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Detection of Multidrug-Resistant *Salmonella enterica* Serotype *typhimurium* DT104 Based on a Gene Which Confers Cross-Resistance to Florfenicol and Chloramphenicol

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Salmonella enterica* serotype *typhimurium* (*S. typhimurium*) DT104 (DT104) first emerged as a major pathogen in Europe and is characterized by its pentadrug-resistant pattern. It has also been associated with outbreaks in the United States. The organism typically carries resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline. The mechanism of chloramphenicol resistance in DT104 was determined by producing antibiotic-resistant *Escherichia coli* host strain clones from DT104 DNA. DNA from chloramphenicol-resistant clones was sequenced, and probes specific for the genes $flo_{S.\ typhimurium}$ (flo_{St}), *int*, *invA*, and *spvC* were produced for colony blot hybridizations. One hundred nine *Salmonella* isolates, including 44 multidrug-resistant DT104 isolates, were tested to evaluate the specificities of the probes. The gene flo_{St}, reported in this study, confers chloramphenicol and florfenicol resistance on *S. typhimurium* DT104. Florfenicol resistance is unique to *S. typhimurium* DT104 and multidrug-resistant *S. typhimurium* isolates with the same drug resistance profile among all isolates evaluated. Of 44 DT104 isolates tested, 98% were detected based on phenotypic florfenicol resistance and 100% had the flo_{St}-positive genotype. Resistances to florfenicol and chloramphenicol are conferred by the gene flo_{St}, described in this paper. Presumptive identification of *S. typhimurium* DT104 can be made rapidly based on the presence of the flo_{St} gene or its resulting phenotype.

Salmonella enterica serotype *typhimurium* (*S. typhimurium*) definitive type 104 (*S. typhimurium* DT104 or DT104) is an increasingly common multiple-antibiotic-resistant strain of *Salmonella* that has rapidly emerged as a world health problem (5, 21). The DT is based on phage typing of the organism. DT104 is characterized by chromosomal resistance to ampicillin (A), chloramphenicol (C), streptomycin (S), sulfonamides (Su), and tetracycline (T) and is commonly referred to as having resistance (R) type ACSSuT (20).

Antibiotic resistance is increasing among many bacterial species and is rapidly becoming a major world health problem (5, 16). The Centers for Disease Control and Prevention (Atlanta, Ga.) maintain several programs designed to monitor antimicrobial resistance. As part of the 1995 National *Salmonella* Antimicrobial Resistance Study, the Centers for Disease Control and Prevention serotyped 3,903 *Salmonella* isolates and determined that 976 (25%) were *S. typhimurium*. Approximately 28% (275 of 976) of the *S. typhimurium* isolates had the R type ACSSuT compared to just 7% in 1990 (7). Two other antibiotic resistance monitoring programs, the National Antimicrobial Resistance Monitoring System and Periodic Surveys of Antimicrobial Drug Resistance in Sentinel Counties, have reported similar sharp increases in multidrug-resistant *Salmonella* (5).

The mechanisms for resistance to sulfonamides, streptomycin, and ampicillin in DT104 have been described previously (15, 18). DT104 contains at least two integrons, one containing the aminoglycoside resistance gene cassette *ant(3″)-Ia*, which encodes resistance to streptomycin, and one containing a β-lactamase gene cassette, *pse-1*, which encodes resistance to ampicillin (15, 18). A gene coding for sulfonamide resistance (*sul-1*) was found in the 3′ conserved sequences of both integrons. The mechanisms for resistance to tetracycline and chloramphenicol have not been reported.

This study reports the discovery of a new gene called $flo_{S.\ typhimurium}$ (flo_{St}) and the use of flo_{St}-based gene probe to detect *S. typhimurium* DT104. Our objectives were to ascertain the mechanism of chloramphenicol resistance in DT104, to determine if DT104 is florfenicol resistant, and to discern if chloramphenicol and florfenicol resistances are linked. In addition, we assessed the utility of the florfenicol-resistant phenotype and the flo_{St} genotype as diagnostic tools for identification of *S. typhimurium* DT104. Finally, we examined DT104 isolates to determine the frequency with which the integrons *invA* and *spvC* occurred, and we sought to determine if a diagnostically useful relationship between these genotypes and DT104 exists.

MATERIALS AND METHODS

Lambda library construction and screening of clones. Standard recombinant DNA procedures were performed as described by Sambrook et al. (17). Chromosomal DNA from *S. typhimurium* DT104 cattle isolate 152N17 was prepared for construction of a lambda library by partial *Sau*IIIA (New England Biolabs, Beverly, Mass.) digestion. The partial digestion yielded DNA fragments ranging from 500 bp to 12 kbp. Digested DNA was extracted with phenol-chloroform and ethanol precipitated.

