Interspecies Recombination between the penA Genes of Neisseria meningitidis and Commensal Neisseria Species during the Emergence of Penicillin Resistance in N. meningitidis: Natural Events and Laboratory Simulation

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The penicillin-binding protein 2 genes (penA) of penicillin-resistant Neisseria meningitidis have a mosaic structure that has arisen by the introduction of regions from the penA genes of Neisseria flavescent or Neisseria cinerea. Chromosomal DNA from both N. cinerea and N. flavescent could transform a penicillin-susceptible isolate of N. meningitidis to increased resistance to penicillin. With N. flavescent DNA, transformation to resistance was accompanied by the introduction of the N. flavescent penA gene, providing a laboratory demonstration of the interspecies recombinational events that we believe underlie the development of penicillin resistance in many meningococci in nature. Surprisingly, with N. cinerea DNA, the penicillin-resistant transformants did not obtain the N. cinerea penA gene. However, the region of the penA gene derived from N. cinerea in N. meningitidis K196 contained an extra codon (Asp-345A) which was not found in any of the four N. cinerea isolates that we examined and which is known to result in a decrease in the affinity of PBP 2 in gonococci.

Isolates of Neisseria meningitidis (meningococci) with increased levels of resistance to penicillin have been reported in the last few years, particularly from Spain and the United Kingdom (10, 11, 15). Resistance is due, at least in part, to the development of altered forms of penicillin-binding protein 2 (PBP 2) that have a decreased affinity for the antibiotic (9, 11).

In contrast to the PBP 2 genes (penA) of susceptible isolates, which are very uniform in sequence (14, 17), those of penicillin-resistant isolates are very variable (17) and have mosaic structures consisting of regions that are essentially identical in sequence to the corresponding regions in penicillin-susceptible isolates alternating with regions that are highly diverged (14).

In most penicillin-resistant meningococci, the diverged regions are the result of the replacement of regions of the penA genes with the homologous regions from the commensal species Neisseria flavescent (13, 14). The penA genes of some penicillin-resistant meningococci have diverged regions that have not been introduced from N. flavescent and appear to have been obtained from Neisseria cinerea (13).

We provide here further evidence that the penA gene of one of these latter meningococcal isolates (K196) has obtained the whole region encoding the transpeptidase domain from N. cinerea and describe experiments that attempt to mimic in the laboratory the recombinational events between N. cinerea and N. flavescent DNA donors and N. meningitidis that we believe underlie the development of penicillin resistance in nature.

MATERIALS AND METHODS

Bacterial strains. The isolates used in this work are described in Table 1. They were grown at 37°C on Oxoid GC agar base plus supplements in an atmosphere of 5% CO₂ and 95% air.

Isolation of chromosomal DNA and genetic transformation. A single colony of each Neisseria isolate was spread over a plate of GC agar plus supplements, and after 18 to 24 h at 37°C the confluent bacterial growth was resuspended in 4 ml of 50 mM Tris-HCl–10 mM EDTA, pH 7.5. The addition of 10 μl of lysozyme (10 mg/ml) for 15 min at room temperature was followed by addition of 4 ml of 2% Triton X-100–50 mM Tris-HCl, pH 7.5, and complete lysis was achieved by two cycles of freezing and thawing. The crude lysates were used directly for the amplification of the penA gene by PCR. For genetic transformation, chromosomal DNA was purified as follows: The lysate was treated with protease K (10 μg/ml, final concentration) for 30 min at room temperature, extracted once with an equal volume of phenol and three times with ether, ethanol precipitated, and resuspended at about 100 μg of DNA per ml in 10 mM Tris-HCl–1 mM EDTA, pH 7.4.

Transformation of N. meningitidis C311 to increased resistance to penicillin was carried out as described previously (3).

Nucleotide sequencing of N. cinerea penA genes. A 1.4-kb region of penA (from codon 183 to 187 bp downstream of the coding region) that included the region encoding the complete penicillin-sensitive transpeptidase domain was amplified from crude lysates of N. cinerea isolates by using the oligonucleotides GCup2 and GCdown3 (14). The amplified fragments were end trimmed with T4 DNA polymerase, digested with EcoRI (to cleave the EcoRI site included at the 5’ end of GCdown3), and inserted into M13mp18 and M13mp19 digested with EcoRI and HindII. The penA gene was sequenced with two sets of oligonucleotides that prime dyeoxy-sequencing reactions from intervals along each DNA strand. Errors introduced by the PCR were eliminated by sequencing each region at least twice from independent M13 clones.

penA gene fingerprinting. HpaII fingerprinting was carried out as described previously (17) except that the 1.4-kb frag-
ment from the penA gene, encoding the transpeptidase domain of PBP 2, was used. Briefly, the penA fragments were amplified by PCR with primers GCup2 and GCdown3, digested with HpaII, end filled with [α-32P]dCTP, fractionated on polyacryl-

Nucleotide sequence accession numbers. The N. cinerea penA sequences have been deposited in the EMBL data library under accession numbers Z17308 to Z17310.

