

Regulation of Type IV Fimbrial Biogenesis in *Dichelobacter nodosus*

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Type IV fimbriae are expressed by several bacterial pathogens and are essential for virulence in *Dichelobacter nodosus*, which causes ovine footrot. We have identified a two-component signal transduction system (PilR/S) and an alternative sigma factor (σ^{54}) that were shown by insertional inactivation to be required for the regulation of fimbrial biogenesis in *D. nodosus*. Western blots showed that in both *pilR* and *rpoN* mutants, fimbrial subunit production was significantly reduced by a process that was shown to occur at a PilR- and σ^{54} -dependent promoter. The mutants lacked surface fimbriae, which were shown to be required for the adherence of *D. nodosus* cells to tissue culture monolayers. The reduction in fimbrial subunit production in these mutants also resulted in a concomitant loss of the ability to secrete extracellular proteases. A maltose binding protein-PilR fusion protein was purified and was shown to bind specifically to a region located 234 to 594 bp upstream of the *fimA* transcriptional start point. To determine additional targets of PilR and σ^{54} , genome-wide transcriptional profiling was performed using a whole-genome oligonucleotide microarray. The results indicated that PilR and σ^{54} regulated genes other than *fimA*; these genes appear to encode surface-exposed proteins whose role in virulence is unknown. In conclusion, this study represents a significant advancement in our understanding of how the ability of *D. nodosus* to cause ovine footrot is regulated, as we have shown that the biogenesis of type IV fimbriae in *D. nodosus* is regulated by a σ^{54} -dependent PilR/S system that also indirectly controls protease secretion.

Dichelobacter nodosus is a fastidious gram-negative anaerobe that is the causative agent of footrot in sheep. Ovine footrot is a debilitating disease that is characterized by necrotic separation of the hoof from the underlying soft tissue and leads to significant economic losses as a result of lameness, loss of body condition, and reduced wool growth (51). The virulence of *D. nodosus* isolates varies and depends on factors such as the production of type IV fimbriae and extracellular proteases and the presence of two genomic regions preferentially associated with virulent isolates (3).

Type IV fimbriae are expressed by a broad range of organisms and are characterized by sequence similarity of the fimbrial subunit, a polar location, and the presence of N-methylated phenylalanine or methionine as the N-terminal residue (13). The type IV fimbriae of *D. nodosus* are required for a flagellum-independent form of motility termed twitching motility, natural competence, and extracellular protease secretion and have been shown to be essential for virulence (31). *D. nodosus* isolates are classified into 10 serogroups based on agglutination to antibodies against these fimbriae (12, 20). Little is known about the regulation of fimbrial biogenesis in *D. nodosus*, but preliminary evidence suggests that it involves a regulatory system based on σ^{54} -dependent promoters (22, 30). The regulation of type IV fimbriae in *Pseudomonas aeruginosa* and *Myxococcus xanthus* has been shown to involve σ^{54} and NtrC family response regulators, and given the mechanistic

similarities of type IV fimbrial biogenesis, similar regulatory systems are likely to exist in other species (21, 25, 36, 60). However, in some bacteria that express type IV fimbriae, there are no functional homologues of the *P. aeruginosa* and *M. xanthus* regulatory systems (9, 34).

Two-component signal transduction systems are able to sense and respond to environmental or growth phase stimuli by a histidine-aspartate phosphorelay using a membrane-bound sensor histidine kinase and a cytoplasmic response regulator. Response regulators are transcriptional regulators composed of a conserved N-terminal receiver domain that is the site of phosphorylation and a C-terminal DNA binding domain. The NtrC family of response regulators possesses an additional central domain, which is responsible for ATP hydrolysis, oligomerization, and interaction with σ^{54} (7). NtrC-like regulators belong to the AAA+ (ATPases associated with a variety of cellular activities) family of proteins and interact with σ^{54} -RNA polymerase (RNAP) to regulate diverse functions such as nitrate utilization, xylene degradation, and flagellar synthesis (7, 56). The association of σ^{54} with core RNAP and its subsequent binding to the promoter produce an activator-dependent, closed transcriptional complex. NtrC regulators bind to regions of DNA upstream of the σ^{54} -dependent promoter and, through ATP hydrolysis, convert σ^{54} -RNAP into an open transcriptional complex, thereby allowing transcription to occur (7, 38, 61, 64).

The overall aim of this study was to determine how the production of fimbriae was regulated in *D. nodosus*. In this report, we describe the successful identification and genetic analysis of a σ^{54} -dependent PilRS system and show that it is

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics	Source or reference
Strains		
<i>E. coli</i>		
DH5 α	F ⁻ <i>endA1 hsdR17</i> (r _k ⁻ m _k ⁻) <i>thi-1</i> λ ⁻ <i>recA1 gyrA96 relA1 phoA supE44 deoR</i> ϕ 80 <i>dlacZ</i> Δ <i>M15</i> Δ (<i>lacZYA argF</i>) <i>U169</i>	Invitrogen
C43(DE3)	F' <i>ompT hsdS_B</i> (r _B m _B) <i>gal dcm</i> (DE3) (C43)	37
<i>D. nodosus</i>		
VCS1703A	Serogroup G, transformable, virulent strain	J. Egerton, University of Sydney
JIR3727	VCS1703A <i>fimA</i> Ω <i>tet</i> (M)	31
JIR3772	VCS1703A <i>pilR</i> Ω <i>erm</i> (B) (<i>pilR1</i>)	Natural transformation
JIR3773	VCS1703A <i>pilR</i> Ω <i>erm</i> (B) (<i>pilR2</i>)	Natural transformation
JIR3887	VCS1703A <i>rpoN</i> Ω <i>erm</i> (B) (<i>rpoN1</i>)	Natural transformation
JIR3888	VCS1703A <i>rpoN</i> Ω <i>erm</i> (B) (<i>rpoN2</i>)	Natural transformation
<i>P. aeruginosa</i>		
PAK	Wild type	M. Hobbs and J. Mattick, University of Queensland
R94	PAK <i>pilR</i> Ω Tn5G	23
Plasmids		
pBluescript SK(+)	Ap ^r <i>lacZ'</i> cloning vector	Stratagene
pUCP18	Ap ^r <i>E. coli</i> - <i>P. aeruginosa</i> shuttle vector	50
pET22b(+)	Ap ^r C-terminal His ₆ expression vector	Novagen
pT7Blue-3	Ap ^r cloning vector	Novagen
pMal-c2	Ap ^r N-terminal <i>malE</i> maltose binding protein expression vector	New England Biolabs
pJIR2586	pBluescript SK(+) containing the <i>erm</i> (B) gene inserted into the ClaI site of <i>pilR</i>	Recombinant
pJIR2596	pBluescript SK(+) containing the <i>erm</i> (B) gene inserted into the EcoRI site of <i>rpoN</i>	Recombinant
pJIR2764	pUCP18 EcoRI/PstI Ω 4.5-kb PCR product containing <i>D. nodosus pilR/S</i> genes and upstream region	Recombinant
pJIR2776	pT7Blue-3 EcoRV Ω 729-bp <i>fimA</i> promoter region	Recombinant
pJIR2824	pMal-c2 EcoRI/XbaI <i>pilR</i> ⁺	Recombinant

responsible for the transcriptional regulation of fimbrial subunit synthesis and therefore fimbrial biogenesis in *D. nodosus*. We have also shown that the fimbriae of *D. nodosus* are required for adherence and that protease secretion is linked to the levels of the fimbrial subunit. In addition to fimbrial biogenesis, this system appears to concomitantly regulate additional, previously unidentified, putative virulence factors.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Bacterial strains and plasmids are listed in Table 1. *Escherichia coli* and *P. aeruginosa* strains were grown on 2 \times YT medium (47) supplemented with 100 μ g ampicillin/ml or 150 μ g erythromycin/ml for *E. coli* strains and 250 μ g carbenicillin/ml for *P. aeruginosa* strains. *D. nodosus* strains were grown on Eugon (Difco) yeast extract (EYE) agar containing 5% defibrinated horse blood (Equicell) and supplemented with 1 μ g erythromycin/ml for selection of transformants or in Eugon (Difco) broth with yeast extract in an anaerobic chamber as described previously (40).

