BEAR
Bacterial Epidemiology and Antimicrobial Resistance

Susceptibility of *Salmonella* to biocides used in processing and its relationship to antimicrobial resistance

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Outline

1. Objectives of biocide project
2. Methodology of detecting biocide resistance
3. Data from on-going research
    1. MICs for Wild Type *Salmonella*
    2. Assay 195 strains for resistance
    3. Genome sequences of the 195 isolates
    4. Linkage of biocide resistance and AR

Objectives

1: Determine the susceptibility of wild type *Salmonella* to the biocides used in poultry processing

2: Identify isolates that have reduced susceptibility or resistance as well as isolates that are more susceptible to biocides

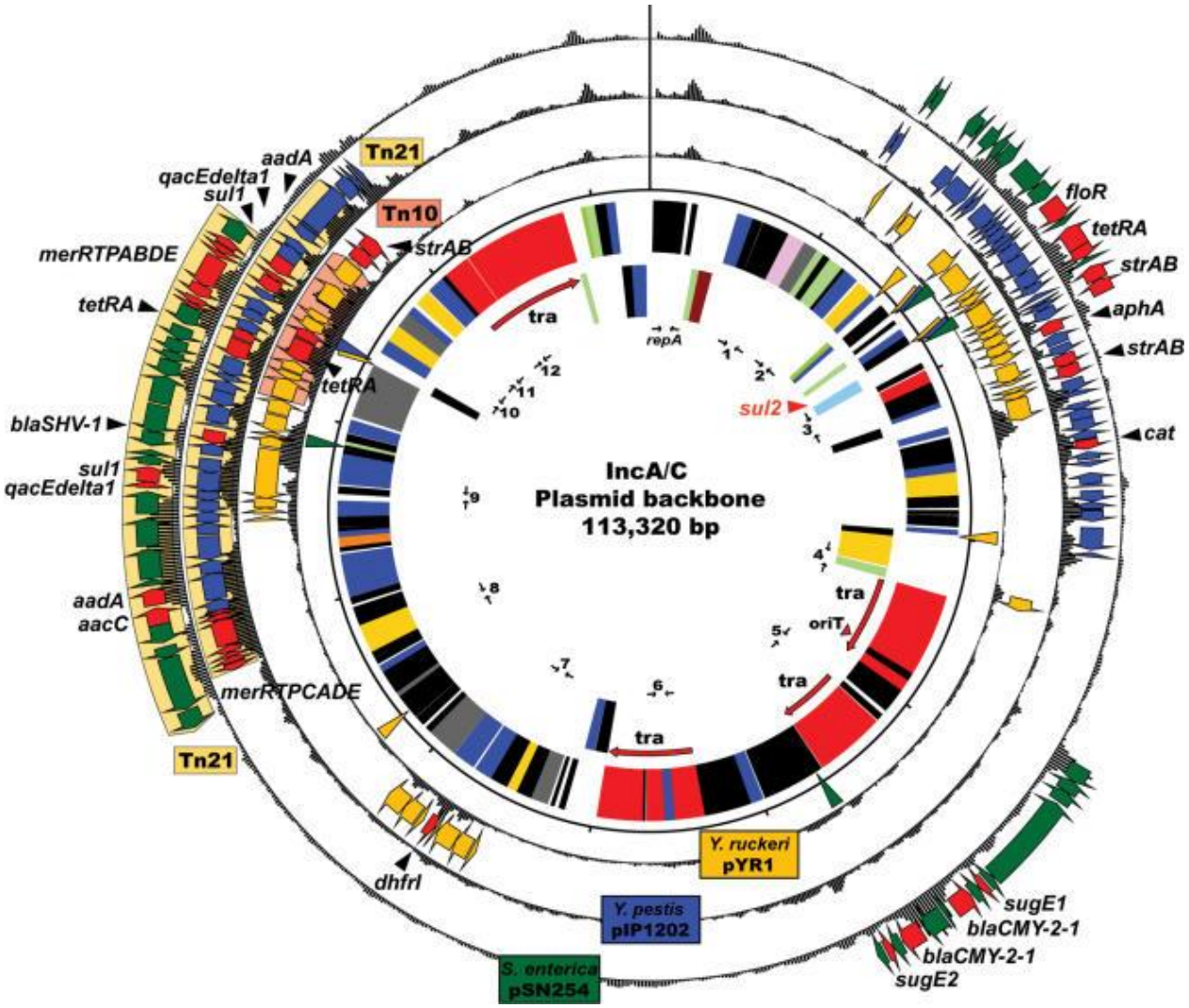
3: Identify any genetic mutations or elements associated with resistance to biocides

4: Determine any correlation or direct linkage of antimicrobial resistance to biocide resistance

Why are we concerned about biocides?

