NOTE

A physical and genetic map of the *Mycoplasma flocculare* ATCC 27716 chromosome reveals large genomic inversions when compared with that of *Mycoplasma hyopneumoniae* strain J^T

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Pulsed-field gel electrophoresis and DNA hybridization data were used to construct a chromosomal map of *Mycoplasma flocculare* ATCC 27716, a non-pathogenic inhabitant of porcine respiratory tracts. Twenty-one genetic markers were placed on the map. Comparison of the genetic map with that of the closely related *Mycoplasma hyopneumoniae* strain J^T, the type strain of the causative agent of enzootic pneumonia in pigs, identified three chromosomal inversions that differentiate these genomes. One of these inversions involves genes that possibly may be involved in *M. hyopneumoniae* pathogenicity.

Keywords: *Mycoplasma flocculare*, *Mycoplasma hyopneumoniae*, physical map, genetic map, inversions

Mycoplasma hyopneumoniae and *Mycoplasma flocculare* are closely related members of the class Mollicutes (Stemke *et al.*, 1992) that inhabit the respiratory tracts of swine. *M. hyopneumoniae* is the causative agent of enzootic pneumonia in pigs, a chronic and highly transmissible disease found in swine herds worldwide, while *M. flocculare* is generally regarded as a commensal organism (Kobisch & Friis, 1996). The genome of *M. hyopneumoniae* was found to be larger (1070 compared with 890 kb) by pulsed-field gel electrophoresis (Robertson *et al.*, 1990) and its pathogenicity is presumably facilitated by genes that are absent in *M. flocculare*. Comparison of detailed maps of the chromosomes of both species could identify chromosomal rearrangements and deletions that contain genes with potential as therapeutic or vaccination targets. Physical maps of the genomes of *M. hyopneumoniae* ATCC 25095 and *M. flocculare* ATCC 27716 and the placement of the separated 5S rRNA and 16S–23S rRNA loci on those maps have been published (Huang & Stemke, 1992). We recently reported the construction of a physical map and partial overlapping cosmid library of the genome of *M. hyopneumoniae* strain J^T (the type strain of the species) with the placement of 42 genetic markers upon that

map (Blank & Stemke, 2000). In this study, we present an improved physical and genetic map of the *M. flocculare* ATCC 27716 chromosome and compare this map with that of *M. hyopneumoniae* strain J^T.

Construction of the *M. flocculare* genomic map

Intact *M. flocculare* chromosomes were prepared, digested with restriction endonucleases and separated by contour-clamped homogeneous electric field (CHEF) electrophoresis as described previously (Blank & Stemke, 2000). Digestion of *M. flocculare* chromosomes by the restriction endonucleases *Apa*I, *Asp*718 and *Sal*I yielded a moderate number of relatively high molecular mass restriction fragments (Fig. 1; Table 1). Fragments were named according to the restriction enzyme that created them and the relative size of the fragment. For example, the largest *Asp*718 band was named As1. Relative band fluorescence intensities and later hybridization data indicated that band Sa5 contained three fragments of similar size; these fragment names are distinguished by the letters a, b and c. Sizes of the bands were determined by averaging the measurements from several gels. The size of the *M. flocculare* genome, as determined by averaging the totals from each of the restriction digests, was determined to be approximately 900 kb; this is in agreement with previous reports (Robertson *et al.*, 1990).

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Abbreviation: CHEF, contour-clamped homogeneous electric field.

Fig. 1. CHEF gel electrophoresis of digested *M. flocculare* DNA. Genomic DNA was digested with *Apal* (lane 1), *Asp718* (lane 2) and *Sall* (lane 3). Pulse times for this gel were 2–15 s over 40 h at 165 V.

