Recruitment of a penicillin-binding protein gene from *Neisseria flavescens* during the emergence of penicillin resistance in *Neisseria meningitidis*

(genetic transformation/hybrid genes/nucleotide sequences/commensal Neisseria/interspecies gene transfer)

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ABSTRACT  Non-β-lactamase-producing, penicillin-resistant strains of *Neisseria meningitidis* produce altered forms of penicillin-binding protein 2 that have decreased affinity for penicillin. The sequence of the penicillin-binding protein 2 gene (*penA*) from a penicillin-resistant strain of *N. meningitidis* was compared to the sequence of the same gene from penicillin-sensitive strains and from penicillin-sensitive and penicillin-resistant strains of *Neisseria gonorrhoeae*. The *penA* genes from penicillin-sensitive strains of *N. gonorrhoeae* and *N. meningitidis* were 98% identical. The gene from the penicillin-resistant strain of *N. meningitidis* consisted of regions that were almost identical to the corresponding regions in the penicillin-sensitive strains (<0.2% divergence) and two regions that were very different from them (≈22% divergence). The two blocks of altered sequence have arisen by the replacement of meningococcal sequences with the corresponding regions from the *penA* gene of *Neisseria flavescens* and result in an altered form of penicillin-binding protein 2 that contains 44 amino acid substitutions and 1 amino acid insertion compared to penicillin-binding protein 2 of penicillin-sensitive strains of *N. meningitidis*. A similar introduction of part of the *penA* gene of *N. flavescens*, or a very similar commensal *Neisseria* species, appears to have occurred independently during the development of altered *penA* genes in non-β-lactamase-producing penicillin-resistant strains of *N. gonorrhoeae*.

Isolates of *Neisseria gonorrhoeae* that are resistant to penicillin have been increasingly encountered during the last 20 years (1). Resistance to penicillin in gonococci is due either to the production of a plasmid-encoded β-lactamase or to mutations in several chromosomal genes that result in the production of altered forms of penicillin-binding proteins (PBPs) 1 and 2 that have decreased affinity for the antibiotic and in a decrease in the permeability of the outer membrane (1–3).

Resistance to penicillin has until recently been very rare in *Neisseria meningitidis*. However, over the last few years there have been several reports of penicillin-resistant strains particularly from Spain, South Africa, and the United Kingdom (4–8). Four strains that have high levels of resistance due to the production of a β-lactamase have been described (4, 6, 8), but the majority have only moderate increases in their minimal inhibitory concentrations (MICs) for benzylpenicillin (MICs of 0.1–1.3 μg/ml compared to 0.02 μg/ml for typical strains) that are not so far associated with treatment failure (5, 7). Resistance in the latter strains is due, at least in part, to the production of altered forms of PBP-2 that have decreased affinity for the antibiotic (9, 10).

The nucleotide sequences of the PBP-2 gene (*penA*) from two penicillin-sensitive, and three penicillin-resistant, non-β-lactamase-producing strains of *N. gonorrhoeae* have been compared (11). The penicillin-resistant strains each possessed altered *penA* genes that appeared to be derived from a common ancestral altered *penA* gene (11, 12). The most extensively altered *penA* gene, from strain CDC84-060418, was almost identical to the *penA* gene of penicillin-sensitive gonococci throughout the first two-thirds of the gene. However, the remainder of the gene was extensively altered in sequence and Spratt (11, 13) has suggested that this part of the gene has been introduced by transformation from a closely related species to produce a hybrid *penA* gene that encodes an altered form of PBP-2 that has a decreased affinity for penicillin.

The development of an altered form of PBP-2 was probably the first step in the emergence of non-β-lactamase-producing penicillin-resistant gonococci (3, 12) and it appears that this step has now occurred in meningococci. We show here that the altered *penA* gene in a penicillin-resistant meningococcus is a hybrid gene formed by the replacement of two parts of the gene with the corresponding regions from the commensal species *Neisseria flavescens*. A very similar event appears to have occurred independently to create the hybrid *penA* genes in penicillin-resistant gonococci.

