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Pilins of *Bacteroides nodosus*: Molecular Basis of Serotypic Variation and Relationships to Other Bacterial Pilins

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INTRODUCTION

Pili are long filamentous appendages found widely among gram-negative bacteria. They may be few in number, as are sex pili, or so numerous as to cover the cell with a dense pile. Their distribution may be over the whole cell, as in the enterobacteria, or polar, as are the common pili of *Pseudomonas aeruginosa*. A classification has been made, on a functional basis, into sex pili which are involved in the transfer of genetic material and other non-sex pili (6, 40). The latter, through bacterial adherence, motility, or antiphagocytic properties, often play important roles in the parasitic invasion of host tissues. Antibodies against these pili are effective in combating bacterial invasion, so that pili are valuable agents in prophylactic immunization and form an important component of several vaccines.

Among the many piliated bacterial species, Henrichsen (27, 28) distinguished a group of diverse organisms which showed "twitching motility," a type of surface translocation associated with piliation. This group of bacteria included *Bacteroides nodosus*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Acinetobacter calcoaceticus*, *Eikenella corrodens*, *Moraxella bovis*, *Moraxella nonliquefaciens*, and several species of *Pseudomonas* including *P. aeruginosa*. Pili from these species were similar in morphology and often polar in distribution. Twitching motility was not shown to be exhibited by the *Enterobacteriaceae* or most other bacteria with peritrichous pili.

Chemical characterization of pilins from many bacterial species has demonstrated remarkable homology between pilins from those organisms which exhibit twitching motility. Pilins from the diverse bacterial species of *B. nodosus* (14, 36), *M. bovis* (34), *M. nonliquefaciens* (23), *N. gonorrhoeae* (38, 50), *N. meningitidis* (39), and *P. aeruginosa* (41, 46–48) are similar in size (M_r of ~16,000 to 18,000) and display extensive similarity in the amino- and carboxy-terminal regions of the amino acid sequence (Fig. 1). The first 32 amino acid residues are nearly identical and include *N*-methylphenylalanine as a modified amino-terminal residue (23, 24, 29). Pilins of this group, termed mePhe pilins (17, 34), are quite unlike those from the many pilus types found among the *Enterobacteriaceae* (32). While the extensively conserved N-terminal region of mePhe pilins is highly hydrophobic, pilins of the *Enterobacteriaceae* either lack a strongly hydrophobic segment or contain a C-terminal hydrophobic segment. These different pilus types appear to demonstrate different yet analogous evolutionary solutions to the construction of a pilus.

PILI OF *B. NODOSUS* IN PROPHYLAXIS OF OVINE FOOTROT

B. nodosus is the causative organism of ovine footrot, a contagious debilitating disease of sheep transmitted through the pasture (3). The disease affects the epidermal tissues of the interdigital skin and hoof and leads to separation of the hoof from the soft tissues. Lameness and morbidity from infection lead to a reduction in productivity and large economic losses. Even severe footrot results in only minimal protection against subsequent seasonal recurrences of infection due to the superficial nature of the disease, with infection restricted to the avascular epidermis. However, effective protection against footrot is achieved through immunization of sheep with killed *B. nodosus* cells (13), an observation which provided the basis for current commercial footrot vaccines. The efficacy of footrot vaccines is highly dependent on the degree of cell piliation (52, 58, 62), and in challenges involving homologous strains of *B. nodosus*, purified pili alone have been shown to confer protection equivalent if not superior to that afforded by killed whole cells (20, 54, 56, 60), demonstrating the importance of pili as the major host-protective immunogen.

Agglutination of bacteria by antibody directed at the pilus appears to constitute the major mechanism of immunity against footrot (62). Consequently, the pattern of cross-protection conferred by vaccination correlates well with serogrouping of *B. nodosus* isolates based on in vitro agglutination reactions involving the pilus antigen (8, 9). Isolates have been classified into nine serogroups designated A to I,

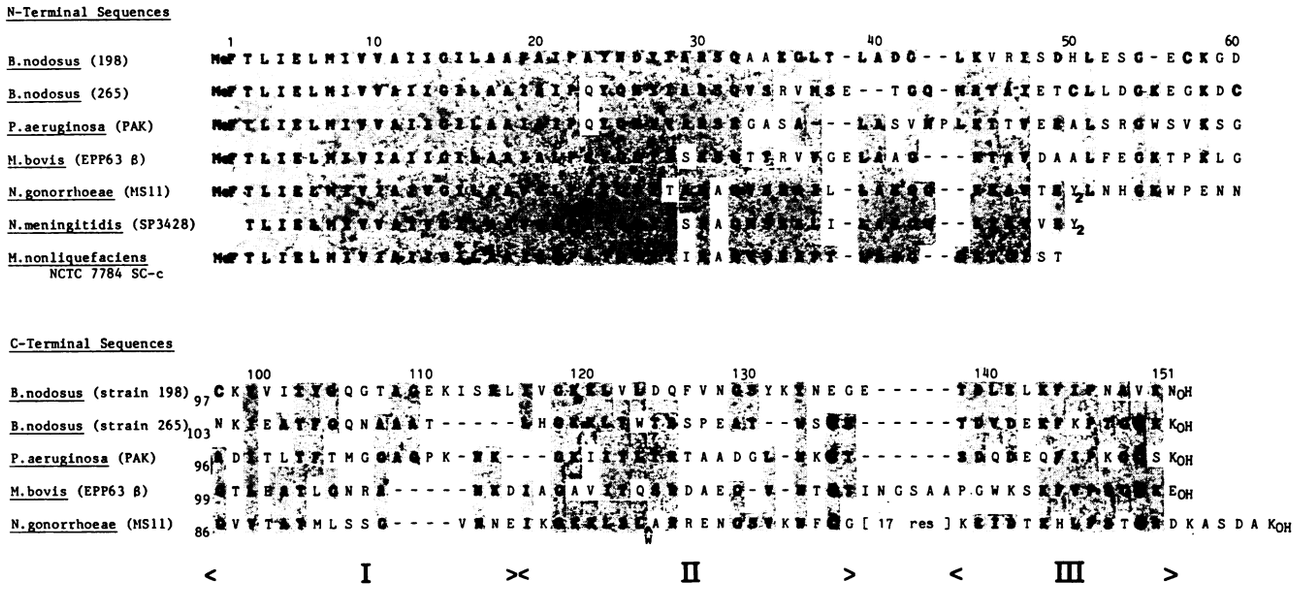


FIG. 1. Alignments of N-terminal and C-terminal regions of mePhe pilins to show sequence homology. Identical or conservatively substituted residues are shaded. In the C-terminal pilin sequence from *N. gonorrhoeae* MS11, a 17-residue segment of the disulfide loop has been omitted, and a single-residue insertion is shown below the sequence. I, II, and III identify regions in the structural prediction profiles of Fig. 13.

the latter serogroup (I) having been defined only recently (8a). Within serogroups, different serotypes can be distinguished which only partially absorb the antibodies produced in response to a heterologous serotype (8) of the same serogroup. Protective immunity conferred by vaccination is restricted, at best, to a serogroup (8, 12, 20 57).

The identification of the pilus as the major host-protective immunogen and the classification of *B. nodosus* isolates on the basis of agglutination reactions involving the pilus antigen have led to a significant improvement in commercial whole-cell vaccines against footrot. Current commercial vaccines now include well-piliated representatives of at least eight serogroups of *B. nodosus*. Unfortunately, the high cost of production of these vaccines, caused by the poor growth characteristics of *B. nodosus*, has inhibited their widespread use. However, the recent demonstration that pili of *B. nodosus* can be expressed in *P. aeruginosa* through the application of recombinant deoxyribonucleic acid technology (17) offers the possibility of pilus-based footrot vaccines prepared in a less fastidious surrogate host having good growth characteristics. In addition, vaccines based on purified pili avoid both dilution of effective immunogen by extraneous cellular antigen and the vaccinal lesions associated with administration of excess extraneous antigen as occurs with whole-cell vaccines (56, 59).

CHARACTERIZATION OF *B. NODOSUS* PILI

Pili of *B. nodosus* are 5 to 6 nm in diameter and up to 10 μm in length (18, 53, 67). Several hundred pili may be present in a polar distribution on the cell (Fig. 2). Each pilus is a homopolymer of the protein pilin of M_r ~16,000, and noncovalent forces between pilin subunits maintain the integrity of the pilus (18). In their physical properties, cellular distribution, and twitching motility (11), the pili of *B. nodosus* closely resemble the more intensely studied common pili of *P. aeruginosa* (5, 40). The homologous relationship of these pili, demonstrated by the extensive amino acid

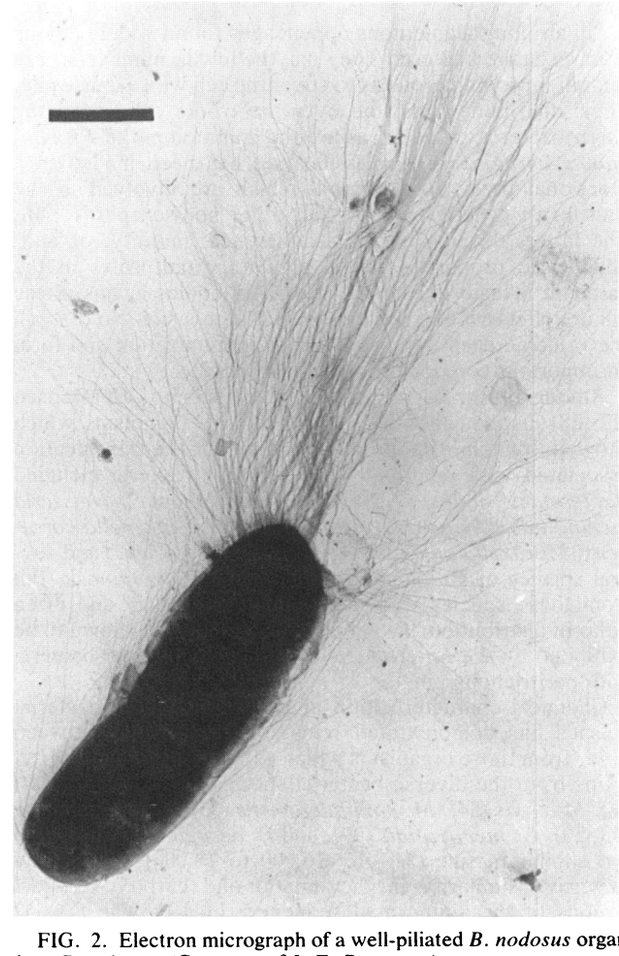


FIG. 2. Electron micrograph of a well-piliated *B. nodosus* organism. Bar, 1 μm. (Courtesy of J. E. Peterson.)

