Design and construction of two genetic tools for use in mycoplasmas

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LIST OF ABBREVIATIONS

A	adenine
AAC	aminoglycoside-*N* -acetyltransferase -
AAD	aminoglycoside-*O*-adenyltransferase
AIDS	acquired immune deficiency syndrome
APH	aminoglycoside-*O*-phosphotransferase
A	adenine
aa	amino acid
bp	base pair
C	cytosine
°C	degrees Celsius
CIP	calf intestinal phosphatase
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
G	guanine
GFP	green fluorescent protein
HEPES	N-[2-hydroxyethyl] piperazine-N′[2-ethanesulfonic acid]
HIV	human immunodeficiency virus
IPTG	isopropyl thio-β-D-galactopyranoside
IS	insertion sequence
kb	kilobase pairs
L	liter
LB	Luria-Bertani media
µF	microFarad
µg	microgram
µl	microliter
M	molar
MLO	mycoplasma like organisms
min	minute
ml	milliliter
mM	millimolar
N	nucleotide
nm	nanometer

nmol	nanomole
O.D.	optical density
ORF	open reading frame
PCR	polymerase chain reaction
pmol	picomole
RNA	ribonucleic acid
rRNA	ribosomal RNA
SB	super broth
T	thymidine
Tn	transposon
tRNA	transfer RNA
U	uracil
10X	10 fold

ABSTRACT

The members of the bacterial class *Mollicutes,* commonly referred to as mycoplasmas have a number of unusual characteristics that make them difficult research subjects. These include complex media requirements, a lack of cell walls, a high genomic AT/GC ratio and frequently a non-standard translational system. In most orders of the *Mollicutes*, the codon UGA, a stop codon in the universal genetic code, codes for the amino acid tryptophan. As a consequence, standard genetic tools frequently do not function properly in these mycoplasmas. The focus of the research described here has been on the construction of two tools with wide applicability for future mycoplasmal research. The first is a derivative of transposon Tn*4001*, mini-Tn*4001*, that can be used to irreversibly insert a variety of markers into mycoplasmal chromosomes. The second is a derivative of the popular genetic marker, green fluorescent protein (GFP). The DNA sequence of this construct, designated GFPmyco was modified to reflect a mycoplasma coding usage pattern. The results of preliminary expression studies have confirmed that mini-Tn*4001*tet is a functional mini-transposon in *Mycoplasma gallisepticum* and that GFPmyco can produce an observable fluorescent product in *E. coli*. Factors effecting GFP fluorescence and possible improvements in GFPmyco construction and expression are discussed. Both mini-transposons and GFP markers have been extremely useful for both eucaryotic and non-mycoplasma bacterial studies. It is hoped that these constructs, or their descendants, will be equally useful for the study of mycoplasmas.

INTRODUCTION

In addition to lacking a cell wall, the bacterial class *Mollicutes*, commonly referred to as mycoplasmas have simple genomes and in most cases, a high genomic AT/GC ratio. Until recently, few researchers chose to work with mycoplasmas because of handling difficulties and the general lack of major human pathogenicity. Mycoplasmas are major economic factors in large-scale livestock production, and they may also impact public health, for example as secondary infections in human immunodeficiency disorders. As a result, there has been an upsurge of interest in mycoplasma research.

While interest in mycoplasmas has increased, the technical difficulties of working with them remain. Mycoplasmas require highly enriched media, are difficult to manipulate genetically and have a nonstandard genetic code that makes the use of virtually any standard genetic tool problematic. Although mycoplasmas have smaller and less complex genomes, much less is known about essential mycoplasma functions and virulence mechanisms than the cellular activities of larger, but easier to manipulate Gram negative or Gram positive bacteria.

The focus of the research described here has been on the construction of two tools with wide applicability for future mycoplasmal research. The first is a derivative of transposon Tn*4001*, mini-Tn*4001*, that can be used to irreversibly insert a variety of markers (including green fluorescent protein) into mycoplasmal chromosomes. The second is a derivative of green fluorescent protein (GFP) that has a mycoplasma-optimized coding usage pattern. This latter construct has been designated GFPmyco.

Mobile genetic elements are powerful tools for the bacterial geneticist. One type of element, the transposon, has been used in a variety of ways including the identification of specific genes or gene control sequences. Transposons have been engineered to make them more useful by either adding useful features (reporter genes) or removing troublesome features. For example, to prevent multiple insertions of the transposon or its spontaneous excision, the transposase has been moved outside the element in some constructs.

Only two transposons are known to function in mycoplasmas, Tn*916* and Tn*4001*. Originally isolated from Gram positive bacteria, both have been used for transposon mutagenesis studies in mycoplasmas. However, either can undergo repeated and multiple insertions and/or excisions. The popular mini-transposon, mini-Tn*5,* was used as a model for the mini-Tn*4001* construct. In the mini-Tn*4001*, the Tn*4001* transposase gene has been shifted to a location on the plasmid outside the transposon. Transposition is only possible in the presence of the original plasmid sequences. Since the plasmid is unable to replicate in mycoplasmas , the transposase gene is quickly lost. The result is a stable, irreversible

chromosomal insert. The prototype mini- Tn*4001* carries only a tetracycline gene for selection purposes, but other markers, such as the GFPmyco construct or other antibiotic resistance markers could easily be inserted.

Green fluorescent protein has been used in a wide range of organisms to identify individual cells, subcellular locations of specific proteins and even follow protein-protein interactions. It also functions well as a fusion protein. While standard GFP genes might be translated in mycoplasmas, the differences in codon usage should prevent efficient translation of the message resulting in a low level fluorescent signal. In these studies we constructed a new GFP gene with the mycoplasma codon usage pattern using synthetic oligonucleotides and polymerase chain reactions. The amino acid sequence of the protein product is very similar to that of other popular GFP mutants, but it has a mycoplasma translation system optimized codon sequence and a mycoplasma promoter.

The goal of this project was to produce two constructs that would be generally useful in mycoplasma research. Their application is beyond the scope of this project. However, it is expected that the first use of mini-Tn*4001*-GFPmyco constructs will be to examine the structure and control of invasion genes in *M. gallisepticum*.

LITERATURE REVIEW

Mycoplasmas

The term mycoplasma is commonly used to denote the class of cell wall-less bacteria in the class *Mollicutes*. Other characteristic features include small genome size (600-2200 kbp) and high genomic AT/GC ratios. The mycoplasmas are grouped into 4 orders and an undefined category. This last group is largely composed of a group of organisms previously referred to as mycoplasma-like organisms (MLO) of plants, now called *Phytoplasmas*. The major genera of the class *Mollicutes* are; *Acholeplasma, Anaeroplasma, Asteroplasma, Spiroplasma, Enteroplasma*, *Mesoplasma*, *Ureaplasma* and, of course, *Mycoplasma* (111). Most mycoplasmas are obligate parasites with narrow host ranges. While some are pathogenic, many are not. It is believed that they share a common ancestor with the Gram positive bacteria, and that the separation was evolutionarily recent. They are particularly close phylogenetically to AT rich Gram positive bacteria such as *Bacillus subtilis* (107) or *Clostridium* (14).

Most mycoplasmas are not considered major pathogens in *homo sapiens*. Of the 150 mollicute species of human origin listed in one review, only four or five are considered true human pathogens (143). Other species were found to be pathogenic only in individuals with immunodeficiencies (51, 143). In the mid 1990's there was a surge of interest in human mycoplasma infections as a result of apparent associations with AIDS and other immune problems such as Stevens-Johnson syndrome, an immune hypersensitivity disease. While mycoplasmas such *Mycoplasma hominis* and *Ureaplasma urealyticum* had long been associated with various diseases (23, 111, 143), a new highly cytopathic species, *Mycoplasma penetrans*, was isolated from AIDS patients. Another species, *Mycoplasma fermentans* was also frequently recovered from patients with AIDS, rheumatoid arthritis, immunosuppressive disorders and multistage oncogenic transformation (141). It was proposed that mycoplasmas might even cause a generalized low level immune suppression. While opportunistic mycoplasma infections are common in the immunosuppressed patient (97), no hard evidence has been found to support the theory that mycoplasma species actually serve as cofactors in HIV disease (11, 12). Still these associations served to spur interest in mycoplasmal research.

The effects of mycoplasma on livestock production are widely recognized. Although it is difficult to obtain exact figures, it is generally agreed that mycoplasmas are a major cause of economic loss in high intensity livestock and poultry production. Two of the most common will be discussed here, *Mycoplasma hyopneumoniae* and *Mycoplasma gallisepticum.*

Mycoplasma hyopneumoniae is endemic in American swine feedlots (117). It causes coughing, respiratory distress and frequently, pneumonia. The infected animals grow more slowly and finishing weights are reduced. The net result is reduced profits (117). It is also frequently associated with other major swine infections such as PRSSV (porcine reproductive and respiratory syndrome virus), an important problem in large-scale pig operations. Current vaccines offer only partial protection (118).

Mycoplasma gallisepticum is a major concern of the poultry industry. In the late 1980's it was estimated that U.S. mycoplasma-related egg production losses were over $95 million/year (15). *Mycoplasma gallisepticum* effects both egg production and bird profitability. It causes a chronic respiratory disease in chickens, turkeys and several other commercially marketed domestic poultry species (71). Like *M. hyopneumoniae*-infected swine (138), *M gallisepticum*-infected birds are more susceptible to opportunistic (and possibly more serious) secondary infections, and dual infection is common. In the absence of a secondary infection, death is rare, but decreased hatchability, growth delays and lowered carcass quality all contribute to significant economic losses (71). It is obvious that organisms of such economic and health significance require further study.

Characterization

Probably the best known molecular property of mycoplasmas is the use of the codon UGA, normally a translational stop signal, to code for the amino acid tryptophan. This codon difference, which is also characteristic of mitochondria, is not found in all mycoplasmas. The phylogenetically early *Acholeplasma* and *Phytoplasma* use UGA as a stop codon (111, 154). Also, unlike mitochondria, the genomes of mycoplasmas do carry a $tRNA^{trp}$ (CCA) gene. At this time the consensus is that these two incidences of identical abnormal codon usage are examples of convergent evolution.

As previously mentioned, another defining characteristic of mycoplasmas is the unusually low G+C content of their genomes. For example, *Mycoplasma capricolum* has a G+C content of 25%, but the nucleotide distribution is not uniform. As might be expected, the non-coding regions are the most AT rich. Protein-coding regions are somewhat less so, and the highest GC content has been retained by the highly conserved tRNA and rRNA genes (50, 103, 104).

The majority of the AT skewing appears to result from an underlying preference for A or T in the 3rd (wobble) codon position. This can be as high as 90% (103, 104, 152). The apparent AT preference is also reflected by amino acid usage frequencies (107). For example, the amino acids phenylalanine, isoleucine, asparagine and lysine are more prevalent in genomes with a low G+C content. There is an associated decrease in the amino acids proline, alanine, arginine and

glycine, all of which have codons with G and/or C in the first 2 positions (107). It is not known what causes this genetic drift in mycoplasmas and/or the G+C richness found in other bacterial species.

AT biased directional mutation

Codon usage is reflected by the tRNA gene pool. Probably the best studied is that of *M. capricolum*. As previously mentioned, the 1070 kilobase (kb) *M. capricolum* genome is only 25% G+C (Yamao 1992). A detailed analysis showed that this genome carries only 29-30 tRNA genes coding for 29 tRNAs (2, 102). In *M. capricolum*, there are groups of codons called family boxes for which the choice of amino acid is the same no matter what base is in the 3rd position. These usually share 1 tRNA, which has the anticodon UNN, and translates all family members with equal frequency (68). For those amino acids coded for by a neighboring pair of codons, the tRNA is typically either GNN or *UNN (*U is a U derivative). These pair with both NNA and weakly with NNG. CNN pairs only with NNG. ANN tRNAs have not been found. Some tRNAs and their associated codons have actually been eliminated, e.g. CGG and the matching arginine tRNA codon CCG in *M. capricolum*.

This dissection of apparent *M. capricolum* tRNA coding rules has also suggested a scenario for the alteration of a stop signal into a tryptophan codon in mycoplasma. Most mycoplasma carry two $tRNA_{trp}$ genes, one for the codons CCA and UCA and a separate one for the triplet UGA (a stop codon in the universal genetic code). In *M. capricolum*, this UGA tryptophan codon is actually translated more efficiently than the prototypical tryptophan codons and also occurs more frequently in the genome. In fact, the classic codon CCA is used for only 10-20% of the tryptophan sequences. (153).

According to this theory, the evolution of a codon goes through three stages. In the first stage as applied to mycoplasma, AT drift led to the preferential use of the more AT rich (in the first two bases) stop codons, UAA and UAG. Over time, the stop codon, UGA, was eliminated from the DNA sequence. Then a mutation occurred, producing a tRNA that could read UGA as a tryptophan codon. Continuing AT drift caused the replacement of most UGG tryptophan codons with UGAs. Eventually the $tRNA_{trp}$ codon, CCA, may be entirely eliminated from the genome and/or evolve into another usage (152).

Mycoplasmas offer more challenges to the researcher than the genomic differences described above. For example, they require complex media, precise growing conditions and careful handling (111). The lack of a cell wall makes them fragile and often polymorphic in shape. Their small sizes make them hard to visualize by light microscopy. They have few natural viruses and no native transposons. Their frequent role as secondary infectious agents

makes defining their unique attributes difficult. However, their economic and health importance will make it necessary to develop tools to overcome these barriers.

Transposons and Transposon Mutagenesis

Transposons

Although credit for the discovery of transposable elements clearly belongs to Barbara McClintock who first observed them in maize over 50 years ago (93), no one individual can be credited with the discovery of bacterial transposable elements. In the early 1960's, Taylor (137) isolated what he referred to merely as a phage (Mu) that introduced a high rate of mutation in host *Escherichia coli*. In fact he was describing a virus-associated transposition system. The first characterization of bacterial IS elements did not take place until the late 1960's (45, 70, 123). Work on more complex transposable units quickly followed (32, 113, 131), but the transposon now referred to as Tn*1*, i.e. the first discovered, was not described by Hedges and Jacob (62) until 1974. Transposable elements have now been found in Gram negative, Gram positive and archebacteria as well as higher eucaryotes. Mycoplasma-native transposons have not been identified.

Transposons are grouped in families. Similarities in inverted repeat sequences, transposition mechanisms and transposases are used as classification criteria. Bacterial range is not. For example, there are a number of Gram positive members of the primarily Gram negative Tn*3* family (124). This suggests that there has been genetic recombination between Gram positive and Gram negative transposons (101). In fact, some transposons function both Gram positive and Gram negative organisms (146). Transposon families differ in insertional sequence specificities, e.g. AT-richness preferences, transposition immunity effects, *cis*-specificity of transposase activity and of course, transposition mechanisms. At this time genetic engineering has also produced many chimeric transposons, combinations of functional segments originally from different families (9).

Mini-Transposons and transpositional mutagenesis

Initially, complete transposons were used to generate insertional mutations for mapping and/or functional studies (9). A native transposon inserted into a genome may continue to transpose around the genome resulting in multiple insertions, and confusing or incorrect experimental data. This is particularly relevant when transposons are used in heterologous genetic systems. To combat this problem, mini-transposons were developed. They typically consist of a replication defective plasmid carrying an antibiotic resistance marker flanked by the

transposon's inner and outer inverted repeat sequences, and an adjacent transposase gene located outside the inverted repeats. The *cis*-acting transposase produced upon transformation acts on the inverted repeats by inserting them into the bacterial genome along with the resistance marker. The transposase gene remains on the plasmid, which is replication defective in the transformed host. The transposon is now trapped at the initial site of integration and without transposase activity cannot move to other locations.

The usefulness of mini transposons has been well established. A large range of transposon derivatives have been developed including, mini-Tn*5* (36, 66), mini-Tn*10* (48, 135), mini-Tn*3*, (67), mini-Tn*7* (7), Tn*1000* (10) and Mu (18) and various combinations thereof. These are common tools in both bacterial and eukaryotic experimental genetic systems. Most studies that employ mini-transposons fall into one of four categories; 1) identification of specific genes and/or their chromosomal localization 2) creation of fusion proteins for protein localization and/or expression studies 3) promoter studies, and 4) use as constitutively expressed cellular or organismal markers. The widely varying insertional specificities, species restrictions, etc. have necessitated the development of this battery of transposon derivatives. There are no mini-transposon derivatives of the only two transposons known to function in mycoplasma, Tn*4001* and Tn*916*.

