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# Multiple Genetic Elements Carry the Tetracycline Resistance Gene *tet*(W) in the Animal Pathogen *Arcanobacterium pyogenes*▽

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**The *tet*(W) gene is associated with tetracycline resistance in a wide range of bacterial species, including obligately anaerobic rumen bacteria and isolates from the human gut and oral mucosa. However, little is known about how this gene is disseminated and the types of genetic elements it is carried on. We examined tetracycline-resistant isolates of the animal commensal and opportunistic pathogen *Arcanobacterium pyogenes*, all of which carried *tet*(W), and identified three genetic elements designated ATE-1, ATE-2, and ATE-3. These elements were found in 25%, 35%, and 60% of tetracycline-resistant isolates, respectively, with some strains carrying both ATE-2 and ATE-3. ATE-1 shows characteristics of a mobilizable transposon, and the *tet*(W) genes from strains carrying this element can be transferred at low frequencies between *A. pyogenes* strains. ATE-2 has characteristics of a simple transposon, carrying only the resistance gene and a transposase, while in ATE-3, the *tet*(W) gene is associated with a streptomycin resistance gene that is 100% identical at the DNA level with the *aadE* gene from the *Campylobacter jejuni* plasmid pCG8245. Both ATE-2 and ATE-3 show evidence of being carried on larger genetic elements, but conjugation to other strains was not observed under the conditions tested. ATE-1 was preferentially associated with *A. pyogenes* strains of bovine origin, while ATE-2 and ATE-3 elements were primarily found in porcine isolates, suggesting that these elements may circulate in different environments. In addition, four alleles of the *tet*(W) gene, primarily associated with different elements, were detected among *A. pyogenes* isolates.**

Bacterial tetracycline resistance is widespread in nature and is particularly prominent in bacterial isolates from animals (8). The latter is a likely consequence of the use of tetracycline and its derivatives as feed additives for livestock, either for the prevention of disease or as growth promotants (34, 35). While several mechanisms of bacterial tetracycline resistance exist, the most common is that conferred by *tet* genes of the ribosomal protection class. Among the commonly found genes in this class is *tet*(M), which is thought to be prevalent because of its association with transposable elements, particularly those of the Tn*916* family (8). More recently, Tet W has emerged as a widespread determinant, particularly among bacterial isolates of mucosal surfaces. This determinant was originally described for *Butyrivibrio fibrisolvens* and several other anaerobes from human and animal gastrointestinal tracts (4, 28, 29, 31) but has since been described for a number of bacterial species isolated from the human oral mucosa (36).

*Arcanobacterium pyogenes* is a common inhabitant of the mucous membranes of the upper respiratory, gastrointestinal, and urogenital tracts of a number of domestic animal species (13). It is also an important opportunistic pathogen, particularly in cattle, capable of causing a number of suppurative infections of the joints and visceral organs (7). As a mucosal commensal, *A. pyogenes* is exposed to antimicrobial feed additives used for growth promotion and disease prevention in animals. Therefore, it is no surprise that nearly 42% of *A. pyogenes* isolates are resistant to tetracycline and the derivatives commonly used in agriculture, chlortetracycline and oxytetracycline (33). We have previously demonstrated that all tetracycline-resistant *A. pyogenes* isolates (MIC $\geq$ 4 μg/ml) carry a *tet*(W) gene (6). A second resistance determinant, Tet 33, has been detected in some isolates, but the resistance it confers results in a tetracycline MIC of only 1 μg/ml; thus, the strains are not considered resistant (14).

Of 20 tetracycline-resistant *A. pyogenes* isolates, five carried *tet*(W) on a genetic element which could be transferred at low frequencies between *A. pyogenes* strains (6), and we have designated this element ATE-1 (*A*rcanobacterium tetracycline resistance *e*lement-*1*). For ATE-1, *tet*(W) is associated with a functional *mob* gene and an origin of transfer, *oriT*, which are presumably responsible for its ability to transfer between strains (6). Despite the widespread nature of *tet*(W), little is known about the genetic elements on which it is carried. In *B. fibrisolvens*, the *tet*(W) gene is associated with the conjugative transposons Tn*B123* and Tn*B1230*, which confer high-frequency transfer of *tet*(W) to recipient strains (4, 28, 29). Nucleotide sequence from Tn*B1230* indicates that this transposon shares transfer genes similar to those of the *Enterococcus faecalis* conjugative transposon Tn*1549* (21). A recent study suggests that, in addition to the *B. fibrisolvens* elements, a *Bifidobacterium longum* element in which *tet*(W) is associated with a transposase can be transferred to other bacterial strains (17). In four other gastrointestinal bacterial species, *tet*(W) is associated with *orfY*, a gene which is found on a number of mobile elements. However, these species were unable to transfer *tet*(W) in the laboratory (17). In this paper we report the presence of *tet*(W) on at least three genetic elements in *A. pyogenes* and carriage of distinct *tet*(W) alleles.

