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Western Blot (Immunoblot) Analysis of the Fimbrial Antigens of *Bacteroides nodosus*

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The roles of the fimbrial subunit and the putative basal protein antigens in the serological classification of *Bacteroides nodosus* have been examined by Western blot (immunoblot)-antibody binding studies of fimbriae isolated from a wide range of strains representative of different serogroups and serotypes. Fimbrial subunits were recognized by antiserum against the homologous serogroup but not generally by heterologous antisera, whereas recognition of the basal antigen was independent of serological classification. Secondary cross-reaction patterns among fimbrial subunits indicated that some serogroups may be more closely related than others. Examples include serogroups C and G and serogroups D and H. Similar analyses of isolates classified within serotypes A1 and A2, with serotype-specific antisera, showed that this subdivision is also determined by the fimbrial subunit and that significant variation does occur even at this level. These studies suggest that the various serogroups and serotypes of *B. nodosus* comprise a series of overlapping sets of antigenically related strains.

Bacteroides nodosus is the primary causative agent of ovine footrot (4, 10). Infectious isolates of this bacterium are characterized by the presence of numerous surface filaments termed fimbriae (or common pili) (12, 33, 39), which appear to play a major role in the colonization of host tissue (24). These fimbriae have been classified as type 4 (26, 29), sharing common physical and molecular characteristics with fimbriae from a range of other bacteria, including many *Moraxella*, *Neisseria*, and *Pseudomonas* species (16, 26). *B. nodosus* fimbriae are major protective antigens against footrot (13, 19, 35, 37). They are also involved in the serological K agglutination reaction (8, 12, 19, 39), which has been used to divide *B. nodosus* isolates into at least eight serogroups and numerous subsidiary serotypes (6–8, 19, 32). These groupings are reflected in the patterns of immunity against different strains, in that the range of protection conferred by vaccination with either isolated fimbriae or whole cells is largely restricted to the serogroup involved (9, 13, 34, 35).

The fimbrial strand of *B. nodosus* is comprised of a small structural protein of about 17,000 molecular weight (2, 12, 15, 27). It has recently become evident that isolated fimbrial preparations also contain significant amounts of another polypeptide, of about 80,000 molecular weight, which appears to represent the basal protein anchoring the fimbrial strand to the cell wall (25). Both proteins are prominent antigens in vivo and in vitro (25, 28) and display variation in size among different *B. nodosus* isolates (2, 15, 19, 25). These observations raise important questions concerning the relative roles of these two proteins in the serological classification of *B. nodosus* and in the generation of immunity against footrot.

A recent survey of fimbriae isolated from a range of isolates classified within different serogroups and serotypes of *B. nodosus* showed a relatively consistent relationship between the electrophoretic mobility of the fimbrial subunit and serological classification, which was not evident with the basal protein (2). However, since electrophoretic behavior may not necessarily correlate with antigenic profile, the immunological relationships among these antigens were examined by Western blot (immunoblot) transfer analyses, wherein fimbriae taken from a range of isolates were electrophoretically displayed and challenged with representative antisera. The results, presented here, demonstrate that the structural subunit of the fimbrial strand is the primary serological antigen. The data also indicate that variation is widespread in the *B. nodosus* population, and that the serogroup-serotype system may actually represent a series of overlapping sets of antigenically related strains. A preliminary report of some of this material was presented at a Commonwealth Scientific and Industrial Research Organization Workshop (1).

MATERIALS AND METHODS

Bacterial strains. The *B. nodosus* strains used in this study were, with two exceptions (Table 1), those designated (2, 7) as the prototype representatives of each of the serogroups A to H and subsidiary serotypes, as classified by K agglutination profiles. A number of independent isolates classified within serotypes A1 and A2 were also examined (Table 1).

Fimbrial isolation and analysis. Fimbriae were isolated from anaerobic agar plate cultures (36) by the isoelectric precipitation method described previously (25) and electrophoresed on sodium dodecyl sulfate-urea gradient (8 to 15%) polyacrylamide gels with a modified Laemmli buffer system (2, 25). Each gel track contained approximately 5 μg of fimbrial subunit protein. Gel displays were then electrophoretically transferred to nitrocellulose paper (BA85; Schleicher and Schuell Co.) by the method of Towbin et al. (38). A mixture of prestained protein standards (Bethesda Research Laboratories, Inc.) and ^{14}C-labeled protein standards (Amersham Corp.) were included in peripheral gel tracks to allow visual verification of transfer and to provide internal molecular weight standards for subsequent autoradiography. After transfer, any remaining protein binding sites were blocked, and the papers were then incubated in rabbit

