Nucleotide sequence and transfer properties of two novel types of Actinobacillus pleuropneumoniae plasmids carrying the tetracycline resistance gene tet(H)

Journal:	*Journal of Antimicrobial Chemotherapy*
Manuscript ID:	JAC-2007-0363
Manuscript Type:	Brief Report
Date Submitted by the Author:	19-Apr-2007
Complete List of Authors:	Blanco, Mónica; Universidad de Cantabria, Biología Molecular Kadlec, Kristina; Bundesforschungsanstalt für Landwirtschaft, Institut für Tierzucht Gutiérrez Martín, César; Universidad de León, Microbiología e Inmunología Martín de la Fuente, Ana; Universidad de León, Microbiología e Inmunología Schwarz, Stefan; Bundesforschungsanstalt für Landwirtschaft, Institut für Tierzucht Navas, Jesus; Universidad de Cantabria, Biologia Molecular
Keywords:	respiratory tract infection, Antimicrobial resistance, gene transfer, mobilization, interspecies transfer

Nucleotide sequence and transfer properties of two novel types of *Actinobacillus pleuropneumoniae* plasmids carrying the tetracycline resistance gene *tet*(H)

Mónica Blanco[1], Kristina Kadlec[2], César B. Gutiérrez Martín[3], Ana Judith Martín de la Fuente[3], Stefan Schwarz[2], and Jesús Navas[1*]

[1] Departamento de Biología Molecular (Unidad Asociada al Centro de Investigaciones Biológicas, C.S.I.C.), Facultad de Medicina, Universidad de Cantabria, 39011 Santander, Spain.

[2] Institut für Tierzucht, Bundesforschungsanstalt für Landwirtschaft (FAL), Höltystr. 10, 31535 Neustadt-Mariensee, Germany

[3] Departamento de Sanidad Animal, Unidad de Microbiología e Inmunología, Facultad de Veterinaria, Universidad de León, 24007 León, Spain.

Key words: respiratory tract infection, antimicrobial resistance, gene transfer, mobilization, interspecies transfer.

Short title: *A. pleuropneumoniae* plasmids carrying the *tet*(H) gene

[*] Corresponding author. Tel. 34-942-201943 Fax. 34-942-201945 E-mail: navasj@unican.es

SYNOPSIS

Objectives: To analyze the sequence and transfer properties of two tetracycline resistance plasmids found in clinical isolates of *Actinobacillus pleuropneumoniae* in order to assert their role in the spread of tetracycline resistance.

Methods: The plasmids designated p9956 and p12404 were purified from *A. pleuropneumoniae* and completely sequenced by primer walking directly or after subcloning into pBluescript®II SK+. Their transfer properties were evaluated by electroporation and/or conjugation into *Pasteurella multocida* and *E. coli.*

Results: Both plasmids showed a function-related modular structure, with three regions involved in either mobilization, tetracycline resistance, or replication. The mobilization regions were composed of different genes whose products are involved in plasmid transfer. The tetracycline resistance regions were closely related and consisted of the *tet*(H) gene and its repressor gene *tetR*(H). The tetracycline resistance phenotype was transferred successfully to *P. multocida* and *E. coli* by electroporation of the plasmids. Moreover, plasmid p9956 could be mobilized in *E. coli* with the assistance of RP4 conjugal transfer functions.

Conclusion: For the first time, the complete sequences of two *tet*(H)-carrying plasmids from *A. pleuropneumoniae* were determined. Sequence comparisons revealed distinct differences to the so far known *tet*(H)-carrying plasmids from *Pasteurella* spp. or *Mannheimia* spp. Structural analysis confirmed that these plasmids consisted of segments which showed similarities to plasmids previously detected in members of the families Pasteurellaceae and Enterobacteriaceae. The results of this study point towards the role of

interplasmid recombination in the development of novel types of resistance plasmids in porcine respiratory tract pathogens.