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TABLE 1. *A. pyogenes* strains

| Strain | Relevant characteristics | Source or reference[a] |
|---|---|---|
| BBR1 | Bovine isolate, Tet$^r$, ATE-1 | 5 |
| OX4 | Porcine isolate, Tet$^r$, ATE-2 | Oxford Laboratories, Worthington, MN |
| OX9 | Porcine isolate, Tet$^r$, ATE-2 and ATE-3 | Oxford Laboratories, Worthington, MN |
| 52785-99 | Porcine isolate, Tet$^r$, ATE-3 | Rollins ADDL, Raleigh, NC |
| 3274 | Bovine isolate, Tet$^s$ | CSU, Fort Collins, CO |
| JGS610 | *nanH*::*erm*(X) derivative of strain 3274, Erm$^r$ | 6 |

[a] ADDL, Animal Disease Diagnostic Laboratory; CSU, Colorado State University.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** *Escherichia coli* DH5αMCR (Gibco-BRL) derivatives used in this study were grown either on LB agar or in LB broth (Difco) at 37°C. *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). All of these are independent isolates and likely represent different strains based on U.S. state and animal host of isolation and the presence or absence of virulence genes such as *cbpA* (9) and *nanP* (15). *A. pyogenes* strains (Table 1) were grown on brain heart infusion (BHI) agar (Difco), supplemented with 5% citrated bovine blood, at 38°C and 5% $CO_2$ 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 aerobically with shaking. Media were supplemented when appropriate with antibiotics at the following concentrations: for *E. coli*, chloramphenicol at 30 μg/ml, kanamycin at 50 μg/ml, and tetracycline at 10 μg/ml; for *A. pyogenes*, erythromycin at 15 μg/ml and tetracycline at 5 μg/ml.

**DNA techniques.** Procedures for *E. coli* transformation and plasmid extraction, DNA restriction, ligation, agarose gel electrophoresis, Southern blotting, and DNA dot blotting were performed essentially as described previously (2). 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 using a digoxigenin DNA labeling and detection kit (Roche Molecular Biochemicals). Primers for construction of an ATE-2 *tnp*-specific gene probe for dot blot analysis and a *tet*(W)-specific gene probe for Southern blot analysis are shown in Table 2. PCR DNA amplification was performed using *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). Primers for delineation of the ATE-1 element, and linkages between *tet*(W) and the ATE-2 *tnp* gene or the ATE-3 *aadE* gene, are shown in Table 2. PCR amplification for nucleotide sequencing over the

TABLE 2. PCR primers used in this study

| Purpose | Primer | Sequence | Region covered (bases) | Amplicon (bp) |
|---|---|---|---|---|
| Delineation of ATE-1 (A)[a] | tetw5 | 5′-CCCGAGGCCAAACACTGCGAGC-3′ | 511-532 of *guaA* | 489 |
| | tetw82 | 5′-CGTGCCCTGCACGAGAAACTTG-3′ | 978-999 of *guaA* | |
| Delineation of ATE-1 (B) | tetw21 | 5′-TGAGTACCGCAGAGTGTCGCAG-3′ | 38-59 3′ of *guaA* | 782 |
| | tetw44 | 5′-ACTACGAGACGGATTTCAGGAC-3′ | 383-404 of *orf171* | |
| Delineation of ATE-1 (C) | tetw33 | 5′-GCAGGAAGGTAACGAAGATGGG-3′ | 464-485 of *int* | 534 |
| | ATE1-1 | 5′-CGATACTTTCATGTCCCACCCG-3′ | 976-997 of *int* | |
| Delineation of ATE-1 (D) | tetw22 | 5′-TGCCTGGCAGCGTCCGTCCGTG-3′ | 260-281 3′ of *tet*(W)-*3* | 522 |
| | tetw4 | 5′-AGGGCCAAGACCGCCGAGTTCC-3′ | 97-118 of *orf110* | |
| Delineation of ATE-1 (E) | tetw24 | 5′-ATAGCCGAGCTCTCCGAGTCTG-3′ | 28-49 of *orf87* | 303 |
| | tetw36 | 5′-ATGTGCATTGAGTGCGAGGCTG-3′ | 309-330 of *orf209* | |
| Delineation of ATE-1 (F) | tetw76 | 5′-TCGCCCAGCCCTACCAGACCTT-3′ | 116-137 of *doc* | 212 |
| | tetw83 | 5′-TCAGCGACACCGAGCATAGCTGAG-3′ | 304-327 of *doc* | |
| *tnp*-specific gene probe | tnp1 | 5′-GACGAGCTGTCTGGTAAACTTC-3′ | 15-36 of *tnp* | 822 |
| | tnp2 | 5′-TAAATCTCAATGTCCTGCCTGC-3′ | 815-836 of *tnp* | |
| *tet*(W)-specific gene probe | tetw18 | 5′-GACAACGAGAACGGACACTATG-3′ | 117-138 of *tet*(W) | 1,246 |
| | tetw3 | 5′-AAGCGGGAGCGGCGTAACAGAC-3′ | 1,341-1,362 of *tet*(W) | |
| *tet*(W)-*tnp* linkage | ATE2-3 | 5′-GTCGGCAAGCCAGTCATCCAGC-3′ | 1,840-1,861 of *tet*(W) | 998 |
| | tnp2 | 5′-TAAATCTCAATGTCCTGCCTGC-3′ | 815-836 of *tnp* | |
| *tet*(W)-*aadE* linkage | tetw10 | 5′-GTGTGCCTGACGGAACTGAAAG-3′ | 1,804-1,825 of *tet*(W) | 1,398 |
| | tetw59 | 5′-ACTACCACTTGCAGAAGCCTAC-3′ | 415-436 of *aadE* | |
| *aadE* sequencing | tetw45 | 5′-AGCCCGAAGATGTTAATGTGCC-3′ | 215-236 5′ of *aadE* | 500 |
| | tetw46 | 5′-ATGAAAATCCCTTTTCTACAGC-3′ | 243-264 of *aadE* | |
| *tet*(W) sequencing | tetw18 | 5′-GACAACGAGAACGGACACTATG-3′ | 117-138 of *tet*(W) | 1,843 |
| | tetw28 | 5′-CGCAATAGCCAGCAATGAACGC-3′ | 18-39 3′ of *tet*(W) | |

[a] Letters in parentheses refer to the bars indicating the fragments amplified in Fig. 1.

region covering the frameshift in the OX9 *aadE* gene was performed with primers tetw45 and tetw46 (Table 2). A 1,771-bp fragment of the *tet*(W) gene from all 20 tetracycline-resistant *A. pyogenes* strains was sequenced from PCR products amplified with primers tetw18 and tetw28 (Table 2).