The complexity of mini-transposons varies. The simplest (and smallest) is probably Tn*5supF*(109). The selective marker for this transposon is the amber suppressor, *supF*, which is a gene that codes for a modified tRNA gene. The total size is 264 bp, and it was designed as a mutagen and for sequencing cloned DNAs in lambda phage. Because they can be modified so easily, the variety of these mini transposons is almost limitless. For example, Rode *et al.* inserted rare restriction sites into mini-Tn*10* derivatives, which were then used for rapid mapping and comparisons of various *E. coli* strains (116). A large number of selectable markers or reporter genes have been used in these constructs. Antibiotic markers are most often used for selection, but other selective markers are readily available (109). Reporter genes have included *lacZ*, GFP (136) or other bioluminescent markers (20, 150). Such markers may carry their own promoters when being used for cell localization studies or for simple selection in culture. Promotorless markers are frequently used to construct mutations in gene control systems. Tang *et al.* used mini-Tn*5*-gfp-km, a bifunctional, promotorless construct to simultaneously select for insertion (viability on kanamycin-containing plates) and promoter expression levels under varying conditions (GFP fluorescence levels) in *A. tumefaciens*. Several pH sensitive promoters were identified. (136). Such dual marker GFP- carrying Tn*5* derivatives have also been used for cell morphology studies (79) or bacterial localization (151).

Mini-transposon design has become quite sophisticated. An early model, the original TnMax mini-transposons (Tn*1721* derivatives), carried antibiotic resistance or other (promoterless) marker genes for the identification of promoter signals (56). In the next generation of TnMax, M13 forward and reverse sequencing primers sequences were added to allow for easy sequencing of flanking host DNA (73). Other more sophisticated or specialized derivatives have followed. Recently Neuveglise constructed mTnYl1, a Tn*3* derivative with an antibiotic resistance marker for selection in *E. coli*, a selectable yeast marker (Ura3), a rare restriction site (*Sce*I) to allow identification of the chromosomal insertion site, and a reporter gene (GFP) for creating translational fusions (105). The resulting construct is a new shuttle mutagenesis system for the yeast *Yarrowia lipolytica*.

Mini-transposons are frequently used in the study of fusion proteins, intracellular interactions and regulatory systems. The mini-transposon Tn*hlyA*, which carries the secretion signal of the *E. coli* hemolysin HlyA, is used to produce fusion proteins that are secreted. This element has been used to determine the causes of variations in secretion level (52, 130). The bi-cistronic reporter transposon mini-Tn*5* *lacZ*-tet/1 was used to identify *E. coli* operons that were activated by extracellular growth media from stationary phase cultures. The corresponding genes and their biochemical functions were then easily identified (6).

Two other (sometimes overlapping) major research interests are regulatory pathway studies and virulence research. Ansaldi *et al.* used classic mini-transposon insertion techniques to identify genes involved in the c-type cytochrome maturation pathway in *E. coli* (4). A similar system was used for virulence studies in *Brucella suis*. Bacteria were tagged with mini-Tn*5* km2, and avirulent (attenuated) mutants were identified. Mini-transposon flanking host sequences were used to assign functions to the associated genes (49). Mini-Tn*10* derivatives were used to identify the gene in a two gene operon coding for a critical virulence factor in *Legionella pneumophila* (145).

Green fluorescent protein cassette-containing mini-transposons have successfully been employed in a number of organisms, for example, yeast (105), marine bacteria (133), salmonella (21) and *Helicobacter* (72). These studies have produced variable results. Not all good promoters function equally well for GFP expression (72). Fluorescence can be media-dependent, and in some systems there can be a high individual variation of GFP fluorescence (expression) within a single bacterial population.

Transposons and mycoplasmas

Although native transposable elements have not been identified in mycoplasmas (even mycoplasmal viruses are rare), at least two Gram positive transposons are functional in

mycoplasmas, Tn*916* and Tn*4001*. Tn*916*, the first transposon found to be capable of transforming mycoplasmas (41, 115), has been shown to be capable of transforming many mycoplasma species. However its large size, 18 kb, can cause difficulties such as reduced transformation efficiency, especially in some microorganisms (89). Its size is due to the complexity of its sequence, which includes conjugal functions. Also, Tn*916* does not function in all mycoplasma species and cannot be used in some species because of tetracycline resistance problems. Tn*4001* is a Gram positive transposon that encodes gentamicin resistance. It also has been shown to function in a number of mycoplasma species, and can also be used without concern for introduction of an antibiotic resistance that is of clinical significance. Because of its small size, *Tn4001* was chosen for the parent for our mycoplasma-specific mini-transposon construction.

Tn*4001*

Tn*4001* is a 4.7kb composite, class 1 transposon, first discovered in Australia during the investigation of a multi-antibiotic resistant outbreak of *Staphylococcus aureus* (85). It confers resistance to the aminoglycoside antibiotics, gentamicin, tobramycin and kanamycin. When the plasmid-based resistance gene was used in mixed culture experiments, resistant *S. aureus* isolates free of plasmid DNA were frequently produced. From this, it was concluded that the resistance gene was actually carried by a transposon (85).

It is believed to be a young transposon. The first gentamicin-resistant *S. aureus* strains were isolated in 1970's (128, 129). Interestingly, the chromosomal insertion found on resistant *S. aureus* strains isolated in 1976 is 1.3 kb larger than that found in resistant strains isolated in the 1980's. It has been suggested that the deletion of the 1.3kb section created an element small enough to be carried by a transposon. Direct repeats, typically due to duplication of flanking sequences during transposon insertion, are found in the plasmid resistance carrier, pSK1. In fact it is probably this transfer to a plasmid carrier that caused the observed increased incidence of antibiotic resistance. Sequence data supports the recent evolution of Tn*4001*. There is virtual sequence homology in the IS*256* ends of multiple isolates. Long-separated populations should have acquired unique (silent) mutations over time (16).

Although highly mobile *in vivo*, Tn*4001* appears to have jumped to a plasmid carrier only once. Older resistant bacterial strains carry a chromosomal insert whereas more recently isolated strains tend to carry the plasmid-based version (53). A plasmid-based transposition-defective version has also been found in North America and Europe (83). While the prototype source is the 27 kb plasmid pSK1, Tn*4001* has also been found in a number of related plasmids. Since both transfer from plasmid to staphylococcal hosts and transposition within a

genome are extremely common *in vivo*, and the incidence of this resistance gene is high, it might have been expected that staphylococcus–to–plasmid transposition would also have been observed. This has not occurred, and no unrelated plasmid carriers have been identified.

The structure of Tn*4001* is well established. A 1.9 central portion carrying the resistance determinants is flanked by two 1.3 kb insertion sequence (IS) inverted repeats (IS*256*)(84, 85), which can also function as autonomous transposable elements (16). A 2.5 kb central *Hin*dIII fragment containing the antibiotic resistance marker and a portion of the IS*256* repeats is frequently used for cloning modifications (53). Another 1.3kb *Taq*I/*Hin*cII fragment is also sometimes used in genetic manipulations of the central coding region.

The 1,918 base pair (bp) central region has two open reading frames (ORFs), a 1,437 bp ORF which codes for the 56.9 kDa bifunctional antibiotic resistance protein and a 2nd smaller ORF. This second ORF does not appear to have translational initiation signals and may be nothing more than a vestigial gene fragment (16). Several potential promoters 5′ to the coding sequence of the first ORF have been identified, with those named P1 and P2 considered the most important.

As usual, the IS elements code for the transposase gene. Each 1,324bp IS*256* element has a single ORF. This large 390 amino acid ORF codes for the transposase gene, designated *tnp*. It is preceded by a potential Shine Dalgarno ribosomal binding site and -35 and -10 promoter sequences. Transposition is controlled by transposase concentration, which in turn is regulated by a combination of weak promoters and/or antisense RNA produced from a highly active promoter p_{out} . No apparent homology with other transposase genes has been found. Like many other IS elements, IS*256* has 26 bp imperfect terminal inverted repeats, which in this case, have a 17 bp match (16).

The G+C content of the antibiotic gene coding region (~24% G+C) is very different from that of the IS*256* elements (~36% G+C), and quite uncharacteristically low for a *S. aureus* sequence. This data supports the cassette assembly model of transposon Tn*4001* construction, i.e. an antibiotic resistance gene was captured from an organism in another genera (16, 120).

Enzymatic activity

While enzymatic modification is characteristic of antibiotic resistance, typically a single enzyme product of a single gene acts upon a unique antibiotic. The enzyme carried by Tn*4001* is an exception. Of the three major types of aminoglycoside resistance enzymatic activities, aminoglycoside-*O*-phosphotransferase (APH), aminoglycoside-*N*- acetyl transferase (AAC) and aminoglycoside-*O*-adenyltransferase (AAD), Tn*4001* carries two, a 6′-acetyltransferase [AAC(6′)] and a 2′-phosphotransferase [APH(2")]. The protein actually is a 56.9 kDa bi-

functional protein with two enzymatic domains. The 5′ end (167-80 codons) codes for the *aacA* gene for tobramycin and kanamycin resistance while the carboxy terminal end encodes for the APH gene, *aphD* for gentamicin resistance (120). Deletion mutation studies have shown that while the 5′ end can function in the absence of the 3′ end, most of the 5′ end is required for APH activity (120).

Although useful as a genetic tool, Tn*4001* has not been used in many transposition studies as, for example, Tn*916*. Previous researchers listed the advantages of using Tn*4001* in Gram positive hosts. They include easily selectable phenotype, multiple chromosomal insertion sites, ability to manipulate chromosome adjacent to sites of interest and capability of precise excision. Less positive characteristics were also listed: instability at any one site (high reversion frequency), insertion hot spots, and the possible requirement for a recombination deficient host to negate a homologous recombination effect for efficient recovery (75). Not all of these negative features may be operative when mycoplasmas are used as the recipient.

In 1989, Mahairas and Minion used a pSK1 derivative, pISM1001, to introduce Tn*4001* into *Acholeplasma oculi* (86, 88). It was found to form stable integrants and be transpositionally active. Probably the most frequent use of Tn*4001* has been for mapping. It has been used to map *S. aureus* (87), *Acholeplasma oculi* 1499 (140), and *M. gallisepticum* (17, 139). A rare restriction site was inserted in the transposon to facilitate mapping,.

Tn*4001* derivatives have also been used in several studies of promoter probes in mycoplasmas. Initially, two restriction sites, *Sma*I and *Bam*HI, were placed 26 bp from one end of the transposon within the IS*256* element to create Tn*4001*mod (75). This construct was used to deliver wildtype genes in *M. pneumoniae* to complement chromosomal mutations (57). The same sites were used for the rare restriction site insertions for the chromosomal physical mapping studies (140). Upstream stop codons in each of the reading frames were removed by site directed mutagenesis to create a protein fusion vector using β-galactosidase as the reporter gene (75). Other reporter genes have also been used (*tetM* and *phoA*)(95). Another construct, Tn*4001*.2065, was constructed with a plasmid origin of replication and a selectable marker within Tn*4001* to allow for the direct rescue of chromosomal sequences adjacent to the insertion site (75). These derivatives of Tn*4001* can be used for a variety of studies in mycoplasmas, but independent transposition of the IS*256* elements and other features that limit their usefulness.

Adherence is an essential component of mycoplasma infectivity. Recently, Tn*4001* was used for complex studies of mycoplasma adherence proteins. Hedreda and Krause used transposition mutagenesis to identify adherence gene regulatory sequences in *M pneumoniae* (63, 77). Almost incidentally, their experiments also established that a previously identified major adherence protein was actually a dimer of another protein (46, 77).

Researchers have only begun to explore the experimental potential of Tn*4001*. In addition to its original host staphylococcus and various mycoplasmas, it has shown promise in *Streptococcus gordonii* transposition mutagenesis studies (82). Other transposons, such as Tn*916*, have been shown to have extremely wide host ranges, and it is possible Tn*4001* or its derivatives will be useful in other bacterial hosts. The development of one such tool is the focus of this work.

Green Fluorescent Protein

Uses

Since the discovery that the green fluorescent protein (GFP) of *Aequorea victoria* can be expressed in heterologous organisms (19), it has become a widely used molecular and cellular research tool. It has been visualized in bacteria (91), yeast (40), *Drosophila* (43, 44), human cell lines (156, 157), various plants and many other higher eucaryotes. A partial list of uses might include: labeling single cells, labeling organelles within cells, identifying changes in organelle pH, cell sorting, and studying individual protein-protein interactions. These are discussed in more detail below.

The most common use of GFP is as a marker for gene expression. Sometimes GFP functions as a reporter gene for protein production where it replaces such standbys as *lacZ* fusions or antibody labeling. The original Chalfie studies on a subpopulation of neuronal cells in the nematode *Caenorhabditis elegans* clearly demonstrated the biological equivalence of antibody and GFP gene fusion labeling, at least in their system (19). It has also been used for more basic studies on gene expression mechanisms.

GFP tagging has become a standard technique for protein localization studies. Chalfie *et al.* stated that native GFP is primarily localized in the cytoplasm, but it may also diffuse into the nucleus (19). By attaching various signal sequences, GFP can be targeted to specific organelles (35) or the cellular membrane (100). Since fixation is not required for GFP visualization, it can be used to monitor organelle changes *in vivo*. One particularly elegant example is the work on nuclear localization signals and nucleus-cytoplasm protein shuttling in yeast by Lee *et al.*, but there have been many other studies (78).

Many recent studies of the cellular cytoskeleton have used GFP as a marker. Endow and Komma used a linked *ncd-gfp* construct (Ncd is a microtubule motor protein) to visualize mitotic spindle attachment and assembly in *Drosophila* oocytes and embryos (43, 44). GFP-α-actinin, GFP-titin, and GFP-myosin are currently being used to deconstruct the actin/myosin cytoskeleton activities of living muscle cells (31). It can even be used to mark specific

chromosomal segments. *In vivo* expression of GFP-*lac*-repressor fusion proteins binding to previously introduced *lac* operator sequences cloned into CHO or yeast chromosomal DNA has been used to study chromosomal structures *in situ* (8, 132).

One of the most exciting new uses for GFP is in the study of protein-protein interactions. FRET (fluorescence resonance energy transfer) is a technique that uses two fluorophores (light emitting proteins), one donor and one acceptor, to measure the distance between the two. In simplest terms, two potentially interactive proteins are each tagged with a different fluorophore i.e. a GFP variant. UV excitation causes the donor protein to emit energy at the optimal excitation wavelength for the second tag. The amount of energy absorbed by this second protein tag (and therefore the amount of energy it emits as fluorescence) is proportional to the distance between the two proteins. The latest technique, PRIM (34) uses the formation of GFP dimers by two GFP tags and the associated emission spectra wavelength shifts to monitor the proximity of the proteins of interest. Although use of GFP has exploded in recent years, researchers have just begun to develop new techniques that utilize its unique properties.

Properties

Green fluorescent protein is a 238 amino acid fluorescent protein. It has two absorbance peaks, the major one at 395 nm and a minor one at 475 and a single emission peak at 508nm (69, 148). Like other green fluorescent proteins, it is one of a pair of chemiluminescent proteins. In *Aequorea* the other protein is aequorin, In the presence of Ca^{2+} *in vivo*, aequorin emits chemiluminescence with a peak around 470 nm, near the absorbance maximum of GFP. This in turn causes GFP to emit at 508nm producing the green fluorescence observed *in vivo*. In the absence of GFP, aequorin fluorescence is blue.

The 3d structure of GFP is the prototypical β barrel or β can consisting of 11 anti parallel β sheets arranged in a cylindrical shape with caps of α helix at each end. The barrel has a diameter of 25-30Å and a length of about 40Å. An irregular α helical strand positions the chromophore (amino acids 65-67) near the center of the hollow barrel (155). This unusual, highly protective structure is conserved in all functional GFP mutants that have as yet been studied, such as the popular S65T mutant (106). Amino acids 2-229 are required for activity (39). Unlike other GFP proteins, e.g. that of *Renilla* the sea pansy, which are obligate dimers, the *Aequorea* GFP protein molecule can function as a monomer in dilute solution, although it is believed to function as a dimer or as an $aequorin_2$ - GFP_2 heterotetramer *in vivo* (30).

