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Short communication

Genomic polymorphism of *Mycoplasma flocculare* revealed by a newly developed multilocus sequence typing scheme

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ABSTRACT

Mycoplasma flocculare is genetically closely related to *M. hyopneumoniae*, the etiologic agent of porcine enzootic pneumonia, and is frequently isolated with this second species. In this article, we report on the development of the first multilocus sequence typing (MLST) scheme for *M. flocculare*, based on three genes (*adk*, *rpoB* and *tpiA*). In total, 5022 bp of sequence were analyzed. MLST was used to characterize seven *M. flocculare* isolates and the reference strain. Eight distinct sequence types were defined, showing the great intraspecies variability of *M. flocculare,* and the high discriminatory power of the new typing method. The relative contribution of recombinations to the genomic evolution of *M. flocculare* was revealed by calculating the index of association (I_A: 0.0185). This MLST scheme is now available for the acquisition of new knowledge on *M. flocculare* epidemiology via an online database comprising the DNA sequences of each allele, available at http://pubmlst.org/mflocculare/.

1. Introduction

Mycoplasma (M.) flocculare is commonly described as a commensal agent of the porcine respiratory tract (Thacker and Minion, 2012). However, this mycoplasmal species can adhere to respiratory epithelium and cohabits in severe gross pneumonia-like lesions with *M. hyopneumoniae*, the etiological agent of enzootic pneumonia and the primary agent of the porcine respiratory disease complex (PRDC). The PRDC is a multifactorial disorder causing financial losses to the pig industry worldwide (Kobisch and Friis, 1996; Fourour et al., 2018). *M. hyorhinis*, also detected in cases of pneumonia, can induce polyserositis (pericarditis, pleuritis, peritonitis), arthritis, ear infections, conjunctivitis and sepsis and may be present in subclinical infections (Thacker and Minion, 2012). A recent study showed that *M. flocculare* and *M. hyorhinis* were found in extensive gross pneumonia-like lesions in association with *M. hyopneumoniae*, but their role is not well known (Fourour et al., 2018). *M. flocculare* and *M. hyopneumoniae* are closely related genetically, and share several virulence factors, raising the question of the potential role of *M. flocculare* as a PRDC agent (Paes et al., 2018). A comparative analysis between soluble secreted protein repertoires (secretome) of *M. hyopneumoniae* and *M. flocculare* strains allowed to identify 12 putative virulence factors in *M. hyopneumoniae* secretome and four putative virulence factors in *M. flocculare* secretome, with two virulence factors in common (Paes et al., 2017). Very recently, this mycoplasmal association appeared to induce an additive effect and to increase the inflammatory status of pigs, possibly involving impairment of the immune system (Fourour et al., 2019).

M. flocculare can be isolated from tracheal and bronchiolar mucus, but isolation and identification are tedious and time consuming. The difficulty in culturing has led to the development of other diagnostic assays, including PCR tests (Stakenborg et al., 2006; Fourour et al., 2018). No typing method has been developed to date for *M. flocculare* strains. Only the comparison of 16S rRNA genes was performed from four strains and showed a certain genomic diversity (Yamaguti et al., 2015). Multilocus sequence typing (MLST) scheme is an unambiguous method currently regarded as the gold standard for typing, able to replace even pulsed-field gel electrophoresis (PFGE) analysis (Gevers et al., 2005). MLST schemes already published have been used

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successfully to characterize *M. hyopneumoniae* and *M. hyorhinis* and demonstrated a great intraspecies diversity (Mayor et al., 2008; Tocqueville et al., 2014; Kuhnert and Overesch, 2014; Trüeb et al., 2016; Michiels et al., 2017; Felde et al., 2018). This method is based on the nucleotide sequences of housekeeping genes, in which mutations are assumed to be largely neutral (Selander et al., 1986). Isolates that share the same sequence type (ST) are assumed to be members of the same clone (Selander et al., 1986), i.e., they have a recent common ancestor. Data obtained can be used to address questions about the evolutionary and population biology of bacterial species (Feil et al., 1999). Moreover, it appears that the MLST scheme can also be used to relate *M. hyorhinis* strains, according to the pathology observed in pig, to a particular phylogenetic group (in addition to providing data on genetic diversity), even though the targets are housekeeping genes (Tocqueville et al., 2014). In this article, we discuss development of the first typing method for *M. flocculare*, an MLST scheme. We also report on findings concerning genomic polymorphism in this mycoplasmal species, obtained with the new method.

