

New Selectable Marker for Manipulating the Simple Genomes of *Mycoplasma* Species[∇]

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Over the past several years, significant advances have been made in the molecular genetics of the *Mollicutes* (the simplest cells that can be grown in axenic culture). Nevertheless, a number of basic molecular tools are still required before genetic manipulations become routine. Here we describe the development of a new dominant selectable marker based on the enzyme puromycin-*N*-acetyltransferase from *Streptomyces alboniger*. Puromycin is an antibiotic that mimics the 3'-terminal end of aminoacylated tRNAs and attaches to the carboxyl terminus of growing protein chains. This stops protein synthesis. Because puromycin conscripts rRNA recognition elements that are used by all of the various tRNAs in a cell, it is unlikely that spontaneous antibiotic resistance can be acquired via a simple point mutation—an annoying issue with existing mycoplasma markers. Our codon-optimized cassette confers pronounced puromycin resistance on all five of the mycoplasma species we have tested so far. The resistance cassette was also designed to function in *Escherichia coli*, which simplifies the construction of shuttle vectors and makes it trivial to produce the large quantities of DNA generally necessary for mycoplasma transformation. Due to these and other features, we expect the puromycin marker to be a widely applicable tool for studying these simple cells and pathogens.

The mycoplasmas, which is the generic name for members of the class *Mollicutes*, are cell wall-less bacteria that are often important pathogens in a variety of species (2, 20). In addition to their importance as pathogens, their small genomes and limited metabolic complexity make them platform organisms for studies on the universal elements of life itself. For example, transposon mutagenesis of the simplest mycoplasma (*Mycoplasma genitalium*) showed that more than 100 of the 485 protein-encoding genes could be individually disrupted under laboratory conditions (5, 7, 18). Although it is unknown if the disrupted genes are simultaneously dispensable, the remaining 370 or so undisturbed genes provide an initial map of the minimum genetic requirements for life.

The simplicity of mycoplasmas also introduces some technical complications. For example, auxotrophic markers may be difficult to establish in organisms that utilize ill-defined media and that cannot synthesize most biological building blocks. Similarly, antibiotics that target the cell wall are not useful because this structure is completely absent. The use of other markers is not advisable because they are clinically relevant and the simplicity of mycoplasmas naturally restricts clinical options in the first place. In our studies, fairly reliable markers include the tetracycline resistance protein (TetM) and, to a lesser extent, chloramphenicol acetyltransferase and aminoglycoside resistance protein (AacA/AphD). The TetM-tetracycline combination is usually preferable because of the potent antimycoplasma activity of tetracyclines and the low levels of spontaneous tetracycline resistance. In comparison, sponta-

neous resistance frequently arises with the other markers (presumably via a point mutation in an rRNA). Such resistance is rarely insurmountable, but screening numerous colonies for authentic transformants wastes valuable resources unnecessarily.

In spite of the limitations imposed by mycoplasma biology, the characteristics and mode of action of puromycin suggested that it was a likely candidate for marker development. Puromycin is readily available, is relatively inexpensive, has no clinical value, and is a potent inhibitor of translation in both prokaryotes and eukaryotes. To our knowledge, no example of rRNA-based resistance to the antibiotic exists. Most likely, this is a consequence of the universality of the acceptor end of tRNA and the mechanism of peptidyl transferase—the two translational features puromycin exploits as a nearly perfect tRNA mimic (25). Moreover, the puromycin *N*-acetyltransferase (PAC) gene from *Streptomyces alboniger* encodes a small protein which provides resistance to the antibiotic. This suggested that a codon-optimized version of the enzyme might be efficiently expressed. The resistance genes for the usual markers have not been codon optimized for production in mycoplasmas, and expression issues likely contribute to the use of low antibiotic concentrations and thus to the establishment of spontaneous resistance. Here we describe the use of the PAC gene as a new selectable marker in several mycoplasma species.

