

## Transcriptional Regulation of Type 4 Pilin Genes and the Site-Specific Recombinase Gene, *piv*, in *Moraxella lacunata* and *Moraxella bovis*

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*Moraxella lacunata* and *Moraxella bovis* use type 4 pili to adhere to epithelial tissues of the cornea and conjunctiva. Primer extension analyses were used to map the transcriptional start sites for the genes encoding the major pilin subunits (*tfpQ/I*) and the DNA invertase (*piv*), which determines pilin type expression. *tfpQ/I* transcription starts at a  $\sigma^{54}$ -dependent promoter (*tfpQ/Ip<sub>2</sub>*) and, under certain growth conditions, this transcription is accompanied by weaker upstream transcription that starts at a potential  $\sigma^{70}$ -dependent promoter (*tfpQ/Ip<sub>1</sub>*). *piv* is expressed in both *M. lacunata* and *M. bovis* from a putative  $\sigma^{70}$ -dependent promoter (*pivp*) under all conditions assayed.  $\sigma^{54}$ -dependent promoters require activators in order to initiate transcription; therefore, it is likely that *tfpQ/Ip<sub>2</sub>* is also regulated by an activator in *Moraxella*. Primer extension assays with RNA isolated from *Escherichia coli* containing the subcloned pilin inversion region from *M. lacunata* showed that *pivp* is used for the expression of *piv*; however, *tfpQ/Ip<sub>2</sub>* is not used for the transcription of *tfpQ/I*. Transcription from *tfpQ/Ip<sub>2</sub>* was activated in *E. coli* when the sensor (PilS) and response regulator (PilR) proteins of type 4 pilin transcription in *Pseudomonas aeruginosa* were expressed from a plasmid. These results suggest that the expression of the type 4 pilin in *M. lacunata* and *M. bovis* is regulated not only by a site-specific DNA inversion system but also by a regulatory system which is functionally analogous to the PilS-PilR two-component system of *P. aeruginosa*.

*Moraxella lacunata* and *Moraxella bovis* are gram-negative human and bovine pathogens, respectively, that primarily infect the conjunctiva and cornea (3, 15, 29, 32). Attachment to the epithelial tissues of these areas is mediated by type 4 pili (28). Other bacteria that express type 4 pili include *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Pseudomonas aeruginosa*, *Vibrio cholerae*, *Aeromonas hydrophila*, and *Dichelobacter nodosus* (10, 13, 23, 25, 37). In bacteria expressing type 4 pili, the major pilin subunit is expressed as a pre-pilin protein with an unusually short 6-amino-acid leader sequence followed by a predominantly hydrophobic domain (35). The hydrophobic domain and the leader sequence are required to deliver the protein to an ATP-dependent, Sec-independent secretory pathway (34). During the process of secretion, a pre-pilin peptidase cleaves the leader sequence and methylates the first amino acid of the mature protein, creating *N*-methylphenylalanine (36). Thus, type 4 pili have also been classified as MePhe pili.

*M. bovis* exhibits antigenic and phase variations of its type 4 pili, alternately expressing Q- or I-type pili and switching between P<sup>+</sup> (piliated) and P<sup>-</sup> (nonpiliated) at a frequency as high as 10<sup>-4</sup> per cell per generation (21). *M. bovis* cells expressing Q-type pili are more efficient at establishing infection than those expressing I-type pili (27, 29), and nonpiliated *M. bovis* cells cannot establish infection (15). Therefore, pili are important virulence factors. The genes encoding the major pilin subunits of *M. bovis* are located on an invertible segment of DNA (Fig. 1) that allows the alternate expression of *tfpQ* or *tfpI* (type four pilin) (12). The shared amino terminus is en-

coded outside the invertible segment, and the genes for the carboxy termini of the Q- and I-type pilin proteins are oriented in opposite directions within the invertible segment. The orientation of the invertible DNA determines which pilin gene will be transcribed. An additional open reading frame (*tfpB*) is found within the invertible segment; the function of *tfpB* is as yet unknown (12). The *piv* gene, located immediately adjacent to the invertible segment, encodes the putative site-specific DNA invertase (19, 20). The pilin genes of *M. lacunata* share considerable homology with the pilin genes of *M. bovis* and are identically arranged (26). However, the *M. lacunata* *tfpI* gene contains a 19-bp perfect tandem repeat near the inversion junction site that creates a frameshift, resulting in a shortened, nonfunctional pilin gene product. Thus, *M. lacunata* phase variation is an on/off switch of Q-type pilin expression.

In *M. lacunata* and *M. bovis*, the *tfpQ/I* pilin genes and *piv* have potential  $\sigma^{54}$ -dependent promoters, based on sequence inspection.  $\sigma^{54}$  is an alternative sigma factor, encoded by *rpoN*, that interacts with core RNA polymerase and binds to the consensus sequence -27TGGCAC-N<sub>5</sub>-TTGCA-11 (24). Unlike that in  $\sigma^{70}$ -dependent promoters, the spacing between the highly conserved GG and GC dinucleotides of  $\sigma^{54}$ -dependent promoters is invariant (24). The  $\sigma^{54}$ -RNA polymerase holoenzyme (E $\sigma^{54}$ ) requires an activator to initiate transcription. The activator catalyzes ATP-dependent isomerization of a closed complex between E $\sigma^{54}$  and the promoter to an open complex that is transcriptionally competent (39, 40).