The order of the restriction fragments from each digest on the *M. flocculare* chromosome was determined by examining hybridization (and therefore overlap) between individual fragments from different digests (Table 1). Individual macrorestriction fragments were purified from gels, labelled and hybridized to Southern blots of CHEF gels as before (Blank & Stemke, 2000). Based upon the overlapping restriction fragments, a physical map of the circular *M. flocculare* chromosome was deduced (represented linearly in Fig. 2). The precise order of fragments As6 and As7 could not be determined because both were contained entirely within two large fragments, Ap1 and Sa1. The genetic markers listed in Table 2 were also used to probe blots of digested *M. flocculare* DNA; their locations are indicated in Fig. 2. The origin of chromosomal replication (*oriC*), as indicated by the *dnaA* gene, was located to the region overlapped by fragments Ap3, As4 and Sa5c. The direction of transcription of the 16S–23S rRNA operon (Stemke *et al.*, 1994) could be determined since the 16S probe hybridized with fragment As4, while the 23S probe hybridized to As8.

Table 1. *M. flocculare* restriction fragment size and hybridization data

Fragment	Size (kb)	Fragments hybridized
<i>Apal</i>		
Ap1	220	As2, As3, As6, As7, Sa1, Sa5, Sa7
Ap2	125	As2, As3, Sa2, Sa5
Ap3	105	As4, As5, As8, Sa5, Sa6
Ap4	95	Sa4, Sa5
Ap5	85	As3, Sa1, Sa2, Sa4, Sa5
Ap6	80	As1, Sa3, Sa5
Ap7	70	As1, Sa3, Sa4
Ap8	60	As1, Sa5, Sa8
Ap9	40	As1, Sa2, Sa5, Sa8
Ap10	20	As2, As5, Sa5
Total	900	
<i>Asp718</i>		
As1	400	Ap2, Ap4, Ap6, Ap7, Ap8, Ap9, Sa2, Sa3, Sa4, Sa5, Sa8
As2	155	Ap1, Ap10, Sa1, Sa5, Sa7
As3	140	Ap1, Ap2, Ap5, Sa1, Sa2, Sa4, Sa5
As4	60	Ap3, Sa5, Sa6
As5	55	Ap3, Ap10, Sa5, Sa6
As6	45	Ap1, Sa1
As7	30	Ap1, Sa1
As8	15	Ap3, Sa6
Total	900	
<i>Sall</i>		
Sa1	220	Ap1, Ap5, As2, As3, As6, As7
Sa2	165	Ap2, Ap5, Ap9, As1, As3
Sa3	90	Ap6, Ap7, As1
Sa4	85	Ap4, Ap7, As1
Sa5*	75	Ap1, Ap3, Ap4, Ap5, Ap6, Ap8, Ap10, As1, As2, As3, As4, As5
Sa6	60	Ap3, As4, As5, As8
Sa7	30	Ap1, As2
Sa8	30	Ap8, Ap9, As1
Total	905	

* Triplet band.

Comparison of the *M. flocculare* and *M. hyopneumoniae* maps

Conservation of rare restriction sites between the two organisms was not apparent. Exceptions to this are the conserved *Asp718* sites in the 16S and 23S genes of both species (GenBank accession numbers E02783, X68421 and L22210). The 5S rRNA gene of *M. flocculare* is separated from the 16S–23S rRNA operon, as in *M. hyopneumoniae* (Taschke *et al.*, 1986). Conservation of the *recA*, *ptsII* and *glyT* genes near the 5S gene in both organisms indicates that this separation is ancestral.

Unfortunately, attempts at probing the *M. flocculare* genome with the ordered cosmid library of *M. hyopneumoniae* strain J^T were unhelpful for genomic comparison, most probably due to the low level (10%)

Fig. 2. Physical and genetic map of the *M. flocculare* genome, as determined by CHEF electrophoresis of macrorestriction fragments and Southern hybridization. Individual restriction fragments are represented by rectangles; horizontal lines are ambiguity bars covering the area within which each element is located. Multiple elements under one ambiguity bar are not listed in any particular order. Refer to Table 2 for identification of gene probes. Arrow indicates direction of transcription of rRNA operon.

Table 2. Hybridization of genetic probes with *M. flocculare* macrorestriction fragments

Probes are of *M. hyopneumoniae* origin unless indicated otherwise. Sources: Blank & Stemke (2000) and references therein.