**RESULTS**

*N. cinerea* and *N. flavescens* as the sources of the diverged regions in the penA genes of penicillin-resistant meningococci. The penA gene of *N. meningitidis* K196 has a mosaic structure consisting of a region between nucleotides 1 and 620 that differs at only two nucleotide sites from the penA gene of the penicillin-susceptible *N. meningitidis* isolate C311 and a region from nucleotide 621 to 1944 that differs at 13.7% of the nucleotide sites (13) (Fig. 1). PBP 2 encoded by the mosaic penA gene of strain K196 differs from PBP 2 of the susceptible isolate by 34 amino acid substitutions and three insertions, all located between residues 202 and 574.

The 13.7% diverged block in the penA gene of *N. meningitidis* K196 has been shown to be 96.5% identical in sequence to the corresponding region of the penA gene of *N. cinerea* NCTC10294 (13). *N. cinerea* was recognized only in 1962 (1), and even at that time the MICs of penicillin for isolates of this species were very variable (0.02 to 0.6 μg/ml). It could be argued that the similarity between the penA genes of *N. meningitidis* K196 and *N. cinerea* NCTC10294 was the result of both isolates having received the penA gene from the same unidentified Neisseria species. The examination of an *N. cinerea* isolate from the preantibiotic era would help to eliminate this possibility, but such strains are not available. To ensure that the penA gene of *N. cinerea* NCTC10294 was typical of the species, we sequenced the gene from a further three isolates.

Table 2 shows that the downstream region of the penA gene of K196 was very similar to that of each of the *N. cinerea* isolates and, in particular, to those of *N. cinerea* LNP2060 and

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**TABLE 1. Bacterial isolates used in this study**

<table>
<thead>
<tr>
<th>Isolate</th>
<th>MIC (μg/ml) of benzylpenicillin</th>
<th>Yr of isolation</th>
<th>Country of isolation</th>
<th>Sourcea</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>N. meningitidis</em> C311</td>
<td>0.02</td>
<td>1986</td>
<td>United Kingdom</td>
<td>J. R. Saunders</td>
</tr>
<tr>
<td><em>N. meningitidis</em> K196</td>
<td>0.32</td>
<td>1989</td>
<td>Ireland</td>
<td>D. M. Jones</td>
</tr>
<tr>
<td><em>N. meningitidis</em> IDA</td>
<td>1.28</td>
<td>1987</td>
<td>Spain</td>
<td>E. Perez-Trallero</td>
</tr>
<tr>
<td><em>N. cinerea</em> NCTC10294</td>
<td>0.04</td>
<td>1962</td>
<td>Germany</td>
<td>NCTC</td>
</tr>
<tr>
<td><em>N. cinerea</em> LNP1646</td>
<td>0.64</td>
<td>1979</td>
<td>France</td>
<td>LNP</td>
</tr>
<tr>
<td><em>N. cinerea</em> LNP2060</td>
<td>0.32</td>
<td>1980</td>
<td>France</td>
<td>LNP</td>
</tr>
<tr>
<td><em>N. cinerea</em> LNP3172</td>
<td>0.16</td>
<td>1982</td>
<td>France</td>
<td>LNP</td>
</tr>
<tr>
<td><em>N. flavescens</em> NCTC8263</td>
<td>0.32</td>
<td>1929</td>
<td>United States</td>
<td>NCTC</td>
</tr>
<tr>
<td><em>N. mucosa</em> NCTC10774</td>
<td>0.64</td>
<td>1971</td>
<td>Germany</td>
<td>NCTC</td>
</tr>
<tr>
<td><em>N. gonorrhoeae</em> FA19</td>
<td>0.004</td>
<td>1970</td>
<td>United States</td>
<td>T. J. Dougherty</td>
</tr>
<tr>
<td><em>N. lactamica</em> NCTC10617</td>
<td>0.02</td>
<td>1968</td>
<td>United States</td>
<td>NCTC</td>
</tr>
</tbody>
</table>

a NCTC, National Collection of Type Cultures; LNP, Laboratoire Neisseria Pasteur.

b T, type strain.

