Anais da Academia Brasileira de Ciências (2016) 88(1 Suppl.): 599-607
(Annals of the Brazilian Academy of Sciences)
Printed version ISSN 0001-3765 / Online version ISSN 1678-2690
http://dx.doi.org/10.1590/0001-3765201620150164
www.scielo.br/aabc

Molecular biology of mycoplasmas: from the minimum cell concept to the artificial cell

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Manuscript received on May 13, 2015; accepted for publication on June 2, 2015

ABSTRACT

Mycoplasmas are a large group of bacteria, sorted into different genera in the *Mollicutes* class, whose main characteristic in common, besides the small genome, is the absence of cell wall. They are considered cellular and molecular biology study models. We present an updated review of the molecular biology of these model microorganisms and the development of replicative vectors for the transformation of mycoplasmas. Synthetic biology studies inspired by these pioneering works became possible and won the attention of the mainstream media. For the first time, an artificial genome was synthesized (a minimal genome produced from consensus sequences obtained from mycoplasmas). For the first time, a functional artificial cell has been constructed by introducing a genome completely synthesized within a cell envelope of a mycoplasma obtained by transformation techniques. Therefore, this article offers an updated insight to the state of the art of these peculiar organisms' molecular biology.

Key words: mycoplasma, molecular biology, microbial genetics, synthetic biology, gene transfer techniques.

INTRODUCTION

It is believed that mycoplasmas originated through degenerative evolution from Gram-positive ancestors, with the loss of non-essential genes (Dybvig and Voelker 1996). The term mycoplasma is often used to refer to all microorganisms in the *Mollicutes* class, regardless of which genus they belong to. In order to prevent confusion with microorganisms specific to the *Mycoplasma* genus, the term mollicutes has come into use as a generic name for all microorganisms in the class (Razin et al. 1998). The criteria for defining the taxonomy of mollicutes are established by the Subcommittee on the Taxonomy of Mollicutes, linked to the International Committee on Systematics of Prokaryotes, and can be found at its website: <http://icsp.org/subcommittee/mollicutes/>.

MOLECULAR BIOLOGY OF MOLLICUTES

Aside from the fundamental differences between these and other prokaryotes, the molecular biology

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of mollicutes is very typical in some respects. Having evolved from Gram-positive bacteria, gene expression and other genetic characteristics of mollicutes are similar to those of their ancestors. On the other hand, because of the limited capacity of their genome, mollicutes do not have many of the enzymatic pathways characteristics of the majority of bacteria (Dybvig and Voelker 1996). These characteristics make mollicutes particularly interesting in molecular and cellular biology studies, as, even with these limitations, they are capable of independent replication and parasitism, causing diseases that lead to losses in agriculture as well as in human health.

GENOME STRUCTURE AND ORGANIZATION

Mollicutes have a chromosome made up of circular double stranded DNA, which can vary between 580 and 1,800 kb (Razin et al. 1998). The 580 kb chromosome of the human pathogen *M. genitalium* is currently the smallest known genome (Lucier et al. 1994). The GC content of the majority of mollicutes' chromosomes is around 35%, with 25% being the smallest (Glass et al. 2000). Even though they have such small genomes, some mollicutes have repeated sequences of DNA in their chromosomes. The repeated elements found in mollicutes are homologous to members of the IS3 Family Insertion Sequence (Westberg et al. 2002, Pilo et al. 2003, Ditty et al. 2003, Thomas et al. 2005, Lysnyansky et al. 2009).

With the exception of spiroplasmas, extrachromosomal DNA is rarely found in mollicutes. Currently, only a few plasmids have been isolated from *M. mycoïdes* subsp. *mycoïdes* and from *S. citri* (Bergemann et al. 1989). On the other hand, numerous replicative forms of viral genomes (phages) have been isolated from *Acholeplasma* and other species of *Mycoplasma* and *Spiroplasma* (Maniloff 1992).

In recent years, the greatest advances in the understanding of mollicute biology were obtained with the sequencing of the complete genome of various species. Currently, the complete genome sequences of 46 species and strains have been published. For a detailed analysis, we suggest visiting MolliGen.org. MolliGen is a database dedicated to the comparative genomics of bacteria belonging to the Mollicutes class. It offers sequence and analysis of data collected on 54 genomes belonging to 37 species. The MolliGen database provides pages with genome and genetic elements, homology relationships defined by various methods, precompiled statistical data, metabolic pathways, and a large collection of tools to explore and compare genomes (Barré et al. 2004). Currently there is an effort, sponsored by the International Organization for Mycoplasmology (IOM), to encourage genome sequencing of all species of mollicutes.