If *tfpQ/I* and/or *piv* uses a  $\sigma^{54}$ -dependent promoter, it is likely that there is regulation of type 4 pilin expression beyond the control mediated by DNA inversion, i.e., at the level of initiation of transcription. As with many  $\sigma^{54}$ -dependent promoters, the activation of transcription may be controlled by a two-component regulatory system. For example, in *P. aeruginosa* a two-component regulatory system controls the initiation

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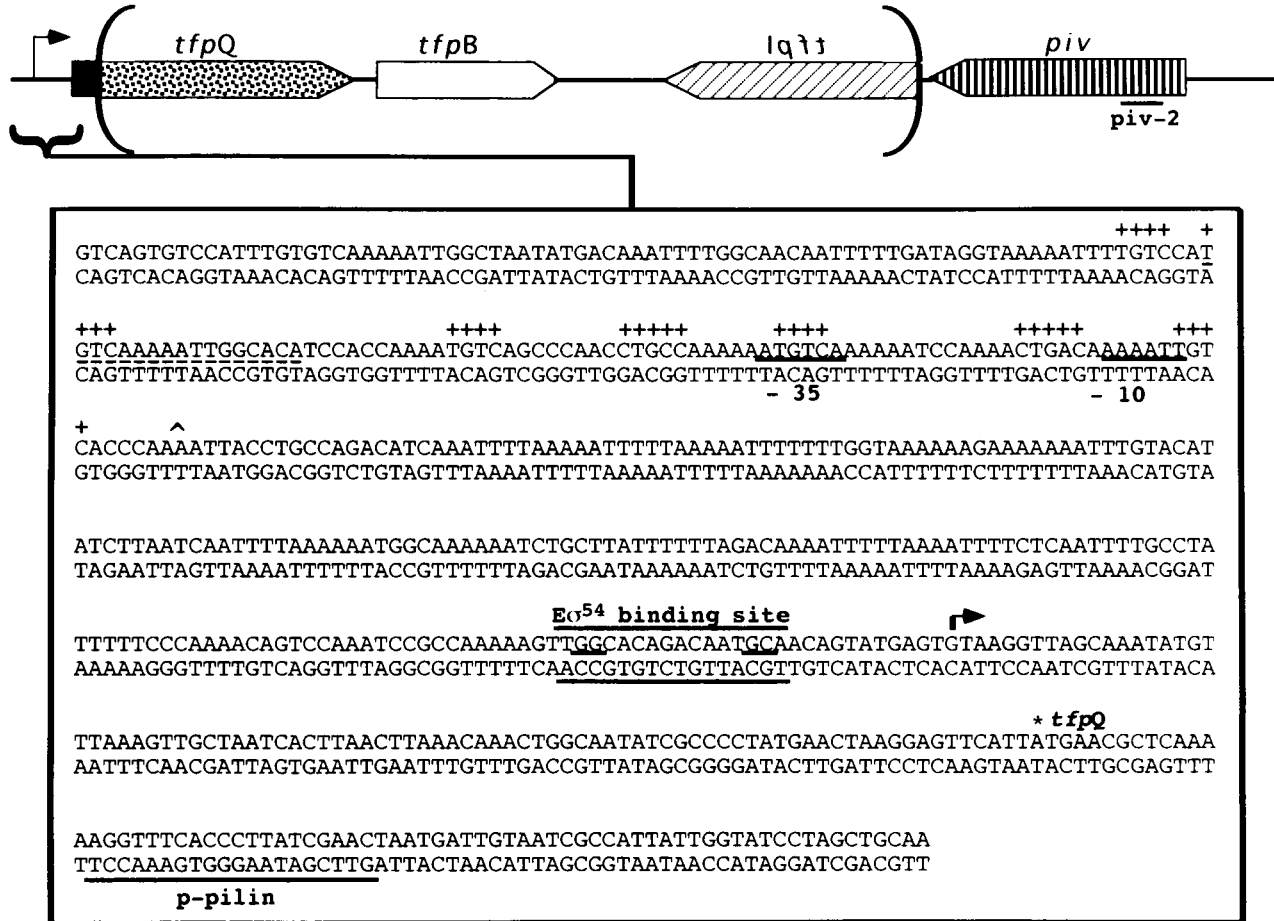


FIG. 1. Schematic representation of the type 4 pilin DNA inversion regions of *M. lacunata* and *M. bovis*. The parentheses denote the invertible segment of DNA. The boxed sequence is that of the *tfpQ/I* promoter region in *M. lacunata*. An arrow marks the start of *tfpQ/lp<sub>2</sub>* transcription. The potential binding site for the RNA polymerase holoenzyme that uses  $\sigma^{54}$  ( $E\sigma^{54}$ ) is marked with two bold lines. The highly conserved  $-24$  GG and  $-12$  GC motifs of the  $\sigma^{54}$ -dependent promoters are underlined. The predicted start of *tfpQ* translation is marked with an asterisk. The *tfpQ/lp<sub>1</sub>* promoter is indicated with an inverted caret, and its potential  $-10$  and  $-35$  sequences are underlined. The sequences similar to the *P. aeruginosa* PiLR binding sites are denoted with plus signs, and the sequence similar to the NifA recognition sequence is underlined with a broken line. The *piv-2* and *p-pilin* primers used in the primer extension assays are indicated.

of transcription from the  $\sigma^{54}$ -dependent promoter of the type 4 pilin gene, *pilA*. The sensor of this two-component system, PilS, is a histidine kinase which autophosphorylates in response to an as-yet-undefined environmental signal and subsequently transfers the phosphate to PilR. The phosphorylated PilR can then interact with  $E\sigma^{54}$  and activate *pilA* transcription (14). PilR interacts with a transcriptional enhancer sequence upstream of the  $\sigma^{54}$ -dependent promoter which contains the sequence 5'<sup>C</sup>/G TGTC3' repeated at the four PiLR binding sites (16). This enhancer region also has homology to the recognition sequence of NifA (5'TGT-N<sub>11</sub>-ACA3'), which is an activator of  $\sigma^{54}$ -dependent transcription of the nitrogen fixation genes in *Klebsiella pneumoniae* (16). The potential  $\sigma^{54}$ -dependent promoter for *tfpQ/I* has the repeated consensus sequence for PiLR DNA binding and the overlapping NifA recognition sequence found in the *pilA* enhancer sequence, suggesting a possible similarity in the regulation of these type 4 pilin genes.

To address the regulation of *tfpQ/I* and *piv*, we used primer extension assays to map the promoters for these genes in *M. lacunata* and *M. bovis*. We also mapped the transcriptional start sites from a subclone of the *M. lacunata* pilin inversion region in *Escherichia coli*. Analyses of transcription in *rpoN*

mutant or wild-type *E. coli* strains in the presence of the PilS and PilR proteins of *P. aeruginosa* further defined transcriptional regulation of the type 4 pilin genes.

