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# Distribution of Tetracycline Resistance Genes in *Actinobacillus pleuropneumoniae* Isolates from Spain†

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***Actinobacillus pleuropneumoniae* is the etiological agent of porcine pleuropneumonia. Tetracycline is used for therapy of this disease, and *A. pleuropneumoniae* carrying the *tet*(B) gene, coding for an efflux protein that reduces the intercellular tetracycline level, has been described previously. Of the 46 tetracycline-resistant (Tc$^r$) Spanish *A. pleuropneumoniae* isolates used in this study, 32 (70%) carried the *tet*(B) gene, and 30 of these genes were associated with plasmids. Eight (17%) isolates carried the *tet*(O) gene, two (4%) isolates carried either the *tet*(H) or the *tet*(L) gene, and all these genes were associated with plasmids. This is the first description of these *tet* genes in *A. pleuropneumoniae*. The last two Tc$^r$ isolates carried none of the *tet* genes examined. Except for *tet*(O)-containing plasmids, the other 34 Tc$^r$ plasmids were transformable into an *Escherichia coli* recipient. Two plasmids were completely sequenced. Plasmid p11745, carrying the *tet*(B) gene, was 5,486 bp and included a *rep* gene, encoding a replication-related protein, and two open reading frames (ORFs) with homology to mobilization genes of *Neisseria gonorrhoeae* plasmid pSJ7.4. Plasmid p9555, carrying the *tet*(L) gene, was 5,672 bp and, based on its G+C content, consisted of two regions, one of putative gram-positive origin containing the *tet*(L) gene and the other comprising four ORF*s* organized in an operon-like structure with homology to mobilization genes in other plasmids of gram-negative bacteria.**

Porcine pleuropneumonia caused by *Actinobacillus pleuropneumoniae* is a worldwide disease of swine that manifests as severe outbreaks, resulting in important economic losses, among fattening and finishing pigs (1). Clinical signs include dyspnea, fever, reduced appetite, and rapidly occurring death, with an elevated mortality rate. A cough and, in peracute cases, a frothy and bloodstained nasal discharge can also be observed. In addition, the lung lesions are characteristic in acute cases, with consolidation and raised cherry-red or black areas of necrotizing and hemorrhagic pneumonia, with interlobular edema on the diaphragmatic lobe and fibrinous pleuritis (27). The change from traditional continuous swine production to the modern "all in/all out" industry has contributed to new epidemiological situations (20).

Tetracyclines are often used for therapy of this disease (2). Tetracyclines belong to a family of broad-spectrum antibiotics that include tetracycline, doxycycline, and other semisynthetic derivatives (4). Tetracyclines have been widely used in veterinary medicine for prophylaxis and growth promotion in food animals. Numerous studies have described the effects of long-term usage of subtherapeutic doses of tetracyclines, which include increased levels of resistant gut bacteria or pathogens (24). The use of antibiotics as growth promoters has been discouraged by the European Union (Council Directive 70/524 EEC, 1977); however, consumption figures suggest that the use of tetracyclines in veterinary practice is still high compared with use of other classes of antibiotics (30).

Several studies have reported the high incidence of tetracycline resistance (Tc$^r$) in *A. pleuropneumoniae.* Forty-two of the 71 (59.2%) isolates collected in Spain in the period 1992 to 1993 were resistant to oxytetracycline (11), whereas 55 of 60 (90%) *A. pleuropneumoniae* strains isolated from pigs in Taiwan during 1985 to 1993 showed tetracycline resistance (3). Previous work identified the *tet*(B) gene in 8 of the 17 (47%) Tc$^r$ *A. pleuropneumoniae* isolates collected in Norway between 1986 and 1992 (34). However, the data of that study suggested that other *tet* genes may be present in these isolates.

The aim of the present study was to determine the type and genomic location of *tet* genes carried in a collection of Spanish Tc$^r$ *A. pleuropneumoniae* isolates recovered from different farms of the "Castilla-León" region between 1997 and 2001.

## MATERIALS AND METHODS

**Isolation and serotyping of *A. pleuropneumoniae*.** Forty-six Tc$^r$ *A. pleuropneumoniae* strains isolated from the "Castilla y León" region of Spain between 1997 and 2001 were studied. The isolates were grown on chocolate agar plates with gonococcus (GC) agar (Oxoid, Basingstoke, United Kingdom) supplemented with 5% sheep blood (bioMerieux, Marcy l'Etoile, France), 12 μg/ml of NAD (Sigma-Aldrich Química., Madrid, Spain), and Kellogg's supplement solution (0.22 M D-glucose, 0.03 M L-glutamine, 0.001 M ferric nitrate, and 0.02 M cocarboxylase) (Sigma-Aldrich Química, Madrid, Spain) as previously described

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† Supplemental material for this article may be found at http://aac.asm.org/.