TABLE 1. Percent identity in sequence comparisons of pilins from *B. nodosus* strains of different serogroups[a]

Serogroup (strain)	% Identity between strains							
	A (198)	B (234)	C (217)	E (216)	F (1017)	G (238)	D (340)	H (265)
A (198)	100	76	59	74	71	64	33	36
B (234)		100	64	72	72	65	33	37
C (217)			100	65	59	78	36	37
E (216)				100	81	64	35	37
F (1017)					100	62	37	34
G (238)						100	36	38
D (340)							100	68
H (265)								100

[a] The number of identities in each comparison is expressed as a percentage of the maximum possible number of aligned residues in that comparison. Gaps were introduced in alignments if the total number of identical residues aligned increased by more than two for each added gap. Comparisons exhibiting a relatively low number of identities are boxed.

sequence similarity of the pilins, suggests further potential similarities in the folding and packing arrangement of pilin subunits within these pili. Data obtained from X-ray studies of *P. aeruginosa* pili suggest that subunits are arranged in a helix with 5.1 subunits per turn and a pitch of 4.1 nm (22, 68). Individual pilin molecules are thought to have closely packed α-helical rods oriented approximately parallel to the pilus fiber axis. Hydrodynamic studies on detergent-dissociated pili of *P. aeruginosa* have further suggested that dimeric units possessing twofold rotational symmetry may comprise the basic building blocks of the pilus helix (68).

The role played by *B. nodosus* pili in bacterial invasion has not been established, although a high degree of piliation is a prerequisite for virulence in footrot infection (21, 51, 52, 61). In many bacterial infections, the specific adhesive properties of pili play a major role in the location and colonization of the appropriate tissue (40, 49); however, an adherence function for *B. nodosus* pili has not been demonstrated (D. J. Stewart, Ph.D. thesis, University of Sydney, Sydney, Australia, 1975). Secreted proteases play an important role in facilitating the penetration and colonization of the hoof epidermis in footrot infection (19, 52, 55), and once the epidermal matrix of the hoof is broken down by proteases, pili may aid the spread of bacteria by twitching motility. Elevated levels of antibody directed against the pilus following vaccination prevent this spread of organisms and consequently control the spread of infection (13).

SEROTYPIC VARIATION IN PILINS OF *B. NODOSUS*

Variation in pilin structure is the basis of serological diversity in *B. nodosus*, and amino acid sequences of pilins permit some insight into evolutionary relationships of strains and define the structural basis of serological differences at the molecular level. Comparison of the amino acid sequences of pilins from representative members of each serogroup indicates that serogroups can be classified into two distinct categories based on the degree of amino acid sequence similarity (Table 1). Although pilins of all serotypes exhibit virtual identity between residues 1 to 32, extensive sequence conservation in the remaining part of the pilin sequence is limited to within each category or subset.

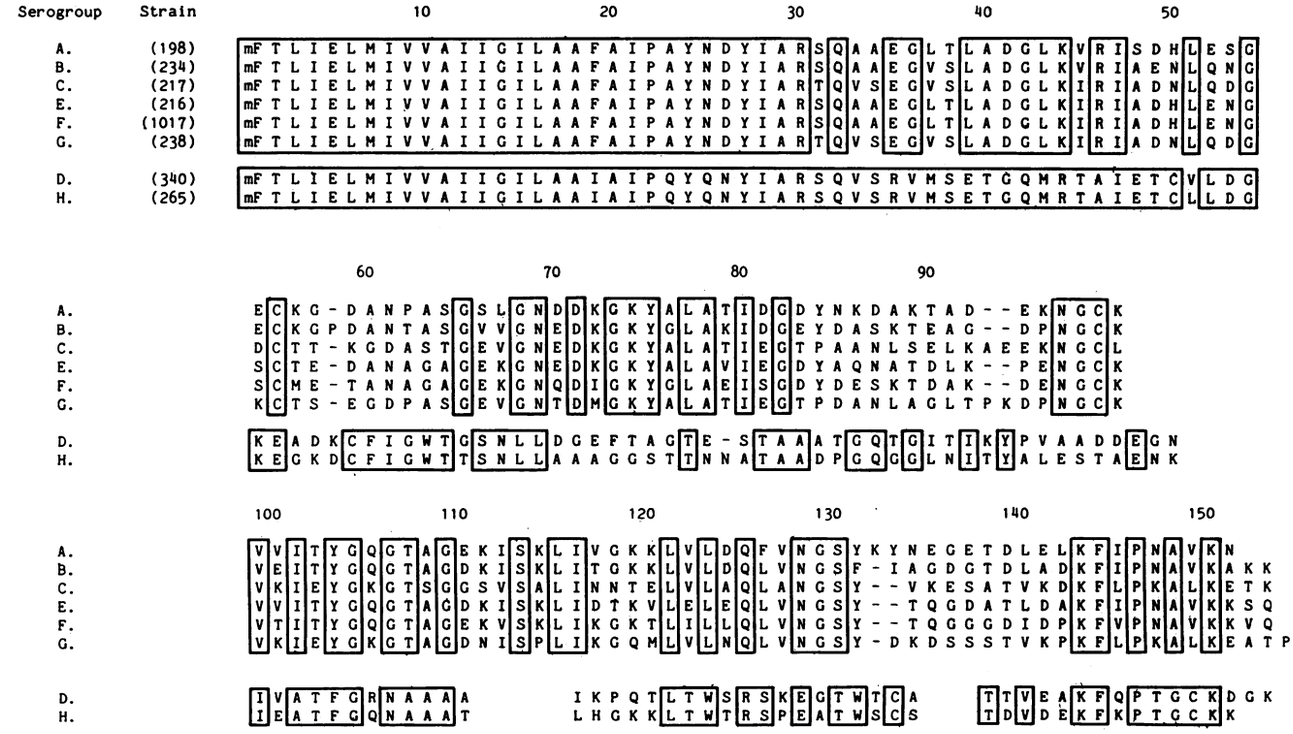
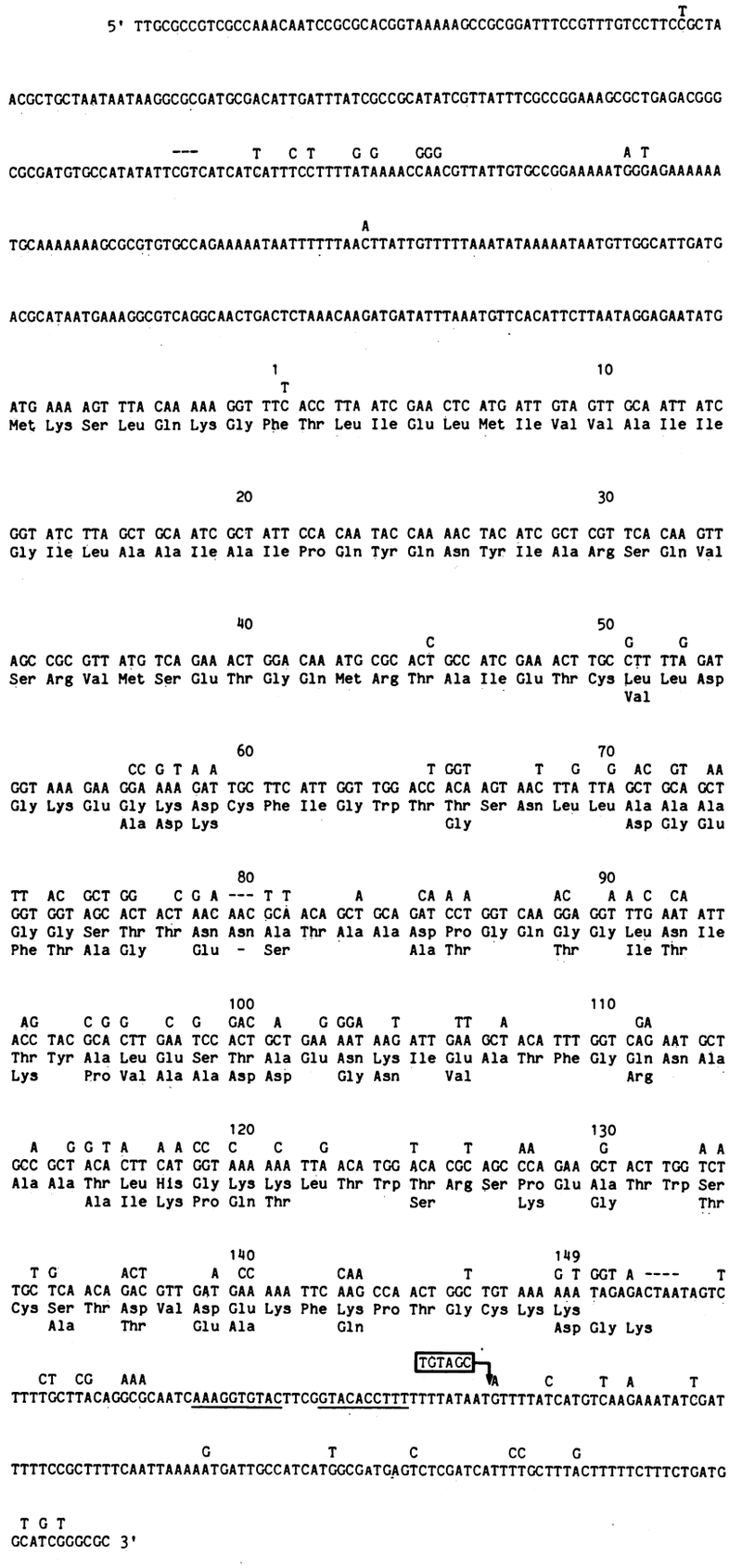


