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Plasmid-located *dfrA14* gene in *Pasteurella multocida* isolates from three different pig-producing farms in Germany

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ABSTRACT

Pasteurella multocida is an important respiratory tract pathogen in intensive livestock farming, especially in pigs. Antimicrobial agents are frequently used to combat infections caused by this pathogen. In a study on antimicrobial resistance among respiratory tract pathogens of pigs from 30 German pig-producing farms, *P. multocida* isolates (n = 9) with high minimal inhibitory concentration (MIC) values of 16/304 mg/L (n = 2), 32/608 mg/L (n = 3) or ≥64/1216 mg/L (n = 4) for trimethoprim/sulfamethoxazole (1:19) and of ≥512 mg/L (n = 9) for trimethoprim (TMP) were detected in three of these farms. The genetic relatedness of the isolates was investigated via capsule-specific PCR and macrorestriction analyses with *Apa*I and *Sma*I. Pulsed-field gel electrophoresis revealed indistinguishable restriction patterns per farm, with slight differences between the three farms. All isolates represented capsular type A. Four representative isolates, that were subjected to whole genome sequencing, shared the multi-locus sequence type (ST) 3. Their plasmids were transformed into *E. coli* TOP10 with subsequent selection on TMP-containing agar plates. Antimicrobial susceptibility testing and plasmid analysis of the transformants confirmed that they were resistant to sulfonamides and trimethoprim and carried only a single small plasmid. This plasmid was completely sequenced and revealed a size of 6050 bp. Sequence analyses identified the presence of a resistance gene cluster comprising the genes *sul2-ΔstrA-dfrA14-ΔstrA-ΔstrB*. Further analysis identified a *dfrA14* gene cassette being integrated into the *strA* reading frame. Neither the gene *dfrA14* nor this gene cluster have been detected before in *P. multocida*.

1. Introduction

Pasteurella multocida belongs to the family *Pasteurellaceae*, which includes several pathogens that are associated most commonly with respiratory tract infections in livestock (Olsen et al., 2005). In pigs, *P. multocida* can be found as a colonizer of the respiratory tract, but can also play a role in diseases, such as the progressive form of atrophic rhinitis or the porcine respiratory disease complex (PRDC) (Brockmeier et al., 2002t). In Germany, β-lactams, aminoglycosides, tetracyclines, macrolides, phenicols or trimethoprim/sulfonamides are approved antimicrobial classes for the treatment of respiratory diseases in pigs (Friendship, 2007). *P. multocida* isolates may harbour several acquired antimicrobial resistance genes e.g. *floR, tet*(H), *sul2, dfrA20, aadA25, aadB, aphA1, strA, strB, erm*(42) and *msr*(E)-*mph*(E) (Kehrenberg et al., 2003; Kehrenberg and Schwarz, 2005; Kehrenberg et al., 2008; Michael et al., 2012a, 2018). These resistance genes are often located on mobile genetic elements, frequently on plasmids of sizes between 4.3–11 kb (Michael et al., 2018). Exchanges of resistance genes are common within the *Pasteurellaceae* family, as well as with other Gram-negative and Gram-positive bacteria (Livrelli et al., 1991; Kehrenberg et al., 2001; Schwarz, 2008). Commonly detected and frequently linked resistance genes in *P. multocida* are *strA* and *strB* that code for

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streptomycin-inactivating enzymes (Kehrenberg and Schwarz, 2001). In addition, *sul2* is often associated with *strA* and *strB* (Kehrenberg and Schwarz, 2001). Sulfonamides and trimethoprim inhibit the enzymes dihydropteroate synthase (DHPS) and dihydrofolate reductase (DHFR), respectively, which are essential components of the folic acid biosynthesis pathway (van Duijkeren et al., 2018). Acquired resistance genes *sul* and *dfr* code for enzymes that displace the original DHPS or DHFR and have a reduced affinity to sulfonamides and trimethoprim, respectively (van Duijkeren et al., 2018). The most common sulfonamide resistance gene in *P. multocida* is *sul2*, whereas *dfrA20*, which is related to *dfr* genes of Gram-positive bacteria, is the only trimethoprim resistance gene detected in *P. multocida* to date (Kehrenberg and Schwarz, 2005; Schwarz, 2008; Michael et al., 2018).