TABLE 1. *A. pleuropneumoniae* isolates and *tet* genes carried

| No. of isolates[a] | Serovar | MIC (μg/ml) of: | | *tet* gene | Location | Plasmid size (kb) | Transformation of *E. coli* |
|---|---|---|---|---|---|---|---|
| | | Tetracycline (0.25–128 μg/ml) | Doxycycline (0.25–32 μg/ml) | | | | |
| 5 | 2 | 64 | 16 | B | Plasmid | 5.5 | + |
| 1 | 2 | 64 | 32 | B | Plasmid | 5.5 | + |
| 4 | 4 | 64 | 16 | B | Plasmid | 5.5 | + |
| 1 | 7 | 64 | 16 | B | Plasmid | 5.5 | + |
| 19 | Nontypeable | 16–64 | 4–32 | B | Plasmid | 5.5 | + |
| 2 | Nontypeable | 16–32 | 2–8 | B | Not done | | − |
| 2 | 6 | 32–64 | 32 | O | Plasmid | 6 | − |
| 1 | 7 | 64 | 32 | O | Plasmid | 6 | − |
| 5 | Nontypeable | 64 | 32 | O | Plasmid | 6 | − |
| 1 | 4 | 32 | 16 | H | Plasmid | 5.7 | + |
| 1 | Nontypeable | 64 | 16 | H | Plasmid | >12 | + |
| 1 | 4 | 32 | 8 | L | Plasmid | 5.6 | + |
| 1 | 1 | 64 | 8 | L | Plasmid | >12 | + |
| 2 | Nontypeable | 16–64 | 2–16 | Unknown | | | |
| 1[b] | 3 | 0.5 | 0.5 | No gene | | | |

[a] The total number of isolates was 47.
[b] *A. pleuropneumoniae* ATCC 27090.

(5). The plates were incubated at 37°C under a 5% $CO_2$ atmosphere. The isolates were serotyped as previously described (11).

**Growth of *Escherichia coli* strains.** *Escherichia coli* S17-1 (31), DH5α (28), and IT1022 (9) were grown on Luria-Bertani (LB) agar (Pronadisa S.A., Madrid, Spain) supplemented with the appropriate antibiotics (300 μg of streptomycin/ml, 20 μg of nalidixic acid/ml, or 10 μg of tetracycline/ml).

**Antimicrobial susceptibility testing.** Bacteria were grown overnight on chocolate agar plates and resuspended in sterilized saline solution, and the turbidity was adjusted to a 0.5 McFarland standard. The MICs of tetracycline and doxycycline were determined by broth microdilution assays in accordance with CLSI (formerly NCCLS) guidelines (23) in veterinary fastidious medium supplemented with 0.025% NAD (Sigma-Aldrich Química, Madrid, Spain). The microplates (Sensititre VAV5; Trek Diagnostic Systems, England) were incubated at 37°C for 24 h, and the MIC of each antimicrobial agent was the lowest concentration that inhibited visible growth. *A. pleuropneumoniae* ATCC 27090 (serovar 3) was included in the study as a quality control.

**Extraction of DNA.** Genomic DNA was obtained as previously described (6, 29). Plasmid DNA was prepared from 100 ml broth culture using a Midi plasmid preparation and purification kit (QIAGEN S.A., Courtaboeuf, France).

**DNA-DNA hybridization.** Whole-cell bacterial dot blots were prepared, and DNA-DNA hybridization was performed as previously described with $^{32}$P-labeled probes (19) or with chemiluminescently labeled probes by using the CDP-Star system according to the manufacturer's instructions (Roche Diagnostics, S.L., Barcelona, Spain). *tet* genes—*tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(G), *tet*(H), *tet*(K), *tet*(L), *tet*(M), *tet*(O), *tet*(S), *tet*(Y), and *tet*(29)—were detected by DNA-DNA dot blot hybridization and/or PCR assays as previously described (21). Positive and negative controls were included in each assay. To determinate the location of *tet* genes, Southern blotting of uncut genomic and plasmid DNA was performed according to reference 28.

**PCR detection of *tet* genes.** The presence of the various *tet* genes was confirmed using PCR assays with primers specific for each gene and by sequencing of the PCR products. The sequences of the primers and their annealing temperatures have been described previously and are available upon request (see the supplemental material). The PCR mixture consisted of 10 μl of DNA template (10 pg/μl), 4 U of *Taq* DNA polymerase (Bioline, London, United Kingdom), 1.5 mM $MgCl_2$, 10 μl of 10× PCR amplification buffer [160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl (pH 8.8), 0.1% Tween 20], 40 pmol of each primer, 0.2 mM each deoxynucleoside triphosphate (Bioline, London, United Kingdom), and double-distilled water to a final volume of 100 μl. DNA was first denatured at 95°C for 5 min and then subjected to 30 cycles of amplification under the following conditions: denaturation at 95°C for 1 min, annealing for 1 min, and extension at 72°C for 1 min. After the final cycle, the reactions were terminated by an extra run at 72°C for 10 min, and the reaction products were then kept at 4°C until analysis.