FIG. 3. Alignment of pilin sequences from *B. nodosus* 198 (serogroup A) (14), 234 (serogroup B), 217 (serogroup C) (McKern, unpublished data), 216 (serogroup E) (37), 1017 (serogroup F) (9a), and 238 (serogroup G) (17b). Residue numbers refer to the strain 198 sequence. A sequence alignment of pilin from strain 340 (K. G. Finney, T. C. Elleman, and D. J. Stewart, J. Gen. Microbiol., in press) with pilin of strain 265 (16) is shown below the A-set pilins and is broken to illustrate the homology in the carboxy-terminal region between the A-set and D-set pilins. Identical residues in either subset are boxed.


5' TTGCGCCGTCGCCAAACAATCCGCGCACGGTAAAAAGCCGCGGATTTCCGTTTGTCCTTCCGCTA
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CGCGATGTGCCATATATTCGTCATCATCATTTCCTTTTATAAAACCAACGTTATTGTGCCGGAAAAATGGGAGAAAAAA
TGCAAAAAAAGCGCGTGTGCCAGAAAAATAATTTTTTAACTTATTGTTTTTAAATATAAAAATAATGTTGGCATTGATG
ACGCATAATGAAAGGCGTCAGGCAACTGACTCTAAACAAGATGATATTTAAATGTTCACATTCTTAATAGGAGAATATG
ATG AAA AGT TTA CAA AAA GGT TTC ACC TTA ATC GAA CTC ATG ATT GTA GTT GCA ATT ATC
Met Lys Ser Leu Gln Lys Gly Phe Thr Leu Ile Glu Leu Met Ile Val Val Ala Ile Ile
GGT ATC TTA GCT GCA ATC GCT ATT CCA CAA TAC CAA AAC TAC ATC GCT CGT TCA CAA GTT
Gly Ile Leu Ala Ala Ile Ala Ile Pro Gln Tyr Gln Asn Tyr Ile Ala Arg Ser Gln Val
AGC CGC GTT ATG TCA GAA ACT GGA CAA ATG CGC ACT GCC ATC GAA ACT TGC CTT TTA GAT
Ser Arg Val Met Ser Glu Thr Gly Gln Met Arg Thr Ala Ile Glu Thr Cys Leu Leu Asp
GGT AAA GAA GGA AAA GAT TGC TTC ATT GGT TGG ACC ACA AGT AAC TTA TTA GCT GCA GCT
Gly Lys Glu Gly Lys Asp Cys Phe Ile Gly Trp Thr Thr Ser Asn Leu Leu Ala Ala Ala
Ala Asp Lys Gly Asp Gly Glu
TT AC GCT GG C G A --- T T A CA A A AC A A C CA
GGT GGT AGC ACT ACT AAC AAC GCA ACA GCT GCA GAT CCT GGT CAA GGA GGT TTG AAT ATT
Gly Gly Ser Thr Thr Asn Asn Ala Thr Ala Ala Asp Pro Gly Gln Gly Gly Leu Asn Ile
Phe Thr Ala Gly Glu - Ser Ala Thr Thr Ile Thr
AG C G G C G GAC A G GGA T TT A GA
ACC TAC GCA CTT GAA TCC ACT GCT GAA AAT AAG ATT GAA GCT ACA TTT GGT CAG AAT GCT
Thr Tyr Ala Leu Glu Ser Thr Ala Glu Asn Lys Ile Glu Ala Thr Phe Gly Gln Asn Ala
Lys Pro Val Ala Ala Asp Asp Gly Asn Val Arg
A G G T A A A CC C C G T T AA G A A
GCC GCT ACA CTT CAT GGT AAA AAA TTA ACA TGG ACA CGC AGC CCA GAA GCT ACT TGG TCT
Ala Ala Thr Leu His Gly Lys Lys Leu Thr Trp Thr Arg Ser Pro Glu Ala Thr Trp Ser
Ala Ile Lys Pro Gln Thr Ser Lys Gly Thr
T G ACT A CC CAA T G T GGT A ---- T
TGC TCA ACA GAC GTT GAT GAA AAA TTC AAG CCA ACT GGC TGT AAA AAA TAGAGACTAATAGTC
Cys Ser Thr Asp Val Asp Glu Lys Phe Lys Pro Thr Gly Cys Lys Lys
Ala Thr Glu Ala Gln Asp Gly Lys
TGTAGC
CT CG AAA A C T A T
TTTTGCTTACAGGCGCAATCAAAGGTGTACTTCGGTACACCTTTTTTTATAATGTTTTATCATGTCAAGAAATATCGAT
G T C CC G
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T G T
GCATCGGGCGC 3'

