Multidrug-Resistant *Salmonella enterica* Serovar Typhimurium Isolates Are Resistant to Antibiotics That Influence Their Swimming and Swarming Motility

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ABSTRACT Motile bacteria employ one or more methods for movement, including darting, gliding, sliding, swarming, swimming, and twitching. Multidrug-resistant (MDR) *Salmonella* carries acquired genes that provide resistance to specific antibiotics, and the goal of our study was to determine how antibiotics influence swimming and swarming in such resistant *Salmonella* isolates. Differences in motility were examined for six MDR *Salmonella enterica* serovar Typhimurium isolates grown on swimming and swarming media containing subinhibitory concentrations of chloramphenicol, kanamycin, streptomycin, or tetracycline. Chloramphenicol and tetracycline reduced both swimming and swarming, though the effect was more pronounced for swimming than for swarming at the same antibiotic and concentration. Swimming was limited by kanamycin and streptomycin, but these antibiotics had much less influence on decreasing swarming. Interestingly, kanamycin significantly increased swarming in one of the isolates. Removal of the *aphA1* kanamycin resistance gene and complementation with either the *aphA1* or *aphA2* kanamycin resistance gene revealed that *aphA1*, along with an unidentified *Salmonella* genetic factor, was required for the kanamycin-enhanced swarming phenotype. Screening of 25 additional kanamycin-resistant isolates identified two that also had significantly increased swarming motility in the presence of kanamycin. This study demonstrated that many variables influence how antibiotics impact swimming and swarming motility in MDR S. Typhimurium, including antibiotic type, antibiotic concentration, antibiotic resistance gene, and isolate-specific factors. Identifying these isolate-specific factors and how they interact will be important to better understand how antibiotics influence MDR *Salmonella* motility.

IMPORTANCE *Salmonella* is one of the most common causes of bacterial foodborne infections in the United States, and the Centers for Disease Control consider multidrug-resistant (MDR) *Salmonella* a “Serious Threat Level pathogen.” Because MDR *Salmonella* can lead to more severe disease in patients than that caused by antibiotic-sensitive strains, it is important to identify the role that antibiotics may play in enhancing *Salmonella* virulence. The current study examined several MDR *Salmonella* isolates and determined the effect that various antibiotics had on *Salmonella* motility, an important virulence-associated factor. While most antibiotics had a neutral or negative effect on motility, we found that kanamycin actually enhanced MDR *Salmonella* swimming in some isolates. Subsequent experiments showed this phenotype as being dependent on a combination of several different genetic factors. Understanding the influence that antibiotics have on MDR *Salmonella* motility is critical to the proper selection and prudent use of antibiotics for efficacious treatment while minimizing potential collateral consequences.
**MULTIDRUG-RESISTANT (MDR) SALMONELLA STRAINS ARE CHARACTERIZED BY HIGH LEVELS OF ANTIMICROBIAL RESISTANCE THAT ARE OFTEN ATTRIBUTED TO THE ACQUISITION OF SPECIFIC MECHANISMS** (1). MDR *Salmonella enterica* SEROVAR TYPHIMURIUM PHAGE TYPES DT104 AND DT193 HAVE VERY SIMILAR RESISTANCE PROFILES DUE TO THEIR SPECIALIZED RESISTANCE GENES, THOUGH THE SAME RESISTANCE PHENOTYPE CAN BE ENCODED BY DIFFERENT GENES. IN OUR PREVIOUS WORK, INVASION OF HEp-2 CELLS WAS INDUCED IN MDR S. TYPHIMURIUM DT193 ISOLATES 1434 AND 5317 BY THE ADDITION OF EITHER TETRACYCLINE OR CHLORAMPHENICOL AT SUBINHIBITORY CONCENTRATIONS DURING THE TYPICALLY NONINVASIVE EARLY-LOG-GROWTH PHASE (2). TRANSCRIPTOME SEQUENCING (RNA-SEQ) ANALYSIS OF THESE TWO ISOLATES INDICATED THAT GENES FROM AT LEAST THREE *Salmonella* PATHOGENICITY ISLANDS (SPI-1, SPI-2, AND SPI-3) WERE UPREGULATED COMPARED TO ISOLATES THAT DID NOT HAVE AN ANTIBIOTIC-INDUCED INVASION PHENOTYPE. INTERESTINGLY, ALL ISOLATES TESTED HAD A SIGNIFICANT AND COMPARABLE DECREASE IN MOTILITY GENE EXPRESSION, INDEPENDENT OF INVASION PHENOTYPE. HOWEVER, THESE ISOLATES WERE ALL GROWN IN LIQUID CULTURES WITH AGITATION, AND SIGNIFICANT GENE EXPRESSION DIFFERENCES BETWEEN *Escherichia coli* GROWN IN LIQUID MEDIUM AND *E. coli* GROWN ON SEMISOLID MOTILITY MEDIUM HAVE BEEN REPORTED (3).