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MATERIALS AND METHODS

**Strains and plasmids.** The strains and plasmids used in this study are listed in Table 1. pAG1206 was created by inserting the *Bam*HI/*Hind*III fragment of pJB300 (7), containing the *P. aeruginosa pilS* and *pilR* genes, into the *Bam*HI/*Hind*III sites of pMPM-K3 (22), carrying the *p15A* origin of replication and a pBluescript multicloning site.

**Media and growth conditions.** *E. coli* was grown on Luria-Bertani (LB) agar plates or in LB media (31) at 37°C with the appropriate antibiotics at the following concentrations: ampicillin, 100 µg/ml; kanamycin, 10 µg/ml; and chloramphenicol, 30 µg/ml. *M. lacunata* and *M. bovis* were grown on sheep blood agar plates (BBL Microbiology Systems, Cockeysville, Md.), heart infusion agar with rabbit blood (BBL), GC agar plates (11), or chocolate agar plates (Difco Laboratories, Detroit, Mich.) or in GC broth (11). All cultures were grown at 37°C unless otherwise indicated.

**RNA isolation.** Bacterial colonies were scraped with a sterile loop and resuspended in 20 mM Tris-HCl (pH 7.6)–10 mM NaCl–1 mM EDTA–0.5% sodium dodecyl sulfate. Liquid cultures (5 ml) were centrifuged in a Beckman J2-21 centrifuge with a Beckman JA20 rotor for 5 min at 4,000 × g and resuspended

TABLE 1. Strains and plasmids

Strain or plasmid	Genotype or description	Source or reference <sup>a</sup>
<b>Strains</b>		
<i>Escherichia coli</i>		
DH5 $\alpha$	<i>endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1 <math>\Delta</math>(lacZYA-argF)</i> <i>U169 <math>\lambda</math>-<math>\phi</math>80 dlacZ<math>\Delta</math>M15</i>	Bethesda Research Laboratories
YMC10	<i>endA thi-1 hsdR17 supE44 <math>\Delta</math>lacU169 hutCk</i>	Steve Lory; 2
TH1	YMC10 $\Delta$ <i>rpoN</i>	Steve Lory; 2
DPB29.1	DH5 $\alpha$ /pMxL1	Laboratory strain
DWH15	DH5 $\alpha$ /pMxL1/pAG1206	This study
DWH16	DH5 $\alpha$ /pMxL1/pMPM-K3	This study
DWH17	YMC10/pMxL1	This study
DWH18	TH1/pMxL1	This study
DWH19	YMC10/pMxL1/pMPM-K3	This study
DWH20	TH1/pMxL1/pMPM-K3	This study
DWH21	YMC10/pMxL1/pAG1206	This study
DWH22	TH1/pMxL1/pAG1206	This study
<i>Moraxella lacunata</i>		
ATCC 17956	Wild-type laboratory strain	20
F9223	Wild-type clinical isolate	CDC, Atlanta, Ga.
<i>Moraxella bovis</i> Tifton	Sm <sup>r</sup> laboratory strain	Carl F. Marrs
<b>Plasmids</b>		
pMxL1	6 kb of <i>M. lacunata</i> DNA in pBluescript from a $\lambda$ Zap (Stratagene) library, containing the pilin inversion region (Fig. 1); Amp <sup>r</sup>	20
pJB300	<i>P. aeruginosa pilS pilR</i> in pBluescript; Amp <sup>r</sup>	Steve Lory; 7
pMPM-K3	Cloning vector with <i>p15A ori</i> and pBluescript multicloning site; Kan <sup>r</sup>	Matthias Mayer; 22
pAG1206	<i>Bam</i> HI/ <i>Hind</i> III segment of pJB300 containing the <i>pilS pilR</i> genes in the <i>Bam</i> HI/ <i>Hind</i> III sites of pMPM-K3; Kan <sup>r</sup>	This study
pBluescript	Cloning vector; Amp <sup>r</sup>	Stratagene, La Jolla, Calif.

<sup>a</sup> CDC, Centers for Disease Control and Prevention.

in the same buffer. The suspension was immediately extracted three times with Tris-EDTA-saturated phenol, three times with acid-phenol-chloroform (Ambion), and once with chloroform-isoamyl alcohol (24:1) and was then precipitated overnight at  $-20^{\circ}\text{C}$  with 1 volume of 8 M LiCl. The preparation was centrifuged in a tabletop microcentrifuge at  $20,000 \times g$  and  $4^{\circ}\text{C}$  for 20 min, washed with 70% ethanol, and dried at ambient temperature in a Savant Speed Vac Plus. RNA was quantitated by spectrophotometry (optical density at 260 nm) and stored at  $-70^{\circ}\text{C}$  in 2 mM vanadyl ribonucleoside complex (GIBCO BRL, Gaithersburg, Md.).

**Analysis of piliation.** *M. lacunata* colonies were prepared for transmission electron microscopy (TEM) analysis as described previously (28). TEM was performed on the samples with a Philips 201 transmission electron microscope.

Since natural transformation of *M. lacunata* requires piliation, the following assay was also used to determine the piliation state of the bacteria. A crude preparation of *M. bovis* Tifton DNA was naturally transformed into *M. lacunata* as described previously (17). Potentially transformed *M. lacunata* was grown on LB agar plates supplemented with 25  $\mu\text{g}$  of streptomycin per ml. *M. bovis* Tifton is naturally resistant to streptomycin (Sm<sup>r</sup>). Therefore, only piliated *M. lacunata* can acquire Sm<sup>r</sup> from the *M. bovis* DNA and grow on LB agar-streptomycin.

**Mapping of *tfpQ/I* and *piv* promoters by primer extension.** RNA from *M. lacunata* ATCC 17956 and F9223 and *M. bovis* Tifton was isolated as described above. Growth conditions were varied. Primer extensions were performed as described previously (30). Primer extension reactions were analyzed on a 6% denaturing polyacrylamide gel with the *tfpQ/I* sequence from pMxL1. Sequencing was performed with the Sequenase Kit (USB/Amersham Life Sciences, Inc., Arlington Heights, Ill.) using the same end-labeled primers as those used in the primer extension assays. The sequence of the p-pilin primer used to map the start of *tfpQ/I* transcription was 5'GTTTCGATAAGGGTGAACCT3'. The sequence of the *piv*-2 primer used to map the start of *piv* transcription was 5'GGTTATTGTGAAATGTACAGTTTTGCCAAG3'.