MATERIALS AND METHODS

Mycoplasma strains. The mycoplasma strains used were *Mycoplasma mycoides* subsp. *mycoides* large colony GM12, which has recently been renamed *M. mycoides* subsp. *capri* GM12 (15), *Mycoplasma capricolum* subsp. *capricolum* California kid (ATCC 27343), *M. genitalium* G37, *Mycoplasma gallisepticum* (ATCC 15302), *Mycoplasma pneumoniae* Eaton (ATCC 15531), and *M. pneumoniae* M129-B170 (ATCC 29343). *M. mycoides* and *M. capricolum* were grown at 37°C in SP4 medium (8, 9, 23). *M. genitalium* and the *M. pneumoniae* strains were

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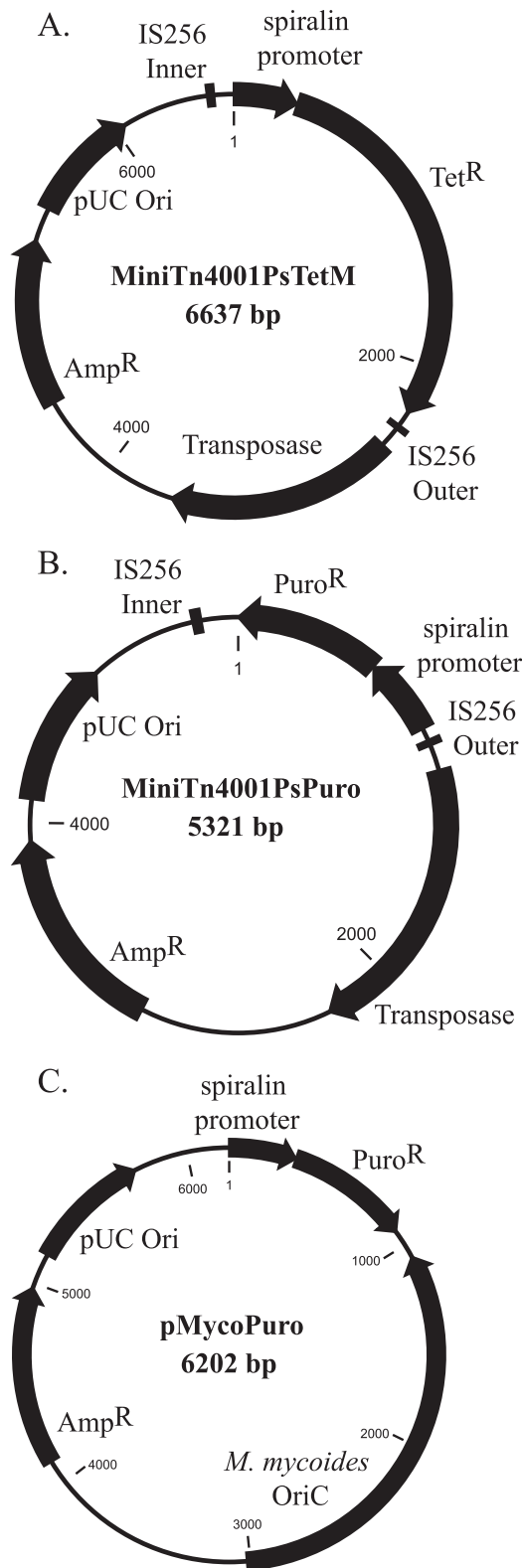


FIG. 1. Maps of plasmids used in mycoplasmas. (A) Map of the Mini-Tn4001PsTetM plasmid. The spiralin promoter is upstream of the TetM open reading frame. The IS256 elements flank the tetracycline resistance cassette with the transposase gene outside of the repeats. (B) Map of the Mini-Tn4001PsPuro plasmid. The features are identical to those of the Mini-Tn4001PsTetM plasmid, except that the

grown at 37°C in the presence of 5% CO₂ in SP4 medium. *M. gallisepticum* was grown in Hayflick's medium (23) in the presence of 5% CO₂ at 37°C.