The VASIB project, funded by the German Federal Ministry of Food and Agriculture, aims at providing guidance to reduce antimicrobial consumption in pig-producing German farms by integrating information from consulting expertise in clinical examination, hygiene, epidemiology, microbiology and pharmacology (Niemann et al., 2018). The acronym VASIB represents an abbreviation of the initial components of the project title in German language. In this project, the resistance profiles of selected respiratory tract pathogens obtained from 30 farms were determined. In three of these farms, nine *P. multocida* isolates showing high trimethoprim/sulfamethoxazole minimal inhibitory concentration (MIC) values were identified. The aim of this study was to investigate these *P. multocida* isolates, for their genetic relatedness and to determine the genetic basis of sulfonamide and trimethoprim resistance.

2. Materials and methods

2.1. Pasteurella isolates

In the context of the VASIB project, bronchoalveolar lavage fluids (BALFs) of pigs from 30 pig-producing farms in the Western region of Germany were examined (Niemann et al., 2018). These farms were chosen because of their history of recurring respiratory tract infections and samples were collected at different time points within a 12-month period during 2016 and 2017 (Table 1). For each farm, BALFs were obtained from four pigs, respectively, at the first visit (time point A) and at the last visit (time point D). In addition, farms were sampled (three weaners selected by clinical symptoms) in case of an acute respiratory tract disease outbreak before starting an antimicrobial therapy (time point B) and the same animals were tested at the last day of treatment (time point C) (Niemann et al., 2018). In 29 out of the 30 examined farms *P. multocida* was identified. In three of these 29 farms, *P. multocida* isolates with high trimethoprim/sulfamethoxazole MICs were detected. Pure cultures were supplied from a diagnostic laboratory (Bio-Diagnostix, Velen, Germany) to the Freie Universität Berlin for further examinations. Five isolates were obtained from Farm 1 from healthy pigs at time points A and D. Two isolates were from Farm 2, obtained from the same diseased pig with respiratory problems at time points B and C. The remaining two isolates were from Farm 3 at time point B and originated from the same pig with respiratory symptoms (Table 1). The species *P. multocida* was confirmed using a species-specific PCR as well as a capsular type PCR (Townsend et al., 1998, 2001). In addition to *P. multocida*, *Haemophilus parasuis* and/or *Streptococcus suis* were isolated from all pigs but one from farms 1 and 2, while *Bordetella bronchiseptica* and *H. parasuis* was additionally isolated from the pig in the third farm.

2.2. Antimicrobial susceptibility testing

The isolates were tested for their MIC values according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2018). The trimethoprim/sulfamethoxazole (1:19) MIC was determined by broth microdilution, whereas the trimethoprim MIC was tested by broth macrodilution. *E. coli* ATCC®25922 served as quality control strain.

Table 1
MICs (mg/L) of trimethoprim/sulfamethoxazole (1:19, SXT) and trimethoprim (TMP) of *P. multocida* isolates and *E. coli* TOP10, with and without the transformed plasmids.

Isolate	Farm	Pig	Time point[a]	MIC [mg/L]	
				SXT[c]	TMP[d]
IMT41689[b]	1	Pig 1	A	≥64/1216	≥512
IMT41690	1	Pig 2	A	≥64/1216	≥512
IMT41691	1	Pig 3	A	≥64/1216	≥512
IMT43692	1	Pig 4	D	16/304	≥512
IMT43698[b]	1	Pig 5	D	≥64/1216	≥512
IMT45639[b]	2	Pig 6	B	32/608	≥512
IMT42012	2	Pig 6	C	32/608	≥512
IMT43844[b]	3	Pig 7	B	32/608	≥512
IMT43848	3	Pig 7	B	16/304	≥512
E. coli TOP10	–	–	–	0.06/1.19	≤0.25
E. coli TOP10 + p41689	–	–	–	≥64/1216	≥512
E. coli TOP10 + p43698	–	–	–	≥64/1216	≥512
E. coli TOP10 + p45639	–	–	–	≥64/1216	≥512
E. coli TOP10 + p43844	–	–	–	≥64/1216	≥512

[a] Sampling time points. A: the pigs were healthy and have not been treated with antimicrobial agents before sampling; B: pigs showed respiratory symptoms; C: same pigs as in B, but sampled at the end of the antimicrobial treatment; D: healthy pigs one year after time point A, without an antimicrobial treatment before sampling.
[b] Isolates that were subjected to plasmid extraction, transformation and WGS.
[c] Trimethoprim/sulfamethoxazole 1:19.
[d] Trimethoprim.