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TABLE 1. *B. nodosus* strains employed for fimbrial isolation and analysis

Serogroup	Serotype	Strain[a]
A	1	**1001**, 1016 (1068, 1293)[b]
	2	**1251**, 1014 (1133)[b]
B	1	**1006**
	2	**1208**
	3	**1190**
	4	1197[c]
C	1	**1008**, 1009, 1245
D		**1172**, 1072, 1216, 1239
E	1	**1137**, 1185
	2	**1114**, 1071
F	1	**1017**, 1067, 1140
	2	**1243**[c]
G	1	**1220**, 1295, 1049
	2	**1004**
H	1	**1215**, 1135, 1200
	2	**1057**

[a] The designated prototype strain of each of the serotypes is in boldface type; the prototype of each serogroup is that of the first serotype. All numbers refer to the VCS classification system (7). (For full details of individual isolates and their serological profiles, see Claxton, Ph.D. thesis.)

[b] Isolates within parentheses were not included in the primary matrix experiment (Fig. 1), but were utilized in the A1 and A2 subtype analysis (Fig. 2).

[c] *B. nodosus* VCS1197 was substituted for the prototype of serotype B4, VCS1125. VCS1243 was substituted for the original F2 prototype strain VCS1244 (7). These strains have the same serological profiles as the original prototypes.

anti-fimbrial antiserum followed by ^{125}I-protein A, as detailed previously (2). The blocking, antibody, and protein A incubation steps were all carried out in a Tris-saline buffer containing 5% (wt/vol) nonfat milk powder at pH 7.5 (2, 18).

Reference antisera were raised in rabbits against purified fimbriae isolated from the prototype strains of each serogroup (or relevant serotype) (25). To minimize low-specificity cross-reactions and to obtain relatively standard conditions, each antiserum was used at 75% of its agglutinin titer against whole cells of the strain from which the fimbriae were originally isolated.

RESULTS

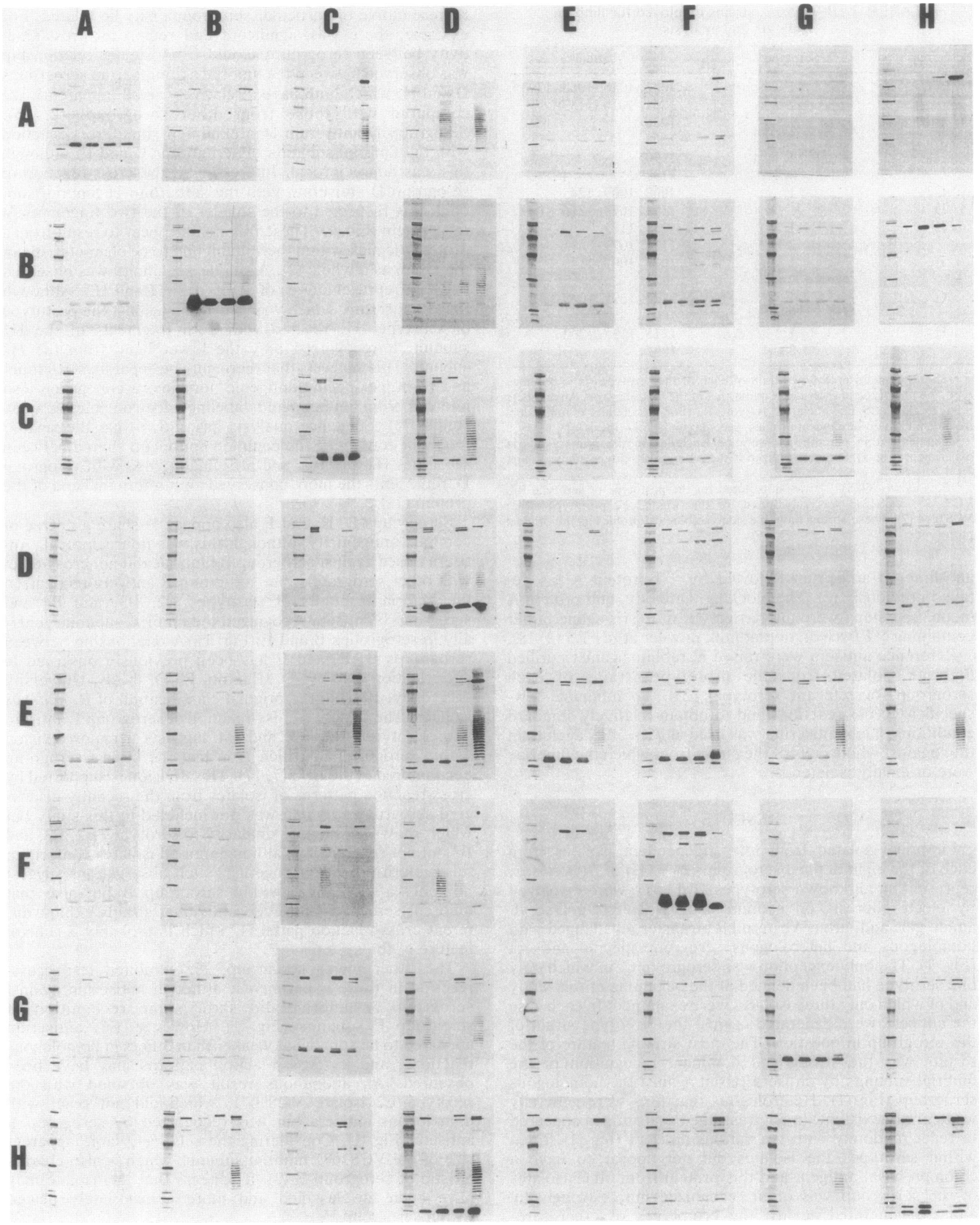
Fimbriae isolated from four independent isolates from each of the eight defined serogroups (A to H) of *B. nodosus*, representing all known serotypes (Table 1), were displayed by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels and, after Western blot transfer, challenged with homologous and heterologous serogroup-specific antisera (Fig. 1). The only exception was serogroup C, in which only one serotype had been defined at the beginning of this study and of which only three isolates were examined. In each case the antisera were generated against the prototype strain of the serogroup in question. The most striking feature of the results was the strong and dominant recognition of the fimbrial subunits by antisera raised against the homologous serogroup (Fig. 1). Heterologous reactions were generally weak or nonexistent. In contrast, the basal antigen appeared to react randomly with the various antisera (Fig. 1). Even within serotypes the isolates did not appear to have a common basal antigen, and this protein from other isolates of the serogroup was often recognized more strongly by antisera generated against the prototypes of other serogroups.