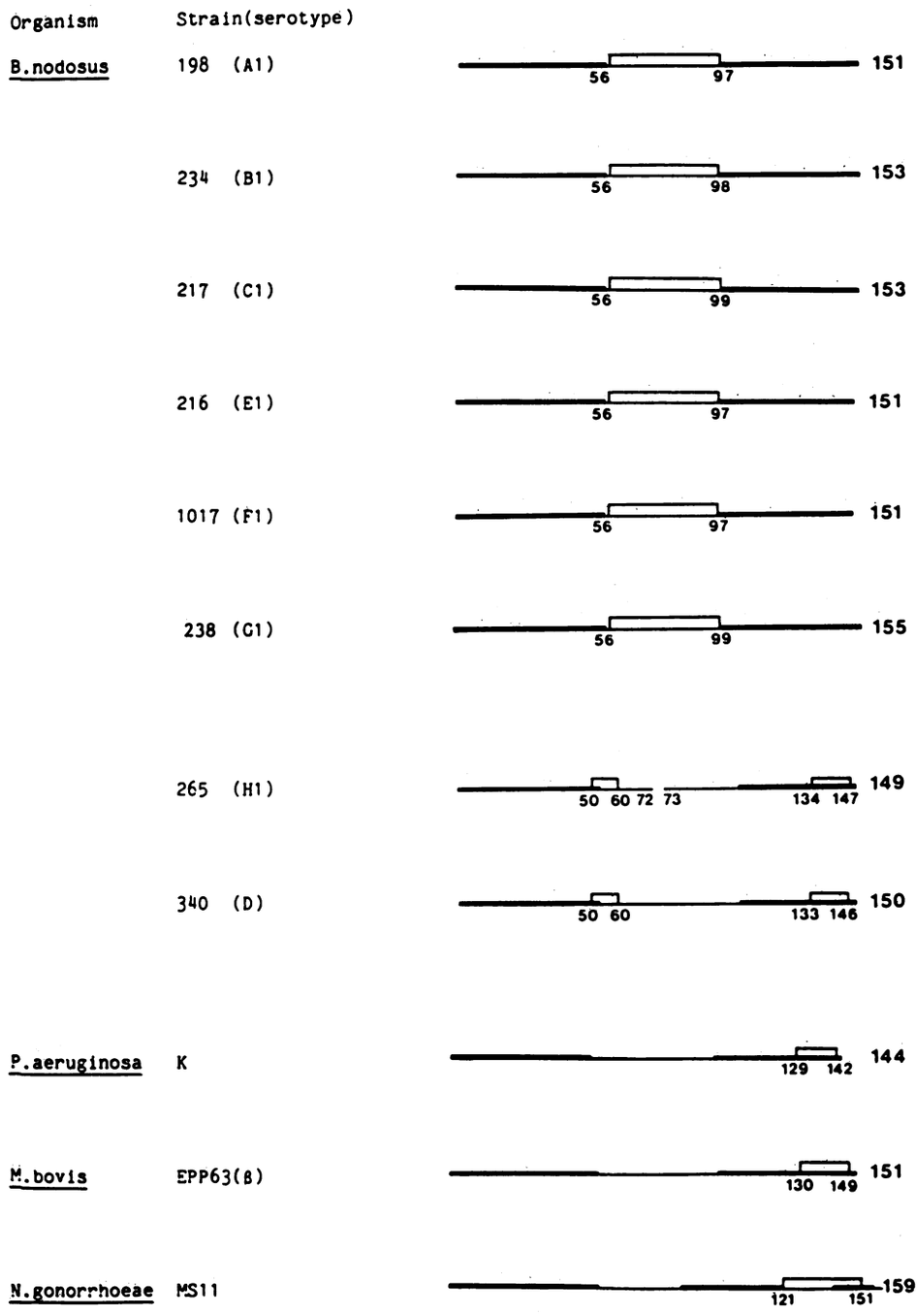
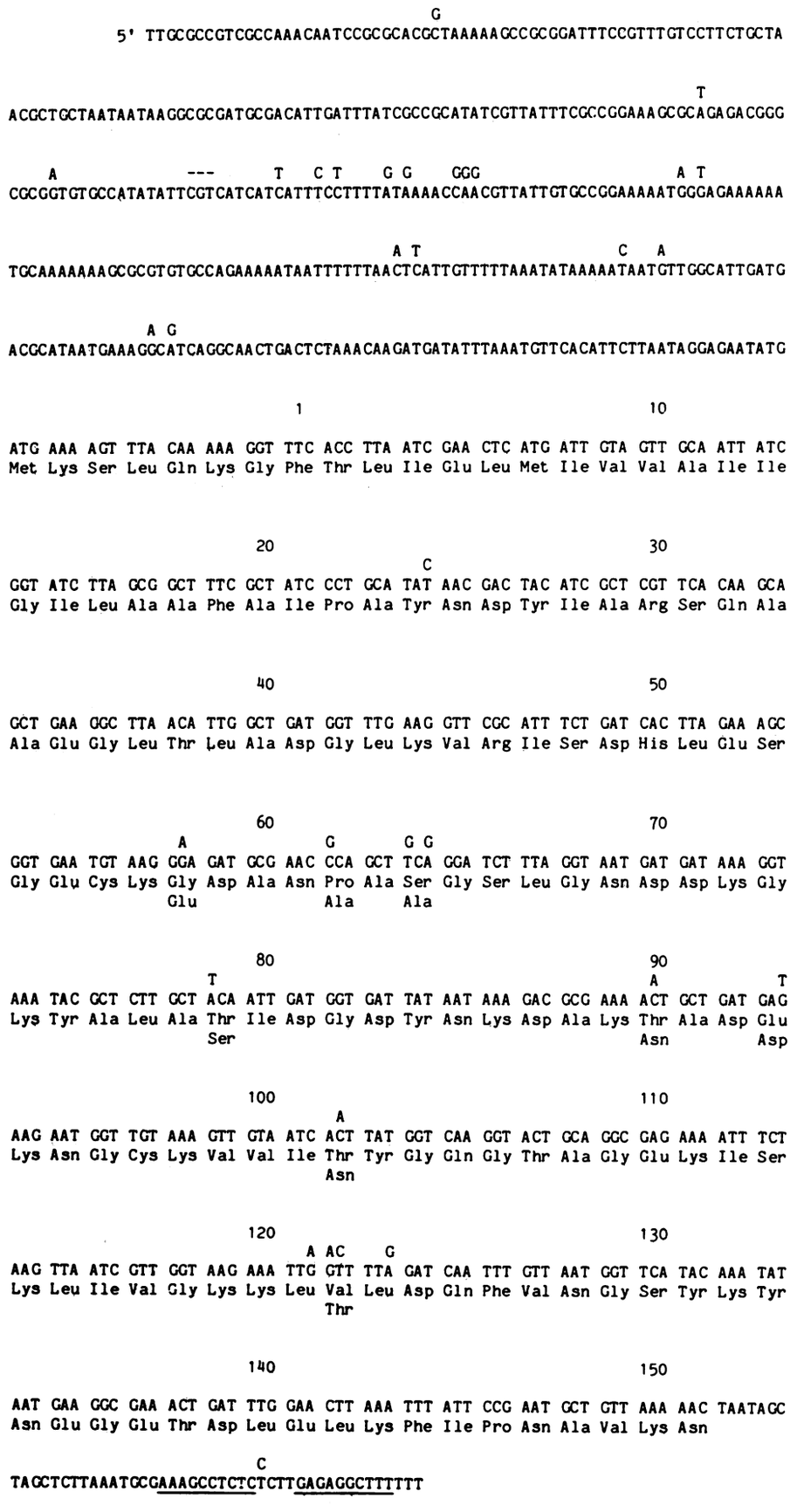


FIG. 5. Diagrammatic representation of mePhe pilin sequences to illustrate the disposition of disulfide bridges and homologous N-terminal and C-terminal sequences (thicker lines). The residue length of each pilin is also shown.

Representatives of pilins from serogroups A, B, C, E, F, and G (A-set pilins) exhibit 60 to 80% identity of sequence in alignments (Fig. 3), and representatives of serogroups D and H (D-set pilins) exhibit 68% identity (Fig. 4). In contrast, alignments of sequence between A-set and D-set pilins exhibit <40% identity despite the insertion of numerous gaps in alignments. Disulfide bridge location also differs between the subsets (Fig. 5): a large central disulfide loop is found in

FIG. 4. Nucleotide sequence and predicted protein sequence of pilin from *B. nodosus* 265 (serogroup H). Differences in the pilin of strain 340 (serogroup D) are indicated, and deletions and insertions are indicated by dashes and boxes, respectively. The predicted protein sequence is numbered from the first amino acid of mature pilin. A region of hyphenated dyad symmetry is underlined.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CGCGGTGTGCCATATATTCGTCATCATCATTTCCTTTTATAAAACCAACGTTATTGTGCCGGAAAAATGGGAGAAAAAA
TGCAAAAAAAAGCGCGTGTGCCAGAAAAATAATTTTTTAACTCATTGTTTTTAAATATAAAAATAATGTTGGCATTGATG
ACGCATAATGAAAGGCATCAGGCAACTGACTCTAAACAAGATGATATTTAAATGTTCACATTCTTAATAGGAGAATATG
1
10
ATG AAA AGT TTA CAA AAA GGT TTC ACC TTA ATC GAA CTC ATG ATT GTA GTT GCA ATT ATC
Met Lys Ser Leu Gln Lys Gly Phe Thr Leu Ile Glu Leu Met Ile Val Val Ala Ile Ile
20
30
GGT ATC TTA GCG GCT TTC GCT ATC CCT GCA TAT AAC GAC TAC ATC GCT CGT TCA CAA GCA
Gly Ile Leu Ala Ala Phe Ala Ile Pro Ala Tyr Asn Asp Tyr Ile Ala Arg Ser Gln Ala
40
50
GCT GAA GGC TTA ACA TTG GCT GAT GGT TTG AAG GTT CGC ATT TCT GAT CAC TTA GAA AGC
Ala Glu Gly Leu Thr Leu Ala Asp Gly Leu Lys Val Arg Ile Ser Asp His Leu Glu Ser
60
70
GGT GAA TGT AAG GGA GAT GCG AAC CCA GCT TCA GGA TCT TTA GGT AAT GAT GAT AAA GGT
Gly Glu Cys Lys Gly Asp Ala Asn Pro Ala Ser Gly Ser Leu Gly Asn Asp Asp Lys Gly
Glu Ala Ala
80
90
AAA TAC GCT CTT GCT ACA ATT GAT GGT GAT TAT AAT AAA GAC GCG AAA ACT GCT GAT GAG
Lys Tyr Ala Leu Ala Thr Ile Asp Gly Asp Tyr Asn Lys Asp Ala Lys Thr Ala Asp Glu
Ser Asn Asp
100
110
AAG AAT GGT TGT AAA GTT GTA ATC ACT TAT GGT CAA GGT ACT GCA GGC GAG AAA ATT TCT
Lys Asn Gly Cys Lys Val Val Ile Thr Tyr Gly Gln Gly Thr Ala Gly Glu Lys Ile Ser
Asn
120
130
AAG TTA ATC GTT GGT AAG AAA TTG GTT TTA GAT CAA TTT GTT AAT GGT TCA TAC AAA TAT
Lys Leu Ile Val Gly Lys Lys Leu Val Leu Asp Gln Phe Val Asn Gly Ser Tyr Lys Tyr
Thr
140
150
AAT GAA GGC GAA ACT GAT TTG GAA CTT AAA TTT ATT CCG AAT GCT GTT AAA AAC TAATAGC
Asn Glu Gly Glu Thr Asp Leu Glu Leu Lys Phe Ile Pro Asn Ala Val Lys Asn
TAGCTCTTAAATGCGAAAGCCTCTCTCTTGAGAGGCTTTTTT

A-set pilins, while two smaller loops are present in D-set pilins. This subdivision of pilins into two distinct subsets explains some instances of low-level serological cross-reactivities between serogroups having pilins in the same subset (1, 17c) and the ability of T cells from sheep primed with either strain 198 (serogroup A) or 265 (serogroup H) pili to respond by proliferation only following stimulation by members of the same subset (17c).