**Cloning of *A. pyogenes tet*(W) genes.** The *tet*(W) gene of a bovine *A. pyogenes* isolate, BBR1, was previously cloned in the plasmid pJGS279, a pBC KS (Stratagene) derivative containing a 12.1-kb insert (6). The *tet*(W) genes from *A. pyogenes* strains OX4, OX9, and 52785-99 were cloned from libraries of SacI-digested genomic DNA from the respective strain constructed in the vector pHSS20, pHSS21 (22), or pBC KS. The ligation mixtures were introduced into *E. coli* strain DH5αMCR by electroporation, and tetracycline-resistant transformants were selected on LB agar containing 10 μg/ml tetracycline. Recombinant plasmids containing *A. pyogenes tet*(W) genes were designated pJGS338 (OX4), pJGS464 (ATE-2, OX9) and pJGS468 (ATE-3, OX9), and pJGS529 (52785-99).

**Nucleotide sequence determination.** Nucleotide sequencing of tetracycline resistance determinants was performed on a 377A DNA sequencer (Applied Biosystems Inc.) at the Genomic Analysis and Technology Core at the University of Arizona. Sequence was determined from both strands, crossing all restriction sites.

**Computer analysis.** Nucleotide sequence data was compiled using the Sequencher 4.5 program (GeneCodes). Database searches were performed using the BlastN, BlastX, BlastP, and CD-search algorithms (1, 20). Sequence analysis was performed using the suite of programs available through the Genetics Computer Group, Inc. (Accelyrs). Similarity was determined from optimized sequence alignments by use of the CLUSTAL W program (32).

***A. pyogenes* filter matings.** Tetracycline-resistant *A. pyogenes* isolates were used in filter matings with the erythromycin-resistant, tetracycline-sensitive *A. pyogenes* recipient strain JGS610, as previously described (6). Briefly, donor and recipient strains were grown under appropriate selection conditions to an optical density at 600 nanometers ($OD_{600}$) of 1.0 (~$10^9$ bacterial cells/ml) in a Beckman DU-64 spectrophotometer with a 1 cm cell path length. The two cultures (0.5 ml of each) were mixed and filtered using a 0.45 μm filter. Following overnight incubation on a BHI–5% blood agar plate, the cells were resuspended from the filter in BHI broth. Serial dilutions were plated onto BHI–5% blood agar supplemented with 5 μg/ml tetracycline and 15 μg/ml erythromycin to select for transconjugants. Bacterial viable counts were obtained from identically treated filters containing either the donor or recipient strain alone plated on the appropriate media. Conjugation frequencies were expressed as transconjugants per donor cell recovered following overnight incubation (~$2 \times 10^9$ bacteria) and determined as averages of the results of at least three independent experiments.

**Nucleotide sequence accession numbers.** The nucleotide sequences of the ATE-1 element from *A. pyogenes* BBR1, the ATE-2 element from *A. pyogenes* OX4, and the ATE-3 elements from *A. pyogenes* OX9 and 52785-99 have been deposited in the GenBank nucleotide sequence database under the accession numbers AY049983, DQ517519, DQ519394, and DQ519395, respectively.

## RESULTS AND DISCUSSION

**Nucleotide sequence of the ATE-1 element.** A gene map of the nucleotide sequence of the *A. pyogenes* BBR1 ATE-1 element is shown in Fig. 1A. This sequence was determined from pJGS279, and an adjacent 4 kb of DNA sequence, identified in a draft genome sequence of strain BBR1 generated at 454 Life Sciences (S. J. Billington and B. H. Jost, unpublished data), was confirmed by sequencing of overlapping 1-kb PCR products. As previously described, immediately upstream of *tet*(W) are a *mob* gene and its concomitant *oriT* site (6). In the sequence 5′ of *oriT* were seven open reading frames (ORFs), all oriented in the same direction as *mob* and *tet*(W) (Fig. 1A). At the left end of the sequence were two putative housekeeping genes, *rluA* and *guaA*, encoding homologues of pseudouridine synthase (conserved domain match PSRA_1; E value = $1 \times 10^{-23}$) and a bifunctional GMP synthase/glutamine amidotransferase (conserved domain matches GMP_synthase_C [E value = $4 \times 10^{-123}$] and GATase1_GMP_synthase [E value = $5 \times 10^{-63}$]), respectively. Given their housekeeping nature, these two genes are likely part of the *A. pyogenes* genome flanking the left boundary of ATE-1.

Downstream of *guaA* were two ORFs, *orf171* and *orf68*. The latter ORF encodes a 68-amino-acid helix-turn-helix motif protein with 23.5% to 32.4% identity to a group of small proteins, including Gp27 from the *Clostridium perfringens* bacteriophage ϕ3626 (38) and hypothetical proteins from *Enterococcus faecalis* (GenBank accession no. AAO82256) and *Haemophilus somnus* (GenBank accession no. ZP_00122065). *int*, which overlapped *orf68*, encodes a protein with similarity to DNA integrases, including those from bacteriophage ϕ3626 (43.9% identity, 65.4% similarity), the *C. perfringens* CW459 *tet*(M) element (28.0% identity, 51.1% similarity), and the *Staphylococcus aureus* pathogenicity island SaPIbov (23.5% identity, 48.1% similarity) (10, 26, 38). These elements use the 3′ end of *guaA* as an integration site, a site now recognized as a hot spot for site-specific recombination (18), which is consistent with the location of ATE-1 downstream of *guaA*.

