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Diverse Tetracycline Resistance Genotypes of *Megasphaera elsdenii* Strains Selectively Cultured from Swine Feces

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A total of 30 *Megasphaera elsdenii* strains, selectively isolated from the feces of organically raised swine by using Me109 M medium, and one bovine strain were analyzed for tetracycline resistance genotypic and phenotypic traits. Tetracycline-resistant strains carried *tet*(O), *tet*(W), or a *tet* gene mosaic of *tet*(O) and *tet*(W). *M. elsdenii* strains carrying *tet*(OWO) genes exhibited the highest tetracycline MICs (128 to >256 μg/ml), suggesting that *tet*(O)-*tet*(W) mosaic genes provide the selective advantage of greater tetracycline resistance for this species. Seven *tet* genotypes are now known for *M. elsdenii*, an archetype commensal anaerobe and model for *tet* gene evolution in the mammalian intestinal tract.

Megasphaera elsdenii is a commensal (mutualist) species in the gastrointestinal tracts of ruminant and nonruminant mammals, including humans (7, 25, 26). This anaerobic bacterium contributes to the overall metabolism that takes place in those microbial ecosystems (2, 5, 13, 15). *M. elsdenii* has been the focus of both prebiotic and probiotic applications for improving animal health (10, 12, 19, 27).

In a recent study of intestinal bacteria resistant to tetracycline, we detected resistant *M. elsdenii* strains at high population levels (approximately 10^7 CFU/g) in cecal samples from healthy swine (24). Eight strains were isolated and characterized. The *M. elsdenii* strains are highly resistant to chlortetracycline (MIC = 256 to >256 μg/ml) and carry one of two "*tet*(OWO)" genes for tetracycline resistance. [Throughout the manuscript, "*tet*(OW)" and "*tet*(OWO)" are used as convenient, practical terms for describing *M. elsdenii* recombinant *tet* genes and genotypes. As noted previously (24), these designations are not recognized under present *tet* classification guidelines (14). It is our hope that future guidelines will be developed to accommodate these novel interclass hybrid genes.] These *tet* genes are interclass mosaic genes apparently formed by double-crossover recombinations between *tet*(O) and *tet*(W) genes.

Our previous study used a nutritionally complex medium with high concentrations of chlortetracycline and, thus, was biased to select tetracycline-resistant *M. elsdenii* strains. In this study, Me109M medium was developed and used to select *M. elsdenii* strains without using chlortetracycline. The goals were twofold: first, to obtain tetracycline-sensitive strains of *M. elsdenii* useful both for investigating *tet* gene transfer and for probiotic applications, and second, to discover whether or not *M. elsdenii* strains have additional *tet* genotypes.

Selective isolation of *M. elsdenii*—Me109M medium. On the basis of previous studies (4–6, 9, 14, 16, 17, 19–21) and preliminary experiments in our laboratory, Me109M medium was developed to selectively culture *M. elsdenii*. Me109M contained (per liter): tryptone-peptone, 4 g; yeast extract, 2 g; salts solution A (6 g of K_2HPO_4/liter of water), 40 ml; salts solution B [6 g of KH_2PO_4, 12 g of $(NH_4)_2SO_4$, 12 g of NaCl, 1.2 g of $MgSO_4$–$7H_2O$, 0.6 g of $CaCl_2$/liter of water], 40 ml; Na-DL-lactate syrup (60% [wt/wt]), 12.7 ml; resazurin solution (0.1% [wt/vol]), 1 ml; L-cysteine-HCl, 0.5 g; distilled water, 900 ml; Difco Bacto agar, 12 g. The pH of the medium was adjusted to 5.0. A total of 10 ml of a monensin solution (5 mg/ml of ethanol) was added to the autoclaved medium. Agar plates of medium were prepared aerobically and, at least 2 days before inoculation, transferred into a Coy anaerobic chamber at room temperature.