2. Materials and methods

One reference strain (ATCC 27399) and seven isolates obtained from seven farms in 2016 (Table 1) were used. All isolates were cultured from lung specimens in Friis liquid medium at 37 ± 2 °C (Friis, 1975). The isolates derived from a single colony, cloned twice maximum, and the purity was ensured by multiplex qPCR (Fourour et al., 2018).

DNA was extracted from a 12 mL culture with the QIAamp® DNA Mini Kit (Qiagen, Courtaboeuf, France), and quantified with a Qubit® 2.0 fluorometer (Invitrogen, Paris, France). DNA was sheared by sonication using a Bioruptor® Plus (Diagenode) apparatus. Libraries were prepared using a NEBNext® Ultra DNA library Prep Kit for Illumina® and NEBNext® Multiplex Oligos for Illumina®, according to the manufacturer's instructions (New England Biolabs, Evry, France). Size selection and purification steps were conducted with magnetic beads (Agencourt AMP pure XP system; Beckman-Coulter, Villepinte, France). Sequencing was performed using MiSeq Illumina technology (paired-end sequencing 2 × 150 cycles, MiSeq Reagent kit v2-300 Cycles, Illumina). Reads were cleaned with Trimmomatic 0.36 (Bolger et al., 2014) (ILLUMINACLIP:oligos.fasta:2:30:5:1:true LEADING:3 TRAILING:3 MAXINFO:40:0.2 MINLEN:36). Cleaned reads were then aligned versus known references (AFCG01000001.1 to AFCG01000014.1) with bwa (0.7.15-r1140, Li and Durbin, 2009). Reads were down-sampled to fit a global coverage estimation of 80×, cleaned with Trimmomatic (same parameters as before), and submitted to SPAdes (v3.10.0, Nurk et al., 2013). The *de novo* contigs were then submitted to MEGABLAST (2.2.26, Chen et al., 2015) on a local copy of the NCBI "Nucleotide" databank (nt). The ordered set of contigs was obtained using MauveReorder (Rissman et al., 2009) on CP007585.1 reference genome. The next step was to annotate the draft genome using the Rapid Annotation using Subsystem Technology (RAST) server with genetic code 4 (Mold, Protozoan, and Coelenterate Mitochondrial Code and the Mycoplasma/Spiroplasma Code) (Aziz et al., 2008). The contigs shorter than 200 bp were removed from the analysis. The *Mycoplasma* species of each strain was verified with the primer sequences described by Fourour et al. (2018) in the insilico.ehu.es website.

Table 1
Main characteristics of the eight *M. flocculare* (MF) strain/isolates and sequence types identified by MLST.

Isolate	Mycoplasmal combinations[a]	Mean pneumonia score (/28)[b]	ST profile[c]	ST[d]
ATCC[e]_27399	NK[f]	NK	1,1,1	1
MF11	MHP/MHR/MF	4.9	2,2,2	2
MF12	MF/MHR	2	4,6,3	3
MF18	MHP/MHR/MF	8.9	4,3,4	4
MF22	MF/MHP	2.3	3,7,5	5
MF29	MHP/MHR/MF	7.3	5,4,6	6
MF30	MF/MHR	1.4	2,8,7	7
MF33	MF/MHP	6.3	6,5,8	8

[a] Mycoplasmal combination observed in pig lungs at the farm level (Fourour et al., 2018). MHP: *M. hyopneumoniae*, MHR: *M. hyorhinis*.

[b] Mean score of gross pneumonia-like lesions by farm, estimated as previously described by Madec and Kobisch (1982).

[c] Results of *M. flocculare* typing obtained with the complete genes *adk*, *rpoB*, and *tpiA* using the MLST scheme described in this study.

[d] ST: sequence type.

[e] ATCC: American Type Culture Collection, Rockville, USA.

[f] NK: Not known.

