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Distribution of a Protein Antigenically Related to the Major Anaerobically Induced Gonococcal Outer Membrane Protein among Other *Neisseria* Species

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The Pan 1 protein of *Neisseria gonorrhoeae* is a novel 54-kDa outer membrane protein expressed only when gonococci are grown in the absence of oxygen. It is a major antigen recognized by sera from patients with gonococcal infection. We raised mouse monospecific polyclonal antiserum to gel-purified Pan 1 from gonococcal strain F62. The antiserum was broadly cross-reactive among gonococcal strains; all strains tested reacted in immunoblot analysis proportionate to the amount of Pan 1 visible in silver-stained sodium dodecyl sulfate (SDS)-polyacrylamide gels. In immunoblot experiments, *N. lactamica* and *N. cinerea* reacted very strongly to the anti-Pan 1 antiserum, whereas *N. sicca*, *N. flava*, and *N. mucosa* did not react at all. The other commensals tested, *N. subflava* and *N. perflava*, exhibited only a minor reaction. These results correlated with the apparent amount of Pan 1 seen on SDS-polyacrylamide gels of outer membranes. SDS-polyacrylamide gel analysis of six meningococcal strains revealed no visible anaerobically induced outer membrane proteins, and the subsequent immunoblots showed only slight or no reaction to the anti-Pan 1 antibody. In the four meningococcal strains that did react slightly with the antiserum, a Pan 1-like protein was seen only in anaerobically grown cells. Thus, meningococci did not express Pan 1 at levels comparable to that found in gonococci; however, when Pan 1 was expressed in meningococcal strains, it was oxygen regulated. This is the first example of a protein found in the gonococcal outer membrane that, under identical growth conditions, is not expressed at similar levels in the meningococcus.

Anaerobic growth of *Neisseria gonorrhoeae* results in the induction of at least three novel outer membrane proteins (8). One of these, Pan 1, is a major antigen recognized by sera from patients with gonococcal infection (9). The fact that patients make antibody against the Pan 1 protein and that the gonococcus can be routinely isolated from mixed infections with facultative and obligate anaerobes suggests that it grows anaerobically in vivo. Moreover, these observations suggest that anaerobiosis may be a significant physiological state for the organism and may be important in pathogenesis.

The genus *Neisseria* includes two human pathogens, *N. gonorrhoeae* and *N. meningitidis*, which have an extraordinarily high degree of homology of the DNA level (13, 16) but which cause quite dissimilar diseases. Many gonococcal proteins have been found to be related to analogous proteins in the meningococci (25). For example, the meningococcus has been found to contain proteins homologous at the DNA and amino acid levels to the gonococcal PI (1, 7, 12, 14), PII (2, 15, 22), and PIII (4, 11) outer membrane proteins as well as the pilin proteins (20, 23), the iron-repressible protein (21), and the H.8 antigen (3, 5, 6, 11). Commensal *Neisseria* species have been less extensively analyzed for the presence of these proteins, but in most cases in which an analogous protein has been found, these were restricted to *N. lactamica* and *N. cinerea* (6, 10), species most closely related to the meningococcus and the gonococcus.

In the study presented here, we investigated the distribution of the Pan 1 protein among the commensal *Neisseria* species and *N. meningitidis*. Our results indicate that the Pan 1 protein is different in its distribution among the *Neisseria* species from previously reported gonococcal proteins.

(Parts of this research were presented previously [G. T. Hoehn, J. C.-R. Chen, and V. L. Clark, Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, D-38, p. 88].)

MATERIALS AND METHODS

Strains and growth conditions. The gonococcal strains used have been described previously (9). All meningococcal strains were relatively nitrite tolerant and were kindly provided by J. S. Knapp, Centers for Disease Control, Atlanta, Ga. Commensal strains were obtained from the Neisseria Reference Laboratory, University of Washington, Seattle, Wash.

Gonococci, meningococci, and commensal species were grown on GC medium base plates with 1% Kellogg supplements (8) either aerobically in a 5% CO_2 incubator or anaerobically in a Coy anaerobe chamber with an atmosphere of 85% N_2–10% H_2–5% CO_2. For anaerobically grown bacteria, sodium nitrite (50 μl of a 20% solution) was added to a sterile cellulose disk placed in the middle of the plate (17). Since *N. gonorrhoeae* is nitrite sensitive, the nitrite disk method allows a continuous supply of nitrite to diffuse into the agar at concentrations that are not lethal to the gonococci. Anaerobic conditions were monitored by using a Coy oxygen detector. All strains were passed two times anaerobically and incubated at 37°C for 18 to 24 h.

