ICEPmu1, an integrative conjugative element (ICE) of Pasteurella multocida: structure and transfer

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Background: Integrative and conjugative elements (ICEs) have not been detected in Pasteurella multocida. In this study the multiresistance ICEPmu1 from bovine P. multocida was analysed for its core genes and its ability to conjugatively transfer into strains of the same and different genera.

Methods: ICEPmu1 was identified during whole genome sequencing. Coding sequences were predicted by bioinformatic tools and manually curated using the annotation software ERGO. Conjugation into P. multocida, Mannheimia haemolytica and Escherichia coli recipients was performed by mating assays. The presence of ICEPmu1 and its circular intermediate in the recipient strains was confirmed by PCR and sequence analysis. Integration sites were sequenced. Susceptibility testing of the ICEPmu1-carrying recipients was conducted by broth microdilution.

Results: The 82214 bp ICEPmu1 harbours 88 genes. The core genes of ICEPmu1, which are involved in excision/integration and conjugative transfer, resemble those found in a 66641 bp ICE from Histophilus somni. ICEPmu1 integrates into a tRNALeu and is flanked by 13 bp direct repeats. It is able to conjugatively transfer to P. multocida, M. haemolytica and E. coli, where it also uses a tRNALeu for integration and produces closely related 13 bp direct repeats. PCR assays and susceptibility testing confirmed the presence and the functional activity of the ICEPmu1-associated resistance genes in the recipient strains.

Conclusions: The observation that the multiresistance ICEPmu1 is present in a bovine P. multocida and can easily spread across strain and genus boundaries underlines the risk of a rapid dissemination of multiple resistance genes, which will distinctly decrease the therapeutic options.

Keywords: horizontal gene transfer, bovine respiratory disease, insertion site, integrases, recombinases

Introduction

Integrative and conjugative elements (ICEs) represent mobile genetic elements that are able to (i) mediate their excision from a host genome by site-specific recombination, (ii) form a circular intermediate and transfer themselves as this circular intermediate by conjugation and (iii) insert into a new host genome. Conjugative transposons differ from ICEs in two major aspects; conjugative transposons are able to transpose within the host cell and often show a low integration specificity. As a consequence, they may be found integrated into various genomic sites. In contrast, many ICEs have been described to be site-specifically inserted into tRNAs and are not able to change their location within the same host cell. Observations have been made that suggest that the integration specificity of ICEs may rely on the different exchanged or acquired transfer modules rather than on the different integrases harboured by the respective ICEs.

Like prophages, ICEs have been described to evolve by exchange of functional modules and display a more conserved structure than genomic islands. ICEs are composed of core genes and accessory genes (or cargo genes). While the core genes are more important for the spreading and maintenance of the ICEs, the accessory genes, which include genes for antibiotic, heavy metal or phage resistance, metabolic activities (sucrose, biphenyl and chlorocatechol degradation) or bacteriocin synthesis, are relevant for the fitness of the host and its survival under specific conditions. With the increasing number of well-characterized ICEs, it has been possible to identify ICE families in which synteny, extensive homology and shared...
modular evolution are seen.\textsuperscript{6} ICEs have been considered as one of the driving forces involved in bacterial evolution.\textsuperscript{5,6}

The aim of this study was to analyse a multiresistance ICE, designated ICEPmu1, detected in the plasmid-free \textit{P. multocida} strain 36950, which originated from a case of bovine respiratory tract infection, for its structure and transfer abilities. The accompanying study focused on the resistance genes associated with ICEPmu1 and the resistance-mediating mutations present in the ICEPmu1-carrying \textit{P. multocida} strain 36950.\textsuperscript{7}