Although the data are qualitative rather than quantitative, these Western blots also indicated that the fimbrial subunits representative of particular serogroups may be related. For example, there was significant and reciprocal cross-reactivity between serogroups C and G. A similar relationship was observed between the fimbrial subunits from serogroups D and H, which both have a relatively small (apparent) size compared with those from other serogroups (2, 15). Serogroup D antiserum also exhibited some cross-reaction with the fimbrial subunits of serogroups C and E, although this was nonreciprocal. Interestingly, the cross-reaction of serogroup D antiserum with the serogroup H fimbrial subunits was restricted to the smaller of the two fragments of this protein (Fig. 1). These fragments appear to result from a proteolytic nick within the subunit and to be characteristic of the group as a whole (2). A similar specificity was observed between representatives of serotypes H1 and H2. Although the H antiserum, which was generated against the prototype of serotype H1, reacted with both fragments of the H1 subunit, it recognized only the lower band from the H2 subunit. This suggests that there may be separate serogroup- and serotype-specific antigenic domains, a conclusion also inferred from immunogold labeling electron micrographic studies (3). Sequence analysis has shown that the smaller fragment containing the common epitope(s) shared between serotypes H1 and H2, and presumably also with serogroup D, represents the more conserved amino-terminal end of the protein (11).

Serogroups B, E, and F also appear to share a degree of common antigenicity, although this was nonreciprocal. Antisera raised against serogroup B fimbriae did not cross-react with other serogroups, but serogroup E antisera recognized the fimbrial subunits of serotypes B2, B3, and B4 and serogroup F antisera recognized the fimbrial subunits generally in serogroups B and E (Fig. 1). A relationship between serogroups B, E, and F has been previously observed in agglutination tests (P. D. Claxton, Ph.D. thesis, University of Sydney, 1981) in the original classification of *B. nodosus* isolates. The selective cross-reaction of serogroup E antisera with serotypes B2, B3, and B4 suggests a major division within serogroup B, which is in fact the largest serogroup encountered in the field (7, 17). This division is supported by cross-reaction patterns of isolates from the recently discovered serogroup I, which was not included in this study but which shows serological similarity to serotypes B2, B3, and B4 but not serotype B1, although not all isolates conform to this pattern (6). Taken together, such observations suggest not only a dichotomy within serogroup B but also that different serotypes and even individual strains exhibit different cross-reaction patterns, which is probably a general feature of the system.

