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Short Communication

Insertion sequences as variability generators in the *Mycoplasma hyopneumoniae* and *M. synoviae* genomes

Elgion Lúcio Silva Loreto[1], Mauro Freitas Ortiz[2] and Jorge Ivan Rebelo Porto[3]

[1]*Departamento de Biologia, Universidade Federal de Santa Maria, Santa Maria, RS, Brasil.*
[2]*Curso de Ciências Biológicas; Universidade Federal de Santa Maria, Santa Maria, RS, Brasil.*
[3]*Coordenação de Pesquisas em Biologia Aquática, Instituto Nacional de Pesquisas da Amazônia, Manaus, AM, Brasil.*

Abstract

We have analyzed the sequenced genomes of three strains of *Mycoplasma hyopneumoniae* and one strain of *M. synoviae*, and have found three and two different transposable element families, respectively in each species. In *M. hyopneumoniae,* the Insertion Sequences of the IS4 family is represented by ISMHp1, a putatively active element. The IS3 family is represented by several degenerated sequences. A third element called tMH was found, which shows some characteristics reminiscent of retrotransposons. In *M. synoviae,* three different possibly active IS4 elements are present (ISMHp1-like; ISMs1 and IS1634-like elements). The IS30 family is represented by the degenerated IS1630-like element. The IS1634-like element is shown to be involved in chromosomal rearrangements and horizontal gene transfer (HGT). The ISMHp1-like element is shown to relate to the HGT of a 25-kb region from *M. gallisepticum* to *M. synoviae.* The fractions of these genomes that correspond to mobile elements varied from 1.35 to 3.13% in *M. hyopneumonia* strains and was 2.08% in *M. synoviae.* Although these species possess reduced genomes, they maintain mobile elements, perhaps as a mechanism for genetic variability production.

Key words: insertion sequences, IS, mollicutes, horizontal gene transfer, mycoplasma.

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Mycoplasmas, apart from being potential pathogens, are considered the best examples of a minimal genome. They are Gram-positive bacteria, but rather than being primitive, they diverged recently - around 65 million years ago - with a drastic reduction in genome size that resulted in the loss of many biosynthetic abilities. Normally, their genome size is smaller than 1 Mb, and whole genome comparisons suggest that the severe genome reduction in mollicutes probably reflects their parasitic lifestyle (Trachtenberg, 2005).

Small genomes, as those of Mycoplasmas, are characterized by progressive gene loss, and are often presumed to be impermeable to mobile DNA, given that only the essential genes would be maintained in these cases (Rocha and Blanchard, 2002). Indeed, mobile elements have been described in many mycoplasma species (Ferrel *et al*., 1989; Hu *et al*., 1990; Bhugra and Dybvig, 1993; Zheng and McIntosh, 1995; Calcutt *et al*., 1999; Chandler and Mahillon, 2002; Ditty *et al*., 2003; Thomas *et al.*, 2005). Complete genome sequencing has also revealed mobile elements in the majority of the *Mycoplasma* studied so far (Chambaud *et al.*, 2001; Sasaki *et al.*, 2002; Papazisi *et al*., 2003; Westberg *et al*., 2004; Vasconcelos *et al*., 2005). In *M. mycoides*, for example, insertion sequences (ISs) represent 13% of the genome (Westberg *et al*., 2004). Nevertheless, there are some exceptions, as no insertion sequence, transposon, or endogenous plasmid was found in *M. mobile* (Jaffe *et al*., 2004) or other bacteria with reduced genomes, such as *Wigglesworthia glossinidia, Buchnera aphidicola,* and *Blochmannia floridanus* (Bordenstein and Reznikoff, 2005).

One interesting question to be answered considers the hypothesis that the mobile elements are maintained in these reduced genomes because they represent important genomic elements, probably as sources of genetic variability. Alternatively, in the light of their selfish nature, these mobile elements may be maintained because the hosts are unable to get rid of these parasites.