Probe	Gene product	Fragments hybridized
23S rRNA	—	Ap3, As8, Sa6
16S rRNA	—	Ap3, As4, Sa6
5S rRNA	<i>M. flocculare</i> 5S rRNA	Ap4, As1, Sa4
<i>dnaA</i>	Chromosomal replication initiator protein	Ap3, As4, Sa5
<i>glyT</i>	<i>Mycoplasma mycoides</i> glycine-tRNA	Ap4, As1, Sa4
<i>hsp60</i>	60 kDa heat-shock protein	Ap6, As1, Sa5
IS1221	Insertion sequence 1221	Ap2, As1, Sa2
<i>ldh</i>	Lactate dehydrogenase	Ap6, As1, Sa5
<i>nrpF</i>	Ribonucleotide reductase R2 subunit	Ap6, As1, Sa5
<i>p46</i>	46 kDa surface antigen	As6
<i>ptsII</i>	Phosphotransferase system enzyme II	Ap4, As1, Sa4
<i>pyk</i>	Pyruvate kinase	Ap6, As1, Sa5
<i>recA</i>	DNA recombinase A	Ap4, As1, Sa4
<i>rpoB</i>	RNA polymerase beta subunit component	Ap3, As4, Sa5
<i>samS</i>	S-Adenosyl-methionine synthetase	Ap2, As3, Sa2
<i>secA</i>	Preprotein translocase	Ap1, As2, Sa1
<i>tuf</i>	<i>M. pneumoniae</i> elongation factor Tu	As6
<i>valS</i>	Valyl-tRNA synthetase	Ap3, As5, Sa5
B5-1b	Putative permease	Ap2, As1, Sa2
B7-1b	—	Ap2, As1, Sa2
13-1a	<i>M. hyopneumoniae</i> repetitive element	Ap1, As2, Sa1

of interspecies DNA–DNA hybridization (Stemke *et al.*, 1985). Comparison of the gene arrangements on the two maps revealed that at least three chromosomal inversions have apparently occurred since the two species diverged. One relatively small inversion (Fig. 3) would involve the *oriC* region (as indicated by the *dnaA* gene) and the 16S–23S rRNA operon and was

flanked by the nearby *rpoB* and *valS* genes. An inversion containing the origin of replication itself would preserve the orientation of these genes relative to the direction of replication, thereby minimizing interference between DNA and RNA polymerases, particularly at highly transcribed loci such as the 16S–23S rRNA operon.

Fig. 3. Schematic diagram of a cross-over event leading to chromosomal inversion and the predicted gene arrangement in the *oriC* region of *M. hyopneumoniae* (Mhp) and *M. flocculare* (Mf). Arrows indicate directions of transcription of the rRNA operons. Note that the actual orders of *dnaA* and *valS* in *M. hyopneumoniae* and of *dnaA* and *rpoB* in *M. flocculare* are ambiguous from the hybridization data.

Two overlapping large inversions can also be deduced by comparing the two genetic maps. Fig. 4 illustrates the genomic organization of a hypothetical intermediate or ancestor of *M. hyopneumoniae* and *M. flocculare* and indicates the inversions that may have occurred since the two species diverged. One involves a region of the *M. flocculare* genome contained in fragments Ap4, Ap7 and Ap6, which could be up to 245 kb in size and includes the genes mapping near the 5S rRNA gene as well as *nrdF* and *ldh*. The second inversion covers up to 370 kb of the *M. hyopneumoniae* genome and again involves the origin of replication region as well as some of the genes involved in the previously mentioned inversion. It cannot be determined from this study which of the inversions occurred first, but genomic analysis of another relatively closely related organism such as *Mycoplasma hyorhinis* (Stemke *et al.*, 1992) may provide clues.

