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The Major Anaerobically Induced Outer Membrane Protein of *Neisseria gonorrhoeae*, Pan 1, Is a Lipoprotein

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Pan 1 is an acidic outer membrane protein of *Neisseria gonorrhoeae* that is expressed only when gonococci are grown anaerobically. On silver-stained sodium dodecyl sulfate-polyacrylamide gels, Pan 1 migrates as an intense but diffuse 54-kDa protein. The deduced amino acid sequence of Pan 1 from the *aniA* (anaerobically induced protein) open reading frame reveals a lipoprotein consensus sequence, Ala-Leu-Ala-Ala-Cys, and a processed molecular mass of 39 kDa. Furthermore, there is strong homology at the N terminus and C terminus of Pan 1 to the termini of the gonococcal outer membrane lipoproteins Lip and Laz. [^{3}H]palmitic acid labeling of gonococci grown under oxygen-limited conditions demonstrated specific incorporation of label into Pan 1, suggesting further that Pan 1 is a lipoprotein.

When grown in the absence of oxygen, *Neisseria gonorrhoeae* expresses several novel outer membrane proteins. One of these, Pan 1, migrates as an intense but diffuse 54-kDa band on silver-stained sodium dodecyl sulfate (SDS)-polyacrylamide gels (4) and reacts strongly with sera from patients with gonococcal infection (5). The presence of specific antibodies to Pan 1 in patient sera not only indicates that Pan 1 is expressed in vivo but, more importantly, strongly suggests that *N. gonorrhoeae* is able to grow anaerobically in vivo as well. Thus, anaerobiosis may be an important physiological condition relevant to the course of gonococcal infection.

Work in our laboratory has focused on the characterization of Pan 1. Using monospecific, polyclonal anti-Pan 1 antiserum in immunoblot analysis, we demonstrated that Pan 1 is uniquely distributed among the *Neisseria* species (10). In particular, we showed that most commensal *Neisseria* species and all gonococcal species produce Pan 1 when grown anaerobically, whereas *N. meningitidis* produced very little if any Pan 1. Another unusual feature of this protein is its intense staining by silver and the diffuse nature of the stained band. This observation, coupled to the fact that Pan 1 is not amenable to N-terminal sequencing, suggests that Pan 1 contains a covalent N-terminal modification that occurs in vivo (11).

Two other described proteins of the gonococcal outer surface, Lip and Laz, have been shown to migrate aberrantly on SDS-polyacrylamide gel electrophoresis (PAGE) and stain unusually with silver (2, 9). Recent cloning of the *lip* and *laz* genes (1, 6, 17, 23) has revealed that the predicted amino acid sequences of both Lip and Laz contain a signal peptide lipoprotein consensus sequence that is recognized by the bacterial enzyme signal peptidase II (15). Processing by signal peptidase II results in an N-terminal acylation of cysteine with fatty acid and glycerol (7, 20). The Lip lipoprotein has a predicted molecular mass of 6.3 kDa (1, 23) and consists almost entirely of pentapeptide repeats of the motif Ala-Ala-Glu-Ala-Pro (AAEAP). The function of Lip is unknown, although sera from patients with gonococcal infection contain antibodies that react to the Lip antigen (2). The mature Laz lipoprotein has a predicted molecular mass of 17 kDa and contains several copies of the AAEAP repeat at the N terminus (6, 17). At the C-terminal region, however, the amino acid sequence of Laz diverges and shows extensive homology to azurins (2, 6, 17), which are small, blue, copper-binding proteins that are believed to function in electron transport.

Here, we present the deduced amino acid sequence of the cloned *aniA* gene (12), which contains a lipoprotein signal peptidase II-processing site and contains regions of homology to the gonococcal lipoproteins Lip and Laz. Homology to Lip and Laz is found at both the N terminus and the C terminus of Pan 1, and the protein shows similarity to the AAEAP repeat motif. We also demonstrate that Pan 1 is specifically labeled with [^{3}H]palmitic acid when gonococci are grown under oxygen-limited conditions, further indicating that Pan 1 is lipid modified.

MATERIALS AND METHODS

Growth of neisserial strains. *N. gonorrhoeae* F62 was grown on GC medium base (Difco) plates with 1% Kellogg's supplements (GCK) 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, and 5% CO_2 as described previously (10, 18). Anaerobic conditions were monitored with a Coy oxygen detector. All strains were passaged two times anaerobically and incubated at 37°C for 18 to 24 h.

