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Target Gene Sequencing To Characterize the Penicillin G Susceptibility of *Neisseria meningitidis*▽

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Clinical isolates of *Neisseria meningitidis* with reduced susceptibility to penicillin G (intermediate isolates, Pen^I) harbor alterations in the *penA* gene encoding the penicillin binding protein 2 (PBP2). A 402-bp DNA fragment in the 3′ half of *penA* was sequenced from a collection of 1,670 meningococcal clinical isolates from 22 countries that spanned 60 years. Phenotyping, genotyping, and the determination of MICs of penicillin G were also performed. A total of 139 different *penA* alleles were detected with 38 alleles that were highly related, clustered together in maximum-likelihood analysis and corresponded to the penicillin G-susceptible isolates. The remaining 101 *penA* alleles were highly diverse, corresponded to different genotypes or phenotypes, and accounted for 38% of isolates, but no clonal expansion was detected. Analysis of the altered alleles that were represented by at least five isolates showed high correlation with the Pen^I phenotype. The deduced amino acid sequence of the corresponding PBP2 comprised five amino acid residues that were always altered. This correlation was not complete for rare alleles, suggesting that other mechanisms may also be involved in conferring reduced susceptibility to penicillin. Evidence of mosaic structures through events of interspecies recombination was also detected in altered alleles. A new website was created based on the data from this work (http://neisseria.org/nm/typing/penA). These data argue for the use of *penA* sequencing to identify isolates with reduced susceptibility to penicillin G and as a tool to improve typing of meningococcal isolates, as well as to analyze DNA exchange among *Neisseria* species.

The natural habitat of *Neisseria meningitidis* is the nasopharynx where it is encountered in ca. 10% of the general population (asymptomatic carriers) (10), but it can also cause serious invasive infections, mainly septicemia and meningitis, provoking public health concern. Prompt treatment is critical to the management of invasive meningococcal diseases. Penicillin G remains, in several countries, the antibiotic of first choice in the treatment of invasive meningococcal diseases, particularly when the bacteriological diagnosis has been established (20, 23). However, isolates with reduced susceptibility to penicillin G (Pen^I) are increasingly being reported worldwide (29) and have led clinicians to use third generation cephalosporins such

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as ceftriaxone for initial treatment (20). The Pen^I isolates are defined phenotypically by showing a MIC of penicillin G ranging between 0.094 and 1 mg/liter (8). A recent questionnaire among European laboratory members of the European Monitoring Group on Meningococci (EMGM) revealed heterogeneous definitions of penicillin susceptibility among these laboratories (9). Consequently, the percentage of agreement on susceptibility to penicillin using Etest (AB Biodisk, Solna, Sweden) was not optimal among these laboratories and varied between 24 and 100% (33).

N. meningitidis is a transformable bacterium that undergoes frequent horizontal DNA transfer. The alteration of the *penA* gene encoding the penicillin binding protein 2 (PBP2) through horizontal DNA transfer was suggested as the major mechanism for the emergence of Pen^I isolates (5, 24, 26, 31). The modifications of PBP2 result in a decrease in the affinity of PBP2 to penicillin G, as well as in modifications in the structure of peptidoglycan in the bacterial cell wall that are responsible for the Pen^I phenotype (5). We have previously reported penicillin-binding experiments using membrane extracts or purified PBP2 proteins to show that modification of PBP2 are correlated with reduction in binding affinity of PBPs for [^{3}H]benzylpenicillin (5). The modifications of PBP2 that confer the Pen^I phenotype are located in the C-terminal half of the protein that binds penicillin and harbors the transpeptidase region (5). Indeed, we have previously reported that transformation with DNA (both genomic DNA or *penA* PCR products) from Pen^I isolates from several countries conferred the Pen^I phenotype on a Pen^S strain, indicating that this phenotype is directly related to changes in *penA* (5, 7). Several polymorphic positions were observed in this part of *penA* with alterations in the corresponding amino acid residues. Transformation of a susceptible isolate by a 3′ fragment of *penA* (encoding the C-terminal half of the protein) harboring these polymorphisms was sufficient to confer the Pen^I phenotype (5).

The impact of these alterations on the spread of Pen^I meningococcal isolates and the structure of the bacterial population is not clear. The aims of the present study were to analyze the *penA* sequences, the corresponding deduced amino acid sequences of the encoded PBP2, as well as the phenotypic susceptibility to penicillin of a large collection of meningococcal isolates to investigate the spread of Pen^I isolates. Such approaches may also allow the establishment of a general molecular scheme to define bacterial susceptibility and/or resistance to different antibiotics by strategies of modifications of key (target) genes involved in this process.

MATERIALS AND METHODS

Meningococcal isolates and conventional bacteriological methods. Meningococcal isolates were received by the participating laboratories and National Reference Centers and had been identified by using standard culture and identification methods. The MIC of penicillin G was determined as previously described (33). Several participating laboratories performed the following analysis themselves, and some laboratories sent isolates for analysis in one of the other participating laboratories. However, all laboratories followed the methods described in the references or in the text below.