Preparation of outer membranes. Outer membrane fractions were prepared as described previously (8). Briefly, cells were harvested in Tris-sucrose buffer, washed, resuspended in the same buffer containing 10 mg of lysozyme per ml, and incubated on ice for 30 min. The cell solution was

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diluted fivefold in 0.5 M Tris buffer (pH 8.0) and subjected to three bursts of sonic oscillation in 30-s intervals. Cell debris was removed by centrifugation, and total membrane was isolated by ultracentrifugation at 100,000 × *g* for 60 min. Outer membranes were isolated by differential solubilization in 1% sodium lauroyl sarcosinate (Sarkosyl; Sigma) and centrifuged at 100,000 × *g* for 60 min. The remaining pellet containing outer membrane was resuspended in sterile, distilled H_2O and stored at −70°C.

Preparation of anti-Pan 1 antibody. Pan 1 protein was purified from outer membrane extracts by electroelution from preparative 13% sodium dodecyl sulfate (SDS)-polyacrylamide gels. Gels were fixed in 10% trichloroacetic acid and stained with Coomassie blue. The band corresponding to the Pan 1 protein was removed with a scalpel and electroeluted from the gel overnight in a Schleicher & Schuell Elutrap System. SDS was then removed by extensively dialyzing the preparation against 10 mM Tris-EDTA (pH 8.0) buffer. For antibody preparation, purified Pan 1 was mixed with an equal volume of Freund complete adjuvant (Difco) and injected intraperitoneally into 6- to 8-week old male BALB/c mice. Mice were boosted 4 weeks later with the same preparation, and serum was collected 2 weeks after boosting.

SDS-PAGE and immunoblots. Samples for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) were mixed with an equal volume of sample buffer and boiled for 5 min. Electrophoresis was performed by using the procedure of Laemmli (19). After electrophoresis, gels were either fixed in trichloroacetic acid and stained with Coomassie blue and silver (9) or transferred to nitrocellulose by the method of Towbin (24) in a Hoefer semidry Transphor system. For immunoblot analysis, nitrocellulose filters were incubated for 60 min in BLOTTO (10% nonfat dry milk in 1× phosphate-buffered saline [PBS]) and washed once in 1× PBS and 0.1% Triton X-100 (Sigma) and once in 1× PBS. Filters were then incubated for 60 min with a 1:500 dilution of anti-Pan 1 antiserum in 5% bovine serum albumin and washed two times with PBS-Triton X-100 mix and two times with 1× PBS. The filters were incubated for another 60 min with ^{125}I-labeled sheep anti-mouse $F(ab')_2$ fragment antibody (Amersham) in BLOTTO, washed as before, and exposed to X-ray film (Kodak X-RP) at −70°C.

RESULTS

Antibody to Pan 1. To investigate the Pan 1 protein in more detail, we prepared mouse polyclonal antiserum to gel-purified Pan 1 from the gonococcal laboratory strain F62. When outer membranes from F62 were probed with the mouse anti-Pan 1 antiserum, a protein found only in membranes from anaerobically grown cells was detected (see Fig. 1B, lane b). However, the antiserum also recognized an outer membrane protein that is not oxygen regulated. This contaminating antibody was selectively removed by incubating the antiserum with nitrocellulose filters containing outer membranes from aerobically grown cells. Subsequent immunoblots (see Fig. 2 and 3) demonstrated the efficiency of absorbing out this antibody.

Reactivity of anti-Pan 1 to gonococcal outer membranes. To show that the monospecific anti-Pan 1 antiserum recognized the Pan 1 protein from heterologous gonococcal strains, we probed nitrocellulose filters containing outer membranes from nine strains grown aerobically and anaerobically (Fig. 1). All gonococcal strains expressed the anaerobically induced Pan 1 protein, although amounts of this protein varied from strain to strain (Fig. 1A; compare lanes n and r with lanes b and d). The immunoblot with the anti-Pan 1 antiserum showed that the antibody recognized Pan 1 epitopes from all gonococcal strains tested (Fig. 1B). Moreover, the extent of this reaction was consistent with the apparent amount of Pan 1 present. For example, F62 and NRL 36329 (lane d) made much more Pan 1 than either A281 (lane n) or RUN 4383 (lane r) as seen on the silver-stained gel. The reaction of these samples on the immunoblot correlated with the amount of Pan 1 visible in silver-stained gels; F62 and NRL 36329 showed a greater reaction than either A281 or RUN 4383. This demonstrated that although Pan 1 is believed to be antigenically heterogeneous among gonococcal strains (9), the Pan 1 antiserum was able to recognize conserved epitopes of this protein from strain to strain.