The chromophore is formed by the cyclization of S65Y66G67 into an imidazolone 5-member ring. It is currently believed that the chromophore is formed in three steps; 3D structure formation, cyclization, and finally, oxidation of the hydroxybenzyl side chain of Y66

(22, 65). While most authors believe that the oxidation is the slow (2-4 hours) rate-limiting step (112), studies on thermal mutants have suggested that, in some cases, folding may be the rate-limiting step (126).

As previously mentioned, wild type GFP has two absorbance peaks, one at 375 nm and one at 495 nm. The ratio of these two peaks is strongly affected by reaction conditions. Increases in pH, temperature, ionic strength, protein concentration (29, 147, 149) decrease the relative size of the 375 nm peak. Part of this shift may be due to changes in spectral properties resulting from dimerization. Of course, mutation is another source of spectral differences (37, 64, 65).

The expression "wild type green fluorescent protein" (WT-GFP) is generally used for proteins with a DNA sequence that matches that of the GFP cDNA first cloned and sequenced in 1992 (110). In fact that clone included an inadvertent (functionally silent) PCR artifact, E80R. Even at that time, there was evidence that there are a number of GFP isoforms in the jellyfish population. Some are the products of multiple GFP genes or alleles with point mutations while other isolates may be artifacts, as the carboxyl end of the protein is highly susceptible to proteolytic cleavage (29).

Background

Green fluorescent protein was first discovered by Shimomura *et al.* in the early 1960's during studies on aequorin, the primary luminescent protein of *Aequorea* (125). As previously mentioned, they observed that purified aequorin produced blue fluorescence, but the fluorescence of *Aequorea in vivo* is green. Another protein in their impure aequorin protein preparations was the source of the green fluorescence. This protein was called "green fluorescent protein". Two years later, the same group published the spectra of both aequorin and GFP (69), but it was not until 1974 that Morise *et al.* actually proved that aequorin could directly transfer its luminescence energy to GFP (99). The exact mechanism of the aequorin-GFP energy transfer has still not been definitively established. It was not cloned and sequenced until 1992 (110).

The source of GFP, the jellyfish, *Aequorea victoria*, is a curved, transparent, lens- shaped creature with fringed edges about 7-10 cm in diameter. The fluorescence pattern is striking. While the center of the body is largely invisible, a disk (the outer margin) has green fluorescent stripes radiating inward from approximately 200 spots on the outer edge (the photogenic organs). Each photogenic organ has 6-7000 photogenic cells (33).

A number of other coelenterates also contain green fluorescent proteins (25, 61, 98). The best studied of these is the sea pansy, *Renilla*. Most have the same core chromophore and emission spectra, but not the same absorption spectra, DNA sequence or 3D structure. All seem

to employ the same basic scheme, requiring excitation by a primary chemiluminescent protein. They differ from *Aequorea* GFP in two important ways. They require external cofactors for chromophore formation, and they are obligate dimers (148). *Aequorea victoria* is the only known source of non-cofactor-requiring GFP. Recently, Matz isolated several other fluorescent proteins homologous to GFP from *Discosoma* species (92). These proteins also appear to be cofactor-independent and have the same β can structure as GFP, but they fluoresce at very different wavelengths. In this paper, as in most studies, the term green fluorescent protein or GFP will refer to the *Aequorea* product unless otherwise specified.

In 1994 Chalfie *et al.* discovered two interesting facts. GFP could be expressed in heterologous organisms, i.e. *E. coli* and *C. elegans* when spliced to organism-specific promoters, and the cellular distribution of these proteins would mimic that of the promoter used (19). Two attributes transformed *Aequorea* green fluorescent protein from an obscure research subject into a major molecular biology tool.

1. No external cofactors are required for its activity.
2. It can be expressed in virtually any organism (19).

Characteristics of GFP as a research tool

There are a number of features that make GFP particularly useful. Once the chromophore has folded, the protein is very stable. The folded protein is highly resistant to proteases, (13, 119), anionic and non-anionic detergents (54), fixatives ((19) and changes in pH (147), although very high pH may change the 470 nm/395 nm absorbance ratio. The folded protein is temperature resistant (Tm=76°C)(13). Refolding and activity are usually recoverable even after harsh denaturing conditions. The fluorescence is unaffected by many cellular fixatives. While wild type GFP also has a few less desirable characteristics.

1. Complex folding is required to form the chromophore and it does not fold well at 37°C (80, 142) even though, once folded it is temperature resistant.
2. The folding process is slow 2-4 hours (64, 65), and a significant proportion of the molecules never fold. This makes its use in real-time *in vivo* gene studies difficult.
3. The fluorescence signal (which is not amplified in the system) is not very bright and may be masked by cellular autofluorescence.
4. The optimal codon usage for jellyfish is not that of other species.

The design of mutant GFP proteins has been a major area of study in the past few years. Researchers have generally focused on increasing brightness, spectral shifting, and/or optimizing codon usage. In the process they have isolated or designed temperature resistant

and/or faster folding mutants, various red, blue and yellow fluorescent proteins, and clones with prokaryotic, human (or mammalian) and plant optimized sequences.

Probably the greatest interest has been in color-shifted mutants. The chromophores of *Renilla* and *Aequorea* GFP are identical, yet the single excitation peak of Renilla GFP is at a much more experimentally useful wavelength than that of *Aequorea* (498 nm vs 395 nm and 475 nm in *Aequorea*). *Aequorea* GFP mutants with *Renilla* GFP-like spectral qualities would be ideal. When Heim *et al.* first subjected a GFP cDNA library to random mutagenesis, they found a number of GFP variants, most with mutations in the C terminal region (65). Delagrave *et al.* used site directed mutagenesis of the 6 amino acid region surrounding the *Aequorea* GFP chromophore and screened the resulting clones for red shifted variants (RSGFP)(37). Several of their (multiple site) mutants had the desired spectral shift. The results of both studies seemed to suggest that changes in the chromophore microenvironment can cause in major modifications in absorbance spectra

Major absorbance shifts can also be produced by simple amino acid substitutions in the chromophore itself. Each amino acid position of the chromophore has unique requirements (28). While there seems to be an absolute requirement for G67, Y66 can be replaced by virtually any aromatic side chain. The less electron-rich the side chain, the more blue-shifted the spectra. Phenylalanine is the least electron rich, followed by histidine, tryptophan, and the charged form of tyrosine found in the wild type sequence. The corresponding GFP excitation peaks are 382 nm for Y66H, 433 nm for Y66W and 475 nm for the charged, wildtype Y66. The uncharged tyrosine emission peak of wildtype is at 395 nm. The emission peaks of the GFP variants, Y66H (488 nm) and Y66W (485 nm), are equally blue-shifted when compared with the 504 nm peak of the Y66 wild type, charged tyrosine. The blue-shifted mutants produce significantly lower levels of fluorescence than wild type. The blue-shifted proteins are commonly referred to as blue fluorescent proteins (65), a term which unfortunately has also been used for activated aequorin.

Changes in serine 65 (small aliphatic amino acids required) are associated with longer (red) wavelength shifts. Probably the best known is S65T which has a single red-shifted absorbance peak with a maximum of 490-510 nm (64), but not all red-shifted mutants carry an S65 mutation (37). There are now also yellow mutants (YBP). The spectral range of GFP mutants is now so great that mutants with differing absorbance and emission spectra can be used to label and differentiate between two proteins or structures in the same cell (114). This was first demonstrated by Heim *et al.* who used changing fluorescence patterns to follow trypsin digestion and the associated loss of dimerization of a GFP-BFP chimera (64). Excited BFP was used to excite neighboring GFP (64). Color mutants have different uses, i.e. red shifted mutants

are good for FACS (flow activated cell sorting) but are hard to see with a fluorescence microscope. Mutants may also have different fluorescent lifetimes. Pepperkok *et al.* recently individually visualized four co-expressed GFP variants using fluorescence lifetime imaging microscopy (108).

Most folding mutations have been isolated indirectly. In many cases so called brighter mutants were actually faster folding mutants. Siemering *et al.* compared cellular fluorescence levels with cellular GFP protein concentrations and found that this ratio (which decreases with temperature) is greater in their bright mutant GFPA (126). The apparent thermo-tolerance is due to improved folding not increased oxidation rates. One of the reasons that the mutant S65T has become so popular is that it folds approximately 4 times faster than wild type, 45 min vs 2 hours under the conditions used in Heim *et al.* 1995 (64). When Cormack *et al.* used FACS sorting to select for increased fluorescence, they also isolated S65T mutants (24). Many folding mutations are far from the chromophore. V163A is a folding mutant that has been independently isolated several times. Amino acid 163 is believed to be an important contributor to a folding intermediate since the presence of V163A in virtually any mutant decreases folding time (126). Another mutation S147P greatly improves GFP folding at 37°C but not at 23°C (74).

Until recently, most studies have utilized the great stability of folded GFP. However this can be a disadvantage for real-time protein-protein or protein-organelle studies. Several unstable variants of GFP with C-terminal extensions that are highly susceptible to degradation have been constructed (3) and are now commercially available (Clontech).

The third area of GFP mutation development focus is species-specific codon optimization. While GFP expression has been possible in virtually all organisms tried, in many cases the wild type gene produces low or non-detectable levels of protein. One of the most extreme examples of this is *Arabidopsis* where a region of the wild type GFP sequence mimics an *Arabidopsis* splice signal causing a 84bp deletion in the protein product found in these plants (60). Some researchers have used strong promoters to increase protein production and compensate for low expression or poor fluorescence signals (114, 134, 144). Others have turned to codon optimization systems.

Optimized variants have been produced for *Arabidopsis* (60), humans (55, 157) and *E. coli* (26). The last two are commercially available as GFPuv and EGFP respectively (Clontech), and have been successfully used in a variety of bacteria, mammals and some plants.

At this time there has been only one published account of GFP use in mycoplasma research. Dhandayuthapani *et al.* used it to retrieve certain promoter sequences from a

mycoplasma cDNA library (38). Since in this study the GFP marker was expressed only in *E. coli*, the question of GFP expression *in vivo* in mycoplasma was not addressed.

As the field of GFP research matures, the focus is shifting from mutant construction and characterization towards sophisticated applications in cell and molecular biology and experimental results. However, there are still cases in which GFP modification must be the preliminary step in an experimental sequence. The construction of a mycoplasma-specific GFP mutant discussed in this paper is one such step.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

The bacterial strains used in this study are listed in Table 1. Bacteria were maintained at –70°C. *E. coli* strains were grown in Luria-Bertani Broth, Super Broth or SOC broth (122). *M. gallisepticum* strain R and *M. pulmonis* strain UAB6510 were maintained in PPLO mycoplasma media (25 gm per L, 0.2 gm per L herring sperm DNA, 15% donor herd horse serum, 5% fresh yeast extract, 0.5% w/v glucose, and 2.5 mg per L Cefobid) (94). *Acholeplasma oculi* 1499 was maintained in the same medium except that agamma horse serum (Gibco BRL) was used. For solid agar, Noble agar was added at 1% prior to autoclaving the base. Table 2 describes the plasmids used in these studies. Plasmids were stored at either -20°C or -70°C. Oligomers used in these studies are listed in Table 3.

Table 1. Bacterial strains.

Strain	Description	Source
E. coli		
LE392	F⁻ *hsdR514* (*rk–, mk–*) *lacY1 supE44 supF58 galK2 galT22 trpR55 metB1*, λ⁻	(58)
TOP10	F- *mcrA* Δ(*mrr-hsa* RMS-*mcr* BC) Φ80*lacZΔM15 ΔlacX74 deoR recA1 araD139* Δ(*ara-leu*)*7697 galU galK rpsL endA1 nupG*; pZerO2 cloning host	Invitrogen
ISM614	LE392 pISM3001; used for expression of GFP	(127)
ISM1246	LE392 pISM1238 (pISM2062.2:GFPuv)	This study
ISM955	LE392 pGFPuv	This study
Mycoplasma		
M. gallisepticum	virulent strain R	F. Goff
A. oculi	1499	(5)
M. pulmonis	virulent strain UAB6510	G. Cassell

Table 2. Plasmids.

Plasmid	Genotype or phenotype	Source
pZerO2	positive selection cloning vector, Cm^R	Invitrogen
pGFPuv	source of GFP gene, Kan^R	Clontech
pSK-	cloning vector, Amp^R	Stratagene
pISM27	pSK- containing outer repeat of Tn*4001*	This study
pISM28	pISM27 containing inner repeat of Tn*4001*	This study
pISM29	pISM28 and transposase of Tn *4001*; mini-Tn*4001*, Amp^R	This study
pISM30	P2GFPmyco in pISM2062, Amp^R, Gmt^R	This study
pISM31	mini-Tn*4001*tet, Amp^R Tet^R	This study
pISM32	GFPmyco in pSK, Amp^R	This study
pISM33	5′ end of GFPmyco gene in pZerO2, Cm^R	This study
pISM34	3′ end of GFPmyco gene in pZerO2, Cm^R	This study
pISM35	GFPmyco in pISM2062.2	This study
pISM36	GFPmyco in pISM2062.2 (reverse orientation)	This study
pISM1001	contains Tn4001, primer for transposase gene PCR, Tet^R	(88)
pISM1002	primer for tetM gene PCR, Tet^R	(87)
pISM2050.2	primer forP2 promoter PCR, Amp^R, Gm^R	(76)
pISM2062	cloning vector, contains Tn*4001* mod, Amp^R, Gm^R	(75)

Table 3. Primers.

Name	Function	Sequence (5' -3')
TNI2-	IS*256* inner repeat sequence	CAGTCAAGTCCAGACTCCTGTGTAAAAA
TNI2+	IS*256* inner repeat sequence	CTAGTTTTTACACAGGAGTCTGGACTTGA CTGAGCT
TNO-	IS*256* outer repeat sequence	CCCCGAATTCTTTTACACAATTATACGGA CTTTATCAACAAGCTTACCC
TNO+	IS*256* outer repeat sequence	GGGTAAGCTTGATAAAGTCCGTATAATTG TGTAAAAGAATTCGGGG
TNPR2	Transposase PCR primer	GGGTGGTACCCCATTTCTACTTATC
TNPL3	Transposase PCR primer	GGGTGTCGACGTGTAAAAGTAAAAAGGCC
IS256	sequencing	GGAAATGTATGTATCAGGCG
CM3F	Construction of GFP-1	ATGGATCCATGAGTAAAGGTGAAGAATTA TTTACAGGTGTAGTACCAATTTTAGTTG
CM4F	Construction of GFP-1	AATTAGATGGTGATGTTAATGGTCATAAA TTTAGTGTAAGTGGTGAAGGTGAAGGTGA TG
CM5F	Construction of GFP-1	CAACATATGGTAAATTAACATTAAAATTT ATTTGTACTACTGGTAAATTACCAGTACC AT
CM6F	Construction of GFP-1	GACCAACTTTAGTAACAACTTTTGGTTAT GGTGTACAATGTTTTGCTAGATATCCAGA TC
CM7Fb	Construction of GFP-2	CTAGATATCCAGATCACATGAAAAGACAC GATTTTTTTAAAAGTGCTATGCCAGAAGG TT
CM8Fb	Construction of GFP-2	ATGTTCAAGAAAGAACAATTAGTTTTAAA GATGATGGTAATTATAAAACAAGAGCAGA AG
CM9R	Construction of GFP-1	CACCTTTACTCATGGATCCAT
CM10R	Construction of GFP-1	GACCATTAACATCACCATCTAATTCAACT AAAATTGGTACTACACCTGTAAATAATTC TT

Table 3. Primers (Continued)

