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Bovine Intestinal Bacteria Inactivate and Degrade Ceftiofur and Ceftriaxone with Multiple β-Lactamases[▽]

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The veterinary cephalosporin drug ceftiofur is rapidly degraded in the bovine intestinal tract. A cylinder-plate assay was used to detect microbiologically active ceftiofur, and high-performance liquid chromatography–mass spectrometry analysis was used to quantify the amount of ceftiofur remaining after incubation with bovine intestinal anaerobic bacteria, which were isolated from colon contents or feces from 8 cattle. Ninety-six percent of the isolates were able to inactivate ceftiofur to some degree, and 54% actually degraded the drug. None of 9 fungal isolates inactivated or degraded ceftiofur. Facultative and obligate anaerobic bacterial species that inactivated or degraded ceftiofur were identified with Vitek and Biolog systems, respectively. A subset of ceftiofur degraders also degraded the chemically similar drug ceftriaxone. Most of the species of bacteria that degraded ceftiofur belonged to the genera *Bacillus* and *Bacteroides*. PCR analysis of bacterial DNA detected specific β-lactamase genes. *Bacillus cereus* and *B. mycoides* isolates produced extended-spectrum β-lactamases and metallo-β-lactamases. Seven isolates of *Bacteroides* spp. produced multiple β-lactamases, including possibly CepA, and metallo-β-lactamases. Isolates of *Eubacterium biforme*, *Bifidobacterium breve*, and several *Clostridium* spp. also produced ceftiofur-degrading β-lactamases. An agar gel overlay technique on isoelectric focusing separations of bacterial lysates showed that β-lactamase enzymes were sufficient to degrade ceftiofur. These results suggest that ceftiofur is inactivated nonenzymatically and degraded enzymatically by multiple β-lactamases from bacteria in the large intestines of cattle.

Ceftiofur is a cephalosporin antibiotic used for treating foot rot caused by *Fusobacterium necrophorum* and *Bacteroides melaninogenicus* and subclinical mastitis in dairy cattle (48, 47) and for certain indications in other animal species. Some reports claim that it may also be used for off-label indications (46). After injection, ceftiofur is rapidly metabolized by kidney and liver esterases to desfuroylceftiofur, which allows it to be conjugated to cysteine and glutathione (33). Some observations suggest that primary metabolites of these polar conjugates may be rapidly degraded further by viable intestinal microbes to inactive metabolites, since fecal slurries from cattle rapidly degrade ceftiofur, but steam-sterilized fecal slurries do not (17). Metabolites of ceftiofur are predominantly eliminated in the urine (26).

The environmental fate of antibiotics after excretion from farm animals has been studied in detail, but their metabolism by commensal microbes in the animals has not been widely explored. In one study, the chemically unrelated antimicrobial drug tylosin was shown to be degraded by bovine fecal microbes with a half-life of 6.2 days (44). The half-life of ceftiofur in feces of cattle was too rapid to measure, and addition of feces to urine containing ceftiofur decreased the half-life from 23 to 17 h, indicating that a fecal component catalyzed degradation of the drug (17).

Ceftiofur is closely related to ceftriaxone, a drug used for human therapy but not veterinary medicine, which may suggest a role for ceftiofur in the development and dissemination of resistance to ceftriaxone (2, 15, 16, 46). The risk of antimicrobial drug resistance selection by an antibiotic may be proportional to its environmental persistence. Ceftiofur does not persist in animal tissues, having a zero-day slaughter withholding interval (20). Ceftiofur breaks down in acidic and alkaline environments (25) and in soils (17). Additionally, the drug may be metabolized by microorganisms, which would also reduce its half-life in the environment.

The principle mechanism of microbial degradation of ceftiofur may be initiated by β-lactamases, which are heat-labile proteins, based on evidence that fresh feces from cattle but not autoclaved feces rapidly degrades ceftiofur (17). Hydrolysis of ceftiofur by β-lactamases is supported by several studies showing that these enzymes confer resistance to the drug (1, 10, 21). A variety of β-lactamases are produced by species in the intestinal microbiota of cattle that can readily pass between animals in a herd (37, 46). These include molecular class A (functional group 2) enzymes, some of the extended-spectrum β-lactamases (ESBLs), carbapenemases that belong to molecular classes A and D, the metallo-β-lactamases that are molecular class B (functional group 3), and the chromosomal cephalosporinases from molecular class C (functional group 1), which may be present in the bovine intestinal microbiome and have activity against ceftiofur (6, 7, 46). Multidrug-resistant *Escherichia coli* strains resistant to ceftiofur are prevalent in dairy calves soon after birth (13). The chromosomal *ampC* gene has traditionally been considered the predominant β-lactamase in bacteria from animals that have not been treated with antibiotics (37), but it is not clear whether natural selection resulting from antibiotic use is really responsible for dissemination of mobile resistance genes (46). Additional mechanisms that inactivate ceftiofur without degradation of the

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drug molecule may exist in bovine intestinal tracts, such as adsorption to bacteria and fecal molecules (11). In fact, adsorption of drug, instead of β-lactamase activity, was the major inactivation mechanism in human feces in the 1993 study (11).

A recent publication (38) reported degradation of ceftiofur by some bacterial isolates from bovine feces, but none of them were recognized commensal bacteria from the bovine intestinal tract (14). In the present study, facultative and obligate anaerobes isolated anaerobically from bovine feces and colon contents were identified and characterized for their ability to functionally inactivate ceftiofur using a cylinder-plate assay, and their ability to degrade ceftiofur was determined using high-performance liquid chromatography (HPLC) and HPLC-mass spectrometry (MS) techniques. The growth inhibition of the bacterial isolates by β-lactam antibiotics was identified. Bacterial lysates were analyzed by isoelectric focusing and nitrocefin reactions to differentiate β-lactamases. Genes with sequence identities to known and putative β-lactamase genes encoded in the bacterial DNA were detected by PCR.

