
20X SSC pH 7.0	88.0 g $Na_3Citrate{\cdot}2H_20$ 175.4 g NaCl bring to 1 L with ddH_2O
Equilibration buffer 1.5 M NaCl, Tris-HCl pH 7.5	60.6 g Tris 87.6 g NaCl bring to 1 L with ddH_2O

[c]Heat on heating plate with stirring to get the reagent into solution, then autoclave.

(PEG) was added and the solution centrifuged for 5 min as described above. The precipitate was resuspended in TE buffer and a phenol and chloroform/isoamyl alcohol extraction was performed with 400 µl of the organic solvents as described above for chromosomal DNA isolation. DNA was precipitated by adding 1 ml of 95% ethanol and incubating the solution on ice for 10 min. After centrifugation for 10 min, the supernatant was discarded and the DNA dried and resuspended in 30 µl of TE.

C. Quantification and purity of DNA

DNA concentration and purity were determined using a Beckman DU™ 530 spectrophotometer. A dilution factor of 1:100 was used. The concentration of double stranded DNA was determined using the following equation: (OD_{260})(Dilution factor)(50) = µg/ml. The purity of the DNA was determined by the OD_{260}/OD_{280} ratio.

III. Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) was performed using a Minicycler™ Thermocycler (MJ Research, Inc., Watertown, MA). Reaction mixtures consisted of 500 ng of chromosomal DNA, 1.5 mM of $MgCl_2$, 50 µM of primers, 0.2 mM of dNTPs, PCR buffer (30 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$, 10 mM DTT, and 1 mM ATP), and 1 unit of Taq polymerase in a final volume of 100 µl (Table 3). In general, amplification consisted of a denaturation step at 94^0C for 1 min, followed by annealing of the primers at

Table 3. Primers and reaction conditions used in PCR

Primers	Primer Sequence	Conditions	
STmarRAB-F	5' GGG AAC AGG TTT CCG GCA GAC GAA 3'	94°C 5 min	one cycle
		94°C 1 min	30 cycles
STmarRAB-R	5' GCT GGC GAG CGC CGC GGT GTT AC 3'	60°C 2 min	30 cycles
		72°C 2 min	30 cycles

60°C for 2 min and extension at 72°C for 2 min. This 3-step cycle was repeated 30 times. PCR products were analyzed by agarose gel electrophoresis.

IV. DNA Extraction from Agarose Gels

Extraction of PCR products from agarose gels was performed using a Qiagen II Extraction kit (Qiagen, Chatsworth, MA) according to the manufacturer's recommendation.

V. Preparation of $CaCl_2$ Competent Cells

Competent cells were prepared using a modification of an established protocol (3,36). Briefly, 2 ml of 2X LB was inoculated with a single bacterial colony of *E. coli* DH5α and incubated overnight at 30°C with shaking at 250 rpm. Following incubation, 0.5 ml of the culture was added to 1 L of prewarmed (30°C) 2X LB. Cells in the new culture were propagated for approximately 5 hr at 30°C to an $O.D_{600}$ of 0.3 at 250 rpm. Four milliliters of 1 M $MgCl_2$ was added and the incubation continued approximately 1 hr until the $O.D_{600}$ was between 0.45 and 0.55. The culture was incubated on ice for an additional 2 hr. Cells were precipitated by centrifugation at 3,000 x g for 5 min and resuspended in 100 ml of ice-cold $CaCl_2$ media. After incubation on ice for 40 min, cells were precipitated as described above. Cellular precipitates were resuspended in 5.1 ml of ice-cold $CaCl_2$-glycerol media. Cells were aliquoted and stored at -70°C.

VI. Ligation Reactions

In general, reaction mixtures consisted of 1 μl of T-vector (Novagen, Inc., Madison, WI) 1.5 μl of buffer (final concentration 30 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$, 10 mM DTT, and 1 mM ATP), 6 μl of DNA, and 1 μl of T4 DNA ligase. After incubation for 2-3 hr in a 15°C water bath, ligation mixtures were immediately transformed into competent cells by heat shock as described below.

VII. Transformation

Ligation mixtures were incubated on ice for 15 min with 100 μl of competent cells. Following incubation, the mixture was immediately placed in a 42°C water bath for 90 sec and then back on ice for 1 min. Nine hundred microliters of LB was added and 100 μl the mixture spread onto LB plates containing the appropriate antibiotic. Plates were incubated overnight at 37°C and examined for growth.

VIII. Southern Blots

Southern Blot and dot blot analysis were performed according to the manufacturer's recommendations with slight modifications as described bellow (Boehringer Mannheim, Germany).

A. Preparation of Dig labeled probe

Gel extracted DNA was added to ddH_2O in a 1.5 ml microfuge tube to a final volume of 15 µl. The tube was incubated in boiling water for 10 min and immediately placed on ice. After a brief centrifugation for 1 sec, 2 µl of hexanucleotide mix, 2 µl of dNTP mix, and 1 µl of Klenow enzyme were added. The mixture was incubated at 37°C for approximately 20 hr and stopped by the addition of 2 µl of 0.2 M EDTA (pH 8) and 1 µl of glycogen. Labeled probe was precipitated upon the addition of 2.5 µl of 4M LiCl and 75 µl of 100% ethanol followed by incubation for 2 hr at -20 °C. After centrifugation for 15 min, the precipitate was washed with 100 µl of 70% of ethanol and dried in a speedvac. The Dig labeled probe was resuspended in 50 µl of TE buffer and stored at -20 °C.