DNA REPLICATION AND REPAIR

Studies in this area have long maintained the idea that mollicutes were very limited with respect to their DNA repair mechanisms. However, at least in *M. pulmonis*, transposon induced mutagenesis in the *recA*, *ruvA*, *ruvB,* and *ruvC* genes produced deficient phenotypes (French et al. 2008). Through *in silico* analysis of sequenced genomes, the only complete repair mechanism found in mycoplasmas is that of repair by nucleotide excision repair (NER), probably complemented by enzymes from the base excision repair (BER) mechanism and the recombination mechanism (Carvalho et al. 2005). The NER mechanism was also identified in *M. genitalium*, in which the RecA protein has an active role in the antigenic variation mechanism but not in DNA repair (Burgos et al. 2012). A histone-like protein that recognizes nucleotide incorporation errors in DNA was identified in *M. gallisepticum*. However, this does not appear to be a rule among mycoplasmas, which, during their evolution, lost the DNA mismatch repair mechanism (MMR) (Kamashev et al. 2011). Nevertheless, despite their

limitations, it is evident that mycoplasmas possess an adequate set of genes and gene products, even if they have not yet been completely identified, to promote DNA repair in a way that makes their development possible, causing significant diseases in a wide range of hosts (Gorbachev et al. 2013).

GENETIC RECOMBINATION

Until recently, there was no evidence that homologous recombination could occur in mollicutes' genomes. In *M. agalactiae*, the necessary genes for homologous recombination appear to be reduced, showing only the components involved in the formation of the presynaptic complex (*recD, recO,* and *recR*), strand exchange (*recA*), branch migration (*ruvA* and *ruvB*), and the Holliday junction resolution (*recU* and *yqgF*); however, it has already been demonstrated in other bacteria that, with the absence of complete sets of genes, homologous recombination can occur (Rocha et al. 2005). Gene disruption by homologous recombination has already been proven in *M. genitalium* (Dhandayuthapani et al. 1999) and in *S. citri* (Duret et al. 1999), and in *Mycoplasma mycoïdes* subsp. *capri*, inclusion of *recA* from *E. coli* in a suicide plasmid favored the occurrence of mutants (Allam et al. 2010). Apparently there are various mechanisms that can be explored to increase the effectiveness of this approach, which may be species-dependent.

GENETIC MATERIAL TRANSFER

Because of the absence of a cellular wall, it can be expected that the introduction of exogenous genetic material to mollicute cells happens without much difficulty. In fact, the chromosomic DNA exchange occurring during direct contact between mollicute cells (Barroso and Labarere 1988), and the spontaneous conjugal transfer of Tn916 between *Enterococcus faecalis* and *M. hominis* (Roberts and Kenny 1987) is probably due to the momentary fusion of cellular membranes in the contact region. Nevertheless, the efficiency of DNA transfer in these cases was low.

VECTORS

Transfection experiments of *S. citri* with the SpV1 virus containing an inserted gene that codes the *M. pulmonis* P1 adhesin resulted in an expression of this spiroplasma fragment. However, this system is inefficient, since there is a rapid loss of the cloned DNA fragment. Transposons have also been used in the transformation of *A. laidlawii* and *M. pulmonis* in antibiotic-resistant mollicutes using the pAM120 plasmid, containing the Tn*916* streptococcus transposon, which confers resistance to tetracycline through the expression of the *tetM* gene (Dybvig and Cassell 1987). Other transposons have also been used with various species of mollicutes since then, such as Tn*1545* and Tn*4001* (Cao et al. 1994, de Barbeyrac et al. 1996, Foissac et al. 1997, Kapke et al. 1994). The transposons undergo excision from the plasmid that contains them, and integrate with the mollicute chromosome in random positions (Foissac et al. 1997). Mutagenesis by transposon is done by inserting it into genes, causing the inactivation of transcription or the formation of truncated products. The genes containing the transposon are then mapped and identified, and the definitive proof of its function is given by the complementation of the mutant with the wild-type gene. This strategy has been used in *M. pneumoniae* (Fisseha et al. 1999), *M. genitalium* (Reddy et al. 1996) and *S. citri* (Jacob et al. 1997). However, in many cases, these elements transpose actively, changing location in the chromosome frequently (Foissac et al. 1997).