Introduction

Actinobacillus pleuropneumoniae is the causative agent of porcine pleuropneumonia, a respiratory disease transmitted by aerosols or direct contact with infected pigs.[1] The incidence of this disease has recently increased due to the intensification of porcine production.[2] Several whole cell bacterin vaccines have been developed, but neither prevents the occurrence of asymptomatic carriers nor provides complete cross-serotype protection for the 15 existing serotypes.[3] Therefore antibiotic therapy is still critical for the treatment and control of pleuropneumonia outbreaks. Tetracyclines are broad-spectrum antibiotics which have been widely used for the treatment and prophylaxis of animal infections, but also as feed additives in pig production. Consumption figures suggest that the use of tetracyclines in veterinary practice is still high compared with use of other classes of antibiotics.[4,5] Reflecting this situation, a recent study of the antimicrobial susceptibility of Spanish *A. pleuropneumoniae* clinical isolates recovered from 1997 to 2004 revealed a high rate (73.8%) of tetracycline resistance.[5] Four resistance determinants, *tet*(B), *tet*(L), *tet*(H) and *tet*(O), were found in a selected group of *A. pleuropneumoniae* isolates showing high MIC values for tetracyclines. In most of them, the *tet* gene was plasmid-encoded, including the two isolates carrying the gene *tet*(H).[6] This gene codes for an energy-dependent 46 kDa membrane-associated protein which exports tetracycline and doxycycline out of the bacterial cell. The gene *tet*(H) has been found as part of the small composite

transposon Tn*5706* [7] on plasmids or in the chromosome of *Pasteurella*, *Mannheimia*, *Acinetobacte*r, and *Moraxella* spp.[8]

Since plasmids play a key role in spreading antibiotic resistance genes, in the present study we have sequenced and analyzed the transfer properties of plasmids p9956 and p12494, carried by two tetracycline-resistant *A. pleuropneumoniae* isolates.

Material and methods

The *A. pleuropneumoniae* isolates APP9956 and APP12494 were isolated from the lung of diseased pigs. Plasmid preparation, hybridization and transformation into *E. coli* S17.1 and *P. multocida* B130 were performed as described.[6,7] Conjugal transfer of the tetracycline resistance plasmids was performed using either the *E. coli* S17.1 or *P. multocida* B130 transformants as donors and *E. coli* DH5α as recipient strain, as previously described.[6] *E. coli* S17.1 carries the RP4 conjugation genes inserted in its chromosomal DNA. The plasmid designated p9956 was amplified using primers complementary to *tet*(H) internal sequences, tetHoutF (5'-CCAATATTACCGGGATCA-3') and tetHoutR (5'-CCAATGGCATCTAATACG-3'), and sequenced by a primer walking strategy. The plasmid p12494 was purified from *A. pleuropneumoniae* APP12494 and transformed into electro-competent *P. multocida* B130. Plasmid DNA from a transformant was prepared and subjected to restriction mapping. *Cla*I and *Hin*dIII restriction fragments were subcloned into pBluescript®SK II (Stratagene, La Jolla, USA) and transformed into *E. coli* JM109. Sequences of p12494 subclones were determined by primer walking and assembled using the ContigExpress Vector NTI Advance 10.1 software (Informax, Bethesda, USA). Homology searches were performed with BLAST and ORF finder tools

(http://www.ncbi.nlm.nih.gov). The complete sequences of plasmids p9956 and p12494 have been deposited in GenBank under accession numbers AY362554 and DQ517426, respectively.

Results and discussion

Isolation of two plasmids carrying the gene tet(H) in A. pleuropneumoniae

The gene *tet*(H) was detected by PCR in the *A. pleuropneumoniae* isolates APP9956 and APP12494.[6] MICs for doxycycline were 16 mg/L for both isolates, whereas MICs for tetracycline were 32 mg/L for APP9956 and 64 mg/L for APP12494. Single plasmids of 5.6 or 14.3 kb were detected in isolates APP9956 and APP12494, respectively. Electroporation of the two plasmids into *P. multocida* B130 produced tetracycline-resistant colonies. Both plasmids hybridized with a *tet*(H) probe consisting of the PCR amplification product from plasmid pVM111.[9]

Nucleotide sequence and genetic organization of plasmid p9956

Plasmid p9956 from isolate APP9956 consisted of 5,674 bp (41.4% G+C content) encompassing five orfs which encode putative proteins highly homologous to proteins of known function (Fig. 1a). Consideration of the G+C content and the presumptive function of the protein encoded by each orf (Table 1) revealed the existence of two regions in the plasmid, the resistance region and the mobilization region. The resistance region included the structural gene *tet*(H) and the repressor gene *tetR*(H). The sequence of this region was mainly identical to that of plasmids pPAT1 [10] and pPMT1 [7] from *Pasteurella aerogenes* and *P. multocida*, respectively. As in these two plasmids, two putative Rho-independent transcriptional terminators were found downstream of the two

genes. However, in plasmid p9956 the tetracycline resistance gene region was associated neither with Tn*5706* nor with other transposable elements.

