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Typing by Serovar, Antibiogram, Plasmid Content, Riboprobing, and Isoenzyme Typing To Determine Whether *Neisseria gonorrhoeae* Isolates Requiring Proline, Citrulline, and Uracil for Growth Are Clonal

LAI-KING NG* AND JO-ANNE R. DILLON

National Laboratory for Sexually Transmitted Diseases, Laboratory Centre for Disease Control, Health and Welfare, Ottawa, Ontario, Canada K1A OL2

Received 20 July 1992/Accepted 12 March 1993

Neisseria gonorrhoeae **isolates requiring proline, citrulline, and uracil for growth (PCU$^-$) have homogeneous phenotypes; most are plasmid-free, belong to few serovars, and are significantly associated with intermediate levels of susceptibility to penicillin, tetracycline, erythromycin, and cefoxitin. Because of their lack of variation by these criteria, molecular typing methods, ribotyping (restriction fragment length polymorphism [RFLP] of rRNA genes), and multilocus enzyme electrophoresis were explored as tools for further distinguishing PCU$^-$ isolates. By ribotyping, selected PCU$^-$ isolates could be separated into four groups on the basis of the hybridization patterns (RFLPs) of *Sma*I- and *Ava*II-digested DNA with probes containing rRNA sequences. Most of the isolates (18 of 23 isolates) belonged to a single RFLP (group I). One isolate each was in groups II and IV, and three isolates were in group III. All isolates except one, isolate NS791, had similar multilocus enzyme electrophoresis patterns. Strain NS791 was unusual in that it contained a variant cryptic plasmid with an insert in the 0.46-kb *Msp*I-*Hin*fI fragment of the 4.2-kb plasmid, it was the only isolate belonging to RFLP group IV, and it differed in its multilocus enzyme electrophoresis pattern, having different mobilities for glyceraldehyde phosphate dehydrogenase, phosphoglucose isomerase, 6-phosphogluconate dehydrogenase, and glutamate dehydrogenase. Serovars of PCU$^-$ isolates appeared to be more indicative of strain divergence than RFLP or isoenzyme typing. Multilocus enzyme electrophoresis indicated that PCU$^-$ isolates are clonal.**

A combination of techniques has been used for the epidemiological differentiation of *Neisseria gonorrhoeae* isolates. These include auxotyping, serological typing with monoclonal antibodies to protein I, antimicrobial susceptibility determinations, and plasmid analysis of isolates (8, 13, 16, 17). Correlation of these variables with geographic areas showed that isolates requiring proline, citrulline, and uracil (PCU$^-$) are mainly found in Canada and Jamaica, but are increasingly reported from areas of the United States and Europe (6, 7, 18). The PCU$^-$ auxotrophs comprised less than 1% of all Canadian gonococcal isolates in 1973, 23% in 1978 (9), and 14% in 1989; they are the most prevalent auxotype in some areas of Canada (7). Therefore, methods capable of differentiating isolates presumed to be epidemiologically unrelated would be useful.

Previous studies have shown that *N. gonorrhoeae* PCU$^-$ auxotrophs comprised only a few serovars, the predominant serovar being IB-2 (5, 7). All PCU$^-$ auxotrophs have been plasmid-free, an unusual phenotype compared with those of other auxotypes, which often may carry one or more plasmids belonging to several categories: the 24.5-MDa conjugative plasmid, *tetM*-containing conjugative plasmids, β-lactamase plasmids of various sizes, and a 2.6-MDa cryptic plasmid (8, 10). PCU$^-$ isolates have a narrow range of intermediate susceptibilities to antibiotics (9, 28) and were reported to be associated with asymptomatic urethral infections in men (2).

On the basis of the small variation in the phenotypic characteristics of PCU$^-$ isolates, they are believed to be a largely homogeneous population that is not easily differentiated by using criteria such as plasmid content and serovar or antibiogram analysis (5). The occurrence of variants among PCU$^-$ isolates has not been thoroughly investigated, and the differentiation of PCU$^-$ isolates on the basis of molecular or genetic variations of these isolates has not been reported. Although multilocus enzyme electrophoresis (MEE) has been used to differentiate *Neisseria* species (14, 15, 26) as well as penicillinase-producing *N. gonorrhoeae* (PPNG), six auxotypes of non-PPNG strains in Spain (3, 4), and 65 isolates of different serovar in Singapore (22), there are no published data on PCU$^-$ strains differentiated by this method. Genetic analysis on the basis of restriction fragment length polymorphism (RFLP) analysis of rRNA genes described by Grimont and Grimont (12) also has not been used for the differentiation of PCU$^-$ *N. gonorrhoeae* isolates (25). In the study described here, the techniques of MEE and RFLP of rRNA genes (riboprobing) were used to differentiate PCU$^-$ isolates and were compared with other differentiation methods such as plasmid analysis and serovar and antibiogram determinations.

