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Fewer Essential Genes in Mycoplasmas than Previous Studies Suggest

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Abstract

We describe here mutants of *Mycoplasma pulmonis* that were obtained by using a minitransposon, Tn*4001TF1*, which actively transposes but is then unable to undergo subsequent excision events. Using Tn*4001TF1*, we disrupted 39 genes previously thought to be essential for growth. Thus, the number of genes required for growth has been overestimated. This study also revealed evidence of gene duplications in *M. pulmonis* and identified chromosome segregation proteins that are dispensable in mycoplasmas but essential in *Bacillus subtilis*.

Introduction

Because of their small genomes, mycoplasmas are commonly chosen as model organisms for the study of the minimal gene set needed to support the growth of free-living bacteria and are ideal for dissecting the role of individual genes in pathogenesis. The most comprehensive work has been performed with *Mycoplasma genitalium*. Using transposon mutagenesis, 387 of the 480 protein-coding regions and all of the 37 genes coding for RNA species were identified as essential for growth of *M. genitalium* (Glass *et al.*, 2006). Because of the lack of gene duplication and the absence of redundant pathways, it should be expected that the 580-kb genome of *M. genitalium* would have more essential genes than a bacterium with a large genome such as *Bacillus subtilis*, a Gram-positive bacterium to which the mycoplasmas are phylogenetically related. Indeed, only 271 of the 4,100 genes of *B. subtilis* are thought to be essential (Kobayashi *et al.*, 2003).

Mycoplasma pulmonis is a pathogen of rats and mice and the etiological agent of murine respiratory mycoplasmosis (Lindsey & Cassell, 1973). In an earlier study of a transposon library of *M. pulmonis*, 321 of the 782 protein-coding regions were identified as dispensable for growth (French *et al.*, 2008). The criteria used to consider a gene to be inactivated were that at least 10% of the coding region from the annotated 5′ start site or at least 15% of the coding region from the 3′ end would be truncated. The size of the library was large enough to conclude that nonessential genes greater than 1 kb were inactivated.

The previous studies relied on Tn*4001T*, which transposes actively in the mycoplasmal genome. A more recent study in *Mycoplasma arthritidis* made use of the Tn*4001TF1* minitransposon, derived from Tn*4001* (Dybvig *et al.*, 2008). The minitransposon readily transposed into the genome, but once inserted, was unable to undergo subsequent transposition events. This minitransposon was applied to *M. pulmonis* in the current study.

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The use of the minitransposon generated a superior library with inactivation of 39 genes that were previously thought to be essential.

Materials and methods

Library construction

M. pulmonis strain CT (Davis *et al.*, 1986) was propagated in mycoplasma broth (MB) and mycoplasma agar (MA) as described (Dybvig *et al.*, 2000; Simmons & Dybvig, 2003). CT was transformed with plasmid pTF85, carrying minitransposon Tn*4001TF1* (Dybvig *et al.*, 2008), by using 36% polyethylene glycol and selecting for resistance to tetracycline as described (Dybvig *et al.*, 2000). Individual colonies were picked, grown in 1 ml MB and stored at −80°C as described (French *et al.*, 2008).

Mapping of transposon insertion sites

The genomic location of the transposon was determined for each library member by DNA sequence analysis of an inverse PCR product containing the junction between the transposon and the adjacent mycoplasmal DNA by using primers and reaction conditions as described (French *et al.*, 2008; Teachman *et al.*, 2002). The sequence of the junction was compared to the complete genome sequence for identification of the transposon insertion site (Chambaud *et al.*, 2001). For each transformant that disrupted a gene in the current library that had not been disrupted in the previous library, the genomic position of the transposon was confirmed by performing two sets of PCR amplifications, analyzed on agarose gels stained with ethidium bromide, as described (French *et al.*, 2008). The first set used a transposon-specific primer paired with a gene-specific primer. The presence of a PCR product of the predicted size indicated that the transposon was at the expected location provided that the same PCR product was absent when using the parental wild-type strain as template. For the second PCR amplification, two gene-specific primers were used that would flank the site of the transposon. If the expected product was obtained with wild-type DNA as template but no product was obtained with the transformant DNA as template, it was concluded that the gene was disrupted and that the transformant lacked a second, intact copy of the gene.

For some transformants, the PCR amplifications confirmed the location of the transposon but also detected the presence of an intact copy of the gene. In these cases, the transformant culture was subcloned and the two PCR reactions performed again on each subclone. Prior to subcloning, cell aggregates were disrupted by sonicating in a sonifier (model 250/450; Branson, Danbury, CT) at a power level of 5 and duty cycle of 10% for 20 sec. These conditions maximally increased the CFU of the cultures. No gene was considered to be disrupted unless the PCR data indicated that at least one subclone had the transposon at the expected site with no intact copy of the gene.

