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DIRECCIÓN DE NUEVAS CREACIONES

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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



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 Evento 378
 Actuación 519
 Oficio 15 - 196 57
 Folios

Patente de Invención ☒ Patente de Modelo de Utilidad ☐

Vía Nacional ☐

Vía PCT (Fase Nacional) ☒ Capítulo I ☒ Capítulo II ☐

No. Solicitud Internacional PCT/US2008/082454
Fecha de Presentación Internacional 05/Noviembre/2008

Como resultado del Examen de Fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	Folios 47 a 92	06/Mayo/2010
Reivindicaciones:	Folios 93 a 97	06/Mayo/2010
	Folios 140 a 142	11/Noviembre/2011
Prioridad:	US 60/985,811	06/Noviembre/2007

2. Análisis de la Prioridad

Se acepta la reivindicación de prioridad porque esta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Objeto de la invención

Título de la solicitud: "Vacuna Viva Avirulenta de *Mycoplasma Hyopneumoniae* con Adyuvante".

El problema planteado en esta solicitud consiste en la necesidad de proporcionar composiciones inmunogénicas o de vacuna, preparadas a partir de *Mycoplasma hyopneumoniae* avirulento con adyuvante, que proporcionen eficacia contra cepas virulentas de *M. hyopneumoniae*, previniendo y/o minimizando de esta manera la gravedad de la enfermedad producida por este organismo o mejorando al menos un síntoma asociado con la enfermedad.

Para resolver este problema técnico la solicitud en estudio proporciona composiciones

inmunogénicas o de vacuna que comprenden una cantidad inmunológicamente eficaz de *Mycoplasma hyopneumoniae* vivo avirulento y un adyuvante biológicamente aceptable.

El objeto de la presente solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): A61K 39/02; C07K 14/30.

4. De la Solicitud de Patente

Reivindicaciones (Art 30 D 486, Art 22 PCT)

No es clara la diferencia entre una composición para inducir una respuesta inmune anti-*Mycoplasma hyopneumoniae* de la reivindicación 1 y una composición inmunogénica de la reivindicación 14, y puesto que en el primer caso se hace referencia a el uso de la misma, se sugiere encabezar la reivindicación principal como: "Una composición inmunogénica".

La reivindicación 1 no es concisa porque se refiere a una cantidad eficaz de una cepa viva avirulenta de *Mycoplasma hyopneumoniae* y un adyuvante biológicamente aceptable, esta forma de reivindicar no es adecuada porque no se están incluyendo las características técnicas de la invención y se da lugar a un sinnúmero de composiciones que no encuentran soporte en la descripción. Se sugiere al solicitante caracterizar la composición inequívocamente teniendo en cuenta que la cepa se define por su material genómico caracterizado por su secuencia de nucleótidos.

El término: "aproximadamente" de las reivindicaciones 2, 3, 9, 10, 17 y 18 es impreciso, por lo cual no permite distinguir la invención del estado de la técnica. Se sugiere sustituirlo por un término preciso o un rango concreto

Los porcentajes de homología y de identidad polimórfica de las reivindicaciones 2 y 3 no son aceptables porque no corresponden a una secuencia o cepa específica.

La reivindicación 2 se refiere a una cepa que comprende una secuencia de ácido nucleico que solo se esta definiendo como la "cepa J", esta forma de definir no es adecuada porque no se están definiendo las características técnicas. Se sugiere al solicitante definir la secuencia de nucleótidos exacta e incluirla en la reivindicación principal puesto que es una característica esencial de la invención.

No se observa ninguna diferencia entre la materia reclamada en la reivindicación 4 y 5. Se sugiere al solicitante eliminar una de estas reivindicaciones o realizar las aclaraciones necesarias.

En las reivindicaciones 7 – 10 y 15 – 18 se utilizan las abreviaturas SL-CD y SP-Oil, respecto a lo cual se requiere al solicitante utilizar los nombres completos o definir su composición.

No se encuentra soporte descriptivo para la composición de las reivindicaciones 11 y 19 que contenga además bacterias vivas, bacterinas y/o toxoides, además esta forma de definir no permite caracterizar inequívocamente los ingredientes de la composición. Debido a la falta de soporte en la descripción de la obtención o prueba de una composición como la reclamada en estas reivindicaciones, se sugiere eliminarlas del alcance del pliego reivindicatorio.

La reivindicación 12, 13, 20 y 21 se refieren a antígenos que no se encuentran caracterizados. Se recuerda al solicitante que un antígeno se define por su secuencia específica de aminoácidos.

Las reivindicaciones 12 y 20 se refieren a una composición que además comprende uno o más antígenos virales que se encuentran definidos de forma general por el tipo de antígeno, en el capítulo descriptivo solo se encuentra soporte para cuando el antígeno de circovirus porcino es una quimera de tipo1 - tipo2 de circovirus porcino, por lo cual se sugiere restringir la solicitud a una razonable generalización de lo que se encuentra debidamente soportado en el capítulo descriptivo, definiendo el antígeno por su secuencia de aminoácidos.

Descripción (Art 28 D 486)

Dado que esta solicitud se refiere a antígenos y material biológico, deben presentarse las listas de las secuencias de aminoácidos y nucleótidos en forma separada de la descripción, bajo el título: "Listado de secuencias" y anexarlas en formato digital.

Debido a que la solicitud se refiere a material biológico, es necesario presentar el Certificado de Depósito para las cepas ATCC 25934 y 27715.

Se requiere al Solicitante hacer las aclaraciones necesarias.

5. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: 06/Noviembre/2007 y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación
D1	WO 02/10343	Temperature-Sensitive Live Vaccine for <i>Mycoplasma Hyopneumoniae</i>	07/Febrero/2002
D2	US 2007/0253981	Canine Influenza Virus	01/Noviembre/2007

D1<http://worldwide.espacenet.com/publicationDetails/originalDocument?CC=WO&NR=0210343A2&KC=A2&FT=D&ND=3&date=20020207&DB=EPODOC&locale=en> EP divulga (Pág. 3, líneas 16 - 24) una preparación de vacuna que contiene una bacterina viva sensible a la temperatura de *Mycoplasma hyopneumoniae* en combinación con un vehículo fisiológicamente aceptable. La preparación puede contener un adyuvante inmunológico y un agente infeccioso que puede ser *Borderella bronchiseptica*, *Pasteurella multocida* tipo A o D, o *Haemophilus parasuis*.

