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Type IV Fimbrial Biogenesis Is Required for Protease Secretion and Natural Transformation in *Dichelobacter nodosus*▽

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The objective of this study was to develop an understanding of the molecular mechanisms by which type IV fimbrial biogenesis, natural transformation, and protease secretion are linked in the ovine foot rot pathogen, *Dichelobacter nodosus*. We have shown that like the *D. nodosus* fimbrial subunit FimA, the pilin-like protein PilE and the FimN, FimO, and FimP proteins, which are homologs of PilB, PilC, and PilD from *Pseudomonas aeruginosa*, are essential for fimbrial biogenesis and natural transformation, indicating that transformation requires an intact type IV fimbrial apparatus. The results also showed that extracellular protease secretion in the *fimN*, *fimO*, *fimP*, and *pilE* mutants was significantly reduced, which represents the first time that PilB, PilC, and PilE homologs have been shown to be required for the secretion of unrelated extracellular proteins in a type IV fimbriate bacterium. Quantitative real-time PCR analysis of the three extracellular protease genes *aprV2*, *aprV5*, and *bprV* showed that the effects on protease secretion were not mediated at the transcriptional level. Bioinformatic analysis did not identify a classical type II secretion system, and the putative fimbrial biogenesis gene *pilQ* was the only outer membrane secretin gene identified. Based on these results, it is postulated that in *D. nodosus*, protease secretion occurs by a type II secretion-related process that directly involves components of the type IV fimbrial biogenesis machinery, which represents the only type II secretion system encoded by the small genome of this highly evolved pathogen.

Dichelobacter nodosus is a slow-growing, anaerobic, gram-negative rod that is the principal causative agent of foot rot in ruminants, especially sheep and goats. Ovine foot rot is characterized by the separation of the keratinous hoof from the underlying tissue, leading to lameness, loss of body weight, and reduced wool growth and quality. Therefore, the economic losses from this disease are very significant (72). The outcome of a *D. nodosus* infection is dependent upon the virulence properties of the *D. nodosus* isolate and the climatic conditions, with virulent disease occurring under warm, wet conditions. The virulence factors of *D. nodosus* include type IV fimbriae (34), extracellular serine proteases (36, 37, 39, 63), and potentially, the genomic islands *vrl* and *vap*, which are preferentially associated with virulent strains (9, 65).

Type IV fimbriae or pili are produced by many pathogenic gram-negative bacteria, including *Neisseria gonorrhoeae*, *Pseudomonas aeruginosa*, *Pseudomonas stutzeri*, *Moraxella bovis*, and *D. nodosus* (46). These fimbriae have a polar location on the surface of the cell and are comprised of a conserved structural subunit that generally has an N-terminal methylated residue, often phenylalanine, a conserved hydrophobic N-terminal domain, and a C-terminal disulfide bond (14). Type IV fimbriae mediate attachment and adherence to epithelial cells (50), twitching motility (46), gliding motility (77), cell agglutination, and biofilm and fruiting body formation (12, 59); they act as receptors for bacteriophages (11); and they are required for extracellular protein secretion (34, 42) and natural transformation (7, 26).

Type IV fimbriae are capable of extension and retraction, processes that are integral to twitching motility (70). Current models suggest that pilus extension and retraction reflect fimbrial subunit polymerization and depolymerization events (49) that are mediated by two antagonistic cytosolic hexameric ATPases (32, 43, 51). One of these proteins, known as PilB in *P. aeruginosa* (4, 57) and PilF in *Neisseria* spp. (22), promotes fimbrial polymerization. An inner membrane protein (known as PilC in *P. aeruginosa* [57] and PilG in *N. gonorrhoeae* [75]) appears to be involved in fimbrial assembly by interacting with PilB. The bifunctional prepilin peptidase PilD is encoded by a gene located in a cluster with *pilB* (*pilF*) and *pilC* (*pilG*) and is required for cleavage of the leader peptide and for methylation of the new N-terminal phenylalanine residue of the pilin subunit prior to its assembly into filamentous fimbriae (22, 57).

In addition to the major pilin subunit, several pilin-like proteins that contain an overall positively charged leader peptide preceding a conserved hydrophobic N-terminal domain are involved in type IV fimbrial biogenesis and its associated functions, such as natural transformability and epithelial cell adherence (2, 3, 5, 6, 27, 67, 81, 82). It has been postulated that these pilin-like proteins form the base of the fimbrial structure or initiate, modulate, or cap pilin assembly into the fimbriae (46, 80). Alternatively, as suggested for *Neisseria*, they may be inhibitors of fimbrial retraction (82). In type II secretion systems, their homologs (known as pseudopilins) are involved in pilus-like macromolecular secretin formation (21, 56).

Another important gene cluster, *pilMNOPQ*, is essential for type IV fimbrial biogenesis in *P. aeruginosa* (44, 45), *N. gonorrhoeae* (16, 17), and *Myxococcus xanthus* (78) and is also required for natural transformation in *N. gonorrhoeae* (16, 17). It has been suggested that the PilQ secretin forms a homo-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or characteristic(s)	Source or reference
Strains		
E. coli		
DH5α	F⁻ *endA1 hsdR17*(r_K^- m_K^-) *thi-1* λ⁻ *recA1 gyrA96 relA1 rhoA supE44 deoR* ϕ80d*lacZ*Δ*M15* Δ(*lacZYA-argF*)*U169*	Invitrogen
D. nodosus		
VCS1703A	Serogroup G, transformable virulent isolate	34
JIR3885	VCS1703A *fimO* Ω *aphA-3* (*fimO1*)	Natural transformation with pJIR2618
JIR3886	VCS1703A *fimO* Ω *aphA-3* (*fimO2*)	Natural transformation with pJIR2618
JIR3889	VCS1703A *fimP* Ω*erm*(B) (*fimP1*)	Natural transformation with pJIR2773
JIR3890	VCS1703A *fimP* Ω*erm*(B) (*fimP2*)	Natural transformation with pJIR2773
JIR3895	VCS1703A *fimN* Ω*aphA-3* (*fimN1*)	Natural transformation with pJIR2798
JIR3896	VCS1703A *fimN* Ω*aphA-3* (*fimN2*)	Natural transformation with pJIR2798
JIR3910	VCS1703A *pilE* Ω*erm*(B) (*pilE1*)	Natural transformation with pJIR3088
JIR3911	VCS1703A *pilE* Ω*erm*(B) (*pilE2*)	Natural transformation with pJIR3088
JIR3913	VCS1703A *pilC* Δ(405–2373) Ω*aphA-3* (*pilC1*)	Natural transformation with pJIR3132
JIR3914	VCS1703A *pilC* Δ(405–2373) Ω*aphA-3* (*pilC2*)	Natural transformation with pJIR3132
Plasmids		
pBluescript II SK(+)	Ap^r *lacZ*$^+$ cloning vector	Stratagene
pWSK29	Ap^r *lacZ*$^+$ low-copy-no. cloning vector	79
pUC18K	pUC18 containing *aphA-3* inserted into SmaI site, nonpolar cassette	48
pGEM7Zf(−)	Ap^r *lacZ*$^+$ cloning vector	Promega
pJIR1532	pBluescript II SK(+) containing *tet*(M) located between *D. nodosus rrnA* promoter and terminator	Recombinant
pJIR2412	pBluescript II SK(+) PstI Ω*erm*(B)	60
pJIR2614	pJIR2412 XbaI/BamHIΩ(935-bp PCR product containing 5′ end of *pilQ*) XhoI/KpnI Ω(927-bp PCR product containing 3′ end of *pilQ*)	Recombinant
pJIR2617	pBluescript II SK(+) XbaI/EcoRI Ω(2.1-kb PCR product containing *fimO*$^+$)	Recombinant
pJIR2618	pJIR2617 HpaI Ω*aphA-3*	Recombinant
pJIR2691	pWSK29 containing *tet*(M) located between *D. nodosus rrnA* promoter and terminator	Recombinant
pJIR2714	pJIR2412 EcoRI/HindIII Ω(940-bp PCR product containing internal *pilQ*)	Recombinant
pJIR2769	pBluescript II SK(+) XbaI/BamHI Ω(1.9-kb PCR product containing *fimP*$^+$)	Recombinant
pJIR2770	pGEM7Zf(−) XbaI/BamHI Ω(1.9-kb *fimP* from pJIR2769 XbaI/BamHI)	Recombinant
pJIR2773	pJIR2770 HindII Ω*erm*(B)	Recombinant
pJIR2797	pGEM7Zf(−) XbaI/EcoRI Ω(2.6-kb PCR product containing *fimN*$^+$)	Recombinant
pJIR2798	pJIR2797 EcoRV Ω*aphA-3*	Recombinant
pJIR3087	pGEM7Zf(−) BamHI/XbaI Ω(2-kb PCR product containing *pilE*$^+$)	Recombinant
pJIR3088	pJIR3087 T4-filled HindIII Ω*erm*(B)	Recombinant
pJIR3124	pUC18K XbaI/BamHI Ω(1.4-kb PCR product containing 3′ fragment of *pilC*)	Recombinant
pJIR3132	pJIR3124 T4-filled EcoRI Ω(1.3-kb PCR product containing 5′ fragment of *pilC*)	Recombinant