Because of low cell yields from anaerobic growth on plates and difficulty in growing most gonococcal strains anaerobically in broth, a culture system was devised to allow growth in broth under oxygen-limiting conditions. A flask half full of GCP supplemented with 0.042% $NaHCO_3$, 1% Kellogg's supplements, and 2 mM $NaNO_2$ was inoculated and shaken very slowly overnight. The large volume of broth created a small surface area which, coupled with slow shaking, prevented good aeration. Under these conditions, Pan 1 was expressed (data not shown).

Palmitic acid labeling of *N. gonorrhoeae*. To determine whether the Pan 1 protein was lipid modified during growth, strain F62 was labeled with [^{3}H]palmitic acid by the procedure of Chen et al. (3). F62 was grown in broth under oxygen-limiting conditions in order to induce the synthesis

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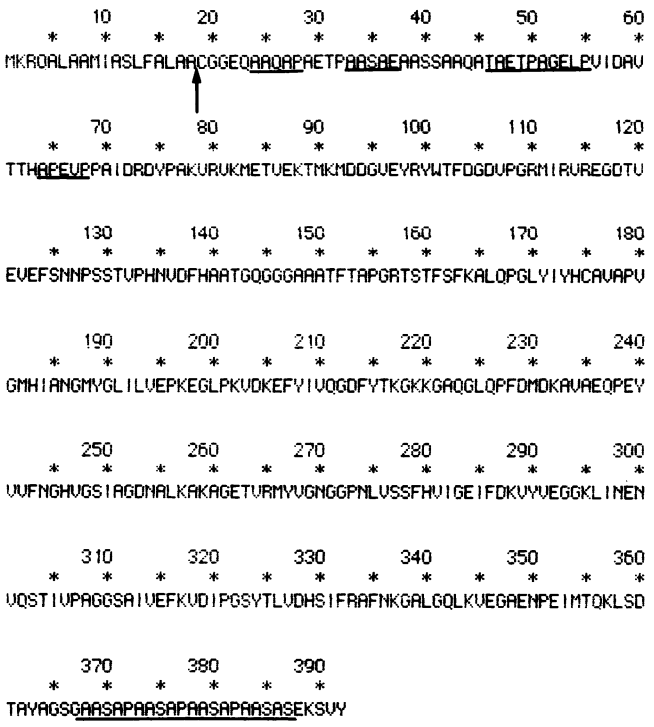

Calculated Molecular Weight = 39091.953

Estimated pI = 4.877

Amino Acid Composition:

Non-polar:	Number	Percent
Ala	54	14.44
Val	35	9.36
Leu	14	3.74
Ile	16	4.28
Pro	25	6.68
Met	9	2.41
Phe	15	4.01
Trp	1	0.27
Polar:	**Number**	**Percent**
Gly	39	10.43
Ser	23	6.15
Thr	21	5.61
Cys	2	0.53
Tyr	14	3.74
Asn	12	3.21
Gln	12	3.21
Acidic:	**Number**	**Percent**
Asp	18	4.81
Glu	26	6.95
Basic:	**Number**	**Percent**
Lys	21	5.61
Arg	9	2.41
His	8	2.14

FIG. 1. Deduced amino acid sequence of the *aniA* gene product and physical characteristics of the encoded protein. The *aniA* open reading frame encoded a protein with a typical lipoprotein processing site (Ala-Leu-Ala-Ala-Cys). The site of cleavage is shown by the arrow. Processing by signal peptidase II results in modification of the N-terminal cysteine residue by glycerol and fatty acid. The underlined amino acids represent a repeat region similar to the motif Ala-Ala-Glu-Ala-Pro (AAEAP). Several physical characteristics of the mature protein encoded by the *aniA* gene, including its predicted molecular weight, isoelectric point (pI), and amino acid composition, are also shown.