D2<http://worldwide.espacenet.com/publicationDetails/originalDocument?CC=US&NR=2007253981A1&KC=A1&FT=D&ND=3&date=20071101&DB=EPODOC&locale=en> EP divulga (Resumen; Pág. 10, párrafo 65) una vacuna para la influenza canina que comprende agentes capaces de aumentar la respuesta inmune celular como SP oil.

6. Examen de Patentabilidad (Art 14 D486)

Novedad (Art 16 D486)

La reivindicación 1 consiste en una composición para inducir una respuesta inmune anti-*Mycoplasma hyopneumoniae* en un animal, comprendiendo dicha composición una cantidad inmunológicamente eficaz de una cepa viva avirulenta de *Mycoplasma hyopneumoniae* y un adyuvante biológicamente aceptable.

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características esenciales	D1
Una composición que comprende:	Una composición que comprende:
Una cantidad inmunológicamente eficaz de una cepa avirulenta de <i>Mycoplasma hyopneumoniae</i>	Una bacterina viva sensible a la temperatura de <i>Mycoplasma hyopneumoniae</i>

Características esenciales	D1
Un adyuvante biológicamente activo	Un adyuvante inmunológico

Las características **esenciales** de la composición de la reivindicación 1 habían sido divulgadas previamente en el documento D1, el cual da a conocer una preparación de vacuna que contiene una bacterina viva sensible a la temperatura de *Mycoplasma hyopeumoniae* y un adyuvante inmunológico (Pág. 3, líneas 16 – 24; Págs. 10 – 12, ejemplo 3; Pág. 16, reivindicaciones 3 – 5).

El documento D1 también revela adyuvantes como el carbopol (Pág. 6, línea 33); y la combinación con otros agentes infecciosos como *Borderella bronchiseptica*, *Pasteurella multocida* tipo A o D, o *Haemophilus parasuis* (Pág. 3, líneas 20 – 24).

En consecuencia, la reivindicación 1 no es nueva según lo divulgado en el documento D1.

Las reivindicaciones dependientes 7, 11, 14 y 19 no mencionan alguna característica que haga nueva a la composición de la reivindicación 1, por lo cual se considera que no son nuevas.

Nivel Inventivo (Art18 D486)

La reivindicación 2 consiste en la composición de la reivindicación 1, en la que la cepa viva avirulenta de *Mycoplasma hyopeumoniae* es una cepa cuyo genoma comprende una secuencia de ácido nucleico que tiene homología de al menos aproximadamente 90% con la secuencia de ácido nucleico de una cepa J de referencia.

Comparación de las características técnicas **esenciales** con las del Estado de la Técnica:

Características esenciales	D1
Una composición que comprende: Una cepa viva avirulenta de <i>Mycoplasma hyopneumoniae</i> cuyo genoma tiene una homología de al menos aproximadamente 90% con la secuencia de ácido nucleico de la cepa J	Una composición que comprende: Una bacterina viva sensible a la temperatura de <i>Mycoplasma hyopneumoniae</i>
Un adyuvante biológicamente aceptable	Un adyuvante inmunológico

El documento D1 se considera el estado de la técnica cercano a la invención definida en la reivindicación 2 porque divulga una preparación de vacuna que contiene una bacterina viva sensible a la temperatura de *Mycoplasma hyopeumoniae* y un adyuvante inmunológico (Pág. 3, líneas 16 – 24; Págs. 10 – 12, ejemplo 3; Pág. 16, reivindicaciones 3 – 5).

El documento D1 también enseña las ventajas de una vacuna viva, refiriéndose a que las mutantes que son sensibles a la temperatura y que no crecen a la temperatura normal de los cerdos puede crecer *in vitro* a temperaturas más frías (Pág. 4, líneas 27 – 33).

La diferencia entre la invención, definida en la reivindicación 1 y la composición del documento D1 consiste en que se utiliza la cepa J de *Mycoplasma hyopeumoniae*.

No se observa ningún efecto técnico generado por la composición de la cepa J de *Mycoplasma hyopeumoniae*.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: “cómo obtener una composición de una cepa viva de *Mycoplasma hyopeumoniae* alternativa a la conocida en el documento D1”.

Sin embargo, el documento D1 enseña que cepas adecuadas de *M. hyopeumoniae* pueden ser obtenidas de diferentes fuentes como los depósitos ATCC y NRRL (Pág. 5, líneas 20 – 22) y la cepa J es conocida por la persona normalmente versada en la materia, así como su depósito ATCC 25934⁽¹⁾. Por lo tanto, la composición de la solicitud corresponde a una alternativa que en ausencia de soporte de algún efecto técnico inesperado o la solución a algún problema técnico carece de nivel inventivo.

Respecto a la composición que comprende además otros antígenos como el virus de la gripe porcina, virus del síndrome respiratorio y reproductivo porcino, virus de la viruela del mapache etc., se aclara que la combinación de varios antígenos en una composición es una practica común en el campo técnico, y puesto que no se observa ningún efecto técnico inesperado tal como sinergia, disminución de dosis, etc., tampoco le otorgan nivel inventivo a la composición de la solicitud.

En consecuencia, la persona del oficio normalmente versada en la materia, incluiría una cepa de *Mycoplasma hyopeumoniae* conocida en la técnica como la cepa J en la composición del documento D1 para llegar así a la composición alternativa de la reivindicación 2 de la solicitud. Por lo cual se considera obvia.

Puesto que las reivindicaciones dependientes 3 – 6, 12 y 13 no mencionan características inventivas adicionales tampoco se pueden considerar inventivas.

Conclusión:
Las reivindicaciones 2 – 6, 12 y 13 cumplen el requisito de novedad (Art. 16), pero no cumplen con el requisito de nivel inventivo (Art. 18).