Materials and methods

Sequence analysis and comparative genomics

The whole genome sequencing, gap closure, prediction of genes and sequence comparisons were performed as described previously.\textsuperscript{1} The sequence of the whole genome of \textit{P. multocida} 36950 was deposited in GenBank under accession number CP003022. The comparison between the ICES was done with the WebACT program.\textsuperscript{5} The genome of strain 36950 was compared with the most similar whole genome sequences of members of the family Pasteurellaceae using the BiBaG software tool for reciprocal BLAST analysis and a global sequence alignment using the Needleman–Wunsch algorithm.\textsuperscript{7} Circular plots of DNA sequences were generated with the program DNAPlotter.\textsuperscript{10} The following genomes were used for comparison: \textit{P. multocida} Pm70 (GenBank accession number AE004439.1), \textit{Haemophilus influenzae} R2866 (CP002277), \textit{H. influenzae} Rd KW20 (L42023.1), \textit{H. influenzae} PtTEE (CP000671.1), \textit{H. influenzae} PtTG (CP000672.1), \textit{H. influenzae} R2846 (CP002276), \textit{Haemophilus ducreyi} 35000HP (AE017143.1), \textit{Haemophilus parasuis} SH0165 (CP001321.1), \textit{H. somnii} somni 129PT (CP000436.1), \textit{H. somnii} 2336 (CP000947.1), \textit{Mannheimia succiniproducens} MBEL55E (AE016827.1), \textit{Actinobacillus succinogenes} 1302 (CP000746.1) and \textit{Actinobacillus pleuropneumoniae} JLO3 (CP000687.1).

Conjugation, susceptibility testing and PCR assays

The plasmid-free \textit{P. multocida} strain 36950 (capsular type A) served as donor and the rifampicin-resistant \textit{Escherichia coli} HK225\textsuperscript{11} or the rifampicin-resistant \textit{P. multocida} E348-08 (capsular type F) and \textit{Mannheimia haemolytica} 39229 were used as recipient cells. The latter two strains were from the strain collection of the Institute of Farm Animal Genetics (FL), and spontaneous rifampicin-resistant mutants of both strains were generated by selection on agar blood plates supplemented with increasing rifampicin concentrations (1–150 mg/L). For the mating assay, overnight cultures of the donor and recipient cells grown in brain heart infusion (BHI) broth were mixed in a ratio of 1:6 or 1:1. Cell mixtures were centrifuged for 15 min at 15000 × g, resuspended in 30 mL of BHI broth, and spotted on sheep blood agar plates, except in the assay with \textit{E. coli} recipient cells, which was performed on Luria–Bertani agar plates. Mating plates were incubated for 6 h at 37°C. Cells were collected in 1 mL of 0.85% NaCl, and serial dilutions were plated onto the appropriate selective media and incubated at 37°C for at least 48 h. The transfer frequency was calculated as the number of transconjugants per donor cell. The phenotypic resistance patterns of the transconjugants were determined by susceptibility testing via broth microdilution according to document M31-A3 of the CLSI.\textsuperscript{12} When the \textit{P. multocida} recipient was used, at least one transconjugant from each mating assay was investigated by PCR assays with a \textit{P. multocida}-specific PCR and a multiplex PCR for the capsular type.\textsuperscript{13,14} Moreover, transconjugants obtained with all recipient strains were checked for the presence of the ICEPmu1-associated resistance genes\textsuperscript{15–21} as well as the relaxase (Pmu_02890) gene (Table 1). In these PCR assays, the original recipient strains served as negative controls.

Extrachromosomal DNA extraction, PCR assays, hybridization experiments, inverse PCR and sequencing

To detect the extrachromosomal circular form of the ICE by agarose gel electrophoresis, DNA was extracted as previously described.\textsuperscript{12,13} For PCR detection of this circular form either the primer set P156 or a nested PCR (P159 followed by P156) was used (Table 1). For this, 2 μL of genomic DNA extracted by thermal lysis (single colonies resuspended in 200 μL of water) were heated for 5 min at 95°C and centrifuged for 10 min at 15000 × g, 1 U of Taq DNA polymerase (Qiagen, Hilden, Germany) in 1× buffer with 1.5 mM MgCl\textsubscript{2}, 120 μM of dNTPs and 0.4 μM of each primer were mixed in a final volume of 50 μL. The PCR conditions used consisted of an initial denaturation for 2 min at 94°C, 30 cycles of 10 s at 94°C, 20 s at the appropriate annealing temperature and 1 min/kb at 72°C, followed by a final extension of 5 min at 72°C. The amplicons obtained were sequenced.

