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Total Variation in the *penA* Gene of *Neisseria meningitidis*: Correlation between Susceptibility to β-Lactam Antibiotics and *penA* Gene Heterogeneity

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In recent decades, the prevalence of *Neisseria meningitidis* isolates with reduced susceptibility to penicillins has increased. The intermediate resistance to penicillin (Pen^i) for most strains is due mainly to mosaic structures in the *penA* gene, encoding penicillin-binding protein 2. In this study, susceptibility to β-lactam antibiotics was determined for 60 Swedish clinical *N. meningitidis* isolates and 19 reference strains. The *penA* gene was sequenced and compared to 237 *penA* sequences from GenBank in order to explore the total identified variation of *penA*. The divergent mosaic alleles differed by 3% to 24% compared to those of the designated wild-type *penA* gene. By studying the final 1,143 to 1,149 bp of *penA* in a sequence alignment, 130 sequence variants were identified. In a 402-bp alignment of the most variable regions, 84 variants were recognized. Good correlation between elevated MICs and the presence of *penA* mosaic structures was found especially for penicillin G and ampicillin. The Pen^i isolates comprised an MIC of >0.094 μg/ml for penicillin G and an MIC of >0.064 μg/ml for ampicillin. Ampicillin was the best antibiotic for precise categorization as Pen^s or Pen^i. In comparison with the wild-type *penA* sequence, two specific Pen^i sites were altered in all except two mosaic *penA* sequences, which were published in GenBank and no MICs of the corresponding isolates were described. In conclusion, monitoring the relationship between *penA* sequences and MICs to penicillins is crucial for developing fast and objective methods for susceptibility determination. By studying the *penA* gene, genotypical determination of susceptibility in culture-negative cases can also be accomplished.

Neisseria meningitidis (meningococci) is a widespread human pathogen causing meningitis and septicemia (16). During the last decades, there have been several reports from different countries of *N. meningitidis* with reduced susceptibility to penicillin G (6, 14). This is of general concern since penicillin is the first-line antibiotic for treatment of meningococcal disease (17). The intermediate resistant isolates, Pen^i, have previously been defined by MICs of >0.064 μg/ml to ≤1.0 μg/ml by using Etest (8) and MICs of >0.064 μg/ml to 0.5 μg/ml by using the agar dilution method (7, 9). This intermediate resistance has been reported to be due mainly to alterations in the structure of penicillin-binding protein 2 (PBP2), encoded by the *penA* gene (3, 20). The *penA* genes of susceptible isolates, the so-called wild-type *penA* gene (1,745 bp total in MC58 [23]), seem to be highly conserved in their DNA sequence. However, the genes of Pen^i isolates are fairly variable and highly divergent from the wild-type *penA* gene (3, 9, 21). These variations have been suggested to be due to genetic exchange through transformation between *N. meningitidis* and nonpathogenic commensal neisserial species, for example, *Neisseria flavescens* (19). Due to this transformation, the *penA* gene of Pen^i isolates has a mosaic structure, consisting of regions that are essentially identical to those in susceptible isolates and regions that are 14% to 23% divergent in sequence (20). The polymorphisms are located mainly in the last two-thirds of the gene that encodes about 400 amino acids at the C-terminal part of the protein (5).

Sweden is a country with low incidence of meningococcal disease at present, i.e., an incidence of 0.5 to 0.9 cases per 100,000 inhabitants in 1997 to 2005 (The Swedish Institute for Infectious Disease Control, http://gis.smittskyddsinstitutet.se/mapapp/build/11-109000/table/Meningococci_eng_year_all.html, accessed 23 March 2006). There has been an increase in the number of invasive *N. meningitidis* isolates with reduced susceptibility to penicillin G during the last decade. Consequently, in 2005, 23% of the Swedish invasive isolates comprised the Pen^i phenotype (MIC > 0.064 μg/ml) compared to 5% in 1996 (12).

The reports of increasing numbers of circulating *N. meningitidis* strains with the Pen^i phenotype emphasize the need for fast and objective methods for the determination of susceptibility to penicillins. Hence, the aims of the present study were to explore the total reported and presently identified variation in the *penA* gene and to describe the detailed association between *N. meningitidis penA* sequences and the MICs of mainly different penicillins. This would also provide a way to determine penicillin susceptibility of culture-negative cases of meningococcal meningitis/septicemia as well as to approach the development of an objective control system for both phenotypic and genetic penicillin susceptibility testing.

