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Identification of Multiresistant *Salmonella* Isolates Capable of Subsisting on Antibiotics[▽]

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This study assessed the ability of *Salmonella* (572 isolates) to subsist on 12 different antibiotics. The majority (11/12) of the antibiotics enabled subsistence for at least 1 of 140 isolates. Furthermore, 40 isolates were able to subsist on more than one antibiotic. Antibiotic resistance and antibiotic subsistence do not appear to be equivalent.

A recent study found that a diverse group of bacteria, including intrinsically resistant microbes (e.g., *Pseudomonadales* and *Burkholderiales*), could subsist on and presumably catabolize antibiotics as a sole carbon source. This group includes bacteria related to the pathogens *Burkholderia cepacia* and *Serratia marcesens* (5). This phenotype has also previously been reported for catabolism of chloramphenicol by *Streptomyces* (1) and beta-lactams by *Leptospira* (8) and *Pseudomonas* (7).

In the current study, we assessed this subsistence phenotype in multiresistant and antibiotic-sensitive *Salmonella* isolates. Five hundred seventy-two isolates of *Salmonella* were obtained from cattle, swine, poultry, equine, beef, pork, chicken, and turkey samples (3, 6). These isolates were obtained from clinical, nonclinical, and food samples. Of the 572 isolates, 179 (31.3%) were *Salmonella enterica* subspecies *enterica* serovar Typhimurium harboring the SGI1 integron, encoding multiple antibiotic resistance (2), i.e., definitive type 104 (DT104). All *Salmonella* isolates were previously derived from single colonies stored at −80°C in 50% glycerol-50% LB broth (Sigma). The isolates were then inoculated into LB broth and grown aerobically for 16 h at 37°C prior to growth on single-carbon-source (SCS) medium.

SCS medium was prepared as previously described (5), with 1 mg/ml of antibiotic. Since the isolates were of animal origin, the antibiotics included drugs either used in veterinary medicine or closely related to veterinary antibacterials. The antibiotics chosen were amikacin, ampicillin, cefepime (an extended-spectrum and beta-lactamase-resistant human cephalosporin chosen because of the pending FDA approval of the use of cefquinome in cattle), ceftiofur, ciprofloxacin (a metabolite of the veterinary fluoroquinolone enrofloxacin [10]), florfenicol, kanamycin, streptomycin, sulfisoxazole, tetracycline, trimethoprim, and vancomycin (chosen because of its relationship to the poultry feed additive avoparcin). Except for cefepime (Bristol-Myers Squibb Company) and florfenicol (Schering-Plough), all antibiotics were obtained from Sigma.

Approximately 10^7 CFU were incubated on SCS agar for 16 h at 37°C. Subsistence was ascribed for isolates in which the corresponding plate contained distinct colonies; typically, 10 to 1,000 colonies were observed, i.e., a relatively low frequency (10^{-6} to 10^{-4}). Representative colonies were then statically incubated in 200 μl SCS broth containing the antibiotic for 16 to 48 h at 37°C. Of the 572 isolates examined, 140 (24.5%) subsisted on at least one antibiotic. Of the 12 antibiotics examined, 11 enabled the growth of at least one *Salmonella* isolate. All antibiotics that provided sustenance on agar also enabled subsistence in broth. Subsistence was observed fastest in the presence the bacteriostatic agents sulfisoxazole and trimethoprim (Table 1). Tetracycline did not enable subsistence for any of the 572 isolates examined, and no isolates grew on or in SCS medium lacking an antibiotic.

Table 2 depicts the 40 isolates that subsisted on multiple antibiotics, and 36 of these isolates possessed the SGI1 integron. Fourteen isolates were capable of subsisting on three different antibiotics, and 13 of these isolates contained SGI1.

Ninety-one of the 179 SGI1-bearing DT104 isolates exhibited subsistence, while this phenotype was also observed in an SGI1-bearing isolate of *Salmonella enterica* subspecies *enterica* serovar Agona (Table 1). Antibiotic subsistence may be linked to the SGI1 structure since this activity was observed in more than half of the isolates bearing this integron. Subsistence on ampicillin was the most common phenotype in the DT104 isolates, despite uniform resistance to this antibiotic and three others (sulfisoxasole, streptomycin, and tetracycline) in these isolates. SGI1 contains nearly 30 genes unrelated to antibiotic resistance (2), and future mutagenesis studies will attempt to identify which of these elements contribute to the subsistence phenotype.

Subsistence was also observed in multiple isolates from seven different non-Typhimurium serovars (*Salmonella enterica* subspecies *enterica* serovars Agona, Dublin, Heidelberg, Montevideo, Oranienberg, Typhisuis, and Worthington). Given the smaller numbers of isolates from outside the Typhimurium serotype, no conclusions can be drawn regarding serotype predilection for antibiotic subsistence. However, these results documented that 14% (56/393) of the isolates were able to subsist on an antibiotic when the data from *S.* Typhimurium were omitted.

