J Antimicrob Chemother 2015; **70**: 93–97
doi:10.1093/jac/dku361 Advance Access publication 18 September 2014

Journal of Antimicrobial Chemotherapy

Analysis and comparative genomics of ICE*Mh1*, a novel integrative and conjugative element (ICE) of *Mannheimia haemolytica*

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Received 21 July 2014; returned 12 August 2014; revised 13 August 2014; accepted 14 August 2014

Objectives: The aim of this study was to identify and analyse the first integrative and conjugative element (ICE) from *Mannheimia haemolytica*, the major bacterial component of the bovine respiratory disease (BRD) complex.

Methods: The novel ICE*Mh1* was discovered in the whole-genome sequence of *M. haemolytica* 42548 by sequence analysis and comparative genomics. Transfer of ICE*Mh1* was confirmed by conjugation into *Pasteurella multocida* recipient cells.

Results: ICE*Mh1* has a size of 92 345 bp and harbours 107 genes. It integrates into a chromosomal tRNA^{Leu} copy. Within two resistance gene regions of ~7.4 and 3.3 kb, ICE*Mh1* harbours five genes, which confer resistance to streptomycin (*strA* and *strB*), kanamycin/neomycin (*aphA1*), tetracycline [*tetR*-*tet*(H)] and sulphonamides (*sul2*). ICE*Mh1* is related to the recently described ICE*Pmu1* and both ICEs seem to have evolved from a common ancestor. A region of ICE*Mh1* that is absent in ICE*Pmu1* was found in putative ICE regions of other *M. haemolytica* genomes, suggesting a recombination event between two ICEs. ICE*Mh1* transfers to *P. multocida* by conjugation, in which it also uses a tRNA^{Leu} as the integration site. PCR assays and susceptibility testing confirmed the presence and activity of the ICE*Mh1*-associated resistance genes in the *P. multocida* recipient.

Conclusions: These findings showed that ICEs, with structurally variable resistance gene regions, are present in BRD-associated *Pasteurellaceae*, can easily spread across genus borders and enable the acquisition of multidrug resistance via a single horizontal gene transfer event. This poses a threat to efficient antimicrobial chemotherapy of BRD-associated bacterial pathogens.

Keywords: bovine respiratory disease, *Pasteurellaceae*, horizontal gene transfer, susceptibility testing

Introduction

The facultative pathogen *Mannheimia haemolytica* represents the major bacterial component of the multifactorial bovine respiratory disease (BRD) complex. BRD causes losses of over three billion US dollars per year in the global cattle industry.[1] BRD-associated *M. haemolytica* infections are commonly treated with antimicrobial agents. However, increasing percentages of resistant *M. haemolytica* have been observed, particularly in the USA and Canada. Portis *et al*.[2] collected MIC data on bacteria involved in the BRD complex in North America and their latest data, for the year 2009, identified resistance rates to enrofloxacin (6.6%), florfenicol (8.6%), tetracycline (43.7%), tilmicosin (27.3%) and tulathromycin (8.9%) among the 304 *M. haemolytica* isolates tested. In 2012, the multiresistant *Pasteurella multocida* isolate 36950, which originated from a case of BRD and carried the 82.2 kb integrative and conjugative element (ICE) ICE*Pmu1*, was described.[3,4] ICEs are mobile genetic elements that usually integrate into a specific site, most frequently a tRNA, in the chromosomal DNA of the host.[5] They are able to excise from the host chromosome, form a circular intermediate by which they conjugatively transfer themselves to a new host bacterium, and finally reintegrate into the chromosome of the new host cell.[6] ICEs contain a set of core genes, that are responsible for maintaining and spreading the element, and a set of accessory genes, such as antimicrobial resistance genes.[3,4,7] During recent years, ICEs or parts thereof have been identified by PCR approaches in *P. multocida*, *M. haemolytica* and *Histophilus somni*.[8,9] However, to date no complete ICE of *M. haemolytica* has been described in detail and investigated in depth. The recently published whole-genome sequence of *M. haemolytica* 42548 appears to harbour an ICE, designated ICE*Mh1*.[10]

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The aim of the present study was to analyse in detail the multiresistance-mediating ICE*Mh1* detected in the plasmid-free *M. haemolytica* strain 42548 for its structure and transfer abilities.