BACTERIAL CHEMOTAXIS AND MOTILITY ARE ASSOCIATED WITH VIRULENCE AS THESE PROCESSES ARE THOUGHT TO BE NECESSARY FOR PATHOGENS TO REACH SPECIFIC NICHEs WITHIN THE HOST (4–8). MANY MODES OF MOTILITY AMONG BACTERIA EXIST, THOUGH *Salmonella* PRIMARILy EMPLOYS SWIMMING AND SWARMING (9–11). SWIMMING IS THE MOVEMENT OF INDIVIDUAL BACTERIA THROUGH A MEDIUM, WHILE SWARMING IS THE MOVEMENT OF A GROUP OF BACTERIA ACROSS A SURFACE (5, 9). *Salmonella* motility is directly linked to invasion as coordinated regulation of the two systems is required for cellular invasion; genes associated with motility are downregulated as the invasion genes are upregulated (12–15). PREVIOUS REPORTS THAT HAVE EXAMINED THE EFFECT THAT ANTIBIOTICS HAVE ON *Salmonella* motility primarily used sensitive isolates and antibiotic gradients to examine the extent to which the motility phenotype affects resistance to antibiotics (7, 16–19). HOW DIFFERENT ANTIBIOTICS INFLUENCE BACTERIAL MOTILITY IN MDR *Salmonella* isolates has not been previously examined. GIVEN THE HIGH LEVEL OF ANTIMICROBIAL RESISTANCE AMONG *Salmonella* isolates (20), THE CLINICAL RELevANCE OF DT104 AND DT193 ISOLATES (21–25), AND THE OBSERVATION THAT MDR *Salmonella* isolates have increased morbidity in patients compared to sensitive isolates (26–28), IT IS IMPORTANT TO ESTABLISH THE POTENTIAL COLLATERAL CONSEQUENCES THAT ANTIBIOTICS MAY HAVE ON VIRULENCE MECHANISMS, INCLUDING MOTILITY. THE GOAL OF THE CURRENT PROJECT WAS TO ASSESS THE EFFECT THAT SUBINHIBITORY LEVELS OF ANTIBIOTICS HAVE ON MOTILITY FOR MDR *Salmonella* isolates grown on semisolid agar that enables swimming and swarming phenotypes.

**RESULTS AND DISCUSSION**

THREE S. TYPHIMURIUM DT193 ISOLATES (1434, 5317, AND 752) AND THREE S. TYPHIMURIUM DT104 ISOLATES (290, 360, AND 530) WERE SELECTED TO IDENTIFY THE EFFECT THAT CHLORAMPHENICOL, KANAMYCIN, STREPTOMYCIN, AND TETRACYCLINE HAVE ON SWIMMING AND SWARMING MOTILITY. THESE SIX ISOLATES ARE RESISTANT TO AMPICILLIN, CHLORAMPHENICOL, STREPTOMYCIN, AND TETRACYCLINE, AS PREVIOUSLY REPORTED (2). ISOLATES 1434, 5317, AND 290 ARE ALSO RESISTANT TO KANAMYCIN, AND PCR WAS USED TO IDENTIFY THE SPECIFIC KANAMYCIN RESISTANCE GENES PRESENT (TABLE 1). THE RESISTANCE GENES FOR CHLORAMPHENICOL, STREPTOMYCIN, AND TETRACYCLINE IN THESE ISOLATES HAVE BEEN IDENTIFIED (2). GROWTH CURVES WITH 2-FOLD DILUTIONS (0 AND 2 TO 512 µg/ml) OF EACH OF THE FOUR ANTIBIOTICS IN LIQUID LB MEDIUM WERE UTILIZED TO DETERMINE CONCENTRATIONS THAT INHIBITED GROWTH (MIC), IF WITHIN THE RANGE TESTED (TABLE 1). ALL MDR ISOLATES RESISTANT TO STREPTOMYCIN AND KANAMYCIN HANd AN MIC THAT EXCEEDED 512 µg/ml. FOR A GIVEN ANTIBIOTIC, THE MIC OF RESISTANT ISOLATES WAS AT LEAST 4-FOLD ABOVE THE CLSI RESISTANCE BREAKPOINT (CHLORAMPHENICOL, ≥32; KANAMYCIN, ≥64; STREPTOMYCIN, ≥64; AND TETRACYCLINE, ≥16 µg/ml).
Antibiotics Influence MDR Salmonella Motility

TABLE 1 Antibiotic resistance genes and MICs for six MDR Salmonella isolates

| Isolate | Chloramphenicol | Streptomycin | Kanamycin | Tetracycline |
|---------|-----------------|--------------|-----------|-------------|
|         | Presence of resistance gene | MIC (µg/ml) | Presence of resistance gene | MIC (µg/ml) | Presence of resistance gene | MIC (µg/ml) | Presence of resistance gene | MIC (µg/ml) |
| 1434    | +    | 128 | +    | >512 | +    | >512 | +    | 256 |
| 5317    | +    | 128 | +    | >512 | +    | >512 | +    | 256 |
| 752     | +    | 128 | +    | >512 | +    | >512 | +    | 256 |
| 530     | +    | 128 | +    | >512 | +    | >512 | +    | 64 |
| 290     | +    | 128 | +    | >512 | +    | >512 | +    | 64 |
| 360     | +    | 256 | +    | >512 | +    | >512 | +    | 64 |

| Gene | MIC (µg/ml) |
|------|-------------|
| floR | 128         |
| cm | 128         |
| aadA2 | >512 |
| strAB | >512 |
| aphA1 | >512 |
| aphA2 | >512 |
| aadB | >512 |
| tetA | 64         |
| tetG | 64         |

*No isolates had tetB, tetC, or tetD.
*Antibiotic sensitive.