Molecular techniques. Unless otherwise stated, molecular techniques were performed as described elsewhere previously (40, 47). Chemically competent *P. aeruginosa* cells were prepared as previously described (16) by using nutrient yeast broth.

Mutant construction and analysis. Suicide vectors for *pilR* (pJIR2586) and *rpoN* (pJIR2596) were constructed by amplifying the *pilR* (primers 18635 [GGGTACCTTAATGCTCAATGCCGTTGCC] and 19397 [GGAATTCATAATGATCGCTCGTCTCGC]) and *rpoN* (primers 19606 [GTTTCCCAGGCATACTTAGG] and 19760 [GGGTACCTATTCGGCACATTATCAACA]) genes by PCR and cloning each product into the Asp718/EcoRI and Asp718/BamHI sites of pBluescript SK(+), respectively. Unique internal restriction sites in *pilR* (ClaI) and *rpoN* (EcoRI) were then used to insert an erythromycin resistance cassette, *erm*(B).

Mutant strains were constructed by inserting the suicide vectors into *D. nodosus* VCS1703A by natural transformation (31). Two independently isolated

mutants were obtained for each gene and analyzed by PCR using 16S rRNA gene primers to confirm that the strains were *D. nodosus* and using *erm*(B) primers to detect the presence of the *erm*(B) gene (40). To confirm that each gene was insertionally inactivated, a combination of *erm*(B) primers and gene-specific primers was used. To confirm that the antibiotic-resistant strains were derived from the wild-type strain, PCR-restriction fragment length polymorphism analysis of the outer membrane protein-encoding gene *omp1* was performed (19).

Phenotypic analysis. Qualitative protease (40) and elastase (52) assays, quantitative azocaseinase assays (31), twitching motility assays (31), and transmission electron microscopy (31) were performed as previously described. Slide agglutination assays were performed using a 10- μ l aliquot of serogroup G fimbrial antibodies (1:10 dilution; John Egerton, University of Sydney) mixed with a loopful of cells from a 2-day-old EYE agar culture.

Adhesion assay. Semiconfluent CHO-K1 cells, grown in 24-well tissue culture plates, were washed three times with prewarmed (37°C) phosphate-buffered saline (PBS) before the addition of 500 μ l of prewarmed α -minimal essential medium (1 \times HEPES buffer [25.9 mM HEPES, 11.5 mM NaOH, and 0.0005% phenol red], 8% fetal calf serum; Gibco) containing 0.5% mannose. *D. nodosus* cells were removed from EYE agar cultures grown for 48 h using 1.5 ml of prewarmed PBS. Fifty microliters of bacterial culture (turbidity at 600 nm of 0.4) was added to each cell, and the mixture was allowed to incubate for 6 h at 37°C in the anaerobic chamber. After incubation, the cells were washed four times with prewarmed PBS, and the wells were resuspended in 100 μ l of 0.1% (wt/vol, in PBS) digitonin and allowed to sit for 5 min to lyse the CHO-K1 cells. The digitonin solution was then removed, serially diluted, subcultured onto EYE blood agar, and incubated for 5 days at 37°C in the anaerobic chamber. Viable counts were then compared to viable counts done on the original cultures. Adhesion ratios were then calculated relative to wild-type levels. Adhesion assays were performed in triplicate on three separate occasions.

RNA extraction and analysis. RNA was extracted from *D. nodosus* cells grown in EYE broth using TRIzol (Invitrogen) according to the manufacturer's instructions. To determine if *pilS* and *pilR* were cotranscribed, reverse transcriptase (RT) PCR was performed using primers 25964 (CGGGGTTAGGTTTATTTTAG) and 26714 (GCAGCAACAAATAACCCATCC) as described elsewhere

previously (31). Quantitative real-time RT-PCR (QRT-PCR) was performed with an ABI PRISM 7700 sequence detector as described previously (40) using primers JRP2044 (ACATCGCTCGTACCCAGGTT) and JRP2045 (AAGTTGTCAGCGATACGGATTTTAA) for *fimA*, JRP2042 (CGGAATGACTGGGC GTAAA) and JRP2043 (CTAGGTTGAGCCAGGATTT) for 16S rRNA, JRP2724 (CGCATCAGTTAATTTCCGTAGGT) and JRP2725 (ACTCCTTGGAATAGCCATTTGC) for *gdn1292*, JRP2728 (GCAATCCGGACCTTTGT TGA) and JRP2729 (CGCCGCTTTATGATTTTTCG) for *gdn1329*, JRP2730 (ACGATTCACGGCGAAGATATG) and JRP2731 (CCAAGCTTTCAATGC GATCA) for *gdn1330*, and JRP2732 (CGCCGCCGAGGAA) and JRP2733 (GATGCGTTCCGGCTTTCATTT) for *gdn1331*. The data were standardized to the control 16S rRNA gene levels, and Student's *t* tests were performed to determine significance ($P < 0.05$).

Primer extension reactions on the *fimA* promoter region were performed using 50 μ g of RNA and primer 22796 (TCATGAGTTCGATTAAGGTGA) with the Primer Extension System-AMV Reverse Transcription (Promega) according to the manufacturer's instructions. Reaction mixtures were ethanol precipitated overnight and run on 8% acrylamide sequencing gels alongside a sequencing ladder generated with primer 22796 and a pJIR2776 template using [α - 35 S]dATP and the T7 sequencing kit (USB) according to the manufacturer's instructions.

Microarray hybridization and analysis. Microarrays consisted of 2,317 custom 70-mer oligonucleotides (designed to reduce cross-hybridization) spotted in triplicate on Ultra-GAPS slides (Corning) using a QArray Mini (Genetix) arrayer, representing 1,343 open reading frames (ORFs) determined from the unpublished *D. nodosus* genome sequence (<http://www.tigr.org>). Labeled cDNA was synthesized using amino-allyl dUTP at a 3:2 ratio with dTTP. Random hexamers (6 μ g) were annealed to 5 μ g of RNA in RT reaction mixtures that contained 20 U of RNasin (Promega); 10 mM dithiothreitol (DTT); 400 U Superscript II (Invitrogen); 500 μ M dATP, dCTP, and dGTP; 200 μ M dTTP; and 300 μ M amino-allyl dUTP. Reaction mixtures were allowed to incubate overnight at 42°C. RNA was hydrolyzed at 65°C for 15 min in the presence of 200 mM NaOH and 100 mM EDTA before neutralization in 300 mM Tris-HCl, pH 7.4. Reaction mixtures were purified with Microcon columns (Millipore) and dried using a Speedvac SVC (Savant). The amino-allyl-labeled cDNA was coupled to mono-reactive Cy3 or Cy5 dye (Amersham Biosciences) for 2 h at room temperature in the dark in 50 mM sodium bicarbonate buffer (pH 9) and 50% dimethyl sulfoxide before purification using Microcon columns and drying under vacuum. Typically, 15 μ g of labeled cDNA (Cy3 and Cy5 cDNA probes) was hybridized to the slides.

