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Genotypic-Phenotypic Discrepancies between Antibiotic Resistance Characteristics of *Escherichia coli* Isolates from Calves in Management Settings with High and Low Antibiotic Use▽†

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We hypothesized that bacterial populations growing in the absence of antibiotics will accumulate more resistance gene mutations than bacterial populations growing in the presence of antibiotics. If this is so, the prevalence of dysfunctional resistance genes (resistance pseudogenes) could provide a measure of the level of antibiotic exposure present in a given environment. As a proof-of-concept test, we assayed field strains of *Escherichia coli* for their resistance genotypes using a resistance gene microarray and further characterized isolates that had resistance phenotype-genotype discrepancies. We found a small but significant association between the prevalence of isolates with resistance pseudogenes and the lower antibiotic use environment of a beef cow-calf operation versus a higher antibiotic use dairy calf ranch (Fisher's exact test, $P = 0.044$). Other significant findings include a very strong association between the dairy calf ranch isolates and phenotypes unexplained by well-known resistance genes (Fisher's exact test, $P < 0.0001$). Two novel resistance genes were discovered in *E. coli* isolates from the dairy calf ranch, one associated with resistance to aminoglycosides and one associated with resistance to trimethoprim. In addition, isolates resistant to expanded-spectrum cephalosporins but negative for bla_{CMY-2} had mutations in the promoter regions of the chromosomal *E. coli ampC* gene consistent with reported overexpression of native AmpC beta-lactamase. Similar mutations in hospital *E. coli* isolates have been reported worldwide. Prevalence or rates of *E. coli ampC* promoter mutations may be used as a marker for high expanded-spectrum cephalosporin use environments.

Antimicrobial resistance in enteropathogens is a public health problem that has increased in both nosocomial (14, 50) and community settings (21), creating barriers to effective therapies (14). There is evidence that changes in antimicrobial use may result in a reduction in the prevalence of resistant bacteria (1, 24). There are also reports of antimicrobial resistance persisting after the reduction or removal of antimicrobial selection pressure (4, 8, 18, 36, 42, 47). One mechanism by which antibiotic resistance genes may be maintained in a population is linkage with other selectively advantageous genes. For example, multidrug resistance genes may be linked on a common element, in which case selection for one resistance trait would lead to the propagation of all of the linked resistance traits (1, 9, 25, 28). In addition, there is evidence that linkage to fitness traits unrelated to antimicrobial resistance may also be a mechanism by which resistance genes are maintained in bacterial populations in the absence of a resistance advantage (29–32, 52). If this is correct, we can predict that bacterial populations growing in the absence of antibiotic selection pressure will accumulate and retain more deleterious mutations in resistance genes than bacterial populations under intense antimicrobial selection pressure. Over time, random mutations should accumulate in gene sequences that encode resistance to rarely used antibiotics because there would be fewer selection events from the use of antibiotics to eliminate them from the population. In that case, the proportion of dysfunctional resistance genes (resistance pseudogenes) could be used to assess the level of antibiotic exposure present in a given production setting or environment. We assayed field strains of *Escherichia coli* for their resistance genotypes using a resistance gene microarray developed in our laboratory and further characterized the resistance genes of those isolates having resistance phenotype-genotype discrepancies.

MATERIALS AND METHODS

Bacterial isolates. The current study used *E. coli* isolated from calf fecal samples obtained during a previous field study (5) and stored in 15% glycerol at −80°C. Isolates were previously characterized for antibiotic resistance based on a breakpoint agar diffusion assay (5). For the purposes of the current study, a single dairy calf raiser and a single beef cow-calf operation were chosen to represent two calf management types that differ greatly with respect to antimicrobial use. Calves in dairy operations are considerably more likely to be treated with antibiotics and to be fed milk supplemented with antibiotics than are calves in beef cow-calf operations (48, 49). To maximize the likelihood of detecting resistance genes, only isolates resistant to tetracycline were included in the study. For each calf management type, isolates were stratified by the resistance profile obtained during the previous study (5) and systematically selected to maximize the number of resistance profiles represented. This selection approach yielded 52 isolates from a dairy calf raiser and 29 isolates from a beef cow-calf operation (total = 81) (see Table S1 in the supplemental material).