Serogroup was determined by agglutination with serogroup-specific antisera according to the standard procedure of each laboratory. Further phenotyping (serotyping and serosubtyping) was performed as previously described (1). Genotyping, using multilocus sequence typing (MLST), *porA* typing, and *fetA* typing were performed as previously described (12, 14, 18, 27, 28, 30, 32). Sequence types (STs) and FetA and PorA types were determined through MLST websites (http://pubmlst.org and http://neisseria.org). Data on geographical location, year, and anatomical site of isolation were obtained.

DNA sequencing and analysis of *penA*. Two primers were designed to amplify the *penA* gene between the positions 4948 and 5459 (according to EMBL/GenBank accession number AE002397). These primers are penA1F (the upstream oligonucleotide; 5′-gttttcccagtcacgacgttgtaATCGAACAGGCGACGATGTC-3′) and penA1R (the downstream oligonucleotide; 5′-ttgtgagcggataacaattcGATTAAGACGGTGTTTTGACGG-3′). The universal forward and reverse sequences were added as adapters to the 5′ end upstream and downstream from the oligonucleotides (shown in lowercase letters). The universal forward and reverse sequences were then used for sequencing. A DNA fragment of 402 bp of the *penA* gene, which corresponds to the residues 441 to 574 of the PBP2, was extracted from the DNA sequence. Alignments were made by using the MacMolly program (Mologen, Berlin, Germany). Sequences differing by at least one nucleotide were assigned a unique *penA* allele sequence number. Some laboratories used other protocols and primers to obtain the same 402-bp fragment of *penA* described above (8, 31).

A new database on *penA* typing was especially created based on the data obtained in the present study, and information regarding all included isolates and DNA sequences are available (http://neisseria.org/nm/typing/penABlast) (3); clustering analyses were made by the PHYML program of maximum-likelihood phylogenies using the *penA* sequences (16) through the website of the Institut Pasteur, Paris, France (http://www.pasteur.fr). The *penA* sequences from *N. perflava* (accession number X76423), *N. mucosa* (accession number X59635), *N. cinerea* (accession number Z17310), and *N. flavescens* (accession number M26645) were also obtained through the website (http://www.pasteur.fr). The identification of potential recombination events between two *penA* sequences was performed by using the maximum chi-squared test in the START package available through (http://pubmlst.org) using an input of two sequences and 1,000 resamplings (17, 19).

Statistical analysis. Qualitative data were analyzed by using the chi-squared test. A P value of ≤ 0.05 was considered to be statistically significant. Geometric means, as well as lower and upper 95% confidence limits, were calculated using GraphPad InStat version 3.06 (GraphPad Software, San Diego, CA). For reliable calculation of the geometric means and 95% confidence intervals, only alleles that were represented by at least five isolates were included. Geometric mean was used since it better evaluates the MIC of different isolates.

RESULTS

General characteristics of meningococcal isolates. A large number (n = 1,670) of clinical isolates of *N. meningitidis* were included from 22 different countries worldwide spanning 60 years (1945 to 2006) (Table 1). Most isolates were from reference laboratories of European countries that are members of the EMGM and represented mostly invasive isolates (n = 1393, 83%). Isolates from the cerebrospinal fluid were the most frequent (n = 808 [48%]), followed by isolates from blood (585 [35%]). Some of the isolates were from United States and several African countries, as well as French overseas territories. Representative isolates for which the penicillin G MIC was ≥0.06 mg/liter were tested. A few isolates with lower penicillin G MICs (n = 153) were also analyzed. For some countries (e.g., Denmark and France), all of the invasive isolates from or since 2005 were included, in addition to several isolates from other years. The distribution of isolates with known MIC (n = 1,644 [98%]) is shown in Fig. 1. According to the phenotypic definition of Pen^I isolates (MICs ranging between 0.094 and 1 mg/liter), 1,072 of 1,644 isolates (65%) showed reduced susceptibility to penicillin G. The number of isolates per country and their MICs are available online (http://neisseria.org/nm/typing/penA).

Serogroups were determined for 1,625 isolates (97%). The serogroup distribution for the five major serogroups was as follows: A, 1.4%; B, 55%;, C, 29%; Y, 4.5%; and W-135, 6.1%. This distribution was similar to the general distribution ob-

TABLE 1. Distribution of the major *penA*^{ps} and altered *penA* alleles according to country[a]

Country	No. of alleles					
	penA^{ps}	*penA14*	*penA9*	*penA12*	Other altered alleles	Total
Austria	61	0	0	3	8	72
Belgium	1	4	1	1	1	8
Congo	1	0	0	0	0	1
Croatia	7	1	0	0	2	10
Czech Republic	113	0	0	0	24	137
Denmark	62	0	0	5	0	67
France (domestic)	463	38	49	20	124	694
France (overseas territories)	19	0	1	0	3	23
Germany	63	2	2	4	9	80
Greece	19	19	1	0	19	58
Ireland	32	10	2	0	13	57
Italy	14	8	11	75	15	123
Madagascar	7	0	0	0	0	7
Niger	18	0	0	0	0	18
Norway	1	1	0	0	0	2
Poland	20	0	1	0	7	28
Romania	2	0	0	0	8	10
Scotland	34	15	0	2	12	63
Slovenia	2	3	0	0	9	14
Spain	21	3	10	30	30	94
Sweden	45	0	0	1	2	48
Tunisia	15	0	3	0	3	21
United States	24	2	1	0	8	35
Total	1,044	106	82	141	297	1,670