Between *int* and *mob* were two ORFs, *nikA* and *orf384* (Fig. 1A). NikA showed similarity to the putative *Listeria monocytogenes* strain 4b H7858 NikA protein (44.3% identity, 66.0% similarity) and MobC (23.8% identity, 42.6% similarity) from the *Lactococcus lactis* plasmid pSK11A (30), suggesting that its function may be in mobilization. Orf384 shared low-level similarity with DNA replication and repair proteins. *orf384* is separated from *mob* by a 510-bp intergenic sequence that contains the *oriT* of ATE-1 (6). The 5.7 kb downstream of *tet*(W) contained five ORFs, including *pcrA*, a likely housekeeping gene, which encoded a protein with similarity to homologues of the PcrA helicases, most notably from *Streptomyces coelicolor* (GenBank accession no. CAB92660; 52.1% identity and 68.5% similarity). The *doc* gene encoded a protein with similarity to "death on curing" prophage addiction proteins, including that from *Clostridium tetani* (GenBank accession no. AAO36443; 33.9% identity and 48.0% similarity), while *orf110*, *orf87*, and *orf209* have no known homologues.

**Delineation of ATE-1 sequences.** The G+C contents of *rluA* and *guaA* (left end) and *doc* and *pcrA* (right end) were consistent with those previously observed for *A. pyogenes* housekeeping genes (27). However, a decreased G+C content of 54.9% from *orf171* through *orf209* (Fig. 1A) was consistent with the integration of ATE-1 at the 3′ end of *guaA*. PCR analysis was performed on the 20 tetracycline-resistant isolates and 10 tetracycline-susceptible isolates with a set of primers designed to cover the putative ATE-1 sequences. While *guaA*-specific primers amplified a product from all 30 strains, primers specific for *int*, or a fragment extending from downstream of *tet*(W) into *orf110*, amplified a product only from the 5 ATE-1 strains (Fig. 1A). *doc*-specific primers amplified a product from all 30 strains, suggesting that this gene is not part of ATE-1 (Fig. 1A). These results, in addition to the hybridization of a *mob*-specific probe only to ATE-1 strains (6), suggest that ATE-1 extends at least from *int* to *orf110*. Primers designed to amplify a fragment extending from immediately downstream of *guaA* into *orf171* amplified this fragment in all tetracycline-resistant isolates, not just ATE-1 strains, but not in tetracycline-susceptible isolates (Fig. 1A). Conversely, all ATE-1 strains gave positive results in a PCR designed to amplify an *orf87-orf209* fragment (Fig. 1A), and all results for tetracycline-resistant, non-ATE-1 isolates were negative, but the results for 4 of 10 tetracycline-susceptible isolates were positive. These results confound the delineation of the element, and while it is likely