**Assay for PilR-dependent *tfpQ/I* transcription in *E. coli*.** pMxL1 was transformed into wild-type *E. coli* DH5 $\alpha$  and YMC10 and into  $\Delta$ *rpoN* *E. coli* TH1 by electroporation as described previously (4), creating strains DPB29.1, DWH17, and DWH18 (Table 1). pMPM-K3 or pAG1206 (containing *P. aeruginosa pilR* and *pilS*) was then transformed into DPB29.1, DWH17, and DWH18 by electroporation, creating DWH15, DWH16, DWH19, DWH20, DWH21, and DWH22 (Table 1). RNA from these strains was isolated, and primer extension was performed as described above with the p-pilin primer. As a control for RNA concentration, a second primer extension was performed on each RNA sample

with the B-lact primer to map the start of  $\beta$ -lactamase transcription (5'GGTGAAGCAAAAACAGGAAGG3'). RNA was quantitated with a Molecular Dynamics PhosphorImager and ImageQuant software. The integrated intensity of the pixels in each band generated by p-pilin primer extension was divided by the integrated intensity of the corresponding bands generated by primer extensions with the B-lact primer. This method normalized the *tfpQ/I* bands to the concentration of RNA loaded onto the gel.

**Quantitation of *M. lacunata tfpQ/I* transcription under various growth conditions.** *tfpQ/I* primer extension was performed on RNA isolated from *M. lacunata* cultures grown under the following conditions: complete GC medium (containing 22 mM glucose, 86 mM NaCl, and 1.2  $\mu\text{M}$  iron) at 25 or 37 $^{\circ}\text{C}$ ; GC medium without NaCl; GC medium with 257 mM NaCl; GC medium with 0.12  $\mu\text{M}$  iron; GC medium with 5.6 mM glucose; and complete GC medium supplemented with 10% human AB serum. Bacteria from an overnight *M. lacunata* culture grown in complete GC medium were pelleted and resuspended in the same volume of 1 mM Tris-HCl (pH 7.6)–10 mM MgCl<sub>2</sub>. The bacteria were diluted 1:100 in each medium to be tested and grown at either 37 or 25 $^{\circ}\text{C}$  with shaking. Growth curves were calculated to ensure that RNA was isolated from log-phase cultures. *tfpQ/I* transcripts were quantitated and normalized to the 16S rRNA primer extension transcripts from the same RNA samples as those described above. The primer used for 16S rRNA extensions was 5'TCCTAAGCATTACTCTCCCG3'. Duplicates of the primer extension reactions for *tfpQ/I* and 16S rRNA and two dilutions of each 16S rRNA extension reaction were analyzed by denaturing gel electrophoresis.

## RESULTS

**Mapping of the *tfpQ/I* and *piv* promoters.** The potential  $\sigma^{54}$ -dependent promoter sequence, located approximately 100 bp from the translational start site of the *tfpQ/I* gene, differs from the consensus E $\sigma^{54}$  binding site sequence by only one base (in bold) (5'TGGCAC-N<sub>5</sub>-ATGCA3') and retains the invariant spacing between the highly conserved GG and GC dinucleotide residues. The potential  $\sigma^{54}$ -dependent promoter sequence for the *piv* gene also retains the 10-base spacing between the GG and GC doublets (5'TGGTAT-N<sub>5</sub>-GAG

