APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1988, p. 760–767
0099-2240/88/030760-08$02.00/0

Vol. 54, No. 3

Comparison of Virulence Factors and R Plasmids of *Salmonella* spp. Isolated from Healthy and Ill Swine

KAREN W. SIMMONS, RICHARD E. WOOLEY,* AND JOHN BROWN

Department of Medical Microbiology, College of Veterinary Medicine, University of Georgia, Athens, Georgia 30602

Received 14 September 1987/Accepted 19 December 1987

The antibiotic resistances and virulence profiles of *Salmonella* spp. isolated from healthy (group 1) and ill (group 2) swine were compared. Parameters studied included colicin and siderophore production; mannose-sensitive hemagglutination of erythrocytes; resistance to the lethal effect of serum complement; resistance to antibiotics; and the transmissibility of these characteristics to recipient organisms. Group 1 (19 isolates) had 14 serotypes, and group 2 (20 isolates) had 2 serotypes. Isolates from group 2 were resistant to more antibiotics and had a greater ability to hemagglutinate erythrocytes and transfer R plasmids to recipient organisms, but a lesser ability to produce siderophore than group 1. All 39 isolates resisted the lethal effects of serum complement. Colicin was produced by 1 of 19 from group 1 and 0 of 20 from group 2. A donor *Escherichia coli* isolated from a pig with enteritis transferred R plasmids to 62% of group 1 and 0% of group 2 *Salmonella* spp. when they were used as recipient organisms. A transconjugant from the mating of donor *E. coli* to a group 1 *Salmonella* spp. was further able to pass an R plasmid to recipient *E. coli* and salmonellae. Plasmid isolation from group 1 yielded 1 of 19 strains with a 56-megadalton plasmid, while 20 of 20 strains from group 2 contained three to five plasmids from 2.4 to 60 megadaltons in size.

Salmonellosis, a major disease of swine, is one of the most economically important of the enteric and septicemic diseases affecting young pigs up to 4 months of age. Traditionally, *Salmonella choleraesuis* has been considered not only the most common but also the most important of the salmonellae producing clinical disease in swine (28). Enteric salmonellosis can also be caused by *S. typhimurium*, and the organism has been reported to be an endemic cause of diarrheic disease in swine (28). While *S. choleraesuis* and *S. typhimurium* are most often associated with clinical illness in swine, other *Salmonella* species, including *S. heidelberg*, *S. anatum*, *S. dublin*, *S. derby*, and *S. enteritidis*, have been isolated (10, 21, 27).

Distinct from yet related to clinical illness caused by *Salmonella* spp. is the occurrence of salmonellae in asymptomatic swine destined for slaughter. A number of *Salmonella* serotypes have been isolated at slaughter from the mesenteric lymph nodes of apparently healthy swine (13). The public health implications of this *Salmonella* reservoir in swine are clear, since almost one-half of the serotypes were also isolated from human salmonellosis outbreaks (4). Therefore, *Salmonella* serotypes in swine are of concern not only because of their disease-causing potential for swine, but also because of their public health significance for humans.

Salmonella virulence factors may contribute to the establishment of disease in a host. These factors include adhesion pili (15), colicin (bacteriocin) production (9), siderophore (enterobactin) production (30), and the ability to resist the lethal effects of serum complement (19). Also of significance is the organism's ability to resist antimicrobial agents and its ability to pass this trait on to other bacteria via R plasmids.

While much information is available about serotypes isolated from healthy swine (27), specific virulence factors and transferable (R) plasmids from these isolates have not been characterized. Therefore, this study was undertaken to characterize the virulence factors and R plasmids of salmonellae isolated from healthy swine and compare them with *Salmonella* strains isolated from clinically ill pigs.

MATERIALS AND METHODS

Organisms. Nineteen *Salmonella* strains (group 1) were isolated over a 6-month period from 166 healthy swine that had passed anti- and postmortem inspections at a U.S. Department of Agriculture-inspected abattoir. Samples containing five to seven mesenteric lymph nodes were collected from the area of the large intestine of each carcass and frozen until processed for *Salmonella* isolation (13). Twenty *Salmonella* strains (group 2) isolated from clinically ill swine with enteritis were supplied by the Athens Diagnostic Assistance Laboratory. These isolates were also collected over a 6-month period from separate outbreaks of swine salmonellosis.

Mating recipients used in the study included *Escherichia coli* 1932 (29), *E. coli* LM-835 (Centers for Disease Control, Atlanta, Ga.), and *Salmonella typhimurium* 475 (Centers for Disease Control). Recipients were susceptible to the action of polymyxin B, chloramphenicol, streptomycin, kanamycin, tetracycline, sulfonamides, gentamicin, and ampicillin, but resistant to penicillin and nalidixic acid. *E. coli* 13515, a mating donor, was isolated from swine with enteritis and supplied by the Athens Diagnostic Assistance Laboratory. It was resistant to tetracycline, sulfonamides, streptomycin, polymyxin B, kanamycin, ampicillin, and gentamicin and susceptible to the action of chloramphenicol and nalidixic acid. *E. coli* V-517 was used as a source of reference plasmids (16).

Serological typing. *Salmonella* isolates were typed by Charles F. Smyser, Department of Veterinary and Animal Sciences, University of Massachusetts at Amherst, Amherst, Mass.

Media. Bacterial strains were maintained in brain-heart infusion broth (BHI) (Difco Laboratories, Detroit, Mich.). Bacteria were mated in PenAssay broth, pH 7.6 (antibiotic medium No. 3; Difco). Transconjugants from most bacterial matings were selected on MacConkey agar with donor- and

---

* Corresponding author.

recipient-inhibiting concentrations of antibiotics. Transconjugants from matings of *E. coli* 13515 (donor) to *Salmonella* isolates (recipients) were selected on plates containing brilliant green agar (Difco) (5.8 g/100 ml) and selenite broth powder (Difco) (2.3 g/100 ml) plus a recipient (*Salmonella*)-inhibiting concentration of antibiotic. This selective medium suppressed the growth of *E. coli* while allowing *Salmonella* transconjugants to grow as large brown colonies after 24 h of incubation at 37°C.