CM11R	Construction of GFP-1	TTAATGTTAATTTACCATATGTTGCATCAC CTTCACCTTCACCACTTACACTAAATTTA T
CM12R	Construction of GFP-1	CAAAAGTTGTTACTAAAGTTGGTCATGGT ACTGGTAATTTACCAGTAGTACAAATAAA TT
CM13R	Construction of GFP-1	GATCTGGATATCTAGCAAAACATTGTACA CCATAAC
CM14F	GFP-1 PCR primer	ATGGATCCATGAGTAAAGGTG
CM15R	GFP-1 PCR primer	GATCTGGATATCTAGCAAAAC
CM16F	Construction of GFP-2	TAAAATTTGAAGGTGATACTTTAGTAAAT AGAATTGAATTAAAAGGTATTGATTTTAA AG
CM17F	Construction of GFP-2	AAGATGGTAATATTTTAGGTCATAAATTA GAATATAATTATAACAGTCATAACGTTTA TA
CM18F	Construction of GFP-2	TTACTGCTGATAAACAAAAAAATGGTATT AAAGCTAATTTTAAAATTAGACATAACAT TG
CM19F	Construction of GFP-2	AAGATGGTAGTGTTCAATTAGCAGATCAC TATCAACAAAATACTCCAATTGGTGATGG TC
CM20F	Construction of GFP-2	CAGTTTTATTACCAGATAATCATTATTTAA GTACTCAAAGTGCTTTAAGTAAAGATCCA A
CM21F	Construction of GFP-2	ATGAAAAAAGAGATCACATGGTATTATTA GAATTTGTAACTGCTGCTGGTATTACTCA CG
CM22F	Construction of GFP-2	GTATGGATGAATTATATAAATAAGAATTC GCTG
CM23R	Construction of GFP-2	CAGCGAATTCTTATTTATATAATTCATCCA TACCGTGAGTAATACCAGCAGCAGTTACA A
CM24R	Construction of GFP-2	ATTCTAATAATACCATGTGATCTCTTTTTT CATTTGGATCTTTACTTAAAGCACTTTGAG
CM25R	Construction of GFP-2	TACTTAAATAATGATTATCTGGTAATAAA ACTGGACCATCACCAATTGGAGTATTTTG TT
CM26R	Construction of GFP-2	GATAGTGATCTGCTAATTGAACACTACCA TCTTCAATGTTATGTCTAATTTTAAAATTA G

Table 3. Primers (Continued)

CM27R	Construction of GFP-2	CTTTAATACCATTTTTTTGTTTATCAGCAG TAATATAAACGTTATGACTGTTATAATTAT
CM28R	Construction of GFP-2	ATTCTAATTTATGACCTAAAATATTACCAT CTTCTTTAAAATCAATACCTTTTAATTCAA
CM29R	Construction of GFP-2	TTCTATTTACTAAAGTATCACCTTCAAATT TTACTTCTGCTCTTGTTTTATAATTACCAT
CM30R	Construction of GFP-2	CATCTTTAAAACTAATTGTTCTTTCTTGAA CATAACCTTCTGGCATAGCACTTTTAAAA A
CM31R	Construction of GFP-2	AATCGTGTCTTTTCATGTGATCTGGATATC TAG
CM32F	GFP-2 PCR primer	CTAGATATCCAGATCACATGAAAAGAC
CM33R	GFP-2 PCR primer	CAGCGAATTCTTATTTATATAATTCATCC
GFPmy.seq	sequencing	AAAGTGCTATGCCAGAAGGTTATGTTC
AcP2R	PCR of P2 promoter	CTTATCACTCCAAATAATCTTATACTTTTT ATA
AcP2F	PCR of P2 promoter	TTGGATCCGTCTTGGATGAAGTCTTAAAG CAC
GFPmyF	Forward primer for GFPmyco	ATGAGTAAAGGTGAAGAATTATTTACAGG TGTA
CM33R.Bam	Reverse primer for GFPmyco	CAGCGGATCCTTATTTATATAATTCATCC
GFPmyR	Reverse primer for GFPmyco	TAGGATCCTTATTTATATATAATTCATCCA TACCGTG
TH111	*bla* specific primer	TTATCCGCCTCCATCCAGTC
TH112	*bla* specific primer	TGGGTGCACGAGYGGGYYAC
tetM.1	*tetM* specific primer	CCCATTATTAATATTGGAGTTTTA
tetM.2	*tetM* specific primer	CCCTTATATAACAACTTAAATTACACTA

Reagents and Buffers

Restriction enzymes, 1 kb plus molecular weight ladder, and proteinase K were obtained from Gibco BRL (Gaithersburg, Md.), New England Biolabs (Beverly, Maine), or Promega Corporation (Madison, Wis.). Calf intestinal alkaline phosphatase was obtained from Qiagen, and *Pfu* DNA Polymerase from Stratagene (La Jolla, Calif.) or Promega. Agarose was purchased from Bio-Rad Laboratories (Hercules, Calif.) or FMC Bioproducts (Rockland, Maine), isopropyl thio-B-D galactoside (IPTG) from Gold Biotechnology, Inc. (St. Louis, Mo.). PCR grade water (Gibco-BRL) and deoxynucleotides (Amersham Pharmacia, Piscataway, N.J.) were used in PCR reactions. Dry media components were obtained from Difco, horse sera from Gibco-BRL, and Cefobid from Pfizer (New York, N.Y.). Other chemicals were obtained from Sigma Chemical Co. (St. Louis, Mo.). A Zero Background/Kan Cloning Kit (Invitrogen, Carlsbad, Calif.) was also used.

A Peltier Thermal Cycler PTC-2000/PTC225 (MJ Research, Watertown, MA) was used for polymerase chain reactions (PCR). An Olympus microscope was used for UV fluorescence studies. Confocal microscope studies were done at the Iowa State University Image Analysis Facility using a Leica TCS-NT Confocal Microscope (Leica Microsystems, Inc., Exton, Penn.).

DNA Manipulations

The strategies used for the assembly of constructs were straightforward. DNA was obtained from *E. coli* using an E.N.Z.A Plasmid Miniprep Kit II from Omega Biotek (Norcross, Ga.) or Qiagen plasmid Midi Kits (Santa Clarita, Calif.). In some cases, PCR products were purified from agarose gels using a QIAquick PCR Purification kit (Valencia, Calif.). Sequence data (1) were used to design oligonucleotides to match the region(s) of interest. Oligonucleotide primers were designed using Oligo 5 software (National Biosciences, Inc, Plymouth, Minn.) and synthesized at the Iowa State University DNA facility using a DNA/RNA Synthesizer (Model 399, Applied Biosystems, The Perkin-Elmer Corporation, Norwalk, Conn.).These oligonucleotides were used to construct GFPmyco gene segments or as PCR primers. The DNA product(s) were then ligated into the appropriate vectors. The resulting constructs were transformed into *E coli* and grown on the appropriate selective media. The resulting putative clones were digested with restriction enzymes, and the fragments were analyzed on agarose gels to determine the restriction pattern of the insert. Those clones with the expected restriction pattern were sequenced to confirm that no base pair changes had been introduced during PCR.

All DNA sequencing was done at the Iowa State University DNA Sequencing Facility using cycle sequencing protocols and an automated DNA sequencer (Applied Biosystems, The Perkin-Elmer Corporation). The data were edited and assembled using Factura, AutoAssembler and Sequence Navigator (Applied Biosystems, The Perkin Elmer Corporation). MacVector 6.0 (Eastman Kodak Company, Rochester, N.Y.) was used for in-lab sequence analysis. After assembly, the constructs were tested for expression by transformation into *E. coli* (122) and in mycoplasmas by electroporation (96) where appropriate.

Transformation

To prepare electrocompetent *E. coli*, 500 ml of LB were inoculated with 10 ml of an overnight broth culture of *E. coli* and grown at 37°C with shaking until the $O.D._{600} = 0.4- 0.7$. After chilling on ice for 20 minutes, the cells were centrifuged at 6-7,000 x *g* for 10 minutes at 4°C and resuspended in 500 ml of sterile, chilled Super Q water. After a 10 minute incubation on ice, the cells were centrifuged as before, resuspended in 250 ml cold water, incubated on ice for 10 minutes and centrifuged again. The pellet was resuspended in 10 ml sterile, cold 10% glycerol, centrifuged one final time. The pellet was resuspended in approximately1ml of cold 10% glycerol. Sixty µl aliquots were frozen in a dry ice-ethanol bath and stored at -70°C until used.

For transformation of *E. coli*, one tube containing frozen competent *E. coli* was thawed on ice, and 2 µl of the DNA ligation reaction or DNA were mixed with the cells and immediately transferred to a cold electroporation cuvette (0.2 mm gap, Bio-Rad Laboratories, Inc.). The cells were subjected to electroporation using a BTX Model 600 electroporator, settings of 2.5 kV charging voltage, 129 ohms, capacitance of 50 µF, which generates a 5 millisecond pulse length. Following electroporation, 1 ml of SOC media was added to the cuvette, which was incubated at 37°C for 1 hour before plating. Transformants (10 µl, 100 µl and 500 µl volumes) were plated on LB plates containing a selective antibiotic and grown overnight in a 37°C incubator.

Electrocompetent mycoplasmas were always prepared just prior to use as described previously (96). Mycoplasma cultures were grown to mid-log phase (slightly orange culture) and harvested by centrifugation at 8,500 x *g* in a J17 rotor for 10 minutes at room temperature. The pellet was resuspended in an equal volume of room temperature electroporation buffer (8 mM HEPES, 272mM sucrose, pH 7.4, autoclaved) and immediately centrifuged. This pellet was resuspended in one half the original volume electroporation buffer and centrifuged. The final pellet was resuspended in 1/300 the original volume room temperature electroporation buffer and placed on ice. Sixty to one hundred µl aliquots were used within 1 hour. Approximately 10

μg of DNA was added to the tube and the cells were immediately transferred to a chilled electroporation cuvette. Electroporation conditions used were the same as for *E. coli*. The electroporated cells were immediately resuspended in cold mycoplasma media and incubated at room temperature for 10 minutes and then at 37°C for 2-3 hours. One hundred and fifty μl of cells were plated onto selective agar (2 μg per ml tetracycline or 40 μg per ml gentamicin). The plates were then incubated at 37°C for 5 – 7 days. Transformation frequency was determined by dividing the number of resistant colonies by the total colony forming units plated.

DNA Constructions

Mini-Tn*4001*

The strategy for the construction of mini-Tn*4001* is shown in Figure 1. For this construction, fragments of the transposon Tn*4001* were serially inserted into the plasmid cloning vector pSK-. Three sets of paired oligonucleotides were used; plus and minus strands with the sequences of the inner and outer repeat sequences of IS*256* and PCR primers for the transposase gene.

The outer repeat sequences were cloned first. The two oligomers, TNO+ and TNO-, were mixed (50pmole each in a final volume of 50 μl, Gibco React buffer 2) , heated at 70°C for 5 minutes and slowly cooled to room temperature. The resulting double-stranded fragment was precipitated by adding 10 μg of yeast tRNA, 1/3 vol 3M potassium acetate and 2 vol 100% ethanol. After centrifugation at 12,000 x *g* for 20 minutes, the precipitant was resuspended in React buffer 2, digested with 20 U of *Eco*RI and *Hin*dIII in a final volume of 50 μl for 1 hour, and precipitated as before. The vector was also digested with *Eco*RI and *Hin*dIII (5 μg DNA in a 100 μl reaction). The two digested DNA products were ligated in a 20 μl final volume containing 500 ng vector, 1 μl of the digested oligomers, 10 U of T4 DNA Ligase, and ligase buffer at 16°C for 16 hours. The product was precipitated, digested with *Eco*RV for 1 hour, precipitated again and resuspended in water. One fifth of this product was used to transform LE392 by electroporation. Twenty clones were picked and cultured. Plasmid DNAs were isolated and digested with *Eco*RV to check for the presence of inserts (the *Eco*RV restriction site should have been lost during the cloning). One plasmid was sequenced and contained the correct outer repeat insert. This plasmid was designated pISM27 and was used for the next stage of cloning.

The same basic protocol was followed for the insertion of the inner repeat sequences. The oligonucleotides TNI+ and TNI- were designed with staggered ends so that the annealed product did not require restriction digestion. Twenty-five pmoles of the oligomers TNI2+ and

Figure 1. Construction of the mini-Tn*4001*tet. A. the multiple cloning site of pSK-; B. the outer repeat DNA sequence of Tn*4001* inserted into the *Hin*dIII and *Eco*RI restriction sites; C. the inner repeat sequence of Tn*4001* inserted into the *Spe*I and *Sac*I restriction sites; D. the transposase gene inserted into the *Kpn*I and *Sal*I restriction sites; and E. the *Hin*cII *tetM* gene-containing fragment from Tn*916* inserted into the *Sma*I restriction site. Inserted sequences are underlined or labeled. Only relevant restriction sites are shown. Diagram is not drawn to scale.

A

```
                                                  EcoRV
 KpnI            XhoI   SalI            HindIII      EcoRI PstI   SmaI
GGGTACCGGGCCCCCCCTCGAGGTCGACGGTATCGATAAGCTTGATATCGAATTCCTGCAGCCCGG
 BamHI SpeI         NotI              SacI
GGGATCCACTAGTTCTAGAGCGGCCGCCACCGCGGTGGAGCTC
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B

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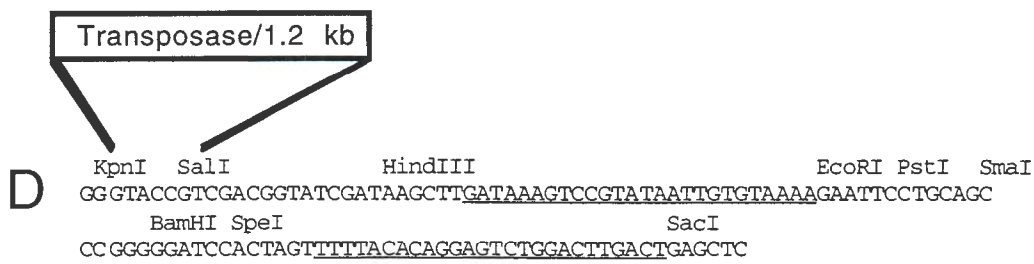
 KpnI            XhoI   SalI            HindIII
GGGTACCGGGCCCCCCCTCGAGGTCGACGGTATCGATAAGCTTGATAAAGTCCGTATAATTGTGTAA
  EcoRI PstI   SmaI   BamHI SpeI         NotI               SacI
AAGAATTCCTGCAGCCCGGGGGATCCACTAGTTCTAGAGCGGCCGCCACCGCGGTGGAGCTC
```

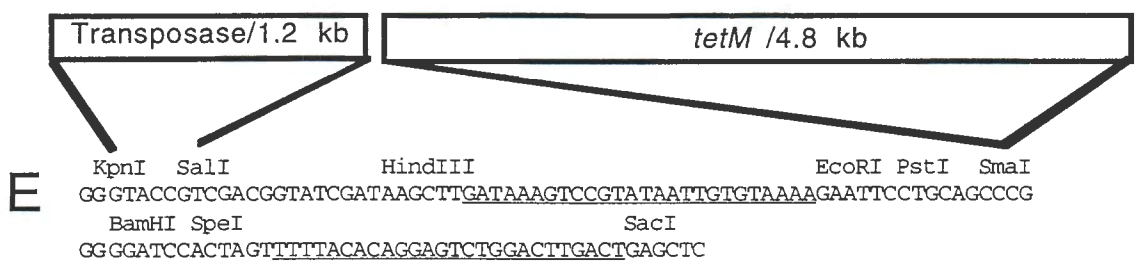
C

```
 KpnI            XhoI   SalI            HindIII
GGGTACCGGGCCCCCCCTCGAGGTCGACGGTATCGATAAGCTTGATAAAGTCCGTATAATTGTGTAAA
 EcoRI PstI   SmaI   BamHI SpeI                                SacI
AGAATTCCTGCAGCCCGGGGGATCCACTAGTTTTTACACAGGAGTCTGGACTTGACTGAGCTC
```

D



E



TNI2- were mixed with ligation buffer in a 10 µl volume, heated to 67°C for 20 minutes, and slowly cooled to room temperature. The vector was prepared in two steps. pISM27 was digested with *Spe*I (7.5 µg DNA, 10 µl NEB buffer II, 1 µl BSA, 10 U *Spe*I in a total volume of 100 µl, 37°C for 1 hour), precipitated, and then digested with *Sac*I (NEB buffer I, other conditions as before). The resulting fragments were purified using a QIAquick PCR Purification Kit. The 20 µl ligation reaction containing 50 ng *SpeI*/*Sac*I-digested pISM27 DNA, 1 µl of the oligomer mix, ligase buffer and 10 U T4 DNA Ligase was incubated at 16°C overnight. Two µl of this reaction mixture was used to transform LE392, and the resulting transformants were plated on LB plus ampicillin (100 µg per ml). Twenty clones were picked and processed as described previously. Three were sent for sequencing and all contained the correct insert. The selected plasmid with the inner and outer IS*256* repeat sequences was designated pISM28.