**Electrotransformation.** Competent *E. coli* S17-1 cells were prepared as previously described (28) and electroporated with 100 ng of *A. pleuropneumoniae* plasmid DNA. Electrotransformation was performed in a Gene Pulser II electroporation system (Bio-Rad, Foster City, CA) by applying an electric pulse as described by the manufacturer (2.5 kV, 25 μFa, 800 Ω) (8). Transformants were selected on LB agar supplemented with tetracycline (10 μg/ml) and streptomycin (300 μg/ml).

**Mobilization experiments.** Conjugal transfer of the tetracycline resistance phenotype was assayed by mating *A. pleuropneumoniae* isolates carrying the various plasmids to an *E. coli* S17-1 recipient. *A. pleuropneumoniae* isolates APP11745 [*tet*(B)], APP13142 [*tet*(O)], APP9956 [*tet*(H)], and APP9555 [*tet*(L)], or *E. coli* S17-1 transformants carrying the Tc$^r$ plasmid p9555, were used as donors, and *E. coli* DH5α was used as the recipient. The bacteria were grown at 37°C overnight and mixed in a donor-to-recipient ratio of 1:8. The resultant suspension was then placed on a 0.45-μm HA membrane (Millipore) and incubated at 37°C overnight. Transconjugants were selected on LB agar plates containing 10 μg of tetracycline and 20 μg of nalidixic acid (Nx) per ml. Tc$^r$ Nx$^r$ colonies were tested for streptomycin resistance in order to confirm that they were true transconjugants. Transfer frequency was expressed as the number of transconjugants per recipient cell obtained after plating on selective medium.

**DNA sequencing and analyses.** Plasmids p11745 and p9555 were amplified using primers tetBoutF (AAA GAG TCA TCA GCA AGG TGC T)–tetBoutR (TAT GCG GTG AAA TCT CTC CTG C) and tetLoutF (TTA CTT GAT CAA AGG TTG TT)–tetLoutR (AAT CAT TTG CAA TAT CAG GT), respectively, which are complementary to the *tet*(B) and the *tet*(L) gene, respectively. The PCR amplicons were sequenced by following a primer-walking strategy at the "Servicio de Secuenciación de DNA HUMV—Unican" of the "Universidad de Cantabria" using a Beckman CEQ 2000XL apparatus. Contiguous sequences were assembled using the ContigExpress Vector NTI Suite 5.5 (Informax, Bethesda, Md.). Homology searches were performed with BLAST and ORF Finder tools at the website of the National Center for Biotechnology Information (Bethesda, MD).

**Nucleotide sequence accession numbers.** The complete sequences of plasmids p11745 and p9555, which contain the *tet*(B) and *tet*(L) genes, respectively, have been deposited in GenBank under accession no. DQ176855 and AY359464. The sequences of the *tet* regions of the *A. pleuropneumoniae* isolates have been deposited in GenBank under accession no. AY987963 for *tet*(O) and AY987962 for *tet*(H).

## RESULTS

**Serotyping of *A. pleuropneumoniae*.** The 46 Tc$^r$ *A. pleuropneumoniae* isolates were serotyped. Among the five serotypes detected, serovar 2 and serovar 4 were predominant (six isolates each), followed by serotypes 6 and 7 (two isolates each); one isolate belonged to serotype 1. Twenty-nine of the isolates were nontypeable.