Slides were prepared for hybridization by incubation at 42°C for 45 min in a solution containing 25% formamide, 5 \times SSC (1 \times SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% sodium dodecyl sulfate (SDS), 10 mg/ml bovine serum albumin, and 1 mg/ml salmon sperm DNA under a coverslip in a humidified chamber. The coverslip was removed in double-distilled water before drying by centrifugation for 10 min at 1,000 \times g. Hybridizations were carried out overnight at 42°C under a coverslip in humidified CMT-hybridization chambers (Corning) in the presence of a solution containing 25% formamide, 5 \times SSC, 0.1% SDS, and 1 mg/ml salmon sperm DNA. Probes were heated at 95°C for 5 min, cooled slightly, and then applied to the slides. After hybridization, the slides were washed once in 2 \times SSC–0.1% SDS at 42°C for 5 min, once in 0.1 \times SSC–0.1% SDS for 10 min at room temperature with mixing, and four times for 30 s each in 0.1 \times SSC and were then rinsed once in double-distilled water, dried by centrifugation, and scanned.

Slides were scanned using a GMS 418 microarray scanner (Genetic Microsystems), and the Cy3 and Cy5 channels were combined and quantified using ImaGene version 5.1 software (Biodiscovery). A minimum of three slides from separate biological replicates, including dye swaps, were used in the data analysis. Expression values for each gene were determined using BASE v1.2.9 software (46). Low-intensity spots were removed before global median ratio and lowess normalizations (63) were performed, and ratios (*n*-fold) were determined. The statistical significance of the derived ratios was determined using a Wilcoxon signed-rank test. Genes that were consistently twofold up- or down-regulated across the majority of slides, with a *P* value of <0.005 , were designated differentially expressed.

Protein expression and purification. To express PilR as a maltose binding protein (MBP) fusion, the *pilR* gene was cloned into pMAL-c2 using primers 25321 (GGAATTC AATCACGCGTTAGTTGTTG) and 25322 (GCTCTAGA TCAATCAATTCCTAATTTTTTAA), which contained EcoRI and XbaI sites, respectively. The resultant plasmid, pJIR2824, was used to transform *E. coli* C43 cells to ampicillin resistance. The cells were grown in 500 ml of 2 \times YT containing ampicillin to a turbidity at 600 nm of 0.5 and then induced with 0.3 mM isopropyl- β -D-thiogalactopyranoside (IPTG). The cultures were grown for an additional 3 h before pelleting at 6,000 \times g for 10 min at 4°C. The pellets were washed in Tris buffer (20 mM Tris-HCl, pH 7.8, 250 mM NaCl) and lysed using a French

press. After centrifugation at 12,000 \times g for 30 min at 4°C, the supernatant was mixed with amylose resin (NEB) equilibrated in the same buffer. The resin mixture was applied to a Poly-Prep chromatography column (Bio-Rad) and washed with 20 ml of Tris buffer. PilR was eluted in 2-ml fractions using Tris buffer containing 10 mM maltose. Elutions containing high concentrations of protein were then pooled and subjected to dialysis overnight at 4°C in dialysis buffer (20 mM Tris-HCl, pH 7.8, 250 mM NaCl, 50% glycerol) using dialysis tubing (Spectra/Por; Spectrum Laboratories) with a 12,000- to 14,000-Da cutoff.

Gel filtration analysis. Proteins (2 to 10 mg) were separated on a Superdex 200 16/60 column (Amersham Biosciences) that was equilibrated in a solution containing 20 mM Tris-HCl, pH 7.8, 300 mM NaCl, and 1 mM DTT. Proteins were eluted at a flow rate of 0.4 ml/min, and fractions of 1 ml were collected and analyzed by 12% SDS-polyacrylamide gel electrophoresis (PAGE). Molecular sizes were determined by calibrating the column with standard proteins (gel filtration calibration kit [Bio-Rad] and gel filtration high-molecular-weight markers [Amersham Biosciences]).

Immunoblot analysis. Samples to be separated by SDS-PAGE were isolated from either whole-cell lysates (31) or surface fimbriae. Surface fimbriae were isolated from vortexed suspensions of 2-day-old EYE blood agar cultures. Samples were resolved on 12% polyacrylamide gels and transferred onto nitrocellulose membranes (PROTRAN; Schleicher & Schuell). Blots were blocked with 5% (wt/vol) skim milk powder, washed in Tris-buffered saline (5 mM Tris-HCl, pH 7.4, 15 mM NaCl) containing 0.05% Tween 20, and probed with polyclonal rabbit anti-FimA antibodies (dilution, 1:1,000; John Egerton, University of Sydney) or polyclonal rabbit anti-*P. aeruginosa* PilR antibodies (1:3,000; Jessica Boyd, Harvard University) (5). The blots were then washed and probed with sheep anti-rabbit antibodies (1:2,000) conjugated to horseradish peroxidase (Chemicon) before detection using the Western Lightning chemiluminescence reagent (Perkin-Elmer) according to the manufacturer's instructions.

Gel mobility shift assays. DNA for gel shift reactions (amplified using primers 22795 [GGCGCGTAAAATGCTGTGA] and 22797 [GCCTATTATTAGC AGCGTTA] for *fimA* and 24915 [GGAATTCATATGCAGCGCGGGAAA ATAAAAT] and 24935 [CCGCTCGAGTCCCTCGATGCAGATGATT] for *pilS*) was labeled using terminal transferase (Roche) using [α - 32 P]dATP (Perkin-Elmer) according to the manufacturer's instructions for 3' end labeling with radioactive nucleotides. Labeling reaction mixtures were then purified with the QIAquick PCR purification kit (QIAGEN) according to the manufacturer's instructions before counting on a TriCarb 2100TR Liquid Scintillation Analyser (Packard). Gel shift reactions were carried out in 20- μ l volumes containing binding buffer (100 mM HEPES, pH 7.6, 50 mM ammonium sulfate, 5 mM DTT, 1% [vol/vol] Tween 20, 150 mM KCl, 5 mM MgCl₂, 1 μ g poly(dI-dC) (Amersham Biosciences), 4,000 cpm of DNA (0.05 to 0.5 fmol) and the desired concentrations of protein (15 to 1,500 nM). Reaction mixtures were incubated for 15 min at room temperature, and 5 μ l of loading buffer (0.25 \times TBE [1 \times TBE contains 89 mM Tris-HCl, pH 8.0, 89 mM boric acid, 2 mM EDTA], 60% glycerol, 0.2% [wt/vol] bromophenol blue) was then added before the samples were loaded onto the gel. Samples were loaded onto 4% acrylamide gels containing 0.25 \times TBE and run for 3 h at 200 V.

Microarray accession numbers. Microarray data have been deposited at the National Center for Biotechnology Information's Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/geo/>) under the accession numbers GSE3465 (*rpoN*) and GSE3466 (*pilR*).

RESULTS

The *D. nodosus* genome contains *pilR* and *rpoN* homologues.

Little is known about the regulation of virulence factors in *D. nodosus*, with a *fur* homologue being the only regulatory gene that has been mutated and analyzed (40). To identify genes whose products may be involved in the regulation of fimbrial biogenesis in *D. nodosus*, the unpublished genome sequence (<http://www.tigr.org>) was analyzed using standard bioinformatic tools. Three genes with a potential coordinated role in fimbrial gene regulation were identified, *pilR*, *pilS*, and *rpoN*.

PilR was identified based on its 55% identity to the PilR regulator of pilin production in *P. aeruginosa* (26). PilR was a putative 528-amino-acid protein with a predicted size of 58.8 kDa, 83 residues larger than its *P. aeruginosa* homologue. These 83 residues were localized to a single C-terminal region.