To verify labeling of the probe, 1 µl was placed on a piece of nylon membrane (Micron Separation Inc., Westborough, MA) and crosslinked using a UV Crosslinker. The nylon membrane was incubated with 10 ml of blocking solution for 5 min. This solution was discarded and replaced with 10 ml of blocking solution containing 2 µl of anti-DIG antibody. After incubation for 10 min, the nylon membrane was washed two times in wash buffer for 5 min each and equilibrated in detection buffer for 1 min. Finally, 10 ml of detection buffer containing 200 µl of Nitro-blue-tetrazolium-phosphate (NBT/X-Phos) was added and the membrane incubated for 16 hr in the dark. Color development was indicative of the DIG-labeled probe binding to DNA contained on the membrane.

B. Agarose gel electrophoresis

Electrophoresis was carried out as described in Table 4. After electrophoresis, the gel was placed in a plastic dish containing 10 ml of acid solution and incubated for 15 min with shaking at room temperature. The acid solution was discarded and the gel was rinsed twice with ddH_2O. Base solution was added to the gel and incubation at room temperature was performed for 30 min followed by rinsing twice with ddH_2O. After incubation for 30 min at room temperature in equilibration buffer, the gel was ready for transfer.

C. DNA transfer to nylon membrane.

Three pieces of chromatography paper (Whatman filter paper) and a piece of MagnaGraph nylon transfer membrane were cut to the size of the agarose gel. A Whatman paper wick was cut and pre-soaked in 10X SSC for 15 min. A baking-dish was filled half way with 10X SSC and two empty pipette tip boxes were centered and placed inside it. A glass plate was placed on top of the boxes. The wick was centered on the glass plate with the ends in the 10X SSC. The agarose gel was centered on top of the wick and a 10X SSC pre-soaked nylon membrane placed on top of the agarose gel. Three pieces of chromatography paper pre-soaked in 10X SSC were centered on the nylon membrane, and a stack of paper towels was placed on top of the chromatography paper. A heavy flat object (e.g books) was stacked on top of the paper towels. Plastic wrap was used to seal the baking-dish without covering the agarose gel. Transfer by capillary

Table 4. Preparation of agarose gels and electrophoresis conditions

Agarose %	Range of Resolution (kb)	Amount of Agarose
0.5[a]	30 to 1	0.15 g
0.7[a]	12 to 8	0.21 g
1.0[a]	10 to 0.5	0.30 g
1.2[a]	7 to 0.4	0.36 g
1.5[a]	3 to 0.2	0.45 g
0.5[b]	30 to 1	2.25 g

[a]30 ml of 1X TAE and 1 µl of EtBr mixed with agarose. Electrophoresis was performed at 80 volts for 1hr in 150 ml TAE buffer

[b]150 ml of 1X TAE and 5 µl of EtBr mixed with agarose. Electrophoresis was performed at 115 volts for 1.5 hr in 1.75 L TAE buffer

action was accomplished in approximately 18 hr. After transfer, the side of the nylon membrane bound by DNA was marked with a pencil along with the positions of the wells. This was followed by UV-cross linking the DNA to the nylon membrane for 1 min. The nylon membrane was stored at -20 °C until needed.

D. Prehybridization and hybridization of nylon membrane

The nylon membrane was placed in a hybridization bottle, 20 ml of hybridization solution added, and rotation performed in a Hybaid hybridization oven (Midwest Scientific., Valley Park, MO) for 2 hr at 55 °C. After incubation, the pre-hybridization solution was discarded and 20 ml of hybridization solution containing the DIG-labeled probe was added. Hybridization of the DIG-labeled probe to the nylon membrane was allowed to proceed overnight at 60 °C.

E. Detection of the DIG-labeled probe bound to the nylon membrane

After hybridization, the membrane was washed two times for 5 min each in 2X SSC-1% SDS at room temperature. The membrane was transferred to a fresh hybridization bottle containing 0.5X SSC-1% SDS and washing continued two times for 15 min each at 40 °C with constant rotation in the hybridization oven. The nylon membrane was placed in a Petri dish and 10 ml of wash buffer was incubated with the nylon membrane for 5 min at room temperature with rocking. Ten milliliter of blocking solution was added and rocking continued for 30 min at room temperature. This solution

was discarded and 10 ml of blocking solution containing 2 µl of anti-DIG antibody was added and the solution rocked for 30 min at room temperature. Two washings with 10 ml of wash buffer each was performed at room temperature for 15 min with constant rocking. After the nylon membrane was equilibrated in detection buffer for 5 min, 10 ml of detection buffer containing 200 µl of NBT/X-Phos was added and development was allowed to proceed for 16 hr in the dark. Following visual analysis, the developed nylon membrane was preserved in water and documented.