Antibiotic concentrations below the MIC chosen for use in the motility assays were as follows: 4, 32, and 64 µg/ml chloramphenicol; 32, 128, and 512 µg/ml kanamycin; 32, 128, and 512 µg/ml streptomycin; and 4, 16, and 32 µg/ml tetracycline. For each antibiotic, the CLSI resistance breakpoint fell within the concentration range that was used in the motility assays. All isolates were grown on solid agar plates at the highest concentration of each antibiotic tested in the motility assays to verify that growth was not inhibited at these levels.

**Effects of antibiotics on MDR isolate motility. (i) Chloramphenicol.** For swimming motility, the lowest level of chloramphenicol tested (4 µg/ml) significantly inhibited swimming of isolate 360 compared to the no-antibiotic control, though the same concentration significantly inhibited swarming for isolates 360, 1434, and 290 (Fig. 1). At 32 and 64 µg/ml, both swimming and swarming were significantly reduced compared to the no-antibiotic control in all isolates tested. A significant difference was observed between 32 and 64 µg/ml for swimming but not for swarming, as there was little to no swarming motility at either of these concentrations. At any given concentration, chloramphenicol had a greater effect on limiting swimming than on limiting swarming; for example, 32 µg/ml substantially reduced swimming and yet only moderately decreased swimming motility.

**(ii) Tetracycline.** Tetracycline at 4 µg/ml significantly decreased swimming in the DT104 isolates (290, 360, and 530) but not the DT193 isolates (Fig. 1). Compared to the control without tetracycline, swimming motility was significantly decreased due to 16 µg/ml of tetracycline for all isolates except 1434, while 32 µg/ml inhibited all isolates tested. Not including isolate 1434, swimming motility was significantly decreased for all isolates due to 4 µg/ml of tetracycline; at 16 and 32 µg/ml of tetracycline, swimming motility was significantly decreased for all isolates. Similarly to chloramphenicol, tetracycline appeared to have a greater effect on reducing swimming than on reducing swimming at the same antibiotic concentration.

**(iii) Streptomycin.** Streptomycin at 128 µg/ml significantly decreased swimming in all isolates compared to the no-antibiotic control except for isolate 1434, while the same concentration had no effect on swarming in any of the isolates (Fig. 1). All isolates had significantly reduced motility at 512 µg/ml of streptomycin, except swarming for isolates 5317 and 752. The level of decreased swimming at 512 µg/ml is noteworthy as DT193 isolates were reduced in swimming by 0 to 31% compared to the control, while at this concentration the three DT104 isolates did not swarm at all. Additionally, isolate 5317 had a nonsignificant increase in swimming at 32 µg/ml compared to the control. The degree to which streptomycin appears to limit swimming was greater than that for swimming at equivalent streptomycin concentrations, which was the converse of the results for chloramphenicol and tetracycline.

**(iv) Kanamycin.** Only three of the six isolates were resistant to kanamycin (1434, 5317, and 290), and the three kanamycin-sensitive MDR isolates did not exhibit any motility at any kanamycin concentration tested (Fig. 1). Kanamycin did not influence...
**FIG 1** Effect of antibiotics on swimming and swarming motility in MDR *Salmonella* isolates. Chloramphenicol, tetracycline, streptomycin, and kanamycin at indicated concentrations were added to swimming and swarming agar media to determine their effect on motility in six MDR *S*. Typhimurium isolates. Growth was normalized to the no-antibiotic control for each isolate, and data were expressed as fold changes for all pairwise comparisons for each antibiotic, isolate, and motility combination. Statistical differences are noted by letters \((P < 0.05)\), where any pairwise combination that shares the same letter is not considered different.
swimming or swarming in isolate 290. Isolate 1434 had reduced swimming at 512 g/ml. All tested concentrations decreased swimming for isolate 5317, while swarming was reduced only at 512 g/ml. Similar to the streptomycin results, isolate 5317 had a nonsignificant increase in swarming at 32 g/ml compared to the control. Notably, kanamycin significantly increased swarming at 32 and 128 g/ml for isolate 1434, but not at 512 g/ml. Isolates 1434, 5317, and 290 all had various motility phenotypes within and between isolates in response to kanamycin, and yet kanamycin resistance in all three isolates was encoded by an aphA1 gene. Genetic factors beyond the presence of the resistance mechanism likely play a role in how kanamycin influences motility between isolates, as well as differences in gene regulation at the various antibiotic concentrations for a specific isolate. Additionally, the highest concentrations of kanamycin may lead to the induction of the SOS response (29, 30).

