In-feed antibiotic effects on the swine intestinal microbiome

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Antibiotics have been administered to agricultural animals for disease treatment, disease prevention, and growth promotion for over 50 y. The impact of such antibiotic use on the treatment of human diseases is hotly debated. We raised pigs in a highly controlled environment, with one portion of the littermates receiving a diet containing performance-enhancing antibiotics [chlortetracycline, sulfamethazine, and penicillin (known as ASP250)] and the other portion receiving the same diet but without the antibiotics. We used phylogenetic, metagenomic, and quantitative PCR-based approaches to address the impact of antibiotics on the swine gut microbiota. Bacterial phylotypes shifted after 14 d of antibiotic treatment, with the medicated pigs showing an increase in *Proteobacteria* (1–11%) compared with nonmedicated pigs at the same time point. This shift was driven by an increase in *Escherichia coli* populations. Analysis of the metagenomes showed that microbial functional genes relating to energy production and conversion were increased in the antibiotic-fed pigs. The results also indicate that antibiotic resistance genes increased in abundance and diversity in the medicated swine microbiome despite a high background of resistance genes in nonmedicated swine. Some enriched genes, such as aminoglycoside O-phosphotransferases, confer resistance to antibiotics that were not administered in this study, demonstrating the potential for indirect selection of resistance to classes of antibiotics not fed. The collateral effects of feeding subtherapeutic doses of antibiotics to agricultural animals are apparent and must be considered in cost-benefit analyses.

intestinal microbiota | microbiome shifts | swine bacteria | BioTrove microarray | metagenomics

Antibiotics are the most cost-effective way to maintain or improve the health and feed efficiency of animals raised with conventional agricultural techniques (1, 2). In addition to improving feed efficiency, antibiotics are commonly given to livestock, poultry, and fish for disease treatment and prevention. The sum of agricultural antibiotic use reportedly accounts for as much as half of all antibiotics produced in the United States (3). Despite the clear benefits of antibiotics to agriculture, liberal antibiotic use combined with rapid and widespread emergence of both animal and human pathogens resistant to multiple antibiotics has led some to question the prudence of current antibiotic use (4, 5). Studies of environmental and intestinal microbial communities reveal enormous diversity of antibiotic resistance genes (6–8). The addition of antibiotics to feed introduces a selective pressure that may lead to lasting changes in livestock commensal microorganisms. Furthermore, reservoirs of antibiotic resistance genes have been shown to be stable in bacterial communities, even in the absence of antibiotics (9–12). A central concern of increased abundance of antibiotic resistance is the transfer of resistance to pathogens (13). As a result, the Food and Drug Administration recently released a draft guidance recommending restrictions on the use of antibiotics in animal agriculture (14). The Infectious Diseases Society of America testified before a Congressional subcommittee in support of such limitations (15).

Bacteria that inhabit the gastrointestinal tract of animals are important for the maintenance of host health. The intestinal microbiota assists the host in nutrient extraction, immune system and epithelium development, and are a natural defense against pathogens (16). Contrary to these benefits, the gut microbiota may antagonize future disease treatment by facilitating the dissemination of resistance alleles across distantly related organisms. For example, commensal bacteria of the human colon harbor antibiotic resistance genes and can transfer these genes to pathogens (17, 18). In fact, horizontal gene transfer is largely the cause of multidrug resistance in Gram-negative bacteria (19). With the identification of antibiotic resistance genes in commensal bacteria in the human food-chain (20–22), the role of the gut microbiota as a reservoir of resistance genes for animal and food-borne pathogens needs to be explored.

Valuable insights have been gained by culture- and PCR-based approaches to study narrow groups of bacteria or genes, such as erythromycin resistance in swine isolates (23); however, the comprehensive effects of daily feeding of subtherapeutic doses of antibiotics on livestock microbiotas have not been studied. We therefore sought to extensively evaluate the effects of in-feed antibiotics on the entire gut microbiota. Phylotyping, metagenomic, and parallel quantitative PCR (qPCR) approaches were used to track changes in microbial membership and encoded functions, enabling the detection of so-called "collateral" effects of antibiotics (i.e., effects outside of the intended growth promotion and disease prevention). These collateral effects included increases in *Escherichia coli* populations and in the abundance of certain antibiotic resistance genes.

Piglets were birthed at the National Animal Disease Center in Ames, IA, and housed together in highly-controlled, decontaminated rooms to avoid cross contamination among the medicated animals, nonmedicated animals, and other resident barn animals. Neither the piglets nor the sow were exposed to antibiotics before the study. This design was to ensure that the inoculum for the piglets would come horizontally from their mother, minimizing variability so that effects of antibiotic treatment could be detected. At 18 wk of age, one group of littermates received ASP250 feed (medicated) and the other received the same but unamended feed (nonmedicated) for 3 wk. ASP250 is an antibiotic feed additive containing chlortetracycline, sulfamethazine, and penicillin that is commonly given to swine for the treatment of bacterial

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Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. SRP004660).

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enteritis and for increased feed efficiency. Fecal samples were collected just before treatment (day 0), and after 3, 14, and 21 d of continued treatment. Day 0 samples were used to describe the swine intestinal microbiome before antibiotic treatment period.

Results

Shifts in Community Membership with ASP250. We collected 133,294 sequences of the V3 region of the 16S rRNA gene from a total of 12 fecal samples. Data from pigs of the same treatment and sampling date were grouped to appraise an antibiotic effect on community membership. As reported for a mammalian intestinal environment (24), and recently in a swine metagenome (25), the majority of classifiable sequences (75–86%) belonged to the *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* phyla (Table S1). Of the *Bacteroidetes*, the *Prevotella* genus was consistently abundant, as was shown to be a feature of the swine microbiome (25). The Bray-Curtis index was calculated for all sample combinations and an analysis of similarities (ANOSIM) was performed. A nonmetric multidimensional scaling (NMDS) plot of these data indicated divergence of the day 14 samples from the day 0 samples ($P < 0.01$), and the medicated microbiome diverged from the nonmedicated ($P < 0.05$) (Fig. 1*A*), demonstrating changes in microbial community membership over time and with treatment.