PilR belonged to the NtrC family of response regulators (53) and had significant identity (40%, over 75% of the protein) to NtrC from *Klebsiella pneumoniae* (15). NtrC-type regulators contain three domains, a response regulator receiver domain (pfam00072), a σ^{54} interaction/ATPase domain (pfam00158) that contains a Walker A motif, and a DNA binding domain, all of which were present in PilR from *D. nodosus*. Only one other putative response regulator was identified with the domain hallmarks of NtrC family proteins, but it did not display similarity to PilR. The NtrC ATPase domain contains a motif, GAFTGA (present as GSFTGA in *D. nodosus* PilR), that is unique to this class of activators and is thought to be required for interaction with the σ^{54} subunit of RNAP (57, 64). The putative DNA binding domain of *D. nodosus* PilR, which in NtrC-like activators is required for dimerization, was shown to contain a helix-turn-helix motif by using established algorithms (14, 42).

The gene encoding the putative sensor of this two-component system, *pilS*, was located upstream of *pilR*. PilS had 26% identity to PilS from *P. aeruginosa* (21), was 581 residues in size, and was predicted to have six transmembrane domains. Similar identity (26%) was also observed with the PilS protein of *Xanthomonas axonopodis*, which is not involved in fimbrial synthesis (62). PilS contained the conserved histidine residue that is the site of phosphorylation (pfam00512) and GXGL, DXGXG, N, and F motifs, which are conserved in sensor histidine kinases and are thought to form a nucleotide binding surface (41, 54). The region containing the putative site of phosphorylation was highly conserved in *D. nodosus* and *P. aeruginosa*, with more variation occurring in the N-terminal sensor region of the protein. Only 9 bp separated the stop and start codons of *pilS* and *pilR*, respectively, which indicated that the two genes may represent an operon. RT-PCR using a primer internal to *pilS* and a second primer located 677 bp into the coding region of *pilR* confirmed that *pilS* and *pilR* were cotranscribed (data not shown).

The alternative σ factor, σ^{54} (encoded by *rpoN*), is associated with the regulation of pilin production in *P. aeruginosa* and flagellar synthesis in numerous organisms (7, 25, 56). The product of *rpoN* from *D. nodosus* was predicted to be a 469-amino-acid protein, 53.8 kDa in size, and was 35% identical to σ^{54} factors of *P. aeruginosa* and *E. coli*. The *D. nodosus* protein was predicted to contain all the domains consistent with its assignment as σ^{54} : an activator interaction domain (pfam00309) that is involved in conformational changes necessary for open complex formation (11), a central core RNA polymerase binding domain (pfam04963) (7), and a C-terminal region required for DNA binding (pfam04552) (7). The DNA binding domain contained a helix-turn-helix motif (14) that in enteric species has been shown to be involved in binding to the -12 promoter element (59). A second motif associated with DNA binding, the RpoN box, is associated with interactions at the -24 promoter region (8) and was conserved in 9 of 10 motif residues in the *D. nodosus* σ^{54} protein.

***pilR* and *rpoN* are required for fimbrial production.** Since type IV fimbriae are an essential *D. nodosus* virulence factor, it was important to determine if *pilR* and *rpoN* were involved in fimbrial biogenesis. Therefore, insertionally inactivated *pilR* and *rpoN* mutants were constructed by allelic exchange. Suicide vectors were constructed and introduced into *D. nodosus*

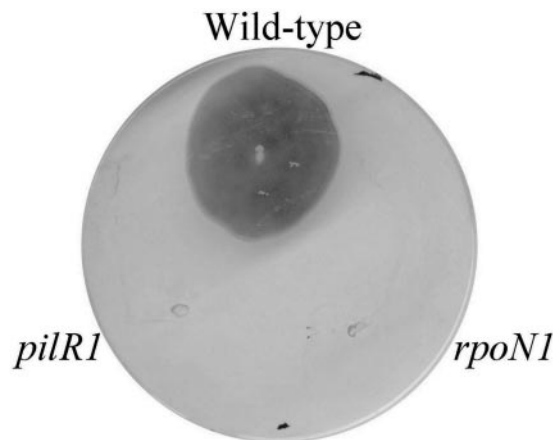


FIG. 1. Twisting motility analysis of *pilR* and *rpoN* mutants. Strains were stab inoculated into 1% EYE agar plates and incubated for 7 days, and the agar plates were then compressed and stained with Coomassie blue.

by natural transformation, and erythromycin-resistant colonies were selected. PCR and restriction fragment length polymorphism analysis confirmed that the genes had been disrupted by double-crossover events (data not shown). Two independently derived mutants were obtained for each gene. Since their phenotypic properties were the same, detailed analysis focused on JIR3772 (*pilR1*) and JIR3887 (*rpoN1*).

Both types of mutants had an altered colony morphology. The colonies were convex, differing from the usual spreading phenotype that is a result of their twitching motility. This altered phenotype was consistent with a loss of fimbriae, which was first confirmed by a slide agglutination assay using fimbria-specific antiserum. Also consistent with a loss of surface fimbriae was the observation that the mutants were no longer able to undertake twitching motility, as determined by using a sub-surface stab assay of agar medium (Fig. 1) (31). Electron microscopy showed that no surface fimbriae were present in the mutants (Fig. 2B), unlike the wild-type strain, which had numerous polar fimbriae (Fig. 2A). This observation was confirmed by Western immunoblotting of sheared surface fimbriae using fimbrial antiserum (Fig. 2C). Since the fimbriae of *D. nodosus* are essential for natural transformation (31), the loss of surface fimbriae made the *pilR* and *rpoN* mutants no longer transformable. Therefore, for technical reasons, it was not possible to complement these mutants, as there are no other genetic exchange methods available for *D. nodosus*.

Western immunoblotting was also performed on whole-cell lysates to determine if the fimbrial subunit FimA was still being produced even though surface fimbriae were absent. The results (Fig. 2D) showed that FimA levels were significantly reduced in both the *pilR* and *rpoN* mutants, with almost negligible levels present in the *pilR* mutants. Determination of the *fimA* transcript levels by QRT-PCR showed that the loss of fimbriae in the *pilR* and *rpoN* mutants was due to a significant reduction in the level of *fimA* (*pilR/pilR*⁺ transcript ratio of 0.00078 ± 0.00043 ; *rpoN/rpoN*⁺ transcript ratio of 0.012 ± 0.0081).

***D. nodosus pilR* does not complement a *P. aeruginosa pilR* mutant.** Previous studies (30) showed that the *D. nodosus*

