

Widespread Distribution of a Tet W Determinant among Tetracycline-Resistant Isolates of the Animal Pathogen *Arcanobacterium pyogenes*

Stephen J. Billington,* J. Glenn Songer, and B. Helen Jost

Department of Veterinary Science and Microbiology, The University of Arizona, Tucson, Arizona 85721

Received 8 August 2001/Returned for modification 9 August 2001/Accepted 8 February 2002

Tetracycline resistance is common among isolates of the animal commensal and opportunistic pathogen *Arcanobacterium pyogenes*. The tetracycline resistance determinant cloned from two bovine isolates of *A. pyogenes* was highly similar at the DNA level (92% identity) to the *tet(W)* gene, encoding a ribosomal protection tetracycline resistance protein, from the rumen bacterium *Butyrivibrio fibrisolvens*. The *tet(W)* gene was found in all 20 tetracycline-resistant isolates tested, indicating that it is a widely distributed determinant of tetracycline resistance in this organism. In 25% of tetracycline-resistant isolates, the *tet(W)* gene was associated with a *mob* gene, encoding a functional mobilization protein, and an origin of transfer, suggesting that the determinant may be transferable to other bacteria. In fact, low-frequency transfer of *tet(W)* was detected from *mob*⁺ *A. pyogenes* isolates to a tetracycline-sensitive *A. pyogenes* recipient. The mobile nature of this determinant and the presence of *A. pyogenes* in the gastrointestinal tract of cattle and pigs suggest that *A. pyogenes* may have inherited this determinant within the gastrointestinal tracts of these animals.

Resistance to tetracycline is the most common bacterial antibiotic resistance found in nature and similarly is the most prominent type of resistance among bacteria isolated from animals (10). Tetracycline binds to the 30S ribosomal subunit and inhibits protein synthesis (32), and resistance to this drug is most commonly mediated either by active efflux of tetracycline from the cell or by ribosomal protection from the action of tetracycline (10). In rare cases, tetracycline resistance is mediated through direct inactivation of the antibiotic (30) or by mutations in the 16S rRNA that prevent binding of tetracycline to the ribosome (26).

Arcanobacterium pyogenes is a common inhabitant of the respiratory and urogenital tracts of domestic animals, including cattle and swine (8). It has also been isolated from the rumen of feedlot cattle (22) and more recently from the gastric mucosa of pigs (B. H. Jost, K. W. Post, J. G. Songer, and S. J. Billington, unpublished data), suggesting that *A. pyogenes* may also be a common inhabitant of the gastrointestinal tract of these species. Under conditions of physical trauma or previous microbial infection, *A. pyogenes* can disseminate to cause a variety of suppurative infections, such as liver abscesses and mastitis in cattle (15, 18, 21) and suppurative pneumonia and polyarthritis in pigs (14, 35). Virulence, at least in an intraperitoneal mouse model, appears to be mediated through a pore-forming toxin, pyolysin (6, 16). The development of antibiotic resistance in normal flora secondary pathogens, such as *A. pyogenes*, is of particular significance, not only because of the ability of resistant *A. pyogenes* to avoid antibiotic therapy, but also because of the potential for transfer of resistance to other pathogens.

Resistance to the tetracycline antibiotics among *A. pyogenes*

isolates is widespread. A French study indicated that 67% of 103, predominantly bovine, *A. pyogenes* isolates were resistant to tetracycline, doxycycline, and minocycline (13), while a study in Japan found that 85.7% of porcine and 57.1% of bovine isolates were resistant to oxytetracycline and doxycycline (40). These results were confirmed by a recent study of predominantly North American isolates of *A. pyogenes*, which indicated that 42% were resistant to tetracycline, chlortetracycline, and oxytetracycline, with more than 70% of porcine isolates being resistant (34).

No information is currently available on the mechanism of tetracycline resistance in *A. pyogenes* or the determinants involved in this resistance. In this work, we present evidence that tetracycline resistance in *A. pyogenes* isolates is predominantly conferred by a Tet W determinant previously found only in obligately anaerobic bacteria isolated from the rumen of cattle and the human gut (2, 5, 27, 28) and that this determinant, in at least some strains, is carried on a mobile genetic element capable of transfer between strains of *A. pyogenes*.

MATERIALS AND METHODS

Bacterial strains, bacteriophage, plasmids, and growth conditions. *Escherichia coli* strains and plasmids are described in Table 1. *E. coli* strains were grown on either Luria-Bertani (LB) agar or in LB broth (Difco) at 37°C. Antibiotics (Sigma) were added as appropriate at the following concentrations: chloramphenicol, 30 µg/ml; kanamycin, 50 µg/ml; nalidixic acid, 10 µg/ml; tetracycline, 10 µg/ml. λGEM12 derivatives were propagated on *E. coli* LE392 as previously described (3). *A. pyogenes* isolates used in this study were obtained from veterinary diagnostic laboratories or personal culture collections in North America and include 20 tetracycline-resistant isolates (7 of bovine origin, 12 of porcine origin, and 1 from a macaw) and 10 tetracycline-susceptible isolates (6 of bovine origin and 4 of porcine origin). Specific *A. pyogenes* strains used in mating experiments are described in Table 1. *A. pyogenes* strains were grown on brain heart infusion (BHI) agar (Difco), supplemented with 5% bovine blood, at 38°C and 5% CO₂ in a humidified incubator. Liquid cultures of *A. pyogenes* were grown in BHI broth supplemented with 5% fetal bovine serum (Omega Scientific, Inc.) at 37°C. Antibiotics were added as appropriate at the following concentrations: erythromycin, 15 µg/ml; kanamycin, 30 µg/ml; nalidixic acid, 10 µg/ml; tetracycline, 5 µg/ml.

* Corresponding author. Mailing address: Department of Veterinary Science and Microbiology, The University of Arizona, 1117 East Lowell St., Tucson, AZ 85721. Phone: (520) 621-2745. Fax: (520) 621-6366. E-mail: sbilling@u.arizona.edu.