The most abundant class of mobile elements in the *Mycoplasma* genomes is the IS. These are mobile genetic parasites of about 800-2500 bp, often present in multiple copies on bacterial genomes and able to carry out transposition. This phenomenon corresponds to the move-

Send correspondence to Elgion L.S Loreto. Departamento de Biologia, Universidade Federal de Santa Maria, 97105-900 Santa Maria, RS, Brazil. E-mail: elgion@base.ufsm.br.

ment of specialized DNA elements, namely transposons and insertion elements, within or between loci mediated either by a transposase or an integrase, the transposition enzymes (Zhou and Reznikoff, 1997; Haren *et al*., 1999). The substrate of transposition is DNA flanked by inverted repeats, which are recognized as the target for transposase. The mobile stretch of DNA bordered by these inverted repeats is called the transposable element, the insertion sequence or the transposon (Zhou and Reznikoff, 1997; Haren *et al*., 1999). Transposition of IS elements can cause deletions, insertions and inversions of genomic loci and consequently contribute to the genetic variability of bacteria. The genome thus acquires greater plasticity from these processes, and quickly adapts to diverse environmental selective pressures (Bordenstein and Reznikoff, 2005).

Mahillon and Chandler (1998) provided a review of Insertion Sequences and, at that time, a total of 17 IS families were recognized based on the following features: IS ORF organization, conserved signature motifs among transposases, similarity of terminal inverted repeat sequences (TIRs), and length of target site duplications (direct repeats, DR). So far, more than 800 IS elements belonging to 19 families have been discovered (http://www-is biotoul.fr/is.html).

Further importance of insertion sequences lies in their utility as genetic markers for diagnosis and epidemiological analyses. This is because IS elements are typically present in multiple copies, rendering assays more sensitive and demanding less DNA for analyses. Also, the IS mobility can contribute to producing variants and subtypes of bacterial species (Stanley *et al*., 1993; Small *et al*., 1994; Frey, 1998).

The present study reports the presence, copy number and functional status of the IS elements in three strains of *Mycoplasma hypneumoniae* (the pathogenic 7448 and 232 and the non-pathogenic J strains) and one strain of *M. synoviae,* aiming to contribute to the understanding of the evolutionary role played by transposable elements in reduced genomes such as those of mollicutes.

The sequences that were annotated as transposase or transposable elements in the *M. hyopneumonia* and *M. synoviae* genomes (data available at http://www.brgene.lncc.br/finalMS for *M. synoviae* and http://www.genesul.lncc.br/finalMH for *M. hyopneumoniae* strain J and http://www.genesul.lncc.br/finalMP for *M. hyopneumoniae* strain 7448) served as the first step for analyses. The annotated sequences as transposable elements were used as seeds for searching for related sequences using BLAST (http://www.ncbi.nlm.nih.gov/blast/). Global alignments were performed using Clustal X (Thompson *et al*., 1997). The Artemis software was used for ORF integrity analyses and visualization of IS distribution in the genomes (Rutherford *et al.*, 2000). The identification of IS in the described families was conducted using the following criteria: *i*) similarities in ORF organization; *ii*) identities or similarities in their Tpases (common domains or motifs); *iii*) similar features of their ends (TIRs); *iv*) direct target duplication characteristics (ISFinder http://www-is biotoul.fr/is.html).

We found three transposable elements (TEs) from three diverse families in *Mycoplasma hyopneumoniae*, while *M. synoviae* had four different TEs from two IS families. Table 1 summarizes the main information about these ISs: location in the genomes, their length, the length of TIRs and of DRs. Also, the elements are classified accord-

Table 1 - Characterization of IS in the *Mycoplasma hyopneumoniae* and *M. synoviae* genomes: location in the genomes (beginning and end), structure (complete, defective or partial), length, the length of putative proteins, TIRs or LTRs and DRs.

Begin	End	Structure	Length (bp)	Putative protein (aa)	TIR or LTR (pb)	DR (bp)
M. hyopneumoniae J						
IS 4-ISMhp1						
379008	377099	Complete	1910	552	21	37
398472	396563	Complete	1910	552	21	6
534875	536784	Complete	1910	552	21	17
622178	624086	Complete	1909	552	21/20	80
662756	664665	Complete	1910	552	21	55
698600	696691	Complete	1910	552	21	45
711560	709651	Complete	1910	552	21	81
729145	731054	Complete	1910	552	21	82/83
820961	821371	Partial	411	ND	ND	ND
410987	411140	Partial	153	ND	ND	ND
IS3-IS1221I						
479702	478148	Defective	1555	233 (partial)	26/27	ND
482258	480745	Defective	1514	165 (partial)	20	ND
tMH						
724865	729143	Complete	4279	589/477	359/356	ND
731055	735051	Partial	3997	589/477	356/356	ND

Table 1 (cont.)

