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Bacteroides nodosus is the specific etiologic agent

of footrot in sheep. Pili must be present on the

B nodosus cells for virulent sheep footrot to occur and

variations in pilin, the structural protein molecule which

polymerizes to form the pili, is the basis for antigenic

diversity of the various B nodosus serotypes. Electron

microscopy of B nodosus cells has shown pili

morphologically typical of those classified as NMePhe

pili. Such pili were present in high numbers, polar in

distribution, and their length often exceeded 10 μm. The

presence of pili was demonstrated on B nodosus cells taken

directly from lesion material and from broth culture after

multiple serial transfers. Also visible by electron

microscopy were the basal proteins which differ from the

pilin proteins and appear to anchor the pili to the outer

cell membrane.

The pili of the 11 most serologically distinct U.S. serotypes of B nodosus were characterized by electrophoretic mobility and monoclonal antibody binding. The molecular weights of the pilin protein of 10 of the 11 serotypes ranged from approximately 17,600 to 19,400, with the mode being 18,500 ± 100. The remaining serotype was distinctive in that its pilin protein was seen as 2 separate bands having approximate molecular weights of 7,800 and 6,200.

Examination of B nodosus pili, using monoclonal antibodies raised against intact pili, demonstrated that some monoclonal antibodies were specific for a single serotype and others were specific for multiple serotypes. Two monoclonal antibodies that were reactive to multiple serotypes were of special interest. One reacted to 9 of the 11 serotypes and the other reacted to the remaining 2 serotypes. The group of 9 reactive U.S. serotypes are similar to the Australian "A-set pilin" group (which is based on amino acid homology), in that all members of both groups were recognized by the same monoclonal antibody. The remaining 2 U.S. serotypes and the Australian "D-set pilin" group are similar in that they were each recognized by the other monoclonal antibody. Therefore, these 2 monoclonal antibodies are reactive to the pili of the known B nodosus serotypes. This finding suggests that a

vaccine containing no more than 2 key epitopes may be efficacious for all cases of footrot in sheep.

Morphologic, Molecular and Antigenic Characteristics
of <u>Bacteroides</u> <u>nodosus</u> Pili

by

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PREFACE

This thesis is presented as a literature review, and three manuscripts which have been submitted for possible publication to: <u>American</u> <u>Journal</u> <u>of</u> <u>Veterinary</u> <u>Research</u>.

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INTRODUCTION

Purpose

Bacteroides nodosus is the specific etiologic agent
of footrot in sheep. The pili of B nodosus are the major
host-protective immunogens as well as the serotype
specific portion of this organism. The purpose of this
study was to characterize pili morphologically, using
Electron Microscopy; molecularly, using SDS-Polyacrylamide
Gel Electrophoresis; and antigenically, using Enzyme-
Linked Immunosorbent Assays and Immunoblot Assays of
monoclonal antibodies directed against the pili. The
first objective was performed on various randomly selected
strains, and the other 2 objectives were performed on the
11 most serologically distinct of the 20 serotypes of
B nodosus known to occur in the United States.

The Disease of Sheep Footrot

Contagious footrot is an important infectious disease
affecting sheep of all ages and every breed in all major
sheep growing areas of the world. The disease itself is
seldom fatal; however, severe economic losses can occur
forcing growers out of business. Under environmental

conditions favoring transmission, the disease spreads rapidly, affecting up to 90% of a flock (264). Some predisposing factors include: wet and warm conditions, breaks in the skin due to chafing and lacerations, overgrown hooves and breed susceptibility. Infected animals become lame and unthrifty resulting in reduced wool and milk production, stunted or cull lambs, rams that are unable to breed effectively, and increased susceptibility to predation.

Footrot is caused by a synergism between a minimum of 2 fastidious anaerobic bacteria, B nodosus and Fusobacterium necrophorum. Corynebacterium pyogenes and other organisms may also contribute to secondary infections (61, 197, 198, 217). F necrophorum is ubiquitous wherever sheep are grown and, conversely, B nodosus is host dependent. The only known habitat of B nodosus is the infected hooves of animals (292). Therefore, the spread of footrot occurs when B nodosus is transmitted by infected animals.

There are 20 known serotypes of B nodosus in the United States (228). Each type is classified on the basis of differences in the pilus antigen. Commercial footrot vaccines, containing 8 piliated B nodosus serotypes, produce only marginal protection even against homogeneous challenge. In addition, the adjuvants used to achieve

high antibody titers have a tendency to cause severe
tissue reactions (14, 33, 92, 138, 164, 203, 228).
Because of these problems and the slim profit margin in
the sheep market, many growers no longer use these
vaccines. Instead, they use labor intensive control
measures including hoof trimming and foot bathing which
provide relative control of footrot, but usually does not
eliminate the disease from their flocks.

The data reported here provide additional information
on the pili which contain the protective antigens of
B nodosus. The objectives of this research were to
provide the information needed for developing efficacious
pili and/or pilus subunit vaccines against footrot.

REVIEW OF PILI LITERATURE

Introduction

Pili are defined as any morphologically distinct, non-flagellar, filamentous appendage of bacterial cells (31). Unlike flagella, pili cannot be viewed directly using light microscopy. No staining method has been developed for viewing pili; their diameter is well below the resolving power of even the best optical lenses. Therefore, it was not until the advent of the electron microscope that these ultramicroscopic structures were discovered and studied. In 1949, 2 researchers, Anderson (10) and Houwink (114, 115) independently described pili as being distinct from the known flagella.

Pili from a number of bacterial species have been studied and their properties described. In general, they range in diameter from 2 to 25 nm, in length from 0.5 to 20 μm and in number per cell from 1 or 2 to several hundred (31, 180, 183).

Terminology

Since their discovery, these non-flagellar filamentous appendages have been referred to as threads, filaments, adhesins, bristles, cilia, colonization factor

antigens, fibrils, fuzz, hemagglutinins, ligands, fimbriae
and pili. Whereas the terms "adhesins", "hemagglutinins"
and "ligands" are commonly used to correctly describe
these structures or their functional regions, it should be
pointed out that these terms could also correctly describe
other non-pili associated molecular components of
bacterial cells.

The expression "fimbriae" (Latin for thread or fiber)
was introduced by Duguid et. al. in 1955 (57). In 1959,
Brinton suggested that the term "fimbriae" was
linguistically incorrect, was not distinctive and would
lead to confusion. He introduced the designation "pili"
(Latin for hair-like structure) and urged that it be used
to describe these structures (31). Ironically, both the
terms "fimbriae" and "pili" are now commonly used
interchangeably. However, some researchers reserve the
term "pili" for the conjugative filaments encoded by self-
transmissible plasmids, and the term "fimbriae" for the
non-conjugative type. Another term, "curli", has been
recently introduced to describe a special structural form
(129, 177).

Since there is no general agreement regarding the
nomenclature of these structures, the term "pili" is used
throughout this paper to describe all non-flagellar

surface appendages, including conjugative and non-
conjugative types.

Distribution

Initially, pili were thought to be restricted to the
gram negative enteric bacteria. It remains true that much
of the pili research is conducted with Escherichia coli.
Several types of pili with differing functions (some of
which will be discussed later) have been described for
this organism. Pili have also been reported on Klebsiella
pneumoniae (89, 90), other Klebsiella species (176),
Salmonella typhimurium (235), other Salmonella species
(5), Shigella flexneri (91), Aeromonas liquefaciens (182),
A hydrophila (12, 210), Proteus mirabilis (6), strains of
Morganella and Providencia (174), Acinetobacter
calcoaceticus (202), Neisseria gonorrhoeae (156),
N meningitidis (191), Pseudomonas aeruginosa (208),
P fluorescens (281), P solanacearum (302), Moraxella bovis
(148), M nonliquefaciens (85) and B nodosus (97). Other
Bacteroides species that have pili include: B fragilis
(193, 280), B gingivalis (49, 255), B loescheii (295) and
B intermedius (48), and other related gram negative
anaerobic bacteria which possess pili are Fusobacterium
necrophorum (221) and F polymorphum (101). Other piliated

gram negative bacteria are: <u>Pasteurella</u> <u>haemolytica</u> (161),

<u>P</u> <u>multocida</u> (274), <u>Yersinia</u> <u>enterocolitica</u>,

<u>Y</u> <u>pestis</u> and <u>Y</u> <u>pseudotuberculosis</u> (225, 288), <u>Serratia</u>

<u>marcescens</u> (158), other <u>Serratia</u> species (175),

<u>Citrobacter</u> <u>freundii</u> (3), <u>Vibrio</u> <u>cholerae</u> (122), <u>V</u> <u>eltor</u>

(275), <u>V</u> <u>vulnificus</u> (87), <u>V</u> <u>parahaemolyticus</u> (113),

<u>Chromobacterium</u> species (55), <u>Erwinia</u> species (131),

<u>Bordetella</u> <u>pertussis</u> (196, 139), other <u>Bordetella</u> species

(160), <u>Hemophilus</u> <u>influenzae</u> (279, 142), <u>H</u> <u>parainfluenzae</u>

(125), other <u>Hemophilus</u> species (11), <u>Campylobacter</u> <u>pylori</u>

(73), <u>Legionella</u> <u>pneumophila</u> (200) and <u>Gardnerella</u>

<u>vaginalis</u> (23).

More recently, pili have been demonstrated on gram

positive bacteria including: <u>Actinomyces</u> <u>viscosus</u> and

<u>A</u> <u>naeslundii</u> (37), <u>Corynebacterium</u> <u>renale</u> and <u>C</u> <u>pilosum</u>

(133), <u>Streptococcus</u> <u>sanquis</u> (82), <u>S</u> <u>salivarius</u> (103),

<u>S</u> <u>pyogenes</u> (15), <u>Staphylococcus</u> <u>saprophyticus</u> (216) and

<u>Clostridium</u> <u>difficile</u> (22).

Other somewhat "unusual" or "special" bacteria which

exhibit pili are: <u>Photobacterium</u> <u>splendidum</u> (115),

<u>Caulobacter</u> <u>crescentus</u> (231), <u>Verrucomicrobium</u> <u>spinosum</u>

(215), <u>Myxococcus</u> <u>xanthus</u> and other "gliding bacteria"

(51, 145), <u>Alysiella</u> <u>bovis</u> (120), <u>Bradyrhizobium</u> <u>japonicum</u>

(282), <u>Synechocystis</u> CB3 (276), other cyanobacteia (277),

Thiobacillus ferrooxidans (50) and the hydrogen-oxidizing

bacterium 2Kl (151).

Quite surprisingly, pili were found emanating

randomly from the spores of Bacillus cereus and

B thuringiensis, but were not seen on the log-phase and

sporulating cells (47). The fungal organisms, Ustilago

violacea, U heufieri and Peronospora hyoscyami f.sp.

tabacina, also have pili (42, 88).

Conjugative pili have been shown on E coli and

species of Salmonella, Shigella, Proteus, Caulobacter and

Pseudomonas (28, 31, 117).

It was incorrectly reported in a review article (229)

that Mycoplasmas possess pili. The author of the original

article discusses the binding sites of Mycoplasmas and

provides evidence that these sites are of a protein

nature, but had not isolated or characterized their

chemical structure nor did he suggest that they may be

pili (40).

Composition and Structure

The chemical composition of the non-conjugative pili

is protein with no detectable amounts of carbohydrate,

lipid or nucleic acid (31). The fine structure of several

of these pili has been determined using a combination of

crystallography, electron microscopy and x-ray diffraction. It was found that the pili are cylindrical and are composed of identical structural subunits arranged in a helical array. The number of subunits per turn varies from 3.125 units to 6.0 units depending upon the molecular weight, outside and inside diameters, and pitch of the helix (31, 183). As yet, B nodosus pili have not been analyzed by these methods. However, pili of P aeruginosa, which share a similar diameter, molecular weight, and a common N-terminal sequence, have been analyzed and exhibit pilin subunits of 5.06 to 5.08 units per turn (293). Strains of E coli that cause infections of the urinary tract produce "Pap-pili". Using immuno-electron microscopy, it was determined that these pili are heteropolymers composed of the "major pilin", 2 "minor pilins" and the "adhesin"; the last 3 proteins located at the tip of the pilus (141).

Conjugative pili consist of a single, repeating subunit protein with carbohydrate and phosphate moieties. The F pili appear to be hollow fibers with an 8.0 nm outer and a 2.0 nm inner diameter. The subunits are related by a fivefold rotation axis around the helix axis. The helix symmetry is 25 units in 2 turns of a helix with a pitch of 16 nm and a crystallographic repeat of 32 nm (183).

Classification

The distinct variation in the morphology and function of pili have stimulated attempts for their classification. To date, there has been no general agreement on any specific classification scheme. In 1965, Brinton (31) originally described 6 types of pili on E coli according to dimension and number per cell which he designated types I-V and F. In 1966, Duguid and co-workers (54) similarly described 7 types of pili on enteric bacteria which were designated types 1-6 and F. Because both classifications were based on data from the enteric bacteria, it is not surprising that other researchers observed and described pili that could not be placed in any of the classes of Brinton or Duguid. The only types in which there was agreement and in which there continues to be common usage is in type 1 (or I) and type F.