M. elsdenii strains were isolated under anoxic conditions from fecal samples of 10 grower-phase (40- to 50-kg) swine purchased from two Iowa farms that have raised animals organically (National Organic Foods Production Act; Alternative Farming Systems Information Center [www.nalusda.gov/afsic/ofp]), without antibiotic feeding, for at least 4 years. The swine were housed at the National Animal Disease Center, with no exposure to other animals, in chemically decontaminated buildings with strict entry requirements for human personnel. The animals were supplied water ad libitum and fed their original organic diet, which was free of detectable tetracycline (3).

M. elsdenii population densities averaged 2.4×10^8 CFU/g of feces (wet weight), and there were no significant differences in counts between animals from the two farms. *M. elsdenii* colonies appeared as large (2- to 3-mm-diameter), yellow-white, dome-shaped colonies after 72 h of incubation at 38°C and contained large cocci in pairs or chains. These colonies represented approximately one-third of the colonies growing on Me109M agar and 0.4% of the total cultivable fecal bacteria (data not presented). Me109M is highly selective for *M. elsdenii* and has been used to detect population levels of this species as low as 10^4 CFU/g of feces (T. B. Stanton, unpublished data). A total of 30 strains (3 from each animal) were cloned by subculturing and confirmed to be *M. elsdenii* on the

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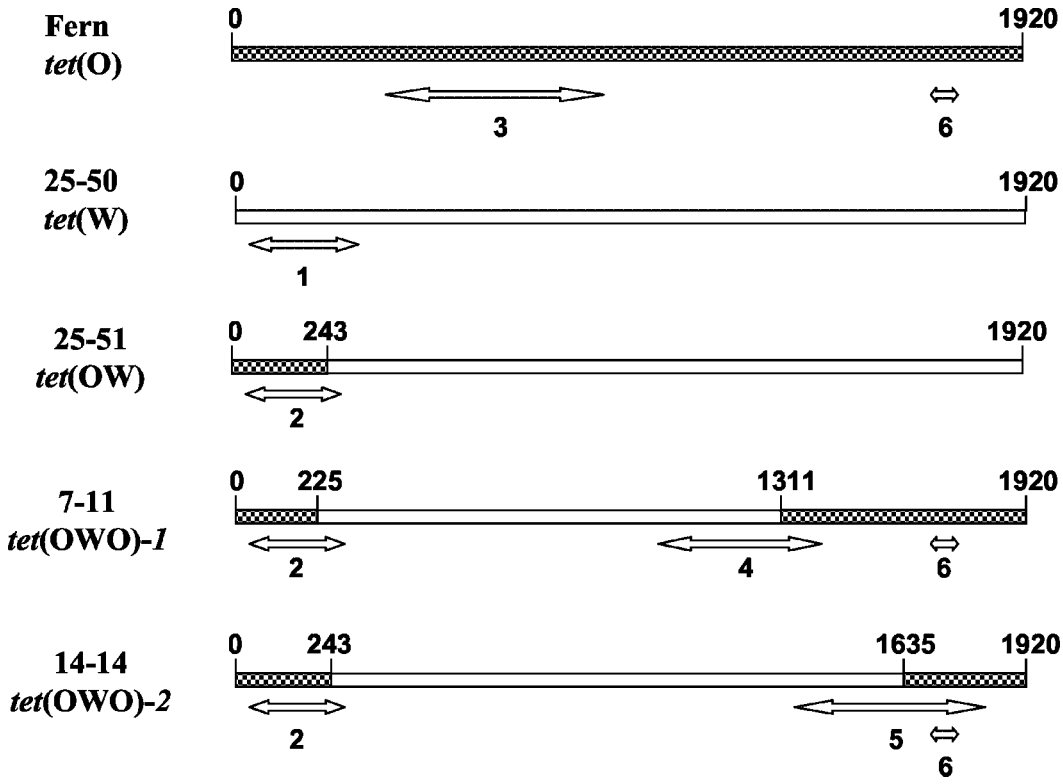

FIG. 1. Schematic depiction of PCR assays for differentiating *M. elsdenii* tetracycline-resistant genotypes. Strain and tetracycline resistance genotype designations are given at the left of the figure. Checkered regions of genes have high-level sequence identity with *tet*(O); open regions have high-level sequence identity with *tet*(W). Products of PCR amplification are depicted by double-headed arrows. Crossover regions of the genes were established by sequence analysis.

basis of physiological properties and 16S rRNA-V3 sequence analysis (24).