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FIG. 1. Diagrammatic representation of the mosaic penA genes of *N. meningitidis* K196 and 1DA. For the penA gene of the penicillin-susceptible *N. meningitidis* C311 (A), the lower arrow shows the coding region for PBP 2. The region encoding the penicillin-sensitive transpeptidase domain and the position of the active-site serine residue of PBP 2 are also shown. The penA genes of *N. meningitidis* K196 (B) and *N. meningitidis* IDA (D) and the sequenced part of the penA genes of *N. cinerea* LNP2060 (C) and *N. flavescens* NCTC8263 (E) are shown. The percent nucleotide sequence divergence between regions of the penA genes compared with the corresponding regions in the penA gene of *N. meningitidis* C311 is shown. V, insertion of a codon; 1, *N. meningitidis* DNA; *1*, *N. cinerea* DNA; *1*, *N. flavescens* DNA.
penA gene of *N. meningitidis* and *N. cinerea*. The divergence between the downstream region in K196 and the corresponding regions in the four *N. cinerea* isolates (1.9 to 3.6%) was less than the intraspecies diversity among the *N. cinerea* penA genes (2.4 to 3.8%). Indeed, the similarity between the diverged region of *N. meningitidis* K196 and those of *N. cinerea* LNP2060 and LNP3172 was greater than that found between any pair of the four *N. cinerea* isolates that we examined (Table 2). We therefore believe that the donor of the diverged region in the penA gene of *N. meningitidis* K196 would be classified as *N. cinerea*.

As a further indication of the sources of the diverged regions in the penA genes of penicillin-resistant meningococci, a dendrogram was constructed using DNA maximum parsimony (5) on the basis of the sequences of nucleotides 546 to 1600 of the penA genes from typical isolates of several *Neisseria* species (13) and those from the penicillin-resistant meningococcal isolates, K196 (13) and 1DA (13), in which this region is proposed to have been obtained, respectively, from *N. cinerea* and *N. flavescens* (Fig. 1). Figure 2 shows that this region of the penA gene of *N. meningitidis* 1DA was grouped on the dendrogram with the *N. flavescens* gene, whereas that from *N. meningitidis* K196 was grouped among those from the *N. cinerea* isolates.

**Transformation of *N. meningitidis* to penicillin resistance by using *N. flavescens* and *N. cinerea* DNA.** Penicillin resistance appears to have emerged in *N. meningitidis* by the replacement of regions of the penicillinococcal penA gene with the corresponding regions from the penA gene of either *N. cinerea* or *N. flavescens*. If this is so, it should be possible to achieve these interspecies recombinational events in the laboratory. Chromosomal DNA from several *Neisseria* isolates was therefore examined for its ability to transform the penicillin-susceptible *N. meningitidis* C311 to an increased level of penicillin resistance. As expected, penicillin-resistant transformants were not obtained with chromosomal DNA from penicillin-susceptible isolates of *N. meningitidis* (C311), *Neisseria gonorrhoeae* (FA19), or *Neisseria lactamica* (NCTC10617) (frequency, <10\(^{-8}\)). They were, however, obtained at a frequency of 10\(^{-4}\) with DNA from the penicillin-resistant *N. meningitidis* K196 and were also obtained, at approximately 100-fold and 1,000-fold lower frequencies, respectively, with DNA from *N. cinerea* LNP2060 and *N. flavescens* NCTC8263.

The region of penA that encodes the transpeptidase domain of PBP 2 was amplified from six of the transformants obtained with *N. meningitidis* K196 chromosomal DNA. The DNA fragments were digested with *Hpa*II, labelled with [\(\alpha\)-\(32\)P]dCTP, fractionated on polyacrylamide, and autoradiographed to give *Hpa*II fingerprints. The penA genes of *N. meningitidis* C311 and K196 could be readily distinguished by *Hpa*II fingerprinting, and all of the transformants had gained the penA gene from strain K196 (Fig. 3A). The mosaic penA gene therefore contributes to the penicillin resistance of K196.