2.3. Macrorestriction analysis and pulsed-field gel electrophoresis (PFGE)

P. multocida DNA extractions were digested with *Sma*I as well as with *Apa*I (Thermo Fisher Scientific Inc., Waltham, USA) for macrorestriction analysis and subsequent PFGE. The running conditions were as previously described (Kehrenberg et al., 2001). Further, relatedness analysis was accomplished with BioNumerics v7.5 (bioMérieux, Applied Math, Sint-Martens-Latem, Belgium) by a cluster analysis with the unweighted pair group method using arithmetic averages (UPGMA) generated from Dice coefficients.

2.4. Plasmid isolation, transformation and analysis

The representative isolates IMT41689, IMT43698, IMT43844 and IMT45639 were selected for plasmid analysis. Plasmids from the wild type isolates were obtained by an adapted alkaline lysis protocol previously described (Kehrenberg et al., 2001). The plasmids of the aforementioned isolates were transformed into electro-competent *Escherichia coli* TOP10 (Thermo Fisher Scientific Inc., Waltham, USA) and transformants were selected on Luria-Bertani agar supplemented with trimethoprim (64 mg/L) (Dower et al., 1988). *E. coli* TOP10 transformants were tested for their antimicrobial MICs. In addition, PCR for the detection of *sul2* in the transformed *E. coli* TOP10 was performed as previously described (Kehrenberg and Schwarz, 2001). The plasmids from the transformants were extracted with the HiPure Plasmid Maxiprep Kit (Thermo Fisher Scientific Inc., Waltham, USA) and their size estimated with both single and double digestion using *Xba*I and *Hin*dIII restriction enzymes (Thermo Fisher Scientific Inc., Waltham, USA).

2.5. Whole genome sequencing (WGS)

Genomic DNA of the selected isolates and the aforementioned *E. coli* transformants was obtained by using the Master Pure™ DNA Purification Kit (Epicentre, Illumina company, Madison, USA). The libraries for WGS were prepared using the Nextera XT library preparation kit (Illumina Inc., San Diego, USA) according to the manufacturer's

recommendations. The 2 × 300 bp paired-end sequencing in 30-fold multiplexes was performed on the Illumina MiSeq (Illumina Inc., San Diego, USA) platform. Genome sequences were *de novo* assembled using SPAdes v3.12.0 (Nurk et al., 2013) and newbler v3.0 (Roche, Basel, Switzerland). The analysis of the nucleotide sequences was performed with Geneious v10.1.3 (Biomatters Ltd, Auckland, New Zealand). Rapid annotations using subsystem technology server (RAST) served for annotations (Aziz et al., 2008) which were compared with BLAST (National Center for Biotechnology Information, Rockville Pike, USA) results. Further investigations were performed by using ResFinder, PlasmidFinder and the *P. multocida* scheme of the multi-host Multi Locus Sequence Typing (MLST) tools of the Center for Genomic Epidemiology website (Davies et al., 2003; Larsen et al., 2012; Zankari et al., 2012; Carattoli et al., 2014). Alignments were performed using the Geneious alignment with default settings. The complete sequence of the plasmid from the *P. multocida* isolate IMT41689 was deposited in GenBank under accession number MH910619.1.

3. Results

3.1. Antimicrobial susceptibility and macrorestriction analysis

The species assignment *P. multocida* and the capsular type A were confirmed by PCR using the primer pairs described by Townsend et al. (1998, 2001). The MIC values of all *P. multocida* isolates as well as those of the transformed and original *E. coli* TOP10 are listed in Table 1. The *P. multocida* isolates showed streptomycin MICs of 4 mg/L (n = 3) and 8 mg/L (n = 6). *E. coli* TOP10 had a streptomycin MIC of ≥1024 mg/L.

