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# 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 flavescens* or *Neisseria cinerea*. Chromosomal DNA from both *N. cinerea* and *N. flavescens* could transform a penicillin-susceptible isolate of *N. meningitidis* to increased resistance to penicillin. With *N. flavescens* DNA, transformation to resistance was accompanied by the introduction of the *N. flavescens 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 flavescens* (13, 14). The *penA* genes of some penicillin-resistant meningococci have diverged regions that have not been introduced from *N. flavescens* 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. flavescens* 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% $CO_2$ 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 *Eco*RI (to cleave the *Eco*RI site included at the 5′ end of GCdown3), and inserted into M13mp18 and M13mp19 digested with *Eco*RI and *Hin*cII. The *penA* gene was sequenced with two sets of oligonucleotides that prime dideoxy-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.** *Hpa*II fingerprinting was carried out as described previously (17) except that the 1.4-kb frag-

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TABLE 1. Bacterial isolates used in this study

| Isolate | MIC (μg/ml) of benzylpenicillin | Yr of isolation | Country of isolation | Source[a] |
|---|---|---|---|---|
| *N. meningitidis* C311 | 0.02 | 1986 | United Kingdom | J. R. Saunders |
| *N. meningitidis* K196 | 0.32 | 1989 | Ireland | D. M. Jones |
| *N. meningitidis* 1DA | 1.28 | 1987 | Spain | E. Perez-Trallero |
| *N. cinerea* NCTC10294[T][b] | 0.04 | 1962 | Germany | NCTC |
| *N. cinerea* LNP1646 | 0.64 | 1979 | France | LNP |
| *N. cinerea* LNP2060 | 0.32 | 1980 | France | LNP |
| *N. cinerea* LNP3172 | 0.16 | 1982 | France | LNP |
| *N. flavescens* NCTC8263[T] | 0.32 | 1929 | United States | NCTC |
| *N. mucosa* NCTC10774 | 0.64 | 1971 | Germany | NCTC |
| *N. gonorrhoeae* FA19 | 0.004 | 1970 | United States | T. J. Dougherty |
| *N. lactamica* NCTC10617[T] | 0.02 | 1968 | United States | NCTC |

[a] NCTC, National Collection of Type Cultures; LNP, Laboratoire Neisseria Pasteur.
[b] T, type strain.

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 *Hpa*II, end filled with [α-$^{32}$P]dCTP, fractionated on polyacrylamide, and autoradiographed.

**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* NCTC 10294 (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

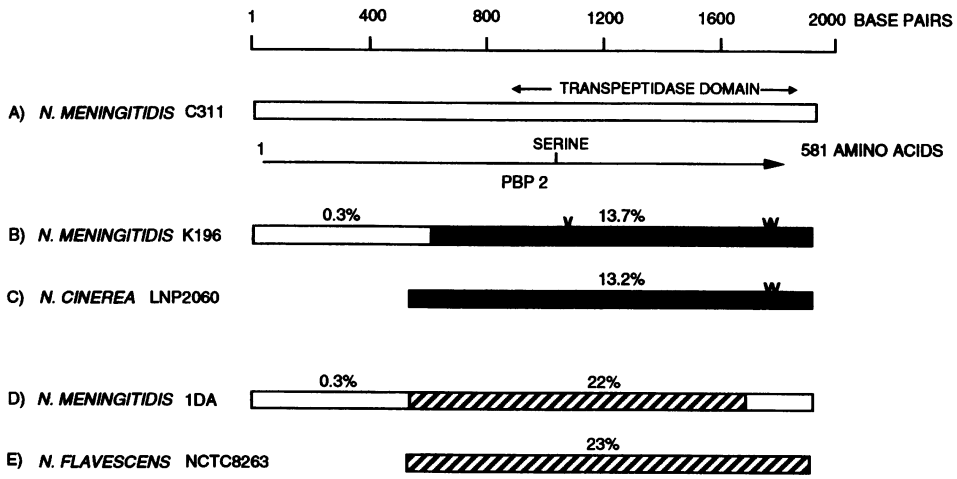


FIG. 1. Diagrammatic representation of the mosaic *penA* genes of *N. meningitidis* K196 and 1DA. For the *penA* gene of the penicillin-susceptible *N. meningitidis* C311 (A), the lower arrow shows the coding region for PBP 2. The region encoding the penicillin-sensitive transpeptidase domain and the position of the active-site serine residue of PBP 2 are also shown. The *penA* genes of *N. meningitidis* K196 (B) and *N. meningiditis* 1DA (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; ▭, *N. meningitidis* DNA; ▩, *N. cinerea* DNA; ▨, *N. flavescens* DNA.

TABLE 2. Percent nucleotide differences between the *penA* genes of *Neisseria* isolates[a]

| Isolate | Divergence (%) | | | | |
|---|---|---|---|---|---|
| | *N. meningitidis* | | *N. cinerea* | | |
| | C311 | K196 | LNP1646 | LNP2060 | LNP3172 |
| *N. meningitidis* K196 | 0.3, 13.7 | | | | |
| *N. cinerea* LNP1646 | 13.0 | 3.6 | | | |
| *N. cinerea* LNP2060 | 13.2 | 1.9 | 3.5 | | |
| *N. cinerea* LNP3172 | 12.9 | 2.1 | 3.3 | 2.4 | |
| *N. cinerea* NCTC10294 | 13.7 | 3.5 | 3.3 | 3.8 | 2.7 |

[a] In all cases, except the comparison between *N. meningitidis* C311 and K196, where the sequences of the entire *penA* genes are available, the percent sequence differences between nucleotides 570 and 1944 are shown. In the comparison between *N. meningitidis* C311 and K196, the two values are the percent sequence differences between nucleotides 1 and 620 and 621 and 1944, respectively. The sequences of the *penA* genes of *N. cinerea* NCTC10294 and *N. meningitidis* C311 and K196 have been reported previously (13).