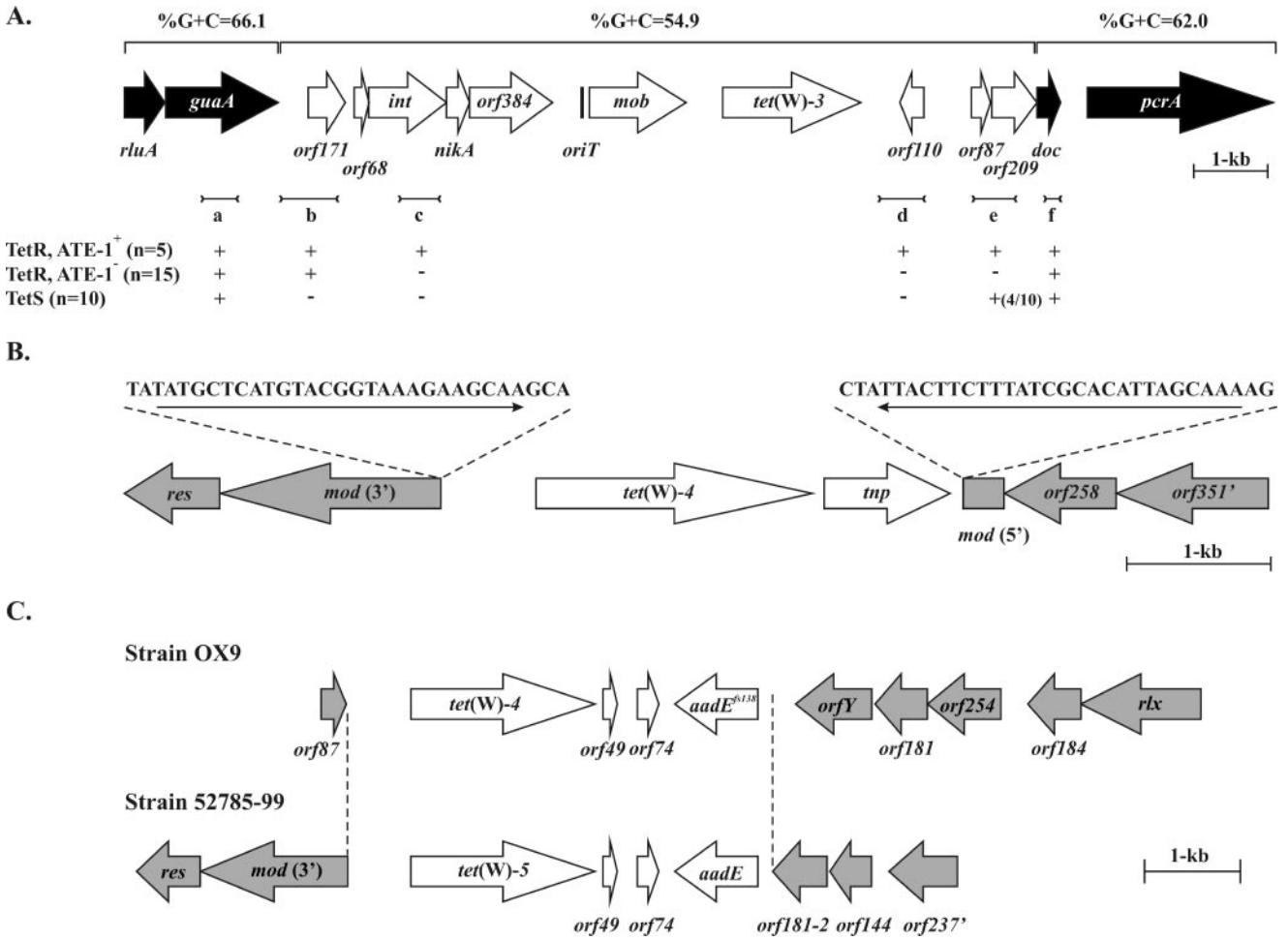


FIG. 1. Genetic organization of the ATE-1, ATE-2, and ATE-3 elements of *A. pyogenes*. (A) Gene map of the BBR1 ATE-1 element. ORFs and their orientations are represented by the arrows. ORFs designated by white arrows are located in the proposed ATE-1 sequence, while black ORFs at the left and right ends of the sequence are likely found in all *A. pyogenes* strains. Above the gene map the percentages of G+C content of the proposed ATE-1 sequence and the flanking sequences are shown. The results of PCRs (a to f) using primers (Table 2) designed to delineate ATE-1 are shown below the gene map. (B) Gene map of the OX4 ATE-2 element. White ORFs are located in the ATE-2 sequence, while shaded ORFs are present in flanking sequences. The sequences of the 26-bp imperfect inverted repeats which delineate ATE-2 are shown above the gene map. The *mod* ORF is disrupted by ATE-2, and both the 5′ and 3′ ends of the ORF are shown. (C) Gene maps of the OX9 and 52785-99 ATE-3 elements. The extent of ATE-3 sequence, as determined by comparison between the OX9 and 52785-99 sequences, is indicated by the dotted lines. The *mod* ORF is truncated at the 5′ end by ATE-3. In each figure the scale is indicated by the 1-kb bar.

that the 3′ end of *guaA* represents the insertion site of ATE-1, remnants of ATE-1 or similar elements may exist in some non-ATE-1 *A. pyogenes* isolates.

The presence of genes involved in site-specific integration and mobilization, but not conjugation, in ATE-1 suggests that it represents a mobilizable transposon, which may explain its low frequency of transfer (6).

**Cloning of the *tet*(W) gene from a tetracycline-resistant, ATE-1-negative *A. pyogenes* isolate.** As only 25% of tetracycline-resistant *A. pyogenes* isolates appear to contain ATE-1 (6), the *tet*(W) gene was cloned from the tetracycline-resistant, but ATE-1-negative, porcine isolate OX4. Nucleotide sequence of the OX4 *tet*(W) gene indicated that it was more similar (99.9% DNA identity) to the *tet*(W) gene of *Bifidobacterium* sp. strain ISO3519 (GenBank accession number AF202986) than it was to that of BBR1 (91.9% DNA identity). This divergence was also reflected at the protein level, with the OX4 Tet(W) protein having 99.8% identity to that of *Bifidobacterium* sp. strain ISO3519 but only 89.7% identity to the BBR1 Tet(W) protein. *tet*(W)-*1* and *tet*(W)-*2* alleles have been designated for *Megasphaera elsdenii* (31) and are substantially different from the two *A. pyogenes* alleles. Therefore, we have designated the BBR1 allele *tet*(W)-*3* and the OX4 allele *tet*(W)-*4*. The differences between *tet*(W)-*3* and *tet*(W)-*4* are evenly distributed over the entire length of *tet*(W), and there is no evidence of a mosaic structure to these alleles. Unlike that of ATE-1, the sequence upstream of *tet*(W) in OX4 shares considerable (96.3%) DNA identity with the core *tet*(W) upstream region previously described for a number of gastrointestinal isolates (17).

**Identification of ATE-2.** Nucleotide sequencing of an 8-kb SacI fragment containing the OX4 *tet*(W)-*4* allele indicated that this gene was arranged in an operon with *tnp* (Fig. 1B). Tnp shared similarity with site-specific recombinases, in par-

ticular, the central regions of the large clostridial resolvases, TnpX from *C. perfringens* Tn*4451* (31.3% identity and 54.6% similarity over amino acids 250 to 537 of TnpX) (3) and TndX from *C. difficile* Tn*5397* (30.1% identity and 53.4% similarity over amino acids 252 to 530 of TndX) (37). However, this region of TnpX is required for dimerization and DNA binding but not for its resolvase activity (19).

*tet*(W) and *tnp* form a genetic element, designated ATE-2, which was bound by 25-bp imperfect inverted repeats (Fig. 1B). ATE-2 was inserted within an ORF, *mod*, which would encode a protein similar to the modification enzymes of type III restriction-modification systems, including that of *Clostridium thermocellum* (GenBank accession no. ZP_00504330; 44.2% identity, 64.0% similarity), and is immediately followed by the gene for its cognate restriction enzyme, *res*. The *mod* and *res* genes and other sequences which flank ATE-2 were not identified in the draft genome sequence of the ATE-1-carrying strain, BBR1, and their G+C contents are similar to that of ATE-2 (49.9%), which is much lower than the genome average. Therefore, while ATE-2 has characteristics of a simple transposon, it is possible that ATE-2 is part of a larger genetic element.