Overall, the effect of different antibiotics on MDR Salmonella swimming and swarming motility was determined by the combination of isolate, antibiotic, and antibiotic concentration. None of the isolates were natively hypermotile, as the motility phenotypes were relatively similar between the isolates. One interesting relationship between chloramphenicol and tetracycline, which have resistance factors encoded by tet and floR efflux pumps, respectively (31, 32), was the greater negative impact on swarming than on swimming at the same antibiotic concentration. This was the opposite of what was observed for kanamycin and streptomycin, as they had more of an inhibitory effect on swimming than on swarming, with swimming being significantly enhanced in one instance. Kanamycin and streptomycin resistance are encoded by genes for aminoglycoside inactivation enzymes (33), and the difference between the effect of these antibiotics on motility and that of chloramphenicol and tetracycline could be due to the difference in the mechanism or regulation of these resistance factors; previous studies have demonstrated a relationship between innate efflux pumps and motility (34–36). However, antibiotic-induced decreases in motility may not necessarily decrease virulence. We previously have found that 16 µg/ml tetracycline and 32 µg/ml chloramphenicol can downregulate genes associated with motility when strains are grown in LB broth but can also induce the invasive phenotype in isolates 1434 and 5317 (2). These tetracycline and chloramphenicol concentrations are at the CLSI resistance breakpoints for Salmonella Typhimurium that are predictive of whether a microorganism will respond to antibiotic therapy.

**Role of the kanamycin resistance gene in swarming.** To evaluate the role of the aphA1 gene in the kanamycin-enhanced swarming phenotype observed for isolate 1434, several mutants that removed the kanamycin resistance gene were created and were subsequently complemented with different resistance genes (Table 2). The aphA1 gene was deleted from the 1434-derived BBS1156 mutant to create 1434ΔaphA1 (BBS1182). Also, because motility plays a role in regulating the type III secretion system required for cellular invasion, the SPI-1 gene hilA was deleted from the BBS1182 strain to create 1434ΔaphA1ΔhilA (BBS1211). Kanamycin resistance was restored to both of these mutants by transformation with the E. coli-derived pACYC177 plasmid that carries aphA1 to create 1434ΔaphA1/aphA1pACYC (strain BBS1213) and 1434ΔaphA1ΔhilA/

### TABLE 2 Kanamycin-sensitive isolates and mutants, as well as the plasmids and genes used to restore resistance

| Strain   | Parent | aphA1 | hilA | Plasmid          | Kan Comp | MIC (µg/ml) |
|----------|--------|-------|------|------------------|----------|-------------|
| BBS1213  | 1434   | ΔaphA1| hilAWT| pACYC177         | aphA1    | >512        |
| BBS1215  | 1434   | ΔaphA1| ΔhilA| pACYC177         | aphA1    | >512        |
| BBS1219  | 1434   | ΔaphA1| hilAWT| pCR              | aphA2    | >512        |
| BBC1501  | 1434   | ΔaphA1| hilAWT| p74S             | aphA1    | >512        |
| BBC1502  | 752    | —     | hilAWT| p74S             | aphA1    | >512        |
| BBS1213  | 1434   | ΔaphA1| hilAWT| pACYC177         | aphA1    | >512        |
| BBS1215  | 1434   | ΔaphA1| ΔhilA| pACYC177         | aphA1    | >512        |
| BBS1219  | 1434   | ΔaphA1| hilAWT| pCR              | aphA2    | >512        |
| BBC1501  | 1434   | ΔaphA1| hilAWT| p74S             | aphA1    | >512        |
| BBC1502  | 752    | —     | hilAWT| p74S             | aphA1    | >512        |

**Kan Comp**, kanamycin complementation.

**WT**, wild type.

**——**, not present in wild type.
aphA1 pACYC (BBS1215). A 1434ΔaphA1 sensitive mutant was also restored using the E. coli-derived pCR plasmid that carries aphA2 to create 1434ΔaphA1/aphA2 pCR (BBS1219). While functionally similar, aphA1 and aphA2 share only ~45% nucleotide identity. A Salmonella-derived plasmid from DT104 isolate 745 that carries aphA1 was transduced into 1434ΔaphA1 to create 1434ΔaphA1/aphA1 p745 (BBC1501). The same plasmid was also transduced into the kanamycin-sensitive DT193 isolate 752 to create 752/aphA1 p745 (BBC1502).

