

## NOTES

# Construction and Use of Derivatives of Transposon Tn4001 That Function in *Mycoplasma pulmonis* and *Mycoplasma arthritidis*

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**Previous attempts to introduce transposon Tn4001 into *Mycoplasma pulmonis* and *Mycoplasma arthritidis* have not been successful, possibly due to functional failure of the transposon's gentamicin resistance determinant. Tn4001C and Tn4001T were constructed, respectively, by insertion of a chloramphenicol acetyltransferase gene and the *tetM* tetracycline resistance determinant into Tn4001. Both Tn4001C and Tn4001T transposed in *M. pulmonis*, and Tn4001T transposed in *M. arthritidis*. The incorporation of a Tn4001T derivative that contained *lacZ* into either *Mycoplasma* species resulted in transformants with readily detectable LacZ activity. Tn4001T may be of general utility for use as a mycoplasma cloning vehicle because *tetM* functions in all species of *Mycoplasma* examined thus far.**

Few strategies exist to genetically manipulate mycoplasmas. There are no plasmids known to replicate in *Mycoplasma* species other than *Mycoplasma mycoides* and *Mycoplasma capricolum* (15, 16). The gram-positive bacterial transposon Tn916 can transpose into the genome of virtually all species of *Mycoplasma* for which transformation methods have been described, and Tn4001 can transpose into a number of *Mycoplasma* species, including *Mycoplasma pneumoniae*, *Mycoplasma genitalium*, and *Mycoplasma gallisepticum* (10). Transformation of these species with plasmids containing either transposon results in insertion of the transposon into the mycoplasma genome at any of numerous sites. Tn916 is large (18 kb) and not amenable to use as a cloning vehicle. In contrast, Tn4001 (4.7 kb) is small enough to be used as a cloning vehicle to insert genes into the mycoplasma chromosome. *Sma*I and *Bam*HI cloning sites have been introduced into Tn4001 to create Tn4001mod, which has been used as a vector in *M. gallisepticum* and *M. pneumoniae* (17, 20).

Tn4001 does not function in several species of *Mycoplasma*. Earlier reports of transposition of Tn4001 in *Mycoplasma pulmonis* were incorrect, and transformation of this species with plasmids containing Tn4001 has not previously been achieved (6, 18). The inability to introduce Tn4001 into *M. pulmonis* by transformation could result from either lack of transposase activity or failure of the transposon's gentamicin resistance determinant to serve as a selectable marker. In the present study, we inserted alternative antibiotic resistance determinants into Tn4001 to construct transposon derivatives that function in *M. pulmonis* and in *Mycoplasma arthritidis*. One of the transposon derivatives (Tn4001T) contains the *tetM* gene and may be used as a broad-host-range mycoplasma vector, as

indicated by the successful expression of a *lacZ* fusion gene in both *M. pulmonis* and *M. arthritidis*.

**Transformation of *M. pulmonis* and *M. arthritidis* with transposons Tn4001C and Tn4001T.** To determine whether Tn4001 would function in *M. pulmonis* if an appropriate antibiotic resistance marker was provided and to develop additional antibiotic resistance markers for this species, a chloramphenicol acetyltransferase gene (*cat*) was developed for use in *M. pulmonis*. One reason a *cat* determinant was chosen was that growth of *M. pulmonis* was found to be highly susceptible to the inhibitory effects of chloramphenicol at a concentration of 15 µg/ml. A chimeric gene consisting of the coding region of the *cat* gene of *Escherichia coli* plasmid pACYC184 (5) and the promoter region from the expression locus of the *M. pulmonis* *vs*a genes (2) was constructed. The *cat* coding region was amplified by PCR using the *cat* forward primer (5'-GGAAGGTACCATGGAGAAAAAATCAC-3') and the *cat* reverse primer (5'-CACTTCTCGAGGCGTAGCACCAGG-3'). A 460-bp portion of the *vs*a locus encompassing the promoter and the Shine-Dalgarno (SD) sequence was amplified by PCR using the *vs*a forward primer (5'-CGTTTCTGCAGTTTTTTTGAACC-3') and the *vs*a reverse primer (5'-TGCATGGTACTCCTATTTTAAAATTATG-3'). PCR amplification conditions were as described previously (11). The *vs*a promoter was chosen for its presumed strength, given that the *vs*a gene products are major surface proteins of *M. pulmonis* (i.e., the V-1 antigens) (2, 14, 21). The *vs*a reverse primer and the *cat* forward primer were designed with *Kpn*I restriction sites incorporated into their 5' ends to facilitate cloning such that the correct spacing between the *vs*a SD sequence and the *cat* translation start codon would be maintained. Accordingly, the *vs*a and *cat* PCR products were digested with *Kpn*I and ligated together to form a hybrid *vs*a-*cat* gene. This gene was PCR amplified using the *vs*a forward primer (which contained an internal *Pst*I site) and the *cat* reverse primer (which contained an internal *Xho*I site). The PCR product was digested with *Pst*I and *Xho*I and inserted into the *Pst*I/*Xho*I site of plasmid pZErO-1 (Invitrogen). Transformation of *E. coli* strain JM109