**Distribution of ATE-2.** Genomic DNAs from the 20 tetracycline-resistant *A. pyogenes* isolates and 10 tetracycline-susceptible isolates were screened by DNA dot blotting using a *tnp*-specific gene probe (Table 2) as a marker for ATE-2 (data not shown). None of the five ATE-1 strains or the tetracycline-susceptible isolates hybridized to the *tnp*-specific probe. Seven of the remaining 15 tetracycline-resistant isolates hybridized, indicating that 35% (7/20) of tetracycline-resistant *A. pyogenes* strains carried the ATE-2 element. Linkage of *tet*(W)-*4* and *tnp* in these strains was confirmed by PCR (Table 2 and data not shown).

**Some ATE-2 strains carry two *tet*(W) genes.** EcoRI-digested genomic DNA from each of the seven ATE-2 strains was subjected to Southern blotting with a *tet*(W)-specific probe (Fig. 2). All seven strains showed a hybridizing band of 5 kb consistent with the presence of the ATE-2 element. However, five of the seven strains also showed an additional band of either 7 kb or 11 kb, indicating the presence of a second copy of *tet*(W).

**Identification of ATE-3.** To investigate the additional *tet*(W) genes in ATE-2 strains, the *tet*(W) genes of the porcine strain OX9 were cloned. Two types of recombinant plasmid were obtained. Nucleotide sequencing of one plasmid type indicated that it contained ATE-2 inserted at a position identical to that found in OX4 (data not shown). In addition, the OX9 ATE-2-associated *tet*(W)-*4* allele differed by only a single base pair from that of OX4. The second plasmid type contained a *tet*(W)-*4* allele identical to that of ATE-2 in this strain. However, this second *tet*(W)-*4* was associated with a defective streptomycin resistance gene, $aadE^{fs138}$, in an element designated ATE-3 (Fig. 1C). This ATE-3 element may be derived from ATE-2, as the sequences from 661 bp upstream of *tet*(W)-*4* to 99 bp downstream of *tet*(W)-*4* are 99.9% identical between the two elements. The potential product of the $aadE^{fs138}$ would be 100% identical to AadE encoded by the *Campylobacter jejuni* plasmid pCG8245 (23), but a frameshift at nucleotide 138 puts the remainder of the coding sequence out of frame. Interestingly, strain OX9 is resistant to streptomycin (MIC > 256 μg/ml), and sequencing of a PCR product that flanked the frameshift gave a sequence consistent with both the frame shift and a functional sequence, suggesting that there is a second functional *aadE* gene in OX9. This suggestion was supported by PCR sequencing of other strains carrying this gene, which gave only sequences consistent with a functional gene. The region of the ATE-3 element, from 150 bp upstream of *aadE* through the end of the gene, is also 100% identical to pCG8245 at the DNA level (with the exception of the *aadE* frameshift base deletion). These data indicate recent horizontal transfer of this *aadE* gene between gram-negative and gram-positive bacteria. In ATE-3, the 3′ ends of *tet*(W) and *aadE* are separated by *orf49* and *orf74*. The genes flanking the *tet*(W)-*aadE* region are of unknown function. However, *orfY*, encoding a putative methyltransferase, has previously been identified in *A. pyogenes* strain OX7 as a gene into which an *erm*(B) element has inserted (16). In OX7, ATE-3 is located at the same position as in OX9 and is thus clustered with the *erm*(B) element (16). *orfY*, with near DNA identity (but designated *mte*), is also present as part of the *Bacteroides uniformis* conjugative transposon CTnBST, where it is interrupted by a Tn*10*-like element (12). Proteins with approximately 28% similarity to OrfY are encoded adjacent to the *tet*(W) genes of several animal and human gastrointestinal bacterial species. These *tet*(W)/*orfY* sequences are presumably mobile, given their high sequence identity between several species (17). *orf181* has an unannotated homologue with 100% DNA identity in CTnBST (GenBank accession no. AY345595) encoded upstream of *mte* and three homologues on pCG8245 (23). These data, along with the lack of ATE-3 flanking genes in the BBR1 draft genome sequence and their relatively low G+C contents, suggests that ATE-3

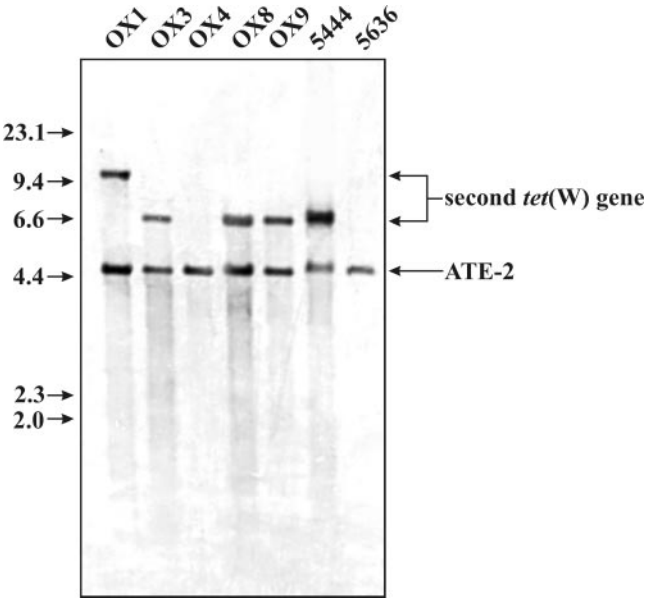


FIG. 2. Some ATE-2 positive *A. pyogenes* isolates carry a second *tet*(W) gene. The results of Southern blot analysis of EcoRI-digested *A. pyogenes* genomic DNA from ATE-2-positive isolates with a digoxigenin-labeled *tet*(W) gene probe are shown. The *A. pyogenes* strains are indicated above the lanes. DNA size standards in kilobases are shown to the left of the blot, while the positions of bands corresponding to the ATE-2 element and the second *tet*(W) gene are indicated on the right.