The penA genes of *N. meningitidis* and *N. flavescens* could also be distinguished easily by *Hpa*II fingerprinting, but only three of the six transformants obtained with *N. flavescens* DNA had replaced the penicillinococcal penA gene with that from *N. flavescens* (Fig. 3B). The penA gene from one of these transformants was partially sequenced to confirm the presence of the *N. flavescens* penA gene. The sequence of a 350-bp region from the downstream GcDown3 PCR primer into the penA gene was identical to that of the *N. flavescens* DNA donor.

The transformants that had gained the *N. flavescens* penA gene consistently showed slightly higher MICs of penicillin than those that did not receive this gene (0.2 compared with 0.1 \(\mu\)g/ml). Two classes of transformants presumably arise because differences in another gene besides penA contribute to the higher MIC for *N. flavescens* NCTC8263 compared with that for *N. meningitidis* C311. An alternative possibility was that the higher MIC for *N. flavescens* was due to a single gene that was about 50% linked to penA. These two possibilities can be distinguished by transforming *N. meningitidis* C311 to increased penicillin resistance with chromosomal DNA from one of the *N. meningitidis* transformants that gained the *N. flavescens* penA gene. If the resistance gene is linked to penA, only 50% of the transformants will gain the *N. flavescens* penA gene. However, if there are two unlinked resistance genes in *N. flavescens*, 100% of the transformants in this experiment will now gain the *N. flavescens* penA gene. In practice, fingerprinting showed that all of the 18 transformants that were examined gained the *N. flavescens* penA gene (data not shown).

The fingerprints of the penA genes of *N. meningitidis* C311 and *N. cinerea* LNP2060 could also be readily distinguished (Fig. 3C). As expected from an examination of their sequences, the *Hpa*II fingerprints from *N. meningitidis* K196 and *N. cinerea* LNP2060 were identical. Surprisingly, none of the 12 penicillin-resistant transformants of *N. meningitidis* C311 obtained by using *N. cinerea* DNA had gained the penA gene from *N. meningitidis* K196.
The penA genes of many penicillin-resistant meningococci contain regions derived from the penA gene of N. flavescens (13, 14). N. flavescens isolates, including those obtained in the preantibiotic era (2), require relatively high MICs of penicillin compared with those for typical gonococci and meningococci; this has been shown to be due, at least in part, to the low affinity for penicillin of the N. flavescens PBP 2 (16). We show here that we can mimic in the laboratory the interspecies recombinational events that we believe occurred in nature between N. flavescens and N. meningitidis. Thus, a typical penicillin-susceptible meningococcal strain could be transformed to an increased level of resistance to penicillin by using chromosomal DNA from an N. flavescens isolate that was recovered in the preantibiotic era. About 50% of the resulting transformants had replaced their penA genes with that from N. flavescens. The penicillin-resistant transformants that retained the meningococcal penA gene almost certainly arose by the introduction of a second gene that contributes to the relatively high MIC for typical N. flavescens isolates. In this case, the availability of genetic transformation has allowed meningococci to obtain increased resistance to penicillin by simply replacing their penicillin-sensitive penA gene (or the relevant parts of it) with the more penicillin-resistant penA gene from N. flavescens. Similar events have also occurred to produce low-affinity forms of PBP 2 in penicillin-resistant isolates of N. gonorrhoeae and N. lactamica (7, 12). Since Neisseria species are naturally transformable, it is likely that these interspecies recombinational events have occurred by this mechanism (8).

In the penicillin-resistant N. meningitidis K196, the region of the penA gene encoding the transpeptidase domain has been introduced from N. cinerea. Knapp and Hook (6) showed that 28% of 202 adults harbored N. cinerea in their oropharynges. N. meningitidis and N. cinerea are thus likely to coexist in the human naso- and oropharynx, providing opportunities for exchange of chromosomal genes by genetic transformation. Indeed, interspecies recombinational events between N. cinerea and N. meningitidis have been detected in the argF gene (18).