of Pan 1. GCP broth containing Kellogg's supplements, $NaHCO_3$, and 2 mM $NaNO_2$ was inoculated with an overnight culture of F62 and grown at 37°C with heavy aeration or minimal aeration. After approximately 10 h of growth, 10 μCi of [^{3}H]palmitic acid (New England Nuclear) per ml was added. Growth was allowed to proceed for an additional 2 h, at which time the gonococci were harvested by centrifugation at 7,700 × *g* and resuspended in a small volume of H_2O. An aliquot (40 μl) of cells was mixed with an equal volume of 2× sample buffer (4), boiled for 5 min, loaded in duplicate onto a 13% PAGE–SDS gel, and electrophoresed at 8 mA overnight. To ensure that labeled bands contained protein, whole-cell extracts were treated with 25 mg of proteinase K at 65°C for 1 h, an equal volume of 2× sample buffer was added, and the samples were boiled for 5 min. After electrophoresis, half the gel was prepared for fluorography by fixing proteins with 10% acetic acid–30% methanol for 1 h and then treating them with En^{3}Hance (New England Nuclear) per the manufacturer's instructions. The gel was then dried under vacuum and exposed to Kodak X-RP film at −70°C. The other half of the gel was transferred to nitrocellulose and immunoblotted with anti-Pan 1 antiserum as described elsewhere (10).

DNA and protein sequence analysis. DNA sequence information obtained from sequencing gels (12) was analyzed by the MacVector program (IBI Technologies, Inc.). DNA and protein homology searches were performed with the Genetics Computer Group computer system and by searching the University of Wisconsin gene bank.

RESULTS AND DISCUSSION

Amino acid sequence of Pan 1. Previously, we reported the cloning and nucleotide sequence of the gonococcal *aniA* gene (12), the structural gene encoding the Pan 1 protein. An ATG start codon at nucleotide 87 initiates an open reading frame that terminates at a TAA stop codon at nucleotide 1263. The deduced amino acid sequence derived from the *aniA* open reading frame encodes a protein (Fig. 1) with a consensus lipoprotein leader peptide sequence of the motif Ala-Leu-Ala-Ala-Cys (7, 21). This pentapeptide sequence is recognized and processed by the enzyme signal peptidase II (15), which results in cleavage between the Ala and Cys residues and modification of the cysteine residue with fatty acid and glycerol (20). The enzyme is present in *N. gonorrhoeae*, as growth of gonococci is inhibited by as little as 1 μg of the antibiotic globomycin (22), a specific inhibitor of signal peptidase II (14), per ml. The processed Pan 1 lipoprotein predicted by the sequence has a molecular mass of 39.2 kDa, considerably smaller than the 54 kDa determined by SDS-PAGE. The deduced isoelectric point of the processed Pan 1 is 4.9, which is consistent with observations that Pan 1 migrated at an acidic pH on two-dimensional gels (4). The amino acid composition data (Fig. 1) for the *aniA*-encoded polypeptide reveal that the protein has a high content of proline and alanine, which may also contribute to its aberrant migration on SDS-PAGE.

Homology to Lip and Laz. Regions at both the N terminus and C terminus of the deduced amino acid sequence of Pan

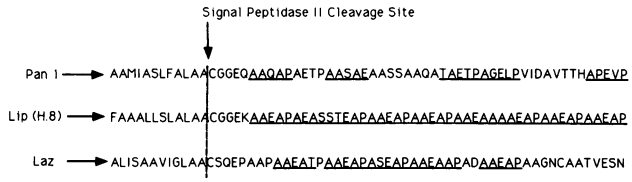

FIG. 2. Predicted amino acid sequence of the N-terminal region of Pan 1 and homology to the gonococcal lipoproteins Lip (H.8) and azurin (Laz). The amino acid sequences of the gonococcal Lip (2) and azurin (10) proteins are shown below the Pan 1 sequence. The dotted line indicates the putative processing site for signal peptidase II. Underlined regions indicate sequences of the motif Ala-Ala-Glu-Ala-Pro (AAEAP).

