JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 2003, p. 4141–4147 Vol. 41, No. 9
0095-1137/03/$08.00+0 DOI: 10.1128/JCM.41.9.4141–4147.2003

Comparison of Serologic and Genetic *porB*-Based Typing of *Neisseria gonorrhoeae*: Consequences for Future Characterization

Magnus Unemo,[1]* Per Olcén,[1] Jan Albert,[2] and Hans Fredlund[1,3]

National Reference Laboratory for Pathogenic Neisseria[1] and Unit for Infectious Disease Control,[3] Department of Clinical Microbiology, Örebro University Hospital, Örebro, and Department of Clinical Virology, Karolinska Institute, Huddinge University Hospital, Stockholm,[2] Sweden

Received 17 March 2003/Returned for modification 26 May 2003/Accepted 23 June 2003

Due to temporal changes in the epidemiology of gonorrhea, a precise characterization of *Neisseria gonorrhoeae* is essential. In the present study genetic heterogeneity in the *porB* genes of *N. gonorrhoeae* was examined, and serovar determination was compared to *porB* gene sequencing. Among 108 *N. gonorrhoeae* isolates, phylogenetic analysis of the entire *porB* alleles (924 to 993 bp) identified 87 unique sequences. By analyzing only the four to six most heterogeneous *porB* gene regions (174 to 363 bp), 86 out of these 87 genetic variants were identified. Consequently, analysis of shorter highly variable regions of the *porB* gene generates high-level discriminatory ability as well as fast, objective, reproducible, and portable data for epidemiological characterization of *N. gonorrhoeae*. Regarding putative antigenic epitopes of PorB for Genetic Systems monoclonal antibodies (MAbs), some of the previous findings were confirmed, but new findings were also observed. For several of the MAbs, however, the precise amino acid residues of PorB critical for single-MAb reactivity were difficult to identify. In addition, repeated serovar determination of 108 *N. gonorrhoeae* isolates revealed discrepancies for 34 isolates, mostly due to nonreproducible reactivity with single MAbs. Thus, the prospects of a genetic typing system with congruent translation of the serovar determination seem to be limited. In conclusion, analysis of short highly variable regions of the *porB* gene could form the basis for a fast molecular epidemiological tool for the examination of emergence and transmission of *N. gonorrhoeae* strains within the community.

Neisseria gonorrhoeae is the etiological agent of gonorrhea, which remains a major sexually transmitted infection worldwide (8). The mainstay in the development of improved control and preventive measures for infection is based on regional, national, and international surveillance of the epidemiological characteristics as well as the antibiotic susceptibility of the bacteria. The phenotypic and genotypic characteristics of circulating *N. gonorrhoeae* strains fluctuate over time from a local as well as global perspective. Thus, it is crucial to have thorough knowledge about the population of strains that circulate in the community to identify temporal and geographic changes as well as emergence and transmission patterns of individual strains. Characterization of *N. gonorrhoeae* isolates for epidemiological and clinical purposes is therefore essential. The widely used phenotypic characterizations of the bacteria are based on auxotyping, serovar determination, and antibiograms. However, these methods have some limitations (10, 12, 13, 18, 26, 30). Various DNA-based methods for revealing genetic relatedness of *N. gonorrhoeae* isolates have therefore been developed (12, 19, 25, 27, 28, 29, 30, 33, 35).

The *N. gonorrhoeae* outer membrane protein PorB is universally present, is constitutively expressed at the cell surface, and does not undergo high-frequency variation during the course of infection in smaller groups of sexual contacts (36). The antigenic expression of PorB within a strain is stable; however, diversities between strains form the basis for serogroup and serovar determination with monoclonal antibodies (MAbs) (14, 23). Attempts to map the epitopes of PorB recognized by serovar-specific MAbs by using amino acid alignments of the mature proteins as well as synthetic peptides have been published, but many epitopes remain unidentified (1, 2, 3, 4, 16). Thus, for several of the widely used MAbs, the exact antigenic epitopes of PorB are not identified. A two-dimensional structural model of the topology of the porin PorB within the outer membrane has previously been predicted (32). The model predicts eight surface-exposed loops, which exhibit an extensive variation in length and amino acid sequence and which are interspaced with nine more conserved predominantly transmembrane regions (referred to as interspacing regions in the text below). The PorB proteins are classified into two different groups, PorB1a or PorB1b, on the basis of immunological and sequence homology. Any individual strain expresses only one of the groups, either PorB1a or PorB1b (3, 5, 11, 32). However, naturally occurring strains that express PorB1a/PorB1b hybrids have been identified (3, 9). The proteins PorB1a and PorB1b are encoded by the mutually exclusive alleles of the *porB* gene, *porB1a* and *porB1b*, respectively (5, 11).

The aims of the present study were to examine the genetic heterogeneity in the *porB* genes of *N. gonorrhoeae* reference strains and clinical isolates of different serovars, to investigate the highly variable regions as well as entire alleles of the *porB* genes for discrimination of the strains, to compare PorB-based serovar determination and *porB* gene sequencing of the strains,

* Corresponding author. Mailing address: National Reference Laboratory for Pathogenic Neisseria, Department of Clinical Microbiology, Örebro University Hospital, SE-701 85 Örebro, Sweden. Phone: 46 19 602 15 20. Fax: 46 19 127 416. E-mail: magnus.unemo@orebroll.se.

