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Penicillin-Resistant Isolates of *Neisseria lactamica* Produce Altered Forms of Penicillin-Binding Protein 2 That Arose by Interspecies Horizontal Gene Transfer

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Isolates of *Neisseria lactamica* that have increased resistance to penicillin have emerged in recent years. Resistance to penicillin was shown to be due to the production of altered forms of penicillin-binding protein 2 (PBP 2) that have reduced affinity for the antibiotic. The sequences of the PBP 2 genes (*penA*) from two penicillin-resistant isolates were almost identical (≤1% sequence divergence) to that of a penicillin-susceptible isolate, except in a 175-bp region where the resistant and susceptible isolates differed by 27%. The nucleotide sequences of these divergent regions were identical (or almost identical) to the sequence of the corresponding region of the *penA* gene of *N. flavescens* NCTC 8263. Altered forms of PBP 2 with decreased affinity for penicillin in the two penicillin-resistant isolates of *N. lactamica* appear, therefore, to have arisen by the replacement of part of the *N. lactamica penA* gene with the corresponding region from the *penA* gene of *N. flavescens*.

Non-β-lactamase-producing, penicillin-resistant isolates of *Neisseria gonorrhoeae* emerged during the 1960s and are now widely distributed (4). Resistance to penicillin in these isolates is due to the production of altered forms of both penicillin-binding proteins (PBPs) 1 and 2 that have decreased affinity for penicillin and to a decrease in the permeability of the outer membrane (2, 3). Isolates of *N. meningitidis* that have increased levels of resistance to penicillin have also been encountered in recent years in Spain (7) and the United Kingdom (12). Resistance to penicillin in *N. meningitidis* is due, at least in part, to the production of low-affinity forms of PBP 2 (5, 6, 11).

In both *N. gonorrhoeae* and *N. meningitidis*, altered forms of PBP 2 appear to have arisen by a novel genetic mechanism in which regions of the PBP 2 genes (*penA*) have been replaced (presumably by genetic transformation) with the corresponding regions from the *penA* gene of either *N. flavescens* or another, so-far-unidentified, *Neisseria* species (1, 9, 11).

Isolates of the commensal species *N. lactamica* that have increased levels of resistance to penicillin have been encountered since the late 1970s. We show here that two penicillin-resistant isolates of *N. lactamica*, one from Spain and one from the United Kingdom, produce altered forms of PBP 2 that have decreased affinity for penicillin. The altered forms of PBP 2 have arisen by the replacement of a 175-bp region of the *N. lactamica penA* gene with the corresponding region from the *penA* gene of *N. flavescens*.

MATERIALS AND METHODS

Bacterial isolates. *N. lactamica* NCTC 10617 (the type strain; MIC, 0.02 μg of benzylpenicillin per ml) was isolated in the United States in 1968 and was obtained from the National Collection of Type Cultures (Central Public Health Laboratory, Colindale, London). *N. lactamica* K183 (MIC, 0.4 μg/ml) and NL2535 (MIC, 0.4 μg/ml) are penicillin-resistant nasopharyngeal isolates that were obtained, respectively, in the United Kingdom in 1989 and Spain in 1979. *N. flavescens* NCTC 8263 (the type strain; MIC, 0.4 μg/ml) was isolated in the United States in 1929 and was obtained from the National Collection of Type Cultures. The penicillin-susceptible isolates *N. gonorrhoeae* LM306 (MIC, 0.004 μg/ml) and *N. meningitidis* C311 (MIC, 0.02 μg/ml) were described previously (9, 11).

Cloning and sequencing of the *penA* gene. Nucleotides 547 to 1980 (using the numbering system of reference 13) of the *penA* genes of the *N. lactamica* isolates were amplified by the polymerase chain reaction by using the primers GCup2 and GCdown3 as described previously (11). The amplified 1.4-kb fragments were cloned in each orientation into the replicative form of bacteriophage M13mp18 and M13mp19 and were sequenced by using a series of oligonucleotide primers as described previously (11).

Assay of PBPs. *N. lactamica* isolates were streaked across three GC agar plates (Oxoid Ltd., Basingstoke, England), and after 16 h at 37°C, the confluent bacterial growth was resuspended in 8 ml of 50 mM Tris hydrochloride–10 mM EDTA (pH 7.5). The bacteria were lysed by the addition of 30 μl of a 20-mg/ml concentration of lysozyme for 10 min, followed by two cycles of freezing in dry ice and slow thawing. The lysates were passed once through a syringe needle (0.5 by 16 mm) to reduce the viscosity, and cell debris was removed by centrifugation at 5,000 × *g* for 10 min at 4°C. The crude cell envelopes were pelleted by centrifugation at 100,000 × *g* for 1 h at 4°C, rinsed with 50 mM sodium phosphate buffer (pH 7.0), resuspended at about 5 mg of protein per ml in the same buffer, and stored at −70°C.