Phenotypic antimicrobial resistance testing. Isolates were assayed using a standard disk diffusion assay (3) according to Clinical and Laboratory Standards Institute guidelines (11). The antimicrobials tested included: ampicillin (10 μg),

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TABLE 1. Isolates that had resistance genes with no corresponding phenotypes detected by disk diffusion ($G^+ P^-$)

Isolate	Herd type[a]	Resistance profile[b]	Resistance genes detected using microarray[c]
145	CC	SuT	***strA***, ***strB***, *sul2*, *tetB*
147	CC	SuT	***aadA2***, ***aadA21***, ***aph(3′)-Ia***, ***aphA7***, ***dfrA23***, *qacΔE*, ***strA***, ***strB***, *sul1*, *sul2*, *tetA*, *tetU*
151	CC	SuT	***strA***, ***strB***, *sul2*, *tetB*
3953	CC	ATK	*aphA7*, ***strA***, ***strB***, bla_{TEM1}, *tetB*
3970	CC	ASTK	*aph(3′)-Ia*, *aphA7*, ***dfrA1***, *strB*, bla_{TEM1}, *tetB*
3972	CC	ATK	***aadA1***, ***aadA2***, ***aadA21***, *aph(3′)-Ia*, *aphA7*, *cmlA*, *qacΔE*, ***sul3***, bla_{TEM1}, *tetA*
3974	CC	ATKS	*aadA2*, *aph(3′)-Ia*, *aphA7*, *qacΔE*, *strA*, *strB*, ***sul1***, ***sul2***, bla_{TEM1}, *tetE*
1011	CR	ACSSuTKSxt	***aac(3)-IVa***, *aadA1*, *aadA2*, *aadA21*, *aph(3′)-Ia*, *aph4*, *aphA7*, *cat4*, *catI*, *dhfrXII*, *qacΔE*, *strA*, *strB*, *sul1*, *sul3*, bla_{TEM1}, *tetA*
2568	CR	ACSSuTGKCazAmc	*aacC1*, *aadA2*, *aadA21*, *aadA7*, *aph(3′)-Ia*, *aph(3′)-IIa*, *aph4*, bla_{CMY-2}, *blaCTX-M-1*, *blaCTX-M-12*, *blaOXA-27*, *cat*, *cmlA*, ***dhfrII***, ***dhfrIII***, *floR*, *mpha*, *qacΔE*, bla_{TEM1}, *tetJ*, *tetK*, *texT*
2587	CR	ACSuTK	***aadA1***, ***aadA2***, ***aadA21***, *aph(3′)-Ia*, *aph(3′)-Ia*, *aph(3′)-Ia*, *aphA7*, *cmlA*, *sul3*, bla_{TEM1}, *tetA*
4034	CR	ACSuTKSxt	***aadA1***, ***aadA2***, ***aadA21***, *aph(3′)-Ia*, *aphA7*, *cmlA*, *dhfrXII*, *dhfrxIII*, *qacΔE*, *sul1*, *sul3*, *sul3*, bla_{TEM1}, *tetA*, *tetM*

[a] CC, cow-calf (beef calves); CR, calf ranch (dairy calves).
[b] A, ampicillin; Amc, amoxicillin-clavulanic acid; Amik, amikacin; C, chloramphenicol; Caz, ceftazidime; G, gentamicin; K, kanamycin; Nal, nalidixic acid; S, streptomycin; Su, sulfisoxazole; Sxt, trimethoprim-sulfamethoxazole.
[c] Genes for which a corresponding phenotype was missing are in boldface.

chloramphenicol (30 μg), gentamicin (10 μg), kanamycin (30 μg), streptomycin (10 μg), tetracycline (30 μg), ceftazidime (30 μg), amoxicillin-clavulanic acid (20 and 10 μg, respectively), nalidixic acid (30 μg), amikacin (30 μg), and sulfisoxazole (250 μg) (Hardy Diagnostics, Santa Maria, CA).

Microarray assay. The microarray was constructed using 203 60-mer oligonucleotide probes, of which 117 were for bacterial resistance genes, 16 for virulence genes, and 25 for plasmid replicon markers; the remainder included *Salmonella enterica* serogroup- and other subtype-specific markers (17). The resistance gene probes were developed *de novo* or chosen from publications and included all drug classes important in resistance surveillance of Gram-negative bacteria.