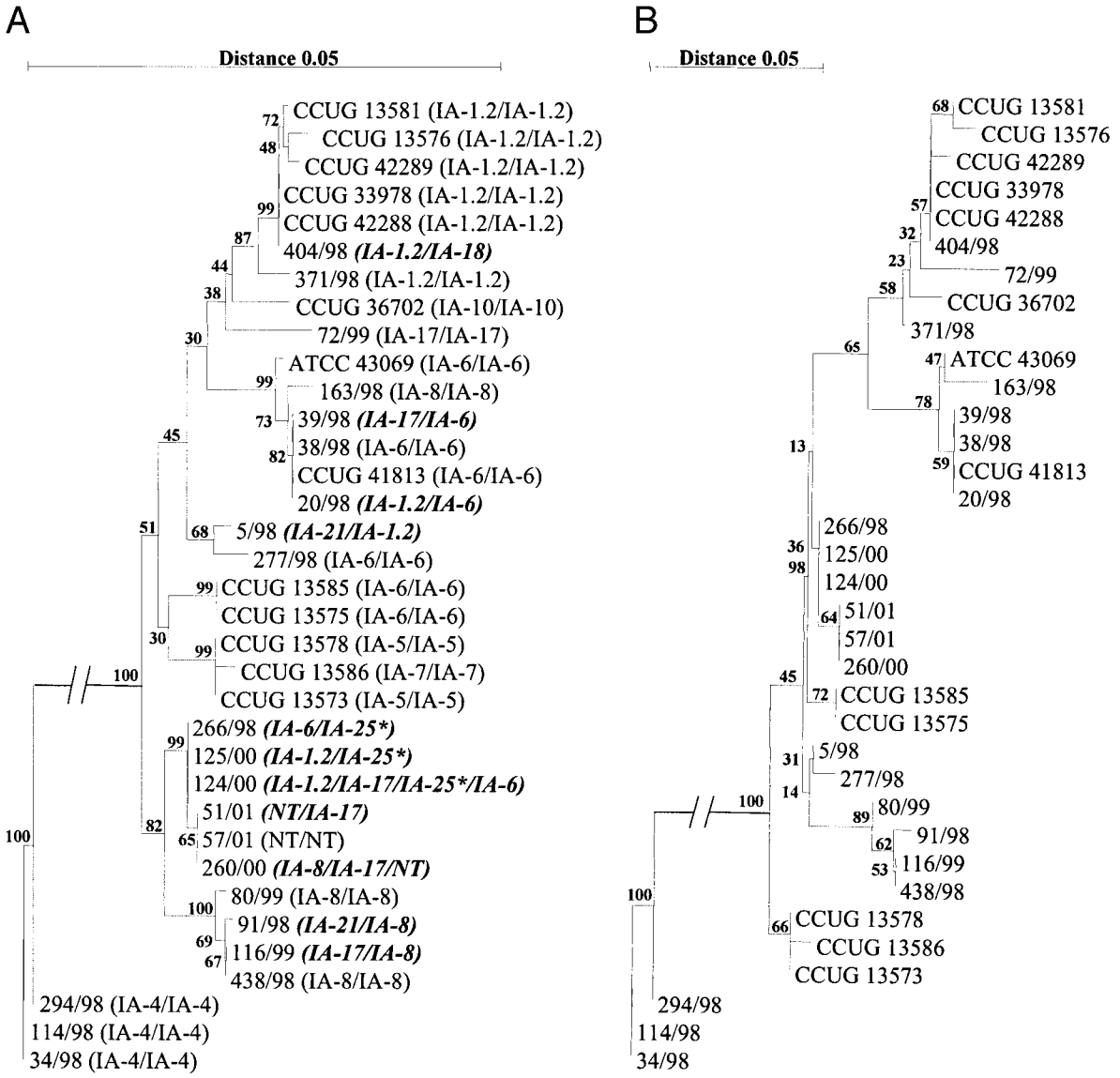

FIG. 1. (A) Neighbor-joining phylogenetic tree of the entire *porB1a* alleles (924 unambiguously aligned nucleotides) coding for the mature PorB1a proteins of *N. gonorrhoeae* isolates ($n = 35$), comprising nine different serovars and two nonserotypeable (NT) serovars according to the initial serovar determination. The serovars of the isolates are included; discrepancies between initial and repeated (one to three times) serovar determination are indicated by boldface italics. *, the isolates were reactive with MAbs 2F12, 5G9, 5D1, and assigned serovar IA-25 in the present study. (B) Neighbor-joining phylogenetic tree of the loop sequences 1, 2, 4, and 8 (174 unambiguously aligned nucleotides) of the *porB1a* alleles in the isolates described above. The entire and partial *porB1a* sequences, respectively, of the isolates 34/98 and 114/98, which represent an outgroup, were used to root the trees. The lengths of the branches leading to the outgroups have been reduced by a factor of three. All bootstrap values (as a percentage of 1,000 resamplings) are shown.

and finally to form the basis for a fast genetic *porB*-based typing system for *N. gonorrhoeae*.

MATERIALS AND METHODS

Bacterial isolates and culture conditions. A total of 108 *N. gonorrhoeae* isolates were examined in the present study (see Fig. 1A and 2A). The clinical isolates ($n = 74$) were cultured from patients in Sweden between 1998 and 2001. The *N. gonorrhoeae* reference strains ($n = 34$) originated from different geographic localities between 1973 and 1997. The selection of the clinical isolates and reference strains was based on the results of the initial serovar determination and on results obtained in a previous study (30). One to three isolates that differed only in the reactivity with a single MAb (of all the 12 used MAbs) were included, as well as all isolates comprising a unique *porB* gene sequence. The isolates comprised 28 different serovars, as well as two nonserotypeable isolates, according to the initial serovar determination performed on *N. gonorrhoeae* isolates at the Swedish Reference Laboratory for Pathogenic Neisseria (see Fig. 1A and 2A). All isolates were cultured as previously described (30) and preserved at −70°C.

Serological characterization. Serovar determination was performed by a coagglutination technique (23) with the Genetic Systems (GS) panel of MAbs (14), which comprises six different MAbs against PorB1a and six different MAbs against PorB1b.