MATERIALS AND METHODS

Microbial isolation and identification of bovine intestinal bacteria and fungi. Bovine feces were collected from cows that had not been treated with cephalosporins. Fecal material was scooped from the center of a freshly defecated bolus into sterile sample cups, which were placed into plastic anaerobe jars with PackAnaero sachets (Mitsubishi Gas Co. via Remel, Lenexa, KS) and transported to the laboratory. Alternatively, colon contents were collected from cows at the abattoir by direct incision into the colon immediately after the cows were slaughtered. The samples were transported anaerobically to the laboratory, where they were immediately transferred to an anaerobic chamber with an atmosphere of 91 to 93% N_2, 2 to 4% H_2, and 5% CO_2 (Coy, Grass Lake, MI) and cultures were set up in broth and agar media. Glycerol was added to the remaining material to a final concentration of 10%, and the samples were frozen at −70°C for later assays. Bacteria were isolated on brucella blood agar, *Bacteroides* bile esculin agar, laked blood kanamycin-vancomycin agar, egg yolk agar, *Bifidobacterium* selective agar, *Bacteroides vulgatus* selective agar, phenylethyl alcohol agar, and chocolate agar (all from Anaerobe Systems, Inc., San Jose, CA). All bacterial isolations were carried out in the absence of ceftiofur selective pressure. Cultures were incubated for 48 h at 35°C in the anaerobic chamber. Anaerobic (MTGE) enrichment broths were inoculated from anaerobic transport swabs collected at the time of sampling (Anaerobe Systems). These were cultured overnight anaerobically at 35°C and then subcultured to the same plate media described above for bacterial isolation. Additional media (Trypticase soy agar with 5% sheep blood, MacConkey agar, phenylethyl alcohol agar, and cetramide agar from Remel) were inoculated, removed from the anaerobic chamber, and incubated in a 5% CO_2 aerobic incubator at 37°C overnight. The isolated bacteria were frozen in sterile SS stabilizer (8% nonfat dry skim milk, 5% sucrose, 0.5% thiourea) for subsequent evaluations.

The obligate anaerobes were verified by failure to grow in aerobic subculture on Trypticase soy agar with 5% sheep blood. All obligate anaerobic isolates were characterized by Gram stain reaction and identified with a Biolog system (Biolog, Hayward, CA). The facultative anaerobes were characterized by Gram stain reaction and identified with a Vitek 2 Compact system (bioMérieux, St. Louis, MO). Facultatively anaerobic fungi were isolated by streak plate culture on Sabouraud's dextrose agar medium (Remel) for 4 days at 42°C in air. Colonies of filamentous fungi were lifted from the plates with cellophane tapes, the fungal elements were stained with lactophenol cotton blue stain, and the tapes were adhered to glass slides for microscopic observation. The fungi were identified according to their colony and microscopic morphologies. Yeasts that grew in colonies on the plates were identified with the Vitek 2 Compact system (bioMérieux).

Cylinder-plate assay. A cylinder-plate assay (25) was used to measure ceftiofur antimicrobial activities in bacterial culture supernatants. Ceftiofur (1 μg/ml) was added to overnight cultures of test bacteria and incubated for 6 h at 37°C. The cultures were centrifuged at 10,000 × *g* for 2 min, and the supernatants were tested for residual ceftiofur activity after inactivation (binding) and degradation. Cultures of cephalosporin-sensitive *Kocuria rhizophila* (*Micrococcus luteus*) ATCC 9341 (American Type Culture Collection, Manassas, VA) were grown in Trypticase soy broth (TSB) at 37°C for 15 h. The cultures were diluted to the concentration of a no. 3 McFarland nephelometer standard (9×10^8 CFU/ml) and added to 6 ml of antibiotic medium 8 agar (5) at a 10% concentration. The agar suspensions were poured into petri dishes containing 10 ml antibiotic medium 8 agar and allowed to solidify at room temperature. Stainless steel cylinders were placed on end on the surface of the plate, and 200 μl of supernatant, control, or ceftiofur standard dilution was added. Controls were culture supernatants without ceftiofur, to assess the inhibitory effects of the supernatants on *K. rhizophila* growth. The plates were incubated at 37°C for 15 h, and the diameters of zones of growth inhibition were measured to the nearest 0.1 mm. Calibration curves were generated using serial dilutions of ceftiofur in culture medium that had been incubated simultaneously with the test samples to control for drug instability over the incubation time. The amounts of biologically active drug remaining in the test samples were estimated from the calibration curves by linear least-squares analysis and are presented as a percentage of the original inhibition of *K. rhizophila* growth remaining in the culture supernatants after incubation. Ceftriaxone inactivation was tested using the same method, but with substitution of ceftriaxone for ceftiofur in both the bacterial and standard samples.

HPLC and HPLC-MS detection of ceftiofur and desfuroylceftiofur in culture supernatants. Residual ceftiofur and desfuroylceftiofur concentrations in the bacterial cultures that were analyzed by the cylinder-plate assay were determined using a modification of the desfuroylceftiofur acetamide assay (25). After 6 h of incubation of 1 μg/ml ceftiofur with test bacteria, the culture was diluted 1:1 with 20% trichloroacetic acid and the pH of the solution was raised to 9.0 with 1 N NaOH. A 1-ml sample of the solution was combined with 5 ml DTE extraction solution (0.4% [wt/vol] dithioerythritol in 0.2 M sodium borate made fresh on the day of extraction) and incubated for 15 min at 65°C. One milliliter iodoacetamide solution (15% [wt/vol] iodoacetamide in 0.025 M phosphate buffer, pH 7.0, made fresh on the day of extraction) was added, and the solution was incubated for another 15 min at 65°C. A 1-g C_{18} solid-phase extraction column (Mega Bond Elute; Varian, Inc., Palo Alto, CA) was washed with 5 ml methanol, 5 ml H_2O and then loaded with the derivatized sample solution. The column was washed twice with 5 ml of 0.1% phosphoric acid, and the sample was eluted with 5 ml C_{18} elution solution (15% aqueous acetonitrile). The eluate was diluted with 10 ml H_2O and applied to a 500 mg SAX anion-exchange solid-phase-extraction column (Bond Elute; Varian, Inc.), which was pretreated with 2 ml methanol, 2 ml methanol–0.1 M NaCl (25:75, vol/vol), and then 2 ml H_2O. The column with loaded sample was washed with 1 ml C_{18} elution solution, 1 ml H_2O, and the sample was eluted with 5 ml 1% acetic acid. The eluate was diluted with 10 ml H_2O, before it was loaded onto a 100-mg SCX cation-exchange SPE column (Bond Elute; Varian, Inc.) prewashed with 2 ml methanol, 2 ml methanol–0.1 M $CaCl_2$ (25:75, vol/vol), and 2 ml H_2O. The column was loaded with sample and washed with 1 ml methanol and 1 ml 0.1% acetic acid. The column was dried with vacuum for 30 s, and the sample was eluted with 1 ml 0.1 M ammonium acetate.