- Biocides are used to control and reduce the level of contamination with spoilage bacteria or pathogens like *Salmonella*
- Biocides and the maximum concentration at which they can be used in contact with meat is approved by FDA and FSIS
- Could using biocides lead to biocide resistant strains thus reducing their efficacy?
- Could biocide resistance be linked to AR?
- Could biocide use select for AR strains contaminating meat?

Mobile Genetic Elements and Salmonella



Welch TJ, Fricke WF, McDermott PF, et al. Multiple Antimicrobial Resistance in Plague: An Emerging Public Health Risk. Petrosino J, ed. PLoS ONE. 2007;2(3):e309. doi:10.1371/journal.pone.0000309.

Methodology

- Determine the susceptibility of wild type *Salmonella* to various biocides
- Develop an assay to detect isolates that have reduced susceptibility or resistance to biocides, and also detect isolates that are more sensitive to biocides
- Screen Salmonella isolates for resistance to biocides
- Investigate the genetics of resistance
- Determine if the biocide resistance genes are linked to antibiotic resistance genes

The Biocides

TABLE 2. Chemicals to be tested.

Chemical	Initial dilution range tested (µg/ml)	Range of MIC (µg/ml)	Average MIC[a] (µg/ml)
Dodecyltrimethylammonium chloride	32-1024	64-512	256
Cetylpyridinium chloride	10-320	20-160	80
Lactic acid	945-15,120	3780-7560	7560
Citric Acid	790-12,640	1580-12,640	3160
Benzalkonium chloride	5-160	10-40	40
Sodium Metasilicate	3160-240,160	6320-120,080	6,320
Arsenite	7-924	7-924	56
Chlorhexidine	0.5-188	8-24	2
Arsenate	26-6578	52-6578	416
1,3 dibromo, 5,5 dimethylal	114-5016	342-3654	1827
Acetic acid	410-6564	820-3280	1640
Sodium Hypochlorite_Bleach	8672-164,768	8672-138,752	138,752
Peroxyacetic acid	100-3500	400-1700	800
Trisodium phosphate	2370-75,840	9480-37,925	18,960
Hexadecyltrimethylammonium Bromide	10-330	40-160	80
Hexadecyltrimethylammonium Chloride	10-330	40-160	80
Acidified Sodium Chlorite	20-1280	80-1280	320

[a]Minimum inhibitory concentration

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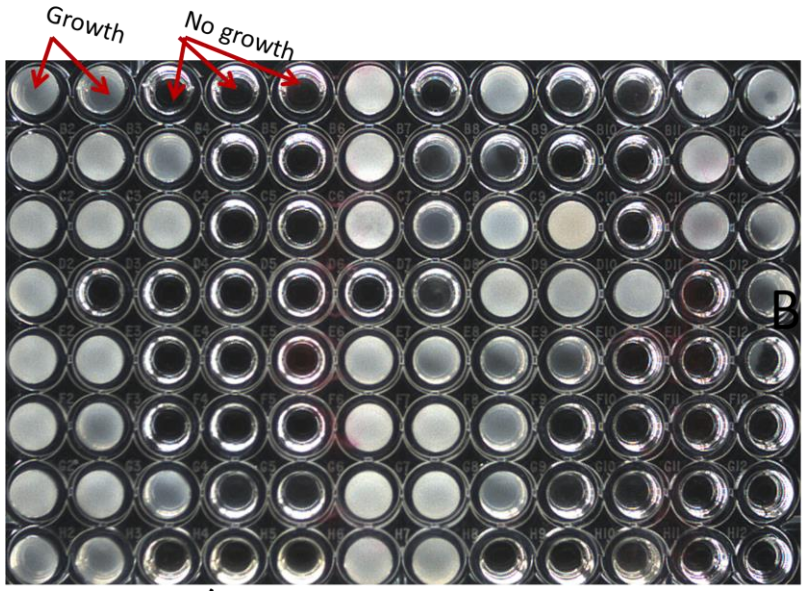
High throughput 96 well pate format of 2-fold broth microdilution assays for determining MICs to 17 biocides