La reivindicación 8 consiste en: La composición de la reivindicación 7, en la que dicho adyuvante biológicamente aceptable comprende SP-Oil.

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características esenciales	D1	D2
Una composición que comprende:	Una composición que comprende:	Una vacuna para influenza canina que comprende:
Una cantidad inmunológicamente eficaz de una cepa avirulenta de <i>Mycoplasma hyopneumoniae</i>	Una bacterina viva sensible a la temperatura de <i>Mycoplasma hyopneumoniae</i>	No se menciona
SP-Oil	Un adyuvante inmunológico	SP oil

El documento D1 se considera el estado de la técnica cercano a la invención definida en la reivindicación 2 porque divulga una preparación de vacuna que contiene una bacterina viva sensible a la temperatura de *Mycoplasma hyopeumoniae* y un adyuvante inmunológico (Pág. 3, líneas 16 – 24; Págs. 10 – 12, ejemplo 3; Pág. 16, reivindicaciones 3 – 5).

La composición de la reivindicación 8 se diferencia de las composiciones de D1 en que como adyuvante se utiliza SP-Oil.

El hecho de utilizar como adyuvante SP-Oil en la composición que se daba a conocer en D1, causa que las composiciones tengan la capacidad de aumentar la respuesta inmune.

(1) VASCONCELOS A. ET AL, "Swine and Poultry Pathogens: The Complete Genome Sequences of Two Strains of *Mycoplasma hyopneumoniae* and a Strain of *Mycoplasma synoviae*", Journal of Bacteriology, Vol. 187, No. 16, 2005, Págs. 5568 – 5577.

Por tanto, el problema técnico objetivo consiste en “cómo modificar la composición del documento D1 para aumentar la respuesta inmune”.

El documento D1 ya divulgaba el uso de adyuvantes para aumentar la respuesta inmune (Pág. 6, línea 25 – Pág. 7, línea 3) y el documento D2 que divulga una vacuna para la influenza canina (Resumen) y revela agentes para aumentar la respuesta inmune celular como el SP oil (Págs. 9 y 10, párrafo 65).

Respecto a la composición que comprende además otros antígenos como el virus de la gripe porcina, virus del síndrome respiratorio y reproductivo porcino, virus de la viruela del mapache etc., se reitera que la combinación de varios antígenos en una composición es una practica común en el campo técnico, y puesto que no se observa ningún efecto técnico inesperado tal como sinergia, disminución de dosis, etc., tampoco le otorgan nivel inventivo a la composición de la solicitud.

La reivindicación es obvia, porque la persona del oficio normalmente versada en la materia enfrentada al problema de aumentar la respuesta inmune, incluiría el adyuvante SP oil que se revela en el documento D2, dentro de las composiciones que se enseñan en D1, logrando de esta forma la composición objeto de la reivindicación 8. Por lo cual la composición de la Reivindicación 8 carece de Nivel Inventivo.

Puesto que las reivindicaciones dependientes 9, 10 y 15 – 21 no mencionan características inventivas adicionales tampoco se pueden considerar inventivas.

Conclusión:
Las reivindicaciones 8 – 10 y 15 – 21 cumplen el requisito de novedad (Art. 16), pero no cumplen con el requisito de nivel inventivo (Art. 18).

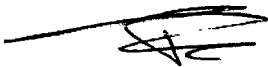
7. Conclusión

Los requisitos de Patentabilidad de la presente Solicitud están afectados así:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	WO 02/10343	1, 7, 11, 14, 19	Novedad
		2 – 6, 8 – 10, 12, 13, 15 - 21	Nivel Inventivo
D2	US 2007/0253981	8 – 10, 15 - 21	Nivel Inventivo

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.


JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (C)

El anterior requerimiento fue notificado por fijación en lista, de fecha 13 O OCT. 2012

Elaborado por: Jeny Paola Villate Becerra *P. Villate*
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Swine and Poultry Pathogens: the Complete Genome Sequences of Two Strains of *Mycoplasma hyopneumoniae* and a Strain of *Mycoplasma synoviae*†

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This work reports the results of analyses of three complete mycoplasma genomes, a pathogenic (7448) and a nonpathogenic (J) strain of the swine pathogen *Mycoplasma hyopneumoniae* and a strain of the avian pathogen *Mycoplasma synoviae*; the genome sizes of the three strains were 920,079 bp, 897,405 bp, and 799,476 bp, respectively. These genomes were compared with other sequenced mycoplasma genomes reported in the literature to examine several aspects of mycoplasma evolution. Strain-specific regions, including integrative and conjugal elements, and genome rearrangements and alterations in adhesin sequences were observed in the *M. hyopneumoniae* strains, and all of these were potentially related to pathogenicity. Genomic comparisons

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† Supplemental material for this article may be found at <http://j.b.asm.org/>.

Mycoplasmata comprise a group of more than 180 species of wallless bacteria that are obligate parasites of a wide range of organisms including humans, plants, and animals (46). Mycoplasmas typically exhibit strict host tissue specificity, probably due to their nutritional requirements (48). A direct consequence of the genomic reduction that likely occurred as a consequence of the metabolic complementarity of the hosts (3). The evolutionary dynamics of these organisms involved population bottlenecks and asexual reproduction leading to accumulation of deleterious mutations, which resulted in further genome contraction (58). A predictable consequence of this process is preservation of a minimal genome comprising essential genes to maintain basic core functions and adaptation to specific environments.

The differences between the sequences have been determined and numbered in the years 1972, 1973, 1974, 1975, 1976, 1977, 1978, 1979, 1980 and 1981. The sequences of the 1972-1973 and 1974-1975 studies and the findings dates have not been mentioned. In order to obtain a more uniform nomenclature, the sequences of the 1972-1973 and 1974-1975 studies have been numbered in the same way as those of the organisms being studied in the recently completed study (1981).

MATERIALS AND METHODS

After the separation of amphoteric protozoans, *M. wrightii* was isolated from the brain of a squirrel in the state of Paraná, in Brazil [9].