F. Dot blot analysis

Dot blot analysis was performed using a modification of the Southern blot analysis procedure described above. Briefly, 5 µl of 100 ng/ml DNA was spot-loaded onto a nylon membrane. After the DNA solution dried, the nylon membrane was UV cross-linked and the steps of pre-hybridization through detection were the same as for the Southern blot protocol.

IX. Induction of the Mar Phenotype in *Salmonella*

Thirty different *Salmonella* species (Table 1) were induced to express the *marRAB* operon according to a previously defined protocol (8). Briefly, the 30 species were individually inoculated into 3 ml of BHI broth and incubated at 30°C for 18 hr with shaking at 250 rpm. Cells were harvested by centrifugation at 4,000 rpm for 3 min and washed with 1.5 ml of physiological saline. After centrifugation at 4,000 rpm for 3 min,

cells were concentrated to 0.1 volume (e.g. 0.3 ml) of the original culture volume in physiological saline.

One hundred microliter of saline-washed cells were spread onto BHI plates containing either tetracycline (3 μg/ml) or chloramphenicol (7 μg/ml) and incubated at 30°C for 3-5 days. Resulting colonies were re-inoculated onto the respective antibiotic media and propagated overnight 30°C.

X. Determination of the Minimum Inhibitory Concentration (MIC)

Organisms grown on BHI plates containing either tetracycline or chloramphenicol were propagated in 3 ml of BHI overnight at 30°C with shaking at 250 rpm. After 18 hr of growth, the OD_{600} of the culture was determined. Cultures were diluted to an OD_{600} of 0.005 by adding the appropriate volume of physiological saline. BHI plates containing either tetracycline, chloramphenicol, or ampicillin at variable concentrations were spot inoculated with 1 μl of the diluted cells and incubated for 16 hr at 30°C. The minimum inhibitory concentration (MIC) of each species was visually determined by recording the concentration of antibiotic that inhibited bacterial growth.

Results

I. Polymerase Chain Reaction (PCR)

PCR was performed on chromosomal DNA from 30 different *Salmonella* species and amplification products visually analyzed by agarose gel electrophoresis (Fig. 2). Primers used in the amplification were based on the *marRAB* operon of *Salmonella typhimurium* (Table 3). Sixteen of 30 organisms amplified a 2.2 kb band indicative of the *marRAB* operon. Four organisms, *Salmonella kentucky* (#14), *Salmonella newport* (#19), *Salmonella stanley* (#26) and *Salmonella uganda* (# 29) amplified a band that appeared slightly larger than the predicted 2.2 kb band, whereas DNA from *Salmonella munester* (#18) resulted in no amplification. All 30 organisms, with the exception of *Salmonella munester*, amplified at least one band smaller than the predicted 2.2 kb band. The most conserved PCR amplification product was observed at approximately 0.5 kb.

II. Cloning and Sequence Analysis of *marRAB*

A 2.2 kb PCR product of *Salmonella enteritidis* was cloned into pT7Blue-3 and its nucleotide sequence determined at the University of Arkansas for Medical Sciences. The nucleotide sequence of *marRAB* is approximately 99% identical to the *marRAB* operon of *S. typhimurium* (Fig. 3).

III. Southern Blot and Dot Blot Analysis

Southern blot analysis was performed on the PCR products illustrated in Figure 2 using a 2.2 kb Dig-labeled *marRAB* probe from *S. enteritidis*. All PCR amplified bands

Fig. 2. Agarose gel electrophoresis of PCR amplified products using primers based on the marRAB operon of *Salmonella enteritidis*. The 30 *Salmonella* isolates are listed in Table 1.