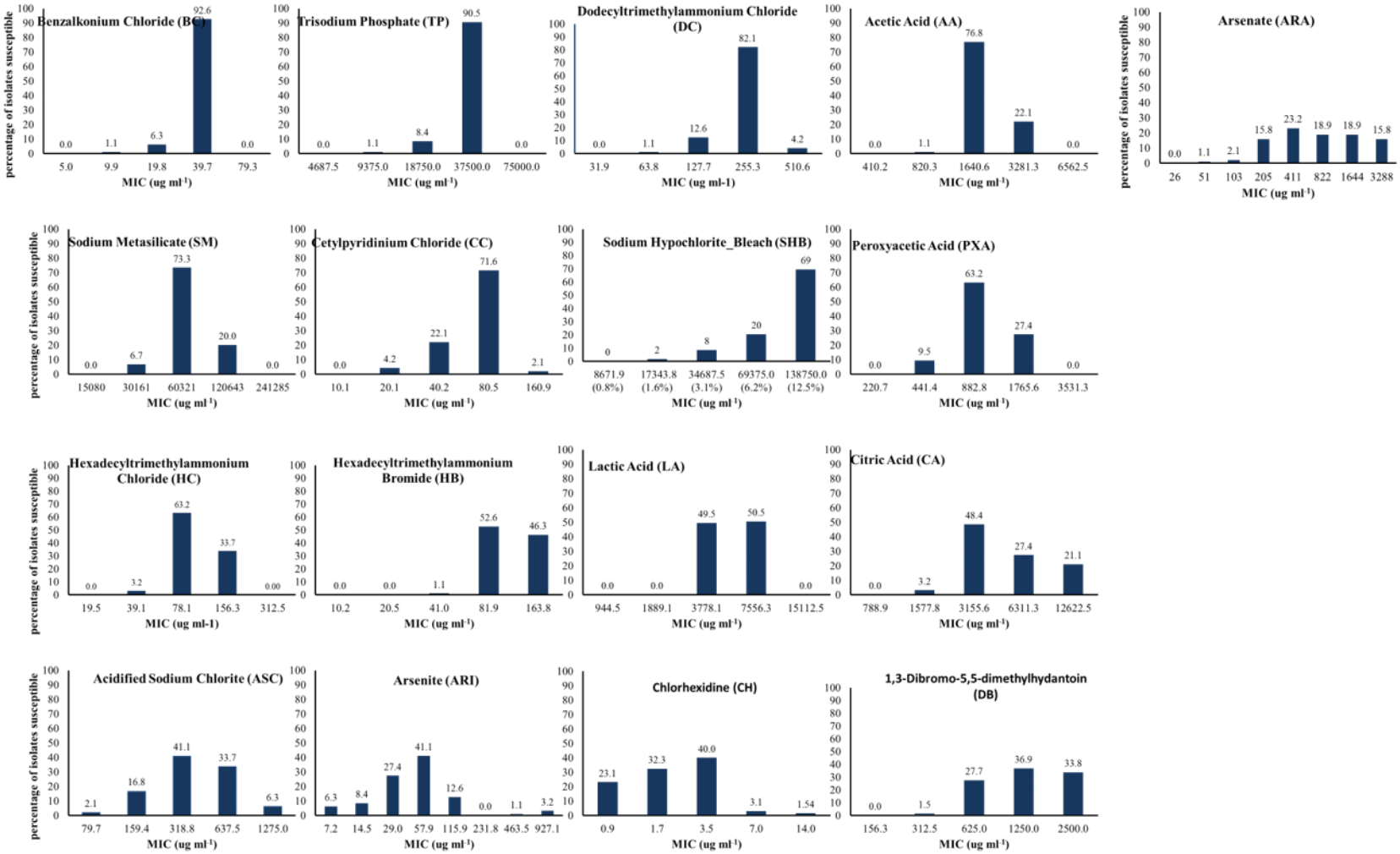


Growth
No growth

	1	2	3	4	5	6	7	8	9	10	11	12
A	DC 63.8	DC 127.7	DC 255.3	DC 510.6	DC 1021.3	CC 10.1	CC 20.1	CC 40.2	CC 80.5	CC 16.9	ARI 7.2	ARA 51.4
B	LA 944.5	LA 1889.1	LA 3778.1	LA 7556.3	LA 15112.5	CA 788.91	CA 1577.81	CA 3155.63	CA 6311.25	CA 12622.5	ARI 14.5	ARA 102.7
C	BC 4.96	BC 9.9	BC 19.8	BC 39.7	BC 79.3	SM 7540	SM 15080	SM 30161	SM 60321	SM 120643	ARI 29.0	ARA 205.5
D	CH 0.87	CH 1.75	CH 3.50	CH 6.99	CH 13.98	CH 27.97	CH 55.94	Blank	Blank	Blank	ARI 57.9	ARA 410.9
E	AA 410.2	AA 820.3	AA 1640.6	AA 3281.3	AA 6562.5	ASC 39.8	ASC 79.7	ASC 159.4	ASC 318.8	ASC 637.5	ARI 115.9	ARA 821.9
F	PXA 220.7	PXA 441.41	PXA 882.81	PXA 1765.63	PXA 3531.25	TP 4688	TP 9375	TP 18750	TP 37500	TP 75000	ARI 231.8	ARA 1643.8
G	HB 20.5	HB 41.0	HB 81.9	HB 163.8	HB 327.7	HC 19.5	HC 39.1	HC 78.1	HC 156.3	HC 312.5	ARI 463.5	ARA 3287.5
H	SHB 8671.9 (0.8%)	SHB 17343.8 (1.6%)	SHB 34687.5 (3.1%)	SHB 69375.0 (6.2%)	SHB 138750.0 (12.5%)	DB 156.25	DB 312.5	DB 625	DB 1250	DB 2500	ARI 927.1	ARA 6575