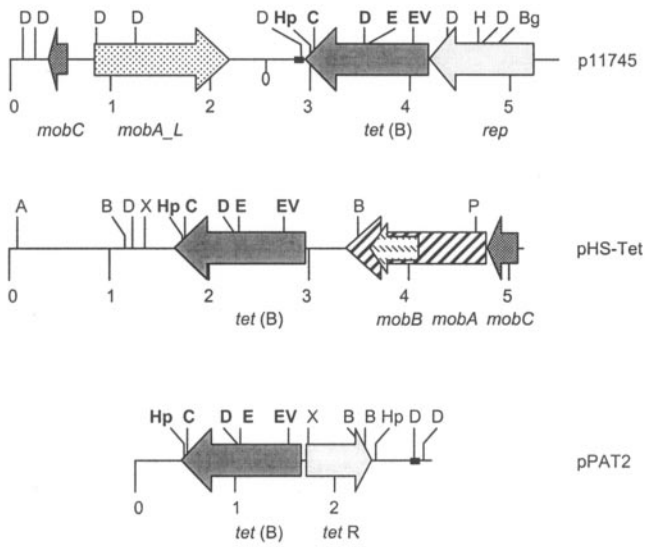


FIG. 1. Plasmid p11745 from *A. pleuropneumoniae* compared to plasmids pHS-Tet from *H. parasuis* and pPAT2 from *P. aerogenes*. Numbers indicate size in kilobases. Striped and stippled arrows correspond to plasmid mobilization genes (*mobA*, *mobA_L*, *mobB*, and *mobC*). Shaded arrows correspond to tetracycline resistance genes or replication genes [*tet*(B), *tet*(R), or *rep*]. The putative transcriptional terminator for p11745 *tet*(B) is depicted. Black boxes represent the 99-bp region of homology between p11745 and pPAT2. Restriction sites are abbreviated as follows: B (BclI), Bg (BglII), C (ClaI), D (DraI), E (EcoRI), EV (EcoRV), H (HindIII), Hp (HpaI), K (KpnI), P (PstI), and X (XbaI). Boldface indicates coincident restriction sites.

**Distribution of the *tet* genes.** Of the 46 Tc$^r$ *A. pleuropneumoniae* isolates tested, 44 (96%) carried one of the *tet* genes tested. These included 32 (70%) isolates carrying the *tet*(B) gene, 8 (17%) isolates carrying the *tet*(O) gene, and 2 isolates each (4% each) carrying the *tet*(H) or the *tet*(L) gene (Table 1). None of the 44 isolates carried more than one *tet* gene (Table 1). Two Tc$^r$ isolates, as well as *A. pleuropneumoniae* ATCC 27090, did not carry any of the 14 *tet* genes examined.

**Characterization of the *tet*(B) genes.** To determine the location of the *tet*(B) genes, Southern blots of uncut genomic and plasmid DNA were hybridized with a *tet*(B) probe consisting of a 1-kb internal region of the gene isolated from *E. coli* IT1022 which contains Tn*10*. In 30 of the 32 *tet*(B)-positive isolates, their plasmids hybridized with the *tet*(B) probe, indicating a plasmid location. The location was verified by transforming the plasmids into *E. coli* and selecting for tetracycline resistance. The location of the *tet*(B) gene in the remaining two isolates is still under investigation.

The plasmid size (5.5 kb) and indistinguishable restriction patterns with the HindIII and DraI endonucleases indicated that the 30 *tet*(B)-positive plasmids were closely related, if not identical. Plasmid p11745, from isolate APP11745, was selected and completely sequenced. This plasmid consisted of 5,486 bp and had a G+C content of 38.1%. The plasmid had four open reading frames (ORFs), with *orf3* containing the 1,209-bp structural gene *tet*(B), which had 99% sequence identity at the DNA and amino acid levels with the *tet*(B) gene from Tn*10* (GenBank accession no. AF162223). The *tet*(B) gene usually has a regulator gene, *tetR*(B), immediately upstream of the structural *tet*(B) gene; the two genes share a common promoter and are read in opposite directions (25). However, the *tetR*(B) gene was not present in plasmid p11745 (Fig. 1). A PCR assay for the *tetR*(B) gene gave a PCR product of the correct size for the control, but the *tet*(B)-positive *A. pleuropneumoniae* isolate did not produce a PCR product from the assay, nor was the sequence found on the plasmid. The 21 bp upstream of the *tet*(B) gene was identical to that found in Tn*10* and included the putative ribosome binding site (RBS) sequence (AGAGAA) 7 bp upstream of the *tet*(B) gene. Beyond this sequence, −35 (TAAAAT) and −10 (TAAAAT) boxes resembling *A. pleuropneumoniae* consensus promoter sequences (7) were found. A putative Rho-independent transcription terminator located 35 bp downstream from the translational stop codon of the *tet*(B) gene was also identified. In the downstream region there was a 75-bp stretch showing identity with pPAT2 (Fig. 1).

Three other ORF*s* (*orf1*, *orf2*, and *orf4*) were identified in plasmid p11745 (Table 2). The *orf4* gene encoded a replication-related protein, whereas *orf1* and *orf2* coded for mobilization-related proteins, which had 70% and 56% homology, respectively, with the MobC and MobA_L proteins of the *Neisseria gonorrhoeae* plasmid pSJ7.4 (GenBank accession no. AY221996) (Fig. 1) (26). The functional map of p11745 was compared with those of two other *tet*(B)-positive plasmids of *Pasteurellaceae*, pHS-Tet from *Haemophilus parasuis* (GenBank accession no. AY862435) (18) and pPAT2 from *Pasteurella aerogenes* (GenBank accession no. AY278685) (15). DNA homology between p11745 and the other two plasmids was limited to the *tet*(B) genes (Fig. 1).