The gene for the ciliary adhesin (*adh*), which is believed to be a virulence factor in *M. hyopneumoniae* (Hsu &

Minion, 1998), was not detected by hybridization in *M. flocculare* in this study, nor was it identified earlier in either *M. flocculare* or *M. hyorhinis* (Wilton *et al.*, 1998). In *M. hyopneumoniae*, the adhesin gene mapped within approximately 100 kb of the genes for lactate dehydrogenase and nucleotide reductase, both strongly antigenic proteins for that organism. In addition, Hsu & Minion (1998) have described an open reading frame encoding a protein of 102 kDa that is part of an operon with *adh* and may be present in four copies in the same region. This suggests a cluster of genes that may be involved in the pathogenicity of *M. hyopneumoniae* and could potentially provide antigenic variation through recombination among the repeated genes, a mechanism seen in other mycoplasmas (Dybvig & Voelker, 1996). This region is part of the largest inversion in the genome: it may have been lost during the chromosomal rearrangement. The distance of *pyk* and *ldh* from *nrdF* and *hsp60* appears to be about 150 kb in *M. hyopneumoniae* (Blank & Stemke, 2000) but is less than 75 kb (the overlap between Ap6 and Sa5c) in *M. flocculare*. This could represent the major difference between *M. hyopneumoniae* and *M. flocculare* in terms of both genome size and pathogenicity. Although no other obvious, large regions of deletion could be observed in *M. flocculare* in this study, attrition of other, presently uncharacterized individual genes could explain further the differences in genome size and pathogenicity between the two species. Even the intergenic distance between the 16S and 23S genes differs between the two species; that of *M. flocculare* is 105 bp smaller than that of *M. hyopneumoniae*, although the remaining sequence is highly conserved (Stemke *et al.*, 1994).

Chromosomal rearrangements are generally the result of homologous recombination between repeated sequences within the genome (Mahan *et al.*, 1990). The

Fig. 4. Schematic diagram of large chromosomal inversions in *M. hyopneumoniae* (Mhp) and *M. flocculare* (Mf), with a hypothetical ancestor or intermediate chromosome. Maps were linearized at the conserved region containing the IS1221 element. 16S–23S rRNA and *dnaA* genes are indicated as *oriC* in the hypothetical chromosome since the gene order cannot be predicted. Grey bars indicate inverted regions and the shaded area has been involved in two inversions. *M. hyopneumoniae* repetitive elements *repJ* (●), B28-1b (■) and 13-1a (▲) are also indicated. *M. hyopneumoniae* gene arrangement adapted from Blank & Stemke (2000).

fully sequenced genomes of *Mycoplasma pneumoniae* and *Mycoplasma genitalium*, which are closely related, can be divided into six segments in which gene organization is very highly conserved, although these segments are arranged differently (Himmelreich *et al.*, 1997). These rearrangements are believed to have been mediated by homologous recombination between repetitive DNA sequences flanking each segment. While some mollicutes may lack this activity (Marais *et al.*, 1996), the presence of the gene for the site-specific recombinase RecA in both *M. hyopneumoniae* and *M. flocculare* indicates that homologous recombination is possible in these organisms. At least three different types of repetitive elements exist in *M. hyopneumoniae* strain J^T (Blank & Stemke, 2000): five copies of one element, designated *repJ*; two copies of another type of element, B28-1b, which appear to be associated with two of the copies of *repJ*; and a third element, 13-1a, also present in two copies. *M. flocculare* may contain additional repetitive elements, as suggested by the mutual hybridization of the non-overlapping fragments Ap5 and Sa5. The two 13-1a elements in *M. hyopneumoniae* are located close to the ends of one of the inversions (Fig. 4), although one is inside the region, with *secA* defining the left-most boundary. Only one copy of 13-1a is present in *M. flocculare*. If recombination between the 13-1a elements has occurred, a more recent, smaller inversion could have switched the positions of *secA* and 13-1a in *M. hyopneumoniae*, with the second copy being deleted in *M. flocculare*. Attempts to detect *repJ* and 28-1b in the *M. flocculare* genome were unsuccessful. However, once the two large inversions are accounted for, the order of genes mapping near these elements in *M. hyopneumoniae* remains conserved in *M. flocculare*, suggesting that they have not been involved in recombination activity since the two species diverged.

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