Acidified Sodium Chlorite (ASC), Acetic acid (AA), Arsenite (ARI), Arsenate (ARA), Benzalkonium chloride (BC), Cetylpyridinium chloride (CC), Citric Acid (CA), 1,3 dibromo, 5,5 Dimethylhydantoin (DBH), Dodecyltrimethylammonium chloride (DC), Hexadecyltrimethylammonium Bromide (HB), Hexadecyltrimethylammonium Chloride (HC), Lactic acid (LA), Peroxyacetic acid (PXA), Sodium Hypochlorite (SHB), Sodium Metasilicate (SM), Trisodium phosphate (TP), Chlorhexidine (CH).

Determine Median MIC for 100 Wild Type *Salmonella* to 17 biocides



Pilot study screening *Salmonella* isolates for resistance to biocides

- Select 195 *Salmonella* animal isolates
- Assay to identify isolates that have two 2-fold higher or lower MICs than median MIC of Wild Type *Salmonella*
- Sequence genomes and identify any mutations or genes that could be responsible for reduced susceptibility

Whole genome sequencing of first 195 *Salmonella*

- **Most from food animals and food production facilities**
- **Isolated 1998 – 2011 in U.S.**
- **74 serotypes identified by serum testing and MLST**
- **60% resistant to at least 1 antibiotic**
- **46% resistant to 3 or more antibiotics**

USDA United States Department of Agriculture

MIC of 195 *Salmonella* isolates

Isolates with greater than one 2-fold dilution higher than the median MIC were categorized as resistant