Antibiotic sensitivity tests. The disk diffusion method (3) was used to test for sensitivity for nalidixic acid, polymyxin B, chloramphenicol, streptomycin, kanamycin, tetracycline, sulfisoxazole, ampicillin, and gentamicin.

Colicin tests. Colicin production was tested by overlaying chloroform-killed colonies of the test organisms with a colicin-sensitive strain of *E. coli* K-12 (ATCC 23559). A colicin-producing control *E. coli* strain (ATCC 23558) was included on each plate (6).

HA tests. The ability of the test strains to hemagglutinate erythrocytes was tested in 96-well round-bottomed microtiter plates with a 3% suspension of guinea pig, chicken, and pig erythrocytes in saline solution. Combinations of 25 μl of culture, erythrocyte suspension, and either saline solution or 2% D-mannose were mixed and incubated at 25°C for 30 min before hemagglutination (HA) activity was monitored. Broth cultures (BHI) of the test organisms were incubated statically in air, with subcultures made every 48 to 72 h for six transfers before testing (12).

Bacterial matings and selection of transconjugants. R plasmids were transferred by mixing 0.2 ml of exponentially grown donor cells with 1.8 ml of an overnight culture of recipient cells in PennAssay broth. Mixtures were incubated at 25 and 37°C for 18 h (26). In trial 1, the 39 *Salmonella* isolates (donors) were mated with *E. coli* 1932, *E. coli* LM-835, and *S. typhimurium* 475. The resulting transconjugants were selected on MacConkey agar plates containing a donor-inhibitory concentration of nalidixic acid (30 μg/ml) and a recipient-inhibiting concentration of sulfisoxazole (500 μg/ml) or streptomycin (25 μg/ml), depending on the antibiotic profile of the donor organism. Samples from selector plates were picked and reidentified, and their antibiotic susceptibility patterns were determined. Frequencies of transconjugants were expressed relative to the number of donor cells in the mating mixtures (26).

In trial 2, matings of *E. coli* 13515 (donor) to 13 *Salmonella* isolates from healthy swine (group 1) and 10 from swine with enteritis (group 2) were handled in a similar manner except that the selector plates were made of brilliant green agar (5.8 g/100 ml) and selenite broth powder (2.3 g/100 ml) plus tetracycline (100 μg/ml) or gentamicin (10 μg/ml). After 18 h at 37°C, *Salmonella* transconjugants appeared as large brown colonies. Transductional passage of R plasmids was determined by inoculating recipient cells with cell-free broth cultures from the donor strains and processing as described above (26).

In trial 3, the transconjugant that resulted from the conjugation of *Salmonella* sp. strain 1-3 and *E. coli* 13515 (designated 1-3×13515) was mated to *E. coli* 1932, *E. coli* LM-835, and *S. typhimurium* 475 as described above. Selector plates were made of MacConkey agar containing nalidixic acid (30 μg/ml) plus gentamicin (10 μg/ml), tetracycline (100 μg/ml), or streptomycin (25 μg/ml). Mating mixtures were incubated at 25 and 37°C. Transconjugants of these matings and mating frequencies were determined as described above.

Plasmid isolation. Plasmid DNA from donor, recipient, and transconjugant cells was isolated and purified by the method of Birnboim and Doly (2) from overnight BHI cultures.

Agarose gel electrophoresis. Samples of 25 μl of plasmid DNA preparation were loaded into wells of a 0.75% agarose gel (MC Corp., Rockland, Maine) and run at 40 V for 10 h on a horizontal electrophoresis apparatus (model MPH; IBI, New Haven, Conn.) with a constant-voltage power source (model 452; E-C Apparatus Corp., St. Petersburg, Fla.). Cells were stained with ethidium bromide and visualized on a UV transilluminator (model TM 36; Ultra-Violet Products, Inc., San Gabriel, Calif.) (2). Photographs were taken with Polaroid type 55 (4 by 5) film (Polaroid Corp., Cambridge, Mass.) with a no. 23A Wratten gelatin filter (Eastman Kodak Co., Rochester, N.Y.) on a Polaroid MP4 Land camera.

Bacterial resistance to serum. Bacterial resistance to the lethal activity of serum (pig and rabbit) was determined by the rapid assay method of Moll et al. (17). The microtiter assay was done by inoculation of 100 μl of peptone-glucose broth with 100 μl of twofold serum dilutions and 25 μl of bacterial culture in log phase into 96-well microtiter plates. Plates were incubated for 3 h at 42°C and observed for color changes as an indication of bacterial growth.

Enterobactin (phenolate siderophore) assay. The bioassay for enterobactin was done in petri dishes (35 by 10 mm) containing 5 ml of low-iron agar with 30 μg of deferrated ethylenediamine-*N*,*N'*-diacetic acid (EDDA) per ml and *S. typhimurium* LT-2 *enb-7* (10^5 CFU/ml) as the indicator organism (5). The organisms used in the test included *S. typhimurium* LT-2 *enb-7*, a mutant unable to synthesize enterobacterin (ENT^-) (20); *E. coli* AN193, an ENT^- mutant unable to synthesize dihydroxybenzoic acid but able to utilize enterobactin produced by other organisms (negative control); and *E. coli* AN194, an ENT^+ strain (positive control). These three organisms were supplied by J. B. Neilands. The test organisms were passed five times in low-iron medium, and an overnight culture was concentrated by centrifugation (5,000 × *g*, 10 min) and filtration (0.45-μm pore size filter). The culure filtrate was concentrated 100 times by an evaporator-concentrator (Savant model SVC-100H; Savant Instruments Inc., Farmingdale, N.Y.). Sterile disks (Difco) were placed on the solidified agar plates and inoculated with 10 μl of the 100× culture filtrates of the test and control organisms and 100 mM 2,3-dihydroxybenzoic acid (positive control). Plates were incubated for 24 and 48 h at 37°C. Growth around the disks was recorded as − (no growth), + (small zone of growth), or ++ (wide zone of growth).