Genome sequencing, assembling, and annotation. Genomes were sequenced using a 150 bp paired-end sequencing strategy on a HiSeq 2500 (Illumina). Sequence reads were assembled using the SOAPdenovo2 software package (Li et al., 2012) with a k-mer size of 25. The assembled genomes were annotated using the Braker software package (Braker et al., 2015). Sequences were then aligned using the SnpEff software (Cingolani et al., 2012). Annotations were performed using the GeneMark-ES software package (Zhu et al., 2010). Gene models were confirmed using manual curation. Genomes were annotated using the GeneMark-ES software package (Zhu et al., 2010). Gene models were confirmed using manual curation. Genomes were annotated using the GeneMark-ES software package (Zhu et al., 2010). Gene models were confirmed using manual curation.

Phylogenetic reconstructions and comparative analyses. Maximum-likelihood (ML) phylogenies, based on individual orthologous proteins, were generated using ProtML (Mophy package [http://www.ism.ac.jp/mophy/orthology.html]) and TREE-PUZZLE 5.1 (51). A data set concatenating all proteins in a single sequence and was analyzed using neighbor-joining (NJ) distance tree with MEGA 2.1 (30), maximum parsimony using PAUP* v0.010 (55), and by ML using PhyML (56). Confidence estimates based on 100 bootstrap replicates were obtained using the same software. Phylogenetic analyses were performed using the following parameters: ML subsequent analysis by NJ base, or ML distance, using the JTT matrix, gamma correction, four categories, and 1000 bootstrap replicates. A detailed description of the methods used in this study is available in the Supplementary Materials and Methods (see Supplementary Materials and Methods, available at <http://www.jmcb.oxfordjournals.org/>).

[illegible]Downloaded from <http://jib.asm.org/> on October 1, 2012 by guest

TABLE 1. General characteristics of the genomes of *M. hyopneumoniae* strains J and 7448 and *M. synoviae*^a

Characteristic	Mhy-J	Mhy-P	Msy
Total length (base pairs)	897,405	920,079	799,476
G + C content (%)	28	28	28
Total no. of CDSs	679	681	694
Genome constituting coding regions (%)	88	88	91
Average CDS length (base pairs)	1,178	1,190	1,058
No. of known proteins	412	421	464
No. of conserved hypothetical proteins	109	105	167
No. of hypothetical proteins	158	155	63
No. of rRNAs			
16S	1	1	2
23S	1	1	2
5S	1	1	3
No. of tRNAs	30	30	34
No. of insertion sequences ^b			
IS3	2	0	0
tMH	(4)	2 (8)	0
ISMhpI	14	11	13

^a Abbreviations: Mhy-J, *M. hyopneumoniae* strain J; Mhy-P, *M. hyopneumoniae* strain 7448; Msy, *M. synoviae*.

^b Number of copies of different insertion sequence families. The number of defective copies is shown in parentheses.

Nucleotide sequence accession numbers. Sequence data reported in this paper were deposited in GenBank (accession nos. AE017243, AE017244, and AE017245). Sequence and annotation data are available at <http://www.brgene.lncc.br/finalMS> (*M. synoviae*), <http://www.genesul.lncc.br/finalMH> (*M. hyopneumoniae* strain J), and <http://www.genesul.lncc.br/finalMP> (*M. hyopneumoniae* strain 7448).

RESULTS AND DISCUSSION

Features of the *M. hyopneumoniae* strain J, *M. hyopneumoniae* strain 7448, and *M. synoviae* genomes. The main features of the three newly sequenced genomes are shown in Table 1. Clustering analysis of *M. synoviae*, *M. hyopneumoniae* strains J, 7448, and 232 (35), and eight other mycoplasma genomes (*Mycoplasma pneumoniae* M129, *Mycoplasma pulmonis* UAB CTIP, *Mycoplasma penetrans* HF-2, *Mycoplasma genitalium* G37, *Mycoplasma gallisepticum* R, *Mycoplasma mycoides* subsp. *mycoides* SC PG1, *Mycoplasma mobile* 163K, and *Urea-*

plasma urealyticum serovar 3) revealed 235 orthologous clusters. A comparison of the number of CDSs in all sequenced mycoplasma genomes is shown in Table 2.

Genome-specific regions and rearrangements in *M. hyopneumoniae* strains. Comparison of the three *M. hyopneumoniae* strains provided evidence of intraspecific rearrangements, resulting in strain-specific gene clusters (Fig. 1). This was the case for a 16-kb region of *M. hyopneumoniae* strain J, containing 15 CDSs, most of which encoded type III restriction-modification (R-M) system components and putative transposases. *M. hyopneumoniae* strain 7448 contained a specific 22.3-kb region similar to the integrative conjugal element (ICEF) of *Mycoplasma fermentans* (7), which was designated ICEH (for integrative conjugal element of *M. hyopneumoniae*). ICEH contained 14 CDSs, four of which similar to *tra* genes, usually associated with bacterial conjugative plasmids, and another encoding a single-strand binding protein (SSB), an essential protein for the transfer process. Direct repeat sequences (TAGATTTT), generated by target site duplications, flanked ICEH. This target site was localized in the homologous region of *M. hyopneumoniae* strain J, pointing to the mobility of this element. Evidences for the presence of circular extrachromosomal forms of ICEH in *M. hyopneumoniae* strain 7448 and another unrelated, pathogenic Brazilian field isolate of *M. hyopneumoniae* were obtained by an inverted PCR assay (results not shown), indicating that this element might be functionally active in these isolates. Moreover, we also observed a region similar to ICEF in the genome of *M. hyopneumoniae* strain 232. It has recently been demonstrated that some pathogenic bacteria use the type IV secretion system, composed of subunits related to the conjugation machinery, for the delivery of effector molecules to host cells (16), and that this system may be involved in pathogenesis (52). However, the involvement of ICEH in pathogenesis through delivery of effector molecules into cells remains to be explored. Other strain-specific differences included an inverted region of 243,104 bp in *M. hyopneumoniae* strain 232 (Fig. 1) and less drastic rearrangements between *M. hyopneumoniae* strains at five genomic regions (Fig. 1; see Tables S1 to S3 in the supplemental mate-

TABLE 2. Comparison of the total number of CDSs in all sequenced mycoplasma genomes and number of exclusive CDSs per species^a