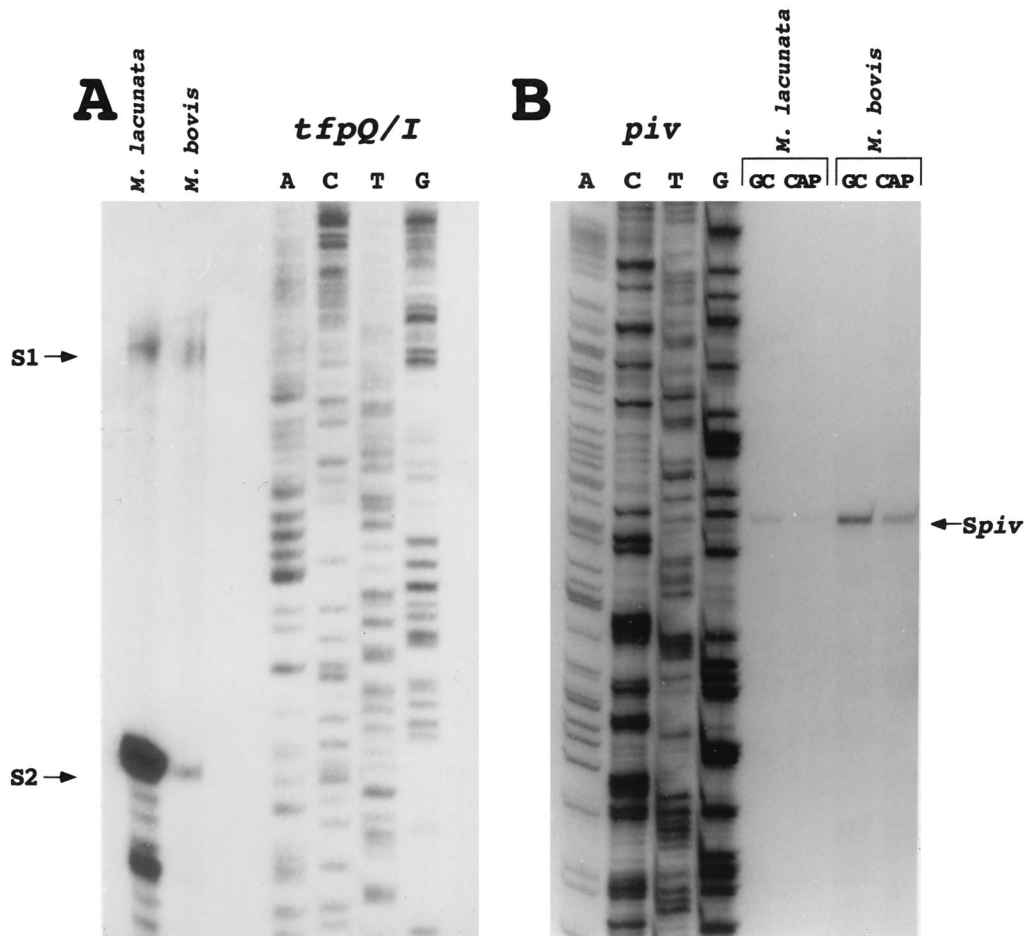


FIG. 2. Primer extension assays mapping the start sites for the *tfpQ/I* and *piv* transcripts. Primer extension assays were performed with *M. lacunata* and *M. bovis* RNA as described in Materials and Methods. (A) Primer extension assays with RNA from *M. lacunata* and *M. bovis* grown on sheep blood agar plates and with the p-pilin primer to detect *tfpQ/I* transcripts. The bands marked S1 (start 1) and S2 (start 2) correspond to the start of the transcripts initiated at *tfpQ/I*<sub>p1</sub> and *tfpQ/I*<sub>p2</sub>, respectively. (B) Primer extension assays with RNA from *M. lacunata* and *M. bovis* grown on either GC agar plates (GC) or chocolate agar plates (CAP) and with the *piv*-2 primer to detect *piv* transcripts. The bands marked *Spiv* correspond to the start of the transcripts initiated at *pivp*. The primers used for the primer extension assays were also used for the DNA sequence reactions for *tfpQ/I* and *piv* from pMxL1.

CA3'); however, it diverges from the consensus sequence in the two nucleotides (in bold) before the GC doublet (from TT to GA), which have been shown to be critical contacts for  $\sigma^{54}$  binding (8, 9). Thus, this sequence is predicted to be a low-affinity  $\sigma^{54}$  binding site. Primer extension assays were used to determine the 5' end of the transcripts for *tfpQ/I* and *piv* with the primers shown in Fig. 1. Piliated colonies, which were distinguished by colony morphology (5), were picked for RNA isolation. Piliated colonies have a corroding morphology with a raised translucent center, and nonpiliated colonies are flat and opaque. TEM and transformation assays for natural competence were used to confirm that these morphologies corresponded to the state of piliation. Indeed, the corroding colonies displayed bundled pili in TEM and were capable of acquiring a chromosomal marker (Sm<sup>r</sup>) in the transformation assays. Flat, opaque colonies were nonpiliated in TEM and not naturally competent (data not shown).

Primer extension analyses of *tfpQ/I* transcripts with RNA isolated from *M. lacunata* and *M. bovis* grown on blood agar plates are shown in Fig. 2A. The primary transcriptional start site was located 12 bases downstream of the GC doublet in the potential  $\sigma^{54}$  binding sequence (Fig. 1) in both *M. lacunata* and *M. bovis*, suggesting utilization of the  $\sigma^{54}$ -dependent promoter

(*tfpQ/I*<sub>p2</sub>). A second, weaker transcriptional start site was identified 301 bp upstream of the start site for *tfpQ/I* translation. This transcript initiated at a possible  $\sigma^{70}$ -dependent promoter (Fig. 1) and was designated *tfpQ/I*<sub>p1</sub>. The shorter transcripts seen in the *tfpQ/I* primer extension assays may have been early termination products due to the possible secondary structure resulting from the repeated tracts of A's and T's downstream of *tfpQ/I*<sub>p1</sub>. The temperature of extension was raised from 43 to 55°C in order to reduce potential secondary structure, but this increase did not eliminate the shorter transcripts (data not shown). These additional transcripts may also represent products of RNA processing. Since *M. lacunata* ATCC 17956 is a laboratory strain and has been passaged many times, *tfpQ/I* primer extension assays were also performed on RNA isolated from the clinical strain F9223 (grown on heart infusion agar with rabbit blood), which has been passaged only three times after being isolated during an outbreak in 1986 (32). The results of this analysis indicated that the *tfpQ/I*<sub>p2</sub> promoter was utilized in this clinical strain; however, transcription from the *tfpQ/I*<sub>p1</sub> promoter was absent (data not shown). The absence of the *tfpQ/I*<sub>p1</sub> transcript was also noted for *M. lacunata* ATCC 17956 and *M. bovis* Tifton grown on heart infusion agar with rabbit blood, GC medium, or chocolate agar (data not shown).

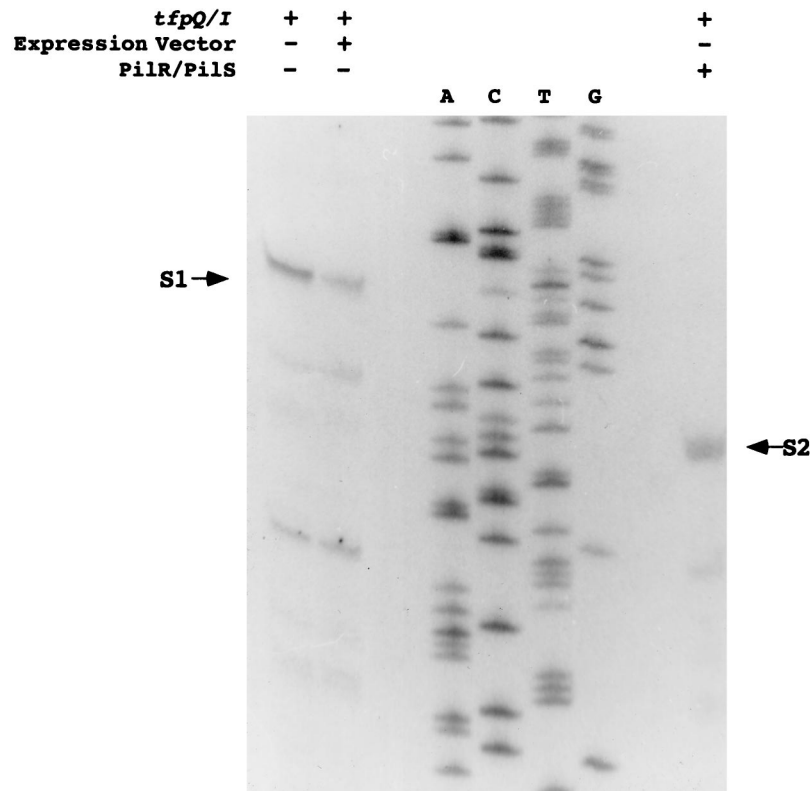


FIG. 3. PilS-PilR activation of *tfpQ/Ip<sub>2</sub>* transcription in *E. coli*. Primer extension assays were performed with RNA expressed from pMxL1 in the presence (*pilS* and *pilR* expressed from pAG1206) or absence (parent expression vector or no additional plasmid present) of PilS-PilR in *E. coli*. The p-pilin primer was used for the extensions and the sequencing of pMxL1. The bands marked S1 and S2 correspond to the start of the transcripts initiated at *tfpQ/Ip<sub>1</sub>* and *tfpQ/Ip<sub>2</sub>*, respectively.

