Polymorphism of *Neisseria meningitidis* penA gene associated with reduced susceptibility to penicillin

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We studied polymorphism of *penA* (which encodes penicillin-binding protein 2) in 13 strains of *Neisseria meningitidis* susceptible to penicillin (*penS*) and 12 strains with reduced susceptibility to penicillin (*penI*). These strains differed in geographical origin. Serological and genetic typing showed that they were highly diverse and belonged to several genetic lineages. Restriction analysis and DNA sequencing of *penA* showed that all *penS* strains had the same *penA* allele regardless of genetic group, whereas *penI* strains harboured various *penA* alleles. Transformation with amplicons of *penA* and genomic DNA from several *penI* strains conferred the *penI* phenotype on a *penS* strain. Thus, reduction in susceptibility to penicillin is directly related to changes in *penA* and analysis of *penA* polymorphisms could be used as a reliable tool for characterizing meningococcal strains in terms of their susceptibility to penicillin.

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<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC (mg/L)</th>
<th>Serogroup:serotype: serosubtype</th>
<th>Country/ year isolated</th>
<th>Site</th>
<th>pilA allele</th>
<th>pilD allele</th>
<th>penA allele</th>
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*Abbreviations: CAR, Central African Republic; CSF, cerebrospinal fluid; LNP, Laboratoire des Neisseria, Institut Pasteur, Paris, France; NT, not serotypable; NST, not serosubtypeable.

aPenicillin-resistant transformants obtained after transfer of penA alleles from penI strains W-39 (TR1, TR2), TH-41 (TR3, TR4) and LNP 16454 (TR5, TR6) to susceptible strain LNP 8013.

bPenicillin- and kanamycin-resistant transformant obtained after mixed growth of penI strain LNP 16454 and penS and kanamycin-resistant strain CAP1.
out membrane porins (PorA and PorB). These antigenic structures are subject to strong selection by the host immune response. They are therefore highly variable and are not good markers of genetic relatedness among meningococcal strains. Genetic typing methods have been developed and shown to be more reliable for the characterization of meningococcal strains. Multilocus enzyme electrophoresis (MLEE), multilocus sequence typing (MLST) and multilocus DNA fingerprinting (MLDF) are now generally used to study population genetics and for epidemiological characterization of meningococcal strains.

The aim of this study was to analyse penA polymorphisms in a genetically well-defined collection of meningococcal strains obtained from three national reference centres and to assess the relationship between these polymorphisms and the reduced susceptibility of these strains to penicillin.

Materials and methods

Bacteria and media

The meningococcal strains used in this study and their geographical origins are listed in Table I. The CAP1 strain was derived from strain LNP 8013, as described previously. It harbours pilA::aph3 and is resistant to kanamycin. Bacteria were grown at 37°C under 5% CO2 on G medium with G supplement (Sanofi Diagnostic Pasteur, Marnes La Coquette, France) and on GCB medium (Difco, Detroit, MI, USA) with Kellogg supplements. Penicillin G susceptibility was tested by the agar dilution method with G medium. The strains were tested using inocula of 108 cfu/mL on plates containing penicillin G at 0.06, 0.125, 0.250, 0.500 and 0.750 mg/L. The MIC was defined as the lowest concentration of penicillin G that inhibited visible growth after 18 h of incubation in 5% CO2 at 37°C. Penicillin G susceptibility was also tested by the diffusion method (Etest) on G medium. When needed, kanamycin was added to the medium at 100 mg/L. β-Lactamase activity was detected using Cefinase discs (bioMérieux, Marcy l’Etoile, France).

Serological typing

Serological typing was performed as previously described.

DNA fingerprinting

For genomic DNA extraction, bacteria were suspended in 500 μL of distilled water, subjected to one freeze–thaw cycle, heated at 100°C for 3 min and then centrifuged for 5 min at 10000g. The pilA, pilD and penA genes were amplified by PCR from the supernatant using the oligonucleotide primers listed in Table II. PCR was performed as previously described. The amplicons obtained by PCR corresponding to the pilA (1.8 kb) and pilD (0.9 kb) genes were then digested with AluI, HpaII or TaqI. For penA amplicons (1.7 kb), the restriction enzymes HaeIII, HpaII and TaqI were used separately. An arbitrary number was assigned to each enzyme-digested restriction fragment.