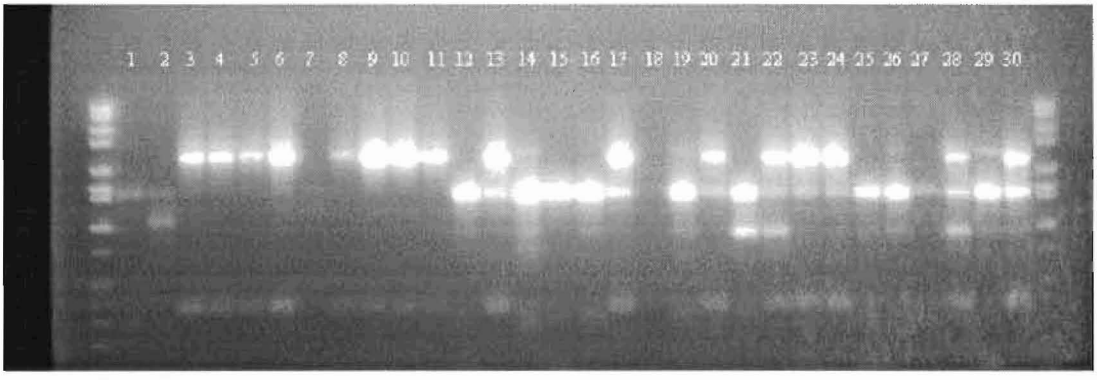

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30

Fig 3. Nucleotide sequence of 2.2 kb *marRAB* of *Salmonella enteritidis.*

1	GGGAACAGG	TTTCCGGCAG	ACGAAAATGC	GCCTTAGTGG	TACGTTTTAA	TAATTTCCAA
61	CACGCCGTTG	ATAATAAATT	GCACGCCCAT	ACACACCAGC	AGGAATCCC	ATTAAGCGGG
121	AGATCGCTTC	AATCCCCCCT	TTACCCACCA	GCCGCATAAT	AGCGCCAGA	ACTGCGTAAG
181	CATCCCCACA	GGATCACCGC	CACGGCAAGG	AAAATAATCG	GCGGCGCGAC	CATAATGACC
241	CAGTCGGGAA	ACTCGCCGCC	ATGACGCACC	GTGGAAGCGG	AACTGATGAT	CATTGCGATG
301	GTCCCCGGTC	CTGCGGTGCT	TGGCATAGCC	AGTGGAACAA	ATGCAATATT	AGCCGTCGGT
361	TCGTCTGCCA	GCTCCTCCGA	TTTGCTTTTC	GCTTCCGGCG	ACTCATGCGC	CTTCTGCTGC
421	GGGAAAAGCA	TTCTGAAGCC	GATAAACGCC	ACGATTAACC	CCCCGGCGAT	CCGTAGCCCT
481	GGAATCGAAA	TACCGAAGGT	GTTCATAACT	AACTGCCCGG	CGTAGTACGC	CACCATCATA
541	ATAGCGAAGA	CATAAACCGA	AGCCATATAG	GACTGCCGGT	TGCGTTCCGC	ACTATTCATA
601	TTGCCCGCAA	GGCCAAGAAA	CAGCGCCACG	GTGGTTAGCG	GATTGGCTAA	CGGGAGCAGT
661	ACGACCAACC	CCAATCCAAT	CGCTTTAAAC	AAATCCATCA	TAATACTATC	TCTTACCCAT
721	CAGCGTTTCA	TGAACCGGAA	GTATAAAGTG	AAATTGCCCA	GGCGCGCCAT	TTCGCCAGTG
781	TGCAAGTTAA	TATCCTCTAC	AACCTATAAC	CTGTAATTAT	CAATTAGTTA	CAAGTTATCA
841	CAGCACAATA	CCCCGGACGC	CTTTTAGCAA	ATCGTGGCAT	CGGCCAATTC	ATTTAGTTGA
901	CTTATACTTG	CCTGGGCAAT	AGTATCTGAC	GAAATTAATT	ACTTGCCGGG	GCAACCATTT
961	TGAAAAGCAC	CAGTGATCTG	TTCAATGAAA	TCATTCCGCT	GGGTCGCTTG	ATCTACATGG
1021	TAAATCAAAA	AAAAGATCGC	CTGTTAAATA	ACTATTTATC	CCCGCTGGAT	ATCACCGCAA
1081	CACAGTTTAA	AGTGCTTTGC	TCGATACGCT	GCGCGGGATG	TATTACCCCG	GTTGAACTTA
1141	AAAAAGTGCT	GTCTGTCGAT	CTCGGCGCAT	TGACGCGAAT	GCTCGACCGC	CTGCTGTGCA
1201	AAGGCTGGAT	CGAAAGACTG	CCGAATCCTA	ATGACAAACG	CGGCGTACTG	GTGAAGCTAA
1261	CGCCGGACGG	CGCGGCAATT	TGTGAGCAAT	GTCATCAACG	ACCAGGGCAA	GACCTGCATC
1321	AGGAATTAAC	AAAAAACTTA	ACGGCGGACG	AAGTGGCAAC	GCTTGAGTAT	TTGCTCAAGA
1381	AAATTCTGCC	GTAGCAAAAA	AGAGGTATGA	CGATGTCCAG	ACGCAACACT	GACGCTATTA
1441	CTATTCATAG	CATTTTGGAC	TGGATCGAGG	ATAACCTGGA	GTCGCCGCTC	TCACTGGAAA
1501	AAGTGTCTGA	GCGTTCAGGA	TATTCCAAAT	GGCACCTGCA	ACGGATGTTT	AAAAAAGAGA
1561	CCGGTCATTC	ATTAGGCCAA	TACATCCGCA	GCCGTAAAAT	GACGGAAATC	GCGCAAAAAT
1621	TAAAAGAGAG	CAACGAGCCC	ATTCTCTATC	TGGCGAACGC	TATGGCTTTG	AGTCACAGCA
1681	AACATTGACC	CGGACGTTCA	AAAACTATTT	TGATGTGCCG	CCACACAAAT	ACCGGATCAC
1741	CAATATGCAT	GGCGAATCAC	GGTATATGCT	GCCGCTGAAC	CATGGCAACT	ACTAGTTTGT
1801	TTATGCGCCA	CGCGAAGAGC	ACCATGAAAA	TGCTGTTTCC	CGCCCTGCCG	GGTCTGTTAC
1861	TTATCGCCTC	CGGATATGGC	ATTGCAGAAC	AAACTTTGTT	ACCTGTGGCG	CAAAATAGCC
1821	GCGATGTGAT	GCTGCTGCCC	TGTGTAGGCG	ATCCGCCAAA	TGACCTTCAC	CCCGTGAGCG
1981	TGAACAGCGA	TAAGTCAGAT	GAATTAGGCG	TGCCCTATTA	TAACGACCAA	CACCTTTAAC
2041	CTCTTTGCCC	CGTTTTGCGG	GGCATTTTTT	TAGTACCGTC	TGGCTATGAC	AAGTCCGCCG
2101	CTATTACCCC	GCGGCATGGT	CTGAAATTAG	CGTTTGTCTT	CTTAGTAACA	TAATGCCTCA
2161	TCCGCTGACA	CGCGCAGTAC	GGCGCACACG	AAATCCAAAC	ACATTGATAT	AGAGTCCAGC
2221	CATAATCAGT	ACTGCGCCCG	CAAGCTGCAT	CCCGGTCAAC	GTTTCCCCGA	GTAACACCGC
2281	GGCGCTCGCC	AGC				

