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Plasmid-Encoded Tet B Tetracycline Resistance in *Haemophilus parasuis*

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The complete sequence of two plasmids, pHS-Tet (5.1 kb) and pHS-Rec (9.5 kb), isolated from *Haemophilus parasuis* strain HS1543 has been obtained. Plasmid pHS-Tet contains four open reading frames including a *tet*(B) tetracycline resistance gene which unusually did not have an associated *tetR* repressor gene. From a total of 45 *H. parasuis* isolates surveyed (15 international reference strains, 15 field isolates selected for their genetic diversity, and 15 recent Australian field isolates), 2 tetracycline-resistant field isolates (HS226 and HS1859) were identified. Analysis of three additional isolates from the same disease outbreak as strain HS1859 revealed a further tetracycline-resistant *H. parasuis* strain (HS1857, serovar 8) and a tetracycline-resistant *Actinobacillus pleuropneumoniae* strain (HS1861). An approximately 10.6-kb plasmid was identified in field isolate HS226 and outbreak strains HS1857, HS1859, and HS1861. Southern hybridization analysis of these plasmids showed that the Tet B determinant was present, and restriction digest comparisons suggest that these plasmids are related. This is believed to be the first report of native *H. parasuis* plasmids and Tet B-mediated tetracycline resistance in this microorganism.

Haemophilus parasuis is the causative agent of Glässer's disease in swine. Common symptoms of this disease include anorexia, pyrexia, and lameness, though some pigs may die suddenly during acute outbreaks (19).

The use of antibiotics in animal production as treatment or prophylaxis against common infections or at subtherapeutic levels in feed to promote growth is under increasing scrutiny (11). Tetracycline has a long history of use in the swine industry (17), and its use generates a strong selective pressure that has resulted in the exchange of tetracycline resistance genes associated with plasmids or transposons within and between bacterial species (13). Tetracycline resistance determinants Tet B, Tet H, and Tet M have previously been found in other members of the *Pasteurellaceae* (7, 9). Tetracycline-resistant isolates of *H. parasuis* have previously been reported in Austria (15) and Denmark (1) although the mechanism of this resistance has not been elucidated.

In this study, we have identified and sequenced two native *H. parasuis* plasmids, one of which encoded the Tet B tetracycline resistance determinant. A survey of tetracycline resistance was undertaken in 45 *H. parasuis* strains, identifying *tet*(B) plasmid-mediated tetracycline resistance in two additional *H. parasuis* strains. One of these tetracycline-resistant field isolates was isolated from a disease outbreak involving both *H. parasuis* and *Actinobacillus pleuropneumoniae*. Tetracycline resistance determinant *tet*(B) was identified in an *A. pleuropneumonie* and a further *H. parasuis* isolate from this outbreak.

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MATERIALS AND METHODS

Bacteria. *Escherichia coli* strains were cultured in Luria-Bertani medium (LB) with appropriate antibiotics. Strain XL1-Blue MR (Stratagene) was used in electroporations using plasmid pHS-Tet, and strain TST-1 (*E. coli* Genetic Stock Center, Yale University) was used to make the *tetR* probe. All *H. parasuis*, *Haemophilus paragallinarum* (HP31 and 221), and *A. pleuropneumoniae* (HS1861) strains used in this study were obtained from the culture collection at the Animal Research Institute, Brisbane, Australia. *H. parasuis* strain HS19, *H. paragallinarum* strains 221 and HP31, and *Haemophilus influenzae* strain Rd were used in electroporations using plasmid preparations containing both plasmids pHS-Tet and pHS-Rec. The *H. parasuis* strains surveyed for tetracycline resistance, including additional outbreak strains, are listed in Table 2. All *Pasteurellaceae* strains used in this study were grown on TM/SN medium prepared as described previously (20). Briefly, TM/SN is prepared from a basal medium known as TM which contains 1% biosate peptone, 1% NaCl, 0.1% starch, 0.05% glucose, and 1.5% agar. Immediately before pouring, TM/SN medium is supplemented with the following sterile additives: 0.0025% NADH, 0.005% thiamine, 1% heat-inactivated horse serum, and 5% oleic acid bovine serum albumin complex consisting of 4.75% bovine serum albumin (fraction V) in normal saline (with the normal saline containing 0.06% oleic acid and 5% 0.05N NaOH). When required, a liquid medium version of TM/SN, termed TMB, was prepared by omitting the agar from TM/SN.

