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

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

Methods: The plasmids designated p9956 and p12494 were purified from A. pleuropneumoniae and completely sequenced. The transfer properties of both plasmids were evaluated by electroporation and/or conjugation into Pasteurella multocida and Escherichia coli.

Results: Both plasmids showed a modular structure consisting of three regions involved in mobilization, tetracycline resistance or replication. The mobilization regions included the mobA gene, encoding a relaxase, a protein involved in plasmid transfer. The tetracycline resistance regions were closely related and consisted of the tet(H) gene and its repressor gene tetR. The tetracycline resistance phenotype was transferred successfully to P. multocida and in the case of p9956 also to E. coli by electroporation of the plasmids. Moreover, plasmid p9956 could be mobilized in E. coli with the assistance of RP4 conjugal transfer functions.

Conclusions: For the first time, the complete sequences of two tet(H)-carrying plasmids from A. pleuropneumoniae were determined. These two plasmids differed from one another and from known tet(H)-carrying plasmids from Pasteurella or Mannheimia spp. Structural analysis confirmed that these plasmids consisted of segments that have been previously detected in members of the families Pasteurellaceae and Enterobacteriaceae.

Keywords: respiratory tract infections, antimicrobial resistance, gene transfer, mobilization, interspecies transfer

Introduction

Tetracyclines are broad-spectrum antibiotics that have been widely used for the treatment and prophylaxis of animal infections, but also as feed additives in pig production.1 Consumption figures suggest that the use of tetracyclines in veterinary practice is still high compared with use of other classes of antibiotics.2,3 Reflecting this situation, a recent study on the antimicrobial susceptibility of Spanish Actinobacillus pleuropneumoniae clinical isolates recovered from 1997 to 2004 revealed a high rate (73.8%) of tetracycline resistance.4 Four resistance determinants, tet(B), tet(L), tet(H) and tet(O), were found in a selected group of A. pleuropneumoniae isolates showing high MICs of tetracyclines.4 In most of them, the tet gene was plasmid-encoded, including the two tet(H)-carrying isolates investigated in the present study.4

The gene tet(H) was first identified in 1993 in an avian Pasteurella multocida isolate.5 Studies on the tet gene...
distribution among members of the family Pasteurellaceae revealed that tet(H) is the dominant tet gene in *P. multocida* and *Mannheimia haemolytica* isolates. However, this gene has also been found in *Acinetobacter* and *Moraxella* spp. The gene tet(H) is part of the small composite transposon Tn5706, which might integrate into the chromosomal DNA as well as plasmids. Previously identified tet(H)-carrying plasmids from *P. multocida* and *M. haemolytica* differed in size and structure and occasionally also carried additional resistance genes. None of them has been sequenced completely.

Since no detailed information is currently available about *A. pleuropneumoniae* plasmids harbouring the gene tet(H), we have completely sequenced the tet(H)-carrying plasmids p9956 and p12494 and analysed them for their transfer properties in the present study.

**Materials and methods**

The *A. pleuropneumoniae* isolates APP9956 and APP12494 were isolated from the lung of diseased pigs. Plasmid preparation, hybridization and transformation into *Escherichia coli* S17.1 and *P. multocida* B130 were performed as described previously. 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. *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'-CCAATGGCATCTAATAG-3'), and sequenced by a primer-walking strategy. The plasmid p12494 was purified from *A. pleuropneumoniae* APP12494 and transformed into electrocompetent *P. multocida* B130. Plasmid DNA from a transformant was prepared and subjected to restriction mapping. Clad and HindIII restriction fragments were subcloned into pBluescript SK+ (Stratagene, La Jolla, CA, 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, MD, 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**

**Nucleotide sequence and genetic organization of plasmid p9956**

Plasmid p9956 consisted of 5674 bp (41.4% G+C content) encompassing five orfs that encode putative proteins showing at least 98% amino acid identity to proteins of known function (Figure 1). 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. The sequence of this region was mainly identical to that of plasmids pPAT1 and pPMT1 from *Pasteurella aerogenes* and *P. multocida*, respectively. As 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 Tn5706 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 at least 98% homology to the corresponding proteins of plasmids pHS-Tet from *Haemophilus parasuis* and p9555 from *A. pleuropneumoniae*. The two regions differed in their G+C content (40% of the resistance region versus 45% of the mobilization region) suggesting a different origin. However, no conserved recombination sequences separating the two regions were detected. The sequence spanning from 4797 to 304 bp was 91% identical to the putative replication region of plasmid pLS88 from *Haemophilus ducreyi* (accession no. L23118).