To measure the affinity of the PBPs for penicillin, a 20-μl volume of cell envelopes was added to 5 μl of a range of concentrations of [^{3}H]benzylpenicillin (26 Ci/mmol/liter; a

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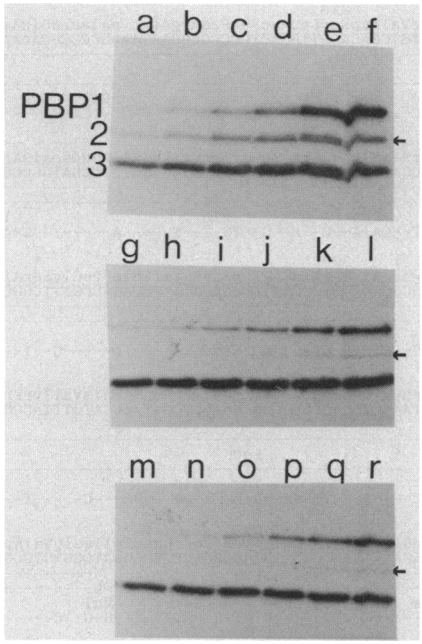

FIG. 1. Affinity of PBPs of penicillin-susceptible and -resistant *N. lactamica* isolates. Increasing concentrations of [^{3}H]benzylpenicillin were incubated for 10 min at 30°C with cell envelopes from penicillin-susceptible *N. lactamica* NCTC 10617 (top panel) or the penicillin-resistant isolates NL2535 (middle panel) and K183 (bottom panel). The PBPs were fractionated on a 12% SDS-polyacrylamide gel and were detected by fluorography. The following concentrations of [^{3}H]benzylpenicillin were used: lanes a, g, and m, 0.015 μg/ml; lanes b, h, and n, 0.03 μg/ml; lanes c, i, and o, 0.06 μg/ml; lanes d, j, and p, 0.12 μg/ml; lanes e, k, and q, 0.25 μg/ml; and lanes f, l, and r, 0.5 μg/ml. The arrows mark the position of PBP 2.

gift of Merck Sharp & Dohme, Rahway, N.J.) for 10 min at 30°C. After the addition of 20 μl of sodium dodecyl sulfate (SDS) gel solubilizer, 5 μl of a 5-mg/ml concentration of benzylpenicillin, and 5 μl of 2-mercaptoethanol, the samples were heated in a boiling water bath for 3 min and fractionated on a 12% SDS-polyacrylamide gel and fluorographed as described previously (8).

Nucleotide sequence accession numbers. The sequences reported in this paper have been deposited in the EMBL Data Library with accession numbers X53712 to X53714.

RESULTS

Low affinity of PBP 2 in penicillin-resistant isolates of *N. lactamica*. The affinities for benzylpenicillin of the PBPs of the two penicillin-resistant isolates of *N. lactamica* (K183 and NL2535) were compared with those of the penicillin-susceptible isolate (NCTC 10617). The affinities of PBP 1 and PBP 3 were almost identical in all three isolates (Fig. 1). In contrast, the affinity of PBP 2 of both of the penicillin-resistant isolates was ≈10-fold lower than that of the penicillin-susceptible isolate. Resistance to penicillin in *N. lactamica* has therefore occurred by the development of altered forms of PBP 2.

Sequences of the *penA* genes of penicillin-susceptible and -resistant isolates of *N. lactamica*. A 1.4-kb region of the *penA* gene, encoding the penicillin-sensitive transpeptidase domain of PBP 2 (residues 240 to 581), was amplified by the polymerase chain reaction by using the oligonucleotides GCup2 and GCdown3. The amplified fragments from the penicillin-susceptible isolate and from the two penicillin-resistant isolates were cloned into M13 phage and sequenced. The sequences of nucleotides 601 to 1920 of the *penA* genes of these isolates are shown in Fig. 2.

The sequence of the *penA* gene of the penicillin-susceptible isolate of *N. lactamica* differed from that of *N. gonorrhoeae* LM306 (9) and *N. meningitidis* C311 (13) at 86 of 1,314 nucleotides (6.5% divergence) and at 84 of 1,314 nucleotides (6.4% divergence), respectively.