The Tn*4001* transposase gene was obtained by PCR. The oligomers TNPL2 and TNPL3 were designed to include the transposase promoter and the translational stop sequences. Plasmid pISM1001, which contains Tn*4001*, was used as the template. The PCR conditions were as follows: 100 ng template DNA, 2 µl 2mM dNTPs, 1 µl 2.5-3 U *Pfu* enzyme, 5 µl of a 5pmole per µl solution of each primer, 10 µl commercial 10X buffer in a final volume of 100 µl. The reaction conditions were 95°C for 1 min, 5 cycles of 95°C for 45 sec, 45°C for 45 sec and 65°C for 2 minutes followed by 35 cycles of 94°C for 45 sec, 45°C for 45 sec, and 70°C for 2 minutes, ending with 70°C for 10 minutes. A portion of the PCR product was resolved on a 1% agarose gel to check for correct size, and then the remaining portion was digested with *Kpn*I (400 ng DNA, 2 µl enzyme, 15 µl 10X React 4, in a final volume of 150 µl). The fragment was precipitated and was then digested with *Sal*I (React 10 buffer, all other conditions the same as above). To clone the transposase gene, *Kpn*I and *Sal*I-digested PISM28 was resolved on a 0.6% Nu-Sieve preparative gel, and the band containing the vector was isolated using a QIAEX II Agarose Gel Extraction Kit. Forty four ng of the double digested pISM28 DNA was ligated overnight at 14°C with 2 µl of the digested transposase product in a 10 µl reaction volume containing 0.5 µl T4 DNA Ligase. Two µl of this ligation mixture was electroporated into LE392 and plated as usual. Twenty colonies were picked, processed, and restriction digested as before. Three were selected for sequencing. To sequence across the entire transposase gene, it was necessary to design an internal oligomer, IS*256*. One clone containing the complete, correct transposase sequence was designated pISM29, also known as mini-Tn*4001*.

The *tetM* marker from Tn*916* was then inserted between the inner and outer repeat sequences in the following way. The *tetM* gene was obtained from plasmid pISM1002 on a *Hinc*II fragment (89). Plasmid pISM1002 DNA was digested with *Hinc*II for 30 minutes at

37°C under the following conditions: 15 µg of plasmid DNA, 50 U *Hin*cII, 10 µl of 10X React 4 buffer in a final volume of 100 µl. The product was purified on a 1% agarose preparative gel and isolated as described above. Plasmid pISM29 was digested with *Sma*I (15 µg DNA, 40 U of *Sma*I, 15 µl of 10X React 4 buffer in a final volume of 150 µl) at 37°C for 30 minutes, precipitated and resuspended in water. The final ligation mixture containing 180 ng pISM29, 440 ng of the *tetM* containing fragment DNA, and 5 U of T4 DNA Ligase in a 25 µl final volume was incubated at 14°C overnight, precipitated, and resuspended in water. Two µl of this ligation reaction were used to transform LE392. Ten µl of the cells were spread on media containing 100 µg per ml ampicillin and 12.5 µg per ml tetracycline and incubated overnight. No colonies were observed, so 50 µl of the same transformation mixture were plated on plates containing only 100 µg per ml ampicillin and grown overnight. The resulting 60 colonies were picked and streaked on Amp/Tet plates, and one colony grew. Restriction digests confirmed that this clone did carry the *tetM* gene. It was given the designation pISM31, also known as mini-Tn*4001*tet.

Green Fluorescent Protein

The goal of these studies was to alter the codon usage pattern of a GFP gene from a Gram negative-optimized sequence to a mycoplasma-specific one. To accomplish this, a GFP DNA sequence was designed that utilized mycoplasma codon usage preferences to code for a GFP protein product. The amino acid sequence of GFPuv was used as the starting sequence for the new gene (Clontech). Two amino acid substitutions were made (S65G and S72A) which are found in the sequence of the highly fluorescent GFP mutant, GFPmut3 (24). Codon usage tables were obtained for *M. gallisepticum* (1) and several other mycoplasma species. This codon usage data was used to derive a DNA sequence employing the most frequently used codons for each amino acid in the modified amino acid sequence described above. There is only one tryptophan in the gene (amino acid 57), and the mycoplasma-preferred codon TGA, a stop codon in the universal genetic code was used. For clarity, the designation "GFPmyco" will refer only to the GFP sequence optimized for mycoplasmas described above.

The GFPmyco sequence was divided into two parts referred to as GFP-1 (5′ end of the gene) and GFP-2 (3′ end of the gene). Each fragment contained an internal *Eco*RV (blunt end) restriction site, which would be used to join the two gene halves. Overlapping 50 mer oligonucleotides were designed to span the fragment, and two PCR primers were designed to amplify portions of the fragment (Table 3).

First, the positive and negative strand oligomers were divided into separate tubes and treated with DNA T4 kinase.(2 µl of each 12.5 pmol oligomer, ligase buffer, 10 U T4 DNA kinase

37°C for 30 minutes). Then 15 µl of each reaction was mixed with that of the opposite strand, heated to 95°C, and slowly cooled to room temperature in a temperature block to allow annealing. Ten µl of each mixture, now largely in the form of nicked, double-stranded DNA, was ligated in a reaction containing10 µl annealed solution, ligase buffer and 5 U T4 Ligase at16°C overnight. The double stranded segment produced was amplified by PCR using the following conditions. The mixture contained100 ng template, 2 µM dNTPs, 25 pmol each primer, 10 µl commercial 10X PCR buffer plus 0.5mm $MgCl_2$ and 4 U *Pfu* enzyme in a 100 µl final volume. The reaction conditions were 35 cycles at 94°C for 45 sec, 55°C for 45 sec, 72°C for 1 min (GFP-1) or 1.15 min (GFP-2) followed by 72°C for 10 min. The PCR products were resolved on a 1% agarose gel and compared with a 1 kb plus standard to confirm their sizes. The GFP-1 fragment was about 300 bp and contained a 5′ *Bam*HI site and a 3′ *Eco*RV site. The 500 bp GFP-2 fragment contained a 5′ *Eco*RV site and a 3′ *Eco*RI site. Each purified fragment was cloned by double digesting and ligating it into the appropriately digested pZerO2™. Following transformation into LE392, the transformation mix was plated on LB plates containing 25 µg per ml kanamycin. DNA was isolated using E.N.Z.A Plasmid Miniprep Kits, and those preparations carrying plasmid inserts of the correct size were sent for sequencing. Two clones with the correct sequences, designated pISM33 and pISM34 were used for the next stage of assembly.

Plasmids pISM33 and pISM34 were digested with *Eco*RV (10 µg DNA, 15 µl 10X buffer, 50 U *Eco*RV in a final volume of 150 µl at 37°C for 1 hour), precipitated and then digested with *Eco*RI (GFP-2) or *Bam*HI (GFP-1). The GFP-1 and GFP-2 fragments were isolated from 2% soft agar gels using QIAX Agarose Gel Extraction Protocol Kits. The vector pSK was double-digested with *Eco*RI and *Bam*HI. The two inserts and the digested vector were ligated together (7.2 ng GFP-1, 16.8 ng GFP-2, 10 ng vector, 2 U T4 Ligase at 16°C for 1 hour), precipitated and digested with *Eco*RI (10 U *Eco*RI in a 50 µl reaction volume at 37°C for 1 hour). The proper clone would be refractory to *Eco*RI digestion. Two µl of this product were used to transform LE392 with selection on ampicillin agar. Colonies were picked, cultured and restriction digested as before. One possible clone was sent for sequencing. It contained the complete, correct GFP for mycoplasma gene sequence (GFPmyco) and was designated pISM32.

In order to maximize gene expression in mycoplasmas, it was decided to attach the P2 promoter, a strong mycoplasma promoter that is also functional in *E. coli* (76). To accomplish this, PCR was used to add a *Bam*HI restriction site to the 5′ end of the promoter (100 ng template pISM2050.2, 2 µM dNTPs, 25 pmol each primer, 10 µl 10X PCR buffer plus 0.5 mM $MgCl_2$, and 4 U *Pfu* enzyme in a 100 µl volume) using conditions of 35 cycles of 94°C for 45

sec, 60-65°C for 45 sec, 72°C for 2 min, followed by 72°C for 10 min. The primers AcP2R and AcP2F were used.

The final P2:GFPmyco construct was assembled in two stages, P2:GFPmyco ligation and amplification was followed by insertion of the P2:GFPmyco fragment into the Tn*4001*mod of plasmid pISM2062. P2 and GFP fragments were blunt end ligated (60 ng each DNA, ligase buffer, 5 U T4 DNA Ligase in final volume of 20 µl at 16°C overnight). This ligation mixture showed no visible product by gel analysis, but was used as the template for a PCR reaction using primers GFPMyF and CM33R.Bam (see conditions above). The resulting PCR product was digested with *Bam*HI and ligated into *Bam*HI-digested, dephosphorylated pISM2062. Dephosphorylation was accomplished with calf intestinal phosphatase (1 µg DNA, 2 U CIP in a final volume of 30 µl for 60 minutes at 37°C) followed by proteinase K digestion (10 µl 10%SDS, 25 µl 1M Tris, pH 7.4, 11 U Proteinase K, 0.5 µl 0.5 M EDTA in a final volume of 100 µl for 1 hr at 65°C), and phenol:chloroform extracted. A final ligation joined the *Bam*HI vector and insert fragments (100 ng *Bam*HI-digested pISM2062 DNA, 120 ng *Bam*HI-digested P2:GFPmyco fragment in a 20 µl reaction volume at 14°C overnight). The product was heat inactivated at 70°C for 15 minutes, precipitated, resuspended in 3 µl of water, and transformed into LE392. Transformants were plated on LB plus 100 µg per ml ampicillin. Plasmid DNAs from twenty of the resulting colonies were analyzed by restriction digestion. One clone containing the complete P2:GFPmyco sequence was designated pISM30. The construct was confirmed by DNA sequence.

Functional Studies

Mini-Tn*4001*tet

The functionality of mini-Tn*4001*tet was tested by transformation of pISM31 into *M. gallisepticum*. Plasmid DNA was prepared using the Qiagen midi-kit, and electrocompetent *M. gallisepticum* were prepared as described above. Ten µg of plasmid DNA were mixed with 100 µl of competent cells and electroporated as previously described. Following incubation at 37°C, the cells were centrifuged at 12,000 x *g* for 2 minutes, resuspended to 150 µl in phosphate buffered saline, and 100 µl of the cell suspension was plated on PPLO agar containing 2 µg per ml tetracycline. Colony forming units were determined by plating serial dilutions in PBS on non-selective PPLO agar. Plates were incubated at 37°C for 10 days prior to counting.

Putative transformants were picked randomly, grown in 1 ml of non-selective PPLO media overnight and then diluted to 10 ml with selective media containing 2 µg per ml tetracycline. Aliquots were frozen for future analysis, and the remaining cells, representing approximately 8

mls of fully grown cells, were centrifuged to pellet the cells. Chromosomal DNA was prepared from these cells using the QIAamp DNA mini kit (Qiagen). The DNA was analyzed for the presence of the transposon using *tetM*-specific primers in a PCR reaction. To control for plasmid insertion, another PCR reaction was run with *bla* primers, which should only produce a product when the plasmid ampicillin resistance gene is present.

GFP

Prior to determining if GFPmyco could function in mycoplasmas, its fluorescent signal was examined in *E. coli.* This seemingly simple experiment was compounded by the presence of a UGA codon in the GFPmyco reading frame. To suppress the UGA codon, pISM32 was transformed into the opal suppressor strain *E. coli* ISM614, and the resulting transformants were examined for fluorescence.

To transform GFPmyco into *M. gallisepticum* R, electrocompetent cells were prepared for transformation as described above. Ten μg of pISM32 DNA was used to transform *M. gallisepticum.* Half were plated on phenol red containing PPLO selective media and half were plated on phenol red-free PPLO selective media. The presence of phenol red complicates analysis since it fluoresces at the same wavelength as GFP. All plates were incubated at 37°C until colonies appeared (7-10 days). After 10 days, the colonies on the phenol red-free plates were viewed with an UV light box. Colonies were picked from the plates using sterile pasteur pipettes and grown in 1 ml of non-selective, phenol red-containing media until the media turned slightly orange (usually 1-2 days). Each culture was then split 1/10 into two tubes of selective media containing 40 μg per ml gentamicin. One of the tubes contained phenol red, and the second did not. Mid log phase phenol red-containing cultures (slightly orange) were pelleted and frozen for future DNA studies. The equivalent phenol red-free cultures were examined for fluorescence.

RESULTS AND DISCUSSION

Mini-Tn*4001*tet

Mini transposons have been used for many kinds of genetic studies including mutant construction, gene expression studies, protein function analyses and cellular tagging. Their compact size and ease of manipulation make them excellent genetic tools (9). The mini transposon structure also eliminates some unfortunate genetic behaviors characteristic of native transposons. In particular, the movement of the transposase to outside the inverted repeats, and its subsequent loss from the cell, does away with multiple transposition events and spontaneous deletions (36).

Mini-transposons offer other advantages that make them ideal genetic tools. They are designed so that they can easily be altered to suit a particular experimental system. For example, genes are easily inserted into the multiple cloning sites located between the inverted repeats. Modifications may include changes in antibiotic resistant markers (36) or the exchange of an antibiotic resistance for another selectable marker (109). Reporter genes are also often used with mini-transposons.

While mini transposons have been developed for analysis of both Gram negative and Gram positive bacteria, no such tools have been available for use with mycoplasmas. In fact, only two transposons are known to function in mycoplasmas, Tn*916* and Tn*4001* (42). When deciding on the starting element for such a construction, the complexity and size of the conjugative transposon Tn*916* was thought to be a difficult barrier to overcome. Tn*4001*, however, is structurally similar to Tn*5* making it an ideal candidate for these studies. In addition, this laboratory has a history of successful genetic manipulation of Tn*4001* (75). Thus, the decision was made to develop a mini-Tn*4001* derivative for use in mycoplasmas.