A-Set Pilins

In comparisons of the similar sequences of pilins from serogroups A, B, C, E, F, and G, especially close similarities are recognized between pilins of serogroups C and G (78% identity) and serogroups E and F (81% identity). This similarity, although accounting for low-level serological cross-reactivity between these serogroups (1) is insufficient to permit cross-protection following vaccination. On the other hand, both shared and serotype-specific antigenic determinants are present on pilins of the same serogroup, and considerable cross-protection can be afforded by the shared determinants (8, 60). The high level of serological cross-reactivity and cross-protection between members of the A serogroup is reflected by the extensively similar amino acid sequences (Fig. 6). The amino acid sequences of pilins from *B. nodosus* 198 (serotype A1) and 286 (serotype A2) exhibit 95% identity, differing in only eight amino acid residues. Nucleotide sequences of the pilin genes from the A1 and A2 serotypes are consistent with the creation of serotypic variants through a process of genetic drift, with the accumulation of point mutations in those regions of the pilin molecule not constrained by structural or functional necessity.

Four serotypes in serogroup B and two serotypes in each of serogroups A, C, E, F, and G have been identified (9), although to date the amino acid sequence of pilin from only a single representative of each serogroup other than serogroup A has been determined. Sequence differences between the pilins of different serogroups are consistent with a process of further accumulation of point mutations together with local in-frame deletions to produce the serologically unrelated serogroups A, B, C, E, F, and G. A single intramolecular disulfide bridge encompasses residues 56 to 97 in A-set pilins, and the location of this bridge is characteristic of these pilins. The average hydrophilicity of residues (30) within the disulfide loop (Fig. 7) is considerably greater than that of residues in the corresponding region of D-set pilins (Fig. 8), and this might reflect the replacement of hydrophobic bonding in this region of the pilin molecule by the disulfide bridge of A-set pilins. No homology is apparent between residues 56 to 97 in the disulfide loop of A-set pilins and the corresponding central region of D-set pilins (Fig. 3), although these central regions are flanked by homologous N-terminal and C-terminal regions (Fig. 1).

Optimal alignments of A-set pilin sequences require the insertion of up to three gaps. These occur at or adjacent to predicted β-bends, loci which connect major secondary structural units of proteins (43) and often the site of deletions because their surface position involves interactions with solvent rather than with other regions of the molecule. Short-range frameshifts may also have contributed to heterogeneity of sequence between serogroups as illustrated by comparison of residues 59 to 62 of strain 217 (serogroup C) and residues 57 to 60 of strain 198 (serogroup A).

A-set pilins show variability in <50% of the positions in the amino acid sequences. Substitutions are distributed throughout the C-terminal two-thirds of the molecule, with regions of extensive substitution corresponding with regions of high hydrophilicity (Fig. 7) and hence potentially at the surface of the molecule. Linear regions of extensive substitution include residues 57 to 64, 83 to 94, 117 to 124, and 132 to 142. The relatively high hydrophilicity of these regions and the extensive substitution between all serogroups suggest them as possible sites of the antigenic determinants in the A-set pilin molecules. The latter region (residues 132 to 142) is identical in serotypes A1 and A2 and might indicate a shared antigenic determinant of these serotypes.

D-Set Pilins

No serological subdivision of serogroup D has been recognized, while two serotypes of serogroup H have been distinguished by cross-agglutination (9). In sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the pilins of serogroup D migrate as a single species of M_r ~16,000 similar to A-set pilins, whereas the pilins of serogroup H display two subunits (2, 16). Electrophoretic mobilities suggest M_r ~6000 and M_r ~10,000 for the subunits of serotype H1 (2), whereas amino acid sequence studies show subunit sizes of 7,875 for the faster-migrating N-terminal subunit and 8,017 for the C-terminal subunit (16). The anomalously high electrophoretic mobility of the N-terminal subunit may relate to the high content of the hydrophobic amino acid residues, while the anomalously low mobility of the C-terminal subunit is presumably associated with overestimation of the molecular weights of small proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (69).

Studies of the pilin gene from serotype H1 indicate that the two subunits arise by posttranslational processing of a single-chain precursor (16). Small quantities of a precursor of M_r ~16,000 are in fact seen in pili preparations from serogroup H, and in surrogate hosts harboring the pilin gene of serogroup H, only single-chain proteins of M_r ~16,000 to 17,000 are produced (Fig. 9). Processing of pilin from serotype H1 involves cleavage between residues 72 and 73, possibly by secreted proteases. The charge change in this region from neutral in pilin of serotype H1 to negative in pilin from serogroup D could explain the absence of cleavage in pilins from serogroup D.

The absence of intersubunit disulfide bridges between N-terminal and C-terminal subunits of pilin from serogroup H (16) and the absence of free thiol in pilins of *B. nodosus* (N. M. McKern, unpublished data) suggest that the four half-cystine residues of the D-set pilins form two small local disulfide loops. Sequence surrounding the half-cystine residues of the disulfide bridges of D-set pilins shows no homology with the sequence surrounding the half-cystines of A-set pilins. However, the C-terminal disulfide loop of D-set pilins is homologous with the single disulfide loop found in pilin from *P. aeruginosa*, *M. bovis*, and *N. gonorrhoeae*.

Differences in sequence between pilins from strains 340 (serogroup D) and 265 (serogroup H) are distributed throughout the C-terminal two-thirds of the molecules, as were the

FIG. 6. Nucleotide sequence and predicted protein sequence of pilin from *B. nodosus* 198 (serotype A1). Differences in the nucleotide and protein sequence of pilin from strain 286 (serotype A2) (P. A. Hoyne, unpublished data) are indicated. The predicted protein sequence is numbered from the first amino acid of mature pilin. A region of hyphenated dyad symmetry is underlined.

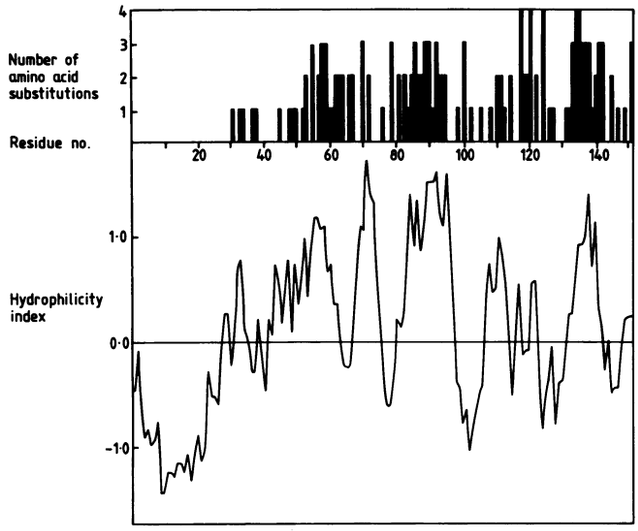

FIG. 7. Hydrophilicity profile of *B. nodosus* 198 (serogroup A), using a span of seven amino acid residues. The number of substitutions occurring at each position in strains 198, 234, 217, 216, 1017, and 238 (i.e., representatives of serogroups A, B, C, E, F, and G) are indicated above the graph.

changes comprising the potential antigenic determinants of the more numerous A-set pilins. Extensive regions of amino acid substitutions again occur in regions of high hydrophilicity which may constitute surface parts of the molecule (Fig. 8). These substitutions are compatible with the generation of diversity through a process of genetic drift. Extensive amino acid substitutions which may correlate with potential antigenic determinants occur at residues 57 to 59, 71 to 79, 94 to 106, 116 to 121, and 137 to 143.

PILIN GENE SEQUENCES

mePhe Pilin Genes

A major difference to emerge from studies of the pilin genes of *B. nodosus*, *M. bovis*, *N. gonorrhoeae*, and *P.*

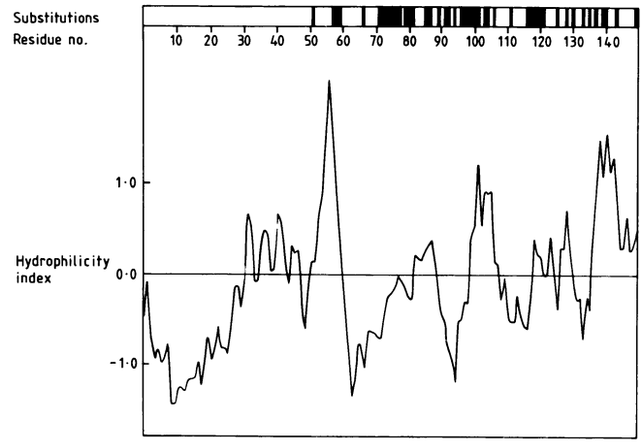

FIG. 8. Hydrophilicity profile of *B. nodosus* 265 (serotype H1), using a span of seven amino acid residues. The positions of substitutions in strain 340 (serogroup D) are indicated above the graph.