The eluates were analyzed by either HPLC or HPLC-MS. HPLC analysis was performed using an Agilent Technologies series 1200 system attached to a G1315D photodiode array detector (Agilent Technologies, Santa Clara, CA). A Phenomenex Luna 5-μm PFP2, 250- by 4.6-mm pentafluorophenyl propyl column (Phenomenex, Inc., Torrance, CA) was used, and the mobile phase was a 15-min gradient of 5 to 90% methanol in water–0.005% formic acid at a flow rate of 1 ml/min. HPLC with photodiode array/electrospray ionization mass spectrometry was performed using a TSQ Quantum Ultra mass spectrometer (Thermo Fisher, Houston, TX) with an Agilent Technologies 1100 series HPLC system. A Phenomenex Prodigy 5 μm ODS-3 analytical column (250 mm by 2.0 mm) was used, and the mobile phase was a 40-min gradient of 5 to 95% acetonitrile in water with 0.1% formic acid at a flow rate of 0.2 ml/min. Photodiode array spectra were acquired from 190 to 550 nm, and quadrapole 1 was scanned from *m/z* 400 to 600 in 0.5 s. The areas under the *m/z* 487 peaks were measured to quantify the ceftiofur or ceftriaxone concentrations remaining in processed culture supernatants after 6-h incubations with bacteria.

Etest. Since many commensal anaerobic bacteria have no susceptibility breakpoints established by international committees, the growth responses of bacterial isolates to β-lactam antimicrobial drugs were measured using Etest strips according to the manufacturer's directions (bioMérieux). Obligate anaerobes were tested on brucella blood agar plates supplemented with vitamin K and hemin (Remel), and facultative organisms were tested on Mueller-Hinton agar plates (Remel), as described in the manufacturer's instructions, which included interpretive criteria. Expected Etest MIC endpoints of control organisms (*E. coli* ATCC 25922, *E. coli* ATCC 35218, *Bacteroides fragilis* ATCC 25285, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 29213, and *Klebsiella pneumoniae* ATCC 700603) were used to verify quality control of the assays.

TABLE 1. PCR primer sequences for β-lactamase gene detection

Oligonucleotide identification	Sequence	Size (bp)	Reference[a]
Bacteroides fragilis bla$_{CepA}$-F	TTTCTGCTATGTCCTGCCC	780[b]	3
Bacteroides fragilis bla$_{CepA}$-R	ATCTTTCACGAAGACGGC		
Bacteroides fragilis bla$_{CfiA}$-F	TCCATGCTTTTCCCTGTCGCAGTTAT	728	45
Bacteroides fragilis bla$_{CfiA}$-R	GGGCTATGGCTTTGAAGTGC		
*Bacillus cereus bla*I-F	GTGGATGAAAGGAAATGCTACG	156[b]	28
*Bacillus cereus bla*I-R	ATTGCGATGATAATTGGTGCTC		
*Bacillus cereus bla*II-F	GCGTCAGCACATTCTCAATCG	165[b]	12
*Bacillus cereus bla*II-R	ATCCAGGGAAAGGACATACAGAAG		
*Bacillus cereus bla*III-F	TGCGGGTGTACCAAAAGGATGGG	464[b]	24
*Bacillus cereus bla*III-R	GGGTGGTGCAAGTGGGAAAGCAA		
Bacteroides vulgatus bla$_{CfxA}$-F	ACTCTAACTATACATCTCCTCTTG	193	34
Bacteroides vulgatus bla$_{CfxA}$-R	TAACCTGAACCTGTCTTATGC		
Bacteroides uniformis bla$_{CblA}$-F	GGTGGAAGAGCAGTTAGG	181	43
Bacteroides uniformis bla$_{CblA}$-R	CTGATAGGCTACCGAAGTC		
*Bacillus mycoides blaC*1-F	ATTGCTGAGGCTGCTGTTC	173[b]	18
*Bacillus mycoides blaC*1-R	TCACGAATGTCTCCTGGAATAG		
Clostridium perfringens bla-F	GAGGAAAAGCAGAAAGAAGAGAAG	145	41
Clostridium perfringens bla-R	CATGAGCAAAGCGGTTACTATTAC		
Bifidobacterium longum bla-F	GGTGCCTTCGGGGATGTG	194	27
Bifidobacterium longum bla-R	CCCTTGACCAAGCGTTCG		
Enterococcus gallinarum bla$_{AmpC}$-F	CGTTACCCGTGGCAGAAG	136	23
Enterococcus gallinarum bla$_{AmpC}$ -R	GCGAGCATCACAATACCG		
Enterococcus gallinarum bla$_{TEM}$-F	TTCCGTGTCGCCCTTATTC	149[b]	22
Enterococcus gallinarum bla$_{TEM}$-R	AGGATCTTACCGCTGTTGAG		
Enterococcus faecalis bla$_{SHV}$-F	TTATATTCGCCTGTGTATTATCTC	186	40
Enterococcus faecalis bla$_{SHV}$-R	ATGGGAAAGCGTTCATCG		
Desulfovibrio vulgatus bla-F	CTGATAGCCCACGAAGAC	172	8
Desulfovibrio vulgatus bla-R	GCCCAACCTGAAATACAC		
Desulfovibrio desulfuricans DES-1-F	CACCGCCAATGATGTAGG	111	32
Desulfovibrio desulfuricans DES-1-R	GATCCCGTGAGGTAGAGG		
Sphingomonas wittichii bla-F	CGCCGTGAGCATCCAATAG	81	9
Sphingomonas wittichii bla-R	GCTTCGCTGTCGTCCTATC		

[a] References to GenBank nucleotide sequences are available at http://www.ncbi.nlm.nih.gov/.
[b] PCR product sequences confirmed by BLAST analysis (49).