In 1975, Ottow (180) attempted a provisional classification of pili based upon function. He classed them into Groups 1-6 (with subtypes 1-4 in Group 1). The following is a brief description of each of his types. Group 1 is comprised of pili as organelles of adhesion which have characteristics as follows: between 100 and 300 pili per cell; peritrichous arrangement; non-conjugative; production determined by the chromosome; and separable

into 4 subtypes by their morphology, hemagglutination of red blood cells, mannose sensitivity, and other adhesive peculiarities. These 4 subtypes correspond to the Duguid types 1-4: subtype 1 pili are approximately 7.0 nm in diameter, responsible for adhesive properties in general, and the hemagglutinating ability in particular. The pili of subtype 2 are found on a few Salmonella species. They differ from the subtype 1 pili in that they lack adhesive and hemagglutinating properties. The subtype 3 class of pili are specific for Klebsiella and Serratia marcescens, and differ from subtype 1 in that they are thinner (4.8 nm in diameter) and more numerous; they exhibit strong mannose-resistant adhesiveness to fungal and plant cells, glass surfaces, and cellulose fibers; they are non-hemagglutinating; and do not adhere to animal tissue cells. The subtype 4 class of pili are specific for Proteus; they are extremely thin (approximately 4.0 nm in diameter); demonstrate mannose-resistant adhesiveness; and exhibit hemagglutination. Group 2 pili are the sex pili; which include F, F-like R, and I-like sex factors and usually have a larger diameter and longer length than other pili. Group 3 pili are the thick, hollow tubes observed on Agrobacterium which are 40-60 nm in diameter, up to 3 μm long, and are "infundibuliformly" attached to the cell surface. The Group 4 pili are flexible, rodlike,

and of a polar arrangement. They are observed on
Pseudomonas and Vibrio, they promote bacterial motion as
opposed to acting as organelles of attachment, and they
appear to have a hollow core. The Group 5 pili are also
polarly arranged, but consist of the contractable tubules
of soil bacteria. These pili pull bacteria together by
contraction into star-forming cell clusters thus allowing
competent cells to conjugate by serving in recognition,
contact and irreversible joining of competent cells,
rather than acting as true transferring bridges. The
Corynebacterium renale are the only members of Group 6
which produce characteristic bundles of several pili.
Each pilus is 2.5-3.0 nm in diameter, non-
hemagglutinating, and non-pellicle forming.

In 1980, Duguid and Old (56) offered an update of the
1966 classification previously proposed by Duguid (54)
based upon the adhesive properties of Enterobacteriaceae.
(Although the term "fimbriae" was used throughout these
classifications, this review continues to substitute the
term "pili" for consistency.) A brief summary of the
types is as follows: i) mannose-sensitive adhesins
(type 1), ii) mannose-resistant and eluting adhesins,
iii) mannose-resistant/klebsiella pili (type 3),
iv) mannose-resistant/proteus pili (type 4), v) non-
hemagglutinating, adhesive or pellicle-forming pili

(type 2--a possible mutant form of type 1), vi) non-hemagglutinating pili which exhibit only 10-20 per cell, are up to 10 μm in length, are approximately 10 nm in diameter, and may be sex pili (type 6), and vii) other thinner non-hemagglutinating pili. Obviously absent from this classification scheme was the previously described conjugative pili and type 5 pili (54).

Also in 1980, Bradley (25) introduced a classification for the conjugative pili. He divided the conjugative pili into 3 morphological groups: thin flexible (I, B and K pili), thick flexible (C, D, F, H, J, T, V and X pili), and rigid (M, N, P and W pili). In each group described there were also subtypes and/or types that were included which did not have a letter designation. Bradley concluded that all pili for plasmids within a incompatibility group are related and that while plasmids of different incompatibility groups usually determine serologically unrelated pili, there are notable exceptions.

In 1983, Orskov and Orskov (179) introduced a new nomenclature based on serology of fimbrial antigens in which only F (fimbrial) designations would be used (F1-Fn). They proposed that the designations F1 to F6 be reserved for renumbering of already established antigens (F1 = type 1 pili, F2 = CFA/I, F3 = CFA/II, F4 = K88,

F5 = K99, F6 = 987P). This attempt to simplify the pili nomenclature has not been widely accepted. It would lead to confusion between conjugative and non-conjugative pili (the most widely studied conjugative pili are of the F type whose incompatibility groups have been designated FI-FV); additionally, these researchers added to the confusion of this system, in that, in the same article that the system was introduced, they reported that F7 (representing the first "new" antigen in this system) actually consisted of 2 entities, $F7_1$ and $F7_2$, each of which may correspond to a separate pilus.

In 1988, Paranchych and Frost (183) attempted to classify pili as follows:

> "Three criteria that lend themselves to the classification of pili are: i) morphology (e.g. thin flexible, thick flexible, rigid), ii) function (e.g. distinct from conjugative as adhesive pili), and iii) biochemical properties (e.g. type-related pili with free amino termini as distinct from those with NMePhe at the amino terminus). The three major groups discussed in this article are termed conjugative, adhesive and NMePhe pili. . ."

After indicating that the 3 criteria for classification are morphology, function and biochemical properties; the authors discuss conjugative pili (a function), adhesive pili (a function) and the NMePhe pili (a biochemical property). They then show representative photomicrographs of conjugative, adhesive and NMePhe pili and describe how

they differ (morphologically). They then describe that
there are 3 morphologically different conjugative pili and
4 morphologically different non-conjugative pili. They go
on to state that the simplest classification of pili on
the basis of function is the division into 2 broad groups:
"conjugative" and "adhesive" pili, and that the
biochemical properties help to identify subpopulations of
these pili. Next they describe the organization and
expression of pilin genes and divide the pili into
6 types: conjugative pili, type 1 pili, pili designated
Pap, pili designated CFA/I and CFA/II (CS1, CS2 and CS3),
pili designated K88 and K99, and pili designated NMePhe.
Finally, they discuss the structure-function relationships
of pili proteins and again discuss the 3 groups:
conjugative, adhesive and NMePhe pili.

The main point of this section of the review on the
classification schemes of pili is to point out the
bewildering array of pili types and the confusion that
arises when various researchers try to bring order into
such a diversified field of study. The disagreement
starts at the most basic level of naming the organelle and
continues as each new type is described. Each
classification system proposed has merit in that each
provides information which groups pili and/or pili
characteristics in some way. However, each system falls

short because the characteristics of pili are so broad, and there is such great diversity, as well as sharing of characteristics, for any given pilus. Therefore, each researcher must carefully define the terms he uses and be consistent in describing his results.

Function

Conjugation

The conjugative pili function as organelles of DNA transfer (25). This may be conjugation between male and female bacteria or between bacteria and bacteriophage (268). There is also evidence that drug resistance plasmids are able to be transferred between bacterial species via conjugative pili (27).

Adherence

The main function of the non-conjugative pili appears to be that of adherence. Extensive adherence mechanisms have been examined with several pilus types of E coli (86, 184, 257). This review will not include an exhaustive study of these mechanisms; it will instead consider specific aspects of E coli pilus adherence in the section "Special Classes of Interest".

The binding of P aeruginosa to mucosal surfaces by means of pili is a common step during the initial stages of pathogenesis (52, 209, 297).

Adherence mechanisms also have been studied at length in the pathogenic Neisseria species, N gonorrhoeae and N meningitidis. The ability of gonococci to attach to the mucosal surfaces of the genital tract and to multiply there, despite the flow of mucus and other body fluids, is the essential first stage in the pathogenesis of gonorrhea. After it was determined that the piliated forms of gonococci were more virulent than the non-piliated forms, many studies confirmed that pili facilitate adhesion to a wide range of different cell types such as tissue culture cells (259), vaginal epithelial cells (146), fallopian tube epithelium (291), and buccal epithelial cells (195). It was further suggested that the pili actually participate in the first stage of a two-stage attachment process. Initially, pili are able to overcome the electrostatic barrier which exists between the negatively charged surfaces of the gonococcus and the host cell (possibly because of their smaller size). This is followed by a more stable adhesion which involves the outer membrane proteins of the cell and may occur even if the cell is non-piliated (107).

Clinical isolates of meningococci are also invariably piliated; the pili mediate adhesion of meningococci to nasopharyngeal cells and may, therefore, be an important factor(s) in establishing the carrier state (238). Isolates cultured from the blood and cerebrospinal fluid of patients with meningococcal disease are also piliated (238, 239), and pili have been directly demonstrated in the cerebrospinal fluid of a child with meningococcal meningitis (236).

Related to the adherence of the <u>Neisseria</u> pili is their ability to undergo variation in pilus expression known as "phase variation", "antigenic variation", and "antigenic shift".

<u>Phase Variation</u>

Phase variation generally refers to reversible switches between alternative states such as pilus-expressing and pilus-nonexpressing states. Genetic analysis of phase variation in the gonococci has revealed complex mechanisms of piliation control. One study revealed 2 classes of pilus minus (P⁻) variants: members of one group are unable to revert to pilus production (P⁺), whereas members of the other group revert at high frequency (261). Another group of researchers identified

and differentiated 3 distinct P⁻ phenotypes: P⁻n, P⁻rp⁻, and P⁻rp⁺ in strain MS11$_{mk}$ Gc, which contains only one complete pilin gene. Non-reverting, P⁻ (P⁻n) Gc carry deletions in their pilin structural gene DNA, produce no pilin, and contain no pilin-encoding mRNA. Neither of the reverting phenotypes (P⁻rp⁺ and P⁻rp⁻) exhibits pilin gene DNA rearrangement, and both synthesize mRNA that hybridizes with pilin-specific DNA probes. P⁻rp⁺ and P⁻rp⁻ phenotypes differ in that P⁻rp⁺ Gc express immunologically detectable pilin protein, while P⁻rp⁻ Gc do not (17). The possible role of the P⁻ phase in infection is unclear, but it appears likely that the reduced attachment associated with loss of piliation may allow the gonococci to leave the site of initial colonization and gain access to new hosts or sites, with reversion to the P⁺ phase allowing subsequent adhesion (17, 240, 261).

Phase variation has also been extensively studied in several of the E coli pilus types. For example, the type 1 and S pili which commonly co-exist on strains of E coli. Phase variation has been shown to occur in either or both pili types (168, 169, 211), and either in vitro (168, 169) or in vivo (169, 211). When E coli strains which expressed only the type 1 pili were studied, it was shown that phase variation also occurs in vitro (119) and in vivo (118), and that the phenomenon is due to the periodic

inversion of a specific DNA sequence which is controlled by the products of 2 regulatory genes (1, 128, 233). Gene regulation also controls the phase variation of the E coli Pap pili (20).

A number of other pilus types, including those expressed by K pneumoniae (144) and M bovis (136), have also demonstrated phase variation.

Antigenic Variation

Antigenic variation refers to alterations in structure that are accompanied by detectable alterations in antigenicity. It has been shown that a single gonococcal strain can produce at least a dozen different pilus types (260). Recent studies of the genetic mechanisms of pilus variation show that the potential repertoire of pili expressed by a single strain may be even greater than that indicated above (157).

Antigenic Shift

Akin to "antigenic variation", antigenic shift refers to alterations during growth of the organism that are accompanied by detectable alterations in antigenicity. This phenomenon is less easily established, but has been confirmed by comparing gonococcal isolates taken from

different sites in sexual partners where differing molecular weight pilin proteins have been found (53, 232). It was also confirmed in a study where human volunteers were subjected to urethral challenge in which the pilins of the gonococci, isolated during the resulting infection, were analyzed. All reisolates were found to express pili which were structurally and antigenically distinct from those expressed by the input gonococci (262). These reports suggest that antigenic shift in pilus expression occurs commonly during the course of the natural infection and must play an important role in pathogenesis of gonococcal disease.

The occurrence of antigenic shift during meningococcal infection has been investigated by comparison of paired isolates obtained from the blood, cerebrospinal fluid, and nasopharynges of patients (267), and variations in pilus molecular weights that were detected following non-selective laboratory subculture of one strain (173). Since meningococci produce 1 of 2 distinct varieties of pili (189, 99), both of which can undergo antigenic shift during infection, their antigenic repertoire appears to be equal to or even greater than that of gonococci.

The antigenic shift among the 3 classes of pili of M bovis, α, β, and Γ, have also been studied. It appears

that pilin gene expression is associated with an inversion of DNA whose endpoints occur within the coding region of the expressed pilin gene (137, 147).

Twitching Motility

Twitching motility is a special kind of bacterial surface translocation that may lead to the production of spreading zones on solid surfaces. The cells move predominantly singly, although smaller moving aggregates occur. The movement appears intermittent and jerky and does not regularly follow the long axis of the cell (109). Twitching motility appears to be a phenomenon directly correlated to the possession of polar pili. In one study, all species which demonstrated polar pili under electron microscopy exhibited twitching, and twitching was not found in variant strains without polar pili or in strains with peritrichous pili (109). This form of motility has been demonstrated to occur in a number of gram negative bacteria including B nodosus (26, 46, 108, 109, 110). It appears that in the case of P aeruginosa PAO phage pili, fully functional retractile pili are required for the presence of twitching (26). However, retractile pili are not required for other systems, and the precise mechanism of twitching motility is still not

fully known. It does not appear to be an autonomous biological process such as swimming or gliding, but rather a result of an effect of physicochemical forces upon polarly piliated bacteria (109).

In a study on B nodosus, an interesting correlation between twitching motility and virulence was suggested. It appears that, even though both strain types appeared to produce similar numbers of pili, virulent strains of B nodosus are highly motile and benign strains show a low level of motility (46).

Resistance to Phagocytosis

Early reports showed that piliated gonococci are more resistant to phagocytosis by polymorphonuclear leukocytes (PMNs) than are the equivalent non-piliated variants, suggesting another role of pili in virulence (107). However, others suggested that the pili had only a minor role in the gonococcus-PMN interaction and that additional outer membrane proteins were dominant factors (263, 286). More recently, the resistance of a piliated gonococcus to intraleukocytic killing by human PMNs was examined; it was determined that even though the gonococci were phagocytized and the majority killed, approximately 2% of the intracellular cells survived (35). It was

subsequently found that these surviving bacteria apparently had their pili degraded by the PMNs (240). A result of this process could be to decrease the ability of intracellular or possibly extracellular, viable gonococci to bind to mucosal surfaces. Although this may serve as an important local host defense, the process might promote release and transmission of non-piliated, but virulent gonococci—organisms which may rapidly produce new pili, to new hosts or to new sites in the same host.

The pili of A viscosus not only initiate the recognition of this organism by PMNs, but also stimulate the respiratory burst in PMNs as well as the release of the contents of the secondary granules which promote the killing of the bacteria (207). Likewise, piliated (and therefore more hydrophobic) P aeruginosa strains were more susceptible to phagocytosis by human PMNs than their non-piliated counterparts (234). On the other hand, piliated and non-piliated H influenzae type b strains opsonized in normal human serum were equally ingested and killed by human PMNs (269).

Serum factors enhance the rate of uptake of microorganisms by PMNs. The serum factors are called opsonins and the process has become known as "opsonophagocytosis". The primary role of the opsonins is to provide a means of recognition between the PMN and

their target bacteria known as "binding". Another term
which describes another type of specific recognition has
recently been adopted. This is the recognition between
proteins (lectins) on the bacteria that combine with
complementary carbohydrates on the PMNs. This type of
recognition, which also leads to phagocytosis, has been
termed "lectinophagocytosis" (170).

