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Genetic Diversity of Penicillin G-Resistant *Neisseria meningitidis* from Spain

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Genotypic and phenotypic diversity among 16 penicillin G-resistant (Pen^r) isolates of *Neisseria meningitidis* recovered from human blood or cerebrospinal fluid in Spain was compared with that among 12 penicillin-susceptible (Pen^s) isolates by the use of multilocus enzyme electrophoresis, serotyping, auxotroph testing in chemically defined media, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis of penicillin-binding proteins (PBPs). Thirteen distinctive multilocus enzyme genotypes (electrophoretic types [ETs]) were identified among the 28 isolates. There was slightly less genetic diversity among the eight ETs of Pen^r isolates (H = 0.385) than among the eight ETs of Pen^s isolates (H = 0.431). Cluster analysis demonstrated two distinctive complexes of ETs and one ET that was not closely related to either complex. The possibility of a singular clonal origin of penicillin G-resistant isolates was excluded by the observations that resistance occurred in isolates of each of the two distantly related complexes of ETs, that three of the four ETs represented by multiple isolates included both susceptible and resistant strains, and that serotypes and growth requirements were not associated with the resistance phenotype. The 28 isolates showed a relatively homogeneous pattern of four PBPs, with apparently reduced penicillin G binding by PBP 3 of the Pen^r isolates.

Because penicillin G is the drug of choice for treatment of patients with meningococcal disease, the emergence of penicillin-resistant (Pen^r) strains of *Neisseria meningitidis* may pose therapeutic problems. Pen^r isolates of *N. meningitidis* which do not produce β-lactamase have recently been reported in South Africa (1) and in Spain, where the frequency of their recovery is increasing (2, 16). The emergence of these apparently chromosomally mediated, relatively resistant strains in a short period suggests a mutational event in a clone, followed by rapid dispersal of this clone with selective advantage.

We have asked whether these Pen^r isolates from Spain are genetically homogeneous as a consequence of recent derivation from a single clone or whether they represent a heterogeneous population of distantly related cell lines. To answer this question, we analyzed a sample of 16 Pen^r and 12 penicillin-susceptible (Pen^s) isolates with respect to variation in four characteristics—multilocus enzyme genotype, serotype, auxotrophic requirements, and penicillin-binding protein (PBP) profile—all of which presumably are controlled by chromosomal genes.

MATERIALS AND METHODS

Strains. All 16 resistant strains (defined as those growing on medium containing 0.1 μg of penicillin G per ml) were isolated from human cerebrospinal fluid or blood in 1985 and 1986 in Spain. They were identified as *N. meningitidis* by standard methods (14); 11 were serogroup B, and 5 were serogroup C (2). Twelve strains identified as susceptible (no growth on medium containing 0.1 μg of penicillin G per ml) and isolated in the same geographic area of Spain were used for comparison; eight were serogroup B and four were serogroup C. All strains were stored in 50% tryptic soy broth (Difco Laboratories, Detroit, Mich.) containing 10% glycerol at −70°C. Five strains previously designated 5655, 5706, 5609, 5959, and 6037 in Spain (10) are now designated R1910, R1912, R1907, R1919, and R1926, respectively.

Controls used for auxotroph testing included *Haemophilus influenzae* CDC 76-039256, which requires arginine, methionine, and hypoxanthine (12); *H. influenzae* A8, which is auxotrophic for lysine, hypoxanthine, and six other growth factors not tested (13); *Neisseria gonorrhoeae* 6392, which requires arginine, hypoxanthine, and uracil; *N. gonorrhoeae* F29, which requires lysine and methionine; and *N. gonorrhoeae* F18, which is prototrophic for all metabolites tested. The strains of *N. gonorrhoeae* were kindly provided by Rose Rice from the Neisseria Reference Laboratory, Harborview Medical Center, Seattle, Wash.