The start site for the *piv* transcript was also determined by primer extension assays with RNA isolated from *M. lacunata* and *M. bovis* each grown on GC agar and chocolate agar plates as indicated in Fig. 2B. In each case, the 5' end of the transcripts initiated at a putative  $\sigma^{70}$ -dependent promoter 111 bp upstream of the start site for the translation of *piv*. The apparent absence of initiation of transcription from the potential  $\sigma^{54}$ -dependent promoter for *piv* is consistent with the prediction, based on chemical interference and base substitution DNA binding assays, that this promoter sequence is a low-affinity  $\sigma^{54}$  and  $E\sigma^{54}$  binding site (8, 9). However, since  $\sigma^{54}$ -dependent transcription is often regulated by environmental signals, it is still possible that this promoter is used under conditions that were not tested.

Primer extension assays were also performed with RNA isolated from *E. coli* DH5 $\alpha$  carrying plasmid pMxL1, which contains the *M. lacunata* inversion region and exhibits inversion in *E. coli* (20). Figure 3 shows that the *tfpQ/I* transcripts did not map to the *tfpQ/Ip<sub>2</sub>* promoter sequence, which is utilized in *M. lacunata* and *M. bovis*. Instead, we detected transcripts which may correspond to processed versions or early terminations of primer extension from the *tfpQ/Ip<sub>1</sub>* promoter in *M. lacunata* and *M. bovis*. It is also possible that these transcripts correspond to additional promoters used in *E. coli*; however, there were no identifiable promoter sequences associated with the 5' ends of the transcripts. Since transcription from a  $\sigma^{54}$ -dependent promoter is dependent on an activator protein, it is likely that *E. coli* lacks a homolog of the *M. lacunata* *tfpQ/I* activator protein and therefore that  $E\sigma^{54}$  cannot initiate transcription from *tfpQ/Ip<sub>2</sub>*.

Primer extension assays for the *piv* transcript performed with

RNA isolated from *E. coli* containing pMxL1 revealed that the  $\sigma^{70}$ -dependent promoter *pivp*, which is used in *M. lacunata* and *M. bovis*, is also used in *E. coli* (18a).

***P. aeruginosa* PilS-PilR can activate *tfpQ/Ip<sub>2</sub>* transcription in *E. coli*.** In the *pilA* transcriptional enhancer sequence of *P. aeruginosa*, four binding sites for the PilR activator are centered approximately 100 bp upstream of the  $E\sigma^{54}$  binding site (16). Approximately 200 bp upstream of *tfpQ/Ip<sub>2</sub>*, there is significant sequence similarity with the PilR binding sites of the *pilA* enhancer sequence (Fig. 1). To determine if transcription from *tfpQ/Ip<sub>2</sub>* could be activated in *E. coli* by PilR, primer extension assays were performed with RNA isolated from an *E. coli* strain containing pMxL1 and a plasmid expressing *pilS* and *pilR* from *plac* (pAG1206). The *pilR* translation product in *E. coli* is full length, but the *pilS* product is missing its transmembrane domain due to initiation of translation at an internal TTG methionine codon instead of the GTG codon used in *P. aeruginosa* (7). Although the activity of this truncated form of PilS was not determined in *E. coli*, Boyd and Lory (6) have shown that an identically truncated gene product from a 5' deletion of *pilS* (PilS-cyt), which is cytoplasmically located, retains its histidine kinase activity in *P. aeruginosa*. Therefore, PilS expressed from pAG1206 may also be an active histidine kinase. In the primer extension assays (Fig. 3), when PilS-PilR was expressed from pAG1206, transcripts initiating at the *tfpQ/Ip<sub>2</sub>* promoter were detected (Fig. 3). *tfpQ/Ip<sub>2</sub>* transcripts were not observed when the vector alone was cotransformed with pMxL1.

**PilS-PilR-dependent *tfpQ/I* transcription in *E. coli* requires the *rpoN* gene product,  $\sigma^{54}$ .** To verify that PilR-stimulated *tfpQ/I* transcription from *tfpQ/Ip<sub>2</sub>* (Fig. 3) was dependent on