Isolation of genomic DNA. Isolation of bacterial DNA was performed as previously described (30). Briefly, bacterial suspensions (approximately 3×10^8 cells/ml) were prepared in sterile 0.15 M NaCl. DNA was isolated from 500 μl of

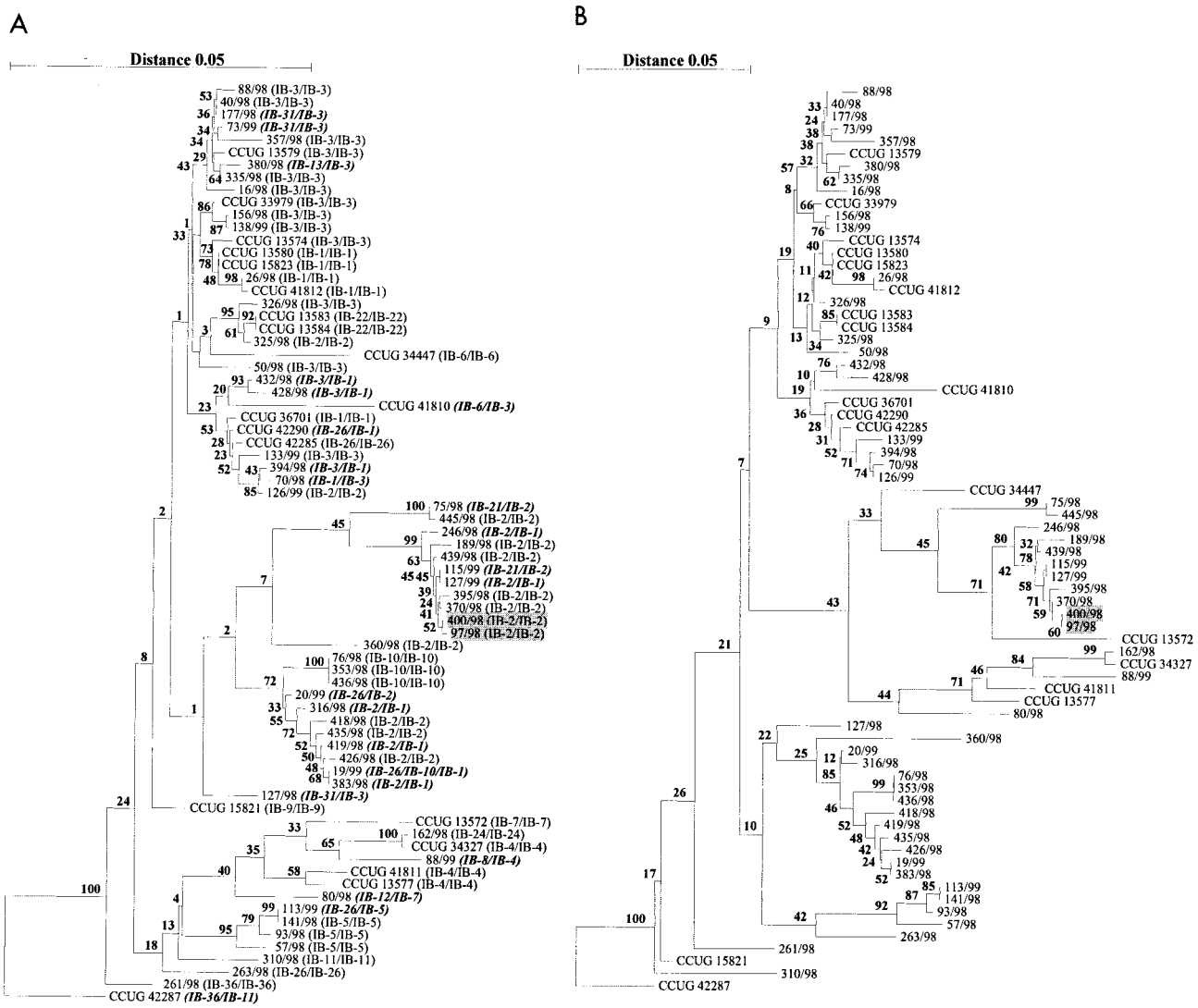

FIG. 2. (A) Neighbor-joining phylogenetic tree of the entire *porB1b* alleles (999 unambiguously aligned nucleotides) coding for the mature PorB1b proteins of *N. gonorrhoeae* isolates ($n = 73$), comprising 19 different serovars according to the initial serovar determination. The serovars of the isolates are included; discrepancies between initial and repeated (one or two times) serovar determination are indicated by boldface italics. (B) Neighbor-joining phylogenetic tree of the loop sequences 1, 3, 5, 6, 7, and 8 (369 unambiguously aligned nucleotides) of the *porB1b* alleles in the isolates described above. The entire and partial *porB1b* sequence, respectively, of the strain CCUG 42287, which represents an outgroup, was used to root the trees. All bootstrap values (as a percentage of 1,000 resamplings) are shown. The isolates that were indistinguishable by using only the loop sequences are shaded.

each suspension using the Dynabeads DNA DIRECT Universal kit (Dynal ASA, Oslo, Norway) according to the manufacturer's instructions. The DNA preparations were stored at 4°C prior to PCR.

***porB* PCR.** The entire *porB* gene was amplified by using the primers PorBU and PorBL, yielding a PCR product of approximately 1.0 to 1.1 kbp, as previously described (30). Briefly, a PCR mixture (50 μl) was prepared by using 1.0 U of *AmpliTaq* Gold DNA polymerase (Applied Biosystems, Foster City, Calif.), 1× PCR Gold buffer (Applied Biosystems), 2.5 mM $MgCl_2$ (Applied Biosystems), 0.1 mM deoxynucleoside triphosphates (Applied Biosystems), 0.5 μM concentrations of each primer, and 1 μl of the DNA template. The cycling parameters of the amplification were as follows: an enzyme activation step at 94°C for 10 min, followed by 30 sequential cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 90 s. At the end of the final cycle, an extension phase at 72°C for 4 min was included before storage at 4°C.

DNA sequencing. The amplified *porB1a* or *porB1b* allele was sequenced using the primers PorBU, PorBL, PorB1bU, PorB1bL, PorB1aU, and PorB1aL as previously documented (30). Briefly, the PCR products were purified with the High Pure PCR product purification kit (Roche Diagnostics, Mannheim, Germany) and cycle sequenced by using the ABI BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems, Warrington, United Kingdom). The sequence extension products were purified with the DyeEx Spin kit (Qiagen, Hilden, Germany), and the nucleotide sequences were determined by using an ABI PRISM 310 genetic analyzer (Applied Biosystems). The sequence of each strand of each compiled sequence was determined.