[a] *penA*^{ps}, penicillin-susceptible alleles.

served in Europe in 2004 (0.4, 70, 18.5, 2.6, and 2.8%, respectively) (see http://www.euibis.org). MLST was performed on 636 isolates representing 193 different STs. Most of these isolates (56%) represented STs that belong to the five major hypervirulent clonal complexes in Europe (11, 13, 35): ST-8 complex/Cluster A4 (16%), ST-11 complex/ET-37 complex (14%), ST-32 complex/ET-5 complex (10%), ST-41/44 complex/Lineage 3 (12%), and ST-269 complex (4%). Isolates belonging to the ST-22 complex represented 3% and were most frequently isolated from elderly individuals (data not shown).

Characteristics of *penA*. The sequence of a 402-bp DNA fragment of the 3′ part of *penA* gene was obtained for all of the 1,670 isolates of the present study and allowed the identification of 139 different alleles of *penA* that were named *penA1* to *penA139*. The frequencies of theses alleles varied from 1 up to 428 isolates per allele and are available online (http://neisseria.org/nm/typing/penA). The most frequent allele among the tested isolates was *penA1* (n = 428 isolates [26%]), and it was distributed globally. When all alleles were aligned with the *penA1* allele, they showed homologies of between 99.75% (1 polymorphic site) and 80.60% (78 polymorphic sites). The maximum-likelihood analysis clustered together a group of 38 alleles that shared at least 98.5% homology (no more than six polymorphic sites). These 38 highly related alleles corresponded to 1,043 isolates (62%). This cluster harbored the two most frequent alleles, *penA1* and *penA3*, together representing 46% of isolates (428 and 337 isolates, respectively). Most of these alleles (23 of 38) had an identical deduced amino acid sequence (only silent, synonymous DNA polymorphisms) for the corresponding 402-bp fragment and accounted for 1,018 isolates. The other 15 alleles belonging to this particular cluster differed by no more than two amino acids and were rare (only 25 isolates). Consequently, this cluster of 38 alleles corresponded to isolates with highly related *penA* sequences with low levels of alterations (if any) of the PBP2 protein. The remaining 101 alleles, corresponding to 627 isolates (38% of the total isolates) were distinctly separated from the first cluster into several other clusters and showed up to 21 amino acid changes from the most frequent sequence described above encoded by the *penA1* allele (data not shown). Forty different altered *penA* alleles were observed among all of the invasive meningococcal strains isolated in France since 2005 (http://neisseria.org/nm/typing/penA). These 101 alleles corresponded to the group of altered alleles with an altered PBP2 protein as deduced from the *penA* allele sequences.

Three alleles (*penA12*, *penA14*, and *penA9*) were the most frequent among these altered alleles and accounted for 8%

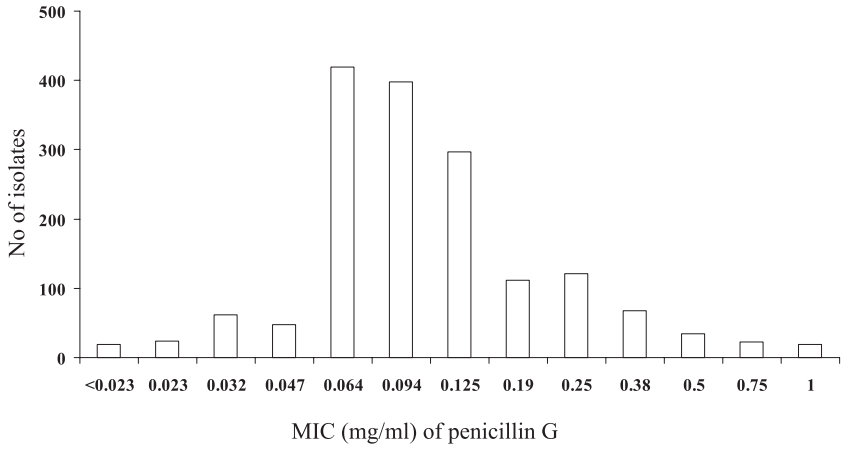

FIG. 1. Phenotype distribution among the tested meningococcal isolates for which the penicillin G MICs are known (n = 1,644, 98% of total isolates).