Organism ^b	GenBank accession no.	No. of CDSs				
		Total	Known	Conserved hypothetical	Hypothetical	Exclusive
Mhy-J	AE017243	679	412	109	158	67
Mhy-P	AE017244	681	421	105	155	53
Mhy-232	AE017332	691	327	227	137	59
Msy	AE017245	694	464	167	63	150
Mpu	NC002771	782	695	86	204	204
Mmo	NC006908	633	571	28	37	121
Mpn	NC000912	689	428	158	73	169
Mge	NC000908	484	318	164	2	8
Mga	NC004829	726	452	152	122	164
Uur	NC002162	614	323	107	184	164
Mpe	NC004432	1,037	647	190	200	433
Mmy	NC005364	1,016	833	26	157	491

^a Data derived from the GenBank database.
^b Abbreviations: Mhy, *Mycoplasma hyopneumoniae* (strains J, 7448 [P], and 232); Msy, *Mycoplasma synoviae*; Mge, *Mycoplasma genitalium*; Mpn, *Mycoplasma pneumoniae*; Mga, *Mycoplasma gallisepticum*; Mpu, *Mycoplasma pulmonis*; Mpe, *Mycoplasma penetrans*; Mmy, *Mycoplasma mycoides*; Mmo, *Mycoplasma mobile*; Uur, *Ureaplasma urealyticum*.

TABLE 3. CDSs encoding adhesion-related proteins in different mycoplasma species or strains

Protein	Presence of CDS encoding protein in mycoplasma ^a												
	Hominis group						Pneumoniae group					Mmv	Other
	Mhy-J	Mhy-P	Mhy-232	Msv	Mpu	Mmo	Mpu	Mge	Mga	Mpe	Uur		
Tip organelle-related proteins													
P1, MgPa	•	•	•	•	•	•	•	•	•	•	•	•	• (Mpi)
GapA													
p30, p32							•	•	•				
Orf6, MgpC							•	•	•				
CrmA													
Hmw1							•	•	•	?	?		
Hmw2					•		•	•	?				
Hmw3							•	•					
Other adhesion-related proteins													
LppS	•	•	•		•								• (Mco)
LppT	•	•	•										•
p97	•	•	•										
p102	•	•	•										
p216	•	•	•										
p146	•	•	•										
p110 (yx1)	•	•	•	•					•				
p140/p110							•	•					
p76	•	•	•										
p29													• (Mfe)
p65 (Mpn)							•	•					
p65 (Mhy)	•	•	•	•		•					•		
p40													• (Mag)
p50													• (Mho)
p69								•					
Vaa				•				•	•			•	• (Mho)
PvpA									•				

^a Presence of CDSs encoding proteins related to the tip organelle or other previously described adhesion-related proteins are indicated by dots. Question marks indicate putative orthologous CDSs with relatively low similarity (between 40 and 55% at the amino acid level). Species names are indicated in parentheses in the case of CDSs identified in mycoplasmas whose genomes had not been previously sequenced (Mag, *Mycoplasma agalactiae*; Mco, *Mycoplasma conjugatae*; Mfe, *M. fermentans*; Mho, *Mycoplasma hominis*; Mpi, *Mycoplasma pirum*). Orthologs were identified by BLAST. Additional abbreviations of mycoplasma species described in Table 2, footnote b.

moniae strains 7448, 232, and J code for proteins with 10, 15, and 9 of the previously described R1 tandem repeats, respectively; all three strains had more than the minimum number of CDSs (8 CDSs) required for cilium binding (34). This indicates that other adhesion determinants, with different characteristics

in *M. hyopneumoniae* strains 7448, 232, and J, must be responsible for their different adhesion properties. Differences between the encoded adhesins and other putative virulence factors in *M. hyopneumoniae* strains 7448, 232, and J include variations in the number of amino acid repeats between orthologous proteins (Ta-

TABLE 4. CDSs encoding proteins showing differences between *M. hyopneumoniae* strains J, 7448, and 232 with respect to repetitive amino acid sequences^a

CDS product ^b	Mhy-J CDS	Mhy-P CDS	Mhy-232 CDS
Known adhesins			
p97 adhesin	MHJ0194	MHP0198	mhp183
p76 membrane protein precursor	MHJ0494	MHP0497	mhp494
p216 surface protein	MHJ0493	MHP0496	mhp493
p146 adhesin-like protein	MHJ0663	MHP0663	mhp684
Membrane or putative membrane proteins			
p95 outer membrane protein	MHJ0096	MHP0099	mhp280
Hypothetical protein	MHJ0441	MHP0443	mhp445
Hypothetical protein	MHJ0444	MHP0445	mhp447
Hypothetical protein	MHJ0350	MHP0355	mhp366
Hypothetical protein	MHJ0032	MHP0036	mhp037
Hypothetical protein	MHJ0662	MHP0662	mhp683
Hypothetical protein	MHJ0442	MHP0444	mhp446
Other			
Conserved hypothetical protein	MHJ0089	MHP0092	mhp287

^a Abbreviations: Mhy-J, *M. hyopneumoniae* strain J; Mhy-P, *M. hyopneumoniae* strain 7448; Mhy-232, *M. hyopneumoniae* strain 232.

^b The membrane character for hypothetical proteins was established by PSORT.