The structure of the mini-Tn*4001* was based on the structure of mini Tn*5* (36). Mini Tn*5* contains the inner and outer repeat sequences of the insertion sequence IS*5* flanking a selective marker. The IS256 of Tn*4001* is the source of the mini-Tn*4001* outer and inner repeats (Figures 1 and 2). The transposase is supplied in *cis* outside the repeat sequences. Since plasmids with *E. coli* compatible origins of replication act as suicide vectors in mycoplasmas, the transposase is lost during cell replication even after insertion of the mini-transposon into the chromosome.

```
GGGTACCGTGTAAAAGTAAAAAGGCCATATAACAGTCCTTTTACGGTACAATGTTTTTAACGACAAAAAC  70

      Transposase>  M  T  Q  V  H  N  T  L  K  S  E  E  I  Q  S  I  I
ATACCCAGGAGGACTTTTACATGACCCAAGTACATTTTACACTGAAAAGCGAAGAGATTCAAAGCATTAT 140

  E  Y  S  V  K  D  D  V  S  K  N  I  L  T  T  V  N  N  Q  L  M  E  N
TGAATATTCTGTAAAGGATGACGTTTCTAAAAATATTTTAACAACGGTATTTAATCAACTAATGGAAAAT 210

 Q  R  T  E  Y  I  Q  A  K  E  Y  E  R  T  E  N  R  Q  S  Q  R  N  G
CAACGAACAGAATATATTCAAGCAAAAGAATATGAACGAACAGAAAACCGACAAAGTCAACGAAATGGCT 280

Y  Y  E  R  S  N  T  T  R  V  G  T  L  E  L  K  V  P  R  T  R  D  G  H
ATTATGAGCGCAGCTTTACGACACGTGTAGGCACGCTAGAATTAAAAGTACCCAGAACACGTGATGGCCA 360

  N  S  P  T  V  N  E  R  Y  Q  R  N  E  K  A  L  M  A  S  M  L  E  M
TTTTTCACCCACAGTGTTTGAACGTTATCAACGAAACGAAAAAGCCCTCATGGCTTCAATGTTGGAAATG 420

 Y  V  S  G  V  S  T  R  K  V  S  K  I  V  E  E  L  C  G  K  S  V  S
TATGTATCAGGCGTTTCAACTCGTAAAGTATCAAAAATTGTGGAAGAACTTTGTGGTAAATCCGTCTCTA 490

K  S  F  V  S  S  L  T  E  Q  L  E  P  M  V  N  E  W  Q  N  R  L  L  S
AGTCCTTCGTTTCTAGCTTAACAGAACAGCTAGAACCTATGGTTAACGAGTGGCAGAATCGTTTATTATC 560

  E  K  N  Y  P  Y  L  M  T  D  V  L  Y  I  K  V  R  E  E  N  R  V  L
AGAAAAAAATTATCCTTACTTAATGACCGATGTACTCTATATAAAAGTACGAGAAGAAAATCGAGTACTC 630

 S  K  S  C  H  I  A  I  G  I  T  K  D  G  D  R  E  I  I  G  F  M  I
TCAAAAAGCTGTCATATAGCGATTGGAATAACCAAAGATGGCGACCGTGAAATTATCGGCTTCATGATTC 700

Q  S  G  E  S  E  E  T  W  T  T  N  N  E  Y  L  K  E  R  G  L  Q  G  T
AAAGTGGCGAAAGCGAAGAGACCTGGACAACATTTTTTGAATACCTAAAAGAACGCGGTTTACAAGGTAC 770

  E  L  V  I  S  D  A  H  K  G  L  V  S  A  I  R  K  S  F  T  N  V  S
GGAACTCGTTATTTCTGATGCGCACAAAGGATTAGTCTCTGCCATTAGAAAATCCTTCACCAACGTAAGT 840

 W  Q  R  C  Q  V  H  F  L  R  N  I  N  T  T  I  P  K  K  N  S  K  S
TGGCAAAGATGCCAAGTTCACTTCCTAAGAAATATCTTTACCACCATTCCTAAAAAAAAATTCAAAATCTT 910

F  R  E  A  V  K  G  I  N  K  F  T  D  I  N  L  A  R  E  A  K  N  R  L
TCAGAGAAGCTGTTAAAGGAATTTTTAAGTTCACAGATATTAACTTAGCGCGTGAGGCTAAAAATCGATT 980

  I  H  D  Y  I  D  Q  P  K  Y  S  K  A  C  A  S  L  D  D  G  F  E  D
GATTCATGATTATATCGATCAACCAAAATATTCAAAAGCTTGCGCATCATTGGATGATGGATTCGAAGAC 1050

 A  N  Q  Y  T  V  Q  G  N  S  H  N  R  L  K  S  T  N  L  I  E  R  L
GCCTTTCAATATACCGTACAAGGAAATTCCCACAATCGACTAAAGAGTACCAATCTAATTGAACGACTGA 1120

N  Q  E  V  R  R  R  E  K  I  I  R  I  F  P  N  Q  T  S  A  N  R  L  I
ATCAAGAAGTACGCAGAAGAGAAAAGATTATTCGCATCTTCCCCAATCAAACATCAGCCAATCGCTTAAT 1190

  G  A  V  L  M  D  L  H  D  E  W  I  Y  S  S  R  K  Y  I  N  N  D  K
TGGAGCCGTTCTTATGGACCTACATGATGAATGGATTTATTCTTCAAGAAAATACATCAATTTTGATAAG 1260

 *                                  O
TAGAAATGGGTCGACGGTATCGATAAGCTTGATAAAGTCCGTATAATTGTGTAAAAGAATTCCTGCAGCC 1330
CGTCAACATACTTCTTGCGATTATACAGGTGGAATCGGGCGGTACTGCGGAAGATGTTATGCAGTCCTCG 1400
```

Figure 2. DNA sequence of mini-Tn*4001*tet. **O,** 5′ end of outer repeat (underlined); **I,** 5′ end of inner repeat (underlined). Amino acid sequences of transposase and TetM proteins are listed under the corresponding DNA sequence. The sequence of the *Hin*cII fragment (containing *tetM*) was taken from Flannagan (47).

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GAATCCCTCGGTCTTCCACCTAATTCATTGAGTACAGAAGAATCCATTAAGCAAGGTGTGAAGTATTTCA  1470
GTGAATTATTAGCCAGTAGCGAAAGGCTCAGTGTAGATTTAGAATCGGTTATCCAGTCCTACAATTATGG  1540
TGGTGGTTTCTTAGGGTATGTGGCTAATCGTGGAAATAAATATACCTTTGAACTGGCTCAAAGTTTCTCA  1610
AAAGAGTATTCAGGTGGCGAAAAAGTGTCTTACCCCAATCCCATAGCCATACCTATCAATGGGGGCTGGC  1680
GATACAACTATGGCAATATGTTTTATGTGCAACTGGTAACGCAGTATCTTGTCACAACAGAGTTTGATGA  1750
TGATACGGTACAAGCCATCATGGACGAAGCACTGAAATATGAGGGCTGGCGATACGTTTACGGTGGAGCT  1820
TCCCCGACTACTTCTTTTGATTGTAGCGGACTGACACAATGGACGTATGGAAAAGCTGGAATTAACTTAC  1890
CACGAACCGCACAACAGCAATATGATGTGACCCAGCATATCCCACTATCGGAAGCACAAGCTGGCGATTT  1960
GGTTTTCTTTCATTCTACCTATAACGCTGGCTCTTATATTACTCATGTTGGGATATACCTTGGCAATAAC  2030
CGTATGTTTCATGCAGGCGACCCAATCGGTTATGCCGACTTAACAAGCCCCTACTGGCAACAGCATTTAG  2100
TGGGAGCAGGACGAATCAAACAATGAGAAAGGAAGATTTAATGATGAAATTTAGAAAAAATCAGAATAAA  2170
GAAAAACAGATACCAAAGGAAAAGAAACCTCGTGTCTATAAGGTCAATCCTCATAAAAAGGTTGTGATTG  2240
CCTTGTGGGTACTTTTAGGGCTTAGTTTCAGCTTTGCGATATTCAAGCACTTTACAGCTATAGATACTCA  2310
TACTATTCACGAAACAACTATCATAGAAAAGGAATACGTTGATACTCATCATGTAGAAAATTTTGTAGAG  2380
AACTTTGCGAAAGTCTACTATTCATGGGAGCAATCCGATAAGTCCATTGATAATCGAATGGAAAGTCTAA  2450
AAGGCTATCTGACAGATGAACTTCAAGCTCTCAATGTTGATACAGTACGCAAAGATATTCCTGTATCGTC  2520
TTCTGTAAGAGGATTTCAGATATGGACGGTAGAGCCAACTGGCGACAATGAGTTTAATGTAACCTACAGT  2590
GTAGACCAGCTCATTACAGAGGGAGAAAATACAAAGACCGTCCACTCTGCTTATATAGTGAGTGTCTATG  2660
TAGATGGTTCTGGAAATATGGTACTGGTTAAGAATCCGACCATTACCAACATACCTAAGAAATCAAGTTA  2730
TAAACCAAAAGCCATTGAAAGTGAGGGGACGGTTGATTCCATTACAACCAATGAAATCAATGAGTTTTTA  2800
ACGACGTTCTTCAAGCTCTATCCTACAGCGACAGCCAGTGAACTTTCCTACTATGTGAATGACGGGATAT  2870
TAAAACCAATCGGAAAAGAGTACATCTTTCAAGAACTGGTAAATCCTATTCACAATCGTAAGGATAATCA  2940
AGTCACGGTATCGCTGACAGTGGAGTATATCGACCAGCAGACCAAAGCAACGCAGGTATCTCAATTTGAT  3010
TTGGTACTTGAAAAGAACGGGAGTAATTGGAAGATTATAGAATAACAAATATTGGTACATTATTACAGCT  3080
ATTTTGTAATCACGTACTCTCTTTGATAAAAAATTGGAGATTCCTTTACAAATATGCTCTTACGTGCTAT  3150
TATTTAAGTATCTATTTAAAAGGAGTTAATAAATATGCGGCAAGGTATTATTAAATAAACTGTCAATTTG  3220
ATAGCGGGAACAAATAATTGGATGTCCTTTTTTAGGAGGGCTTAGTTTTTTGTACCCAGTTTAAGAATAC  3290
CTTTATCATGTGATTCTAAAGTATCCGGAGAATATCTGTATGCTTTGTATGCCTATGGTTATGCATAAAA  3360
                                                                    tetM>
ATCCCAGTGATAAGAGTATTTATCACTGGGATTTTTATGCCCTTTTGGGCTTTTGAATGGAGGAAAATCA  3430
  M  K  I  I  N  I  G  V  L  A  H  V  D  A  G  K  T  T  L  T  E  S  L
CATGAAAATTATTAATATTGGAGTTTTAGCTCATGTTGATGCAGGAAAAACTACCTTAACAGAAAGCTTA  3500
 L  Y  N  S  G  A  I  T  E  L  G  S  V  D  K  G  T  T  R  T  D  N  T
TTATATAACAGTGGAGCGATTACAGAATTAGGAAGCGTGGACAAAGGTACAACGAGGACGGATAATACGC  3570
L  L  E  R  Q  R  G  I  T  I  Q  T  G  I  T  S  N  Q  W  E  N  T  K  V
TTTTAGAACGTCAGAGAGGAATTACAATTCAGACAGGAATAACCTCTTTTCAGTGGGAAAATACGAAGGT  3640
  N  I  I  D  T  P  G  H  M  D  F  L  A  E  V  Y  R  S  L  S  V  L  D
GAACATCATAGACACGCCAGGACATATGGATTTCTTAGCAGAAGTATATCGTTCATTATCAGTTTTAGAT  3710
 G  A  I  L  L  I  S  A  K  D  G  V  Q  A  Q  T  R  I  L  N  H  A  L
GGGGCAATTCTACTGATTTCTGCAAAAGATGGCGTACAAGCACAAACTCGTATATTATTTCATGCACTTA  3780
```

Figure 2. (Continued)

```
R K M G I P T I N N I N K I D Q N G I D L S T V
GGAAAATGGGGATTCCCACAATCTTTTTTATCAATAAGATTGACCAAAATGGAATTGATTTATCAACGGT  3850

  Y Q D I K E K L S A E I V I K Q K V E L Y P N
TTATCAGGATATTAAAGAGAAACTTTCTGCCGAAATTGTAATCAAACAGAAGGTAGAACTGTATCCTAAT  3920

 V C V T N N T E S E Q W D T V I E G N D D L L
GTGTGTGTGACGAACTTTACCGAATCTGAACAATGGGATACGGTAATAGAGGGAAACGATGACCTTTTAG  3990

E K Y M S G K S L E A L E L E Q E E S I R N Q N
AGAAATATATGTCCGGTAAATCATTAGAAGCATTGGAACTCGAACAAGAGGAAAGCATAAGATTTCAGAA  4060

  C S L F P L Y H G S A K S N I G I D N L I E V
TTGTTCTCTGTTCCCTCTTTATCATGGAAGTGCAAAAAGTAATATAGGGATTGATAACCTTATAGAAGTT  4130

 I T N K N Y S S T H R G P S E L C G N V F K I
ATTACTAATAAATTTTATTCATCAACACATCGAGGTCCGTCTGAACTTTGCGGAAATGTTTTCAAAATTG  4200

E Y T K K R Q R L A Y I R L Y S G V L H L R D S
AATATACAAAAAAAAGACAACGTCTTGCATATATACGCCTTTATAGTGGAGTACTACATTTACGAGATTC  4270

  V R V S E K E K I K V T E M Y T S I N G E L C
GGTTAGAGTATCAGAAAAAGAAAAAATAAAAGTTACAGAAATGTATACTTCAATAAATGGTGAATTATGT  4340

 K I D R A Y S G E I V I L Q N E N L K L N S V
AAGATTGATAGAGCTTATTCTGGAGAAATTGTTATTTTGCAAAATGAGTTTTTGAAGTTAAATAGTGTTC  4410

L G D T K L L P Q R K K I E N P H P L L Q T T V
TTGGAGATACAAAACTATTGCCACAGAGAAAAAAGATTGAAAATCCGCACCCTCTACTACAAACAACTGT  4480

  E P S K P E Q R E M L L D A L L E I S D S D P
TGAACCGAGTAAACCTGAACAGAGAGAAATGTTGCTTGATGCCCTTTTGGAAATCTCAGATAGTGATCCG  4550

 L L R Y Y V D S T T H E I I L S F L G K V Q M
CTTCTACGATATTACGTGGATTCTACGACACATGAAATTATACTTTCTTTCTTAGGGAAAGTACAAATGG  4620

E V I S A L L Q E K Y H V E I E L K E P T V I Y
AAGTGATTAGTGCACTGTTGCAAGAAAAGTATCATGTGGAGATAGAACTAAAAGAGCCTACAGTCATTTA  4690

  M E R P L K N A E Y T I H I E V P P N P F W A
TATGGAGAGACCGTTAAAAAATGCAGAATATACCATTCACATCGAAGTGCCGCCAAATCCTTTCTGGGCT  4760

 S I G L S V S P L P L G S G M Q Y E S S V S L
TCCATTGGTTTATCTGTATCACCGCTTCCGTTGGGAAGTGGAATGCAGTATGAGAGCTCGGTTTCTCTTG  4830

G Y L N Q S N Q N A V M E G I R Y G C E Q G L Y
GATACTTAAATCAATCATTTCAAAATGCAGTTATGGAAGGGATACGCTATGGTTGTGAACAAGGATTGTA  4900

  G W N V T D C K I C N K Y G L Y Y S P V S T P
TGGTTGGAATGTGACGGACTGTAAAATCTGTTTTAAGTATGGCTTATACTATAGCCCTGTTAGTACCCCA  4970

 A D N R M L A P I V L E Q V L K K A G T E L L
GCAGATTTTCGGATGCTTGCTCCTATTGTATTGGAACAAGTCTTAAAAAAAGCTGGAACAGAATTGTTAG  5040

E P Y L S N K I Y A P Q E Y L S R A Y N D A P K
AGCCATATCTTAGTTTTAAAATTTATGCGCCACAGGAATATCTTTCACGAGCATACAACGATGCTCCTAA  5110

  Y C A N I V D T Q L K N N E V I L S G E I P A
ATATTGTGCGAACATCGTAGACACTCAATTGAAAAATAATGAGGTCATTCTTAGTGGAGAAATCCCTGCT  5180

 R C I Q E Y R S D L T F N T N G R S V C L T E
CGGTGTATTCAAGAATATCGTAGTGATTTAACTTTCTTTACAAATGGACGTAGTGTTTGTTTAACAGAGT  5250
```

Figure 2. (Continued)

```
L  K  G  Y  H  V  T  T  G  E  P  V  C  Q  P  R  R  P  N  S  R  I  D  K
TAAAAGGGTACCATGTTACTACCGGTGAACCTGTTTGCCAGCCCCGTCGTCCAAATAGTCGGATAGATAA  5320

  V  R  Y  M  F  N  K  I  T  *
AGTACGATATATGTTCAATAAAATAACTTAGTGTATTTTATGTTGTTATATAAATATGGTTTCTTGTTAA  5390
ATAAGATGAAATATTTTTTAATAAAGATTTGAATTAAAGTGTAAAGGAGGAGATAGTTATTATAAACTAC  5460
AAGTGGATATTGTGTCCTGTATGTGGAAATAAAACACGATTAAAGATAAGGGAAGATACTGAATTAAAAA  5530
AATTCCCCCTCTATTGTCCGAAATGCAGACAAGAAAATTTAATTGAAATAAAGCAGTTCAAAGTAACTGT  5600
GATTACAGAGCCAGACGCAAAGACGCAGAGCCGATAAAATGAGATTAATACAATCTCATTTTATCGGCTC  5670
TTTCCGTTATGTATGGATTCTTTTAATTAGTCTTCGATGTTTCTTGCTTCGTTGATACCGCTGGCTAAAG  5740
ATTCCATTAAGGATAGTTCTTTGTCTGTAAAGCTATCCATGTATTTCTCTATCTGTAATCGTCGGGTGCT  5810
TTTTACCAAGTTATTAGCAGGTAAGAAAAATTCATCAACGGAAACATGAAGTAACGATACAAGGTCATAA  5880
AGAACTTGTATGCTGGGGTGTTGCCCTTTATTTTCAATATTAGTTAAGTACCGTGGGTCAATTTCAATCA  5950
ATGCTCCCACTTGTTCACGAGTTAAACCTCGTTGCAATCGAGCTTCTTTAATGGCTAAACCAAAGGCTCT  6020
AAAATCATATTTATCTTCTTTTTTACGCATAGTAGACCACCTCTATACATTTTATTGTTCCTACTGAATT  6090
AAAAACAGGTATAGAAAAACGTGTTATATGGTTTATAGGTTTATATTTAATAAAAAGCACTACTAAACGC  6160
CAATAAAAAAAAACCGTTATATGGTAGTGCTATTTACGCTGTTAAAATATTGTATATTACTTCCAAATGGC  6230