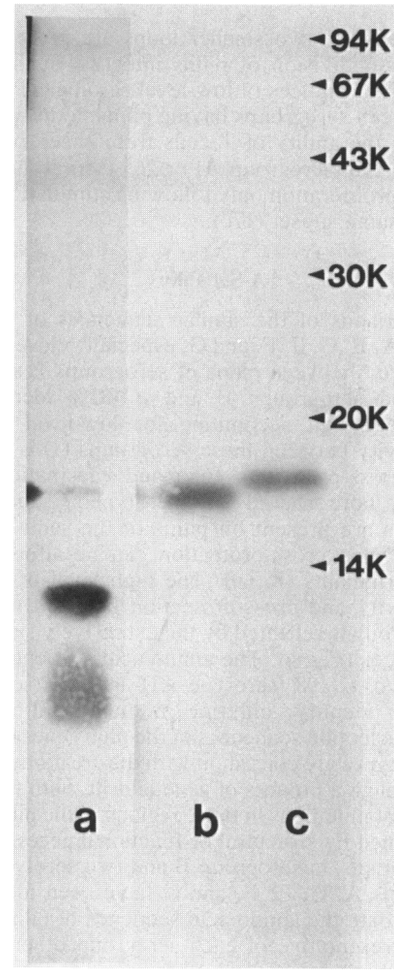

FIG. 9. Immunoblot, using anti-*B. nodosus* 265 pili antisera following sodium dodecyl sulfate-15% polyacrylamide gel electrophoresis of cellular products. Lanes: a, *B. nodosus* 265 (serogroup H) pili; b, *P. aeruginosa* PAODB2 harboring the pilin gene of *B. nodosus* 265 and induced for expression: c, *E. coli* C600 harboring the pilin gene of *B. nodosus* 265 and induced for expression. An incompletely processed pilin and prepilin are present in lanes b and c, respectively.

aeruginosa is the copy number of the pilin gene in the genome. The pilin genes of *B. nodosus* and *P. aeruginosa* are present as single copies within the genome (15, 41), whereas multiple copies of pilin genes are present in the chromosomes of *N. gonorrhoeae* and *M. bovis* (34, 38). This multiplicity of pilin genes in the latter organisms is thought to be associated with gene switching mechanisms for evading the immunological defenses of the host through a changed antigenic profile of the pilus (26). In other respects pilin gene sequences from these organisms show similarities which indicate conservation in the mechanism of pilin biosynthesis between organisms additional to the amino acid sequence homology of pilins required for pilus structural assembly. The pilins are all encoded as prepilins having similar short leader sequences of six or seven residues preceded by a well-defined ribosome-binding site (Fig. 10A). These leader sequences are considerably smaller than the signal peptides which confer export status on other procaryotic proteins destined for secretion (66), but in overall appearance the prepilins resemble other preproteins since a short positively charged N-terminal segment precedes a hydrophobic seg-

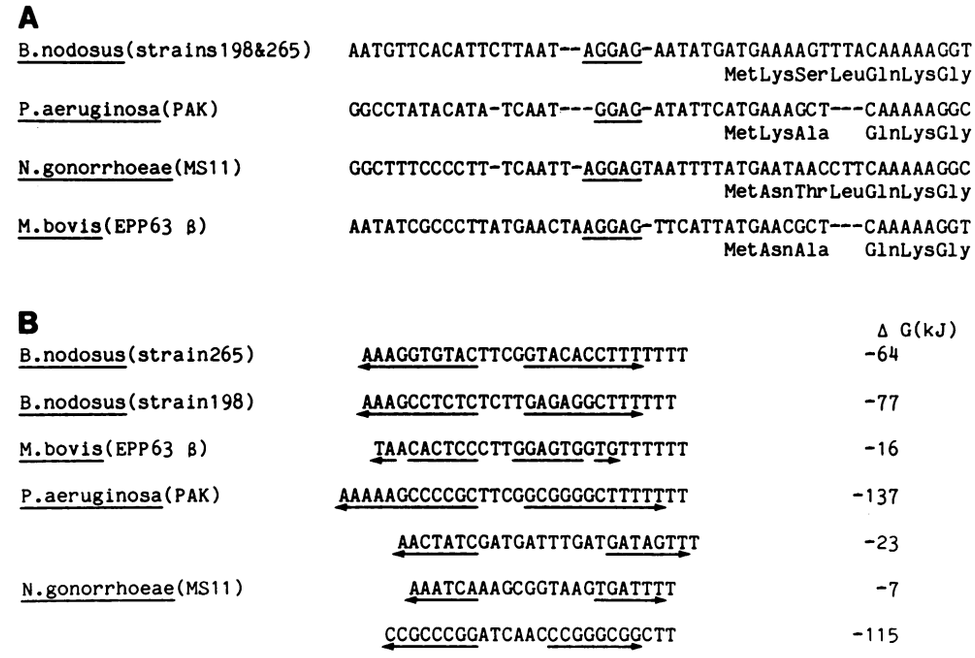

FIG. 10. Ribosome-binding sites and signal peptides in pilin gene sequences (A) and regions of hyphenated dyad symmetry located downstream of pilin genes (B). (A) Shine-Dalgarno sequences are underlined. (B) The free energies, $\Delta G_{25°C}$, of possible secondary structures are shown (63).

ment. The prepilins differ from other preproteins in the position of posttranslational cleavage, which is prior to rather than following the hydrophobic segment. Processing of prepilin to mature pilin involves both removal of the leader sequence and N-methylation of phenylalanine at the amino terminus. However, in all mePhe pilins a proportion of molecules are found which lack the *N*-methylphenylalanine residue and begin with threonine, the residue encoded after phenylalanine.

A large body of data indicates that the signal peptides of secreted or membrane proteins function to bring the polysome to a site of export on the membrane where further elongation of the polypeptide chain occurs (42). Signal sequences are generally removed following translation, thus avoiding the necessity to shield the hydrophobic segment of the signal peptide from water. The hydrophobic segment of pilin is retained in the mature protein, where it is presumably well shielded in inter- or intrasubunit contacts and may play a role in the aggregation of subunits. In prepilin the role of the leader sequence may be to confer a conformation which prevents aggregation of pilin prior to pilus assembly. The removal of the short leader sequence may be necessary to permit the correct conformation of pilin to be assumed for pilus assembly.

Adjacent to the coding region of mePhe pilins are found features of nucleotide sequence characteristic of transcriptional initiation and termination sites for ribonucleic acid polymerase. Promoters have been demonstrated upstream of the pilin-coding sequence in *P. aeruginosa* and *N. gonorrhoeae* (31, 38), and these resemble the consensus promoter sequence of ribonucleic acid polymerase requiring the *ntrA* gene product rather than the *Escherichia coli* consensus promoter sequence. Sequences similar to the *ntrA*-activated promoter consensus sequences are also found upstream of the pilin-coding sequences of *B. nodosus* and *M. bovis* (31). Immediately downstream of the pilin-coding sequences are found structures characteristic of transcription terminators for ribonucleic acid polymerase, a region of hyphenated dyad symmetry followed by a string of thymidine residues (Fig. 10B).

B. nodosus Pilin Genes

The available nucleotide sequences of pilin structural genes from *B. nodosus* confirm the simple relationships between pilins within each subset, base substitutions largely accounting for the sequence divergence between serogroups (cf. Fig. 4). However, comparison of the coding regions available for pilins representative of the different subsets (Fig. 11) gives no indication of the derivation of subsets. A relationship between the coding sequences from the C-terminal residues of pilins from *B. nodosus* 198 (serogroup A) and 265 (serogroup H) suggests that in A-set pilins a C-terminally located disulfide bridge in a location characteristic of many other mePhe pilins may have been lost through a frameshift mutation (Fig. 12).

Nucleotide sequences downstream of the pilin-coding sequence from representatives of serogroups A, G, D, and H confirm the close relationship within subsets recognized from the comparison of pilin sequences. While homology between strains 198 (serogroup A) and either 265 (serogroup H) or 340 (serogroup D) is limited to the vicinity of the potential transcriptional unit (Fig. 12), homology between strains 265 and 340 (Fig. 4) or between strains 198 and 238 (serogroup G) (data not shown) extends well beyond this unit. This limited downstream sequence homology contrasts with extensive homology (95% identity) present in nucleotide sequence upstream of the pilin-coding region of these *B. nodosus* strains. The homology in this region may relate to the presence of a long open reading frame initiated just upstream of the pilin gene in the opposite orientation (T. A. A. Dopheide, unpublished data). Clustering of pilin

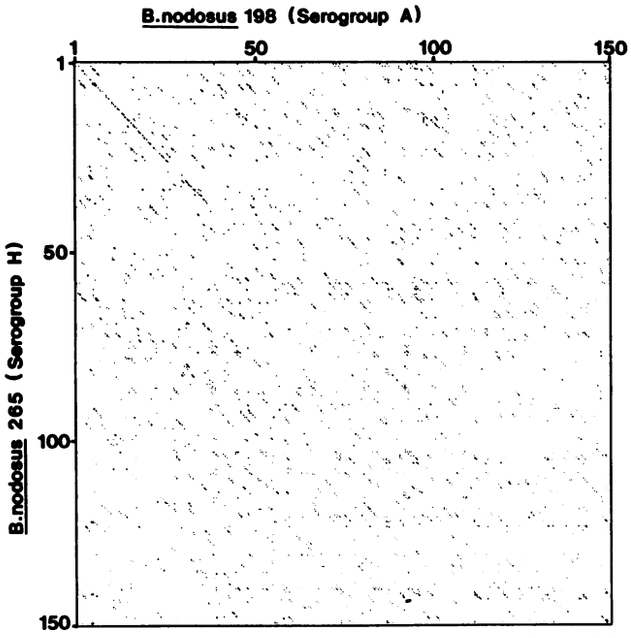

FIG. 11. Nucleotide sequence comparison matrix of the pilin-coding sequences of *B. nodosus* 198 and 265. A dot in the matrix indicates five or more identities in a span length of seven nucleotides.

genes with those of auxiliary proteins of pilus biosynthesis in other organisms (32) suggests a potential auxiliary function in pilus biosynthesis for the product of this open reading frame.