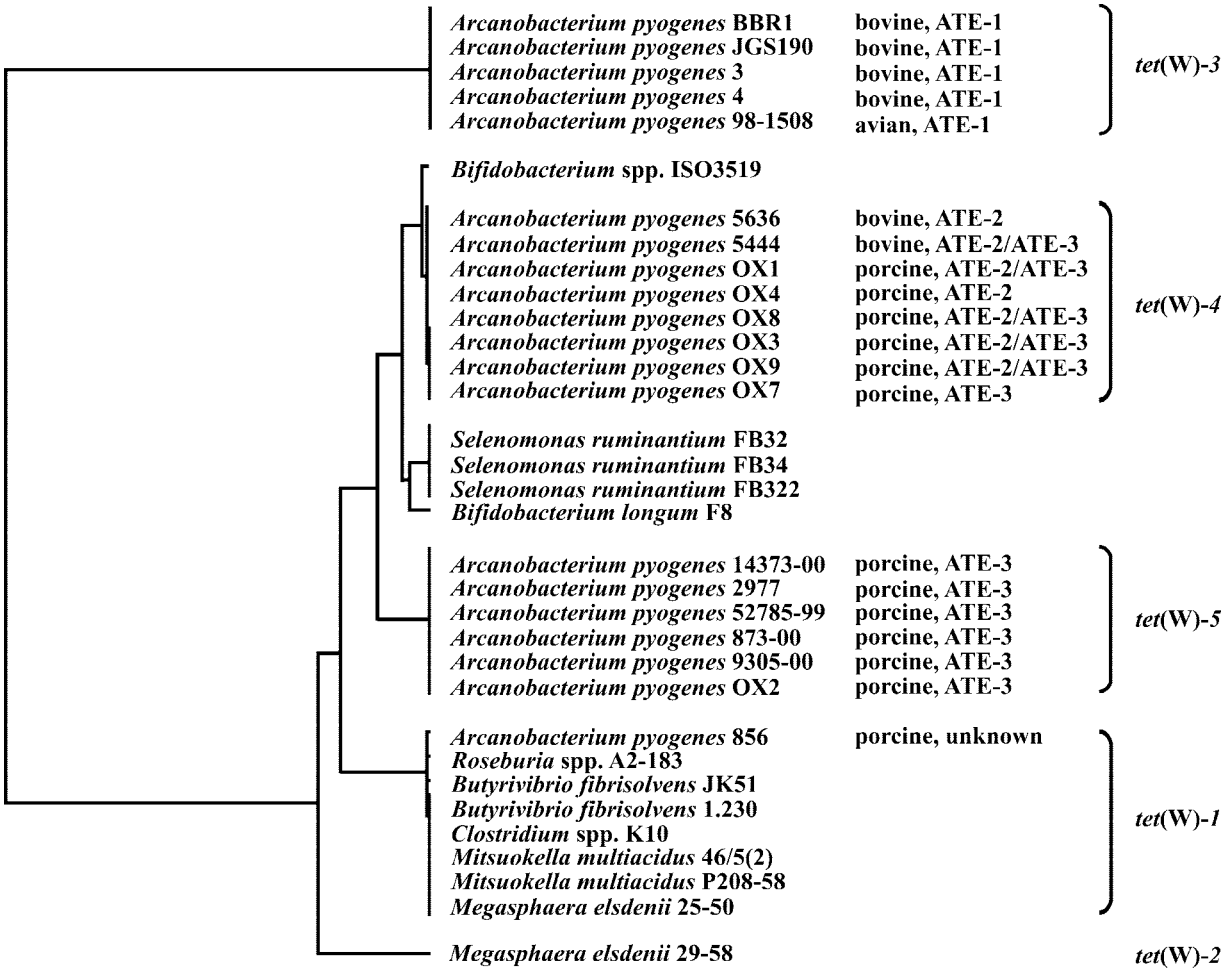


FIG. 3. Phylogenetic analysis of a 1,771-bp fragment of *tet*(W), showing the relationships between the *tet*(W) genes of *A. pyogenes* isolates and those from other bacterial species. The figure shows an unrooted dendrogram of *tet*(W) sequences from *A. pyogenes* strains determined in this study, *Bifidobacterium* sp. strain ISO3519 (GenBank accession number AF202986), *Bifidobacterium longum* F8 (GenBank accession number DQ294299), *Butyrivibrio fibrisolvens* strains JK51 (GenBank accession number AJ427421) and 1.230 (GenBank accession number AJ222769), *Clostridium* sp. strain K10 (GenBank accession number AY601650), *Megasphaera elsdenii* strains 25-50 (GenBank accession AY485125) and 29-55 (GenBank accession number AY485124), *Mitsuokella multiacidus* strains 46/5(2) (GenBank accession number AJ427422) and P208-58 (GenBank accession number AY603069), *Roseburia* sp. strain A2-183 (GenBank accession number AJ421625), and *Selenomonas ruminantium* strains FB32 (GenBank accession number DQ294296), FB34 (GenBank accession number DQ294297), and FB322 (GenBank accession number DQ294295) compiled using PAUPsearch and Genetics Computer Group software. The host of origin of each *A. pyogenes* strain is indicated along with the associated ATE elements. Groups of strains carrying each of the *tet*(W) alleles are also indicated.

may be part of a larger element. This suggestion is given some credence by the presence of a putative mobilization gene, *rlx*, ~3 kb downstream of ATE-3 (Fig. 1C). Rlx has a conserved domain associated with relaxases (conserved domain match pfam03432 relaxases; E value = $1 \times 10^{-9}$) and low similarity to a number of Mob and Rlx proteins, including that of the *S. aureus* plasmid pS194 (25).