Begin	End	Structure	Length (bp)	Putative protein (aa)	TIR or LTR (pb)	DR (bp)
M. hyopneumoniae 7448						
IS 4-ISMhp1						
152999	151090	Complete	1910	552	21	37
256966	258875	Complete	1910	552	21	24
266975	265066	Complete	1910	552	21	151
281834	283743	Complete	1910	552	21	56
396888	398796	Complete	1909	552	21	64
558791	560699	Complete	1909	435	21	7
647224	645315	Complete	1910	552	21	79
837648	838058	Partial	411	46 (partial)	ND	ND
856828	854919	Complete	1910	552	21	47
913975	912066	Complete	1910	552	21	32
427479	427631	Partial	153	ND	ND	ND
tMH						
745696	749941	Complete	4246	590/149/309	362/358	ND
749584	753877	Complete	4294	589/477	358/358	ND
753520	757793	Complete	4274	589/477	358/356	ND
742506	742849	Partial	344	ND	ND	ND
M. hyopneumoniae 232						
IS 4-ISMhp1						
99736	97827	Complete	1910	552	21	44
732002	730091	Complete	1912	286	21	17
813968	814375	Partial	408	46 (partial)	ND	ND
417561	417713	Partial	153	ND	ND	ND
tMH						
726261	732486	Complete	4297	589/477	367/356	ND
732131	736123	Complete	3993	589/149/185	356/356	ND
M. synoviae 53						
IS 4-ISMhp1-like						
80102	81967	Complete	1866	562	20	19
192128	193159	Defective	1864	185/343	20	74
325739	327600	Defective	1862	162/149	20	ND
556638	554775	Defective	1864	376 (partial)	20	ND
531181	531509	Partial	329	ND	ND	ND
IS4- ISMsy1						
311850	309982	Complete	1869	552	12	ND
691602	693452	Defective	1851	166 (partial)	19	ND
IS4- IS1634-like						
176215	178015	Complete	1801	283	17	284
IS30- IS1630-like						
302572	303851	Defective	1280	ND	ND	ND
306766	308005	Defective	1240	ND	28	24/23
319536	320773	Defective	1238	ND	28	ND
602294	603572	Defective	1279	149	26	33
365937	367175	Defective	1239	ND	28	ND
498002	499294	Defective	1293	ND	28	ND
504938	506213	Defective	1276	ND	28	ND
139289	140583	Defective	1295	ND	28	ND
34556	35843	Defective	1288	ND	28	ND

ND = not determined. The Genbank accession number of the respective genome is: *M. hyopneumoniae* 7448 - AE017244; *M hyopneumoniae* J - AE017243; *M. hyopneumoniae* 232 - AE017332; *Mycoplasma synoviae* - AE017245.

ing to their structure as: *i*) complete-or IS without nonsense mutations or indels. These are putatively active elements, and when the IS is considered complete, the length of putative protein is also described; *ii*) defective-or IS with almost the complete length of putative active elements but showing nonsense mutations or indels, suggesting that these sequences are inactive; *iii*) partial-or short sequences showing high similarity with part of the IS (more than 95%).

Elements of the IS4 family are the most representative in these analyzed genomes. This IS family is a heterogeneous group and is present in various bacterial taxa. Usually, these ISs present approximately 50-bp Terminal Inverted Repeats (TIRs) along with direct repeats (DR) that correspond to target sequence duplication from 9 to 12 bp, and contain a single ORF coding for a transposase with a DD_E motif. In *M. hyopneumoniae* the sole representative of this family is ISMHp1 (Calcutt and Wise, 2000; direct submission to NCBI/GenBank). This element is present in an elevated copy number in the J and 7448 strains (11 and 10 copies), and in a lower number (4) in the 232 strain. A complete ISMHp1 element is 1910 bp long, codifies a 552-aa transposase, and has 21-bp long TIRs. The various complete elements present in the genomes are almost identical (98-99% nucleotide similarity), showing polymorphism of insertion sites in different strains (Figure 1-A) and probably corresponding to the most active IS in this species.

Furthermore, partial ISMHp1 copies are detected in the *M. hyopneumoniae* genomes. These partial sequences are, probably, footprints of complete ISs that have recently occupied these genomic positions. A remarkable characteristic of ISMHp1 is its extraordinary variability in the length of target sequence duplication generated by transposition events. This can sometimes result in extremely long DRs. For example, DR length varied between 8 and 82 bp, with an average of 50 bp in the J strain. In the 7448 strain, it varied between 8 and 151 bp (64.7 on average). Although the mechanism involved in generating such long and variable DRs remains unknown, they have been identified as well in two other IS4 elements, IS1549 from *Mycobacterium smegmatis* and IS1634 from *Mycoplasma mycoide* (Vilei *et al*., 1999; Chandler and Mahillon, 2002).