ANALYSIS OF PILIN STRUCTURE IN RELATION TO POTENTIAL FUNCTION

The morphological and functional similarities of pili from *B. nodosus*, *M. bovis*, *N. gonorrhoeae*, and *P. aeruginosa* and the homologous origins as evidenced by sequence similarity of pilins suggest that pilin subunits may share other similarities, including peptide chain folding and packing arrangements. Also, the compatibility of the pilin processing, translocation, and assembly systems of *P. aeruginosa* and *B. nodosus* (17) indicates conservation of those regions of the molecule interacting with the assembly apparatus for pili. Analysis of the sequences of the various mePhe pili permits the identification of conserved features and allows the speculative assignment of potential functional roles to these features. Three major regions can be recognized for this purpose in comparisons of mePhe pilins: a highly conserved amino-terminal region (residues 1 to 54), a variable central region, and a partly conserved carboxy-terminal region (Fig. 1).

Conserved Amino-Terminal Region

Residues 1 to 20 are identical in all mePhe pilins except for the highly conservative substitutions of isoleucine, valine, and phenylalanine at residues 10, 13, and 19. In prepilin, this highly hydrophobic region, in concert with the short positively charged leader sequence, might act as a topogenic signal (4) by which nascent prepilin molecules are recognized for transfer to a specific destination. The conservation of this putative signal sequence between prepilins from different bacterial families contrasts with the general diversity in bacterial signal sequences and implies a highly discriminating recognition system for prepilin molecules. The demonstration that the prepilin of *B. nodosus* when expressed in *P. aeruginosa* is both processed and assembled into pili (17) indicates conservation of recognition signals in the prepilins of these species. The highly conserved N-terminal sequence most likely constitutes this signal, and this extensive conservation might indicate the extreme functional pressure to retain this sequence unchanged.

In mature pili the highly hydrophobic N-terminal segment would need to be buried, either within the subunit, so contributing to the maintenance of subunit structure, or between subunits at a highly hydrophobic interface, providing interactions which could maintain the quarternary structure of the pilus. In either case, the hydrophobic character of this region would be important to the structural integrity of the pilus. Structural predictions suggest a high potential for an α-helical configuration in this region (Fig. 13). This configuration permits clustering of the most highly hydrophobic residues which may favor hydrophobic interactions with similar segments of other molecules to form a polymeric array. A dual role of this N-terminal segment as a topogenic signal and structural element would undoubtedly contribute to conservation of the sequence between pilins from diverse bacterial species.

Residues 21 to 32 of the amino-terminal regions constitute a somewhat more hydrophilic segment, with many positions

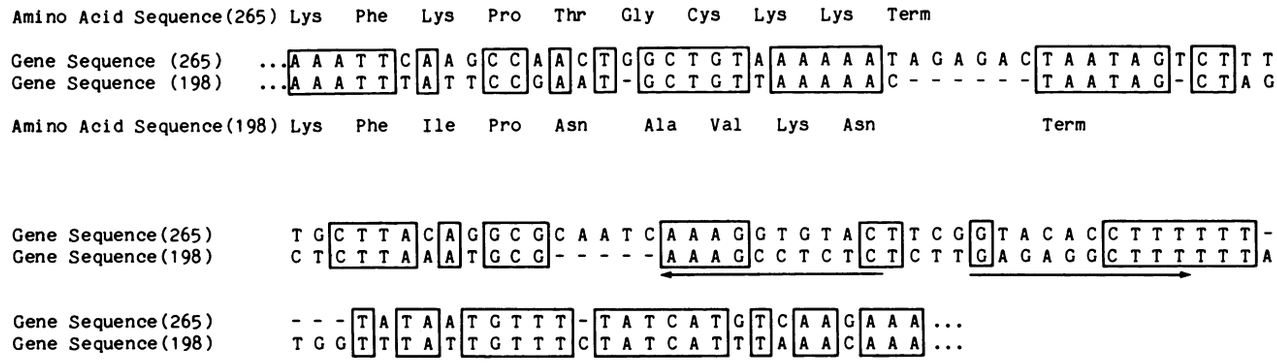

FIG. 12. Nucleotide sequence alignment to demonstrate homology in the C-terminal coding regions and 3′ noncoding regions of the pilin gene from *B. nodosus* 198 (serogroup A) and 265 (serogroup H). Identical bases are boxed. The arrows indicate a region of hyphenated dyad symmetry (potential transcription terminator).

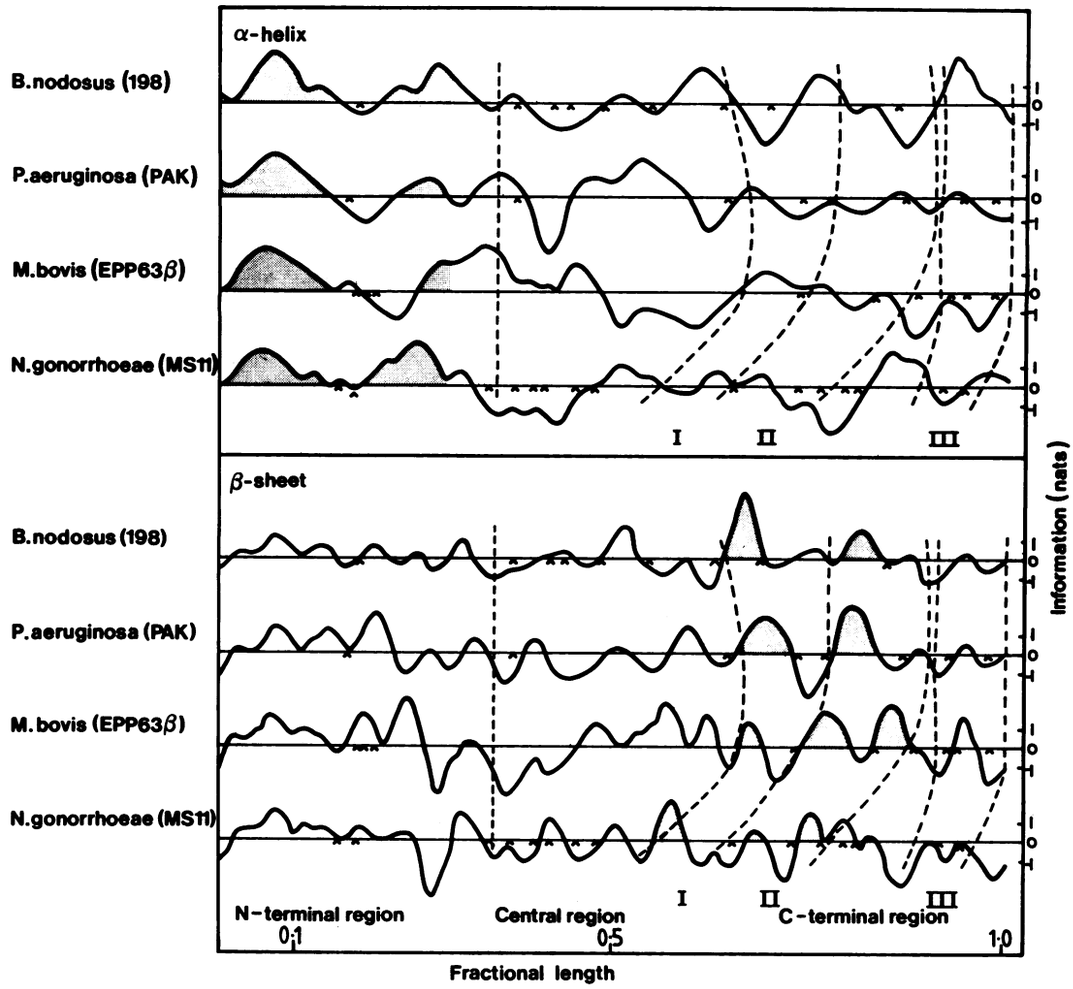

FIG. 13. Predictive analysis (25) for α-helix and β-sheet in the subunits of mePhe pili. The positions of predicted β-turns are indicated by arrowheads. Potential secondary structure assignments common to all pilins are denoted by shaded areas. Broken lines indicate the regions of homology shown in Fig. 1, and lines are displaced between sequences due to amino acid deletions or insertions.

conservatively substituted between pilins of different species. Invariant residues are found at positions 22, 24, and 27. Conserved tyrosine residues at positions 24 and 27 are inaccessible to titration in *P. aeruginosa* pili, but are exposed following detergent dissociation of pili to nondenatured dimeric units (68), suggesting that this region of the pilin molecule is located at a hydrophobic interface between subunits. Structural predictions indicate that residues 24 to 27 have the potential to form a β-bend, characteristically a surface element joining two major secondary structures (43) and compatible with the exposure of the tyrosines at a subunit interface where hydrophobic interactions maintain the polymeric structure.

Residues 33 to 55 are considerably less well conserved between pilins of different bacterial species (20 to 50% identity) than are residues 1 to 32, suggesting that less stringent structural constraints are imposed on these residues. The receptor-binding site of gonococcal and meningococcal pili has been identified between residues 31 and 92 (50), while antiserum generated against residues 41 to 50 of these pili inhibit receptor binding (45), indicating that these residues are part of, or contiguous with, the receptor-binding site of pili in the *Neisseriae*. The conservatively substituted positions of this segment are mainly occupied by hydrophobic residues (Fig. 1), which if juxtaposed, might form a hydrophobic cleft for interaction with the carbohydrate receptors of epithelial cells.