Single bacterial colonies were used to inoculate LB broth and then incubated overnight at 37°C with shaking. Genomic DNA was extracted from bacteria using the Qiagen DNeasy Tissue Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. If the DNA concentration after this step was less than 25 ng/μl, DNA was ethanol precipitated and resuspended in PCR grade water (50 μl) to ≥25 ng/μl. Elution buffer or resuspended DNA (1 μg) was diluted to 30 μl with PCR grade water and used in the nick translation labeling step. Hybridization, signal amplification, imaging, and data normalization were carried out as previously described (17).

Characterization of inactive resistance genes. We PCR amplified and sequenced resistance genes when the microarray analysis indicated that the gene was present but the expected corresponding antibiotic resistance phenotype was absent (genotype no phenotype [$G^+ P^-$]). PCR primers were designed based on accession sequences from which the array probes were designed using Primer3 software (http://primer3.sourceforge.net/). PCR constituents included Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA). Primer sequences and PCR conditions for each isolate-gene combination are listed in Table S2 in the supplemental material. PCR products were sequenced at Amplicon Express (Pullman, WA), and sequence data were analyzed using Sequencher (Gene Codes, Ann Arbor, MI). The accession sequence that was used to design the original microarray probe was used as the reference sequence to identify potential point mutations, deletions, and insertions that may contribute to gene dysfunction.

Investigation of unexplained resistance phenotypes. Plasmid profiles of isolates showing a resistance phenotype with no corresponding gene detected by array hybridizations (phenotype no genotype [$P^+ G^-$]) were obtained by using a modification of previous methods (27). Briefly, plasmid DNA was extracted using a QIAprep kit (Qiagen, Valencia, CA) according to the manufacturer's instructions and electroporated into competent *E. coli* cells (GeneHog; Invitrogen, Carlsbad, CA). Transformants were grown on solid medium containing the antibiotic of interest. Large plasmids were extracted from transformants using phenol-chloroform extraction and polyethylene glycol precipitation. Plasmid DNA was sonicated, and after blunt-end repair and dephosphorylation, the resulting DNA fragments were ligated into a pCRII-Blunt-TOPO vector (Invitrogen, Carlsbad, CA) and chemically transformed into One Shot TOP10 cells (Invitrogen). Transformants were then plated on LB supplemented with gentamicin (10 μg/ml), trimethoprim (20 μg/ml), or ampicillin (16 μg/ml), depending on the resistance phenotype of interest. Insert DNA was PCR amplified using flanking M13 primer binding sites, and products were sequenced at Amplicon Express. The resulting sequences were compared to NCBI online protein databases using the blastx query (2). When plasmids were not present, total genomic DNA was extracted, fragmented by sonication, and cloned as described above.

Amplification of the chromosomal *ampC* gene was carried out using primers Int-Hn (5′-AAAAGCGGAGAAAAGGTCCG-3′) and Int-B2 (5′-TTCCTGATGATCGTTCTGCC-3′) (35) with the following PCR protocol: 15 min at 95°C; 35 cycles including 1 min of denaturation at 95°C, 1 min of annealing at 55°C, and 1 min 30 s of extension at 72°C; and a final extension at 72°C for 10 min. Amplification products were sequenced at Amplicon Express, and resulting DNA sequences were compared to published *E. coli ampC* sequences (35) using the blastn algorithm (53).

Novel gene discovery and PCR identification. A novel aminoglycoside resistance gene was discovered by transforming fragmented DNA, followed by growth on LB medium supplemented with gentamicin (10 μg/ml). The primers and PCR conditions for the identification of this gene have been previously reported (16). A second novel gene, apparently encoding resistance to trimethoprim, was also discovered in a transformant from the same donor isolate that grew on LB medium supplemented with trimethoprim (20 μg/ml). The primers used for the identification of this gene were TMP-F (5′-CACCGTGAACACCGTGGACGCTGC-3′) and TMP-R (5′-TGCGCGCTTGCGGGTCCATTTA-3′). PCR cycling was carried out with a 15-min initial denaturation at 95°C, followed by 30 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 30s with a final extension of 7 min at 72°C.