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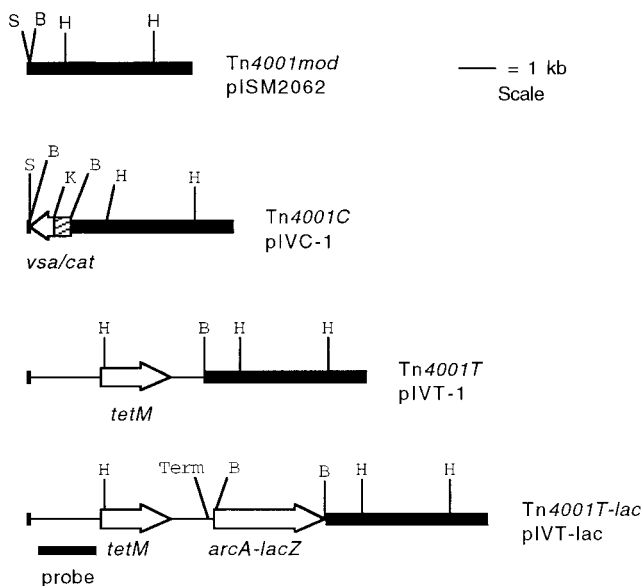


FIG. 1. Schematic diagram of Tn4001mod in plasmid pISM2062, Tn4001C in pIVC-1, Tn4001T in pIVT-1, and Tn4001T-lac in pIVT-lac. Dark regions indicate Tn4001mod sequences. Unshaded regions indicate the *cat*, *tetM*, and *arcA-lacZ* genes. The hatched region of Tn4001C indicates the *vsa* promoter region. Thin lines in Tn4001T and Tn4001T-lac indicate streptococcal sequences originating from pJ13 that flank *tetM*. Arrows represent direction of gene transcription. Abbreviations: S, *Sma*I; B, *Bam*HI; H, *Hind*III; K, *Kpn*I; Term, putative transcription terminator derived from *M. arthritidis* sequences upstream of *arcA*. The bar represents the 1.5-kb PCR product used to probe transformants containing Tn4001T or Tn4001T-lac.

resulted in chloramphenicol-resistant transformants (selected at 25  $\mu$ g of chloramphenicol/ml), indicating that the *vsa-cat* gene functions in *E. coli*. To incorporate the *cat* determinant into Tn4001, the *vsa-cat* gene was excised from pZErO-1 by digestion with *Pst*I and *Xho*I, and the ends of the DNA fragment were made flush by digestion with T4 DNA polymerase. *Bam*HI linkers were attached, and the *vsa-cat* gene was inserted into the *Bam*HI site of plasmid pISM2062 (17), encoding chloramphenicol resistance, to generate plasmid pIVC-1 (Fig. 1).