Another type of vector was constructed by inserting the *oriC* region of *S. citri* in the commercial plasmid pBS (Stratagene, La Jolla, Ca, USA), and the resulting recombinant plasmid was efficiently introduced into the spiroplasma cells by electroporation, where it was able to replicate

and maintain stability as an extrachromosomal element (Renaudin et al. 1995). Vectors derived from this plasmid show themselves to be capable of inactivating specific *S. citri* genes (Duret et al. 1999). The great advantage of these types of replicative plasmids is the possibility to direct the insertion of the vector to a specific region of the chromosome. In this way interference from the random insertion of the vector in other mollicute genes can be avoided, which can happen with viral particles and transposons. This method also makes it possible to promote complementation of the mollicute genes in mutant strains (Gaurivaud et al. 2000), definitively proving its function. Vectors derived from the pKMK1 plasmid were also used in the transformation of *M. mycoïdes* and *M. capricolum* (King and Dybvig 1993). However, these plasmids produce few copies, they are incapable of transforming other species of mollicutes, and the stability of the inserts in these vectors does not appear to be adequate (Dybvig and Voelker 1996).

The cloning of the fragment containing the DnaA boxes located near the *M. capricolum* subsp. *capricolum dnaA* gene, through the pSRT2 vector, initially developed to transform *S. citri* (Lartigue et al. 2002), made possible the construction of two new plasmids, pMCO2 and pMCO3. The pMCO2 plasmid was constructed using the *oriC* region of *M. capricolum* inserted at the *Bam*HI multiple cloning sites oriented in the same direction as the *tetM* gene. Construction of the pMCO3 plasmid was done in a similar manner to that of pMCO2, although the cloned fragment was oriented in the opposite direction of the *tetM* gene. By cloning the amplified fragments of the *spoJ-dnaA* and *dnaN-spoJ* intergenic regions of *M. genitalium* in the pSRT2 vector, it was possible to obtain the pMGO1 and pMGO2 plasmids. In the same way, cloning the fragments containing the putative *M. pulmonis* UAB CTIP DnaA boxes in the pSRT2 vector made it possible to create the new plasmid named pMPO1. This plasmid contains the *M. pulmonis oriC* region inserted at the *Bam*HI site of the multiple cloning regions in the same orientation as the *tetM* gene. By amplifying the 327 bp fragment containing the DnaA boxes located in the *dnaA-dnaN* intergenic regions of *M. pulmonis* and cloning it in the pSRT2 vector, the pMPO2 plasmid was constructed. In the same way, by cloning a 262 bp fragment containing the DnaA boxes located in the *recD-dnaA* intergenic region, the pMPO3 plasmid was constructed. The pMPO4 plasmid was constructed cloning both of these fragments side by side at the *Bam*HI site of the pSRT2 vector. The pMPO5 plasmid was constructed cloning these two fragments with the *tetM* gene in between (Cordova et al. 2002). Further studies using the results of this work were able to produce the specific oriC plasmids capable of transforming the *M. mycoïdes* subsp. *mycoïdes* SC and *M. mycoïdes* subsp. *capri* (Lartigue et al. 2003). Suicide plasmids were constructed by amplifying a 717 bp internal fragment of the *M. pulmonis* hemolysin A gene and inserting it in the pSRT2, pMPO1 and pMPO5 plasmids, by which disruption vectors were obtained, with the names pSRT2-Δ*hlyA*, pMPO1-Δ*hlyA* and pMPO5-Δ*hlyA*, respectively (Cordova et al. 2002).

Plasmids with the *M. capricolum* subsp. *capricolum* oriC region are not capable of replicating in *M. mycoïdes* subsp. *mycoïdes* SC or *M. mycoïdes* subsp. *capri*, all phylogenetically related and belonging to the Mycoïdes group. On the other hand, plasmids with the oriC region from *M. mycoïdes* subsp. *mycoïdes* SC, *M. mycoïdes* subsp. *capri* or even from *S. citri* are capable of transforming and replicating in *M. capricolum*. *M. mycoïdes* subsp. *mycoïdes* SC and *M. mycoïdes* subsp. *capri* are capable of being transformed with each other's plasmids (Lartigue et al. 2003). The use of plasmids with the *M. mycoïdes* subsp. *capricolum* oriC region was shown to be viable for heterologous gene expression in this mollicute, but gene disruption by homologous recombination was

only possible with plasmids containing the *S. citri* oriC region (Janis et al. 2005).