Consequently, in the present study, the total variation of the *penA* gene in *N. meningitidis* was examined. In addition, the MICs of penicillin G, penicillin V, ampicillin, cefuroxime, and cefotaxime were determined to explore the correlation with altered *penA* genes. Penicillin G was chosen, as being consid-

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ered a first-line antibiotic, along with penicillin V as well as ampicillin to evaluate whether the Pen^i phenotype would be easier to distinguish when their MICs were compared to *penA* sequences. Another reason to include penicillin V, which normally is not used for treatment of meningococcal disease, was that all commensal or carrier strains of *N. meningitidis* are exposed to a high pressure of antibiotics in the society, including penicillin V. Cefotaxime was included as a representative of effective cephalosporins and cefuroxime as a less satisfactory cephalosporin.

MATERIALS AND METHODS

Bacterial isolates and clinical samples. Sixty clinical isolates, invasive ($n = 55$) and carrier isolates ($n = 5$), of *N. meningitidis* collected in Sweden between 1996 and 2004 and 17 *N. meningitidis* strains, previously used in an antibiotic susceptibility study performed by the European Monitoring Group on Meningococci (EMGM) (26), were examined. The clinical isolates were selected to represent all of the different MICs of penicillin G found in Sweden (Fig. 1a). Additional isolates comprising MICs in close association to the phenotypical breakpoint for Pen^i were also included. Of the 60 clinical isolates, 27 were phenotypically determined to be susceptible to penicillin G (MIC $\leq$ 0.064 μg/ml) and 33 comprised a reduced susceptibility (Fig. 1a). For comparison, two *N. meningitidis* reference strains, i.e., MC58 (23) and OR173/87 (10), and one *Neisseria gonorrhoeae* reference strain (CCUG 15821) were included in the study.

Five cerebrospinal fluid (CSF) samples derived from patients suffering from meningococcal meningitis were also examined in order to analyze whether the protocol was suitable for direct sequencing from CSF.

Phenotypical antibiotic susceptibility testing. The MICs of penicillin G, penicillin V, ampicillin, cefuroxime, and cefotaxime were determined using the Etest method (AB Biodisk, Solna, Sweden) on Mueller-Hinton agar (Becton, Dickinson and Company, Franklin Lakes, NJ) supplemented with 5% sheep blood at 37°C in 5% CO_2 for 16 to 18 h. The breakpoints used for penicillin G were described by Hughes et al. (susceptible [S], MIC $\leq$ 0.064 μg/ml; resistant [R], MIC $>$ 1 μg/ml) (8). For ampicillin, the breakpoint was described by Jorgensen et al., i.e., an MIC of $<$0.25 μg/ml (S) (9). For the remaining antibiotics, the breakpoints were as follows: penicillin V, MIC $\leq$ 1 μg/ml (S) and MIC $>$ 1 μg/ml (R); cefuroxime, MIC $\leq$ 0.25 μg/ml (S) and MIC $>$ 1 μg/ml (R); and cefotaxime, MIC $\leq$ 0.064 μg/ml (S) and MIC $>$ 1 μg/ml (R) (in accordance with the Swedish Reference Group for Antibiotics, http://www.srga.org, accessed 23 March 2006).

Isolation of genomic DNA. Isolation of bacterial DNA from *N. meningitidis* isolates was performed using a MagNA Pure LC system with DNA isolation kit III (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. For isolation of DNA from the five CSF samples, the samples (100 to 500 μl) were initially centrifuged (8000 $\times$ *g* for 10 min) and then processed in the same way as the bacterial isolates. The DNA preparations were stored at 4°C prior to PCR.

***penA* PCR.** The PCR was performed as previously described by Arreaza and Vázquez (5), with minor modifications. The 50-μl PCR mixture contained 1.25 U AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA) and 1 μl of the genomic DNA template. One of the primers, Mod-Gcdown3, was slightly modified compared to the primer used by Arreaza and Vázquez (Table 1) (5). The PCR conditions were as previously described except that an initial enzyme activation step at 94°C for 10 min was included and, for the CSF samples, there were 40 cycles of amplification instead of 30. MC58 was used as a positive control and distilled water as a negative control. The PCR products were analyzed by electrophoresis through a 2% agarose gel and by ethidium bromide staining. DNA molecular weight marker VI (Roche Diagnostics, Mannheim, Germany) was included on each gel.

DNA sequencing. The PCR products were purified using the High Pure PCR product purification kit (Roche Diagnostics, Mannheim, Germany) and then cycle sequenced. The primers used for the cycle sequencing PCR are shown in Table 1. Each sequencing reaction (10 μl) contained 4 μl of a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Warrington, United Kingdom), 1.6 pmol primer, and 1 μl purified PCR product. The cycle sequencing PCR consisted of 25 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. Subsequently, the products were purified using ethanol-sodium acetate precipitation and resuspended in 10 μl formamide (Applied Biosystems, Warrington, United Kingdom) according to the manufacturer's instructions. The nucleotide sequences were determined using an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Foster City, CA). The sequence of each strand of each compiled sequence was determined.