MATERIALS AND METHODS

Bacterial Strains. The penicillin-sensitive *N. gonorrhoeae* strain LM306 (MIC of 0.004 μg of benzylpenicillin per ml) and the penicillin-resistant strain CDC84-060418 (MIC of 1 μg/ml) have been described (11). *N. meningitidis* C311 is a penicillin-sensitive serogroup B isolate (MIC of 0.02 μg/ml), kindly provided by J. R. Saunders (Liverpool University). Strain S738 is a serogroup B, non-β-lactamase-producing penicillin-resistant isolate of *N. meningitidis* (MIC of 1.28 μg/ml isolated in the United Kingdom in 1978 from a blood culture). The following *Neisseria* strains were obtained from the U.K. National Collection of Type Cultures (Central Public Health Laboratory, Colindale, London): *N. animalis* (NCTC10212); *N. canis* (NCTC10296); *N. caviae* (NCTC10293); *N. cinerea* (NCTC10294); *N. cuniculi* (NCTC10297); *N. dentrificans* (NCTC10295); *N. elongata* (NCTC10660); *N. elongata* subsp. *ghibolycia* (NCTC11059); *N. flavescens* (NCTC8263); *N. lactamica* (NCTC10617); *N. meningitidis* group A

Abbreviations: PBP, penicillin-binding protein; PCR, polymerase chain reaction; MIC, minimal inhibitory concentration.

†In ref. 9 the altered PBP in penicillin-resistant meningococci was referred to as PBP-3, but as it is clearly the homolog of gonococcal PBP-2, we propose it should be referred to as PBP-2.

*The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M26644 and M26645).*

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Isolation and Sequencing of the penA Gene of Neisseria Species. Chromosomal DNA was isolated by a lysozyme/EDTA/Triton X-100 lysis procedure, as described (12), and was purified on a cesium chloride/ethidium bromide gradient. N. flaveiscens could not be lysed using Triton-X-100 and a modified procedure using 1% sarkosyl was used. The penA genes of Neisseria species were amplified from chromosomal DNA using the polymerase chain reaction (PCR; ref. 14) and oligonucleotide primers based on the sequence of the penA gene of N. gonorrhoeae, as described (15). The complete coding region of the penA gene from the N. meningitidis strains C311 and S738 could be amplified as a 2.0-kilobase fragment using GC11 (dGCCGTGTGCCG- GAGAG), which hybridizes to a region 55 base pairs upstream of the start of translation of penA, and GCdown3 (dTCGTTGATCCTGGATATATACGTCCGGCTC), which hybridizes 187 base pairs downstream of penA. The GC11 primer did not hybridize to DNA from N. polysacchareae or N. flaveiscens and was replaced with GCup2 (dTTGACACGTATCGGATTAC; nucleotides 547–569), which corresponds to a region that is highly conserved between the penA gene of N. gonorrhoeae and the homologous pbpB gene of Escherichia coli. A 1.4-kilobase fragment, encoding the complete transpeptidase domain of PBP-2, was amplified from N. polysacchareae and N. flaveiscens using GCup2 and GCdown3.

The amplified fragments were cloned in each orientation into M13mp18–mp19 cut with EcoRI and HincII, and plaques containing the amplified fragment were detected by hybridization. The penA gene was sequenced on both strands using a series of oligonucleotides that prime at intervals along each strand. The initial set of primers was based on the gonococcal penA sequence; additional primers were synthesized in those cases where the sequences of particular penA genes were too different from the gonococcal sequence for the above primers to anneal. Errors arising from the PCR (14) were eliminated by sequencing at least two independent M13 templates for each penA gene.

DNA Hybridization. The NCTC strains were inoculated onto blood agar plates plus supplements and, after overnight incubation at 37°C in a 5% CO2/95% air atmosphere, the bacterial growth was lifted onto nitrocellulose filters. Hybridization with 32P-labeled probes was performed by standard techniques using stringent conditions (16). One probe was a 235-base-pair region of the penA gene of the penicillin-resistant meningococcal strain S738. The fragment was amplified by PCR from chromosomal DNA by using the primers GC2 (dCGCAACGACGCGTTGTCG; nucleotides 1343–1360) and MN2 (dGTACCGACTTGTGGTGTC; nucleotides 1577–1558). The fragment was purified on an 8% polyacrylamide gel and was oligolabeled with [32P]dCTP. The second probe was the 32P-end-labeled oligonucleotide GC20 (dATGCGCCGTATGGTGC; nucleotides 1543–1560 from the penA gene of strain S738).

RESULTS

Sequences of the penA Genes of Penicillin-Sensitive Isolates of N. gonorrhoeae and N. meningitidis. The sequences of the penA genes from two penicillin-sensitive strains of N. gonorrhoeae have been reported (11). These sequences and those of two further penicillin-sensitive strains (unpublished data) were almost identical and differed from the sequence of strain LM306 (Fig. 1, line a) at a maximum of three sites. Similarly, the sequences of the penA genes from three penicillin-sensitive strains of N. meningitidis (serogroup B and C strains) were almost identical and differed from the sequence of strain C311 (Fig. 1, line c) at a maximum of four sites. N. gonorrhoeae and N. meningitidis are known to be very closely related species and, as expected, the sequences of their penA genes differed at only 37 of 1800 sites (98% identity), resulting in eight alterations in the amino acid sequence of PBP-2.