1 show significant homology to the termini of two other gonococcal lipoproteins, Lip (H.8) and Laz (Fig. 2). Lip is a lipoprotein that consists almost entirely of 14 pentapeptide repeats of the motif AAEAP (1, 23). The function of the 71-amino-acid Lip protein is unknown. Laz has four of these AAEAP pentapeptide repeats at the N terminus before the sequence diverges (6, 17). The rest of the protein has 77% homology to azurin proteins, which are small, blue, copper-binding proteins that function in electron transfer during respiration (2, 6). The N-terminal region of Pan 1 is high in alanine and proline content and contains several imperfect repeats of the AAEAP motif. Unlike the azurin protein, however, the Pan 1 protein has four direct contiguous copies of this pentapeptide repeat in the form AASAP at the C terminus as well (Fig. 1). The significance of these repetitive regions is unknown, but it is interesting that Pan 1 contains several copies of the pentapeptide motif at both the N terminus and C terminus. Interestingly, the epitope for a monoclonal antibody that was used to clone the *lip* and *laz* genes has been localized to a 20-amino-acid stretch, consisting of four repeats of the AAEAP motif (17). However, this monoclonal antibody failed to recognize the Pan 1 protein in immunoblot analysis (data not shown). It is also important to note that the *aniA* gene maps to a single chromosomal locus that is clearly distinct from the *lip* and *laz* genes (1a).

[^{3}H]palmitic acid labeling of *N. gonorrhoeae*. The deduced amino acid sequence suggested that Pan 1 is modified at the N terminus by a lipid moiety. To investigate whether Pan 1 is a lipoprotein, cultures of *N. gonorrhoeae* were grown either aerobically or under oxygen-limited conditions in the presence of [^{3}H]palmitic acid, and whole-cell extracts were prepared and analyzed by SDS-PAGE and fluorography (Fig. 3). When gonococci were grown with limited oxygen (Fig. 3A, lane b), a 54-kDa protein that was not present in cells grown aerobically (Fig. 3A, lane a) was specifically labeled. This was the only labeled protein that was unique to cells grown with limited oxygen; all other labeled proteins were present in cells grown under both conditions and were similar to the pattern described by Chen et al. (3). To confirm that these bands contained polypeptides, labeled extracts were treated with proteinase K (Fig. 3A, lanes c and d). All radiolabeled bands disappeared except for some lower-molecular-weight species that probably represented gonococcal lipopolysaccharide (LPS). It is unlikely that incorporated label represents metabolic products of palmitic acid because little incorporation into protein I, the most abundant gonococcal outer membrane protein, was observed. The possibility that contamination or association of LPS is responsible for the labeling was excluded because the anti-Pan

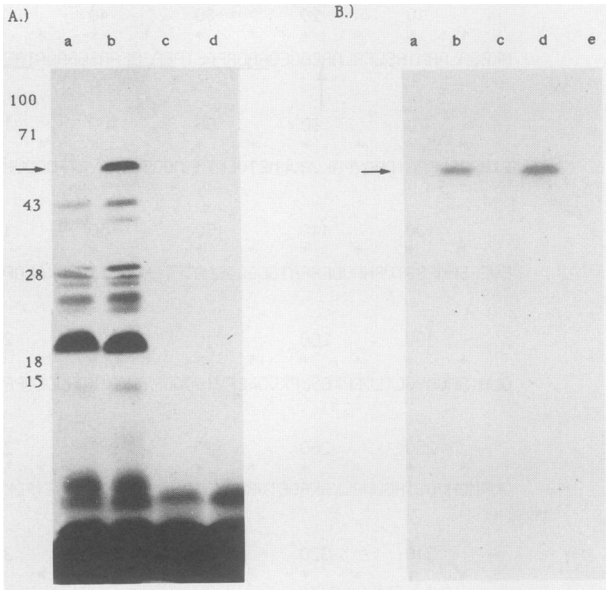

FIG. 3. [^{3}H]palmitic acid labeling of *N. gonorrhoeae* F62 grown aerobically or under oxygen-limited conditions. Approximately equal amounts of whole-cell extracts were prepared and then loaded in duplicate onto a 13% PAGE–SDS gel. After electrophoresis, half the gel was prepared for fluorography (A) by treatment with En^{3}Hance and drying under vacuum, and the other half was transferred to nitrocellulose and immunoblotted with anti-Pan 1 antiserum (B). (A) Lanes: a, ^{3}H-labeled extracts of cells grown aerobically; b, ^{3}H-labeled extracts of cells grown under oxygen-limited conditions; c, ^{3}H-labeled extracts of cells grown aerobically and treated with 25 mg of proteinase K; d, ^{3}H-labeled extracts of cells grown under oxygen-limited conditions (anaerobically) and treated with 25 mg of proteinase K. An anaerobically induced protein that migrates at about 54 kDa (arrow) is specifically labeled by [^{3}H]palmitic acid. (B) Lanes: a, F62 outer membranes from aerobically grown cells; b, F62 outer membranes from anaerobically grown cells; c, ^{3}H-labeled extracts of cells grown aerobically; d, ^{3}H-labeled extracts of cells grown under oxygen-limited conditions; e, ^{3}H-labeled whole-cell extracts of cells grown under oxygen-limited conditions and treated with 25 mg of proteinase K. Molecular size markers are shown on the left (in kilodaltons). A protein that is specifically labeled by [^{3}H]palmitic acid, present only in the anaerobically grown cells and migrating at a position comparable to that of Pan 1, is recognized by the anti-Pan 1 antiserum (arrow).