Nitrocefin disk detection of β-lactamase activity. The presence of β-lactamase production by bacterial isolates was detected with nitrocefin-impregnated paper disks according to the manufacturer's specifications (Remel). This test provided a screening method prior to nitrocefin detection of β-lactamases on isoelectric focusing electrophoresis gels run on cell lysates. Bacterial cell lysates were incubated with 1 mM EDTA for 1 h at 4°C prior to testing with the nitrocefin disk assay to observe inhibition of metallo-β-lactamase activity. The positive-control organism for the nitrocefin reaction was *Staphylococcus aureus* ATCC 29213, and that for metallo-β-lactamase inhibition by EDTA was *Stenotrophomonas maltophilia* ATCC 13636.

Analytical isoelectric focusing of β-lactamases. An analytical isoelectric focusing procedure (19, 30) was used to determine the isoelectric points of some of the β-lactamases detected in the bovine bacterial isolates, using a nitrocefin staining technique (35) to visualize the location of enzyme on the gel. Briefly, bacteria were grown in 200-ml cultures overnight with ceftiofur selective pressure at a concentration of 10 μg/ml. The cultures were centrifuged at 12,000 × *g* for 10 min. The pellets were washed with 20 mM potassium phosphate buffer (pH 7.0) and suspended in 3 to 5 ml of the same buffer. The cells were disrupted with a French press at 12,000 lb/in^2. Protein concentrations were measured with a bicinchoninic acid protein assay kit (Pierce, Rockford, IL) and adjusted to 10 mg/ml with the phosphate buffer. Twenty microliters of the lysates along with isoelectric point (pI) standards and β-lactamase controls (*Bacillus cereus bla* II; Sigma Chemical Co., St. Louis) was applied to a pH 3.5 to 9.5 PAG-plate gel using an isoelectric focusing (IEF) applicator strip on a Multiphor apparatus (GE Healthcare Biosciences, Watertown, MA). Isoelectric focusing was run with 1 M H_3PO_4 in the anode strip and 1 M NaOH in the cathode strip at 10°C, and electrophoresis was performed at a constant power of 25 W for 1.5 h (30). The gels were stained with 1 μM nitrocefin (Thermo Fisher, Houston, Tx) in 20 mM potassium phosphate buffer, pH 7.0, with 1 mM $ZnSO_4$ for 30 min, and the gels were electronically imaged through a 530-nm filter on an Image Station 440CF (Eastman Kodak, Rochester, NY). The gels were counterstained with Coomassie blue R-250 and imaged again to compare the active bands with isoelectric focusing markers (Eastman Kodak).

To confirm that β-lactamases in the microbial lysates degraded ceftiofur, tempered antibiotic medium 8 agar containing 0.1 μg/ml ceftiofur was pipetted onto a rapid IEF gel for separation of crude lysates after visualization of positive nitrocefin reactions. Rapid electrophoresis yielded low-resolution bands but maintained optimal enzyme activity. The agar was spread with 9×10^8 CFU/ml *K. rhizophila* and incubated overnight at 37°C. Patches of growth over the bands in the IEF gel confirmed degradation of the ceftiofur.

β-Lactamase gene detection. The presence of β-lactamase genes in total DNA isolated from bovine bacterial isolates was detected using a PCR assay modified from methods described by Almeida and Avila-Campos (3) and Mendes et al. (31). The 25-μl reaction mixtures contained 15 ng DNA (isolated with a Wizard genomic DNA isolation kit; Promega, Madison, WI), 0.5 U recombinant *Taq* DNA polymerase (Thermo Fisher, Atlanta, GA), 200 μM deoxynucleotide triphosphate mix, 1.5 mM $MgCl_2$, and 0.4 μM each primer. Primers were commercially prepared (MWG Biotech, Inc., High Point, NC) from sequences obtained from literature sources or from GenBank sequences and were analyzed using Beacon Designer software (Premier Biosoft International, Palo Alto, CA) to construct PCR primers that detected β-lactamase genes in the bacterial isolates, as indicated in Table 1. Sequences from isolates of the same species were expected to have a greater probability of having close primer sequence identities to optimize the PCRs. Sequences that are diversely distributed throughout multiple bacterial genera (e.g., *bla*$_{TEM}$, *bla*$_{SHV}$, and *bla*$_{AmpC}$) were tested in most of the isolates of ceftiofur degraders, whereas genes like *bla*$_{CepA}$, which are unique to *Bacteroides* spp., were tested only in *Bacteroides* sp. and *Hallella* sp. isolates. The PCR cycling parameters were 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min. The temperature cycling was followed by a dwell at 72°C for 5 min. The PCR products were analyzed by agarose gel electrophoresis on 1.2% agarose Flashgels (Lonza, Rockland, ME). Sequences of PCR product DNA were checked by BLAST

analysis for identity with the source β-lactamase gene sequence (49). The DNA sequencing was provided by the DNA Sequencing Laboratory at the Department of Microbiology/Immunology, University of Arkansas Medical School, Little Rock, AR.

RESULTS

Bacterial and fungal identification. A total of 166 anaerobic bacterial isolates from intestinal and fecal samples from eight cows were evaluated by colony and Gram stain morphologies. Biochemical identifications were made for bacterial isolates that inactivated and degraded ceftiofur, and 71 unique isolates were further characterized (Table 2). The genus *Escherichia* contributed 20% of the identified isolates (Fig. 1). At 17% of isolates each, *Bacteroides* and *Clostridium* were the next most abundant genera (Fig. 1). There were 14 other genera represented in the identified isolates, including *Abiotrophia*, *Actinomyces*, *Arcanobacterium*, *Bacillus*, *Bifidobacterium*, *Desulfovibrio*, *Enterococcus*, *Eubacterium*, *Hallella*, *Lactobacillus*, *Paenibacillus*, *Proteus*, *Sphingomonas*, and *Streptococcus*. There were 9 isolates of facultative anaerobic fungi, identified as *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus terreus*, *Acremonium* sp., *Absidia* sp., *Penicillium* sp., *Paecilomyces* sp., *Cryptococcus laurentii*, and *Rhodotorula glutinis*.