Materials and methods

Bacterial strain and susceptibility testing

The *M. haemolytica* strain 42548 was obtained from a case of bovine respiratory tract infection in a feedlot from Pennsylvania, USA, in 2007. Antimicrobial susceptibility testing was performed by broth microdilution using custom-made microtitre panels (MCS Diagnostics, Swalmen, The Netherlands) or by broth macrodilution for the antimicrobial agents for which resistance genes have been detected in the whole-genome sequence.[10] Performance of the tests and the evaluation of MIC values followed the recommendations given in documents VET01-A4 and VET01-S2 of the CLSI.[11,12] *Staphylococcus aureus* ATCC 29213 served as a quality control strain.

Whole-genome sequence analysis and comparative genomics

Genomic DNA preparation and whole-genome sequencing were performed as previously described.[10] Detailed sequence comparisons were performed using various tools, including the BLASTn and BLASTp programs of NCBI (http://blast.ncbi.nlm.nih.gov/), ResFinder 2.1 and the Artemis Comparison Tool.[13,14] The GC content of ICE*Mh1* was determined using the Artemis genome browser and annotation tool.[15] The genome sequence of *M. haemolytica* 42548 has been deposited at DDBJ/EMBL/GenBank under the accession number CP005383.

Comparison between ICE*Mh1* and ICE*Pmu1* and their resistance gene regions was conducted with the genome comparison visualizer Easyfig.[16] Orthologues were identified for strain 42548 and the most similar whole-genome sequences of members of the family *Pasteurellaceae* were compared using the BiBaG software tool, which utilizes a reciprocal BLAST heuristic,[17] and a global sequence alignment using the Needleman–Wunsch algorithm. The following genomes were used for comparison: *Bibersteinia trehalosi* USDA-ARS-USMARC-190 (CP006956.1), *M. haemolytica* USDA-ARS-USMARC-183 (NC_020833.1) and USDA-ARS-USMARC-185 (NC_020834.1) and *P. multocida* 36950 (NC_016808.1).

Conjugation, susceptibility testing and PCR assays

The plasmid-free *M. haemolytica* strain 42548 served as an ICE donor for the rifampicin-resistant recipient strain *P. multocida* E348-08 (capsular type F).[4] For the mating assay, overnight cultures of the donor and recipient cells grown in brain heart infusion broth were mixed in a ratio of 1:1. The assay was conducted as previously described.[4] The transfer frequency was calculated as the number of transconjugants per recipient cell. Obtained transconjugants were checked for the presence of the ICE*Mh1*-associated resistance genes as well as the relaxase (MHH_c22640) gene (Table 1). In these PCR assays, the original recipient strain served as a negative control, while *M. haemolytica* strain 42548 served as a positive control. Furthermore, all transconjugants were subjected to species-specific PCR.[18]

Detection of the circular intermediate of ICEMh1

To detect the extrachromosomal circular form of the ICE, DNA was extracted with a modified thermolysis technique.[4] For PCR detection of the circular intermediate form, a nested PCR (323×389 followed by 295×388) was used (Table 1). For this, 1 μL of genomic DNA (100 ng/μL), 2.5 U of Bio-X-act DNA polymerase (Bioline Reagents Ltd, London, UK) in 1× Opti buffer with 3 mM $MgCl_2$, 1 mM dNTPs, 200 mM betaine and 0.3 μM of each primer were mixed in a final volume of 50 μL. The PCR conditions for the amplicons of 695–5409 bp consisted of an initial denaturation for 2 min at 98°C, 30 cycles of 20 s at 96°C, 20 s at the annealing temperature of 56 or 57°C and 1 min to 5.5 min at 68°C (Table 1), followed by a final extension of 10 min at 68°C. The obtained amplicons were sequenced using Sanger sequencing, BigDye 3.0 chemistry and an ABI3730XL capillary sequencer (Applied Biosystems, Life Technologies GmbH, Darmstadt, Germany).

The ICE's integration site in the *P. multocida* E348-08 transconjugants was determined via PCRs for each terminus (MAN_394 and MAN_395; Table 1) and subsequent sequence analysis. Previously designed outward primers (MAN_295 and MAN_388; Table 1) were used and combined with inward primers (past0257 and past0256; Table 1)[4] designed from sequences in the left or right flanking region of the $tRNA^{Leu}$. The amplified fragments were gel purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) and sequenced.