Sequence alignments and phylogenetic analysis. Multiple-sequence alignments of the *porB* gene segments encoding the mature PorB, according to the model of van der Ley et al. (32), as well as of the deduced amino acid sequences of the mature proteins, were performed with BioEdit (version 5.0.9) software and by manual adjustment. Phylogenetic trees were constructed with TREECON (version 1.3b) software by using the Jin and Nei substitution model, the Kimura evolutionary model, an α-value of 0.5, and the neighbor-joining method (31). Bootstrap analysis (6) with 1,000 resamplings was performed by using TREECON software with the same settings described above. The *porB1a* sequences of the isolates 34/98 and 114/98 (see Fig. 1) and the *porB1b* sequence of the reference strain CCUG 42287 (see Fig. 2) were used as outgroups to root the trees. The alignments were not stripped of gaps before phylogenetic analysis

TABLE 1. The numbers of nonsynonymous and synonymous substitutions, the numbers of nonsynonymous and synonymous sites, and the *p* distances of substitutions per 100 potential sites in the alignments of *porB1a* alleles and *porB1b* alleles

Parameter	Value for region[d]					
	Total[a]		Loop region[b]		Interspacing region[b]	
	Nonsyn.	Syn.	Nonsyn.	Syn.	Nonsyn.	Syn.
porB1a alleles						
No. of substitutions	17.0	1.9	12.8	0.16	4.2	1.7
No. of sites	702.7	221.3	280.3	79.7	422.4	141.6
p distances (*p*N or *p*S)[c]	2.4	0.9	4.6	0.2	1.0	1.2
porB1b alleles						
No. of substitutions	25.4	10.2	20.9	4.0	4.5	6.1
No. of sites	745.6	239.6	325.3	95.9	420.4	143.6
p distances (*p*N or *p*S)[c]	3.4	4.2	6.4	4.2	1.1	4.3

[a] The total numbers of substitutions in the multiple-sequence alignments of the *porB* gene regions (indels excluded) encoding the mature PorB proteins. The single *porB1a* alleles consisted of 924 bp, and the single *porB1b* alleles comprised 981 to 993 bp.

[b] The gene regions encoding the predicted surface-exposed loops or the interspacing sequences in the mature protein according to the model of van der Ley et al. (32).

[c] The number of substitutions (nonsynonymous or synonymous) is normalized using the number of potential sites for nonsynonymous and synonymous substitutions, respectively.

[d] Nonsyn., nonsynonymous; Syn., synonymous.

because most indels occurred in the variable loops. Thus, important phylogeny would have been lost if gap stripping had been used.

The numbers of synonymous and nonsynonymous substitutions, the numbers of each type of site, and the *p* distances (*p*S and *p*N [for synonymous and nonsynonymous, respectively]) were calculated as overall means by the method of Nei and Gojobori (17) using the MEGA (version 2.1) software (15) with pairwise deletions of indels.

This study was performed in the National Reference Laboratory for Pathogenic Neisseria, Department of Clinical Microbiology, Örebro University Hospital, Örebro, Sweden.

RESULTS

Sequencing of *porB1a* genes from PorB1a strains ($n = 35$). In the alignment of the *porB1a* gene sequences encoding the mature PorB1a proteins (924 unambiguously aligned nucleotides), a total of 67 polymorphic nucleotide sites (a ratio of 7.2 per 100 sites) were identified. The regions of the gene encoding the amino acid loops of the porin exhibited 50 polymorphic sites (a ratio of 13.9) compared with 17 (a ratio of 3.0) in the regions between the surface-exposed loops. The *p* distances of nonsynonymous substitutions (substitutions that result in amino acid replacements per 100 nonsynonymous sites) were 4.6 and 1.0 in loops and interspacing regions, respectively. Synonymous (silent) *p* distances were 0.2 and 1.2 in the loops and interspacing regions, respectively (Table 1). Thus, the *p*S/*p*N ratio was 0.043 in the loops and 1.2 in the interspacing regions. The regions of the gene encoding loops 1, 2, 4, and 8 of the proteins were the most heterogeneous ones, whereas regions encoding loops 3, 5, 6, and 7 were more conserved (Table 2). The alignment of the deduced amino acid sequences of the mature PorB1a proteins contained 36 (30 per 100 sites) heterogeneous sites located in the predicted surface-exposed loops and 12 (6.4 per 100 sites) in the interspacing sequences.

The phylogenetic analysis of the entire *porB1a* alleles (a total of 924 bp) identified 22 unique gene sequences in the 35 PorB1a isolates. Identical discrimination between the isolates was obtained when the analysis was based only on the four most variable gene regions (a total of 174 bp) (Fig. 1). However, when only the four loop sequences were used, the phylogenetic association was somewhat weaker, as indicated by lower bootstrap values (Fig. 1).

Sequencing of *porB1b* genes from PorB1b strains ($n = 73$). The individual *porB1b* alleles were 57 to 69 nucleotides larger than the *porB1a* alleles, mostly due to a longer loop 5 in the mature protein (Tables 1 and 2). The alignment of the *porB1b* sequences encoding the mature PorB1b proteins (999 unambiguously aligned nucleotides) exhibited a total of 145 polymorphic nucleotide sites (a ratio of 14.5). The regions of the gene encoding the loops of the porin exhibited 103 polymorphic sites (a ratio of 23.7) in comparison to 42 (a ratio of 7.4) in the interspacing regions. Nonsynonymous substitutions existed in *p* distances of 5.9 and 1.1 in loops and interspacing sequences, respectively. Synonymous *p* distances, however, were similar in loops (4.2) and interspacing regions (4.3) (Table 1). Thus, the *p*S/*p*N ratio was 0.7 in the loops and 3.9 in the interspacing regions. The regions of the *porB1b* gene encoding loops 1, 3, 5, 6, 7, and 8 of the proteins exhibited the most heterogeneity, whereas regions encoding loops 2 and 4 were more homologous (Table 2). The alignment of the deduced amino acid sequences of the mature PorB1b proteins contained 50 (34.5 per 100 sites) heterogeneous sites located in the predicted surface-exposed loops and 15 (8.0 per 100 sites) in the interspacing sequences.