When bacteria bind poorly to PMNs because the latter
lack the appropriate receptor sugar, lectinophagocytosis
can be induced by precoating the PMNs with suitable
carbohydrates. This has been demonstrated for E coli with
Pap pili, which binds poorly to human PMNs. Human PMNs
are deficient in Galα4Gal-containing glycolipids, which
the Pap type lectin recognizes. However, after the human
PMNs were coated by globotetraosylceramide, there was
increased binding of the bacteria to the phagocytes,
followed by their activation (256).

Lectinophagocytosis of E coli mediated by the
mannose-specific lectin associated with type 1 pili has
been more thoroughly investigated (93, 178, 223, 230).
There is strong evidence that the recognition of type 1-
piliated E coli by phagocytes is mediated by interaction
of the pilus lectin with mannose-containing glycoproteins
on the surfaces of the phagocytes (170). However, the
rate of ingestion which varies among different strains or

conditions, is usually greatly reduced (93, 171, 172, 178).

A model was developed using a piliated mutant to provide a possible explanation why piliated cells can resist phagocytosis: pili with recognizing receptors impede phagocytosis by physically putting distance between the cells; pili mutants that are unable to bind to the receptors cannot "hold" the PMN away and the cell is phagocytosed (178). However, given the much larger size of the PMN in relation to the bacterial cell, this explanation does not appear plausible. There is, however, evidence to suggest that constituents other than the type 1 pili, which modify the physicochemical surface properties of the bacteria, may interfere with ingestion (171, 172).

In another study, addition of type 1 pili monoclonal antibodies to type 1-piliated E coli resulted in increased association, phagocytosis, and metabolic activation of the granulocytes indicating that both opsonophagocytosis and lectinophagocytosis may be involved (230).

Bacteriophage Transport

Retractile pili are thought to be the initial site to which certain phage adsorb. After a phage is attached by

means of lateral phage tail fibers, the pilus retracts into the cell, pulling the phage tail tip to the cell surface. Upon contact with the cell, the phage DNA is injected into the cell through the pilus port (29).

Pilus Expression (Environmental Factors)

Most bacteria have been shown to lose pili production upon cultivation in vitro; however, under certain strict culture conditions, this can be circumvented (152).

It has been shown that media ingredients such as alanine, glucose, and sodium acetate can be inhibitory to pili production (45, 80, 84). Temperature has also been shown to effect pili production: lowering the production at suboptimal temperatures in E coli (45, 80, 127, 201), and lowering the production at temperatures higher than optimal in Y enterocolitica (79). Piliation is also effected by the pH of the environment both in vitro (30) and in vivo (83)--expression being generally depressed at a lower pH. The redox potential also effects the expression of the E coli F pilus--production tending to be favored by aerobic conditions; however, as one mutant strain hyperproduced the pilus anaerobically, this difference was not as clear cut (121).

Subminimal inhibitory concentrations of antimicrobial agents may also inhibit the expression of pili. Aminoglycosides block protein synthesis by interacting with both initiating and elongating ribosomes. Streptomycin, the most extensively studied aminoglycoside, inhibits the production of E coli pili (213). The tetracyclines, which act upon exported proteins, have also been shown to inhibit E coli pili production (213, 237). Chloramphenicol inhibits peptide chain elongation; however, the results of pili production are unpredictable (213). In fact, chloramphenicol has been shown to actually increase pili production in E coli which were growing under other conditions, inhibitory to pili production (127). Lincomycin, which can also block the initiation of protein synthesis (213), has been shown to inhibit N meningitidis pili expression (132). The sulfonamides and trimethoprim are both antimetabolites. Trimethoprim has reportedly reduced pili production in several E coli strains (44, 278), and sulfamethoxazole and trimethoprim used together acted synergistically on type 1 pilus subunit synthesis preventing pili formation (212). Finally, penicillin, which inhibits cell wall synthesis, disorganized the membrane-bound machinery responsible for pilus assembly and anchorage in N gonorrhoeae and

N meningitidis, thereby inhibiting pili production
(214, 237).

Pilus Vaccines

Since pili are important mediators of adherence for
many pathogenic bacteria, blocking their adherence and
thereby preventing disease, would be an important means of
prophylaxis. Experiments most supportive of using pili as
vaccines have been done in animals and these studies show
that antibody-mediated blockage of attachment is
correlated with protection from disease.

Pilus vaccines prepared from diarrheagenic strains of
E coli K88, K99 or 987 and given parenterally to pregnant
cows, ewes and gilts, in the hope that suckling offspring
would be protected against disease by antibodies secreted
in the colostrum of the vaccinated dams, have proven quite
successful (4, 32, 162, 165, 194, 206, 272). Likewise, a
monoclonal antibody to K99 antigen administered orally to
calves during the first 12 hours of age prevented fatal
diarrhea (220).

Piliated M bovis whole cell vaccines are effective in
preventing experimental infectious bovine
keratoconjunctivitis (123). And, as will be discussed in

a later section, both B nodosus pilus and piliated whole
cell vaccines can protect against sheep footrot.

Many experiments to determine the potential of pili
for vaccination against human diseases such as gonorrhea
have also been conducted. Antisera against gonococcal
pili produced in laboratory animals has been shown to be
protective in a variety of biological systems. Such
antisera reduce the adhesion of both piliated gonococci
(270), and purified pili (188) to human buccal epithelial
cells. Anti-pilus monoclonal antibodies to gonococcal
pili have been shown to inhibit adhesion, opsonize pili
for phagocytosis by PMNs, and protect against infection
(284, 285). Immunization with pili can also protect
guinea pigs against infection following gonococcal
challenge (135). However, in many of these studies, the
immunizing and challenge strains were identical, and
little or no protection was observed with heterologous
strains.

Anti-pilus antibodies which will inhibit the
attachment of the infecting strain of gonococcus to buccal
epithelial cells can be detected in genital secretions
from patients with gonorrhea (271); also, human volunteers
immunized with pili produce detectable adherence
inhibiting anti-pilus antibodies in both serum and genital
secretions (273). However, vaccinated human male

volunteers develop gonorrhea despite high levels of serum antibody directed against their infecting strain; therefore it appears that the levels of antibody present in genital secretions either were insufficient or the antibody was directed against a conserved but nonprotective epitope (107). An alternative to immunization with intact pili is the use of synthetic pilus peptide vaccines. A synthetic peptide vaccine has shown promise for the prevention of gonorrhea of various antigenic types (204, 205); however, further studies have shown that the peptide selected is not conserved in all antigenic variants of N gonorrhoeae (199, 262).

The problems that remain with pilus vaccines are: i) lack of broad cross reactivity of the vaccines thus far developed, ii) poor immunogenicity of the important binding ligands both in terms of quality and quantity of antibody produced, and iii) inadequate antibody at the local site of infection (272).

Special Classes of Interest

Pili with Specialized "Tip" Adhesins

As discussed earlier, it was originally thought that each pilus type consisted of identical monomeric polypeptide subunits of pilin protein. Genetic studies of

several pilus types have revealed that the bacterial properties of adherence and piliation may be determined at distinct chromosomal sites. Immuno-gold electron microscopic studies of 2 other pilus types have revealed the location of adhesins.

The pap (pili associated with pyelonephritis) operon encoding a Pap pilus adhesin has been cloned from chromosomal DNA of the pyelonephritic E coli (143). The Pap pili encoded by the pap operon are heteropolymers consisting of numerous major pilin subunits (PapA) and a few copies of minor pilin subunits located either at the base (PapH) or at the tip of the pilus (PapE, PapF and PapG) (141, 143). A single copy of PapG appears to be the actual adhesin which interacts with the receptor (143). The PapC and PapD proteins are required for the polymerization and transport of the pilus subunit, respectively (166). The PapB and PapI proteins are implicated in regulation, and it is postulated that PapH is a pilus-growth terminator that is incorporated as the last monomer at the pilus base (141).

The S pili (α-sialyl-β-2,3-galactosyl-specific adhesin) which are present on certain E coli strains were studied using immuno-gold electron microscopy to attempt to localize the adhesin. The gold label appeared to be

preferentially associated with the tips of the pili, indicating a distal location of the adhesin (159).

Likewise, 2 distinct types of pilus-associated adhesins of B loescheii were studied by immuno-gold electron microscopy. This study confirmed that both adhesins were pilus-associated and that they both appeared to be located, for the most part, at the distal portion of the organelle. Neither adhesin appeared to be arranged in any consistent fashion on the pili. They occasionally were found in a linear array along a segment of the pili, but more often appeared in pairs, triads, or clusters of up to 10 gold particles (295).

The type 1 pili have been extensively studied. The results suggest that the PilG and PilH gene products are components of the type 1 pili (PilH being the specific adhesin). Although PilH is found mainly at the tips (104, 105, 181), it is also found at long intervals along the length of the pilus (2). In another study, antibodies directed against PilH were cross-reactive not only with type 1 pili of heterologous E coli strains but also with type 1 pili isolated from strains of K pneumoniae, S marcescens, and C freundii (3).

The K88ab and K99 pili of E coli have also been shown to have single minor pilus subunits at their tips; however, it appears that these tip proteins are not

adhesins and that the adhesive capability of these pili is an intrinsic property of the major pilus subunit (181).

Finally, the most widely used methods for the purification of pili from many bacterial strains have relied upon dissociation and reassociation of pilin subunits, a process which yields highly purified pilin preparations. However, this process could also result in the loss of pilus-associated proteins. A procedure was developed for the isolation of intact native pili of N gonorrhoeae in a special buffer in which the pili were fractionated on the basis of size and hydrophobicity (163). A number of proteins copurified with pilin which appeared to be associated exclusively with piliated cells. Although there is a possibility that the production of these proteins is coregulated with pilin production and that the proteins are not physically associated with the pilus, the results indicate that they are pilus-associated (163). These proteins may have a critical role in adhesion of the gonococcus to host cells, as in the "tip" adhesins discussed here.

In summary, it appears from the current knowledge that the adhesins of pili may be confined strictly to the tips, as in the Pap pili; found mainly at the tip, but with an additional random distribution mainly at the distal portion of the pili, as in the S pili and the pili

of <u>B</u> <u>loescheii</u>; found mainly at the tip, but also at long
intervals along the length of the pili, as in the type 1
pili; found on the major pilus subunit, even though there
is a distinctive tip protein, as in the K88ab and K99
pili; or found in some pilus-associated way that has yet
to be determined, as possibly in <u>N</u> <u>gonorrhoeae</u> and other
species. Also, the minor adhesin protein of <u>E</u> <u>coli</u> type 1
pili is conserved in other genera and species of the
<u>Enterobacteriaceae</u> which express type 1 pili.

<u>Pili with a NMePhe Amino Terminus</u>

The pili of this class have been designated in
several publications as belonging to the "type 4" class of
Ottow (180). This designation is confusing for several
reasons: Ottow uses the terms "group" and "subtype" in his
classification system (not "type") so that "group 4" would
be a more correct designation of these pili (to be
differentiated from "group 1, subtype 4" for instance).
Also, referring to these pili as "type 4" brings confusion
among the pili of this class, the "type 4" pili of Duguid
(54), and the "type IV" pili of Brinton (31), as discussed
in the classification section above. These pili have more
popularly been termed "NMePhe" (or sometimes "MePhe") pili
(183). An inherent problem with this designation is that,

before a newly isolated pilus can be classified, at least a portion of its N terminal amino acids must be determined.

The bacterial members of this group have polar pili which produce the unique form of locomotion called "twitching motility" (see above). These pili are produced by a number of gram negative bacteria, including B nodosus, M bovis, M nonliquefaciens, N gonorrhoeae, N meningitidis, and P aeruginosa. Each of these species is a pathogen: B nodosus causes contagious footrot in sheep; M bovis causes infectious bovine keratoconjunctivitis; M nonliquefaciens is an opportunistic pathogen; N gonorrhoeae causes human venereal disease; N meningitidis causes meningococcal meningitis; and P aeruginosa is an opportunistic pathogen which may infect hosts who have been immunocompromised by cancer, burns, or cystic fibrosis or cause surgical, corneal, urinary tract, or respiratory tract infections.

The amino acid sequence has been determined for representative pilins from each of the bacteria in this class: B nodosus (discussed separately below), M bovis (148), M nonliquefaciens--N-terminus portion (85), N gonorrhoeae (156), N meningitidis (191), and P aeruginosa--both the PAK and PAO strains (208).

Members of the NMePhe pili class are so called because their pilins are characterized by the unusual N-terminal residue, N-methylphenylalanine (NMePhe). Pilins of this group are also characterized by a highly conserved, hydrophobic stretch of 30 amino acids at the N-terminus (183). These pilins are translated as a precursor (prepilin) with an amino-terminal extension of 6 or 7 amino acids characteristic to all NMePhe pili in general. The positively charged leader peptide is removed enzymatically, leaving phenylalanine (Phe) as the new N-terminal amino acid. Subsequent methylation produces NMePhe (187).

The highly conserved N-terminus of the NMePhe pili suggests that this region is involved in an important function(s). It is likely that the N-terminus acts as a signal sequence for membrane targeting. Another possibility is that the necessary stabilizing forces for subunit-subunit interaction during pilus assembly are provided by this part of the molecule (41, 293). Studies involving mutations within the N-terminal region of the pilin gene and their effects on pilin stability and processing within P aeruginosa have been performed. The results indicate that NMePhe or the glutamate residue at position 5, or both, play an integral role in the polymerization of the pilin subunits (186, 187).

The pilin genes of <u>B</u> <u>nodosus</u> (65) and <u>P</u> <u>aeruginosa</u> (185) appear to be present as single copies within the genome. Contrarily, multiple copies of pilin genes have been demonstrated in the chromosomes of <u>N</u> <u>gonorrhoeae</u> (156) and <u>M</u> <u>bovis</u> (148); these multiple genomic pilin copies appear to be the basis for the diverse antigenic variants in these 2 genus groups.