TABLE 1. Relevant characteristics of bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source ^a or reference
Strains		
<i>E. coli</i>		
DH5 α MCR	F ⁻ <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) Φ 80 <i>dlacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>) U169 <i>deoR recA1 endA1 thi-1 phoA supE44 gyrA96 relA1</i> λ ⁻	Gibco-BRL
LE392	F ⁻ e14 ⁻ <i>mcrA hsdR514</i> (r _K ⁻ m _K ⁺) <i>supE44 supF58 lacY1 galK2 galT22 metB1 trpR55</i>	Promega
S17-1	<i>recA pro hsdR</i> RP4-2-Tc::Mu-Kan::Tn7 Tmp ^r Spt ^r Str ^r	29
<i>A. pyogenes</i>		
BBR1	Bovine isolate, Tet ^r	6
JGS190	Bovine isolate, Tet ^r	CSU
3	Bovine isolate, Tet ^r	25
4	Bovine isolate, Tet ^r	25
98-1508	Macaw isolate, Tet ^r	AZ VDL
3274	Bovine isolate, Tet ^s	CSU
JGS478	<i>nanP</i> ::Kan derivative of strain 3274	This study
JGS603	Tet ^r Kan ^r BBR1 \times JGS478 transconjugant	This study
JGS605	Tet ^r Kan ^r JGS190 \times JGS478 transconjugant	This study
JGS610	<i>nanH</i> ::Erm derivative of strain 3274	This study
Plasmids		
pBC KS	Chl ^r <i>lacZ'</i> , ColE1 origin	Stratagene
pCR-Script Cam SK(+)	pBC SK with incorporated <i>SrfI</i> site	Stratagene
pWSK129	Kan ^r <i>lacZ'</i> , p15A origin	38
pJRD215	Kan ^r <i>mob</i> ⁺ , RSF1010 origin	12
pJGS259	pBC KS <i>BamHI</i> Ω 4-kb partial <i>Sau3AI</i> fragment containing the <i>tet(W)</i> gene of strain 4	This study
pJGS264	pJGS259 Δ 1.3-kb <i>NorI</i> fragment	This study
pJGS279	pBC KS <i>BamHI</i> Ω 12-kb <i>BamHI</i> fragment from λ JGS9 containing the <i>tet(W)</i> gene of BBR1	This study
pJGS324	pCR-Script Cam SK <i>SfiI</i> Ω 1,710-bp PCR product containing <i>mob</i> and <i>oriT</i>	This study
pJGS416	pBC KS <i>SacI-KpnI</i> Ω 1,524-bp PCR product containing <i>mob</i>	This study
pJGS417	pWSK129 <i>SacI-KpnI</i> Ω 202-bp PCR product containing <i>oriT</i>	This study

^a CSU, Colorado State University; AZ VDL, Arizona Veterinary Diagnostic Laboratory.

Determination of MICs of tetracycline antibiotics. A broth microdilution technique was used to determine the MICs of tetracycline antibiotics for *A. pyogenes* isolates. The inoculum was prepared by resuspending several isolated colonies in sterile Mueller-Hinton Broth (Difco) containing 2% lysed, defibrinated horse blood (BBL Microbiology Systems) and adjusting the suspension to an optical density at 600 nm (OD₆₀₀) of 0.001 (approximately 10⁶ CFU/ml). The antimicrobial agents tetracycline, chlortetracycline, and oxytetracycline (Sigma) were prepared according to the guidelines of the National Committee for Clinical Laboratory Standards (23). The antibiotics were diluted in a doubling dilution pattern over the range 0.06 to 64 μ g/ml in the wells of sterile, 96-well, round-bottom microtiter plates (Falcon) in 50- μ l volumes. Fifty microliters of inoculum was dispensed into each well, and the plates were sealed and incubated at 38°C and 5% CO₂ for 24 h. The MIC was read visually as the lowest concentration of the antibiotic to prevent growth (turbidity) compared with that of the control (no antibiotic added). All MICs were tested in duplicate on at least two independent cultures. MICs for *A. pyogenes* isolates were also determined after growth as described above on BHI-5% bovine blood agar, supplemented with 1 μ g of tetracycline per ml, to determine the inducibility of the determinant. MICs for *E. coli* strains were performed essentially as described above, except that Mueller-Hinton broth was used without supplementation, and plates were incubated at 37°C for 18 to 24 h.

DNA techniques. Procedures for *E. coli* transformation and plasmid extraction, bacteriophage DNA extraction, DNA restriction, ligation, agarose gel electrophoresis, and DNA dot blotting were performed essentially as described previously (3). Genomic DNA was prepared from *A. pyogenes* strains by the method of Pospiech and Neumann (24). Preparation of DNA probes, DNA hybridization, and probe detection were performed with the digoxigenin (DIG) DNA labeling and detection kit (Roche Molecular Biochemicals). For dot blot analysis against genomic DNA from *A. pyogenes*, a *tet(W)*-specific gene probe spanning bases 117 to 1362 of *tet(W)* was generated by PCR with primers (Sigma-Genosys), 5'-AAGCGGGAGCGGCGTAACAGAC-3' and 5'-GACAA CGAGAACGGACACTATG-3', and a *mob*-specific gene probe spanning bases 450 to 1177 of *mob* was generated by PCR with primers 5'-CTACCCTCCA TGTGTCTAT-3' and 5'-GCGCAGACCTCGTAAATCCTGG-3'. PCR DNA amplification was performed with *Taq* DNA polymerase (Promega) for 35 cycles consisting of 1 min at 94°C (DNA denaturation), 1 min at 55°C (primer annealing), and 1 min/kb at 72°C (DNA synthesis).

Cloning of the *A. pyogenes* tetracycline resistance genes. A library of partially *Sau3AI*-digested genomic DNA from *A. pyogenes* strain 4 was prepared in the plasmid vector pBC KS (Stratagene). The ligation mixture was introduced into *E. coli* strain DH5 α MCR by electroporation, and tetracycline-resistant transformants were selected on LB agar containing 10 μ g of tetracycline per ml. One tetracycline-resistant transformant contained the plasmid pJGS259 with a 4-kb insert.

A DIG-labeled probe was prepared from pJGS259 and used to screen a λ GEM12 (Promega) library of partial *Sau3AI* fragments from our standard laboratory *A. pyogenes* strain, BBR1. A 12-kb *BamHI* fragment from one probe-positive λ GEM12 recombinant, λ JGS9, was cloned into the *BamHI* site of pBC KS to create pJGS279.

Nucleotide sequence determination. Nucleotide sequencing of tetracycline resistance determinants was performed on a 377A DNA sequencer (Applied Biosystems, Inc.) at the Automated DNA Sequencing Facility at the University of Arizona. Sequence was determined from both strands, crossing all restriction sites. Sequence data were determined from primary clones and appropriate subclones by using vector- and insert-specific primers. To determine the sequence of the strong *rho*-independent terminator of *tet(W)*, it was necessary to design primers to the sequence immediately upstream and downstream of the stem-loop. These primers were used in two-step (96°C, 60°C) cycle sequencing reactions performed in the presence of 5% dimethyl sulfoxide.