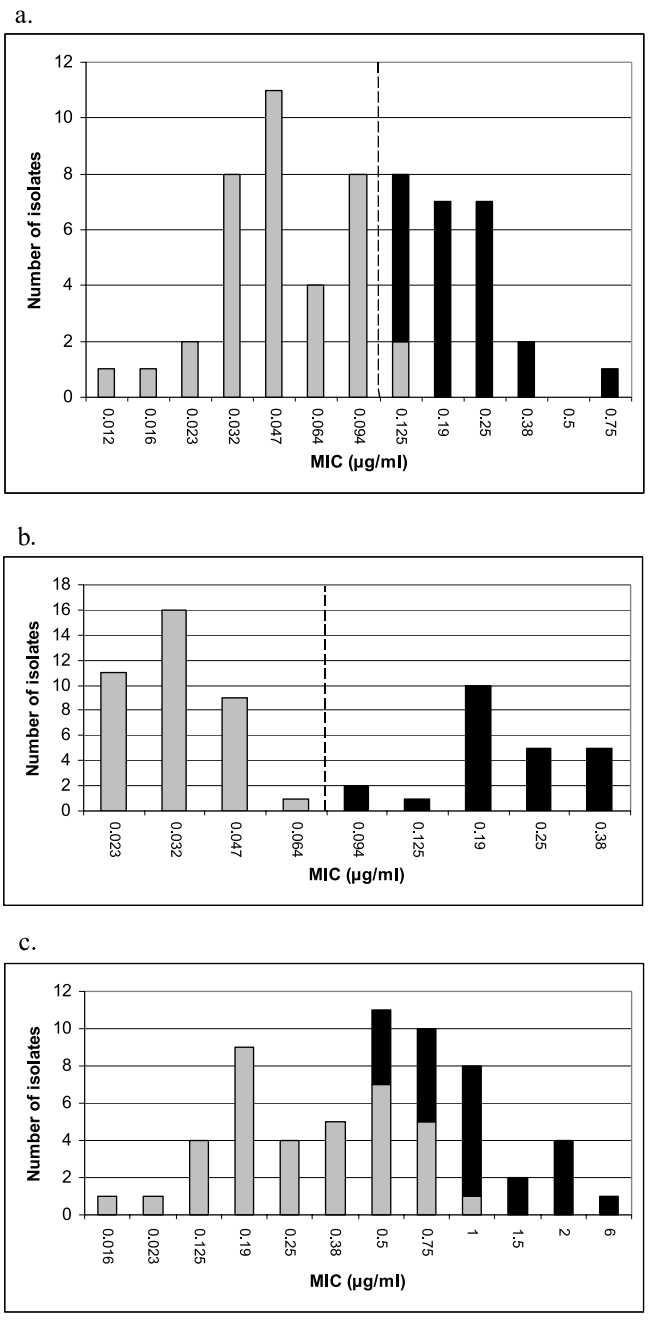


FIG. 1. MICs of penicillin G (a), ampicillin (b), and penicillin V (c) for 60 selected clinical *N. meningitidis* isolates collected in Sweden between 1996 and 2004. Gray bars indicate wild-type *penA* genes, and black bars indicate mosaic structures in the *penA* gene. The broken line in panels a and b indicates the suggested breakpoint for Pen^i isolates (MIC $>$ 0.094 μg/ml for penicillin G and MIC $>$ 0.064 μg/ml for ampicillin).

Sequence analysis. Multiple-sequence alignments (1,149 unambiguously aligned nucleotides) of the final part of the *penA* gene and the corresponding deduced amino acid sequences were performed using the software BioEdit (version 5.0.9) and by manual adjustment. For comparison, *penA* gene sequences from 26 different Pen^i meningococcal isolates with MICs of penicillin G ranging from 0.094 μg/ml to 1.28 μg/ml and 211 *penA* gene sequences from meningo-

TABLE 1. Primers used in PCR and sequencing of the *penA* gene

Primer[a]	Sequence (5′→3′)	Use(s)	Nucleotide location[b]	Source and/or reference
Gcup2	TTTGCACACGTCATCGGATTTAC	PCR and sequencing	523–542	5
Mod-Gcdown3	CGGGGATATAACTGCGGCCGTC	PCR and sequencing	187–166 bp downstream of the stop codon of the *penA* gene	Shortened Gcdown3; 5
Fo	TATACCGCACTGACGCACGAC	Sequencing	1301–1323	5
Ro	GCCGTCGTGCGTCAGTGC	Sequencing	1326–1309	5
AA-1[c]	ATCGAACAGGCGACGATGTC	Sequencing	1237–1256	3
PenA-R2[c]	GCCTGTTTTTCAAAGCTGACC	Sequencing	1358–1338	Present study

[a] Synthesized by Scandinavian Gene Synthesis AB, Köping, Sweden.
[b] According to the nucleotide sequence of the *penA* gene in MC58 (23).
[c] For some of the isolates, the primers Fo and Ro did not work due to polymorphism in the annealing site and AA-1 and PenA-R2 were used instead.

coccal isolates without depicted MICs, deposited in GenBank, were also included. In fact, all *penA* sequences with a minimum length of the final 1,143 bp deposited in GenBank (1 March 2006) were included in the alignment. A shorter multiple-sequence alignment of 402 bp in the end of the *penA* gene sequences, which has been suggested to be sufficient for the identification of mosaic-structured *penA* genes (EMGM Working Group on Antibiotics and M.-K. Taha, personal communication), was also performed.