The sequence of the *penA* gene of penicillin-resistant *N. lactamica* NL2535 was almost identical to that of the susceptible isolate between nucleotides 601 and 1379 and between nucleotides 1555 and 1920 (≤1% nucleotide sequence divergence). However, between nucleotides 1380 and 1554 the sequence of the resistant isolate differed extensively from the corresponding sequence in the susceptible isolate. Within this region, the two sequences differed at 49 of 175 nucleotides (28% divergence). The *penA* gene of the second resistant *N. lactamica* isolate (K183) showed a similar mosaic structure, differing in sequence from that of the susceptible isolate by ≈1% between nucleotides 601 and 1379 and 1555 and 1920 but by 27% (48 of 175 sites) between nucleotides 1380 and 1554. Both of the penicillin-resistant isolates, therefore, contained an island of highly divergent DNA within the region encoding the penicillin-sensitive transpeptidase domain of PBP 2 (Fig. 3).

The presence of an island of highly divergent sequence within the *penA* genes of the resistant *N. lactamica* isolates suggests that the formation of altered *penA* genes, encoding forms of PBP 2 with decreased affinity for penicillin, has occurred by recombinational events that have replaced part of the *penA* gene of *N. lactamica* with the corresponding region from the *penA* gene of a closely related species. Recombinational events of this type have been implicated in the development of low-affinity forms of PBP 2 in penicillin-resistant isolates of both *N. gonorrhoeae* (9) and *N. meningitidis* (11).

The divergent regions in the *penA* genes of most penicillin-resistant isolates of *N. gonorrhoeae* and *N. meningitidis* appear to have been introduced from *N. flavescens* (11). The latter species also appears to be the source of the divergent regions in the two penicillin-resistant *N. lactamica* isolates. Thus, in *N. lactamica* NL2535, the sequence of the divergent region (nucleotides 1380 to 1554) is identical to the sequence at the corresponding position of the *penA* gene of *N. flavescens* NCTC 8263 (Fig. 2). Similarly, in isolate K183 the sequence of the divergent region only differs from that of *N. flavescens* at 8 of 175 nucleotides.

DISCUSSION

The data presented here demonstrate that the *penA* genes of two penicillin-resistant isolates of the commensal species *N. lactamica* each contain a small region of 175 bp from the *penA* gene of *N. flavescens*. These recombinational events result in the production of altered forms of PBP 2 that have decreased affinity for penicillin and which provide *N. lactamica* with increased resistance to penicillin.

The production of low-affinity forms of PBP 2 by the replacement of part(s) of the *penA* gene with the corresponding part(s) of the *penA* gene of *N. flavescens* has also been shown to have occurred in penicillin-resistant isolates of *N. gonorrhoeae* (9), *N. meningitidis* (11), and *N. polysaccharea* (1). *N. flavescens* isolates, including the type strain