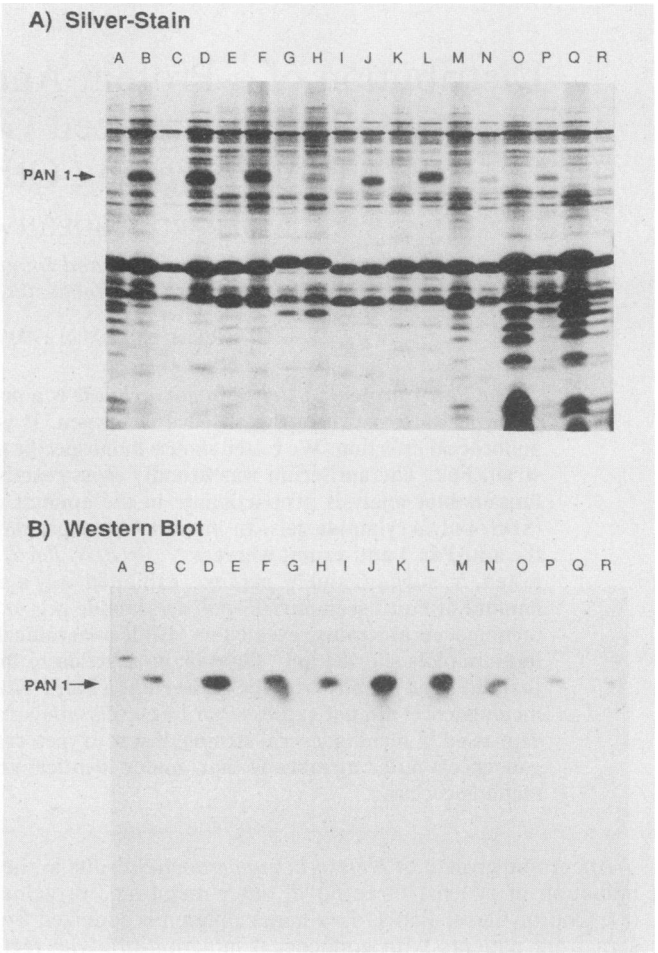

FIG. 1. SDS-PAGE and immunoblot analysis of outer membranes of nine different gonococcal strains. (A) Silver-stained gel; (B) immunoblot of the same gel. Gonococcal strains used were as follows: F62 (lanes a and b), NRL 36329 (lanes c and d), A277 (lanes e and f), NRL 36591 (lanes g and h), NRL 36325 (lanes i and j), CDC 86-039627 (lanes k and l), A281 (lanes m and n), NRL 37264 (lanes o and p), and RUN 4383 (lanes q and r), grown aerobically (lanes a, c, e, g, i, k, m, o, and q) or anaerobically (lanes b, d, f, h, j, l, n, p, and r). The immunoblot (B) shows different reactions to the Pan 1 protein of the different gonococcal strains which are approximately equal to the staining intensity of Pan 1 seen in panel A.

Pan 1 in commensal *Neisseria* strains. We sought to determine if commensal *Neisseria* strains express an anaerobically induced outer membrane protein that would react to