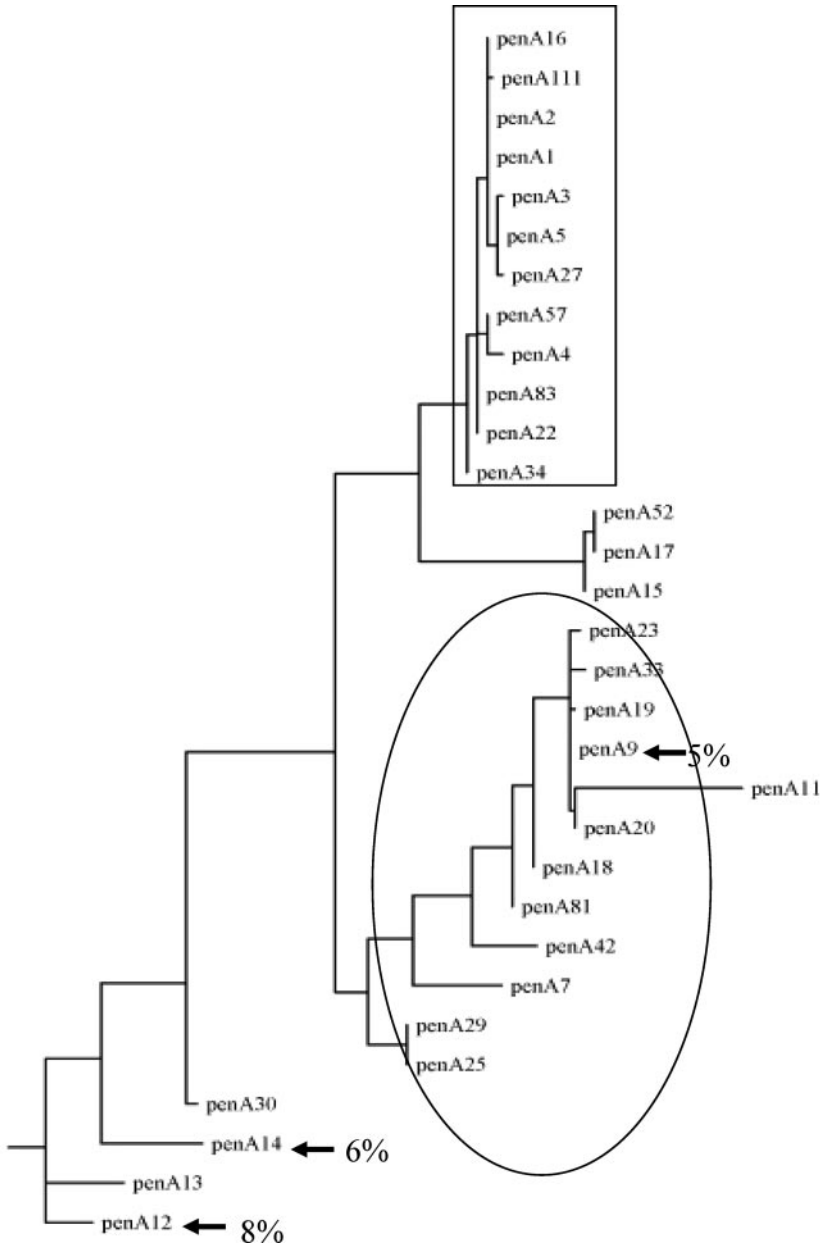

FIG. 2. Schematic illustration of phylogenic analysis by maximum likelihood of the 31 *penA* alleles that were identified among the tested isolates and were represented by at least five isolates. The group of *penA*^{ps} alleles that were highly related and corresponded to susceptible isolates is indicated in a box. The most frequent altered alleles (*penA12*, *penA14*, and *penA9*) are indicated by arrows, and their percentages are indicated. The "*penA9* cluster" is shown in an ellipse.

(n = 141), 6% (n = 106), and 5% (n = 82) of the total isolates, respectively. The maximum-likelihood clustering of *penA* alleles is shown in Fig. 2 for the 31 *penA* alleles that were represented by at least five isolates. The three frequent altered alleles showed disparity in their geographical distribution, *penA9* and *penA14* being frequent in France and Denmark, respectively, while *penA12* was frequent in Spain and Italy. These three alleles were not observed in, for example, the Czech Republic (Fig. 3 and Table 1). The *penA9* allele was observed in isolates of several different phenotypes and genotypes. Moreover, the maximum-likelihood analysis clustered several other altered *penA* alleles together with the allele *penA9* with identical deduced amino acid sequences for some of them (*penA9*, *penA19*, *penA20*, *penA23*, and *penA33*) (Fig. 2).

Correlation between MIC and alterations of PBP2. Transformation using PCR products of altered *penA* alleles (including the most frequent alleles *penA12*, *penA14*, and *penA9*) confers PenI phenotype and reduction of [3H]benzylpenicillin binding to PBP2 (5, 7; data not shown). We therefore analyzed the associations between *penA* alleles and susceptibility to penicillin G; only *penA* alleles that were represented by at least five isolates were considered (see Materials and Methods). The

