

ICEPmu1, an integrative conjugative element (ICE) of *Pasteurella multocida*: analysis of the regions that comprise 12 antimicrobial resistance genes

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Background: In recent years, multiresistant *Pasteurella multocida* isolates from bovine respiratory tract infections have been identified. These isolates have exhibited resistance to most classes of antimicrobial agents commonly used in veterinary medicine, the genetic basis of which, however, is largely unknown.

Methods: Genomic DNA of a representative *P. multocida* isolate was subjected to whole genome sequencing. Genes have been predicted by the YACOP program, compared with the SWISSProt/EMBL databases and manually curated using the annotation software ERGO. Susceptibility testing was performed by broth microdilution according to CLSI recommendations.

Results: The analysis of one representative *P. multocida* isolate identified an 82 kb integrative and conjugative element (ICE) integrated into the chromosomal DNA. This ICE, designated ICEPmu1, harboured 11 resistance genes, which confer resistance to streptomycin/spectinomycin (*aadA25*), streptomycin (*strA* and *strB*), gentamicin (*aadB*), kanamycin/neomycin (*aphA1*), tetracycline [*tetR-tet(H)*], chloramphenicol/florfenicol (*floR*), sulphonamides (*sul2*), tilmicosin/clindamycin [*erm(42)*] or tilmicosin/tulathromycin [*msr(E)-mph(E)*]. In addition, a complete *bla*_{OXA-2} gene was detected, which, however, appeared to be functionally inactive in *P. multocida*. These resistance genes were organized in two regions of approximately 15.7 and 9.8 kb. Based on the sequences obtained, it is likely that plasmids, gene cassettes and insertion sequences have played a role in the development of the two resistance gene regions within this ICE.

Conclusions: The observation that 12 resistance genes, organized in two resistance gene regions, represent part of an ICE in *P. multocida* underlines the risk of simultaneous acquisition of multiple resistance genes via a single horizontal gene transfer event.

Keywords: multiresistance, horizontal gene transfer, bovine respiratory disease, plasmids, transposons

Introduction

Bovine respiratory disease (BRD) is an economically important disease. Global losses of the feedlot industry are estimated to be >\$3 billion/year.¹ BRD is a multifactorial and multiagent disease whose colloquial designation, 'shipping fever', refers to some of the factors that play a relevant role in the development of the disease. Transportation over long distances, often associated with exhaustion, starvation, dehydration, chilling or overheating, depending on weather conditions, serves as a stressor. Additional stressors include passage through auction markets, commingling of animals from different herds, dusty environmental conditions in

the feedlot and nutritional stress associated with changes in diet.² Initial viral infections may pave the way for subsequent bacterial infections, in which *Mannheimia haemolytica*, *Pasteurella multocida* and *Histophilus somni* are the most important pathogens.¹ Antimicrobial agents are commonly used to combat bacteria involved in BRD.^{3,4} Analysis of the susceptibility status of bovine *P. multocida* isolates from respiratory tract infections conducted in different European countries during the ARBAO-II study 2003–05 revealed, in general, low percentages of resistance, which proved to be particularly true for newer antimicrobial agents such as ceftiofur, cefquinome, florfenicol and fluoroquinolones.⁵ Corresponding data from the GERM-Vet programme

2004–05 in Germany confirmed the low MIC₉₀ values and the corresponding low percentages of resistant strains—if present at all—not only for the aforementioned antimicrobial agents, but also for the triamylide tulathromycin.⁶ Tulathromycin was approved in 2005 in the European Union and the USA for use in the treatment and control of BRD and the treatment of swine respiratory disease.⁷

Pfizer Animal Health has conducted continuous surveillance of the tulathromycin susceptibility status of the target pathogens. Single tulathromycin-resistant strains have been detected, which mostly showed resistance to numerous other antimicrobial agents commonly used in the treatment of cattle and other animals. To identify the gene(s) responsible for resistance to tulathromycin and the macrolide tilmicosin in the representative multiresistant and plasmid-free isolate *P. multocida* 36950, a whole genome sequencing approach was chosen followed by a matched assembly against the *P. multocida* strain Pm70.⁸ This approach resulted in the identification of the gene *erm*(42), which conferred resistance to macrolides and lincosamides, as well as *msr*(E)-*mph*(E), which mediate resistance to macrolides and triamylides.⁹

The aim of the present study was to: (i) determine the complete genetic basis of the expanded multiresistance phenotype of *P. multocida* 36950; (ii) investigate whether *erm*(42) and *msr*(E)-*mph*(E) are linked to other resistance genes; and (iii) determine whether the resistance genes identified in *P. multocida* 36950 are part of a transferable element.