Electrophoresis of enzymes. Each isolate was grown overnight at 33°C in 100 ml of tryptic soy broth (Difco). Bacteria were harvested by centrifugation, and pellets were suspended in 1 ml of buffer (0.01 M Tris hydrochloride, 0.001 M EDTA [pH 6.8]). Cells were disrupted by freezing at −25°C for 24 h and then thawing. After centrifugation at 20,000 × *g* for 20 min at 4°C, the supernatants were sterilized by filtration and stored at −70°C.

Methods of starch gel electrophoresis and selective enzyme staining have been described by Selander et al. (17). The 14 enzymes assayed were malic enzyme, glucose 6-phosphate dehydrogenase, peptidase, isocitrate dehydrogenase, aconitase, NADP-linked glutamate dehydrogenase, NADP-linked glutamate dehydrogenase, alcohol dehydroge-

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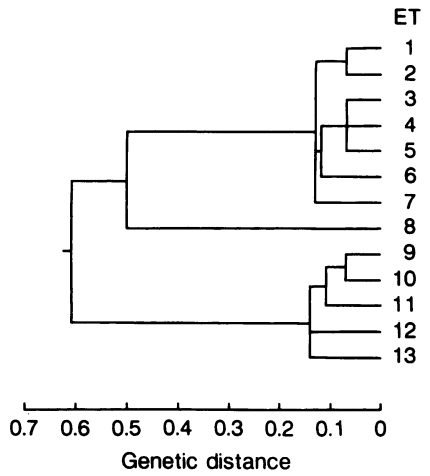

FIG. 1. Genetic relationships of ETs of *N. meningitidis* strains. The dendrogram was generated from a matrix of coefficients of pairwise genetic distances for 14 enzyme loci by the average-linkage method of clustering.

nase, fumarase, alkaline phosphatase, two indophenol oxidases, adenylate kinase, and an unknown dehydrogenase.

Electromorphs of each enzyme, numbered in order of decreasing anodal mobility, were equated with alleles at the corresponding structural gene locus. Numerical allele designations are cognate with those previously recorded for *N. meningitidis* (6). Each isolate was characterized by its combination of alleles at the 14 enzyme loci, and distinctive multilocus genotypes were designated as electrophoretic types (ETs).

ETs were numbered sequentially according to their position in the dendrogram (Fig. 1). Except for ET 5 (5), ET numbers were not cognate with those previously assigned (6).

Serotyping. Isolates were serotyped with monoclonal antibodies against antigens 2a, 2b, 4, 15, P1.2, P1.15, and P1.16 by coagglutination, as described by Frøholm et al. (7), and by dot blotting on nitrocellulose paper (8) (technique modified as described by Wedege and Frøholm [19]). Strains that were nonserotypeable were further serotyped by dot blotting with monoclonal antibodies against antigens 1, 2c, 5, 6, 8, 9, 11, 14, P1.1, and P1.3.

Auxotroph testing. After being grown overnight on chocolate agar plates in 5% CO_2 at 37°C, cultures were suspended in 0.9% phosphate-buffered saline to an optical density of 0.6 at 600 nm (10^8 CFU) and plated onto Catlin completely defined medium (4) supplemented with β-NAD^+ (V factor) and hemin (X factor) and solidified with 1.5% agarose. Catlin completely defined medium (as the control plate) and medium lacking the single growth factor methionine or the combined growth factors lysine and methionine; cysteine, cystine, and glutathione; or arginine, hypoxanthine, uracil, and histidine were used for auxotroph testing. An inoculum of 10^5 CFU of each strain was spotted onto the media with a Steers replicator. Duplicate plates were examined after 18 to 24 h in the presence of 5% CO_2 and room air at 37°C. Absence of growth or limited growth (isolated colonies or a slight haze) was recorded as a negative result.