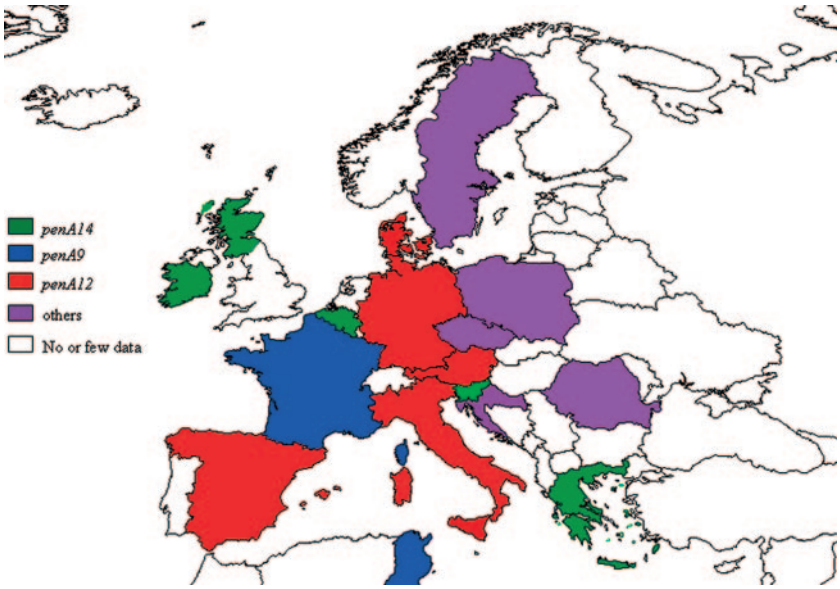

FIG. 3. Geographic distribution of the most frequent altered *penA* alleles (*penA12*, *penA14*, and *penA9*). The colors correspond to the most frequent allele in each country. "Other" indicates countries where other alleles were observed, such as *penA132* (Czech Republic), *penA32* (Romania), and *penA52* (Sweden).

selected alleles ($n = 31$) corresponded to 1,520 isolates (91% of the total isolates). Among these alleles, 12 were from the cluster of the highly related *penA* that encoded one identical PBP2, except for *penA111*, which encoded a PBP2 with one amino acid change (P551 to S), while the remaining 19 *penA* alleles encoded 15 distinct altered PBP2 (Fig. 2 and Table 2). The geometric mean MICs (MICgm) of penicillin G were calculated for the corresponding isolates of each selected allele. The MICgm for isolates harboring the alleles from the cluster of highly related *penA* varied between 0.055 and 0.094 mg/liter (Table 2). This range was 0.055 to 0.076 mg/liter if only alleles with more than 10 isolates were considered. The narrow 95% confidence intervals had an upper limit of 0.090 mg/liter (Table 2). This correlation suggests that the cluster of highly related *penA* is linked to meningococcal susceptibility to penicillin G. The corresponding *penA* alleles are designated henceforth "penicillin-susceptible alleles $penA^{ps}$," and the corresponding PBP2 are designated "$PBP2^{ps}$." In contrast, the isolates of other clusters (with altered PBP2) showed higher MICgm ranging from 0.112 to 0.511 mg/liter; an MIC of amoxicillin of >0.25 mg/liter was also correlated with altered PBP2 (data not shown). When these altered PBP2 were aligned to $PBP2^{ps}$, five positions corresponding to residues F504, A510, I515, H541, and I566 in the wild-type PBP2 were always modified in all altered PBP2 (Fig. 4). These positions were also always modified in the PBP2 encoded by altered *penA* alleles that were represented by three and four isolates (data not shown). These residues are located in the transpeptidase domain of PBP2. However, the KTG motif was always conserved in all PBP2 encoded by the 139 *penA* alleles (Fig. 4).

Distribution of altered *penA* alleles. Association between serogroups and alterations of PBP2 was analyzed for the 1,625 isolates with determined serogroups. A significant association was observed between isolates of serogroup W135 and alterations of PBP2 at the five amino acid positions ($P < 0.00001$)

TABLE 2. Characteristics of penicillin G MIC and PBP2 alterations among the tested isolates ($n = 1,520$)[a]

penA allele	No. of isolates	MIC range (mg/liter)	Geometric mean MIC (mg/liter)	95% Confidence interval	No. of alterations in PBP2[b]
83	17	0.023–0.380	0.055	0.040–0.076	0
57	19	0.008–0.125	0.058	0.041–0.084	0
5	38	0.015–0.190	0.066	0.056–0.077	0
2	39	0.015–0.125	0.066	0.056–0.077	0
34	20	0.032–0.125	0.067	0.057–0.079	0
3	333	0.004–0.250	0.070	0.067–0.074	0
27	34	0.032–0.125	0.070	0.060–0.081	0
4	16	0.032–0.125	0.072	0.060–0.087	0
16	8	0.047–0.125	0.074	0.055–1.004	0
1	423	0.008–0.250	0.076	0.073–0.079	0
22	43	0.023–0.250	0.077	0.064–0.090	0
111[c]	7	0.094–0.094	0.094	0.094–0.094	0
25	5	0.064–0.190	0.112	0.073–0.193	5
29	12	0.094–0.125	0.119	0.111–0.128	5
52	20	0.064–0.500	0.123	0.096–0.157	5
15	24	0.094–0.250	0.145	0.126–0.167	5
12	141	0.064–1.000	0.162	0.148–0.176	5
30	6	0.125–0.380	0.170	0.102–0.281	5
23	5	0.064–0.380	0.171	0.073–0.340	5
42	10	0.094–0.250	0.172	0.137–0.216	5
14	102	0.064–1.000	0.173	0.155–0.194	5
19	11	0.064–0.500	0.174	0.115–0.262	5
7	17	0.064–0.750	0.193	0.146–0.254	5
17	5	0.125–0.380	0.201	0.122–0.330	5
20	7	0.094–0.380	0.227	0.124–0.414	5
81	5	0.190–0.750	0.321	0.149–0.691	5
9	81	0.064–1.000	0.328	0.282–0.381	5
33	14	0.125–1.000	0.340	0.242–0.477	5
13	14	0.125–0.750	0.351	0.250–0.493	5
18	12	0.094–1.000	0.388	0.250–0.603	5
11	12	0.094–1.000	0.511	0.331–0.787	5

[a] Only data for alleles that were represented by at least five isolates are shown.
[b] There were no alterations in the five amino acid positions F504, A510, I515, H54,1 and I566.
[c] The PBP2 encoded by *penA111* differs from susceptible PBP2 by one amino acid residue (P551).