Results and discussion

Identification of ICEMh1 and comparative genomics

The bidirectional BLAST analysis with other similar genomes, including the genome of *P. multocida* 36950, revealed that ICE*Mh1* was located in a region of *M. haemolytica* 42548 similar to the location of ICE*Pmu1* in the genome of *P. multocida* 36950. ICE*Mh1* had a size of 92 345 bp and was integrated into the second

Table 1. PCR primers used for the characterization of ICE*Mh1*

PCR assay	Primer name	Primer sequence (5′ to 3′)	Amplicon (bp)	Annealing temperature (°C)	Elongation time at 68°C (min)
Relaxase	ICE-relaxase-fw	CTGGTTCAACGTCCTGTCAA	695	57	1
	ICE-relaxase-rv	ATCGTTGCAATTTCCTGTCC			
323×389	MAN_323	CGGTAGTGCAGGAAAGTTAG	5409	56	5.5
	MAN_389	GAGAGTATGAAGGTAGTCGG			
295×388	MAN_295	CCTTGAGATAGTGGTACTGG	2436	56	2.5
	MAN_388	GCCAATACAGACATATCGAC			
MAN_394	past0257	TGAATTTGAATAAGGGCATCG	2232	56	2.5
	MAN_295	CCTTGAGATAGTGGTACTGG			
MAN_395	MAN_388	GCCAATACAGACATATCGAC	1654	56	2
	past0256	GTAGCGAGCATGACTGTTTTAC			

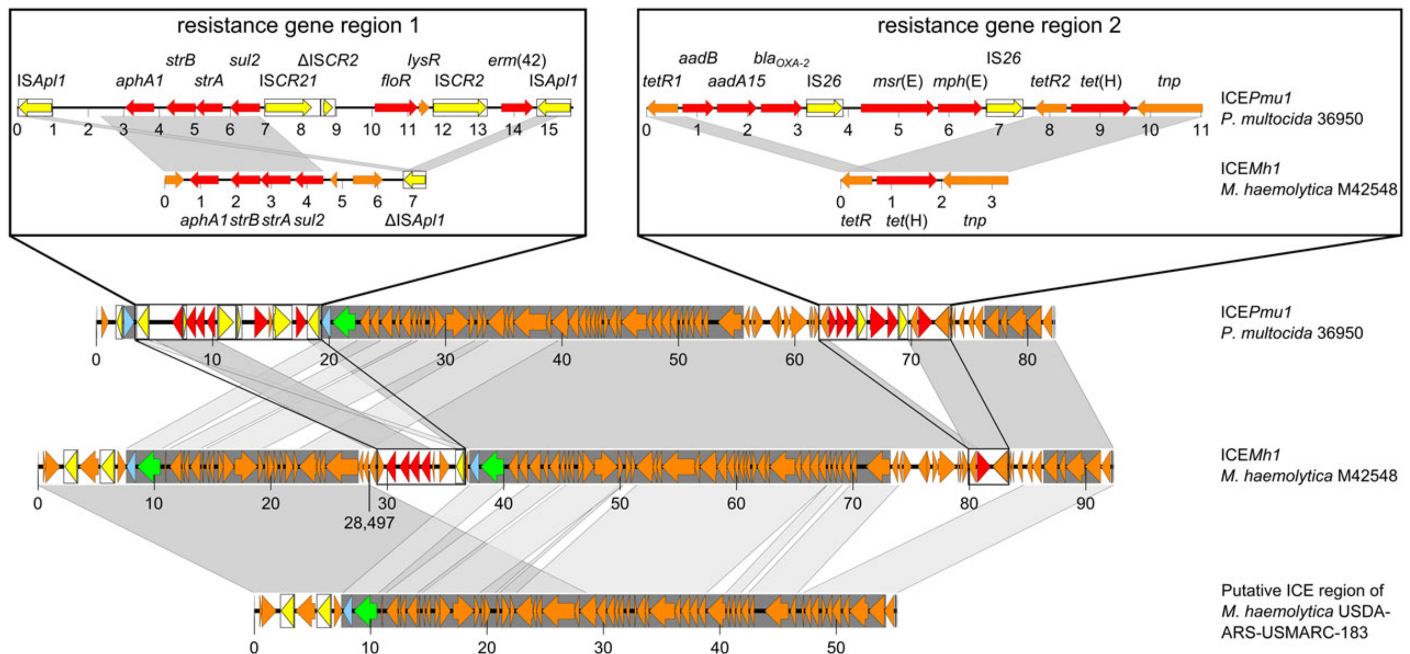

Figure 1. Schematic comparison of the resistance gene regions 1 (upper left) and 2 (upper right) of ICE*Mh1* and ICE*Pmu1* and a schematic comparison of the entire ICE*Mh1* with ICE*Pmu1* and the putative ICE region of *M. haemolytica* USDA-ARS-USMARC-183. Genes are presented as arrows, with the arrowhead indicating the direction of transcription. Integrase genes are represented by blue arrows, green arrows depict relaxase genes and red arrows indicate resistance genes. ISs and IS elements are shown as boxes, with the yellow arrows inside the boxes indicating the transposase genes. A dark grey background behind the arrows indicates a core region.[7] Areas between the ICEs shaded in light grey indicate regions of ≥67% sequence identity between ICE*Mh1*, ICE*Pmu1* and the putative ICE region of *M. haemolytica* USDA-ARS-USMARC-183, while areas shaded dark grey indicate ≥99% sequence identity. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