Detection of PBPs. Whole-cell labeling of 12 Pen^s and 16 Pen^r *N. meningitidis* strains with [^{3}H]penicillin G (0.006 μg/100 μl) was compared with that of whole cells taken directly from solid medium after overnight growth and subsequently with that of cells at the logarithmic phase of growth (optical density, 0.6 at 600 nm) after incubation for 30 min. Cell lysis, electrophoresis in a sodium dodecyl sulfate-polyacrylamide gel, fixation, enhancement, and autofluorography were performed as previously described (10). X-ray films were developed after exposure for 4 weeks. For a single isolate, PBP comparisons were made with cells in the logarithmic as well as the early and late stationary phases of growth.

Statistical analyses. Genetic diversity at an enzyme locus among ETs or isolates was calculated as $h = (1 - x_i^2)(n/n - 1)$, where x_i is frequency of the ith allele and n is number of ETs or isolates. Mean genetic diversity (H) is the arithmetic average of h values over all loci. Genetic distance between pairs of ETs was expressed as the proportion of enzyme loci at which dissimilar alleles occurred (mismatches), and clustering was performed from a matrix of genetic distances by the average-linkage method (18).

RESULTS

Multilocus enzyme genotypes. In the collection of 28 isolates (Table 1), 4 of the 14 enzyme loci were monomorphic and 10 were polymorphic for two to five alleles (Table 2). The average number of alleles per locus was 2.5.

A total of 13 distinctive multilocus genotypes (ETs) were identified (Table 2), for which mean genetic diversity per

TABLE 1. Characteristics of the 28 isolates of *N. meningitidis*

ET	Reference isolate	Serogroup	Serotype	Penicillin MIC (μg/ml)	Auxotype[a]
1	R1915	B	4:P1.15	0.20	
	R1923	B	14:P1.1	0.20	
2	R1919	B	15:P1.16	0.30	Cystine$^-$ cysteine$^-$ glutathione$^-$
3	R1911	B	4:P1.15	0.06	Cystine$^-$ cysteine$^-$ glutathione$^-$
4	R1920	B	4:P1.15	0.20	
5	R1904	B	15:P1.16	0.03	Cystine$^-$ cysteine$^-$ glutathione$^-$
	R1907	B	4:P1.15	0.10	Cystine$^-$ cysteine$^-$ glutathione$^-$
	R1908	B	14:P1.1	0.03	
	R1912	B	4:P1.15	0.03	
	R1914	B	15:−	0.06	Cystine$^-$ cysteine$^-$ glutathione$^-$
	R1916	B	4:P1.15	0.03	Cystine$^-$ cysteine$^-$ glutathione$^-$
	R1922	B	4:P1.15	0.30	Cystine$^-$ cysteine$^-$ glutathione$^-$
	R1929	B	4:P1.15	0.20	
	R1930	B	15:P1.16	0.30	
	R1931	B	4:1.15	0.20	
6	R1905	B	4:P1.15	0.03	Cystine$^-$ cysteine$^-$ glutathione$^-$
7	R1917	B	4:P1.15	0.06	
	R1921	B	4:P1.15	0.20	Cystine$^-$ cysteine$^-$ glutathione$^-$
8	R1909	C	NT:−[b]	0.06	
9	R1910	C	2b:−	0.06	
	R1918	B	2b:−	0.30	
	R1924	C	2b:−	0.50	Cystine$^-$ cysteine$^-$ glutathione$^-$
	R1926	C	2b:−	0.70	
	R1927	C	2b:−	0.30	
10	R1925	C	2b:−	0.40	
11	R1906	C	2b:−	0.06	
12	R1928	C	2b:−	0.50	
13	R1913	C	2a:−	0.06	

[a] An entry indicates a requirement for those factors.
[b] NT, Not typeable.