The mobilization region included three orfs organized in an operon-like structure, coding for MobA, MobB and MobC proteins respectively (Table 1). The Mob proteins exhibited more than 98% identity to the corresponding proteins of plasmids pHS-Tet from *H. parasuis* [11] and p9555 from *A. pleuropneumoniae.* [6] The two regions differed in their G+C content (40% the resistance region, versus 45% G+C of the mobilization region) suggesting a different origin. However, no recombination sequences separating the two regions were detected. The sequence spanning from 4797 bp to 304 bp was highly homologous to the putative replication region of plasmid pLS88 from *Haemophilus ducreyi* (accession number L23118). Plasmid p9956 can be considered as a broad-host range plasmid, since it replicated stably in *E.coli* and *P. multocida*.

Structure and organization of plasmid p12494

The plasmid p12494 is 14,393 bp in size and has a G+C content of 32.9 %. Analysis of the p12494 sequence revealed the presence of 10 orfs, nine of them coding for proteins homologous to proteins registered in GenBank (Fig. 1b). Based on the presumptive functions of the orfs three different regions associated with replication, resistance and mobilization were found in p12494. The replication region included the origin of replication (*oriV*), comprising five 22-bp iterons, and a *rep* protein. This protein was 85% similar to the RepB protein of *H. parasuis* plasmid pHS-Rec [11] and possessed several leucine residues at the N-terminus and secondary structure motifs (leucine zipper and a HTH motif) characteristic of type θ replication proteins.

The resistance module comprised the structural gene *tet*(H) and the repressor gene *tetR*(H) as in plasmid pPMT1 [7] and in other plasmids found in *Pasteurella* spp. When compared to previously described regions, the gene *tet*(H) was identical, but on the *tetR*(H) gene a 100 bp insertion produced the loss of 60 amino acids at the C-terminus of the repressor protein. The MIC for tetracycline of 64 mg/L of isolate APP12494, however, suggested that the truncated TetR(H) protein had no negative impact on tetracycline resistance. Immediately downstream of the resistance region, we found a unique copy of an insertion sequence identical to IS*1592* from the *Pasteurella trehalosi* plasmid pCCK13698 and similar to IS*1596* and IS*1597* from the transposon Tn*5706.*[7] The IS*1592*-like element was followed by the 7 bp integration sequence (TATGATA). The putative transposase encoded by this insertion sequence was closely related to those encoded by the IS*982* family which has been described in Gram-positive bacteria and also in some *Pasteurella* spp.[7] Further downstream of the IS element and transcribed in the opposite direction, we located an *orf* encoding an IS*607*-like transposase belonging to the Serine-Recombinase family. This Rec protein was similar (64% identity) to the corresponding protein of *H. parasuis* plasmid pHS-Rec and also to the resolvase/integrase-like protein of *Haemophilus influenzae* (accession no. YP_247803). Serine-recombinases catalyze site-specific recombination of DNA molecules and are functionally versatile, including resolvases, invertases, integrases and transposases. The gene *parA* was detected 186 bp downstream of the *rec* gene. It encoded a protein homologous to the partition protein of the *H. parasuis* plasmid pHS-Rec and belonging to a family (pfam00991) of bacterial ATPases involved in DNA segregation.

The mobilization region of plasmid p12494 comprised a single orf. The protein encoded, designated MobA, showed conserved domains with proteins of the MobA_L relaxase family (pfam03389), mainly on the C terminus where its nicking activity is located. This family includes the MobA protein from the *E. coli* plasmid RSF1010 and the MobL protein from the *Thiobacillus ferrooxidans* plasmid pTF1, among others.

Functionally relevant orfs of p12494 involved in resistance are constrained in an 8.5 kb region of the plasmid. The remaining 6 kb include orf8 and two small orfs organized in an operon-like structure, *vapD* and *vapX* (Fig. 1b). The function of the putative protein (442 aa) encoded by orf8 is unknown. Proteins VapD and VapX are homologous to components of the toxin-antitoxin system of non-typeable *Haemophilus influenzae*.[12] The protein VapD is assumed to be involved in the modulation of bacterial persistence in human cells. A homologue of p12494 VapD encoded by the *Actinobacillus actinomycetemcomitans* plasmid pVT736-1 (accession no. L24000) is implicated in plasmid maintenance. Homologues of VapD and VapX are also present among the hypothetical proteins of the *Neisseria gonorrhoeae* plasmid pJD1 (accession no. NC_001377).