Specific changes in the microbial community associated with ASP250 treatment included a decrease in the abundance of *Bacteroidetes*, along with members of *Anaerobacter*, *Barnesiella*, *Papillibacter*, *Sporacetigenium*, and *Sarcina* genera. Members of the *Deinococcus-Thermus* and *Proteobacteria* phyla increased with ASP250 treatment as well as *Succinivibrio* and *Ruminococcus* genera (Table S1). The increase in *Proteobacteria* abundance with in-feed ASP250 was particularly striking: from 1% of the population in nonmedicated animals to 11% of the population with antibiotic treatment (Fig. 1*B*). Specifically, *E. coli* populations were the major difference between medicated and nonmedicated animals, comprising 62% of the *Proteobacteria* in medicated animals (Fig. 1*C*). The increase in *E. coli* was confirmed in the metagenomic data (Fig. 1*D*) and by qPCR targeting the *uidA* gene of *E. coli* ($P < 0.05$). A separate study using 12 pigs similarly treated but with analysis by culture-based techniques further established that swine fed ASP250 have an increased *E. coli* population at 14 d posttreatment, showing a 20- to 100-fold greater *E. coli* abundance in medicated than nonmedicated swine (Fig. S1).

Shifts in Functional Gene Abundance with ASP250. DNA samples from the feces of nonmedicated and medicated pigs at days 0 and 14 were isolated, and samples of like treatment and sampling date were pooled for pyrosequencing. Metagenome sequences (1,202,058 total) were analyzed in MG-RAST for SEED subsystems (26), and in-house for clusters of orthologous groups (COGs). All metagenomes showed functional stability over time by both COG and subsystem analyses (Fig. S2). The most abundant SEED subsystem of known function was carbohydrate metabolism, mirroring what was previously reported for the swine metagenome (25). A statistical analysis of COGs revealed shifts in microbial community functions with ASP250: the medicated metagenome contained 169 COGs that were significantly more abundant than in the nonmedicated metagenomes (Table S2). Three COGs (0477, permeases of the major facilitator superfamily; 1289, predicted membrane protein; 3570, streptomycin 6-kinase) contain swine metagenomic genes that are annotated as resistance genes in the antibiotic resistance gene database (ARDB). Three of the COGs with the lowest *P* value (3188, 3539, and 3121) contained genes

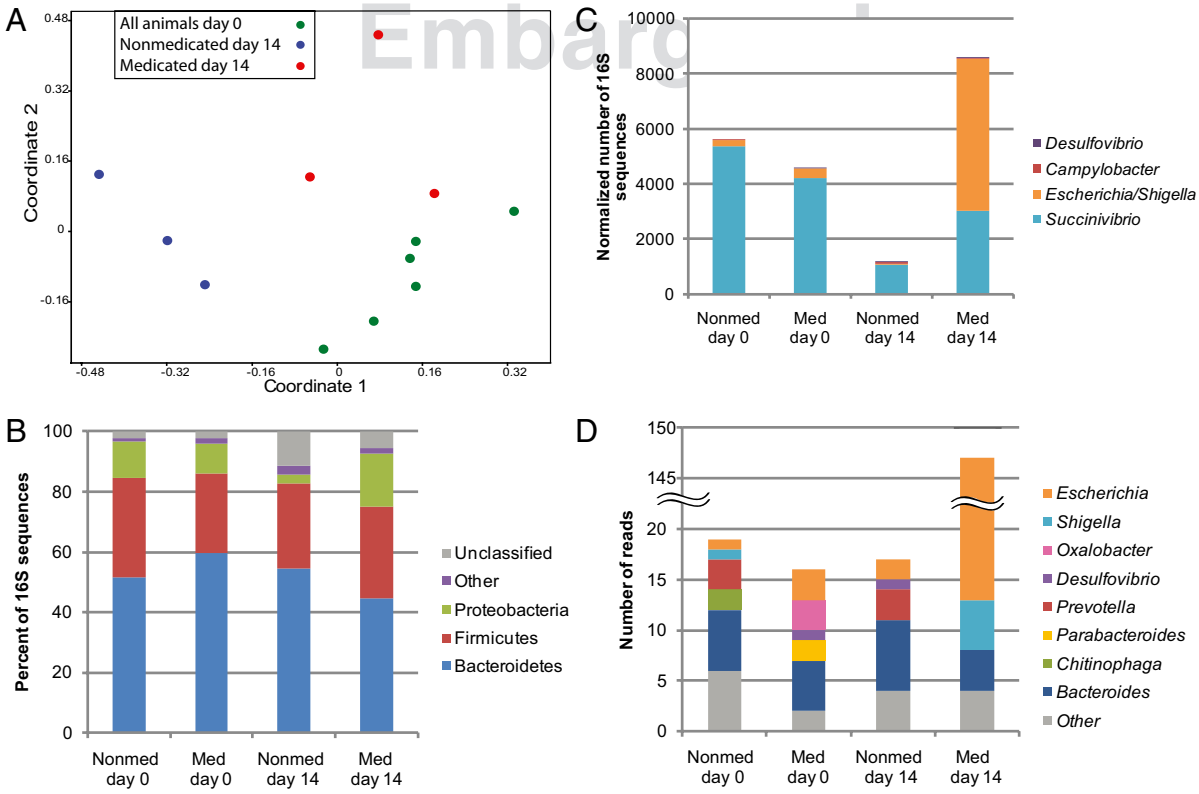

Fig. 1. Shifts in fecal bacterial community membership with antibiotic treatment. (*A*) NMDS analysis of Bray-Curtis similarity coefficients calculated from 16S rRNA gene sequence data from individual animals at days 0 and 14 shows the similarity among replicate pig fecal samples. (*B*) Phylum-level composition of fecal microbial communities. Data were pooled for a given treatment and time point and are shown as percentage of abundance. (*C*) Genus-level composition of *Proteobacteria*, shown as the total number of sequences (normalized to 50,000 total reads). (*D*) Predicted genera of COG3188 homologs found in the swine metagenomes based on BLASTx analysis. COG3188 was overrepresented in the medicated metagenome vs. the nonmedicated metagenomes.

related to P pilus assembly, and additionally among the statistically significant COGs are transposases (0675, 1662, and 4644).

To identify themes among differentially represented COGs between the medicated and nonmedicated metagenomes, COGs of Table S2 were clustered by their respective COG category. Only one COG functional category, energy production and conversion (C), was found more frequently ($P < 0.05$) in the medicated metagenome than in the nonmedicated metagenomes (Table S3).