Transformation of *M. pulmonis* with pIVC-1 resulted in chloramphenicol-resistant transformants, indicating that *vsa-cat* functions as a selectable marker in this species of *Mycoplasma*. *M. pulmonis* strain KD735-15 (1) was propagated in mycoplasma broth medium consisting of 2.1% PPLO broth without crystal violet (Difco Laboratories, Detroit, Mich.) supplemented with 20% whole horse serum (Gibco BRL Life Technologies, Grand Island, N.Y.), 0.5% IsoVitalax (VWR), 0.02% degraded free-acid DNA (Sigma), 100  $\mu$ g of ampicillin/ml, and 0.5% glucose. *M. pulmonis* was transformed with 10  $\mu$ g of pIVC-1 by the polyethylene glycol method as described previously (8). After incubation of transformed cells at 37°C for 2 h in nonselective medium, transformants were selected on mycoplasma agar (mycoplasma broth medium supplemented with 1.4% agar) containing 15  $\mu$ g of chloramphenicol/ml. Transformants were obtained at a frequency of  $2 \times 10^{-7}$  per CFU, which is about 10-fold less efficient than transformation of *M. pulmonis* with the Tn916-containing plasmid pAM120 (7).

As expected, *M. pulmonis* cells transformed with pIVC-1 had Tn4001C inserted in the mycoplasma chromosome. Total DNA was isolated from strains of *Mycoplasma* as described previously (7). Extrachromosomal plasmid was not detected in

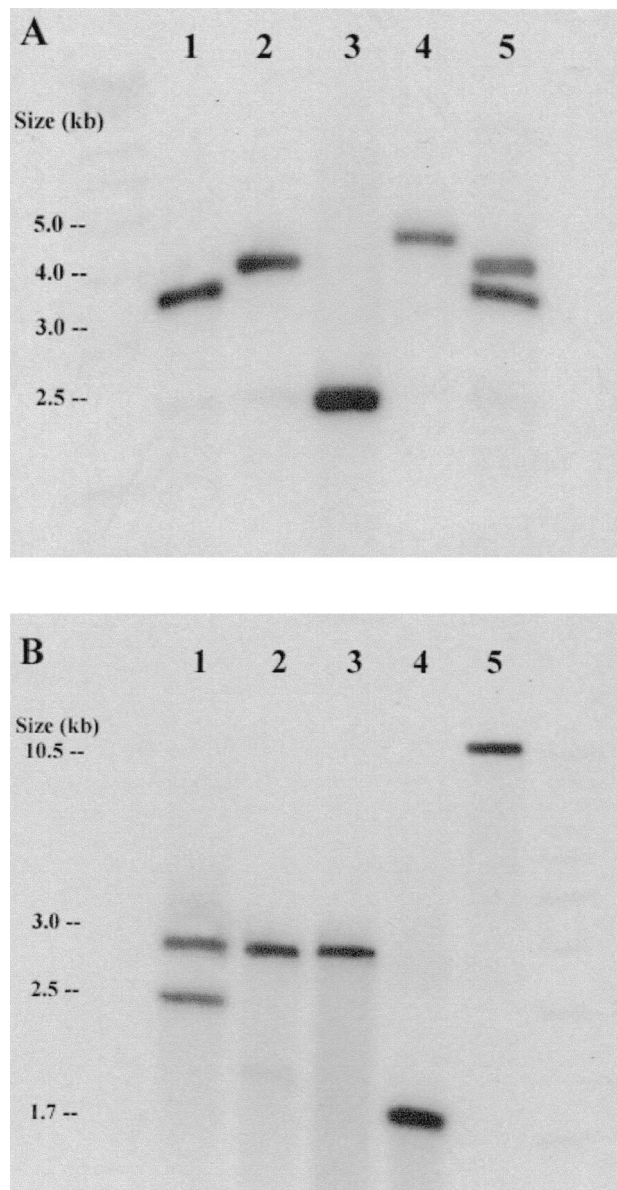


FIG. 2. Southern analysis of mycoplasma transformants. (A) Autoradiogram of a Southern blot hybridized with a *cat*-specific probe. Lanes 1 through 5 are *Hind*III-digested genomic DNAs from independent transformants of *M. pulmonis* containing Tn4001C. (B) Autoradiogram of a Southern blot hybridized with the probe derived from sequences upstream of *tetM* (Fig. 1). Lanes 1 through 5 are *Hind*III-digested genomic DNAs from five independent isolates of *M. arthritidis* transformed with pIVT-lac.