Ceftiofur inactivation and degradation. The cylinder-plate assay measures the functional inactivation (e.g., blocking of substrate binding by adsorption to microbial surface molecules) and enzymatic degradation of ceftiofur. The desfuroylceftiofur acetamide assay measures the amount of intact ceftiofur and desfuroylceftiofur derivatives remaining after biodegradation of the drug by converting them to the more stable desfuroylceftiofur acetamide, which was quantified by high-performance liquid chromatography–mass spectrometry (6). The two methods together differentiated the organisms that had the ability to inactivate ceftiofur from those that could contribute to the degradation of the molecule itself (Table 2). Most of the bacteria isolated from the bovine intestinal microbiota inactivated some amount of ceftiofur (96% of 166 isolates tested). Fifty-four percent actually degraded ceftiofur to some extent. All of the genera identified in this study had a representative in the list of ceftiofur inactivators (Table 2). Among all the genera that could inactivate ceftiofur, *Abiotrophia* sp. and *Actinomyces* sp. isolates did not exhibit any capacity to degrade this drug. Isolates from some genera were better than others at the ability to degrade ceftiofur within a 6-h period. *Bacillus* spp. were the most efficient ceftiofur degraders (Fig. 1). *Bacteroides* spp. as a whole made up the group that was the second most efficient at ceftiofur degradation. Although more *E. coli* isolates were collected and analyzed, the *Bacillus* sp. isolates were significantly more efficient at degrading ceftiofur than the *E. coli* isolates ($P < 0.05$ by the Mann-Whitney test). None of the fungal isolates displayed any activity against ceftiofur in the cylinder-plate assay (data not shown).

Ceftriaxone inactivation and degradation. The ability of the bovine isolates to inactivate or degrade ceftriaxone, a closely related cephalosporin with a thiotriazinedione side chain in place of the thiofuroic acid group of ceftiofur, was tested. A higher concentration of ceftriaxone (30 μg/ml) than ceftiofur (1 μg/ml) was needed to conduct the cylinder-plate assay to get similar *K. rhizophila* growth inhibition zone sizes in both assays. Most of the isolates tested for ceftriaxone degradation digested the drug to a similar extent as ceftiofur, but 58% of *Bacteroides* spp. degraded less of the ceftriaxone than the ceftiofur in 6 h (Table 2).

Characterization of β-lactamases. (i) Gram-negative facultative anaerobes. In the present study, the β-lactamases from some of the ceftiofur-degrading bacterial isolates were characterized for sensitivity to EDTA to detect metallo-β-lactamases, by isoelectric focusing to differentiate some enzymes with similar substrate specificities, and by bacterial growth inhibition using the epsilometer test (Etest) to assess substrate specificities within the β-lactam drug families. The results are summarized in Tables 3 and 4. Evidence suggests that β-lactamases were present in Gram-negative facultative anaerobes that degraded ceftiofur. For example, *Sphingomonas paucimobilis* isolate 11-025 and *Escherichia coli* isolate 04-059 grew well in the presence of penicillins, but they did not grow well in the presence of several cephalosporins (Table 3). *E. coli* 04-059 contained a possible β-lactamase, based on the positive nitrocefin reaction of a protein with a pI of 5.4 (the pI of TEM), and its DNA produced an amplification product with DNA sequence similarity to the bla_{AmpC} gene (Table 4). The expected pI for *E. coli* AmpC is 9.0 (35), but a β-lactamase was inconsistently detected at that isoelectric point by IEF (Fig. 2). A β-lactamase from the *E. coli* isolate hydrolyzed nitrocefin on an IEF gel but could not remove sufficient ceftiofur from the agar overlay to allow growth of the sensitive indicator organism in 18 h (Fig. 2). Cumulatively, these results suggest that *E. coli* 04-059 may produce a β-lactamase that can slowly degrade ceftiofur.

(ii) Gram-positive facultative anaerobes. Facultative anaerobic Gram-positive bacteria that had the capacity to degrade ceftiofur and ceftriaxone were also isolated. *Bacillus mycoides* isolate 11-019 and *Bacillus cereus* isolate 11-022 are examples of the *Bacillus* sp. isolates that degraded ceftiofur and grew in the presence of β-lactam antibiotics, especially cephalosporins (Table 3). *B. mycoides* 11-019 and *B. cereus* P-41 yielded PCR products with sequences similar to those of *B. cereus* β-lactamases I, II, and III and *B. mycoides* β-lactamase C1.

(iii) Gram-negative obligate anaerobes. Many of the obligate anaerobic isolates that degraded ceftiofur were species of *Bacteroides* (Table 2). They had nitrocefin-hydrolyzing β-lactamases detected by IEF (Table 4). Some isolates, such as *B. fragilis* isolate 04-020, *Bacteroides helcogenes* isolate 04-021, and *Bacteroides stercoris* 04-092, grew in the presence of extended-spectrum cephalosporins like cefotaxime and had PCR bands for bla_{CepA} and bla_{CfiA} and β-lactamases with the expected isoelectric points (4.9 to 5.1) for these enzymes (Table 4). Other ceftiofur-degrading *Bacteroides* sp. isolates had nitrocefin-reactive β-lactamases with isoelectric points in the range of 6.8 to 8.6, which are different than expected for CepA or CfiA (Table 4). *Bacteroides suis* isolate 04-042, *Bacteroides thetaiotaomicron* 04-049, and *Bacteroides uniformis* 04-036 are examples of these isolates. They had β-lactamases that degraded ceftiofur in the IEF agar overlay experiment (Fig. 2). These results show that the *Bacteroides* sp. isolates expressed several different β-lactamases that can degrade ceftiofur.

(iv) Gram-positive obligate anaerobes. The obligate anaerobic Gram-positive isolates that degraded ceftiofur were *Eu-*

TABLE 2. Representative bacterial isolates that inactivate or degrade ceftiofur and ceftriaxone

