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Diverse Antibiotic Resistance Genes in Dairy Cow Manure

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ABSTRACT Application of manure from antibiotic-treated animals to crops facilitates the dissemination of antibiotic resistance determinants into the environment. However, our knowledge of the identity, diversity, and patterns of distribution of these antibiotic resistance determinants remains limited. We used a new combination of methods to examine the resistome of dairy cow manure, a common soil amendment. Metagenomic libraries constructed with DNA extracted from manure were screened for resistance to beta-lactams, phenicols, aminoglycosides, and tetracyclines. Functional screening of fosmid and small-insert libraries identified 80 different antibiotic resistance genes whose deduced protein sequences were on average 50 to 60% identical to sequences deposited in GenBank. The resistance genes were frequently found in clusters and originated from a taxonomically diverse set of species, suggesting that some microorganisms in manure harbor multiple resistance genes. Furthermore, amid the great genetic diversity in manure, we discovered a novel clade of chloramphenicol acetyltransferases. Our study combined functional metagenomics with third-generation PacBio sequencing to significantly extend the roster of functional antibiotic resistance genes found in animal gut bacteria, providing a particularly broad resource for understanding the origins and dispersal of antibiotic resistance genes in agriculture and clinical settings.

IMPORTANCE The increasing prevalence of antibiotic resistance among bacteria is one of the most intractable challenges in 21st-century public health. The origins of resistance are complex, and a better understanding of the impacts of antibiotics used on farms would produce a more robust platform for public policy. Microbiomes of farm animals are reservoirs of antibiotic resistance genes, which may affect distribution of antibiotic resistance genes in human pathogens. Previous studies have focused on antibiotic resistance genes in manures of animals subjected to intensive antibiotic use, such as pigs and chickens. Cow manure has received less attention, although it is commonly used in crop production. Here, we report the discovery of novel and diverse antibiotic resistance genes in the cow microbiome, demonstrating that it is a significant reservoir of antibiotic resistance genes. The genomic resource presented here lays the groundwork for understanding the dispersal of antibiotic resistance from the agroecosystem to other settings.

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The accumulation of antibiotic resistance (AR) determinants in human pathogens is currently one of the most significant threats to public health (reviewed in reference 1). The extensive use of antibiotics in animal agriculture is thought to have created a selection pressure for resistant organisms and made farms an important source of antibiotic-resistant bacteria and AR genes (2–5), including in human commensal bacteria (6). Antibiotic-resistant bacteria can be transferred directly from animals to humans (7, 8) and can spread to soil, food, and groundwater through the application of manure to agricultural fields (reviewed in 9, 10). Manure bacteria that do not persist in the environment may transfer resistance genes to resident soil microorganisms, including pathogens, via horizontal gene transfer (11, 12). Therefore, it is essential to understand the contribution of animal agricultural practices to the environmental AR gene pool in more detail.

Cows in the United States generate between 1.9 and 14.2 billion pounds of manure each day (13), which is applied to fields as

fertilizer and may serve as reservoirs of zoonotic pathogens and AR genes (reviewed in 14, 15). Valuable insight into the manure resistomes of pigs, cattle, and chickens has been gained from direct culturing of antibiotic-resistant bacteria (10, 16, 17), quantitative PCR (qPCR) amplification of AR genes (5, 18–20), and the exogenous isolation of plasmids carrying AR genes (21, 22). These approaches have shown that animal microbiomes are a substantial source of AR genes, especially of those conferring resistance to tetracycline and sulfonamide, which are often carried on plasmids (18, 21–26). Some of these approaches are limited by the recalcitrance of certain bacteria to growth in culture and lack of AR gene expression under the culture conditions used. For example, among the culturable bacteria, which represent nearly half of the species in the gut microbiota of animals and humans, most are restricted to growth under anaerobic conditions (27). However, most AR screens rely on aerobic culturing conditions. Similarly, qPCR is limited by the sequences of primers used to amplify target

TABLE 1 Functional metagenomic libraries constructed with DNA from dairy cow manure

Cow no.	Library name	Library type	No. of clones	Average insert size (kb)	Gb of cloned DNA	Antibiotic treatment history between September 2011 and March 2012
01	Cow01_fos	Fosmid	90,000	24.1	2.1	No treatment
	Cow01_si	Small insert	480,000	3.2	2.5	
02	Cow02_fos	Fosmid	80,000	29.1	2.3	No treatment
	Cow02_si	Small insert	1,044,780	2.5	2.6	
03	Cow03_fos	Fosmid	60,000	28.1	1.6	Repeatedly treated prior to sampling: January 2012, 125 mg ceftiofur, 5×; February 2012, 200 mg cephalirin, 20×
	Cow03_si	Small insert	1,427,800	3.1	4.3	
04	Cow04_before_fos	Fosmid	60,000	32.3	1.9	No antibiotic treatment history; sample obtained before antibiotic treatment
	Cow04_before_si	Small insert	1,304,000	2.1	2.8	
	Cow04_after_fos	Fosmid	142,800	31.3	4.4	Sample obtained after antibiotic treatment in March 2012: 125 mg ceftiofur, 5×
	Cow04_after_si	Small insert	1,428,000	2.3	1.4	
Total	10 libraries		6,117,380		25.9	

AR genes. More recently, sequence-based metagenomics has been used to explore the resistome in diverse environments, including cattle manure (28). However, sequence-based discovery is limited to those genes with similarity to known AR genes and therefore cannot identify novel AR genes. In addition, the short read length of many novel sequencing platforms prevents characterization of the genomic context of the putative AR genes.