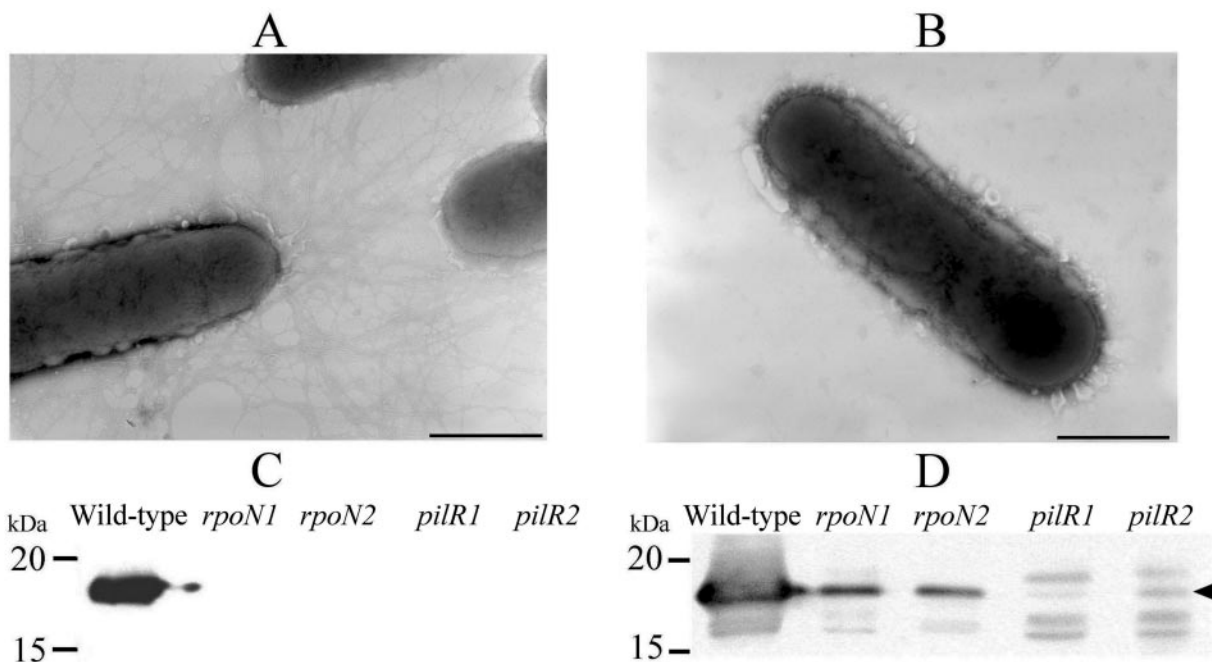


FIG. 2. Absence of fimbriae in *pilR* and *rpoN* mutants. The presence of surface fimbriae was observed using electron microscopy. (A and B) Wild-type cells showing the presence of fimbriae (A) and the *pilR* mutant lacking surface fimbriae (B). Note that the *rpoN* mutants appeared similar to those shown in B. Bars equal 1 μ m. The presence of surface fimbriae was also determined using Western immunoblotting with fimbrial antisera on sheared fimbriae (C) and whole-cell lysates (D).

prepilin peptidase gene, *fimP*, can complement its homologous *P. aeruginosa pilD* mutant. To determine if the sequence identity between the PilR proteins of *D. nodosus* and *P. aeruginosa* was sufficient to enable genetic complementation, the *pilSR* region of *D. nodosus*, including 900 bp upstream of *pilS*, was cloned into the shuttle vector pUCP18 and introduced into *P. aeruginosa pilR* mutant R94 (21). The results showed that the *pilR* mutant was not complemented by the *D. nodosus pilSR* operon since the recombinant strain did not produce fimbriae, nor was it able to undergo twitching motility. RT-PCR was used to confirm that the *D. nodosus pilR* gene was being transcribed. Western immunoblotting using antisera against *P. aeruginosa* PilR revealed a cross-reacting band, approximately 60 kDa in size, which was consistent with the size of *D. nodosus* PilR. This band was observed in the complemented strain but was absent in the wild-type and *pilR* *P. aeruginosa* strains (data not shown). These data indicate that sequence similarity does not necessarily correlate with the ability to carry out functional complementation in these different bacterial species. Alternatively, the sequence variation between the PilS homologues may mean that the environmental signal required to activate the *D. nodosus* PilS protein was not present.

***D. nodosus* fimbriae are required for adherence.** Type IV fimbriae are involved in many biological functions, including the attachment to various surfaces and epithelial cells (36, 55). The type IV fimbriae of *D. nodosus* are essential for virulence (31), but their role in attachment is yet to be determined. To determine if the type IV fimbriae were required for adherence, an assay was established using Chinese hamster ovary epithelial cells (CHO-K1). The adherence capabilities of the wild-type strain, the *pilR* and *rpoN* mutants, and a *fimA* mutant (31)

were determined in an adherence assay undertaken for 6 h in an anaerobic chamber. The *fimA* mutant was included as a control for any pleiotropic effects caused by the *pilR* or *rpoN* mutations. The CHO-K1 cells were examined microscopically to confirm that they were not affected by the anaerobic conditions. The results showed that wild-type *D. nodosus* cells were adherent and that this adherence was dependent on the presence of the type IV fimbriae (Fig. 3). The *pilR*, *rpoN*, and *fimA* mutants, which all lacked surface fimbriae, showed a decreased ($\geq 90\%$ reduction) ability to adhere compared to the wild-type strain.

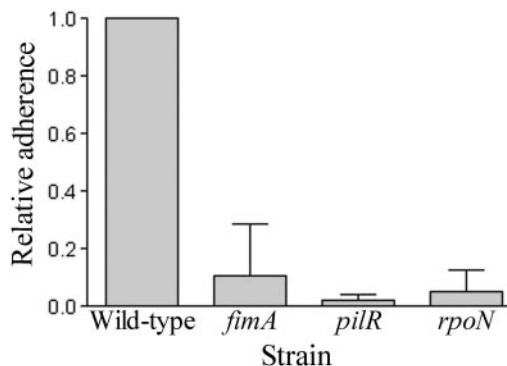


FIG. 3. Fimbriae mediate adherence to epithelial cells. *D. nodosus* cells were incubated with CHO-K1 cell monolayers for 6 h anaerobically and washed, and viable counts were determined. Levels of adherent bacteria are shown relative to the wild-type level of adherence.

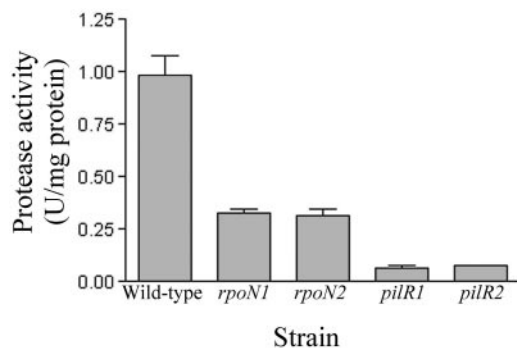


FIG. 4. Reduced extracellular protease activity in *pilR* and *rpoN* mutants. Culture supernatants from wild-type, *rpoN*, and *pilR* strains were assessed for protease activity using azocasein as the substrate.

Extracellular protease activity correlates with levels of the fimbrial subunit. Type IV fimbriae play a role in protein secretion, and it has been postulated that the fimbrial extension and retraction reactions involved in twitching motility may act to pump proteases out of the cell (48). As mutants lacking fimbriae in both *D. nodosus* (31) and *P. aeruginosa* (35) have reduced extracellular protease activity, this property was examined with the *pilR* and *rpoN* mutants. Qualitative plate assays detecting elastase (52) and caseinase activity revealed an absence of elastase activity and a significant reduction in caseinase activity in both types of mutant. This reduction in activity was quantitated using culture supernatants and an azocasein substrate (Fig. 4). Extracellular protease activity levels were significantly reduced ($P < 0.05$) in all of the *pilR* and *rpoN* mutants compared to that of the wild type, consistent with their loss of fimbriae (31). Extracellular protease activity levels were also different in the *pilR* mutant compared to that of the *rpoN* mutant ($P < 0.05$). Protease secretion by the *pilR* mutants was less than that by the *rpoN* mutants (Fig. 4), commensurate with the different levels of fimbrial subunit produced by the two types of mutant (Fig. 2D). RNA dot blots that were carried out using separate probes specific for the three known *D. nodosus* extracellular protease genes (*aprV2*, *aprV5*, and *bprV*) did not reveal any significant differences, indicating that the reduction in extracellular protease levels was not due to direct regulatory effects on the transcription of the protease genes by PilR and σ^{54} . Note that no extracellular protease activity was observed in a triple *aprV2 aprV5 bprV* mutant (R. M. Kennan, D. Parker, D. W. Wong, and J. I. Rood, unpublished results).