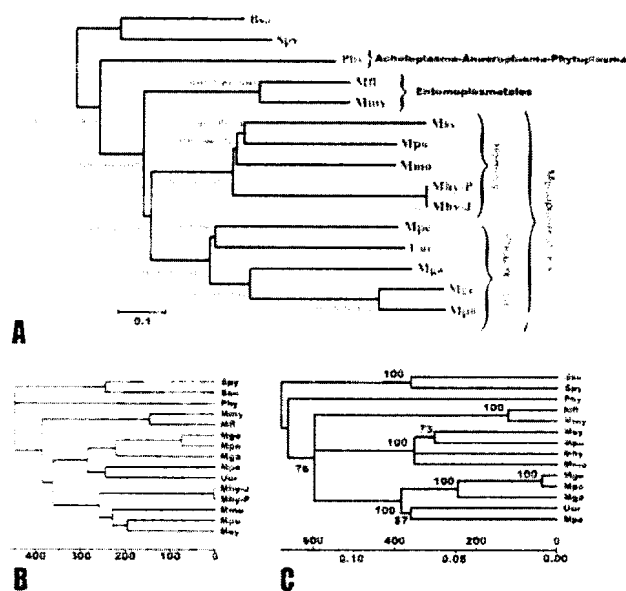


Fig. 2. Phylogenetic analysis of *Mollicutes*. (A) Phylogenetic tree of the *Mollicutes* based on the concatenated alignment of deduced amino acid sequences of the CDSs. Bootstrap support values based on 1000 bootstraps are indicated near each node. For neighbor joining with JTT, distance, maximum parsimony, and TREE-PUZZLE, maximum likelihood, respectively (the asterisk indicates an unconfirmed node). The main taxonomic groups within *Mollicutes* are shown on the right. The *Mollicutes* phytoplasma (*Candidatus* Phytoplasma asteris) OY strain AF066281 (Phy) from the Achaeoplasma-Aneroplasmia-Phytosplasma clade, *Mycoplasma fortuiti* (NC_006055) (Mf) from the *Entomoplasmatales*, as well as *Bacillus subtilis* (NC_003964) (Bs) and *Neisseria meningitidis* (NC_002122) (Sp) were included as outgroups. (B) Phylogenetic tree with branch lengths proportional to divergence from the root of the CDSs as estimated with the maximum likelihood method. (C) Phylogenetic tree with branch lengths proportional to divergence from the root of the CDSs as estimated with the maximum likelihood method. The scale bar represents 0.1 substitutions per site. The scale bar represents 0.1 substitutions per site. The scale bar represents 0.1 substitutions per site.

the 5'. These insertions/deletions result from variations in the number of tandem nucleotide repeats within coding regions, which is indicative of a mutational mechanism generating functional and/or antigenic variants. This variation in surface proteins is likely to be a key determinant of different pathogenic properties observed in *M. mycoides* strains.

Phylogenetic reconstructions. Phylogenetic relationships were established on the basis of a maximum of 200 single-copy CDSs comprising 15,500,000 aligned deduced amino acid positions. The results of phylogenetic analyses based on individual orthologous proteins were subsequently compared to the ML, maximum parsimony, and distance topologies based on the concatenated amino acid sequence alignment of a single sequence unit. In contrast to the highly incongruent topologies resulting from separate analyses of individual orthologous CDSs, the concatenated alignment generated a single, highly supported tree (Fig. 2A). Phylogenies were also estimated with nucleotide data using a wide variety of methods, based on either gene content or gene order. Only the tree generated with the concatenated protein sequence unit was presented, as all methods gave essentially the same results. A tentative time

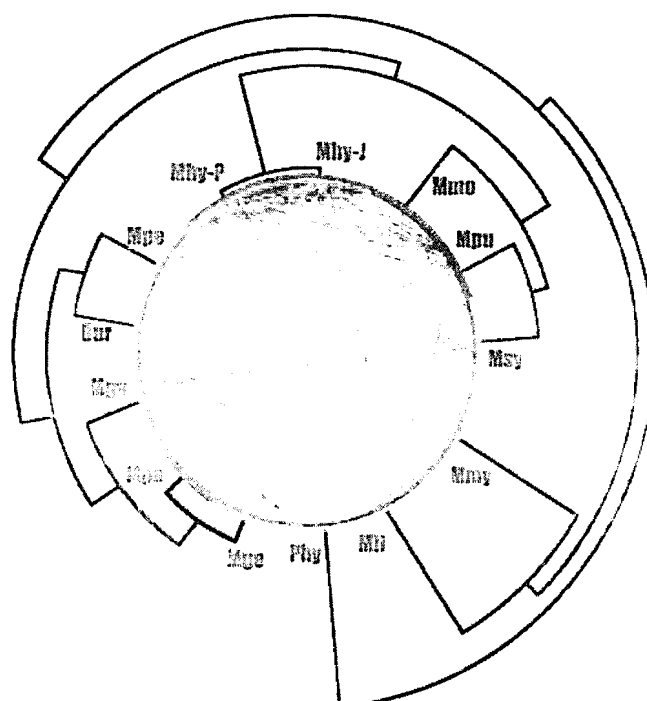


Fig. 3. Similarity matrix between 12 *Mollicutes* genomes. Connecting lines inside the circle denote BLAST matrix similarity nodes with each node representing a protein sequence. Unrooted nodes are shown in green. The color of the circle indicates the degree of similarity between nodes as follows: 0.9 (dark gray), 0.8 (medium gray), 0.7 (light gray), and 0.6 (white). The color of the circle indicates the degree of similarity between nodes as follows: 0.9 (dark gray), 0.8 (medium gray), 0.7 (light gray), and 0.6 (white). The color of the circle indicates the degree of similarity between nodes as follows: 0.9 (dark gray), 0.8 (medium gray), 0.7 (light gray), and 0.6 (white).

scale of genomic evolution was estimated (Fig. 2B) and was only 0.001 substitutions per site for *Mollicutes* and *Entomoplasma* species. Splitting times between about 400 MYBP would most of the species have diverged some 400 to 500 MYBP ago, largely in the time of the emergence and diversification of tetracytes. *M. phagocytum* and *M. genitalium* diverged quite recently, however, at 1–15 MYBP, and *M. mycoides* strains 1 and 7+8 diverged about 2 MYBP.