Table II. Oligonucleotides used in this study

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<th>Amplicon length (kb)</th>
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<td>A-212</td>
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assigned to each restriction endonuclease pattern and an allele was defined by three numbers corresponding to the restriction endonuclease patterns obtained for the three enzymes. Restriction digests were analysed by electrophoresis in 5% non-denaturing polyacrylamide gels prepared with 89 mM Tris–borate and 2 mM EDTA (pH 8.0). Restriction profiles were analysed as described previously using the Taxotron package (PAD Grimont, Institut Pasteur, Paris, France).

Analysis of penA nucleotide sequence
The penA gene was amplified by PCR using oligonucleotides 99-1 and 99-2 (Table II). The amplicons were purified on a Sepharose CL-6B column (Pharmacia, Uppsala, Sweden) and sequenced using oligonucleotides 99-16, 99-22, 99-24, 99-27, AA-1, AA-5 and AA-8 (Table II) and a Sequenase PCR product sequencing kit (USB–Amersham, Cleveland, OH, USA). Sequences were aligned using the Multiple Alignment Program (MAP).

Transformation of N. meningitidis
Genomic DNA was prepared from three penI strains of N. meningitidis (W-39, TH-41 and LNP 16454) and used to transform a penicillin-susceptible (pen5) strain, LNP 8013, as described previously. For penA amplicons to be used for transformation, PCR was performed with oligonucleotides 99-14 and 99-23. Oligonucleotide 99-23 contains the uptake sequence necessary for DNA transformation in Neisseria spp. Transformants were selected on GCB medium containing penicillin G at concentrations of 0.125, 0.25, 0.50 and 0.75 mg/L. For genetic transfer during bacterial growth (co-culture experiments), meningococcal strains CAP1 and LNP 16454 were suspended in GCB liquid medium supplemented with 5 mM MgCl2. The optical density at 600 nm was adjusted to 0.6. Suspensions of each strain were mixed together (1:1) and cultured at 37°C under 5% CO2 for 3 h. The mixture was then plated on selective GCB medium containing kanamycin 100 mg/L and penicillin G at 0.125, 0.25, 0.50 or 0.75 mg/L.

Results
Phenotypic analysis of meningococcal strains
To analyse the structure of penA in N. meningitidis, we first set up a collection of 25 meningococcal strains corresponding to various MICs of penicillin G and to various
Polymorphism of meningococcal penA gene

geographical and anatomical sites of isolation (Table I). On the basis of MIC, these strains fell into two categories: penS strains \((n = 13, \text{MIC} < 0.125 \text{mg/L})\) and penI strains \((n = 12, 1 \text{mg/L} \geq \text{MIC} \geq 0.125 \text{mg/L})\). Strains were isolated over a period of 10 years in several countries (Table I). No \(\beta\)-lactamase activity was detected in these strains using Cefinase discs. Serological typing showed that these strains also differed in antigenic formula (serogroup:serotype:serosubtype). All three major serogroups (A, B and C) were represented in our collection (Table I).

Molecular characterization of meningococcal strains

Strains were then typed by MLDF, which involves amplification by PCR of several chromosomal loci and analysis of restriction fragment length polymorphisms (RFLPs). MLDF of the \(\text{pilA}\) and \(\text{pilD}\) genes has been shown to be reliable for characterizing strains and its resolution is as good as that of MLEE.\(^{19,22}\) Distance matrices for \(\text{pilA}\) and \(\text{pilD}\) were used to construct a dendrogram using the Taxotron package. Fifteen different groups were identified among the strains tested in this study (Figure 1). The distribution of pen\(^5\) and pen\(^1\) strains on this dendrogram did not depend on MIC. These results indicated that our collection was composed of different genetic lineages and that both types of strain (pen\(^5\) and pen\(^1\)) were present in several genetic groups of \(\text{N. meningitidis}\). Several strains corresponded to major epidemic complexes as shown by MLEE analysis. For instance, strain LNP 10824 belongs to clone IV-1 which has been involved in large epidemics in Africa. Strains LNP 13302 and LNP 13408 belong to the ET-37 complex which is frequently encountered in Europe and North America.\(^{19,25}\)