A complementary strategy, functional metagenomics, provides a culture-independent tool that relies on expression of resistance in a surrogate host, enabling characterization and discovery of AR genes (29–31). Functional screens of metagenomic libraries constructed with DNA from human and avian feces have found that the majority of AR genes in gut microbial communities share only 40 to 60% identity with genes previously deposited in public databases, and as such might not have been recognized as resistance genes based on sequence alone (3, 32–34).

In this study, we used functional metagenomics to investigate the dairy cow microbiome as a reservoir for AR determinants to four antibiotic classes: beta-lactams, phenicols, aminoglycosides, and tetracyclines. We discovered a new clade of chloramphenicol resistance genes and a great diversity of resistance determinants originating from a diverse set of phyla. The study combined a novel sequencing technology, PacBio, with a classical functional metagenomics approach, which afforded more insight than was possible with either method alone.

RESULTS

Resistant clones in metagenomic libraries. Dairy cows are treated with antibiotics such as beta-lactams to treat and prevent mastitis (35). To assess the dairy cow microbiome as a potential source of AR genes, we constructed fosmid and small-insert metagenomic libraries from each of five manure samples (Table 1). Libraries containing a total of 25.9 Gb of DNA were screened for resistance to beta-lactam antibiotics (carbenicillin, representing the penicillin class of beta-lactam antibiotics, and ceftazidime,

representing the cephalosporin class) and kanamycin (an aminoglycoside). Fosmid libraries were also screened for resistance to tetracycline, and small-insert libraries were also screened for chloramphenicol resistance. The frequency of carbenicillin-, chloramphenicol-, kanamycin-, and tetracycline-resistant clones was consistent across libraries constructed from different cows and between small-insert and fosmid libraries (see Fig. S1 in the supplemental material). In contrast, the frequency of ceftazidime resistance was variable and detected in five of the ten libraries close to the detection limit (10^{-7}). Four of the five libraries containing ceftazidime-resistant clones were derived from manure of cows that had been treated with beta-lactam antibiotics prior to the initiation of this study (Table 1).

Identification of resistance genes. Of 87 clones with genes conferring resistance to chloramphenicol, kanamycin, tetracycline, or beta-lactam antibiotics, 80 carried unique AR genes (see Table S1 in the supplemental material). Further screening tetracycline and beta-lactam resistance rediscovered the same genes, but each new round for kanamycin and chloramphenicol resistance identified unique AR genes. This suggests a high diversity of kanamycin and chloramphenicol resistance genes in the libraries, which was corroborated by the finding that the majority of the 80 unique AR genes conferred resistance to either chloramphenicol or kanamycin (35% and 53%, respectively) (Fig. 1a). Chloramphenicol and kanamycin resistance was highly represented in all libraries (see Table S1 in the supplemental material).

The predicted protein sequences of the AR genes shared between 32% and 100% amino acid sequence identity with proteins deposited in public databases (Fig. 1b), and the average sequence identity differed among the types of AR genes. For example, all of the tetracycline resistance genes contained high (97% and 100%), and all of the chloramphenicol resistance genes shared low amino acid sequence identity (46% to 52%) (Fig. 1b) with sequences previously deposited in public databases. In contrast, the beta-

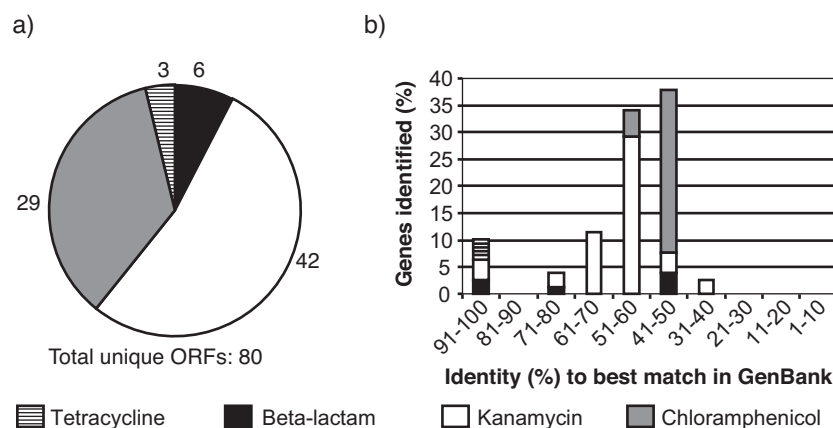


FIG 1 Antibiotic resistance genes from dairy cow manure. (a) Distribution of 80 unique antibiotic resistance (AR) genes among four classes of antibiotics. (b) Distribution of similarity of 80 unique AR genes from manure compared to homologues in GenBank.

lactamases ranged from 41 to 100% sequence identity with sequences previously deposited in the databases. Notably, all sequences were either very similar to (>96% identity) or very different from (<52% identity) sequences deposited in GenBank. This might indicate that the cow microbiome is a reservoir of AR genes which have not been identified in other environments yet.

We identified approximately the same number (21 to 26) of unique AR genes from each of the four cows, demonstrating that manure samples from different cows harbor similar-sized pools of resistance genes despite different prior exposure to antibiotics. This was reinforced by qPCR in total DNA of six of the AR genes reported here in cow manure from the pens of antibiotic-treated and untreated cows, which contained the same number of copies compared with 16S rRNA genes as the internal standard (Fig. 2). 16 rRNA copy numbers were very high, yielding low but highly sensitive detection of the AR genes.