The *Sau*IIIA-digested DNA was ligated into Lambda Zap Express Vector (Stratagene, La Jolla, Calif.) digested with *Bam*HI according to instructions provided by the manufacturer. The library was then packaged into lambda phage (MaxPlax Packaging Extract; Epicentre Technologies, Madison, Wis.) and used to transduce the *Escherichia coli* XL1-Blue host strain. The vector was excised from *E. coli* XL1-Blue in the form of plasmid pBK-CMV and used to transform the *E. coli* XLOLR host strain according to the instructions provided in the Lambda Zap Express Vector kit. Plasmid preparations from colonies that grew

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TABLE 1. PCR primers

Name	Target	Sequence	Expected product size (bp)	Reference
Flo 1	flo_{St}	AATCACGGGCCACGCTGTATC	215	This study
Flo 2		CGCCGTCATTCTTCACCTTC		
Int 1	*int*	CCTCCCGCACGATGATC	280	10
Int 2		TCCACGCATCGTCAGGC		
Inv 1	*invA*	TTGTTACGGCTATTTTGACCA	521	19
Inv 2		CTGACTGCTACCTTGCTGATG		
Spv 1	*spvC*	CGGAAATACCATCTACAAATA	669	19
Spv 2		CCCAAACCCATACTTACTCTG		

on Luria-Bertani agar (Difco Laboratories, Detroit, Mich.) supplemented with chloramphenicol (25 μg/ml) were made. Purified plasmid DNA was used to transform competent Library Efficiency *E. coli* DH5αMCR (Gibco-BRL, Grand Island, N.Y.) according to the manufacturer's instructions. The transformed cells were screened for chloramphenicol resistance at a concentration of 25 μg/ml as described above, and plasmid was purified from chloramphenicol-resistant isolates. Plasmid pLB510 was determined to be chloramphenicol resistant and stable. Insert DNA from plasmid pLB510 was excised by digestion with *Eco*RI and *Sal*I, yielding a 3.3-kb fragment. DNA was also excised by digestion with *Eco*RI and *Pst*I, yielding a 2.1-kb fragment and a 1.2-kb fragment. Plasmid pLB510 was purified and insert DNA was sequenced by the dideoxy chain termination method at the University of Georgia Molecular Genetics Instrumentation Facility (Athens, Ga.) (MGIF).

Isolates for probe specificity and antibiotic resistance testing. One hundred nine *Salmonella* isolates were selected from the banked culture collection which is part of the National Antimicrobial Susceptibility Monitoring System (22). Isolates were obtained from cattle, swine, chickens, turkeys, carcass rinses and washes, swine and cattle feeds, exotics (lizards, snakes, and iguanas), dogs, and cats from both clinically ill and nonclinical animals. All isolates were serotyped and tested for resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides, tetracycline, and florfenicol. Testing, for all but florfenicol, was done with a semiautomated system (Sensititre; Accumed, Westlake, Ohio) according to the manufacturer's directions. A custom-designed microtiter plate panel with the minimal inhibitory concentration format was used for all isolates. All isolates were maintained at −70°C. Florfenicol resistance was determined by disk diffusion assay on Mueller-Hinton agar plates (Schering-Plough Animal Health, Kenilworth, N.J.) according to the manufacturer's instructions. Florfenicol and chloramphenicol MIC tests were conducted for selected *Salmonella* isolates by the microtiter broth dilution technique (8). *S. typhimurium* strains with R types characteristic of DT104 were phage typed. Serotyping and phage typing were conducted at the National Veterinary Services Laboratory, Ames, Iowa.