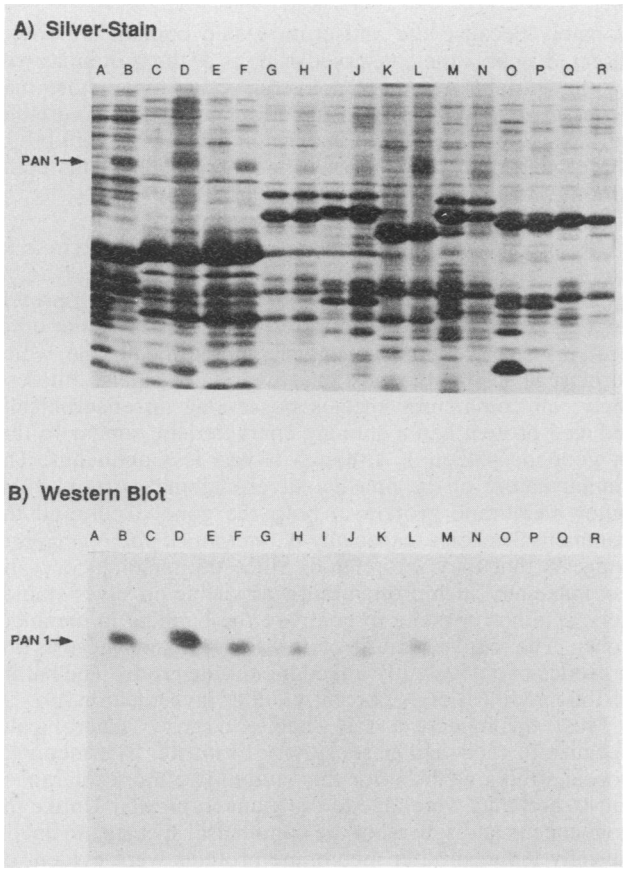

FIG. 2. SDS-PAGE and immunoblot analysis of outer membranes from commensal *Neisseria* species. (A) Silver-stained gel; (B) immunoblot of the same gel. The strains used were as follows: *N. gonorrhoeae* F62 (lanes a and b), *N. lactamica* NRL 30011 (lanes c and d), *N. lactamica* NRL 8828 (lanes e and f), *N. cinerea* NRL 30003 (lanes g and h), *N. cinerea* NRL 32165 (lanes i and j), *N. subflava* NRL 30017 (lanes k and l), *N. perflava* NRL 30015 (lanes m and n), *N. sicca* NRL 30016 (lanes o and p), and *N. flava* NRL 30008 (lanes q and r), grown aerobically (lanes a, c, e, g, i, k, m, o, and q) or anaerobically (lanes b, d, f, h, j, l, n, p, and r). The immunoblot (B) shows that *N. lactamica* and *N. cinerea* reacted strongly to moderately with the anti-Pan 1 antiserum; *N. subflava* and *N. perflava* reacted weakly, and *N. sicca* and *N. flava* did not react at all. These results correlate with what was seen on the silver-stained gel in panel A.

the gonococcal anti-Pan 1 antiserum. We prepared outer membrane extracts from various commensal strains that were grown aerobically or anaerobically and assayed for a Pan 1-like protein by SDS-PAGE and immunoblot analysis. Most of the commensals displayed an anaerobically induced outer membrane protein with a molecular weight similar to that of the gonococcal Pan 1 (Fig. 2A), although this band was more diffuse and less abundant than that of the Pan 1 from F62. In comparison, both strains of *N. lactamica* produced approximately equivalent amounts of the Pan 1-like protein, while the two *N. cinerea* strains, *N. subflava*, and *N. perflava* appeared to make less than F62. *N. sicca* and *N. flava* did not produce any detectable anaerobically induced proteins (compare lanes d, f, h, j, l, and n with lanes r and p). The anaerobically induced proteins in *N. lactamica*, *N. cinerea*, *N. subflava*, and *N. perflava* were shown to react to the gonococcal anti-Pan 1 antiserum in immunoblots (Fig. 2B) at an intensity approximate to the amount of Pan 1-like protein present in silver-stained gels. *N. lactamica* strains exhibited the strongest reaction to the anti-Pan 1 antiserum, whereas *N. sicca*, *N. flava*, and *N. mucosa* (data not shown) showed no reaction. Moderate to weak reactions were seen with both *N. cinerea* strains, *N. subflava*, and *N. perflava*. Therefore, most of the commensal strains (six of nine tested) expressed an anaerobically induced outer membrane antigenically similar to the gonococcal Pan 1. These results further demonstrated the ability of the anti-Pan 1 antiserum to recognize Pan 1 epitopes from species different from the gonococcus, indicating that the anti-Pan 1 antiserum was broadly cross-reactive.

Pan 1 in meningococci. We wished to determine whether meningococcal strains express anaerobically induced outer membrane proteins analogous to the gonococcal Pan 1. Outer membrane extracts were prepared from six different meningococcal strains that were grown aerobically or anaerobically and assayed for the presence of Pan 1-like proteins by SDS-PAGE and immunoblot analysis (Fig. 3). Silver-stained gels revealed no apparent anaerobically induced outer membrane proteins (Fig. 3A; compare meningococcal strains with the gonococcal control). However, when these gels were probed with gonococcal anti-Pan 1 antiserum, four of the six strains reacted very slightly, whereas the other two strains did not react at all (Fig. 3B). In those meningococcal strains in which a Pan 1-like protein was detected, it was present only in anaerobically grown cells. Similar results were obtained with whole cell extracts (data not shown).