To confirm the circular form of the ICE detected by agarose gel electrophoresis and to determine the copy number of the ICE in the donor strain \textit{P. multocida} 36950 and in the transconjugants, Southern blot hybridization experiments were performed in which ICE-specific PCR products were labelled enzymatically (Dig-High prime DNA labelling and detection system; Roche, Mannheim, Germany) and used as probes. For the detection of the extrachromosomal circular form, the amplicon of the ICE-specific relaxase gene served as the probe. For the determination of the ICE copy number, genomic DNA of \textit{P. multocida} 36950, transconjugants and recipient cells (used as negative controls) was prepared\textsuperscript{12,14} and digested with HindIII (Fermentas, St. Leon-Rot, Germany). Two hybridization experiments were conducted in which labelled PCR products of genes located at the left (Pmu_02680–PCR P157; Table 1) and the right (Pmu_03610–PCR P158; Table 1) terminus of the ICE were used as probes. Standard PCRs, one for each terminus of the ICE, were performed to determine the integration point of the ICE in the \textit{P. multocida} E348-08 (PCRs P160 and P161; Table 1) and \textit{E. coli} HK225 (PCRs P162 and P163; Table 1) transconjugants, followed by sequence analysis of the amplicons. Outward primers were designed from sequences at the left or right terminus of the ICE (Table 1) and were combined with inward primers designed from sequences at the left or right flanking region of the trNA\textsuperscript{18} (Table 1), which contained the sequence for the site-specific recombination of the ICE (5’-GATTTTGAACT-3’). For the \textit{E. coli} and \textit{P. multocida} transconjugants the sequences of \textit{E. coli} DH1 (accession number AP012030.1) and \textit{P. multocida} 36950 (accession number CP003022), respectively, were used for the primer design. For the \textit{M. haemolytica} transconjugant, two inverse PCRs—one for each terminus—were performed. The genomic DNA of this transconjugant was digested with HindIII for 4 h, self-ligated by overnight incubation at 14°C in the presence of a T4 DNA ligase (Fermentas) and used as template in the inverse PCR assays with outward primers (PCRs—left terminus P164 and right terminus P165; Table 1). The amplified fragments were gel-purified using the QIAquick PCR purification kit (Qiagen) and sequenced.

Results and discussion

Designation and structure of ICEPmu1

Analysis of the \textit{P. multocida} 36950 genome sequence revealed the presence of an ICE. The results of reciprocal BLAST analysis and global sequence alignment of the genome of \textit{P. multocida} 36950 with other similar genomes of members of the family Pasteurellaceae are shown in Figure 1. This ICE, designated ICEPmu1\textsuperscript{i}, is located exactly in the region that revealed no striking homology to most of the genomes used for comparison. The designation ICEPmu1\textsuperscript{i} was based mainly on the nomenclature proposal by Burrus et al.\textsuperscript{2} This proposal suggested using the initials of the name of the bacterium from which it was isolated and
ICE_Pmu1 is 52214 bp in size and was found to be integrated into the second of six genomic copies of a tRNA Leu, which was located on the lagging strand. As a result of the integration, it is flanked by 13 bp direct repeats (5′-GATTTTGAATCAA-3′). In contrast to reports, in which the disrupted tRNA gene is restored upon integration, only the fragments of the tRNA Leu were seen. However, a copy of an integral tRNA Leu (Pmu_03620) proved to be part of the ICE_Pmu1 and was located close to the right terminus (Figure 2). The definition of the left and right termini was based on the orientation of the ICE_Pmu1 in the genome of P. multocida 36950.

Within ICE_Pmu1, a total of 88 open reading frames were identified, among which a function was predicted by sequence comparisons or—in the case of the resistance genes—confirmed phenotypically for 56 of them. Analysis of the coding sequences revealed the presence of the essential genes for the functionality of an ICE, like the genes involved in excision/integration and conjugative transfer (Figure 2). Two genes coding for phase integrases were identified close to the left attachment site (attL). The first integrase gene (Pmu_02700) was located 2319 bp and the second (Pmu_02880) 19284 bp from the left terminus. Identities of 44% or 49% were seen when the amino acid sequences of the whole protein or only the catalytic C-terminal region of these two integrases were aligned. Both integrase proteins harboured in the C-terminus the three strongly conserved residues, the arginine residues in BOX A and BOX B (major clusters of similarity) and the active site tyrosine residue in BOX C. Additional experiments are necessary to show which of them or if both are necessary for the excision/integration of ICE_Pmu1. Structural comparisons suggested that these enzymes are tyrosine recombinases of the Xer family, which mediate integration by site-specific recombination. Since 7 bp (5′-TTTTGAA-3′) from the sequence of the recircularization point correspond to the sequence of the anticodon loop of the disrupted tRNA Leu, the responsible integrase may use this sequence for integration of the ICE.