Pervasive Antibiotic Resistance in the Absence of Antibiotic Exposure. The discovery that resistance-related COGs fluctuated with antibiotic treatment led to further scrutiny of the metagenomes by BLAST against the ARDB (27). All metagenomes, regardless of antibiotic treatment, harbored sequences similar to diverse antibiotic resistance genes representing most mechanisms of antibiotic resistance: efflux pumps, antibiotic-modifying enzymes, and modified or protected targets of the antibiotic (Fig. 2*A*). This analysis detected 149 different resistance genes in the day 0 metagenomes.

The finding of diverse fecal antibiotic resistance genes in the nonmedicated metagenomes was supported by parallel qPCR analysis. A rich array of 57 resistance genes was detected at least once in the swine fecal samples by qPCR. Samples from nonmedicated animals showed a total of 50 different resistance genes, but few were shared between animals: only five [*ermA*, *ermB*, *mefA*, *tet*(32), and *aadA*] were detected in 66% of the samples and none were found in more than 80% of the samples. No enrichment of these genes was observed in the medicated animals, even though *tet*(32), a ribosomal protection protein, is known to confer resistance to an administered antibiotic (tetracycline). Samples from medicated animals yielded more homogenous resistance gene diversity: 38 genes were detected in at least one medicated sample, 19 were detected in 66% of samples, and 10 [*mefA*, *ermA*, *ermB*, *tet*(32), *tet*(O), *aadA*, *aph(3′)-ib*, *bcr*, *acrA*, and *bacA*] were detected in at least eight of nine of the samples.

qPCR and Metagenomic Analyses Reveal Shifts in Resistance Gene Richness and Abundance in Medicated Pigs. Statistical analysis of the ARDB results showed 23 genes to be differentially represented in the medicated and nonmedicated metagenomes (Table 1). The 20 genes that were more abundant in the medicated metagenome were associated with efflux, sulfonamide resistance, and aminoglycoside resistance, the latter of which represents resistance to a class of antibiotics not present in ASP250 (Table 1).

The qPCR results mirrored the metagenomic analysis, revealing six resistance-gene types with statistically significantly greater abundance in the medicated animals than in the nonmedicated animals ($P < 0.05$): tetracycline efflux pumps, class A β-lactamases, sulfonamide resistance genes, aminoglycoside phosphotransferases, and two types of multidrug efflux (Fig. 2*B* and Table 1). No statistical difference in abundance was found for these six resistance gene types between the medicated and nonmedicated microbiomes on day 0 (Fig. 2*B*), suggesting that in-feed ASP250

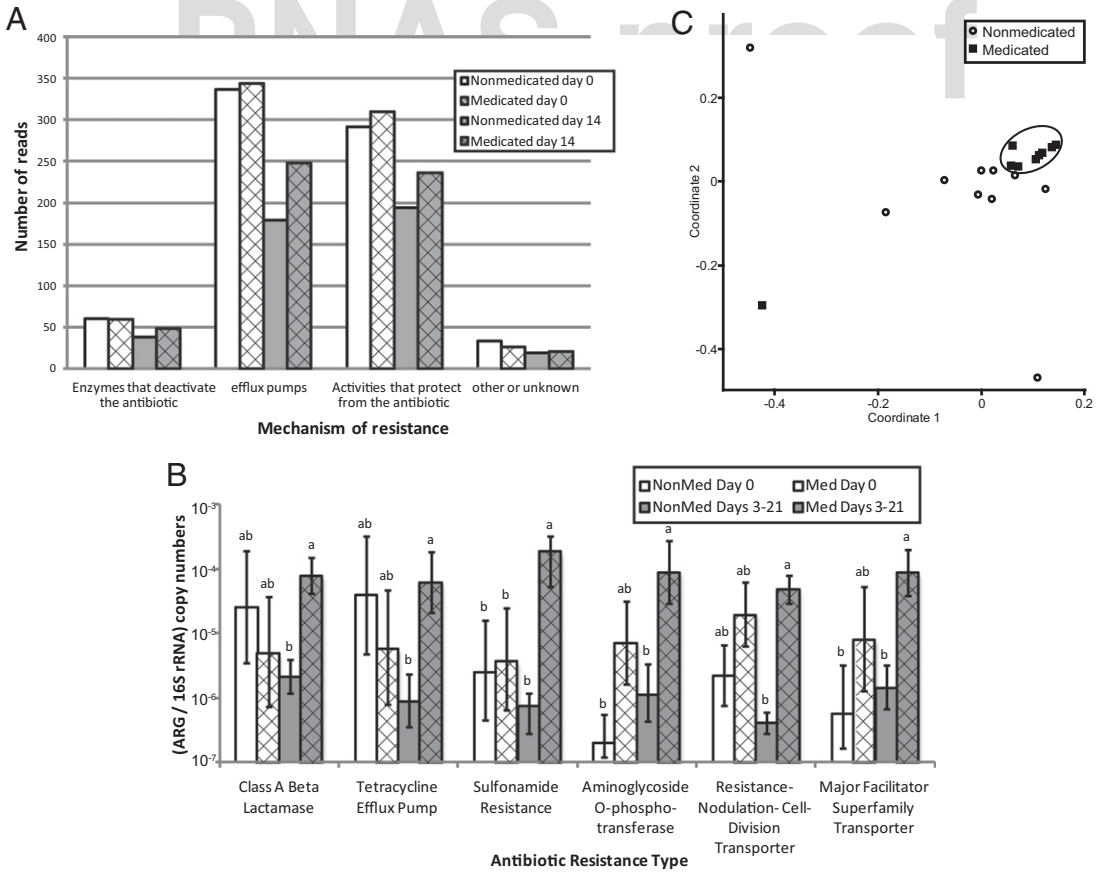

Fig. 2. Changes in diversity and abundance of antibiotic resistance genes (ARG) in swine feces with antibiotic treatment. (*A*) Metagenomes were analyzed by BLASTx against the ARDB, and the number of reads were normalized to 100,000 total reads per metagenome. (*B*) Differences in the abundance of resistance genes were assessed by calculating the ratio of resistance gene copy number (ARG) to 16S rRNA gene copy number per sample as detected by qPCR. Columns denoted by the same letter are not statistically significant ($P > 0.05$) within each resistance type. Error bars represent the SEM. (*C*) Bray-Curtis similarity coefficients were calculated from qPCR-derived resistance gene abundance data and plotted in a multidimensional scaling graph. The distance between points indicates the degree of difference in the diversity of resistance genes between samples. The medicated sample outlier (square) is from one medicated pig on day 21. Measures for day 0 samples are not shown.