MICs were determined for all of the isolates that subsisted

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TABLE 1. *Salmonella* isolates that subsisted on SCS agar or in SCS broth containing 1 or more of the 12 antibiotics[a]

Antibiotic or other carbon source[b]	Serovar(s) or subspecies of isolate(s) in indicated MIC group[c]	
	MIC > 1 mg/ml	MIC ≤ 1 mg/ml
Ampicillin or ampicillin-clavulanic acid (63)[d]	Abortus-equi, Clackamas, Dublin (2), Minnesota, Panama, Typhimurium (52)^{SGI1}	Newport, Norwich, Nottingham, Ordonez, Worthington
Kanamycin (51)		Aqua, Berta, Choleraesuis, Duisburg, Enteriditis, Gatow, Heidelberg, Infantis, Oranienburg, Paratyphi B, Paratyphi B var. L(+) tartrate, Paratyphi C, London var. 15^{+}, Ohio, Typhimurium (36)^{SGI1}, Typhisuis
Sulfisoxazole (38)[e]	Agona^{SGI1}, Amager, Clackamas, Derby, Infantis, Montevideo, Typhimurium (30)^{SGI1}, Worthington	Typhimurium^{SGI1}
Trimethoprim (21)[e]		Agona, Aqua, Choleraesuis var. Decatur, Heidelberg, Montevideo, Oranienburg, Saintpaul, Typhimurium (14)^{SGI1}
Vancomycin (11)		Derby, Mbandaka, Schwarzengrund, Sendai, Typhimurium (7)^{SGI1}
Streptomycin (7)		Typhimurium (7)^{SGI1}
Ceftiofur (5)		Aqua, Bietri, Durham, Havana, subsp. *houtanae*
Ciprofloxacin (2)	Kingston	Hartford
Amikacin (1)		Typhisuis
Florfenicol (1)		Typhisuis
Cefepime (1)		Typhimurium^{SGI1}
Tetracycline (0)		

[a] Subsistence is defined by the observation of individual distinct colonies on SCS agar or an OD_{600} of >0.1 for SCS broth. The Mueller-Hinton-associated MIC is also presented for each antibiotic and isolate.

[b] Numbers in parentheses represent the cumulative number of isolates exhibiting the subsistence phenotype for a given antibiotic. No isolates exhibited the subsistence phenotype for tetracycline or antibiotic-free SCS agar or broth. (Growth in antibiotic-free broth was assessed only for certain isolates that subsisted on an antibiotic.)

[c] Numbers in parentheses represent the number of isolates exhibiting the corresponding subsistence phenotype when more than one isolate was involved. "SGI1" indicates an isolate bearing SGI1. Of the 201 isolates exhibiting the subsistence phenotype for any antibiotic, 99 were associated with MICs of >1 mg/ml (82 of which were serovar Typhimurium isolates bearing SGI1), and 102 were associated with MICs of ≤1 mg/ml (66 of which were serovar Typhimurium isolates bearing SGI1).

[d] None of these isolates were able to subsist on clavulanic acid alone.

[e] All assessed isolates were capable of growth in SCS broth after overnight incubation (16 h), whereas all other isolates demonstrated growth after 48 h in SCS broth.

on SCS medium. Approximately 2×10^5 CFU were used in microbroth (200 μl per well; 96-well microtiter plates) 2-fold serial dilutions in Mueller-Hinton broth (Difco) per CLSI guidelines (4). MICs were ascribed to the lowest concentration of antibiotic that inhibited overnight growth as determined visually, with an approximate optical density at 600 nm (OD_{600}) of <0.1 indicating growth inhibition. *Escherichia coli* ATCC 25922 was used as the control strain.

TABLE 2. *Salmonella* isolates that subsisted on two or more antibiotics

Antibiotics that enabled subsistence	Serovar(s)[a]
Amikacin and florfenicol	Typhisuis (1)
Ampicillin and kanamycin	Typhimurium (7)^{SGI1}
Ampicillin, kanamycin, and streptomycin	Typhimurium (7)^{SGI1}
Ampicillin, kanamycin, and trimethoprim	Typhimurium (5)^{SGI1}
Ampicillin and sulfisoxasole	Typhimurium (5)^{SGI1}, Clackamas (1)
Ampicillin and trimethoprim	Typhimurium (2)^{SGI1}
Ceftiofur, kanamycin, and trimethoprim	Aqua (1)
Kanamycin and sulfisoxasole	Typhimurium (1)^{SGI1}
Kanamycin and trimethoprim	Typhimurium (2)^{SGI1}, Oranienburg (1)
Kanamycin, sulfisoxasole, and trimethoprim	Typhimurium* (1)^{SGI1}
Sulfisoxasole and vancomycin	Typhimurium (6)^{SGI1}

[a] Numbers in parentheses represent the number of isolates exhibiting the specified multisubsistence phenotype. "SGI1" indicates an isolate bearing SGI1. An asterisk indicates that the isolate subsisted on the combination of sulfisoxasole and trimethoprim after 48 h of incubation in SCS broth but that subsistence was observed after 16 h in the presence of either drug alone.