***M. elsdenii* tetracycline-resistant genotypes.** The 30 swine strains and bovine strain Fern, previously isolated during unrelated studies, were analyzed by PCR assays (24) to differentiate known *M. elsdenii* tetracycline resistance genes (Fig. 1; Table 1). Nine strains did not contain detectable *tet* genes. The *tet*(OWO)-*1* gene identified previously in swine *M. elsdenii* strain 7-11 (Fig. 1) was not detected in any of the isolates. A total of 11 strains had the *tet*(OWO)-*2* genotype reported previously for *M. elsdenii* strain 14-14 (Fig. 1; Table 1). Another 11 strains carried genes—*tet*(O), *tet*(W), and *tet*(OW)—not previously detected for *M. elsdenii*. The *tet*(OW) genes apparently originate from single-crossover recombinations between *tet*(O) and *tet*(W).

Sequence comparisons revealed differences between the strain 27-51 and 25-51 *tet*(OW) genes; these have been designated *tet*(OW)-*1* and *tet*(OW)-*2*, respectively. In the strain 25-51 *tet*(OW)-*2* gene, the *tet*(O)-to-*tet*(W) crossover position occurs at base position 243, resembling that of strain 14-14 (Fig. 1). The corresponding crossover position of the strain 27-51 *tet*(OW)-*1* gene at base position 225 is similar to that of strain 7-11 (Fig. 1).

The *tet*(W) genes of *M. elsdenii* strains 25-50 and 29-55 likely

TABLE 1. *M. elsdenii* tetracycline resistance genotypes determined by PCR analyses[a]

M. elsdenii strain(s)	PCR[b]						*tet* genotype[c]
	1	2	3	4	5	6	
24-50; 26-50; 27-50; 28-50; 29-54; 30-54; 31-54; 32-54; 33-54	−	−	−	−	−	−	No *tet*
Fern	−	−	+	−	−	+	*tet*(O)
25-50; 29-55	+	−	−	−	−	−	*tet*(W)
24-51; 25-51; 27-51; 28-53; 29-57; 30-55; 31-55; 33-57	−	+	−	−	−	−	*tet*(OW)
24-53; 25-52; 26-51; 26-52; 27-52; 28-52; 30-56; 31-57; 32-56; 32-57; 33-56	−	+	−	−	+	+	*tet*(OWO)-2

[a] All *M. elsdenii* strains are swine fecal isolates except strain Fern, which was isolated from bovine rumen contents.
[b] "+" indicates product and "−" indicates no product for PCR depicted in Fig. 1.
[c] Additional *tet* genotypes were discovered upon sequencing *tet*(OW) and *tet*(W) genes, as described in the text.