1 antiserum did not react with LPS moieties and because the protein I band, which is known to be associated with LPS (8), did not appear to incorporate much label. If either of these possibilities were true, then we would have expected that protein I, which comprises approximately 60% of the total protein content of the gonococcal outer membrane (16), would have been labeled much more intensely than Pan 1.

To show that this specifically labeled protein was Pan 1, immunoblot analysis with monospecific, polyclonal anti-Pan 1 antiserum (10) was performed. A protein that was found only in the lanes containing extracts from cells grown under oxygen-limiting conditions (Fig. 3B, lanes b and d) and that comigrated with the protein labeled by [^{3}H]palmitic acid reacted to the anti-Pan 1 antibody. These results indicated that the unique [^{3}H]palmitic acid-labeled band from extracts grown under oxygen-limiting conditions was Pan 1.

The presence of a covalently linked lipid moiety is consis-

tent with our difficulty in sequencing the N terminus of the Pan 1 protein. Failure to obtain any amino acid sequence information whatsoever, even when methods to prevent artifactual blocking were used, indicated that the N terminus is blocked in vivo (11). These experiments were performed in duplicate on two separate gas sequencers, suggesting that the blocking was not a technical problem but rather was due to the nature of the N terminus of Pan 1.

The predicted molecular mass of the mature gonococcal Pan 1 protein is 39.2 kDa, substantially different from the apparent molecular mass of 54 kDa determined by SDS-PAGE. Presumably, this discrepancy is due to the presence of the covalently attached lipid moiety, which influences the electrophoretic mobility of the Pan 1 protein. Other bacterial lipoproteins display aberrant migration on SDS-PAGE as well. For example, Braun's lipoprotein migrates at a molecular weight that is 30% greater than its predicted molecular weight (13). Similarly, the neisserial Lip antigen, which migrates as a broad smear at about 20 to 25 kDa, has a predicted molecular mass of 6.3 kDa (1, 23).

Other than the homology at the N terminus and C terminus to Lip and Laz, Pan 1 failed to reveal any significant similarities to any known proteins, and thus we were unable to assign a putative function to this protein. The fact that Pan 1 is expressed in many of the commensal *Neisseria* species (10) seems to indicate that the Pan 1 protein may not be a virulence determinant. However, bacterial pathogenesis is a multicomponent phenotype that requires the synthesis of many different gene products, some of which are coordinately regulated by environmental factors (19). Some clues about the potential in vivo role of Pan 1 in pathogenesis may be deduced from its lack of expression in *N. meningitidis*. To date, Pan 1 is the only protein that has been investigated that is found in *N. gonorrhoeae* but is not expressed at similar levels in *N. meningitidis* (10). All other surface proteins, including those that have purported roles in pathogenesis, such as pilin, Opa, and protein I, are expressed in both species. Despite this similarity, these two organisms cause quite disparate diseases, each colonizing unique niches within the human host. It is possible that the presence of Pan 1 in *N. gonorrhoeae* allows the organism to survive anaerobically in vivo. In this regard, Pan 1 would not be a specific virulence determinant but rather an accessory protein that is needed for establishing infection. Since *N. meningitidis* does not express much Pan 1, it would have a reduced capacity to survive within the reduced areas of the urogenital tract and hence would not cause urogenital infections. Alternatively, Pan 1 might confer some novel adhesive properties on the gonococcus which allow it to colonize reduced sites within the urogenitary tract, thus acting as a specific adhesin molecule. *N. meningitidis* would not be able to attach to and colonize these tissues because it lacks Pan 1. Mutant strains that are deficient in *aniA* expression are being constructed in our laboratory and may assist in establishing the function of Pan 1.

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