Materials and methods

Bacterial strain and susceptibility testing

The *P. multocida* strain 36950 was obtained from a case of bovine respiratory tract infection in a Nebraska feedlot in 2005. Antimicrobial susceptibility testing was performed by broth microdilution using custom-made microtitre panels (MCS Diagnostics, Swalmen, The Netherlands). For this, 18 antimicrobial agents and two combinations of antimicrobial agents were tested in 10–12 concentrations in 2-fold dilution series. These comprised penicillins [penicillin G, ampicillin, amoxicillin/clavulanic acid (2:1) and oxacillin], cephalosporins (cefalotin, cefotaxime, cefquinome and ceftiofur), tetracyclines (tetracycline), macrolides (erythromycin, tilmicosin and tulathromycin), lincosamides (clindamycin), folate pathway inhibitors [trimethoprim and sulfamethoxazole/trimethoprim (19:1)], an aminoglycoside (gentamicin), an aminocyclitol (spectinomycin), phenicols (chloramphenicol and florfenicol) and a fluoroquinolone (enrofloxacin). In addition, MICs of streptomycin, kanamycin and neomycin were determined by broth microdilution using a 2-fold dilution series of 0.5–32 mg/L (streptomycin) or 0.5–16 mg/L (kanamycin and neomycin). Performance of the tests and the evaluation of MIC values followed the recommendations given in document M31-A3 of the CLSI.¹⁰ The reference strains *Staphylococcus aureus* ATCC 29213 and *Escherichia coli* ATCC 25922 served as quality control strains.

Whole genome sequence analysis and analysis of resistance genes and mutations

Genomic DNA was prepared as previously described.¹¹ For whole genome sequencing, a shotgun library and a long-tag paired end library were generated using the 454 Life Sciences (Roche) GS-FLX system (Eurofins MWG, Ebersberg, Germany). PCR assays were performed and the amplicons sequenced to close the gaps between the different contigs. Genes were predicted by the YACOP¹² and the ORF Finder programs ([\[ncbi.nlm.nih.gov/projects/gorf/\]\(http://ncbi.nlm.nih.gov/projects/gorf/\)\). The predicted coding sequences \(CDSs\) were compared with the SWISSProt/EMBL databases and manually curated using the annotation software ERGO.¹³ For detailed sequence comparisons the BlastN and BlastP programs \(<http://blast.ncbi.nlm.nih.gov/>\) were also used. The GC content of ICEPmu1 was determined using the Artemis genome browser and annotation tool.¹⁴](http://www.</p>
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The whole genome sequence of *P. multocida* 36950 has been deposited in GenBank under accession number CP003022.