**Distribution of ATE-3.** An ATE-3-specific PCR, linking *tet*(W) and *aadE*, was performed with each of the 20 tetracycline-resistant isolates (data not shown). Products were obtained from each of the five ATE-2 isolates which contained a second *tet*(W) gene and from seven of eight strains which lacked both ATE-1 and ATE-2. Products were not obtained from any ATE-1-containing strain. The clustering of tetracycline and streptomycin resistances on ATE-3, along with the frequency of ATE-3 among tetracycline-resistant *A. pyogenes* isolates (60%), may explain the high correlation of tetracycline and streptomycin resistance previously observed (11).

**ATE-3 can be found at multiple genetic loci.** To examine the characteristics of an ATE-3 element from a non-ATE-2 strain, the ATE-3 element was cloned from strain 52785-99. Nucleotide sequencing indicated that the organization of ATE-3 in 52785-99 was identical to that in OX9 (Fig. 1C), with similarity between the two sequences extending 4,433 bp from 663 bp 5′ to *tet*(W) to 154 bp 5′ to *aadE*, with only 51 bp of divergence between the two sequences. One of these base changes included correction of the *aadE* frameshift present in the OX9 ATE-3 element. The 52785-99 *tet*(W) gene differed nearly 1% in DNA sequence from the *tet*(W)-*4* alleles of strains OX4 and OX9 and was thus designated *tet*(W)-*5*. In strain 52785-99, the ATE-3 element is flanked by the 3′ end of *mod*, as is ATE-2 in OX4. However, the 5′ end of *mod* is not located at the other

end of ATE-3, suggesting some rearrangement, perhaps associated with ATE-3 insertion. The other end of ATE-3 is flanked by a homologue of *orf181* which is 89.2% identical to that in strain OX9. Sequences flanking the 52785-99 ATE-3 element also have G+C contents of <50% and are not present in the BBR1 draft genome sequence, suggesting that ATE-3 may be carried by more than one larger genetic element.

**Transferability of ATE-2 and ATE-3 elements.** To assess the transferability of ATE-2 and ATE-3, strains OX4 (ATE-2), OX9 (ATE-2 and ATE-3), and 52785-99 (ATE-3) were used as donors in mating experiments with the recipient strain, JGS610 (Table 1). Tetracycline-resistant, erythromycin-resistant transconjugants were not detected from any of these mating experiments, suggesting that ATE-2 and ATE-3 are not capable of conjugative transfer under the conditions used or that the transfer frequency was $<5 \times 10^{-10}$ transconjugants/donor cell. Transconjugants were detected at a frequency of $9 \times 10^{-9}$ transconjugants/donor cell from matings between the ATE-1-positive strain BBR1 and JGS610.

**Relationships between ATEs and *tet*(W).** Nucleotide sequence variations in the *tet*(W) genes from different tetracycline-resistant *A. pyogenes* strains led to the identification of three *tet*(W) alleles. The *tet*(W) gene was amplified and sequenced from each of the 20 tetracycline-resistant *A. pyogenes* isolates, and a comparison of these sequences is shown as a dendrogram in Fig. 3. In general, particular *tet*(W) alleles were associated with particular ATEs. All strains which carried ATE-1 had *tet*(W)-*3*, while all strains which carried ATE-2 had *tet*(W)-*4*. Strains which carried both ATE-2 and ATE-3 gave a single unambiguous *tet*(W)-*4* sequence, suggesting that both the ATE-2 and ATE-3 elements in these strains carried this allele. With the exception of strain OX7, ATE-3 strains consistently carried *tet*(W)-*5*. One *A. pyogenes* tetracycline-resistant isolate, 856, which did not carry ATE-1, ATE-2, or ATE-3, carried a *tet*(W) gene which differed by a single base from the *M. elsdenii* 25-50 *tet*(W)-*1* allele (31). No obvious differences in levels of resistance between strains carrying different alleles, including those that carry two copies of *tet*(W), have been observed (33).

**Relationship between ATEs and the host source of the isolate.** A general association exists between the host species of tetracycline-resistant *A. pyogenes* isolates and the type of ATE present (Fig. 3). ATE-1 was present in four of seven bovine isolates but in no porcine isolates. ATE-2 was present in 5 of 12 porcine isolates and 2 of 7 bovine isolates, while ATE-3 was present in 11 of 12 porcine isolates but only 1 of 7 bovine isolates. ATE-2 and ATE-3 were found in combination in 4 of 12 porcine isolates and 1 of 7 bovine isolates. Of the seven bovine isolates examined, four contained ATE-1, one contained ATE-2 alone, and one contained ATE-2 in combination with ATE-3. Of the 12 porcine isolates, 1 contained ATE-2 alone and 7 contained ATE-3 alone, with the remaining 4 possessing both ATE-2 and ATE-3. The single avian isolate contained ATE-1.

**Conclusions.** *tet*(W) has recently been recognized as a widely distributed gene conferring tetracycline resistance to a number of bacterial species (4, 28, 29, 31, 36). However, little information is available about the types of genetic elements involved in its dissemination (17, 21). We have identified three genetic elements which carry *tet*(W) in *A. pyogenes* isolates. These elements are transferable, or show genetic signs of mobility, but differ from those elements that carry *tet*(W) in other species and carry *tet*(W) alleles not identified in other bacteria. These data suggest that there may be some constraints on the transfer of these elements to other bacteria. Indeed, there appears to be considerable host species preference for *A. pyogenes* isolates to carry certain ATE elements, suggesting that these elements may be circulating predominantly in certain environments.

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