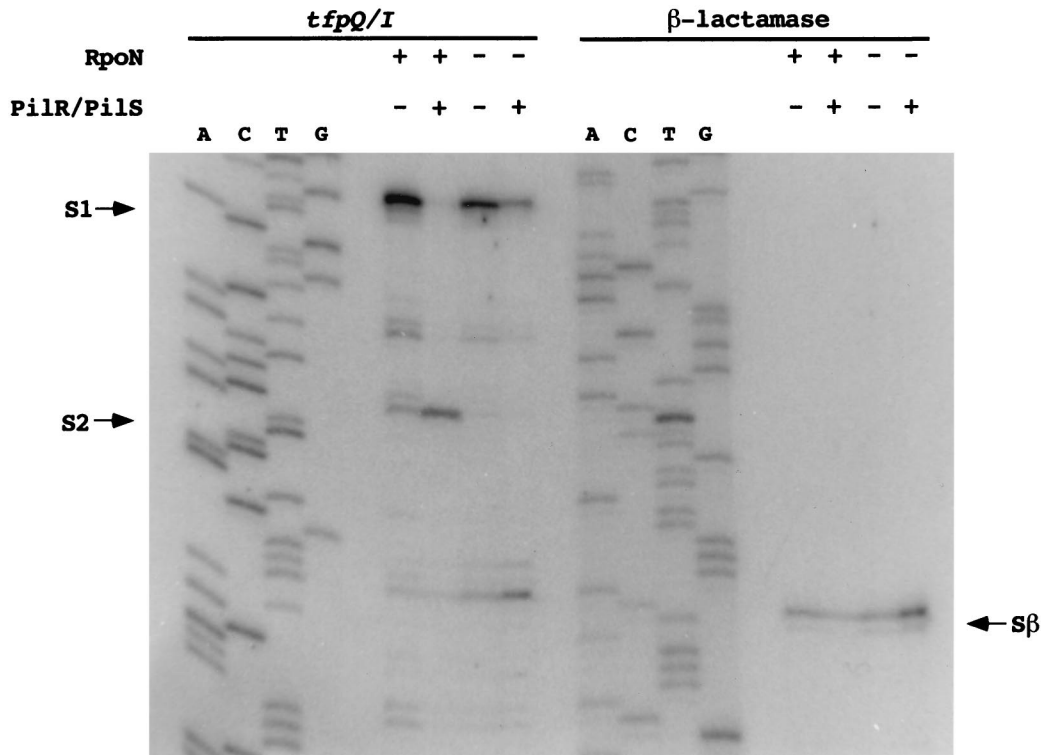


FIG. 4. RpoN dependence of PilS-PilR-activated *tfpQ/Ip<sub>2</sub>* transcription in *E. coli*. Primer extension assays were performed with RNA expressed from pMxL1 in the presence or absence of PilS-PilR in *rpoN* mutant and wild-type *E. coli*. The p-pilin primer and the  $\beta$ -lact primer were used for the indicated *tfpQ/Ip<sub>1</sub>* and  $\beta$ -lactamase primer extensions, respectively, and the sequencing of pMxL1. The bands marked S1 and S2 correspond to the start of the transcripts initiated at *tfpQ/Ip<sub>1</sub>* and *tfpQ/Ip<sub>2</sub>*, respectively; S $\beta$  indicates the start of the transcripts initiated at the  $\beta$ -lactamase promoter.

$\sigma^{54}$ , pMxL1 and pAG1206 were transformed into the *rpoN* mutant *E. coli* strain TH1 and its parent strain, YMC10. RNA from the resulting strains (DWH17 to DWH22) was used in primer extension assays for *tfpQ/Ip<sub>2</sub>* transcription. As a control for RNA levels, primer extension assays were performed with the same RNA preparations to measure  $\beta$ -lactamase transcription from plasmid pMxL1. Figure 4 shows that PilS-PilR did not activate transcription from *tfpQ/Ip<sub>2</sub>* in the absence of  $\sigma^{54}$ . As was seen with DH5 $\alpha$  (Fig. 3), no *tfpQ/Ip<sub>2</sub>* transcripts were detected in the *rpoN*<sup>+</sup> *E. coli* parent strain (YMC10) unless PilS-PilR was expressed. Primer extension assays with RNA from *rpoN* mutant and wild-type strains containing the vector pMPM-K3 and pMxL1 revealed no *tfpQ/Ip<sub>2</sub>* transcripts; the primer extension products that were present may have represented early termination products of transcription from *tfpQ/Ip<sub>1</sub>*. Interestingly, in both *rpoN* mutant and wild-type strains containing PilS-PilR, it appeared that the presence of pAG1206 downregulated *tfpQ/Ip<sub>1</sub>* transcription.

In Figure 5, the relative amounts of transcription from *tfpQ/Ip<sub>1</sub>* and *tfpQ/Ip<sub>2</sub>*, determined from the primer extension assays in Figure 4, are shown normalized for RNA concentrations with the corresponding  $\beta$ -lactamase primer extension assays. This comparison of transcript levels indicated that transcription from *tfpQ/Ip<sub>1</sub>* was decreased in strains in which PilS-PilR was present. Three of the potential PilR binding sites in the region upstream of *tfpQ/Ip<sub>2</sub>* overlap *tfpQ/Ip<sub>1</sub>* (Fig. 1); therefore, binding of PilR to the enhancer-like sequence for *tfpQ/Ip<sub>2</sub>* may repress transcription from *tfpQ/Ip<sub>1</sub>*. A similar pattern of repression and activation by an activator protein regulates transcription from the  $\sigma^{70}$ - and  $\sigma^{54}$ -dependent promoters of the *glnA* (glutamine synthetase) gene in *E. coli* and *Salmonella*

*typhimurium* (33; see Discussion). Figure 5 also shows that transcription from the *tfpQ/Ip<sub>2</sub>* promoter was detected at significant levels only in the strain in which RpoN and PilS-PilR (pAG1206) were present.

**Effect of growth conditions on *tfpQ/Ip<sub>1</sub>* transcription.** In an attempt to identify environmental factors that would influence *tfpQ/Ip<sub>1</sub>* transcription, RNA was isolated from *M. lacunata* ATCC 17956 grown under a variety of conditions. To mimic conditions of the eye, bacteria were grown in 257 mM NaCl (1) and compared with bacteria grown in either 86 mM NaCl or no NaCl. The iron concentration was also reduced 10-fold, since there are lactoferrins in tears that limit iron accessibility (38). A low-glucose environment was created by reducing D-glucose from 22 to 5.6 mM, which was previously shown to decrease piliation in *N. gonorrhoeae* (18). The effects of 10% human AB serum and growth temperature (25 versus 37°C) were also assayed. Under all conditions tested, there were no significant changes in the levels of *tfpQ/Ip<sub>1</sub>* transcription (data not shown).

## DISCUSSION

In order to adjust to changing conditions in the progression of infection, many pathogenic bacteria regulate the expression of virulence factors in response to environmental signals and/or use programmed genetic alterations which provide selective advantages to a subgroup of the infecting population. Pili are important virulence factors for many bacteria, mediating adherence to host cells, defining tissue tropism, and providing a means of evading the host immune system by antigenic variation of the major pilin subunits. It has been demonstrated that type 4 pili are essential virulence factors for a number of