The affinity of PBP 2 of N. cinerea did not appear to be significantly lower than that of N. meningitidis C311 (data not shown). Since it is difficult to detect small differences in affinity by PBP assays, we used transformation to examine whether the higher MICs for most N. cinerea isolates compared with those for typical N. meningitidis isolates were due to the production of a lower-affinity form of PBP 2. If this is the case, transformation of N. meningitidis C311 to an increased level of penicillin resistance, with chromosomal DNA from N. cinerea, should be accompanied by the replacement of the meningococcal penA gene with that from N. cinerea. These experiments showed that penicillin-resistant transformants of N. meningitidis could readily be obtained by using DNA from N. cinerea LNP2060 (MIC, 0.32 μg/ml) but the transformants, unexpectedly, had not gained the N. cinerea penA gene. Similarly, second-level penicillin-resistant transformants obtained with N. cinerea LNP2060 DNA did not receive the N. cinerea penA gene. Further increase in penicillin resistance by a third round of transformation with N. cinerea DNA was not possible. The cloned penA fragment from N. cinerea LNP2060 was also not able to transform N. meningitidis C311 to increased resistance to penicillin, but this experiment was not informative, since the resistance to penicillin (also) failed to transform resistance. Presumably, the sequence divergence and small size of the cloned DNA fragment lead to very inefficient transformation.

**DISCUSSION**

The penA genes of many penicillin-resistant meningococci contain regions derived from the penA gene of N. flavescens (13, 14). N. flavescens isolates, including those obtained in the preantibiotic era (2), require relatively high MICs of penicillin compared with those for typical gonococci and meningococci; this has been shown to be due, at least in part, to the low affinity for penicillin of the N. flavescens PBP 2 (16). We show here that we can mimic in the laboratory the interspecies recombinational events that we believe occurred in nature between N. flavescens and N. meningitidis. Thus, a typical penicillin-susceptible meningococcal strain could be transformed to an increased level of resistance to penicillin by using chromosomal DNA from an N. flavescens isolate that was recovered in the preantibiotic era. About 50% of the resulting transformants had replaced their penA genes with that from N. flavescens. The penicillin-resistant transformants that retained the meningococcal penA gene almost certainly arose by the introduction of a second gene that contributes to the relatively high MIC for typical N. flavescens isolates. In this case, the availability of genetic transformation has allowed meningococci to obtain increased resistance to penicillin by simply replacing their penicillin-sensitive penA gene (or the relevant parts of it) with the more penicillin-resistant penA gene from N. flavescens. Similar events have also occurred to produce low-affinity forms of PBP 2 in penicillin-resistant isolates of N. gonorrhoeae and N. lactamica (7, 12). Since Neisseria species are naturally transformable, it is likely that these interspecies recombinational events have occurred by this mechanism (8).

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same fragment from N. meningitidis K196, which is known to be able to provide resistance, also failed to transform resistance. It is likely that the 13% divergence in sequence between the donor and recipient DNAs prevented successful recombination with this small fragment. Presumably, the higher MICs for typical N. cinerea isolates compared with those of N. meningitidis isolates are due to differences in genes other than penA, for example, differences in genes influencing outer membrane permeability.

The failure of N. cinerea to show a low-affinity form of PBP 2 was unexpected, since N. meningitidis K196, which has replaced the entire region of penA that encodes the transpeptidase domain with that from N. cinerea, does produce a low-affinity form of PBP 2, as demonstrated by direct affinity measurements (16). Furthermore, the low-affinity form of PBP 2 in strain K196 contributes to resistance, since transformation of N. meningitidis C311 to increased resistance to penicillin, with chromosomal DNA from K196, resulted in the replacement of the meningococcal penA gene with that from K196. A crucial difference between the penA genes of N. meningitidis K196 and the four N. cinerea isolates that we examined was the presence in the former of an additional Asp-345A codon (GAT). An additional aspartic acid codon at exactly the same position is found in the penA genes of all penicillin-resistant gonococci that have been examined (although in this case the codon is GAC rather than GAT [3, 4, 12]). The insertion of Asp-345A is known to reduce the affinity of PBP 2 for penicillin and to provide gonococci with increased resistance to the antibiotic (3).

There are two main possibilities for the events that led to the mosaic gene of strain K196. Firstly, the donor in the interspecies recombinational event may have been an N. cinerea isolate that, unlike those we examined, possessed the Asp-345A codon, either as a preexisting polymorphism or as a recent mutational event selected by pressures for the emergence of penicillin resistance within the nasopharyngeal commensal flora. In this case, the interspecies event that resulted in the mosaic penA gene of N. meningitidis K196 would have led directly to increased resistance to penicillin. Alternatively, the original interspecies recombinational event may have occurred with an N. cinerea isolate that lacks the Asp-345A codon. According to this scenario, the original interspecies recombinational event would have little or no effect on penicillin resistance and a low-affinity form of PBP 2 emerged within N. meningitidis by a subsequent mutational event that introduced the Asp-345A codon.

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