LNP3172 (98.1 and 97.9% sequence identity, respectively). 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 by 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.

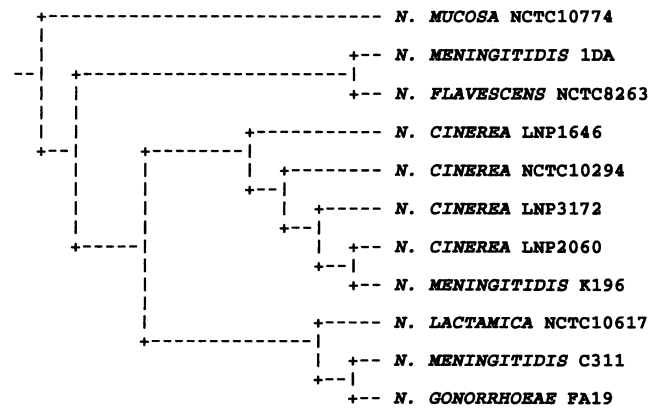


FIG. 2. Dendrogram showing the relatedness of the regions encoding the transpeptidase domain of PBP 2 of *Neisseria* isolates. A dendrogram was constructed from the sequences of nucleotides 546 to 1600 of the *penA* genes (13) by using DNA maximum parsimony (5). The single most parsimonious tree is shown.

**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 meningococcal *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 meningococcal *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 μg/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.*

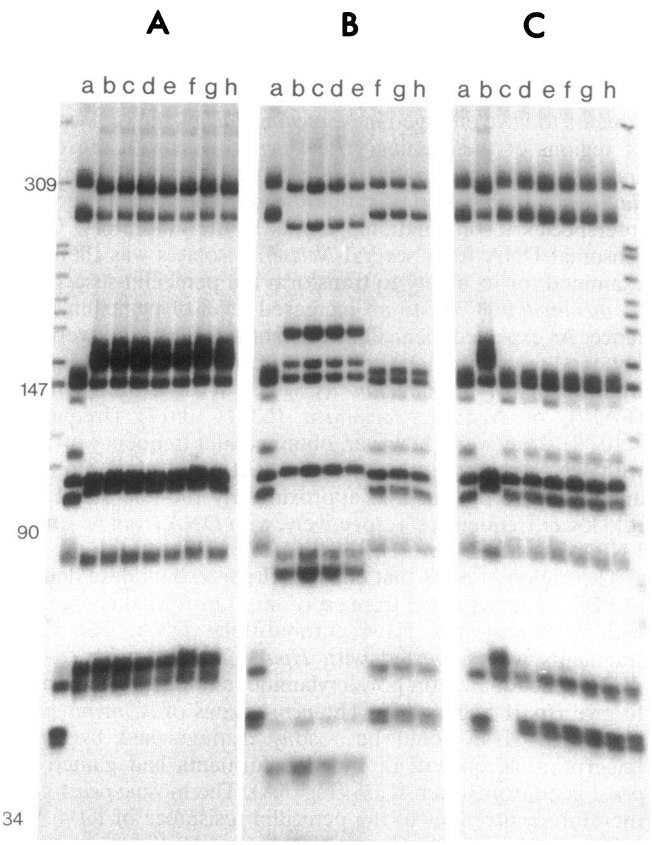


FIG. 3. Fingerprinting of the *penA* genes of penicillin-resistant transformants of *N. meningitidis*. (A) *Hpa*II fingerprints from the *penA* genes of *N. meningitidis* C311 (a), *N. meningitidis* K196 (b), and penicillin-resistant transformants of *N. meningitidis* C311 (c through h) obtained with *N. meningitidis* K196 DNA. (B) Fingerprints from the *penA* genes of *N. meningitidis* C311 (a), *N. flavescens* NCTC8263 (b), and penicillin-resistant transformants of *N. meningitidis* C311 (c through h) obtained with DNA from *N. flavescens* NCTC8263. (C) Fingerprints from the *penA* genes of *N. meningitidis* C311 (a), *N. cinerea* LNP2060 (b), first-level penicillin-resistant transformants (c through e), and second-level penicillin-resistant transformants of *N. meningitidis* C311 (f through h) obtained with DNA from *N. cinerea* LNP2060. The outside lanes are size markers (pBR322 digested with *Hpa*II). Sizes of fragments are shown on the left in base pairs.

*cinerea*. The MIC of penicillin for these first-level transformants (0.1 μg/ml) was not as high as that for the *N. cinerea* DNA donor, and second-level transformants with a further twofold increase in penicillin resistance were obtained from a first-level transformant by using *N. cinerea* LNP2060 DNA. None of the three second-level penicillin-resistant meningococcal transformants that we examined received the *penA* gene from the *N. cinerea* DNA donor (Fig. 3C).

A direct test of the ability of the *penA* gene of *N. cinerea* to provide *N. meningitidis* with increased resistance to penicillin would be to demonstrate that the cloned *N. cinerea penA* gene transforms resistance. Replicative-form DNA of M13 phage containing the region encoding the transpeptidase domain of PBP 2 (amplified with the primers GCup2 and GCdown3) from *N. cinerea* LNP2060 was unable to transform *N. meningitidis* C311 to penicillin resistance. However, no conclusions can be made from this result, as the same region of the *penA* gene of *N. meningiditidis* K196 cloned in M13 (which is known to express a low-affinity form of PBP 2 that provides increased 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 meningococcus 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

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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