DNA techniques. Restriction and DNA modification enzymes were purchased from New England Biolabs. *Taq* polymerase for amplifying probes by PCR was purchased from Fisher Biotech. Molecular biology methods used were as previously described (3). QIAGEN plasmid midi kits were used for large-scale plasmid purifications.

DNA sequencing, analysis, and annotation of plasmids. DNA fragments from a midi-prep (QIAGEN) isolation of plasmids derived from strain HS1543 digested with either Sau3A (cloned fragment sizes, 0.3 and 0.6 kb) or MspI (cloned fragment sizes, 1.0 and 1.6 kb) were cloned into pUC19 digested with BamHI or AccI, respectively, and sequenced using universal M13 forward and reverse primers. Oligonucleotides were designed at the ends of the cloned fragments. Purified plasmid DNA from strain HS1543 was used as a template in subsequent sequencing reactions. After each round of sequencing, new primers were designed until a complete double-stranded sequence of the plasmid was obtained. ABI Prism BigDye Terminator version 3.1 (PE Applied Biosystems) was used for DNA sequencing. Following EDTA/ethanol precipitation, samples were sent to

the Australian Genome Research Facility for automated sequencing using an ABI 3730xl 96-capillary automatic sequencer (PE Applied Biosystems).

Sequence data were aligned and annotated using MacVector version 7.2 (Accelrys). Open reading frames (ORFs) were identified using MacVector, and gene identities were assigned using searches against the nucleotide and protein databases at the National Center for Biotechnology Information using the tBLAST-n algorithm (2). Similarity between ORFs was calculated using BLAST analysis of two sequences with filters off.

Electroporation of pHS-Tet into *H. parasuis*, *H. paragallinarum*, *H. influenzae* strain Rd, and *E. coli*. pHS-Tet was isolated from strain HS1543 using a QIAGEN midi-prep kit. *H. paragallinarum* strains 221 and HP31 and *H. parasuis* strains HS19 and HS29 were grown to an optical density at 600 nm of 0.5 in TMB medium, washed three times with sucrose/glycerol (SG) buffer (15% [vol/vol] glycerol, 272 mM sucrose), and resuspended in a final volume of 0.5 ml of SG buffer. *H. influenzae* strain Rd was heavily inoculated onto 10 brain heart infusion (BHI) plates, harvested into 50 ml of SG buffer and washed as described above. Cells (100 μl) were mixed with 10 μg of pHS-Tet DNA. After electroporation (Bio-Rad micropulser electroporator, 2.5 kV, 0.2-cm cuvettes), cells were suspended in 1 ml of TMB (*H. parasuis* and *H. paragallinarum*) medium or BHI broth supplemented with 10 μg/ml Hemin and 2 μg/ml NAD (*H. influenzae*) and grown for 1 h at 37°C before plating on TM/SN (*H. parasuis* and *H. paragallinarum*) or BHI (*H. influenzae*) plates supplemented with 5 μg/ml tetracycline and incubated overnight at 37°C with 5% CO_2. Tetracycline-resistant colonies were inoculated into appropriate liquid media and grown overnight, and plasmid DNA was isolated using a mini-prep kit. Restriction digests using AccI and ClaI confirmed the presence of pHS-Tet.

One microgram of plasmid pHS-Tet isolated from an *H. parasuis* HS19/pHS-Tet transformant was electroporated in *E. coli* strain XL1-Blue MR as described above. Cells were resuspended in 1 ml of LB broth and incubated for 1 h at 37°C with shaking and then plated on LB agar with tetracycline (10 μg/ml). The presence of pHS-Tet in transformants was confirmed using restriction digests as described above.