In the *M. synoviae* genome we have found three different ISs belonging to the IS4 family. Previously, Vasconcelos *et al.* (2005) have identified and annotated as IS4 the ISMHp1-like and IS1634-like elements. Here, we include a third IS4 to this list, which we have called ISMsy1. This element was classified as IS4 due to the characteristics of DD_E motif and to TIRs similarity with other IS4 elements. ISMsy1 is a 1881-bp long putatively active element and presumed to potentially codify a protein with 552 amino acids. Two copies were found in the genome, one complete and one partial, showing TIRs with 12 and 19 bp, respectively.

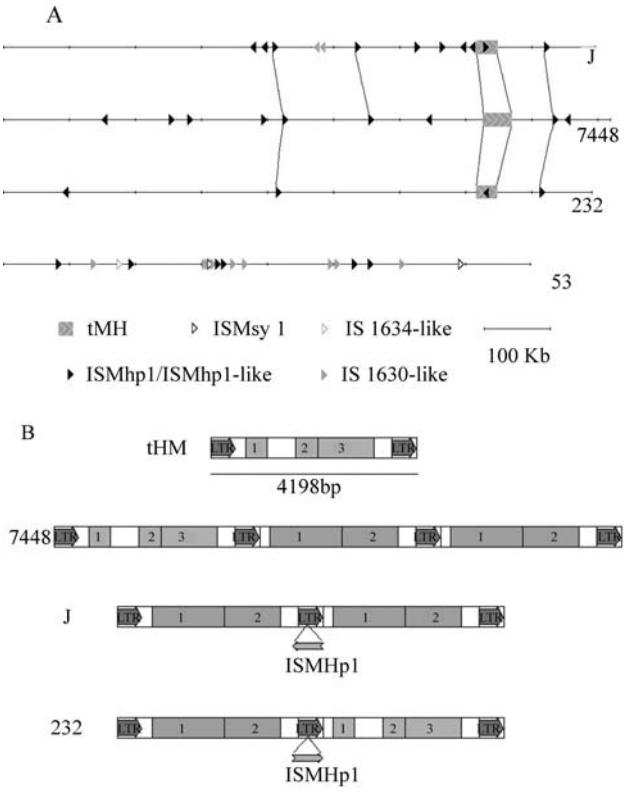

Figure 1 - **A**- An overview location of diverse transposable elements in J, 7448, 232 *M. hyopneumoniae* and *M. synoviae* genomes. Different arrowheads symbolize the various ISs. The line connecting IS among genomes corresponds to elements that are homologous in the different genomes. **B** - Structural features of the tMH element in *M. hyopneumoniae*. In the upper diagram the tHM is depicted as described by Harasawa (1995) with the Long Terminal Repeats (LTRs) and three ORFs. The complete element has 4193 base pairs. The remaining diagrams depict the arrangement of tMH in: the 7448 strain - in which three tMH elements are arranged in tandem and share adjacent LTRs; in the J and 232 strains - in which two tMH elements are arranged in tandem, and in the internal shared LTR, an ISMHp1 element is inserted. Note the opposite directions of the inserted ISMHp1 element for each strain.

The second IS4 element present in *M. synoviae* was called IS1634-like by Vasconcelos *et al.* (2005) due to the similarity with IS1634 from *M. mycoides* (Vilei *et al*., 1999). The general nucleotide similarity between these elements is 71%, but the TIRs are almost identical. The unique IS1634-like copy found is 1801 bp long and has perfect 18-bp TIRs. The ORF has some indels and frameshifts, and has no potential for coding a transposase. However, it is likely that this copy has mobilized recently, because its long 284-bp DR is almost completely conserved. As previously mentioned, it is known that the IS1634 element is able to generate long DRs (Vilei *et al*., 1999).

The third IS4 element in the *M. synoviae* genome was denominated ISMHp1-like by Vasconcelos *et al.* (2005) due to the similarity with the described *M. hyopneumoniae* ISMHp1 element. Although they show a low general nucleotide similarity (65%), they share TIRs and some remark-

ably well conserved parts of the sequences. The complete element is 1862 bp long and encodes a protein of 562 aa. It possesses 20-bp TIRs, generally differing in two nucleotides in the 3' and 5' TIRs. As the ISMHp-1 element of *M. hyopneumoniae,* the DRs showed variations in length, varying between 19 and 73 bp. Five copies are present in the *M. synoviae* genome, of which one is complete, putatively active, three are defective, and one is partial (Table 1).