Results and discussion

P. multocida 36950 exhibited resistance to most antimicrobial agents approved for the control of bovine respiratory diseases. This included resistance to older antimicrobial agents such as tetracyclines (32 mg/L), chloramphenicol (16 mg/L), sulphonamides (≥ 512 mg/L) and spectinomycin (≥ 512 mg/L), but also to newer agents such as enrofloxacin (2 mg/L), florfenicol (8 mg/L), tilmicosin (≥ 128 mg/L) and tulathromycin (≥ 128 mg/L). Moreover, high MICs of the aminoglycosides streptomycin (≥ 64 mg/L), gentamicin (128 mg/L), kanamycin and neomycin (≥ 32 mg/L each) were detected. The resistance genes found in *P. multocida* 36950 were located in resistance gene regions 1 and 2, which were located 42526 bp apart from each other. Comparisons of the whole genome sequence of *P. multocida* 36950 with the genome sequence of *P. multocida* Pm70 identified an 82 kb element that was present in *P. multocida* 36950, but absent in *P. multocida* Pm70. This element included the resistance gene region 1 at its left terminus and the resistance gene region 2 close to its right terminus. A schematic presentation of the ICE of *P. multocida* 36950, designated ICEPmu1, is shown in Figure 1(a), whereas detailed presentations of the resistance gene regions 1 and 2, including their relationships to sequences of previously described plasmids, transposons, insertion elements and genomic islands, are shown in Figure 1(b and c). ICEPmu1 showed a G+C content (41.9%) different from that of the genome of its host (40.4%). The G+C content graphic clearly shows that the higher G+C content of ICEPmu1 resulted from the higher G+C content of the sequences present in the two resistance gene regions (Figure 1a). While this study focused on the identification and the linkage of the resistance genes, the accompanying study concentrated on the characterization of ICEPmu1, its mobility across genus boundaries and its integration in different hosts.¹⁵ Data for the expression of ICEPmu1-associated resistance genes in bacteria of the same and other species and genera (e.g. *M. haemolytica* and *E. coli*) after conjugative transfer of ICEPmu1 are presented in the accompanying study. These data suggest that certain resistance genes are either functionally inactive (e.g. *bla*_{OXA-2}) or associated with distinctly lower MIC values (e.g. *floR*) for Pasteurellaceae as compared with *E. coli*.¹⁵

Resistance gene region 1

Resistance gene region 1 is 15711 bp in size and is bracketed by copies of the insertion element ISAp1 originally identified in the chromosomal DNA of the porcine respiratory tract pathogen *Actinobacillus pleuropneumoniae*.¹⁶ This insertion sequence is 1070 bp in size and exhibits 27 bp imperfect inverted repeated sequences at its boundaries as well as a single reading frame for a transposase of 307 amino acids. ISAp1 belongs to the IS30 family and has been reported to produce a 2 bp direct duplication

at its integration site.¹⁶ Upon inspection of the sequences immediately up- and downstream of each of the two copies of IS*Apl1*, the repeated sequence GT was detected upstream of the right-hand copy and downstream of the left-hand copy of IS*Apl1*. This might suggest that the entire resistance gene region 1 was inserted via an IS*Apl1*-mediated integration or recombination process. It should be noted that the left-hand IS*Apl1* is complete, whereas a single bp exchange generated an early stop codon after codon 104 in the *tnp* sequence of the right-hand IS*Apl1*. Almost in the middle of resistance gene region 1, a novel ISCR element designated ISCR21 was detected. ISCR21 is 1751 bp in size and has a single reading frame for a 430 amino acid transposase, which is next related (83.5% identity and 89.1% homology) to the recently described transposase of ISCR20 from *E. coli*.¹⁷

Upstream of ISCR21, the four resistance genes *sul2*, *strA*, *strB* and *aphA1*, all oriented in the same direction, were identified. The gene *sul2* codes for a dihydropteroate synthase of 281 amino acids that confers sulphonamide resistance. It should be noted that the start codon and the adjacent 10 codons in the 5' terminus of the gene differed completely from the sequences of any other known *sul2* gene. The amino acid sequence deduced from codons 12–281 was indistinguishable from that of the 271 amino acid Sul2 proteins commonly found among Pasteurellaceae and other organisms.³ A 168 bp spacer separated the *sul2* gene from the *strA* gene. An identical spacer sequence was seen in plasmid pB1003 from *P. multocida*,¹⁸ pPASS1 from *Pasteurella aerogenes*¹⁹ and pMS260 from *A. pleuropneumoniae*.²⁰ The gene *strA* codes for a 267 amino acid aminoglycoside 3''-phosphotransferase. The terminal 'A' in the translational stop codon of the *strA* gene represented the initial 'A' in the start codon of the gene *strB* (–TGATG–). The gene *strB* codes for a 278 amino acid aminoglycoside 6-phosphotransferase. Both genes are involved in streptomycin resistance. The deduced StrA and StrB amino acid sequences were indistinguishable from those found in a wide variety of bacteria. However, it is noteworthy that complete *strB* genes have been detected rarely in *P. multocida*,^{21,22} whereas truncated *strB* genes are commonly seen.^{3,4,18,19} Another 335 bp downstream of *strB*, a third aminoglycoside resistance gene, *aphA1* [also known as *aph(3'')-I*], was detected. This gene codes for a different type of aminoglycoside 3'-phosphotransferase, which confers resistance to kanamycin and neomycin. The 271 amino acid AphA1 protein showed 99.6%–100% identity to the corresponding proteins of *Avibacterium paragallinarum* and *A. pleuropneumoniae*.^{23,24} The *sul2-strA-strB-aphA1* segment showed 99.8% nucleotide sequence identity to the corresponding sequence of the IncQ-like plasmid pIE1130 from an uncultured eubacterium (accession number AJ271879) (Figure 1b).