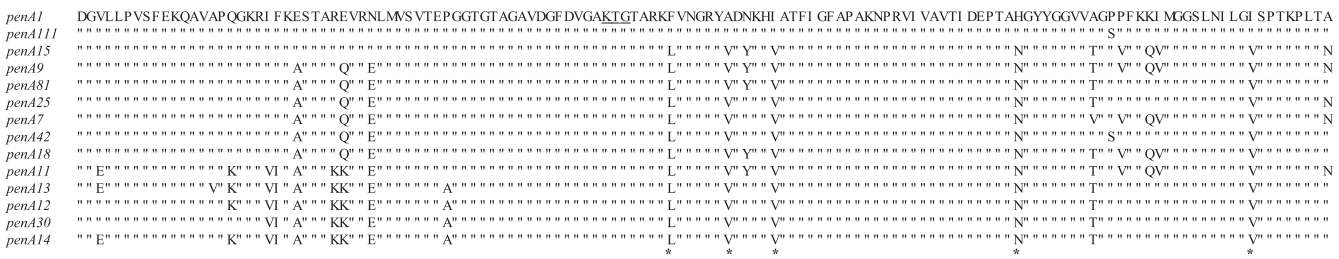

FIG. 4. Partial sequences of the C-terminal part of PBP2 (amino acids 441 to 574) deduced from the sequences of *penA* genes from isolates of *N. meningitidis* tested in the present study. Only the PBP2 encoded by the alleles that were represented by at least five isolates are shown. Ditto symbols (″) indicate identical residues. Polymorphic residues are indicated in one-letter code. The five positions that were always modified in Pen^{I} isolates are indicated by asterisks (F504, A510, I515, H541, and I566). The KTG motif is underlined. The corresponding alleles are indicated on the left. PBP2 from susceptible *penA* alleles were identical except for PBP2 encoded by *penA111* that differed by one residue (P551). Both of these wild-type PBP2 sequences are shown.

(Fig. 5A). Indeed, 65 of the 99 isolates belonging to the serogroup W135 (66%) harbored altered PBP2 proteins. Moreover, 59 of these isolates shared the same altered allele (*penA14*), which was also one of the most frequent altered *penA* alleles in the present study and was detected in 106 isolates. Isolates of serogroup A were mostly from Africa and harbored *penA*^{ps} alleles (particularly *penA83*). Only one serogroup A isolate (from Romania) harbored an altered *penA* allele (*penA32*) that was the most frequent altered *penA* allele in Romania.