Gene duplication

Rarely, the PCR data indicated that all subclones of a transformant had an intact copy of the gene that was disrupted by the transposon. The presence of both a disrupted and an intact copy of the gene suggested gene duplication. In these cases, the identity of the PCR products was confirmed by performing another PCR amplification. The products from the first amplification that had used primers that flanked the insertion site of the transposon were used as template, and an internal set of primers were used for amplification. In cases where there was doubt about the results, the PCR products were also sequenced.

Results and discussion

Disrupted genes

A total of 1210 different minitransposon insertion sites were mapped. Thus, the library is smaller than the original Tn*4001T* library for which 1856 different insertion sites were mapped (French *et al.*, 2008). Combined, the libraries provide excellent coverage with on average a transposon insertion site every 300 bp in the 960-kb genome of *M. pulmonis*.

Using the same criteria for considering a gene to be inactivated as described above for the previous library, 84 genes were inactivated by the minitransposon but not by Tn*4001T* in the original library (see Table S1) with 39 of these genes previously identified as essential for growth (French *et al.*, 2008). For each of the 84 genes, PCR analyses confirmed the location of the transposon and demonstrated the absence of an intact copy of the gene. The 321 genes inactivated in the original library and the 84 additional genes inactivated in the minitransposon library bring the total number of inactivated genes in *M. pulmonis* to 405. None of the genes coding for RNA species were disrupted in the transposon libraries.

The 1.4-kb NADH oxidase gene (MYPU_0230) was disrupted in the minitransposon library. In the original library transposon insertions mapped to this gene in 27 transformants, but in each case additional PCR analyses failed to confirm the position of the transposon in MYPU_0230 (French *et al.*, 2008). Because the minitransposon inactivated genes thought to be essential such as MYPU_0230, the distribution of the transposon insertion sites was examined for both libraries. The distribution found to be highly similar (Fig. 1). Most of the differences may be due to random chance, with the exception of two hot spots for transposon insertion that were identified in the original library as HS1 and HS2 (French *et al.*, 2008). In the minitransposon library, the density of transposon insertion sites within HS1 and HS2 was not higher than for other regions and hence the distribution of transposon insertions may be more uniform.

Because there were no substantial differences in the distribution of transposon insertion sites in the libraries, alternative explanations for the inactivation of what were previously thought to be essential genes were considered. One possibility was that some nonessential genes are required for optimal growth and mutants with these genes disrupted were lost from the original library due to transposon excision, which is known to occur precisely at a high frequency (Krause *et al*., 1997; Mahairas *et al.*, 1989). Growth curves were performed and the doubling times calculated as described (Dybvig *et al.*, 1989). The wild-type parent and a transformant that contained the minitransposon at an intergenic site had doubling times of 2.0 hours with a standard deviation (SD) of 0.1 hours. The minitransposon mutant with a disruption in the NADH oxidase gene had a doubling time of 3.2 hours (SD = 0.1 hours). With a reduction in growth rate by 50%, ample opportunity exists for revertants to eventually dominate a culture. Tn*4001* excision is often precise (Mahairas *et al.*, 1989) and occurs at a high frequency in *M. pulmonis* (Dybvig *et al.*, 2000). Thus, reversion due to loss of the transposon would be commonplace when using Tn*4001T* but not when using the minitransposon. Orthologs of 18 of the 84 genes knocked out in the minitransposon library but not the original library were identified previously (Glass *et al.*, 2006) as being essential in *M. genitalium* (Table 1). These 18 genes lack any obvious paralog in *M. pulmonis* that might have compensated for the gene loss. Many of these 18 genes may be similarly nonessential in *M. genitalium* but not inactivated previously due to the reversions that occur when using an active transposon.

The essential genes of mycoplasmas have been compared often to those of *Bacillus subtilis* because of their phylogenetic relationship (Dybvig *et al.*, 2008; French *et al.*, 2008; Glass *et al.*, 2006). Three of the *M. pulmonis* genes knocked out by the minitransposon have essential

orthologs in *B. subtilis* (Table 1). Interestingly, orthologs of these three genes are nonessential in *M. genitalium*. The *tkt* gene coding for transketolase is essential in *B. subtilis* for growth in minimal medium when using glucose as the sole carbon source (Kobayashi *et al.*, 2003) but is nonessential when alternative carbon sources and aromatic amino acids are available (Sasajima & Yoneda, 1974; Sasajima & Kumada, 1981). The finding that *tkt* (MYPU_5110) is nonessential in mycoplasmas is not surprising because of the rich medium required for growth. The other two genes that are essential for growth of *B. subtilis* but not the mycoplasmas coded for SMC (MYPU_ 7140 gene product) and the segregation and condensation protein ScpA (MYPU_ 1150 gene product). These proteins co-localize in *B. subtilis* and are required for growth at temperatures above 23°C and for normal chromosome segregation (Mascarenhas *et al.*, 2002). The *M. pulmonis* mutants used in this study were grown at 37°C, the optimal growth temperature for this organism. Perhaps the processes of chromosome segregation and cell division differ in mycoplasmas from those of other bacteria because of the lack of a cell wall, rendering the SMC and ScpA proteins dispensable under normal growth conditions.