Antibiotic resistance genes in clusters on fosmid clones. To assess the genomic context of genes conferring resistance to kanamycin, tetracycline, and beta-lactam antibiotics, we used the Pa-

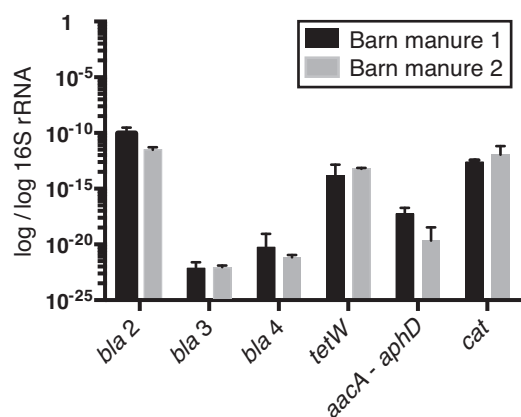


FIG 2 Quantitative PCR detection of the abundance of antibiotic resistance genes in manure. Values are expressed as the ratio of resistance gene copy number to 16S rRNA gene copy number. Genes: *bla*, beta-lactamases; *tetW*, tetracycline resistance; *aacA-aphD*, bifunctional aminoglycoside-modifying enzyme; *cat*, chloramphenicol acetyltransferase. Barn manure 1 is a sample from a pool of untreated cows, and barn manure 2 originated from a pool of antibiotic-treated cows.

cific Biosciences (PacBio) RS platform to sequence 12 fosmids. After error correction using the hierarchical genome assembly process (HGAP) pipeline (36), we obtained six completely assembled inserts ranging in size from 22.6 to 37.9 kb (Fig. 3). The other six inserts were only partially assembled, and two scaffolds smaller than 10 kb were excluded from further analyses. BLASTX analysis of the genes flanking the antibiotic resistance genes indicated that they originated from phyla that are characteristic of the cow microbiome (28, 37). *Firmicutes* were predominant (50%), followed by *Bacteroidetes* (23%), *Proteobacteria* (14%), and *Actinobacteria* (6%). Less abundant phyla included *Spirochaeta*, *Planctomyces*, *Thermotogae*, *Fusobacteria*, *Chlorobi*, *Verrucomicrobia*, and *Cyanobacteria*. One gene on one scaffold most closely matched (47% identity) an archaeal sequence affiliated with the *Euryarchaeota* (Fig. 3). Two of the clones (DCM001Kan16 and DCM001Kan17) harboring kanamycin resistance genes contained eight and five open reading frames (ORFs), respectively, with predicted functions related to AR or mobile genetic elements. One gene revealed high sequence identity (99%) to a gene encoding a bifunctional aminoglycoside-modifying enzyme, *aacA-aphD* from *Ruminococcus flavefaciens*. Although previously reported homologues in this family structurally modify a wide spectrum of aminoglycosides, including kanamycin, gentamicin, and amikacin (38, 39), the gene reported here confers resistance to kanamycin (MIC, 256 μ g/ml) and amikacin (MIC, 32 μ g/ml) and none of six other aminoglycosides tested. This clone also carries a beta-lactamase homologue but does not confer resistance to beta-lactam antibiotics. The abundance and arrangement of genes on these fosmid clones suggest that some manure bacteria harbor clusters of AR genes that may be transferred across species via mobile genetic elements. The genes putatively encoding mobile genetic elements were also diverse. These included insertion sequence (IS) elements belonging to different families (e.g., IS3 and IS116) and genes encoding other mechanisms of DNA mobility, such as excisionases, phage integrases, site-specific recombinases, and a plasmid-partitioning protein (*parB*).

Narrow- and extended-spectrum beta-lactamases. Dairy cows are often treated with beta-lactam antibiotics, making resistance to them of special interest in the cow microbiome. Of the four beta-lactamases that we identified, one (beta-lactamase 2 [*bla* 2]) was found in two different clones from the same metagenomic