Biocides	Concentration tested (µg/ml)	Median MIC (µg/ml)	Isolates susceptible to median MIC (%)	Isolates susceptible to 1-fold higher than median MIC (%)	Isolates Resistant (%)
BC	10-160	40	100	0	0
ASC	159-2544	1272	100	0	0
LA	1889-30224	7556	100	0	
SM	7540-120640	60320	99	1	0
DC	64 - 1024	256	99	1	0
HB	41-656	82	93	7	0
DB	312-4992	1248	89	11	0
CC	20-320	40	79	21	0
CA	1579-25264	6316	72	28	0
AA	820-13120	1640	68	32	0
PXA	221-3536	884	61	39	0
TP	4744-75904	37952	98	1	1
HC	39-624	78	93	6	1
CH	0.2-56	3.5	65	34	1
SHB	13875-222000 (1.25%-20%)	55500 (5%)	51	34	15
ARI	15-1920	30	52	26	22
ARA	51-6258	408	64	14	22

Acidified Sodium Chlorite (ASC), Acetic acid (AA), Arsenite (ARI), Arsenate (ARA), Benzalkonium chloride (BC), Cetylpyridinium chloride (CC), Citric Acid (CA), 1,3 dibromo, 5,5 Dimethylhydantoin (DBH), Dodecyltrimethylammonium chloride (DC), Hexadecyltrimethylammonium Bromide (HB), Hexadecyltrimethylammonium Chloride (HC), Lactic acid (LA), Peroxyacetic acid (PXA), Sodium Hypochlorite (SHB), Sodium Metasilicate (SM), Trisodium phosphate (TP), Chlorhexidine (CH).

Median MIC of 4 Biocides (CH, SHB, ARI, and ARA) for Different Serotypes of *Salmonella*

Serotype	Median MIC (µg/ml)			
Biocides	CH	SHB	ARI	ARA
All 84 serotypes (195)	3.5	55500	30	408
Typhimurium (6)	3.5	111000	30	306
Newport (4)	3.5	111000	60	1632
Infantis (5)	3.5	55500	30	408
Dublin (4)	5.2	33300	30	306
Kentucky (7)	7.0	111000	60	816
Heidelberg (34)	3.5	27750	30	204
Enteritidis (16)	3.5	83250	30	408

Comparison of isolates resistant to ARA with antibiotic resistance pattern, source of origin and serotypes.

Isolates	Year	Origin	AR	ARA	ARI	Serotype
MH38582	2000	Chicken	0	>6528	960	Schwarzengrund
MH54710	2004	Ground beef	0	>6528	960	Orion var. Binza
MH41983	2007	Ground beef	0	6528	480	IIIb 61:-:1,5,7
MH23894	2007	Ground chicken	2	>6528	1920	Braenderup
MH37151	2007	Ground turkey	3	>6528	1920	Anatum
MF87944	2008	Ground veal	0	>6528	1920	Oranienburg
FL117658	2004	Horse	0	>6528	480	Saintpaul
MH72995	2009	Market hog swab	5	>6528	960	Havana
MH98631	2006	Market hog swab	2	>6528	960	Alachua
TN2515b	2004	Snake	0	>6528	960	IIIb 35:i:z35
IA8590	2004	Swine	2	>6528	1920	Cubana
IA2313	2005	Swine	10	>6528	960	Ohio
MH34024	2006	Turkey carcass swab	1	>6528	960	Orion var. Binza
MB00404	2002	Whole eggs	0	>6528	960	Alachua
MH31332	2008	Young chicken rinse	5	>6528	1920	Orion
MH92313	2004	Young chicken rinse	1	>6528	1920	Ouakam

Metal and biocide resistance genes predicted from WGS of 195 *Salmonella* isolates.