The mutant isolates, along with the 1434 wild-type isolate, were inoculated onto swarming medium containing 0, 32, or 128 μg/ml of kanamycin (Fig. 2). The swarming data indicated that deletion and replacement of the aphA1 gene carried on a Salmonella-derived plasmid restored kanamycin-enhanced swarming at 128 μg/ml in strain 1434ΔaphA1/aphA1 p745, though 32 μg/ml had a nonsignificant increase. However, adding the same plasmid to a different DT193 kanamycin-sensitive isolate in strain 752/aphA1 p745 did not enhance swarming motility, indicating that more than just the presence of the aphA1 gene is required to observe kanamycin-enhanced swarming. Therefore, genetic factors encoded within 1434, but absent from 752, are necessary for the kanamycin-induced motility phenotype. To assess if a non-Salmonella-derived plasmid carrying aphA1 could restore the kanamycin-enhanced swarming phenotype, strain 1434ΔaphA1/aphA1 pACYC was tested and found to have a significantly enhanced swarming phenotype due to kanamycin at both 32 and 128 μg/ml. The genetic factor required for kanamycin enhancement may require the hila gene, as strain 1434ΔaphA1 Δhila/aphA1 pACYC had a small but nonsignificant increase in swarming in the presence of kanamycin. Finally, restoring resistance to the sensitive 1434 mutant with the aphA2 gene in strain 1434ΔaphA1/aphA2 pCR actually led to a significant decrease in swarming in the presence of kanamycin. Despite pCR/aphA2 being a higher-copy-number plasmid than pACYC/aphA1 (~100 copies compared to ~15, respectively), pCR/aphA2 still failed to complement the kanamycin-enhanced swarming phenotype. Together, these data indicate that the aphA1 gene, along with unknown Salmonella genetic factors, was required for the kanamycin-enhanced swarming phenotype.

Screening additional kanamycin-resistant isolates. To establish if the kanamycin-enhanced motility phenotype occurred in other MDR isolates similar to 1434, 25 additional kanamycin-resistant DT193 isolates were tested at 128 μg/ml in swarming medium. Out of these 25 isolates, three isolates demonstrated an increase in motility, with two isolates being significantly enhanced (Table 3). Isolates 413, 9853, and 18599 all carry the kanamycin resistance gene aphA1, while 9853 and 18599 also carry aadB.
We observed a significant kanamycin-induced motility phenotype in 3 of 28 (~11%) DT193 isolates from our collection, and it would be interesting to identify the presence of this phenotype in a larger and more diverse *Salmonella* population. It would also be worthwhile to ascertain if this effect is more prevalent in *S. Typhimurium* isolated from turkeys, as turkeys have a higher level of kanamycin-resistant *S. Typhimurium* (34%) than do cattle (24%), pigs (10%), chickens (8%), and humans (6%) over a 10-year period (20).

**Effect of antibiotics on motility of an antibiotic-sensitive isolate.** *S. Typhimurium* isolate SARB65 is sensitive to the antibiotics used in this study and was tested to identify similarities and differences between the MDR isolates. As sensitive isolates typically utilize innate resistance mechanisms (i.e., *AcrAB*-ToIC) that have different and lower inhibitory thresholds than those of isolates with specific resistance mechanisms (1), the MIC for SARB65 was determined for chloramphenicol (4 µg/ml), kanamycin (64 µg/ml), streptomycin (64 µg/ml), and tetracycline (2 µg/ml). Based on this, swimming and swarming assays were performed at various subinhibitory concentrations relative to the MIC of each antibiotic (Fig. 3). Chloramphenicol had no effect on swimming but significantly decreased swarming, similar to the trend observed in the MDR isolates. Tetracycline prevented all swimming and swarming motility, which diverges from what was observed in the MDR isolates. Streptomycin did not influence swimming but significantly reduced swarming, while 16 and 32 µg/ml limited or inhibited both motility phenotypes. Finally, kanamycin did not influence motility at 4 or 16 µg/ml but significantly reduced both swimming and swarming at 32 µg/ml. A significantly reduced swimming phenotype for SARB65 in the presence of 32 µg/ml kanamycin is consistent with MDR isolates 752, 360, and 530, which are also kanamycin sensitive, but differs from the kanamycin-resistant MDR isolates containing *aphA1* (1434, 5317, and 290) where swimming either remained similar to the no-antibiotic control or was enhanced. While there is some overlap of antibiotic-influenced motility between the sensitive and MDR isolates, it is likely that the different resistance mechanisms and genetics of the isolates influence the observed swimming and swarming phenotypes.

**Conclusion.** In this study, we assessed the effect that four different antibiotics had on MDR *Salmonella* motility when grown on swimming and swarming medium. In general, these antibiotics either decreased or had no effect on motility. A decrease in motility, however, may not always be beneficial for reducing virulence, as our previous work demonstrated that tetracycline and chloramphenicol can downregulate motility gene expression while simultaneously inducing cellular invasion *in vitro*. Notably, kanamycin significantly enhanced swarming in a subset of isolates. Further analysis indicated that the *aphA1* resistance gene and a *Salmonella*-specific genetic component were both required in order to observe this phenotype. Given the relationship between motility and virulence in *Salmonella*, it is important to better understand the role that antibiotics may have in promoting potential collateral effects in MDR isolates.

**MATERIALS AND METHODS**

**Isolates and growth conditions.** The isolates and mutants utilized in the study are listed in Table 4. *Salmonella* DT104 and DT193 isolates were from our archive of clinical samples originally cultured from cattle. The antibiotic-sensitive *S. Typhimurium* isolate SARB65 is from the *Salmonella* Reference Collection.
B (37). Each Salmonella isolate was streaked onto solid LB (Lennox L) agar plates (Invitrogen, Carlsbad, CA). A colony was grown in LB broth at 37°C with agitation for 6 h followed by a 1:1,000 dilution in LB broth that was grown overnight at 37°C with agitation. The overnight culture was diluted 1:200 in LB broth and grown at 37°C with agitation to an approximate optical density at 600 nm (~OD₆₀₀) of 0.3.