**Characterization of the *tet*(O) genes.** Eight isolates carried the *tet*(O) gene (Table 1), which was associated with a 6-kb plasmid in each isolate. From plasmid p13142, 2,489 bp was sequenced, which included the complete *tet*(O) gene (GenBank accession no. AY987963) and shared 99% identity at the DNA and amino acid levels with the *tet*(O) gene from *Campylobacter jejuni* (GenBank accession no. AY394561) (33). The upstream

TABLE 2. Plasmid p11745 ORFs[a]

| ORF | Gene | % G+C | Size (aa) | Identity of predicted protein | |
|---|---|---|---|---|---|
| | | | | % Identity | Closest predicted protein (GenBank accession no.) |
| 1 | *mobC* | 33.7 | 97 | 70 | MobC of *Neisseria gonorrhoeae* pSJ7.4 (AAO45531) |
| 2 | *mobA_L* | 39.2 | 450 | 56 | MobA of *Neisseria gonorrhoeae* pSJ7.4 (AAO45530) |
| 3 | *tet*(B) | 42.9 | 411 | 99 | TetB of *Haemophilus parasuis* pHS-Tet (AAW51465) |
| 4 | *rep* | 35.1 | 337 | 47 | Replication protein of *Mannheimia varigena* pMVSCS1 (NP_573540) |

[a] See Fig. 1.