Data analysis. Proportions of isolates with genotype-phenotype discrepancies of both types were compared using Fisher's exact test (20). Alignments of the chromosomal *ampC* sequences were performed using Mega4 (46).

RESULTS

Resistance profiles of dairy calf isolates were more variable and were more likely to be resistant to five or more antimicrobials (92.3%) than those from the beef cow-calf operation (10.3%, $P < 0.01$). Correspondingly, isolates from the dairy calf raiser harbored more resistance genes than did the isolates from the beef cow-calf operation (Tables 1 and 2). There were no isolates with resistance to ceftazidime from the cow-calf operation (0/29), compared to 17/52 from dairy calves.

As predicted, the proportion of isolates with inactive resistance genes was higher among cow-calf isolates (Fisher exact one-tailed test, $P = 0.04$) and the proportion of isolates with unexplained resistance phenotypes was higher among isolates

TABLE 2. Isolates that had resistance phenotypes with no corresponding gene detected by array hybridization (P^+ G^-)

Isolate	Herd type[a]	Resistance profile[b]	Unexplained phenotype[b,c]	Resistance genes detected by array hybridization
189	CC	ASSuTGK	A	*aadE*, *aph(3′)-Ia*, *aphA7*, *strA*, *strB*, *sul2*, *tetB*
1010	CR	SSuTGK	G	*aph(3′)-Ia*, *aphA7*, *strA*, *strB*, *sul2*, *tetB*
1013	CR	ACSTGK	A, G	*aph(3′)-Ia*, *aphA7*, *cat4*, *strB*, *tetM*
1026	CR	ASSuTGK	A	*aphA7*, *qacΔ*, *strA*, *strB*, *sul1*, *sul2*, *tetB*
1030	CR	ACSSuTKSxtNalCazAmc	A, Caz, Amc	*aph(3′)-Ia*, *aphA7*, *cat4*, *catI*, *dfr1*, *dfrA1*, *dhfrI*, *qacΔ*, *strA*, *strB*, *sul1*, *sul2*, *tetB*
1039	CR	ASSuTGKNalCazAmc	A, Caz, Amc	*aadA1*, *aphA7*, *tetB*, *aadA2*, *qacΔE*, *strA*, *strB*, *sul1*, *sul2*, *aph(3′)-Ia*
1040	CR	ASSuTGKNalAmc	A, Amc, G	*aadA1*, *aph(3′)-Ia*, *aphA7*, *qacΔE*, *strA*, *strB*, *sul1*, *sul2*, *tetB*
1063	CR	ASSuTGKSxt	A, G, Sxt	*aadA1*, *aadA2*, *aadA21*, *aadA7*, *aph(3′)-Ia*, *aphA7*, *qacΔE*, *strA*, *strB*, *sul1*, *tetB*
1067	CR	ASSuTKCazAmc	A, Caz, Amc	*aph(3′)-Ia*, *aphA7*, *strA*, *strB*, *sul2*, *tetB*
1090	CR	ACSSuTGKSxtAmikNalAmc	G, Amik	*aadA2*, *aadA21*, *aph(3′)-Ia*, *aphA7*, *bla*$_{CMY-2}$, *dhfrXII*, *floR*, *qacΔE*, *strA*, *strB*, *sul1*, *bla*$_{TEM1}$, *tetA*, *tetM*
2517	CR	ASSuTKGSxtAmikAmc	G, Amik, Sxt	*aph(3′)-Ia*, *aphA7*, *bla*$_{CMY-2}$, *qacΔE*, *strA*, *strB*, *sul1*, *sul2*, *tetB*, *tetJ*