Transfer of the two A. pleuropneumoniae tet(H) plasmids

Plasmids p9956 and p12494 were successfully electroporated into *P. multocida* B130 where they expressed tetracycline resistance. When the two plasmids were transformed into *E. coli* S17.1, resistant colonies appeared only in the case of plasmid p9956. However a pBluescript®SK II clone carrying the resistance region from plasmid p12494 conferred resistance to tetracycline, suggesting that the *tetR*(H)-*tet*(H) systems of both plasmids are functionally

active in *E. coli*. The absence of tetracycline-resistant p12494 transformants in *E. coli* could be explained by the inability of this plasmid to replicate in this host.

Plasmids p9956 and p12494 could not be mobilized from their original *A. pleuropneumoniae* isolates into *E. coli* S17-1. However p9956 was mobilized from *E. coli* S17.1 to *E. coli* DH5α at a frequency of 10^{-3} colonies per recipient. Plasmid profiling and a *tet*(H)-specific PCR assay confirmed that all tetracycline-resistant *E. coli* DH5α colonies carried p9956. This result demonstrated that Mob proteins of p9956 are functionally active. Mobilizable plasmids can contribute to the intraspecies transfer of tetracycline resistance among *A. pleuropneumoniae* strains causing an outbreak and also to the interspecies transfer of tetracycline resistance among pathogens inhabiting the respiratory tract of pigs.

Acknowledgements

We thank Vera Nöding for excellent technical support. This work was supported by grants from the Spanish Ministry for Science and Technology (AGL2002-04585) and the Spanish Network for Research in Infectious Pathology (REIPI). M. Blanco was a recipient of fellowships from the University of León and the University of Cantabria.

Transparency declaration

None to declare.

References

1. Torremorell M, Pijoan C, Janni K *et al*. Airborne transmission of *Actinobacillus pleuropneumoniae* and porcine reproductive and respiratory syndrome virus in nursery pigs. *Am J Vet Res* 1997; **58**: 828-32.

2. Gilchrist MJ, Greko C, Wallinga DB *et al*. The potential role of concentrated animal feeding operations in infectious disease epidemics and antibiotic resistance. *Environ Health Perspect* 2007; **115**: 313-6.

3. Bosse JT, Janson H, Sheehan BJ *et al*. *Actinobacillus pleuropneumoniae*: pathobiology and pathogenesis of infection. *Microbes Infect* 2002; **4**: 225-35.

4. EMEA. Antibiotic Resistance in the European Union associated with Therapeutic Use of Veterinary Medicines- Report and Qualitative Risk Assessment by the Committee for Veterinary Medicinal Products (EMEA/CVMP/342/99-FINAL). http://www.emea.eu.int/pdfs/vet/regaffair/034299ENC.pdf, last accessed 18-4-2007.

5. Gutierrez-Martin CB, del Blanco NG, Blanco M *et al*. Changes in antimicrobial susceptibility of *Actinobacillus pleuropneumoniae* isolated from pigs in Spain during the last decade. *Vet Microbiol* 2006; **115**: 218-22.

6. Blanco M, Gutierrez-Martin CB, Rodriguez-Ferri EF *et al*. Distribution of tetracycline resistance genes in *Actinobacillus pleuropneumoniae* isolates from Spain. *Antimicrob Agents Chemother* 2006; **50**: 702-8.

7. Kehrenberg C, Werckenthin C, Schwarz S. Tn*5706*, a transposon-like element from *Pasteurella multocida* mediating tetracycline resistance. *Antimicrob Agents Chemother* 1998; **42**: 2116-8.

8. Roberts MC. Update on acquired tetracycline resistance genes. *FEMS Microbiol Lett* 2005; **245**: 195-203.

9. Hansen LM, Blanchard PC, Hirsh DC. Distribution of *tet*(H) among *Pasteurella* isolates from the United States and Canada. *Antimicrob Agents Chemother* 1996; **40**: 1558-60.

10. Kehrenberg C, Schwarz S. Identification of a truncated, but functionally active *tet*(H) tetracycline resistance gene in *Pasteurella aerogenes* and *Pasteurella multocida*. *FEMS Microbiol Lett* 2000; **188**: 191-5.