of the four $tRNA^{Leu}$ copies. The direct repeats (DRs) flanking the ICE were 2 bp shorter than the DRs of ICE*Pmu1* (5′-GATTTTGAATCAA-3′) and consisted of only 11 bp (5′-GATTTTGAATC-3′). The $tRNA^{Leu}$ in the chromosomal DNA of *M. haemolytica* 42548, which was disrupted by the ICE integration, was replaced by an intact $tRNA^{Leu}$ (MHH_c23290) copy that was part of ICE*Mh1*. A total of 107 genes were identified within ICE*Mh1*, one of which was $tRNA^{Leu}$, one was a pseudogene (MHH_c22610), three coded for transposases of IS elements and 45 coded for hypothetical proteins. ICE*Mh1* included a set of genetic elements related to genome plasticity. The pseudogene was an IS*Apl1* transposase disrupted by an internal stop codon, while the intact IS elements were identified as IS*Shes15* (MHH_c22230), IS*Vvu4* (MHH_c22250) and IS*Pst9-like* (MHH_c23170). Furthermore, two genes (MHH_c22270 and MHH_c22630) coded for XerD-like recombinases that differed in only 10 amino acids, while another two genes (MHH_c22280 and MHH_c22640) coded for relaxase proteins with 90% amino acid similarity. The recombinase and relaxase genes were assumed to participate in excision/integration and conjugative transfer. ICE*Mh1* had a GC content of 40.18%, which is slightly lower than the GC content of 41.05% of the entire genome of *M. haemolytica* 42548.[10]

Comparative genomics showed that ICE*Mh1* shared the highest identity with ICE*Pmu1*, as well as putative ICE regions found in *B. trehalosi* USDA-ARS-USMARC-190 and *M. haemolytica* USDA-ARS-USMARC-183.[19] Whereas *M. haemolytica* 42548 was isolated in Pennsylvania in 2007, *B. trehalosi* USDA-ARS-USMARC-190 originated from Nebraska in 2010 and *M. haemolytica* USDA-ARS-USMARC-183 from Kansas in 1991. Thus, these isolates were collected at different times in different parts of North America, displaying both a wide geographical and intergenus distribution of ICEs harbouring multiple antimicrobial resistance genes. Up to base 28497, ICE*Mh1* shared 100% sequence identity with the putative ICE region of *M. haemolytica* USDA-ARS-USMARC-183, followed by ≥99% sequence identity—excluding the resistance gene regions—to ICE*Pmu1* starting from base 28497 (Figure 1). There were neither IS elements in the vicinity nor overlapping sequences, except for the single base at position 28497, at the point where the homologies switched.

A direct sequence comparison between the entire ICE*Mh1* and the closely related ICE*Pmu1* showed that ICE*Pmu1* lacked 15 of the genes found in ICE*Mh1*, while ICE*Mh1* lacked 19 of the genes found in ICE*Pmu1*. Only one of the recombinase genes of ICE*Pmu1* (Pmu_02700) was present in ICE*Mh1*, while only one of the relaxase genes found in ICE*Mh1* (MHH_c22280) was present in ICE*Pmu1*. The core genes involved in DNA cleavage (Pmu_02900 to Pmu_03290) were found within the area homologous between both ICEs. Moreover, no IS*CR* elements were present in ICE*Mh1*.

Antimicrobial resistance gene regions

M. haemolytica 42548 was classified as intermediate to tetracyclines (4 mg/L) and had elevated MICs of the aminoglycosides kanamycin (≥512 mg/L), neomycin (64 mg/L) and streptomycin (256 mg/L) and the sulphonamide sulfamethoxazole (512 mg/L). When induced, the MIC of tetracyclines increased one step to 8 mg/L.