Southern hybridization analysis. Bacterial genomic DNA was isolated as previously described (3). Restriction endonuclease (EcoRV or AccI for strain HS1858 only)-digested plasmid DNA [for *tet*(B) blot] or MspI-digested *H. parasuis* strain 1543 genomic DNA and EcoRV-digested pHS-Tet ad pHS-Tet/pHS-Rec plasmid preparations (for *tetR* blot) were separated on 0.7% agarose gels and transferred to GeneScreen Hybridization Transfer membrane (NEN Life Science Products) by capillary action (3). A DNA fragment containing the *tet*(B) gene was amplified from pHS-Tet using primers BF and BR as previously described (22). A DNA fragment containing the Tn*10 tetR* gene was amplified from *E. coli* strain TST-1 genomic DNA using primers TetRfwd (5′-ATGATG TCTAGATTAGATAAAAGTAAAG) and TetRrev (5′-TTAAGACCCACTTT CACATTTAAGTTG). Blots were hybridized with digoxigenin (DIG)-labeled PCR products for 16 h at 68°C. Washes and detection were carried out (DIG DNA Labeling and Detection Kit; Roche) as recommended by the manufacturer.

Antibiotic susceptibility assays. The tetracycline MIC test described by Blackall (5) was used. Isolates were regarded as being resistant to tetracycline if the MIC was ≥16 μg/ml and sensitive if the MIC was ≤4 μg/ml (5). *A. pleuropneumoniae* strain ATCC 27090 was used as the control strain. Serial dilutions of bacterial dilutions were also grown on TM/SN medium with and without 5 μg/ml tetracycline to confirm the MIC results.

Nucleotide sequence accession numbers. The sequences of plasmids pHS-Tet and pHS-Rec have been deposited in the GenBank database under accession numbers AY862435 and AY862436, respectively.

RESULTS AND DISCUSSION

Sequence analysis and genetic organization of plasmids pHS-Tet and pHS-Rec. Examination of whole genomic DNA preparations of *H. parasuis* strain HS1543 digested with MspI revealed distinct DNA bands that were confirmed to be plasmids by restriction endonuclease digests of small-scale plasmid isolation preparations.

The genetic map of plasmid pHS-Tet is shown in Fig. 1. The plasmid is 5,147 bp in size, and detailed analysis of the sequence revealed a *tet*(B) tetracycline resistance gene and *mobABC* plasmid mobilization genes (see Table 1). The plasmid is similar in structure to the *Mannheimia haemolytica* plasmid pAB2 (23), which differs from pHS-Tet by containing a β-lactamase (*bla*) resistance gene in place of *tet*(B). The *tet*(B) gene has 99% amino acid similarity to the *Salmonella enterica* serovar Typhi Tn*10 tet*(B) efflux pump and is flanked by a duplicated 53-bp sequence. Unusually, there is no *tetR* gene encoding the Tn*10*-associated repressor protein located downstream of the pHS-Tet *tet*(B) gene. This is in contrast to plasmid pPAT2 (14), previously isolated from *Pasteurella aerogenes*, that contains the *tet*(B) gene and its repressor *tetR*. Tn*10*