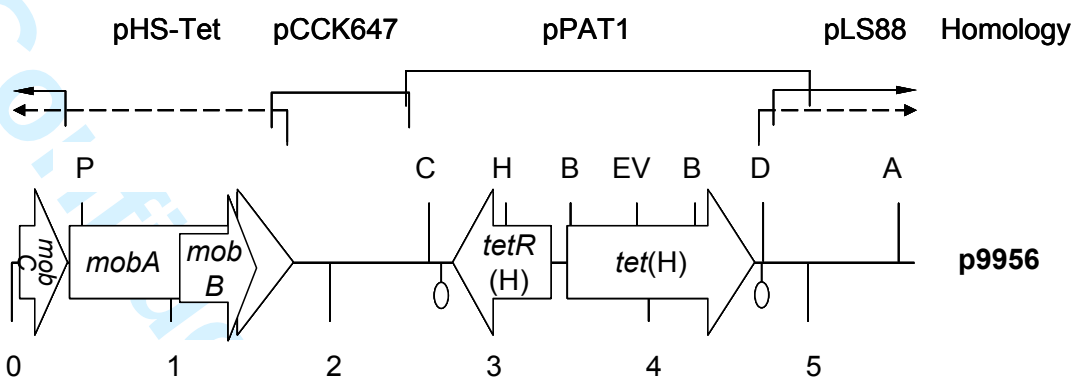
11. Lancashire JF, Terry TD, Blackall PJ *et al*. Plasmid-encoded Tet B tetracycline resistance in *Haemophilus parasuis*. *Antimicrob Agents Chemother* 2005; **49**: 1927-31.

12. Daines DA, Jarisch J, Smith AL. Identification and characterization of a nontypeable *Haemophilus influenzae* putative toxin-antitoxin locus. *BMC Microbiol* 2004; **4**: 30.

253 **Table 1:** Coding regions from plasmids p9956 and p12494.

