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Two Novel Antibiotic Resistance Genes, *tet*(44) and *ant(6)-Ib*, Are Located within a Transferable Pathogenicity Island in *Campylobacter fetus* subsp. *fetus*[▽]

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New tetracycline and streptomycin resistance genes, *tet*(44) and *ant(6)-Ib*, were identified in *Campylobacter fetus* subsp. *fetus* within a transferable pathogenicity island that is typically unique to *Campylobacter fetus* subsp. *venerealis*. The 640-amino-acid tetracycline resistance determinant, Tet 44, belongs to a class of proteins that confers resistance to tetracycline and minocycline by ribosomal protection. The 286-amino-acid streptomycin resistance determinant, ANT(6)-Ib, belongs to a family of aminoglycoside nucleotidyltransferases. The resistance phenotypes were demonstrated by gene inactivation and expression.

Campylobacter fetus contains two closely related subspecies, *Campylobacter fetus* subsp. *fetus* and *Campylobacter fetus* subsp. *venerealis*, which exhibit distinct host and tissue specificities (19, 21). *C. fetus* subsp. *venerealis* is associated with genital tract infections in cattle and causes endemic abortion, embryonic death, and infertility. *C. fetus* subsp. *fetus* is associated with intestinal tract colonization in cattle and sheep and has been reported only in sporadic cases of abortion in these animals (12). *C. fetus* subsp. *fetus* is most frequently related to human infections causing several types of diseases (14, 22). The host specificity of *C. fetus* subsp. *venerealis* has been attributed to the presence of a unique pathogenicity island containing a type IV secretory pathway operon (6, 11). In our study, we describe the identification of new tetracycline and streptomycin resistance genes that were located within the *C. fetus* subsp. *venerealis*-specific pathogenicity island present in *C. fetus* subsp. *fetus*. The tetracycline- and streptomycin-resistant *C. fetus* subsp. *fetus* isolates were collected from veal calves in Switzerland (5) and were identified by PCR (1) and their tolerance to glycine (13, 21).

The nature of the tetracycline resistance mechanism was first investigated in the *C. fetus* subsp. *fetus* strain IMD523-06 by PCR using degenerate primers that amplify ribosome protection-type genes as previously described (2). Sequence comparison of the resulting 1,207-bp PCR product using the BLAST algorithm revealed homologies with tetracycline resistance determinants. The sequences of the 5′ and 3′ ends of the tetracycline resistance gene and the flanking regions (40,622 bp) were then obtained using the Illumina/Solexa sequencing technology (Fasteris SA, Geneva, Switzerland). Sequence analysis showed that the tetracycline resistance gene was preceded by two genes, a 471-bp putative regulatory gene (*tr*) and an 807-bp gene (*glo*) that was homologous to genes encoding glyoxalase, and followed by a novel streptomycin resistance gene (Fig. 1). This antibiotic resistance gene cluster was integrated within a pathogenicity island that was almost identical to the pathogenicity island described in *C. fetus* subsp. *venerealis* (6). The genomic regions 23,844 bp upstream of the glyoxalase gene and 12,286 bp downstream of the streptomycin resistance gene showed DNA alignment coverages of 86% and 87%, respectively, with the genomic island of *C. fetus* subsp. *venerealis* strain ATCC 19438 (Fig. 1). Differences were mainly due to an insertion sequence element, five additional small hypothetical proteins in *C. fetus* subsp. *venerealis*, and variable nucleotide polymorphisms (Fig. 1). Importantly, the genetic structures of the gene locus encoding the type IV secretion system were identical in the two subspecies, and the involved proteins VirB2 to VirB11, VirD4, and CagT (6) shared at least 95% identity.

The tetracycline resistance gene encoded a 640-amino-acid (aa) protein, which shares less than 80% overall identity with amino acid sequences of other known tetracycline resistance determinants (Table 1) and was assigned the new name of *tet*(44) using the nomenclature of the tetracycline resistance determinants (10). Based on its amino acid similarity and resistance to tetracycline and minocycline, *tet*(44) is likely to confer resistance by ribosomal protection. The streptomycin resistance gene encoded a 286-aa protein that shares homology with aminoglycoside nucleotidyltransferase ANT(6)-I (Fig. 2). Based on its amino acid homology to ANT(6)-I and the resistance to streptomycin (16) (Fig. 2), the new aminoglycoside resistance gene was named *ant(6)-Ib* using the nomenclature proposed for genes encoding aminoglycoside-modifying enzymes (16, 20). GenBank sequence comparisons showed that *tet*(44) and *ant(6)-Ib* were also present in the *Clostridium perfringens* strain JGS1495, which is currently being completely sequenced (contig GenBank accession number NZ_ABDU01000081).