***fimA* is transcribed from a PilR- and σ^{54} -dependent promoter.** The transcriptional start point of *fimA* has been determined previously from strains of two different serogroups (serogroups A and H) (22). This work showed the presence of a major transcript starting 63 bp upstream of the ATG start codon and two less intense bands 45 and 46 bp upstream. Primer extension analysis of our type G wild-type strain indicated the presence of one major product that corresponded to a start point 63 bp upstream of ATG (Fig. 5), indicating that the same start site is used in this strain. No additional products were observed. Upstream of the start site, we identified a σ^{54} -dependent promoter with the conserved -12 GC and -24 GG elements. The position of the -12 element was 13 bp upstream of the start site, which occurs most frequently (27%)

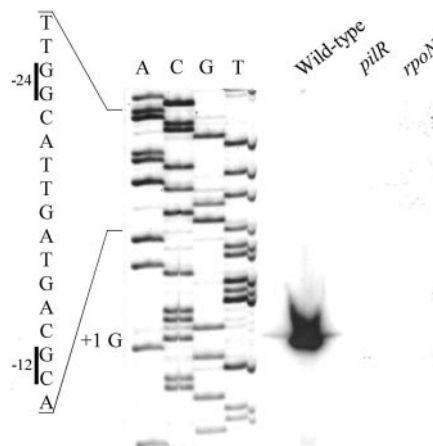


FIG. 5. *fimA* is transcribed from a PilR- and σ^{54} -dependent promoter. Primer extension reactions were performed on 50 μ g of RNA from wild-type, *pilR1*, and *rpoN1* strains using a primer 42 bp downstream of the translational start site. Shown is the σ^{54} promoter region with the -12 and -24 elements indicated along with the transcriptional start point. Product sizes were determined by comparison to a sequencing ladder generated using the same primer (lanes A, C, G, and T).

among σ^{54} -dependent promoters (2). The guanine start site is also the most common: 64% of the starting bases are purines (2). In lieu of this putative σ^{54} -dependent promoter (22), we went on to determine if transcription from this start site was dependent on σ^{54} and PilR. Primer extension analysis was repeated on RNA isolated from the *rpoN* and *pilR* mutants. The results revealed an inability to obtain extension products from these mutants (Fig. 5), even upon longer exposure, which indicated that the *fimA* promoter was dependent on both σ^{54} and PilR.

PilR binds to a region upstream of the *fimA* promoter. To investigate the role of PilR in the regulation of fimbrial biogenesis in *D. nodosus* further, PilR was overproduced and purified. PilR was initially expressed with a hexahistidine tag, but this fusion protein was insoluble. To increase its solubility, PilR was produced with an N-terminal MBP tag. Upon induction, this construct was overexpressed, and the soluble fusion protein was purified using amylose affinity chromatography to $>90\%$ purity (Fig. 6A).

NtrC-like proteins are typically present as dimers in solution (32) and are able to oligomerize into tetramers and bind at tandem recognition sites upon phosphorylation (61). To determine the multimeric state of nonphosphorylated PilR, the fusion protein was applied to a Superdex 200 16/60 gel filtration column. The elution profile indicated that there were four reproducible and distinct peaks (Fig. 6B) that all gave a dominant protein band corresponding to the size of MBP-PilR when analyzed by SDS-PAGE (Fig. 6C). When these peaks were compared to gel filtration standards, the peaks corresponded to an aggregate at the void volume (Fig. 6B, peak 1), an octamer (Fig. 6B, peak 2), a tetramer (Fig. 6B, peak 3), and a dimer (Fig. 6B, peak 4). Most of the protein was present in the higher multimeric states as well as in the aggregate. The presence of these multiple forms is consistent with the need for

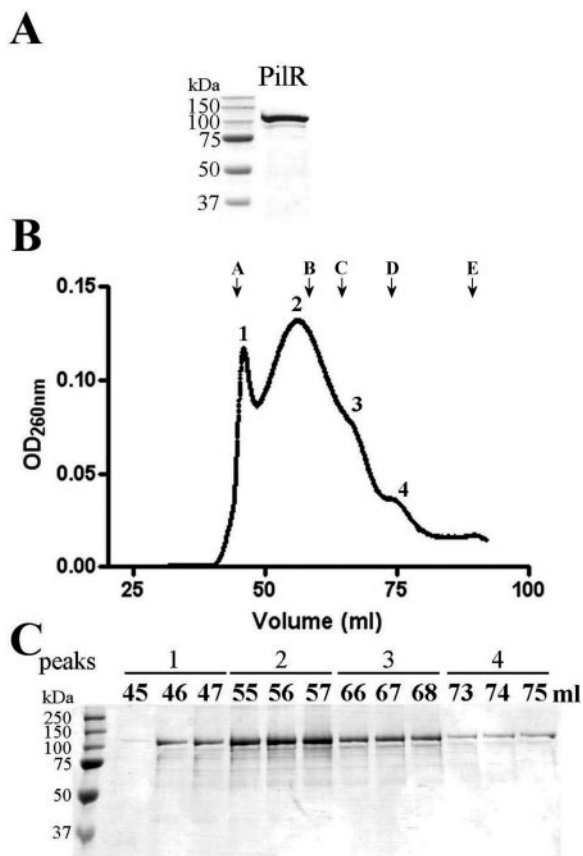


FIG. 6. Purification and gel filtration analysis of PilR. (A) PilR purification. PilR was purified as an MBP-tagged fusion using affinity chromatography. Samples were run on SDS-PAGE gels and stained with Coomassie blue. (B) Gel filtration elution profile of MBP-PilR. MBP-PilR was separated on a Superdex 200 gel filtration column, and peaks 1 to 4 correspond to the aggregated, octameric, tetrameric, and dimeric forms, respectively, based on comparisons to standard proteins (see Materials and Methods). Elution volumes of standards are indicated as follows: A, blue dextran (2 MDa); B, thyroglobin (670 kDa); C, ferritin (440 kDa); D, gamma globulin (158 kDa); E, ovalbumin (44 kDa). OD_{260nm}, optical density at 260 nm. (C) SDS-PAGE analysis of selected fractions corresponding to the different peaks (1 to 4) from the gel filtration elution profile and stained with Coomassie blue. Markers are Precision Plus protein standards (Bio-Rad).

NtrC-like transcriptional activators to have the ability to oligomerize.

To identify the site at which PilR binds to the *fimA* promoter region, gel mobility shift assays were carried out. NtrC proteins typically bind from 100 bp (29) to 1 kb (38) upstream of the target promoter. Two ³²P-labeled DNA fragments were used as targets: a 370-bp fragment encompassing the transcriptional start site and promoter region and a 359-bp fragment that was upstream of the promoter and corresponded to a region 234 to 593 bp upstream of the *fimA* transcriptional start site. These DNA fragments were incubated with various concentrations of PilR (15 to 1,500 nM) and separated on 4% polyacrylamide gels. A concentration-dependent shift of DNA was observed with the 359-bp fragment but not with the 370-bp promoter-containing fragment (data not shown). Binding appeared to be specific for PilR, since purified MBP did not bind (data not

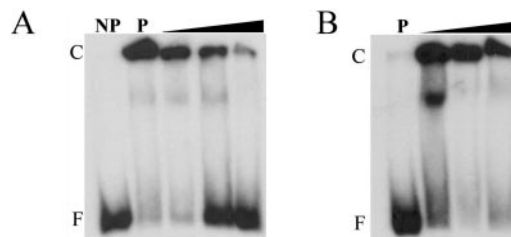


FIG. 7. DNA binding of PilR to the *fimA* promoter region. (A) A 359-bp ³²P-labeled DNA fragment located upstream of the *fimA* promoter was incubated with PilR and increasing concentrations (0.005, 0.05, and 0.5 fmol) of unlabeled competitor DNA and analyzed using gel mobility shift analysis on 4% polyacrylamide gels. Lanes are as follows: NP, no protein; P, 150 nM PilR and no competitor DNA and increasing concentrations (10 to 10,000 times) of specific competitor DNA, compared to labeled DNA. The locations of free DNA (F) and protein-DNA complexes (C) are indicated. (B) Gel mobility shift analysis of PilR and the *fimA* promoter region incubated with increasing concentrations of nonspecific unlabeled competitor DNA.