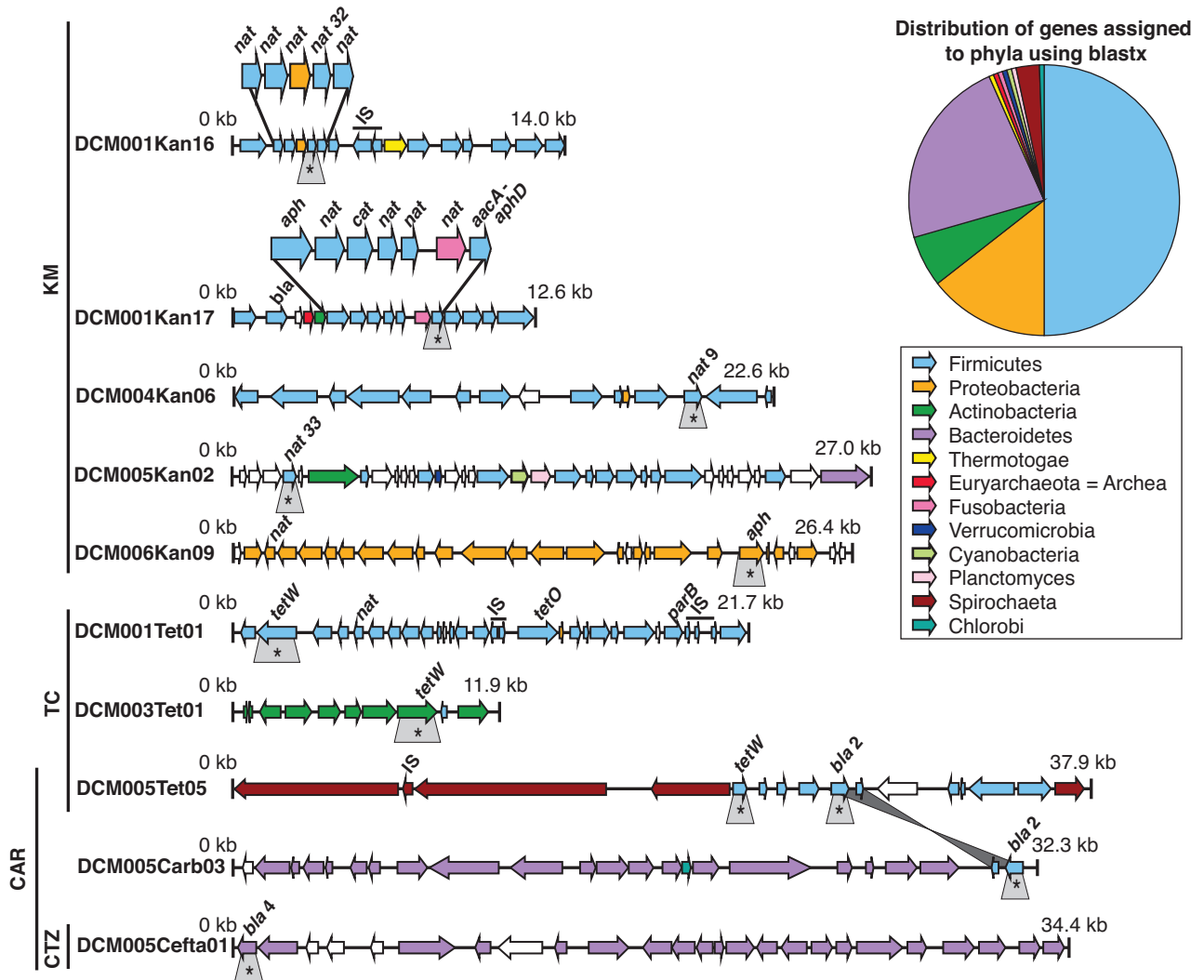


FIG 3 Organization of genes on ten metagenomic fosmid clones conferring resistance to kanamycin (KM), tetracycline (TC), carbenicillin (CAR), or ceftazidime (CTZ) assessed using Pacific Biosciences RS sequencing technology. Genes are as follows: *nat*, *N*-acetyltransferases; *aph*, aminoglycoside phosphotransferases; *aacA-aphD*, bifunctional aminoglycoside-modifying enzyme; *tetW* and *tetO*, tetracycline resistance genes; *cat*, chloramphenicol acetyltransferase; *bla*, beta-lactamases. Genes with annotations related to mobile genetic elements are labeled as IS (insertion sequence elements/transposases) or *parB* (plasmid-partitioning protein). Identical genes are connected with gray lines. Antibiotic resistance genes for which we demonstrated a function by subcloning are indicated with gray boxes and asterisks. All other labels were assigned based on sequence homology.

library and showed high sequence identity (99%) to a beta-lactamase previously found only in *Firmicutes* (Fig. 3; also, see Tables S2, S3, and S4 in the supplemental material). Although both *bla 2* genes were flanked by the same genes downstream, the two clones appear to have originated from different phyla, most likely *Bacteroidetes* and *Spirochaeta*, based on other genes on the fosmids (Fig. 3). This suggests that horizontal gene transfer distributed these beta-lactamase genes among various phyla within the cow microbiome.

To determine the relationships among all four unique beta-lactamase sequences, we constructed a phylogenetic tree (Fig. 4a). The range of similarity between the beta-lactamases from manure and previously identified sequences is striking—beta-lactamase 1 was highly identical (99%) to a beta-lactamase from *Bacillus*, whereas the highly divergent beta-lactamase 3 was 41% identical

to a beta-lactamase from the genus *Bacteroides*. Beta-lactamase 4 clustered most closely with a cephalosporinase from a *Parabacteroides* sp. and provided high-level resistance against both penicillins and third-generation cephalosporins (see Table S5 in the supplemental material). All four identified beta-lactamases belong to Ambler class A (40).

Novel clade of chloramphenicol acetyltransferases in dairy cow manure. The low sequence identity with sequences in GenBank of the chloramphenicol acetyltransferases and *N*-acetyltransferases prompted us to explore the phylogenetic affiliations of these genes. A total of 29 unique chloramphenicol resistance genes and 33 unique *N*-acetyltransferases were identified in the manure libraries (see Table S1 in the supplemental material). Phylogenetic trees based on protein sequences (Fig. 4b; also, see Fig. S2 in the supplemental material) revealed a high