Antibiotic resistance characterization. Selected isolates were tested for resistance to kanamycin by streaking on solid LB agar plates with 50 μg/ml kanamycin. The inhibitory concentrations for kanamycin

FIG 3 Effect of antibiotics on swimming and swarming motility in an antibiotic-sensitive isolate of Salmonella. Chloramphenicol, tetracycline, streptomycin, and kanamycin were added at various subinhibitory concentrations to swimming and swarming agar media to determine their effect on motility in the antibiotic-sensitive S. Typhimurium isolate SARB65. Growth was normalized to the no-antibiotic control for each isolate, and data were expressed as a fold change for all pairwise comparisons for each antibiotic and motility combination. Statistical differences are noted by letters (P < 0.05), where any pairwise combination that shares the same letter is not considered different.
were evaluated by growth with serial 2-fold dilutions of the antibiotic (0 and 2 to 512 μg/ml) using a Bioscreen C instrument (Growth Curves Ltd., Raisio, Finland), where optical density measurements were taken once an hour for 24 h to establish a growth curve for each isolate and antibiotic concentration. The inhibitory concentrations for chloramphenicol, streptomycin, and tetracycline were determined previously using the same methodology for the MDR isolates (2). For the antibiotic-sensitive SARB65 isolate, the inhibitory concentrations were determined by growth with serial 2-fold dilutions of chloramphenicol (0 and 0.125 to 32 μg/ml), streptomycin (0 and 2 to 512 μg/ml), and tetracycline (0 and 0.125 to 32 μg/ml). Isolates were streaked onto solid LB agar containing antibiotics at the concentrations selected for use in the motility assays to confirm normal growth on nonmotility medium. Genes associated with kanamycin resistance were assessed in all resistant isolates by PCR (Table 5). MDR isolates were previously screened for the following resistance genes: cat, cml, and floR (chloramphenicol); aadA2, strA, and strB (streptomycin); tetA, -B, -C, -D, and -G (tetracycline) (2).

**Motility assays.** Based on the growth curve data, motility assays were performed in swimming and swarming media containing antibiotics at the indicated subinhibitory concentrations for the MDR isolates: chloramphenicol, 0, 4, 32, and 64 μg/ml; kanamycin, 0, 32, 128, and 512 μg/ml; streptomycin, 0, 32, 128, and 512 μg/ml; and tetracycline, 0, 4, 16, and 32 μg/ml. The following antibiotics and concentrations were used for the antibiotic-sensitive SARB65 isolate: chloramphenicol, 0, 0.5, 1, and 2 μg/ml; kanamycin, 0, 0.5, 1, and 2 μg/ml; streptomycin, 0, 0.5, 4, 16, and 32 μg/ml; and tetracycline, 0, 0.25, 0.5, 2, and 4 μg/ml. The 0.3% agar LB swim medium contained 10 g Bacto tryptone, 5 g Bacto yeast extract, 5 g NaCl, and 3 g Bacto agar in 1 liter deionized water. The 0.5% agar LB swim medium contained 10 g Bacto tryptone, 5 g Bacto yeast extract, 5 g NaCl, and 5 g Bacto agar in 975 ml deionized water (3, 38). Motility medium was autoclaved and placed in a 56°C water bath. After the medium reached 56°C, antibiotics at the appropriate concentrations were added. For the swimming medium, 25 ml of a 20% stock glucose solution per liter was added (for a final glucose concentration of 0.5%). Twenty-five milliliters of medium was pipetted into 100- by 15-mm culture plates, which were covered and allowed to cool and solidify for 30 min.

A 5-μl aliquot of *Salmonella* culture at an OD<sub>600</sub> of 0.3 (as described above) was placed in the center of each plate, covered, and allowed to rest for 5 to 10 min. The plates were placed in a 37°C humid incubator for ~5 h (swimming) or ~10 h (swarming). Two technical replicates were performed for each condition. Diameters of bacterial swimming and swarming were measured and normalized to the no-antibiotic control. Each condition was tested three or more separate times and analyzed for differences using a one-way analysis of variance (ANOVA) with a Tukey posttest for all pairwise comparisons for each antibiotic and isolate combination.