Table 1. Antibiotic resistance genes differentially represented ($P < 0.05$) in the medicated vs. nonmedicated pig fecal samples as detected by metagenomics [number of sequences in the medicated ($n = 1$) vs. nonmedicated ($n = 3$) metagenomes per resistance gene] and qPCR (gene copy number/16S rRNA gene copy number) during the treatment period

Mechanism of resistance	Gene(s) detected by		Confers resistance to
	Metagenomics	qPCR	
More prevalent in the treated metagenome			
ABC transporter system. Macrolide-lincosamide-streptogramin B efflux pump.	*lmrA*		Lincomycin
Aminoglycoside O-phosphotransferase. Modifies aminoglycosides by phosphorylation.	*aph(3′′)-Ib*, *aph(6′)-Ic*, *aph(6′)-Id*	*aph(3′′)-Ib*	Streptomycin
Class A β-lactamase. Cleaves the β-lactam ring.		bla_{TEM-1}, bla_{SHV-2}	β-Lactams
Major facilitator superfamily transporter, tetracycline efflux pump. Multidrug resistance efflux pump.	*emrD*, *mdfA*, *mdtH*, *mdt*L, *rosA*, *tet*(B)	*tet*(B), *bcr*	Chloramphenicol, tetracycline, deoxycholate, fosfomycin, Florfenicol, sulfathiazole
Resistance-nodulation-cell division transporter system. Multidrug resistance efflux pump.	*adeA*, *amrB*, *mdtF*, *mdtN*, *mdtO*, *mdtP*, *oprA*, *tolC*	*acrA*	Fluoramphenicol, aminoglycoside, macrolide, acriflavine, doxorubicin, erythromycin, puromycin, β-lactams
Ribosomal protection protein. Protects ribosome from inhibition by tetracycline.	*tet*(M)	*tet*(O)	Tetracycline
Sulfonamide-resistant dihydropteroate synthase. Cannot be inhibited by sulfonamide.	*sul2*	*sul2*	Sulfonamide
More prevalent in the control metagenomes			
Resistance-nodulation-cell division transporter system. Multidrug resistance efflux pump.	*mexF*		Chloramphenicol, fluoroquinolone
Ribosomal protection protein. Protects ribosome from inhibition by tetracycline.	*tetB*(P), *tet*(Q)		Tetracycline

caused the effect. Resistance-gene abundance increased most dramatically in the 3- and 14-d samples (Fig. S3), indicating that antibiotic treatment induced a rapid shift in the abundance of resistance genes.

ASP250 treatment increased the diversity of resistance gene types as detected by qPCR [Shannon indices 1.4 (medicated) and 0.8 (nonmedicated); $P = 0.04$]. A t test comparing the mean number of resistance genes in the metagenomes at day 14 to the corresponding nonmedicated metagenome confirms this result ($P < 0.05$). Additionally, the structure of the resistance-gene communities (β-diversity) was altered by antibiotic treatment, as determined by a two-way ANOSIM ($P < 0.01$) of Bray-Curtis measures; however, the comparison R-value was 0.25, indicating that the degree of separation is limited. Nevertheless, resistance gene diversity converges with ASP250 treatment, presumably because of the selective pressure of the antibiotics (Fig. 2*C*). Taken together, these results show that feeding antibiotics increases the diversity of resistance genes within an individual sample and homogenizes that diversity between treated samples.

Discussion

We assessed the effect of ASP250 on the swine antibiotic resistome using phylotype, metagenomic, and qPCR approaches. The results show that the swine microbiome harbors diverse resistance genes even in the absence of selective pressure. Five genes in particular were detected at high frequency in both the medicated and nonmedicated microbiomes. These genes could represent a core antibiotic resistome for this cohort of swine. Indeed, it was suggested that *tet*(32) is abundant in farm animals (28), and our data support that conclusion for swine. The constant selective pressure of 50 y of in-feed antibiotics appears to have established a high background level of resistance in the swine microbiome.

Antibiotic treatment caused a detectable increase in the abundance of resistance genes even above the high background of resistance, and many of these were likely enriched because of direct interaction with the antibiotics in ASP250. For example, sulfamethazine presumably selected for the sulfonamide resistance genes *sul2* or *sul1*, present in eight of the nine medicated samples. Additionally, class A β-lactamases were overrepresented in the medicated animals and confer resistance by cleaving such β-lactam antibiotics as penicillin. Many of the other enriched resistance genes function by exporting chemicals. Such efflux includes but is not limited to antibiotics and may allow bacteria that lack specific resistance genes to survive antibiotic pressure. Multidrug efflux is frequently associated with the medically alarming issue of multiple-drug resistance and can be found on mobile genetic elements (29). In addition to the effects on specific gene families, in-feed antibiotics homogenized the richness of resistance genes among individuals over time. The breadth of the current study enabled the visualization of this intriguing phenomenon despite the tremendous resistance gene heterogeneity across samples.

One type of resistance, the aminoglycoside O-phosphotransferases, increased in abundance with in-feed ASP250, although they do no confer resistance to the antibiotics therein. This finding suggests an indirect mechanism of selection, perhaps by co-occurrence on mobile elements conferring resistance to ASP250 antibiotics. Ten of the 13 phosphotransferases identified in the medicated swine metagenome are homologous (7 of 10 have 100% amino acid identity) with the streptomycin phosphotransferase on the pO86A1 plasmid in *E. coli* O86:H- (accession number YP_788126). Resistance genes aggregate on plasmids in response to selective pressure (30), and pO86A1 carries at least two other resistance genes (accession number NC_008460). This congregation of resistance genes on mobile genetic elements could offer a fitness advantage to a bacterium living in the constant presence of antibiotics. However, this would be an undesirable collateral effect of in-feed antibiotics because these resistance gene clusters could be transferred to *E. coli* or other potential human pathogens in the swine gut or in the agriculture environment. Regardless of the mechanisms of selection, the results show that antibiotic use increased the abundance of resistance genes specific to and beyond the administered antibiotics from a diverse pool of

background resistance genes in the swine microbiome, and that this increase was detectable even above a high background of resistance-gene diversity.