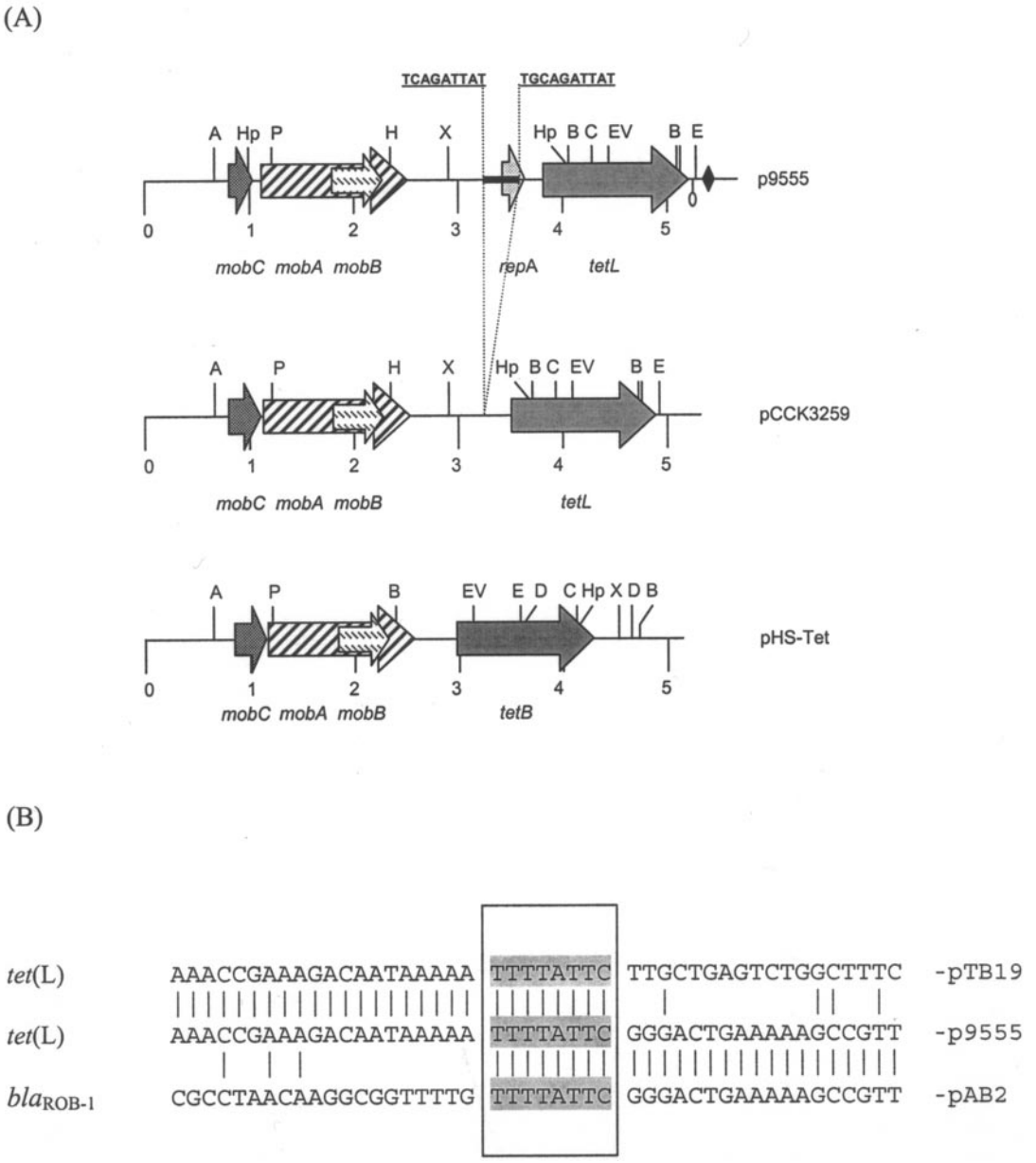


FIG. 2. (A) Plasmid p9555 from *A. pleuropneumoniae* compared to plasmids pCCK3259 from *Mannheimia haemolytica* and pHS-Tet from *H. parasuis*. Numbers indicate size in kilobases. Striped and stippled arrows correspond to plasmid mobilization genes (*mobA*, *mobB*, and *mobC*). Shaded arrows correspond to tetracycline resistance genes [*tet*(B) and *tet*(L)]. The putative transcriptional terminator (oval) for the p9555 *tet*(L) gene and the potential recombination site (diamond) detected in this plasmid are depicted. Restriction sites are abbreviated as follows: B (BclI), Bg (BglII), C (ClaI), D (DraI), E (EcoRI), EV (EcoRV), H (HindIII), Hp (HpaI), K (KpnI), P (PstI), and X (XbaI). The pCCK3259 357-bp deletion is represented by a black box flanked by the 10-bp direct repeats. (B) Potential recombination site (boxed) downstream of *tet*(L) in p9555 and comparison with the corresponding sequences of plasmid pTB19 from *Geobacillus stearothermophilus* and plasmid pAB2 from *Mannheimia haemolytica*. The comparison includes the following bases in their respective plasmid sequences: bp 3222 to 3268 in the *G. stearothermophilus* pTB19 sequence (GenBank accession no. M63891), bp 1971 to 2017 in the *A. pleuropneumoniae* p9555 sequence (GenBank accession no. AY359464), and bp 3229 to 3274 in the *M. haemolytica* pAB2 sequence (GenBank accession no. Z21724).

(270-bp) and downstream (299-bp) sequences were identical to that of *Campylobacter coli* and included a putative RBS sequence 12 bp upstream of the start codon. The −35 and −10 regions corresponding to the putative *tet*(O) promoter were identified.

**Characterization of the *tet*(H) genes.** The *tet*(H) genes were located on a 5.6-kb plasmid (p9956) (isolate APP9956) and a >12-kb plasmid (p12494) (isolate APP12494). One-kilobase PCR amplicons from these plasmids were sequenced, and their DNA and amino acid sequences were 100% identical to those of the *tet*(H) gene from the *Pasteurella* plasmid pPAT1 (GenBank accession no. AJ245947) (14). The *tet*(H) structural gene and the *tetR*(H) repressor gene were identified in a 1,179-bp region by partial sequencing (3 kb) of plasmid p9956. These gene sequences were identical to the *tet*(H) gene from plasmid

TABLE 3. Plasmid p9555 ORFs[a]

| ORF | Gene | % G+C | Size (aa) | Identity of predicted protein | |
|---|---|---|---|---|---|
| | | | | % Identity | Closest predicted protein (GenBank accession no.) |
| 1 | *repA* | 34.8 | 67 | 50 | RepB of *Lactococcus lactis* plasmid pFX2 (Q04138) |
| 2 | *tet*(L) | 35.5 | 451 | 99 | TetL of *Bacillus cereus* plasmid pJH1 (AAN05731) |
| 3 | *mobC* | 49.4 | 76 | 91 | MobC of *Mannheimia haemolytica* plasmid pMHSCS1 (CAC20662) |
| 4 | *mobA* | 43.7 | 468 | 89 | MobA of *Haemophilus parasuis* plasmid pHS (AAW51466) |
| 5 | *mobB* | 45.1 | 160 | 89 | MobB of *Haemophilus parasuis* plasmid pHS (AAW51467) |

[a] See Fig. 2A.

pPAT1 but were 24 bp shorter than the complete *tet*(H) gene (12). This deletion produced a truncated structural *tet* protein that was missing the last 8 residues in the C terminus. The deleted protein is functional, since the tetracycline MIC was 32 μg/ml for isolate APP9956, and it is also functional in plasmid pPAT1 (14). The *tetR*(H) gene was 624 bp long, and its DNA and amino acid sequences were 100% identical with those of the *tetR*(H) gene from *Pasteurella* plasmids (14).