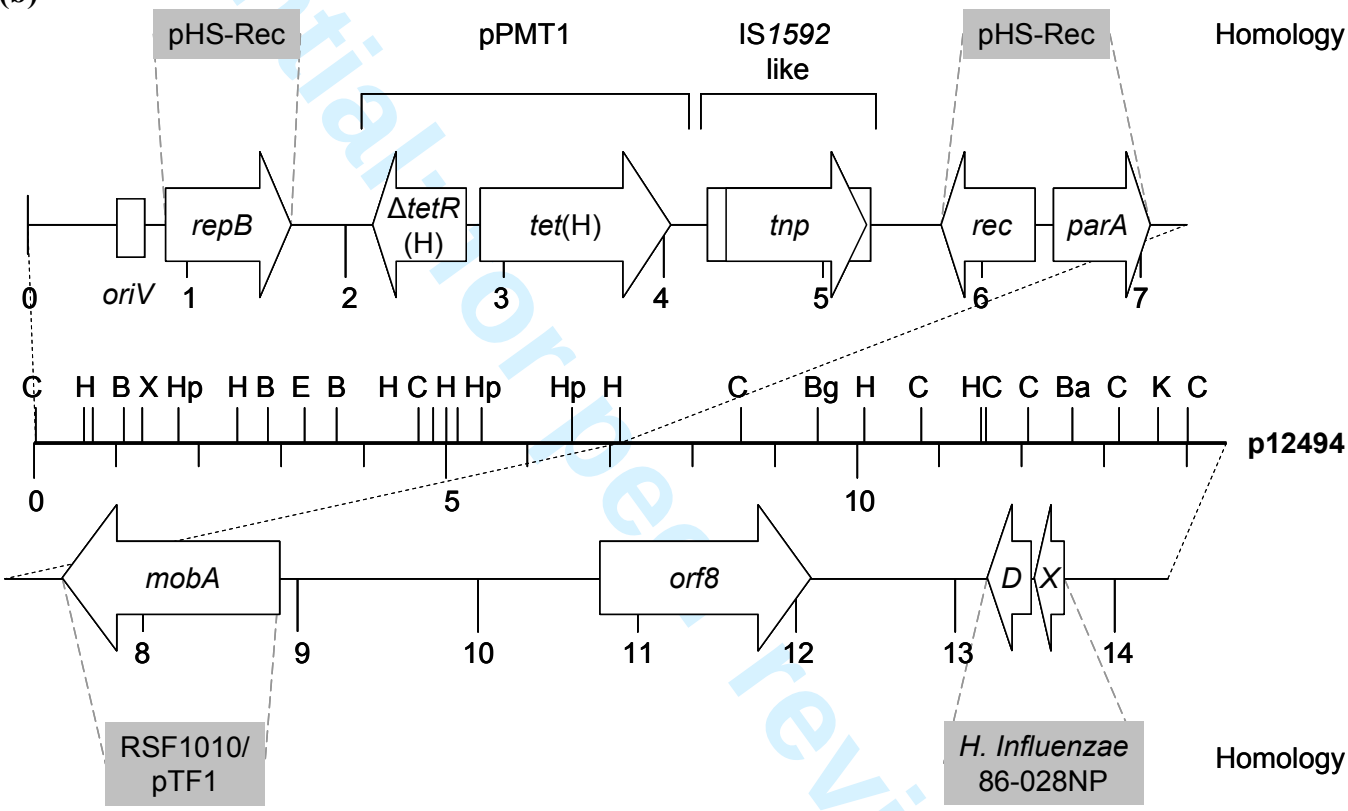
Plasmid	orf	Gene	G+C content (%)	Size (aa)	Identity to proteins in the databases (GenBank accession no.)
p9956	1	*mobC*	45.1	101	100 % MobC of *H.parasuis* plasmid pHS-Tet (AAW51468)
	2	*mobA*	44.2	469	98 % MobA of *H. parasuis* plasmid pHS-Tet (AAW51466)
	3	*mobB*	44.9	160	98 % MobB of *H. parasuis* plasmid pHS-Tet (AAW51467)
	4	*tetR*(H)	39.7	207	100 % TetR(H) of *P. aerogenes* plasmid pPAT1 (CAC08219)
	5	*tet*(H)	41.3	392	100 % Tet(H) of *P. aerogenes* plasmid pPAT1 (CAC08220)
p12494	1	*repB*	31.2	264	85 % RepB of *H. parasuis* plasmid pHS-Rec (AAW51472)
	2	*ΔtetR*(H)	38.8	147	100 % TetR(H) of *P.multocida* plasmid pPMT1 (CAA75662)
	3	*tet*(H)	41.4	400	100 % Tet(H) of *P. multocida* plasmid pPMT1 (CAA75663)
	4	*tnp*	39.7	294	100 % Tnp of *P. trehalosi* plasmid pCCK13698 (CAJ65905)
	5	*rec*	39.6	197	79 % Rec of *H. parasuis* plasmid pHS-Rec (AAW51474)
	6	*parA*	33.8	205	79 % ParA of *P. trehalosi* plasmid pCCK13698 (CAJ65900)
	7	*mobA_L*	36.3	457	44 % MobA of *C. coli* plasmid pCC178 (EAL55997)
	8	*orf8*	30.8	442	
	9	*vapD*	30.4	92	88 % VapD of *H. influenzae* 86-028NP (YP_248162)
	10	*vapX*	34.9	63	86 % VapX of *H. influenzae* 86-028NP (YP_248163)

254 **(a)**



255

256 **(b)**



257

Figure 1 (a) Schematic diagram of plasmid p9956 (accession no. AY362554). The reading frames are presented as arrows with the arrowhead indicating the direction of transcription [*mobA, mobB, mobC:* mobilisation; *tet*(H): tetracycline resistance; *tetR*(H): tetracycline resistance repressor]. The putative transcription terminators for the *tet*(H) and *tetR*(H) genes are indicated downstream of the two genes. **(b)** Schematic diagram of plasmid p12494 (accession no. DQ517426). A distance scale in kb is shown below the restriction map in the middle. The reading frames are presented as arrows in more detail either above or below the map with the arrowhead indicating the direction of transcription (*repA*: plasmid replication; *tnp*: transposition; *mobA*: mobilisation; *rec*: recombination functions; *par*: DNA partition; *orf8*: unknown function; *vapD, vapX:* virulence associated proteins; *tet*(H): tetracycline resistance; *tetR*(H): tetracycline resistance repressor. The white box indicates the limits of the insertion sequence. The Δ symbol indicates a truncated gene. Restriction sites are abbreviated as follows: A (*Ava*I), C (*Cla*I), B (*Bcl*I), Bg (*Bgl*II), Ba (*Bam*HI), D (*Dra*I), E (*Eco*RI), EV (*Eco*RV), H (*Hind*III), Hp (*Hpa*I), K (*Kpn*I), P (*Pst*I) and X (*Xba*I). Gray boxes indicate protein homology. Delimited lines indicate sequence identity.