The macrorestriction patterns obtained with *Apa*I and *Sma*I revealed enzyme-specific indistinguishable fragment patterns for the isolates from the same farm and differences between the patterns of the isolates of the three farms were limited to one to two fragments (Fig. 1). Further analysis with BioNumerics revealed that the *Apa*I pattern of the isolates obtained from Farm 1 showed a similarity of 92.4% when compared to those of the isolates of Farms 2 and 3 (Fig. 1). Moreover, the *Apa*I patterns of the isolates of Farms 2 and 3 were similar at 94.7%. However, isolates of Farms 1 and 3 were indistinguishable from one another in their *Sma*I macrorestriction pattern. Moreover, the *Sma*I restriction pattern of the isolates from Farms 1 and 3 showed a similarity of 95.7% to the isolates of Farm 2.

The MLST results of isolates IMT41689, IMT43698, IMT43844 and IMT45639, as extracted from the whole genome sequences, identified all of them as ST3.

3.2. Plasmid transformation and digestion

The alkaline lysis preparation revealed the presence of only one plasmid of approximately 6 kb in all isolates, except IMT41689, which also harboured a second smaller plasmid. The plasmids of isolates IMT41689, IMT43698, IMT43844 and IMT45639 were transformed into *E. coli* TOP10. The presence of the resistance gene *sul2* was confirmed by PCR (Kehrenberg and Schwarz, 2001) in all the tested transformants. Restriction analysis of the transformed plasmids revealed a single *Xba*I fragment of approximately 6 kb and two *Hin*dIII fragments of approximately 2 kb and 4 kb. The double digestion with *Xba*I and *Hin*dIII showed three fragments of approximately 1.1 kb, 2 kb and 2.9 kb for all transformed plasmids.

3.3. Sequence analysis

The WGS confirmed the expected number and size of plasmids in the original *P. multocida* isolates. A single plasmid was identified in each *E. coli* TOP10 transformant. All plasmids had a common size of 6050 bp and a nucleotide sequence identity of 99.9% according to the Geneious alignment. ResFinder results revealed the presence of the resistance genes *sul2* and *dfrA14* on the same or directly adjacent contigs in every isolate. Moreover, disrupted *strA* and *strB* genes were detected. PlasmidFinder analysis did not lead to the identification of any known Inc group in the sequences. Further sequence analysis displayed a G + C content of the whole *P. multocida* genome of approximately