Comparison of the Altered penA Genes from Penicillin-Resistant Strains of N. gonorrhoeae and N. meningitidis. The sequence of the penA gene from the penicillin-resistant meningococcal strain CDC84-060418 is shown in Fig. 1, line b. This gene differs from the penA gene of penicillin-sensitive gonococci by the extra Asp-345a codon and by a block of altered sequence from nucleotides 1524 to 1800 (11). One of the two blocks of altered sequence in the penA gene of N. meningitidis S738 overlapped with the altered block of N. gonorrhoeae CDC84-060418. In this overlap region (nucleotides 1524–1800), the altered blocks were strikingly similar (Fig. 1, lines b and d); 31 of the 40 nucleotide substitutions in the altered block of strain CDC84-060418 were also found in the penA gene of strain S738. However, the divergence of the altered block in CDC84-060418 was only 14%, compared to the divergence of 23% found in the altered blocks of strain S738. Consequently, many of the nucleotide differences found in strain S738 were not found in the altered block from strain CDC84-060418 (Fig. 1).

A schematic representation of the penA genes of penicillin-sensitive and penicillin-resistant meningococci and gonococci is shown in Fig. 2.

Origin of the Altered Blocks in the penA Gene of Penicillin-Resistant Gonococci and Meningococci. The altered blocks in the penA genes of penicillin-resistant strains of N. gonorrhoeae and N. meningitidis are believed to have been introduced by the uptake and integration of homologous DNA from a closely related species (see Discussion). We therefore obtained a collection of Neisseria strains that included an example of each species represented in the UK National Collection of Type Cultures and used two DNA probes...
corresponding to parts of the altered blocks to identify the species that don these regions.

Both of the probes hybridized under stringent conditions to the same two strains, *N. flavescens* (NCTC8263) and *N. polysacchareae* (NCTC11858). The region of the penA genes encoding the transpeptidase domain of *N. polysacchareae* and *N. flavescens* were amplified by PCR, cloned into M13 phage, and sequenced.

The sequence of the penA gene of *N. flavescens* was ~77% similar to that of penicillin-sensitive strains of *N. gonorrhoeae* and *N. meningitidis*. The sequences of the altered blocks in the meningococcal strain S738 were 97% identical to the sequences in the corresponding parts of the penA gene of *N. flavescens* (Fig. 1, line e); 152 of the 166 differences found in the altered blocks in strain S738 were present in the penA gene of *N. flavescens* NCTC8263.

The penA gene from *N. polysacchareae* was ~91% similar to that of *N. gonorrhoeae* and *N. meningitidis* and contained a region (nucleotides 1512–1695) that was sufficiently similar to the corresponding region in penicillin-resistant gonococci and meningococci to hybridize to the probes (data not shown).

**DISCUSSION**

**Altered Blocks in the penA Genes of Resistant Gonococci and Meningococci Have Arisen by Recombination.** The penA gene of the penicillin-resistant meningococcal strain S738 consists of regions that are almost identical to those in penicillin-sensitive meningococci, alternating with blocks of sequence that are ~22% different in these regions. The most feasible explanation for the data is that these regions of the meningococcal penA gene have been replaced, by genetic transformation, with the homologous regions from the penA gene of a source that shows ~78% nucleotide sequence similarity with *N. meningitidis*. The two blocks of altered sequence may have arisen either from the incorporation into the meningococcal genome of only parts of the DNA taken up during the initial transformation event or, alternatively, by the incorporation of a continuous block from nucleotide 895 to some position downstream of nucleotide 1800, which has subsequently been interrupted by a secondary transformation event that has replaced the region from nucleotides 1195 to 1358 with meningococcal DNA.

A similar situation is found in the penA gene of the penicillin-resistant gonococcal strain CDC84-060418 where the sequence from nucleotides 1 to 1523 was almost identical to that of the penA gene of penicillin-sensitive strains. Similarly, the sequence of a region ~1.7 kilobases downstream of penA was also almost identical between CDC84-060418 and penicillin-sensitive gonococci (unpublished data). However, from nucleotides 1524 to 1800 the sequence of the penA gene of strain CDC84-060418 diverged by ~14% from that of penicillin-sensitive strains (11). As in the case of the penicillin-resistant meningococcus, the most likely explanation for the island of altered sequence is that a part of the penA gene of a related species has been introduced by transformation and has replaced the corresponding region of the gonococcal penA gene (11).