Presence of DNA Restriction-Modification Systems

Restriction-modification systems are very common in mollicutes and can obstruct gene transfer. Some studies have shown that the transformation of *S. citri* with isolated *E. coli* plasmids are only successful when the *E. coli* strain used had type I *Eco*K methyltransferase, which suggests that the methylation by *Eco*K is necessary to protect plasmid DNA from the *S. citri* restriction system (Bové 1993). Because of this, transformation errors of certain mollicutes with certain vectors can be due to restriction enzyme activity (Dybvig and Voelker 1996, Sirand-Pugnet et al. 2007).

Horizontal Gene Transfer

The possibility of horizontal gene transfer between different mollicutes, at least for more phylogenetically related species, has long been investigated, considering the small genome and probable degenerative evolution mechanism, which in theory doesn't contribute to the acquisition of new genes. One of the first studies to demonstrate horizontal gene transfer was between different serotypes of *Ureaplasma* sp., isolated from clinical samples (Xiao et al. 2011). These studies explained the persistent difficulty in relating different levels of pathogenicity and thus different clinical presentations to different microorganism serotypes. In this research, it was demonstrated that not less than 40% of the isolated *U. urealyticum/parvum* strains were genetic mosaics. Evidence of horizontal gene transfer had already been reported in species located in the same ecological site (Sirand-Pugnet et al 2007) and in mycoplasmas of the same species (Teachman et al. 2002). It was also demonstrated that certain strains of *Ureaplasma* sp. are capable of forming biofilms, just as various mollicutes that infect animals, such as *M. pulmonis*, a characteristic acquired from other bacterial species (Garcia-Castillo et al. 2008). In addition to biofilms, the presence of an integrative conjugative element (ICE) in *M. agalactiae*, as multiple copies in the chromosome or in free circular form, is worthy of attention because it is a microorganism responsible for a disease that can also be caused by *M. capricolum* subsp. *capricolum* and *M. mycoïdes* subsp. *capri* (Marenda et al. 2006). ICEs can also be called CONSTINS or conjugative transposons and are capable of conferring various metabolic or resistance characteristics to their hosts. They are self-transmissible elements, which carry the genes necessary for excision, conjugative transfer, and integration with the host (Burrus and Waldor 2004). There are indications that at least 18% of the *M. agalactiae* genome was transferred horizontally from mycoplasmas of the Mycoïdes group, since the former, even though it can inhabit the same hosts and ecological sites, is phylogenetically located in the Hominis group (Sirand-Pugnet et al. 2007). Nevertheless, half of these genes' functions are still unknown, and the mass transfer of genes is not believed to have had a very significant role in the evolution of mollicutes. However, this data indicates that, in addition to being able to survive against the host's defenses, mollicutes are also capable of promoting chromosomic exchange to a certain degree, which avoids genome stagnation, making possible the continued adaptation to new sites and even new hosts (Sirand-Pugnet et al. 2007). Nevertheless, the mechanism by which gene transfer occurs between mollicute cells is still not well documented (Dordet-Frisoni et al. 2014).

THE MINIMAL GENOME CONCEPT

Because *M. genitalium* has the smallest bacterial genome currently known (Fraser et al. 1995), some authors have investigated the hypothesis that its genome represents the minimum gene set sufficient for life in a microorganism capable of self-replication. Mushegian and Koonin (1996)

compared this mycoplasma's genome with that of *Haemophilus influenzae*, identifying 240 common genes. Adding 22 genes related to essential metabolic pathways not identified in these microorganisms, and subtracting 6 redundant genes specific to parasites, the authors came to a total of 256 genes, which they classified as the approximate minimum number of genes necessary for the functioning of a cell. However, *M. genitalium* has double this number of genes. Maniloff (1996) suggests that the difference is probably reflected in that the comparison was made between a minimal genome produced by billions of years of evolution and a minimal gene set produced by a computer. Other authors attempted to determine the minimum number of essential genes through mutagenesis experiments with *M. genitalium* (Hutchinson et al. 1999). In a study involving global transposon mutagenesis, the authors determined that 387 genes is the minimum set necessary for life (Glass et al. 2006). In a comparison of 186 genomes, including *M. genitalium*, other authors determined that 702 is the minimum number for Gram-negative bacteria and 959 for Gram-positive bacteria. Naturally, the necessity for genes in mycoplasma cells is lower, since they maintain a parasitic life in their hosts (Huang et al. 2013). In *M. pneumoniae*, phylogenetically related to *M. genitalium*, 33% of its 816 kpb genome, including 0.9% of intergenic regions, were considered essential (Lluch-Senar et al. 2015). In *M. mycoïdes*, using gene cloning in yeast cells and carrying out transposon mutagenesis to reduce the genome, making use of yeast's sexual cycle, 368 genes were determined as non-essential gene candidates. From these, 11 of 14 genes were of the DNA restriction-modification system, 3 of 5 were *vlc* genes, and 7 of 22 were integrative conjugative elements (Suzuki et al. 2015).