dodecameric outer membrane complex that is essential for the translocation of the fimbriae across the outer membrane (13).

Type IV fimbrial biogenesis in *D. nodosus* is poorly understood. Previous studies in this laboratory (30) identified three genes in the virulent *D. nodosus* strain A198, *fimN*, *fimO*, and *fimP*, which encode homologs of the *P. aeruginosa* PilB, PilC, and PilD proteins, respectively (57). At the time, due to the lack of a genetic manipulation system in *D. nodosus*, genetic studies were carried out with *P. aeruginosa*. The *D. nodosus fimP* gene complemented a *pilD* mutant of *P. aeruginosa* and was shown to have prepilin peptidase activity, but neither *fimN* nor *fimO* could complement their homologous *pilB* or *pilC* mutations (31). Our recent discovery of natural transformation in *D. nodosus* led to the construction of *fimA* mutants and the finding that the fimbrial subunit protein, FimA, is essential for virulence in sheep and for natural transformation and is involved in protease secretion (34).

In this study, we describe the identification and characterization of genes involved in type IV fimbrial biogenesis in *D. nodosus* and report the functional genetic analysis of the *fimN*, *fimO*, *fimP*, and *pilE* (encodes one of five putative pilin-like proteins) genes. The results demonstrate that *D. nodosus* has most of the genes known to be required for type IV fimbrial biogenesis but that there is a significant difference between *D. nodosus* and other type IV fimbriate organisms, including *Pseudomonas* and *Neisseria* spp. We provide experimental evidence to support the hypothesis that the type IV fimbrial assembly apparatus is part of the only type II secretion system in this bacterium.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* DH5α cells used for plasmid propagation and cloning experiments were grown at 37°C in 2× YT medium (68). The transformable virulent *D. nodosus* strain VCS1703A and its derivatives were grown in a Coy anaerobic chamber (Coy Laboratory Products Inc.) in an atmosphere of 10% H_2, 10% CO_2, and 80% N_2 on Eugon (BBL) yeast extract (EYE) agar with 5% defibrinated horse blood (Bio-Lab) or in EYE broth, as described previously (34). When required, media were supplemented with the following antibiotics at the indicated concentrations: for *E. coli*, ampicillin, 100 μg/ml; erythromycin, 150 μg/ml; kanamycin, 20 μg/ml; and tetracycline, 10 μg/ml; and

for *D. nodosus*, erythromycin, 1 μg/ml; tetracycline, 1 μg/ml; and kanamycin, 10 μg/ml. *D. nodosus* cells used for azocasein assays, transmission electron microscopy, twitching motility assays, elastase assays, and protease zymograms were grown in TAS media (71).

DNA manipulations and molecular techniques. Unless otherwise stated, standard procedures were applied for DNA manipulations and molecular techniques (68). *D. nodosus* genomic DNA was prepared by using a QIAGEN DNeasy kit according to the manufacturer's instructions. Southern hybridizations were performed as described previously (33), with probes specific for the relevant antibiotic resistance genes, the target genes, or the 16S *rrnA* gene. PCR-restriction fragment length polymorphism analysis (24) of the *omp1* gene, which encodes an outer membrane protein of *D. nodosus* (53), was conducted to confirm that all mutants were derivatives of the wild-type strain VCS1703A. Reverse transcriptase PCR (RT-PCR) was performed as previously described (34), except that the *D. nodosus* cells were grown in TAS broth. Quantitative RT-PCR (qRT-PCR) was carried out as previously described (60) using RNA prepared as for RT-PCR. DNA sequencing was performed using an A3730S capillary sequencer (Applied Biosystems) and analyzed by using Sequencher version 3.0 (Gene Codes Corporation).

Nucleotide and amino acid sequence comparisons were carried out using the National Center for Biotechnology Information BLAST server (http://www.ncbi.nlm.nih.gov/BLAST) and resources available at TIGR (http://www.tigr.org) and the Victorian Bioinformatics Consortium (http://vbc.med.monash.edu.au/). Homologous amino acid sequences were aligned with the CLUSTAL W program at the Network Protein Sequence Analysis (NPS@) server provided by Pole Bioinformatique Lyonnais (http://npsa-pbil.ibcp.fr). The prediction of subcellular localization was carried out using PSORTb (http://www.psort.org/psortb).

***D. nodosus* mutant construction.** To construct a *fimN* suicide vector, a 2.6-kb PCR product that contains the *fimN* gene was cloned into the XbaI/EcoRI sites of pGEM7Zf(−). An 800-bp *aphA-3* kanamycin resistance cassette from pUC18K (48) was inserted into the EcoRV site of the resultant plasmid pJIR2797 (within *fimN*) to construct the *fimN* suicide vector pJIR2798, where the *aphA-3* cassette was flanked on each side by 1.3-kb fragments of *D. nodosus*-derived DNA. The *fimO* suicide vector pJIR2618 was constructed by cloning a 2.1-kb *fimO* PCR product into the XbaI/EcoRI sites of pBluescript II SK(+) followed by introducing the *aphA-3* cassette into the HpaI site, which was located in the middle of the 2.1-kb *D. nodosus*-derived sequence. To construct the *fimP* suicide vector pJIR2773, a 1.9-kb PCR fragment containing the *fimP* gene was cloned into pBluescript II SK(+) and then subcloned into pGEM7Zf(−) followed by the insertion of a 1.1-kb fragment containing the *erm*(B) erythromycin resistance cassette from pJIR2412 into the HindII site within *fimP*. In this construct, a 954-bp and a 967-bp *fimP*-derived fragment was located upstream and downstream, respectively, of *erm*(B). To construct the *pilE* suicide vector pJIR3088, a 2.1-kb PCR product containing the *pilE* gene was cloned into pGEM7Zf(−), and an *erm*(B) cassette was inserted into the T4 polymerase-filled HindIII site located within *pilE*, so that *erm*(B) was flanked by about 1 kb of *D. nodosus* DNA on each side. Since *pilC* is the first gene in an operon, a nonpolar cassette from pUC18K was used to construct the *pilC* mutants. A 1.42-kb PCR product comprising the 3′ end of *pilC* and its downstream region was amplified and cloned into the XbaI/BamHI sites of pUC18K. Then a 1.33-kb PCR fragment containing the 5′ end of *pilC* was cloned into the T4 polymerase-filled EcoRI site of the resultant plasmid pJIR3124 to construct the *pilC* suicide vector pJIR3132, in which the *aphA-3* cassette was located between two *pilC* fragments and replaced a 1.9-kb internal part of the *pilC* gene.