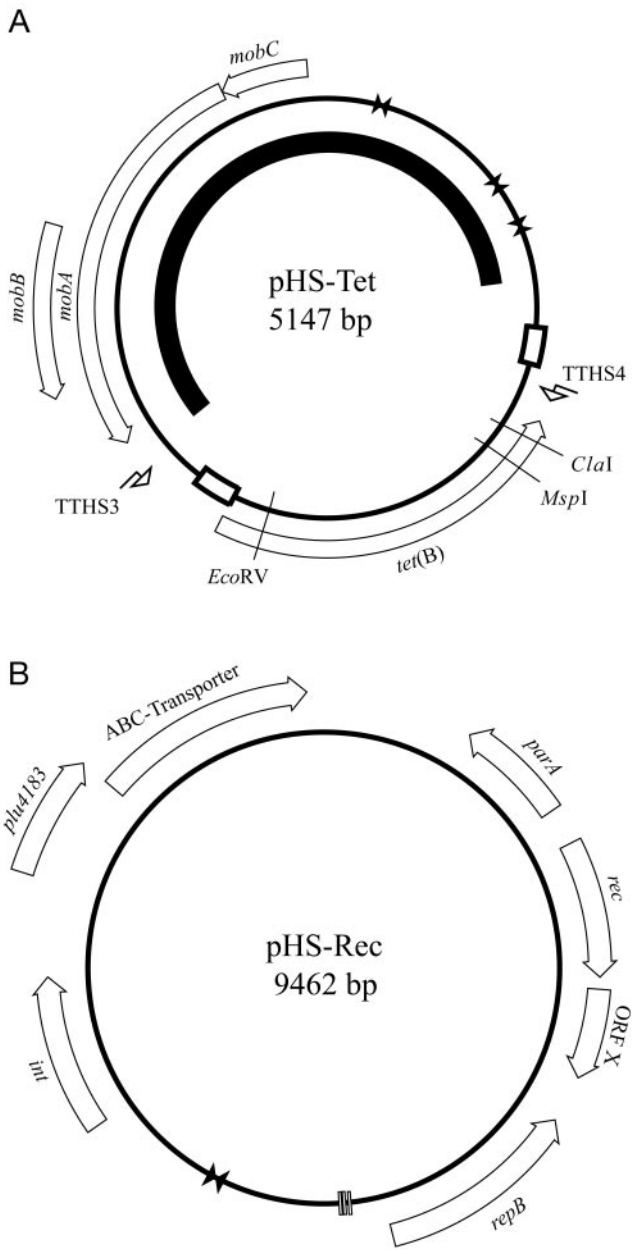

FIG. 1. Schematic map of plasmid pHS-Tet (A) and pHS-Rec (B). White arrows indicate putative ORFs, vertical bars indicate direct repeats, bow ties (opposing triangles) indicate inverted repeats, and bent arrows indicate primer binding sites. The black arc inside the map of pHS-Tet indicates the region of pHS-Tet sharing similarity with plasmid pAB2. The white boxes on the map of pHS-Tet indicate regions of duplicated sequence.

TABLE 1. Summary of genes found on the pHS-Tet and pHS-Rec plasmids of *H. parasuis*

Plasmid and genes	Nearest homology	Nucleotide accession no.	Putative function	% DNA identity	% Protein identity/similarity
Plasmid pHS-Tet genes[a]					
tet(B)	*S. enterica* serovar Typhi Tn*10*	AY150213	Tetracycline efflux pump	99	99/99
MobA	*MobA* pAB2	Z21724	Plasmid mobilization	99	99/100
MobB	*MobB* pAB2	Z21724	Plasmid mobilization	99	98/98
MobC	*MobC* pAB2	Z21724	Plasmid mobilization	100	100/100
Plasmid pHS-Rec genes[b]					
rec	*Haemophilus somnus*	NC_002947	Recombinase	44	45/54
int	*H. paragallinarum* p250	AY300023	Integrase	42	68/78
repB	*H. paragallinarum* p250	AY300023	Replication	67	23/38
ABC-transporter	*Pseudomonas putida* KT2440	NC_002947	Transporter	46	29/55
parA	*Actinobacillus actinomycetemcomitans*	AF302424	DNA partitioning	42	28/48
*plu*4183	*Photorhabdus luminescens*	NC_005126	Unknown	57	11/16
ORFX	None		Unknown		

[a] GenBank accession no. AY862435.
[b] GenBank accession no. AY862436.

and plasmid pHS-Tet diverge 15 bp upstream and 78 bp downstream of the *tet*(B) gene. Plasmid pHS-Tet has a single partial repressor binding site, in contrast to the two binding sites necessary for full gene repression located between the *tet*(B) and *tetR* genes in Tn*10*. Southern hybridization analysis of the HS1543 genome (including plasmids pHS-Tet and pHS-Rec) using a *tetR* probe confirmed the absence of *tetR* either on pHS-Tet, pHS-Rec, or in the chromosome (data not shown). Constitutive expression of Tn*10*-like tetracycline resistance has previously been reported in *Haemophilus parainfluenzae*, mediated by an inactivating point deletion mutation in the *tetR* gene (10). Tetracycline sensitivity profiling revealed that strain HS1543 was tetracycline resistant, indicating that the *tet*(B) gene product is functional. Tetracycline resistance determinants Tet A and Tet B have previously been reported in transposons on a conjugative multidrug-resistant plasmid in *E. coli* of the normal enteric flora of pigs (21). The tetracycline resistance determinant *tet*(B), the most common determinant found in *Enterobacteriaceae* (12), has also been found in *Pasteurella multocida* isolates from swine (13). In addition, it has been demonstrated that in chickens given feed with tetracycline, the transfer of tetracycline resistance genes occurred between *E. coli* strains (16). Hence, it is possible that the use of tetracycline as a therapeutic agent and in-feed additive in the swine industry may select for *H. parasuis* strains resistant to tetracycline.