HGT. We analyzed the mosaicity of HGT in the 12 genomes putative to have occurred by the characteristics of protein-coding genomes, and the transfer of genes related to pathogenesis (13). A comparison of mollicute genomes using both comparative and phylogenetic strategies showed that HGT was most likely to have occurred between *M. mycoides* and *M. genitalium* (Fig. 3). Fourteen putative transferred regions were identified (Table 5); the largest comprising 5.9 kb and encoding not only hypothetical CDSs but also CDSs coding for an ABC transporter, a signal peptidase I, and a putative EF-G elongation factor. This region was almost identical in both genomes, indicating a recent transfer event. Additionally, another region containing a relevant pathogenesis-related CDS (coding for a putative sialidase) might also have been horizontally transferred (Table 5). The presence of a putative sialidase in both genomes is noteworthy, since this enzyme had been identified only in *Mycoplasma agalactiae* elsewhere among the

TABLE 5. Horizontally transferred regions between *M. synoviae* and *M. gallisepticum*

Species and region	Region			Product
	Start	End	Length	
<i>M. synoviae</i>				
1	41155	47098	5943	ABC transporter, signal peptidase I, elongation factor EF-G, conserved hypothetical proteins
2	80242	80947	705	Transposase
3	185374	186994	1620	Conserved hypothetical protein
4	221238	224926	3688	Putative sialidase, glyceraldehyde 3-phosphate dehydrogenase
5	311024	311541	517	Transposase
6	320763	325527	4764	Conserved hypothetical proteins
7	533311	534827	1516	Conserved hypothetical proteins (compositional bias)
8	535709	537101	1392	Conserved hypothetical proteins (compositional bias)
9	537179	537545	366	Conserved hypothetical proteins (compositional bias)
10	546230	547733	1503	Conserved hypothetical proteins (compositional bias)
11	549723	554763	5040	Conserved hypothetical proteins (compositional bias)
12	693080	693615	535	Conserved hypothetical protein (transposase)
13	693616	695386	1770	Conserved hypothetical proteins (transposase)
14				Hemagglutinin cluster (codon bias) ^a
Total			29359 (3.21) ^b	
<i>M. gallisepticum</i>				
1	356997	362986	5989	ABC transporter, signal peptidase I, elongation factor EF-G, conserved hypothetical proteins
2	372501	373205	704	Transposase
3	370747	372370	1623	Conserved hypothetical protein
4	771294	774961	3667	Putative sialidase, glyceraldehyde 3-phosphate dehydrogenase
5	400149	400682	533	Transposase
6	365139	369905	4766	Conserved hypothetical proteins
7	969572	971087	1515	Conserved hypothetical proteins
8	951348	952740	1392	Conserved hypothetical proteins
9	950904	951270	366	Conserved hypothetical proteins
10	947932	949436	1504	Conserved hypothetical proteins
11	349890	354930	5040	Conserved hypothetical proteins
12	398943	399492	549	Conserved hypothetical protein (transposase)
13	401897	403633	1736	Conserved hypothetical protein (transposase)
14				Three hemagglutinin CDSs ^c
Total			29384 (2.58)	

^a Due to the large extent of recombination in hemagglutinin clusters, a single region of horizontal transfer could not be identified. However, region 14 in this study contains CDSs listed in Table 1 at <http://www.brgene.ncc.br/finalMS/table1> as cluster number 2.

^b The genome percentage is shown in parentheses after the total length.

mycoplasmas (6). Sialidase cleaves terminal sialic acid residues from sialoglycoconjugates, generating free sialic acid (a likely nutrient). The *M. synoviae* genome region (216417 to 224099) contains five CDSs encoding enzymes involved in sialic acid catabolism (sialic acid lyase, *N*-acetylmannosamide kinase, *N*-acetylmannosamine-6-phosphate epimerase, glucosamine-6-phosphate isomerase, and *N*-acetylglucosamine-6-phosphate deacetylase). Some of these enzymes are found in selected mycoplasmas (e.g., *M. hyopneumoniae*, *M. mycoides*, and *M. penetrans*), but none of them contains the CDS cluster found in *M. synoviae*. This suggests that sialic acid could be a substrate for *M. synoviae* growth, a hypothesis that can be addressed experimentally.

Hemagglutinins play a fundamental role in the pathogenesis of *M. synoviae* and *M. gallisepticum*, and their genes could have been transferred between these species (5, 40). However, the organization of hemagglutinin genes differs sharply in *M. synoviae* and *M. gallisepticum*, the former with a single locus (1) comprising 70 CDSs (see Fig. 1 in <http://www.brgene.ncc.br/finalMS/fig1>) and the latter containing 43 genes organized in

five loci (44). Whole-genome alignments of *M. synoviae* and *M. gallisepticum* allowed the identification of three *M. gallisepticum* hemagglutinin genes showing a strong similarity to the *M. synoviae* hemagglutinin CDS cluster. Using the Tribe-MCL algorithm, based on similarity data of the whole set of hemagglutinin CDSs of *M. gallisepticum* and *M. synoviae*, three hemagglutinin CDS groups could be identified (see Table 1 at <http://www.brgene.ncc.br/finalMS/table1>). The first one contained 41 *M. synoviae* CDSs, the second contained 35 *M. gallisepticum* CDSs, and the third contained 29 *M. synoviae* CDSs and the 3 *M. gallisepticum* highly homologous CDSs found in whole-genome alignments. These results, and additional phylogenetic and codon usage analyses, support the postulation of a likely transfer event of hemagglutinin genes from *M. gallisepticum* to *M. synoviae*.

Comparative genomics and evolution. A deeper understanding of the genomic diversity and evolution of *Mycoplasma*tales is now possible by analyzing the available sequenced genomes of species belonging to the Hominis clade (*M. pulmonis*, *M. hyopneumoniae*, *M. synoviae*, and *M. mobile*) and the Pneu-

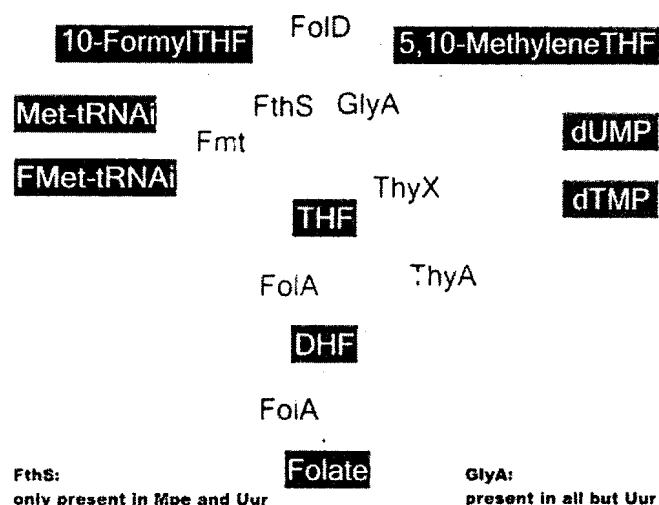


Figure 3. Sedimentary deposition of ^{137}Cs in the distribution area, 1986–1990. A: 1986; B: 1987; C: 1988; D: 1989; E: 1990. The values show the activity of the total sediment sample (NA) and of the ^{137}Cs in the sediment (BNA). The values of NA and BNA are expressed in Bq/kg of dry weight. The values of NA and BNA are expressed in Bq/kg of dry weight. The values of NA and BNA are expressed in Bq/kg of dry weight.