In the *Mycoplasma* species studied, the only representative of the IS3 family found was IS1221I in *M. hyopneumoniae*. This element was previously described for J strains by Ferrell *et al*., (1989) as a related element to the IS1221 from *M. hyorhinis*. It is present in the J strain as two defective copies with roughly 1500 bp, showing 20-27 bp TIRs but without any detected DRs. These features indicate that these copies are probably inactive and ancient. This element is absent in the 7448 and 232 strains.

Elements of the IS30 family were found exclusively in *M. synoviae*. A total of 9 copies was found, all of which had indels and frameshift mutations in the transposase gene. These sequences were called IS1630-like because they are similar to the IS1630 element from *Mycoplasma fermentans* (Calcutt *et al*., 1999). The TIRs are 28 bp long and show slight differences between TIRs of the same element. One noteworthy feature of this element is the fact that the DRs of one element are, sometimes, found in other IS. This fact can simply result from homologous inter- or intra-molecular recombination between two IS elements, each with a different DR sequence, or from the formation of adjacent deletions resulting from duplicative intramolecular transposition (Turlan and Chandler, 1995; Ohtsubo and Ohtsubo, 1978). As an example of this fact, the DR of IS1360-like element located in position 302572 is exchanged by the element located in position 498002. The same kind of exchange can be seen in the ISs located in positions 365937 and 504938. This is illustrated by the capacity of these ISs to act as agents of chromosomal rearrangements.

The tMH element is neither an IS nor related to any described prokaryotic transposable element. However, it presents features resembling those of LTR retrotransposons. This element was described by Harasawa *et al.* (1995) as organized into three ORFs flanked by 272-bp LTRs (Figure 1-B). The putative encoded peptides do not show similarity to any described protein. Nonetheless, when the genetic code is changed from *Mycoplasmas* to universal, the third ORF shows weak similarity to RNA polymerases. Complete and potentially active and partial copies are present in *M. hyopneumoniae* strains. Recently, Wu *et al.* (2004) described the presence of retrotransposon-like elements in the genome of *Wolbachia pipientis* wMel. However, even though these elements cannot be classified as "classical" retrotransposons, they indicate that this class of TEs may also be present in prokaryotes. In the genomes we have analyzed herein, the tMH elements are arranged in tandem and the copy share the same LTR. In the 7448 strain, three tMH elements are arranged in tandem, while in the J and 232 strains there are two copies, respectively. Nevertheless, in the three strains, the elements occupy the same genomic location (Fig 1-A). This means the particular element was present in the ancestor of these strains and has been maintained by vertical transfer. In J and 232 strains an ISMHp1 element is inserted in the shared internal LTR (Fig 1-B). Nevertheless, in these different strains the ISMHp1 elements are located in an opposite orientation and occupy a diverse location in the LTRs, thus showing different DRs. So, we can conclude that this represents an independent ISMHp1 insertion in these strains.

An overview of the transposable element location in the analyzed genomes can be seen in Figure 1-A. Searching for flanking sequences of each element we are able to show that tMH elements and two copies of ISMph1 share the same position in the three *M. hyopneumoniae* strains. A third ISMph1 copy is in the same genomic position, in the J and 7448 strains. An analysis of these insertion site polymorphisms backs the suggestion that these elements are active or were active until recently. Also, some regions where there are accumulations of ISs can be observed in the *M. synoviae* genome, preponderantly of ISMph1-like and 1630-like elements. Alternatively, these results could also suggest the existence of hot-spot regions for these ISs in this genome.

Vasconcelos *et al*. (2005) have described the horizontal gene transfer (HGT) of fourteen regions between *Mycoplasma synoviae* and *M. gallisepticum*, the largest with 5.9 kb and encompassing various CDSs. Some CDSs are hypothetical, while others code for an ABC transporter, a signal peptidase I, and a putative EF-G elongation factor. These fourteen regions were almost identical in both genomes, indicating a recent transfer event. The HGT between these species could have been facilitated by the fact that both are bird parasites and, therefore, keep close contact. Furthermore, these *Mycoplasma* species belong to different Mycoplasmatales clades that diverged about 350 MYA ago (Vasconcelos *et al*., 2005). The nucleotide similarity observed in conserved genes such as rRNA 16S is only 79%, while the observed similarity to two different ISs is significantly higher. For example, the similarity shared by *M. synoviae* ISMHp1-like elements and a related element found in *M. gallisepticum* is 97%. Also, an element similar to ISMsy1 found in *M. synoviae* is present in the *M. gallisepticum* genome, with identical 12-bp TIRs and a general similarity of 96%. HGT for these ISs has been suggested by Vasconcelos *et al*. (2005), and four of the fourteen putative transfered regions correspond to these ISs. Transposable elements have been pointed out in the literature as potential promoter agents to HGT (Lawrence, 2002). For this reason, we have decided to look for the putative association between ISs and the 14 regions previously mentioned and described as involved in HGT by Vascon-