For the identification of different sequence variants, phylogenetic trees were constructed with TREECON (version 1.3b) software as previously described (24).

RESULTS

Total variability in *penA* gene. In total, 321 meningococcal *penA* gene sequences were examined. The phylogenetic tree of the different sequence variants (based on the 1,149-bp alignment) identified one highly homogeneous group, with only a few nucleotide polymorphisms (Fig. 2a). Within this homogeneous group, 138 sequences were found. Between these 138 sequences, there were a maximum of 1% nucleotide difference and no recognizable mosaic structures, and all of the Pen^s isolates were found in this group. Based on these observations, the *penA* gene sequences included in this group were designated wild-type *penA* gene sequences. Among the remaining *penA* gene sequences (n = 183), 22% of the nucleotides differed between the two most divergent sequences (A30 and A97) (Fig. 2a). All of these sequences (n = 183) comprised obvious mosaic structures; however, the extent of mosaic structures varied substantially between the sequences, spanning from over about 100 bp to the whole sequence examined.

When the 1,149-bp multiple-sequence alignment of the mosaic-structured *penA* genes (n = 183) was studied, 87 different nucleotide sequence variants, which coded for 74 PBP2 amino acid sequence variants, were identified (Fig. 2a). In the alignment, a total of 409 polymorphic nucleotide sites were identified. When the deduced amino acid sequences (383 unambiguously aligned amino acids) were studied, a total of 103 polymorphic amino acid sites were identified. In the 402-bp multiple-sequence alignment of the mosaic sequences (n = 182; one sequence was excluded due to lack of mosaic structure in this shorter segment), 66 different nucleotide sequence variants, which coded for 36 PBP2 amino acid sequence variants, were identified (Fig. 2b). One hundred forty-two polymorphic nucleotide sites and 36 polymorphic amino acid sites were identified (134 unambiguously aligned amino acids in total). For the wild-type *penA* sequences (n = 138), 43 different nucleotide sequence variants and 17 PBP2 amino acid sequence variants were found when the 1,149-bp alignment was studied and 18 nucleotide and 7 PBP2 variants were found when the 402-bp alignment was studied. Thirty-eight polymorphic nucleotide sites and 16 polymorphic amino acid sites were identified in the 1,149-bp alignment, and 15 polymorphic nucleotide sites and 6 polymorphic amino acid sites were identified in the 402-bp alignment.

The sequences of the divergent mosaic alleles differed by 3% to 24% compared to the wild-type sequence, represented by MC58, when 1,149 bp of the *penA* gene was studied. When we studied 402 bp, the difference was 7% to 23% compared to the wild-type sequence.

***penA* gene versus MIC.** Of the clinical isolates with the Pen^i phenotype (n = 33), all isolates with an MIC of >0.125 μg/ml (n = 17) and 75% of the isolates with an MIC of 0.125 μg/ml of penicillin G had mosaic structures in the *penA* gene (Fig. 1a). For ampicillin, all clinical isolates with an MIC of >0.064 μg/ml (n = 23) had mosaic-structured *penA* genes (Fig. 1b). For penicillin V, a somewhat lower correlation between MICs and mosaic-structured *penA* genes was found (Fig. 1c). For cefuroxime, no absolute correlation was found either; all isolates with an MIC of >0.094 μg/ml (n = 21) showed mosaic structures in the *penA* gene, but mosaic structures were also found in one of three isolates with an MIC of 0.094 μg/ml and one of eight isolates with an MIC of 0.064 μg/ml. All of the isolates were fully susceptible to cefotaxime, and hence, no correlation with *penA* gene sequences was possible to determine.

In addition, all 11 "EMGM strains" with reduced susceptibility to penicillin G also had mosaic structures in the *penA* gene. For ampicillin and penicillin V, all isolates with an MIC of >0.047 μg/ml (n = 11) and an MIC of >0.38 μg/ml (n = 11), respectively, had mosaic structures in the *penA* gene. For cefuroxime, all isolates with an MIC of >0.125 μg/ml (n = 11) showed mosaic structures, and as for the clinical isolates, no correlation was possible to determine between the low MICs of cefotaxime and *penA* gene sequences.