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MATERIALS AND METHODS

Strains and cultural conditions. The culture collection (1973 to 1989) of the National Laboratory for Sexually Transmitted Diseases, Laboratory Centre for Disease Control (LCDC), Ottawa, Ontario, Canada, was scanned, and 854 submitted gonococcal isolates were found to have been typed as PCU$^-$. All of these 854 PCU$^-$ isolates were analyzed for their plasmid contents. For antimicrobial sus-

* Corresponding author.

ceptibility testing, 480 *N. gonorrhoeae* PCU$^-$ isolates collected during 1988 to 1989 were tested to compare the results for these isolates with those from previous studies (9). The serovars of these 480 isolates were determined (7), with most classified as serovar IB-2 (88%); this was followed by IB-1 (8%) and a few (4%) IB-3, IB-6, IB-07, IB16, IB-25, and IB-26. Nineteen isolates (NS8, NS13, NS54, NS97, NS211, NS213, NS263, NS304, NS348, NS361, NS384, NS571, NS576, NS697, NS724, NS739, NS791, NS873, 2152) of the 480 isolates as well as 4 other isolates collected from 1973 to 1981 (C3, WC41, WC42, and WC59) were selected for ribotyping. This sample of 23 isolates reflected serovar prevalence, comprising 15 isolates of serovar IB-2, 4 isolates of serovar IB-1, and 1 isolate each of serovars IB-3, IB-07, IB-16, and IB-25. These were apparently epidemiologically unrelated isolates from a broad geographic spectrum (i.e., all Canadian provinces and territories except Manitoba) collected in different years and from different isolation sites (i.e., urethra, five isolates; endocervix, six isolates; vagina, one isolate; pharynx, one isolate) or unknown sites (six isolates). Except for isolates WC41, WC59, and C3, the same isolates were used for MEE analysis.

Control strains for auxotyping, serovar determination, and antimicrobial susceptibility determination have been described previously (13, 17, 19). *N. gonorrhoeae* C1 was used as the source of the reference 2.6-MDa cryptic plasmid, pLCDC1 (23), and *N. gonorrhoeae* isolate B266 was used as the source of the 2.8-MDa variant cryptic plasmid. Two *N. meningitidis* isolates (FA1 and FA2), whose enzyme profiles were well characterized by MEE (1), were included in each gel as positive controls for enzyme detection.

All *N. gonorrhoeae* isolates were grown on GC medium base (GCMB; Difco, Detroit, Mich.) supplemented with 1% modified defined supplement (5) in 5% CO_2 at 37°C for 24 h. Stock cultures were stored at −70°C in GC broth (Difco) with 20% glycerol.

Auxotyping. The auxotypes of the *N. gonorrhoeae* isolates were determined (at least twice for confirmation) by the modification by Dillon et al. (6) of the method of Hendry and Stewart (13).

Plasmid analysis. All the PCU$^-$ isolates (n = 854) were screened for their plasmid contents as described previously (8).

Restriction endonuclease analysis of pNS791. Plasmid DNA from strain NS791 was prepared by an alkaline sodium dodecyl sulfate (SDS) method (24). Plasmid DNAs from strains NS791 and B266 were digested with *Hin*fI and *Msp*I for comparison with the DNAs of plasmid pLCDC1 (4.2 kb) and a 4.4-kb variant cryptic plasmid (p266 from *N. gonorrhoeae* B266) which had the same restriction endonuclease pattern (unpublished data) as the 4.4-kb plasmid previously described by Roy et al. (23). The fragments were resolved by 2% agarose gel electrophoresis in TA buffer (24) at 150 mA for 2 to 3 h with bacteriophage λ *Hin*dIII fragments or a 1-kb ladder (size ranges, 0.75 to 12.2 kb; Bethesda Research Laboratories/GIBCO Canada, Burlington, Ontario, Canada) as molecular size markers.