DISCUSSION

The gonococcal outer membrane has long been the focus of much research in an effort to understand factors involved in pathogenesis. Taxonomic studies have revealed a high degree of similarity at the genetic and biochemical level between the gonococcus and the meningococcus (13). Two commensal species, *N. cinerea* and *N. lactamica*, which have been reported to cause infection, also show a high degree of similarity to the pathogens *N. gonorrhoeae* and *N. meningitidis*. Recent work by Mietzner et al. (21) and Cannon et al. (6) have identified gonococcal outer membrane proteins that were present in *N. lactamica* and *N. cinerea* but not in the other commensal species. It has been argued that the absence of these proteins in the commensal species suggests that they may be determinants of potential virulence. The genes for many other gonococcal outer membrane proteins such as the PI (1, 7, 12, 14), PII (2, 15, 22), PIII (4, 11), the pilus protein (20, 23), and the immunoglobulin A (IgA) protease (18) have also been cloned and found to have meningococcal analogs. The distribution of these genes among various commensal species has not been investigated, although a majority of these proteins have a purported role in pathogenesis.

Previous work in our laboratory has shown that the Pan 1 protein is a major antigen recognized by sera from patients with gonococcal infection (9). The differential reactivity of these sera to a panel of gonococcal strains suggested that Pan 1 is antigenically heterogeneous, consisting of both common and unique epitopes. Amino acid composition data and proteolytic digestion analysis of pure Pan 1 (data not shown) from several gonococcal strains further demonstrated this heterogeneity. In this respect at least, Pan 1 is similar to many of the other defined outer membrane proteins of the gonococcus. The mouse monospecific anti-Pan 1 antiserum reacted proportionately to the apparent amount of Pan 1 seen in the silver staining of SDS-polyacrylamide gels

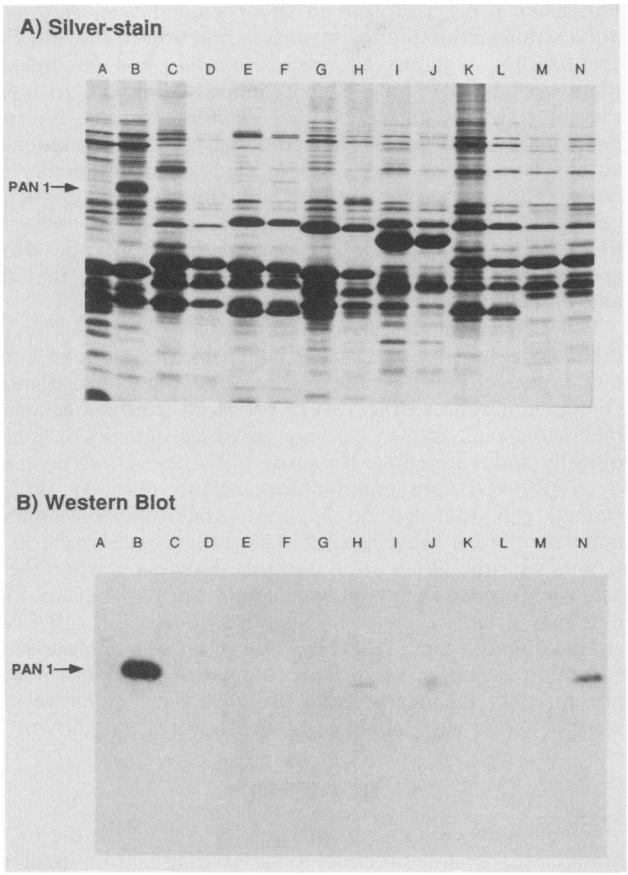

FIG. 3. SDS-PAGE and immunoblot analysis of outer membranes from six different meningococcal strains. (A) Silver-stained gel; (B) immunoblot of the same gel. The strains used were as follows: *N. gonorrhoeae* F62 (lanes a and b), *N. meningitidis* RUN 5645 (serogroup A) (lanes c and d), *N. meningitidis* RUN 5646 (serogroup B) (lanes e and f), *N. meningitidis* RUN 5647 (serogroup B) (lanes g and h), *N. meningitidis* RUN 5648 (serogroup C) (lanes i and j), *N. meningitidis* RUN 5649 (serogroup Z) (lanes k and l), and *N. meningitidis* RUN 5650 (serogroup Z) (lanes m and n), grown aerobically (lanes a, c, e, g, i, k, and m) or anaerobically (lanes b, d, f, h, j, and l). Meningococci did not make any apparent anaerobically induced proteins visible on silver-stained SDS-polyacrylamide gels (A). In the immunoblot (B), reaction to anti-Pan 1 is indicated. Note the strong reaction to the homologous gonococcal strain but little or no reaction to an analogous meningococcal Pan 1.

in immunoblots of outer membranes from a panel of gonococcal strains. This suggests that the anti-Pan 1 antiserum contained broadly cross-reactive antibody species that were able to recognize a variety of different epitopes.