Mutants were constructed by homologous recombination between these double-crossover suicide vectors and the *D. nodosus* chromosome, as described previously (34). Either circular or linearized plasmid DNA or PCR products derived from suicide vectors were introduced into *D. nodosus* by natural transformation. Transformants were initially screened by resistance to the appropriate antibiotics and then by capillary PCR analysis (33). Potential mutants were further analyzed by PCR with 16S *rrnA* gene primers to confirm they were derivatives of *D. nodosus* (38) and with antibiotic resistance gene primers to verify that the correct resistance genes were present. PCR analysis and Southern hybridization were used to confirm that the genes had been insertionally inactivated by double-crossover events.

SDS-PAGE and immunoblotting. Whole-cell extracts of *D. nodosus* strains were prepared as previously described (34). Cell surface fimbriae were purified by isoelectric precipitation (47). The fimbrial subunits were detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) after staining with Coomassie brilliant blue and also by immunoblotting with serogroup G-specific fimbrial antisera (J. Egerton, University of Sydney) at a 1:1,000 dilution (34).

Analysis of fimbriae and twitching motility. Transmission electron microscopy was performed on 3-day TAS agar cultures as previously described (34). Twitching motility assays were carried out with *D. nodosus* cells inoculated through the agar layer of 1% TAS medium plates, as previously described (34).

Extracellular protease assays. Caseinase and elastase activities were detected qualitatively by growing *D. nodosus* colonies on EYE agar containing 2% skim milk powder or on TAS agar containing 0.3% (wt/vol) insoluble elastin (73), respectively. Total protease activity in the culture supernatant was determined quantitatively using azocasein (Sigma) as the substrate, as previously described (34). One unit of protease activity is defined as the amount of enzyme required to digest 1 μg of azocasein per min (34). Protease zymograms were carried out as previously described (40).

Proteomics, MS, and protein identification. The proteins present in culture supernatants derived from the wild-type and mutant strains were TCA precipitated and analyzed by two-dimensional gel electrophoresis, as previously described (60). Protein spots of interest were excised from gels stained with colloidal Coomassie blue, and SDS was removed by incubation in 5% acetic acid in 40% methanol for 15 min at room temperature followed by washing for 5 min in deionized H_2O. Dye was removed by incubation for 20 min in 50 mM ammonium bicarbonate in 50% acetonitrile (high-performance liquid chromatography grade; BDH); this step was repeated when necessary. The gel pieces were washed twice for 1 h in 20 mM ammonium bicarbonate, dehydrated for 10 min in acetonitrile, and dried under vacuum. After rehydration in 5 μl of 100 ng/μl sequencing grade trypsin (Promega) in 0.1% (vol/vol) acetic acid, 15 mM ammonium bicarbonate, and 1 mM $CaCl_2$ for 10 min at 4°C, 20 mM ammonium bicarbonate was added to immerse the gel pieces followed by incubation at 37°C overnight. The supernatant was retained. Further extractions were performed by adding an equal volume of 5% trifluoroacetic acid (TFA; Pierce) and incubating for 30 min at room temperature with shaking, followed by a 5% TFA-50% acetonitrile extraction with a volume sufficient to cover the gel pieces. Both extracts were pooled with the earlier supernatant and then vacuum dried. The material was resuspended in a small volume of 50% acetonitrile-0.1% TFA. One microliter of Matrix solution (10 mg/ml of α-cyano-4-hydroxycinnamic acid [Laser Biolab, France] in 50% acetonitrile-0.1% TFA) was added to the target plate together with 1 μl of peptide sample prior to matrix-assisted laser desorption ionization–time of flight (mass spectrometry [MS]) analysis. MS and MS/MS were performed with an Applied Biosystems 4700 Proteomics Analyzer in reflection mode using standard procedures. Peptide mass fingerprint spectra and MS/MS spectra were submitted to the Mascot-based GPS explorer software version 3.0 and then searched against a *D. nodosus* database predicted from the then-unpublished genome sequence (http://www.tigr.org). The mass protein score was utilized to determine if the observed match was likely to be a random event. A match with a score greater than 100 was accepted as a positive hit.

RESULTS

Identification of genes potentially involved in fimbrial biogenesis in *D. nodosus*. The availability of the *D. nodosus* strain VCS1703A genome sequence (54) enabled the putative identification of genes involved in fimbrial biogenesis by carrying out BLAST searches with known type IV fimbrial biogenesis components from *P. aeruginosa*. This analysis led to the identification of more than 20 potential homologs (Table 2), at several different genomic locations, in addition to the fimbrial genes previously identified (30, 61). There were several regions that contained more than two putative fimbrial genes, particularly the *fimNOP*, *pilCVWX-fimU-pilE*, *pilMNOPQ*, and *pilGHIJ-chpA* loci. The genetic organization of these loci was similar but not identical to that previously observed in *P. aeruginosa* (80).

***fimN*, *fimO*, and *fimP* are required for type IV fimbrial assembly.** Comparative analysis revealed that the *fimNOP* cluster (equivalent to *pilBCD* from *P. aeruginosa*) of the transformable *D. nodosus* strain VCS1703A was identical to that previously observed in *D. nodosus* strain A198 (30) in terms of its genetic organization, nucleotide sequence, and the deduced amino acid sequences of the protein products. Two adjacent genes (*DNO_1127* and *DNO_1122*), which correspond to *orfM* and

TABLE 2. Genes identified as being potentially involved in fimbrial biogenesis in *D. nodosus*

Locus[a]	Designation[a]	No. of amino acids	Potential function	Rationale[b]
DNO_0110	*fimA*	163	Major pilin	66% similarity to *P. aeruginosa* PilA/COG4969
DNO_0238	*fimX*	684	Signal transduction protein	59% similarity to *P. aeruginosa* FimX/COG4943
DNO_0344	*pilM*	337	Fimbrial biogenesis	60% similarity to *P. aeruginosa* PilM/COG4972
DNO_0345	*pilN*	215	Fimbrial biogenesis	59% similarity to *P. aeruginosa* PilN/COG3166
DNO_0346	*pilO*	222	Fimbrial biogenesis	61% similarity to *P. aeruginosa* PilO/COG3167
DNO_0347	*pilP*	206	Fimbrial biogenesis	57% similarity to *P. aeruginosa* PilP/COG3168
DNO_0348	*pilQ*	734	Secretin	67% similarity to *P. aeruginosa* PilQ/COG4796
DNO_0434	*pilS*	581	Two-component system/sensor protein	59% similarity to *P. aeruginosa* PilS/COG0642
DNO_0435	*pilR*	528	Two-component system/response regulator	70% similarity to *P. aeruginosa* PilR/COG2204
DNO_0515	*pilF*	253	Fimbrial assembly	60% similarity to *P. aeruginosa* PilZ/COG3063
DNO_0532	*rpoN*	469	σ_{51} factor	70% similarity to *P. aeruginosa* RpoN/COG1508
DNO_0675	*pilT*	357	Twitching motility ATPase	86% similarity to *P. aeruginosa* PilT/COG2805
DNO_0676	*pilU*	406	Twitching motility ATPase	76% similarity to *P. aeruginosa* PilU/COG5008
DNO_0712	*fimV*	959	Fimbrial biogenesis	53% similarity to *P. aeruginosa* FimV/COG3170
DNO_0890	*pilE*	151	Pilin-like protein	69% similarity to *P. aeruginosa* PilE/COG4968
DNO_0891	*fimU*	159	Pilin-like protein	58% similarity to *P. aeruginosa* FimU/T/COG4970
DNO_0892	*pilX*	221	Pilin-like protein	53% similarity to *P. aeruginosa* PilX/COG4726
DNO_0893	*pilW*	381	Pilin-like protein	46% similarity to *P. aeruginosa* PilW/COG4966
DNO_0894	*pilV*	189	Pilin-like protein	52% similarity to *P. aeruginosa* PilV/COG4967
DNO_0895	*pilC*	1272	Fimbrial biogenesis/adhesin	52% similarity to *N. gonorrhoeae* PilC/COG3419
DNO_1093	*chpA*	2554	CheA/CheY hybrid, chemosensory	55% similarity to *P. aeruginosa* ChpA/COG0643
DNO_1094	*pilJ*	436	PilJ homolog, chemosensory	54% similarity to *P. aeruginosa* PilJ/COG0840
DNO_1095	*pilI*	173	CheW-like protein, chemosensory	54% similarity to *P. aeruginosa* PilI/COG0835
DNO_1096	*pilH*	120	CheY-like protein, chemosensory	87% similarity to *P. aeruginosa* PilH/COG0745
DNO_1097	*pilG*	127	CheY homolog, chemosensory	82% similarity to *P. aeruginosa* PilG/COG0745
DNO_1106	*ppk*	722	Polyphosphate kinase	70% similarity to *P. aeruginosa* Ppk/COG0855
DNO_1124	*fimP*	286	Prepilin peptidase	77% similarity to *P. aeruginosa* PilD/XcpA/COG1989
DNO_1125	*fimO*	407	Fimbrial assembly	78% similarity to *P. aeruginosa* PilC/COG1459
DNO_1126	*fimN*	564	Fimbrial assembly	80% similarity to *P. aeruginosa* PilB/COG2804
DNO_1215	*pilZ*	120	Fimbrial assembly	76% similarity to *P. aeruginosa* PilZ/COG3215