Antimicrobial susceptibility. The MICs of penicillin, tetracycline, erythromycin, and cefoxitin for the isolates were determined by the agar dilution method (18). Interpretive criteria (susceptible, penicillin, erythromycin, and cefoxitin MICs, ≤0.032 mg liter^{-1}; tetracycline MIC, ≤0.25 mg liter^{-1}; moderately susceptible, penicillin, erythromycin, and cefoxitin MICs, 0.063 to 1.0 mg liter^{-1}; tetracycline, 0.5 to 1.0 mg liter^{-1}; resistant, MICs, ≥2.0 mg liter^{-1} for all antimicrobial agents) were those described by the National Committee for Clinical Laboratory Standards (19).

Serotyping. Serovars were determined (at least twice for confirmation) as described by Knapp et al. (17). Monoclonal antibodies were provided by Syva, Palo Alto, Calif.

Riboprobing. To isolate genomic DNAs, confluent (18 to 24 h) growth of gonococcal cells was harvested from GCMB plates and suspended in 20 ml of TE buffer (10 mM Tris [pH 8.0], 1 mM sodium EDTA) with 25% sucrose. Lysozyme was added to a final concentration of 1 mg ml^{-1}. After the mixture was held on ice for 30 min, SDS was added at a final concentration of 1%. Sodium thiosulfite (final concentration, 1 M) was added; this was followed by chloroform-isoamyl alcohol (24:1 [vol/vol]) extraction. The chloroform-isoamyl alcohol extraction was repeated until no visible debris was observed at the interface. Then, 2 volumes of 95% ethanol were added and the DNA was precipitated at −20°C for 16 h. Genomic DNA was further purified on cesium chloride-ethidium bromide gradients (24).

Chromosomal DNA was digested with *Ava*II, *Hin*dIII, *Pst*I, and *Sma*I, and the restricted fragments were separated by electrophoresis on 0.8% agarose gels in Tris-borate (TB) buffer (24) at 50 V for 16 h, a condition which allowed the separation of fragments greater than 20 kb. Chromosomal DNA fragments were transferred to nylon membranes (Amersham, Oakville, Ontario, Canada) from agarose gels by the method of Southern (27). The blot was hybridized with labeled probe at 68°C for 18 to 24 h. The probe was either a mixture of γ-^{32}P-end-labeled 16S and 23S rRNAs from *Escherichia coli* (Boehringer-Mannheim, Laval, Quebec, Canada) or the 7.5-kb *Bam*HI-*Pst*I fragment of pKK3535 containing the 5S, 16S, and 23S rRNA genes of the *rrnB* operon of *E. coli* (1a), which was labeled nonradiometrically by random priming (24) with digoxigenin-dUTP (BMC) according to the manufacturer's instructions. The sizes of the hybridized fragments were determined by comparison with the mobilities of *Hin*dIII fragments of lambda DNA. Fragments larger than 23 kb and smaller than 0.56 kb were estimated by extrapolation from a standard curve. The sizes from at least six blots were averaged, and RFLPs and the results were recorded as described previously (20).

The hybridization solution for ^{32}P-labeled probes contained 6× SSC (1× SSC contained 0.15 M sodium chloride and 15 mM sodium citrate), 0.5% SDS, and 10× Denhardt's solution (1× Denhardt's solution contained 0.02% [wt/vol] Ficoll, 0.02% [wt/vol] polyvinylpyrrolidone, and 0.02% [wt/vol] bovine serum albumin). The hybridization solution for nonradiometrically labeled probe was prepared by the protocol suggested by the manufacturer (BMC).

MEE. After 24 h of incubation, *N. gonorrhoeae* cells were harvested from 10 GCMB plates and suspended in 1 ml of buffer (10 mM Tris, 1 mM EDTA, 0.5 mM NADP [pH 6.8]). The cells were lysed by vigorous vortexing with glass beads (diameter, 5 μm) for 3 min. The lysate was cleared by centrifugation at 30,000 × g for 20 min, and the supernatant was filtered through a 0.2-μm-pore-size filter membrane (Millex GS; Gelman Sciences) and stored at −70°C until used for electrophoresis in a horizontal starch gel. The choices of enzymes and buffers used were based on those reported previously (14, 15, 26) and are listed in Table 1. Because of the hazardous nature of lithium hydroxide, additional buffers (Table 1) were also used to analyze FUM, GD2, and PGM (see Table 1 for abbreviation definitions). The specific enzymes on starch gels were detected by the staining techniques described by Selander et al. (26). Additional buffers (on the basis of results with *N. meningitidis*