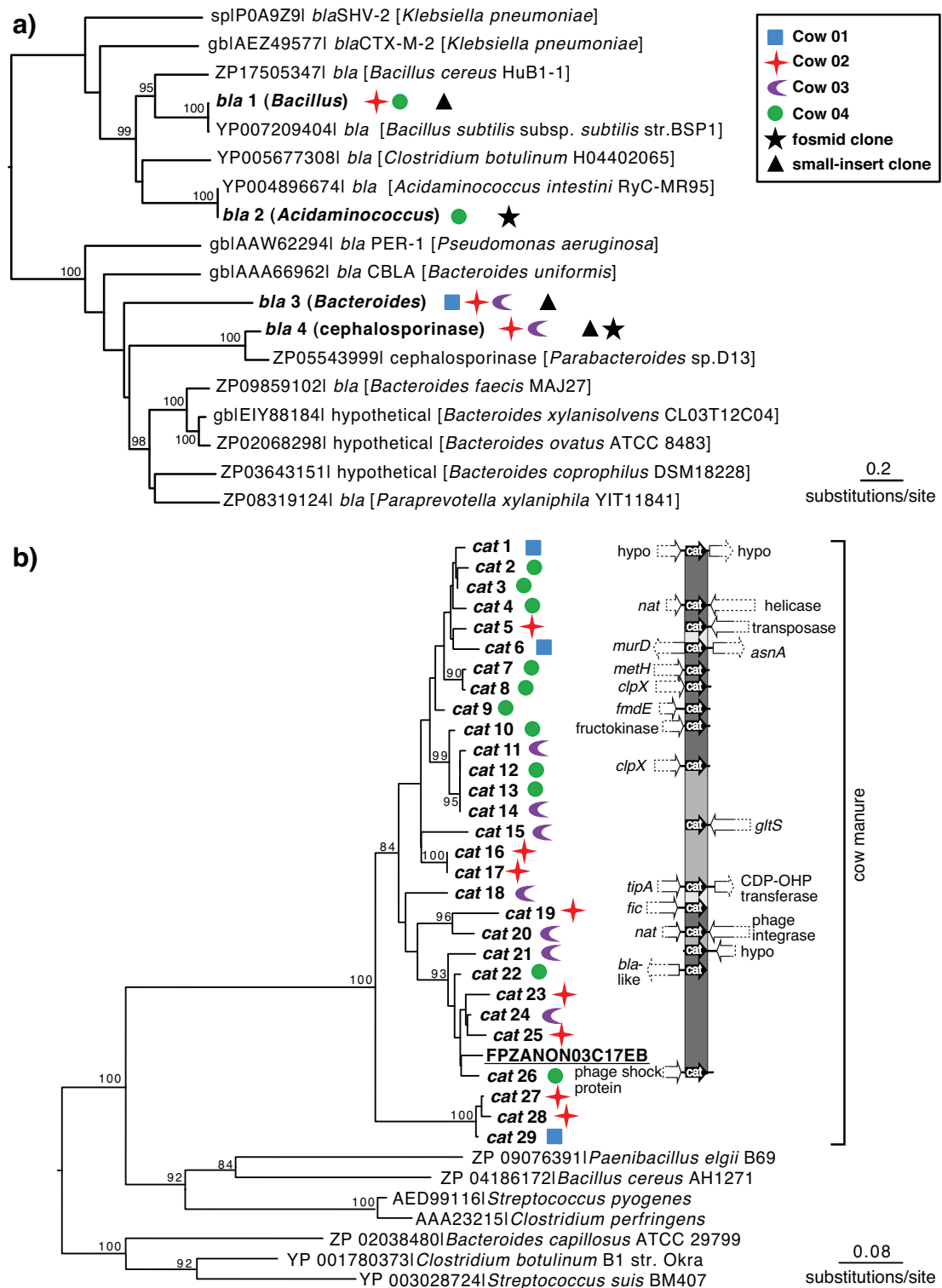


FIG 4 (a) Phylogenetic tree inferred with maximum likelihood for the predicted amino acid sequences of four different beta-lactamases (*bla*) identified in metagenomic libraries constructed from the dairy cow microbiome and related sequences retrieved from GenBank. Numbers represent bootstrap values for 100 replications. Bootstrap values of >80 are shown. (b) Phylogenetic affiliations and genomic organization of chloramphenicol acetyltransferases (*cat*) identified in dairy cow manure small-insert libraries. The phylogenetic tree was inferred with maximum likelihood based on the predicted amino acid sequences of 29 chloramphenicol acetyltransferases and the most similar sequences retrieved from GenBank; one sequence was from the MG-RAST cow fecal metagenome data set 4444130.3 and is underlined (27). The aligned sequence was 148 amino acids. The genomic organization was assessed by a BLASTX search of the available flanking DNA. Dashed parts of arrows indicate regions for which only partial DNA sequences were available. Node tips with no arrows indicate that an insufficient amount of flanking DNA was present or that there were no significant BLASTX matches found. Percentage identity between *cat* sequences is indicated by a black/gray color gradation.

diversity and divergence of the manure-derived genes. Furthermore, the phylogenetic tree strongly supported a divergent clade of chloramphenicol acetyltransferases originating from the manure samples from the four cows. The results show that dairy cow manure contains novel chloramphenicol resistance determinants that are only distantly related to previously known genes.