2521	CR	ACSSuTGKSxtNalAmikCazAmc	G, Amik, Sxt	*aadA21*, *aph(3′)-Ia*, *aphA7*, *bla*$_{CMY-2}$, *floR*, *qacΔE*, *sul1*, *sul2*, *tetA*
2531	CR	SSuTGKSxt	T, G, Sxt	*aphA7*, *aph(3′)-Ia*, *sul1*
2534	CR	SSuTGKSxtAmik	Amik	*aac(6′)-II*, *aadA1*, *aadA2*, *aph(3′)-Ia*, *aphA7*, *dfr1*, *dfrA1*, *dhfrI*, *qacΔE*, *strA*, *strB*, *sul1*, *sul2*, *tetA*
2537	CR	ACSSuTGKSxtAmikCazAmc	Sxt	*aac(3)-III*, *aac(6′)-II*, *aacC2*, *aph(3′)-Ia*, *aphA7*, *bla*$_{CMY-2}$, *floR*, *qacΔE*, *strA*, *strB*, *sul1*, *sul2 bla*$_{TEM1}$, *tetA*
2538	CR	ASSuTGKSxtAmikCazAmc	Amik	*aac(3)-III*, *aacC2*, *aadA2aadA21*, *aph(3′)-Ia*, *aphA7*, *bla*$_{CMY-2}$, *dhfrXII*, *qacΔ*, *strA*, *strB*, *sul1*, *sul2bla*$_{TEM1}$, *tetB*
2540	CR	SSuTGKSxt	T, C, Sxt	*aadA1*, *aadA2*, *aadA21*, *aph(3′)-Ia*, *aphA7*, *qacΔE*, *sul1*
2545	CR	ACSSuTGKSxtAmikCazAmc	Amik	*aac(3)-IVa*, *aadA1*, *aadA2*, *aadA21*, *aph(3′)-Ia*, *aph4*, *aphA7*, *bla*$_{CMY-2}$, *dfrA23*, *dhfrXII*, *dhfrxIII*, *floR*, *qacΔE*, *sul1*, *sul2*, *sul3*
2550	CR	ACSSuTGKSxtAmikCazAmc	G, Amik	*aadA5*, *aph(3′)-Ia*, *aphA7*, *bla*$_{CMY-2}$, *dhfrVII*, *floR*, *qacΔE*, *strA*, *strB*, *sul1*, *sul2*, *bla*$_{TEM1}$, *tetA*, *tetM*
2551	CR	CSSuTGKSxtAmik	Amik	*aac(3)-III*, *aac(6′)-IIa*, *aadA1*, *aadA2*, *aadA21*, *aph(3′)-Ia*, *aphA7*, *bla*$_{CMY-2}$, *dfr1*, *dfrA1*, *dhfrI*, *floR*, *qacΔE*, *strA*, *strB*, *sul1*, *sul2*, *tetA*
2577	CR	ASSuTGKSxtAmik	G, Amik, Sxt	*aphA7*, *qacΔ*, *strA*, *strB*, *sul1*, *sul2*, *bla*$_{TEM1}$, *tetA*, *tetM*
2586	CR	ASSuTGKSxtCazAmc	A, Caz	*aadA1*, *aadA2*, *aadA21*, *aadB*, *aph(3′)-Ia*, *aphA7*, *dfr1*, *dfrA1*, *dhfrI*, *qacΔ*, *strA*, *stx1A*, *stx1B*, *sul1*, *tetA*
2612	CR	ASSuTGKSxtAmik	Amik	*aac(3)-III*, *aac(6′)-IIa*, *aacC2*, *aadA5*, *aph(3′)-Ia*, *aph4*, *aphA7*, *blaCTX-M-12*, *blaOXA-27*, *dhfrIII*, *mpha*, *qacΔE*, *strA*, *strB*, *sul1*, *sul2*, *sul3*, *bla*$_{TEM1}$, *tetB*, *tetK*, *tetM*, *tetX*
2614	CR	ASSuTGKSxtAmikNal	Amik	*aac(3)-III*, *aac(6′)-IIa*, *aacC2*, *aadA5*, *aph(3′)-Ia*, *aphA7*, *cat4*, *catI*, *dhfrVII*, *mphA*, *qacΔE*, *strA*, *strB*, *sul1*, *sul2*, *bla*$_{TEM1}$, *tetB*
4046	CR	ACSSuTGSxtNalCazAmc	G, Sxt	*aadA1*, *aadA2*, *aadA21*, *bla*$_{CMY-2}$, *dhfrXII*, *floR*, *qacΔE*, *strA*, *strB*, *sul1*, *sul2*, *sul3*, *bla*$_{TEM1}$, *tetA*