Computer analysis. Nucleotide sequence data were compiled by using the Sequencher 3.1 program (GeneCodes, Ann Arbor, Mich.). Database searches were performed with the BlastN, BlastX, and BlastP algorithms (1). Sequence analysis was performed with the suite of programs available through the Genetics Computer Group, Inc. (University of Wisconsin). Similarity was determined from optimized sequence alignments by using the CLUSTAL W program (33).

Construction of plasmids for mobilization experiments. The *mob* gene and *oriT* of pJGS279 were amplified by PCR with the primers 5'-CTGGGGGAGG CAACCGCACACC-3', which binds 150 bp upstream of *oriT*, and 5'-GTCCAC GATTTCCGCCGCACAC-3', which binds 265 bp downstream of *mob*. The 1,710-bp PCR product was polished with the PCR-Script Cam cloning kit (Stratagene) and inserted into the *SfiI* site of pCR-Script Cam SK(+). To create pJGS324 (Table 1). The *mob* gene was amplified from pJGS324 with the M13 reverse primer (5'-AGCGGATAACAATTTTCACACAGGA-3') and the primer 5'-TCCCGCAGGAGGTACCTCCCCTGAAC-3', containing a *KpnI* site. The

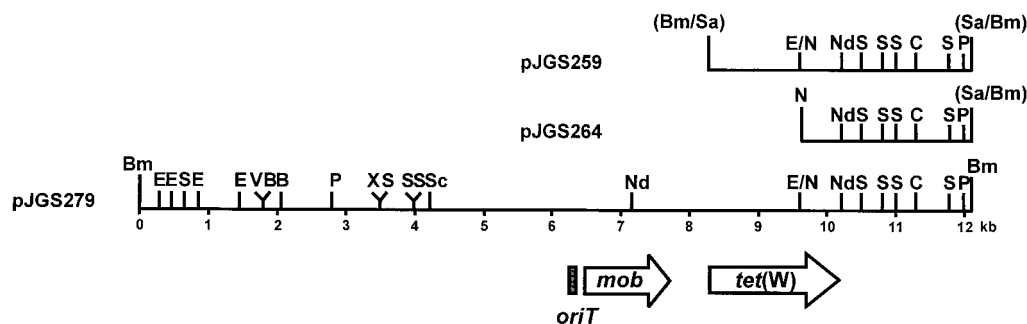


FIG. 1. Restriction maps of plasmids containing the *tet(W)* genes of strains 4 (pJGS259) and BBR1 (pJGS279). pJGS264, the *NotI* deletion derivative of pJGS259 that does not confer tetracycline resistance in *E. coli*, is also shown. Only the *A. pyogenes*-derived insert is shown for each plasmid. The large arrows indicate the positions of ORFs, which are labeled. The *oriT* upstream of *mob* is indicated by the solid rectangle. Restriction enzyme sites are as follows: B, *Bgl*II; Bm, *Bam*HI; C, *Cl*AI; E, *Eag*I; N, *Not*I; Nd, *Nde*I; P, *Pst*I; Sa, *Sau*3AI; S, *Sac*I; Sc, *Sc*AI; V, *Eco*RV; and X, *Xho*I. Coordinates (in kilobases) are given below the pJGS279 map.

1,524-bp PCR product, containing only the *mob* gene, was digested with *Kpn*I and *Sac*I and inserted into similarly digested pBC KS to create pJGS416 (Table 1). *oriT* was amplified from pJGS324 by using the M13 universal primer (5'-ACGTTGTAACGACGCGCCAGT-3') and the primer 5'-TCGAGGACGGGAGCTCAGGGGAGGC-3', containing a *Sac*I site. The 202-bp PCR product, containing only *oriT*, was digested with *Kpn*I and *Sac*I and inserted into similarly digested pWSK129 (Table 1) to create pJGS417.

***E. coli* mobilization experiments.** Recombinant plasmids to be assessed for mobilization were introduced into *E. coli* S17-1 (Table 1) by transformation. S17-1 supplies conjugative functions *in trans* from a chromosomal copy of RP4. These strains were then used as donors in filter matings with the nalidixic acid-resistant recipient strain DH5 α MCR. Donor and recipient strains were grown overnight under appropriate selection, diluted to an OD₆₀₀ of 0.05 in the same medium, and grown to an OD₆₀₀ of 1.0. The two cultures (0.5 ml of each) were mixed and filtered through a 0.45- μ m-pore-size filter. The filter was incubated for 2 h on an LB agar plate at 37°C, and the cells were resuspended in LB broth. Serial dilutions were plated onto LB agar containing the appropriate antibiotics to select for transconjugants. Bacterial viable counts were obtained from identically treated filters containing either the donor or recipient strain alone. Mobilization frequencies were expressed as transconjugants per donor cell and were determined as an average of at least three independent experiments.

***A. pyogenes* filter matings.** Tetracycline-resistant *A. pyogenes* isolates were used in filter matings with the kanamycin-resistant *A. pyogenes* recipient strain JGS478. JGS478 is a derivative of the tetracycline-susceptible isolate 3274, which contains a kanamycin resistance cassette inserted into the neuraminidase gene, *nanP* (Table 1). Donor and recipient strains were grown overnight under appropriate selection, diluted to an OD₆₀₀ of 0.1 in the same medium, and grown to an OD₆₀₀ of 1.0. The two cultures (0.5 ml of each) were mixed and filtered through a 0.45- μ m-pore-size filter. Following overnight incubation on a BHI-5% blood agar plate at 37°C under 5% CO₂, the cells were resuspended from the filter in BHI broth. Serial dilutions were plated onto BHI-5% blood agar supplemented with 5 μ g of tetracycline and 30 μ g of kanamycin per ml to select for transconjugants. Bacterial viable counts were obtained from identically treated filters containing either the donor or recipient strain alone. Conjugation frequencies were expressed as transconjugants per donor cell and were determined as an average of at least three independent experiments.

Selected transconjugants from the conjugations described above were used as donors in separate conjugation experiments with JGS610 (Table 1). JGS610 is an erythromycin-resistant derivative of strain 3274, which contains an erythromycin resistance cassette inserted into the *nanH* gene, constructed as previously described (17).

Nucleotide sequence accession number. The nucleotide sequences of the *A. pyogenes* BBR1 *mob* and *tet(W)* genes have been deposited in the GenBank nucleotide sequence database under accession no. AY049983.