mycoplasma DNA because pIVC-1 does not replicate in mycoplasmas. *Hind*III-digested genomic DNAs isolated from five independent, Tn4001C-containing *M. pulmonis* transformants were analyzed on Southern blots probed with the *cat* gene (obtained by PCR amplification of pACYC184 using the *cat* forward and reverse primers). Conditions for Southern hybridization and preparation of  $^{32}$ P-labeled probe by the random primer method were as described previously (9). The five transformants exhibited different hybridization banding patterns, indicating that Tn4001C can insert into the chromosome of *M. pulmonis* at any of numerous sites (Fig. 2A). One of the transformants (Fig. 2A, lane 5) had two DNA fragments that hybridized with the probe, suggesting either that the initial

transformant was simultaneously transformed with two copies of pIVC-1 or that Tn4001C was transposed to secondary sites in the chromosome after the initial insertion event. In related hybridization experiments, it was shown that the *cat* probe did not hybridize with *M. pulmonis* DNA isolated from wild-type cells that had not been transformed (data not shown). To further explore the issue of whether derivatives of Tn4001 insert into the *M. pulmonis* chromosome at a diversity of sites, the precise transposon insertion site in about 150 transformants of *M. pulmonis* was identified by determining the DNA sequence of the mycoplasma-Tn4001 junction region (K. Dybvig, unpublished data). No two transformants had the transposon inserted at the same site.

Whether Tn4001 could transpose in *M. arthritidis* was examined. Attempts to transform *M. arthritidis* with pISM2062 (which contains Tn4001mod) and pIVC-1 (which contains Tn4001C) were not successful. It is likely that the *vs*a-*cat* gene does not function as a selectable marker in this species, suggesting that the *vs*a promoter may be species specific. In related experiments, attempts to transform *M. pulmonis* and *M. arthritidis* with plasmid pKV98 were not successful. pKV98 contains Tn4001 with an alternative *cat* gene that has been shown to function in *M. pneumoniae* (12), but this *cat* gene evidently fails to confer a selectable level of resistance in either *M. pulmonis* or *M. arthritidis*. To develop a derivative of Tn4001 that might function in *M. arthritidis*, the *tetM* gene was inserted into Tn4001 to generate Tn4001T. *tetM* was chosen because this gene functions as a selectable marker in many *Mycoplasma* species, including *M. arthritidis* (22). A 5-kb *Hinc*II fragment from plasmid pJ13 (4) that contained the *tetM* gene was isolated and inserted into the *Sma*I site of pISM2062 to generate plasmid pIVT-1 (Fig. 1). *M. arthritidis* strain H606 was propagated in the same medium as was *M. pulmonis* except that 0.5% arginine was used instead of glucose. Transformation of *M. arthritidis* with 10  $\mu$ g of pIVT-1 was done as described previously (22). Transformation of *M. arthritidis* (selection at 5  $\mu$ g of tetracycline/ml) and *M. pulmonis* (with 3  $\mu$ g of tetracycline/ml) with pIVT-1 resulted in transformants at frequencies of about  $2 \times 10^{-9}$  and  $3 \times 10^{-7}$ , respectively. Southern blot analysis indicated that Tn4001T had transposed into the chromosome of either species at any of numerous sites, as expected. The results for *M. arthritidis* transformed with pIVT-lac (i.e., pIVT-1 containing *lacZ*, described below) are shown in Fig. 2B. As was the case for *M. pulmonis* transformed with pIVC-1, one transformant (Fig. 2B, lane 1) out of five had two DNA fragments that hybridized with the probe.

Although Tn4001C and Tn4001T have an intact gentamicin resistance determinant, transformants of *M. pulmonis* containing either transposon and transformants of *M. arthritidis* containing Tn4001T had no change in susceptibility to gentamicin compared to wild-type cells lacking the transposon. The failure of the gentamicin resistance determinant to confer a selectable level of resistance in *M. pulmonis* and *M. arthritidis* explains why these species could not be transformed with pISM2062.