**Characterization of the *tet*(L) genes.** Two isolates (APP9555 and APP13213) carried the *tet*(L) genes, which were located on plasmids, p9555 and p13123, of 5.6 and >12 kb, respectively. The 0.9-kb amplicons were sequenced and were 100% identical at the DNA and amino acid levels to the *tet*(L) gene from *Bacillus subtilis* (GenBank accession no. AY129652). Plasmid p9555 was completely sequenced and consisted of 5,672 bp with a G+C content of 39.4%. The plasmid had five ORFs with significant homology to proteins of known function (Fig. 2; Table 3). *orf2* was the *tet*(L) structural gene and consisted of 1,356 bp which had 99% identity at the DNA and amino acid levels to other plasmid-borne *tet*(L) genes, including the *Mannheimia* plasmid pCCK3259 (EMBL database accession no. AJ966516) (17) and the *Bacillus cereus* plasmid pJHI (GenBank accession no. AY129652). The *tet*(L) gene in plasmid p9555 is preceded by a RBS sequence (AGAAGG) at positions 417 to 422 but lacks the translational attenuator which is found upstream in other *tet*(L) gene sequences along with the promoter sequence (Fig. 2). The intergenic region between *orf1* and the *tet*(L) gene does not present significant homologies with DNA sequences registered in databases. However, −35 (TTAGCT) and −10 (TTTAAT) regions resembling *A. pleuropneumoniae* consensus promoter sequences at positions 3109 to 3114 and 3140 to 3145 are potential promoters for *tet*(L) gene expression (7). A putative Rho-independent transcription terminator was found 9 bp downstream of the translational stop codon. This terminator is very similar to that previously found in all *tet*(L) genes of gram-positive bacteria (GenBank accession no. AY129652).

Four other *orfs* (*orf1* and *orf3* to *orf5*) were identified in plasmid p9555. The *orf1* gene codes for a truncated 67-amino-acid replication-related protein, whereas the other three showed homology at the DNA and amino acid levels with previously described mobilization-related genes organized in an operon structure (Fig. 2) (22). A potential recombination site was found downstream of the *tet*(L) gene (Fig. 2).

Two regions were identified in plasmid p9555. The first region consists of the *tet*(L) gene and has a G+C content of 35%. The second contains the *mob* genes (*mobC*, *mobA*, and *mobB*), with a G+C content of 40 to 45%, and is indistinguishable from the recently described *Mannheimia* plasmid pCCK3259 (17) with the exception of an additional 363-bp region that is absent in pCCK3259 (Fig. 2). This 363-bp region is flanked by 10-bp direct repeats which could possibly be easily deleted. The *mob* region of plasmid p9555 is highly homologous (>90%) to the *mob* region of the *H. parasuis* plasmid pHS-Tet (Fig. 2).

**Transfer of *A. pleuropneumoniae* plasmids.** The *tet*(B)-, *tet*(H)-, and *tet*(L)-positive plasmids were electroporated into *E. coli* S17-1, and $Tc^r$ transformants were shown to carry the different tetracycline resistance plasmids, indicating that these plasmids replicate and that their associated *tet* genes are expressed in *E. coli.* In contrast, no $Tc^r$ transformants occurred when plasmids from isolates carrying the *tet*(O) gene were electroporated into *E. coli* S17-1.

Mobilization of plasmids p11745, p13142, p9956, and p9555 from their original *A. pleuropneumoniae* isolates into *E. coli* S17-1 was examined, but no transconjugants were obtained. However, analysis of the plasmid p9555 sequence revealed the existence of a *mob* operon encoding MobA, a relaxase belonging to the ColE1 superfamily of relaxases (10), suggesting that p9555 is mobilizable. Plasmids encoding relaxases of this family can be mobilized with the assistance of IncP plasmid conjugation machinery. Mobilization of p9555 was assayed from *E. coli* S17-1, which carried a derivative of the conjugative RP4 plasmid integrated into the chromosome, to the *E. coli* DH5α recipient, and $Tc^r$ colonies were obtained at a frequency of $10^{-3}$ per recipient. The PCR assay with *tet*(L)-specific primers determined that all 10 $Tc^r$ transconjugants carried p9555, confirming the transfer of p9555 from *E. coli* S17-1 to *E. coli* DH5α.

## DISCUSSION

This is the first description of the *tet*(L), *tet*(H), and *tet*(O) genes in *A. pleuropneumoniae* or the genus *Actinobacillus*. All three of these *tet* genes were on small plasmids, as were the majority (94%) of the *tet*(B) genes. This is also the first time the *tet*(O) gene has been identified on small nonconjugative plasmids (4). The *tet*(O) gene is of gram-positive origin but has been identified in gram-negative *Campylobacter*, *Neisseria*, and *Megasphaera* spp. and has been associated with conjugative plasmids in some of these isolates (http://faculty.washington.edu/marilynr/) (25). In the eight *tet*(O)-positive *A. pleuropneumoniae* isolates, the gene is on a 6-kb nonmobilizable plasmid.