The phenotypes of *tet*(44) and *ant(6)-Ib* were demonstrated by gene inactivation and expression. For expression, *tet*(44) was amplified by PCR using primers tet(44)-F (5′-aatagatctAAAATAA

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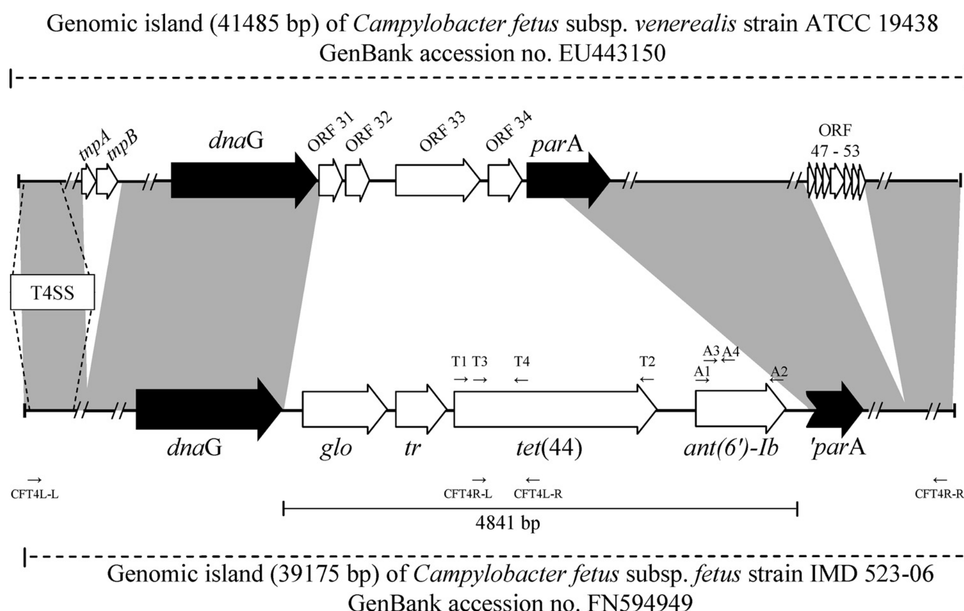

FIG. 1. Schematic organization of the antibiotic resistance genes *tet*(44) and *ant(6)-Ib* found in the genomic island of *C. fetus* subsp. *fetus* compared to the genomic island of *C. fetus* subsp. *venerealis.* Gray areas represent domains sharing more than 92% DNA identity. Open reading frames (ORFs) that are found in only one of the two genomic islands are represented by white arrows. *tnpA* and *tnpB*, transposases; ORF 31 to 34 and ORF 47 to 53, hypothetical open reading frames; *dnaG*, hypothetical DNA gyrase gene; *glo*, hypothetical glyoxalase gene; *parA*, ParA-like protein gene; *'parA*, truncated *parA* gene. T4SS indicates the location of the type IV secretion system (T4SS) within the pathogenicity island and includes genes *virB2* to *virB11*, *virD4*, and *cagT* (6). Primers are indicated by arrows: T1 [tet(44)-F] and T2 [tet(44)-R] and A1 (ant6Ib-F) and A2 (ant6Ib-R) indicate primer pairs used for the genetic cloning of *tet*(44) and *ant(6)-Ib*, respectively; T3 (CfetTetKO-L) and T4 (CfetTetKO-R) and A3 (CfetstreptKO-L) and A4 (CfetstreptKO-R) indicate primer pairs used for the disruption of *tet*(44) and *ant(6)-Ib* by homologous recombination; and CFT4L-L and CFT4L-R and CFT4R-L and CFT4R-R indicate primer pairs used for the PCR restriction analysis of the genomic island.