Analysis of the resistance genes found in the whole-genome sequence of *M. haemolytica* 42548—*aphA1* (MHH_c22550), *strA* (MHH_c22570), *strB* (MHH_c22560), *sul2* (MHH_c22580) and *tetR-tet*(H) (MHH_c23150-23160)—revealed that all of them were located in ICE*Mh1*. The resistance genes could be identified in two resistance gene regions corresponding to two accessory gene regions also found in ICE*Pmu1*. Within resistance gene region 1 (Figure 1), the resistance genes *strA* and *strB* confer resistance to streptomycin, *aphA1* to kanamycin and neomycin and *sul2* to sulphonamides. Of these antimicrobial resistance genes of ICE*Mh1*, only one—*sul2*—differed from its counterpart in ICE*Pmu1*. While the deduced amino acid sequence of *sul2* from ICE*Mh1* had a size of 271 amino acids, which corresponds to what is commonly found among *Pasteurellaceae*,[20] the Sul2 protein from ICE*Pmu1* had a size of 281 amino acids.[3] Resistance gene region 2 (Figure 1) of ICE*Mh1* contained only the resistance gene *tet*(H), which confers resistance to tetracyclines, and one copy of its repressor gene *tetR*. However, the *tet*(H) of ICE*Mh1* did not have the amino acid substitution N258H found in ICE*Pmu1*. A transposase, also present in the whole-genome sequence of *Mannheimia succiniciproducens* MBEL55E, was located downstream of *tet*(H). In contrast to ICE*Pmu1*, ICE*Mh1* lacked the following seven resistance genes: *floR* (resistance to phenicols) and *erm*(42) (resistance to macrolides and lincosamides) in resistance gene region 1 and *aadB* (resistance to gentamicin), *aadA15* (resistance to streptomycin and spectinomycin), $bla_{OXA\text{-}2}$ (resistance to β-lactams) and *msr*(E) and *mph*(E) (both conferring resistance to macrolides) in resistance gene region 2.

Activity of ICEMh1

It was possible to transfer ICE*Mh1* to *P. multocida* E348-08 by conjugation and to visualize the circular intermediate form of the ICE in *M. haemolytica* 42548 as well as in the *P. multocida* E348-08 transconjugants. The transfer frequency was calculated to be $\sim 6.25 \times 10^{-9}$ transconjugants per recipient cell. Furthermore, the resistance pattern and the PCR results of the *P. multocida* transconjugants confirmed the transfer of all resistance genes. The transconjugants carrying ICE*Mh1* showed, in comparison with *P. multocida* E348-08, increased MICs of neomycin (from 8 to $\geq$128 mg/L), streptomycin (from 8 to $\geq$1024 mg/L) and tetracycline (from 0.5 to $\geq$8 mg/L). Analysis of the integration site of ICE*Mh1* revealed the same DRs in the transconjugants as could be found in *M. haemolytica* 42548.

Conclusions

In summary, the two resistance gene regions of ICE*Mh1* contained only 5 resistance genes, in contrast to the 12 resistance genes found in ICE*Pmu1*. Based on the structural similarities observed between the resistance regions of these two ICEs (Figure 1), it can be suggested that the resistance gene regions found in ICE*Mh1* might represent precursors of the resistance gene regions found in ICE*Pmu1*. From an evolutionary perspective, it is tempting to speculate that several recombination and IS-mediated integration events in ICE*Pmu1* resulted in the acquisition of additional resistance genes. However, comparative analysis of ICE*Mh1* with related ICEs revealed that ICEs may have a modular structure with shared features of genome plasticity and specific resistance modules, which resembles the modular organization of other mobile genomic elements, such as prophages and pathogenicity islands, and thus may contribute to the rapid evolution of pathogenic bacteria. The future will show whether and which additional resistance genes can be incorporated into the ICE*Mh1*-associated resistance gene regions under *in vivo* conditions. The observation that multiresistance ICEs are present in naturally occurring bovine *M. haemolytica* and can spread between BRD-associated pathogens underlines the threat that these elements pose to the efficient antimicrobial therapy of BRD.

Acknowledgements

We thank Kathleen Gollnow for her excellent technical assistance.

Funding

This study was supported by grant SCHW382/10-1 of the German Research Foundation (DFG) and Zoetis, Kalamazoo, MI, USA. C. E. received a scholarship of the Gesellschaft der Freunde der Tierärzlichen Hochschule Hannover e.V.

Transparency declarations

M. T. S., R. W. M. and J. L. W. are employees of Zoetis (formerly Pfizer Animal Health) and own Zoetis stocks. All other authors: none to declare.

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