Polymorphism of penA in pen\(^1\) and pen\(^5\) strains

It was necessary to have various genetic lineages represented among the meningococcal strains in our collection for a thorough analysis of the genetic diversity of penA among these strains. The polymorphism of penA was analysed by investigating restriction endonuclease patterns for amplified penA following digestion with three enzymes, \(\text{HaeIII}, \text{HpaII}\) and \(\text{TaqI}\). The entire open reading frame of penA was amplified and digested by these enzymes. Distance matrices were constructed and used to draw a dendrogram for the strains tested. All pen\(^5\) strains had identical restriction endonuclease patterns for the three enzymes (Figure 2). The penA allele harboured by these strains was named penA\(1\). One susceptible strain (LNP 13129) harboured a slightly different allele (penA\(2\)), the HpaII profile of which differed from that of penA\(1\) by only two bands. However, penA\(1\) and penA\(2\) clustered together on the dendrogram (Figure 1). The pen\(^1\) strains showed a high degree of polymorphism for penA (Figure 2): eight different penA alleles were observed among the 12 strains tested in this study (Figure 1, Table I). The \(\text{pilA/pilD}\)-based dendrogram and penA-based dendrogram cannot be directly superimposed (Figure 1 compares the two dendrograms). Several strains that clustered together on the basis of \(\text{pilA/pilD}\) polymorphism (LNP 16454, LNP 16467, LNP 16519, W-39 and W-88) harboured different penA alleles, penA\(3\) (W-39 and W-88) and penA\(7\) (LNP 16454, LNP 16467 and LNP 16519) (Figure 1, Table I). Two pen\(^1\) strains (LNP 16308 and 99/93) had the penA\(1\) allele despite having an MIC of 0.190 and 0.125 mg/L, respectively (Figure 1, Table I).

The DNA sequences of the penA amplicons were determined and aligned. All strains harbouring the penA\(1\) allele had identical sequences. The sequence of the penA\(2\) allele was identical to that of penA\(1\) (these are the two alleles found in pen\(^5\) strains) except for one synonymous change (Figure 3). The penA sequence from a pen\(^5\) strain of \(\text{Neisseria gonorrhoeae}\)\(^{26}\) was also very similar to that of pen\(^5\)
Figure 3. See legend on p. 294.
Polymorphism of meningococcal penA gene

Figure 3. See legend on p. 294.

<table>
<thead>
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Figure 3. See legend on p. 294.
Figure 3. See legend on p. 294.
strains of \textit{N. meningitidis} (Figure 3). We compared the sequences of \textit{penA} alleles from \textit{pen} strains with each other and with that of \textit{pen} \textit{N. meningitidis} observed here (regardless of phenotype) (Figure 3).

These results clearly demonstrate a high degree of polymorphism in \textit{penA} that seems to modify the PBP2 transpeptidase structure.

**Correlation between reduced susceptibility to penicillin and alteration of the \textit{penA} gene**

We investigated the correlation between \textit{penA} polymorphism and reduced susceptibility to penicillin by transferring \textit{penA} alleles from three \textit{pen} strains (W-39/\textit{penA5}, TH41/\textit{penA6} and LNP 16454/\textit{penA7}) to a susceptible strain (LNP 8013/\textit{penA1}). Transformation was performed using genomic DNA or PCR-amplified \textit{penA} from donor strains. Penicillin-resistant transformants were obtained at a high frequency (10⁻⁴/cfu). The \textit{penA} alleles of these transformants (TR1–TR6) were identical to the alleles in the donor strains (Table I). Transformants also had the \textit{pilA/pilD} alleles of the recipient strain, LNP 8013 (\textit{pilA10} and \textit{pilD3}) (Table I), indicating that the altered \textit{penA} allele had been acquired by the recipient strain. It is unlikely that, in these experiments, changes at another chromosomal locus in transformants were involved in the resistance of transformants to penicillin, as similar results were obtained with genomic DNA and PCR-amplified DNA. These results strongly suggest that the \textit{pen} phenotype in our collection of strains is directly related to \textit{penA} polymorphism. One transformant (TR2), which bore the \textit{pilA10} and \textit{pilD3} alleles (Table I), had a new \textit{penA} allele (\textit{penA10}) different from those of both the donor strain (W-39/\textit{penA5}) and the recipient strain (LNP 8013/\textit{penA1}). However, \textit{penA10} differed from \textit{penA1} only in its TaqI profile by the presence of an additional TaqI site (profile 8, Table I, Figure 2). This transformant may have originated through a partial recombination between \textit{penA1} and \textit{penA5}. However, it could also have resulted from a point mutation in \textit{penA1} of the recipient strain LNP 8013.