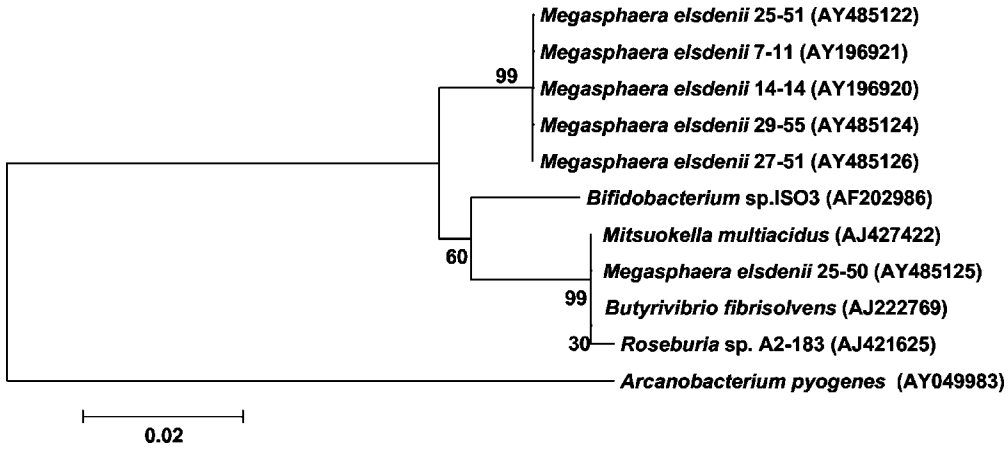

FIG. 2. Phylogenetic analysis of a Tet(W) common region (365 amino acids) shared by various species. GenBank accession numbers for nucleotide sequences used to derive amino acid sequences are given in parentheses. The unrooted neighbor-joining tree was generated using Mega2 (11). A Poisson substitution model was applied to distance calculations. Branches are labeled by bootstrap values from 10,000 replicates. Bar, number of substitutions per amino acid.

have different origins and have been designated, respectively, *tet*(W)-*1* and *tet*(W)-*2*. When the Tet(W) portions (356-amino-acid segments) common to *M. elsdenii* Tet(W), Tet(OW), and Tet(OWO) proteins are compared, the amino acid sequences fall into two main clusters marked by a bootstrap support value of 99 (Fig. 2). Sequences between the two clusters differ by 11 amino acids. One cluster of segments is comprised of *M. elsdenii* Tet(W), Tet(OW), and Tet(OWO) sequences. The sequences are identical, suggesting that recombination between a *tet* gene identical to *tet*(W)-*2* of *M. elsdenii* strain 29-55 and a *tet*(O) gene produced the presently known mosaic genes of *M. elsdenii*. These sequences do not match any known Tet(W) sequences of other intestinal species. By contrast, not only the *M. elsdenii* 25-50 Tet(W)-1 segment (Fig. 2) but also the complete 25-50 Tet(W)-1 sequence is identical to those of Tet(W) proteins of *Butyrivibrio fibrisolvens* and *Mitsuokella multiacidus*, suggesting that these *tet*(W) genes share a common origin and may be communicable among these intestinal anaerobes. A larger sample of *tet*(W) sequences from diverse bacterial species will undoubtedly increase the robustness of the tree and could provide further insight into horizontal gene flow among species in the intestinal tract.

Seven tetracycline resistance genotypes of *M. elsdenii* have now been detected in these and previous studies (24). *M. elsdenii* appears to be a commensal "warehouse" for *tet*(W), recombinant *tet*(O)-*tet*(W), and perhaps *tet*(O) genes in the mammalian intestinal tract. Whether *M. elsdenii* cells are the site for *tet*(O) and *tet*(W) recombination or are the recipients of mosaic *tet* genes remains unclear (24).

Tetracycline MICs of *M. elsdenii* strains. Tetracycline MICs for *M. elsdenii* strains were determined by the agar dilution method according to NCCLS recommendations (18). All strains carrying *tet* genes exhibited tetracycline antimicrobial MICs that were 4- to >100-fold greater than those exhibited for strains lacking the genes (Table 2). Of the strains, 11 carrying mosaic *tet*(OWO) genes consistently exhibited the highest tetracycline MICs (128 to >256 μg/ml) (Table 2). This finding implies that recombinant *tet*(O)-*tet*(W) genes provide a