According to the phylogenetic analysis of the entire *porB1b* alleles (a total of 981 to 993 bp), the 73 PorB1b isolates comprised 65 unique gene sequences. Nearly identical phylogenies were obtained when the analysis was based only on the six most heterogeneous regions, encoding six of the loops, of the gene (a total of 351 to 363 bp); 64 out of the 65 genetic variants were identified (Fig. 2). The entire *porB1b* alleles of these two indistinguishable isolates differed by a single A→T transversion at nucleotide site 826, located in the region encoding the amino acid sequence between loops 6 and 7. However, when only the six loop sequences were used, the phylogenetic asso-

TABLE 2. The numbers of polymorphic nucleotide sites, the numbers of sites, and the ratios of polymorphic sites per 100 sites in the gene regions encoding the predicted surface-exposed loops of the mature protein (32) in the alignments of *porB1a* alleles and *porB1b* alleles

Parameter	Value for loop:							
	1	2	3	4	5	6	7	8
porB1a alleles								
No. of polymorphic sites[a]	13	8	7	5	2	4	4	7
No. of sites	69	39	72	30	27	51	36	36
Ratio (per 100 sites)	18.8	20.5	9.7	16.7	7.4	7.8	11.1	19.4
porB1b alleles								
No. of polymorphic sites[a]	15	2	20	2	31	17	7	9
No. of sites	69	39	84	27	81	60	36	39
Ratio (per 100 sites)	21.7	5.1	23.8	7.4	38.3	28.3	19.4	23.1

[a] Polymorphic sites due to substitutions, insertions, or deletions.

TABLE 3. Amino acid residues critical for single-MAb reactivity according to previous studies or identified in the present study

Protein or MAb	Sequence identified in:	
	Previous studies (reference[s])	Present study[c]
PorB1a		
5D1[a]	^{18}VAYHG^{22} and ^{291}GTEKF^{295} (3, 16)	^{18}VA(Y/H)HG^{22} and ^{291}GTEKF^{295}
5G9[a]	?[b] and ^{291}GTEKF^{295} (3)	?[b] and ^{291}GT(E/Q)KF^{295}
6D9	^{211}DAKLTWRND^{219} (3, 16)	^{211}DAKLTWR(N/D)D^{219}
4G5[a]	?[b] and ^{118}IAQPEE^{123} (3)	?[b] and ^{118}IA(Q/R)PEE^{123}
4A12		^{215}TWRD^{218}, ^{255}DA(D/G)H^{258} and ^{288}KGKGA^{292}
2F12		—
PorB1b		
2D4	^{233}K(L/Y)YQNQLVRD^{242} (3, 4, 20)	^{233}KLYQNQLVRD^{242} or ^{233}KLYQNQIVR(D/G)^{242}
3C8	^{197}YSIPS^{201} (3, 4)	^{197}YS(I/M/V/T)PS^{201} or ^{197}YNIPS^{201}
2H1		—
1F5		—
2D6		—
2G2		—

[a] The MAb is suggested to recognize a conformational epitope.
[b] Unknown sequence.
[c] —, no amino acid residues critical for reactivity with the MAb were identified. Underlining denotes amino acids identified in the present study.

ciation was somewhat weaker, as indicated by lower bootstrap values (Fig. 2).

Comparison of PorB-based serovar determination and *porB* gene sequencing. The 35 PorB1a isolates (representing 9 different serovars and 2 nonserotypeable isolates according to the initial serovar determination) and the 73 PorB1b isolates (initially representing 19 different serovars) comprised 22 unique *porB1a* gene sequences and 65 unique *porB1b* sequences, respectively. Overall, a substantial genetic heterogeneity was identified in the *porB* sequences of isolates determined to be different serovars as well as identical serovars. However, a few isolates designated as different serovars in the same assay in most cases differing in reactivity to single MAbs, displayed identical *porB* gene sequences (Fig. 1A and 2A).

In the present study, differences in the multiple-sequence alignments of the deduced PorB amino acid sequences of strains that differed only by the recognition of a single MAb in the serovar determination were also examined. This strategy was used in an attempt to identify amino acid residues critical for single-MAb reactivity. The results of the present study as well as those of previous publications, which in most cases are in agreement, are summarized in Table 3. Discrepancies and new findings were identified, however (Table 3). Consequently, in contrast to the findings of Mee et al. (16), the sequence ^{18}VAHHG^{22}, in combination with ^{291}GTEKF^{295}, was identified solely in isolates reactive with the MAb 5D1. The sequence ^{291}GT(E/Q)KF^{295} was identified in all 5G9-reactive isolates but also in nonreactive isolates that did not show any sequence disparities in any other amino acid loops. The sequence ^{211}DAKLTWRDD^{219} was identified only in 6D9-reactive isolates. The amino acid residues ^{118}IAQPEE^{123}, critical for recognition by the MAb 4G5, probably as part of a conformational epitope (3), were identified also in nonreactive isolates that did not differ in the amino acid sequences of any other loop. In addition, the sequence ^{118}IARPEE^{123} was found only in 4G5-reactive isolates. In the present study, the amino acid sequences ^{215}TWRD^{218}, ^{255}DA(D/G)H^{258}, and ^{288}KGKGA^{292} were merely found in isolates recognized by the MAb 4A12. The amino acid sequence ^{233}KLYQNQIVRD^{242}, in contrast to the findings of Poh et al. (20), and ^{233}KLYQNQIVRG^{242} were also identified solely in 2D4-reactive isolates. According to previous studies, the amino acid residues ^{197}YSIPS^{201} were critical for the reactivity with MAb 3C8 and the two residues ^{198}SI^{199} comprised the minimum epitope (3, 4). In the present study, the amino acid residues ^{197}YSIPS^{201} but also ^{197}YS(M/T/V)PS^{201} or ^{197}YNIPS^{201} were exclusively found in 3C8-reactive isolates (Table 3). No amino acid residues critical for the reactivity with MAb 2F12 (PorB1a) and the MAbs 2H1, 1F5, 2D6, and 2G2 (PorB1b) could be identified, due to the absence of distinct amino acid residue disparities, at least in the extracellular amino acid loops, between reactive and nonreactive strains.