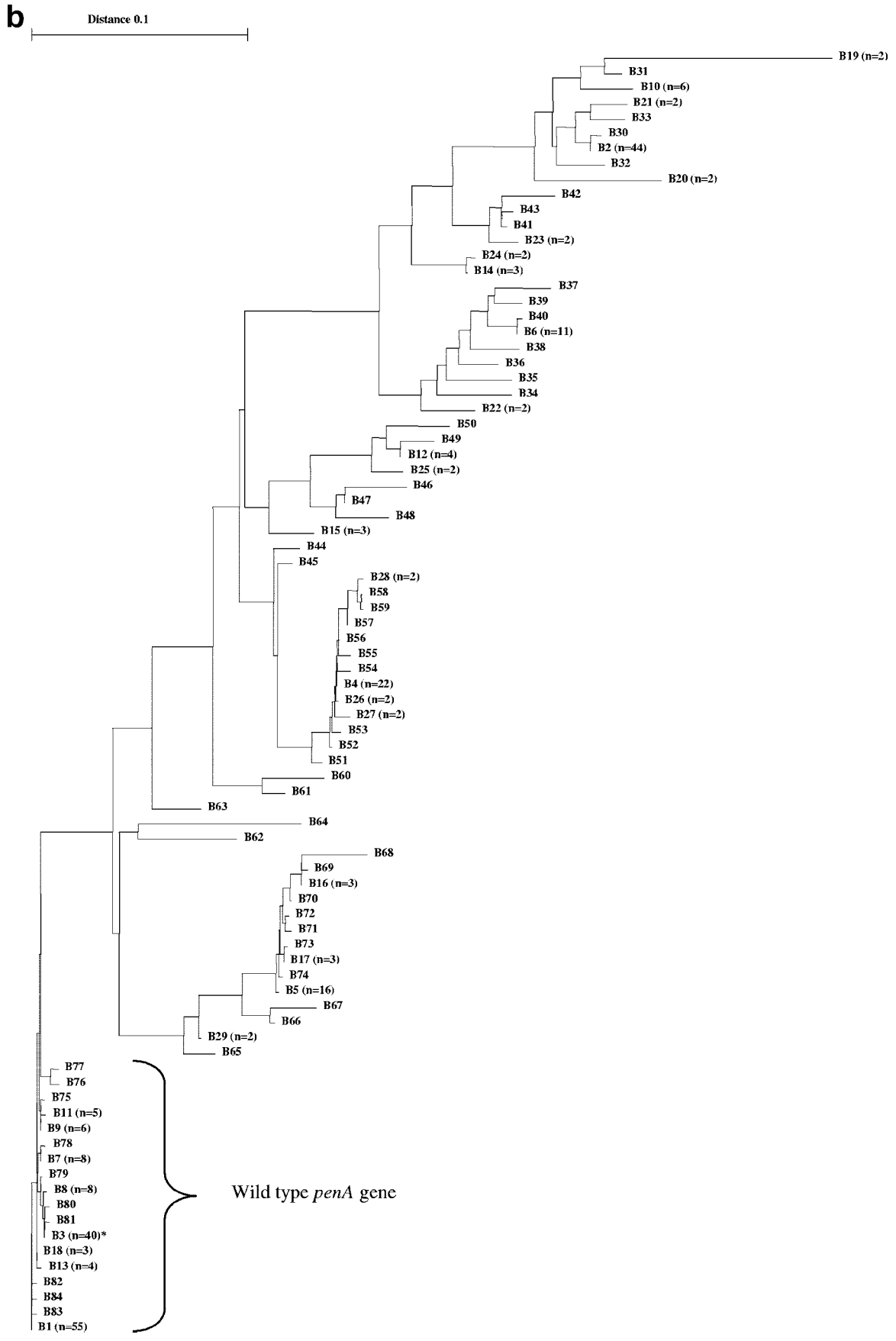
Covariation between elevated MICs of penicillin G, penicillin V, ampicillin, and, to some extent, cefuroxime could be observed. The low MICs of cefotaxime of the isolates were unaffected by the elevated MICs for the other antibiotics.

Based on the reported correlation between MIC and *penA* gene sequence in the present study, Pen^i isolates could be defined as the ones comprising an MIC of >0.094 μg/ml for penicillin G and an MIC of >0.064 μg/ml for ampicillin by using the Etest method.