RESULTS

Cloning of an *A. pyogenes* tetracycline resistance determinant. The tetracycline resistance determinant from *A. pyogenes* strain 4 was cloned, by direct selection, on a 4-kb partial *Sau*3AI fragment, in the vector pBC KS, to generate pJGS259

(Fig. 1). The MICs of tetracycline, chlortetracycline, and oxytetracycline for *E. coli* DH5 α MCR carrying pJGS259 were significantly higher than that for a control strain carrying the vector pBC KS (Table 2). A *Not*I deletion derivative, pJGS264 (Fig. 1), did not confer tetracycline resistance upon DH5 α MCR (Table 2), suggesting this deletion removed all or part of the tetracycline resistance determinant. Nucleotide sequence data from this region of pJGS259 allowed the identification of a 1,920-bp open reading frame (ORF) that spanned the *Not*I site of pJGS259 and showed 92% DNA sequence identity to the *tet(W)* gene of the rumen bacterium *Butyrivibrio fibrisolvens* (5). The putative product of this ORF had 91% identity and 96% similarity to the *B. fibrisolvens* TetW protein. Since current convention insists that 80% amino acid identity is the cutoff between tetracycline resistance determinants (19), the *A. pyogenes* determinant was also designated Tet W. Like the TetW protein of *B. fibrisolvens* and other ribosomal protection tetracycline resistance proteins, the *A. pyogenes* TetW protein shared similarity with the elongation factors, particularly in N-terminal regions associated with GTP binding (32).

Since the *tet(W)* gene in pJGS259 abuts one ends of the clone, a *tet(W)*-specific probe was used to identify homologous sequences in a λ GEM12 library of our standard laboratory strain, BBR1, which is also tetracycline resistant. Homologous sequences were subcloned into pBC KS to create pJGS279 (Fig. 1), which overlaps considerably in restriction sites with pJGS259 and also confers tetracycline, chlortetracycline, and oxytetracycline resistance to DH5 α MCR (Table 2). Nucleotide

TABLE 2. MICs of tetracycline, chlortetracycline, and oxytetracycline for *E. coli* clones and *A. pyogenes* strain BBR1

Strain	MIC (μ g/ml) of:		
	Tetracycline	Chlortetracycline	Oxytetracycline
DH5 α MCR(pBC KS)	0.25	0.12	1
DH5 α MCR(pJGS259)	16	8	32
DH5 α MCR(pJGS264)	0.25	0.12	0.5
DH5 α MCR(pJGS279)	32	32	64
BBR1	8	8	8
BBR1 + tetracycline ^a	8	8	8

^a BBR1 was grown on BHI-blood agar supplemented with 1 μ g of tetracycline per ml prior to determination of the MIC.

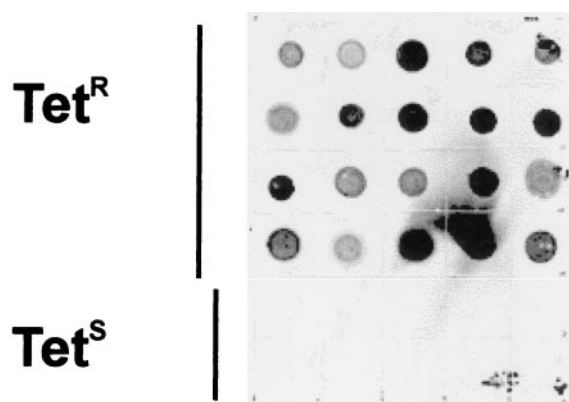


FIG. 2. Dot blot hybridization of tetracycline-resistant (Tet^R) and tetracycline-susceptible (Tet^S) isolates with a *tet(W)*-specific probe. Approximately 500 ng of genomic DNA from 20 tetracycline-resistant and 10 tetracycline-sensitive isolates was spotted onto a nylon membrane in the arrangement indicated and hybridized with the 1,246-bp *tet(W)*-specific probe.

sequencing of the BBR1 *tet(W)* gene indicated that it was identical to that of strain 4. However, pJGS279 contains at least 6 kb upstream of the *tet(W)* gene (Fig. 1).

The percent G+C content of the *tet(W)* genes of strain 4 and BBR1 was 52.2, which is considerably lower than that of the *A. pyogenes* housekeeping genes we have sequenced (average G+C = 62.5%) (S. J. Billington, S. T. Gilbert, and B. H. Jost, unpublished data). Downstream of *tet(W)* was a strong *rho*-independent terminator ($\Delta G = -35.7$ kcal/mol) composed of a 14-bp stem of completely base-paired G and C residues. Upstream of *tet(W)* were sequences similar to those that control the regulation of the inducible *tet(M)* gene of Tn916 (31), including the presence of a significant secondary structure ($\Delta G = -26.3$ kcal/mol) 36 bp upstream of *tet(W)*, which could act as a *rho*-independent terminator. However, the MIC of tetracycline for BBR1 was not increased following incubation on plates containing tetracycline (Table 2), suggesting that this *tet(W)* gene is not induced by tetracycline.

Distribution of the *tet(W)* gene among *A. pyogenes* isolates. Genomic DNA from 20 tetracycline-resistant and 10 tetracycline-susceptible isolates were subjected to dot blot analysis with a *tet(W)*-specific gene probe (Fig. 2). All 20 tetracycline-resistant isolates, but none of the tetracycline-susceptible isolates, hybridized to the *tet(W)*-specific probe. To confirm these results, oligonucleotide primers internal to the *tet(W)* gene were used to screen all 30 isolates by PCR for the presence of the gene. Again, a direct correlation was obtained between resistance of the isolate to tetracycline and the amplification of a 694-bp DNA fragment internal to *tet(W)* (data not shown). These results indicate that *tet(W)* is widely distributed among tetracycline-resistant *A. pyogenes* strains.

Identification of a *tet(W)*-associated mobilization gene. A 1,194-bp ORF, designated *mob*, was identified 657 bp upstream of the BBR1 *tet(W)* gene (Fig. 1). The product of the *mob* gene had significant similarity to members of the Pre/Mob family of gram-positive mobilization proteins (4). Pre/Mob proteins can effect mobilization of DNA elements, such as plasmids and transposons, in the presence of conjugative machinery by creating a nick at an origin of transfer, *oriT* (11).