PCR probes and specificity for DT104. The *Salmonella* isolates described above were tested with probes designed to detect the 2.1-, 1.2-, and 3.3-kb fragments from pLB510, the chloramphenicol and florfenicol resistance gene (flo_{St}), the integrase gene (*int*), the *Salmonella* invasion gene (*invA*), and the *Salmonella* virulence plasmid gene (*spvC*). A single colony of each *Salmonella* isolate was prepared by detergent lysis on nylon filters according to standard methods (17). Hybridization probes were purified as restriction endonuclease DNA fragments from recombinant plasmid pLB510 and/or synthesized as PCR amplification products (Table 1). The PCR-derived integrase probe was produced from *E. coli* SK1592(pDU202) with Int 1 and Int 2 primers specific for the integrase gene (10). The PCR-derived flo_{St} probe was produced from *S. typhimurium* DT104 isolate 152N17. Primers were designed (with GeneRunner version 3.04 [Hastings Software Inc.]) from nucleotide sequence of the gene flo_{St} (Table 1). Primers were synthesized by the MGIF with an ABI model 394 DNA synthesizer. The flo_{St} PCR product was sequenced by dideoxy chain termination to ensure its identity (MGIF). PCR-derived probes for *invA* and *spvC* were produced from *S. typhimurium* SR11 (19). All probes were labeled with digoxigenin deoxynucleoside triphosphates by either PCR or random primer extension (DIG High Prime labeling and detection starter kit; Boehringer Mannheim, Indianapolis, Ind.). Hybridizations and washes were carried out at high stringency (0.1% sodium dodecyl sulfate and 0.1% SSC at 68°C) (17). Positive and negative controls were included for all hybridization colony blots. The positive controls were pLB510 and *S. typhimurium* DT104 152N17 for the flo_{St} blot, *E. coli* SK1592(pDU202) for the *int* blot, and *S. typhimurium* SR11 for the *invA* and *spvC* blots. The negative controls for all blots were *E. coli* XL1-Blue, *E. coli* XLOLR, and *E. coli* DH5α; *S. typhimurium* SR11 and *S. typhimurium* pACYC184 also served as negative controls for the flo_{St} and *int* blots.

Nucleotide sequence accession number. The GenBank accession number of the flo_{St} gene is AF097407.

RESULTS

The following results pertain to the gene flo_{St}, which confers florfenicol and chloramphenicol resistance on *S. typhimurium* DT104. Nearly all R type ACSSuT *S. typhimurium* DT104 isolates also have resistance to florfenicol (Table 2; also see Table 4). Of 44 multidrug-resistant DT104 isolates tested, 43 were resistant to florfenicol and 1 had intermediate resistance (Table 2). The MIC of florfenicol ranged from 37.5 to 150 μg/ml, while chloramphenicol MICs ranged from 100 to greater than 200 μg/ml for the DT104 isolates tested.

Plasmid pLB-510 contained a 3.3-kb fragment of DNA from *S. typhimurium* DT104 isolate 152N17. This fragment conferred chloramphenicol and florfenicol resistance (data not shown). Plasmid pLB510 was negative by both PCR and colony blot for the integrase gene (*int*). The 3.3-kb fragment was digested with *Pst*I and *Eco*RI to form 1.2- and 2.1-kb fragments. The 2.1-kb fragment was strongly associated with both chloramphenicol and florfenicol resistance; however, no correlation between the 1.2-kb fragment and chloramphenicol or florfenicol resistance was found. Of 64 chloramphenicol- and florfenicol-resistant isolates tested, all were positive by colony blot hybridization for the 2.1-kb fragment. However, of 42 isolates tested which were sensitive to chloramphenicol or florfenicol, 41 were negative by colony blot hybridization for the 2.1-kb fragment.

We sequenced the 2.1-kb fragment from the insert DNA of plasmid pLB510. We found within the fragment an open reading frame of 1,202 bp. We examined that DNA sequence for identity with other published gene sequences by using the BLAST search function located in the NCBI database. The

TABLE 2. Summary analysis of antibiotic resistance

Phenotype[a]	Total no. (%) positive				
	Multidrug-resistant DT104[b]	Antibiotic-sensitive DT104[c]	Multidrug-resistant *S. typhimurium* not DT104[d]	Chl[r] *Salmonella* not *typhimurium*	Chl[s] *S. typhimurium*
Chl[r]	44 (100)	0 (0)	18 (100)	11 (65)	0 (0)
Chl[i]	0 (0)	0 (0)	0 (0)	6 (35)	0 (0)
Chl[s]	0 (0)	5 (100)	0 (0)	0 (0)	25 (100)
Flo[s]	43 (98)	0 (0)	15 (83)	3 (18)	0 (0)
Flo[i]	1 (2)	0 (0)	0 (0)	5 (29)	0 (0)
Flo[s]	0 (0)	5 (100)	3 (17)	9 (53)	25 (100)
Total	44	5	18	17	25

[a] Chl, chloramphenicol; Flo, florfenicol. A superscript i indicates intermediate resistance.
[b] *S. typhimurium* DT104 isolates with R type ACSSuT, except for seven isolates with R type ACSuT.
[c] *S. typhimurium* DT104 isolates with no known antibiotic resistance.
[d] *S. typhimurium* isolates with R type ACSSuT or ACSuT phage typed as other than DT104.