TABLE 2. Allele profiles of 14 enzyme loci for 13 ETs of *N. meningitidis*

ET	Reference isolate	Allele no. at indicated enzyme locus[a]													
		MAE	G6P	PEP	IDH	ACO	GD1	GD2	ADH	FUM	ALP	IP1	IP2	ADK	XDH
1	R1915	1	1	7	12	4	2	3	2	1	1	2	3	2	3
2	R1919	1	1	7	5	4	2	3	2	1	1	2	3	2	3
3	R1911	1	1	7	8	4	2	3	3	1	1	2	3	2	3
4	R1920	1	1	7	8	4	2	3	0	1	1	2	3	2	3
5	R1907	1	1	7	8	4	2	3	2	1	1	2	3	2	3
6	R1905	1	1	7	8	4	2	3	2	1	8	2	3	2	3
7	R1917	1	4	7	8	4	2	3	2	1	1	2	3	2	3
8	R1909	3	4	0	7	4	1	3	2	1	8	2	5	2	3
9	R1918	4	3	4	5	5	1	4	0	1	8	2	3	2	3
10	R1925	4	3	4	5	5	1	4	1	1	8	2	3	2	3
11	R1906	4	3	4	5	0	1	4	0	1	8	2	3	2	3
12	R1928	4	3	1	5	1	1	4	0	1	8	2	3	2	3
13	R1913	4	3	5	5	5	1	4	0	1	8	2	3	2	3

[a] MAE, Malic enzyme; G6P, glucose-6-phosphate dehydrogenase; PEP, peptidase; IDH, isocitrate dehydrogenase; ACO, aconitase; GD1, NADP-dependent glutamate dehydrogenase; GD2, NAD-dependent glutamate dehydrogenase; ADH, alcohol dehydrogenase; FUM, fumarase; ALP, alkaline phosphatase; IP1 and IP2, two indophenol oxidases; ADK, adenylate kinase; XDH, unknown dehydrogenase.

locus was 0.405 (Table 3). There was less genetic diversity per locus among isolates (H = 0.330), reflecting the circumstance that four of the ETs were represented by multiple isolates (range, 2 to 10 isolates) (Table 1).

Estimates of genetic relationships among the 13 ETs are shown in Fig. 1. At a genetic distance of 0.50, corresponding to the occurrence of dissimilar alleles at an average of 7 of the 14 loci, there were three lineages, two represented by a cluster of closely related ETs (ETs 1 through 7 and 9 through 13) and one represented by ET 8. ETs 1 through 7 represented clones of the ET 5 complex (5).

There was only slightly less genetic diversity among the ETs of isolates that were relatively resistant to penicillin (H = 0.385) than among those that were sensitive (H = 0.431) (Table 3), and multilocus enzyme genotypes of penicillin-resistant organisms were represented in both clusters. Of the 16 strains that were relatively resistant to penicillin, 10 belonged to the ET 5 complex, but MICs greater than 0.30 were restricted to isolates of ETs in the cluster formed by ETs 9 through 13.

TABLE 3. Genetic diversity among isolates and ETs of *N. meningitidis*

Enzyme locus[a]	Genetic diversity in:					
	Isolates			ETs		
	Susceptible (n = 12)	Resistant (n = 16)	All (n = 28)	Susceptible (n = 8)	Resistant (n = 8)	All (n = 13)
MAE	0.530	0.500	0.500	0.679	0.536	0.603
G6P	0.621	0.575	0.579	0.750	0.679	0.667
PEP	0.561	0.542	0.540	0.750	0.607	0.692
IDH	0.530	0.642	0.601	0.679	0.679	0.679
ACO	0.439	0.542	0.492	0.607	0.607	0.603
GD1	0.485	0.500	0.476	0.571	0.536	0.538
GD2	0.409	0.500	0.452	0.536	0.536	0.513
ADH	0.530	0.575	0.545	0.679	0.679	0.679
FUM	0.000	0.000	0.000	0.000	0.000	0.000
ALP	0.530	0.500	0.495	0.536	0.536	0.538
IP1	0.000	0.000	0.000	0.000	0.000	0.000
IP2	0.167	0.000	0.071	0.250	0.000	0.000
ADK	0.000	0.000	0.000	0.000	0.000	0.000
XDH	0.000	0.000	0.000	0.000	0.000	0.000
Mean	0.343	0.348	0.330	0.431	0.385	0.405

[a] For abbreviations, see Table 2, footnote *a*.