The BBR1 Mob protein had most similarity to the TnpZ proteins of the clostridial mobilizable transposons Tn4451 (35.4% identity, 65.8% similarity) (11) and Tn4453a (35.6% identity, 67.5% similarity) (20) (Fig. 3A). TnpZ nicks the circular form of these transposons at an upstream *oriT*, to create the leading strand for mobilization. Immediately upstream of the BBR1 *mob* gene is a sequence with identity of 22 of 24 bp to the *oriT* of Tn4451 and Tn4453a (11, 20) (Fig. 3B).

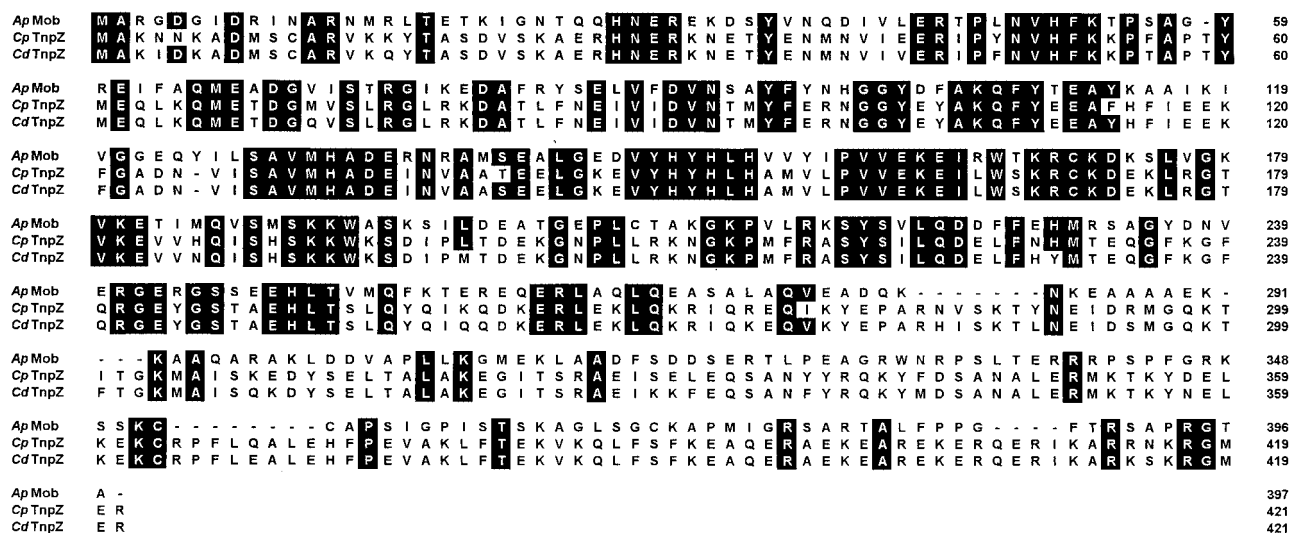
The *tet(W)*-associated *mob* gene and *oriT* can function in mobilization. *E. coli* plasmids carrying the BBR1 *mob* gene and *oriT* were assessed for their ability to be mobilized from *E. coli* strain S17-1 to DH5 α MCR (Table 3). pJGS279 could be mobilized at a frequency >4 logs more efficiently than the negative control plasmid pBC KS, with which no transconjugants were obtained. pJGS324, containing only the *mob* gene and *oriT*, could be transferred at a frequency similar to that of pJGS279, suggesting that the *mob* gene and *oriT* can function in DNA mobilization. When the *mob* gene (pJGS416) or *oriT* (pJGS417) was cloned independently, neither of these plasmids could be mobilized. However, if these plasmids were present in the same cell, pJGS417, but not pJGS416, could be mobilized to the recipient strain. Thus, the product of the *mob* gene expressed from pJGS416 is able to act in *trans* on *oriT* to effect mobilization of pJGS417.

The *mob* gene is only present in 25% of tetracycline-resistant *A. pyogenes* strains. A *mob*-specific gene probe was used to screen genomic DNA from 20 tetracycline-resistant and 10 tetracycline-susceptible *A. pyogenes* isolates (Fig. 4). Despite the fact that each of the 20 tetracycline-resistant isolates carries *tet(W)*, only 5 (25%) of these isolates hybridized to the *mob*-specific probe. The five *mob*⁺ strains represent four of the seven tetracycline-resistant bovine isolates examined as well as the single avian isolate, 98-1508. Thus, none of the 12 tetracycline-resistant porcine isolates examined carried the *mob* gene. The tetracycline-susceptible isolates did not hybridize to the *mob* probe (Fig. 4).

Conjugative transfer of a *tet(W)* element from *mob*⁺ *A. pyogenes* isolates. Each of the five *mob*⁺ strains, BBR1, JGS190, 3, 4, and 98-1508, was used in filter mating experiments with a kanamycin-resistant recipient strain, JGS478 (Table 4). Small numbers of putative tetracycline-resistant, kanamycin-resistant transconjugants were obtained with BBR1, JGS190, or 98-1508 as the donor. The frequency of conjugative transfer for these strains was very low (Table 4), and the possibility existed that colonies with resistance to both tetracycline and kanamycin could have arisen through spontaneous mutation. However, mock conjugations involving only donor or recipient cultures did not yield tetracycline-resistant, kanamycin-resistant colonies. Genomic DNA from transconjugants resulting from matings with each of the donors were probed in DNA dot blots with probes specific for the kanamycin resistance gene of the recipient and the *tet(W)* gene of the donor. DNA from each putative transconjugant hybridized with both probes, confirming that these transconjugants result from the transfer of *tet(W)* from the donor strain to JGS478 (data not shown). In addition, each of these transconjugants also hybridized to a *mob*-specific gene probe (data not shown), indicating that *mob* is part of the transferred genetic element.

Transconjugants resulting from BBR1 \times JGS478 (JGS603) and JGS190 \times JGS478 (JGS605) crosses were used as donors

A.



B.



FIG. 3. (A) Amino acid sequence alignment of the *A. pyogenes* Mob protein (*Ap Mob*) with the clostridial TnpZ proteins from Tn4451 of *Clostridium perfringens* (*Cp TnpZ*) (11) and Tn4453a of *C. difficile* (*Cd TnpZ*) (20). Amino acids identical to those in Mob are boxed. Amino acid numbers are shown on the right. (B) Nucleotide sequence comparison of the *oriT* upstream of *mob* with the *oriT* of Tn4451 and that of Tn4453a are identical (11, 20). Identical nucleotides are boxed, and the region of dyad symmetry is indicated by the divergent arrows.

in conjugation experiments with JGS610, an erythromycin-resistant derivative of 3274 (Table 4). Both transconjugants were able to transfer tetracycline resistance to JGS610 at frequencies approximately 10-fold higher than that of the original donor strains.