All of the 26 *penA* sequences, collected from GenBank, from


a
Distance 0.1
A30 (n=2)
A9 (n=6)
A29 (n=2)
A75
A74
A73*
A101
A28 (n=2)
A72
A71
A70
A69
A68
A26 (n=2)
A27 (n=2)
A25 (n=2)
A67
A40
A22 (n=2)
A41
A90
A18 (n=2)
A100
A19 (n=2)
A39
A20 (n=2)
A21 (n=2)
A42
A16 (n=3)
A43
A44
A45
A12 (n=4)
A46
A49
A50
A48
A47
A6 (n=8)
A51
A52
A53
A54
A57
A58
A55
A56
A59
A61
A60
A62
A63
A64
A65
A66
A24 (n=2)
A23 (n=2)
A76
A78
A80
A79
A31 (n=2)
A77
A32 (n=2)
A87
A86
A13 (n=4)
A2 (n=39)
A85
A7 (n=8)
A84
A83
A82
A81
A93
A92
A88
A89
A38 (n=2)
A91
A4 (n=16)
A95
A96
A94
A97
A98
A99
A114
A113
A115
A15 (n=4)
A110
A109
A14 (n=4)
A111
A10 (n=5)
A112
A11 (n=5)
A116
A36 (n=2)
A117
A118
A5 (n=9)
A121
A120
A8 (n=6)
A122
A17 (n=3)
A119
A123
A125
A124
A104
A34 (n=2)
A103
A102
A130
A129
A33 (n=2)
A108
A35 (n=2)
A107
A106
A105
A126
A3 (n=19)
A37 (n=2)
A128
A127
A1 (n=44)
Wild type penA gene

FIG. 2


b
Distance 0.1
B19 (n=2)
B31
B10 (n=6)
B21 (n=2)
B33
B30
B2 (n=44)
B32
B20 (n=2)
B42
B43
B41
B23 (n=2)
B24 (n=2)
B14 (n=3)
B37
B39
B40
B6 (n=11)
B38
B36
B35
B34
B22 (n=2)
B50
B49
B12 (n=4)
B25 (n=2)
B46
B47
B48
B15 (n=3)
B44
B45
B28 (n=2)
B58
B59
B57
B56
B55
B54
B4 (n=22)
B26 (n=2)
B27 (n=2)
B53
B52
B51
B60
B61
B63
B64
B62
B68
B69
B16 (n=3)
B70
B72
B71
B73
B17 (n=3)
B74
B5 (n=16)
B67
B66
B29 (n=2)
B65
B77
B76
B75
B11 (n=5)
B9 (n=6)
B78
B7 (n=8)
B79
B8 (n=8)
B80
B81
B3 (n=40)*
B18 (n=3)
B13 (n=4)
B82
B84
B83
B1 (n=55)
Wild type penA gene