celos *et al.* (2005). We have found a 25-kb region in the *M. synoviae* genome, encompassing five of the regions described as implicated in HGT and that are flanked by ISMhp1-like elements. It is remarkable that the same DRs are shared by these flanking IS elements (Figure 2). This finding could be explained by a homologous recombination between two IS elements, as previously described for the IS1630-like element. However, the fact that the regions described as 7, 8, 9 and 10 by Vasconcelos *et al*. (2005) are syntenic in the *M. gallisepticum* genome, corresponding to a conserved sequence between both species, makes the homologous recombination hypothesis strongly improbable. The most parsimonious suggestion is that the 25 kb encompassing the regions 7, 8, 9, 10 and 12 (Vasconcelos *et al.*, 2005) flanked by the ISMhp1-like element in *M. gallisepticum* genome has been mobilized by a specific transposase. In *M. synoviae*, this 25-kb region was inserted probably by means of the ISMHph1-like transposase acting over TIRs of both ISs. This transposition could generate the DRs as depicted in Figure 2. It is notable that region 12, present in *M. synoviae*, is not syntenic in the sequenced *M. gallisepticum* strain. However, as the 12 region is also involved in the HGT event, the most parsimonious hypothesis is that region was included in the 25-kb region in the *M. gallisepticum* donor strains implicated in the HGT.

Noteworthy is the fact that among the fourteen regions identified by Vasconcelos *et al*. (2005) as involved in HGT, we were able to demonstrate the association with an IS in nine. Five are genomic regions mobilized by using the IS in the flanking sequences, and the other four regions are the proper ISs that are involved. This result strongly backs the evidence that the transposable elements are probably the most important HGT promoting agents.

Mobile elements correspond to 2.08% of the *M. synoviae* genome, and for *M. hyopneumoniae* the figure varies among the strains (3.13% for 7448, 3.08% for J, and 1.35% for 232). It is known that the portion of mobile DNA per genome significantly increases with genome size. There is a significant, positive correlation between genome size and the percentage of genomic mobile-DNA in bacteria (Bordenstein and Reznikoff, 2005). The proportion of mobile elements in the species we have analyzed is similar to that of free-living bacteria with larger genomes. However, the maintenance of transposable elements-even in a reduced genome such as in *Mycoplasma*-suggests that these sequences could be important to produce the genetic diversity necessary for evolution, by generating chromosomal rearrangements, altering gene expression, or promoting HGT. From an evolutionary standpoint, the possibility that transposable elements might be one of the necessary components for a minimal genome is not to be ruled out. Considering these findings for the genomes described as devoid of mobile elements, it is necessary to clarify whether such an absence corresponds to the reality for these genomes, or whether it is a peculiarity of the sequenced strain. If the transposable elements are part of a minimal genome, we would expect to find them in other strains of those species believed to be devoid of TEs.

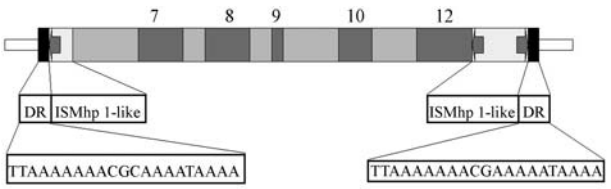

Figure 2 - The 25 kb region supposed horizontally transferred to *M. synoviae.* The ISMhp1-like elements are flanking the sequence and the DRs are present in the extremity of the region. The regions marked as 7, 8, 9, 10 and 12 correspond to those described by Vasconcelos et al (2005). The genomic positions of these regions are: 7 (533311); 8 (535709); 9 (537179); 10 (546230); 12 (693080). The regions 7 to 10 correspond to CDS to conserved hypothetical proteins and 12 to an ISMhp1-like element.

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