                                  I
GGTTTGTTGGAGGTCAACGGGGGATCCACTAGTTTTTACACAGGAGTCTGGACTTGACTGAGCTC        6295
```

Figure 2. (Continued)

To test the functionality of min-Tn*4001*tet, it was transformed into *M. gallisepticum* R by electroporation. The results of those experiments are shown in Table 4. The transformation frequency of the mini-transposon ranged from 1.8×10^{-7} to $2.4\ 10^{-7}$, which is in the range of the wild type Tn*4001* ($1 \times 10^{-6} - 1 \times 10^{-7}$)(17). For unknown reasons, the plasmid containing mini-Tn*4001*tet replicated at a very low copy number, and obtaining sufficient concentrations of plasmid DNA for the transformation experiments was problematic. This may explain the somewhat lower transformation frequency observed.

Table 4. Results of transformation experiments with mini-Tn*4001*tet in *M. gallisepticum.*

Exp. #	CFUs per ml	# Transformants per vol	Tf*
1	5.3×10^{8}	25 per 20 µl	2.4×10^{-7}
2	6.2×10^{9}	35 per 30 µl	1.8×10^{-7}
3	3.9×10^{9}	71 per 100 µl	1.8×10^{-7}

Tf = transformation frequency as determined by dividing the number of resistant colonies by the total colony forming units plated

PCR were used to confirm the insertion of mini-Tn*4001*tet into the *M. gallisepticum*. Transformed colonies were picked as described earlier and chromosomal DNA was isolated. The PCR primers tetM.1 and tetM.2 were used to screen for the *tetM* marker in the mini-transposon, and the primers TH111 and TH112 were used to screen for the ampicillin resistance marker. The presence of the latter would indicate that the plasmid had integrated into the chromosome by homologous recombination. The results of the PCR experiments are shown in Figure 3. All of the transformants demonstrated the presence of the *tetM* marker indicating insertion of mini-Tn*4001*tet into the chromosome. Two of the 9 transformants tested also had *bla* sequences (the ampicillin resistance marker) indicating that in these transformants, the plasmid may also be integrated into the chromosome. Further experiments will be needed to determine if integration of the plasmid occurs at a high frequency and whether this will be a problem with plasmid pISM31.

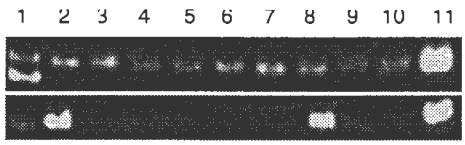


Figure 3. PCR analysis of *M. gallisepticum* mini-Tn*4001*tet transformants. **Upper**. tetM-specific primers. **Lower**. *bla*-specific primers. Lane 1, 1kb+ base pair ladder; lanes 2-10, *M. gallisepticum* transformants; lane 11, control (pISM31).

Although mini-transposons have been constructed for use in many bacterial systems, few have been used in Gram positive bacteria, and they have not been available for mycoplasma researchers. Our prototype, mini-Tn*4001*tet is functional in mycoplasmas and should be useful for a variety of genetic and protein expression studies. The replacement of the tetracycline gene by other markers should be technically trivial and allow for the construction of a family of mini-Tn*4001* derivatives as a needed for specific experimental systems. Several resistance markers could be substituted including gentamicin, erythromycin, and chloramphenicol acetyl transferase. In addition at least two reporter genes can be used, β-galactosidase (*lacZ*), PhoA (95), and *tetM* (95). Eventually, GFP and many other reporter genes will be developed for use in mycoplasmas.

GFPmyco

Green fluorescent protein variants, while relatively new, have already been shown to be exceptionally flexible markers, especially for *in vivo* labeling. A number of groups have obtained interesting results expressing two variants in the same cell. In some cases a single mini-transposon system has transported the genes for two different GFP markers (72, 105, 133, 136). However, GFP has not been expressed in mycoplasma systems. In the one published account of the use of GFP for mycoplasmal studies, Dhandayuthapani *et al.* used it as a marker for retrieving potential promoter sequences from a mycoplasma cDNA library (38) but the GFP was visualized in *E. coli*,. They presented no data on GFP expression in mycoplasma.

GFPuv (Clontech), is a commonly used GFP variant with an efficient promoter and sequence optimized for translation in *E. coli*. An overnight *E. coli* culture containing this plasmid has a glowing yellow green color under fluorescent or daylight conditions. Initial attempts to express this protein in *M. gallisepticum* were unsatisfactory (95). There was some protein production, but the fluorescence levels were so low that they were only faintly visible with a confocal microscope. Since the promoter system, plasmid carriers, etc. used in these experiments were known to function in *M. gallisepticum*, it was hypothesized that the codon usage of the GFPuv gene was negatively impacting protein translation rates and preventing the production of observable quantities of translated product.

To overcome this problem, a new GFP gene sequence was designed utilizing codons favored by mycoplasma translation systems. The design of the coding sequence is shown in Figure 4. GFPuv, which has a prokaryote-optimized DNA sequence was used as the starting sequence. This sequence differs from wild type in three amino acids. These substitutions which increase the fluorescence signal were also incorporated into the GFPmyco sequence. Two more mutations were introduced into the sequence at amino acids 65 (S65G) and 72 (S72A). These mutations have been shown to enhance folding and fluorescence in other GFP variants. All are shown, along with the final amino acid sequence, in Figure 4. The final DNA sequence is shown in Figure 5. The gene assembly strategy is summarized in Figure 6. The GFPmyco gene was constructed using an overlap technique in which the genome was divided into sets of long, overlapping, bi-directional oligonucleotides as shown in Figure 7.

The construction of the mycoplasma-optimized sequences was complicated by the AT richness of the sequences. This caused difficulties in PCR primer design. Theoretically the long runs of adenines and thymidines could also cause misalignments in the annealing stage preventing proper assembly. In fact, several clones that were sequenced did appear to be the

products of aberrant assembly. For assembly of the final GFP gene, only clones with the correct sequence were used.

The second phase of the construction, the addition of the P2 promoter to the GFPmyco gene, required a final PCR step to obtain sufficient material for cloning. It was not possible to identify the correct sized band in the ligation mixtures. In fact, the only visible bands were the unligated products. This could have resulted from damaged fragments or may have simply been due to the fact that it was a blunt end ligation. In any event, a final PCR step was included to obtain a sufficient quantity of the correct product for the final cloning into pISM2062. PISM262 carries a Tn*4001* derivative, Tn*4001*mod, which has unique *Sma*I and *Bam*HI sites just inside of the inverted repeat (75). *Bam*HI sites had previously been added to each end of the P2:GFP fragment during PCR manipulations. As always, *Pfu* polymerase was used in this final PCR reaction to reduce the error rate. Initial attempts to use *Taq* polymerase in the construction process had resulted in multiple sequence errors, and it was replaced with *Pfu*.

```
                        10          20          30          40
GFPwt       MSK GEE LFT GVV PIL VEL DGD VNG HKF SVS GEG EGD ATY GKL TLK FIC
GFPuv       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...>
GFPmyco     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...>

             50          60          70          80          90
GFPwt       TTG KLP VPW PTL VTT FSY GVQ CFS RYP DHM KRH DFF KSA MPE GYV QER
GFPuv       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...>
GFPmyco     ... ... ... ... ... .G. ... ..A ... ... ... ... ... ... ... ...>

               100         110         120         130         140
GFPwt       TIF FKD DGN YKT RAE VKF EGD TLV NRI ELK GID FKE DGN ILG HKL EYN
GFPuv       ..S ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...>
GFPmyco     ..S ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...>

                150         160         170         180         190
GFPwt       YNS HNV YIM ADK QKN GIK VNF KIR HNI EDG SVQ LAD HYQ QNT PIG DGP
GFPuv       ... ... ..T ... ... ... A.. ... ... ... ... ... ... ... ... ...>
GFPmyco     ... ... ..T ... ... ... A.. ... ... ... ... ... ... ... ... ...>

                   200         210         220         230
GFPwt       VLL PDN HYL STQ SAL SKD PNE KRD HMV LLE FVT AAG ITH GMD ELY K*
GFPuv       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .>
GFPmyco     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .>
```

Figure 4. Comparison of the amino acid sequences of wild type GFP (GFPwt), GFPuv (a popular *E. coli*-optimized variant, Clontech), and the GFP sequence used for the GFPmyco gene. The amino acids are given as the single letter codes. Exact amino acid matches are shown as (.). Changes are indicated below the wild type sequence.

Figure 5. Comparison of the DNA gene sequences of GFPuv and GFPmyco. 162 base pair substitutions were necessary to convert the *E. coli*-optimized GFPuv sequence to the mycoplasma-optimized GFPmyco sequence. The translated amino acid sequence is shown above the corresponding DNA sequence. Homology is shown by (.). Base pair differences are shown in the GFPmyco sequence.

```
          M   S   K   G   E   E   L   F   T   G   V   V   P   I   L   V   E   L   D   G>
                      10          20          30          40          50          60
GFPuv     ATG AGT AAA GGA GAA GAA CTT TTC ACT GGA GTT GTC CCA ATT CTT GTT GAA TTA GAT GGT
GFPmyco   ... ... ... ..T ... ... T.A ..T ..A ..T ..A ..A ... ... T.A ... ... ... ... ...>

          D   V   N   G   H   K   F   S   V   S   G   E   G   E   G   D   A   T   Y   G>
                      70          80          90          100         110         120
GFPuv     GAT GTT AAT GGG CAC AAA TTT TCT GTC AGT GGA GAG GGT GAA GGT GAT GCA ACA TAC GGA
GFPmyco   ... ... ... ..T ..T ... ... AG. ..A ... ..T ..A ... ... ... ... ... ... ..T ..T>

          K   L   T   L   K   F   I   C   T   T   G   K   L   P   V   P   W   P   T   L>
                      130         140         150         160         170         180
GFPuv     AAA CTT ACC CTT AAA TTT ATT TGC ACT ACT GGA AAA CTA CCT GTT CCA TGG CCA ACA CTT
GFPmyco   ... T.A ..A T.A ... ... ... ..T ... ... ..T ... T.. ..A ..A ... ..A ... ..T T.A>

          V   T   T   F   S   Y   G   V   Q   C   F   S   R   Y   P   D   H   M   K   R>
                      190         200         210         220         230         240
GFPuv     GTC ACT ACT TTC TCT TAT GGT GTT CAA TGC TTT TCC CGT TAT CCG GAT CAT ATG AAA CGG
GFPmyco   ..A ..A ... ..T GG. ... ... ..A ... ..T ... G.T A.A ... ..A ... ..C ... ... A.A>

          H   D   F   F   K   S   A   M   P   E   G   Y   V   Q   E   R   T   I   S   F>
                      250         260         270         280         290         300
GFPuv     CAT GAC TTT TTC AAG AGT GCC ATG CCC GAA GGT TAT GTA CAG GAA CGC ACT ATA TCT TTC
GFPmyco   ..C ..T ... ..T ..A ... ..T ... ..A ... ... ... ..T ..A ... A.A ..A ..T AG. ..T>

          K   D   D   G   N   Y   K   T   R   A   E   V   K   F   E   G   D   T   L   V>
                      310         320         330         340         350         360
GFPuv     AAA GAT GAC GGG AAC TAC AAG ACG CGT GCT GAA GTC AAG TTT GAA GGT GAT ACC CTT GTT
GFPmyco   ... ... ..T ..T ..T ..T ..A ..A A.A ..A ... ..A ..A ... ... ... ... ..T T.A ..A>

          N   R   I   E   L   K   G   I   D   F   K   E   D   G   N   I   L   G   H   K>
                      370         380         390         400         410         420
GFPuv     AAT CGT ATC GAG TTA AAA GGT ATT GAT TTT AAA GAA GAT GGA AAC ATT CTC GGA CAC AAA
GFPmyco   ... A.A ..T ..A ... ... ... ... ... ... ... ... ... ..T ..T ... T.A ..T ..T ...>

          L   E   Y   N   Y   N   S   H   N   V   Y   I   T   A   D   K   Q   K   N   G>
                      430         440         450         460         470         480
GFPuv     CTC GAG TAC AAC TAT AAC TCA CAC AAT GTA TAC ATC ACG GCA GAC AAA CAA AAG AAT GGA
GFPmyco   T.A ..A ..T ..T ... ... AGT ..T ..C ..T ..T ..T ..T ..T ..T ... ... ..A ... ..T>

          I   K   A   N   F   K   I   R   H   N   I   E   D   G   S   V   Q   L   A   D>
                      490         500         510         520         530         540
GFPuv     ATC AAA GCT AAC TTC AAA ATT CGC CAC AAC ATT GAA GAT GGA TCC GTT CAA CTA GCA GAC
GFPmyco   ..T ... ... ..T ..T ... ... A.A ..T ... ... ... ... ..T AGT ... ... T.. ... ..T>

          H   Y   Q   Q   N   T   P   I   G   D   G   P   V   L   L   P   D   N   H   Y>
                      550         560         570         580         590         600
GFPuv     CAT TAT CAA CAA AAT ACT CCA ATT GGC GAT GGC CCT GTC CTT TTA CCA GAC AAC CAT TAC
GFPmyco   ..C ... ... ... ... ... ... ... ..T ... ..T ..A ..T T.A ... ... ..T ..T ... ..T>

          L   S   T   Q   S   A   L   S   K   D   P   N   E   K   R   D   H   M   V   L>
                      610         620         630         640         650         660
GFPuv     CTG TCG ACA CAA TCT GCC CTT TCG AAA GAT CCC AAC GAA AAG CGT GAC CAC ATG GTC CTT
GFPmyco   T.A AGT ..T ... AG. ..T T.A AGT ... ... ..A ..T ... ..A A.A ..T ... ... ..A T.A>