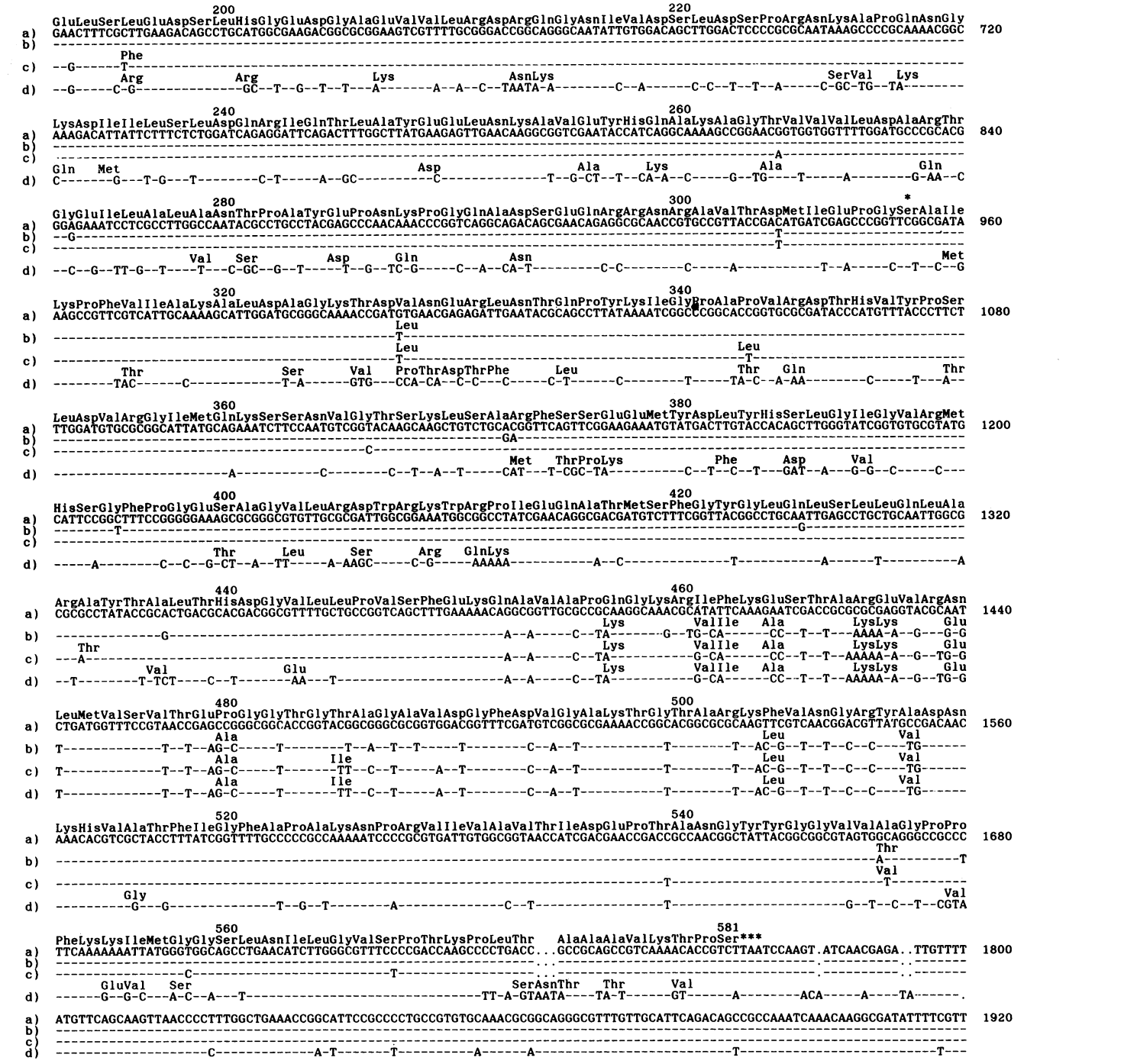

FIG. 2. Sequence of part of the *penA* gene of penicillin-susceptible and -resistant *N. lactamica* isolates. The sequences of nucleotides 601 to 1920 from the *penA* gene of *N. lactamica* NCTC 10617 (type strain) is shown in full in line a. Positions where the sequences of the *penA* genes of the other isolates differ from those in line a are shown. Lines: b, *N. lactamica* K183 (penicillin resistant); c, *N. lactamica* NL2535 (penicillin resistant); d, *N. flavescens* NCTC 8263 (type strain). The numbering system is from reference 13. Asterisk indicates the active-site serine residue.

NCTC 8263 which was obtained in the preantibiotic era, are at least 10-fold less susceptible to penicillin than typical isolates of *N. lactamica*, *N. meningitidis*, or *N. gonorrhoeae*. The intrinsically low susceptibility of *N. flavescens* to penicillin appears to be due to the production of a PBP 2 that fortuitously has lower affinity for penicillin than PBP 2 from typical isolates of the other *Neisseria* species (unpublished experiments). Genetic transformation provides a mechanism by which those *Neisseria* species that are highly susceptible to penicillin, because they produce forms of PBP