The new MLST scheme was developed for *M. flocculare* using the complete genes *adk*, *rpoB* and *tpiA* (Table 2). We chose to work on these three genes alone because they have been used successfully for the development of MLST *M. hyopneumoniae* scheme. These housekeeping genes were searched with blastn (NCBI BLAST + blastn). For each *M. flocculare* strain (including the reference strain ATCC 27399, Accession: CP007585.1 and the seven isolates of this study), the alleles at each of the three loci (e.g. 2,8,7) defined the allelic profile or ST (e.g. ST7) (Calcutt et al., 2015). The STs were assigned arbitrary numbers in order of description. The allelic profile of *M. flocculare* ATCC 27399 is 1,1,1 (ST1). A web-accessible database has been set up for the MLST scheme for *M. flocculare* at http://pubmlst.org/mflocculare/ (Jolley and Maiden, 2010). The number of nucleotide polymorphic sites was determined by using specially designed software, BIGSdb (http://pubmlst.org/software/database/bigsdb/) (Jolley and Maiden, 2010). The degree of clonality within the data set was estimated by calculating the index of association (I_A). The standardized I_A was used to test the null hypothesis of linkage equilibrium for multilocus data, and therefore determine the relative contribution of mutation and recombination to the diversity seen by MLST. An I_A value significantly different from 0 indicates that a population is clonal (linkage disequilibrium), while I_A equal to zero indicates a recombining population structure (linkage equilibrium). Analysis was performed with the LIAN program (http://guanine.evolbio.mpg.de/cgi-bin/lian/lian.cgi.pl) by using 10,000 resampling of the data. The calculation of H (representing genetic diversity) was also performed with LIAN. To investigate whether positive or negative selection had occurred at the protein level, the average non-synonymous/synonymous substitution rate ratio (dN/dS) was calculated using START2 software (https://pubmlst.org/software/analysis/start2/webstart/start2.jnlp). Low ratios indicate a lack of or a very limited contribution of environmental selection to the sequence variation in the housekeeping genes analyzed, which are thus assumed to be suitable for a population genetics study. DnaSP genetic software, version 5.10.01 was used to calculate Tajima's D statistic in order to test the neutrality of the observed DNA polymorphisms (http://www.ub.es/dnasp/software/dnasp51001.msi).

The MLST sequences concatenated head-to-tail of each strain were aligned and one phylogenetic tree was drawn on "Phylogeny.fr" (http://phylogeny.lirmm.fr/phylo_cgi/index.cgi) with the "One Click" mode. BURST analysis was carried out to reveal the relationships between MLST sequence types, and to analyze clonal complexes (http://pubmlst.org/analysis/), then to (i) divide strains into groups according to their allelic profiles, and (ii) count the number of single locus variants (SLVs), double locus variants (DLVs), and satellites (SATs) for each ST, and identify the potential ancestral type (AT). The original BURST algorithm was previously developed (Feil et al., 2004). The version used was adapted as a plugin for BIGSdb database software by Keith Jolley (http://pubmlst.org/software/database/bigsdb/) (Jolley and Maiden, 2010).

The relationships between ST patterns, ST phylogenetic groups, and BURST groups detected in this study and the mycoplasmal combinations or pneumonia-like macroscopic lesion scores observed on the origin farms (Fourour et al., 2018) were analyzed. The statistical

Table 2

Characteristics of the three genes used in the *M. flocculare* (MF) typing scheme.

Locus	Putative function of gene product	Position[a]	Seq. size (bp)	No. of alleles identified	No. of polymorphic nucleotide sites (%)	Genetic diversity (H)	d_N/d_S[b]	Tajima's D[c]
adk	Adenylate kinase	555204	639	6	6 (0.94)	0.9286	0.1482	−0.351
rpoB	RNA polymerase β-subunit	675017	3654	8	44 (1.20)	1.0000	0.0306	−0.126
tpiA	Triose-phosphate isomerase	120812	729	8	10 (1.37)	1.0000	0.0164	0.459

[a] Position in MF ATCC 27399 (accession CP007585.1).

[b] Ratio of nonsynonymous to synonymous mutations.