TABLE 1. Buffer systems used for MEE

Enzyme	Abbreviation	Buffer system used (buffer abbreviation)[a]
Aconitase	ACO	Tris-citrate (pH 8.0) (A)
Alcohol dehydrogenase	ADH	Borate (pH 8.2) (C)
Alkaline phosphatase	ALP	Tris-citrate (pH 8.0) (A)
Fumerase[b]	FUM	Lithium hybroxide (pH 8.1) (D)
Glucose 6-phosphate dehydrogenase	G6P	Tris-maleate (pH 7.4) (E)
Glutamate dehydrogenase, NADP[c]	GD2	Lithium hydroxide (pH 8.1) (D)
Glyceraldehyde phosphate dehydrogenase	GPI	Tris-citrate (pH 6.3) (B)
Isocitrate dehydrogenase	IDH	Tris-citrate (pH 8.0) (A)
Leucine aminopeptidase	LAP	Borate (pH 8.2) (H)
Malate dehydrogenase	MDH	Potassium phosphate (pH 6.7) (G)
Malic enzyme	ME	Potassium phosphate (pH 6.7) (G)
Peptidases	PEP	Borate (pH 8.2) (H)
6-Phosphogluconate dehydrogenase	6PG	Tris-maleate (pH 7.4) (E)
Phosphoglucomutase[d]	PGM	Lithium hydroxide (pH 8.1) (D)
Phosphoglucose isomerase	PGI	Potassium phosphate (pH 6.7) (G)

[a] The buffer systems have been described previously (24).
[b] Buffers A, B, and E were also evaluated.
[c] Buffers A, C, E, and H were also evaluated.
[d] Buffers B and E were also evaluated.

[1]) were also tested for some enzymes to determine whether the number of buffers could be reduced, consequently reducing the number of runs for future studies. In addition to visual comparisons, differences in mobilities of migration, i.e., distances of migration from the origin (26), were expressed as the ratio of the mobilities (R_f) of the isoenzyme from a specific isolate to WC42. Isoenzymes with the same mobility have a R_f value of 1.

Genetic diversity (h) on the basis of the distinctive enzyme profiles was calculated as follows: $h = 1 - \Sigma x_i^2(n/n - 1)$, where x is the frequency of the ith allele, n is the number of isolates, and $n/n - 1$ is a correction for bias in small samples (26). Mean genetic diversity (H) at a genetic locus is the arithmetic mean of h values over all loci.

Genetic distance (D) between each pair of isolates is defined as the proportion of loci at which dissimilar alleles occur (3).

RESULTS

Antimicrobial susceptibilities and plasmid content analysis. The 480 PCU⁻ isolates were associated with moderate susceptibilities to penicillin (100%), tetracycline (83.5%), erythromycin (99.6%), and cefoxitin (99.4%) (Tables 2 and 3).

From 1973 to 1989, 854 PCU⁻ isolates analyzed in our laboratory were plasmid-free except for a single isolate, NS791 (serovar IB-3; sent to our laboratory from Regina, Saskatchewan, Canada, in 1988), which contained a plasmid that was 4.3 kb in size.

Restriction endonuclease analysis of pNS791. Because strain NS791 was the only PCU⁻ isolate found to contain a plasmid, further analysis was carried out to characterize its plasmid (pNS791). Hybridization of the *Msp*I-*Hin*fI fragments of plasmid pNS791 with pLCDC1 as the DNA probe confirmed that all the fragments contained sequences of pLCDC1 (data not shown). Restriction endonuclease analysis of the plasmid from NS791 (pNS791) showed that it was a variant of pLCDC1, a 4.2-kb plasmid with a 0.14-kb insert in the 0.46-kb *Hin*fI-*Msp*I fragment of the 4.2-kb cryptic plasmid (data not shown).

Riboprobing. Chromosomal DNAs from 23 selected isolates were digested with *Hin*dIII, *Pst*I, *Ava*II, and *Sma*I. Hybridization of *Hin*dIII fragments with riboprobes was negative, indicating that the ribosomal sequences were contained in small fragments which ran off the gel. With *Pst*I, a single RFLP pattern with four bands (29.3, 17.5, 8.5, and 1.65 kb) was observed for all isolates (data not shown). Different RFLP patterns were observed when chromosomal DNAs were digested with either *Ava*II or *Sma*I and hybridized with labeled rRNA probes. The average sizes of the hybridized fragments for *Ava*II and *Sma*I digestions are shown in Fig. 1a.