TCAACATTGGTATTCTTGCTCA) and tet(44)-R (5′-aatactagtTAGTAACTTAATTTTCTTTTTATTAAACATATGGCG), and *ant(6)-Ib* was amplified using primers ant6Ib-F (5′-aatggatccAAAATGAGAACAGAGAAACAAATATATGATACT) and ant6Ib-R (5′-aatactagtTTATCTTTGATATTTTTCTTTTTGTCTTATAACA) (sequences in lowercase indicate synthetic linkers that contain restriction sites [underlined]). The *tet*(44) primers contained BglII and SpeI restriction sites and the *ant(6)-Ib* primers contained BamHI and SpeI restriction sites to allow site-directed cloning into the *Escherichia coli-Campylobacter* shuttle vector pRYSK3, which contains a *Campylobacter* surface array protein A (SAP) promoter (9). The resulting plasmids, pCA73 and pCA75 (Table 2), were first transformed into *E. coli* S17-1 λ*pir* (4) by heat shock and subsequently transferred from *E. coli* into the ciprofloxacin-resistant *C. fetus* subsp. *fetus* strain 1516477 by filter mating, as described previously (9). Transconjugants were obtained on heart infusion agar plates (Becton Dickinson, Franklin Lakes, NJ) containing 8 μg/ml of ciprofloxacin and either 8 μg/ml of tetracycline or 10 μg/ml of streptomycin after 48 h of incubation at 37°C under microaerophilic conditions (80% N_2, 10% CO_2, and 10% H_2). The *tet*(44) and *ant(6)-Ib* genes were disrupted in *C. fetus* subsp. *fetus* strain IMD523-06 by single crossover homologous recombinations (15) using the suicide kanamycin resistance plasmid pCA48, which is a pRYSK3 derivative lacking a 90-bp AflII fragment in the *Campylobacter* replication region. Internal fragments of *tet*(44) were amplified by PCR using primers CfetTetKO-L (5′-tgcgcggccgcATGTTTTTAGAACGTCAGCG) and CfetTetKO-R (5′-atcgagctcAGCACTTCCATGGTATATAGG), while internal fragments of *ant(6)-Ib* were amplified using primers CfetstreptKO-L (5′-tgcgcggccgcGGTTACTTTAGAAGGTTCA

TABLE 1. Nucleotide and amino acid identities of the new Tet 44 determinant compared with closely related Tet determinants conferring resistance to tetracycline and minocycline by ribosomal protection

Determinant	GenBank accession no.	% of amino acid (aa) and nucleotide (nt) identity									
		Tet 44		Tet W		Tet O		Tet S		Tet M	
		nt	aa	nt	aa	nt	aa	nt	aa	nt	aa
Tet 44	FN594949	100	100	63.6	67.0	69.1	67.5	69.0	64.6	69.5	66.0
Tet W	AJ222769			100	100	62.7	67.0	61.0	66.0	64.1	68.4
Tet O	M18896					100	100	70.3	70.9	75.5	76.8
Tet S	L09756							100	100	77.7	77.9
Tet M	X04388									100	100