In order to determine whether these divergent chloramphenicol resistance genes are generally found in feces or are instead specific to the manure of cows raised at this particular farm, we interrogated metagenomic data sets of cow and buffalo rumens, animal and human fecal samples, and farm soils accessible through MG-RAST. A local TBLASTN search for the 29 newly discovered chloramphenicol resistance genes revealed significant matches to three reads in the cattle fecal data set (28) (MGRASP 4448367.3) (FQ16D9T05FSHBS, 85% identity; FPZANON03DEQ8T, 85% identity; and FLU3MGX162UVCV, 79% identity). No significant hits were found in the other MG-RAST data sets that we examined. From the cattle fecal data set, we included the nearly complete ORF on one of the reads, FQ16D9T05FSHBS, in the phylogenetic tree (Fig. 4b). This sequence clustered very closely with the 29 predicted protein sequences from the dairy cow manure metagenomic analysis, demonstrating that this type of chloramphenicol resistance is also found in cow microbiomes from another locations.

Based on sequence alignment with CLUSTALW, the chloramphenicol resistance genes shared between 71% and 99% sequence identity. Analysis of the flanking regions suggests that these AR genes likely originated from different genomic contexts (Fig. 4b). In addition, some chloramphenicol resistance genes were flanked by phage integrases or transposases, indicating that these genes may also be highly mobile within bacterial populations, which could contribute to the substantial diversity among these genes.

DISCUSSION

Using a combination of functional metagenomics and third-generation PacBio sequencing, we characterized the resistome of cow manure. The study provides a comprehensive profile of genes conferring resistance to various classes of antibiotics, including a novel clade of chloramphenicol resistance genes specific to the cow microbiome of cow manure. The resistance genes originated from diverse phyla, including *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, and *Actinobacteria*, indicating that the resistome resides in phylogenetically diverse organisms. We also found that the same AR genes are present in different taxa, and many resistance genes are flanked by mobile genetic elements such as transposases and IS elements (reviewed in reference 41). That resistance genes are present on mobile genetic elements in cow manure has been discovered previously using other approaches, including the exogenous isolation of plasmids and the use of conjugative transposons (42, 43). Together, these data indicate that these AR genes are likely horizontally transferred between bacterial species in the cow microbiome and therefore are candidates for dissemination to other bacteria in the environment, including pathogens.

Functional metagenomics in *Escherichia coli* captured diverse AR genes, some of which differed in expression, substrate specificity, and their identity as AR genes from predictions based on homology. First, although all of the genes were discovered based on their functional expression in *E. coli*, analysis of sequence alone would not have predicted their expression, as they most closely match (e.g., 58% identity) alleles from Gram-positive bacteria (in

particular, the genera *Streptococcus* and *Clostridium*). Second, the spectrum of activity (against both penicillins and third-generation cephalosporins but not first-generation cephalosporins) of beta-lactamase 4 was surprising, because it shares significant sequence identity (79%) with a broad-spectrum cephalosporinase. Similarly, the *aacA-aphD* gene on the DCM001Kan17 clone shares high similarity (99%) with a bifunctional aminoglycoside-modifying enzyme, which would predict a broad activity spectrum (38), but in fact, this gene conferred resistance to a narrow spectrum of aminoglycosides. This limited activity could be due to the specificity of different domains in bifunctional proteins that are not always active, as demonstrated previously (29). Finally, many of the genes may not have been identified as AR genes based on their sequence alone, because they deeply diverge from AR genes with demonstrated function and their closest matches are frequently annotated as hypothetical proteins. Each of these examples demonstrates the power of functional metagenomics to assign functions to new genes.

Based on the amount of DNA and diversity of antibiotics screened, this work is the most comprehensive functional metagenomic screen performed from farm microbiomes to date (3, 33, 44). We found resistance genes in cow manure at a lower frequency (3 AR genes/Gb DNA) than was observed in a previous study of chicken (14 AR genes/Gb DNA). Both studies used a replicated experimental design, but such calculations must be treated cautiously, because many factors might skew these frequencies. However, the results are consistent with animal husbandry practice in the United States, in which dairy cows are treated with antibiotics less frequently and usually to treat and prevent diseases, whereas chickens are fed antibiotics to promote growth, resulting in approximately 4-fold more antibiotics being used in chickens than cows (45).

A key finding from this work is that different cows contain similar AR genes and gene families. This result indicates that we screened to completeness and isolated the full complement of manure-derived genes that are active in *E. coli*, contrasting with studies in soil, which contains a far more diverse set of AR genes based on the observation that every soil metagenomic library appears to contain AR genes not found in other soils (29, 46). Although only five cows were analyzed, the widespread distribution of the same genes is indicated by their detection in two additional samples of pooled manure from the barns housing the cows in this study (Fig. 2) and in the cattle fecal-pool data set available at MG-RAST (MGRASP 4448367.3). Therefore, the sequences and primers found and developed in this study may be used in the future to investigate factors driving abundance, diversity, and transferability of AR genes in cow manure from additional farms administering different antibiotics or feeding different diets. To understand in more detail how agricultural antibiotic use can affect the abundance, diversity, evolution, and horizontal gene transfer of novel AR genes identified in functional metagenomic screens, larger-scale studies with manures of animals raised with heavy usage of antibiotics (e.g., pigs) are necessary.

In conclusion, this study deepens our understanding of the diversity of AR gene classes in an important farm animal whose manure is often used as fertilizer. The combination of functional metagenomics and PacBio sequencing, which produces much longer reads than 454 pyrosequencing or Illumina, generated sufficient information without too-high sequence coverage about the genomic context of AR genes to infer the taxonomic affiliation of

the origin of the DNA. This is critical for drawing conclusions about horizontal transfer of AR genes in bacterial populations. Further quantitative analysis is needed to assess the movement of AR genes between agriculture and hospitals or the food system. Such studies will provide a robust basis for management of antibiotic resistance in the future.