Irrespective of the rRNA probe used (i.e., ^{32}P-end-labeled 16S and 23S rRNAs from *E. coli* or the digoxigenin-labeled 7.5-kb *Bam*HI-*Pst*I fragment of pKK3535), the RFLPs produced by hybridization with genomic DNA from *N. gonorrhoeae* were identical (data not shown). Since the 7.5-kb *Bam*HI-*Pst*I fragment contains an additional 5S rRNA sequence which is absent from the ^{32}P-labeled probe, this suggested that there were no internal *Sma*I, *Pst*I, or *Ava*II

TABLE 2. Interpreted antimicrobial susceptibilities of 480 PCU⁻ isolates

Interpretive criteria[a]	No. of isolates and their susceptibilities to:			
	Penicillin	Tetracycline	Erythromycin	Cefoxitin
Susceptible	0	78	0	0
Moderately susceptible	480	401	478	477
Resistant	0	1	2	3

[a] See Materials and Methods and reference 19 for interpretive criteria.

TABLE 3. MICs for 480 PCU⁻ isolates

Antimicrobial agent	MIC (μg/ml)	
	Range	90%[a]
Penicillin	0.063–0.5	0.5
Tetracycline	0.125–2.0	1.0
Erythromycin	0.063–2.0	1.0
Cefoxitin	0.25–2.0	1.0

[a] 90%, MICs for 90% of isolates tested.