[a] Locus names and designations are as previously described (30, 34, 54, 61).
[b] Percentage of overall similarity as determined by CLUSTAL W alignments.

orf197 from strain A198 (30), were part of this gene cluster. These genes appear to encode glutamine amidotransferase and dephosphocoenzyme A kinase, respectively. Although these genes are associated with *pilBCD* homologs in *P. aeruginosa*, *N. gonorrhoeae*, and *P. putida* (30), there is no evidence that they are involved in type IV fimbrial biogenesis (22). In addition to their similarity to their respective PilBCD homologs, the FimNOP proteins have similarity to the type II secretion components $GspE_R$ (COG2804), $GspF_S$ (COG1459), and $GspO_A$ (COG1989), respectively.

Homologous recombination with suicide vectors containing insertionally inactivated genes was used to construct separate chromosomal *fimN*, *fimO*, and *fimP* mutants of strain VCS1703A after double-crossover events. The genotypes of these mutations were confirmed by PCR analysis and Southern hybridization (data not shown), and independently derived *fimN* (JIR3895 and JIR3896), *fimO* (JIR3885 and JIR3886), and *fimP* (JIR3889 and JIR3890) mutants were used for phenotypic characterization. Since *orfM*, *fimNOP*, and *orf197* comprise an operon in strain A198 (31), it was important to ensure that the disruption of these genes did not result in polarity effects on the downstream genes. Accordingly, RNA from the *fimN* and *fimO* mutants was analyzed by RT-PCR, using primers specific for the *fimOP* and *fimP-DNO_1122* intergenic regions. The results (data not shown) confirmed that the downstream genes were expressed in both mutants, either from read-through from the antibiotic resistance genes or from secondary promoters.

Compared to the large spreading colonies produced by the wild-type strain VCS1703A, the *fimN*, *fimO*, and *fimP* mutants all had small, nonspreading colonies (data not shown), which correlated with the loss of twitching motility as determined by an agar stab assay (Fig. 1) and the absence of surface fimbriae

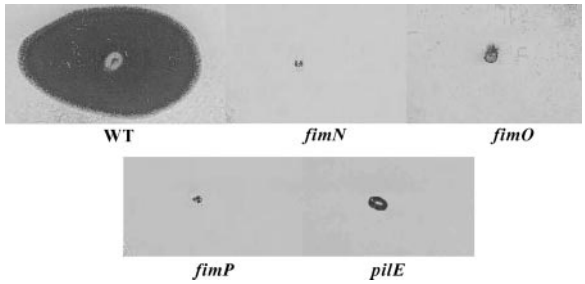

FIG. 1. Analysis of twitching motility. Assays were carried out on 1% TAS agar with stab-inoculated *D. nodosus* cultures incubated for 5 to 7 days. After compression and staining with Coomassie brilliant blue, a large, dark zone around the point of inoculation is indicative of twitching motility. Assays of wild-type strain VCS1703A (WT), the *fimN2* mutant JIR3896, the *fimO2* mutant JIR3886, the *fimP2* mutant JIR3890, and the *pilE2* mutant JIR3911 are shown. The profile obtained from the second mutant of each gene was identical to that of each mutant represented here.

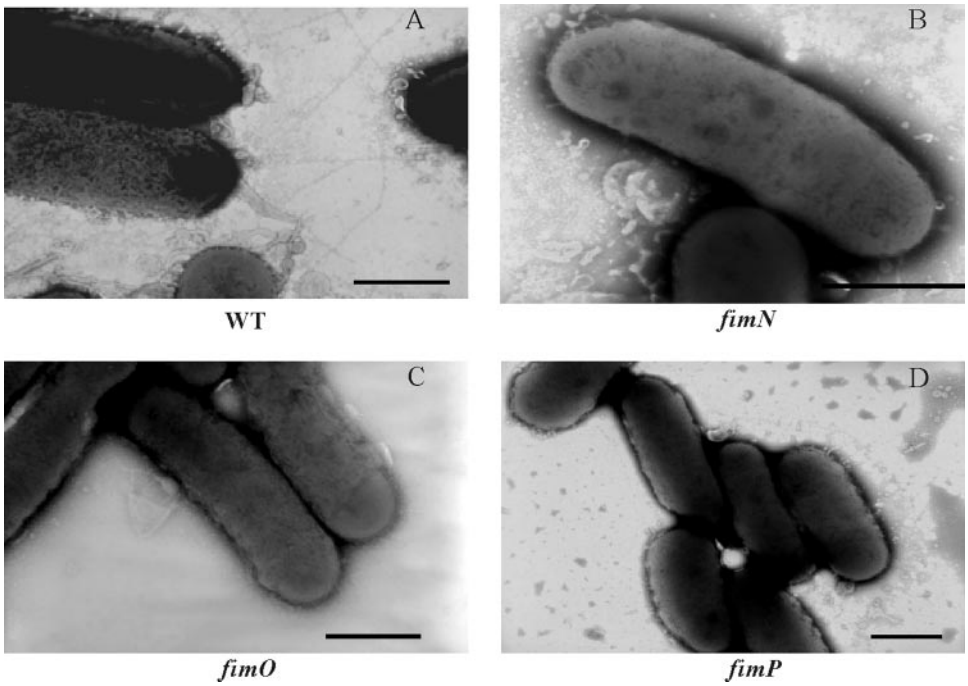

FIG. 2. Absence of cell surface fimbriae in the *fimN*, *fimO*, and *fimP* mutants. Three-day-old cultures of the wild-type (WT) strain VCS1703A (A), the *fimN1* mutant JIR3895 (B), the *fimO2* mutant JIR3886 (C), and the *fimP1* mutant JIR3889 (D) were removed from TAS agar with phosphate-buffered saline, negatively stained, and analyzed by transmission electron microscopy Bars = 1 μm. Note that the fimbriae, which were generally observed at one pole of the wild-type cells, were not observed in any of the cells examined in the *fimN*, *fimO*, or *fimP* mutants. The profile of the second mutant of each gene was identical to that of each mutant shown here.

in transmission electron micrographs (Fig. 2). To determine the role of these gene products in fimbrial biogenesis, whole-cell lysates of these mutants were analyzed by SDS-PAGE and Western immunoblotting using antisera raised against *D. nodosus* type G fimbriae. The results showed that although these mutants did not have extracellular fimbriae, they still expressed the fimbrial subunit protein FimA (Fig. 3), indicating that these proteins are required for FimA polymerization. In addition, compared to the FimA proteins produced by the wild-type strain and the *fimN* and *fimO* mutants, the FimA subunits produced by the *fimP* mutants were larger (Fig. 3), consistent with the prediction that they represented uncleaved prepilin proteins. These results provided direct evidence in *D. nodosus* to support the previous finding that FimP is the prepilin peptidase responsible for the cleavage of the immature FimA prepilin (30, 31). In addition, these data confirmed the RT-PCR results since, if the *fimN* and *fimO* mutations did have polar effects, then all of the mutants would have unprocessed FimA protein.