We investigated whether \textit{penA} could be transferred from a \textit{pen} strain to a susceptible strain during bacterial growth in a culture medium. A penicillin-susceptible, kanamycin-resistant strain, \textit{CAP1} (\textit{penA1}), derived from strain LNP 8013 (Table I; see Material and methods),²⁰ and a \textit{pen} \textit{N. meningitidis} strain, LNP 16454 (\textit{penA7}), were grown to mid-logarithmic growth phase and mixed together. This co-culture was performed to investigate how gene exchange could occur \textit{in vivo} during mixed carriage. Colonies resistant to kanamycin and penicillin were obtained when the mixture was plated on medium containing these antibiotics, at a frequency of 10⁻³/cfu of the \textit{CAP1} recipient strain. These transformants (represented by TR7 in Table I) harboured the \textit{penA7} allele (the allele of the donor strain, LNP 16454) but had the \textit{pilA/pilD} alleles of the recipient strain \textit{CAP1} (\textit{pilA10} and \textit{pilD3}), indicating that the altered \textit{penA} allele (\textit{penA7}) had been acquired by the recipient strain (Table I).

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<td>(Ser-X-X-Lys) as well as the Lys-Thr-Gly and the Ser-X-Asn motifs are indicated by thick lines. Accession numbers are AF306699 (strain LNP 16503, \textit{penA1}), AF306700 (strain LNP 13129, \textit{penA2}), AF306701 (strain W-46, \textit{penA3}), AF306702 (strain LNP 16454, \textit{penA7}), AF306703 (strain TH-41, \textit{penA6}), AF306704 (strain W-39, \textit{penA5}), AF306705 (strain LNP 16504, \textit{penA8}) and AF306706 (strain 214/97, \textit{penA9}).</td>
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\textbf{Figure 3.} Partial sequences of \textit{pen} genes from \textit{pen} \textit{N. meningitidis} strains and from susceptible and \textit{pen} \textit{N. meningitidis} strains. Numbers on the top are according to reference 10 (positions from the start codon of \textit{penA}). The \textit{N. gonorrhoeae} sequences are from reference 4. Polymorphic bases in the other strains that differ from those in the susceptible strain are indicated. Changes in amino acid sequence due to polymorphism are indicated (three-letter code). The insertion of an extra aspartate (Asp345) in the \textit{pen} strain of \textit{N. gonorrhoeae} is indicated in bold. Strains and the corresponding \textit{penA} alleles are shown on the left of each sequence. The active site serine residue (Ser-X-X-Lys) as well as the Lys-Thr-Gly and the Ser-X-Asn motifs are indicated by thick lines. Accession numbers are AF306699 (strain LNP 16503, \textit{penA1}), AF306700 (strain LNP 13129, \textit{penA2}), AF306701 (strain W-46, \textit{penA3}), AF306702 (strain LNP 16454, \textit{penA7}), AF306703 (strain TH-41, \textit{penA6}), AF306704 (strain W-39, \textit{penA5}), AF306705 (strain LNP 16504, \textit{penA8}) and AF306706 (strain 214/97, \textit{penA9}).

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A. Antignac \textit{et al.}
Polymorphism of meningococcal penA gene

Discussion

Bacterial resistance to penicillin may evolve either by the acquisition of inactivating enzymes (β-lactamases) or by target modification (alteration of PBPs). The acquisition of β-lactamases seems to be the most frequent of these two mechanisms in bacteria, but target modification is responsible for the reduced susceptibility of *N. meningitidis* and *S. pneumoniae* to penicillin. PBP2 is usually the protein involved in this phenomenon in *N. meningitidis*. The alteration of only one PBP in *N. meningitidis* suggests that pen1 strains are still evolving, so penicillin-resistant strains may be expected in the near future, by analogy with *S. pneumoniae*. The development of molecular methods of surveillance is hence warranted.