A number of studies using the <u>Neisseria</u> pilus structural genes from variants of a number of different strains have been conducted. Pilins can be considered to contain 3 major regions: a region encompassing approximately the first 53 amino acids which is highly conserved between pilins, a semivariable region (approximately residues 54 to 114), and a hypervariable region at the carboxy terminus. Thus, despite distinct antigenic specificity, variant pilins show a considerable degree of structural homology (107, 157, 191). In another study, synthetic peptides were synthesized corresponding to a series of regions of a specific gonococcal pilin. Immunization with intact pili was found to produce antibodies directed predominantly against peptides equivalent to residues corresponding to the hypervariable region within the disulfide loop. Low levels of antibodies were also directed against a weakly immunogenic determinant between residues 48 and 60. These results

suggested that the most immunogenic domain, the disulfide loop, was located on the surface of the pilin molecule and that amino acid substitutions could occur in this region, altering antigenic specificity without disrupting regions critical for pilus function (204).

The immunochemistry of intrastrain antigenic variation has been investigated by using monoclonal antibodies also raised against variant pili of gonococcal strains. The immunodominance of type-specific epitopes was confirmed by screening over 200 monoclonal antibodies. Only one was obtained which reacted equally well with all strains tested (283, 287).

In another study, a N gonorrhoeae pilin specific synthetic oligonucleotide probe that would hybridize to gonococcal genomic DNA in crude cell lysates was found. Although this probe also recognized N meningitidis, this organism rarely occurs in the human urogenital tract. It was felt, therefore, that this probe could be useful as a rapid and sensitive diagnostic test for the presence of N gonorrhoeae (130).

Pili of the Genus **Bacteroides** (Excluding **B nodosus**)

It appears that the pili of Bacteroides species other than B nodosus are considerably different from B nodosus pili. Although the leader sequence for the pilin of B gingivalis has been shown to be remarkably similar to those of the NMePhe group of pilus subunit proteins (49), and although the N-terminal amino acid is Phe (albeit non-methylated), no significant homology was found in the remainder of the pilus gene sequences (49, 300).

Additionally, it has been shown from electron microscopy studies that the pili of B gingivalis (299), B fragilis (192, 280), B loescheii (294, 295), and B intermedius (48) do not have the typical polar arrangement of the NMePhe pili, but are peritrichously arranged. Since there is extreme heterogeneity of the genus Bacteroides (219), it is not surprising that these differences occur. A recent proposal has been presented which would restrict the genus Bacteroides to B fragilis and "closely related species" (219). In this proposal, B loescheii and B intermedius would be members of a new genus; B gingivalis would be reclassified into the genus Porphyromonas; and the generic position of B nodosus would be uncertain (219).

Pili of **B nodosus**

Morphology

Characteristic of the NMePhe pili group, the pili of
B nodosus are polar in distribution on the cell. This
fact became established (76, 97) after conflicting initial
reports described the distribution as being peritrichous
or mostly polar (100, 222, 242, 252, 290). Their diameter
is approximately 5 to 6 nm (74), and their length often
exceeds 10 μm (97, 242).

Function

The role played by B nodosus pili in the disease of
footrot has not been clearly established, but a high
degree of piliation is associated with virulence
(78, 224). Conversely, some benign strains of B nodosus
have also been shown to be highly piliated (46, 245, 254);
while benign strains from a cattle origin were shown to be
consistently less piliated than those from a virulent
sheep origin (60). Pilus associated twitching motility
has also been correlated to the virulence of B nodosus
(46). Once B nodosus is established on the hoof, secreted
proteases aid in further penetration and colonization of
the hoof (75, 224, 245). At this point, the hoof may be

broken down enough so that pili may aid the spread of
B nodosus by twitching motility.

Pilus mediated adherence has been clearly established
as a factor in the virulence of other organisms with
NMePhe pili (see above); and, although researchers have
stated that adherence is not a function of B nodosus pili
(62, 74), a preliminary report from our laboratory
indicates that B nodosus pili can adhere to epithelial
surfaces (227).

Even though the precise function for B nodosus pili
in the pathogenesis of footrot is not known, it has been
demonstrated by vaccine trials that they are the primary
protective immunogens in footrot (59, 72, 77, 243, 244,
250, 251, 253).

Antigenic Structure

The B nodosus pilus has been described as a
homopolymer made up of pilin protein molecules each with a
weight of 17,000 to 19,400, depending upon serotype
(8, 74, 98, 102, 254). Serotype-specific epitopes are
located on the structural subunit of the pilus strand in
B nodosus (see below).

Another antigen with a much higher molecular weight
range of 77,000 to 88,000 is associated with the pili and

was found to be the basal protein which apparently links
the pilus strand to the cell surface (8, 9, 97, 149).
There is no relationship between the electrophoretic
behavior of the basal protein antigen and the serological
classification of the strain (8, 149). Even within
serotypes, isolates did not appear to have a common basal
antigen, and were often recognized more strongly by
antisera generated against the prototypes of another
serotype (9).

The presence of protein(s) analogous to the "tip" or
adhesin proteins of the pili of E <u>coli</u> has not been shown
for B <u>nodosus</u>.

<u>Serology</u>

The serological classification of B <u>nodosus</u> is based
upon antigenic differences in the pili expressed. Three
separate classification systems from different
geographical locations are currently in use. We, in the
United States, have a total of 20 serotypes of B <u>nodosus</u>
(218, 228), suggesting that 11 of these serotypes are "the
most serologically distinct" (94, 98). Researchers from
England have classified strains of B <u>nodosus</u> into 17
serotypes (43, 265); whereas, the Australian researchers
have organized strains of B <u>nodosus</u> into 9 serogroups with

subtypes occurring within several of these serogroups
(38, 39).

Further research with the Australian serogroups has
indicated that there are some B nodosus strains that did
react with more than one serogroup (38) and that cross
reactions can be seen in checkerboard immunoblotting
experiments (9). This has lead these Australian
researchers to conclude that both structural and antigenic
variation is wide-spread in the B nodosus population, and
that classification into serogroups and serotypes
represents a series of overlapping sets of antigenically
related strains (9).

The sharing of epitopes between pili of different
B nodosus serotypes has been shown using immunogold
labeling techniques (16). A recent study using monoclonal
antibodies directed against pili of B nodosus has also
shown that 6 of 8 Australian serogroups share a common
epitope (301). Comparisons between the amino acid
sequences of the pilin molecules of several B nodosus
serotypes has demonstrated 2 distinct categories
(see below). Finally, a DNA probe has been constructed
that will recognize all B nodosus strains regardless of
serotype (34).

Amino Acid Sequence

The amino acid sequences for the pilin of each of the Australian serogroups A through H have been determined: A (63), B (154), C (154), D (81), E (153), F (41), G (70), H (66); and compared (62). There is exact identity among all 8 serogroups for the first 18 amino acids, and virtual identity between residues 1 to 32; however, 2 serogroups, D and H, have exact identity between residues 1 to 50 and share regions of homology distinct from the other 6 serogroups throughout the remaining sequences. Therefore, Elleman has divided the 8 serogroups into 2 distinct categories which he termed "A-set" and "D-set" pilins (9). The Australian monoclonal antibody study (301) showed that their monoclonal antibody reacted with the 6 serogroups of the A-set pilins; whereas the study reported herein showed monoclonal antibodies that reacted individually with both the A-set and the D-set pilins (94). Recently, the pilin molecule from a subtype of the Australian serogroup H was sequenced. Although there was homology between this strain and the prototype strain of serogroup H for residues 1 to 55, there was considerable differences between the strains in the remaining sequences. It was therefore suggested that this strain be assigned as the prototype strain of a new serogroup (116).

Vaccine Production

The efficacy of B nodosus whole cell vaccines is
highly dependent on the degree of piliation of the cells
(249, 250, 266); and in challenges involving homologous
strains of B nodosus, purified pili alone have been shown
to confer protection equivalent to, if not superior to,
that afforded by the whole cell vaccine (72, 77, 243, 246,
251). In contrast, a vaccine prepared with purified
B nodosus pilin did not protect sheep against a challenge
with the homologous strain, and it was concluded that the
structural integrity of pili is required to elicit
protective immunity (72). This becomes important when
considering genetically-engineered expression of pilus
protein. The assembly of pilin into pili with the same
quaternary structure as that produced by B nodosus may be
required to elicit protective antibodies.

The pilin of B nodosus A198 has been cloned and
expressed in E coli. The quantity of pilin produced was
greater than that of the pilin synthesized by B nodosus.
However, none was expressed as pili on the E coli; all was
membrane bound. A vaccine prepared from the membrane
fraction of sonicated cells did not elicit protection from
subsequent B nodosus A198 challenge (7, 64).

The B nodosus A198 pilin gene was also expressed in
P aeruginosa. In this case, the protein was expressed as
pili. Harvested recombinant-DNA-derived pili were
inoculated into sheep; subsequent challenge of the sheep
with B nodosus A198 indicated that the recombinant-DNA-
derived pilus vaccine provided similar levels of
protection against footrot as a vaccine produced from
native B nodosus pili (59, 67, 150, 253). Native pilus
vaccines reportedly have immunotherapeutic as well as
immunnoprophylactic value against footrot, and it is
noteworthy that these recombinant-DNA-derived pilus
vaccines appear to have the same value (59, 253).
B nodosus pilin genes of A198 and H265 were also expressed
individually or in tandem as a single transcription unit
in P aeruginosa; expression of the pilins from the 2
serogroups produced 2 distinct populations of pili on a
P aeruginosa cell, and each pilus was composed of a single
type of subunit (68, 69).

Finally, the B nodosus A198 pilin gene (complete or
as a carboxy-terminal portion) has also been cloned into
the foot-and-mouth disease virus. A portion of foot-and-
mouth disease virus genome containing the B nodosus pilin
gene was subsequently expressed in either E coli or
P aeruginosa. Examination of these E coli and
P aeruginosa cells showed that complete and partial

B nodosus pilin proteins were produced in E coli; however, only the carboxy-terminal portion could be demonstrated in P aeruginosa and this was associated with an impaired growth rate and reduced pilus yield (124).

Pilus Associated Proteins

Several experiments have provided evidence that immunogens associated with, but distinct from, the B nodosus pilus may be involved in cross-protective antigens (71, 246, 247, 248). These proteins may be the basal protein (see above) or other outer membrane proteins.

AN ELECTRON MICROSCOPIC STUDY OF

BACTEROIDES NODOSUS PILI AND ASSOCIATED STRUCTURES

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SUMMARY

The surface structures of <u>Bacteroides</u> <u>nodosus</u> were examined with the electron microscope. Collodion film and chrome shadowing were used for maximizing the visualization of <u>B</u> <u>nodosus</u> pili and ring structures. The occurrence of <u>B</u> <u>nodosus</u> pili in footrot lesions was confirmed. Contrary to previous reports, it was found that <u>B</u> <u>nodosus</u> pili production can be retained through serial broth transfers under certain conditions. Capsule production in <u>B</u> <u>nodosus</u> was irregular in that it can either be absent or vary in its thickness. Additionally, one culture of <u>B</u> <u>nodosus</u> was shown to be infected with a bacteriophage.

INTRODUCTION

Bacteroides nodosus is the specific etiological agent of ovine footrot, a contagious debilitating disease of sheep (19, 61, 198). Virulent in vitro isolates of this organism possess pili which are important immunogens in the protection of sheep against footrot. Initial attempts to visualize these pili under electron microscopy were confusing because few pili were seen attached to bacterial cells. The pili may have been detached and washed away during sample preparation or simply left in the background where their attachment site was difficult to determine. Therefore the superabundance, extreme length, and polar distribution of the pili were not initially observed. Although researchers assume that B nodosus pili occur in active footrot lesions, this had not been demonstrated.

Vaccinations with purified pili have conferred protection against challenges involving homologous strains of B nodosus, thus demonstrating that the pili are major host-protective immunogens (72, 77, 243, 244, 250, 251, 253). The presence of pili on B nodosus have been reported to be an unstable characteristic especially in liquid media where they become irreversibly lost (224). This becomes an important consideration when producing piliated whole-cell vaccines.

It is now well established that pili are the serotype specific portion of B nodosus and that they are composed of small polypeptide subunits of about 18,000 molecular weight (74, 98, 102). Isolated pili may also contain another significant component, a polypeptide of about 77,000 to 88,000 molecular weight--the basal protein, which apparently links the pilus strand to the cell surface (8, 9, 149). Basal proteins (also termed "ring structures", "pores" and "holes"), have been visualized on B nodosus pili that were negatively stained (149), or, alternatively, the surface of B nodosus cells treated by freeze etch techniques or negatively stained (76, 78, 100, 252). These structures have also been shown on the cell surfaces of other piliated bacteria although their association with pili was not always made (151, 167, 258).

The purpose of the study reported here was to examine various components of the ultrastructural morphology of B nodosus cells, to demonstrate the presence of B nodosus pili in active footrot lesions, and to determine if pili production could be maintained in serial broth transfers of B nodosus.

MATERIALS AND METHODS

Bacteria

The bacterial strains used were: the Australian
prototype of serotype A (A198, the type strain of
B nodosus (36)), Australian strain B206, and strains of
B nodosus isolated in this laboratory from virulent cases
of ovine footrot--designated by an Oregon State University
(OSU) number. These strains were cultured on media
previously described (218).

Preparative Agar Plates

Enriched (218) Eugonbroth (BBL Microbiology Systems,
Cockeysville, MD) was diluted 1:1.5 in ddH_2O and 2.3% agar
(Difco Laboratories, Detroit, MI) added. After
autoclaving for 15 minutes, 25 ml of this agar mixture was
added aseptically to 100 x 15 mm Petri plates. The plates
were weighed and then dried at 37 C until the weight was
decreased by 10 g. When the desired length of drying time
and surface appearance of the agar were established,
weighing was no longer necessary and the more easily
handled 60 x 15 mm Petri plates were used. Agar was
poured into these smaller plates until they were
approximately half full; after cooling, they were stored

at 4 C. When needed, the plates were dried at 37 C until
visual assessment showed them to be adequately dried. A
further simplification of 1.5% agar in ddH_2O without the
addition of Eugonbroth was subsequently found to be
equally effective.