The collateral effects of antibiotics extend beyond influencing resistance genes. Statistical analysis of COGs in the swine metagenomes showed that genes encoding virulence, gene-transfer, and energy production and conversion functions are selected by in-feed antibiotics. Specifically overrepresented COGs included some relating to P pilus assembly; the P pilus has been described for attachment and virulence in *E. coli* (31). Additional COGs of interest in the medicated metagenome included transposases, which are known to participate in the transfer of antibiotic resistance genes (32). These functions could enhance the stability and spread of resistance genes in microbial communities. Additionally, an increase in the abundance of genes encoding energy production and conversion functions could be a factor in growth-promoting properties of at least some antibiotics, but further experiments are required to test this. Antibiotics are thought to improve feed efficiency in agricultural animals primarily by decreasing the bacterial load, which is beneficial to the host by reducing competition for nutrients and decreasing the host's cost of responding to the microbes (2). Analysis of the swine metabolome after antibiotic treatment showed an effect on various biosynthetic pathways, including sugar, fatty acid, bile acid, and steroid hormone synthesis (33). COGs may therefore be useful signposts for identifying microbes and functions important to the performance-enhancing effects of antibiotics like ASP250.

Changes in microbial functions result from changes in microbial membership, and interesting membership shifts were detected. The decrease in *Bacteroidetes* in the treated animals may relate to the growth-promoting benefits obtained from feeding swine ASP250 as part of their diets. Obese mice have lower levels of *Bacteroidetes* relative to *Firmicutes* in their feces compared with lean mice (34). The obese mice have improved energy-harvesting capacity, presumably because of this shift, and perhaps this shift is related to improved feed conversion in swine. In addition, an increase in *E. coli* prevalence in response to oral antibiotic treatment has been reported for amoxicillin, metronidazole, and bismuth (35), metronidazole (36), and vancomycin and imipenem (37) in the mammalian gut microbiota. However, amoxicillin plus the β-lactamase inhibitor clavulanic acid administered both in the feed and intramuscularly resulted in decreased *E. coli* in pigs (38), and oral ciprofloxacin yielded decreased *Proteobacteria* populations in humans during treatment (39). These results are an important reminder of the varying collateral effects of different antibiotics. *E. coli* are both commensal and pathogenic inhabitants of mammalian gastrointestinal tracts; an increase in *E. coli* could be beneficial or harmful, either to the host or to the food chain. Additionally, increased *E. coli* populations associated with excessive weight gain in pregnant women (40) is an unfavorable result in this host but parallels a potential growth-promoting role for this bacterium in livestock. The cost and benefit of a given antibiotic for a desired outcome must therefore be carefully weighed.

Differences among the rarer members of the microbial communities between treatment and control animals are less understood and invite further investigation. Of those that increased with treatment, members of the *Deinococcus-Thermus* phylum are known for being resistant to environmental stress; these organisms have only recently been identified in the human gut (41). In addition, *Ruminococcus* spp. are common in ruminants and are frequently found in the hindgut of pigs (42). Adept at degrading cellulose, an increase in *Ruminococcus* spp. after antibiotic treatment may aid in feed conversion in swine. Taken together, the data suggest numerous possibilities for how the swine gut microbiota might be involved with the improved feed efficiency afforded by certain in-feed antibiotics.

Conclusions

The results show that even a low, short-term dose of in-feed antibiotics increases the abundance and diversity of antibiotic resistance genes, including resistance to antibiotics not administered, and increases the abundance of *E. coli*, a potential human pathogen. Additionally, analysis of the metagenomes implicated functions potentially involved with improved feed efficiency. The study design featured environmental control in a single uniform inoculum source (the mother), control of the host genetics, no exposure of the sow or piglets to antibiotics except for the treatment, and identical diet except for the inclusion of ASP250 in one group. Future studies should include other in-feed antibiotics, multiple litters of swine with robust replication, and the identification of the antibiotic-induced mechanisms that lead to increased feed efficiency. Implications of antibiotic resistance on human and animal health need to be taken into account when discussing agricultural management policies and evaluating alternatives to traditional antibiotics. With the use of antibiotics in animal agriculture at a crossroads, studies like this and others that highlight the collateral effects of antibiotic use are needed.

Materials and Methods

Full protocols are available in *SI Materials and Methods*.

Swine. Six pigs (siblings) were used in this study and were split into two groups of three: a group to receive antibiotics and a group to receive no antibiotics. Animals were raised in accordance with National Animal Disease Center Animal Care and Use Committee guidelines. The rooms housing the pigs were decontaminated before the beginning of the study. A pregnant sow was obtained from a hog farm at which she had no prior exposure to antibiotics. The piglets shared a pen with the sow for 3 wk after birth; her feces were therefore the primary bacterial inocula for the piglets. After weaning, all pigs were fed the same diet (TechStart 17–25; Kent Feeds) until the start of the study, at which point the medicated pigs were moved to a new clean room and given the above diet but containing ASP250 (chlortetracycline 100 g/ton, sulfamethazine 100 g/ton, penicillin 50 g/ton). Freshly voided feces was collected from nonmedicated and medicated animals just before treatment (medicated and nonmedicated day 0) and 3, 14 and 21 d after treatment.

DNA Sequencing. Fecal DNA was isolated by bead-beating, and the V3 region of the 16S rRNA gene was amplified and sequenced. PCR products were sequenced on a 454 Genome Sequencer FLX, using the manufacturer's protocol for FLX chemistry (Roche Diagnostics). For sequencing the metagenome, DNA from the feces was pooled by treatment group (nonmedicated, medicated) for each time point (day 0, day 14). Day 14 samples were sequenced using FLX chemistry and day 0 samples were sequenced using Titanium chemistry (Roche Diagnostics).

Phylotype Analysis. Only sequences longer than 50 bp were used for phylotype analysis (phylotyping), which totaled 133,294 sequences (70,667 unique sequences) from 12 fecal samples. After binning the samples by barcode, phylogenetic analysis and taxonomic assignments of the V3 portion of the 16S rRNA gene were made using the Ribosomal Database project Web tools (43). Additional phylotype comparisons and hypothesis testing were performed with the software package mothur (44). Bray-Curtis similarity coefficients were calculated from 16S rRNA gene sequence data from individual animals at 0 and 14 d and plotted in an NMDS graph to show the similarity among samples. MDS plots and analysis of similarities statistical tests were done in PAST (45).