          L   E   F   V   T   A   A   G   I   T   H   G   M   D   E   L   Y   K   *>
                      670         680         690         700         710
GFPuv     CTT GAG TTT GTA ACT GCT GCT GGG ATT ACA CAT GGC ATG GAT GAG CTC TAC AAA TAA
GFPmyco   T.A ..A ... ... ... ... ... ..T ... ..T ..C ..T ... ... ..A T.A ..T ... ...>
```

Figure 6. Construction strategy for P2:GFPmyco. Two sets of overlapping oligomers, GFP1 (5′ end of the gene) and GFP2 (3′ end of the gene) were used to assembly each half of the GFPmyco gene. Each set was slowly annealed, ligated, used as the templates in PCR reactions and cloned into pZERO as described in the text. Each half gene (gray bar and right hatched bar) was blunt-end digested with *Eco*RV, ligated to the other half and cloned into pSK to produce GFPmyco (pISM32). The GFPmyco gene was then PCR amplified with *Pfu* polymerase to create a 3′ *Bam*HI restriction site (and 5′ blunt end). The P2 promoter sequence was PCR amplified in similar fashion to create a 5′ *Bam*HI restriction site (and 3′ blunt end). The two fragments were ligated and then PCR amplified to create P2:GFPmyco. This PCR product was digested with *Bam*HI and inserted into pISM2062 to create Tn*4001*:P2:GFPmyco.

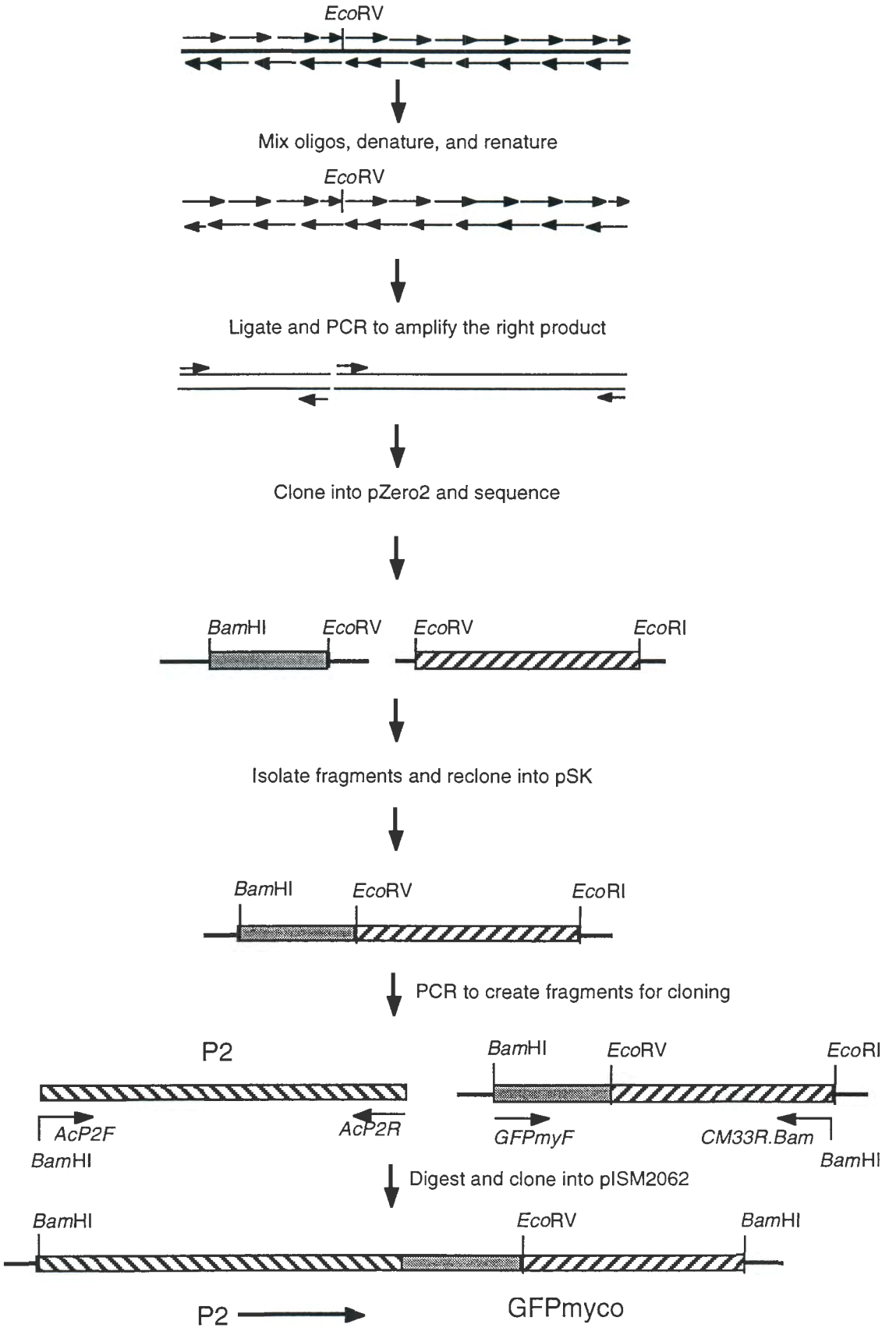
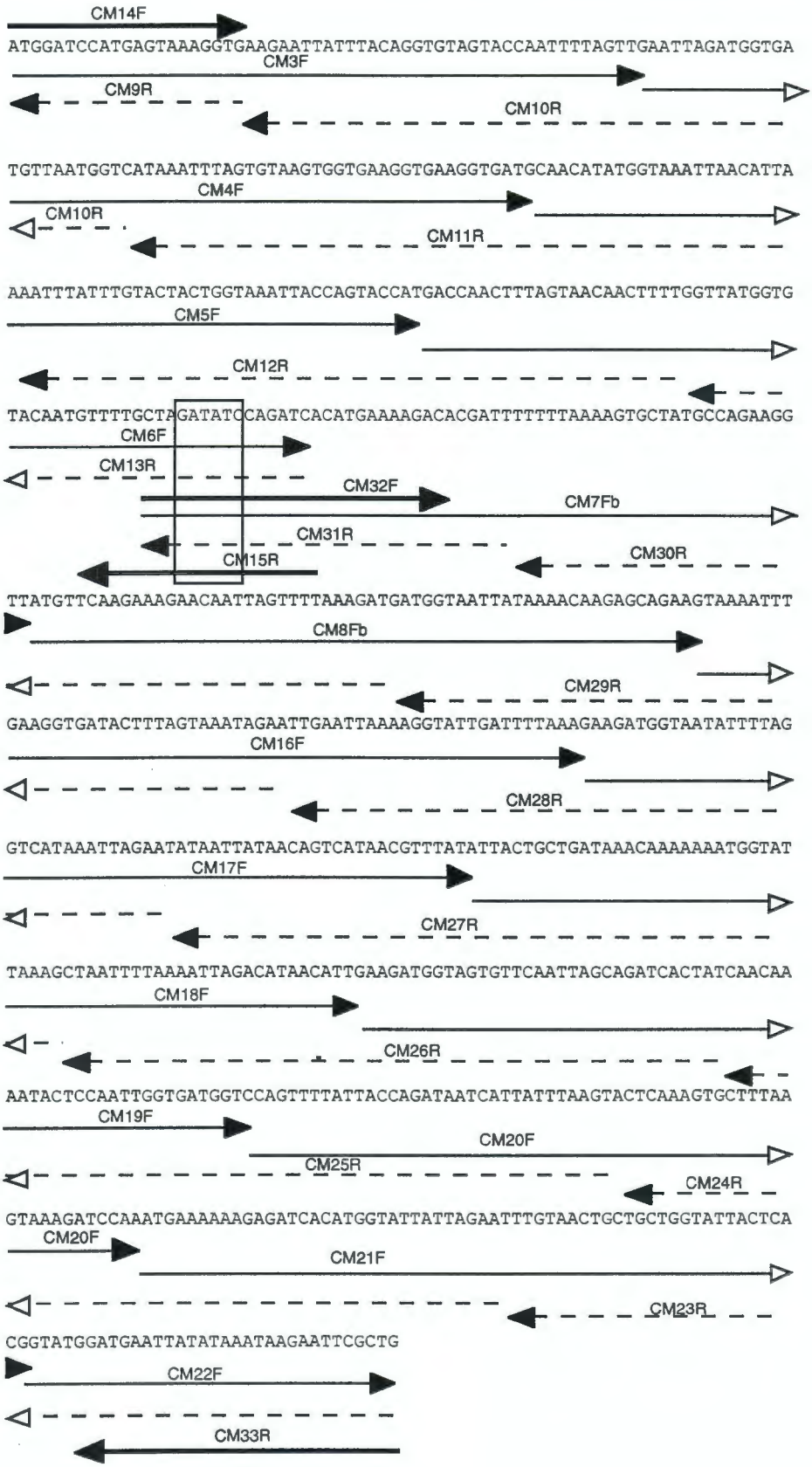

EcoRV
Mix oligos, denature, and renature
EcoRV
Ligate and PCR to amplify the right product
Clone into pZero2 and sequence
BamHI
EcoRV
EcoRV
EcoRI
Isolate fragments and reclone into pSK
BamHI
EcoRV
EcoRI
PCR to create fragments for cloning
P2
AcP2F
BamHI
AcP2R
BamHI
EcoRV
EcoRI
GFPmyF
CM33R.Bam
BamHI
Digest and clone into pISM2062
BamHI
EcoRV
BamHI
P2
GFPmyco

Figure 7. Mapping of oligonucleotides onto the GFPmyco gene sequence. Labeled arrows indicate location and orientation of each oligomer on the corresponding sequence. Bold arrows indicate PCR primers. The *Eco*RV assembly site is shown as a box.


CM14F
ATGGATCCATGAGTAAAGGTGAAGAATTATTTACAGGTGTAGTACCAATTTTAGTTGAATTAGATGGTGA
CM3F
CM9R
CM10R
TGTTAATGGTCATAAATTTAGTGTAAGTGGTGAAGGTGAAGGTGATGCAACATATGGTAAATTAACATTA
CM4F
CM10R
CM11R
AAATTTATTTGTACTACTGGTAAATTACCAGTACCATGACCAACTTTAGTAACAACTTTTGGTTATGGTG
CM5F
CM12R
TACAATGTTTTGCTAGATATCCAGATCACATGAAAAGACACGATTTTTTTAAAAGTGCTATGCCAGAAGG
CM6F
CM13R
CM32F
CM7Fb
CM31R
CM30R
CM15R
TTATGTTCAAGAAAGAACAATTAGTTTTAAAGATGATGGTAATTATAAAACAAGAGCAGAAGTAAAATTT
CM8Fb
CM29R
GAAGGTGATACTTTAGTAAATAGAATTGAATTAAAAGGTATTGATTTTAAAGAAGATGGTAATATTTTAG
CM16F
CM28R
GTCATAAATTAGAATATAATTATAACAGTCATAACGTTTATATTACTGCTGATAAACAAAAAAATGGTAT
CM17F
CM27R
TAAAGCTAATTTTAAAATTAGACATAACATTGAAGATGGTAGTGTTCAATTAGCAGATCACTATCAACAA
CM18F
CM26R
AATACTCCAATTGGTGATGGTCCAGTTTTATTACCAGATAATCATTATTTAAGTACTCAAAGTGCTTTAA
CM19F
CM20F
CM25R
CM24R
GTAAAGATCCAAATGAAAAAAGAGATCACATGGTATTATTAGAATTTGTAACTGCTGCTGGTATTACTCA
CM20F
CM21F
CM23R
CGGTATGGATGAATTATATAAATAAGAATTCGCTG
CM22F
CM33R

Clones from the P2:GFPmcyo:pISM2062 ligation were examined for *Eco*RV restriction patterns. Some were partially sequenced to confirm the presence of the P2:GFPmyco fragment in the *Bam*HI restriction site and to confirm the integrity of the P2:GFPmyco junction region. Plasmid pISM30 contains the P2:GFPmyco construct in Tn*4001*mod (pISM2062).

Plasmid pISM30 caused weak fluorescence in *E. coli*. However no fluorescence was observed when it was used to transform *M. gallisepticum*. A number of factors may have contributed to these results. The low-level of GFPmyco fluorescence observed in *E. coli* could due to low protein levels and/or a marginally functional product. Inefficient opal suppressor activity in *E. coli*, which is required because of the UGA codon at position 171 in the GFPmyco sequence (Figure 5), would produce a truncated protein byproduct. Even a highly functional opal suppressor can be affected by multiple factors including the context of the UGA codon within the sequence (127). Alternatively, the codon usage frequencies could be effecting the translation rate. Many of the codons are extremely rare in *E. coli*, and the pools of charged tRNAs may be quickly depleted.

One obvious explanation for the poor performance in both *E. coli* and mycoplasma is that GFPmyco is a particularly ineffective GFP variant. The translation product of the designed GFPmyco gene differed from that of the GFPuv parent by two amino acids. Although either is located in the chromophore and the alternate amino acids have been found in other highly functional GFP mutants, it is possible that the new sequence introduced a deleterious intra-protein interaction. This could have effected folding, chromophore excitation or fluorescence.

One factor not taken into account in the mycoplasma expression studies was the impact of the media environment on fluorescence. It is well known that changes in pH, salt concentration, etc. affect GFP folding (29, 90). For example, toxic effects have been associated with high media salt concentration in *Helicobacter* species (72). The highly enriched mycoplasma medium is quite different from the standard *E. coli* media. One obvious difference is that mycoplasma media is titrated to an initial pH of 7.8 as opposed to the 7.3-7.4 for *E. coli* media. Potentially even more problematic are the media supplements. Yeast extract, horse serum and even Cefobid could inhibit GFP folding or chromophore activity. It would be interesting to see the effect of these factors on GFP fluorescence levels in *E. coli*.

It is less likely that the lack of GFP expression in mycoplasma is a temperature effect. Although GFP folds relatively poorly at 37°C (142), GFPuv grown at 37°C can still be observed by the naked eye. However temperature effects cannot be excluded completely. Different GFP variants have different temperature sensitivities as well as sensitivities to media components (72, 74). It is possible that the increased temperature in combination with reduced pH, for example, could prevent the formation of a necessary folding intermediate.

Although GFP has generally been considered nontoxic even in large concentrations, this is not always the case. In 1995, Davis *et al.* reported that GFP effected cell growth (27). More recently, Liu *et al.* demonstrated a link between GFP expression and induction of apoptosis in several eukaryotic cell lines (81). Studies of GFP labeled retroviruses had previously shown that producer cells expressing high levels of GFP appeared to be at a selective disadvantage (59). Similar apparent GFP concentration maxima have also been observed in plant cells (121). Since mycoplasmas are in general much more fragile than *E. coli,* a minor toxic effect in *E. coli* might be almost completely lethal in mycoplasma cells expressing GFP. This effect could have been accentuated by the use of P2, an extremely strong mycoplasma promoter for the mycoplasma expression studies.

Another factor that must be considered is the (currently unknown) orientation of the P2GFP construct in the host plasmid. P2 is a strong promoter but pISM2062 has promoters of its own. The production of antisense RNA could certainly reduce the amount of protein product.

It is also possible that protein degradation may have effected either protein levels or apparent fluorescence. GFP is normally an extremely very stable protein. Both 5′ and 3′ GFP fusion proteins are commonly used for protein expression or interaction studies. However, it can be destabilized by the addition of a protease sensitive tail sequence (3) The original construct had an error in its stop codon. This should not have effected folding or fluorescence as the last 9 amino acids of the gene are expendable. It is theoretically possible, however, that the run-on tail may have accidentally duplicated a protease-sensitive sequence. The result would be a reduced GFP half-life and a correspondingly reduced fluorescent signal. Because the (weak) signal observed in E. coli was quite stable, this is unlikely to be a primary cause of reduced fluorescence but it may be a minor contributing factor.

Finally the basic premise of the sequence design itself might be flawed. It was based on codon usage frequency data since detailed tRNA analysis and/or translation efficiencies are unavailable for most mycoplasma species, including *M. gallisepticum.* It is already known that codon usage frequencies differ from mycoplasma to mycoplasma. So do the number of tRNAs and their arrangement on the genome (111). It may be that the synthetic sequence, which was derived from multi-species consensus codon usage data, is not easily processed by the *M. gallisepticum* translational machinery.

It is obvious that work has only begun on the development of a mycoplasma-specific GFP variant. Media and sequence change effects will be easy to isolate. Other factors will be harder to eliminate. There is still a great need for an easily visualized, individual cell, *in vivo* mycoplasma label. More important, the development of this marker may provide interesting and useful information about both mycoplasma cellular activity and GFP functions.

CONCLUSIONS

The goal of this project was to design and assemble two genetic tools for use in mycoplasmas. The first tool was a mini-transposon for the easy delivery of a variety of markers to the mycoplasma chromosome. The second tool was a mycoplasma-specific GFP marker that would allow easy visualization of individual cells, organelles or proteins. The resulting mini-Tn*4001* does function relatively efficiently in *M gallisepticum.* Unfortunately, the mycoplasma-specific GFPmyco construct failed to give a useful signal in preliminary expression studies for undetermined reasons.

There are several potential applications for the miniTn*4001*. The *tetM* gene was inserted into the mini-Tn*4001* cloning site for these studies, but other markers could also be used. The next step will be the insertion of alternative antibiotic resistance markers and hopefully a cell visualization signal (such as GFP) for use in studies of mycoplasma virulence.

The GFPmyco construct must be considered still under development. Work is currently underway to express the gene from a different promoter. Site-directed mutagenesis could be used to modify the GFPmyco gene sequence so that the amino sequence of the protein produced is identical to a known high signal GFP mutant. Additional experiments such as assessing the effect of mycoplasma media supplements on the fluorescence level of well-studied GFP variants could provide useful information about both GFP expression levels in mycoplasma and basic GFP biochemistry.

Historically mycoplasma research has lagged due to the technical difficulties of genetic manipulations and lack of suitable biological tools. Hopefully the constructs described here will help bridge this gap.

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