A comparison between ICE_Pmu1 and the next closely related ICE, a 66641 bp ICE from H. somni strain 2336 (formerly Haemophilus somnus; GenBank accession number NC_010519.1), revealed that 66 of the 88 genes found in ICE_Pmu1 are also present in the ICE from H. somni. However, the ICE from H. somni lacks the second integrase gene and most of the two ICE_Pmu1-associated resistance gene regions (Figure 3). Of the ICE_Pmu1-associated resistance gene region 1, only one copy of the insertion sequence IS_Apl1 was seen in the ICE of H. somni; of the resistance gene region 2, only one copy of the tetracycline repressor gene tetR (HSM_1734) and the tetracycline resistance gene tet(H) (HSM_1735) were seen in the ICE of H. somni. However, the ICE of H. somni carried a number of genes coding for hypothetical proteins, transcriptional regulators and membrane proteins (HSM_1729–HSM_1733 and HSM_1736–HSM_1744) in its accessory gene region. These proteins may be involved in resistance to copper, heavy metals and other toxic elements.

**Table 1.** PCR primers used for the characterization of ICE_Pmu1

<table>
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<tr>
<th>PCR assay</th>
<th>name</th>
<th>sequence (5′ to 3′)</th>
<th>Amplicon (bp)</th>
<th>Annealing temperature (°C)</th>
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<tr>
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<td>57</td>
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<td></td>
<td>ICE-relaxase-rv</td>
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<tr>
<td>P156</td>
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<td>59</td>
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<tr>
<td>P157</td>
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<td>1783</td>
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<td>2334</td>
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<tr>
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</table>

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compounds. Similarities among the ICE from *H. somni* and ICEs found in other families, such as Burkholderiaceae, Enterobacteriaceae and Pseudomonadaceae, were described by Mohd-Zain et al. These authors identified 33 core genes, 15 of which were present in all of these ICEs, and two regions of accessory genes. These accessory gene regions were located close to the termini of the ICEs, as observed in ICE *Pmu1*. A comparison of the core genes of ICE *Pmu1* with those of other ICEs among members of the family Pasteurellaceae revealed the highest identities with the ICEs of *H. somni* 2336 (80.3%–100% identity), followed by *H. somni* 129PT (41.2%–90.0%), *H. influenzae* R2866 and *H. influenzae* 86-028NP (both with the same identities, 24.2%–82.6%) and *H. ducreyi* 35000HP (4.9%–78.1%). However, it should be noted that not all core genes found in ICE *Pmu1* and in the ICE from *H. somni* 2336 were seen in the other ICEs.

A relaxase gene (Pmu_02890) was found upstream of the second integrase gene in the central region (Figure 2). This region harboured most of the core genes that encode the proteins involved in DNA cleavage (putative type I restriction-modification system methyltransferase subunit (Pmu_02900)), proteins necessary for a conjugative transfer (a protein for the formation of type IV pilus (Pmu_03230), TraD (Pmu_03190), TraG (Pmu_03040), TraC-like (Pmu_03070) proteins) and a protein involved in DNA replication [DNA topoisomerase III (Pmu_03290)]. Moreover, genes encoding a protein with a lysozyme-like domain (Pmu_03210) and a multicopper oxidase protein (Pmu_03360) and two other genes encoding enzymes potentially involved in the metabolism of alcohol as well as aldehydes and ketones were detected (Pmu_03370 and Pmu_03330) (Figure 2).

Downstream of resistance gene region 2, genes coding for proteins involved in DNA replication, such as the single-stranded DNA-binding protein (Pmu_03540) and an ATPase involved in chromosome partitioning (Pmu_03610), were found. The analysis of this right-hand terminal region also revealed the presence of the gene dnaB (Pmu_03600), coding for the DNA helicase DnaB, and a gene encoding a ParB family protein (Pmu_03590) with a predicted DNA nuclease function (Figure 2). This final core gene-containing region has been reported as the most conserved region among diverse proteobacterial ICEs.