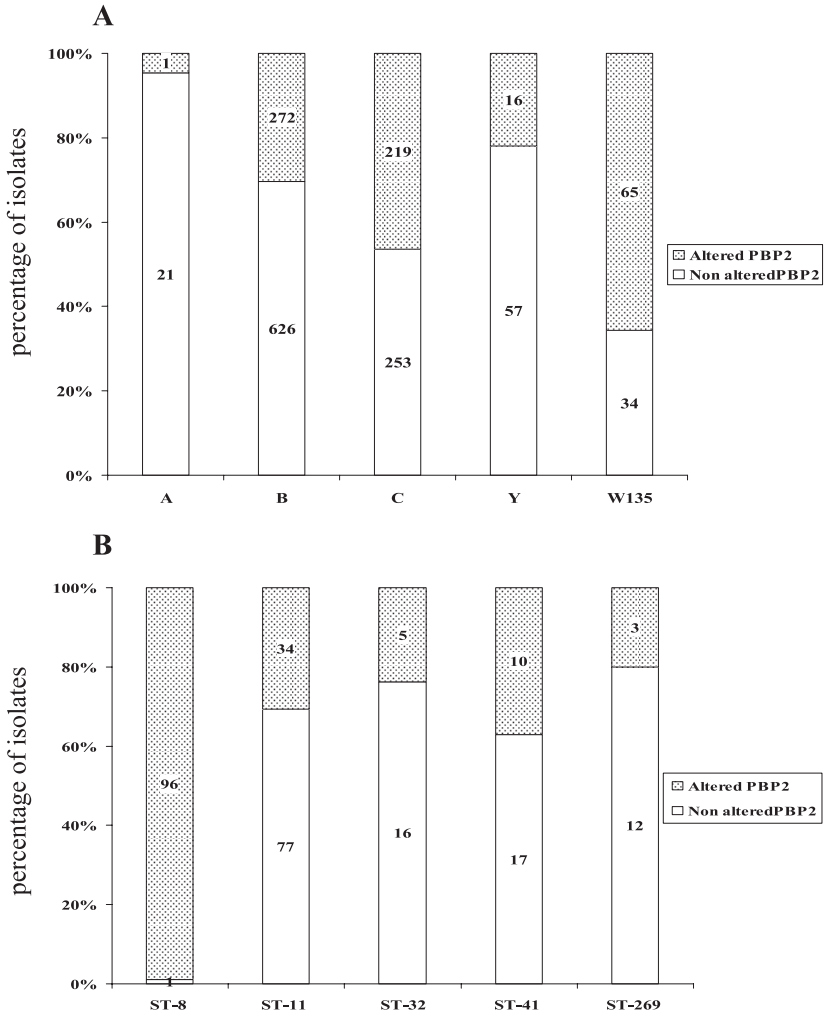

FIG. 5. Distribution of isolates with altered or nonaltered PBP2 according to serogroup (A) or according to ST (B). The number of isolates is also indicated for each corresponding category of isolates.

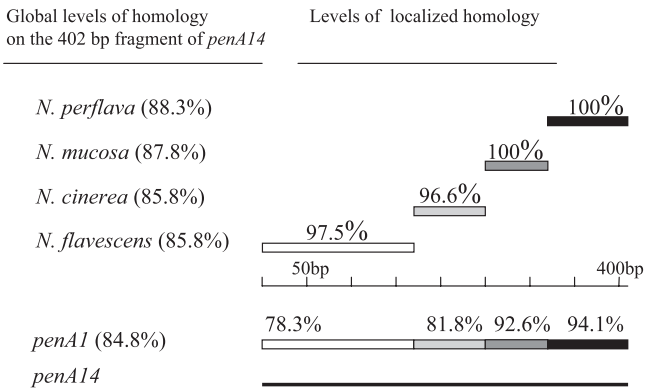

FIG. 6. Schematic and combined representation of DNA homology between *penA14* and the corresponding *penA* genes from other *Neisseria* species: *N. perflava* (accession number X76423), *N. mucosa* (accession number X59635), *N. cinerea* (accession number Z17310), and *N. flavescens* (accession number M26645). The global homology on the 402-bp fragment between *penA14* and each of these *penA* genes, as well as with meningococcal *penA1*, are indicated in parentheses on the left. The levels of localized homology with different segments of *penA* are indicated on the right.

The association between an altered PBP2 and the major invasive STs (ST-8, ST-11, ST-22, ST-32, ST-41, and ST-269) was analyzed for the 636 isolates with determined STs. A significant association with alterations of PBP2 was observed in the case of ST-8 isolates ($P < 0.0001$) (Fig. 5B). These isolates were mostly of the phenotype C:2b:P1.5,2. Moreover, all of these isolates shared the same altered *penA* allele (*penA12*). Most of these isolates were from Spain and Italy.

A higher percentage of altered PBP2 was observed in ST-22 isolates, but a statistical test could not be performed due to the low number of isolates. Several of the ST-22 isolates were of the phenotype W135:NT:NST. No other obvious association was observed among a particular ST and altered *penA* alleles.

FetA types were obtained for 208 isolates. No obvious correlation was observed between FetA types and *penA* alleles. For instance, 16 different FetA types were with *penA1* allele. The same FetA type was observed with several *penA* alleles. For instance, FetA type 1-5 was observed in 19 isolates showing $penA^{ps}$ alleles (*penA1*, *penA3*, *penA5*, *penA22*, and *penA57*) or altered alleles (*penA7*, *penA19*, *penA131*, and *penA135*).

Mosaic structure of altered *penA* alleles. BLAST analysis was performed on the altered *penA* alleles and detected substantial homologies with *penA* genes from other *Neisseria* species, in particular with commensal species (data not shown). For instance, one of the most frequent altered allele (*penA14*) showed identity scores of 88.3, 87.8, 85.8, and 85.8% with the corresponding 402 bp of *N. perflava*, *N. mucosa*, *N. cinerea*, and *N. flavescens*, respectively (Fig. 6). However, this 402 bp corresponding to *penA14* shared an identity of 84.8% with the most frequent allele (*penA1*). Analysis for identity and recombination sites between *penA14* and the four *penA* genes from *N. perflava*, *N. mucosa*, *N. cinerea*, and *N. flavescens* allowed the construction of a combined illustration demonstrating regions of high homology and even complete identity between *penA14* and the other four *penA* genes from the commensal *Neisseria* species (Fig. 6). Analysis using the START package revealed significant putative recombination sites after nucleotides 87 and 147 on *penA14* with *penA* genes of *N. cinerea* and *N. perflava*, respectively. These results strongly suggest a mosaic structure of *penA14*. Similar results were obtained when other altered *penA* alleles were analyzed (data not shown).