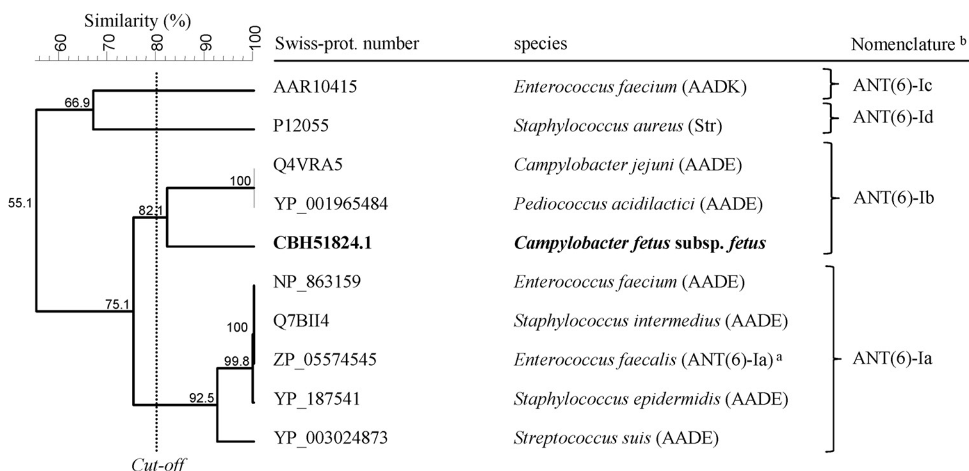

FIG. 2. Alignment of ANT(6)-Ib from *C. fetus* subsp *fetus* (in bold) with other characterized aminoglycoside 6-nucleotidyltransferase enzymes conferring streptomycin resistance. The alignment of the protein sequences was performed by UPGMA (unweighted-pair group method using average linkages) (pairwise, open gap penalty, 100%; unit gap penalty, 0%; FAST, 2.10; Gapcost, 0%) using Bionumerics 5.10 (Applied Maths, Kortrijk, Belgium). Original nomenclature is indicated in parentheses. *a*, nomenclature proposed by Shaw et al. (16); *b*, nomenclature proposed in this study based on clusters with similarity levels higher than 80% and as a continuation of the proposed nomenclature (16, 20).

AGAACA) and CfetstreptKO-R (5′-atcgagctcCCTTTCGA CATAATCCTTTCAC). The PCR products were then cloned into the NotI and SacI restriction sites of pCA48. Single crossover recombinants were obtained by filter mating on heart infusion agar plates containing 25 μg/ml of kanamycin and 8 μg/ml of nalidixic acid, as described above.

Resistance phenotypes were determined by MIC measurements in Mueller-Hinton broth supplemented with 5% laked horse blood (3). When expressed from pRYSK3 in *C. fetus* subsp. *fetus*, *tet*(44) mediated at least a 5-fold increase in tetracycline, doxycycline, and minocycline resistance (Table 2). Similarly, *ant(6)-Ib* expressed from pRYSK3 in *C. fetus* subsp. *fetus* also mediated at least a 5-fold increase in streptomycin resistance (Table 2). On the other hand, a 3- to 5-fold decrease in resistance to these drugs was observed when the genes were disrupted (Table 2). MICs of ciprofloxacin, nalidixic acid, meropenem, ampicillin, amoxicillin-clavulanic acid, chloramphenicol, florfenicol, gentamicin, neomycin, and erythromycin remained unchanged (data not shown).

The pathogenicity and resistance island was localized on the chromosome by DNA hybridization using a digoxigenin (DIG)-labeled probe for *tet*(44). It was transferred from *C. fetus* subsp. *fetus* strain IMD523-06 into *C. fetus* subsp. *fetus* strain 1516477 by filter mating. Transconjugants were selected on plates containing 10 μg/ml of streptomycin and 8 μg/ml of ciprofloxacin. Transconjugants were identified by the detection of *tet*(44) and *ant(6)-Ib* and by multilocus sequence typing (19) (http://pubmlst.org/cfetus/) with the donor strain belonging to ST2 and the recipient strain and the transconjugants belonging to ST35. ClaI restriction digests of two large PCR fragments (21,752 and 14,078 bp) covering the regions situated between *tet*(44) and both ends of the pathogenicity island (Fig. 1) generated identical restriction profiles in both the donor and transconjugants, demonstrating that the complete genomic island was transferred by conjugation. The fragments were amplified using the Expand Long Template PCR system (Roche Diagnostics AG, Rotkreuz, Switzerland) (extension time, 20 min; annealing temperature, 58°C) with primers specific to the 5′ end of the genomic island and to *tet*(44) (CFT4L-L, 5′-TGTTAGTCAAAAAAGATGATATGGCT TTTAGGC, and CFT4L-R, 5′-CATCTTTAGCAGAAATT ACTAAAATTGCTCCATC) and primers specific to *tet*(44) and to the 3′ end of the genomic island (CFT4R-L, 5′-AT AATCAACATTGGTATTCTTGCTCATGTAGATG, and

TABLE 2. Characteristics and MICs for tetracyclines and streptomycin for the *C. fetus* subsp. *fetus* strains used in this study[a]

Strain	Characteristic(s)	MIC (μg/ml)			
		TET	DOX	MIN	STR
IMD523-06	*tet*(44) *ant(6)-Ib*; Nal^r; bovine isolate	16	8	16	>64
1516477	Recipient strain; Cip^r; human isolate	1	0.5	1	≤1
CA73	1516477 containing *tet*(44) on pCA73	>32	32	32	≤1
CA74	IMD523-06 with Δ*tet*(44)::pCA64; Kan^r	1	0.5	2	>64
CA75	1516477 containing *ant(6)-Ib* on pCA75	1	0.5	1	64
CA76	IMD523-06 with Δ*ant(6)-Ib*::pCA76; Kan^r	16	8	16	≤1
CA80	1516477 containing IMD523-06 pathogenicity island with *tet*(44) and *ant(6)-Ib*; Cip^r	16	4	16	64

[a] TET, tetracycline; DOX, doxycycline; MIN, minocycline; STR, streptomycin. Resistance markers used for transformation: Nal^r, intrinsic resistance to nalidixic acid; Cip^r, resistance to ciprofloxacin; Kan^r, resistance to kanamycin.