Preparation of Shadowed Grids

Two or three Pasteur pipet drops of log phase
B nodosus cells grown in enriched Eugonbroth were added to
dried preparative agar plates and spread by gently tilting
the plate. As soon as the surface of the agar appeared
dry, several drops of collodion (1% collodion in technical
grade amyl acetate) were added to the area of the plate
where bacteria were placed. After 75 seconds the plate
was tilted and the excess collodion removed (this
prevented the collodion film from becoming too thick).
After drying, the film (with attached bacteria) was
transferred to a pan of ddH_2O. This was accomplished by
breathing on the plate to loosen the collodion film,
adding a drop of ddH_2O to the film edge, then slowly
immersing the plate (loosened edge first) into the H_2O.
Copper grids (400 mesh) were placed right side down onto
areas of the floating film that were both hazy from the
culture and a gold iridescence from the proper film

thickness. A clean microscope slide was used to retrieve the grids from the H₂O inverting them so that the film was on top with the bacterial cells uppermost. After drying, the grids were viewed under phase contrast microscopy for quality of cell transfer. Grids with many bacteria and few holes in the collodion film were selected and shadowed with chrome at a 10-13 degree angle.

Serial Broth Transfer

Serotype A198 was cultured for 55 broth to broth transfers in 3 ml volumes of enriched Eugonbroth. Each transfer was incubated anaerobically at 37 C for 2-3 days. Pili were harvested and quantified as previously reported (98) from broth subcultures 1 and 55. A sample of broth subculture 55 was also prepared for electron microscopy examination as above.

Lesion Smear Examination

Impression smears of footrot lesion material were made directly onto formvar coated grids. The grids were then examined under phase contrast microscopy and a map made of cells typical of B nodosus morphology. The grids were then shadowed as above and these specific cells were then located and examined under electron microscopy.

Preparation of Negatively Stained Grids

Some grids of <u>B</u> <u>nodosus</u> were stained with 4% uranyl acetate prior to viewing in the electron microscope.

Electron Microscopy

Some electron microscopy preparation techniques were adaptations of previously described methods (115, 126). All specimens were viewed and photographed using a Phillips 300 Electron Microscope.

RESULTS AND DISCUSSION

Chrome-shadowed B nodosus cells that adhered to collodion typically displayed numerous pili attached to the poles of the cells. Frequently these pili fanned out evenly from the cell, were quite long, and there were more attached to one end of the cell than to the other (Fig 1). Occasionally cells were seen in which the pili appeared to "rope" together (Fig 2).

Cells serially cultured in broth for 55 transfers retained their ability to produce pili (Fig 3). Although the pili appeared somewhat shorter and reduced in number, 82 μg of purified pili were obtained from 800 ml of liquid subculture 55. Therefore it appears that, contrary to a previous report (224), pili production can be a relatively stable function of B nodosus cells if certain nutrients are supplied.

Almost all the B nodosus cells in one set of chrome-shadowed grids clearly demonstrated "ring structures" or "basal proteins" on one or both poles of the cells (Figs 4-7). Some researchers have questioned the association between these structures and pili (78, 252), whereas others have described them as grommet-like structures through which the pili pass (100) or as basal attachment sites which anchor the pili to the outer membrane (8). Negatively stained preparations of purified
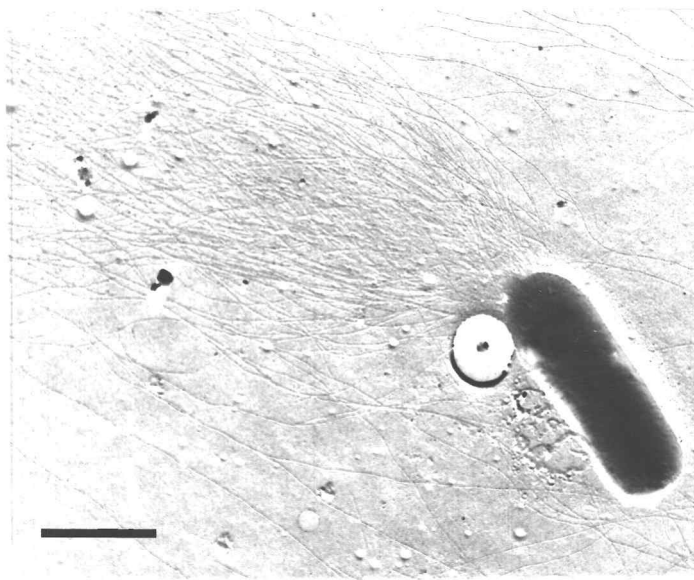
FIG. 1. Heavily piliated <u>B</u> <u>nodosus</u> cell of strain OSU 84. Numerous pili emerge from the poles of the cells (usually more from one pole than the other). Bar = 2 μm.
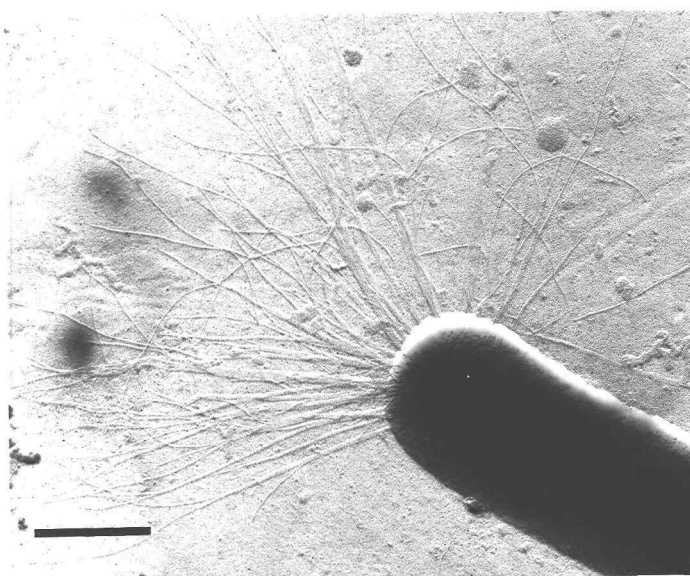
FIG. 2. Heavily piliated <u>B</u> <u>nodosus</u> cell of strain OSU 11.
The pili emerging from the pole of this particular cell
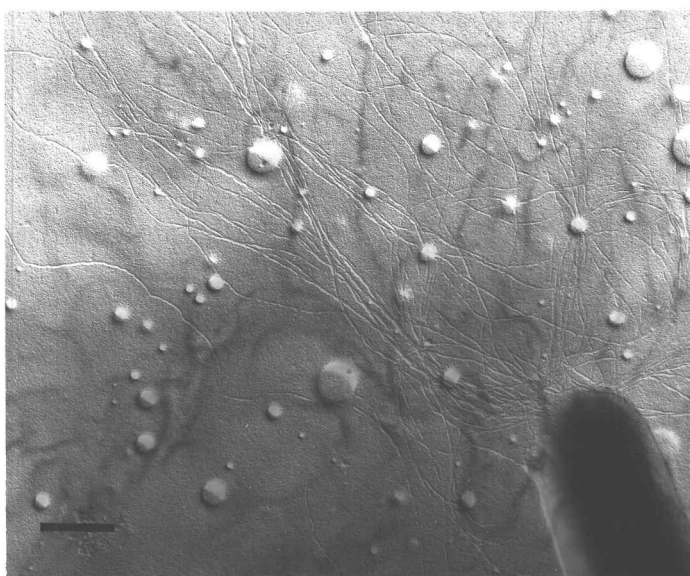appear to have "roped" together.   Bar = 1 μm.

FIG. 3. Piliated B̲ nodosus cell of strain A198 transferred
serially in liquid culture 55 times. Note that although
there are less pili, they are still present. Bar = 1 μm.

pili have revealed an occasional cap-like structure
attached to pili which is assumed to be the ring structure
(149). In the present study, these structures were
clearly associated with the pili and sometimes the pili
actually appeared to be emerging through them (Fig 4);
also, they were restricted to the polar regions of the
cells (Figs 5-6)--the area to which pili are also
restricted. It was previously reported (76) that these
structures are found within the outer membrane and inside
the "additional layer". However, it appeared from chrome-
shadowed preparations that these structures were outside
the "additional layer" (Fig 7). Some have reported that
these structures are arrayed hexagonally (76); whereas
others maintain that they are not arranged in any
particular geometric pattern (100). In the present study
the ring structures appeared to have a pentagonal shape
and were randomly tightly packed (Fig 6). Ring structures
could also be observed upon close examination of chrome-
shadowed preparations of B nodosus cells of several other
serotypes.

B nodosus cells occasionally appeared to be covered
with a thick slime layer or capsular material (Fig 8);
while in other instances the capsule or "additional layer"
appeared quite thin and rigid and had the appearance of an
additional membrane (Fig 9). The existence of a capsule
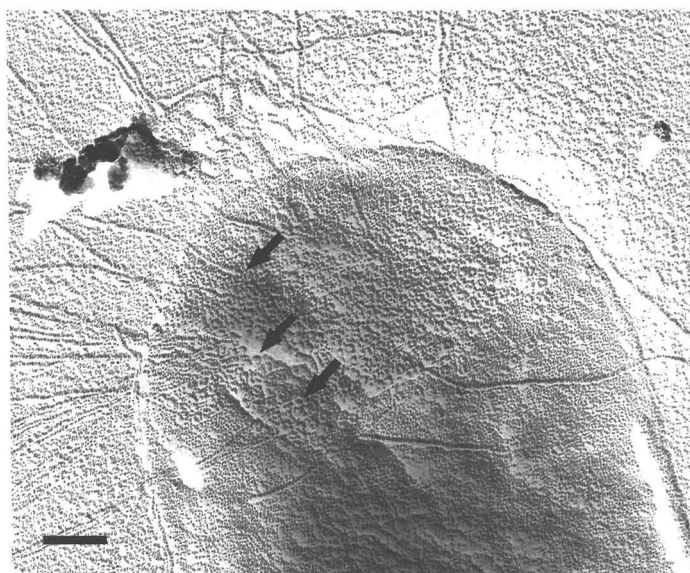
FIG. 4. <u>B nodosus</u> cell of strain OSU 84. Ring structures are clearly visible at the pole. Some pili appear to be emerging through ring structures (arrows). Bar = 0.2 μm.
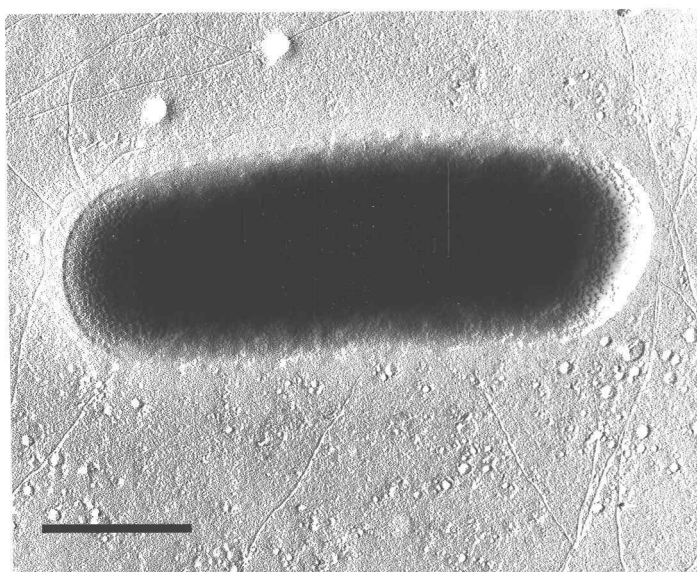
FIG. 5. B nodosus cell of strain OSU 84 showing numerous ring structures at both poles. Bar = 1 μm.
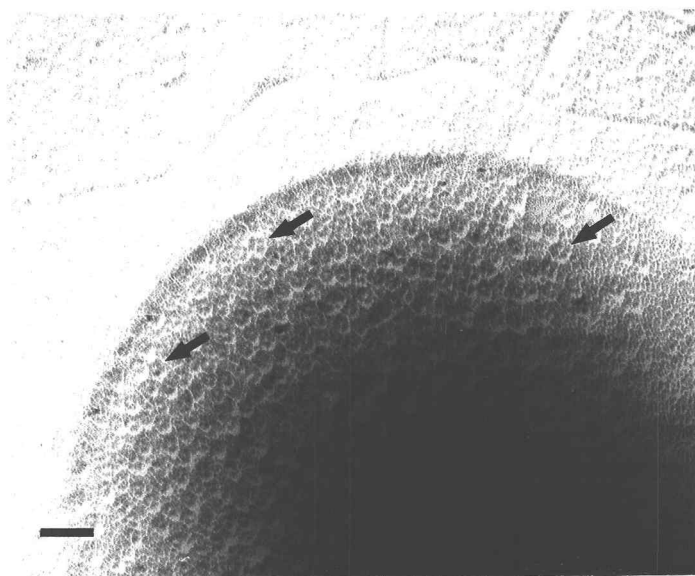
FIG. 6. Enlargement of one pole of the cell shown in Fig. 5. Note that the ring structures appear to be non-circular but pentagonal in shape (arrows). Bar = 0.1 μm.
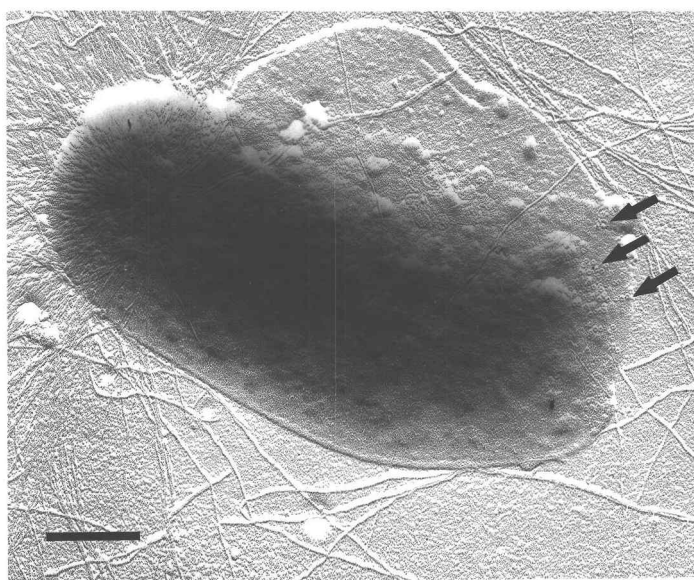
FIG. 7. B nodosus cell of strain OSU 84 showing ring
structures which appear to be on capsular material
(arrows). Bar = 0.5 μm.
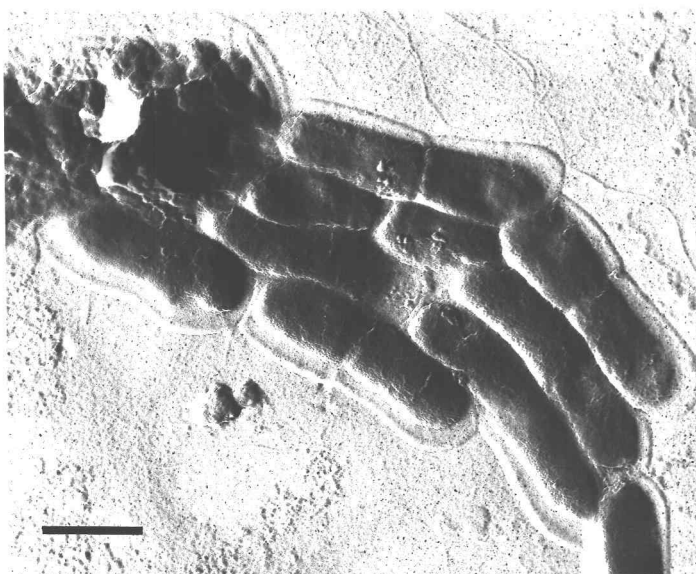
FIG. 8. B nodosus cells of strain OSU 11 showing a thick slime layer or capsular material. Bar = 1 μm.
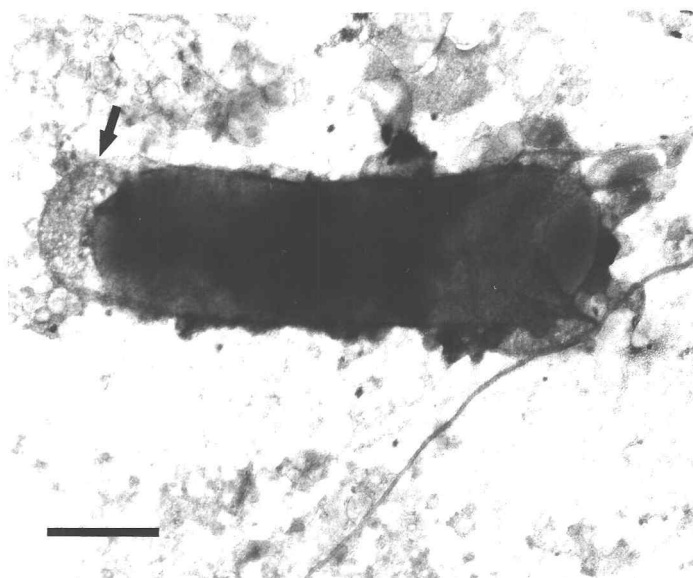
FIG. 9. B nodosus cell of strain B206 grown in liquid
culture and negative stained with uranyl acetate showing
an "additional layer" or thin capsular material (arrow).
Bar = 1 μm.