appearing on the agarose gel in Figure 2 hybridized with the probe. Dot blot analysis was also performed on the chromosomal DNA of all 30 *Salmonella* species. Using the same probe, DNA from all 30 organisms hybridized.

IV. Minimum Inhibitory Concentration (MIC) Determination

The minimum inhibitory concentrations (MIC) of all 30 *Salmonella* species were determined prior to induction with tetracycline (Tet) or chloramphenicol (Chl). Most organisms demonstrated low levels of resistance to Tet, Chl, and Ampicillin (Amp) (Fig. 4). *S. agona* (#1), *S. anatum* (#2), *S. derby* (#7), *S. muenster* (#18), *S. reading* (#23), and *S. uganda* (#29) had innately high tetracycline resistance of 64 μg/ml.

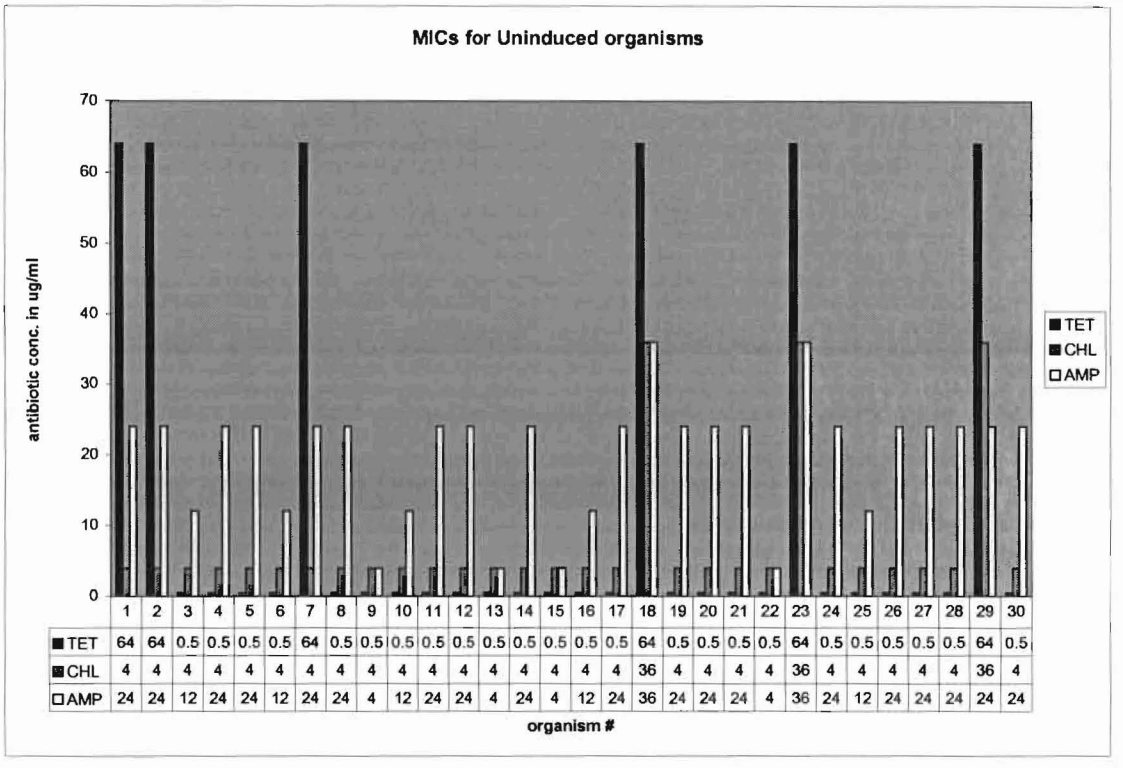
Following induction with tetracycline, *S. agona* (#1), *S. anatum* (#2), *S. derby* (#7), *S. muenster* (#18), *S. reading* (#23), and *S. uganda* (#29) did not increase their Tet resistance and remained at 64 μg/ml (Fig. 5). The majority of organisms however, increased their Tet resistance from 0.5 to 2 μg/ml. Additionally, most Tet induced organisms demonstrated an overall increased resistance to Chl and Amp. *S. anatum* (#2), *S. cholerasesuis* (#6), *S. derby* (#7), *S. gallinarium* (#9), and *S. give* (#10) did not change their level of resistance to Chl after Tet induction whereas *S. arizonae* (#3) and *S. newport* (#19) demonstrated a slight decrease in resistance. Following induction with Tet, the resistance of *S. anatum* (#2), *S. arizonae* (#3), *S. derby* (#7), *S. pullorum* (#22), and *S. uganda* (#29) to Amp decreased.