TABLE 3. Summary analysis of colony blot hybridization results

Probe	No. (%) positive				
	Multidrug-resistant DT104[a]	Antibiotic-sensitive DT104[b]	Multidrug-resistant *S. typhimurium* not DT104[c]	Chl[r] *Salmonella* not *typhimurium*	Chl[s] *S. typhimurium*
flo_{St}	44 (100)	0 (0)	16 (89)	2 (12)	0 (0)
int	44 (100)	0 (0)	18 (100)	14 (82)	18 (72)
invA	43 (98)	4 (80)	18 (100)	17 (100)	25 (100)
spvC	44 (100)	4 (80)	11 (61)	3 (18)	22 (88)
Total	44	5	18	17	25

[a] *S. typhimurium* DT104 isolates with R type ACSSuT, except seven isolates with R type ACSuT.
[b] *S. typhimurium* DT104 isolates with no known antibiotic resistance.
[c] *S. typhimurium* isolates with R type ACSSuT or ACSuT phage typed as other than DT104.

sequence had the greatest identity with flo_{pp}, a 1,122-bp florfenicol resistance gene described in *Pasteurella piscicida* (14). There is 97% identity between the DNA sequences of the gene found in the 2.1-kb fragment and flo_{pp}. Deduced amino acid sequence homology between the two is 84%, but if insertions and deletions resulting in frame shifts are ignored, the identity between the two is 99%. Identity (57%) was also identified between the 1,202-bp gene and *cmlA* (1) which is a 1,549-bp gene that codes for nonenzymatic resistance to chloramphenicol. Sequence identity at the amino acid level was 67% between *cmlA* and the 1,202-bp gene from the 2.1-kb fragment. The gene *cmlA* has been observed as a gene cassette in numerous gram-negative organisms (1). Due to the high level of identity with flo_{pp} and the fact that this gene has been described in *S. typhimurium*, we suggest the nomenclature flo_{St} to describe the gene.

PCR and Southern hybridization of the 2.1-kb fragment support our previous conclusion that the 2.1-kb fragment contained a gene coding for resistance to chloramphenicol and florfenicol. The presence of flo_{St} in the 2.1-kb fragment was confirmed by Southern hybridizations with the flo_{St} PCR amplicand as a probe and by PCR analysis with the Flo 1 and Flo 2 primers (Table 1).

One hundred nine *Salmonella* isolates were tested. Sixty-two were flo_{St} positive, and 60 of these had the florfenicol-resistant phenotype while one had intermediate resistance (Table 3). All 62 flo_{St}-positive isolates were chloramphenicol resistant. All 62 flo_{St}-positive isolates were multidrug-resistant, and both DT104 ($n = 44$) and non-DT104 ($n = 18$) strains were identified within this group. Of 62 multidrug-resistant *S. typhimurium* isolates, 94% (58 of 62) had florfenicol resistance and 97% (60 of 62) tested positive for the flo_{St} gene (Table 4). All 44 confirmed DT104 isolates tested positive for the flo_{St} gene (Tables 3 and 4), and 43 were florfenicol resistant while 1 had intermediate resistance (Table 2). Only 6% (3 of 47) of other *Salmonella* isolates tested were florfenicol resistant, and only 4% (2 of 47) were flo_{St} positive, although 23% (11 of 47) of the other isolates tested were chloramphenicol resistant (Table 4).

The gene *invA* was found in 98% (107 of 109) of the total isolates tested (Table 3). However, *spvC* showed some specificity for *S. typhimurium*. Seventeen percent (3 of 18) of non-*typhimurium Salmonella* isolates were *spvC* positive, while 88% (81 of 92) of *S. typhimurium* isolates were *spvC* positive (Table 3). All multidrug-resistant *S. typhimurium* DT104 isolates tested positive for *spvC*. However, 88% (22 of 25) of the chloramphenicol-sensitive *S. typhimurium* isolates were also positive for *spvC*.

DISCUSSION

Florfenicol is a fluorinated analog of chloramphenicol approved by the FDA in 1996 for the treatment of bovine respiratory pathogens. Previous studies have shown that bacterial isolates which were resistant to chloramphenicol were sensitive to inhibition by fluorinated analogs (2, 3). Neither chloramphenicol acetyltransferase (*cat*) genes nor nonenzymatic chloramphenicol resistance (*cmlA*) genes provide resistance to florfenicol (1, 3, 11). *Salmonella* isolates with *cat* or *cml* genes mediating chloramphenicol-resistance also occur, and these isolates are not resistant to florfenicol. Conversely, flo_{St}-mediated resistance to chloramphenicol also confers resistance to florfenicol. Our research shows that flo_{St} has become a common genotype of chloramphenicol- and florfenicol-resistant *Salmonella*. Because the same gene, flo_{St}, confers resistance to both of these antibiotics on DT104, use of florfenicol may compromise use of chloramphenicol in treatment.