Downstream of ISCR21, the terminal 257 bp of an ISCR2-associated transposase gene as well as the adjacent 234 bp of the ISCR2 element were detected. Downstream of this ISCR2 relic, the gene *floR* for a 404 amino acid phenicol-specific exporter protein of the major facilitator superfamily (MFS) was located. The FloR protein differed by 1–4 amino acids from the FloR proteins previously described, including those found in *P. multocida*,^{21,25} *Bibersteinia* (formerly *Pasteurella*) *trehalosi*²⁶ and *Vibrio cholerae*.²⁷ The *floR* gene was followed by a gene for a 101 amino acid LysR transcriptional regulator protein whose reading frame overlapped by 6 bp with the sequence of a complete

ISCR2 element of 1845 bp. Another 185 bp downstream of ISCR2, the rRNA methylase gene *erm(42)* was detected. This gene has recently been shown to confer resistance to 14- and 16-membered macrolides, such as erythromycin and tilmicosin, as well as to lincosamides.⁹ Database searches revealed that the 301 amino acid Erm(42) protein is only distantly related (<30% identity) to other Erm proteins, but is indistinguishable from the 301 amino acid protein Erm(42) of *P. multocida* and *M. haemolytica*²⁸ and shows 99.3% identity to an erythromycin resistance protein of 303 amino acids from plasmid pPDP9106b (accession number AB601890) of a fish-pathogenic *Photobacterium damsela* subsp. *piscicida* strain (formerly known as *Pasteurella piscicida*). The entire *floR-lysR-ISCR2-erm(42)* region showed 96.2% sequence identity to that of plasmid pPDP9106b (Figure 1b). Moreover, the *sul2-strA-strB* segment as well as the Δ ISCR2-*floR-lysR-ISCR2* segment were present in the SXT element of *V. cholerae*,²⁷ even if in different orientations and not interrupted by an ISCR21 element (Figure 1b).

Resistance gene region 2

Resistance gene region 2 is 9789 bp in size and comprises a total of six different resistance genes as well as two regulatory genes and two complete insertion sequences (Figure 1c). The left-hand part is characterized by a largely truncated transposon Tn5706,²⁹ of which only the repressor gene *tetR*, including 95 bp of the downstream region and 133 bp of the upstream region, remained. The 133 bp, however, included the spacer region between *tetR* and the tetracycline resistance gene *tet(H)*, with the promoters required for *tetR* and *tet(H)* transcription as well as the 5' end of the *tet(H)* reading frame. Detailed analysis revealed that a recombination between the initial part of the *tet(H)* gene and the *att1* site of a class 1 integron occurred (Figure 2a). As a consequence, the three resistance gene cassettes present in this class 1 integron also became integrated into the chromosomal DNA of *P. multocida* 36950. The first gene cassette is 591 bp in size, has a 59-base element of 60 bp and contains an *aadB* gene encoding a 177 amino acid aminoglycoside 2''-O-adenyltransferase, which confers gentamicin resistance (Figure 2b). The AadB protein was indistinguishable from a wide variety of AadB proteins from Gram-negative bacteria deposited in the databases. However, to the best of our knowledge, this is the first report of a gentamicin resistance gene in *P. multocida*. The second gene cassette is 856 bp in size, also has a 59-base element of 60 bp and harbours a novel *aadA* gene variant, designated *aadA25*, for combined resistance to streptomycin and spectinomycin (Figure 2b). The deduced sequence of the 259 amino acid AadA25 protein differed by five amino acid exchanges from the next related variants AadA21 or AadA3c.^{30,31} The third gene cassette is 876 bp in size, has a 59-base element of 70 bp and contains the gene *bla_{OXA-2}*, which codes for a narrow-spectrum β -lactamase of 275 amino acids (Figure 2b). While database searches identified *bla_{OXA-2}* genes indistinguishable from that of *P. multocida* 36950 mainly in Enterobacteriaceae and *Pseudomonas aeruginosa*, this gene has not been seen before in *P. multocida*. However, it has been described as part of a plasmid-borne gene cassette in the porcine respiratory tract pathogen *Bordetella bronchiseptica*.³² Although sequence analysis does not give a hint towards functional inactivity, this *bla_{OXA-2}* gene obviously does not confer resistance to β -lactam antibiotics in *P. multocida* 36950. The corresponding