Serotypes. Seven serotypes (serotype-serosubtype combinations) were represented among the 28 isolates (Table 1). The 18 isolates of the ET 5 complex were serotypes 4:P1.15 (12 isolates), 15:P1.16 (3 isolates), 14:P1.1 (2 isolates), and 15:− (1 isolate). The single isolate of ET 8 was nontypeable. The cluster formed by ETs 9 through 13 corresponded to clones that were genetically similar to those of many serotype 2a isolates from diverse geographic origins (6). However, only one of the nine isolates belonging to that cluster was serotype 2a (R1913); the other eight isolates were serotype 2b (Table 1).

Penicillin resistance was not associated with specific serotypes. For example, the two isolates of ET 1 were resistant, but one was serotype 4:P1.15 and the other was 14:P1.1; among isolates of ET 5, both 4:P1.15 and 15:P1.16 strains were resistant.

Auxotroph analyses. Catlin medium containing all the indicated growth factors supported growth of all 28 isolates of *N. meningitidis* when incubated in 5% CO_2. In contrast, identical cultures incubated simultaneously in room air were unable to support the growth of 15 of the 28 isolates (3 Pen^s and 12 Pen^r); poor growth was observed for 11 of the 28 isolates (7 Pen^s and 4 Pen^r), and adequate growth was observed for only 2 Pen^s isolates. All meningococcal strains grew on Catlin medium lacking methionine, lysine and methionine, or arginine, hypoxanthine, uracil, and histidine, which apparently are all nonessential. In contrast, Catlin medium lacking cysteine, cystine, and glutathione was unable to support the growth of 10 of the 28 isolates (5 Pen^s and 5 Pen^r), indicating that one, two, or all three factors may be essential for growth. All three *N. gonorrhoeae* control strains and *H. influenzae* CDC 76-039256 were also unable to grow on Catlin medium lacking cysteine, cystine, and glutathione. Lysine and methionine were required for growth of *N. gonorrhoeae* F29, and as expected, *N. gonorrhoeae* 6392 required the combination of arginine, hypoxanthine, uracil, and histidine. *H. influenzae* CDC 76-039256 required the latter combination as well as methionine.

PBP detection. Four PBPs with similar mobilities were detected in all 28 strains (Fig. 2). Labeling cells after overnight growth on solid medium revealed variable binding to the PBPs, with no correlation with resistance (data not shown). The single isolate in the early and late stationary phases of growth (Fig. 2B, lanes 9 and 10) showed more binding to all PBPs than the same strain in the log phase (lane