**ICE*Pmu1* is an active element**

The ability of ICE*Pmu1* to transfer to *P. multocida* strain E348-08, *M. haemolytica* 39229 and *E. coli* HK225 by conjugation was confirmed experimentally. Similar transfer frequencies to the...
Figure 2. Organization of ICEPmu1. The regions in grey represent the flanking regions of this ICE when inserted into the genome of *P. multocida* 36950. The different genes are depicted and regions or genes of particular relevance are indicated. The position and orientation of the primers described in Table 1, the HindIII restriction sites used in hybridization experiments and inverse PCR, and the regions of amplicons that were used as probes are also indicated. The resistance gene regions 1 and 2 are shown as boxes. Numbers above the various genes are in agreement with the database entry of the *P. multocida* 36950 whole genome sequence.
different hosts ranging from $1.4 \times 10^{-6}$ to $2.9 \times 10^{-6}$ were observed (Table 2). Although only a gene (Pmu_03323) coding for a putative PilL protein from the type IV pI/p operon was identified, various other genes were present in ICEPmu1, which might also be involved in the transfer and responsible for the host specificity of the ICE. Although no specific function could be assigned by comparison with the proteins deposited in the InterPro databases (http://www.ebi.ac.uk/Tools/pfa/iprscan/), some matches were found in the TIGRfam database, which suggested that they were related to members of protein families found in ICEs of the plant-associated Pseudomonas fluorescens Pf-5 and of human clinical Pseudomonas aeruginosa strains carrying the P. aeruginosa genomic island 1. 30,31

Screening of the transconjugants by susceptibility testing and PCR assays confirmed the transfer of all resistance genes. Moreover, the higher MIC values seen with the E. coli transconjugant, especially for chloramphenicol (32-fold), florfenicol (64-fold) and ampicillin (16-fold), point towards a better functional activity of the floR and blaOXA-2 genes in the E. coli host (Table 2). In this regard, it should be noted that most of the resistance genes found in ICEPmu1 are not indigenous Pasteurellaceae genes, but have been found in various members of the Enterobacteriaceae.32

Detection of the circular intermediate form
The circular form of ICEPmu1 was only visible by agarose gel electrophoresis in the E. coli transconjugant. In contrast, the standard or nested PCR assays were positive for all transconjugants and donor strain 36950 and, as expected, negative for the original recipient cells (Figure 4). The nested PCR was developed to overcome the lower specificity of the left outward primer as recognized when the standard PCR was performed with the E. coli transconjugant. In this case, the left outward primer annealed also with the right-hand flanking region of the ICE in the E. coli genome. Analysis of the sequences of the specific amplicons identified the sequence of the recircularization point (5′-GATTTTGAATCAA-3′), which was in agreement with the sequence of the direct repeats found immediately up- and downstream of the termini of ICEPmu1.

Detection of ICEPmu1 by hybridization experiments
The presence of ICEPmu1 in the transconjugants and as the circular form visualized by agarose gel electrophoresis was also confirmed by the hybridization experiments. The relaxase-specific gene probe hybridized to the expected 6 kb HindIII fragment of extrachromosomal DNA from P. multocida E348-08 transconjugant or genomic DNA from P. multocida E348-08, E. coli HK225 and M. haemolytica 39229 transconjugants, as well as the donor strain. The additional two hybridization experiments with the probes P157 and P158 also resulted in only a single hybridizing fragment. The sizes of these fragments in P. multocida 36950 were 4.4 kb and 4.6 kb. With the P. multocida E348-08 and M. haemolytica 39229 transconjugants, larger fragments of ~5.5 kb and 5 kb, respectively, were observed (data not shown). The E. coli HK225 transconjugant showed hybridizing fragments of 4.6 and 5.1 kb for the probes P157 and P158,
Structure and transfer of ICE