All the pen1 strains tested here harboured an altered penA allele (and hence a modified PBP2) except for two strains which had the penA1 allele (LNP 16308 and 99/93). For these strains, another PBP or bacterial target may be involved. However, we cannot exclude the possibility that these two strains were misclassified, as their MICs were close to the cut-off point (Table I). Strain LNP 16308 (penA1, MIC 0.19 mg/L) was epidemiologically linked to strain LNP 16325 (penA1, MIC 0.04 mg/L). Both strains belonged to the same genetic group (Table I, Figure 1). Strain LNP 16308 (pen1 strain) was isolated from a 4 year old boy suffering from meningococcal infection. Strain LNP 16325 (pen1) was isolated 10 days later from his sister, who also developed meningococcal infection. As the LNP 16325 strain was shown to be susceptible to penicillin and the two strains were epidemiologically linked and genetically identical, it seems most likely that both strains are susceptible to penicillin. This example clearly indicates the technical difficulties involved in interpreting a classical antibiogram.

Our study proposes a reliable and rapid molecular approach for analysing reduced susceptibility to penicillin in *N. meningitidis*. It overcomes problems such as differences in medium or inoculum size, which necessitate the careful standardization of antibiogram determination in different laboratories.

The penA gene seems to be stable, as all susceptible strains harboured the same penA allele (penA1 or the closely related penA2) regardless of genetic group. The similarity in the penA sequences of pen1 strains of *N. gonorrhoeae* (which is closely related to *N. meningitidis*) and pen5 strains of *N. meningitidis* also provides evidence of the stability of penA in pathogenic *Neisseria* before the era of antibiotics. These data suggest that penA was acquired before the two species separated. The widespread use of penicillin leads to selection pressure resulting in the emergence of pen1 strains; penA evolution under this pressure seems to be independent in *N. meningitidis* and *N. gonorrhoeae*. Indeed, whereas the acquisition of an extra aspartate residue (Asp345) is a common mechanism in *N. gonorrhoeae* for reducing the affinity of PBP2 for penicillin, no such extra aspartate residue was observed in the pen1 strains of *N. meningitidis* tested here.

The prevalence of pen1 strains increased in France from 4% of all meningococcal isolates in 1994 to 28% in 1998 (data not shown). The frequency of *N. meningitidis* with reduced susceptibility to penicillin is very low in the Czech Republic: of 655 strains isolated during 1991–1997, only four (0.6%) showed reduced susceptibility to penicillin. Our results indicate that the selection pressure acts directly on the evolution of penA and results in a high degree of penA polymorphism in strains with reduced susceptibility to penicillin. As the strains tested in this study were genetically diverse, the emergence of pen1 strains does not seem to be due to the expansion of one particular clone. Similar results were obtained with pen1 strains in Spain. A group of five strains (LNP 16454, LNP 16467, LNP 16519, W-39 and W-88), which clustered together on the basis of pilA and pilD, harboured two different penA alleles: penA5 (W-39 and W-88) and penA7 (LNP 16454, LNP 16467 and LNP 16519) (Figure 1, Table I). These strains may originate from two closely related clones that acquired two different penA alleles.

The results regarding penA transfer by transformation and during co-culture are consistent with a model of penA evolution by transformation and homologous recombination rather than mutation. Indeed, *N. meningitidis* and *N. gonorrhoeae* are naturally competent for transformation and undergo autolysis throughout their growth phase, facilitating horizontal DNA exchange between these species and even with other species of the genus *Neisseria*. Transformation has been used previously to demonstrate inter-species recombination between the penA genes of *N. meningitidis* and commensal species during the emergence of pen1 strains of *N. meningitidis*.

Acknowledgements

This work was supported by the Institut Pasteur, by research grant no. 310/96/K102 from the Grant Agency of the Czech Republic and by the Greek Ministry of Health. A. A. is supported by a fellowship from the Caisse National d’Assurance Maladie.

References


Received 19 July 2000; returned 13 September 2000; revised 26 September 2000; accepted 19 October 2000

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