**Mutants.** Due to the MDR genotype of *S. Typhimurium* 1434, we were unable to utilize gene knockout cassettes that relied on chloramphenicol or kanamycin antibiotic selection that we have previously designed and constructed for generating numerous recombineering mutants. Instead, we modified our previous universal FLP recombination target (FRT) primers to incorporate nucleotide sequences for the amplification of the zeo gene encoding resistance to zeomycin as a substitution for cat or neo (Table 5). The first 58 nucleotides of the o8BI 465 primer were identical to o8BI 88 and contained a universal primer binding region for cassette amplification and an FRT site for FLP recombinase (39). The o8BI 465 primer had a similar design as o8BI 465 with the first 58 nucleotides being identical to o8BI 89 and containing a universal primer binding region and an FRT site. The universal primer binding sites had

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**TABLE 4 Strains used in the study**

| Strain no | S. Typhimurium strain background | Genotype | Resistance phenotype |
|-----------|----------------------------------|----------|----------------------|
| DT193 1434 | 1434 Wild type | Ap<sup>+</sup> Cam<sup>+</sup> Strep<sup>+</sup> Tet<sup>+</sup> Knr<sup>+</sup> | |
| DT193 5317 | 5317 Wild type | Ap<sup>+</sup> Cam<sup>+</sup> Strep<sup>+</sup> Tet<sup>+</sup> Knr<sup>+</sup> | |
| DT193 752 | 752 Wild type | Ap<sup>+</sup> Cam<sup>+</sup> Strep<sup>+</sup> Tet<sup>+</sup> | |
| DT104 530 | 530 Wild type | Ap<sup>+</sup> Cam<sup>+</sup> Strep<sup>+</sup> Tet<sup>+</sup> Knr<sup>+</sup> | |
| DT104 290 | 290 Wild type | Ap<sup>+</sup> Cam<sup>+</sup> Strep<sup>+</sup> Tet<sup>+</sup> Knr<sup>+</sup> | |
| DT104 360 | 360 Wild type | Ap<sup>+</sup> Cam<sup>+</sup> Strep<sup>+</sup> Tet<sup>+</sup> | |
| DT104 413 | 413 Wild type | Ap<sup>+</sup> Cam<sup>+</sup> Strep<sup>+</sup> Tet<sup>+</sup> Knr<sup>+</sup> | |
| DT193 18599 | 18599 Wild type | Ap<sup>+</sup> Cam<sup>+</sup> Strep<sup>+</sup> Tet<sup>+</sup> Knr<sup>+</sup> | |
| DT193 9853 | 9853 Wild type | Ap<sup>+</sup> Cam<sup>+</sup> Strep<sup>+</sup> Tet<sup>+</sup> Knr<sup>+</sup> | |
| BB9596 | 1434 pKD46-Gm | Ap<sup>+</sup> Cam<sup>+</sup> Strep<sup>+</sup> Tet<sup>+</sup> Knr<sup>+</sup> | |
| BB51156 | 1434 aphA1:zeo | Ap<sup>+</sup> Cam<sup>+</sup> Strep<sup>+</sup> Tet<sup>+</sup> Zeo<sup>+</sup> | |
| BB51182 | 1434 ΔaphA1 | Ap<sup>+</sup> Cam<sup>+</sup> Strep<sup>+</sup> Tet<sup>+</sup> | |
| BB51206 | 1434 ΔaphA1/pKD46-Gm | Ap<sup>+</sup> Cam<sup>+</sup> Strep<sup>+</sup> Tet<sup>+</sup> Gmr<sup>+</sup> 30°C<sup>+</sup> | |
| BB51208 | 1434 ΔaphA1 hiliA:neo | Ap<sup>+</sup> Cam<sup>+</sup> Strep<sup>+</sup> Tet<sup>+</sup> Knr<sup>+</sup> | |
| BB51211 | 1434 ΔaphA1 hiliA | Ap<sup>+</sup> Cam<sup>+</sup> Strep<sup>+</sup> Tet<sup>+</sup> | |
| BB51213 | 1434 ΔaphA1/pACYC177 | Ap<sup>+</sup> Cam<sup>+</sup> Strep<sup>+</sup> Tet<sup>+</sup> Knr<sup>+</sup> | |
| BB51215 | 1434 ΔaphA1ΔhiliA/pACYC177 | Ap<sup>+</sup> Cam<sup>+</sup> Strep<sup>+</sup> Tet<sup>+</sup> Knr<sup>+</sup> | |
| BB51219 | 1434 ΔaphA1/pCR | Ap<sup>+</sup> Cam<sup>+</sup> Strep<sup>+</sup> Tet<sup>+</sup> Zeo<sup>+</sup> | |
| BBC1501 | 1434 ΔaphA1/p745-aphA1 | Ap<sup>+</sup> Cam<sup>+</sup> Strep<sup>+</sup> Tet<sup>+</sup> Knr<sup>+</sup> | |
| BBC1502 | 752 p745-aphA1 | Ap<sup>+</sup> Cam<sup>+</sup> Strep<sup>+</sup> Tet<sup>+</sup> Knr<sup>+</sup> | |
| SAR685 | SAR685 Wild type | Sensitive | |