The other major serogroup, A, exhibited less cross-reaction in these experiments, although antiserum against serogroup A fimbriae did show some recognition of serogroup E subunits (Fig. 1). However, this antiserum appeared to be somewhat weaker than others in homologous reactions, and its cross-reaction patterns may have been obscured. An anomolous result was obtained with the serotype E2 isolate VCS1071, which did not react with homologous antisera but was recognized by serogroup F antisera (Fig. 1). Considering the relatively large apparent size of the VCS1071 fimbrial subunit, which is also characteristic of serogroup F (2), it appears that this isolate may have been misclassified and more appropriately placed within serogroup F.

Previous studies had shown that some degree of structural variation occurs in the fimbrial subunits of isolates classified within the same serotype (2). To investigate further the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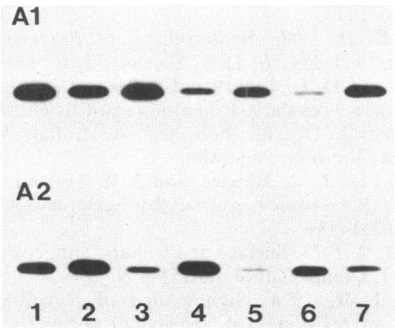

FIG. 2. Western blot transfer analysis of isolates and antisera representative of serotypes A1 and A2. Fimbriae purified from various *B. nodosus* isolates classified in serotypes A1 and A2 (Table 1) were electrophoresed, transferred to nitrocellulose paper, and challenged with antisera against the prototype strains of serotype A1 (panel A1) and serotype A2 (panel A2) as described in Materials and Methods. Lanes 1 to 7 contained approximately 5 μg of fimbriae from strains VCS1001 (A1), 1251 (A2), 1016 (A1), 1014 (A2), 1068 (A1), 1133 (A2), and 1293 (A1), respectively. Only that portion of the gels containing the fimbrial subunit is shown.

question of serotype specificity and variation we carried out a two-by-two matrix analysis of several isolates classified within serotypes A1 and A2 (Fig. 2). All isolates reacted more strongly with the homologous antiserum than with the heterologous antiserum. However, this experiment also showed a significant variation in response within each serotype. For example, the prototype strains of A1 and A2, VCS1001 and VCS1251, cross-reacted well, whereas other isolates such as VCS1016 (A1) and VCS1014 (A2) exhibited relatively poor cross-reaction. Moreover, some isolates such as VCS1068 (A1) and VCS1133 (A2) showed less reaction with their type antisera than the respective prototypes to which the antisera were generated (Fig. 2). It therefore appears that both structural and antigenic variation is widespread in the *B. nodosus* population, and that classification into serogroups and serotypes represents a series of (overlapping) sets of antigenically related strains.

DISCUSSION

The results presented in this paper demonstrate that the serogroup- and serotype-specific epitopes are located on the structural subunit of the fimbrial strand, and therefore that it is this antigen which basically defines the serological profile of *B. nodosus* and the corresponding range of effective immunity engendered by vaccination. The role of this protein as the primary protective antigen has been recently confirmed by the demonstration that *B. nodosus*-type fimbriae produced from a cloned subunit gene in *Pseudomonas aeruginosa* (26) are as effective as either whole cells or purified fimbriae from *B. nodosus* in eliciting protective immunity against footrot (J. R. Egerton, P. T. Cox, B. J. Anderson, C. L. Kristo, M. Norman, and J. S. Mattick, Vet. Microbiol., in press).

The different cross-reaction patterns observed in this study indicate that the fimbrial subunits from different serogroups and serotypes share various degrees of antigenic relatedness. An approximate linear order of relatedness of the serogroups would seem to be (A, E, F, B) (G, C) (D, H), although such relationships are clearly multidimensional, especially when individual serotypes are taken into account. The cross-reaction patterns reported here were detected by using representative antisera at 75% of maximum titer, but more subtle relationships may be revealed at lower dilutions.