The *pilE* gene is essential for type IV fimbrial biogenesis, and the *pilC* mutants are unstable. The first gene product of the putative *pilCVWX-fimU-pilE* operon had similarity to PilC from *N. gonorrhoeae* and PilY1 from *P. aeruginosa* and was named after its *N. gonorrhoeae* homolog. The products of the last five genes of this operon had N-terminal sequence identity to the FimA fimbrial subunit (Fig. 4); these genes were designated after their homologs in *P. aeruginosa*. Many of the homologous genes in *Pseudomonas* (46) and *Neisseria* (82) species are also clustered at a single locus, but with a different genetic organization.

The putative PilE, FimU, PilV, and PilW proteins all have a conserved glutamate residue at the +5 position relative to the predicted prepilin peptidase cleavage site (Fig. 4), which is common for fimbrial subunits (14), pilin-like proteins (82), and pseudopilins from type II secretion systems (21). However, the putative PilX protein had a threonine residue at this position (Fig. 4); the absence of glutamate at +5 appears to be a conserved feature of PilX proteins (3, 82) and the type II secretion pseudopilin $GspK_X$ family (21). Since it has been shown that this conserved glutamate is required for fimbrial assembly (62), the incorporation of atypical pilins or pseudopilins into a growing fiber is thought to control its length by terminating fiber elongation (10).

To examine the biological function of at least some of these proteins in *D. nodosus*, we decided to mutate the first and last genes in this putative operon, *pilC* and *pilE*. Like its orthologs, the *D. nodosus* PilC protein was predicted by bioinformatic analysis to be located in the outer membrane and to have a conserved C-terminal region of unknown function. To determine the role of this protein, two independently derived nonpolar *pilC* mutants, JIR3913 and JIR3914, were constructed by natural transformation of the wild-type strain VCS1703A with the suicide vector pJIR3132 and shown by PCR analysis and Southern hybridization to be derived from double-crossover events (data not shown).

The colony morphology of these mutants was initially between that of the wild-type strain and nonfimbriate *fimA* mutants. The colonies had a domed center but only a moderate spreading edge, indicating that these mutants may have reduced levels of fimbriae. However, it was found that upon

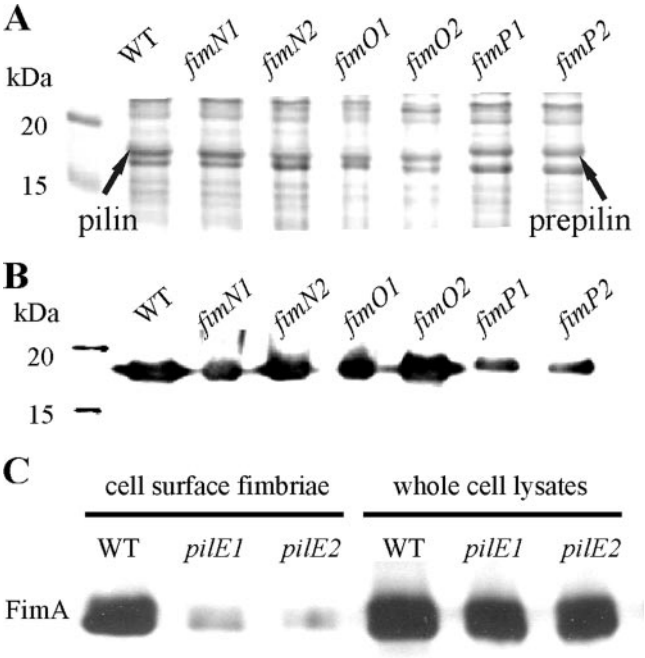

FIG. 3. Western immunoblotting of whole-cell lysates and purified cell surface fimbriae. Shown is the production of FimA in whole-cell lysates from the wild-type strain VCS1703A (WT) and the mutants JIR3895 (*fimN1*), JIR3896 (*fimN2*), JIR3885 (*fimO1*), JIR3886 (*fimO2*), JIR3889 (*fimP1*), and JIR3890 (*fimP2*). Samples were separated by 12 to 15% SDS-PAGE and analyzed by Coomassie brilliant blue staining (A) and Western immunoblotting with fimbrial serogroup G-specific antisera at a 1:1,000 dilution (B). Note that the FimA-specific protein is larger in the *fimP* mutants. (C) Reduced extracellular fimbrial levels of *pilE* mutants. Purified cell surface fimbriae and whole-cell lysates of wild-type strain VCS1703A (WT) and the mutants JIR3910 (*pilE1*) and JIR3911 (*pilE2*) were analyzed.

further subculture, the phenotypes of these mutants frequently switched to a nonfimbriate state, as indicated by the nonspreading domed colonies. SDS-PAGE analysis showed that these cells either had greatly reduced FimA production or no longer produced FimA (data no shown). Since there is no evidence from other bacteria that PilC may regulate fimbrial subunit expression, we concluded that the *pilC* mutants were unstable and that their continued subculture and growth led to the selection of secondary mutations that dramatically reduced FimA production. The instability of the *pilC* mutants meant that no further phenotypic analysis could be carried out on these strains.

Independently derived *pilE* mutants were constructed by natural transformation with the *pilE* suicide vector pJIR3088. Several potential *pilE* mutants were obtained, but they were all derived from a single transformation experiment. To obtain an independently derived mutant, additional transformation experiments were performed using both circular and linearized plasmid DNA, but unfortunately, no more mutants were obtained. Analysis of genomic DNA from two *pilE* mutants (JIR3910 and JIR3911) from the initial transformation experiment confirmed that they were derived from double-crossover events that insertionally inactivated the *pilE* gene (data not shown). The *pilE* mutants had slightly spreading to nonspreading domed colonies, which was consistent with the observation that they did not have detectable twitching motility zones in agar stab assays (Fig. 1). Further investigation using SDS-PAGE and Western immunoblotting with fimbrial antisera showed that the *pilE* mutants exhibited significantly reduced levels of surface fimbriae compared to the wild-type strain, although they produced approximately wild-type levels of FimA, as shown in whole-cell lysates (Fig. 3C). These results suggested that PilE was involved in fimbrial assembly and/or stabilization, with the latter possibility being supported by the presence of trace amounts of surface fimbriae in the *pilE* mutants.