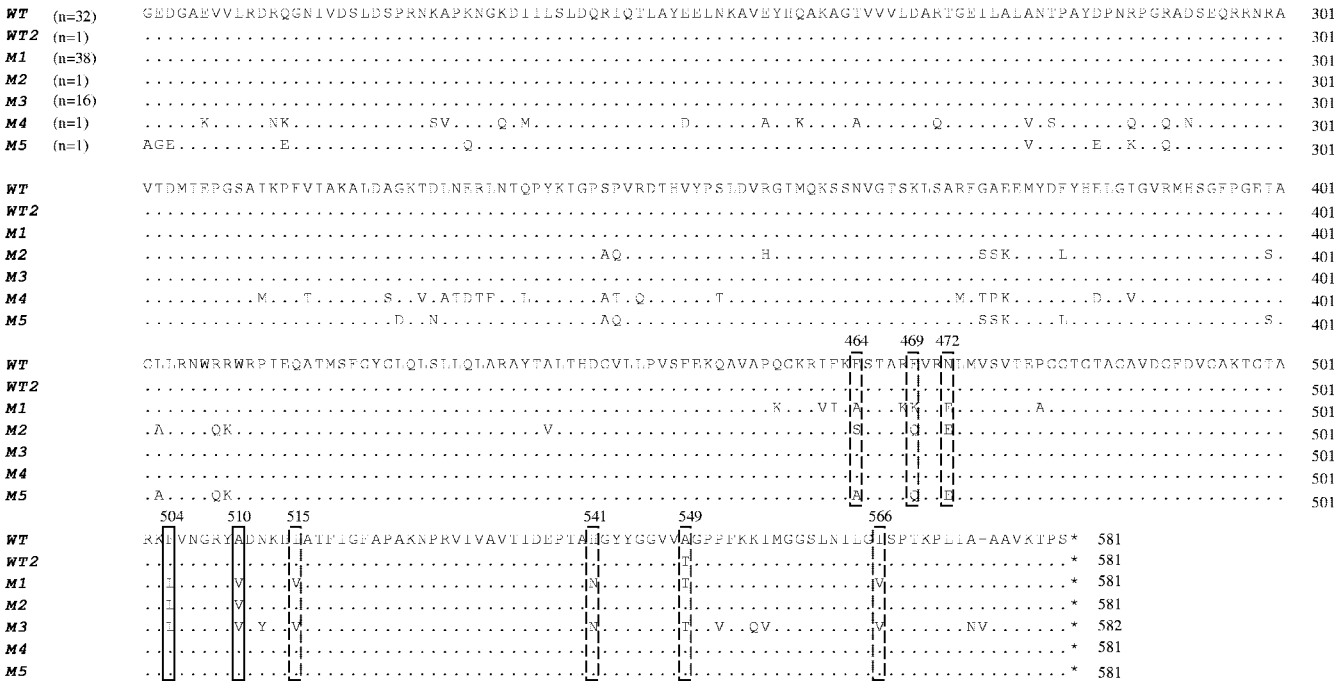


FIG. 3. Multiple-sequence alignment of seven different partial PBP2 amino acid sequences, which include amino acids 202 to 581 of the native protein in MC58 (23), comprising different numbers of the amino acid alterations (in boxes) found in Pen^i isolates (2, 21, 22). The two consistently lined boxes indicate the alterations found in all of the phenotypically confirmed Pen^i isolates. The wild-type sequences (WT) are represented by MC58. WT2 is a wild-type sequence containing one of the alterations (accession no. AY292992). Mosaic sequence 1 (M1) comprises all of the alterations, M2 lacks alterations 515, 541, 549, and 566, and M3 lacks alterations 464, 469, and 472. M4 (accession no. AY127670) comprises wild-type structure in the final 550 bp of the *penA* gene, and M5 (accession no. AF515100) contains wild-type structure in the final 250 bp. For confirmation of the sequences, one representative of each sequence variant was PCR amplified and sequenced twice. No confirmation was possible to perform for M4 and M5, which were downloaded from GenBank.

Pen^i strains proved to have mosaic-structured *penA* genes. Consequently, a total of 70 isolates with both the Pen^i phenotype and mosaic structures in the *penA* gene were examined. Among these isolates, 34 different *penA* nucleotide sequence variants and 31 different PBP2 amino acid sequence variants were identified when the 1,149-bp alignment was examined. In the 402-bp alignment, 27 *penA* nucleotide sequence variants and 15 PBP2 variants were identified. No obvious correlation between individual MICs and any specific *penA* sequence variants or cluster of variants was found. Consequently, no *penA* mosaic allele was predominant to give a particular MIC.

Pen^i-specific sites. According to previous studies (2, 21, 22), in comparison with that of the wild-type *penA* gene, between five and nine specific nucleotides are altered in the *penA* mosaic allele of all Pen^i isolates. All of these polymorphic nucleotides result in alterations in the corresponding encoded amino acids, which are located in the C-terminal part of PBP2. In the present study, none of these five to nine specific Pen^i sites was altered in any of the identified divergent *penA* mosaic alleles. Two of the specific Pen^i sites were altered in all except two (accession no. AF515100 and AY127670) of the *penA* mosaic-structured sequences. These sequences lacked mosaic patterns in the end of the *penA* gene, i.e., in the last 250 bp and 550 bp of the gene, respectively, and hence, none of the specific Pen^i sites was altered (Fig. 3).

Cerebrospinal fluid samples. Of the five CSF samples, four were culture negative and hence could not be phenotypically analyzed regarding antibiotic susceptibility. The single culture-positive isolate was susceptible to penicillin G. The *penA* gene of *N. meningitidis* in the five CSF samples was successfully amplified and sequenced. One of the culture-negative samples had an altered *penA* gene with mosaic structure.

DISCUSSION

The *penA* genes of Pen^i isolates are fairly variable, in most cases, all over the last two-thirds of the gene. The large amount of more or less evenly spread alterations indicates that the mosaic-structured *penA* genes are most likely due to many

FIG. 2. (A) Phylogenetic tree based on a 1,149-bp alignment of *penA* gene sequences of *N. meningitidis* (n = 321). (B) Phylogenetic tree based on a 402-bp alignment of *penA* gene sequences of *N. meningitidis* (n = 321). In both trees, one homogeneous group, comprising wild-type *penA* genes, was identified. The remaining sequences in both trees were highly divergent and displayed obvious mosaic patterns. One single sequence (*, accession no. AY127670) was identified as a mosaic allele when the larger segment was examined but as the wild type when only 402 bp was examined.

different genetic events and/or genetic exchange with many different donors.

In the present study, two different sequence alignments were made, one of the final 1,149 bp of the *penA* gene and one of 402 bp in the end of the gene. One single sequence was not properly categorized as a *penA* mosaic allele in the 402-bp alignment because it lacked mosaic patterns in the final 550 bp of the gene. However, when the longer alignment was examined, this sequence (accession no. AY127670) was correctly categorized as a mosaic *penA* allele (Fig. 2a and b). This clearly illustrates the risks associated with studying only a shorter segment of a gene.