Metagenomic Analysis. Sequences were dereplicated and analyzed by BLAST against the nonredundant database and ARDB (27). The BLAST reports were parsed to extract COG information, and COG frequencies were analyzed in ShotgunFunctionalizeR (46). The ARDB was kindly provided by Liu and Pop (27) so that we could perform BLASTx analyses locally. In both analyses, differences with $P < 0.05$ were significant, and the significant COGs were labeled with their respective COG category to visualize trends. For ecological analyses, the number of hits was normalized to 100,000 submitted reads and analyzed using NMDS and cluster analyses with the Bray-Curtis similarity measurement in PAST (45).

Quantitative PCR. Primer sets were grouped into 18 resistance types by subjecting all primer sets to the ARDB BLAST tool (Table S4) or by the BLAST tool in the National Center for Biotechnology Information when no results were obtained by the ARDB BLAST (Table S5). Quantitative PCR primers, reagents, and DNA samples were loaded into six subarrays of OpenArray plates (Applied Biosystems) (47). For each 33 nL qPCR reaction, 1 ng of extracted DNA was added as template. Quantitative PCR reagents and conditions were preformed as previously described (47). Relative gene copy numbers were calculated as follows: gene copy

number = $10^{(26-Ct)/(10/3)}$, where Ct equals the threshold cycle (Table S6). Amplification curves were manually inspected using quality control measures. The abundance of the 16S rRNA gene was determined (48), and *E. coli* was quantified by using a *uidA* primer set (49). Copy numbers of the *uidA* and 16S rRNA genes were calculated in relation to a standard curve, which was generated by using 10-fold dilutions of 10^8 to 10^0 copies as template, in triplicate reactions. Those reactions targeting 16S rRNA and *uidA* were preformed separately from the OpenArray platform.

Statistical Analysis of qPCR Results: Abundance and Diversity. All qPCR data were normalized between samples by dividing the gene copy number by 16S rRNA copy number and subsequently natural log-transformed to achieve normal distribution. A repeated-measures ANOVA model was used to determine if treatment or time was significantly related to the abundance of antibiotic resistance genes and Shannon diversity in different samples. The best covariance structure of the residuals for each response variable was determined and used for repeated measures ANOVA testing (SAS v9.2; SAS Institute). A Bonferroni adjustment was not used in the comparison of resistance genes or resistance gene types because of excessive reduction in power of tests; therefore, the reported *P* values were not corrected for multiple comparisons.

Shannon diversity was calculated using PAST ver. 1.87 (45) using data normalized between samples (resistance gene copy number/16S rRNA gene copy number). Bray-Curtis coefficients were calculated for each of the samples using the natural log-transformed data (50). A two-way ANOSIM was calculated using these data, considering treatment and time as the two factors. Two-way ANOSIM analysis and NMDS plots were completed using the Bray-Curtis measure for β-diversity.

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Supporting Information

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SI Materials and Methods

DNA Extractions. Feces were processed as follows for phylotype and metagenomic analysis. Ten grams of fresh feces per sample were collected and blended in 300 mL sterile PBS. After suspension, the feces were centrifuged at 250 × *g* for 5 min to remove the large particles (such as insoluble food) from the sample. The supernatant was retained and centrifuged at 10,000 × *g* for 30 min at 4 °C to pellet the bacterial cells. The supernatant was poured off and the pellet was washed by suspending it in PBS and spinning it again at 10,000 × *g* for 30 min at 4 °C. Two grams of the washed pellet was used for DNA extractions using the Power Max Soil DNA Isolation Kit following the manufacturer's protocol (MO BIO Laboratories). DNA samples were quantified on a Nanodrop ND-1000 UV-Vis spectrophotometer (Nanodrop Technologies). DNA integrity was determined by gel electrophoresis. Extracted DNA was stored at −20 °C.

16S rRNA Gene Amplification. Amplification of the V1-V3 region of bacterial 16S rRNA genes was carried out with the conserved primers 8F (5′-AGAGTTTGATCCTGGCTCAG) (1) and 518R (5′-ATTACCGCGGCTGCTGG) (2) with attached unique eight-nucleotide sequence barcodes (3). The V3 region was chosen because it was shown to be highly informative (4). PCR reactions contained 200 μM of each deoxyribonucleotide triphosphate, 2.0 μM of each primer, 2.0 U Ampligold Taq polymerase (Applied Biosystems), 2.5 mM $MgCl_2$, 50 ng template DNA, Ampligold Taq buffer (Applied Biosystems), and water to 50 μL. PCRs were performed in a PTC-225 thermal cycler (MJ Research) with the following protocol: 3 min at 95 °C, 21 cycles of (1 min at 95 °C, 30 s at 56 °C, 45 s 72 °C), and a final elongation step for 3 min at 72 °C. PCR products were separated by gel electrophoresis and purified using MinElute kit (Qiagen).

Metagenomic Analysis. Sequence replicate artifacts were removed using a local version of the 454 Replicate Filter (5) and specifying a sequence identity cutoff of 0.9, a length difference requirement of 0, and a check for a three-base identical sequence at the beginning of each cluster. The clustered sequences were assigned to clusters of orthologous groups (COGs) by using BLASTx to compare the nucleic acid sequences to the database of proteins that was originally used to identify COGs. The BLAST reports were parsed to extract COG information, and COG frequencies were calculated and tabulated using SAS (SAS Institute). COG frequencies were subsequently analyzed in ShotgunFunctionalizeR (6) using the testGeneFamilies.dircomp function and Poisson group statistics to perform gene-centric analysis between two groups [nonmedicated ($n = 3$) and medicated ($n = 1$) swine metagenomes]. Differences with $P < 0.05$ were significant, and the significant COGs were labeled with their respective COG category to visualize trends. Metagenomic sequences belonging to select significantly different COGs were analyzed to infer phylogeny. Phylogeny assignments were made by extracting sequences belonging to the COGs of interest, BLASTx comparison of those sequences to the GenBank nonredundant protein database, extraction of the top-hit accession, and retrieval of the phylogeny for that accession. COG counts were also corrected for differences in the estimated average genome size of each metagenome and reanalyzed as above, invoking the eff.nseq adjustment using the testGeneFamilies2.dircomp function (7). Because different methods of average genome size calculations could affect the outcome, COG counts were also corrected with the average genome sizes that were calculated by GAAS (8). These adjustments did not dramatically affect the results, and therefore only the results of the original ShotgunFunctionalizeR calculations are reported.