*M. pulmonis* containing Tn4001T could be transformed with pIVC-1 at a relatively high frequency,  $3 \times 10^{-6}$  transformants per CFU. Therefore, the presence of one endogenous copy of Tn4001 in the mycoplasma does not preclude and may actually stimulate transposition of a second copy into the chromosome. Stimulation of transposition may result from the presence of transposase activity in the cell prior to acquisition of the second copy of the transposon. Whether the mechanism of Tn4001C insertion into the genome of cells that already contained Tn4001T was by transposition or by homologous recombination was not examined. Regardless of the mechanism, however, the ability to insert both transposons into the genome

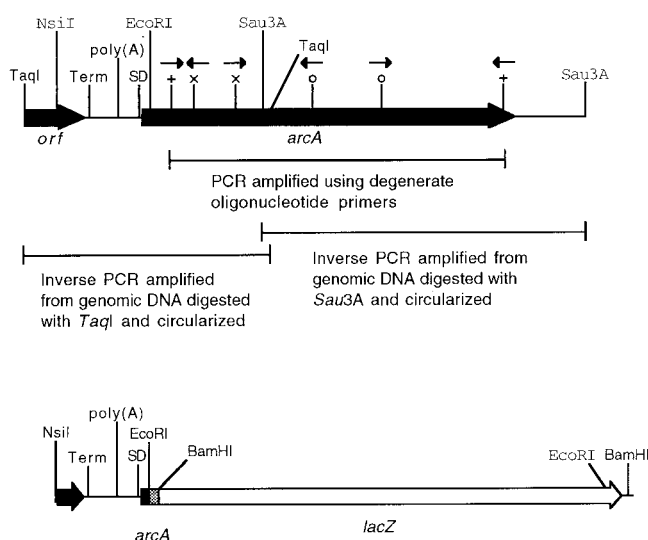


FIG. 3. Schematic diagrams of the region of the *M. arthritidis* chromosome containing the *arcA* gene (top) and the chimeric *arcA-lacZ* gene (bottom). Coding regions are indicated by thick lines, with arrows indicating direction. Regions shaded in black originate from the *M. arthritidis* chromosome. The stippled region is derived from the pZErO vector used for gene construction. The unshaded region is the *lacZ* coding region derived from pISM2062.2lac. The location and direction of primers used for PCR amplification are illustrated as follows: +, degenerate primers used for the initial amplification of an internal portion of *arcA*, and x and o, primers used to amplify the 5' and 3' ends, respectively, of *arcA* by inverse PCR. The location of the putative transcription terminator (Term), the 15-nucleotide poly(A) tract, and the *arcA* SD sequence are also shown. For clarity, the 3-kb *lacZ* coding region is drawn at one-half scale.

should make it possible to complement mutants generated by insertional activation with one of the transposons by using the other transposon as a vehicle to introduce a functional wild-type copy of the inactivated gene.

**Use of Tn4001T as a vector in *M. pulmonis* and *M. arthritidis*.** A *lacZ* gene was used to investigate whether Tn4001T can be used as a vector in *M. pulmonis* and *M. arthritidis*. Little is known about mycoplasmal gene expression signals, and the choice of a promoter that might drive expression of the *lacZ* gene was uncertain. Because the *vs*a promoter evidently did not function in *M. arthritidis* (i.e., the *vs*a-*cat* gene did not function as a selectable marker), a promoter originating from *M. arthritidis* was sought. We reasoned that the *arcA* gene (encoding arginine deiminase) promoter might be relatively easy to isolate and have strong activity.

Degenerate oligonucleotide primers were designed to amplify an internal region of *arcA*. The predicted amino acids of the ArcA proteins of *Mycoplasma hominis* and *Mycoplasma arginini* (13, 19), two species of *Mycoplasma* that are phylogenetically related to *M. arthritidis*, were aligned, and conserved amino acid regions were identified from which primers with minimal degeneracy could be designed. The *arcA* forward primer (5'-CGCTCGAGAYTAYATHACNCCNGC-3') and *arcA* reverse primer (5'-GCCTCGAGCRTTNCATNCC-3') were used to amplify a PCR product of the expected size (1.1 kb) from *M. hominis* strain PG21 (3) and *M. arthritidis* strain PG6 (23). The *M. arthritidis* PCR product was cloned into the TA cloning vector (Invitrogen), and the amino acids predicted from its nucleotide sequence were significantly similar to ArcA of *M. hominis* and *M. arginini*, as expected.