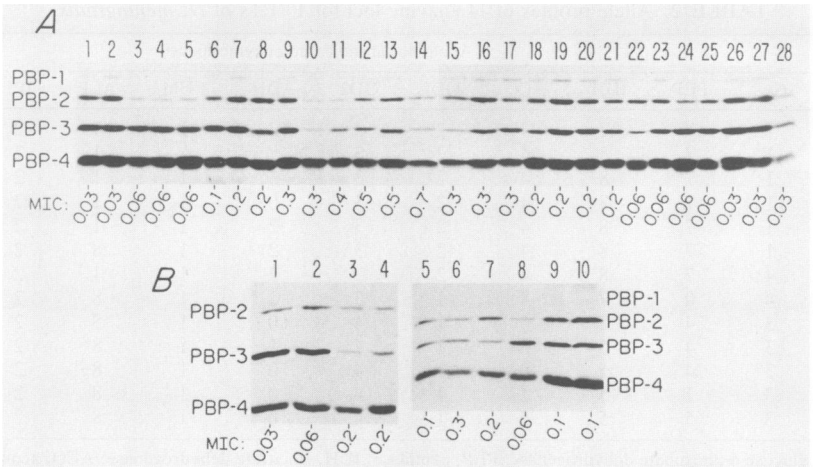

FIG. 2. Detection of the PBPs of penicillin G-susceptible and relatively resistant *N. meningitidis*. ^{3}H-labelled penicillin G (final concentration, 0.06 μg/ml) was incubated for 30 min with whole cells, which were grown to mid-log phase (optical density, 0.6 at 600 nm; 10^8 CFU/ml). Inner membranes were solubilized with Sarkosyl and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. (A) Films developed after fluorography for 48 days at −70°C. Lanes 1 to 5 and 22 to 28, PBPs of 12 penicillin G-susceptible isolates; lanes 6 to 21, PBPs of 16 relatively penicillin G-resistant isolates. The MIC of penicillin, in micrograms per milliliter, is indicated at the bottom of the lane for each strain. The numbers to the left of lane 1 indicate major PBPs 1 through 4, with apparent molecular masses of 108, 81, 59, and 44 kilodaltons, respectively. Strains represented in lanes 1 to 28 are R1908, R1912, R1913, R1914, R1906, R1907, R1915, R1920, R1927, R1918, R1925, R1928, R1924, R1926, R1930, R1922, R1919, R1929, R1923, R1931, R1921, R1917, R1909, R1910, R1911, R1916, R1905, and R1904, respectively. (B) Films developed after fluorography for 20 days (lanes 1 to 4) or 37 days (lanes 5 to 10) at −70°C. Lanes 1, 2, and 8 (corresponding to lanes 28, 22, and 24, respectively, in panel A), PBPs of three penicillin G-susceptible isolates; lanes 3, 4, 5, 6, and 7 (corresponding to lanes 19, 20, 6, 9, and 7, respectively, in panel A), PBPs of five relatively penicillin G-resistant isolates; lanes 9 and 10, PBPs detected in the early and late stationary phases of growth for the strain indicated in lane 5 (lane 6 in panel A), for which the PBPs were detected in the logarithmic phase of growth.

5). Thus, penicillin binding does appear to be dependent on phase of growth in meningococcus. With cells grown to the log phase, the amount of penicillin G bound to PBPs 1, 2, and 4 did not correlate with the MIC for both Pen^r and Pen^s strains. In contrast, PBP 3 of 10 of the 12 Pen^s isolates bound more penicillin G (excluding those strains represented in lanes 22 and 28 in Fig. 2A) than 11 of 16 Pen^r isolates (excluding those strains represented in lanes 6, 7, 9, 19, and 20). Repeat comparative analysis of penicillin G binding in these seven strains (Fig. 2B) revealed that PBP 3 of the two Pen^s strains (Fig. 2A, lanes 22 and 28, and 2B, lanes 2 and 1) did bind more penicillin G than PBP 3 of the five Pen^r isolates (Fig. 2A, lanes 6, 7, 9, 19, and 20, and 2B, lanes 5, 7, 6, 3, and 4). The initial differences in binding may represent strain differences in the relative amount of PBPs produced as the cells enter the stationary phase of growth (11). In addition, the discrepancies may be due to problems in pipetting the small reaction volumes or in loading the samples.

DISCUSSION

Compared with other *Neisseria* species, meningococcus is relatively homogeneous and biosynthetically competent (4). In contrast, *N. gonorrhoeae* exhibits marked diversity of nutritional requirements (4), and it has been established that in certain locales strains causing disseminated gonococcal infection have a unique nutritional profile, that is, a requirement for arginine, hypoxanthine, and uracil (9). Catlin observed a requirement for methionine in certain strains of *N. meningitidis* (3), and we previously found an association between chromosomally mediated, non-β-lactamase- producing, ampicillin-resistant (Amp^r) *H. influenzae* and a requirement for methionine (12). Our nutritional analysis of the Pen^r and Pen^s isolates of *N. meningitidis* from Spain revealed that all strains required CO_2 for optimal growth, that they were prototrophic for methionine, and that an equal number of strains (five) in each subset required a combination of cystine, cysteine, and glutathionine for growth. These auxotrophic markers were distributed among 6 of the 13 ETs and could not be used to differentiate Pen^r and Pen^s strains.