Genes	Family	Function	Compound	Genes in isolates (%)
*ars*A	*ars*A ATPase family	Maintenance of a low intracellular concentration of oxyanion produces resistance to the toxic agents	As, Sb	33
*bae*SR	Regulator	Two-component regulatory system	Cu, Zn, W, Sodium deoxycholate	100
*cor*A	*cor*A metal ion transporter (MIT) family	Mediates both influx and efflux of magnesium ions, cobalt and nickel uptake	Mg, Co, Ni, Mn	100
*cor*C	UPF0053 family. Contains 2 CBS domains	Transport magnesium and cobalt ions	Co, Mg	100
*cui*D	Multicopper oxidase (MCO) family	Copper homeostasis and the copper resistance response in the presence and in the absence of oxygen	Cu, H_2O_2	100
*ges*ABC	RND superfamily	Gold (Au^{3+}) resistance efflux pump, exports a variety of organic chemicals including chloramphenicol	Au, Methylene blue , Crystal violet, Chloramphenicol,	94
*gol*ST	Regulator/Cation transport ATPase	*gol* gene regulon	Cu, Au	93
*mgt*A	Cation transport ATPase (P-type) family	Mediates magnesium influx to the cytosol	Co, Mg	100
*mer*AB	Oxidoreductase family/ *mer*B family	Volatizing mercury as HgO	Hg, Phenylmercury acetate	15
*mod*ABCD	ABC superfamily,	Molybdenum import	W, Mo	100
*pco*ABCD	Multi-copper oxidase family (copA subfamily)	Required for the copper-inducible expression of copper resistance	Cu	22
*sil*PABCRS	*acr*B/ *acr*D / *acr*F family	Component of the sil cation-efflux system (*sil*ABC) that confers resistance to silver	Ag	20
*slt*ABCD	ABC superfamily	Iron/ Mn transport protein	Mn, Fe, H_2O_2	100
*sod*A	Iron/manganese superoxide dismutase family	Destroys superoxide anion radicals which are normally produced within the cells and which are toxic to biological systems.	Se, H_2O_2	100
*teh*AB	Membrane transporter	Over-expression of genes confer potassium tellurite resistance	Te, Proflavin	100
*ter*WZAB-CDE	*ter* family	Response to tellurium ion	Te	11
*ydg*g	Drug/metabolite transporter (DMT) superfamily	Probable efflux pump. Required for resistance to methyl viologen	Methyl viologen	100
*znu*ABC	ABC Superfamily/Membrane Transporter	Involved in the high-affinity zinc uptake transport system	Zn	100
*zra*SR	Regulatory system	Regulates high concentrations of zinc or lead	Zn, Pb	100
*zup*T	ZIP transporter family, *zup*T subfamily	Mediates zinc uptake and also transport other divalent cations	Zn, Fe, Co, Ni, Cu, Cd	100

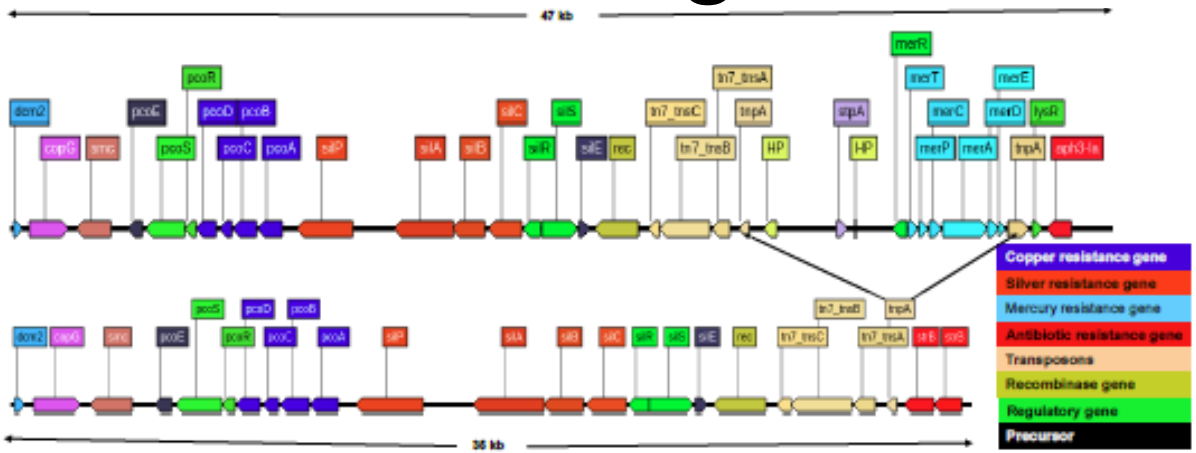
Antibiotic resistance genes predicted from WGS of 195 *Salmonella* enterica isolates