Notably, repeated serovar determination for the 35 PorB1a isolates and the 73 PorB1b isolates determined 11 PorB1a isolates and 23 PorB1b isolates, respectively, to be different serovars than in the initial testing. This was mostly due, however, to nonreproducible reactivity with single MAbs (Fig. 1 and 2). One PorB1a isolate (isolate 57/01) was repeatedly nonserotypeable despite a *porB1a* gene sequence that was identical to that of two other isolates that occasionally expressed serovar-specific epitopes (isolates 51/01 and 260/00) (Fig. 1A).

DISCUSSION

Phylogenetic analysis of the entire *porB* alleles and the four to six most heterogeneous *porB* gene regions, in a total of 108 *N. gonorrhoeae* isolates, exhibited practically identical discrimination between the isolates. The single discrepancy consisted of two PorB1b isolates that were indistinguishable by analysis of the six most variable *porB1b* gene regions. The entire *porB1b* alleles of these two isolates, however, differed only by a single transversion. Other combinations of *porB* gene regions were also analyzed; however, the four (for the *porB1a* allele) to six (for the *porB1b* allele) most heterogeneous regions were needed to receive a practically identical discrimination between the isolates (data not shown). Due to the fact that the *porB1b* allele of the gene is larger than the *porB1a* allele, it is possible to determine the homology group or "genogroup" of

the porin that each strain exhibits by the size of the *porB* gene amplicon. Consequently, the PCR amplification and sequencing of the entire *porB* gene followed by analyzing of exclusively shorter highly variable regions of the gene comprise a powerful method for genetic typing of *N. gonorrhoeae*. This method could be refined by sequencing of only the highly variable *porB* gene regions and thereby could be used as a fast molecular epidemiological tool for *N. gonorrhoeae* strains within the community. Pyrosequencing, a recently described fast real-time DNA sequence analysis technology (22), could be a suitable method for this strategy (Unemo et al., unpublished data). The strategy of sequencing and analysis of exclusively shorter highly variable regions of the *porB* gene would exhibit a high level of discriminatory ability as well as excellent typeability. In addition, the method generates fast, objective, and reproducible sequence data, portable for comparison between different laboratories, for epidemiological characterization of *N. gonorrhoeae* strains. The method also continuously identifies new genetic variants with differences consisting not only of nonsynonymous substitutions but also of synonymous substitutions and can be optimized to amplify the *porB* gene from clinical samples without culturing.

The present study furthermore identified a substantial genetic heterogeneity in the *porB* genes in isolates of the same serovars as well as between different serovars of *N. gonorrhoeae* in accordance with previous findings (4, 7, 12, 30). The highest degree of diversity was identified in the gene regions encoding the surface-exposed loops of the mature PorB, in full agreement with previous studies (1, 3, 4, 7, 12, 27, 32, 34). Overall, the sequencing revealed a higher degree of heterogeneity in the *porB1b* alleles than in the *porB1a* alleles, which is also found for serovars of PorB1b in comparison with PorB1a (14). However, the polymorphic nucleotide sites in the *porB1a* alleles were more evenly scattered over the entire gene in concordance with results in a previous study (16). The *porB1a* and *porB1b* alleles also revealed considerably lower *p*S/*p*N ratios in the surface-exposed loops (*p*S/*p*N ratios of 0.043 and 0.7, respectively), indicating that they both, but especially *porB1a*, are subjected to positive Darwinian selection for amino acid replacement (*p*S/*p*N < 1). This selection is probably driven by the immunological response of the infected host (7, 21, 24). In contrast, synonymous substitutions predominated in the nonloop regions of the *porB1a* and *porB1b* alleles (*p*S/*p*N ratios of 1.2 and 3.9, respectively), suggesting moderate purifying selection against amino acid replacement (*p*S/*p*N > 1) in these regions (7). The differences in *p*S/*p*N ratios between the *porB1a* and *porB1b* sequences suggest that the selective pressures may differ between the two alleles. Consequently, a higher degree of positive selection for amino acid replacement may drive the evolution of *porB1a* sequences, with a higher degree of purifying selection in *porB1b* (21). However, it is also possible that the differences, at least in part, may be due to the fact that the *p*-distance measure does not correct for multiple mutations at single nucleotide sites. The finding that the *p*S and *p*N distances were larger in the *porB1b* allele is in agreement with this possibility. The results of the present study agree with previous suggestions that the evolution of the *porB* gene in the *N. gonorrhoeae* population is driven by the simultaneous action of positive Darwinian selection, purifying selection, mutations, and recombination (7, 21).

The present study confirmed some of the previous findings regarding putative antigenic epitopes of PorB reacting with the GS MAbs (3, 4, 16), but new findings were also observed (Table 3). However, for several of the MAbs used in the serovar determination, the precise amino acid residues of PorB that are critical for single-MAb reactivity were difficult to identify. The difficulties of identifying the antigenic epitopes of PorB for the complete panel of MAbs are probably due to the extensive heterogeneity, existence of not only simple linear epitopes but also conformational epitopes, and limitations of the serovar determination, e.g., problems with reproducibility, as previously described (3, 4, 7, 10, 12, 13, 16, 30). Thus, the prospects of developing a genetic typing system with congruent translation of the serovar determination seem limited.