shown). To confirm that binding was specific for the target site, PilR (150 nM) was incubated with the 359-bp fragment and various levels (10 to 10,000 times) of specific and nonspecific (*pilS* coding sequence) competitor DNA. When competed with nonspecific DNA, no significant changes were observed (Fig. 7B). However, the addition of specific unlabeled DNA resulted in a consistent decrease in protein-DNA complexes and an increase in free, unbound DNA, confirming the specificity of the binding reaction (Fig. 7A). PilR was also shown to be able to bind, with slightly reduced affinity, to a 217-bp fragment that corresponded to a region 376 to 595 bp upstream of the transcriptional start site (data not shown). Further attempts to reduce the size of the PilR target site did not lead to the formation of PilR-DNA complexes.

Global transcriptional profiling of *D. nodosus pilR* and *rpoN* mutants. To determine the full complement of genes regulated by PilR and σ^{54} in *D. nodosus*, the transcriptome of *D. nodosus pilR* and *rpoN* mutants was compared to that of the wild-type strain. To profile the transcription of *D. nodosus* genes, 70-mer oligonucleotide microarrays were constructed using 2,317 gene-specific probes, representing 1,343 ORFs predicted from the genome sequence. The ORFs were determined from the unpublished *D. nodosus* genome sequence (<http://www.tigr.org>), and, where possible, two gene probes per ORF were synthesized.

Microarray analysis was carried out for at least three biological replicates (including dye swaps) using a conservative selection criterion of a more-than-twofold difference with a *P* value of <0.005. Genes that were less-than-twofold regulated were included if they were part of a highly regulated operon. The observation that there was a more-than-fourfold decrease in *fimA* transcript in both mutants (Table 2) agreed with our previous observations that *fimA* transcription was significantly reduced. The microarray data were further validated by performing QRT-PCR on RNA from the wild-type and the *pilR* and *rpoN* mutants by using four of the genes differentially expressed in both strains as targets. The QRT-PCR results confirmed the microarray data, showing significantly reduced transcript levels. As expected, QRT-PCR was a more sensitive

TABLE 2. Differentially expressed genes in the *pilR* and *rpoN* mutants

Gene ID ^a	Putative function	<i>pilR</i> /WT expression ratio		<i>rpoN</i> /WT expression ratio		σ^{54} promoter distance (bp) ^e	Evidence and/or function
		Microarray ^b	QRT-PCR ^c	Microarray	QRT-PCR		
<i>gdn1074</i>	FimA	0.23	0.00078 ± 0.00043	0.15	0.012 ± 0.0081	75	FimA <i>D. nodosus</i>
<i>gdn1291</i>	Outer membrane protein	0.29	NT ^d	0.39	NT	383	Signal sequence, outer membrane OprF of <i>Pseudomonas stutzeri</i> , 37% identity
<i>gdn1292</i>	Outer membrane RTX protein	0.58 (<i>P</i> < 0.15)	0.12 ± 0.06	0.39	0.07 ± 0.02		PSI-BLAST: RTX toxin, leukotoxins
<i>gdn1328</i>	Lipoprotein	0.58 (<i>P</i> < 0.007)	NT	0.64	NT	43	Lipoprotein signal sequence with 61% identity to NMA1549
<i>gdn1329</i>	Unknown	0.57 (<i>P</i> < 0.006)	0.12 ± 0.03	0.46	0.09 ± 0.04		Conserved hypothetical protein
<i>gdn1330</i>	Inner membrane protein	0.31	0.12 ± 0.05	0.44	0.11 ± 0.02		COG1585 membrane protein implicated in regulation of membrane protease activity
<i>gdn1331</i>	Inner membrane protease	0.39	0.16 ± 0.04	0.63	0.19 ± 0.11		COG0330 HflC membrane protease subunit

^a Numbers refer to the annotation at the time of this writing.

^b All expression ratios presented are significant (*P* < 0.005) unless otherwise stated.

^c QRT-PCR ratios are significant (*P* < 0.02).

^d NT, not tested.

^e Distance of the potential σ^{54} binding site from the ATG of the first gene in the operon.

method than microarray analysis for quantifying expression levels.

NtrC family response regulators require σ^{54} for transcriptional activation to occur (7). Therefore, since any genes regulated by PilR should also be differentially expressed in an *rpoN* background, we used this property as a filter to determine the genes of interest. In addition to *fimA*, only two transcriptional units were identified as being coregulated (*gdn1291-2* and *gdn1328-31*) (Table 2). The operon containing *gdn1328-31* was observed as being down-regulated in both the *pilR* and *rpoN* mutants compared to the wild-type. The proteins encoded by this operon were largely hypothetical; however, bioinformatic analysis suggested that they may be involved in protein turnover, with the products of *gdn1331* and *gdn1330* having domains associated with membrane protease activity and the regulation of membrane protease activity, respectively. The second operon regulated by both PilR and σ^{54} had potential virulence functions. The products encoded by *gdn1292* and *gdn1291* were similar to RTX (repeats in toxin) proteins and their accessory proteins, respectively. The product of *gdn1291* showed similarity to outer membrane proteins with OmpA domains. This protein may play a role similar to that of the FrpD protein of *Neisseria meningitidis*, which is involved in an interaction with the RTX protein FrpC of *N. meningitidis* (43). PSI-BLAST (1) analysis indicated that the 300-kDa product of *gdn1292* had similarity to numerous RTX toxins. RTX toxins are cytotoxic proteins with a characteristic repeated motif (G GXGXDX[L/I/V/W/Y/F], where X is any amino acid) (33). Since the putative RTX protein identified here has only two such motifs, less than other family members described to date (33), further studies are required to determine if the GDN1292 protein is a functional RTX protein.

The genes that were observed to be down-regulated in the *rpoN* and *pilR* strains were likely to be directly regulated by σ^{54} . Subsequently, we attempted to identify potential binding sites for σ^{54} by searching the promoter regions of the three transcriptional units for matches to the σ^{54} binding consensus (2). σ^{54} binding sites were identified in all three operons (Table 2). These sites had, as a minimum, the correct spacing and the highly conserved -24 GG and -12 GC elements.

There were also some genes that were differentially expressed in only one of the mutants. Two genes that were only down-regulated by PilR encoded two characterized Omp1 outer membrane proteins of *D. nodosus*. A gene with potential stress response functions was also observed to be up-regulated in the *rpoN* mutant, suggesting a potential role for σ^{54} in the reaction of *D. nodosus* to physiological stress. The putative protein product of this gene had some domain similarity to OstA-like proteins. In *E. coli*, OstA or Imp is σ^E regulated and functions as an outer membrane protein involved in envelope biogenesis and envelope remodeling and may be involved in responding to periplasmic stress (6). Further studies would be required to determine the function of the *D. nodosus* OstA-like protein.

In *D. nodosus*, the *rpoN* gene was located at the end of the putative operon encoding the OstA-like protein. Interestingly, *rpoN* transcription was shown to be increased over twofold in the *rpoN* mutant. The position of the two microarray oligonucleotides, 5' and 3' of the *erm(B)* promoter, excluded the explanation that the altered transcription levels resulted solely from readthrough from the *erm(B)* promoter. The increase in transcription (also observed by RT-PCR) suggested that the cell was compensating for the loss of σ^{54} protein by up-regulating the transcription of this operon.