Among the 46 $Tc^r$ *A. pleuropneumoniae* isolates analyzed, 32 (70%) carried *tet*(B). The *tet*(B) gene is widespread among

gram-negative bacteria; it has been found in 24 other genera (http://faculty.washington.edu/marilynr/) and is the only *tet* gene previously identified in the *A. pleuropneumoniae* literature (34). However, unlike most *tet*(B)-containing bacteria, none of the *tet*(B)-positive *A. pleuropneumoniae* isolates appeared to carry a *tetR*(B) gene on the small 5-kb nonmobilizable plasmid. Similar findings have been reported for a *Neisseria meningitidis* isolate recovered from Japan (32), three strains of *Haemophilus parainfluenzae* (13), and an *H. parasuis* 5.1-kb plasmid carrying *tet*(B) (GenBank accession no. NC006828) (18). All the 5-kb nonmobilizable *tet*(B) plasmids had identical restriction patterns, suggesting that they were highly related or identical to each other. The RBS and transcription terminator sequences of the *tet*(B) gene are conserved in *A. pleuropneumoniae tet*(B) plasmids, whereas the original promoter was not found. Instead, upstream of the *tet*(B) gene there were two sequences that resemble *A. pleuropneumoniae* promoters (Fig. 1). Therefore, the absence of *tetR*(B) and sequence features of the *tet*(B) upstream region suggest that the promoter and transcriptional regulatory signals have been adapted for expression in *A. pleuropneumoniae*.

Sequence analysis of *tet*(B)-positive p11745 indicated that this plasmid codes for RepB, a replication-related protein homologous to a family of proteins involved in initiation of plasmid replication and widespread in plasmids of both gram-negative and gram-positive bacteria. Plasmid p11745 was transformed into both *E. coli* and *H. parasuis* (unpublished results). The development of plasmid vectors based on the p11745 replication region for use in *Pasteurellaceae* is currently in progress in our laboratory.

Plasmid p9956 carried the gram-negative *tet*(H) gene, which has previously been found as part of the small composite transposon Tn*5706* on plasmids or in the chromosome of *Pasteurella*, *Mannheimia* spp., *Acinetobacter radioresistens*, and *Moraxella* spp. (15, 16, 21). The *tet* regions of p9956 and the *Pasteurella* plasmid pPAT1 (14) are identical and, in contrast to *tet*(B) plasmids, include the regulator gene *tetR*(H) and a truncated copy of *tet*(H) which is functional in both *A. pleuropneumoniae* and *E. coli*.

Sequence analysis of p9555 suggests that this plasmid was built by insertion of a region of gram-positive origin containing *tet*(L) into a *Pasteurellaceae* plasmid. The p9555 *tet*(L) was homologous to previously sequenced *tet*(L) genes found in plasmids of several low-GC-content gram-positive bacteria. The upstream region of *tet*(L) lacks the regulatory sequences characteristic of gram-positive *tet*(L) determinants but contains two 6-bp boxes resembling the *A. pleuropneumoniae* consensus promoter. However, the translational terminator downstream of *tet*(L) is conserved. Thus, as in the case of *tet*(B) of p11745, sequences upstream of *tet*(L) seem to be suited for its expression in *A. pleuropneumoniae.* The *tet*(L) gene has been found in other gram-negative bacteria such as *Salmonella*, *Morganella*, *Fusobacterium*, and *Veillonella* spp. (http://faculty.washington.edu/marilynr/). The presence of a *mob* operon including a ColE1 superfamily relaxase indicated that p9555 can be mobilized in *E. coli* if transfer functions from a conjugative IncP plasmid are provided. Mobilization of the plasmids in the *E. coli* strains using a donor-containing plasmid, RP4, demonstrated that p9555 Mob proteins are functional. This plasmid could have been transmitted to *A. pleuropneumoniae* from other bacteria inhabiting the respiratory tracts of pigs and encoding the appropriate transfer machinery for assisted mobilization.

The *tet*(L)-carrying plasmids identified in bovine *Mannheimia* and *Pasteurella* isolates were virtually identical to p9555 (more than 99% homologous) except in a 363-bp region absent in pCCK3259, suggesting that plasmid pCCK3259 might be a deleted derivative of p9555 (17). Supporting this hypothesis are the dates of isolation of the *A. pleuropneumoniae* strain in 1998 and the *Mannheimia* isolates in 2003 (17), with plasmid pCCK3259 created from the parental plasmid p9555 by a 363-bp deletion mediated by the 10-bp repeats flanking the 363-bp stretch. The distinct geographical origins of bacteria carrying p9555 and pCCK3259 could reflect the fact that movement of pigs between different European countries is a common practice for pig breeding in Europe.

The two *A. pleuropneumoniae* $Tc^r$ plasmids sequenced in this study, p11745 and p9555, encoded mobilization-related proteins. The recent finding of a plasmid carrying a *tet*(B) gene and a *mob* region in an isolate of *H. parasuis* (18), another respiratory swine pathogen, suggests that mobilizable plasmids are vehicles for active DNA exchange among bacteria inhabiting the upper respiratory tracts of pigs, contributing to the spread of resistance genes.

Our data suggest that the number of different *tet* genes in *A. pleuropneumoniae* has expanded. What impact this might have on therapy of this disease is not clear, but continued monitoring of this important animal pathogen is warranted.

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