Structural predictions suggest a largely α-helical conformation in this region, and this helix together with the predicted helices of the earlier N-terminal region and other regions may play major roles in maintaining the structure of the pilus subunit. Analogy has been drawn between the up-and-down bundle of antiparallel α-helices in the tobacco mosaic virus coat protein and the predicted secondary structure of gonococcal pilin (10). Circular dichroism and X-ray diffraction studies on the pili of *P. aeruginosa* suggesting an α-helix content of 40% (68), with helices running roughly parallel to the fiber axis (22), are compatible with this proposed tertiary structure motif.

Among pilins from a single species, the amino-terminal region generally remains constant. Residues 1 to 55 of gonococcal pilins from strain R10, MS11, and MS11 variants are identical (26, 50), α and β serotypes of *M. bovis* pilins display 98% identity (34), and pilins of the PAO and PAK

strains of *P. aeruginosa* display 94% identity (46). In contrast, the amino-terminal regions of A-set and D-set pilins of *B. nodosus* display only 60% identity. This difference between *B. nodosus* pilins and other mePhe pilins may reflect greater functional constraints on those pili for which a well-defined adherence function has been demonstrated.

Central Region (Residues 55 to ca. 100)

The extensive difference in the central regions of pilins from *B. nodosus*, *M. bovis*, *N. gonorrhoeae*, and *P. aeruginosa* contrasts with the homologous relationship shown in the amino- and carboxy-terminal regions of these pilins. No statistically significant homology is apparent between the central regions of pilins from different species. A hexapeptide sequence conserved within the receptor-binding site of gonococcal pili variants, viz., $_{73}$IKGKYV (26), is similar to sequence at the same location in many *B. nodosus* A-set pilins, viz., $_{72}$KGKYA, but this correspondence may be fortuitous since no similar sequence is present in D-set pilins of *B. nodosus*, and pilin of strain 1017 (serogroup F) differs in two of the five positions. Residues 69 to 84 of gonococcal pili which include the conserved hexapeptide sequence have been implicated as comprising or contiguous with the receptor-binding site of gonococcal pili since antiserum to these residues prevents bacterial attachment to eucaryotic cells even by heterologous pili and precipitates larger peptide fragments which bind to eucaryotic cells (45).

Despite extensive interspecies differences, sequence similarity is generally evident between the central regions of pilins within a species. The MS11 and R10 serotypes of *N. gonorrhoeae* display 50% identity in this region (50), the α and β serotypes of *M. bovis* display 50% identity (34), and the PAK and PAO strains of *P. aeruginosa* display 38% identity (46). In contrast again, no significant similarity is detected between the central regions of the A-set and D-set pilins of *B. nodosus*.

The constraints on the central region of the pilin molecule as regards structural integrity of the pilus appear to be minimal, and in this respect it is noteworthy that gonococcal pilins have undergone a deletion of at least 11 residues in this region relative to other mePhe pilins.

Conserved Carboxy-Terminal Region

Sequence homology (Fig. 1) and potential similarity of secondary structure (Fig. 13) are exhibited between the C-terminal regions of mePhe pilins, including the A-set pilins of *B. nodosus* which lack the homologous disulfide loop in this region. Sequence predictions for the early C-terminal region prior to the disulfide loop (sections I and II of Fig. 1 and 13) suggest an extensive β-sheet conformation interspersed by β-turns. A relatively high β-sheet content of 38% is predicted from circular dichroism studies of *P. aeruginosa* pili (68), and the absence of extensive regions of predicted β-sheet elsewhere in this or other pilin molecules supports assignment of β-structure to this region, possibly as an antiparallel β-sheet structure. A conserved β-sheet network may contribute to structural identity in the C-terminal region of pilins, despite considerable sequence variations and antigenic diversity in this region.

A disulfide bridge is conserved in the C-terminal region of pilins from several species, and this bridge presumably maintains the local structural integrity in a disulfide loop which often shows extensive substitution between serotypic variants (26, 34, 44). Extensive substitution also occurs in the corresponding region (residues 132 to 142) of the A-set pilins from *B. nodosus*, and the conservation of hydrophobic residues adjacent to this region (Fig. 3) might indicate that disulfide bonding is replaced by hydrophobic bonding in A-set pilins.

Sequence similarity between the C-terminal regions of A-set and D-set pilins of *B. nodosus* (28% identity) is less than that of either A-set or D-set pilins in this region (>48% identity) and considerably less than between the PAK and PAO serotypes of *P. aeruginosa*, the MS11 and R10 serotypes of *N. gonorrhoeae*, and the α and β serotypes of *M. bovis* (all >57% identity), further reflecting the dissimilarity between the two pilus sets of *B. nodosus*.

CONCLUDING REMARKS

A remarkable feature to emerge from the study of bacterial pili is the extensive conservation in the N-terminal region of pilins from several widely divergent bacterial species including *B. nodosus*, *M. nonliquefaciens*, *M. bovis*, *N. gonorrhoeae*, *N. meningitidis*, and *P. aeruginosa*. This conservation of sequence, including that of leader sequences of the prepilins, contrasts with the diversity among the pilin sequences and associated signal peptides of the *Enterobacteriaceae*. It suggests that severe constraints must be imposed on the N-terminal regions of mePhe pilins through a mechanism of pilus biosynthesis fundamentally different from that of the many pilus types of *Enterobacteriaceae*. Incompatibility in pilus biosynthesis between these groups of organisms has been confirmed by the inability of *E. coli* to assemble structured pili from an expressed prepilin of *B. nodosus*, whereas structured pili were formed in *P. aeruginosa* (17).

In addition to the conserved amino-terminal sequences of mePhe pilins, a common structure of the pilin subunits is suggested by further amino acid sequence homology in the C-terminal region of the molecules and the similarity of prediction profiles for secondary structure. Structural domains composed of an α-helix bundle and an antiparallel β-sheet may constitute the basis of a common structural motif for the subunits of all mePhe pili.

This extensive conservation of pilin sequence and predicted structure between species contrasts with the divergence of sequence between the A-set and D-set pilins of *B. nodosus*, the degree of sequence divergence between these subsets being comparable more to that between mePhe pilins from different bacterial families than to that between different strains of a single species.

The possible low structural constraints on the central region of pilin molecules and the apparent absence of an adherence function in *B. nodosus* pili may have contributed to the diversity of *B. nodosus* pilins. The central disulfide bridge of A-set pilins may perform a function similar to the C-terminally located disulfide bridge of gonococcal pilins (44), serving to isolate a region of the molecule which is then better able to tolerate extensive mutational change. Relatively minor substitutions to introduce the central disulfide bridge in a progenitor of A-set pilins may have paved the way for more extensive sequence variations in the central region of the pilin molecule, without disruption of pilus structure and function.

Although the disulfide bridges of *B. nodosus* A-set pilins and gonococcal pilins may perform an analogous function, the means whereby mutational changes are generated and propagated within the disulfide loops must differ. In gonorrheal infection, antibodies directed against gonococcal pili

block mucosal adherence so that antibody selection pressure provides a driving force for the establishment of antigenic variation (35, 64, 65). This is evidenced by the location of most changes in the disulfide loop containing the epitopes for neutralization (44). Variations in gonococcal pili upon which antibody selection pressure acts is generated through recombinational events between multiple pilus loci in the chromosome of *N. gonorrhoeae*. In footrot infection natural immunity is not a consequence of infection, and antibody selection pressure for antigenic diversity is therefore presumably negligible. In addition, a single pilin gene only is present in the *B. nodosus* genome, ruling out recombinational events between homologous intrachromosomal sequences in the generation of diversity. The variation within either A-set or D-set pilins is suggestive of random genetic drift in balance with those constraints imposed by the structural and functional requirements of the pilus. However, recombination with sequences of low homology or with foreign deoxyribonucleic acid transferred from other bacterial species may have contributed to variation in ancestral pilin genes from which the A-set and D-set pilins have arisen. Expansion in sheep population with domestication and geographical isolation of sheep breeds may have contributed to the propagation of variants within the pilins of *B. nodosus*.

Notwithstanding the extensive differences in A-set and D-set pilins, a sufficiently common topogenic signal is retained in each to permit recognition by the foreign pilus biosynthetic mechanism when pilin genes of *B. nodosus* are expressed in *P. aeruginosa* (17, 17a). *B. nodosus* pili produced in *P. aeruginosa* are indistinguishable from the indigenous pili of *B. nodosus* (except for the absence of posttranslational cleavage of serogroup H pilins). Since pilins of *B. nodosus* and *P. aeruginosa* share no greater similarity than other mePhe pilins, the conservation of similar sequence between pilins of several bacterial species may indicate compatibility in pilin processing and assembly mechanisms for all bacteria producing mePhe pili. This possibility of an interchangeable host/pili relationship could have an important application in vaccine development since pili are valuable agents in immunoprophylaxis against several diseases in which vaccine preparation is often hampered by difficulties in culturing the infectious organism. Already a *B. nodosus* pili vaccine prepared from *P. aeruginosa* harboring a plasmid-borne *B. nodosus* pilin gene has proven effective in both prophylactic and therapeutic treatment of footrot in sheep (60), and this approach to a footrot vaccine offers a means whereby problems associated with culturing the fastidious anaerobe *B. nodosus* may be avoided. Potential exists for the production of vaccines against other diseases of medical and veterinary importance; pili from *N. gonorrhoeae* have been shown to protect human volunteers against gonorrhea (7), and pili from *M. bovis* protect cattle against infection with pinkeye (33). The protective properties of pili and the potential compatibility of pilus expression between several bacterial species appear to offer a route to improved pilus-based vaccines, using surrogate hosts which are more easily cultured than the natural organism.

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