DISCUSSION

Type IV fimbriae are present in many microorganisms and are required for diverse biological functions, including virulence, cell adhesion, natural transformation, and twitching motility (36). Previous studies suggested that the type IV fimbriae of *D. nodosus* are expressed from a σ^{54} -dependent promoter (22, 30). In this study, we have provided direct evidence that the *fimA* fimbrial subunit gene is regulated at the transcriptional level by a σ^{54} -dependent two-component signal transduction system that requires the PilR response regulator. We also show that the type IV fimbriae of *D. nodosus* are required for adherence, that the levels of subunit expression correlate with extracellular protease activity, and that two other operons are regulated by this system. This study represents a significant

advancement in our understanding of how the ability of *D. nodosus* to cause ovine footrot is regulated.

In *P. aeruginosa* and *M. xanthus*, there is a definite link between the regulation of type IV fimbrial biogenesis and the presence of NtrC-like response regulators and σ^{54} (21, 25, 26, 60). By constructing chromosomal mutants by allelic exchange and then carrying out functional assays and transcriptional analysis, we have shown that this association extends to the regulation of type IV fimbriae in *D. nodosus*. By contrast, although there is an *rpoN* gene in the pathogenic neisseriae, it is not functional, and no biological activity has been attributed to a regulator that has similarity to PilR (9, 34).

In *D. nodosus*, a basal level of *fimA* transcription occurred even in the absence of PilR or σ^{54} , and some fimbrial subunit was still produced in the mutants. The *rpoN* mutant produced more of the fimbrial subunit and the *fimA* transcript than did the *pilR* mutant (Fig. 2D). In the *rpoN* mutant, a low level of *fimA* transcription may occur by σ^{70} -based transcription, analogous to that observed in *Neisseria gonorrhoeae* and *Neisseria meningitidis* (10, 18). In the *pilR* mutant, there may be a basal level of *fimA* transcription that occurs in a PilR-independent manner. Although an additional transcriptional start site was not detected in these studies, there is evidence for an alternative minor promoter in this system (22).

PilR was shown to bind specifically to a region 234 to 593 bp upstream of the *fimA* start site. This binding region is much further upstream than that observed for *P. aeruginosa* PilR, which bound to the region from positions -120 to -80 (29) and to NtrC-like regulators in general. However, studies with NtrC have shown that it is able to activate transcription when its promoter is some distance from the transcriptional start site (38, 44). The significant distance of the PilR binding region from the *fimA* transcriptional start site may connote a role for the integration host factor (IHF) in our system. We have identified a potential IHF binding site upstream of *fimA* (positions -58 to -46) as well as genes encoding IHF in the *D. nodosus* genome (G. S. Myers, D. Parker, R. M. Kennan, J. G. Songer, I. T. Paulsen, and J. I. Rood, unpublished data). IHF may act to bend the DNA to allow the interaction between PilR and σ^{54} -RNAP. It is proposed that σ^{54} binds to core RNA polymerase and that the resultant holoenzyme then binds to the promoter region. At this stage, transient interactions between σ^{54} -RNAP and PilR may occur, a process facilitated through intrinsic DNA curvature or DNA bending induced by factors such as IHF. Phosphorylation of PilR may induce the conformational change necessary for the hydrolysis of ATP and the conversion of σ^{54} -RNAP into an open transcriptional complex, allowing transcription to occur. Phosphorylation in similar systems normally induces activator oligomerization and DNA binding (44, 58, 61). Phosphorylation may also alter the monomer/oligomer ratio, leading to a higher proportion of larger multimers.

One function of type IV fimbriae is to mediate adherence to various surfaces and cell types (36, 55). Although type IV fimbriae are essential for virulence in *D. nodosus* (31), their precise role in the disease process is yet to be determined. This study has shown for the first time that the fimbriae of *D. nodosus* are essential for adherence. A requirement for type IV fimbriae in adherence to epithelial cells also has been demonstrated in *P. aeruginosa*, *N. gonorrhoeae*, and *Moraxella bovis*

(24, 27, 45). Loss of adherence was observed in both the *pilR* and *rpoN* mutants at levels similar to those of the *fimA* mutant. However, in both mutant strains, some fimbrial subunit was still being produced, albeit at highly reduced levels. In *M. xanthus*, a threshold level of the subunit protein was required before functional fimbriae were formed (28). This result agrees well with our observations and implies that in *D. nodosus*, a threshold level of fimbrial subunits is also required before fimbriae are produced. In vivo, it is likely that the fimbriae adhere to the epithelium of the interdigital skin, enabling the cells to penetrate into the skin-horn junction by twitching motility and to grow and produce extracellular subtilisin-like proteases that contribute to tissue destruction (3).

There is clearly an association between type IV fimbriae and extracellular protease secretion. It has been observed that inactivation of the fimbrial subunit in *D. nodosus* (31, 35) and *P. aeruginosa* (31, 35) leads to a loss of extracellular protease activity. Previous hypotheses have suggested that as some type II secretion proteins share similarity to fimbrial subunits, the type IV fimbriae may act as a secretion portal, expelling proteases through the motive force of fimbrial extension and retraction (39, 48, 49). In this study, analysis of the *pilR* and *rpoN* mutants showed that the loss of extracellular protease activity was proportional to the levels of the fimbrial subunit, which suggests that the fimbrial subunit interacts with components of the type IV fimbrial apparatus to provide a type II secretion pathway through which the proteases are secreted.

To determine additional regulatory targets of PilR and σ^{54} , the unpublished *D. nodosus* genome sequence was utilized to construct an oligonucleotide microarray and to carry out global transcriptional profiling of the *pilR* and *rpoN* mutants. These experiments represent the first whole-genome transcriptomic study of a type IV fimbrial regulator such as PilR. It appears that this regulatory system, in addition to regulating a known virulence factor, the type IV fimbriae, may also coordinate the regulation of additional putative virulence factors. Reduced expression of genes encoding a putative RTX protein and its outer membrane accessory protein was observed in the *pilR* and *rpoN* mutants. The RTX family includes cytotoxic proteins, hemolysins, leukotoxins, lipases, and metalloproteases (33). The RTX toxins are calcium binding proteins that act on various cells types, are involved in cell lysis, and have been shown to be necessary for virulence in some organisms (4, 33). However, these proteins are not always involved in virulence. For example, the RTX protein of *N. meningitidis* was not required for virulence, and based on this result, it was suggested that it does not have toxic activity (17). Since there is no evidence that *D. nodosus* exhibits any hemolytic activity, further studies are required to determine if the putative RTX protein of *D. nodosus* is an RTX toxin. The outer membrane protein whose expression was differentially regulated with the putative RTX protein is likely to anchor the protein to the cell surface (43). The other putative operon that was down-regulated in the *pilR* and *rpoN* mutants appeared to encode a hypothetical membrane-bound protein turnover system whose role in virulence is unknown.

In summary, we were able to use genomic data to identify the genes involved in the regulation of type IV fimbriae in *D. nodosus*. This system regulates the fimbrial subunit gene *fimA* at the level of transcription using a PilR transcriptional acti-

vator and a σ^{54} -dependent promoter, with PilR binding specifically to a region upstream of this promoter. The fimbriae of *D. nodosus* were shown to be required for adherence, and extracellular protease secretion was correlated with the levels of the fimbrial subunit. Finally, using global transcriptional profiling, for the first time in *D. nodosus*, we were able to show that PilR and σ^{54} regulate additional genes, including two putative operons that may encode other virulence factors.

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