Antibiotics classes	Gene	Gene Variants	Phenotypes**	No. of Genes	Genes in No. of isolates
Aminoglycosides	*aac3*	*aac* (3)-IId, *aac* (3)-IVa, *aac* (3)-VIa,	GEN	36	35
	*aac6**	*aac* (6)-Iy, *aac* (6)-Ia	AMK	190	190
	aad	*aad*A, *aad*A2, *aad*A3, *aad*A7, *aad*A12, *aad*A22, *aad*B,	GEN, SPT, STR	60	59
	ant	*ant* (2`)Ia	GEN	1	1
	aph	*aph*A2, *aph*3-Ia, *aph*4-Ia	GEN	40	37
	sph	*sph*	STR	15	15
	str	*str* A, *str* B	GEN, SPT	131	67
Betalactamase	*carb*	carb-3	CAR	3	3
	cmy	*cmy*-58, *cmy*-59, *cmy*-60, *cmy*-90, *cmy*-94	TIO	44	44
	tem	*tem*1D, *tem*-147, *tem*-148, *tem*-150, *tem*-166, *tem*- 186	AMX, AMP	37	37
Chloramphenicol	*cat*	*cat*A1	CHL	4	4
	cml	*cml* A1, *cml* A5	CHL	11	11
	flo	*flo*R	CHL	27	27
Fosfomycin	*fos*	*fos* A	FOS	45	45
Macrolide	*ere*	*ere* A	ERY, AZM	2	2
	mef	*mef* B	ERY, AZM	1	1
	mph	*mph* A	ERY	2	2
Rifampicin	*arr*	*arr* 2	RIF	2	2
Sulfonamide	*sul*	*sul*-I, *sul*-II, *sul*-III	FIS, SXT	91	67
Tetracycline	*tet*	*tet* A, *tet* B, *tet* C, *tet* M, *tet* R	TET	163	96
Trimethoprim	*dfr*	*dfr* A1, *dfr* A14	SXT	8	8

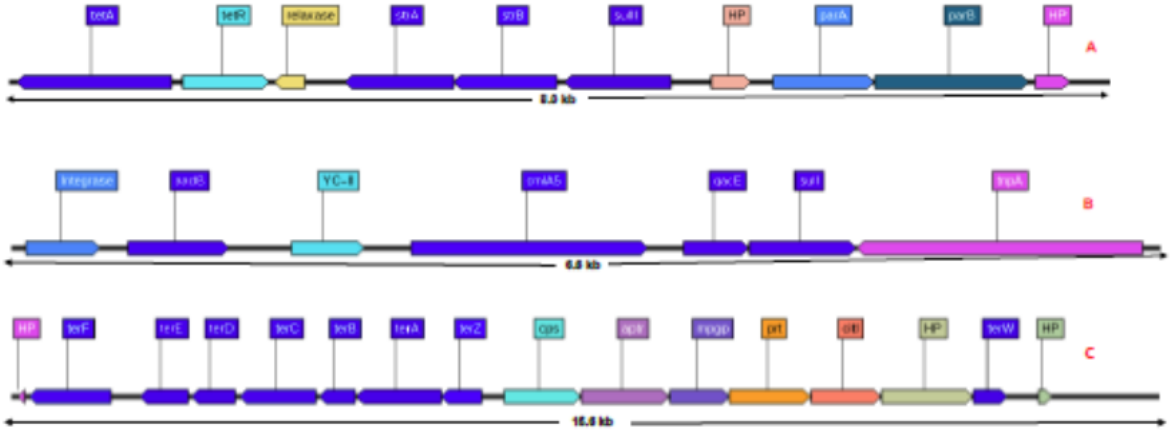
*gene *aac6* has genomic origin and these genes are cryptic in *Salmonella* sp.