To clone the complete *M. arthritidis arcA* gene, including the promoter, an inverse PCR strategy was employed using the primers illustrated in Fig. 3. To obtain the 5' end of *arcA*,

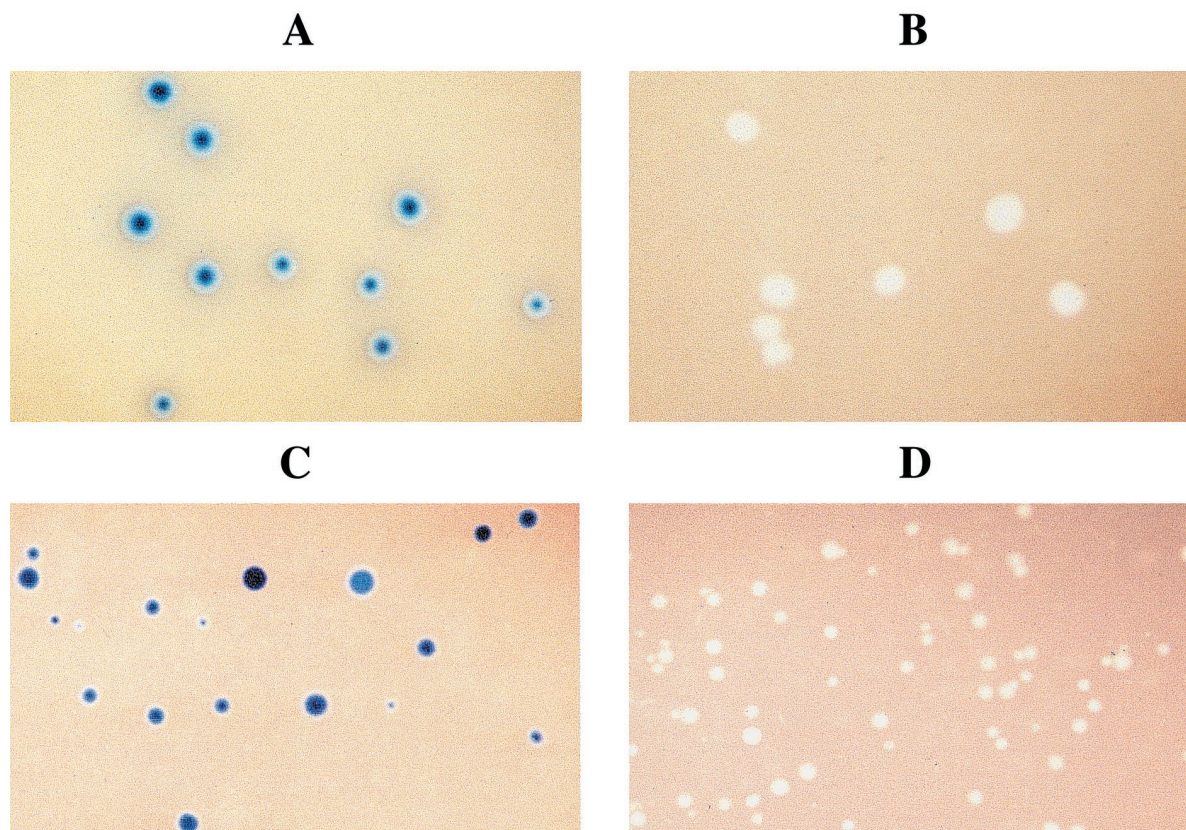


FIG. 4. Blue ( $\text{LacZ}^+$ ) *M. pulmonis* (A) and *M. arthritis* (C) colonies transformed with pIVT-lac, assayed on mycoplasma agar plates supplemented with X-Gal. Control colonies ( $\text{LacZ}^-$ ) arising from cells that had not been transformed are shown for *M. pulmonis* and *M. arthritis* in panels B and D, respectively.

genomic DNA was digested with *TaqI*, ligated to generate circular molecules for PCR templates, and amplified using primers 5'-TGTGTTCTTTTCTTGCATCGTGGC-3' and 5'-AGTTCTATCAGACGAACACCGTGC-3'. To obtain the 3' end of *arcA*, genomic DNA was digested with *Sau3A*, ligated, and amplified using primers 5'-CGGCTAAATAATGTTTCA CGTTGTC-3' and 5'-GAACAAACCTAATGCACTTAGAC AC-3'. The resulting PCR products were cloned into plasmid pGEM-T (Promega) and their nucleotide sequences were determined, permitting assembly of the sequence of the complete *arcA* gene of *M. arthritis*. The predicted *M. arthritis* ArcA protein (arginine deiminase) contains 409 amino acids with extensive overall sequence identity, 87 and 80%, to the ArcA proteins of *M. arginini* (GenBank accession no. X54141) and *M. hominis* (GenBank accession no. D13314), respectively.