The plasmid pHS-Tet also contains genes encoding proteins homologous to the *MobABC* family of proteins that facilitate plasmid mobilization. These genes share 99% nucleotide identity (1,666/1,668; one gap) with the mobilization genes in plasmid pAB2, which was shown to use host proteins to conjugate *E. coli* and *Pasteurella haemolytica* although it was unable to mediate transfer out of *E. coli* (8). The same region shares 72% identity (1,197/1,668) with plasmid pIG1, a streptomycin- and sulfonamide-resistant plasmid isolated from *P. multocida* (24). The presence of mobility proteins has implications for disease outbreaks on farms where treatment with antibiotics gives rise to a selective pressure that favors resistant strains that are capable of transferring resistance within and between bacterial species.

There was approximately 1.2 kb of DNA sequence obtained from plasmid pHS-Tet which had 99% similarity (1,192/1,200; 6 gaps) to the replication region of plasmid pAB2 (23) and 88% similarity (1,060/1,200; 12 gaps) to the replication region of plasmid pIG1 (24). In this region, pHS-Tet contained 20-, 16-, and 38-bp inverted repeats, similar to the 20-, 16-, and 38-bp inverted repeats found in the pIG1 replication region. The absence of large ORFs, with the presence of inverted repeats, and homology to known replication origins suggest that the origin of replication for plasmid pHS-Tet may be located in this region. Like plasmids pAB2 and pIG1, plasmid pHS-Tet is able to replicate in *E. coli* after electroporation into strain XL1-Blue MR.

The genetic map of plasmid pHS-Rec is shown in Fig. 1. The plasmid is 9,462 bp in size, and detailed analysis of the sequence revealed eight ORFs (Table 1). Although plasmid pHS-Rec does not share any DNA or protein similarity to any known plasmid families, the association between *repB* and *parA* has been previously reported in the backbone of self-replicating plasmids (4). There appears to be no antibiotic resistance marker or *tetR* repressor gene on pHS-Rec.

Survey of tetracycline resistance in *H. parasuis*. A survey of the prevalence of tetracycline resistance was undertaken using a panel of *H. parasuis* strains including the 15 international reference strains, a genetically diverse collection of Australian field isolates (15 strains) selected by electropherotype (6), and a group of 15 recent Australian field isolates of different serovars (Table 2). No formally approved methodology exists for a MIC test with *H. parasuis*. The method we have used has been previously used on another difficult bacterium (*H. paragallinarum*) and has been shown to give the expected MIC results with formal control strains (5). The modifications to this procedure were validated by the *A. pleuropneumoniae* control strain ATCC 27090 (18).

H. parasuis strains HS226 and HS1859 were tetracycline resistant (MIC of 64 μg/ml) as was strain HS1543 (MIC of 16 μg/ml). Additional clinical isolates from the same disease outbreak as strain HS1859 were examined, and a further tetracycline-resistant *H. parasuis* strain (HS1857) and a resistant *A. pleuropneumoniae* strain (HS1861) were identified along with a