The results of phenotypic antibiotic susceptibility testing of *N. meningitidis* are hard to value in, for example, interlaboratory comparisons. This is due mainly to significant differences in the media and critical MICs used in different laboratories (26). In the present study, we found a good correlation between elevated MICs and the presence of mosaic-structured *penA* genes, especially for penicillin G and ampicillin but to a somewhat smaller extent for penicillin V and cefuroxime. Based on our findings, using Etest and *penA* gene sequencing, Pen^i isolates could be defined as the ones comprising an MIC of >0.094 μg/ml for penicillin G and an MIC of >0.064 μg/ml for ampicillin. However, these are not clinical breakpoints but epidemiologic definitions. In addition, it is important to note that the number of tested clinical isolates is rather limited ($n = 60$) and that the difference between MICs for penicillin G and ampicillin is very small in the present study. In an earlier study (4), Arreaza and coworkers proposed an MIC of >0.064 μg/ml to be a suitable breakpoint for penicillin G. Their breakpoint is slightly lower than ours, but again, the results of the phenotypic antibiotic susceptibility testing are hard to compare between laboratories.

Both in the present study and in earlier studies (4, 9), the correlation between elevated MICs and the presence of mosaic-structured *penA* genes was even higher for ampicillin than for penicillin G (Fig. 1). Determining the MIC of ampicillin might therefore be a sharper way to categorize *N. meningitidis* isolates into the Pen^s or Pen^i group.

PCR-based methods are increasingly being used for diagnosing meningococcal infection and for characterizing bacteria (10, 11, 13, 25). The protocol for *penA* sequencing, used in the present study, also proved to be effective for culture-negative CSF samples. Hence, in addition to the already existing genetic methods, the present *penA* sequencing can be used to accomplish further characterization of culture-negative samples from patients suffering from meningococcal disease. Another advantage with the *penA* sequencing protocol is that it can also be utilized for *N. gonorrhoeae*. In the *N. gonorrhoeae* population, only a small part is still fully susceptible to penicillins and altered *penA* genes have been described previously (1, 18).

Previous studies have proposed that in comparison with those of the wild-type *penA* gene, between five and nine specific nucleotide positions, and the corresponding amino acids, are altered in all Pen^i isolates (2, 21, 22). In our *penA* sequence collection, none of those specific Pen^i sites was altered in any of the identified divergent *penA* mosaic alleles. Two of the alterations ($Phe_{504} \rightarrow Leu_{504}$ and $Ala_{510} \rightarrow Val_{510}$) were found in all mosaic-structured *penA* genes, except for one sequence having no mosaic patterns in the final 550 bp of the gene and one having no mosaic patterns in the final 250 bp (Fig. 3). However, we have no evidence that these two isolates really belong to the Pen^i group, since we have only the *penA* sequences and no MICs. In addition, one of the clinical Pen^i isolates had no mosaic patterns in the final 220 bp of the gene and hence lacked three of the alterations found in all other mosaic-structured sequences ($Ile_{515} \rightarrow Val_{515}$, $His_{541} \rightarrow Asn_{541}$, and $Ile_{566} \rightarrow Val_{566}$), except for the two discrepant sequences mentioned above. Besides the lack of absolute correlation between alterations in Pen^i-specific sites and mosaic structure, it is worth mentioning that one of the sites was also altered in a wild-type sequence (accession no. AY292992) (Fig. 3).

When individual MICs and *penA* sequence variants were compared within the group of Pen^i isolates, no obvious correlation was found for any of the antibiotics. This lack of correlation could be due to the fact that we studied larger segments of the gene instead of trying to identify "hot spots" particularly important for penicillin susceptibility, e.g., active sites that directly affect the affinity to penicillin. In addition, a limited effect on the MIC in individual isolates due to other factors, such as *mtr*, *penB*, *penC*, and *ponA* (15, 18), which influence the penicillin susceptibility in *N. gonorrhoeae*, cannot be excluded. The somewhat lower correlation between the elevated MIC of penicillin V and the mosaic *penA* gene sequence could also be due to these or similar factors. Since penicillin V is a less potent antibiotic, compared to penicillin G and ampicillin, for *N. meningitidis*, other pharmacokinetic mechanisms may also be involved. In addition to the genetic factors mentioned above, the drawbacks of phenotypic determination of susceptibility must be taken into account. The results of both the agar dilution method and Etest are highly dependent on the media used (26). A previous study has shown that the sources and batches of the ingredients, e.g., blood, in the medium can also affect the MICs (22). It is therefore very important to standardize the media used, especially to enable interlaboratory comparisons.

In conclusion, an up-to-date description of *penA* gene variability and the relation between *penA* gene sequence and the MIC of penicillins made it possible to identify mosaic structures clearly associated with reduced susceptibility. The level of correlation slightly varied between different antibiotics, and in the present study, ampicillin proved to be the best antibiotic for precise categorization of *N. meningitidis* isolates as Pen^s or Pen^i. By studying the *penA* gene, genotypical determination of susceptibility in culture-negative cases and hence further characterization of these samples can be accomplished.

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