DISCUSSION

In the United States, tetracyclines, predominantly chlortetracycline and oxytetracycline, are fed extensively to food animals (36, 37), so commensal organisms like *A. pyogenes* are constantly exposed to these antibiotics. Tetracyclines tend to be fed on a long-term basis for growth promotion in pigs (36), whereas they are fed on a shorter-term basis to cattle, primarily for the prevention of certain diseases upon entry of cattle into feedlots (37). These data may partially explain the high prevalence of tetracycline resistance among porcine isolates of *A.*

pyogenes (34). It is now recognized that bacteria commensal on animals provide a reservoir of antibiotic resistance genes that are frequently mobile and capable of being transferred to other bacteria entering the host.

In this paper, we report the cloning of the tetracycline resistance determinant from two bovine isolates of *A. pyogenes*, a frequent commensal organism on the mucous membranes of domestic animals. The predicted tetracycline resistance protein from *A. pyogenes* is greater than 80% identical to TetW of

TABLE 3. Mobilization frequencies of *mob* and *oriT* plasmids

Donor	Mobilization frequency ^a
S17-1(pJRD215)	3.5 × 10 ⁻²
S17-1(pBC KS)	<7.5 × 10 ⁻⁸
S17-1(pWSK129)	<7.4 × 10 ⁻⁸
S17-1(pJGS279)	1.0 × 10 ⁻³
S17-1(pJGS324)	3.2 × 10 ⁻⁴
S17-1(pJGS416)	<6.6 × 10 ⁻⁸
S17-1(pJGS417)	<2.3 × 10 ⁻⁸
S17-1(pJGS416)	<2.9 × 10 ⁻⁷
+ pJGS417)	1.4 × 10 ⁻⁴

^a Number of transconjugants per donor cell.

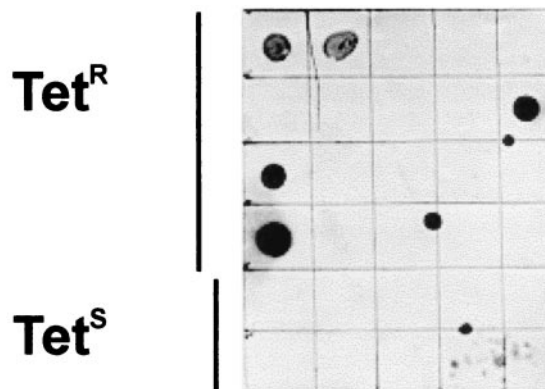


FIG. 4. Dot blot hybridization of tetracycline-resistant (Tet^r) and tetracycline-susceptible (Tet^s) strains with a *mob*-specific probe. Approximately 500 ng of genomic DNA from 20 tetracycline-resistant and 10 tetracycline-sensitive isolates was spotted onto a nylon membrane in the arrangement indicated and hybridized with the 728-bp *mob*-specific probe.

TABLE 4. Conjugation frequencies of Tet W determinants among *A. pyogenes* strains

Donor	Recipient	Conjugation frequency ^a
BBR1	JGS478	2.5×10^{-10}
JGS190	JGS478	2.2×10^{-10}
3	JGS478	$<7.6 \times 10^{-11}$
4	JGS478	$<4.2 \times 10^{-11}$
98-1508	JGS478	9.0×10^{-11}
JGS603	JGS610	5.4×10^{-9}
JGS605	JGS610	4.4×10^{-9}

^a Number of transconjugants per donor cell.

the obligately anaerobic rumen bacterium *B. fibrisolvens*, and thus by the criteria of Levy et al. (19), the *A. pyogenes* determinant was also classified as Tet W. Tet W determinants have previously been described only in obligately anaerobic bacteria isolated from the rumen of sheep and cattle, pig feces, or the human gut (2, 5, 28). *A. pyogenes* is commensal on the ruminal wall of cattle (22) and has been isolated from the stomach of pigs (B. H. Jost, K. W. Post, J. G. Songer, and S. J. Billington, unpublished data), and it may have acquired the determinant from obligately anaerobic rumen or gastric bacteria. The *tet(W)* genes of the obligate anaerobes diverge in DNA sequence by less than 1% over the coding region, consistent with proposed recent intergeneric transfer of the determinant within the gastrointestinal environment (5, 28). The *A. pyogenes tet(W)* gene diverges by approximately 8% from these sequences, but is still highly homologous, consistent with its potential inheritance from the normal flora anaerobes. The greater divergence of the *tet(W)* gene of *A. pyogenes* may be due to a longer residence of this gene in *A. pyogenes*. However, inconsistent with this hypothesis is the fact that less than half of the base changes between the *B. fibrisolvens* and the *A. pyogenes tet(W)* sequences are A/T to G/C transitions. The percent G+C content of the *A. pyogenes tet(W)* gene and the associated *mob* gene are lower than those of *A. pyogenes* housekeeping genes, suggestive of an origin outside of *A. pyogenes*. A similar argument has been used to suggest that *tet(W)* did not originate in *B. fibrisolvens*, which has a much lower percent G+C content. However, several of the gastrointestinal species that carry *tet(W)* have G+C contents in the range of 50 to 55% (28).

The presence of Tet W in the facultative anaerobe *A. pyogenes* gives this determinant a life outside the gastrointestinal tract of animals and humans and establishes a bridge between the *tet(W)*-carrying anaerobic bacteria found in different animal hosts. While *A. pyogenes* is likely capable of survival in the environment, it is still relatively fastidious and is generally found associated with animals. However, even on the respiratory mucosa, *A. pyogenes* will interact with a different set of microbiological flora from that present in the gastrointestinal tract. The ability of *A. pyogenes* to transfer the Tet W determinant to other bacteria may play a role in the dissemination of this determinant. Recent studies of the distribution of *tet(W)* genes suggest that while they contribute significantly to the resistance of the gastrointestinal flora of animals (2), the ability of these genes to be disseminated even in effluent is limited (9). Despite this fact, *tet(W)* genes were detected in food components and even antibiotics fed to swine (2).