Biostatistics. Mating frequencies are essentially percentages. For example, a mating frequency of $10^{-2.2}$ means that there is a mating once in every 158.48 chances (1/158.49 = 0.006; 0.006 × 100 = 0.63%). These data were analyzed by a chi-square–one-way analysis of variance. If statistically significant, the percentages were transformed to arcsins, and the arcsins were compared by Tukey's test (31). Comparison of two percentages was done by using the *Z* test of proportions. Analysis concerned with the effect of temperature and recipient organism (two-factor analysis of variance), the mating frequencies were transformed by using the arcsin transformation prior to analysis. Subsequently, the Tukey test was used. Arcsin means were retransformed into the familiar logarithic form for tabular presentation.

RESULTS

Serotypes and antibiotic profiles. *Salmonella* spp. isolated from the lymph nodes of healthy swine (group 1) were

TABLE 1. Serotypes and antibiotic resistance[a] profiles of *Salmonella* isolates from healthy swine (group 1)

Isolate no.	Species	Antibiotic resistance profile
1-1	*S. derby*	Sulfisoxazole
1-2	*S. indiana*	Sulfisoxazole
1-3	*S. typhimurium*	Sulfisoxazole
1-4	*S. heidelberg*	Streptomycin, tetracycline, sulfisoxazole
1-5	*S. indiana*	Tetracycline, sulfisoxazole
1-6	*S. derby*	Sulfisoxazole
1-7	*S. manhattan*	Sulfisoxazole
1-8	*S. infantis*	Tetracycline, sulfisoxazole
1-9	*S. ohio*	Sulfisoxazole
1-10	*S. montevideo*	Sulfisoxazole
1-11	*S. infantis*	Sulfisoxazole
1-12	*S. muenchen*	Sulfisoxazole
1-13	*S. muenchen*	Sulfisoxazole
1-14	*S. kentucky*	Sulfisoxazole
1-15	*S. anatum*	Sulfisoxazole
1-16	*S. anatum*	Tetracycline, sulfisoxazole
1-17	*S. london*	Sulfisoxazole
1-18	*S. agona*	Sulfisoxazole
1-19	*S. worthington*	Tetracycline, sulfisoxazole

[a] Antimicrobial agents tested were ampicillin, chloramphenicol, gentamicin, kanamycin, nalidixic acid, polymyxin B, streptomycin, sulfisoxazole, and tetracycline.

serologically typed into 14 different serotypes. Antibiotic resistance patterns showed that all 19 isolates were resistant to sulfisoxazole, 4 were resistant to tetracycline and sulfisoxazole, and 1 was resistant to streptomycin, sulfisoxazole, and tetracycline (Table 1).

Salmonella spp. isolated from swine with enteritis (group 2) were serologically typed into *S. choleraesuis* subsp. *kunzendorf* (19 isolates) and *S. muenchen* (1 isolate). Twelve isolates were resistant to ampicillin, tetracycline, streptomycin, and sulfisoxazole; 4 isolates were resistant to streptomycin and sulfisoxazole; 3 isolates were resistant to ampicillin, streptomycin, and sulfisoxazole; and 1 isolate was resistant to ampicillin, streptomycin, sulfisoxazole, tetracycline, chloramphenicol, and kanamycin (Table 2).

Colicin, HA, and serum resistance tests. Colicin production was observed in one isolate (no. 1-11) from group 1 and none from group 2. Mannose-sensitive (MS) HA of erythrocytes was observed in 63.2% (12 of 19) of the isolates in group 1 (1, 3, 4, 8 to 10, 12 to 14, and 16 to 18). In group 2, 90% (18 of 20) were MS positive (isolates 7 and 16 were negative). The percentage of MS positives in group 2 (90%) was significantly larger ($Z = 1.99$, $P < 0.05$) than the percentage of positives in group 1 (63.2%). All *Salmonella* isolates from groups 1 and 2 were resistant to the lethal activity of pig and rabbit serum. Recipient *E. coli* strains 1932 and LM-835 were sensitive to the action of pig and rabbit serum. Transconjugants resulting from the matings of 1932 and LM-835 to the *Salmonella* donors were also sensitive to the action of both types of serum. Recipient *S. typhimurium* 475 was resistant to the action of serum; therefore, passage of serum resistance from donors to recipient 475 could not be determined.

Mating frequencies and antibiotic profiles of transconjugants. In trial 1, one isolate from group 1 (no. 1-4) mated with the recipient organisms. All isolates from group 2 mated with at least one of the three recipients. Results of these matings, showing mating frequencies and antibiotic resistance markers transferred, are shown in Table 3.

The data for the mating frequencies of group 2 were analyzed by a two-factor analysis of variance. Mating temperature (factor 1) was not significant ($F = 0.11$, $df = 1/114$) and recipient (factor 2) was highly significant ($F = 12.28$, $df = 2/114$, $P < 0.001$). The mean frequencies of mating of the recipients were compared by Tukey's test (Table 4). For each temperature, the *E. coli* strains had mean frequencies of mating not significantly different from each other but significantly lower than those of *S. typhimurium* 475.

The data on the number of antibiotic markers transferred to each recipient at each temperature were analyzed the same way. Neither temperature ($F = 0.12$, $df = 1/114$) nor recipient ($F = 0.57$, $df = 2/114$ was significant.