CONSTRUCTION OF AN ARTIFICIAL CELL

With the relative success of the studies attempting to determine the minimal gene set for necessary for life, at least for a bacterium, the following dream began taking shape, making use of synthetic biology: to construct a cell from the available genetic information. Creating life has not yet been accomplished, as one of the major obstacles is completely synthesizing a viable cell envelope. Nature is still very mysterious. However, constructing a new cell from the genome of a different bacterium has been done, by inserting the genome in the cell envelope (membrane) of the other. It all began with cloning experiments and transposing the entire *M. mycoïdes* genome into a yeast cell (from which the original DNA was removed) (Benders et al. 2010). Keeping this cloned genome in a plasmid has also been done (Karas et al. 2013), and later transposing it again into the cell membrane of another mycoplasma species *(M. capricolum*) (Lartigue et al. 2009). This mycoplasma to yeast genome transfection technology has also made possible new methods of reducing the genome, in the continuous search for the minimal essential gene set. In this way, it was possible to create strains with reduced genomes but normal growth, without defective phenotypes (Suzuki et al. 2015). The recombination of mycoplasma genomic elements in yeasts may make possible not only the arrangement of deleted regions (non-essential), but also a combination of deletions, insertions and substitutions of genetic elements. It is possible to recombine two entire synthetic genomes and recombine gene cassettes between them, or to recombine synthetic genome segments through homologous recombination (Suzuki et al. 2015).

A major step towards the creation of life was the synthesis of a complete genome from the *M. genitalium* sequence information in genetic databases, first cloned in *E. coli* and *Saccharomyces cerevisiae* (Gibson et al. 2008). The next step, labeled genome synthesis technology, was to construct a new genome from the *M. mycoïdes* sequence, including 'watermark' sequences, dele-

tions, polymorphisms and mutations, and then inserting this new genome into the cell envelope of *M. capricolum*. This new cell was shown to be completely controlled by the new synthetic genome (Gibson et al. 2010). This created a new microorganism, neither *M. mycoïdes* nor *M. capricolum*, which became widely known as *Mycoplasma laboratorium*.

The cloning of genomes in yeast, in addition to having been recreated with other bacteria, was also demonstrated with eukaryotic chromosomes (Karas et al. 2013).

If, or better yet, when it becomes truly possible to create life in a laboratory from the synthesis of complex molecules using only chemical precursors based on the available genetic information, uniting it all in a synthetic cell envelope, many paradigms will be broken, not only in science, but also in philosophy, theology, ethics and sociology.

RESUMO

Os micoplasmas constituem um grande grupo de bactérias, ordenadas em diferentes gêneros na classe Mollicutes, cuja principal característica em comum, além do genoma reduzido, é a ausência de parede celular. São considerados modelos de estudo de biologia celular e molecular. Apresentamos uma revisão atualizada da biologia molecular destes microrganismos modelo e do desenvolvimento de vetores replicativos para a transformação dos micoplasmas. Estudos de biologia sintética inspirados nestes trabalhos pioneiros se tornaram possíveis e ganharam a atenção da grande mídia. Pela primeira vez, um genoma artificial foi sintetizado (um genoma mínimo produzido a partir das sequências de consenso obtidas de micoplasmas). Pela primeira vez, uma célula artificial funcional foi artificialmente construída, por introdução de um genoma completamente sintetizado dentro de um envelope celular obtido de um micoplasma, por técnicas de transformação. Desta forma, este artigo oferece um olhar atualizado sobre estado da arte da biologia molecular destes peculiares microrganismos.

Palavras-chave: micoplasma, biologia molecular, genética microbiana, biologia sintética, técnicas de transferência de genes.

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