[a] CC, Cow-calf (beef calves); CR, calf ranch (dairy calves).
[b] A, ampicillin; Amc, amoxicillin-clavulanic acid; Amik, amikacin; C, chloramphenicol; Caz, ceftazidime; G, gentamicin; K, kanamycin; Nal, nalidixic acid; S, streptomycin; Su, sulfisoxazole; Sxt, trimethoprim-sulfamethoxazole.
[c] Resistance phenotype(s) for which a corresponding gene was not detected on the array.

from dairy calves (Fisher exact one-tailed test, $P = 0.00003$) (Table 3). Among the 11 G^+ P^- isolates, 7 were characterized further. DNA sequences of inactive resistance genes were compared to accession sequences, and nonsynonymous changes were found in genes for streptomycin resistance (*aadA2* and *strA*), gentamicin resistance [*aac(3)-IVa*], kanamycin resistance [*aph(3′)-Ia*], and phenicol resistance (*floR*). Among the G^+ P^- isolates with *strA* mutations, none had base changes or other mutations in the *strB* sequence (Table 4).

Characterization of unexplained resistance phenotypes revealed a novel aminoglycoside resistance gene in the 16S rRNA methylase gene family designated *rmtE* (GenBank

TABLE 3. Numbers and percentages of *E.* coli isolates with genotype-phenotype discrepancies

Category	No. (%) of isolates		Total
	Dairy calf raiser	Beef cow-calf	
G^+ P^{-a}	4 (7.7)	7 (24.1)	11
Match (no discrepancy)	24 (46.2)	21 (72.4)	45
G^- P^{+b}	24 (46.2)	1 (3.4)	25
Total	52 (100.0)	29 (100.0)	81

[a] Fisher's exact test one-tailed *P* value, 0.044.
[b] Fisher's exact test one-tailed *P* value, <0.001.

TABLE 4. Characteristics of inactive genes in isolates with positive array hybridization but lacking the corresponding phenotype

Isolate	Gene (accession no.)	Nucleotide change[a]	Amino acid change	Management	GenBank accession no.
E145	*strA* (AY055428)	A578C	Asp → Ala	Cow-calf herd	HQ380039
E147	*aph*(*3′*)-*Ia* (V00359)	A55C A80G T377C G434A A747T	Met → Leu Lys → Arg Val → Ala Arg → His Gln → His	Cow-calf herd	HQ380034
	floR (AF118107)	A1045G A1087G C1166T	Iso → Val Thr → Ala Ala → Val	Cow-calf herd	HQ380038
	strA (AY055428)	A578C	Asp → Ala	Cow-calf herd	HQ380040
E151	*strA* (AY055428)	A578C	Asp → Ala	Cow-calf herd	HQ380041
E1011	*aac*(*3*)-*IVa* (X01385)	G734_(Deletion)	Frameshift	Dairy calf raiser	HQ380033
E2538[b]	*strA* (AY055428)	A313_(Deletion)	Frameshift	Dairy calf raiser	HQ380042
	aph(*3′*)-*Ia* (V00359)	A55C A80G T377C G434A A747T	Met → Leu Lys → Arg Val → Ala Arg → His Gln → His	Dairy calf raiser	HQ380035
E2587	*aadA2* (AF071555)	A214G	Lys → Glu	Dairy calf raiser	HQ380031
	aph(*3′*)-*Ia* (V00359)	A55C A80G T377C G434A A747T	Met → Leu Lys → Arg Val → Ala Arg → His Gln → His	Dairy calf raiser	HQ380036
E3974	*strA* (AY055428)	A578C	Asp → Ala	Cow-calf herd	HQ380044

[a] Nucleotide changes are given according to the base number of the accession sequence. The leading letter represents the nucleotide at that position in the accession sequence, and the following letter represents the nucleotide at that position in the tested sequence.
[b] This isolate had a matching phenotype (i.e., kanamycin resistance) but also multiple mutations in the *aph*(*3′*)-*Ia* gene. The presence of another gene (*aphA7*) that codes for kanamycin resistance was detected by array hybridization.