Swine metagenomes were also examined for the presence of known antibiotic resistance genes. MG-RAST (9) was used to bin sequences by subsystems. In addition, sequences were locally analyzed by BLASTx comparison of the sequences against the Antibiotic Resistance Gene Database (ARDB) (10), which was kindly provided by the ARDB authors. The BLASTx parameters were optimized for short reads and diversity by using a bitscore cutoff of ≥60 and an identity cutoff of 35%. Antibiotic resistance gene-centric analysis was carried out in R using the testGeneFamilies function as described above. Differences with $P < 0.05$ were significant. For ecological analyses, the number of hits was normalized to 100,000 submitted reads and analyzed using multidimensional scaling (MDS) and cluster analyses with the Bray-Curtis similarity measurement in PAST (11).

Design of Primers for Quantitative PCR Targeting Antibiotic Resistance Genes in Biotrove Array. Antibiotic resistance-gene reference sequences were collected using: (*i*) the Antibiotic Resistance Genes Online database, which contained 555 β-lactamase and 115 vancomycin resistance-gene sequences at the time of collection (12); (*ii*) a National Center for Biotechnology Information (NCBI) search for resistance-gene sequences; and (*iii*) literature search. Reference sequence protein IDs were used as seeds to harvest all closely related alleles from GenBank using the FunGene pipeline and repository (FGPR) (http://fungene.cme.msu.edu/index.spr). Aligned sequences from the FGPR were used to create consensus sequences using BioEdit (13). Primer sets were designed from consensus sequences and then selected or rejected following criteria previously described (14). Overall, 174 antibiotic resistance genes were targeted with 272 primer sets designed from 5,241 sequences. Primer sets were grouped into 18 resistance types by subjecting all primer sets to the ARDB (10) BLAST tool (Table S4), or by the BLAST tool in the NCBI when no results were obtained by the ARDB BLAST (Table S5). Abundance of the resistance type was the sum of individual genes within the resistance type. Antibiotic resistance gene categories used to group the results include (family: type): beta-lactamase: (*i*) class A, (*ii*) class B, and (*iii*) class C; tetracycline resistance: (*iv*) ribosome protection protein and (*v*) tetracycline efflux; (*vi*) sulfonamide resistance; macrolide-lincosamide-streptogramin B resistance: (*vii*) erm rRNA methylases, (*viii*) ATP-binding transporters, (*ix*) major facilitator family transporters, (*x*) hydrolases, and (*xi*) transferases; aminoglycoside resistance: (*xii*) acetylation, (*xiii*) adenylylation, and (*xiv*) phosphorylation; multidrug transporters: (*xv*) multidrug and toxic compound extrusion family, (*xvi*) major facilitator superfamily transporter, (*xvii*) resistance-nodulation-cell division transporter, and (*xviii*) small multidrug resistance transporter.

Validation of the BioTrove System for Quantitative PCR of Antibiotic Resistance Genes. Randomly-selected genes were tested in parallel with published PCR primers that target the same gene (Table S6). If no amplification curve was observed using the previously published primer set or if the threshold cycle was high (greater than 35), the results were confirmed further by running the quantitative PCR (qPCR) product on a 1% agarose gel and confirming presence or absence of the gene by visualization of a band of the correct length.

Validation of the BioTrove Antibiotic Resistance Genes Primer Set. Results obtained using the BioTrove platform were validated by probing samples in parallel with primers that were previously published. Antibiotic resistance genes were randomly selected for this validation insofar as a published qPCR primer set using SYBR as the dye could be obtained. Samples were probed by qPCR and in some cases by traditional PCR and gel imaging. The result obtained using the BioTrove platform was considered "true" if the previously published primer set confirmed the result. In validation of the results, in total, there were 29 instances of true positives, 46 instances of true negatives, 2 instances of false-positives, and 7 instances of false-negatives; these results translate to an 89% success rate, which we consider satisfactory. We used strict interpretation of the PCR to determine if the BioTrove platform result was accurate or not. For example, three of the false-negative instances resulted in a very faint band on a gel, or a high threshold cycle (14). It is possible that the BioTrove primers did not fail in these instances but is simply less sensitive than other PCR reactions because of the small reaction volume. We also observed that some of the BioTrove individual primer sets may be much more broad than previously published primer sets.

Culturing *Escherichia coli.* The antibiotic feed trial was repeated with an independent set of pigs. Twelve pigs (offspring from three sows) were housed and maintained as described above. Six pigs received antibiotics (ASP250) and six receive no antibiotics continuously for 21 d before being sampled.

E. coli was cultured from fresh pig intestinal contents at necropsy, from both medicated and nonmedicated animals, after 21 d of feed. Serial dilutions were plated on MacConkey plates with lactose and incubated overnight at 39 °C. Colony forming units were enumerated for each animal (Fig. S2).

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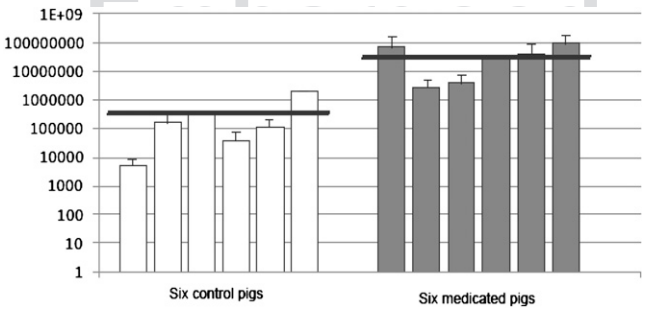

Fig. S1. *E. coli* enumerations from swine gut contents in a repeated ASP250 study. *E. coli* was cultured on MacConkey's agar from fresh gut contents from both medicated and nonmedicated animals after 21 d of feed. Wide black horizontal bars show the average colony forming units per treatment group, which are significantly different ($P = 0.04$).