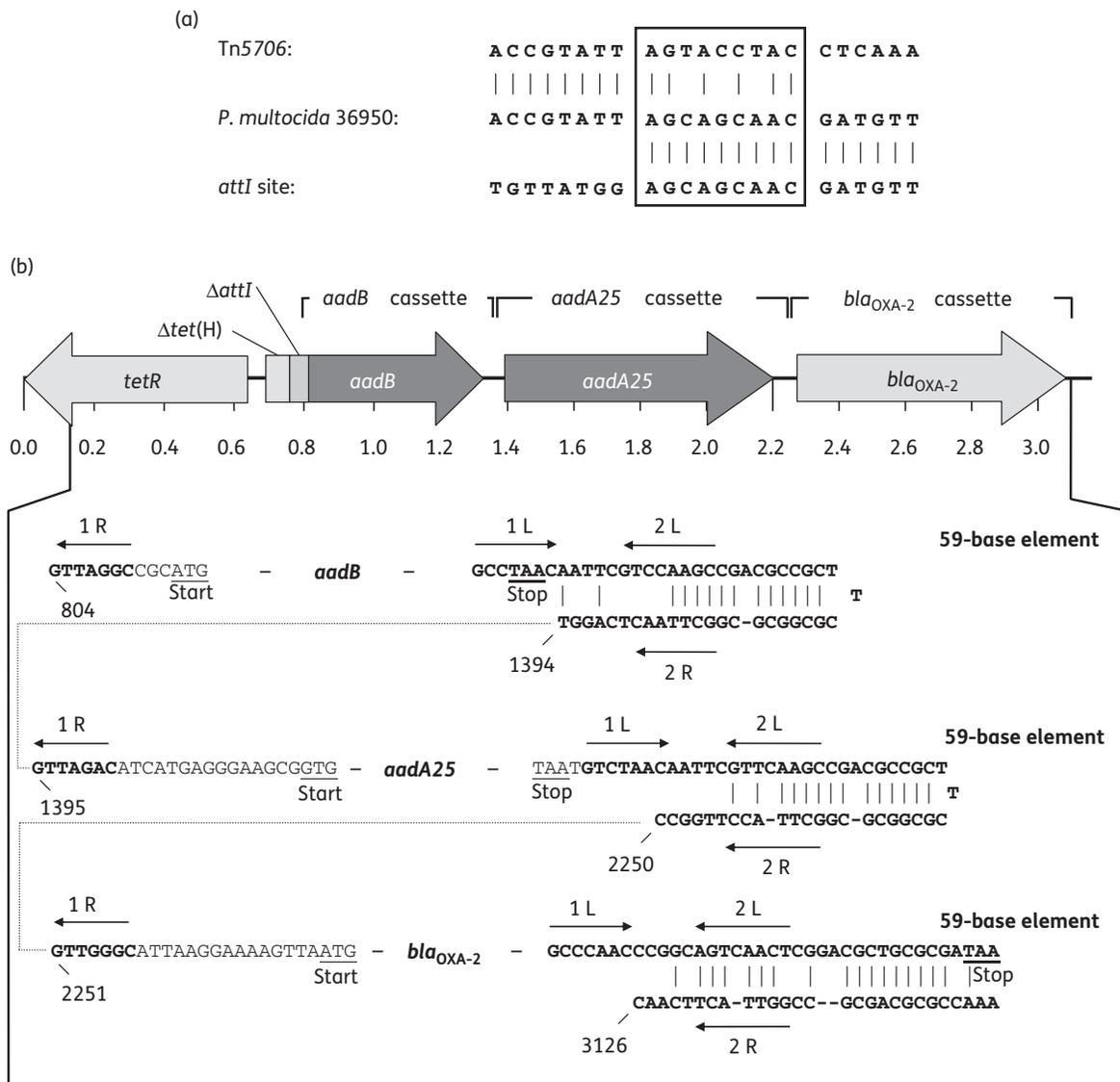


Figure 2. (a) Comparison of the sequence detected in *P. multocida* 36950 at the junction of $\Delta tet(H)$ and $\Delta attI$ with the corresponding sequences of the *tet(H)*-carrying transposon Tn5706 and the *attI* site of a class 1 integron. Vertical bars indicate identical bases. The 9 bp area, in which the crossover is believed to have taken place, is boxed. (b) The 3150 bp segment that contains the *tetR* gene and the three gene cassettes identified in *P. multocida* 36950. The beginning and the end of the gene cassettes are shown in detail. The translational start (GTG and ATG) and stop (TAA) codons are underlined. The 59-base elements shown in bold type are depicted as stem-loop structures and the integrase 1 binding domains 1L, 2L, 2R and 1R are indicated by arrows. The dotted line indicates the connection between the three gene cassettes. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

MICs of penicillin G, ampicillin, amoxicillin/clavulanic acid (2:1) and oxacillin were 0.25, 0.25, 0.25/0.12 and 4 mg/L, respectively. Transfer experiments as conducted in the accompanying study confirmed these observations for *P. multocida* and *M. haemolytica* hosts, but also showed that the *bla_{OXA-2}* gene was functionally active in *E. coli*.¹⁵

Immediately downstream of the 59-base element of the *bla_{OXA-2}* gene cassette, a 4386 bp segment was found that consisted of the genes *msr(E)*-*mph(E)* bracketed by two IS26 elements located in the same orientation (Figure 1c). Insertion sequences of the type IS26 are widespread among Enterobacteriaceae, but have rarely been seen in Pasteurellaceae.²⁶ IS26 is 859 bp in size,