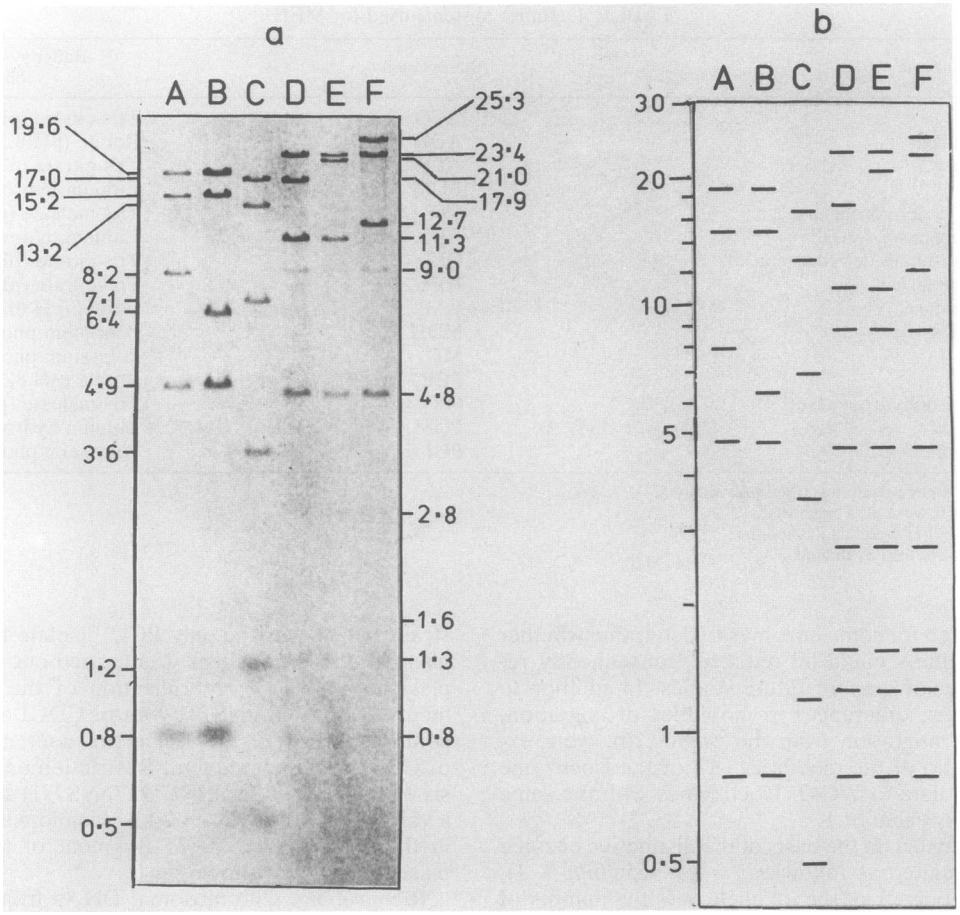

FIG. 1. (a) Hybridization patterns with digoxigenin-labeled rRNA probe of *Ava*II-digested (lanes A to C) and *Sma*I-digested (lanes D to F) chromosomal DNAs with rRNA. The molecular sizes (in kilobases) of the *Ava*II and *Sma*I fragments are indicated on the left and right margins, respectively. The 23.4- and 21.0-kb *Sma*I fragments of isolate 2152 may appear as a single band when a high quantity of DNA was loaded onto the gel or when a radioactive probe was used. The small differences in the mobilities of the larger fragments were observed in all repeated runs. (b) Schematic representation of the RFLP patterns shown in panel a to illustrate the presence of bands that were weak and that did not reproduce well in the photograph. The left margin shows the $\log_{10}$ molecular size (in kilobases).

restriction endonuclease sites around the 5S rRNA gene within the *rrn* operons of *N. gonorrhoeae* PCU$^-$ isolates.

Three RFLP patterns (Table 4) were noticed with *Ava*II-digested chromosomal DNA (Fig. 1, lanes A to C). Twenty-one of the 23 PCU$^-$ isolates exhibited pattern A (Fig. 1, lanes A) with five hybridized bands (19.5, 15.2, 8.2, 4.9, and 0.8 kb); isolate 2152 comprised pattern B, which differed from pattern A by the absence of the 8.2-kb band and the presence of a 6.4-kb band (Fig. 1, lanes B). Pattern C from isolate NS791 differed significantly from the other patterns, having bands 17.0, 13.2, 7.1, 3.6, 1.2, and 0.5 kb in size (Fig. 1, lanes C).

Three RFLPs patterns (Table 4) were also observed when *Sma*I-digested chromosomal DNAs were hybridized with

TABLE 4. Grouping of 23 PCU$^-$ isolates on the basis of RFLPs of rRNA genes from *Ava*II and *Sma*I digests

Group	RFLP pattern[a]	Serovar	PCU$^-$ isolates
I	*Ava*II, A; *Sma*I, 1	IB-2	WC41, WC42, WC59, C3, NS13, NS54, NS97, NS304, NS384, NS576, NS697, NS739, NS873
		IB-1	NS8, NS213, NS724, NS263
		IB-25	NS361
II	*Ava*II, B; *Sma*I, same as group I	IB-2	2152
III	*Ava*II, same as group I; *Sma*I, 2	IB-2	NS571
		IB-07	NS348
		IB16	NS211
IV	*Ava*II, C; *Sma*I, 3	IB-3	NS791[b]

[a] See Fig. 1 for molecular sizes (in kilobases) of the bands in RFLP patterns.
[b] In MEE analysis, NS791 was the only isolate that differed in 4 of the 15 enzymes (GPI, PGI, GD2, 6PG [see Table 1]) compared with the other isolates.

rRNA probes (Fig. 1, lanes D to F). Nineteen of the 23 PCU⁻ isolates demonstrated pattern 1 (Fig. 1, lane D), with nine hybridized *Sma*I fragments (23.4, 17.9, 11.3, 9.0, 4.8, 2.8, 1.6, 1.3 and 0.8); 3 isolates (NS211, NS348, and NS571) had pattern 2 (Fig. 1, lanes E), which differed from pattern 1 in that the 17.9-kb *Sma*I fragment was absent and a 21.0-kb fragment was present. Isolate NS791 demonstrated pattern 3 and lacked the 17.9- and 11.6-kb *Sma*I fragments and had 25.3- and 12.6-kb fragments (Fig. 1, lanes F). Some of the smaller fragments (2.8- and 1.6-kb *Sma*I fragments) did not reproduce well in the black and white photograph; therefore, the RFLP patterns were illustrated schematically in Fig. 1b.

On the basis of the combination of the RFLPs generated by both endonucleases *Sma*I and *Ava*II digestion, the 23 isolates could be divided into four groups (Table 4). Group I comprised 18 of 23 isolates, with 13 of the 18 isolates belonging to serovar IB-2. The other five isolates in group I belonged to serovars IB-1, IB-25, and IB-07 (Table 4). Group II comprised isolate 2152 (serovar IB-2). Group III comprised isolates NS211 (IB-16), NS348 (IB-07), and NS571 (IB-2), and group IV comprised isolate NS791 (serovar 1B-3).