TABLE 2. Tetracycline MIC values for *M. elsdenii* strains

M. elsdenii strain(s)[a]	*tet* genotype[b]	MIC (μg/ml)[c]		
		Tet	Oxytet	Chlortet
24-50; 27-50; 33-54 (LC-1^T; B159; T81)	No gene	4–8	8–16	2–8
Fern	*tet*(O)	64	64	256
25-50; 29-55	*tet*(W)	32–64	64	64–128
24-51; 27-51; 25-51; 31-55	*tet*(OW)	64	64–128	128–256
7-11 (2–9)	*tet*(OWO)-1	128	256	>256
24-53; 27-52; 33-56 (4-13; 7-12; 14-14; 15-5; 19-3; 20-11)	*tet*(OWO)-2	128	256–>256	256–>256

[a] Strain 7–11 was used as a *M. elsdenii* tetracycline-resistant reference strain in the assays. Tetracycline MIC values for *M. elsdenii* strains enclosed by parentheses have been reported previously (26).

[b] Genotypes are based on PCR assay results (Fig. 1; Table 1). Strains 27-51 and 25-51 had *tet*(OW)-*1* and *tet*(OW)-*2* genotypes, respectively, as identified by sequence analysis. Strains 25-50 and 29-55 had *tet*(W)-*1* and *tet*(W)-*2* genotypes, respectively.

[c] Tetracycline (Tet), oxytetracycline (Oxytet), and chlortetracycline (Chlortet) MIC values for NCCLS reference strain *Bacteroides fragilis* ATCC 25285 (Tc^s) were 2, 8, and 4 μg/ml, respectively. For *B. thetaiotaomicron* ATCC 29741 (Tc^r), the MIC value of all three antibiotics was 64 μg/ml.

selective advantage of increased tetracycline resistance to *M. elsdenii* cells. An important test of this hypothesis will be to examine the resistance properties of the various *tet* genes in an isogenic background in, for example, the same *M. elsdenii* strain.

Both tetracycline-sensitive and tetracycline-resistant *M. elsdenii* strains were isolated from the same fecal dilutions from every organically raised animal, an indication that both types are present at population levels of 10^7 to 10^8 CFU/g of feces. Previously, *M. elsdenii* strains exhibiting high tetracycline MIC levels and carrying *tet*(OWO) genes were conservatively estimated at 10^7 CFU/g of cecal contents of conventionally raised swine regardless of whether or not the animals were fed chlortetracycline (24). Tetracycline-resistant *M. elsdenii* strains persist at high population levels in swine in the absence of antibiotic use.

Due to the large diversity of uncultivated or difficult-to-culture commensal anaerobes, it has been suggested that one or a few bacterial species might serve as useful indicators in the analysis of reservoir populations (7a). *Escherichia coli* and *Bacteroides* and *Enterococcus* spp. have been used to monitor the flow and persistence of antibiotic resistance determinants within and between intestinal ecosystems (1, 8, 22, 23, 28, 29). *M. elsdenii* is a common anaerobe in the intestinal tracts of both ruminant and nonruminant mammals, including humans. Currently known intestinal *Megasphaera* comprise only one species, *M. elsdenii*. *M. elsdenii* strains grow rapidly on simple anaerobic culture medium, can be selectively isolated on Me109M agar, and have easily recognizable cell and colony morphologies. *M. elsdenii* strains are among the most numerous tetracycline-resistant populations in swine intestinal tracts (24). On the basis of these considerations, *M. elsdenii* offers several attractive features as an indicator or archetype commensal species for monitoring the antibiotic resistance status and reservoir potential of the intestinal microbiota.

Nucleotide sequence accession numbers. The *tet* gene sequences of five *M. elsdenii* strains were determined and have been deposited in GenBank under the following accession numbers: strain Fern, *tet*(O) (AY485123); strain 25-50, *tet*(W)-*1* (AY485125); strain 29-55, *tet*(W)-*2* (AY485124); strain 27-51, tet(OW)-*1* (AY485126); strain 25-51, *tet*(OW)-*2* (AY485122).

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