In the present study, discrepant results were obtained in repeated serovar determination for 34 of 108 *N. gonorrhoeae* isolates. This was mostly due to nonreproducible reactivity with single MAbs, and previous findings, particularly related to problems in reproducibility for the MAbs 2D6, 2G2, and 6D9, were confirmed (10). However, the MAbs 4G5 and 1F5 exhibited even lower reproducibility. Overall, the discrepancies may reflect a suboptimal quality or interbatch variation of the MAbs reagents used, a subjective interpretation of the coagglutination reaction, or perhaps some antigenic epitopes not accessible due to the conformational effects of PorB (4, 10, 13, 16, 20, 30). In addition to these and other methodological problems, mixed infection and mixup of strains cannot be excluded as possible explanations for some of the discrepancies.

It should also be kept in mind that the GS MAbs were produced several years ago and that the evolution of the *porB* gene and consequently the PorB proteins in the population of *N. gonorrhoeae* strains has selected a lot of new antigenic variants of PorB. The prevalence of nonserotypeable strains as well as new serovars, identified in the present study and in previous studies (3, 10, 30; H. Young, A. Moyes, I. B. Tait, A. C. McCartney, and G. Gallacher, Letter, Lancet **335:**604, 1990), will increase over time. However, in some laboratories the serovar determination may still be useful as a primary epidemiological marker due to the fact that the intra-assay variability, in the present study, was substantially lower than the interassay variability. The genetic typing based on sequencing of the entire *porB* gene or of shorter highly variable gene regions could, when required, be used to improve the discriminatory ability within serovars, increase the typeability, or resolve discrepancies. The questions asked in relation to each issue should guide the use of the most appropriate method for epidemiological characterization.

In conclusion, the present study suggests a strategy based on PCR amplification and sequencing of the entire alleles of the *porB* gene for molecular characterization of *N. gonorrhoeae*. The method could be refined by sequencing and analyzing exclusively the shorter highly variable regions of the *porB* gene and thereby could be used as a fast molecular epidemiological tool for the examination of emergence and transmission of *N. gonorrhoeae* strains within the community.

ACKNOWLEDGMENTS

We are grateful for the technical assistance of Angela Steen in serological characterization. We are also grateful to Dan Danielsson for critical reading and discussions of the manuscript.

The present study was supported by grants from the Research Committee of Örebro County, the Örebro University Hospital Research Foundation, and the National Institute for Public Health, Sweden.