The percentage of transfer of the tetracycline marker at the different temperatures for each of the three recipients was analyzed by using a Z test of proportion. No significant differences were found. For recipient strains 1932, 835, and 475, the calculated Z values were 0.33 ($P = 0.74$), 0.64 ($P = 0.52$), and 1.01 ($P = 0.31$), respectively.

In trial 2, mating of donor *E. coli* 13515 to 13 isolates from group 1 and 10 from group 2 resulted in eight successful matings when group 1 acted as the recipient (strains 1-2, 1-3, 1-9, 1-10, 1-12, 1-15, 1-17, and 1-18) and no matings with group 2. All successful matings occurred at 25°C but not 37°C, and all involved transfer of the antibiotic markers for streptomycin, tetracycline, kanamycin, and gentamicin resistance. Donor *E. coli* 13515 was resistant to ampicillin, streptomycin, sulfisoxazole, tetracycline, polymyxin B, kanamycin, and gentamicin and susceptible to the action of nalidixic acid and chloramphenicol (Table 5).

An analysis of the mating frequencies of group 1 as recipients showed that significant ($F = 477.2$, $df = 7/\infty$, $P < 0.001$) differences existed between the serotypes (Table 5). A Tukey's test to locate these differences demonstrated a stepwise effect of significant differences. Only isolate 1-18 had a mating frequency significantly smaller than the other serotypes.

TABLE 2. Serotypes and antibiotic resistance[a] profiles of *Salmonella* isolates from ill swine (group 2)

Isolate no.	Species	Antibiotic resistance profile[b]
2-1	*S. choleraesuis* subsp. *kunzendorf*	A
2-2	*S. choleraesuis* subsp. *kunzendorf*	A
2-3	*S. choleraesuis* subsp. *kunzendorf*	A
2-4	*S. choleraesuis* subsp. *kunzendorf*	B
2-5	*S. choleraesuis* subsp. *kunzendorf*	A
2-6	*S. choleraesuis* subsp. *kunzendorf*	A
2-7	*S. choleraesuis* subsp. *kunzendorf*	A
2-8	*S. choleraesuis* subsp. *kunzendorf*	A
2-9	*S. choleraesuis* subsp. *kunzendorf*	A
2-10	*S. choleraesuis* subsp. *kunzendorf*	C
2-11	*S. choleraesuis* subsp. *kunzendorf*	C
2-12	*S. choleraesuis* subsp. *kunzendorf*	B
2-13	*S. choleraesuis* subsp. *kunzendorf*	A
2-14	*S. muenchen*	D
2-15	*S. choleraesuis* subsp. *kunzendorf*	C
2-16	*S. choleraesuis* subsp. *kunzendorf*	B
2-17	*S. choleraesuis* subsp. *kunzendorf*	A
2-18	*S. choleraesuis* subsp. *kunzendorf*	A
2-19	*S. choleraesuis* subsp. *kunzendorf*	B
2-20	*S. choleraesuis* subsp. *kunzendorf*	A

[a] See Table 1, footnote *a*.

[b] A, Ampicillin, streptomycin, sulfisoxazole, tetracycline; B, streptomycin, sulfisoxazole; C, ampicillin, streptomycin, sulfisoxazole; D, ampicillin, chloramphenicol, kanamycin, streptomycin, sulfisoxazole, tetracycline.

TABLE 3. Trial 1: mating frequencies and antibiotic resistance markers transferred from *Salmonella* isolates to recipient organisms

Donor strain	Recipient strain[a]	Mating frequency[b] ($\log_{10}$)		Antibiotic markers transferred[c]	
		25°C	37°C	25°C	37°C
1-4	1932	−4.6	—	A	
	835	−4.8	−5.9	A	A
	475	−4.7	−6.7	A	A
2-1	1932	−5.7	−7.3	B	C
	835	−3.9	−5.1	C	C
	475	−5.4	−5.9	B	B
2-2	1932	—	—		
	835	—	—		
	475	−6.4	−6.8	B	B
2-3	1932	−5.6	—	C	
	835	−3.3	−5.3	C	C
	475	−5.9	—	B	
2-4	1932	—	—		
	835	—	—		
	475	−5.8	−6.8	B	B
2-5	1932	−2.7	−2.4	C	C
	835	−4.7	−4.1	C	C
	475	−4.0	−4.1	B	B
2-6	1932	−5.5	−7.2	C	C
	835	−6.5	−6.0	C	C
	475	−4.9	−6.6	B	B
2-7	1932	−7.2	—	D	
	835	−7.0	—	D	
	475	−5.3	−6.8	C	B
2-8	1932	−6.4	−8.6	C	C
	835	−7.3	−6.6	C	C
	475	−7.3	−7.3	B	B
2-9	1932	—	−7.3		C
	835	−5.6	—	C	
	475	—	−5.8		B
2-10	1932	—	—		
	835	—	—		
	475	−5.9	−6.0	B	B
2-11	1932	−4.6	−4.2	B	E
	835	−3.9	−4.7	E	E
	475	−4.0	−2.9	B	B
2-12	1932	—	—		
	835	—	—		
	475	—	−8.3		B
2-13	1932	−1.2	−1.4	B	C
	835	−0.9	−2.2	C	C
	475	−0.8	−0.7	B	B
2-14	1932	—	—		
	835	—	—		
	475	−5.3	−5.9	B	B
2-15	1932	—	—		
	835	—	—		
	475	−6.5	−5.7	B	B
2-16	1932	—	—		
	835	—	—		
	475	−6.0	−7.3	B	B
2-17	1932	−3.5	−5.0	B	D
	835	−3.0	−4.3	C	D
	475	−3.8	−3.3	B	B
2-18	1932	−2.3	−3.7	F	E
	835	−3.9	−3.8	C	C
	475	−2.2	−3.0	B	B
2-19	1932	—	—		
	835	—	—		
	475	−5.1	−5.0	B	B
2-20	1932	—	—		
	835	—	—		
	475	−3.6	−4.7	B	B

[a] Recipients were *E. coli* 1932, *E. coli* LM-835, and recipient *S. typhimurium* 475.

