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REVIEW

Tools for the genetic analysis of *Mycoplasma*

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Abstract

The mycoplasmas have attracted much scientific attention since they contain the smallest known genomes of any independently viable bacterial species. However, the detailed genetic analysis of these bacteria has lagged behind the well-analyzed bacterial model organisms for a long time. This is due to the use of the UGA codon for tryptophan rather than as stop codon, which had often prevented the expression of full-length *Mycoplasma* proteins in heterologous hosts. Additionally, insufficient efficiency of homologous recombination prevented the targeted disruption of genes in some species such as *M. pneumoniae* whereas homologous recombination is operative in other mycoplasmas. Only recently, efficient screening systems for the use of transposon-based mutagenesis have been developed to circumvent this problem and to allow the targeted isolation of desired transposon insertion mutants. With the availability of several *Mycoplasma* genome sequences, artificial plasmids based on the chromosomal origin of replication were constructed that can now be used for complementation studies and for the stable introduction of foreign genetic material. In this review, we give an overview on recent developments in *Mycoplasma* genetics that facilitate the genetic manipulation of these interesting organisms.

Keywords: Mollicutes; Transposon; Gene knockout; Multiple mutation reaction; Reporter system

Introduction

The mycoplasmas are Gram-positive bacteria that lack a cell wall and that are characterized by extremely reduced genomes. In addition to the cell wall biosynthesis apparatus, mycoplasmas have lost a huge portion of the metabolic and regulatory capacities of an average Gram-positive bacterium. Thus, their genomes range in size from 580 kb (*Mycoplasma genitalium*) to 1358 kb (*Mycoplasma penetrans*) (Fraser et al., 1995; Sasaki et al., 2002). This feature made the mycoplasmas a suitable research object for the determination of the minimal gene set required for independent life and prompted attempts to create an artificial cell that is modelled based on the paradigm of *M. genitalium* devoid of all non-essential genes (Hutchison et al., 1999; Check, 2002; Glass et al., 2006). Since, the genes for the tricarboxylic acid cycle and a functional respiration chain are absent in their genomes, energy conservation is restricted to substrate level phosphorylation via glycolysis and, in some species, arginine hydrolysis (for review see Miles, 1992). As observed for the pathways of the central metabolism, only those regulatory systems have been maintained in mycoplasmas during their reductive evolution that are thought to be indispensable to survive in their natural environment. Among the very few remaining regulators are the heat-shock transcription factor HrcA and the metabolite-controlled HPr kinase/phosphorylase of the phosphoenolpyruvate:

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sugar phosphotransferase system. The two systems are involved in the regulation of heat shock gene expression and in the regulated modification of the signal transduction protein HPr, respectively (Weiner et al., 2003; Halbedel et al., 2004; Madsen et al., 2006; Musatovova et al., 2006). An additional consequence of the reduced coding capacity is the combination of unrelated enzymatic activities in single enzymes as it was shown for some glycolytic kinases that had acquired nucleoside diphosphate kinase activity to function also in nucleotide metabolism (Pollack et al., 2002).

Beside these interesting peculiarities, the mycoplasmas are pathogens of humans and animals. The human pathogen *M. pneumoniae* colonizes the surfaces of the respiratory tract resulting in mostly uncomplicated and mild infections, especially in young and elder patients (Jacobs, 1997). In contrast, *M. mycoides* subsp. *mycoides* small colony variant is the etiological agent of contagious bovine pleuropneumoniae and causes severe infections leading to major losses in African livestock of cattle (Thiaucourt et al., 2003). The pathogenicity of several *Mycoplasma* species was attributed to the production of hydrogen peroxide during normal cell metabolism that causes harm to host tissues by oxidative damage (Almagor et al., 1984; Pilo et al., 2005). To persistently colonize their hosts, mycoplasmas have developed a set of surface-exposed adhesins that may undergo phase and size variation to efficiently evade the hosts immune system (for review see Razin (1999).

The detailed genetic analysis of these organisms has been hampered for a long time by the lack of genetic tools that allow (i) the efficient expression of UGA-containing *Mycoplasma* genes in heterologous hosts for purification and subsequent biochemical analysis, (ii) the stable introduction of foreign genetic material into a *Mycoplasma* cell, and (iii) either the targeted construction or the targeted isolation of desired mutant strains.

During the last few years' considerable progress has been made in the field of *Mycoplasma* genetics that made these organisms accessible for genetic studies. Here, we will give a brief overview on the classical and recently developed tools for mycoplasmal genetics. This review is accompanied by additional material available at the URL http://wwwuser.gwdg.de/~genmibio/mycoplasma_tools.html.

Heterologous expression of *Mycoplasma* genes containing UGA codons

Until the end of the 1970s the genetic code was considered to be universal. With the discovery that the opal stop codon UGA is used to incorporate tryptophan in yeast mitochondria and in the mycoplasmas, this dogma had to be abandoned (Macino et al., 1979; Yamao et al., 1985). This peculiar modification of the genetic code was thought to be the result of an optimization process in response to the low genomic G/C content: in a first step, the UGA opal codon was not longer used leaving UAA to serve as the stop codon of choice besides UAG that occurs with a minor frequency in *M. pneumoniae* (Himmelreich et al., 1996). Subsequently, the UGG tryptophan codon was sequentially replaced by the released UGA codon to further optimize the G/C content (Jukes et al., 1987). This occurrence of UGA codons in *Mycoplasma* genes has often prevented their expression in heterologous hosts for detailed biochemical analysis, since they serve as stop codons in *Escherichia coli* and other expression hosts. To circumvent this problem, a variety of different but rather dissatisfying strategies had been employed, including the expression of UGA-containing *Mycoplasma* genes in opal suppressor strains of *E. coli* (Smiley and Minion, 1993), or in *Spiroplasma citri*, which also reads UGA as a tryptophan codon (Stamburski et al., 1991). As long as only few UGA codons are present in a mollicute gene, their sequential replacement by standard site-directed mutagenesis strategies might also be taken into consideration (Robino et al., 2005). However, the latter approach is time consuming and cost-intensive with an increasing number of UGA codons.

Recently, we designed a strategy referred to as multiple mutation reaction (MMR) that allows the simultaneous replacement of multiple UGA codons in a single-step reaction (Hames et al., 2005). This strategy is based on the concept of the combined chain reaction (CCR) described by Bi and Stambrook (1997), where 5′-phosphorylated oligonucleotides containing the desired mutations are included in a polymerase chain reaction. During the elongation steps the external amplification primers are extended. Since, the mutation primers are designed to hybridize more strongly to their targets, the elongated amplification primers can then be ligated to the 5′ ends of the mutation primer by a thermostable DNA ligase, yielding a DNA strand that contains the desired mutation. Using this principle more than one mutation had been introduced simultaneously in *ptsG* promoter fragments of *Bacillus subtilis* (Schilling et al., 2004). In fact, we improved this strategy for the simultaneous introduction of up to 9 A→G transitions to replace 9 UGA codons of the *M. pneumoniae glpK* gene by UGG codons in a single step (Hames et al., 2005). We expect that the number of 9 UGA codons does not mark the upper limit of simultaneous replacements that can be obtained by MMR.

Transposons

The majority of genetic tools which are well-established in model organisms such as *B. subtilis* or *E. coli*