Isolate	Identification	% drug remaining			
		Ceftiofur		Ceftriaxone	
		CYPA[a]	DCA[b]	CYPA	DCA
11-024	*Arcanobacterium bernardiae*	70	91	NT	NT
11-016	*Escherichia coli*	60	100	NT[c]	NT
11-018	*Escherichia coli*	20	100	30	44
11-021	*Escherichia coli*	48	92	NT	NT
03-040	*Escherichia coli*	60	82	NT	NT
03-096	*Escherichia coli*	90	100	NT	NT
03-107	*Escherichia coli*	90	100	NT	NT
03-113	*Escherichia coli*	60	100	NT	NT
03-115	*Escherichia coli*	50	99	NT	NT
04-024	*Escherichia coli*	15	63	60	43
04-059	*Escherichia coli*	20	75	40	53
04-066	*Escherichia coli*	22	96	50	38
04-071	*Escherichia coli*	22	84	63	51
04-109	*Escherichia coli*	22	76	33	57
04-143	*Proteus mirabilis*	22	85	40	56
11-025	*Sphingomonas paucimobilis*	45	52	NT	NT
11-027	*Sphingomonas paucimobilis*	45	100	NT	NT
04-020	*Bacteroides fragilis*	20	50	67	33
04-043	*Bacteroides fragilis*	10	64	53	83
04-021	*Bacteroides helcogenes*	0	27	53	47
04-098	*Bacteroides ovatus*	20	34	93	75
04-093	*Bacteroides* sp.	0	18	12	18
04-086	*Bacteroides stercoris*	0	66	50	38
04-092	*Bacteroides stercoris*	0	19	0	5
04-042	*Bacteroides suis*	20	3	60	74
04-055	*Bacteroides suis*	0	22	53	100
04-049	*Bacteroides thetaiotaomicron*	0.05	43	40	73
04-036	*Bacteroides uniformis*	10	47	42	47
03-041	*Desulfovibrio vulgaris*	0	22	67	22
03-117	Obligate Gram-negative rod	85	100	NT	NT
03-013	*Eubacterium biforme*	10	42	87	42
03-100	*Eubacterium budayi*	85	100	NT	NT
04-076	*Hallella seregens*	38	44	63	69
04-017	*Actinomyces howelli*	80	100	NT	NT
04-124	*Actinomyces* sp.	80	100	NT	NT
P-41	*Bacillus cereus*	0	0	0	0
P-42	*Bacillus cereus*	0	0	0	0
11-022	*Bacillus cereus*	0	0	30	0
11-019	*Bacillus mycoides*	0	3	35	44
03-023	*Bacillus mycoides*	0	12	30	12
03-044	*Bacillus mycoides*	0	15	27	35
03-046	*Bacillus mycoides*	40	17	27	30
03-008	*Enterococcus casseliflavus*	50	44	NT	NT
03-106	*Enterococcus casseliflavus*	85	100	NT	NT
03-119	*Enterococcus casseliflavus*	60	100	NT	NT
03-077	Facultative Gram-positive coccus	85	100	NT	NT
03-024	*Paenibacillus alvei*	70	72	NT	NT
03-004	*Streptococcus bovis*	70	81	NT	NT
03-042	*Streptococcus bovis*	50	36	NT	NT
03-090	*Streptococcus bovis*	85	100	NT	NT
03-121	*Streptococcus bovis*	60	100	NT	NT
04-080	*Streptococcus suis*	20	59	63	49
03-001	*Streptococcus* sp.	70	68	NT	NT
03-088	*Abiotrophia defectiva*	90	100	NT	NT
03-126	*Bifidobacterium breve*	50	43	NT	NT
03-078	*Clostridium absonum*	90	100	NT	NT
03-080	*Clostridium absonum*	90	100	NT	NT
03-063	*Clostridium cellobioparum*	85	100	NT	NT
04-008	*Clostridium cocleatum*	20	37	50	27
04-127	*Clostridium perfringens*	60	100	NT	NT
03-129	*Clostridium sartagoformum*	85	100	NT	NT
03-124	*Clostridium scatalogenes*	90	100	NT	NT
03-049	*Clostridium* sp.	5	100	70	100
03-110	*Clostridium* sp.	0	41	80	41
03-127	*Clostridium* sp.	50	66	NT	NT
03-097	*Clostridium sporogenes*	85	100	NT	NT
04-018	*Lactobacillus hamsteri*	8	98	50	54
03-091	Obligate Gram-positive rod	80	74	NT	NT
03-104	Obligate Gram-positive rod	85	100	NT	NT
03-109	Obligate Gram-positive rod	85	100	NT	NT
03-120	Obligate Gram-positive rod	85	100	NT	NT

[a] CYPA, cylinder-plate assay, which is a biological assay that measures inactivation and degradation of the drug. Percentages of activities of 1 μg/ml ceftiofur or 30 μg/ml ceftriaxone remaining after 6 h of anaerobic incubation at 35°C in overnight cultures of bacteria grown in brucella broth supplemented with vitamin K and hemin.

[b] DCA, desfuroylceftiofur acetate or desthiotriazinedione ceftriaxone acetate assay, which uses HPLC-MS peak areas to quantify the mass of intact drug left after 6 h of degradation.

[c] NT, not tested. Most weak ceftiofur degraders were not further analyzed for ceftriaxone degradation.

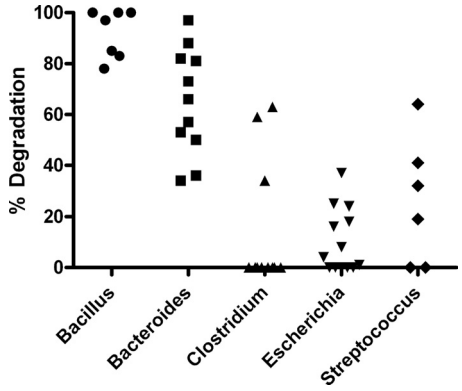

FIG. 1. Variation in capacities of bacterial isolates to degrade ceftiofur. Each column contains the percentage of ceftiofur degraded by isolates of bacterial genera from the bovine intestinal microbiota after 6 h incubation.

bacterium biforme isolate 03-013, *Bifidobacterium breve* isolate 03-126, *Clostridium* sp. isolates 03-110 and 03-127, and *Clostridium cocleatum* isolate 04-008. Most of these isolates grew in the presence of cephalosporin drugs (Table 3). *Clostridium cocleatum* isolate 04-008 had a gene sequence similar to that of a *Clostridium perfringens* β-lactamase gene (Table 4). Gene sequences similar to bla_{TEM}, bla_{SHV}, bla_{AmpC}, and *B. cereus bla* II were detected in some of these isolates (Table 4). The cumulative results from these obligate anaerobic Gram-positive bacteria show that they, too, possibly deploy a mixture of β-lactamase enzymes that metabolize ceftiofur.