Twenty-two of the Chl induced organisms increased their Tet resistance from 0.5 μg/ml to 3 μg/ml or greater. Resistance of *S. agona* (#1), *S. anatum* (#2), *S. derby* (#7), *S. muenster* (#18) and *S. reading* (#23) to Tet remained at 64 μg/ml

(Fig. 6). *S. uganda* (#29) decreased its resistance to Tet from 64 μg/ml to 16 μg/ml following induction with Chl.

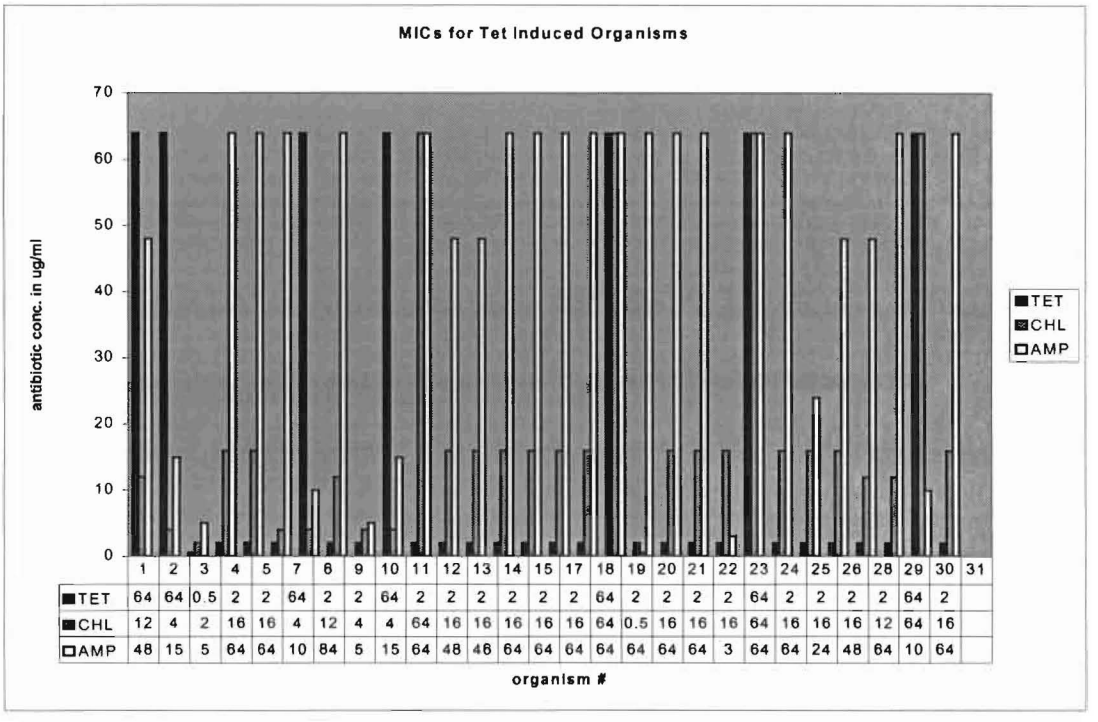
All organisms induced with Chl demonstrated an increased resistance to Chl. Specifically 27 organisms increased their resistance to Chl from 4 μg/ml to greater than 12 μg/ml. *S. arizonae* (#3) increased its resistance from 4 μg/ml to 6 μg/ml. *S. munester* (#18), *S. reading* (#23), and *S. uganda* (#29) increased their resistance to Chl from 36 μg/ml to 64 μg/ml. Following induction with Chl, all 30 organisms had an increase in resistance to Amp ranging from 36 μg/ml to 64 μg/ml.

Fig. 4. Minimum inhibitory concentrations (MIC) for the 30 uninduced *Salmonella* isolates. Antibiotics used were tetracycline, chloramphenicol and ampicillin. All 30 individual *Salmonella* isolates listed in Table 1 had some level of resistance to these antibiotics before induction.