CFT4R-R, 5′-GAAGTGGGGTAATGTTGTTTCATAG GAATT) (Fig. 1).

An identical PCR restriction profile was also obtained with 10 additional tetracycline- and streptomycin-resistant *C. fetus* subsp. *fetus* strains (data not shown), indicating that this genomic island has already disseminated into the *C. fetus* subsp. *fetus* population. Given the location of *tet*(44) and *ant(6)-Ib* within this pathogenicity island, it is likely that the use of antibiotics has been contributing to the spread of this element. Resistance to tetracycline has been reported in *C. fetus* subsp. *fetus* from both human and animal origins (8, 17, 18). Whether *tet*(44) and the pathogenicity island are present in these isolates remains to be determined.

The use of antibiotics poses the risk of selecting a transferable element in *C. fetus* subsp. *fetus*, which has so far been associated only with the virulence and host specificity of *C. fetus* subsp. *venerealis*. The presence of the *C. fetus* subsp. *venerealis* island in *C. fetus* subsp. *fetus* may also lead to the false identification of *C. fetus* subspecies because some PCR methods have been based on genes present in the genomic island to discriminate between the *C. fetus* subspecies (7, 11). This discovery gives a new insight into the host specificity and pathogenicity potential of *C. fetus* subsp. *fetus* and also into the diagnosis of diseases caused by *C. fetus* infections.

Nucleotide sequence accession number. The sequences of *tet*(44), *ant(6)-Ib*, and the flanking genomic island of *C. fetus* subsp. *fetus* IMD523-06 have been deposited in the EMBL/GenBank/DDBJ database under accession number FN594949.