DISCUSSION

One concern about antimicrobial drug use in food-producing animals is that these compounds may promote the selection of antimicrobial drug-resistant bacteria that could persist in the food products or in the environment. Most cephalosporin drugs are excreted primarily in the urine (29), although the injected ceftiofur and ceftriaxone are excreted by both renal and biliary pathways (20, 29). Ceftiofur is excreted in the urine as desfuroylceftiofur conjugates and polar metabolites, but little or no intact ceftiofur or desfuroylceftiofur is detected in the feces, which agrees with our observations of intestinal microbial degradation of the drug (20). Ceftriaxone reversibly binds serum proteins in a dose-dependent manner, and the fraction excreted in the urine is primarily the intact molecule (36). Our observation of intestinal metabolism of ceftiofur to antimicrobially inactive metabolites suggests that urinary excretion of the drug is the primary concern for environmental contamination.

In this work, ceftiofur was more susceptible to biodegradation by bovine bacterial isolates than ceftriaxone. Pharmacologically, ceftriaxone has a long half-life in serum compared to other cephalosporins because of its propensity to bind to serum proteins (29). Ceftiofur also has a propensity for serum binding, because of the action of tissue esterases that leave a reactive free sulfhydryl group on the molecule (33). The results of the present study indicate that while there are bacteria in the bovine gut capable of degrading cephalosporins, a greater variety of organisms that are capable of degrading ceftiofur than ceftriaxone are present.

Antimicrobial drug resistance in *E. coli* is medically impor-

TABLE 3. Etest MICs of β-lactam drugs for select bacterial isolates

Isolate	Organism name	MIC[a] (μg/ml)									
		FOX[b]	CRO[c]	CTX	FEP	IPM[d]	AMP[d]	SAM	AMC	PIP	TZP
04-059	*Escherichia coli*	6	0.064	0.064	0.125	0.19	>256	12	6	128	2
11-025	*Sphingomonas paucimobilis*	16	0.75	0.75	1	0.125	0.38	1.5	1	8	0.032
04-020	*Bacteroides fragilis*	6	64	32	32	0.19	48	0.75	0.38	0.22	0.75
04-021	*Bacteroides helcogenes*	4	64	32	48	0.125	32	0.50	0.38	32	0.75
04-098	*Bacteroides ovatus*	6	0.5	0.25	>256	0.064	32	0.75	0.5	0.25	3
04-092	*Bacteroides stercoris*	16	>256	96	48	0.19	24	1	0.5	>256	0.75
04-042	*Bacteroides suis*	6	16	12	12	0.064	8	0.19	0.064	0.19	0.75
04-049	*Bacteroides thetaiotaomicron*	8	2	0.5	0.25	4	>256	32	32	>256	1.5
04-036	*Bacteroides uniformis*	24	12	4	>256	0.75	12	0.25	0.19	32	4
03-041	*Desulfovibrio vulgaris*	3	64	32	128	0.125	32	0.75	0.5	24	4
04-076	*Hallella seregens*	4	0.75	0.25	0.032	0.25	0.25	0.064	0.032	0.75	0.023
P-41	*Bacillus cereus*	16	128	64	>256	0.094	24	3	6	4	4
11-022	*Bacillus cereus*	>256	>256	>256	>256	8	16	6	8	>256	1
11-019	*Bacillus mycoides*	>256	>256	>256	>256	2	12	4	4	64	0.5
03-008	*Enterococcus casseliflavus*	128	>256	>256	24	1.5	0.19	0.125	0.19	192	1.5
03-013	*Eubacterium biforme*	>256	1	0.38	>256	0.75	>256	>256	>256	>256	>256
03-126	*Bifidobacterium breve*	4	0.75	0.38	0.125	0.25	0.032	0.023	0.032	0.75	0.023
03-110	*Clostridium* sp.	4	48	32	>256	0.19	48	1	96	32	2
03-127	*Clostridium* sp.	1	6	2	1	0.125	0.094	0.064	0.047	0.5	0.25
04-008	*Clostridium cocleatum*	6	0.75	0.38	4	0.38	1.5	0.064	>256	0.75	0.023

[a] Abbreviations: FOX, cefoxitin; CRO, ceftriaxone; CTX, cefotaxime; FEP, cefepime; IPM, imipenem; AMP, ampicillin; SAM, ampicillin-sulbactam; AMC, amoxicillin-clavulanic acid; PIP, piperacillin; TZP, piperacillin-tazobactam.

[b] Interpretive standards from Etest product literature for obligate anaerobes: cefoxitin, 32 μg/ml; ceftriaxone, 64 μg/ml; cefotaxime, 64 μg/ml; imipenem, 16 μg/ml; ampicillin-sulbactam, 32 μg/ml; amoxicillin-clavulanic acid, 16 μg/ml; piperacillin, 128 μg/ml; piperacillin-tazobactam, 128 μg/ml.

[c] Ceftiofur is chemically similar to ceftriaxone. A ceftiofur MIC_{90} of 16 μg/ml was reported in one study for 41 *Bacteroides* sp. isolates in cattle (39).

[d] Interpretive standards were unavailable for the use of cefepime (aerobe MIC = 32 μg/ml) or ampicillin (*Enterobacteriaceae* MIC = 32 μg/ml) with obligate anaerobes.

TABLE 4. β-Lactamase characterizations by nitrocefin hydrolysis with or without EDTA, isoelectric focusing, and PCR detection of gene sequences