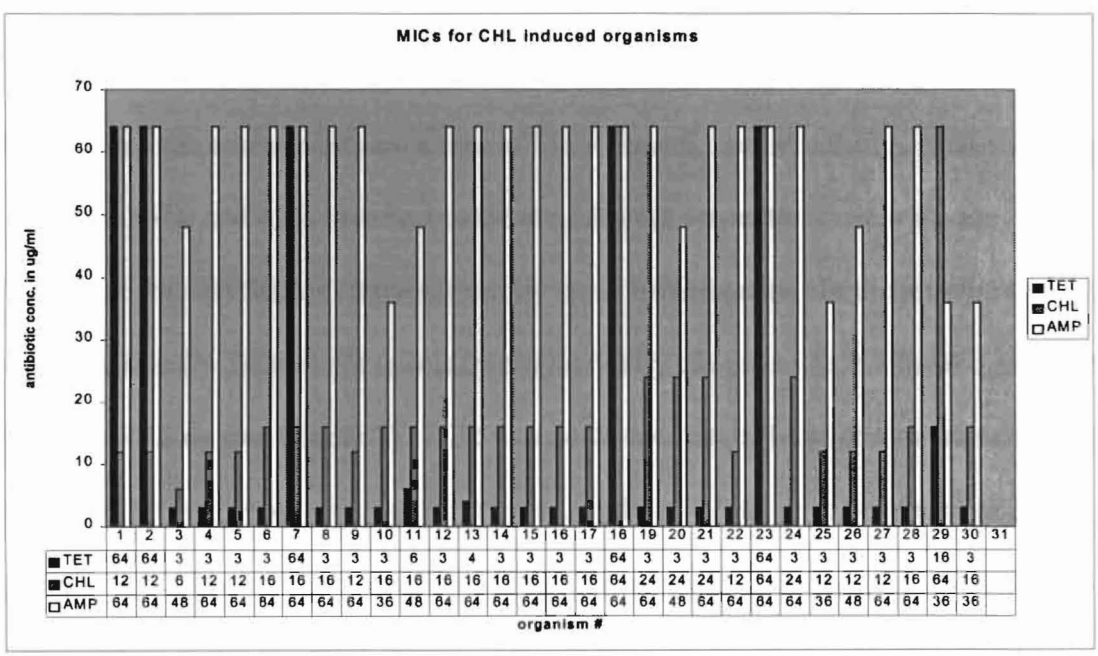
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
TET	64	64	0.5	0.5	0.5	0.5	64	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	64	0.5	0.5	0.5	0.5	64	0.5	0.5	0.5	0.5	0.5	64	0.5
CHL	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	36	4	4	4	4	36	4	4	4	4	4	36	4
AMP	24	24	12	24	24	12	24	24	4	12	24	24	4	24	4	12	24	36	24	24	24	4	36	24	12	24	24	24	24	24

Fig. 5. Minimum inhibitory concentrations (MIC) for tetracycline induced *Salmonella* isolates. MIC was performed using the antibiotics tetracycline, chloramphenicol, and ampicillin. The 30 individual *Salmonella* isolates are listed in Table 1.



	1	2	3	4	5	7	8	9	10	11	12	13	14	15	17	18	19	20	21	22	23	24	25	26	28	29	30	31
TET	64	64	0.5	2	2	64	2	2	64	2	2	2	2	2	2	64	2	2	2	2	64	2	2	2	2	64	2	
CHL	12	4	2	16	16	4	12	4	4	64	16	16	16	16	16	64	0.5	16	16	16	64	16	16	16	12	64	16	
AMP	48	15	5	64	64	10	84	5	15	64	48	46	64	64	64	64	64	64	64	3	64	64	24	48	64	10	64	

Fig. 6. Minimum inhibitory concentrations (MIC) for chloramphenicol induced *Salmonella* isolates. MIC was performed using the antibiotics tetracycline, chloramphenicol, and ampicillin. The 30 individual *Salmonella* isolates are listed in Table 1.



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
TET	64	64	3	3	3	3	64	3	3	3	6	3	4	3	3	3	3	64	3	3	3	3	64	3	3	3	3	3	16	3	
CHL	12	12	6	12	12	16	16	16	12	16	16	16	16	16	16	16	16	64	24	24	24	12	64	24	12	12	12	16	64	16	
AMP	64	64	48	64	64	64	64	64	64	36	48	64	64	64	64	64	64	64	64	48	64	64	64	64	36	48	64	64	36	36	

Discussion

Using the *E. coli marRAB* operon as a probe, Cohen *et al* (8) showed through dot blot analysis that this genetic locus is prevalent in the Enterobacteriaceae family. Genera that hybridized with the probe at an annealing temperature of 65^0C were *Salmonella*, *Klebsiella*, *Citrobacter*, *Hafnia*, and *Enterobacter*. Interestingly, they also reported within the genus *Salmonella*, *S. typhimurium* was positive for the *marRAB* operon while *S. arizonae* was negative. These data suggested this operon may not be conserved throughout *Salmonella*. Thus, these findings led to the basis of the current study to determine the prevalence of the *marRAB* operon in the genus *Salmonella*.