TABLE 2. Tetracycline resistance of bacterial strains used in this study

Group[a]	Bacterial strain[b]	Origin[c]	Associated pathology[d]	Serovar[e]	Tetracycline MIC (μg/ml)
I	No. 4	Japan	Healthy	1	4
	SW 140	Japan	Healthy	2	4
	SW 114	Japan	Healthy	3	4
	SW 124	Japan	Healthy	4	4
	Nagasaki	Japan	Septicemia	5	4
	131	Switzerland	Healthy	6	4
	174	Switzerland	Healthy	7	4
	C5	Sweden	NK	8	4
	D74	Sweden	NK	9	2
	H367	Germany	Healthy	10	2
	H465	Germany	Pneumonia	11	4
	H425	Germany	Polyserositis	12	4
	IA-84-17975	United States	NK	13	8
	IA-84-22113	United States	NK	14	4
	SD-84-15995	United States	Pneumonia	15	4
II	HS306	VIC	Septicemia	NT	2
	HS68	QLD	Septicemia	5	4
	HS202	VIC	Septicemia	4	4
	HS14	QLD	NK	5	4
	HS357	SA	Septicemia	5	2
	HS356	SA	Septicemia	13	8
	HS333	VIC	Septicemia	5	4
	HS16	QLD	Septicemia	5	4
	HS307	VIC	Septicemia	NT	4
	HS72	QLD	Healthy	9	4
	HS145	TAS	Septicemia	1	4
	HS45	WA	Respiratory	4	4
	HS58	WA	Respiratory	NT	4
	HS29	VIC	NK	2	4
	HS226	VIC	Septicemia	NT	64
III	HS1919	NSW	Trachea	4	4
	HS1911	QLD	NK	4	8
	HS1913	NK	NK	14	2
	HS1906	VIC	Pneumonia	13	4
	HS1910	QLD	NK	1	2
	HS1895	NK	Pneumonia	2	2
	HS1859*	VIC	NK	8/9	64
	HS1854	WA	NK	4	4
	HS1848	VIC	Pneumonia	3	4
	HS1939	NK	NK	5	4
	HS1915	VIC	NK	5	4
	HS1905	QLD	NK	NT	2
	HS1889	VIC	Septicemia	NT	4
	HS1921	WA	Pneumonia	4	4
	HS1916	NSW	Pneumonia	4	2
IV	HS1857*	VIC	NK	8/9	64
	HS1858*	VIC	NK	5	4
	HS1543	VIC	NK	10	16
	HS1861*	VIC	NK	NK	64
	ATCC 27090	NK	NK	NK	4

[a] Group I, international reference strains; group II, genetically diverse Australian isolates; group III, recent Australian field isolates; group IV, additional strains used in this study.

[b] Asterisk, strains isolated from the same outbreak. Underlined strains are tetracycline resistant. Strains HS1861 and ATCC 27090 are *A. pleuropneumoniae.*

[c] NSW, New South Wales; QLD, Queensland; SA, South Australia; TAS, Tasmania; VIC, Victoria; WA, Western Australia.

[d] NK, not known.

[e] NT, nontypeable.