Isolate	Species identification	Nitrocefin in EDTA[a]	β-Lactamase pI	Gene(s) identified by PCR[b]
04-059	*Escherichia coli*	+	5.4, 9.0	bla_{AmpC}
11-025	*Sphingomonas paucimobilis*	+	8.2, 8.8–9.3	ND[c]
04-020	*Bacteroides fragilis*	−	ND	bla_{CepA}, bla_{CfiA}
04-021	*Bacteroides helcogenes*	+	5.0, 7.3	bla_{CepA}, bla_{CfiA}
04-098	*Bacteroides ovatus*	+	7.4	bla_{CepA}
04-092	*Bacteroides stercoris*	−	5.1	bla_{CepA}, bla_{CfiA}, bla_{CblA}
04-042	*Bacteroides suis*	+	7.4, 7.8, 8.6	ND
04-055	*Bacteroides suis*	−	4.3, 7.8	bla_{CepA}
04-049	*Bacteroides thetaiotaomicron*	−	7.3	ND
04-036	*Bacteroides uniformis*	−	6.8, 7.3, 8.3	bla_{CepA}
03-041	*Desulfovibrio vulgaris*	−	4.9	ND
04-076	*Hallela seregens*	−	4.5	ND
P-41	*Bacillus cereus*	—[d]	ND	*bla* I, *bla* II, *bla* III, bla_{TEM}, *bla* C1
P-42	*Bacillus cereus*	—[d]	ND	*bla* I, *bla* II, *bla* III, bla_{TEM}, *bla* C1
11-022	*Bacillus cereus*	+	9.1	*bla* I, *bla* II, bla_{TEM}
11-019	*Bacillus mycoides*	−	ND	*bla* I, *bla* II, *bla* III, bla_{TEM}, *bla* C1
03-008	*Enterococcus casseliflavus*	−	ND	*Clostridium perfringens bla*, bla_{TEM}
03-126	*Bifidobacterium breve*	+	ND	bla_{TEM}
04-008	*Clostridium cocleatum*	−	4.5	*Clostridium perfringens bla*, bla_{TEM}
03-110	*Clostridium* sp.	+	5.2	ND
03-127	*Clostridium* sp.	+	ND	*bla* II, bla_{AmpC}, bla_{TEM}
03-013	*Eubacterium biforme*	+	ND	bla_{AmpC}, bla_{SHV}

[a] Nitrocefin hydrolysis prevented by EDTA shows inactivation of metallo-β-lactamases.
[b] β-Lactamase gene designations are the same as noted for oligonucleotides in Table 1.
[c] ND, none detected.
[d] The P-41 and P-42 isolates did not show positive nitrocefin reactions.

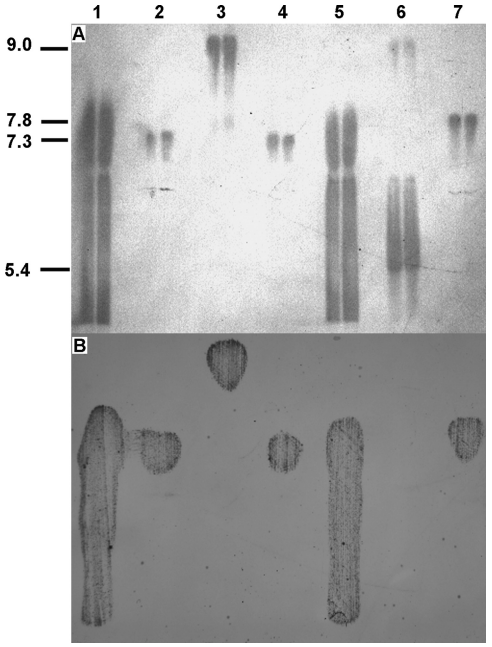

FIG. 2. Agar overlay of *K. rhizophila* growing over nitrocefin-reactive protein bands separated by isoelectric focusing. (A) Nitrocefin-stained proteins separated by IEF; (B) growth of *K. rhizophila* colonies grown 15 h at 37°C on an antibiotic medium 8 agar overlay containing 0.1 μg/ml ceftiofur over the same locations as the bands in panel A. Lanes 1 and 5, commercially prepared β-lactamase II from *Bacillus cereus* (Sigma); lane 2, 04-036 lysate (pI 7.3); lane 3, 11-025 lysate (pI 9.0); lane 4, 04-049 lysate (pI 7.3); lane 6, 04-059 lysate (pI 5.4, 9.0); lane 7, 04-042 lysate (pI 7.8).

tant, so the β-lactamases of this species have been best characterized. In the present study, although bovine *E. coli* isolates were able to inactivate ceftiofur, most of the isolates were unable to efficiently degrade the ceftiofur molecule. Thus, these results suggest that *E. coli* was not the major species involved in ceftiofur degradation but, instead, that numerous isolates of *Bacillus* spp. and *Bacteroides* spp. were more involved. As Fig. 1 shows, all of the *Bacillus* sp. isolates and most of the *Bacteroides* sp. isolates degraded more than 40% of the ceftiofur added to cultures for 6 h, whereas most of the *E. coli* isolates degraded less than 20% of the ceftiofur present in the same amount of time. Isolates of *Streptococcus* spp. and *Clostridium* spp. degraded ceftiofur less well than *Bacillus* spp. and *Bacteroides* spp. but better than most of the *E. coli* isolates. Differences in efficiency of ceftiofur degradation appear to occur at the strain level within species of these genera.

A study of ceftiofur-treated dairy cattle failed to show a correlation between ceftiofur use and selection for resistant strains of bacteria (42). Those investigators observed bla_{CMY-2}-positive *E. coli* isolates in fecal samples from the ceftiofur-treated cattle, but the resistant bacteria did not persist. Most attention regarding selection of antimicrobial drug resistance is focused on pathogenic bacterial species, such as *E. coli*, *Klebsiella pneumoniae*, and *Salmonella enterica* (29), while the potential for commensal microbes to respond to similar selective pressures has not been routinely evaluated. The *Bacteroides* spp. isolated in the present study are components of the normal bovine intestinal microbiota, and they are generally considered to be commensal organisms (14). They contributed much of the capacity of the microbiota to degrade ceftiofur. If the genes for the enzymes involved in ceftiofur degradation by these organisms could be passed to other bacteria, then com-

mensal bacteria could be an important reservoir for resistance. One caveat to this idea is that many of the β-lactamases that degraded ceftiofur in this study did not impart resistance to other extended-spectrum cephalosporins.

Our results suggest that a diverse population of bacteria that produce β-lactamases exists in the normal bovine enteric microbiota. It is not known what selective pressure maintains the expression of β-lactamase genes in bacteria from animals not treated with β-lactam-containing drugs, but resistance gene recycling within the environment may be involved (4).

The present survey has revealed that a variety of commensal bovine intestinal bacteria have the capacity to inactivate ceftiofur and ceftriaxone. Many of them can degrade the drugs with the involvement of β-lactamase enzymes. The enzymes from these bacterial isolates do not necessarily hydrolyze other cephalosporins similar to ceftiofur or ceftriaxone and are not easily characterized by descriptive schemes used for clinically important species. Further characterization of the β-lactamases isolated from these commensal *Bacillus* spp. and *Bacteroides* spp. of cattle will help assess whether these enzymes have been reservoirs for antimicrobial resistance in clinically important bacteria.

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