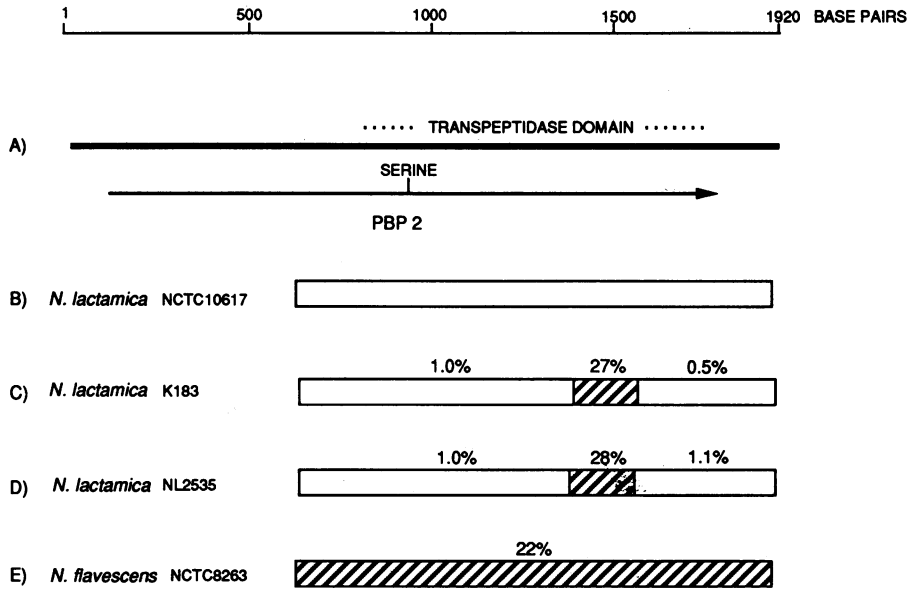

FIG. 3. Schematic representation of the *penA* genes of penicillin-susceptible and -resistant *N. lactamica* isolates. The *penA* gene of *N. lactamica* and the coding region for PBP 2 are represented in diagram A. The extent of the region encoding the transpeptidase domain and the location of the active-site serine residue are marked. The regions of the *penA* gene that were sequenced from penicillin-susceptible *N. lactamica* NCTC 10617 (B) and penicillin-resistant *N. lactamica* K183 (C) and NL2535 (D) are shown. Line E represents the *penA* gene of *N. flavescens* NCTC 8263. The numbers represent the percentage of divergence of regions of the *penA* genes from those of the corresponding regions of the *penA* gene of penicillin-susceptible *N. lactamica* NCTC 10617. The presence of a small region from the *penA* gene of *N. flavescens* in the *penA* genes of penicillin-resistant *N. lactamica* isolates is illustrated. Symbols: ☐, *N. lactamica* DNA; ▨, *N. flavescens* DNA.

2 that have high affinity for the antibiotic, can obtain increased resistance to penicillin by replacing their *penA* genes with the *penA* gene of *N. flavescens*.

In most cases where these interspecies horizontal gene transfer events have occurred, only regions of the *penA* gene have been replaced. These regions presumably encode the amino acid substitutions that are responsible for the difference in the affinity of PBP 2 from the recipient *Neisseria* species and *N. flavescens*. In support of this view, all of the recombinational events have involved regions encoding the penicillin-sensitive transpeptidase domain of PBP 2.

The islands of the highly divergent sequence occur in precisely the same position in the *penA* genes of the two penicillin-resistant *N. lactamica* isolates. Some of the penicillin-resistant isolates of *N. meningitidis* that we have examined also have a replacement of precisely the same region of their *penA* genes with *N. flavescens* DNA (unpublished results). The replacement results in 10 amino acid substitutions in *N. lactamica* K183 and 11 substitutions in isolate NL2535. Presumably these substitutions are the cause of the decreased affinity of PBP 2 from the penicillin-resistant *N. lactamica* isolates. Interestingly, this region includes the conserved lysine-threonine-glycine (KTG) motif (residues 497 to 499 of PBP 2) which is part of a β-strand that forms one side of the penicillin-binding site in the three-dimensional structure of PBPs and serine β-lactamases (10).

It is at present unclear whether mosaic *penA* genes arose first in one *Neisseria* species and were then transferred horizontally by transformation into the other species. The available evidence favors the view that altered *penA* genes have arisen repeatedly, and probably independently, in each of the *Neisseria* species. The evidence for multiple origins is particularly clear in *N. meningitidis*, in which both gene fingerprinting (14) and nucleotide sequencing (1, 11) have shown extensive diversity in the mosaic *penA* genes in different penicillin-resistant isolates. Similarly, it is likely that the mosaic *penA* genes in the two resistant *N. lactamica* isolates described in this paper arose independently, since the sequences of the divergent regions differ by 8 of 175 nucleotides (4.6% divergence). The most likely explanation for these sequence differences is that the same region of the *N. lactamica penA* gene has been replaced on separate occasions with the corresponding regions from the *penA* genes of two genetically diverse isolates of *N. flavescens*.

Whether mosaic *penA* genes arose in one *Neisseria* species and have spread horizontally into other species is an important epidemiological question that is unfortunately difficult to answer. If a mosaic *penA* gene arose, for example, by the replacement of part of an *N. lactamica penA* gene with part of an *N. flavescens penA* gene and then spread horizontally into *N. meningitidis*, we might expect the region of highly divergent *N. flavescens* DNA in the resulting mosaic *N. meningitidis penA* gene to be flanked by sequences characteristic of the *penA* gene of *N. lactamica*. However, the close similarity between the sequences of the *penA* genes of *N. meningitidis* and *N. lactamica* (and *N. gonorrhoeae* and *N. polysaccharea*e) and genetic diversity within these species make it difficult to obtain convincing evidence of this type.

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