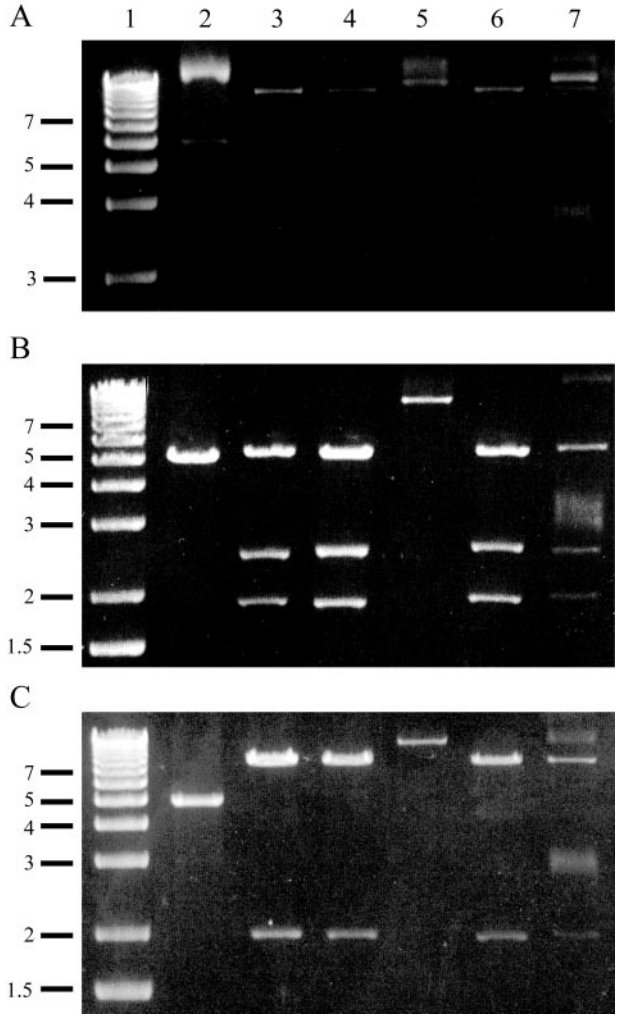

FIG. 2. Restriction endonuclease characterization of plasmids uncut (A), digested with AccI (B), or digested with ClaI (C). Lanes 1, 1-kb ladder (Invitrogen); lanes, 2, pHS-Tet; lanes 3, HS226 plasmid; lanes 4, HS1857 plasmid; lanes 5, HS1858 plasmid; lanes 6, HS1859 plasmid; lanes 7, HS1861 plasmid.

tetracycline-sensitive *H. parasuis* strain (HS1858). An approximately 10.6-kb plasmid was identified in these strains, and characterization by restriction digests (Fig. 2) suggests that the plasmids from strains HS226 and HS1861 are related. Similarly, plasmids from strains HS1857 and HS1859 appear related, though these two strains are both serovar 8/9, suggesting possible clonality.

Southern hybridization analysis (Fig. 3) using a *tet*(B) probe showed that plasmid pHS-Tet and plasmids isolated from strains HS226, HS1857, HS1859, and HS1961 contained the Tet B determinant. However, the Tet B determinant was not found in strain HS1858, which is consistent with the tetracycline-sensitive phenotype of this strain. Strain HS226 was isolated in 1991 from central nervous system tissue of a 24-week-old pig that died from Glässer's disease on farm A. Breeding stock was supplied from farm A to farm B, where strains HS1857, HS1858, HS1859, and HS1861 were isolated from a Glässer's disease outbreak in 2003. Thus, it appears that the

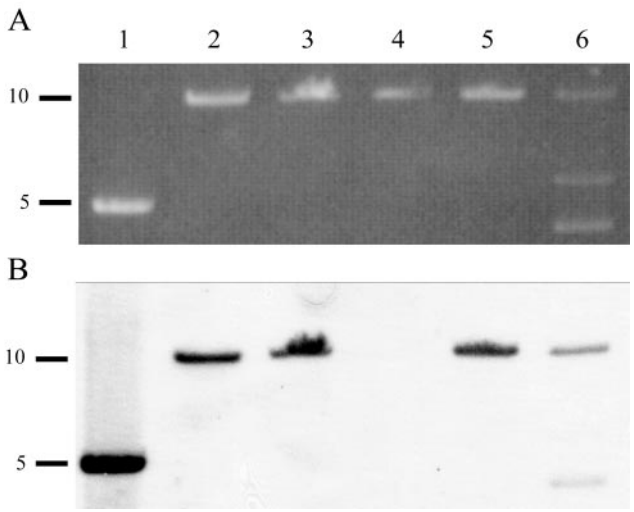

FIG. 3. Agarose gel of restriction endonuclease linearization of plasmids using EcoRV (except that AccI was used to linearize plasmid from HS1858) (A) and Southern hybridization analysis of gel shown in panel A transferred to nitrocellulose membrane and probed with a DIG-labeled PCR product containing the *tet*(B) gene (B). Lanes 1, pHS-Tet; lanes 2, HS226 plasmid; lanes 3, HS1857 plasmid; lanes 4, HS1858 plasmid; lanes 5, HS1859 plasmid; lanes 6, HS1861 plasmid. DNA fragment sizes are indicated in kb.

same plasmid has been identified from two clinical isolates (*H. parasuis* HS226 and *A. pleuropneumonie* HS1861) from epidemiologically connected disease outbreaks occurring 12 years apart on two different farms.

The occurrence of tetracycline resistance among *H. parasuis* isolates in this study (4 resistant strains from 48 strains examined) is comparable to earlier work (15), where 6.4% of isolates from Austria were tetracycline resistant. However, the occurrence of tetracycline resistance in *H. parasuis* is considerably lower than that reported in a study of *A. pleuropneumoniae*, a related pathogenic porcine *Pasteurellaceae*, where 12.1% of clinical isolates from Austria were tetracycline resistant (15).

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