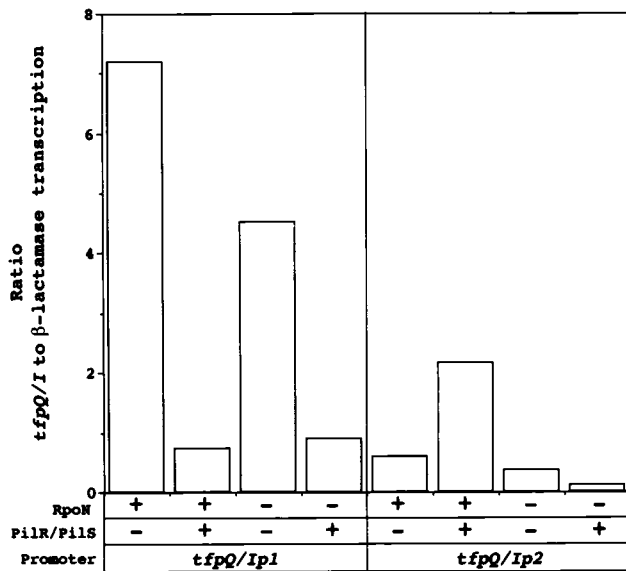


FIG. 5. Comparison of levels of *tfpQ/Ip1* and *tfpQ/Ip2* transcripts in *E. coli*. *tfpQ/I* transcription levels were quantitated from the primer extension assays shown in Fig. 4, normalized for RNA concentrations with the  $\beta$ -lactamase primer extension assays (ratio of *tfpQ/I* to  $\beta$ -lactamase transcription). *tfpQ/Ip1* and *tfpQ/Ip2* transcripts in *rpoN* mutant or wild-type *E. coli* strains in the presence and absence of PilS-PilR were compared.

pathogenic bacteria, including *M. bovis* (28, 35). Results from in vivo infections of calf eyes with *M. bovis* suggest that not only are type 4 pili essential for initiation of infection, but also switching between Q-type and I-type pili is important for the progression of disease (29). This switching is controlled by Piv-mediated site-specific DNA inversion of the chromosomal DNA segment containing the carboxyl-terminal regions of the Q- and I-type pilin genes (20, 21). Transcription of the recombinase gene, *piv*, and/or transcription of the pilin genes, *tfpQ/I*, may provide another level of type 4 pilus regulation in *M. bovis* and *M. lacunata*. The results of this study indicate that transcription of *tfpQ/I* is directed by the alternative sigma factor,  $\sigma^{54}$ , and may be regulated by factors that are required for the activation of  $\sigma^{54}$ -holoenzyme transcription. Alternatively, *piv* appears to be constitutively expressed from a  $\sigma^{70}$ -dependent promoter in *M. lacunata* and *M. bovis*.

The primary *tfpQ/I* transcript in *M. bovis* and *M. lacunata* was mapped by primer extension assays to a  $\sigma^{54}$ -dependent promoter (*tfpQ/Ip2*). When RNA was isolated from bacteria grown on sheep blood agar plates, this transcript was accompanied by a second, weaker transcript that initiated at an upstream putative  $\sigma^{70}$ -dependent promoter (*tfpQ/Ip1*); this *tfpQ/Ip1* transcript was not seen under any other growth conditions, including growth on GC media, heart infusion agar with rabbit blood, and chocolate agar (data not shown). When the *M. lacunata* pilin genes were expressed in *E. coli*, the *tfpQ/Ip2* promoter was not used for *tfpQ/I* transcription, although transcripts that potentially corresponded to the *tfpQ/Ip1* promoter seen in *M. lacunata* were detected. The lack of a *tfpQ/I* transcript corresponding to the *tfpQ/Ip2* promoter suggests that there are no *E. coli* homologs for the activator required for  $\sigma^{54}$ -dependent *tfpQ/I* transcription in *M. lacunata*. Examination of the *tfpQ/I* promoter region revealed several potential binding sites for the PilR activator protein from the *P. aeruginosa* type 4 pilin system. In *E. coli*, the *P. aeruginosa* sensor and activator proteins, PilS and PilR, were able to substitute for the

missing *M. lacunata* factors and activate transcription from the *tfpQ/Ip2* promoter. This activated transcription was abolished in an *rpoN* mutant *E. coli* strain, demonstrating that transcription is actually dependent on  $\sigma^{54}$ .

Another possible role for a PilR homolog regulating *tfpQ/I* transcription in *Moraxella* was indicated by the primer extension assays, which revealed a decreased level of *tfpQ/Ip1* transcription in both wild-type and *rpoN* mutant *E. coli* strains in the presence of PilS-PilR. This result suggests that in *M. bovis* and *M. lacunata*, the protein which serves as an activator for *tfpQ/Ip2* may also block transcription from *tfpQ/Ip1* when bound to a transcriptional enhancer sequence. This dual function as a repressor and an activator for a response regulator protein is seen in the regulation of transcription of the *glnA ntrBC* operon in *E. coli* and *S. typhimurium* (for a review, see reference 33). The expression of *glnA* is regulated from two promoters, designated *glnAp1* and *glnAp2*. *glnAp1*, located 114 bases upstream of *glnAp2*, is active at low levels in a nitrogen-rich environment and is dependent on  $\sigma^{70}$ . The *glnAp2* promoter is activated at high levels when nitrogen is limited and is  $\sigma^{54}$  dependent. The response regulator protein (NtrC) activates transcription from *glnAp2* when it is phosphorylated by the histidine kinase (NtrB) in response to a complex signal transduction system regulating nitrogen utilization. When phosphorylated NtrC binds the transcriptional enhancer sequence to activate *glnAp2* transcription, it binds two sites overlapping the -35 sequence and transcriptional start site of *glnAp1*, thus repressing *glnAp1* transcription. This similarity of the organization of the transcriptional regulatory elements for *tfpQ/I* and *glnA* is striking and suggests future directions for characterization of the regulation of *tfpQ/I* in *M. lacunata* and *M. bovis*.

In summary, our results indicate that *piv* and *tfpQ/I* are not coordinately regulated. Although *piv* is transcribed from a  $\sigma^{70}$ -dependent promoter, *tfpQ/I* is transcribed from a  $\sigma^{54}$ -dependent promoter. As with all other genes whose transcription is dependent on  $\sigma^{54}$ , *tfpQ/I* transcription from the  $\sigma^{54}$ -dependent promoter requires an activator protein. Thus, the expression of the type 4 pili in *M. lacunata* and *M. bovis* is controlled by both a site-specific DNA inversion system that specifies which pilin gene will be transcribed (*tfpQ* or *tfpI*) and a regulatory system that controls the initiation of transcription of *tfpQ/I*.

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