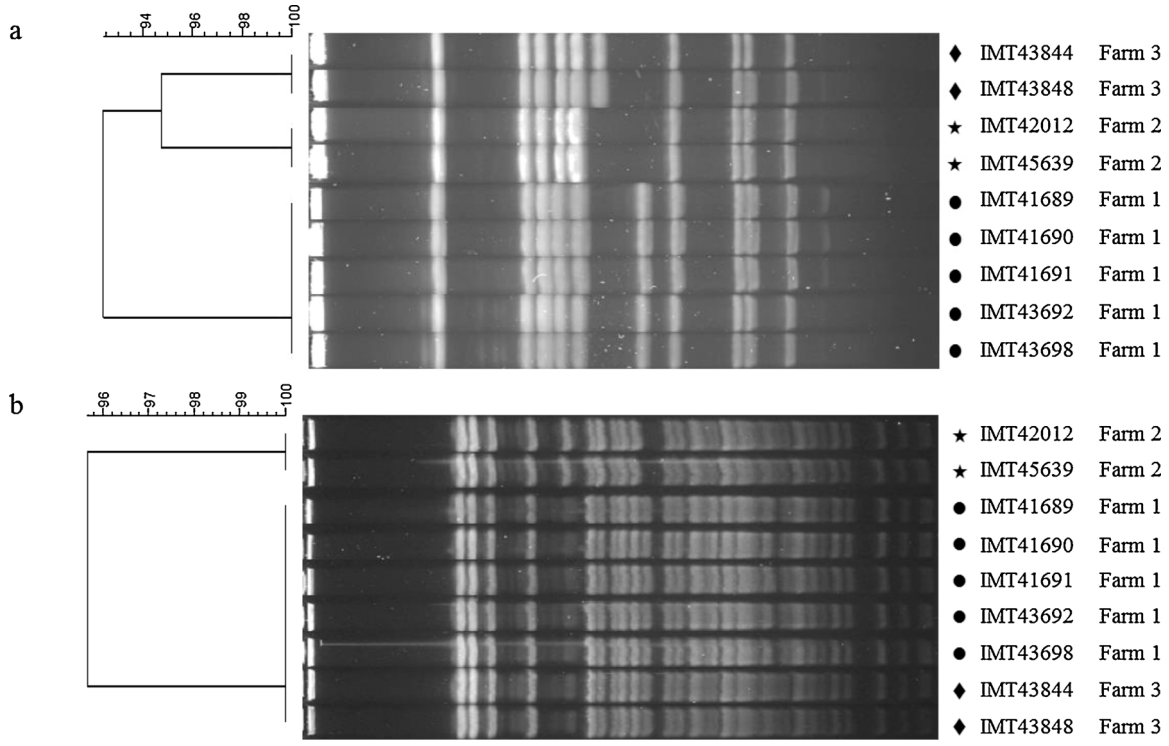

Fig. 1. PFGE macrorestriction patterns of the *P. multocida* isolates that originated from three different farms, digested with (a) *Apa*I and (b) *Sma*I. Cluster analysis was generated by UPGMA based on Dice coefficients.

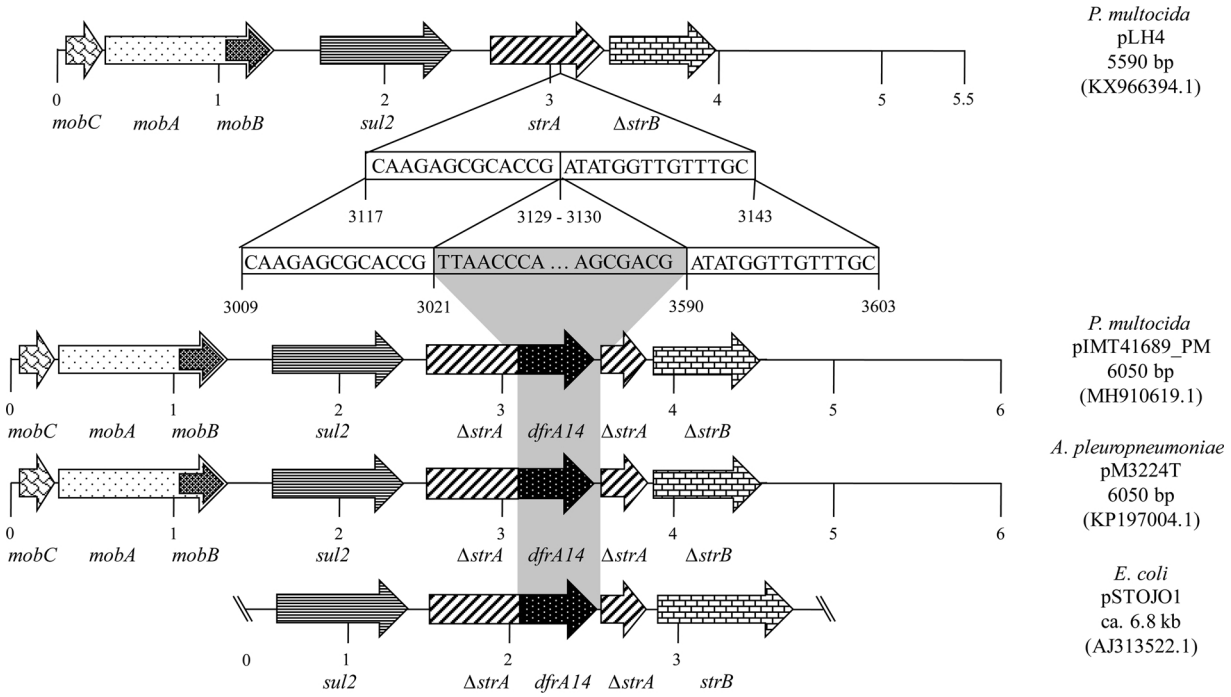

Fig. 2. Sequence alignment of the studied plasmid with similar plasmids identified in other species. The open reading frames of the genes *mobA, mobB, mobC, sul2, strA, dfrA14* and *strB* are shown as arrows, with the arrowhead indicating the direction of transcription, truncated genes are indicated by the Δ before gene names. The distance scale in kb is given below each map. Grey shaded background indicates the inserted gene cassette harbouring *dfrA14*.

40.2–40.4%, while plasmids showed a G + C content of 48.3%. The Geneious alignment analysis of the plasmid sequence revealed only one 99.9% identity match with the plasmid pM3224 T isolated from *Actinobacillus pleuropneumoniae* (Bossé et al., 2015). Another closely related plasmid identified by BLAST was the plasmid pLH4 from *P. multocida* isolate (GenBank accession no. KX966394.1), which, however, lacked the *dfrA14* gene.