accession number GU201947) (16) (Table 5). We also examined a P^+ G^- isolate that was trimethoprim resistant but had no dihydrofolate reductase gene. A sequence from this isolate was cloned into transformants that grew on trimethoprim-supplemented medium. The predicted amino acid sequence from this DNA sequence (GenBank number HQ398305) was homologous to dihydrofolate reductase enzymes from diverse bacterial species. Nine isolates had phenotypic resistance to β-lactams but no β-lactamase gene identified by the array. Five of these isolates were further characterized, and of these five, all had mutations in the promoter-attenuator regions of the *E. coli* chromosomal *ampC* gene that are known to cause overexpression of the AmpC β-lactamase (Table 6) (35, 39). A single P^+ G^- isolate with gentamicin resistance carried an *aac*(*3*)-*II* gene that codes for a gentamicin acetyltransferase and that was not represented among the original microarray probes.

DISCUSSION

Our findings support the hypothesis that lower antibiotic use settings would have more G^+ P^- strains of *E. coli* than higher antibiotic use settings. The G^+ P^- strains appeared to harbor pseudogenes, which are defined as "Inactive but stable components of the genome derived by mutation of an ancestral active gene" (33) or "…DNA sequences homologous to known genes but that have undergone one or more mutations eliminating their ability to be expressed." (23). In this study, we identified putative pseudogenes but more work is needed to determine whether these mutations prevent gene expression. The true frequency of pseudogenes, however, may have been underestimated because of the presence of multiple genes that confer similar resistance characteristics. For example, isolates E2538 and E2587 had multiple mutations in a kanamycin resistance gene [*aph*(*3′*)-*Ia*] but also had a second, distinct

TABLE 5. *E. coli* isolates with resistance phenotypes not explained by detection of a corresponding gene by microarray

Unexplained resistance phenotype(s)[a]	No. of isolates	Molecular finding(s) (no. of isolates tested)
A	3	Chromosomal *ampC* mutation[b] (2)
A, Amc	1	Chromosomal *ampC* mutation
A, Amc, G	1	ND[c]
A, Caz	1	Chromosomal *ampC* mutation
A, Caz, Amc	1	Chromosomal *ampC* mutation
A, G	1	ND
A, G, Sxt	1	ND
Amik	6	*rmtE*[d]
G	1	ND
G, Amik	2	*rmtE*
G, Amik, Sxt	3	*rmtE* and novel *dhfr* gene (2) *rmtE*, Sxt resistance unexplained (1)[e]
G, Sxt	1	ND (PCR negative for novel *dhfr* gene)
Sxt	1	Novel *dhfr* gene
T, C, Sxt	1	ND (PCR negative for novel *dhfr* gene)
T, G	1	Probe for *aac(3)-II* was not on the array, T unexplained

[a] A, ampicillin; Amc, amoxicillin-clavulanic acid; Amik, amikacin; C, chloramphenicol; Caz, ceftazidime; G, gentamicin; K, kanamycin; Nal, nalidixic acid; S, streptomycin; Su, sulfisoxazole; Sxt, trimethoprim-sulfamethoxazole.
[b] Reference 35.
[c] ND, not determined.
[d] Reference 16.
[e] Not PCR assayed yet for novel *dhfr* gene.

kanamycin resistance gene (*aphA7*) (Table 3). The proportion of phenotype-genotype discrepancies presented here is therefore likely to be an underestimate of the true prevalence of pseudogenes in the sampled *E. coli* populations. This represents a serious limitation of an assay dependent on the detection of phenotype-genotype discrepancies with the intent to discover pseudogenes; nevertheless, low antibiotic selection environments are less likely to be affected because of the lower probability that isolates will harbor multiple resistance genes (Table 2). A high prevalence of pseudogenes therefore could still be useful to identify low-selection environments, which can provide important information for targeting interventions. Moreover, the P^+ G^- findings are significant and may represent a feasible avenue to identify sites of high antibiotic selection pressure. Although we made every effort to prevent sampling bias when choosing isolates for this study, the necessity of preselecting those with at least tetracycline resistance may have biased our findings toward the null hypothesis. Tetracycline is more likely to be used among beef cattle than some other antibiotics, so inactive tetracycline resistance genes would theoretically be present in low numbers among beef breeds, as well as dairy breeds. Our findings did not include evidence of tetracycline resistance pseudogenes in either population of *E. coli*, so the impact of this potential bias is difficult to assess.