** AMK: Amikacin, AMP: Ampicillin, AMX: Amoxicillin, AZM: Azithromycin, CAR: Carbenicillin, CHL: Chloramphenicol, ERY: Erythromycin, FIS: Sulfisoxazole, FOS: Fosfomycin, GEN: Gentamicin, RIF: Rifampin, SPT: Spectinomycin, STR: Streptomycin, SXT: Trimethoprim/Sulfamethoxazole, TET: Tetracycline, TIO: Ceftiofur

Maps of biocide, metal, and antimicrobial resistance genes



Biocide, metal, and antimicrobial resistance genes



Resistance gene organization in different *Salmonella* isolates, (A) most common gene organization, (B) chloramphenicol and quaternary ammonium compounds on same gene cassette, (C) tellurite resistance gene cassette. Resistance genes are blue

United States Department of Agriculture

Biocide assays for FSIS isolates

Chemicals tested								DC µg/ml	CC µg/ml	LA µg/ml	CA µg/ml	BC µg/ml	AA µg/ml	TP µg/ml	HB µg/ml	HC µg/ml	ARI µg/ml	ARA µg/ml	SM µg/ml	SHB µg/ml	PXA µg/ml	ASC µg/ml	DB µg/ml	CH µg/ml	SHB %
Median MIC for 100 Salmonella from our collection								256	80	3778	6311	40	1640	37500	82	78	58	822	60320	138750	883	318	1248	3.50	12.5
Isolates	Serotype	Original source	Isolation source	Substrate of isolation	Location	Date	Resistance Profile	MIC of Salmonella enterica isolates received from FSIS																	
FSIS 1400139	Newport	Animal-Calf	Animal-Calf	Veal Carcass Swab	USA:CA	2014	Aug Amp Fox Tio Axo Chl Str Fis Tet Cot	256	40	3778	6316	40	1640	37952	82	78	120	1648	120640	111000	884	636	1248	7.03	10
FSIS 1400142	Newport	Product-Raw-Ground, Comminuted or Otherwise Nonintact-Beef		Bovine	USA:			128	40	3778	6316	40	1640	37952	82	78	120	1648	60320	111000	884	636	1248	3.52	10
FSIS 1400129	Newport	Product-Raw-Ground, Comminuted or Otherwise Nonintact-Beef	Comminuted Beef	Bovine	USA:CA	2011		128	40	7556	6316	40	1640	37952	82	39	60	1648	60320	55500	884	636	1248	3.52	5
FSIS 1400125	Typhimurium var. O 5 - (Copenhagen)	Porcine		Swine Carcass Swab	USA:			128	80	7556	6316	40	1640	37952	82	78	30	3296	120640	55500	884	636	1248	1.76	5
FSIS 1400138	Newport	Animal-Calf	Animal-Calf	Veal Carcass Swab	USA:CA	2014	Aug Amp Fox Tio Axo Chl Str Fis Tet Cot	256	80	7556	6316	40	1640	37952	82	78	120	1648	120640	55500	884	636	1248	3.52	5
FSIS 1400122	Typhimurium	Bovine	Comminuted Beef	Ground Beef	USA:CA	2011		128	80	7556	6316	40	1640	37952	82	78	30	412	120640	111000	884	636	2496	3.52	10
FSIS 1400121	Typhimurium	Bovine	Comminuted Beef	Ground Beef	USA:CA	2011		128	40	7556	6316	40	1640	37952	82	78	15	206	60320	55500	884	2544	1248	1.76	5
FSIS 1400124	Typhimurium	Bovine	Comminuted Beef	Ground Beef	USA:SD	2012		128	80	7556	6316	40	1640	37952	82	78	15	206	60320	111000	884	636	1248	1.76	10
Average MIC								160	60	6612	6316	40	1640	37952	82	73	64	1339	90480	83250	884	875	1404	3.30	7.5
Median MIC of FSIS strains								128	60	7556	6316	40	1640	37952	82	78	45	1648	90480	83250	884	636	1248	3.52	7.5

Note: Highlighted in red ones where the MIC was more than one 2 fold dilution greater than that base line, and highlighted in blue ones where the MIC was more than one 2 fold dilution lower than the base line and Number in red indicate less than >

Future work

- Assay 1,000 isolates from the 65,000 NARMS animal isolate collection to determine level of biocide resistance and detect any linkage to AR
- Sequence isolates with biocide resistance to identify linkages between biocide and AR
- Expand into other bacteria including Escherichia coli and Enterococcus spp. as indicators of resistance in commensal bacteria
- Develop experiments that can confirm any selection of AR by biocides in processing plant model

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