In the plasmids of the present study, we identified the insertion of a gene cassette containing *dfrA14* within the *strA* gene that resulted in the disruption of the *strA* gene (Fig. 2). The *dfrA14* gene cassette had a size of 568 bp, coded for a DHFR of 157 aa, and harboured a typical 59-base element. Neither inverted nor direct repeats were found at the integration site. The high trimethoprim MIC of the transformants indicated that the *dfrA14* gene is functionally active. In addition, a complete sulfonamide resistance gene *sul2*, coding for a DHPS of 271 aa, was present upstream of the Δ*strA-dfrA14-*Δ*strA* segment. Moreover, a reading frame for a truncated 236-aa ΔStrB protein was detected immediately downstream of the 3′ end of Δ*strA* with the translational stop codon of Δ*strA* overlapping the translational start codon of Δ*strB*. In the plasmids of this study, three in part overlapping *mob* genes, *mobA*, *mobB*, and *mobC*, were identified, coding for proteins of 323 aa, 86 aa, and 101 aa, respectively (Fig. 2).

4. Discussion

Trimethoprim resistance is a rare resistance property in *P. multocida* (Michael et al., 2018). So far, only two trimethoprim resistance genes have been described in members of the genus *Pasteurella*, including *dfrA20* in *P. multocida* (Kehrenberg and Schwarz, 2005) and *dfrA1* in *Pasteurella aerogenes* (Kehrenberg and Schwarz, 2011). While *dfrA20* was located on an 11-kb plasmid, the *dfrA1* gene was found on a smaller plasmid of 5.4 kb and was part of a gene cassette located in a truncated class 2 integron. Trimethoprim resistance genes in Gram-negative bacteria are often part of gene cassettes, which commonly integrate into class 1 or class 2 integrons (Recchia and Hall, 1995). This is also true for the *dfrA14* gene. However, class 1 and class 2 integrons have rarely been found in members of the family Pasteurellaceae (Michael et al., 2018). Previous studies have shown that the *dfrA14* gene cassette is occasionally found to be integrated into a secondary site within the streptomycin resistance gene *strA* mainly in Enterobacteriaceae (Ojo et al., 2002; Kikuvi et al., 2007; Anantham and Hall, 2012; Huang et al., 2014). More recently, two small plasmids were identified in porcine *A. pleuropneumoniae*, which also carried a *dfrA14* gene cassette inserted into the *strA* reading frame. However, for *P. multocida*, the identification of a plasmid-borne Δ*strA-dfrA14-*Δ*strA* segment is a new observation. Immediately downstream of the 3′-end of the Δ*strA* gene a truncated Δ*strB* gene was found. The complete StrB protein has a size of 278 aa (Doublet et al., 2008), whereas in the plasmids identified in this study, the StrB protein was predicted with a size of 236 aa. The first 235 aa were identical to many StrB database entries (e.g. GenBank accession no. NG_047469.1) while codon 236 was already changed (threonin instead of prolin) and codon 237 represented a translational stop codon. However, it should be noted that StrB proteins of the same size and aa sequence as the ones found in the plasmids of this study have also been detected in *E. coli* (GenBank accession no. WP_069915930.1) and *Actinobacillus rossi* (GenBank accession no. SSX80666.1) during a BLAST search on GenBank.

The complete analysis of the 6050-bp plasmids in this study also revealed the presence of three *mob* genes coding for plasmid mobilization proteins. Mobilization enables a non-conjugative plasmid to use the conjugative transfer apparatus provided by a conjugative element in the same bacterial cell to move together with the conjugative element into a new recipient cell (Lawley et al., 2004). Among them, the 101-aa MobC protein sequence corresponded exactly to numerous MobC proteins previously described in members of the family Pasteurellaceae, including *P. multocida*, *Mannheimia haemolytica*, *A. pleuropneumoniae*, and *Haemophilus influenzae*. The 323-aa MobA protein is distinctly smaller than the 376-aa MobA proteins usually found among Pasteurellaceae. However, a 323-aa MobA protein with the same sequence has also been found in *A. rossi* (GenBank accession no. SSX80658.1). A similar situation was seen with the 86-aa MobB protein. In most Pasteurellaceae, the MobB protein has a size of 90 aa. However,