Colony immunoblotting and preliminary experiments with protease treatment of whole gonococcal cells grown anaerobically suggest that the Pan 1 protein is surface exposed (G. Hoehn, unpublished data). If Pan 1 is indeed a surface-exposed protein, it is reasonable to assume that there would be conserved domains located in the membrane, while variable domains would be exposed to the environment. This may help to explain the differences in reactivity between the mouse polyclonal anti-Pan 1 antiserum and human sera. Since the mouse anti-Pan 1 antiserum is made from gel-purified, denatured protein, there will be antibodies generated against all possible linear epitopes. Although the Pan 1 may vary from strain to strain, our mouse antiserum would probably recognize the Pan 1 from different strains proportionately because the antiserum would contain antibodies directed against the conserved regions of the protein as well as the variable regions. Conversely, a human host may generate an immune response predominantly to the variable, surface-exposed region of the protein and not to epitopes of the protein that are conserved between strains. This could account for the differences seen in the reactivity to the individual antisera previously reported (9).

An interesting feature concerning the Pan 1 protein is its unusual banding pattern on silver-stained SDS-polyacrylamide gels. In all gonococcal strains tested, the Pan 1 protein appears as a very broad, diffuse band. This is unlike other proteins found in the gonococcal outer membrane which form regular, tight bands on silver-stained gels. Interestingly, all commensal species possessing an anaerobically induced protein had a staining characteristic similar to that of gonococcal Pan 1, although it was less abundant. The similar nature of the unusual silver-staining pattern of this outer membrane protein in both the gonococcus and the commensal species, especially *N. lactamica* and *N. cinerea*, suggests that they are related. Since the meningococci did not make any such protein that was visible on silver-stained gels, it is not possible to compare the banding in meningococci. The diffuse nature of the Pan 1 band suggests the presence of a covalently linked modifying group. The nature of this modification is currently under investigation.

Most meningococci are unable to grow anaerobically because of their higher sensitivity to nitrite. The meningococcal strains we used for this study were more tolerant to nitrite and thus were able to grow anaerobically. Unlike the gonococcus and several of the commensal species, no anaerobically induced outer membrane proteins were evident on silver staining of SDS-polyacrylamide gels of meningococcal outer membranes. Since many of the commensal species expressed an anaerobically induced protein antigenically related to the gonococcal Pan 1, it was expected that *N. meningitidis*, the species most closely related to the gonococcus, would express a similar protein. Most (four of six) of the meningococcal strains tested did show a minor reaction to the gonococcal anti-Pan 1 antisera, suggesting that these meningococci did make a Pan 1-like analog. To exclude the possibility that the meningococci make Pan 1 but do not transport it efficiently to the outer membrane, we performed immunoblot analysis of whole-cell extracts; no additional reaction was observed. Thus, the level of expression of this anaerobically induced outer membrane protein was much lower in meningococci, under the conditions used, than in gonococci.

Unlike other gonococcal surface proteins, the Pan 1 protein seems to be conserved and expressed in many commensal strains, including *N. subflava* and *N. flava*, as well as *N. lactamica* and *N. cinerea*, but poorly expressed in the meningococci. This is the first example of such a distribution among known neisserial proteins. The fact that oxygen regulation of Pan 1 was conserved among all neisserial strains, including the meningococci in which Pan 1 was found, indicates that the Pan 1 structural gene is present in these strains. The effects of anaerobic conditions and of the subsequent expression of Pan 1 on pathogenesis are unknown. The function of Pan 1 and its relevance to gonococcal infection remain to be elucidated.

We are currently attempting to clone the gonococcal Pan 1 gene to investigate the differences in expression of Pan 1 between the meningococcus and the gonococcus. Clearly, oxygen regulation of Pan 1 is maintained in the meningococci that express Pan 1, and, therefore, factors that govern the

regulation of Pan 1 must be present in these strains. An understanding of the difference in expression of Pan 1 will require sequencing the Pan 1 gene and its corresponding upstream regulatory region from both the meningococcus and the gonococcus.

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