FimN, FimO, FimP, and PilE are essential for natural transformation. To determine the effect of the *fimN*, *fimO*, *fimP*, and *pilE* mutations on natural competence, we carried out transformation experiments using the *rrnA*-specific *D. nodosus* suicide vector pJIR2691, which contains a *tet*(M) tetracycline resistance cassette (Table 1). Natural transfor-

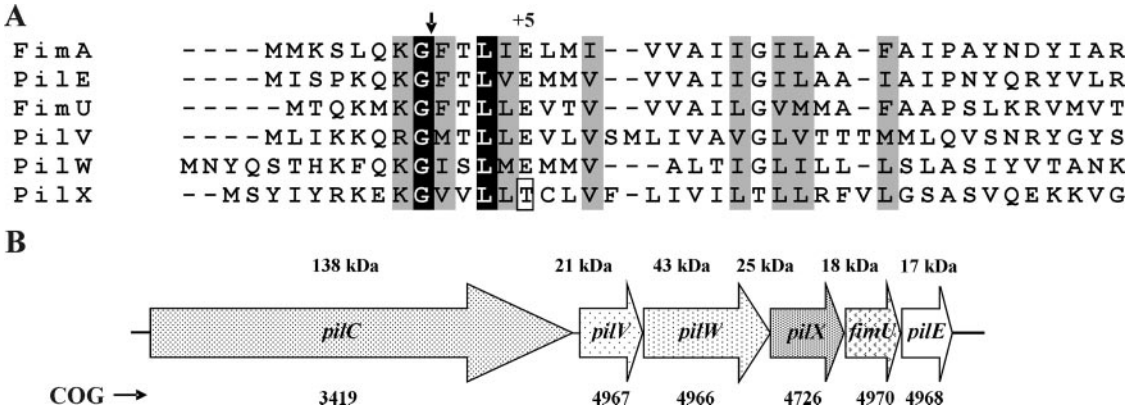

FIG. 4. Sequence alignment of FimA and pilin-like proteins and genetic organization of the *pilCVWX-fimU-pilE* gene cluster. (A) N-terminal sequence alignment. The arrow indicates the prepilin processing site of FimA and the predicted processing sites of the other proteins. Identical residues are shaded in black, while residues with strong and weak similarity are shaded in gray. The atypical threonine residue at +5 in PilX is boxed. (B) Genetic organization of the *pilC* to *pilE* cluster. The direction of transcription, predicted molecular sizes of the corresponding proteins, and clusters of orthologous groups (COG) as determined by an NCBI (http://www.ncbi.nlm.nih.gov) conserved domain search are shown. The genes are shown to scale.

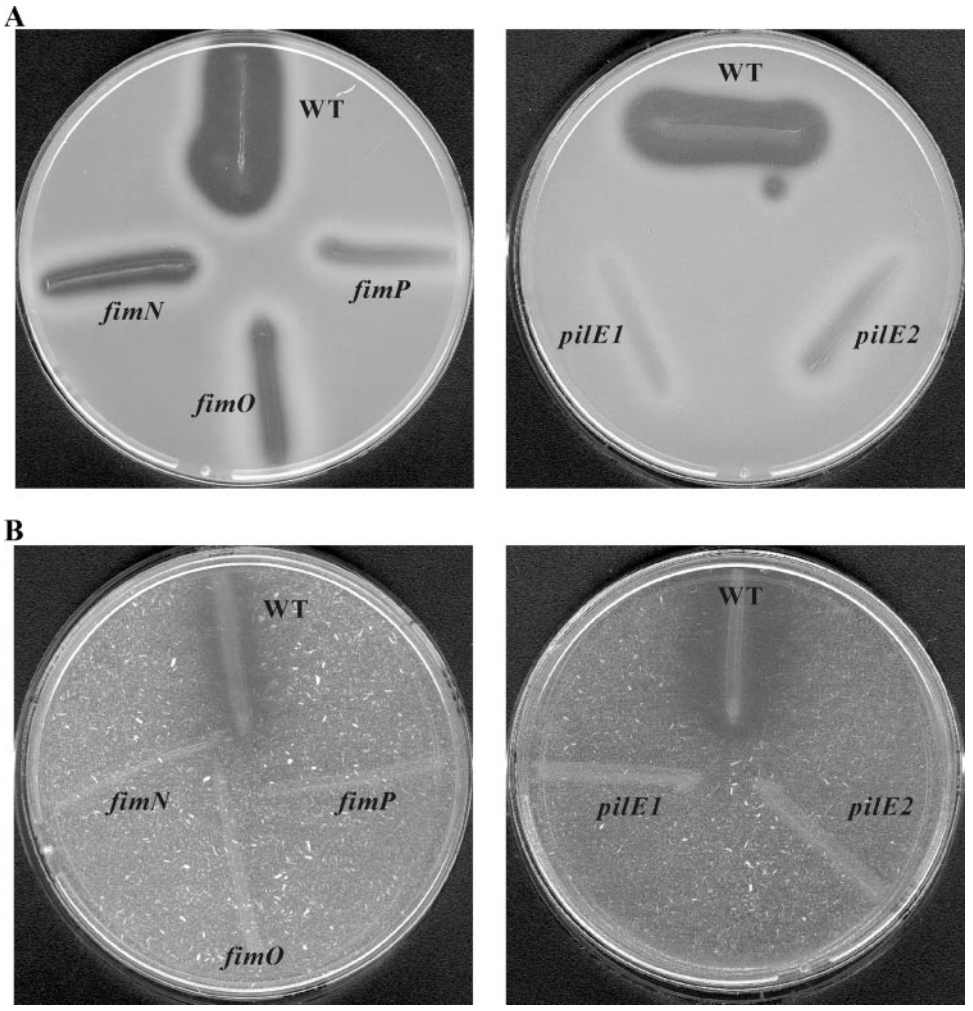

FIG. 5. Qualitative protease activity. (A) Caseinase activity was determined by growing the strains indicated on skim milk agar for 2 days. (B) Elastase activity was determined by growing the strains indicated on TAS agar containing 0.3% elastin for 28 days. The wild-type strain VCS1703A (WT), the *fimN2* mutant JIR3896 (*fimN*), the *fimO2* mutant JIR3886 (*fimO*), the *fimP2* mutant JIR3890 (*fimP*), and the *pilE* mutants JIR3910 (*pilE1*) and JIR3911(*pilE2*) are shown. The profile of each of the second *fimN*, *fimO*, and *fimP* mutants was identical to that of each mutant shown here.

mation of the wild-type strain VCS1703A consistently produced tetracycline-resistant colonies that resulted from homologous recombination between pJIR2691 and one of the three chromosomal *rrnA* operons, as confirmed by PCR analysis. However, despite repeated attempts, no transformants were obtained from the *fimN*, *fimO*, *fimP*, or *pilE* mutants. The ability to undergo natural transformation is a prerequisite for the complementation of chromosomal mutants of *D. nodosus*, since there are no electroporation or conjugation methods available for this bacterium (34). Therefore, for technical reasons, it was not possible to complement these mutants by introducing a wild-type copy of the genes onto the chromosome.

FimN, FimO, FimP, and PilE are involved in extracellular protease secretion. Previous studies have shown that the fimbrial subunit protein FimA is required for efficient extracellular protease secretion in *D. nodosus* (34), indicating that there may be a relationship between the type IV fimbrial system and the protein secretion apparatus in this organism. By performing qualitative caseinase and elastase assays on agar plates, it was initially observed that the *fimN*, *fimO*, *fimP*, and *pilE* mutants had reduced extracellular protease and elastase levels (Fig. 5). These results were confirmed by quantitative protease assays carried out on culture supernatants, using azocasein as the substrate. Each of the mutants had significantly reduced ($P < 0.05$) extracellular protease activity compared to the wild-type strain VCS1703A (Fig. 6A).