[b] —, No mating occurred.

[c] Antibiotic resistance markers transferred: A, streptomycin, sulfisoxazole, tetracycline; B, streptomycin, sulfisoxazole; C, ampicillin, streptomycin, sulfisoxazole, tetracycline; D, ampicillin, streptomycin, tetracycline; E, ampicillin, streptomycin, sulfisoxazole; F, ampicillin, tetracycline.

In trial 3, mating of donor 1-3×13515 to *E. coli* 1932, *E. coli* LM-835, and *S. typhimurium* 475 resulted in successful matings at 25°C for *E. coli* 1932 and *S. typhimurium* 475 but not at 37°C. Successful matings of 1-3×13515 to *E. coli* LM-835 occurred at 25 and 37°C (Table 6). Antibiotic markers passed to recipients were resistance to tetracycline, streptomycin, kanamycin, and gentamicin via a 60-megadalton (MDa) plasmid.

Plasmid isolation. Recipient organisms contained no plasmids. Only isolate 1-4 from group 1 showed one 56-MDa plasmid. *Salmonella* strains from group 2 had three to five plasmids, ranging from 60 to 2.4 MDa in size. Donor *E. coli* 13515 had four plasmids from 60 to 2.0 MDa. Table 7 lists the donor antibiotic resistance profiles and their plasmids along with the R markers and plasmids transferred via conjugation to the recipient organisms. Figures 1 and 2 show plasmid screens of matings between salmonellae and recipients (Fig. 1) and *E. coli* 13515 and recipient salmonellae (Fig. 2).

Enterobactin. In group 1, 15 of 19 (78.9%) isolates produced enterobactin. Wide zones of growth occurred in 8 of 19 (42.1%) and small zones occurred in 7 of 19 (36.8%) of the

TABLE 4. Comparison[a] of the frequencies of transfer from *Salmonella* isolates from clinically sick swine to recipient organisms at 25 and 37°C in trial 1

Temperature	$\log_{10}$ mean frequency of mating		
	E. coli		*S. typhimurium* 475
	Strain 1932	Strain LM-835	
25°C	−2.235a	−2.5a	−4.41
37°C	−2.355a	−2.105a	−5.145

[a] Data were analyzed initially by a two-factor analysis of variance: temperatures ($F = 0.11$, df = 1/114, not significant), recipients ($F = 12.28$, df = 2/114, $P < 0.001$), and interaction ($F = 0.48$, df = 2/114, not significant). Means were compared by a Tukey test.

[b] Frequencies followed by the letter a are not significantly different at the 5% level of significance.

TABLE 5. Trial 2: mating frequencies[a] of *E. coli* 13515 with *Salmonella* isolates of group 1 at 25°C

Donor	Recipient	Mating frequency ($\log_{10}$)[b]
E. coli 13515	1-15	−2.2a
	1-3	−2.7ab
	1-17	−2.9abc
	1-9	−3.4abc
	1-2	−3.6bcd
	1-12	−3.6bcd
	1-10	−4.2cd
	1-18	−6.4d

[a] Data were initially analyzed by a chi-square–one-way analysis of variance ($F = 477.2$, df = 7/∞, $P < 0.001$). Proportions were compared by a Tukey test (5% level of significance).

[b] Antibiotic resistance markers transferred included streptomycin, kanamycin, tetracycline, and gentamicin. Frequencies followed by the same letter are not significantly different at the 5% level of probability.

filtrates. Four of 19 (21.1%) culture filtrates produced no detectable enterobactin. Group 1 filtrates that produced wide zones included 1-2, 1-3, 1-6, 1-8, 1-10, 1-17, 1-18, and 1-19. Those producing small zones included 1-1, 1-5, 1-7, 1-9, 1-13, 1-14, and 1-15. Group 1 filtrates that did not produce enterobactin included 1-4, 1-11, 1-12, and 1-16.

In group 2, 7 of 20 (35%) produced enterobactin, with 1 of 20 (5%) producing wide and 6 of 20 (30%) producing small zones. Thirteen of 20 filtrates (65%) did not produce detectable enterobactin. Group 2 filtrate 2-4 produced wide zones, and small zones were produced by 2-2, 2-3, 2-5, 2-10, 2-12, and 2-15. No enterobactin was detected from filtrates 2-1, 2-6, 2-7, 2-8, 2-9, 2-11, 2-13, 2-14, 2-16, 2-17, 2-18, 2-19, or 2-20.

A *Z* test of proportions comparing 78.9% with 35% established that these percentages were significantly different ($Z = 2.76$, $P = 0.006$).

DISCUSSION

Virulence factors and R plasmids of *Salmonella* spp. isolated from healthy (group 1) and ill (group 2) swine were studied to determine their potential public health significance. The parameters studied included serotypes of isolates; antibiotic resistance profiles; colicin and enterobactin production; ability to hemagglutinate erythrocytes; ability to resist serum complement; presence of plasmids; and the ability to transfer R plasmids to other recipient organisms.

Apparently healthy swine harbor a wide variety of *Salmonella* serotypes (13, 27). Of the 12 most common serotypes found in swine in the United States, 5 are among the 12 most common types in humans (4). These serotypes, which were isolated from group 1, included *S. heidelberg*, *S. typhimurium*, *S. infantis*, *S. agona*, and *S. montevideo*. The predominant serotype isolated from ill swine was *S. choleraesuis* subsp. *kunzendorf*, which is of low incidence in human salmonellosis (4).