**Structure and organization of plasmid p12494**

The plasmid p12494 is 14393 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 which were homologous to proteins registered in GenBank (Figure 1). Three different regions associated with replication, resistance or mobilization were found in p12494. The replication region included the origin of replication (oriV), comprising five 22 bp repeats, and a rep gene. The Rep protein was 85% similar to the RepB protein of *H. parasuis* pHS-Rec and possessed several leucine residues at the N-terminus and secondary structure motifs (leucine zipper and a HTH motif) characteristic of type 1 replication proteins.

The resistance module of p12494 comprised the structural gene tet(H) and the repressor gene tetR as in plasmid pPMT1 and in other plasmids found in *Pasteurella* spp. While the gene tet(H) was identical to that of pPMT1, a 100 bp insertion in the tetR gene was detected, which resulted in the loss of 60 amino acids at the C-terminus of the repressor protein. The MICs of tetracycline for isolate APP12494 were 64 mg/L both in the presence and in the absence of 0.1–0.5 mg/L tetracycline, suggesting that the gene tet(H) was constitutively expressed. Immediately downstream of the resistance region, we found a unique copy of an insertion sequence identical to IS1592 from *Pasteurella trellosis* plasmid pCC13698 and similar to IS1596 and IS1597 from the transposon Tn5706. The IS1592-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 IS982 family, which has been described in Gram-positive bacteria and also in some *Pasteurella* spp. Further downstream of the IS element and transcribed in the opposite direction, we located an orf encoding an IS607-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 resolvas/integrase-like protein of *Haemophilus influenzae* (accession no. YP_247803). Serine-recombinases catalyse site-specific recombination of DNA molecules and are functionally versatile, including resolvases, invertases, integrase and transposases. The gene porA 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 (pafm00991) 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.

The *orf5* of p12494 involved in resistance, plasmid replication and transfer 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* (Figure 1). The function of the putative protein (442 amino acids) encoded by *orf8* is unknown. Proteins VapD and VapX are homologous to components of the toxin–antitoxin system of non-typeable *H. influenzae*. 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 pID1 (accession no. NC_001377).

The *orf8* of p12494 involved in resistance, plasmid replication and transfer 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* (Figure 1). The function of the putative protein (442 amino acids) encoded by *orf8* is unknown. Proteins VapD and VapX are homologous to components of the toxin–antitoxin system of non-typeable *H. influenzae*. 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 pID1 (accession no. NC_001377).

**Transfer of the two tet(H)-carrying plasmids from *A. pleuropneumoniae***

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. Although it was stated in a previous publication that plasmid p12494 also replicated in *E. coli*, this statement needs revision as the detailed analysis of the presumably correct transformants identified them as false positive transformants. Repeated transformation and electrotransformation experiments confirmed that plasmid p12494 does not replicate in *E. coli*.

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

**Figure 1.** Comparative diagram of the so far known tet(H)-carrying plasmids pPMT1 and pVM111 from *P. multocida*, pMHT1 from *Mannheimia haemolytica*, pPAT1 from *P. aerogenes* and the two novel plasmids from *A. pleuropneumoniae*, p9956 (accession no. AY362554) and p12494 (accession no. DQ517426), described in this study. Only plasmids p9956 and p12494 have been completely sequenced. The reading frames are presented as arrows with the arrowhead indicating the direction of transcription [mobA, mobB, mobC, mobA_L: mobilization; tet(H): tetracycline resistance; tetR: tetracycline resistance repressor; strA, strB: streptomycin resistance; sul2, sulphonamide resistance; repB: plasmid replication; rec: recombination functions; parA: DNA partition; orf8: unknown function; vapD, vapX: virulence associated proteins]. The delta symbol indicates a truncated gene. The white boxes indicate the limits of the insertion sequences IS1592, IS1596 and IS1597; the arrows within these boxes indicate the reading frames of the corresponding transposase genes. Grey shaded areas indicate the tetR-tet(H) gene region common to all these plasmids despite their differences in structure and organization. A distance scale in kilobase pairs is given below each map.
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.

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### Transparency declarations

None to declare.

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