Sulavik *et al* (40) further characterized the *marRAB* operon in *S. typhimurium*. They showed the amino acid sequences of MarR, MarA, and MarB of *S. typhimurium* were 91%, 86%, and 42% identical to those of *E. coli*, respectively. However, *S. arizonae* was not further characterized by these investigators. In the present study, 30 clinical veterinary *Salmonella* species were randomly chosen to determine the prevalence of the *marRAB* operon (Table 1). PCR was performed on isolated chromosomal DNA from these 30 organisms using primers based on the *marRAB* gene sequence of *S. typhimurium*. These primers amplify a 2.2 kb region in the genome of *S. typhimurium* corresponding to the *marRAB* operon. After PCR, agarose gel electrophoresis was performed to visually identify amplification products (Fig. 2). Sixteen organisms had the predicted 2.2 kb band. To verify whether the 2.2 kb PCR product was indicative of the *marRAB* operon, the amplification product from *Salmonella enteritidis* (#8) was sequenced. Nucleotide sequence data revealed the genes *marC, marR, marA and marB* where greater than 99% identical to those of *S. typhimurium* (Fig 2). Some organisms

also amplified a 0.5 kb band (Fig. 2). To determine if this was the result of internal binding of the primers to the *marRAB* operon, PCR was performed on the cloned 2.2 kb *marRAB* operon. Primers based on the *marRAB* operon of *S. typhimurium* amplified both a 2.2 kb and a 0.5 kb fragment. These results suggested the approximately 0.5 kb amplification product was an internal fragment of *marRAB* .

Further confirmation was achieved by performing a Southern blot on an agarose gel containing the PCR products of all 30 *Salmonella* species (Fig. 2). The probe used was the sequenced 2.2 kb PCR product of *Salmonella enteritidis*. Southern blot analysis revealed that all bands appearing on the agarose gel hybridized with the probe. These data suggested that all additional amplification products resulting from PCR using *S. typhimurium marRAB* primers are a result of internal binding sites within the operon. The variation in banding patterns suggest sequence divergence at the primer binding sites. Thus, the lack of a 2.2 kb amplification product does not rule out the existence of the operon. Dot blot analysis was also performed on the chromosomal DNA of all 30 organisms at a stringency of both 55^0C and 60^0C using the *marRAB* operon from *S. enteritidis* as a probe. All 30 organisms hybridized with the probe at both temperatures. *S. arizonae* also hybridized with the gene probe which is contrary to prior a study (8) that suggested the *marRAB* operon is not found in *S. arizonae*. The negative *S. arizonae* results by Cohen *et al* (8) may in part be explained the use of an *E. coli marRAB* probe. PCR, Southern blot, and dot blot analysis generated in this study strongly suggest the *marRAB* operon is well conserved in the genera *Salmonella*.

Induction experiments were performed on the 30 organisms to determine if they have a functional *marRAB* operon. Prior to induction, the background minimum

inhibitory concentration (MIC) of all 30 organisms to tetracycline (Tet), chloramphenicol (Chl), and ampicillin (Amp) was determined. As predicted, the MIC demonstrated all 30 *Salmonella* isolates had some level of innate resistance to Tet, Chl, and Amp prior to induction. These three antibiotics were chosen because they are structurally and functionally unrelated. Prior research (8) has shown the *marRAB* operon is induced by subinhibitory concentrations of either Tet or Chl. This results in an organism that is resistant to several unrelated antibiotics (15,16).

In this study, most Tet induced organisms increased their resistance to Tet, Chl, and Amp. The exceptions were *S. agona* (# 1), *S. anatum* (#2), *S. derby* (#7), *S. muenster* (#18), *S. reading* (#23), and *S. uganda* (#29) which maintained a Tet resistance of 64 μg/ml (the highest concentration tested). *S. anatum* (# 2), *S. cholerasesius* (#6), *S. derby* (#7), *S. gallinarium* (#9), and *S. give* (#10) maintained the same level of resistance to Chl after Tet induction, whereas *S. arizonae* (#3) and *S. newport* (#19) had a slight decrease in resistance to Chl. The organisms *S. anatum* (#2), *S. arizonae* (#3), *S. derby* (#7), *S. pullorum* (#22), and *S. uganda* (#29) decreased their resistance to Amp following induction with Tet.

Most Chl induced organisms demonstrated increased resistance to Tet, Chl, and Amp. *S. agona* (#1), *S. anatum* (#2), *S. derby* (#7), *S. muenster* (#18), and *S. uganda* (#29) remained resistant to high levels of Tet (64 μg/ml). *S. uganda* (#29) was the only organism that decreased its resistance to Tet following induction with Chl. However, *S. uganda* (#29) did increase its level of resistance to Amp and Chl following induction with Chl.

Various reasons exist as to why some of the organisms did not increase or why some decreased their level of resistance to the three antibiotics following induction. It is probable that these organisms have other mechanisms involved in antibiotic influx which are not regulated by the *marRAB* operon. Alternatively, experimental error could account for some of the differences.

As previously discussed, *E. coli* has various multidrug resistant (Mdr) genes that confer resistance to either Tet, or Chl, or both. Following induction with these antibiotics, the organisms become more resistant to a wide array of structurally and functionally unrelated antibiotics. Genetically, *E. coli* is very similar to organisms comprising the genus *Salmonella*. This makes it highly probable that the 30 *Salmonella* species used in this study may have the same Mdr genes that are found in *E. coli*. Although this study suggests the *marRAB* operon is conserved in these 30 organisms, further analysis of this and other mechanisms of antibiotic resistance in these organisms needs to be examined.

LITERATURE CITED

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Prevalence of the Multiple Antibiotic Resistance Operon (*marRAB*) in the Genus *Salmonella*

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