The region upstream of the *A. pyogenes tet(W)* gene is similar to a region upstream of the *tet(M)* gene of Tn916, which is involved in transcriptional attenuation of the *tet(M)* gene (31). This observation has previously been made for the *B. fibrisolvens tet(W)* gene, although no inducibility data were described (5). The tetracycline resistance of strain BBR1 was not inducible by previous exposure to tetracycline. This is also true of the resistances of the other tetracycline-resistant isolates examined in this study (34). Therefore, it is unlikely that these upstream sequences are involved in *tet(W)* induction. Other ribosomal protection *tet* genes, such as *tet(O)*, also have upstream sequences similar to the *tet(M)* upstream region, although these determinants are expressed constitutively (39). However, for *tet(O)*, this upstream region is required for full expression of the gene (39).

The association of *tet(W)* with a functional *mob* gene is also indicative of the mobile nature of this determinant. The *mob* gene encodes a protein of the Mob/Pre family of gram-positive mobilization proteins, which function in mobilization by single-stranded cleavage at an *oriT* sequence. The *tet(W)*-associated Mob protein is most similar to the TnpZ proteins of the clostridial mobilizable transposons Tn4451 and Tn4453a (11, 20), and its associated *oriT* shares identity of 22 of 24 bp to the *oriT* of these transposons. The Mob protein appears capable of acting in *trans* at its cognate *oriT* to effect mobilization, at least in *E. coli*. While the direct effect of Mob on the transmissibility of *tet(W)* was not addressed in this study, the transfer of *tet(W)* and *mob*, from at least some of the *mob*⁺ tetracycline-resistant *A. pyogenes* strains, is consistent with the presence of these genes on a mobile genetic element. The exact nature of the genetic element is not yet known, since *mob* genes can be present on either mobilizable or conjugative plasmids or transposons. These strains have previously been examined for the presence of plasmids, and the only plasmid identified was the small, broad-host-range plasmid pAPI1, which lacks conjugative or mobilization functions, in BBR1 and JGS190 (7). However, the techniques used in those studies may not have allowed the identification of large plasmids. Preliminary sequence data from one end of pJGS279 indicated the presence of the putative housekeeping gene *rluC*, which encodes pseudouridylyl synthase (data not shown). The presence of a housekeeping gene on pJGS279 suggests that the insert from this recombinant plasmid is of chromosomal, rather than plasmid, origin. A chromosomal location for *tet(W)* is consistent with the low transfer efficiency, since this adds additional rate-limiting steps that could affect conjugative transfer, such as excision, and in the case of nonreplicating DNA, chromosomal insertion. The *tet(W)* genes of *B. fibrisolvens* strains 1.23 and 1.230 have been reported to be capable of being transferred by conjugation (27), although the frequency of conjugative transfer was considerably higher than was observed in this study. The *B. fibrisolvens* element also appears to be chromosomally located, although no additional sequence information is available to determine if it is an element similar to that described here. The lack of transconjugants observed with strains 3 and 4 as donors may reflect a defect in some part of the transfer process, or, given the low transfer frequency observed with the other three *mob*⁺ strains, the transfer frequency could be below the level of detection in the assays performed. Interestingly, onward transfer of the element from transconjugants appeared to be

more efficient than the initial transfer event. This result may reflect transfer into a strain with the same background, since JGS478 and JGS610 are both derivatives of 3274. Recent evidence suggests that some strains of *A. pyogenes* carry different restriction-modification mechanisms (S. J. Billington and B. H. Jost, unpublished data), which may ultimately affect transfer frequencies.

The presence of *tet(W)* in each of the tetracycline-resistant isolates examined in this study suggests that Tet W is a widespread determinant of tetracycline resistance in *A. pyogenes*. However, since no other determinants were examined in this study, it is possible that some or all of these isolates may carry more than one tetracycline resistance determinant. Despite the fact that all tetracycline-resistant *A. pyogenes* isolates carry *tet(W)*, only 25% of these strains carry the *mob* gene, which appears to be part of the genetic element carrying *tet(W)*. Incidentally, the majority of *mob*⁺ isolates are of bovine origin, and none of the 12 tetracycline-resistant isolates of porcine origin carries this gene. This difference may reflect the way in which bovine and porcine isolates have inherited this gene, with bovine isolates primarily receiving *tet(W)* on the element described in this study, but with porcine isolates receiving *tet(W)* by an alternative route, perhaps through a separate genetic element.

ACKNOWLEDGMENTS

We thank Hien Trinh and Dawn Bueschel for excellent technical assistance and Stefani Gilbert for construction of the *A. pyogenes* BBR1 λGEM12 library.