The proteases encoded by the *aprV2*, *aprV5*, and *bprV* genes (34) are responsible for all of the extracellular protease activity observed in *D. nodosus* (R. M. Kennan and J. I. Rood, unpublished results). To determine whether downregulation of protease gene expression was responsible for the reduction in extracellular protease activity, qRT-PCR experiments were performed using primers specific for each individual protease gene. The results showed that there were no significant differences in *aprV2*, *aprV5*, or *bprV* transcript levels between the wild-type strain and the various mutants (Fig. 6B), with the exception of a slight reduction in the *aprV2* transcript level in

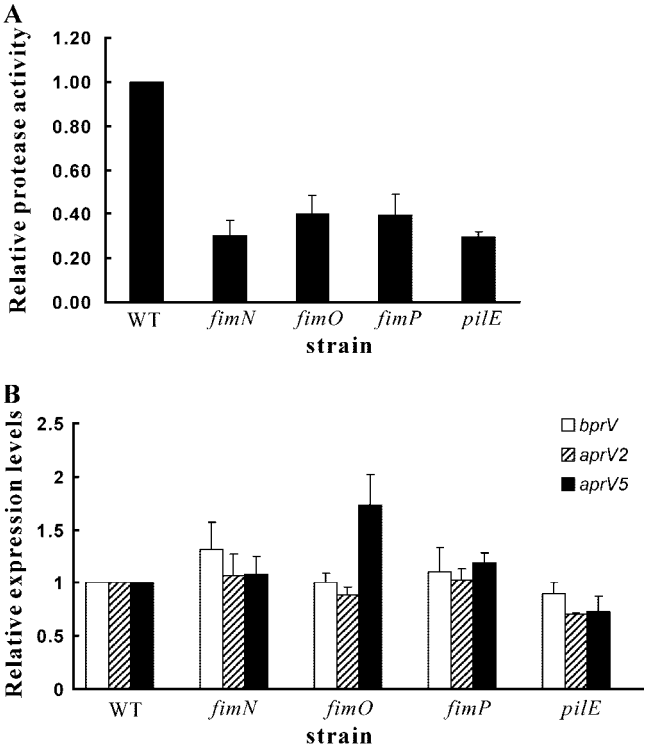

FIG. 6. Quantitative analysis of protease activity and protease gene expression. (A) Protease activity in culture supernatants was determined with an azocasein substrate. (B) Expression of the extracellular protease genes *aprV2*, *bprV*, and *aprV5* was analyzed by qRT-PCR. All values were obtained from at least three independent biological samples and are expressed relative to the wild-type (WT) strain. Strains used were WT (VCS1703A), the *fimN2* mutant (JIR3896), the *fimO2* mutant (JIR3886), the *fimP2* mutant (JIR3890), and the *pilE2* mutant (JIR3911).

the *pilE* mutant. This reduction would not account for the reduction in protease and elastase activity observed in the *pilE* mutants. Therefore, it was concluded that the FimN, FimO, FimP, and PilE proteins are all involved in extracellular protease secretion.

Proteomic analysis confirms that the mutants secrete reduced amounts of the AprV2, AprV5, and BprV proteases. To determine whether there was a reduction in the secretion of all three extracellular proteases, protease zymogram analysis was carried out with culture supernatants from the *fimN*, *fimO*, and *fimP* mutants. The results showed that each of these mutants had reduced AprV2 and AprV5 activity (data not shown). Note that the basic protease BprV cannot be observed by this method (R. M. Kennan and J. I. Rood, unpublished results).

To confirm these results, and to see whether the secretion of any other proteins was affected by these mutations, proteomic studies were carried out. Culture supernatants from the wild-type and mutant strains were TCA precipitated and subjected to two-dimensional gel electrophoresis. Comparative analysis identified six protein spots (P1 to P6) that were present at reduced levels in each of the mutants (Fig. 7). Analysis by MS revealed that these spots were derived from the AprV5 (P1 and P2), AprV2 (P3 to P5), and BprV (P6) proteases, providing evidence that the secretion of BprV was also affected by the

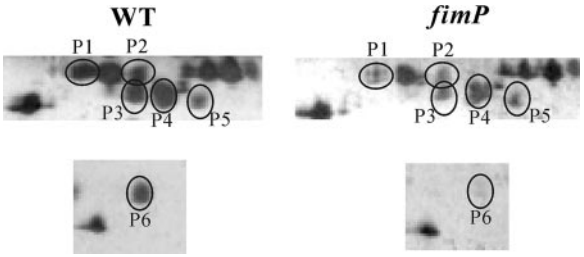

FIG. 7. Two-dimensional gel electrophoresis of extracellular extracts of wild type (WT) and mutants. Culture supernatants were concentrated by TCA precipitation and subjected to two-dimensional gel electrophoresis. Sections of representative two-dimensional gels of the wild-type strain VCS1703A and the *fimP2* mutant JIR3890 are shown. Proteins with reduced intensity in the mutant are circled and numbered from P1 to P6, as indicated.

mutations (Table 3). No other consistent differences in protein spot intensity were observed, suggesting that in the culture supernatants, only the extracellular protease levels were affected by mutations in the fimbrial biogenesis system.

***pilQ* may be an essential gene in *D. nodosus*.** The 2,205-bp *pilQ* gene was identified by searching the products of the *D. nodosus* genome with the PilQ sequence from *P. aeruginosa*, and the gene encoded a putative 81-kDa (734 amino acids) protein that was predicted to be located in the outer membrane. Analysis of the putative *D. nodosus* PilQ protein showed that it contained the typical functional domains of secretins involved in type IV fimbrial biogenesis and type II secretion systems. The *pilQ* gene region also had a similar genetic organization to its homologs in *P. aeruginosa* and *N. gonorrhoeae*, being clustered with four other type IV fimbrial component genes in a putative *pilMNOPQ* operon.

It was postulated that the *pilQ* gene encoded an outer membrane protein that could be polymerized into a secretin complex that had a role in fimbrial extrusion and potentially in natural transformation and protease secretion. To examine this hypothesis, attempts were made to construct a chromosomal *pilQ* mutant in *D. nodosus*. A *pilQ*-specific suicide vector, pJIR2614, was constructed and introduced into VCS1703A by natural transformation. However, despite repeated attempts, when using this vector and PCR products derived from it, only single crossovers that did not mutate *pilQ* were observed. No double-crossover events that mutated *pilQ* were obtained. An alternative strategy that involved transfor-

TABLE 3. Identification of extracellular proteins with altered expression in the *fimP2* mutant

Spot	Protein identification	Mascot protein score[a]	Peptide count (% sequence coverage)[b]
P1	AprV5, acidic serine protease	244	7 (20)
P2	AprV5, acidic serine protease	147	7 (16)
P3	AprV2, acidic serine protease	433	11 (36)
P4	AprV2, acidic serine protease	805	13 (43)
P5	AprV2, acidic serine protease	407	12 (45)
P6	BprV, basic serine protease	193	9 (31)

[a] A score of greater than 100 was accepted as a positive hit.
[b] Number of peptides obtained from MS + MS/MS spectra that match to the protein (degree of matched residues).

mation with pJIR2714, which contained a 940-bp PCR product internal to *pilQ*, was then employed. Incorporation of this plasmid into the chromosome by a single-crossover event would lead to the mutation of the *pilQ* gene. Again, despite repeated attempts, no mutants were obtained. In this study and several others carried out in this laboratory, *pilQ* remains the only gene that we have been unable to mutate in *D. nodosus*. Based on these experiments and our analysis of the genome sequence, where *pilQ* is the only outer membrane secretin gene that can be identified, it is postulated that *pilQ* is an essential gene in *D. nodosus*.

DISCUSSION

D. nodosus is unique in that, not only does it exhibit the twitching motility that is typical among type IV fimbriate bacteria, but also it is virulent and competent for natural transformation, and it secretes extracellular proteases, all by processes that have been shown to be dependent on the amount of the fimbrial subunit protein that is produced (34, 61). In this study we have shown, to our knowledge for the first time in any one bacterium, that fimbrial biogenesis, not just the presence of the fimbrial subunit FimA, is required not only for twitching motility, but also for natural competence and extracellular protease secretion. Both the experimental and bioinformatics data are consistent with the hypothesis that the extracellular proteases of *D. nodosus* are secreted primarily by the fimbrial biogenesis system, which represents the only type II secretion system in this organism.