exhibits 14 bp terminal perfect inverted repeats and produces 8 bp direct repeats at its integration site.³³ The *msr(E)* gene codes for an ABC transporter protein of 491 amino acids while the *mph(E)* gene codes for a macrolide phosphotransferase protein of 294 amino acids. These two genes are organized in an operon-like structure and are separated by a non-coding spacer sequence of 55 bp. A recent study confirmed that the genes *msr(E)*-*mph(E)* confer resistance not only to 14- and 16-membered macrolides, but also to the triamidine tulathromycin.⁹ Database searches identified these genes on plasmids in *Klebsiella pneumoniae* and other Enterobacteriaceae³⁴⁻³⁶ as well as in *Acinetobacter baumannii*,^{37,38} where they have been

referred to as *mel* or *mef(E)* and *mph* or *mph2*. No direct repeats were detectable, neither up- and downstream of each of the two IS26 copies, nor upstream of the left IS26 copy and downstream of the right IS26 copy.

The sixth resistance gene in region 2, the tetracycline resistance gene *tet(H)* accompanied by its repressor gene *tetR*, was located in another truncated Tn5706 element, which was found 106 bp downstream of the right-hand IS26. Both terminal insertion sequences IS1596 and IS1597 present in the composite transposon Tn5706²⁹ were absent. The Tn5706-homologous sequence in the part downstream of *tetR* stopped exactly at the position where otherwise the IS1596 sequence was found. In the part downstream of *tet(H)*, the Tn5706-homologous sequence stopped 65 bp after the translational stop codon of *tet(H)*. Immediately thereafter, perfect nucleotide sequence identity to the whole genome sequence of *Mannheimia succiniciproducens* MBEL55E was observed (Figure 1c). The *tet(H)* gene found in *P. multocida* 36950 codes for a 400 amino acid tetracycline efflux protein of the major facilitator family. It differed by a single homologous amino acid exchange, N258H, from the Tet(H) protein of Tn5706.