MATERIALS AND METHODS

Manure and fecal samples. Cows were housed at the Kellogg Dairy Unit of the University of Connecticut, and sample collection was approved by their Institutional Animal Care and Use Committee (IACUC; approval no. A11-041). Fecal samples were obtained by rectal grabbing of approximately 70 g of fecal material from four different lactating dairy cows in March 2012. A fresh palpation sleeve was used for each sample, which was immediately inverted and sealed. The cows were selected by age and antibiotic treatment history to represent a broad spectrum of manure samples (Table 1). From one cow, we obtained two samples, one before antibiotic treatment and one 4 days after treatment (Table 1). The antibiotic concentration was not quantified in the fecal samples, since this cow was given intramammary treatment for mastitis. Therefore, the antibiotic concentration is likely low, and if antibiotic is present at all in the cow gut, it is likely degraded quickly due to the omnipresence of beta-lactamases in manure (47). All animals were fed the same lactating-cow diet consisting of 71% corn silage, 14% hay silage, 3% alfalfa hay, 3% beet pulp, 4% crude protein concentrate, 4% crude protein supplement, and 1% rumen-protected fat. Cows ranged from two to 11 years old.

Cows recently treated with antibiotics for mastitis prevention were housed separately from healthy cows. To compare AR gene abundance present in manure of pens, we collected random manure samples from the ground of each of the pens occupied by untreated and antibiotic-treated cows. Both rectal grab and pen manure samples were transported on ice to Yale University and stored at -80°C until DNA was extracted.

DNA isolation and library construction. Fosmid libraries in the pCC2Fos vector (Epicentre, Madison, WI), which contains a chloramphenicol selection marker, were constructed using methods previously described for soil (46, 48), with a phenol-chloroform extraction followed by an ethanol-sodium acetate precipitation after end repair.

Manure DNA for small-insert libraries was extracted using the ZR fecal DNA miniprep kit (Zymo Research Corp., Irvine, CA) according to the manufacturer's recommendations and cloned into pCF430, which encodes a tetracycline resistance marker (49). Insert DNA was digested with PstI and ligated into the linearized vector. The reaction mix was dialyzed using 0.2- μm filter membranes (Millipore, Billerica, MA), and the plasmids were electroporated into *E. coli* DH10 β (Invitrogen, Carlsbad, CA) cells using a Micropulser (Bio-Rad, Hercules, CA). Cells were allowed phenotypic expression in super optimal broth with catabolite repression (SOC) medium for 1 h and plated on LB plates supplemented with tetracycline (5 $\mu\text{g}/\text{ml}$).

To estimate the average insert size for each library, we isolated DNA from ten randomly picked clones using QIAprep miniprep buffers (Qiagen, Valencia, CA) and chloroform extraction followed by isopropanol precipitation. For fosmids, fragments resulting from digestion with NotI (New England Biolabs, Beverly, MA) were separated on a 1% pulsed-field agarose gel (Bio-Rad) in 0.5 \times Tris-acetate-EDTA (TAE). For clones from small-insert libraries, fragments resulting from digestion with BamHI were separated on a 1% agarose gel in Tris-borate-EDTA (TBE) buffer. Clones were pooled and stored at -80°C in LB broth supplemented with 30% glycerol and chloramphenicol (20 $\mu\text{g}/\text{ml}$) or tetracycline (5 $\mu\text{g}/\text{ml}$).

Identification of antibiotic-resistant clones. Metagenomic libraries (100 μl of the pooled clones) were grown in 50 ml of LB supplemented with the appropriate marker antibiotic (either chloramphenicol or tetracycline) for 2 h at 37°C and 200 rpm. Library mixtures were serially diluted and cultured on LB plates containing an antibiotic of interest: carbenicillin (50 $\mu\text{g}/\text{ml}$), ceftazidime (4 $\mu\text{g}/\text{ml}$), chloramphenicol (12.5 $\mu\text{g}/\text{ml}$), kanamycin (50 $\mu\text{g}/\text{ml}$), or tetracycline (10 $\mu\text{g}/\text{ml}$). Fosmid and small-

insert libraries were not screened on the antibiotic used to select for the vector (chloramphenicol and tetracycline, respectively). The average frequency of resistant clones was determined by culturing each library on three replicate plates of each antibiotic. For subcloning, the resistant clones were scraped from the plates, pooled, and stored at -80°C in LB supplemented with 30% glycerol.

Subcloning or direct sequencing using primers designed to the pCF430 vector was used to investigate active genes, depending on the library and the antibiotic used for the screen (see Table S6 in the supplemental material). For subcloning, we followed previously published protocols using the pBluescript II KS(+) or the pET28b vector (46). Primers used for sequencing are listed in Table S7 in the supplemental material.

When subcloning from pooled clones as described in Table S6 in the supplemental material, twenty-four antibiotic-resistant subclones were randomly chosen and grown in 96-well square well plates. Plasmid DNA from these antibiotic-resistant subclones was purified using the Nucleospin 96 plasmid kit (Macherey-Nagel, Bethlehem, PA) and sequenced using T7 promoter and T3 primers at the DNA Analysis Facility at Yale University (<http://dna-analysis.research.yale.edu>). Antibiotic-resistant subclones containing the pET28b vector were sequenced using the T7 promoter and terminator primers.