DISCUSSION

The prevalence of resistance due to beta-lactamase production is very low among isolates of *N. meningitidis*, and the modifications of PBP2 appear to be the most common mechanism of resistance to penicillin G (25). This mechanism of resistance is clinically highly relevant since it is being detected in an increasing number of invasive isolates (6). Moreover, the collection of isolates tested in the present study represents isolates with similar phenotypic and genotypic characteristics to invasive strains currently circulating worldwide, particularly in Europe (21). Amoxicillin and cefotaxime (a broad-spectrum cephalosporin) MICs were higher among Pen^I isolates, suggesting that cross-resistance to other beta-lactams may be of clinical concern (6). This cross-resistance was also reported in the closely related species, *N. gonorrhoeae*, where resistance to penicillin G and cefixime has been described to be associated with alterations in *penA* (4). The alterations of *penA* that seem to be directly linked to the Pen^I phenotype are located in the transpeptidase-encoding domain (5). Five polymorphic sites that changed their corresponding amino acid residues were detected in all isolates with MICgm ranging between 0.112 and 0.511 mg/liter (Table 2). Additional polymorphic sites were also observed, but not in all altered *penA* alleles, and may correspond to allelic association with some genotypes. A significant finding of the present study is a molecular structure-based definition of Pen^I isolates. Indeed, for alleles represented by at least five isolates, an MICgm of >0.094 mg/liter always correlated with altered *penA* alleles and five modified amino acid positions in PBP2 (F504, A510, I515, H541, and I566). The isolates showing lower MICgm correlated well with the presence of $penA^{ps}$ alleles (Table 2). One definition of Pen^I isolates could be a combination of an MIC of >0.094 mg/liter with alterations of *penA* encoding the five modified positions in PBP2. The amoxicillin MIC, when available, also correlated well with this definition. The MICs of penicillin G and amoxicillin can thus be used together to better define Pen^I isolates (see http://neisseria.org/nm/typing/penA). Alleles that were represented by three or four isolates also fit well with this breakpoint (data not shown). However, discrepancies were observed for alleles that were represented by less than three isolates. Some rare intermediate isolates may also lack some of the alterations as was previously described (31). The low number of isolates may be responsible for disagreement in the correlation between wild-type/altered *penA* and a susceptible or intermediate susceptibility to penicillin G. Moreover, disagreement in MIC determination between laboratories may also account for this discrepancy (33). Alternatively, other mechanisms may be responsible for reduced susceptibility to penicillin G among rare isolates that showed an MIC of >0.094 mg/liter with no alteration in *penA* (22).

The most common alleles (*penA1* and *penA3*) were found in isolates belonging to the major hypervirulent genotypes (in particular ST-11 isolates). However, several susceptible isolates of ST-11 showed different susceptible $penA^{ps}$ alleles, sug-

gesting a higher diversity of *penA*, most likely through horizontal DNA exchanges, compared to the housekeeping genes used in MLST analysis. The three most frequent altered *penA* alleles (*penA12*, *penA14*, and *penA9*) were found in several different genotypes. The preferential association between isolates of ST-8 and *penA12* may reflect a particular geographic spread of these isolates. Indeed, several of these isolates were from Spain and were responsible for an epidemic wave of meningococcal disease during 1996 and 1997 (2). The association of *penA14* and ST-22 isolates needs to be confirmed. ST-22 isolates are frequently encountered in carriage state (http://pubmlst.org), suggesting that the distribution of *penA* alleles among carriage isolates may differ from that observed among invasive isolates. Altered alleles were genetically diverse and harbored fewer isolates in each allele than susceptible alleles. The majority of these altered alleles (61 of 101) were represented by only one isolate. The distribution of these altered alleles is unlikely to be due to inclusion bias as was observed for France, where all invasive isolates were included since the year 2005. These results suggest frequent emergence of altered alleles without clonal expansion of a particular altered *penA* allele among invasive isolates. This may be due to a biological cost of the modification of *penA* that may decrease meningococcal survival by, for example, enhancing bacterial clearance in blood and hence reducing meningococcal virulence. This would diminish clonal expansion. Supporting this hypothesis are the heterogeneous phenotypes and genotypes of the isolates that harbored altered *penA* alleles of the cluster of *penA9* allele (Fig. 2). Indeed, modifications of PBP2 in meningococci have been shown to provoke changes in peptidoglycan structure, with increasing amounts of pentapeptide-containing muropeptides indicating a defect in peptidoglycan biosynthesis (5). Meningococcal muropeptides showed variable activation of the transcription factor NF-κB pathway (15).

Our data suggest that altered *penA* alleles are most likely to appear through interspecies recombination with other *Neisseria* species, as also suggested by previous studies (26, 34). Interestingly, the allele *penA14* showed higher levels of homology with *penA* genes of *N. perflava*, *N. mucosa*, *N. cinerea*, and *N. flavescens* than with the most common meningococcal *penA* susceptible allele (*penA1*). Evidence of a mosaic-like structure in the *penA14* allele was detected (Fig. 6), suggesting that the *penA14* allele evolved by recombination with *penA* from other *Neisseria* species. In *N. gonorrhoeae*, some regions in the transpeptidase-encoding domain in this *penA* gene were also similar to those in the *penA* genes of *N. meningitidis*, *N. perflava*, *N. cinerea*, and *N. flavescens* (4). These latter three commensal species are intrinsically less susceptible to penicillin G than meningococcal isolates. The detection of several points of crossovers on *penA14* may indicate independent events of recombination and random crossovers points. The data presented here are valuable in establishing the molecular basis for the phenotypic and/or genotypic surveillance of meningococcal resistance to beta-lactam antibiotics. These findings also provide a database that may contribute to the analysis of genetic exchange among the *Neisseria* species.

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