Colicin was produced by one isolate from group 1 and none from group 2. This closely approximates previously reported figures of 2% (22) and 10% (9) production of colicin in *Salmonella* spp. Colicin production by *Salmonella* spp. in the intestinal tract results in suppression of resident flora and increased growth of *Salmonella* organisms (9). In systemic infections, colicins may confer on their host salmonellae a greater ability to survive in the blood, peritoneal fluid, and alimentary tract of the infected animal (7). In addition, colicinogeny may occur in association with drug resistance and is often cotransferable with drug resistance plasmids (9, 22).

All the *Salmonella* isolates in groups 1 and 2 were resistant to the lethal effects of pig serum, but the phenotype was not transferred to a serum-sensitive *E. coli* recipient. Serum is normally bactericidal for a wide range of both smooth and rough gram-negative bacteria by a system involving antibody, complement activation, and, possibly, other serum proteins (24). Some smooth strains, however, are insensitive to this system, and serum resistance may contribute to the pathogenicity of the enterobacterial strains. Surface components such as capsules, O antigens, peptidoglycan, proteins, and pili all play a role in increasing the virulence of bacteria. In addition, certain antibiotic resistance plasmids may also encode an outer membrane protein which interacts with other bacterial surface structures in a highly specific way to provide protection against serum (18, 24). The outer membrane protein has the potential not only to increase serum resistance, but also to increase phagocytosis resistance independently of the bacterial capsule (1).

MS HA of erythrocytes was observed in both group 1 (63.2%) and group 2 (90%) isolates. Approximately 80% of all *Salmonella* isolates exhibit MS activity; that is, they

TABLE 6. Trial 3: mating frequencies and antibiotic resistance markers transferred by the 1-3×13515 transconjugant conjugated with *E. coli* 1932, *E. coli* LM-835, and *S. typhimurium* 475

Donor[a]	Recipient	Selector plate	Temp of mating (°C)	Mating frequency[b] ($\log_{10}$)
1-3×13515	*E. coli* 1932	Nalidixic acid + streptomycin	—[c]	
		Nalidixic acid + tetracycline	25	−5.5
		Nalidixic acid + gentamicin	25	−4.8
	E. coli LM-835	Nalidixic acid + streptomycin	25	−4.4
		Nalidixic acid + tetracycline	25	−6.7
		Nalidixic acid + gentamicin	25	−4.2
			37	−6.7
	S. typhimurium 475	Nalidixic acid + streptomycin	25	−5.0
		Nalidixic acid + tetracycline	25	−4.9
		Nalidixic acid + gentamicin	25	−4.8

[a] Donor 1-3×13515 transconjugant antibiotic resistance profile: resistant to penicillin, gentamicin, streptomycin, kanamycin, sulfisoxazole, and tetracycline; susceptible to nalidixic acid, ampicillin, chloramphenicol, and polymyxin B.

[b] Successful matings passed streptomycin, sulfisoxazole, tetracycline, and gentamicin resistance to recipient organisms by a 60-MDa plasmid.

[c] —, No mating occurred.

TABLE 7. Antibiotic resistance and associated R plasmids transferred via conjugation between donors and recipient organisms

Donor			Recipient			
Isolate no.	Resistance profile	Plasmid(s) (MDa)	Strain no.	Mating temp (°C)	Resistance markers transferred[a]	Plasmid(s) transferred (MDa)
1-4	Streptomycin, sulfisoxazole, tetracycline	56.0	1932	25	A	56.0
			835	25 + 37	A	56.0
			475	25 + 37	A	56.0
2-11	Ampicillin, streptomycin, sulfisoxazole	30.0, 26.0, 12.0, 5.5, 2.4	1932	25	B	26.0, 12.0, 5.5, 2.4
			1932	37	C	30.0, 26.0, 12.0, 5.5, 2.4
			835	25 + 37	C	30.0, 26.0, 12.0, 5.5, 2.4
			475	25 + 37	B	12.0, 5.5, 2.4
2-16	Streptomycin, sulfisoxazole	32.0, 12.0, 5.5, 2.4	475	25	B	5.5, 2.4
2-17	Ampicillin, streptomycin, sulfisoxazole, tetracycline	40.0, 32.0, 26.0, 12.0, 5.5, 2.4	1932	25	B	12.0, 5.5, 2.4
			1932	37	C	40.0, 26.0
			835	25	D	40.0, 26.0, 12.0, 5.5, 2.4
			475	25	B	12.0, 5.5, 2.4
E. coli 13515	Ampicillin, streptomycin, sulfisoxazole, tetracycline, polymyxin B, kanamycin, gentamicin	60.0, 37.0, 3.4, 2.0	1-2	25	E	60.0
			1-3	25	E	60.0
			1-9	25	E	60.0
			1-10	25	E	60.0
			1-12	25	E	60.0
			1-15	25	E	60.0
			1-17	25	E	60.0
			1-18	25	E	60.0

[a] Antibiotic resistance markers transferred: A, streptomycin, sulfisoxazole, and tetracycline; B, streptomycin and sulfisoxazole; C, ampicillin, streptomycin, and sulfisoxazole; D, ampicillin, streptomycin, sulfisoxazole, and tetracycline; E, streptomycin, tetracycline, kanamycin, and gentamicin.

possess type 1 pili. Since mannose is ubiquitous in mammalian cell membranes, MS adhesion to mammalian host cells by type 1-piliated bacteria is widespread. Although many *Salmonella* isolates produce type 1 pili, their role in intestinal colonization is subject to debate. It has been proposed that type 1 pili mediate the MS adherence of *S. typhimurium* to intestine and to both human buccal and rat urinary tract epithelial cells and therefore play a significant role in intestinal colonization and in urinary tract infections (6, 14). However, the definitive physiological functions of the type 1 pili in *Salmonella* spp. are still unknown.