MEE. The 20 isolates tested (except isolate NS791) had similar MEE profiles. Isolate NS791 differed in 4 of the 15 enzymes tested (GPI, PGI, GD2, and 6PG), with average R_f values of 0.83, 0.79, 0.86, and 0.96, respectively (see Table 1 for definitions of abbreviations).

Because lithium hydroxide is a hazardous substance, replacement buffer systems were evaluated (Table 1) for the analysis of FUM, GD2, and PGM. All buffers produced similar results.

The genetic diversity of the loci GPI, PGI, GD2, and 6PG was the same (0.047); the other 11 enzymes had zero genetic diversity. The mean genetic diversity (H) was 0.012. The genetic distance of all PCU⁻ isolates, except isolate NS791, was the same. Isolate NS791 was at a genetic distance of 0.27 from the other 19 isolates.

An anomaly with MEE analysis pertained to isolate NS361, which did not show detectable PGM enzyme activity. Further experiments were not carried out to explain this result and to fully characterize the allele as a null result (3, 26).

DISCUSSION

In a recent Canadian study, 14.8% of *N. gonorrhoeae* isolates were classified as auxotype PCU⁻ (7). These isolates comprised eight serovars, with most (88%) belonging to serovar IB-2. However, only 50% of isolates of serovar IB-2 were PCU⁻. The other 50% of the serovar IB-2 isolates belonged to 14 other auxotypes. By comparison, in the same study, isolates of the nonrequiring (NR) auxotype were grouped into 30 serovars, with the most prevalent serovars being IB-3 (49%) and IB-1 (12%), with only 8% of NR isolates classified as IB-2 (7). Gonococcal isolates with the PCU⁻ auxotype of non-Canadian origin also showed little variation in serovar, with IB-2 being the predominant serovar (16, 18).

Plasmid analysis is a useful molecular typing method for strain differentiation of other gonococcal auxotypes; NR isolates can be classified into at least seven categories on the basis of their plasmid contents (7). This method was not useful for differentiating PCU⁻ isolates because they are plasmid-free (8). This report documents the only PCU⁻ isolate, NS791, ever reported to contain a plasmid. This variant cryptic plasmid, 4.3 kb in size, had an insert in the 0.46-kb *Msp*I-*Hin*fI fragment of the 4.2-kb cryptic plasmid smaller than that in a previously reported variant plasmid (23). This strain also differed from the other PCU⁻ isolates in its RFLP, MEE, and serovar patterns.

Unlike prototrophic (NR) gonococci, for which MICs cover a wide range and which may also demonstrate plasmid-mediated resistance to penicillin and/or tetracycline (7), PCU⁻ isolates have similar antibiograms and MICs for these isolates cover a narrow range. Naturally occurring plasmid-mediated resistance has not been detected in strains of this auxotype. Hendry and Stewart (13) reported that PCU⁻ isolates are similar in their susceptibilities to penicillin (MIC peak, 0.16 mg/liter); therefore, susceptibilities are not useful for differenting PCU⁻ isolates. Intermediate susceptibilities to penicillin, cefoxitin, tetracycline, and erythromycin were also noted in other studies (5, 9, 28). Our data with recently isolated PCU⁻ strains confirmed previous observations on their narrow susceptibility range, reinforcing the conclusion that antibiograms are of little value in differentiating PCU⁻ isolates.

Although restriction endonuclease patterns have been used as a typing method (25), the restriction endonuclease patterns generated by *Ava*II, *Pst*I, and *Sma*I digestion in the present study could not be compared accurately because too many fragments were produced. In order to compare the restriction endonuclease patterns from different isolates, gel electrophoresis should be carried out under several running conditions. Ribotyping has the advantage over restriction endonuclease analysis in that the RFLP patterns contain fewer bands for comparison because only fragments with rRNA sequences are visualized. We are evaluating other restriction endonucleases, which cleave the *N. gonorrhoeae* chromosome at few sites, in combination with pulsed-field gel electrophoresis as a method of differentiating PCU⁻ isolates.

It has been shown previously that the RFLP patterns generated by *Hin*cII digests of *N. gonorrhoeae* could be used to differentiate 43 *N. gonorrhoeae* isolates (21) into nine groups. Since this previous study provided no data on the auxotypes of the isolates and because the restriction enzymes used were different, we were unable to compare our data with those obtained previously. However, both studies (21; this study) showed that common DNA bands of the same sizes were present in the RFLP patterns for all the *N. gonorrhoeae* isolates used, reflecting the conserved nature of the rRNA genes within the species. As with our results, some isolates from the same serovar may show different RFLPs or isolates from different serovars may have identical RFLP patterns (21).