The observed cross-reaction patterns will also be somewhat dependent on the particular antisera used, as well as on the choice of strains designated as the prototypes of the different serogroups and serotypes. The former variable is probably not significant, since the results reported here are generally consistent with earlier K agglutination serological studies (with both sheep and rabbit antisera; Claxton, Ph.D. thesis) as well as with comparative sequence analyses of the fimbrial subunit genes (J. S. Mattick, B. J. Anderson, M. M. Bills, P. T. Cox, and J. R. Egerton, manuscript in preparation). The isolates designated as the prototypes of new serogroups (6, 7) were generally those which showed no significant cross-reaction with the reference antisera of predefined serogroups, and there may well be alternative choices which would provide more balanced representation of the field population. New isolates allocated to existing serogroups were those which did show significant cross-reaction in the K agglutination test with antiserum to the reference strain and were placed in the same or different subsidiary serotype depending on the degree of that cross-reaction. There do nevertheless appear to be definable groups in the population, which have not only distinguishing antigenic characteristics, but also distinct physical properties such as the proteolytically susceptible bond in the fimbrial subunits of serogroup H or the relatively large apparent size of fimbrial subunits from serogroup F (2). However, these groups are not discrete, and there are some variants that cross serological boundaries (2, 6).

The serological classification of *B. nodosus* may thus be viewed as a type of Venn diagram with the prototype strains being the focal point of each set. The immunoprotective status of vaccinated animals appears to follow the same principles, being a function of the intensity of response and the degree of relatedness of the challenge strain to that used for vaccination. A K agglutination titer of approximately 5,000 is regarded as the minimum commensurate with good protective immunity (19, 35, 37; Egerton et al., in press). To what extent cross-reactions are synergistic or additive in a multivalent vaccine remains to be determined.

Despite the fact that antigenic variation is widespread in the *B. nodosus* population, it is also clear that this variation

FIG. 1. Western blot transfer analysis of fimbrial antigens from *B. nodosus* isolates representative of serogroups A to H. Fimbriae from a number of isolates from each serogroup were electrophoresed on sodium dodecyl sulfate-gradient polyacrylamide gels, transferred to nitrocellulose paper, and challenged with homologous and heterologous antisera as described in Materials and Methods. Antibody binding was visualized by autoradiography after incubation with ^{125}I-protein A. Each row of panels contained fimbriae (5 μg) from isolates of the designated serogroup in order from left to right as listed in Table 1, with the exception of serogroup H, in which the last two isolates VCS1200 (H1) and VCS1057 (H2) were reversed. Each column of panels was challenged with the designated antisera, raised against fimbriae from the prototype strain of the serogroup in question. The track on the left of each panel contained ^{14}C-labeled methylated protein molecular weight markers (Amersham Corp.): phosphorylase b (92,500), bovine serum albumin (68,000), ovalbumin (43,000), carbonic anhydrase (29,100), and lysozyme (14,300). Differences in the intensity of these markers among panels is largely due to the use or otherwise of intensifying screens in autoradiographic development. The laddered patterns observed in some fimbrial tracks are due to lipopolysaccharide contamination of these fractions (25).

is (in a broader sense) limited, presumably as a result of constraints imposed by the assembly, structure, and function of the fimbrial strand in vivo. A large number of *B. nodosus* isolates have now been examined and classified into just 9 or 10 serogroups, depending upon whether serogroup B is regarded as one or two serogroups. The structural subunit of *B. nodosus* fimbriae, which is approximately 150 amino acids in length, consists of a conserved hydrophobic amino-terminal domain, followed by the more hydrophilic carboxy-terminal two-thirds of the protein, wherein regions of variability are interspersed with more conserved elements (27). A similar arrangement is observed in the structural subunits of other type 4 fimbriate bacteria, such as *Moraxella bovis* (23), *Neisseria gonorrhoeae* (14), and *P. aeruginosa* (31). Indeed, recent serological analyses of *M. bovis* isolates have thus far identified just six or seven serogroups (21; L. J. Moore, Ph.D. thesis, University of Reading, 1985). Multivalent vaccines (containing a representative of each serogroup) are effective against *B. nodosus* infection in the field (22), and it seems feasible that, given the proper antigenic context, analogous vaccines may be designed and produced against other type 4 fimbriate pathogens. Preliminary evidence suggests that isolated fimbriae are immunoprotective against infection by *M. bovis* (20, 30) and *N. gonorrhoeae* (5). In such cases, the ability of the bacteria to evade host-protective responses would be effectively checkmated by a multivalent vaccine which provided coverage against a relatively complete set of allowable possibilities. Thus, prophylactic or therapeutic vaccination against these related pathogens may be a realistic proposition.

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