Enterobactin production occurred in a significantly greater

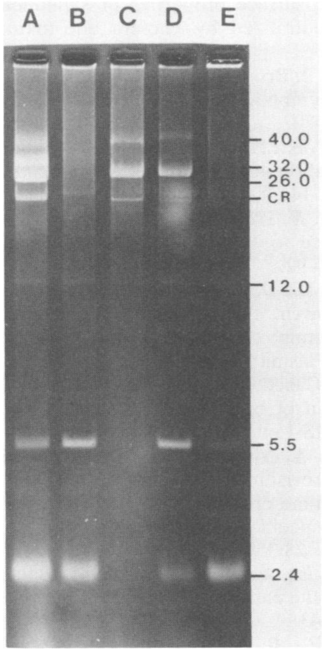

FIG. 1. Plasmid screen of *Salmonella* sp. strain 2-17 (A) and transconjugants resulting from conjugation with *E. coli* 1932 at 25°C (B), *E. coli* 1932 at 37°C (C), *E. coli* LM-835 at 25°C (D), and *S. typhimurium* 475 at 25°C (E). Sizes are indicated in megadaltons. CR, Chromosomal.

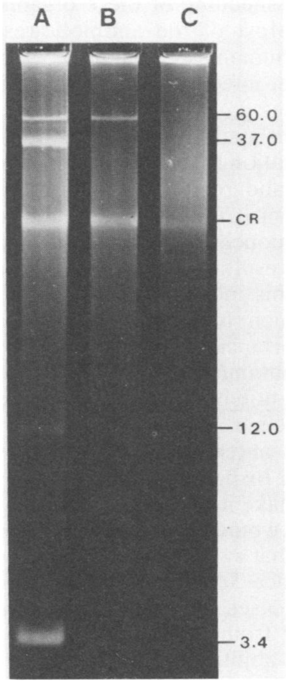

FIG. 2. Plasmid screen of *E. coli* 13515 donor (A), recipient Salmonella sp. strain 1-3 (C), and transconjugant 1-3×13515 (B). Sizes are indicated in megadaltons. CR, Chromosomal.

number of group 1 isolates (78.9%) than group 2 isolates (35%). Enterobactin production by *Salmonella* spp. has been shown to be related to virulence in mice (30).

Plasmids were observed in a greater number of isolates from group 2 than those from group 1. In mating studies, the *Salmonella* isolates from group 2 acted as donors of R plasmids to other enteric bacteria at a greater rate than those from group 1. From 51 to 85% of *Salmonella* species isolated from swine may contain conjugative R plasmids, and they most commonly encode resistance to tetracycline, streptomycin, and sulfonamides (11, 25). In this study, the most common R plasmid in the *Salmonella* isolates encoded resistance to streptomycin and sulfonamides by a low-molecular-weight plasmid. In further studies, the isolates in group 1 acted as recipient bacteria to an *E. coli* swine isolate at a greater frequency than those from group 2. A transconjugant resulting from the mating of the *E. coli* swine isolate with a group 1 *Salmonella* isolate was able to pass R plasmids to both *E. coli* and *Salmonella* recipient bacteria.

Enteropathogenic *E. coli* strains routinely harbor many distinct virulence plasmids that may encode a variety of phenotypes, such as enterotoxin production, colonization antigens, colicin synthesis, and hemolysin production (7). The genes involved are usually found on separate plasmids, but several large conjugative plasmids encoding both toxin production and antibiotic resistance have been reported and probably arose by recombination between an enterotoxin plasmid (ENT) and an R plasmid or by transpositional events (7). The occurrence of enterotoxin-drug resistance plasmids may be important in increasing the number of enterotoxigenic *E. coli* in environments where antibiotics are used as feed additives or in therapy.

One-half of all antibiotics produced in the United States are fed to food-producing animals in subtherapeutic doses as a feed supplement (8). The use of these antibiotics disrupts the normal flora of the intestine, resulting in an increase in and emergence of antibiotic-resistant *Salmonella* strains and prolonged fecal shedding of these organisms into the environment (23). Most of the antibiotic-resistant *Salmonella* spp. that infect humans are of animal origin (23). The fatality rate for people infected with drug-resistant *Salmonella* strains is 21 times greater than for individuals infected with non-antibiotic-resistant *Salmonella* strains (23).

The demonstration that *E. coli* and *Salmonella* isolates act as both donor and recipient organisms in the transfer of antibiotic resistance is of public health importance. Large numbers of salmonellae are required to produce food poisoning, usually resulting from the growth of the organism in food. In turn, this indicates that the environmental conditions under which food is processed and stored are of paramount importance. Temperatures which permit the multiplication of contaminating salmonellae would also permit the transfer of antibiotic resistance between and among *E. coli* and salmonellae.

The rigorous antemortem inspection standards of the Food Safety and Inspection Service of the U.S. Department of Agriculture make it unlikely that swine with clinical signs of salmonellosis would enter the human food chain (13). This would indicate that swine without clinical signs of salmonellosis can introduce *Salmonella* strains into the human food chain. Human cases of salmonellosis are caused by serotypes isolated from healthy swine. These are serotypes whose virulence abilities are expressed by MS HA of erythrocytes, resistance to complement, enterobactin production, and lastly the ability to act as both donor and recipient in the transfer of antibiotic resistance.

ACKNOWLEDGMENT

This work was supported by the Veterinary Medical Experiment Station, College of Veterinary Medicine, The University of Georgia.