[c] Tajima's D, none of the values significantly deviated from zero ($p > 0.10$).

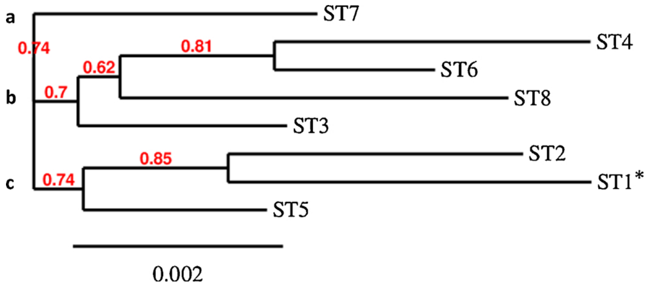

Fig. 1. Genetic relationships between seven *M. flocculare* STs and the reference ST1* (strain *M. flocculare* ATCC 27399), as estimated by clustering analysis of STs revealed by MLST, with loci of genes *adk*, *rpoB* and *tpiA* concatenated (5,022 bp) for *M. flocculare*. The phylograms were constructed using tools at www.phylogeny.fr and the "one click" mode. Bootstrap support values are in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

analyses were performed using the Fisher exact test ($n \le 5$) on independence in two-by-two tables (https://marne.u707.jussieu.fr/biostatgv/?module=tests/fisher). Differences were considered to be significant when $p < 0.1$.

3. Results and discussion

The assemblies of *M. flocculare* isolates yielded 29 to 71 contigs, covering a total of 739,183 to 782,983 bp (mean genome size, 762,578 ± 13,290 bp), with an N_{50} value of 67,566 to 123,263 bp (mean N_{50} value, 82,858 ± 18,873 bp), an average coverage of 74.8× to 93.4× (mean coverage, 83.8 ± 7.7×), and an overall G + C content of 29.0% to 29.6% (mean G + C content, 29.1 ± 0.2%). The seven *M. flocculare* isolates yielded 620 to 669 coding sequences (CDSs), whereas the previously published *M. flocculare* ATCC 27399 genome contained only 585 CDSs (Siqueira et al., 2013). One hypothesis would be that this difference might be related to the presence of more CDSs encoding, for example, virulence factors in our current isolates. The reference strain cultivated many times on culture medium may have lost non-essential genetic elements for *in vitro* growth, while these elements were essential to the colonization of the animal. For our seven *M. flocculare* isolates and the reference strain ATCC27399, the discriminatory ability of the different loci, measured as number of alleles, varied from six (*adk*) to eight (*rpoB* and *tpiA*) (Table 2). The genetic diversity (H) obtained from the three genes varied from 0.9286 (*adk*) to 1.000 (*rpoB* and *tpiA*) (Table 2). The standardized I_A was calculated at 0.0185. Most polymorphisms resulted in nonsynonymous substitutions, with the ratio of nonsynonymous to synonymous substitutions (dN/dS) varying from 0.0164 (for *tpiA*) to 0.1482 (for *adk*), and Tajima's D values varying from -0.351 (for *adk*) to 0.459 (for *tpiA*) (with none of these values significantly deviating from zero, $p > 0.10$). These values show that the genetic variation was roughly in linkage equilibrium, and seemed to be due to recombination (with mutation playing a much smaller role) (Haubold et al., 1998). Homologous recombination was also high for *M. hyopneumoniae* and *M. hyorhinis* (Mayor et al., 2008; Tocqueville et al., 2014). In *M. hominis*, the frequency of recombination is not correlated with the level of variability, and recombination does not induce more variation in the genes but rather shuffles the existing mutations, thereby creating new alleles (Søgaard et al., 2002). Recombination in *M. hyopneumoniae*, *M. hyorhinis* and *M. flocculare* is particularly interesting since no phages or plasmids have been described in these species. However, for *Mycoplasma* species sharing the same hosts, a significant number of genes undergoing horizontal transfer have been described (Sirand-Pugnet et al., 2007).