We have shown that in *D. nodosus*, FimN, FimO, and PilE are required for both fimbrial biogenesis and protease secretion, unlike their counterparts in *P. aeruginosa*, PilB, PilC, and PilE, respectively, which appear to be exclusively involved in type IV fimbrial biogenesis (67, 74, 76). Apart from the fimbrial subunit protein PilA and the prepilin peptidase PilD (8, 42), no other *Pseudomonas* proteins involved in fimbrial biogenesis have been reported to be required for extracellular protein secretion. In *Vibrio cholerae*, the colonization factor TcpF is secreted by the Tcp pathway, but unlike the *D. nodosus* proteases, this protein is encoded within the Tcp fimbrial locus and is part of the Tcp system, although it is not essential for fimbrial biogenesis (35, 58). A recent study with *Francisella* demonstrated that several type IV fimbrial homologs mediate protein secretion, but there is no evidence to show that this bacterium produces surface fimbriae (25). A role for FimP in protease secretion in *D. nodosus* was more predictable, since its homolog in *P. aeruginosa*, PilD (XcpA or $GspO_A$), cleaves the signal sequences of both the pilin subunit PilA and the pseudopilins involved in type II secretion (21).

The fact that both FimP and FimA (34) were required for extracellular protease secretion in *D. nodosus* provided evidence that these proteases may be secreted via a type II secretion pathway. Type II secretion systems have 12 to 16 components (21, 29, 64, 69) that include several proteins with similarity to components of the type IV fimbrial biogenesis pathway, such as $GspD_Q$ (PilQ homolog), $GspE_R$ (FimN/PilB homolog), $GspF_S$ (FimO/PilC homolog), $GspO_A$ (PilD/FimP homolog), $GspG_T$-K_X (pilin homologs), and several proteins ($GspL_Y$, $GspM_Z$, and $GspC_P$) with no homologs in fimbrial biogenesis. *P. aeruginosa* has both systems, which share a prepilin peptidase, PilD/XcpA, and presumably the fimbrial subunit PilA, whose function in type II secretion is not known, although together with the pseudopilins, it has been predicted to form an intermediate structure of the secretion apparatus (42). Bioinformatic analysis indicated that the seemingly essential *pilQ* gene appeared to encode the only outer membrane secretin in *D. nodosus* and that apart from the genes involved in fimbrial biogenesis, no other type II secretion genes were present. There were no identifiable homologs of the type IV fimbria-independent components and no FimN, FimO, FimP, or pseudopilin-like paralogs. *D. nodosus* does have an entire complement of Sec components, which are mainly utilized for the inner membrane translocation of proteins targeted for type II secretion (64). Taken together, these data suggest that proteases may be secreted by a novel type II secretion-related pathway, which utilizes the components of the type IV fimbrial apparatus but does not require $GspL_Y$, $GspM_Z$, and $GspC_P$ homologs. In support of this view is the fact that the novel type II-related secretion pathway Xcm, which is required for the secretion of a manganese-oxidizing factor in *P. putida* strain GB-1, also lacks $GspL_Y$, $GspM_Z$, and $GspC_P$ (15).

We postulate that the growing periplasmic fimbrial polymer may act to push the proteases through the PilQ secretin in the outer membrane, a process that resembles the extrusion of proteins through $GspD_Q$ by the pseudopilus in the type II secretion pathway (21, 29, 69). Therefore, we suggest that in *D. nodosus*, the PilQ secretin is utilized by both the fimbrial biogenesis and protein secretion systems, which may explain why it is difficult, if not impossible, to insertionally inactivate. It follows that retraction of the fimbriae might also be required for protease secretion, being required to release PilQ secretin complexes that are blocked by protruding fimbriae. Since there are no amino acid biosynthesis pathways encoded on the *D. nodosus* genome (54), the extracellular proteases may have an essential physiological role in the provision of the amino acids that are required for growth. Therefore, our inability to construct a *pilQ* mutant may be related to the key role of PilQ in protease secretion.

It was previously suggested, based purely on bioinformatic analysis, that AprV2, AprV5, and BprV may be autotransporter proteins whose passage through the outer membrane is dependent upon their carboxy-terminal domains and does not require any other proteins (28). However, there is no experimental evidence to support this hypothesis. We have now provided clear evidence that protease secretion does require other proteins, specifically, those involved in fimbrial biogenesis. It is possible that the putative autotransporter properties of these enzymes may have been responsible for the residual levels of extracellular proteases that were observed in the various mutants. Alternatively, this activity may simply have resulted from the lysis of some cells in the bacterial population.

PilE is one of five pilin-like proteins identified from the *D. nodosus* genome sequence. In *P. aeruginosa*, mutation of *pilE* leads to a loss of type IV fimbriae (67), whereas in *N. gonorrhoeae*, the PilE homologs PilV and ComP appear to play a more important role in specific DNA binding during natural transformation (PilV and ComP) and adherence (PilV) than in fimbrial biogenesis (1, 81, 83). In *D. nodosus*, the *pilE* mutant had greatly reduced levels of surface fimbriae, although wild-type levels of FimA were still produced. We suggest that PilE

is essential for fimbrial assembly and/or stabilization, perhaps by being incorporated into the fimbrial polymer as a minor but critical subunit.

The *pilC* gene encodes a putative outer membrane protein with similarity to PilC from *Neisseria* spp., which appears to be a fimbrial tip adhesin and is also involved in fimbrial biogenesis and fimbrial retraction (52, 55, 66), and PilY1 from *P. aeruginosa*, which is required for fimbrial structure and twitching motility (3) and is suggested to be involved in the regulation of fimbrial retraction (80). Although we were able to construct *pilC* mutants in *D. nodosus*, and these mutants were altered in colony morphology, they were highly unstable and rapidly lost the ability to produce FimA. These results suggest that PilC is involved in fimbrial biogenesis, but further research would be required to confirm this hypothesis.

Other studies have shown that type IV fimbrial biogenesis and natural transformation are closely related, with some proteins having a dual function in both processes (7, 26). Our findings, both in this study and previously (34), have shown that mutation of any of the fimbrial biogenesis genes eliminates the ability of *D. nodosus* to undergo natural transformation. Natural transformation has been intensely studied in *N. gonorrhoeae* and a model involving type IV fimbrial components and some type IV fimbriae-associated proteins proposed (26). Comparative studies on the potential DNA transformation components demonstrated that despite their common properties, such as three ComEA homologs and one ComEC homolog (18, 20), which are presumably involved in DNA uptake, and a lipoprotein ComL homolog, which is thought to be involved in penetrating the peptidoglycan (19, 23), there are differences between *D. nodosus* and *N. gonorrhoeae*. Apart from the different roles of the PilE homologs, which have already been discussed, *pilR* and *rpoN* mutants of *D. nodosus* express residual levels of FimA but do not elaborate surface fimbriae and are not transformable (61), whereas in *N. gonorrhoeae*, limited amounts of pilin that do not produce fully extended fimbriae are sufficient for natural transformation (41). Although the *D. nodosus* results may reflect the low level of natural transformability in this organism, it appears that in *D. nodosus*, the same apparatus may be responsible for natural transformation and fimbrial protrusion and retraction. Current models suggest that natural transformation utilizes the PilQ secretin pore for DNA uptake (7, 26). We postulate that in *D. nodosus*, DNA uptake occurs through the PilQ secretin and also is dependent upon extension or retraction of its type IV fimbriae.

In conclusion, we have demonstrated that in *D. nodosus*, several proteins that are required for fimbrial biogenesis, FimN, FimO, FimP, and PilE, are also required for both natural transformation and extracellular protease secretion. It is postulated that in *D. nodosus*, natural transformation requires an intact type IV fimbrial apparatus and that the extracellular proteases AprV2, AprV5, and BprV are secreted via a type II secretion-like pathway that also utilizes the type IV fimbrial apparatus. Further genetic and biochemical studies will be required to determine the molecular mechanisms by which type IV fimbrial biogenesis, natural transformation, and protease secretion are mediated.

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