Pmu1

Transfer frequency of ICE and MICs for the donor P. multocida 36950 strain, recipient P. multocida E348-08 RifR, M. haemolytica 39229 RifR and E. coli HK225 RifR strains and transconjugants harboring ICE

| Strain                           | MICs (mg/L) | TET | CHL | FFC | SUL | STR | KAN | NEO | GEN | SPE | XNL | CQN | AMP | NAL | ENR | TUL | TIL | CLI |
|---------------------------------|-------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| P. multocida 36950 donor        |             | 32  | 16  | 8   | 8   | 8   | 32  | 4   | 8   | 8   | 8   | 8   | 8   | 8   | 8   | 8   | 8   | 8   | 8   |
| P. multocida E348-08 RifR recipient |           | 0.5 | 0.25 | 0.25 | 0.03 | 0.03 | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 |
| M. haemolytica 39229 RifR recipient |         | 0.5 | 1   | 4   | 4   | 16  | 8   | 8   | 8   | 8   | 8   | 8   | 8   | 8   | 8   | 8   | 8   | 8   | 8   |
| E. coli HK225 RifR recipient     |             | 0.5 | 1   | 4   | 4   | 16  | 8   | 8   | 8   | 8   | 8   | 8   | 8   | 8   | 8   | 8   | 8   | 8   | 8   |


**Table 2.** Transfer frequency of ICEPmu1 and MICs for the donor P. multocida 36950 strain, recipient P. multocida E348-08 RifR, M. haemolytica 39229 RifR, and E. coli HK225 RifR strains and transconjugants harboring ICEPmu1.

**Insertion points of ICEPmu1 and flanking regions**

Sequence analysis of the amplicons obtained by standard and inverse PCRs proved that the insertion point of ICEPmu1 in all transconjugants was located in a tRNA\textsubscript{Leu}. The tRNA\textsubscript{Leu}, in which the ICE was inserted in the P. multocida E348-08 transconjugant, showed the same sequence as the one in the P. multocida strain 36950. The same tRNA\textsubscript{Leu}, but non-disrupted by ICE integration, is also present in the P. multocida Pm70 genome where it is part of the first RNA cluster. Furthermore, a 5S rRNA and the gene for a haem iron utilization-like protein were detected immediately up- and downstream of this tRNA\textsubscript{Leu} in both P. multocida Pm70 (PM0298) and P. multocida 36950 (Pmu_03640). In the E. coli HK225 transconjugant, the ICE was inserted into the tRNA\textsubscript{Leu} between the genes intB (coding for a putative prophage P4 integrase) and yigB [coding for a flavin mononucleotide (FMN) phosphatase]. In the M. haemolytica transconjugant, the sequence of the inverse PCR from the right-hand flanking region showed 100% identity with the sequence found in contigs 83–31 (Ctg83 Ctg31; GenBank accession number AASAO0000058.1) from M. haemolytica PHL213. This region contained a partial tRNA\textsubscript{Leu} and the xseA gene (coding for the large subunit of exodeoxyribonuclease VII). Analysis of the sequence of these contigs showed that this strain also harboured at least part of an ICE related to ICEPmu1. Comparative analysis revealed 89% identity between a 5565 bp segment of ICEPmu1 from P. multocida 36950 and the ICE fragment of M. haemolytica PHL213. Analysis of the sequences around the integration site showed an exchange of two consecutive adenines for a cytosine and a guanine (5'-GATTTTGAATCCG-3') in the direct repeat at the right terminus of the M. haemolytica PHL213. These exchanges were also present in the direct repeats flanking the ICE in H. somnii 2336. The analysis of the region flanking the right terminus of ICEPmu1 in the M. haemolytica 39229 transconjugant revealed the presence of at least the terminal part of a second ICEPmu1 copy.

The tRNA\textsubscript{Leu} into which ICEPmu1 was inserted in P. multocida 36950 was flanked on one side by a tRNA cluster and on the other side by a clustered regularly interspaced short palindromic repeats (CRISPR) system (Figure 2). This CRISPR system proved to be a CRISPR/Cas Ypest-subtype, composed of a cluster of two CRISPR-associated (Cas) protein families (Cas1 and Cas3) and a repeat-spacer array. In this array, 28 bp repeats (5'-GTTCACCATCGTGTAGATGGCTTAGAAA-3') and 17 spacers were found. The CRISPR-associated module, formed by the repair-associated mysterious protein (RAMP) Cys1-4, was also identified. CRISPR systems have a defence function, as they confer resistance to infection by extrachromosomal agents like phages and plasmids, depending on the sequences present in the spacers. It is interesting to note that such a system is located ~11 kb away from the right terminus of the ICEPmu1. This observation suggested that this tRNA\textsubscript{Leu} is located in a hypervariable region in P. multocida 36950, as previously described in other bacteria.
tRNA\textsubscript{Leu} from strains:

- **P. multocida** 36950
- **P. multocida** E348-08
- **M. haemolytica** 39229
- **E. coli** HK225

Strains:

- **P. multocida** 36950
- **P. multocida** E348-08
- **M. haemolytica** 39229

ICE\textsubscript{Pmu1} inserted into tRNA\textsubscript{Leu}:

<table>
<thead>
<tr>
<th>Strains</th>
<th>DR-L</th>
<th>ICE\textsubscript{Pmu1} inserted into tRNA\textsubscript{Leu}</th>
<th>DR-R</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>P. multocida</strong> 36950</td>
<td>-attL- GATTTTGAATCAA-</td>
<td>-GATTTTGAATCAA-</td>
<td>-attR-</td>
</tr>
<tr>
<td><strong>P. multocida</strong> E348-08</td>
<td>-attL- GATTTTGAATCAA-</td>
<td>-GATTTTGAATCAA-</td>
<td>-attR-</td>
</tr>
<tr>
<td><strong>M. haemolytica</strong> 39229</td>
<td>-attL- GATTTTGAATCAA-</td>
<td>-GATTTTGAATCAA-</td>
<td>-attR-</td>
</tr>
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</table>

**Figure 4.** (a) Nested PCR for the detection of the circular form of ICE\textsubscript{Pmu1}. In lanes 2–5, the amplicons of 990 bp, obtained with the donor **P. multocida** strain 36950 carrying the ICE\textsubscript{Pmu1} and the **P. multocida** E348-08, **M. haemolytica** 39229 and **E. coli** HK225 transconjugants carrying the ICE\textsubscript{Pmu1}, are shown. Lanes 6–8 contain the negative results obtained with the original recipient cells **P. multocida** E348-08, **M. haemolytica** 39229 and **E. coli** HK225. (b) Site-specific recombination of ICE\textsubscript{Pmu1} into the tRNA\textsubscript{Leu} of different strains. The sequences of the tRNA\textsubscript{Leu} are shown in the orientation that matches the orientation of the ICE\textsubscript{Pmu1} sequence. The left attachment sites (attL) and the right attachment sites (attR), the sequences involved in the crossover and the resulting direct repeats located on the left termini (DR-L) and on the right termini (DR-R) of the inserted ICE\textsubscript{Pmu1} are also shown. Due to the presence of at least part of a second ICE\textsubscript{Pmu1} copy in the same site, the true attL site was not identified in the **M. haemolytica** transconjugant.
Structure and transfer of ICE$_{Pmu1}$

Conclusions
The ICE$_{Pmu1}$ described in this study is, to the best of our knowledge, the first ICE identified in $P$. multocida. It was closely related in its core genes to a family of diverse proteobacterial ICES, but also harboured two regions of accessory genes that consisted mainly of insertion sequences and antimicrobial resistance genes. A matter of concern is the great similarity among ICE$_{Pmu1}$ found in $P$. multocida 36950, the ICE in $H$. somni 23364 and the ICE segment available in the incomplete $M$. haemolytica PHL23 genome sequence (strain ATCC BAA-410).37 $P$. multocida, $M$. haemolytica and $H$. somni represent the major pathogens involved in bovine respiratory disease, and the aforementioned three strains were isolated from cases of respiratory tract infections in cattle. These observations corroborate the results of our in vitro transfer experiments and show that horizontal intergeneric transfer of closely related ICES has obviously already happened in vivo. Since ICES are among the most important elements mediating horizontal gene transfer between a wide range of bacterial hosts, the spreading of multiresistance ICES, such as ICE$_{Pmu1}$, may seriously decrease the therapeutic options for bovine respiratory disease. Moreover, the particular structure of the resistance gene regions may allow for the incorporation of not only additional cassette-borne resistance genes, but also for the acquisition of resistance genes via insertion sequence-mediated recombination processes. Further studies will provide information on the stability and potential structural variations in the accessory gene regions of ICE$_{Pmu1}$ in $P$. multocida and other host bacteria.

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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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