Determination of MICs. MIC assays were conducted in Mueller-Hinton Broth (DIFCO, Franklin Lakes, NJ) according to previously published protocols (46). pCC2Fos in Epi300 and pCF430 in DH10 β were used as empty-vector controls. The MIC was defined as the antibiotic concentration that inhibited growth of 10^5 cells after 16 h at 37°C . The optical density at 600 nm (OD_{600}) was measured using a Synergy HT plate reader (Bio-Tek, Winooski, VT). All MIC assays were performed in triplicate, and the MIC was reported as the concentration at which the average OD_{600} indicated reduced visible growth ($\text{OD}_{630} < 0.1$).

Quantitative PCR. To obtain sufficiently pure DNA for quantitative PCR (qPCR), DNA was extracted from manure samples as described above for small-insert libraries and further purified using the nucleic acid purification instrument Aurora (Boreal Genomics, Los Altos, CA) (50). The primers used are listed in Table S7 in the supplemental material. Fourteen nanograms of purified DNA was used for qPCR amplification with the SsoFast EvaGreen Supermix (Bio-Rad) in a total reaction volume of 20 μl . Thermal-cycling conditions were as follows: initial denaturation step at 98°C for 3 min and then 45 cycles of 5 s at 98°C and 10 s at 60°C . The specificities of primer pairs were verified by melting curve analysis.

Sequencing of fosmids carrying AR genes. To better understand the genomic context of twelve distinct clones conferring AR, we sequenced the entire fosmid in each using Pacific Biosciences (PacBio) RS technology. DNA was prepared using the QIAprep kit (Qiagen) from 10 ml of bacterial culture in LB supplemented with chloramphenicol and 1 \times CopyControl induction solution (Epicentre). From each fosmid clone, 300 ng of DNA was pooled into a single reaction mixture and submitted to the Yale high-throughput sequencing center (<http://medicine.yale.edu/keck/ycga>). Assembly was performed using Celera (51) and trimmed for fosmid vector sequence using the ContaminationTrimmer.py command (<https://github.com/PacificBiosciences/HBAR-DTK>). The resulting scaffolds were assigned to the fosmids by performing a BLASTN search for the resistance genes and end sequences generated by Sanger sequencing of the fosmid vector using the primers recommended by the manufacturer. The assembly of 5,037 high-quality reads resulted in 45 scaffolds with an average coverage of 7.88 \times . Twelve of these scaffolds contained the expected antibiotic resistance genes.

Phylogenetic analyses and sequence comparisons to metagenomic data sets. The Geneious software (version 6.0.5) was used for sequence comparisons and phylogenetic analyses. For sequence alignments, we used CLUSTALW (52), and the phylogenetic trees were inferred using maximum likelihood. Bootstrap values were calculated based on 100 replications. To compare our sequences with other data sets, we retrieved metagenomic sequence data sets accessible through the MG-RAST server. These included the following data sets: pooled cattle feces (28) (MGRAS

number 4448367.3), chicken cecum (53) (MGRAST number 4440283.3), cow and buffalo rumens (54) (MGRAST numbers 4441679.3, 4441680.3, 4441682.3, and 4446901.3), human feces (MGRAST numbers 4440946.3 and 4444130.3), and farm soil (MGRAST number 4441091.3).

Nucleotide sequence accession numbers. All nucleotide sequences are publicly available in GenBank and shown in Table S1 in the supplemental material. Sequences were deposited in GenBank under the following accession numbers: KJ512900, KJ512901, KJ512902, KJ512903, KJ512904, KJ512905, KJ512906, KJ512907, KJ512908, KJ512909, KJ512910, KJ512911, KJ512912, KJ512913, KJ512914, KJ512915, KJ512916, KJ512917, KJ512918, KJ512919, KJ512920, KJ512921, KJ512922, KJ512923, KJ512924, KJ512925, KJ512926, KJ512927, KJ512928, KJ512929, KJ512930, KJ512931, KJ512932, KJ512933, KJ512934, KJ512935, KJ512936, KJ512937, KJ512938, KJ512939, KJ512940, KJ512941, KJ512942, KJ512943, KJ512944, KJ512945, KJ512946, KJ512947, KJ512948, KJ512949, KJ512950, KJ512951, KJ512952, KJ512953, KJ512954, KJ512955, KJ512956, KJ512957, KJ512958, KJ512959, KJ512960, KJ512961, KJ512962, KJ512963, KJ512964, KJ512965, KJ512966, KJ512967, KJ512968, KJ512969, KJ512970, KJ512971, KJ512972, KJ512973, KJ512974, KJ512975, KJ512976, KJ512977, KJ512978, KJ512979, KJ512980, KJ512981, KJ512982, KJ512983, KJ512984, KJ512985, KJ512986, KJ512987, KJ512988, KJ512989, KJ512990, and KJ512991.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01017-13/-DCSupplemental>.

- Figure S1, EPS file, 0.8 MB.
- Figure S2, EPS file, 1.3 MB.
- Table S1, DOCX file, 0.1 MB.
- Table S2, DOCX file, 0.1 MB.
- Table S3, DOCX file, 0.1 MB.
- Table S4, DOCX file, 0.1 MB.
- Table S5, DOCX file, 0.1 MB.
- Table S6, DOCX file, 0.1 MB.
- Table S7, DOCX file, 0.1 MB.

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