Many of the G^+ P^- isolates had inactive streptomycin resistance genes, particularly *strA-strB* and *aadA2* (Table 3). The gene pair *strA-strB* is widespread in bacteria associated with plants, humans, and animals, including bacteria from >15,000-year-old permafrost sediments (41, 44). When *strA* and *strB* are cloned separately, *strB* is not expressed because of a secondary structure barrier to the Shine-Dalgarno sequence and start codon (10), a finding consistent with our observations that nonsynonymous changes in *strA* also affect the phenotype caused by *strB*. Synonymous single-base polymorphisms observed in our *strA* sequences (C204G, T467A, A470T) have also been reported in *strA* sequences from plant pathogens (43–45). At the same time, the associated *strB* sequences in our isolates are 100% identical to each other and to published bacterial *strB* sequences from diverse bacterial species (44, 51).

The strong association between P^+ G^- isolates and the dairy calf raiser also supports the hypothesis that antimicrobial use selects for bacteria with novel or unusual resistance determinants. Many of the P^+ G^- isolates had β-lactam resistance and lacked a $bla_{\text{-CMY-2}}$ gene, which is the most common genetic determinant of expanded-spectrum cephalosporin resistance in *Enterobacteriaceae* in the United States (15). We found that these isolates had mutations in the promoter and attenuator sequences of the *E. coli* chromosomal *ampC* gene that have also been reported from human clinical *E. coli* isolates in South Africa (39), Spain (7, 40), France (12, 13, 35), Belgium (6), Norway (22), and Denmark (26). They have also been described in animal source *E. coli* isolates, including those from cattle in Canada (38) and the United Kingdom (34) and pigs in Spain (19) and in *E. coli* collected from recreational water samples in

TABLE 6. Nucleotide differences in the chromosomal *ampC* gene of *E. coli*

Isolate	Resistance [a]	Nucleotide at position:				
		−42	−18	−1	+17	+58
E. coli K-12[b]		C	G	C	G	C
ECB33[c]		T	A	T	G	T
E160	SSuT	C	A	T	G	T
E1026	ASSuTGK	T	A	T	G	T
E1030	ACSSuTKSxtNalRcazAmc	T	A	T	G	T
E1039	ASSuTGKNalRcazAmc	T	A	T	G	T
E1067	ASSuTKCazAmc	T	A	T	G	T
E2534	SSuTGKSxtAmik	C	G	C	G	C
E2537[d]	ACSSuTGKSxtAmikCazAmc	C	G	C	G	C
E2586	ASSuTGKSxtCazAmc	T	A	T	C	T

[a] See Table 5 for antibiotic abbreviations.
[b] gi|169887498:c4477499-4476196, *Escherichia coli* strain K-12 substrain DH10B complete genome.
[c] Reference 35.
[d] Isolate E2537 was positive for $bla_{\text{CMY-2}}$ according to microarray hybridization results.

Canada (37). The locations of base changes were variable, but they had the effect of creating a functional promoter and sometimes affecting the attenuator region as well (6, 7, 13, 35). The most consistently reported changes were at −42 (C → T) and −18 (G → A), creating a strong promoter (35), and this change was found consistently in our isolates (Table 5).

The relative frequency of pseudogenes could provide a biologically valid marker for overall antimicrobial selection pressure in a given environment. The prevalence of *ampC* mutations that result in AmpC overexpression in *E. coli*, however, may be a more accessible and immediate marker for environments with high selection pressure from the use of expanded-spectrum cephalosporins. The continuing debate over the public health impact of antibiotic use in agricultural animals could, in part, be resolved by molecular and ecological approaches. The ability to identify locations where selection for antimicrobial resistance is most intense would allow a correlation between antimicrobial use and the biological significance of such use. These data could guide the development of effective interventions against the development and transmission of resistant pathogens. While assays involving DNA hybridization to detect gene mutations (such as DNA microarray technologies) may become irrelevant in the near future because of rapid developments in DNA sequencing technologies, the microarray used in the present study provides a proof of concept for future studies that may take advantage of those technologies.

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