The altered blocks found in the penA genes of the penicillin-resistant gonococcus and the penicillin-resistant meningococcus were strikingly similar and appear to have been introduced into these species from a similar source, as discussed below.

**Altered Blocks in Gonococcal and Meningococcal penA Genes Have Been Introduced from Commensal Neisseria Species.** DNA probes corresponding to parts of the altered blocks in the penA genes of strains S738 and CDC84-060418 identified two commensal *Neisseria* species as possible sources of these blocks. One of the species, *N. polysacchareae*, possessed a penA gene that showed some similarity to the altered blocks in both the penicillin-resistant meningococci and gonococci but was not sufficiently like them to be the donor species. The other species, *N. flavescens*, was almost certainly the source of the altered blocks in the penA gene of the penicillin-resistant meningococcal strain S738. The sequences of the altered blocks in strain S738 were almost identical to the corresponding regions in the penA gene of *N. flavescens*. Furthermore, as expected of the donor species, the entire 1.4-kilobase region of the penA gene of *N. flavescens* NCTC8263 that we sequenced showed the same level of divergence as found in the altered blocks of strain S738 (~23%).

*N. flavescens* NCTC8263 was isolated in 1928 as one of a cluster of strains causing an outbreak of meningitis in Chicago that were distinguished from previously described *Neisseria* species on the basis of pigment production and bio-
chemical and serological criteria (17). Although N. flavescens was first isolated from cerebrospinal fluid, it is generally regarded as a commensal Neisseria species whose natural habitat, like that of N. meningitidis, is the nasopharynx.

The sequence of the altered block in the gonococcal strain CDC84-060418 was also similar to that of the corresponding region from N. flavescens. Although 31 of the 40 alterations in the altered block of CDC84-060418 were also found in the penA gene of N. flavescens, there were many additional nucleotide differences in the latter that were not found in the altered block. Since penicillin-resistant gonococci emerged several years before penicillin-resistant meningococci, it is possible that a hybrid penA gene arose first in gonococci and was subsequently transferred by transformation into meningococci. However, we think this is very unlikely as the altered blocks in the penA gene of the meningococcal strain S738 were more extensive and more diverged than those found in any of the penicillin-resistant gonococci we have examined (ref. 11 and unpublished results).

A more likely possibility is that part of the penA gene of N. flavescens was introduced very recently into meningococci, whereas a similar independent introduction into gonococci occurred several years earlier, and some of the differences between the introduced N. flavescens sequences and the N. gonorrhoeae penA gene have subsequently been eliminated by mismatch repair processes as the altered penA gene spread by transformation into other gonococci. An equally plausible alternative possibility is that the divergence in the altered block of CDC84-060418 has not been reduced by mismatch repair processes since its introduction and that the source of the altered block in gonococci was an unidentified Neisseria species that was similar to N. flavescens but about 14% diverged from N. gonorrhoeae.

**Mechanism of Reduction in Affinity of PBPs for Penicillin.**

The formation of hybrid PBP genes by the replacement of part of the resident gene with the homologous region from a related species has now been documented in N. gonorrhoeae, N. meningitidis, and Streptococcus pneumoniae (11, 18). It is, however, unclear how this process generates PBPs with decreased affinity for penicillin. All of these species are naturally transformable and can therefore incorporate, albeit at very low frequency, DNA from related species as long as there is sufficient sequence similarity for homologous recombination to occur. If we choose a figure of 70% similarity as the limit at which interspecies transformation can occur, a transformable species could recruit PBP genes from a considerable number of other species. Species that differ by 70% in nucleotide sequence will produce forms of the same PBP that differ by perhaps 15% in amino acid sequence, and this variation in the sequence of the PBP will inevitably result in differences in kinetic parameters, including the affinities for benzylpenicillin.

Under the intense selective pressures of penicillin therapy, a species that produces a PBP with high affinity for penicillin can become more resistant to penicillin by recruiting the homologous PBP gene from any related species that produces a lower affinity form of the enzyme. Apparently in the three transformable species we have studied this event is more significant in nature than the accumulation of mutations that gradually reduce the affinity of the PBP for penicillin (19), although the latter process probably also contributes to the further reduction in the affinity of PBPs as a consequence of continued selective pressures for increasing penicillin resistance.

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