PAC gene construction. The 597-bp PAC gene was synthesized with overlapping oligonucleotides as described previously (22). Briefly, 5'-phosphorylated oligonucleotides encoding both strands of a codon-optimized version (for expression in *M. genitalium*) were ordered from IDT (Coralville, IA). The oligonucleotides were 48 bases long with an overlap of 24 bases. The top-strand and bottom-strand oligonucleotides were mixed, heated to 95°C, and slowly cooled to allow annealing of the overlaps. The reaction products were ligated for 12 h and used as the template for PCR. The PCR amplicon was cloned into pGEM-3Zf(+) (Promega, Madison, WI) and sequenced to identify correct PAC clones. The optimized PAC gene was then cloned under the control of the *Spiroplasma citri* spiralin promoter (Ps) (12) and used to replace the *tetM* gene in a derivative of Mini-Tn4001tet (17, 19) (GenBank accession no. GQ420676), as well as the *tetM* gene in pMyco1 (11). The new plasmid (Mini-Tn4001PsPuro; GenBank accession no. FJ872396) was used to transform *M. genitalium*, *M. gallisepticum*, and *M. pneumoniae*, while the pMyco1 derivative (pMycoPuro; GenBank accession no. GQ420675) was used to transform *M. mycooides* and *M. capricolum*.

Mycoplasma transformations. *M. genitalium* and *M. pneumoniae* were transformed by electroporation as previously described (1, 6, 21). Polyethylene glycol-mediated transformation was used for *M. mycooides*, *M. gallisepticum*, and *M. capricolum* (8, 9, 13, 17).

Puromycin inhibitory concentration determination. Wild-type *M. capricolum* cells and cells bearing the pMycoPuro plasmid were grown in SP4 medium containing various concentrations of puromycin to determine bacteriostatic activity. After 24 h of exposure to the antibiotic, aliquots were removed, diluted 10,000-fold, and plated without antibiotic to evaluate bactericidal activity. A similar procedure was used for the other organisms, but bactericidal activity was not determined.

RESULTS AND DISCUSSION

All five mycoplasma species were grown in SP4 or Hayflick's medium supplemented with various concentrations of puromycin. All of the wild-type mycoplasmas that were tested were unable to grow in the presence of puromycin when the concentration exceeded 1 to 3 µg/ml. Thus, distantly related mycoplasmas from both the mycooides group and the pneumoniae group are sensitive to the antibiotic. This sensitivity may be commonplace within the *Mollicutes*, as they have streamlined genomes unlikely to contain the sophisticated efflux pumps or the modifying enzymes typically required to establish puromycin resistance.

Mini-Tn4001PsPuro (Fig. 1) established resistance in *M. genitalium*, *M. gallisepticum*, and *M. pneumoniae* by transposition. The pMycoPuro plasmid replicates as a free DNA in *M. capricolum*, while in *M. mycooides* the plasmid integrates by single crossover at the *oriC* gene of the *M. mycooides* genome (data not shown). The puromycin constructs have not been used for directed mutagenesis, although we presume it would work in mycoplasmas, where such mutagenesis has been successful with a tetracycline marker. In general, expression of the PAC gene confers wild-type growth at concentrations beyond

TetM open reading frame has been replaced with the puromycin *N*-acetyltransferase open reading frame. After the spiralin promoter was cloned upstream of the PAC gene in pGEM-3Zf(+), the entire cassette was cloned into the backbone of the Mini-Tn4001PsTetM plasmid that had the promoter and TetM open reading frame removed by digestion with PstI and BamHI. (C) Map of the pMycoPuro plasmid. The puromycin resistance unit was cloned into pMyco1, which had the promoter and TetM open reading frame removed by digestion with PstI. *M. mycooides oriC* allows propagation in *M. mycooides*. The pUC *ori* and β-lactamase-encoding genes are used for propagation in *E. coli*.

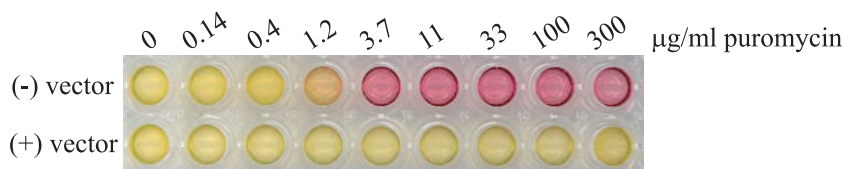


FIG. 2. The pMycoPuro plasmid confers puromycin resistance. *M. capricolum* was grown in SP4 medium with a range of puromycin concentrations in the presence or absence of the pMycoPuro plasmid. Without the plasmid, *M. capricolum* grew slowly at a puromycin concentration of 1.2 µg/ml and no growth was observed at 3.7 µg/ml. In this color-changing unit assay, the change of the pH indicator phenol red in the medium from red to yellow was used as an indicator of growth. In the absence of cell growth, the SP4 medium does not change color over the time course of the experiment (24 h). *M. capricolum* cells containing the vector were resistant to puromycin at concentrations of at least 300 µg/ml.