As shown in Table 1, antibiotic-subsisting isolates of *Salmonella* exhibited a variety of phenotypes in regard to *in vitro* antibiotic susceptibility in nutrient-rich medium. Subsistence on seven antibiotics (amikacin, cefepime, ceftiofur, florfenicol, kanamycin, trimethoprim, and vancomycin) was not related to growth at the same concentration (1 mg/ml) of the antibiotic in Mueller-Hinton broth. The majority of sulfisoxazole- and ampicillin-subsisting isolates (37/38 and 58/63, respectively) were capable of growing in Mueller-Hinton medium supplemented with the respective antibiotic at a concentration that equaled or exceeded the concentration used in the SCS medium (1 mg/ml). Streptomycin resistance was noted to occur in all seven isolates capable of subsisting on this drug, although the MIC values were less than 1 mg/ml, the concentration of drug used in the subsistence assay. Conversely, ceftiofur sensitivity was observed in the five isolates that subsisted on this antibiotic.

Of the 201 individual subsistence phenotypes, 95 (57 for ampicillin, 37 for sulfisoxazole, and 1 for ciprofloxacin) coincided with growth in the presence of these antibiotics in standard medium containing 1 mg/ml of the antibiotic. The majority of ampicillin-subsisting and sulfisoxazole-subsisting isolates could grow in the presence of the respective antibiotic in nutrient-rich medium. Unlike the beta-lactamase-dependent ampicillin subsistence demonstrated in the Dantas et al. study (5), beta-lactamase activity was not relevant to ampicillin subsis-

tence, since the inclusion of clavulanic acid (16 μg/ml) had no effect on the phenotype (Table 1). In 30 of the 37 sulfisoxazole-subsisting isolates exhibiting resistance to sulfisoxazole, resistance corresponds with the presence of *sul1* on SGI1 (3), as confirmed by PCR (not shown). This gene encodes a sulfonamide-insensitive version of the enzyme targeted by this class of antibacterial agents. That is, antibiotic degradation is not required for sulfonamide resistance. Therefore, resistance mechanisms do not appear to be related to the subsistence activities, especially considering that tetracycline resistance is widespread in *Salmonella* (near 100% in the isolate collection used herein; data not shown), yet no isolates subsisted on tetracycline. Subsistence may relate only to certain antibiotics, and the subsistence mechanisms, e.g., those involving catabolic enzymes, may be active only during nutrient deprivation in just a small subpopulation of a given isolate. These novel putative enzymes appear to be unrelated to beta-lactamases and do not use tetracycline as a substrate. That is, subsistence and resistance do not appear to be equivalent, as also demonstrated in the Dantas et al. study (5).

It is interesting that subsistence in SCS broth ensued fastest in the presence of the bacteriostatic drugs that perturb folate biosynthesis in bacteria. That is, these drugs are unable to kill *Salmonella*, but all of the other antibiotics examined are bactericidal. This premise is consistent with the longer incubation time required for subsistence of the single sulfisoxasole- and trimethoprim-subsisting isolate in the presence of both antibiotics (Table 2). That is, the combination of these two antibiotics is bactericidal and thus hinders the expression of subsistence, as observed with the other bactericidal antibiotics.

From a clinical perspective, subsistence would be unnecessary in a host due to abundantly available carbon sources. Nonetheless, this finding raises some issues. First, subtherapeutic antibiotics may actually select for these antibiotic-subsisting bacteria, although the rock-paper-scissors paradigm (9) of intestinal microbial dynamics suggests otherwise. That is, multiple opposing forces often maintain long-term equilibria of the profiles of bacteria present in the intestines. Second, antibiotic dispersal into the environment could result in propagation of bacteria that subsist on antibiotics, although this propagation may help to minimize environmental antibiotics and other xenobiotics, including pesticides, toxins, etc. (9). Third, antibiotic-resistant/antibiotic-subsisting bacteria are especially troubling. These microbes can resist the antibiotic while also protecting sensitive cohorts. That is, the "resistant-subsistent" strains can modify and inactivate therapeutic antibiotics until the concentration drops below the MIC for neighboring microbes sensitive to the antibiotic.

In summary, *Salmonella* spp. are able to subsist on antibiotics as a sole carbon source. The DT104 isolates were more likely to display this phenotype than all of the other isolates. This study provides insight into the ability of a food-borne enteric pathogen to subsist on antibiotics.

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