LITERATURE CITED

1. **Aguero, M. E., L. Aron, A. G. DeLuca, K. N. Timmis, and F. C. Cabello.** 1984. A plasmid-encoded outer membrane protein, *traT*, enhances resistance of *Escherichia coli* to phagocytosis. Infect. Immun. **46:**740–746.
2. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513–1523.
3. **Carter, G. R.** 1973. Diagnostic procedures in veterinary microbiology, 2nd ed. Charles C. Thomas, Springfield, Ill.
4. **Centers for Disease Control.** 1982. Salmonella surveillance annual summary, 1980. Centers for Disease Control, Atlanta, Ga.
5. **Davis, B. D., and E. S. Mingioli.** 1980. Mutants of *Escherichia coli* requiring methionine or vitamin B_{12}. J. Bacteriol. **60:**17–28.
6. **Duguid, J. P., E. S. Anderson, and I. Campbell.** 1966. Fimbriae and adhesive properties in salmonellae. J. Pathol. Bacteriol. **92:**107–138.
7. **Elwell, L. P., and P. L. Shipley.** 1980. Plasmid-mediated factors associated with virulence of bacteria to animals. Annu. Rev. Microbiol. **34:**465–496.
8. **Food and Drug Administration Task Force on the Use of Antibiotics in Animal Feeds.** 1972. Appendices on animal and human health hazards. *In* Report to the Commissioner of the Food and Drug Administration by the Task Force on the Use of Antibiotics in Animal Feeds. Publication no. 72-6009. Food and Drug Administration, Rockville, Md.
9. **Fredericq, P.** 1957. Colicins. Annu. Rev. Microbiol. **11:**7–22.
10. **Ikeda, J. S., D. C. Hirsh, S. S. Jang, and E. L. Biberstein.** 1986. Characteristics of *Salmonella* isolated from animals at a veterinary medical teaching hospital. Am. J. Vet. Res. **47:**232–235.
11. **Ishiguro, N., J. Goto, and G. Sato.** 1980. Genetic relationship between R plasmids derived from *Salmonella* and *Escherichia coli* obtained from a pig farm, and its epidemiological significance. J. Hyg. (Cambridge) **84:**365–379.
12. **Jiwa, S. F. H., and I. Mansson.** 1983. Hemagglutinating and hydrophobic surface properties of salmonellae producing enterotoxin neutralized by cholera anti-toxin. Vet. Microbiol. **8:**443–458.
13. **Keteran, K., J. Brown, and E. B. Shotts.** 1982. *Salmonella* in the mesenteric lymph nodes of healthy sows and hogs. Am. J. Vet. Res. **43:**706–707.
14. **Koshonen, T. K., K. Lounatmaa, H. Ranta, and N. Kuusi.** 1980. Characterization of type 1 pili of *Salmonella typhimurium* LT2. J. Bacteriol. **144:**800–805.
15. **Lindberg, A. A.** 1980. Bacterial virulence factors—with particular reference to *Salmonella* bacteria. Scand. J. Infect. Dis. Suppl. **24:**86–92.
16. **Macrina, F. L., D. J. Kopecko, K. R. Jones, D. J. Ayers, and S. M. McCowen.** 1978. A multiple plasmid-containing *Escherichia coli* strain: convenient source of size reference plasmid molecules. Plasmid **1:**417–420.
17. **Moll, A., F. Cabello, and K. N. Timmis.** 1979. Rapid assay of the determination of bacterial resistance to the lethal activity of serum. FEMS Lett. **6:**273–276.
18. **Moll, A., P. A. Manning, and K. N. Timmis.** 1980. Plasmid-determined resistance to serum bactericidal activity; a major outer membrane protein, the *traT* gene product, is responsible for plasmid-specified serum resistance in *Escherichia coli*. Infect. Immun. **28:**359–367.
19. **Peterson, P. K., and P. G. Quie.** 1981. Bacterial surface components and the pathogenesis of infectious diseases. Annu. Rev. Med. **32:**29–43.
20. **Pollack, J. R., B. N. Ames, and J. B. Neilands.** 1970. Iron transport in *Salmonella typhimurium*: mutants blocked in the biosynthesis of enterobactin. J. Bacteriol. **104:**635–639.
21. **Reed, W. M., H. J. Olander, and H. L. Thacker.** 1985. Studies on the pathogenesis of *Salmonella heidelberg* infection in weanling pigs. Am. J. Vet. Res. **46:**2300–2310.

22. **Sharma, P. L., K. B. Sharma, and K. Prakash.** 1984. Colicin production and coexistence of Col plasmid with R plasmid in salmonellae. Indian J. Med. Res. **79:**591–593.
23. **Sun, M.** 1984. In search of *Salmonella's* smoking gun. Science **226:**30–32.
24. **Taylor, P. W., and C. Hughes.** 1978. Plasmid carriage and the serum sensitivity of enterobacteria. Infect. Immun. **22:**10–17.
25. **Terakado, N., T. Ohya, H. Ueda, Y. Isayama, and K. Ohmae.** 1980. A survey of drug resistance and R plasmids in *Salmonella* isolated from domestic animals in Japan. Jpn. J. Vet. Sci. **42:**543–550.
26. **Watanabe, T.** 1964. Selected methods of genetic study of episome-mediated drug resistance in bacteria. Methods Med. Res. **10:**202–220.
27. **Wilcock, B. P.** 1981. Salmonellosis, p. 445–456. *In* A. D. Leman, R. D. Glock, W. L. Mengeling, R. H. C. Penny, E. Scholl, and B. Straw (ed.), Diseases of swine, 5th ed. Iowa State University Press, Ames, Iowa.
28. **Wilcock, B. P., C. H. Armstrong, and H. J. Olander.** 1976. The significance of the serotype in the clinical and pathological features of naturally occurring porcine salmonellosis. Can. J. Comp. Med. **40:**80–88.
29. **Wooley, R. E., H. W. Dickerson, K. W. Simmons, E. B. Shotts, and J. Brown.** 1986. Effect of EDTA-Tris on an *Escherichia coli* isolate containing R plasmids. Vet. Microbiol. **12:**65–75.
30. **Yancey, R. J., S. A. L. Breeding, and C. E. Lankford.** 1979. Enterochelin (enterobactin): virulence factor for *Salmonella typhimurium*. Infect. Immun. **24:**174–180.
31. **Zar, J. H.** 1984. Biostatistical analysis, 2nd ed. Prentice-Hall, New York.