in B nodosus cultures has historically been quite
controversial. Some researchers have stated that there is
no capsule (76), others theorize that the "capsule"
observed in light microscopy is actually pili (242) or
state that a fragile capsule can be demonstrated in colony
impression smears but not from suspensions of organisms
(252). Still others describe fuzzy projections (222),
distinct envelope-like structures (60) or diffuse polar
material (78). In another study some degree of capsule
production was shown in most Bacteroides strains belonging
to the species B fragilis, B vulgatus, B thetaiotaomicron,
and B ovatus, whereas five B distasonis strains were not
encapsulated. The proportion of encapsulated cells varied
among strains but represented approximately 10% or less of
the total cell number for most isolates. The size of the
capsule also varied among strains and within a single cell
suspension (13). In the present study it appeared that at
least thick capsule production in B nodosus is usually
absent; the expression of a thin capsule, on the other
hand, could be easily overlooked.

Examination of a direct smear of lesion material
clearly showed that pili are attached to B nodosus cells
during the disease process (Fig 10). Because B nodosus
pili are fragile, they were found only in low numbers and
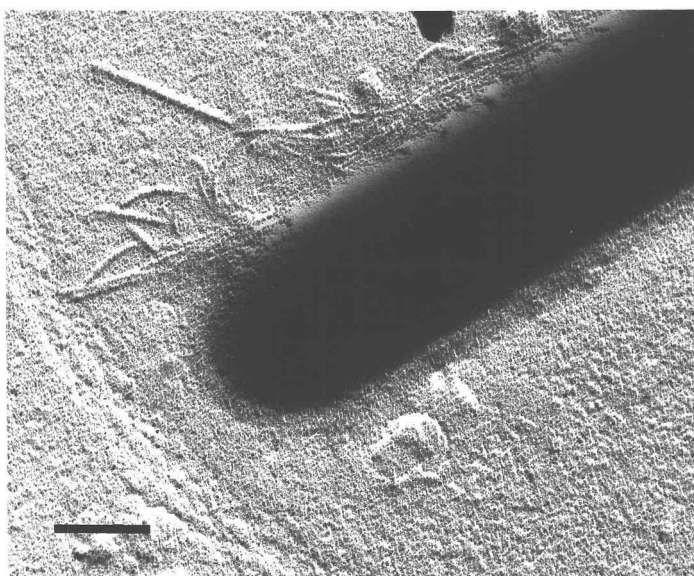
FIG. 10. <u>B</u> <u>nodosus</u> cell from a direct smear of lesion
material showing attached pili (mostly folded back against
the cell). Chrome shadowed. Bar = 0.5 μm.

were not very long. The precise functional significance
of pili in the lesion remains unknown.

B nodosus cells from one broth culture were found to
be infected with bacteriophage. Examination of this
culture clearly showed that some of these bacteriophage
were attached to B nodosus cells, that such cells were
unhealthy, or that they had lysed (Fig 11a and Fig 11b).
A single bacteriophage revealed some of its ultrastructure
(Fig 11c). Phage-like particles were shown attached to
the polar region of cells in a B nodosus broth culture in
a previous study (252), and structures resembling
incomplete phage were shown in another study (290). Other
Bacteroides have been found to be infected with species-
specific bacteriophage (21). The significance of the
presence of phage in B nodosus cultures is unknown,
however it was recently hypothesized that phage may be
associated with the intracellular crystal structures of
B nodosus (111).
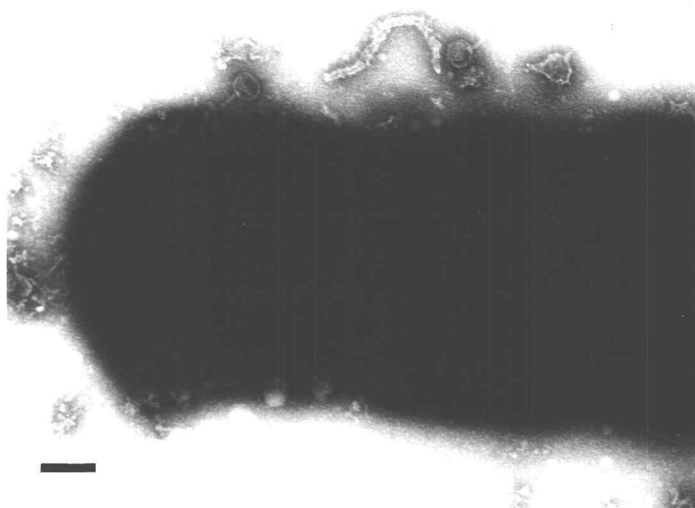
FIG. 11a. Uranyl acetate negative stain of B nodosus
strain OSU 50 grown in liquid culture. An unhealthy
B nodosus cell with associated bacteriophage.
Bar = 0.1 μm.
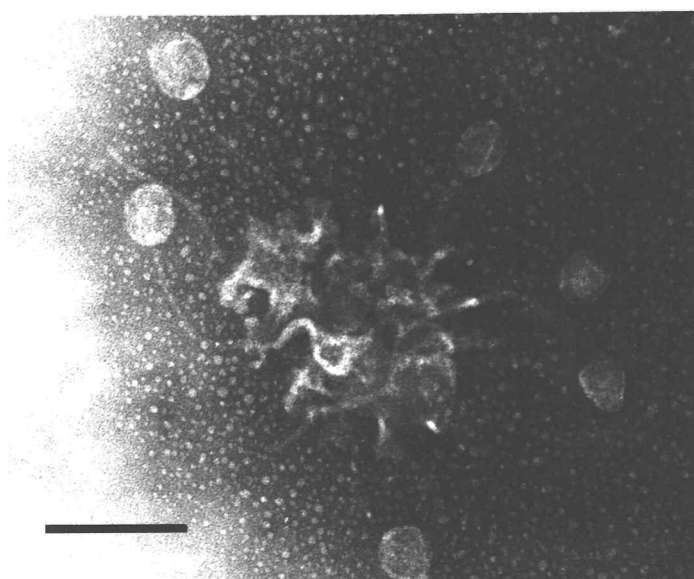
FIG. 11b. Uranyl acetate negative stain of B nodosus
strain OSU 50 grown in liquid culture. A piece of
membrane from a lysed B nodosus cell with several attached
bacteriophage. Bar = 0.1 μm.
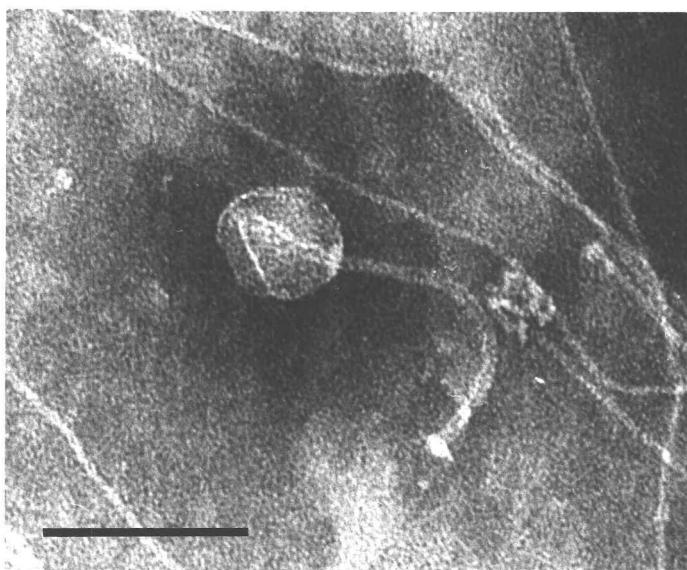
FIG. 11c. Uranyl acetate negative stain of B nodosus strain OSU 50 grown in liquid culture. A single bacteriophage complete with base plate. Bar = 0.1 μm.

DIVERSITY OF PILIN OF SEROLOGICALLY DISTINCT

BACTEROIDES NODOSUS

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SUMMARY

Pili from 11 distinct serotypes of <u>Bacteroides</u> <u>nodosus</u> were examined for diversity of pilin polypeptide subunits between serotypes and for purity of the pilin preparations. The pilin of all 11 samples were shown to be homogeneous. The molecular weights of the pilin of 7 serotypes (A198, IV, V, VI, IX, XVII and XVIII) were 18,500 ± 100. The pilin of serotypes I, III and VIII had molecular weights of 17,600, 19,400 and 19,000 respectively. Serotype XV differed greatly from the other 10 serotypes in that there were 2 distinct polypeptide bands with molecular weights of approximately 7,800 and 6,200. We suggest that these 2 low molecular weight bands resulted from proteolytic cleavage of the pilin protein.

INTRODUCTION

Ovine footrot is a major disease problem of sheep producers worldwide. It continues to plague the industry because treatment is laborious and prevention demanding (33, 138). The specific causative agent of footrot is _Bacteroides_ _nodosus_ (198), a gram negative anaerobic bacterium which cannot survive for more than 2 weeks in the soil (292). The disease must therefore be transmitted from the feet of infected animals or asymptomatic carriers. A comprehensive review of footrot pathology has been presented (217), and the pathogenesis and rationale for immune resistance to _B_ _nodosus_ has been discussed (226). Current treatment can include any one or all of the following: trimming the hooves, treating the feet topically with antimicrobial agents or foot bathing with zinc sulfate, copper sulfate or formalin and intramuscular injection of antibiotics (96). Commercial footrot vaccines have been developed but do not always provide adequate protection under field conditions and sometimes cause undesirable reactions at the site of injection (14, 33, 92, 138, 164, 203).

Serotyping of _B_ _nodosus_ strains has been complicated by differing classification systems from various geographical locations. Researchers in England have

reported 17 serotypes of B nodosus (43). Claxton et al have used a different classification system, suggesting that the 8 original Australian "serogroups" could be further divided into 16 or more subgroups or "serotypes" (39); with an additional, ninth serogroup that has recently been added to their classification system (38). Our classification system in the United States is similar to the system used by the British researchers. The original 14 U.S. serotypes reported by our laboratory (218) have been updated to a total of 20 serotypes (228).

Investigations with the Australian serotypes show that the pili are the serotype-specific immunogens (243, 244), that both protective immunity and therapeutic potential may result from vaccination with pili antigens (59, 72, 250), and that this protection does not effectively extend to heterologous serotypes (266).

The purpose of the present study was to determine the molecular weights of pilin of 11 representative U.S. serotypes of B nodosus. These serotypes were selected because they have little or no cross-reactivity and are therefore the most antigenically distinct U.S. strains.

MATERIALS AND METHODS

Bacteria

There were 11 strains of B nodosus used in this
study. One was the Australian strain, 198. This strain
is the prototype of Australian serotype A and has been
designated the type strain of the species (36). The
remaining 10 strains used were the prototype isolates of
the U.S. B nodosus serotypes I, III, IV, V, VI, VIII, IX,
XV, XVII and XVIII. These 10 strains were isolated from
virulent cases of ovine footrot. They, along with U.S.
serotype XIV which was not included in this study but
which appears identical to strain A198, represent the most
serologically distinct 11 serotypes of B nodosus found in
the United States (218, 241).