Gene duplication

PCR analysis of minitransposon mutants provided evidence for gene duplication. For some mutants, the PCR amplifications performed to verify that a gene was disrupted yielded a product confirming that the transposon disrupted the gene but also yielded a second product indicative of an intact copy of the gene. The discrepancy could be resolved usually by subcloning the mutant. In most cases, when individual subclones were analyzed by PCR, at least one subclone had the gene disrupted with no intact copy present. Thus, the gene was mutable. In a few cases, the PCR analyses indicated that all subclones, five were analyzed, had both a disrupted and an intact copy of the gene (Table 2). The duplications were not necessary to maintain viability due to the inactivation of essential genes. The genes disrupted in transformants JS003 and JS170 are not essential because other transformants in the library had the same genes inactivated without an intact copy being present, and transformant JS620 has the transposon inserted into an intergenic region with apparent duplication. Little is known about the frequency and size of duplications in mycoplasmal genomes, but several examples of duplicated sequences have previously been described in *M. pulmonis* (Bhugra & Dybvig, 1993;Dybvig *et al.*, 1998;Dybvig *et al.*, 2007;Shen *et al.*, 2000). Duplication events may result from DNA replication errors or involve gene transfer between cells of *M. pulmonis* (Teachman *et al.*, 2002).

Concluding remarks

These results underscore the important consideration that past studies have inferred the essentiality of a mycoplasmal gene based on the use of elements that transpose actively in the genome and thus have overestimated the minimal gene set. The use of minitransposons that are stable once inserted into the genome give a more accurate appraisal of gene essentiality.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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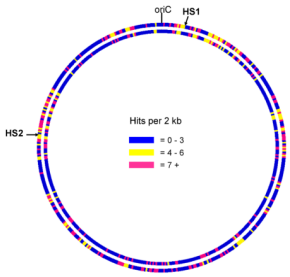

Fig. 1.

Map illustrating the density of transposon insertion sites in the original Tn*4001T* library (outer circle) and the Tn*4001TF1* minitransposon library (inner circle). The genome was divided into 480 blocks of 2 kb each with the number of transposon insertion sites color coded as indicated. *oriC* refers to the chromosomal origin of replication. HS1 and HS2 refer to hot spots for transposon insertion in the Tn*4001T* library that are absent in the Tn*4001TF1* library.

Table 1

Genes disrupted by Tn*4001TF1* but reportedly essential in *M. genitalium* or *B. subtilis*

Gene	Product	Gene length (bp)	Mg ortholog	Essential gene in*	
				Mg	Bs
230	NADH oxidase	1434	MG275	Yes	
250	Lipoprotein, nuclease	768	MG186	Yes	
870	DNA-binding protein Hu	378	MG353	Yes	
1060	Protoporphirogen oxidase HemK	690	MG259	Yes	
1150	Segregation and condensation protein A ScpA	747	MG213	No	Yes
2370	Phosphate acetyltransferase Oligopeptide ABC transporter permease protein	954	MG299	Yes	
2840	OppC Oligopeptide ABC transporter ATP-binding protein	1152	MG078	Yes	
2850	OppD	1101	MG079	Yes	
2980	Pseudouridylate synthase, D	888	MG209	Yes	
3010	Conserved hypothetical protein	921	MG265	Yes	
3140	Deoxyribose-phosphate adolase DeoC	687	MG050	Yes	
3220	Adenine phosphoribosyltransferase	510	MG276	Yes	
3640	Glucose permease	1806	MG069	Yes	
3800	GTP-binding protein	879	MG387	Yes	
5060	Methionine sulfoxide reductase B	435	MG448	Yes	
5110	Transketolase	1845	MG066	No	Yes
6210	Endonuclease IV	831	MG235	Yes	
6990	ATP synthase beta chain	1404	MG399	Yes	
7140	Chromosome segregation ATPase SMC	2937	MG298	No	Yes
7260	Leucine aminopeptidase	1362	MG391	Yes	
7620	Pyruvate dehydrogenase E2 component	945	MG272	Yes	

*Essential genes in Mg (*M. genitalium*) and Bs (*B. subtilis*) as reported by Glass *et al.*, 2006 and Kobayashi *et al.*, 2003.

Table 2

Transformants containing possible gene duplication

Transformant	Nucleotide position	Gene	Product	Gene length (bp)	Gene inactivated in other transformants of *M. pulmonis*
JS003	187277	MYPU_1780	tRNA/rRNA methyltransferase	687	Yes
JS620	642673	Intergenic	NA	NA	NA
JS170	927936	MYPU_7570	Hypothetical protein	294	Yes

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