Before our recent study evaluating the PBP saturation kinetics of two Pen^s and two Pen^r strains, which showed that the mechanism of penicillin G resistance is decreased affinity of PBP 3 for penicillin (10), PBP analysis for *N. meningitidis* had been limited to a single penicillin-susceptible isolate (15). We also compared the PBP profiles of 10 strains (6 Pen^r and 4 Pen^s isolates) (10) and have now studied an additional 18 isolates (10 Pen^r and 8 Pen^s). Our results appear to confirm our previous observation that PBP 3 may bind less penicillin among the Pen^r isolates than among the Pen^s strains. Variability in penicillin binding to certain meningococcal PBPs, both in the amount bound and in the number of PBPs detected with longer incubation times, has previously been described (10). In addition, the phase of growth appears to play a role, with more penicillin being bound to all four PBPs in the stationary phase. The variability in penicillin binding to PBP 3 of two Pen^s and five Pen^r isolates in this study was probably methodologically based, because when these isolates were retested, the amount of penicillin bound to PBP 3 appeared to be uniformly reduced in all the Pen^r strains. Thus, one must be cautious in interpreting the results of single-PBP comparisons for unrelated strains. Precise definition of the role of PBP 3 in resistance would require PBP saturation kinetic studies as previously performed (10) or transformation of the DNA encoding resistance into a susceptible recipient and PBP comparison of the "isogenetic" pairs. The relatively homogeneous pattern of 4

PBPs observed in all 28 meningococcal isolates contrasts with the extensive heterogeneity in PBP number observed among Amp^r, non-β-lactamase-producing *H. influenzae* (5 to 10 PBPs detected [12]).

By multilocus enzyme electrophoresis, Caugant et al. (5) demonstrated that serogroup B and C meningococci are highly heterogeneous but basically clonal in structure; analysis of 460 isolates revealed 192 distinctive multilocus genotypes, with a mean genetic diversity per locus of 0.536. Our analysis of 16 Pen^r and 12 Pen^s serogroup B and C isolates from Spain showed that these strains represented 13 distinctive multilocus genotypes, among which mean genetic diversity per locus was less than that recorded for *N. meningitidis* as a whole. However, the clonal composition of this sample of isolates was representative of that of strains recovered from patients in Spain (D. A. Caugant, unpublished data).

Comparison of Pen^r and Pen^s isolates revealed that the emergence of Pen^r in Spain did not result from the acquisition of resistance by a single resident clone or from the introduction of a new, resistant clone. Apparently, resistance appeared more or less concomitantly in various unrelated clones preexisting in the Spanish population. Genetic mechanisms leading to the acquisition of penicillin G resistance are unknown. It is possible that Pen^r isolates have been present in low frequency for a long time and that recent changes in therapeutic measures (not necessarily against meningococcal disease) led to a rapid increase in the frequency of Pen^r strains of *N. meningitidis*. An alternative is that a gene for penicillin G resistance was acquired by a single meningococcal clone and that this gene is now spreading to other clones through genetic recombination. Only by nucleotide sequence analyses of the gene(s) involved in resistance to penicillin G could these alternative hypotheses be tested.

We conclude that Pen^r meningococci in Spain (i) did not arise from a single clone but are genetically diverse and are similar to Pen^s strains, (ii) are not associated with a unique nutritional requirement, (iii) have four PBPs with M_rs similar to those of Pen^s isolates, and (iv) apparently have a PBP 3 with a uniformly lower penicillin-binding capacity than that of the PBP 3 in Pen^s strains.

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