REFERENCES

1. **Butt, N. J., M. Virji, F. Vayreda, P. R. Lambden, and J. E. Heckels.** 1990. Gonococcal outer-membrane protein PIB: comparative sequence analysis and localization of epitopes which are recognized by type-specific and cross-reacting monoclonal antibodies. J. Gen. Microbiol. **136:**2165–2172.
2. **Chow, V. T. K., Q. C. Lau, and C. L. Poh.** 1994. Mapping of serovar-specific monoclonal antibody epitopes by DNA and amino acid sequence analysis of *Neisseria gonorrhoeae* outer membrane protein IB strains. Immun. Infect. Dis. **4:**202–206.
3. **Cooke, S. J., K. Jolley, C. A. Ison, H. Young, and J. E. Heckels.** 1998. Naturally occurring isolates of *Neisseria gonorrhoeae*, which display anomalous serovar properties, express PIA/PIB hybrid porins, deletions in PIB or novel PIA molecules. FEMS Microbiol. Lett. **162:**75–82.
4. **Cooke, S. J., H. de la Paz, C. L. Poh, C. A. Ison, and J. E. Heckels.** 1997. Variation within serovars of *Neisseria gonorrhoeae* detected by structural analysis of outer-membrane protein PIB and by pulsed-field gel electrophoresis. Microbiology **143:**1415–1422.
5. **Derrick, J. P., R. Urwin, J. Suker, I. M. Feavers, and M. C. J. Maiden.** 1999. Structural and evolutionary inference from molecular variation in *Neisseria* porins. Infect. Immun. **67:**2406–2413.
6. **Felsenstein, J.** 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution **39:**783–791.
7. **Fudyk, T. C., I. W. Maclean, N. J. Simonsen, E. N. Njagi, J. Kimani, R. C. Brunham, and F. A. Plummer.** 1999. Genetic diversity and mosaicism at the *por* locus of *Neisseria gonorrhoeae.* J. Bacteriol. **181:**5591–5599.
8. **Gerbase, A. C., J. T. Rowley, D. H. Heymann, S. F. Berkley, and P. Piot.** 1998. Global prevalence and incidence estimates of selected curable STDs. Sex. Transm. Infect. **74**(suppl. 1)**:**S12–S16.
9. **Gill, M. J., J. Jayamohan, M. P. A. Lessing, and C. A. Ison.** 1994. Naturally occurring PIA/PIB hybrids of *Neisseria gonorrhoeae.* FEMS Microbiol. Lett. **119:**161–166.
10. **Gill, M. J.** 1991. Serotyping *Neisseria gonorrhoeae*: a report of the Fourth International Workshop. Genitourin. Med. **67:**53–57.
11. **Gotschlich, E. C., M. E. Seiff, M. S. Blake, and M. Koomey.** 1987. Porin protein of *Neisseria gonorrhoeae*: cloning and gene structure. Proc. Natl. Acad. Sci. USA **84:**8135–8139.
12. **Hobbs, M. M., T. M. Alcorn, R. H. Davis, W. Fischer, J. C. Thomas, I. Martin, C. Ison, P. F. Sparling, and M. S. Cohen.** 1999. Molecular typing of *Neisseria gonorrhoeae* causing repeated infections: evolution of porin during passage within a community. J. Infect. Dis. **179:**371–381.
13. **Ison, C. A., L. Whitaker, and A. Renton.** 1992. Concordance of auxotype/serovar classes of *Neisseria gonorrhoeae* between sexual contacts. Epidemiol. Infect. **109:**265–271.
14. **Knapp, J. S., M. R. Tam, R. C. Nowinski, K. K. Holmes, and E. G. Sandström.** 1984. Serological classification of *Neisseria gonorrhoeae* with use of monoclonal antibodies to gonococcal outer membrane protein I. J. Infect. Dis. **150:**44–48.
15. **Kumar, S., K. Tamura, I. B. Jakobsen, and M. Nei.** 2001. MEGA2: molecular evolutionary genetics analysis software. Bioinformatics **17:**1244–1245.
16. **Mee, B. J., H. Thomas, S. J. Cooke, P. R. Lambden, and J. E. Heckels.** 1993. Structural comparison and epitope analysis of outer-membrane protein PIA from strains of *Neisseria gonorrhoeae* with differing serovar specificities. J. Gen. Microbiol. **139:**2613–2620.
17. **Nei, M., and T. Gojobori.** 1986. Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. Mol. Biol. Evol. **3:**418–426.
18. **Ng, L.-K., M. Carballo, and J.-A. R. Dillon.** 1995. Differentiation of *Neisseria gonorrhoeae* isolates requiring proline, citrulline, and uracil by plasmid content, serotyping, and pulsed-field gel electrophoresis. J. Clin. Microbiol. **33:**1039–1041.
19. **O'Rourke, M., C. A. Ison, A. M. Renton, and B. G. Spratt.** 1995. Opa-typing: a high resolution tool for studying the epidemiology of gonorrhoea. Mol. Microbiol. **17:**865–875.
20. **Poh, C. L., Q. C. Lau, and V. T. K. Chow.** 1995. Differentiation of *Neisseria gonorrhoeae* IB-3 and IB-7 serovars by direct sequencing of protein IB gene and pulsed-field gel electrophoresis. J. Med. Microbiol. **43:**201–207.
21. **Posada, D., K. A. Crandall, M. Nguyen, J. C. Demma, and R. P. Viscidi.** 2000. Population genetics of the *porB* gene of *Neisseria gonorrhoeae*: different dynamics in different homology groups. Mol. Biol. Evol. **17:**423–436.
22. **Ronaghi, M., M. Uhlén, and P. Nyrén.** 1998. A sequencing method based on real-time pyrophosphate. Science **281:**363, 365.
23. **Sandström, E., and D. Danielsson.** 1980. Serology of *Neisseria gonorrhoeae.* Classification by co-agglutination. Acta Pathol. Microbiol. Scand. Sect. B **88:**27–38.
24. **Smith, N. H., J. Maynard Smith, and B. G. Spratt.** 1995. Sequence evolution of the *porB* gene of *Neisseria gonorrhoeae* and *Neisseria meningitidis*: evidence of positive Darwinian selection. Mol. Biol. Evol. **12:**363–370.
25. **Spaargaren, J., J. Stoof, H. Fennema, R. Coutinho, and P. Savelkoul.** 2001. Amplified fragment length polymorphism fingerprinting for identification of a core group of *Neisseria gonorrhoeae* transmitters in the population attending a clinic for treatment of sexually transmitted diseases in Amsterdam, The Netherlands. J. Clin. Microbiol. **39:**2335–2337.
26. **Tam, M. R., T. M. Buchanan, E. G. Sandström, K. K. Holmes, J. S. Knapp, A. W. Siadak, and R. C. Nowinski.** 1982. Serological classification *of Neisseria gonorrhoeae* with monoclonal antibodies. Infect. Immun. **36:**1042–1053.
27. **Thompson, D. K., C. D. Deal, C. A. Ison, J. M. Zenilman, and M. C. Bash.** 2000. A typing system for *Neisseria gonorrhoeae* based on biotinylated oligonucleotide probes to PIB gene variable regions. J. Infect. Dis. **181:**1652–1660.
28. **Trees, D. L., A. J. Schultz, and J. S. Knapp.** 2000. Use of the neisserial lipoprotein (Lip) for subtyping *Neisseria gonorrhoeae.* J. Clin. Microbiol. **38:**2914–2916.
29. **Unemo, M., T. Berglund, P. Olcén, and H. Fredlund.** 2002. Pulsed-field gel electrophoresis as an epidemiologic tool for *Neisseria gonorrhoeae*; identification of clusters within serovars. Sex. Transm. Dis. **29:**25–31.
30. **Unemo, M., P. Olcén, T. Berglund, J. Albert, and H. Fredlund.** 2002. Molecular epidemiology of *Neisseria gonorrhoeae*: sequence analysis of the *porB* gene confirms presence of two circulating strains. J. Clin. Microbiol. **40:**3741–3749.
31. **van de Peer, Y., and R. De Wachter.** 1994. TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. Comput. Appl. Biosci. **10:**569–570.
32. **van der Ley, P., J. E. Heckels, M. Virji, P. Hoogerhout, and J. T. Poolman.** 1991. Topology of outer membrane porins in pathogenic *Neisseria* spp. Infect. Immun. **59:**2963–2971.
33. **van Looveren, M., C. A. Ison, M. Ieven, P. Vandamme, I. M. Martin, K. Vermeulen, A. Renton, and H. Goossens.** 1999. Evaluation of the discriminatory power of typing methods for *Neisseria gonorrhoeae.* J. Clin. Microbiol. **37:**2183–2188.
34. **Viscidi, R. P., J. C. Demma, J. Gu, and J. Zenilman.** 2000. Comparison of sequencing of the *por* gene and typing of the *opa* gene for discrimination of *Neisseria gonorrhoeae* strains from sexual contacts. J. Clin. Microbiol. **38:**4430–4438.
35. **Viscidi, R. P., and J. C. Demma.** 2003. Genetic diversity of *Neisseria gonorrhoeae* housekeeping genes. J. Clin. Microbiol. **41:**197–204.
36. **Zak, K., J.-L. Diaz, D. Jackson, and J. E. Heckels.** 1984. Antigenic variation during infection with *Neisseria gonorrhoeae*: detection of antibodies to surface proteins in sera of patients with gonorrhea. J. Infect. Dis. **149:**166–174.