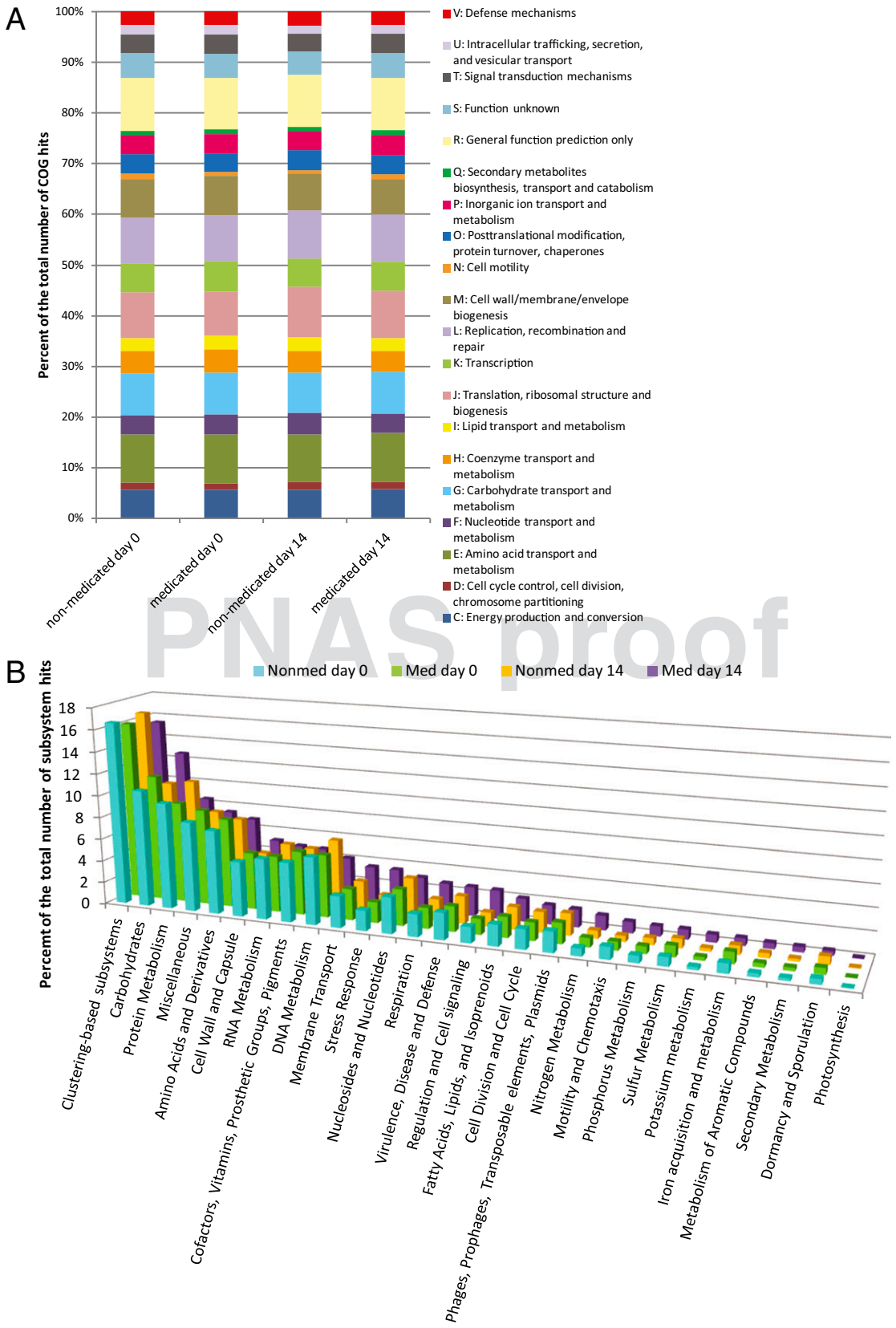

Fig. S2. Microbial functions encoded by the swine metagenomes. (*A*) COGs in the metagenomes. The following COGs were less than 0.02% of the total number of COGs per metagenome and therefore cannot be visualized on the graph: A, RNA processing and modification; B, chromatin structure and dynamics; W, extracellular structures; Y, nuclear structure; Z, cytoskeleton. (*B*) SEED subsystems in the metagenomes.

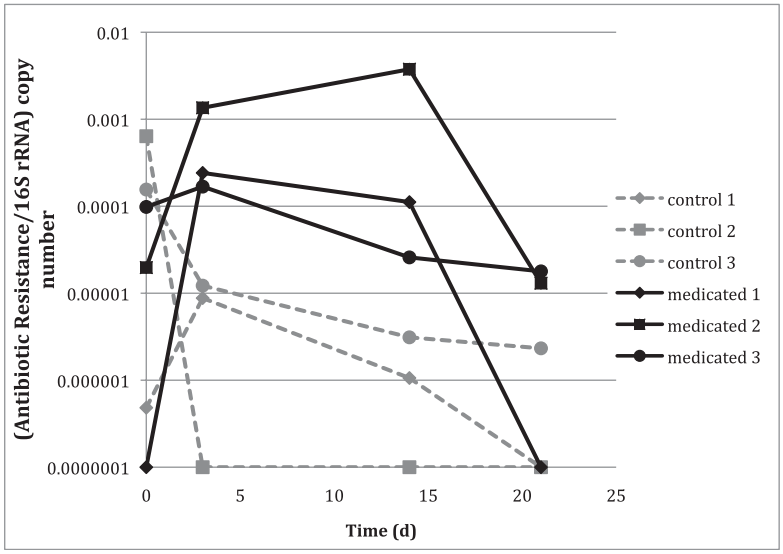

Fig. S3. Tetracycline efflux abundance trends for each treatment animal. This is a representative figure; similar trends were observed for each of the six treatment-enriched gene types. Black lines are medicated animals and gray dashed lines are nonmedicated animals.

Table S1. Phylotypes present in the swine microbiota as determined by 16S rRNA sequence analysis and grouped by treatment

Table S1 (DOCX)

Table S2. COGs that are differentially represented in the medicated (*n* = 1) vs. nonmedicated (*n* = 3) swine fecal metagenomes

Table S2 (DOCX)

Table S3. Individual COGs of the energy production and conversion COG category that were significantly more prevalent in the medicated metagenome (*n* = 1) than the nonmedicated metagenomes (*n* = 3)

Table S3 (DOCX)

Table S4. Primer sets targeting antibiotic resistance genes (specificity classified by ARDB)

Table S4 (DOCX)

Table S5. Primer sets targeting antibiotic resistance genes (specificity classified by NCBI)

Table S5 (DOCX)

Table S6. Quantitative PCR primers used in validation of the BioTrove platform antibiotic resistance results

Table S6 (DOCX)

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