<sup>*Temperature-sensitive phenotype.*</sup>
| Primer purpose | Gene (GenBank accession no.) | Primer name | Sequence (5' to 3') |
|----------------|-----------------------------|-------------|---------------------|
| Detection of kanamycin resistance genes | \( \text{aphA1 (AF093572)} \) | \( \text{aphA1-F} \) | \text{CGTCAGCCAGTTTAGTCTG} |
|                          |                             | \( \text{aphA1-R} \) | \text{CAATCAGCTGGCACAATC} |
|                          | \( \text{aphA2 (U66885)} \) | \( \text{aphA2-F} \) | \text{GGCTATTCCGCTATGACTG} |
|                          |                             | \( \text{aphA2-R} \) | \text{GGACAGTGGTCCTTGAC} |
|                          | \( \text{aadB (JN119852)} \) | \( \text{aadB-F} \) | \text{TACTATGCCGATGAACTCC} |
|                          |                             | \( \text{aadB-R} \) | \text{CAAGACCTCAACCTTTCC} |
| Amplification of \( \text{zeo} \) in pCR-Blunt | \( \text{zeo} \) | \( \text{oBB1 465} \) | \text{CGATAGCTGAAATGAGCGCTGAAATTCTTCTATGAGATAGGAACCTCAGTTCAAATTCATGATGC} |
|                          |                             | \( \text{oBB1 466} \) | \text{GCAATACGAGCGGTAGTGAAGCTGGAATGAACTCAGTTCAAATTCATGATGC} |
| Synthesis of an \( \text{aphA1} \) knockout fragment by amplification of \( \text{oBB1 465/466-zeo} \) | \( \text{aphA1} \) | \( \text{oBB1 467} \) | \text{CAAGACTGCAAAATATCATCATGAACAATAAAACTGTCTGCTTAC} |
|                          |                             | \( \text{oBB1 468} \) | \text{GGATACTGCCAGATATCTGGCCATCGGGAGATGCTCTGGAAGCATAAGGCCAGTGCAGTGC} |
| Synthesis of a \( \text{hilA} \) knockout fragment by amplification of \( \text{oBB1 92/93-neo} \) | \( \text{hilA} \) | \( \text{oBB1 490} \) | \text{GAATAACTATTATCATGCGAGCCCGTTTTTAACTCGTGATGATGAGTGCAGATCTGGAAGCATAAGGCCAGTGCAGTGC} |
|                          |                             | \( \text{oBB1 491} \) | \text{CAAATATTTGTCTTCCGAGATGAGTGCAGATCTGGAAGCATAAGGCCAGTGCAGTGC} |

*The 5' end contains a universal binding region and FRT sites. The underlined sequences bind pCR-Blunt to amplify \( \text{zeo} \).

*Boldface indicates homology to \( \text{aphA1} \) for gene knockout. Underlined sequences bind \( \text{oBB1 465/466-zeo} \).

*Boldface indicates homology to \( \text{hilA} \) for gene knockout. Underlined sequences bind \( \text{oBB1 92/93-neo} \).
stop codons in all three translational reading frames to truncate protein synthesis of the target gene. The 3’ end of oBBI 465 had 20 nucleotides that bind ~197 to 178 bp upstream of the start codon for the zeo gene in pCR-Blunt (Invitrogen). Likewise, the 3’ end of oBBI 466 contained 19 nucleotides of pCR-Blunt sequence that overlaps the zeo stop codon. The zeo gene in pCR-Blunt was amplified by PCR to synthesize an oBBI 465/466-zeo template. To construct an aphA1 mutant of S. Typhimurium 1434, primers oBBI 467 and oBBI 468 were used to PCR amplify the oBBI 465/466-zeo template, and the knockout fragment was gel purified and transformed into arabinose-induced BBS996 containing pKD46-Gm (40). A zeomycin-resistant derivative of 1434 was selected and named BBS1156. The zeo gene was deleted from BBS1156 by transformation with pCP20-Gm and screening for a zeomycin-sensitive derivative which was subsequently named BBS1182 (40). The 1434ΔaphA1Δhia1 mutant (BBS1211) was constructed using similar methods except that an oBBI 490/491-neo gene knock-out fragment was constructed by amplifying oBBI 92/93-neo (41), followed by transformation and selection for kanamycin. Complementation of the kanamycin-sensitive phenotype for BBS1156 and BBS1182 was performed by using one of several plasmids that carried either aphA1 or aphA2 kanamycin resistance genes from pACYC177 (New England Biolabs [42] and a derivative of pCR-Blunt (Invitrogen), respectively. A plasmid native to S. Typhimurium DT104 isolate 745 carrying aphA1 and conferring kanamycin resistance was previously described (43). The kanamycin resistance plasmid from 745 (p745) was isolated and transformed into BBS1182 and a kanamycin-sensitive DT193 isolate, 752, to construct strains BBC1501 and BBC1502. The mutant isolates were inoculated on swarm medium, as described above, that contained 0, 32, or 128 μg/ml kanamycin. A one-way ANOVA with the Dunnett posttest was used to determine differences in motility compared to the no-antibiotic control for each isolate.

Screening of additional DT193 isolates for kanamycin-enhanced swarming. Thirty-one additional DT193 isolates from our collection were streaked on LB plates containing 50 μg/ml kanamycin to screen for resistance. Twenty-five isolates were identified as resistant, and these were inoculated on swarm medium, as described above, that contained 0, 32, or 128 μg/ml kanamycin. A one-way ANOVA with the Dunnett posttest was used to determine differences in motility compared to the control and antibiotic-treated isolates.

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