This MLST scheme of three housekeeping genes, *adk*, *rpoB* and *tpiA* (concatenated 5022 nt) was able to type seven *M. flocculare* isolates and the *M. flocculare* reference strain and to identify eight distinct sequence types, showing the great intraspecies variability of *M. flocculare*, and offering a very highly discriminatory typing method. The MLST scheme for *M. hyopneumoniae* was also based on the same three housekeeping genes (Mayor et al., 2008). This simple scheme has been found to be sufficient for epidemiological investigations of *M. hyopneumoniae*, and also seems to be sufficient for *M. flocculare*. However, more strains should be typed to confirm these preliminary results. Our MLST scheme confirms that sequence variations occur within housekeeping genes, which indicates that the core genome is also variable.

The genetic relationships between the eight *M. flocculare* STs are shown in the phylogram in Fig. 1. Three ST groups, named "a" to "c", were identified. The BURST analysis revealed two groups of two *M. flocculare* isolates, BURST groups A and B. BURST group A was composed of DLVs ST2 and ST7. BURST group B was composed of DLVs ST3 and ST4. ST1, 5, 6 and 8 were not linked to groups and were singletons. The BURST analysis did not highlight a clonal complex for *M. flocculare* strains, whereas several clonal complexes were identified for *M. hyopneumoniae* and *M. hyorhinis* (Mayor et al., 2008; Tocqueville et al., 2014; Kuhnert and Overesch, 2014; Trüeb et al., 2016; Michiels et al., 2017; Felde et al., 2018). *Helicobacter pylori* and *Staphylococcus aureus* populations, also with rich histories of interstrain recombinations, are structured in clonal complexes (Achtman et al., 1999; Day et al., 2001). The analysis of more *M. flocculare* isolates should make it possible to determine whether such clonal structures are found in this species.

This is the first time that *M. flocculare* strains isolated from pneumonia have been typed. All *M. flocculare* isolates from farms with a mean gross pneumonia-like lesions score above 5/28 are in the same ST group (group "b") (Table 3), showing genomic homogeneity. However, with a p value of 0.142, this result must be confirmed. No relationships between the combination of *M. flocculare* with *M. hyopneumoniae* and/or *M. hyorhinis* and ST groups or BURST groups were observed ($p > 0.1$) (Table 3).

An online database for the *M. flocculare* MLST scheme, comprising DNA sequences of each allele, was developed and is available in Pubmlst (http://pubmlst.org/mflocculare/). New data concerning *M. flocculare* genomes, obtained or not by next generation sequencing, can now be included in the database for comparison between laboratories. This genomic approach, certainly coupled with other omics techniques (proteomic, secretomic), will facilitate epidemiological investigations and the acquisition of new knowledge about the porcine respiratory disease complex, in connection with the presence of *M. flocculare*.

Table 3
Distribution of ST patterns, ST groups, and BURST groups in relation to *M. flocculare* isolate origins.

	n	Mycoplasmal combinations[a]				Pneumonia-like lesion score[b]		
		MHP/MHR/MF	MHP/MF	MHR/MF	NK[c]	≤5/28	> 5/28	NK[c]
No. of strains/isolates	8	3	2	2	1[d]	4	3	1[d]
No. of STs	8	3	2	2	1	4	3	1
No. of strains/isolates in ST groups								
a	1	0	0	1	0	1	0	0
b	4	2	1	1	0	1	3	0
c	3	1	1	0	1	2	0	1
No. of strains/isolates in BURST groups								
group 1	2	1	0	1	0	2	0	0
group 2	2	1	0	1	0	1	1	0
singletons	4	1	2	0	1	1	2	1

[a] Mycoplasmal combinations observed on the farms of origin (Fourour et al., 2018).

[b] Mean score of gross pneumonia-like lesions by farm, estimated as previously described by Madec and Kobisch (1982).

[c] NK: not known.

[d] *M. flocculare* strain ATCC 27399 with genome available in GenBank (accession CP007585.1).

Authors' contributions

The experiments were designed by SF, IK and CMC. The experiments were performed by SF and VT. The data were analyzed by SF, PL, FT and CMC. Reagents/materials/analysis tools were contributed by SF, VT, IK and CMC. Contributions to the writing of the manuscript were made by SF, PL, FT, VT, GBAV, IK and CMC.

Declaration of Competing Interest

The authors have no conflicts of interest.

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