Media

The agar medium used was Eugonagar (BBL) supplemented
with 10% defibrinated horse blood and 0.2% yeast extract
(95). Incubation was at 37 C in anaerobic jars evacuated
and filled 4 times with 10% CO_2 in H_2 gas. The liquid
medium was a modification of the enriched Eugonbroth (BBL)
medium described by Schmitz and Gradin (218). Briefly,
the Eugonbroth was enriched with 0.5% yeast extract (BBL);

0.25% arginine, 0.25% lysine and 5% gelatin hydrolysate
(Sigma Chemical Co, St Louis, MO). Resazurin solution
(0.4%) was added as an anaerobic indicator. The medium
was then adjusted to pH 8.5, dispensed in 160 ml volumes
and prereduced. The final pH after autoclaving was
approximately 7.5. During the course of this study, the
availability of Oxyrase (Oxyrase Inc, Ashland, OH) to
achieve anaerobiosis simplified the broth preparation.
The medium could now be initially adjusted to pH 7.5,
160 ml dispensed into 250 ml flasks and sterilized
aerobically. As needed, 266 μl of Oxyrase was added to
each flask (pre-warmed to 37 C). A sterile stopper was
inserted into the flask and the flask was then incubated
at 37 C for a minimum of 60 minutes before inoculation as
previously described (218).

Pili Purification

The procedure used for purification of pili was a
modification of the method of McMichael and Ou (155), as
follows: broth cultures, 2 to 5 days old, were harvested
by centrifugation for 30 minutes at 9,000 x g. The cells
were resuspended in cold 5 mM TES buffer, (Sigma) pH 7.0
and homogenized on ice for 2 minutes at speed 60 in a
Virtis homogenizer using 4 pulses of 30 seconds each.

Cell debris was pelleted at 9,000 x g for an additional

30 minutes. Solid $MgCl_2$ was added to the resulting

supernatant to 0.1 M $MgCl_2$. The pili were allowed to

aggregate at 4 C (usually overnight) and then collected by

centrifugation at 27,000 x g for 40 minutes. The pelleted

pili were resuspended in 0.5 mM TES, pH 7.0 by forcing

them through a 20 gauge needle. The solution was again

clarified, and the supernatant fluid brought to 0.1 M

$MgCl_2$ with a solution of 1 M $MgCl_2$. Three more cycles of

precipitation and clarification were performed. The

amount of protein was estimated by the method of Bradford

(24) using the Bio-Rad Protein Assay Kit (Bio-Rad

Laboratories, Richmond, CA). The pili were stored at

-70 C as a suspension of 1 mg/ml in ddH_2O.

SDS-PAGE Electrophoresis

The discontinuous gel system described by Laemmli

(134) and the Mini-Slab electrophoresis apparatus

(Idea Scientific, Corvallis, OR) were used with

15% acrylamide separating gel (8 cm x 10 cm x 0.5 mm)

and 5% acrylamide stacking gel. Pilin samples of each

serotype were prepared by diluting the purified protein

200-fold into sample buffer (62.5 mM Tris-Cl pH 6.8,

2% sodium dodecyl sulfate, 10% glycerol and

5% 2-mercaptoethanol) giving 5 μg/ml of pilin protein.
The low molecular weight protein standards (Bethesda
Research Laboratories, Gaithersburg, MD) were diluted
100-fold into sample buffer yielding approximately
10 μg/ml of each protein. The electrode buffer
(approximate pH of 8.2) contained 25 mM Tris, 192 mM
glycine and 1% sodium dodecyl sulfate. One microliter of
sample (5 ng) or of standard (60 ng) was applied to the
wells with a 10 μl syringe (Hamilton Co, Reno, NV) and the
protein was electrophoresed at a constant 100 V through
the stacking gel. The protein was then subjected to a
constant 250 V through the separating gel for 105 minutes.

The protein was fixed by soaking the gel in
50% methanol (reagent grade) overnight at room
temperature. The gel was stained with a solution of
ammoniacal silver according to Wray et al (298) for
30 minutes. The bands were allowed to develop for
10 minutes, then the developer was discarded and
50% methanol was added to stop the stain development.
The gel was swelled in ddH$_2$0 and dried on a plexiglass
frame (Idea Scientific) with cellophane membrane backing
(Bio-Rad) and photographed.

LPS Staining

Two identical pilin protein gels were run as described above, except that several concentrations of phenol-extracted lipopolysaccharide from E coli (Sigma) were applied to additional lanes as positive controls in a test for the possible presence of LPS. One gel was fixed in 200 ml of 25% (V/V) isopropanol in 7% (V/V) acetic acid and was preferentially stained with an ammoniacal silver solution (112). The other gel was placed directly into a saturated solution of Oil Red O in 60% (V/V) methanol and 10% acetic acid for 48 hours (190).

RESULTS AND DISCUSSION

Sequential MgCl$_2$ precipitation of pili from $\underline{B}$ $\underline{nodosus}$ produced protein samples that appeared very pure. This was shown by a single band of pilin in SDS-polyacrylamide gels (Fig 12) using a silver-staining procedure which is sensitive to proteins at the nanogram level (298).

The molecular weights of pilin of 7 serotypes (A198, IV, V, VI, IX, XVII and XVIII) were determined to be 18,500 ± 100. This agreed with the molecular weight determination for A198 of approximately 18,400 made by Every (74). Two pilin serotypes, III and VIII, exhibited slightly higher molecular weights of 19,400 and 19,000, respectively; serotype I pilin had a lower molecular weight of 17,600. This range of values essentially agreed with the values reported by others in Australia for serogroups A through G of from 17,000 to 19,000 (8, 102, 254). The only serotype in the present study which differed greatly from the other 10 was XV which gave distinct bands of approximately 7,800 and 6,200. This appeared analogous to Australian serogroup H which also has a similar banding pattern (8, 66, 102, 254).

The 6,200 molecular weight band noted for serotype XV pilin may not have resulted from homogeneous polypeptides. There may be at least two reasons for this possibility.
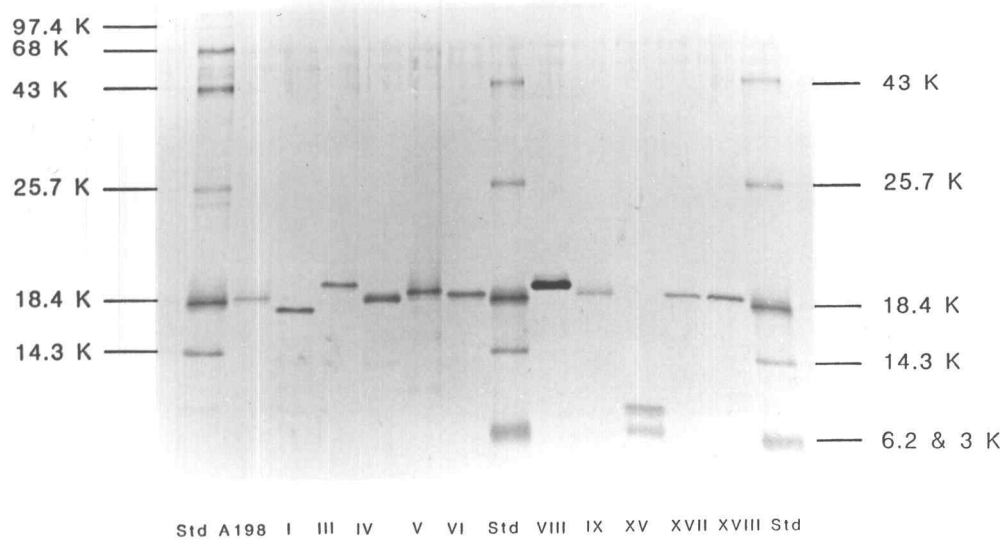
FIG. 12. SDS-PAGE gel (15% acrylamide) of pilin from 11 serotypes of B nodosus, silver stained.

First, Williams and Gratzer have shown that the SDS
complexes formed by all proteins having a molecular weight
of less than 6,000 migrate with the same mobility; the
frictional coefficients of SDS complexes of this size are
no longer dependent on molecular weight (296). Secondly,
we speculate that serotype XV pili are proteolytically
cleaved at some time. Since the sum of the molecular
weights of the 2 bands for serotype XV were much less than
that of the other serotypes, there may be other smaller
fragments present. We have not yet been able to determine
if these pili are cleaved while attached to the bacteria
or during the purification procedure. We have tried
blocking potential cleavage by adding the protease
inhibitor ethylenediaminetetra-acetic acid (EDTA) to the
bacteria while harvesting and again at all resuspension
stages of pili purification. The EDTA appeared to have no
effect on the suspected protease. The EDTA-treated
samples separated on SDS-PAGE gave a banding pattern
identical to those samples purified without EDTA
(unpublished data). Further experimentation using several
classes of protease inhibitors added at different stages
of bacterial growth and of pili purification may help to
clarify this matter.

Studies of the nucleotide sequence of the gene
encoding pilin from the Australian strain H265 and the

mature protein sequence have revealed an internal cleavage which produces 2 noncovalently linked subunits in this strain. The position of cleavage is identified between alanine residues at positions 72 and 73 of the mature pilin protein (62, 66). Further analysis of 2 distinct fractions obtained by HPLC from pili of this strain demonstrates that the faster migrating band on the gels corresponds to an N terminal-derived subunit, whereas the slower migrating band corresponds to a C terminal-derived subunit. Calculated subunit molecular weights of 7,875 and 8,017 are obtained from the amino acid compositions of the N terminal and C terminal subunits, respectively. Since the sum of these values approximate the molecular weight of the nonprocessed, single-chain prepilin these researchers conclude that a single polypeptide chain undergoes further processing to produce 2 noncovalently linked subunits (66). Further studies are necessary to determine if a similar mechanism is involved in the processing of serotype XV pilin.

The majority of pili in our broth culture preparations remained intact and attached to the B nodosus cells until they were harvested by homogenization; other researchers have reported finding the greatest amount of pili in the supernatant of cell harvests (74, 149). Furthermore, researchers using a Coomassie blue R250

stain, have reported polypeptides of 77,000-88,000 molecular weight associated with the pili preparations that appear to be the basal proteins linking the pili strand to the cell surface (8, 149). Even though we used the more sensitive silver stain, we did not demonstrate such polypeptides associated with pili in the current study. In addition, Every reported lipopolysaccharide contamination in pili preparations (74), and although we used similar purification procedures, LPS contamination was absent from the purified pili preparations in this study. The absence of LPS was established by 2 staining techniques (periodate-silver and Oil Red O) which preferentially stain lipoproteins in polyacrylamide gels (112, 190). LPS was not demonstrated by either staining technique except in the lanes containing an E coli LPS standard.

The amino acid sequence of pilin protein from each of the prototype strains of the original 8 Australian serogroups of B nodosus has now been determined. The number of amino acid residues in each serogroup ranges from 149 in H to 155 in G; A, E and F, have 151 amino acid residues each, while B and C each have 153 and D has 150 (62, 153, 154). It remains to be seen if the differences in pilin molecular weights for some of the serotypes reported in the present study are due to a difference in

the number of residues making up the pilin polypeptides.
Serotypic differences in B nodosus may be due to
differences both in amino acid sequences of the same
length and in sequences of differing lengths. Minor
changes in primary structure may make major changes in the
three-dimensional configuration, thereby exposing
differing antigenic determinants (9).

These studies have shown that the major pilin protein
of different serotypes of B nodosus vary in molecular
weight and that some serotypes which are widely separated
antigenically have major pilin subunits of nearly
identical molecular weights. Furthermore, the methods
reported in this study demonstrated only single
homogeneous pilin subunits within serotypes. These
polypeptides can be readily demonstrated and purified with
standard laboratory procedures.

MONOCLONAL ANTIBODIES AGAINST PILI OF

SEROLOGICALLY DISTINCT BACTEROIDES NODOSUS

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SUMMARY

Several monoclonal antibodies against pili of
Bacteroides nodosus were examined to determine their
reactivity with 11 different serotypes. One monoclonal
was identified by Enzyme-Linked Immunosorbent Assay
(ELISA) analysis that binds to 9 of the 11 serotypes and
another that binds to the remaining 2 serotypes tested.
In addition, some monoclonals demonstrated specificity for
a single serotype, while others displayed specificities
for up to 5 other serotypes. Comparison of immunoblot
analysis with the ELISA revealed that the former method
was not as sensitive in that all monoclonals were positive
by the ELISA, whereas not all were positive by immunoblot.
Possible explanations of these findings are discussed.
There appear to be several antigenic determinants on
B nodosus pili and considerable sharing of these
determinants between pili types.

INTRODUCTION

Sheep footrot is a complex disease resulting from a synergistic infection of a minimum of 2 anaerobic bacteria: <u>Bacteroides</u> <u>nodosus</u> and <u>Fusobacterium</u> <u>necrophorum</u> (198). <u>B</u> <u>nodosus</u> is regarded as the primary etiological agent of footrot. Unlike <u>F</u> <u>necrophorum</u> which remains viable and transmissible apart from the host for extended periods; <u>B</u> <u>nodosus</u> cannot survive away from the host for more than 2 weeks, but persists on the hooves of infected sheep (292). Therefore, if <u>B</u> <u>nodosus</u> could be eliminated from a flock, footrot would not occur (106). The multiplicity of <u>B</u> <u>nodosus</u> serotypes is a major obstacle in the control of footrot through immunization (218). Although it has been shown that homogeneous <u>B</u> <u>nodosus</u> vaccines can be both prophylactic and therapeutic (58, 59, 140), current British vaccines which include 8 serogroups of <u>B</u> <u>nodosus</u> may afford only 60-80% protection even in homogeneous challenge situations. Furthermore, these marginally protective levels are acquired using oil adjuvants which can cause severe tissue reactions in some sheep (14, 33, 92, 138, 164, 203).

Currently 20 serotypes of <u>B</u> <u>nodosus</u> have been identified in the United States (98), suggesting that effective control of footrot using conventional polyvalent

vaccines is impractical. Since pili have been demonstrated to be the protective immunogens for B nodosus (72) and because serotyping is based on antigenic variations of the pili, the present study was designed to determine if there are pilin protein antigens shared by various B nodosus serotypes.

MATERIALS AND METHODS

Bacteria

The bacterial strains used for the production of pili were: the Australian prototype of serotype A (A198, the type strain of B nodosus (36)), and the prototypes of the U.S. B nodosus serotypes I, III, IV, V, VI, VIII, IX, XV, XVII and XVIII (98, 218) which were isolated in this laboratory from virulent cases of ovine footrot. These strains were cultivated on the media previously described (218).