In summary, the two resistance gene regions contained a total of 12 different resistance genes, some of which—e.g. *tet(H)*, *sul2*, *strA* and *strB*—have previously been reported to be present mostly on plasmids in *P. multocida* or other members of the family Pasteurellaceae.^{3,4} Other resistance genes—e.g. *florR* and *aphA1*—have rarely been seen in *P. multocida* or other bovine respiratory tract pathogens. The third group of resistance genes—e.g. *erm(42)*, *msr(E)* and *mph(E)*, as well as the cassette-borne genes *aadB*, *aadA25* and *bla_{OXA-2}*—are novel genes in *P. multocida*. The structural comparisons as shown in Figure 1(b and c) strongly suggest that both resistance gene regions have developed as a result of integration and recombination processes in which insertion sequences and ISCR elements seem to have played a key role. Moreover, analysis of the two resistance gene regions clearly showed that *P. multocida* is able to: (i) acquire resistance genes from other Gram-negative bacteria; (ii) incorporate them into its chromosomal DNA; and (iii) use these genes to gain resistance to the respective antimicrobial agents.

Resistance-mediating mutations in *P. multocida* 36950

Besides the resistance genes identified in resistance gene regions 1 and 2 of ICEPmu1, *P. multocida* 36950 exhibited resistance to antimicrobial agents such as spectinomycin and enrofloxacin, for which resistance is often based on mutations in specific target genes.

In addition to enzymatic inactivation via AadA proteins, high-level resistance to spectinomycin in *P. multocida* can also be due to mutations in a specific region of the 16S rRNA and/or in the gene *rpsE* coding for the ribosomal protein S5.³⁹ Comparisons of the 16S rRNA sequence of all six operons and analysis of the *rpsE* sequence did not reveal changes that point towards an involvement of mutations in these targets in the observed spectinomycin resistance.

Fluoroquinolone resistance in *P. multocida* and other bovine respiratory tract pathogens has very rarely—if at all—been observed. As in many other bacteria, quinolone/fluoroquinolone resistance is most likely due to mutations in the genes *gyrA* and *parC* encoding DNA gyrase and topoisomerase IV.⁴⁰ Analysis of

the quinolone resistance-determining regions (QRDRs) within the genes *gyrA* and *parC* identified in *P. multocida* 36950 showed two bp exchanges in the QRDR of *gyrA*, which resulted in amino acid alterations; GGT→AGT (Gly75 to Ser75) and AGC→AGA (Ser83 to Arg83). In addition, a single bp exchange in the QRDR of *parC*, TCA→TTA, which resulted in a Ser80-to-Leu80 exchange, was also seen in *P. multocida* 36950. While single amino acid exchanges within the QRDR of GyrA are usually only associated with resistance to the quinolone nalidixic acid, two or more amino acid exchanges in the QRDRs of GyrA and ParC accompany resistance to fluoroquinolones such as enrofloxacin. While alterations at codon 75 in *gyrA* have rarely been detected,⁴¹ alterations at codon 83 in *gyrA* and at codon 80 in *parC* have frequently been described in connection with fluoroquinolone resistance in other bacteria.^{42–44} In *P. multocida*, only a single *gyrA* mutation, AGC→ATC, which results in a Ser83-to-Ile83 exchange, has been associated with high-level resistance to nalidixic acid (MIC >256 mg/L), but susceptibility to ciprofloxacin (MIC 0.12 mg/L).⁴⁰ The mutations detected in *gyrA* and *parC* of *P. multocida* 36950 are, to the best of our knowledge, the first examples of fluoroquinolone resistance-mediating mutations in *P. multocida*.

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Transparency declarations

M. T. S., R. W. M. and J. L. W. are employees of Pfizer Animal Health and own Pfizer stocks. Other authors: none to declare.

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