While numerous studies have reported on chloramphenicol resistance, only one previous study has described a florfenicol-resistant bacterium. This isolate was a multidrug-resistant isolate of the fish pathogen *P. piscicida* (9). Possibly due to the lack of previously published data in this area, the identification of 61 *Salmonella* isolates with a florfenicol-resistant phenotype suggests that florfenicol resistance is emerging in *Salmonella*. Interestingly, florfenicol-resistant *E. coli* isolates we have tested are also flo_{St} positive (unpublished information). The previous report (9) and this observation suggest that florfenicol resistance is likely to be described in other bacterial species. It is interesting to note that all DT104 isolates tested have been flo_{St} positive; this group includes isolates from cattle, swine, chickens, turkeys, dogs, horses, cats, etc., indicating that the use of florfenicol in cattle since 1996 may not be an important selective agent for this genotype.

Seven of the 44 multidrug-resistant DT104 isolates evaluated (Tables 2 and 3) were streptomycin sensitive. These *S. typhimurium* isolates were phage typed as DT104, indicating that

TABLE 4. Reliability of markers for detection of multidrug-resistant *S. typhimurium* or *S. typhimurium* DT104

Isolate group[a]	% Positive for:		
	Chloramphenicol resistance	Florfenicol resistance	flo_{St}-positive genotype
All *S. typhimurium* isolates with R types ACSSuTF or ACSuTF ($n = 62$)	100	94	97
Only multidrug-resistant *S. typhimurium* DT104 ($n = 44$)	100	98	100
All other *Salmonella* isolates tested ($n = 47$)	23	6	4

[a] F, florfenicol.

the R type ACSuT may also be characteristic of DT104. The streptomycin-resistant isolates were also florfenicol resistant, and they tested positive for the virulence determinants *invA* and *spvC*. No evidence suggests that streptomycin-sensitive DT104 is less virulent than other *S. typhimurium* DT104 isolates with R type ACSSuT.

The invasion gene operon, *invA*, is essential in *Salmonella* for full virulence; it is thought to trigger internalization required for invasion of deeper tissues (4). *Salmonella* virulence plasmid, *spvC*, is associated with an increased growth rate in host cells and interaction with the host immune system (6). Ninety-eight percent of the isolates tested were positive for *invA*. A similar result was reported in a study where 245 *Salmonella* isolates from poultry, wastewater, and human sources were all positive for *invA* (19). The same study found only 37 of the 245 (15%) *Salmonella* isolates positive for *spvC*; however none of the isolates were serotyped, which makes a comparison of these studies difficult. Interestingly, the high percentage (88%) of *S. typhimurium* isolates that were *spvC* positive in contrast to only 18% of non-*typhimurium Salmonella* isolates suggests that some relationship between *spvC* and *S. typhimurium* may be present. Further investigation is warranted to determine the extent of this relationship and the effect it may have on virulence.

Integrons capture and express mobile genes known as cassettes, which are, in most cases, antibiotic resistance genes (13, 14). The integrase gene is an essential part of all integrons; it encodes a site-specific recombinase that catalyzes the insertion of gene cassettes into the integron. The results shown in Table 3 indicate that integrons are common in *Salmonella*, as 94 of 109 (86%) isolates tested were positive for the integrase gene (*int*). However, sequence data from the 3′ flanking region of flo_{St} indicate that it is not contained within an integron; this agrees with the findings of Ridley and Threlfall (15). Integrons have been documented in other gram-negative organisms, such as *E. coli*, *Pseudomonas*, and *Shigella* (10, 12). Therefore, it is unlikely that integrons will serve as useful tools for identification of *Salmonella* DT104.

Phenotypic identification of *Salmonella* isolates based first on serotyping as *S. typhimurium* and secondly on phenotyping as florfenicol resistant gives a simple method of classification as presumptive positive for DT104. Genotypic identification by PCR may be even quicker, as an isolate containing flo_{St} and *spvC* would also be considered presumptive positive for DT104.

This study has described a gene, now called flo_{St} which confers resistance to both florfenicol and chloramphenicol. Additionally, rapid phenotypic and genotypic tests for presumptive identification of multidrug-resistant *S. typhimurium* DT104 have been described.

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