50 µg/ml (*M. capricolum* had wild-type growth at 500 µg/ml). In practice, we typically selected for transformants on solid medium at lower concentrations (3 µg/ml for *M. genitalium*, *M. gallisepticum*, and *M. pneumoniae* and 8 µg/ml for *M. capricolum* and *M. mycoides*). Isolated colonies of *M. capricolum* or *M. mycoides* were subsequently grown in liquid culture at 8 µg/ml for the first passage and 50 µg/ml for later passages. *M. genitalium*, *M. gallisepticum*, and *M. pneumoniae* were maintained at 3 µg/ml during passage. As shown in Fig. 2, the inhibitory concentration for *M. capricolum* is approximately 3 µg/ml.

We wanted to determine if the antibiotic action of puromycin on these mycoplasma species was bactericidal or bacteriostatic. After *M. capricolum* cells were exposed to 300 µg/ml puromycin for 24 h, they could recover when plated in the absence of the antibiotic (data not shown). Apparently, puromycin is not lethal, even at high concentrations. It is possible that the stringent response, which is active in the organism, induces a persistent state and thereby death is avoided (4, 10, 24). If this supposition is correct, the presence of stringent-response proteins (RelA/SpoT family proteins) in most sequenced mycoplasmas suggests that these bacteria might also survive puromycin exposure even if they are incapable of growth. From a practical standpoint, this issue does not affect the usefulness of the marker. For example, puromycin has become our marker of choice when working with the very-slow-growing organism *M. genitalium*. Over the 3-week period required to produce clones, no special procedures are required to prevent the appearance of untransformed colonies.

There are several minor features of the expression cassette that were designed to broaden its usefulness. Transcription of the PAC gene is driven by the promoter for spiralin (Ps), which is an abundant protein from *S. citri*. We have found this promoter to be widely applicable. The efficacy of the spiralin promoter in the range of mycoplasmas examined here further documents its usefulness. Because the promoter also functions in *E. coli* (16), we designed the codon-optimized PAC gene so that it is free of TGA codons (TGA codes for tryptophan in most mycoplasmas, rather than a stop codon, as in the standard genetic code). In the mycoplasmas that use TGA as a tryptophan codon, its use is heavily preferred over the other tryptophan codon (TGG). Coding the Trp residues in the PAC gene with only TGG is a minor violation of codon optimization rules, but together with the Ps promoter, it allows for expression in *E. coli*. This simplifies the construction of shuttle vectors, as only a single marker is required. Puromycin sensitivity in *E. coli* begins at 10 to 50 µg/ml, and the cassette provides

resistance beyond 200 µg/ml. We typically use 125 µg/ml puromycin for selection with *E. coli*. For puromycin expression, as well as the expression of numerous other proteins, we have used a large region upstream of the spiralin start codon (324 nucleotides). In the past, such a large fragment could complicate its movement into various constructs. Now, with the advent of new DNA assembly methods, this is no longer a concern as we can seamlessly join 2 to 10 sequences without regard to restriction sites (3, 14).

Our main objective with this study was to identify a more consistent marker than tetracycline for our work on genome transplantation in *M. genitalium* (2). The finding that the puromycin-PAC gene combination is broadly applicable for mollicute research suggests that it may be generally preferable to the current antibiotic generally used by molecular mycoplasmaologists, tetracycline. This is in part due to the recombinant DNA guidelines that regulate the insertion into pathogens of resistance genes for antibiotics in clinical or veterinary use. Tetracyclines remain a clinical option for some mycoplasma infections and are additives in some animal feeds. The efficacy of puromycin also introduces the possibility of generating double knockouts to further refine the minimal gene content able to support replication and life itself.

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