Purification of Pili

Pili from these 11 serotypes of B nodosus were purified as previously described (98). Briefly, the serotypes were grown anaerobically in broth cultures, harvested and homogenized in a Virtis homogenizer. Each homogenate was centrifuged and the supernatant, containing the pili, was subjected to 4 cycles of $MgCl_2$ precipitation and the concentration determined by the Bio-Rad Protein Assay. Purity of the pili preparation was confirmed by SDS-PAGE (98).

Antisera Production

Adult female BALB/c mice (Simonsen Laboratories, Gilroy, CA) were immunized with purified pili emulsified in Freund's complete adjuvant (Difco). Five different immunization regimens were used (Table 1). Each mouse received an intraperitoneal injection of 100 μg purified pili protein. Three days before fusion (2-7 weeks after the initial injection) the mice were boosted intravenously with 20 μg pili protein without adjuvant.

Fusion of BALB/c Spleen Cells and SP2/0 Myeloma Cells

The mice were euthanized by cervical dislocation and their spleen cells were harvested, washed in RPMI 1640 medium (Flow Laboratories, McLean, VA) and fused with SP2/0 myeloma cells in 50% polyethylene glycol (M. W. 1450, Sigma Chemical Co, St Louis, MO). Fused cells were seeded into Linbro (Flow) 96-well plates at 10^6 cells per well. Cells were fed every other day with RPMI 1640 medium containing 15% defined fetal bovine serum (Hyclone Laboratories, Logan, UT), 10 μg/ml gentamicin, and hypoxanthine-aminopterin-thymidine (HAT Media Supplement, Sigma Chemical Co, St Louis, MO). The hybridomas were tested for antibody production by ELISA on days 10-16. At this point HT media supplement (Sigma) was substituted for

TABLE 1 -- Mouse immunization schedule

Fusion	Initial injection[1]	Second injection[2]
I	A198, I, XV	IV
J	I	I
N	IV, VI	IV, VI
O	III, IV, V, VI	III, IV, V, VI
P	III, IV, V, VI	III, IV, V, VI
R[3]	III, IV, V, VI	XVII, XVIII

[1]The total initial injection of 100 μg was comprised of equal amounts of each listed pili serotype. This preparation was administered intraperitoneally.

[2]The total second injection was 20 μg also comprised of equal amounts of the listed pili serotypes but administered intravenously.

[3]The R fusion received a third injection 20 days after the second injection of 10 μg each of serotypes I and XV administered intravenously.

the HAT media supplement. After 2-3 feedings, the cells were maintained in RPMI 1640 medium without supplementation. Positive hybridomas were cloned by limiting dilution in medium containing endothelial cell growth supplement (Collaborative Research, Lexington, MA) and were examined microscopically to determine monoclonality. Monoclones were transferred to Linbro 24-well plates and retested using ELISA. Clones of interest were expanded and frozen under liquid nitrogen until they could be injected into pristane-primed (Sigma) BALB/c mice to obtain ascites fluid.

ELISA for Hybridoma Screening and Monoclonal Specificity

The ELISA used was a modification of the procedure of Voller et al (289). Appropriate dilutions of all reactants were determined by checkerboard titrations, and the test was optimized. The optimal amount of antigen used throughout was 100 μl/well of a 1 mg/ml concentration of purified pilin (98) diluted 1:1000 with carbonate-bicarbonate coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 0.2% NaN_3, pH 9.6). After adding the antigen to appropriate wells of Costar serocluster 96-well half-area EIA plates (Bellco Glass, Vineland, NJ), they were sealed and incubated at 4 C overnight. The plates were then

emptied and excess moisture was removed from the wells by slapping the plates on a solid surface padded with paper towels. 125 μl/well of a Blocking Solution of 1% Bovine Albumin, Fraction V, RIA grade (US Biochemical Corp, Cleveland, OH) in phosphate buffered saline (PBS) (1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 0.8% NaCl, 0.2% KCl, 0.2% NaN_3, pH 7.4) was added to each well, the plates were sealed and then incubated at room temperature for 1 hour. The plates were then washed 4 times with 125 μl/well of PBS with 0.05% Tween-20 (Sigma) and 4 times with PBS alone. Excess moisture was removed as before. Monoclonal antibody (100 μl) was added to appropriate wells, the plates were again sealed and incubated at 4 C overnight. After washing as described above, rabbit anti-mouse IgG conjugated with alkaline phosphatase (Miles Scientific, Naperville, IL) diluted 1:2000 in Blocking Solution (1% BSA in PBS) was added (100 μl/well). The plates were again sealed and incubated at room temperature for 90 minutes. After another washing, 100 μl/well of Substrate Solution (0.1% paranitrophenylphosphate (Sigma) in diethanolamine buffer (Sigma), 1 mM $MgCl_2$, pH 9.8) was added to each well. The plates were then sealed and incubated at room temperature for 1 hour. Absorbance at 405 nm was determined with a Titertek multiskan ELISA plate reader (Flow), the reader having been first

programed to subtract the background optical density
values of unreacted Substrate Solution.

Immunoblot Analysis of Monoclonal Specificity

Eleven pieces of 0.1 μm pore size nitrocellulose
paper (Schleicher and Schuell, Keene, NH) approximately
40 mm X 40 mm were wetted in Tris Buffered Saline
(TBS, 20 mM Tris (Bio-Rad), 0.5M NaCl, pH 7.5) and then
allowed to air dry. A 1 μg spot of purified pili protein
(98) of each of the 11 serotypes was then allowed to dry
on the nitrocellulose, a separate piece of nitrocellulose
being prepared for each monoclonal antibody to be tested.
After blocking with TBS + 3% W/V gelatin (Bio-Rad) for
60 minutes, 10 ml of the Primary Antibody Solution
(1:500 dilution of monoclonal culture supernatant in TBS,
1% gelatin) was incubated with the nitrocellulose in a
60 mm diameter Petri dish at room temperature with gentle
agitation. After 24 hours the nitrocellulose papers were
rinsed with ddH$_2$O and subjected to four 10 minute washes:
twice with TBS + 0.05% Tween-20 (Bio-Rad) followed by
2 washes with Tween-free TBS. Each membrane was then
incubated at room temperature for 60 minutes in the
Secondary Antibody Solution (goat anti-mouse IgG
conjugated with horseradish peroxidase (Bio-Rad) diluted

1:2000 in TBS, 1% gelatin). The nitrocellulose papers were rinsed and washed as before and then developed in Horseradish Peroxidase Color Development Reagent (Bio-Rad). A second immunoblot test was conducted in the same manner as the first test, except that 2 μg of purified pili protein of each of the 11 serotypes was spotted onto the nitrocellulose papers.

Isotyping of Monoclonals

The monoclonals generated were typed according to class using a mouse monoclonal sub-isotyping kit (Hyclone).

RESULTS AND DISCUSSION

The hybridoma supernatants each contained antibody of
a single sub-isotype. All 4 sub-isotypes of the IgG class
were represented by the clones selected for this study.
The supernatants were also screened for immunoreactivity
to specific B nodosus serotype pili antigens using both
ELISA and immunoblot. Eleven of 20 U.S. pili serotypes
were tested. Based upon tube agglutination tests with
rabbit antisera, these were the most serologically
distinct of the 20 serotypes. Each clone tested had a
unique reaction pattern (Table 2).

The analysis by immunoblot consistently revealed
reactivity with pili of fewer serotypes than did the
analysis by ELISA. Immunoblots are reported to be less
sensitive than ELISA tests (18). The results in this
study may be due to a difference in sensitivity between
tests; however, it appears that the pilus reaction epitope
may have been altered or masked when the pili were bound
to the nitrocellulose rendering them unreactive. In each
case where there was a positive reaction by immunoblot,
there was also a positive reaction by ELISA.

The ELISA reaction of clones I2B10 and O2D1 were
identical, each recognizing 9 of the 11 serotypes;
however, the immunoblot reaction of these 2 clones showed
differing reactions (Table 2). This may indicate that

TABLE 2 -- Characterization of monoclonal antibody supernatants

Antibody	Isotype	B nodosus serotype										
		A198	I	III	IV	V	VI	VIII	IX	XV	XVII	XVIII
I2B10[1]	IgG 1	+[2]/+[3]		+/+	+/+	+/+	+/	+/+	+/+		+/+	+/+
I2C4	IgG 2b	+/+			+/	+/					+/	+/
J1A5	IgG 1		+/+									
J1D3	IgG 1		+/+							+/+		
N1D10	IgG 2b				+/+							
N1E12	IgG 1	+/		+/+	+/+	+/+			+/+			
N3H12	IgG 1	+/+					+/+		+/+		+/+	+/+
O2B4	IgG 2a				+/+	+/+						
O2D1	IgG 2a	+/		+/	+/	+/+	+/	+/	+/		+/+	+/+
P1F8	IgG 1				+/+	+/+	+/+				+/+	+/+
R2B1	IgG 3									+/		

[1]The cell line for this clone has been lost. A limited amount of culture supernatant and ascites fluid is all that remains.

[2]ELISA reaction: +, absorbance >0.200. No entry, absorbance <0.140.

[3]Immunoblot reaction: +, positive. No entry, negative.

these 9 serotypes all have at least 2 common epitopes on
their pili--I2B10 binding to an epitope altered on
nitrocellulose in only one serotype, but O2D1 binding to
an epitope altered in 6 of the 9 serotypes. Three other
clones differed in their reactions by the 2 methods of
analysis: I2C4 reacted to 5 pili types by ELISA but was
monospecific by immunoblot, N1E12 reacted to 5 pili types
by ELISA but only to 4 types by immunoblot, and R2B1,
although monospecific by ELISA, was negative to all
11 types by immunoblot (Table 2).

Since the ELISA analysis gave the most reactions,
this information was used to investigate the cross-
reactivity between serotypes. As mentioned previously,
clones I2B10 and O2D1 each recognized 9 of the 11
serotypes. Clone J1D3 recognized the remaining 2 pili
types; thus, when taken together, clones J1D3 and I2B10 or
O2D1 recognized all 11 of the pili serotypes tested. This
separation of the U.S. pili serotypes into 2 divisions is
analogous to the finding by other researchers that the
Australian serogroups could also be divided into
2 distinct categories based on the degree of amino acid
sequence homology. These researchers designate the
2 categories as A-set and D-set pilins (62, 81). Clones
I2B10 and O2D1 recognized A-set pilins and clone J1D3

recognized D-set pilins; tube agglutination reactions verified that the serotypes recognized by clones I2B10 and O2D1 belong to the A-set category and those recognized by clone J1D3 belong to the D-set category (data not shown). Therefore, the division of B nodosus serotypes into 2 separate categories is substantiated both by amino acid sequence homology and by monoclonal reaction.

Among the selected clones, 3 (J1A5, N1D10 and R2B1) were each monospecific for different serotypes (Table 2). This suggests that the binding site for each of these clones may be the serotype specific epitope.

Seven clones (I2B10, I2C4, N1D10, N1E12, O2B4, O2D1 and P1F8) reacted with serotype IV pili. Each of these clones were either monospecific or had a different set of other pili serotypes that they also recognized (except for I2B10 and O2D1 which were identical by ELISA analysis). This indicates that there is a multiplicity of antigenic determinants on the pili and that there is considerable sharing of these determinants between pili types. A similar conclusion was reached by researchers using polyclonal rabbit antisera suggesting that the serotypes of B nodosus comprise a series of overlapping sets of antigenically related strains (9).

These results provide encouragement that a bivalent vaccine containing only 2 key epitopes could be produced which may be efficacious for all cases of footrot in sheep. Further tests are necessary to isolate these epitopes and to determine if they will be recognized by sheep and be of a protective nature.

SUMMARY AND CONCLUSIONS

B nodosus pili and associated structures were
characterized in this study. The examination of B nodosus
cells by electron microscopy revealed pili typical of the
NMePhe classification. They were present in high numbers,
polar in distribution, and their length often exceeded
10 μm. Pili were found on B nodosus cells harvested
directly from lesion material, establishing that they are
not an artifact of in vitro culture. Pili were also found
in broth culture after multiple serial transfers,
demonstrating that their production can be maintained
under specific culture conditions. The "ring structures"
or basal proteins were visible on B nodosus cells and
their association with the pili was confirmed.

Variation in pilin structure is the basis of
serological diversity in B nodosus. There are 20
serotypes of B nodosus found in the United States;
11 of which are the most serologically distinct. The
electrophoretic mobilities of the pilin of each of the
11 distinct serotypes in this study were determined; the
resulting molecular weights of 10 of the 11 serotypes
ranged from approximately 17,600 to 19,400, with the mode
being 18,500 ± 100. These values generally agree with the
published results from B nodosus pilin molecular weight

determinations from other countries. The remaining
serotype was very distinctive in that the pilin from this
serotype displayed 2 subunits with approximate molecular
weights of 7,800 and 6,200; this appeared analogous to
some members of the B nodosus D-set pilins which have a
similar banding pattern.

The monoclonal antibodies in this study which were
prepared against pili of B nodosus reacted with
distinctive types of epitopes on the various pilin
molecules. Some monoclonal antibodies were specific for a
single serotype suggesting that they were directed against
the serotyping epitope of that serotype. The monoclonal
antibodies reacting to more than one serotype were
directed against cross-reactive or shared epitopes.

Two monoclonal antibody reactions were of special
interest. One reacted with 9 of the 11 serotypes and the
other reacted with the remaining 2 serotypes. This
division of the B nodosus serotypes into 2 distinct
populations is analogous to the division of the Australian
B nodosus serotypes into 2 categories based on the degree
of amino acid sequence homology (A-set and D-set pilins).
Reactions of these latter 2 monoclonal antibodies with
members of the A-set and D-set pili revealed that both
methods of categorizing these serotypes (by degree of

amino acid sequence homology or by monoclonal antibody reactivity) divided them into the identical 2 sets.

These results provide encouraging information regarding the ultrastructure of B nodosus pili. The 11 serotypes analyzed by the monoclonal antibodies in this report are representative of all 20 U.S. serotypes as well as the A-set and D-set categories of Australia. Therefore, the 2 epitopes recognized by these 2 monoclonal antibodies encompass all of the currently characterized B nodosus serotypes. If these 2 epitopes could be made antigenic in sheep, and if sheep would elicit an antibody response that was protective against B nodosus challenge, these peptide sequences would provide the basis for an efficacious bi-valent vaccine against all cases of footrot in sheep.

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