REFERENCES

- Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**:3389–3402.
- Aminov, R. I., N. Garrigues-Jeanjean, and R. I. Mackie. 2001. Molecular ecology of tetracycline resistance: development and validation of primers for detection of tetracycline resistance genes encoding ribosomal protection proteins. *Appl. Environ. Microbiol.* **67**:22–32.
- Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl. (ed.) 1994. *Current protocols in molecular biology*, vol. 1. Greene Publishing Associates and John Wiley and Sons, Inc., New York, N.Y.
- Bannam, T. L., P. K. Crellin, and J. I. Rood. 1995. Molecular genetics of the chloramphenicol-resistance transposon Tn451 from *Clostridium perfringens*: the TnpX site specific recombinase excises a circular transposon molecule. *Mol. Microbiol.* **16**:535–551.
- 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.
- Billington, S. J., B. H. Jost, W. A. Cuevas, K. R. Bright, and J. G. Songer. 1997. The *Arcanobacterium (Actinomyces) pyogenes* hemolysin, pyolysin, is a novel member of the thiol-activated cytolytic family. *J. Bacteriol.* **179**:6100–6106.
- Billington, S. J., B. H. Jost, and J. G. Songer. 1998. The *Arcanobacterium (Actinomyces) pyogenes* plasmid pAP1 is a member of the pIJ101/pJV1 family of rolling circle replication plasmids. *J. Bacteriol.* **180**:3233–3236.
- Carter, G. R., and M. M. Chengappa. 1991. *Essentials of veterinary bacteriology and mycology*, 4th ed. Lea and Febiger, Philadelphia, Pa.
- Chee-Sanford, J. C., R. I. Aminov, I. J. Krapac, N. Garrigues-Jeanjean, and R. I. Mackie. 2001. Occurrence and diversity of tetracycline resistance genes in lagoons and groundwater underlying two swine production facilities. *Appl. Environ. Microbiol.* **67**:1494–1502.
- Chopra, I., and M. Roberts. 2001. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiol. Mol. Biol. Rev.* **65**:232–260.
- Crellin, P. K., and J. I. Rood. 1998. Tn451 from *Clostridium perfringens* is a mobilizable transposon that encodes the functional Mob protein, TnpX. *Mol. Microbiol.* **27**:631–642.
- Davison, J., M. Heusterspreute, N. Chevalier, H.-T. Vinh, and F. Brunel. 1987. Vectors with restriction site banks. V. pJRD215, a wide-host-range cosmid vector with multiple cloning sites. *Gene* **51**:275–280.
- Guérin-Faubleé, V., J. P. Flandrois, E. Broye, F. Tupin, and Y. Richard. 1993. *Actinomyces pyogenes*: susceptibility of 103 clinical animal isolates to 22 antimicrobial agents. *Vet. Res.* **24**:251–259.
- Hoie, S., K. Falk, and B. M. Lium. 1991. An abattoir survey of pneumonia and pleuritis in slaughter weight swine from 9 selected herds. IV. Bacteriological findings in chronic pneumonic lesions. *Acta Vet. Scand.* **32**:395–402.
- Jonsson, P., S.-E. Olsson, A.-S. Olofson, C. Fälth, O. Holmberg, and H. Funke. 1991. Bacteriological investigations of clinical mastitis in heifers in Sweden. *J. Dairy Res.* **58**:179–185.
- Jost, B. H., J. G. Songer, and S. J. Billington. 1999. An *Arcanobacterium (Actinomyces) pyogenes* mutant deficient in production of the pore-forming cytolytic pyolysin has reduced virulence. *Infect. Immun.* **67**:1723–1728.
- Jost, B. H., J. G. Songer, and S. J. Billington. 2001. Cloning, expression, and characterization of a neuraminidase gene from *Arcanobacterium pyogenes*. *Infect. Immun.* **69**:4430–4437.
- Lechtenberg, K. F., T. G. Nagaraja, H. W. Leipold, and M. M. Chengappa. 1988. Bacteriologic and histologic studies of hepatic abscesses in cattle. *Am. J. Vet. Res.* **49**:58–62.
- 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 determinants. *Antimicrob. Agents Chemother.* **43**:1523–1524.
- Lyas, D., and J. I. Rood. 2000. Transposition of Tn4451 and Tn4453 involves a circular intermediate that forms a promoter for the large resolvase, TnpX. *Mol. Microbiol.* **38**:588–601.
- Nagaraja, T. G., S. B. Laudert, and J. C. Parrott. 1996. Liver abscesses in feedlot cattle. I. Causes, pathogenesis, pathology, and diagnosis. *Comp. Cont. Edu. Pract. Vet.* **18**:S230–S241, S256.
- Narayanan, S., T. G. Nagaraja, N. Wallace, J. Staats, M. M. Chengappa, and R. D. Oberst. 1998. Biochemical and ribotypic comparison of *Actinomyces pyogenes* and *A. pyogenes*-like organisms from liver abscesses, ruminal wall, and ruminal contents of cattle. *Am. J. Vet. Res.* **59**:271–276.
- National Committee for Clinical Laboratory Standards. 1999. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals. Approved standard M31-A. National Committee for Clinical Laboratory Standards, Wayne, Pa.
- Pospiech, A., and B. Neumann. 1995. A versatile quick-prep of genomic DNA from gram-positive bacteria. *Trends Genet.* **11**:217–218.
- Reddy, C. A., C. P. Cornell, and A. M. Fraga. 1980. Chemically defined growth medium for *Corynebacterium pyogenes*. *Am. J. Vet. Res.* **41**:843–845.
- Ross, J. I., E. A. Eady, J. H. Cove, and W. J. Cunliffe. 1998. 16S rRNA mutation associated with tetracycline resistance in a gram-positive bacterium. *Antimicrob. Agents Chemother.* **42**:1702–1705.
- Scott, K. P., T. M. Barbosa, K. J. Forbes, and H. J. Flint. 1997. High-frequency transfer of a naturally occurring chromosomal tetracycline resistance element in the ruminal anaerobe *Butyrivibrio fibrisolvens*. *Appl. Environ. Microbiol.* **63**:3405–3411.
- Scott, K. P., C. M. Melville, T. M. Barbosa, and H. J. Flint. 2000. Occurrence of the new tetracycline resistance gene *tet(W)* in bacteria from the human gut. *Antimicrob. Agents Chemother.* **44**:775–777.
- Simon, R., U. Priefer, and A. Pühler. 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram negative bacteria. *Bio/Technology* **1**:784–791.
- Speer, B. S., L. Bedzyk, and A. A. Salyers. 1991. Evidence that a novel tetracycline resistance gene found on two *Bacteroides* transposons encodes an NADP-requiring oxidoreductase. *J. Bacteriol.* **173**:176–183.
- Su, Y. A., P. He, and D. B. Clewell. 1992. Characterization of the *tet(M)* determinant of Tn916: evidence for regulation by transcription attenuation. *Antimicrob. Agents Chemother.* **36**:769–778.
- Taylor, D. E., and A. Chau. 1996. Tetracycline resistance mediated by ribosomal protection. *Antimicrob. Agents Chemother.* **40**:1–5.
- Thompson, J. D., D. G. Higgins, and T. J. Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**:4673–4680.
- Trinh, H. T., S. J. Billington, A. C. Field, J. G. Songer, and B. H. Jost. 2002. Susceptibility of *Arcanobacterium pyogenes* to tetracyclines, macrolides and lincosamides. *Vet. Microbiol.* **85**:353–359.
- Turner, G. V. 1982. A microbiological study of polyarthritis in slaughter pigs. *J. S. Afr. Vet. Assoc.* **53**:99–101.
- U.S. Department of Agriculture. 1996. Swine '95. II. Reference of 1995 U.S. grower/finisher health & management practices. USDA APHIS VS CEAH. National Animal Health Monitoring System, Fort Collins, Colo.
- U.S. Department of Agriculture. 2000. III. Health management and biosecurity in U.S. feedlots 1999. No. N336.1200. USDA APHIS VS CEAH. National Animal Health Monitoring System, Fort Collins, Colo.
- Wang, R. F., and S. R. Kushner. 1991. Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in *Escherichia coli*. *Gene* **100**:195–199.
- Wang, Y., and D. E. Taylor. 1991. A DNA sequence upstream of the *tet(O)* gene is required for full expression of tetracycline resistance. *Antimicrob. Agents Chemother.* **35**:2020–2025.
- Yoshimura, H., A. Kojima, and M. Ishimaru. 2000. Antimicrobial susceptibility of *Arcanobacterium pyogenes* isolated from cattle and pigs. *J. Vet. Med. B* **47**:139–143.