Sequence analysis of the region upstream of the *arcA* structural gene failed to reveal a likely promoter candidate (resembling the consensus -10 and -35 sequences characteristic of promoters recognized by  $\sigma^A$ ). An open reading frame (ORF) was identified upstream of *arcA* that may represent the 3' end of another gene. The amino acids predicted from this ORF have no significant similarity to sequences deposited in the protein and nucleotide databases. Immediately downstream of this ORF are sequences capable of forming a stem-loop structure that may serve as a transcription terminator. Thus, it seems likely that an *arcA* promoter should exist in the area between the putative transcription terminator and the start of the *arcA* coding region. The sequences in this area are interesting and contain a poly(A) sequence of 15 reiterated nucleotides. Assuming that an *arcA* promoter is present in this

region, its structure would be unusual and worthy of further study.

The putative *arcA* promoter region was isolated from the cloned inverse PCR product containing the 5' end of the *arcA* gene and combined with *lacZ* (Fig. 3). The plasmid containing the *arcA* promoter region was linearized by digestion with *NsiI*, the ends were made flush by digestion with T4 DNA polymerase, *XhoI* linkers were attached, and a 300-bp region containing the putative promoter and the beginning of the *arcA* structural gene was excised by digestion with *XhoI* and *EcoRI*. This fragment was cloned into the *XhoI/EcoRI* site of plasmid pZErO-2.1. The promoterless *lacZ* gene from plasmid pISM2062.2*lac* (17) was excised as a 3-kb *BamHI* fragment and inserted into the *BamHI* site of pZErO-2.1 downstream of the *arcA* promoter fragment. This resulted in construction of an in-frame fusion gene containing the first 33 nucleotides of the *arcA* coding region, 31 nucleotides derived from the pZErO-2.1 polylinker, and *lacZ*. The *arcA-lacZ* gene was excised from pZErO-2.1 by digestion with *XhoI* and *KpnI*, and the ends were made flush by digestion with T4 DNA polymerase. *BglIII* linkers were attached, cohesive ends were generated by digestion with *BglIII*, and the gene was inserted into the *BamHI* site (*BglIII* and *BamHI* ends are compatible) of pIVT-1 to generate pIVT-lac. As anticipated, *E. coli* colonies containing either the *arcA-lacZ* gene in pZErO-2.1 or pIVT-lac were blue when assayed on agar supplemented with 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-Gal), indicating that the fusion gene was functional.

Transformation of *M. pulmonis* and *M. arthritis* with pIVT-lac resulted in tetracycline-resistant colonies that were found

to possess  $\beta$ -galactosidase activity when assayed on mycoplasma agar supplemented with X-Gal at a concentration of 150  $\mu\text{g/ml}$  (Fig. 4). Colonies of wild-type mycoplasmas that had not been transformed with pIVT-*lac* were white, indicating that the  $\beta$ -galactosidase activity resulted from the ArcA-LacZ fusion protein and was not endogenous to the mycoplasmas. Although it has not been determined whether transcription of *arcA-lacZ* is initiated from an *arcA* promoter or from outside the *arcA* promoter region, it is clear that *arcA-lacZ* is expressed both in *M. pulmonis* and in *M. arthritidis*, demonstrating the utility of pIVT-1 as a vector in these species. Because the *tetM* marker functions in all species of *Mycoplasma* in which it has been examined, Tn4001T should be used as a general mycoplasmal cloning vector.

**Nucleotide sequence accession number.** The *arcA* nucleotide sequence of *M. arthritidis* has been deposited in GenBank under accession no. AF182646.

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