86-aa MobB proteins that were indistinguishable to the MobB proteins in our study have been described in "*Actinobacillus porcitonsillarum*" (Matter et al., 2008) and in *P. multocida* (San Millan et al., 2009). Whether these MobA and MobB proteins represent truncated versions or novel functionally active Mob proteins, remains to be answered. Moreover, the functionally active domains in these MobA and MobB proteins have not yet been elucidated and hence, it is questionable whether C-terminal deletions as observed in the plasmids of this study have an impact on the function of these proteins.

A closer analysis of the entire plasmid sequence revealed that our 6050-bp prototype plasmid pIMT41689_PM from porcine *P. multocida* isolated in 2016 in Germany corresponded in 99.9% of its nucleotide sequence to plasmid pM3224 T from porcine *A. pleuropneumoniae* isolated in 2007 in the UK (Bossé et al., 2015). The five single nucleotide polymorphisms between these two plasmids were all located in the non-coding region between the Δ*strB* gene and the *mobC* gene. This sequence comparison strongly suggests that these two plasmids have not developed independently. Most likely a transfer of such a plasmid between *A. pleuropneumoniae* and *P. multocida* has happened, although it is impossible to determine in retrospect where, when, in which direction and under which conditions this plasmid exchange has occurred. However, both pathogens (i) play a role in porcine respiratory disease, (ii) may occur together in the same diseased pig, and (iii) have to face a selection pressure by the use of antimicrobial agents including the combination sulfonamide/trimethoprim (Brockmeier et al., 2002t). In Germany, the most frequently described antimicrobial agents for respiratory tract diseases of pigs are β-lactams, tetracyclines and sulfonamides, the latter of which are only available in combination with trimethoprim (van Rennings et al., 2015). As such, all three key requirements for an efficient horizontal gene transfer are fulfilled, including the close contact of the bacteria in the respiratory tract, the location of the resistance genes on a mobile genetic element, e.g. a plasmid, and a selection pressure by the usage of antimicrobial agents. Small plasmids, such as pIMT41689_PM or pM3224 T, cannot move on their own from one bacterial host to another (Schwarz, 2008). However, natural competence and uptake of DNA is known to occur in members of the family *Pasteurellaceae* (Redfield et al., 2006), especially in the genera *Haemophilus* (Dai et al., 2018) and *Actinobacillus* (Bossé et al., 2014), but also in others, such as *Aggregatibacter* (Tanaka et al., 2012) and *Gallibacterium* (Kristensen et al., 2012). In contrast, phages that transduce plasmids within and/or beyond different genera within the family *Pasteurellaceae* have rarely been described (Willi et al., 1997). Mobilization in the presence of a conjugative element might be a good way by which small plasmids can be exchanged across genus boundaries. While conjugative plasmids have also only rarely been described in *P. multocida* and *A. pleuropneumoniae* (Michael et al., 2018), integrative and conjugative elements (ICEs) have been described in both species, including ICE*Pmu1* (Michael et al., 2012a, b), ICE*Apl1* (Bossé et al., 2016) and ICE*Apl2* (Li et al., 2018).

In the present study, we identified closely related *P. multocida* isolates that carried virtually the same resistance plasmid in three different pig-producing German farms. How these isolates have spread between the three farms remains to be clarified. Since all the sampled weaners were born in the respective farms, an import of piglets or weaners, that carried the *dfrA14*-positive *P. multocida*, from other breeding farms can be excluded. However, as all three farms were "open" farms that purchased and introduced replacement sows from at maximum two different suppliers, it is possible that the respective sows were colonized with the *dfrA14*-positive *P. multocida* isolates and then transferred on farm the *P. multocida* isolates with the *dfrA14*-carrying plasmids to their piglets. All three farms, but also the 27 remaining farms in the VASIB study, were under veterinary care by the same veterinarians. If the veterinarians were the vehicle of transfer of these isolates between the three farms, one may ask why these *P. multocida* isolates were not present in any of the other farms. To find out the mode(s) of transfer, a detailed analysis of multiple other factors, including the hygienic and the management conditions, in these three farms versus the other 27 farms will be necessary.

Conflict of interest statement

None to declare.

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