REFERENCES

1. **Abril, C., E. M. Vilei, I. Brodard, A. Burnens, J. Frey, and R. Miserez.** 2007. Discovery of insertion element IS*Cfe1*: a new tool for *Campylobacter fetus* subspecies differentiation. Clin. Microbiol. Infect. **13:**993–1000.
2. **Barbosa, T. M., K. P. Scott, and H. J. Flint.** 1999. Evidence for recent intergeneric transfer of a new tetracycline resistance gene, *tet*(W), isolated from *Butyrivibrio fibrisolvens*, and the occurrence of *tet*(O) in ruminal bacteria. Environ. Microbiol. **1:**53–64.
3. **Clinical and Laboratory Standards Institute.** 2009. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 8th ed., vol. 29, no. 2. Approved standard M07-A8. Clinical and Laboratory Standards Institute, Wayne, PA.
4. **de Lorenzo, V., and K. N. Timmis.** 1994. Analysis and construction of stable phenotypes in gram-negative bacteria with Tn*5*- and Tn*10*-derived minitransposons. Methods Enzymol. **235:**386–405.
5. **Di Labio, E., G. Regula, A. Steiner, R. Miserez, A. Thomann, and U. Ledergerber.** 2007. Antimicrobial resistance in bacteria from Swiss veal calves at slaughter. Zoonoses Public Health **54:**344–352.
6. **Gorkiewicz, G., S. Kienesberger, C. Schober, S. R. Scheicher, C. Gully, R. Zechner, and E. L. Zechner.** 2010. A genomic island defines subspecies-specific virulence features of the host-adapted pathogen *Campylobacter fetus* subsp. *venerealis*. J. Bacteriol. **192:**502–517.
7. **Hum, S., K. Quinn, J. Brunner, and S. W. On.** 1997. Evaluation of a PCR assay for identification and differentiation of *Campylobacter fetus* subspecies. Aust. Vet. J. **75:**827–831.
8. **Inglis, G. D., D. W. Morck, T. A. McAllister, T. Entz, M. E. Olson, L. J. Yanke, and R. R. Read.** 2006. Temporal prevalence of antimicrobial resistance in *Campylobacter* spp. from beef cattle in Alberta feedlots. Appl. Environ. Microbiol. **72:**4088–4095.
9. **Kienesberger, S., G. Gorkiewicz, M. M. Joainig, S. R. Scheicher, E. Leitner, and E. L. Zechner.** 2007. Development of experimental genetic tools for *Campylobacter fetus*. Appl. Environ. Microbiol. **73:**4619–4630.
10. **Levy, S. B., L. M. McMurry, T. M. Barbosa, V. Burdett, P. Courvalin, W. Hillen, M. C. Roberts, J. I. Rood, and D. E. Taylor.** 1999. Nomenclature for new tetracycline resistance determinants. Antimicrob. Agents Chemother. **43:**1523–1524.
11. **Moolhuijzen, P. M., A. E. Lew-Tabor, B. M. Wlodek, F. G. Aguero, D. J. Comerci, R. A. Ugalde, D. O. Sanchez, R. Appels, and M. Bellgard.** 2009. Genomic analysis of *Campylobacter fetus* subspecies: identification of candidate virulence determinants and diagnostic assay targets. BMC Microbiol. **9:**86.
12. **Office International des Epizooties.** 2008. Bovine genital campylobacteriosis, p. 661–670. *In* Manual of diagnostic tests and vaccines for terrestrial animals. Office International des Epizooties, Paris, France.
13. **On, S. L., and C. S. Harrington.** 2001. Evaluation of numerical analysis of PFGE-DNA profiles for differentiating *Campylobacter fetus* subspecies by comparison with phenotypic, PCR and 16S rDNA sequencing methods. J. Appl. Microbiol. **90:**285–293.
14. **Pacanowski, J., V. Lalande, K. Lacombe, C. Boudraa, P. Lesprit, P. Legrand, D. Trystram, N. Kassis, G. Arlet, J. L. Mainardi, F. Doucet-Populaire, P. M. Girard, and J. L. Meynard.** 2008. Campylobacter bacteremia: clinical features and factors associated with fatal outcome. Clin. Infect. Dis. **47:**790–796.
15. **Pei, Y., V. Parreira, V. M. Nicholson, and J. F. Prescott.** 2007. Mutation and virulence assessment of chromosomal genes of *Rhodococcus equi* 103. Can. J. Vet. Res. **71:**1–7.
16. **Shaw, K. J., P. N. Rather, R. S. Hare, and G. H. Miller.** 1993. Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. Microbiol. Rev. **57:**138–163.
17. **Tremblay, C., and C. Gaudreau.** 1998. Antimicrobial susceptibility testing of 59 strains of *Campylobacter fetus* subsp. *fetus.* Antimicrob. Agents Chemother. **42:**1847–1849.
18. **Tremblay, C., C. Gaudreau, and M. Lorange.** 2003. Epidemiology and antimicrobial susceptibilities of 111 *Campylobacter fetus* subsp*. fetus* strains isolated in Quebec, Canada, from 1983 to 2000. J. Clin. Microbiol. **41:**463–466.
19. **van Bergen, M. A., K. E. Dingle, M. C. Maiden, D. G. Newell, L. van der Graaf-van Bloois, J. P. van Putten, and J. A. Wagenaar.** 2005. Clonal nature of *Campylobacter fetus* as defined by multilocus sequence typing. J. Clin. Microbiol. **43:**5888–5898.
20. **Vanhoof, R., E. Hannecart-Pokorni, and J. Content.** 1998. Nomenclature of genes encoding aminoglycoside-modifying enzymes. Antimicrob. Agents Chemother. **42:**483.
21. **Véron, M., and R. Chatelain.** 1973. Taxonomic study of the genus *Campylobacter* Sebald and Véron and designation of the neotype strain for the type species, *Campylobacter fetus* (Smith and Taylor) Sebald and Véron. Int. J. Syst. Bacteriol. **23:**122–134.
22. **Zonios, D. I., G. D. Panayiotakopoulos, E. O. Kabletsas, E. L. Tzima, I. Stefanou, and A. J. Archimandritis.** 2005. *Campylobacter fetus* bacteraemia in a healthy individual: clinical and therapeutical implications. J. Infect. **51:**329–332.