the archaeal *M. marburgensis*, *M. pyrodicticus*, *M. caldococcus*, *M. thermophilus*, and *M. thermoautotrophicus*. Apparently, the evolutionary reduction of archaeal genomes resulted in preservation of a common set of essential metabolic capabilities, rather than a minimum set of specific genes or pathways. The one-carbon metabolism, involving CO_2 /HIF metabolism clearly illustrates the evolutionary retention of redox and metabolic pathways (Fig. 1). The CO_2 /HIF metabolism, conserved in both Bacteria and Eucarya, involves results in formation of the methionine-tRNA adduct, Met-tRNA^{Met}, as well as de novo dTMP synthesis. *M. pyrodicticus* is the only exception, because it is apparently unable to form Met-tRNA^{Met} due to the absence of an essential tRNA formyltransferase of histidine and peptide deformylase (Dfr).

Escherichia coli is also devoid of enzymes involved in the C₁ cycle, except for pyruvate hydroxymethyltransferase (PdhX). Several mutants exist, except *M. leishmania*, which possess an alternative transhydroxymethyltransferase (NADPH-dependent) to PdhX, and a CysA and a CysB homologous to those of *E. coli* may be conserved and probably occur in *Leishmania*, *M. guinea*, *M. parvum*, *M. mageritensis*, *M. leishmania*, *M. fortuitum*, and *M. goodii*.¹⁶ However, CysA and CysB are absent in *E. coli* and *Leishmania*. In both cases, a minimum set of metabolic interventions is suggested for Met tRNA formation and dUMP synthesis has been conserved through the pathway, but has not. *M. parvum*, which has the largest known mycoplasma genome, is the only species tested that has retained a recondent C₁-H₂-H₂ pathway. The absence of initiator Met tRNA provides selection for a methionine codon (42) and also blocks the binding of the elongation factor EF-Tu to the initiator tRNA (39, 54). We noticed the absence of FldD, Fmt, and Fln in a recently sequenced pathogenic mycoplasma (42). As far as we know, *M. mycrophilum* and *Phytoplasma* sp. are the first naturally occurring eubacteria potentially unable to formylate methionyl-tRNA^{Met}. The physiological consequences, if any, to

bacterial growth behavior remain to be studied. Interestingly, disruption of the *hut* gene severely impairs growth of *Escherichia coli* [13]. This could be related to the higher binding affinity of IF-1 to Met-tRNA^{Met} than to Met-tRNA^{Leu} [14]. However, a *hut*-deficient strain of *Thalassothrix weissii* is not only viable, but also undergoes initiation of protein synthesis very rapidly, because IF-1 is not a substrate for the *hut* gene [15].

Genomic reduction resulted in a complex pattern of deletions and relocations in the purine and pyrimidine metabolism pathways. Except for *M. pneumoniae* and *M. genitalium*, no other pair of mycoplasma species shares an identical set of pyrimidine metabolism pathways. Uridine kinase was found to be specific for the Pneumoniae clade, while 5' nucleotidase is restricted to the Hominis clade. The lack of both enzymes was previously reported for *M. penetrans* (50). was also observed in *M. vinayana*. Although UMP may be derived from uridine and/or carbamoyl phosphate in *M. penetrans* (50), *M. vinayana* is exclusively dependent for UMP synthesis. The lack of both enzymes is also true for *M. penetrans* and *M. vinayana* and the absence of uridine and carbamoyl phosphate is also true for *M. penetrans* and *M. vinayana*. Although *M. penetrans* and *M. vinayana* lack UMP synthase, they possess UMP decarboxylase (51).

Intero-de-reliance on TS-THF is an interestingly novel feature of thymine-dependent thymine cytosine syntheses (ThyX) that produce THF. It is reduced forms widely distributed in various genomes [37]. Mycoplasmas lacking ThyA (*M. hyarum* and *C. urealyticum*) and *M. mycoides* might have an alternative mechanism for de novo dTMP synthesis involving an enzyme that ThyX activates, alternatively, the dTMP pool could be directly dependent on a thymidylate kinase, which is present in all mycoplasmas sequenced so far.

[illegible]

Conclusions. Comparative analyses of rickettsial genomes allowed the identification of adaptive mechanisms occurring

for perpetuation across a wide host range. Despite their small genomes, mycoplasmas showed a heterogeneous gene composition, with several species-specific genes whose identification and functional characteristics might be helpful for the prevention and treatment of diseases caused by these bacteria. Our analyses confirmed the occurrence of high rates of genomic rearrangements in mycoplasmas, which was demonstrated by the presence of strain-specific regions in the three *M. hyopneumoniae* strains; some of these regions were probably involved in rearrangements or pathogenesis. In fact, a variety of CDSs coding for putative, outer membrane proteins, or adhesins, were found to contain motifs of repetitive sequences which might have a role in their biological function or in antigenic variation. The presence of an ICEH element restricted to pathogenic strains suggests its possible role in pathogenicity. For the first time, phylogenetic relationships of all sequenced mycoplasma genomes were established on the basis of a concatenated data set, which resulted in a single, highly supported tree. These data were used for estimating a time frame of genome evolution, which added new insights to the evolution of the *Mycoplasmatales-Entomoplasmatales* group. Our studies provide evidence pointing to HGT as the process that provided *M. synoviae* and *M. gallisepticum* with the capacity of infecting the same host.

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