On the basis of the combined *Ava*II-*Sma*I-generated RFLP patterns, the 23 PCU⁻ isolates examined in the present study could be classified into four groups, with 78.3% of the isolates belonging to group I. Of the 15 PCU⁻ IB-2 isolates, only 2 (2152 and NS571) showed different RFLP patterns. Other isolates that showed divergence from the group I pattern belonged to serovar IB-07 (NS348), IB-16 (NS211), and IB-3 (NS791). The RFLP patterns of isolates belonging to serovars IB-1 and IB-25 were not distinguished from the group I RFLP pattern. Therefore, on the basis of the restriction endonucleases used and the small number of PCU⁻ isolates with serovars other than IB-2, it was not possible to evaluate the relationship between rRNA gene arrangements on the chromosome and the expression of outer membrane protein I. Although rRNA typing may add a small level of differentiation of PCU⁻ isolates from serovar IB-2, the method has a low probability of differentiating

PCU^- isolates from different geographic locations or epidemiologically unrelated isolates, and is therefore not economical for use in routine analyses.

MEE had been used to study the genetic diversities of PPNG strains and chromosomal mediated penicillin-resistant isolates from Spain (3, 4) belonging to several auxotype and serovar groups, but those studies did not include PCU^- isolates. The study on non-PPNG strains indicated that these strains were genetically highly variable, with 17 electrophoretic types found among 41 strains analyzed with seven enzymes (4). A clonal origin of strain types was also observed for strains for which the penicillin MIC was ≥1 mg/liter (4). Clonal origins were also observed for PPNG isolates of the NR auxotype isolated in Spain, with 15 of the 18 isolates studied belonging to a single electrophoretic type (3).

In the present study, MEE showed that only one gonococcal isolate (NS791) was different from the other isolates on the basis of enzyme mobilities in starch gels. Although previous studies by others reported the heterogeneity of enzymes among *N. gonorrhoeae* isolates (3, 4, 14, 15, 26), all PCU^- isolates in the present study had identical mobilities and only four enzymes, ME, GD2, GPI, and 6PG from isolate NS791, showed a difference in mobility. Therefore, the ability of MEE to detect genetic differences among PCU^- isolates was inferior to that of RFLP analysis and, hence, was less useful as a typing scheme.

Besides using MEE as a typing or differentiation scheme, MEE has been used to measure genetic relatedness among strains or to identify clones (3, 26). Isolates with identical electrophoretic types or low genetic distances are considered to be clonal (3, 4). The small variability in the enzyme profiles (mean genetic diversity per locus, 0.012; all 20 isolates were clustered at genetic distance of 0.2) supports the hypothesis that PCU^- isolates are clonal.

The application of other characteristics of enzyme differences such as their optimum pH, specific activity, and quantity of enzyme produced under defined growth conditions was not explored in the present study. Since cultural conditions may affect the quantity of enzyme produced (14, 15), the recording of enzyme locus variation (i.e., null results) is difficult to interpret. In order to score the absence of enzyme activity in isolate NS361 as a true null allele, and not a result of an inadequate concentration of enzyme in the crude lysate or denaturation of the enzyme during preparation, storage, or electrophoresis, additional analysis is required (14, 26).

Isolate NS791 differed significantly from the other PCU^- isolates tested, as measured by serotyping, plasmid analysis, RFLP analysis of rRNA genes, and MEE. It has been demonstrated in our laboratory that PCU^- isolates can acquire plasmids in vitro (11). The PCU^- isolate which could acquire plasmids (isolate 2152) was the sole isolate belonging to RFLP group II. Since the plasmid found in NS791 was a variant cryptic plasmid, the stability of the plasmid in its PCU^- host may be related to the inserted sequences in the plasmid.

The present study showed the limited phenotypic and molecular variation among PCU^- isolates. Thus, these isolates are likely to be clonal and the occurrence of variants is infrequent. Serovar appears to be more indicative of strain divergence than RFLP analysis of rRNA or isoenzyme typing because PCU^- isolates can be grouped into eight serovars. The variation in serovars may reflect the higher selective pressure on the expression of antigens exposed to the cell surface, thereby accounting for the greater serovar variability.

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