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Transfer of Penicillin Resistance Between *Neisseriae* in Microcosm

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ABSTRACT

Horizontal gene transfer between commensal and pathogenic *Neisseriae* is the mechanism proposed to explain how pathogenic species acquire altered portions of the *penA* gene, which encodes penicillin binding protein 2. These changes resulted in a moderately penicillin-resistant phenotype in the meningococci, whose frequency of isolation in Spain increased at the end of the 1980s. Little has been published about the possibility of this gene transfer in nature or about its simulation in the laboratory. We designed a simple microcosm, formed by solid and liquid media, that partially mimics the upper human respiratory tract. In this microcosm, penicillin-resistant commensal strains and the fully susceptible meningococcus were co-cultivated. The efficiency of gene transfer between the strains depended on the phase of bacterial growth and the conditions of culture. Resistance of penicillin was acquired in different steps irrespective of the source of the DNA. The presence of DNase in the medium had no effect on gene transfer, but it was near zero when nicked DNA was used. Cell-to-cell contact or membrane blebs could explain these results. The analysis of sequences of the transpeptidase domain of PBP2 from transformants, and from donor and recipient strains demonstrated that the emergence of moderately resistant transformants was due to genetic exchange between the co-cultivated strains. Finally, mechanisms other than *penA* modification could be invoked to explain decreased susceptibility.

INTRODUCTION

HORIZONTAL GENE TRANSFER between bacteria could presumably play an important role in evolution of bacterial populations. All mechanisms involved (conjugation, transduction, and transformation) take place both in the laboratory and in nature. Those mechanisms can be investigated from three different perspectives: (i) their occurrence in nature, (ii) their impact on the dynamics of natural bacterial populations, and (iii) their molecular basis. Here we focus on the occurrence of horizontal transmission between microorganisms of the genus *Neisseria* and its impact on the dynamics of population.

Neisseria, like other genera, is "naturally" transformable.[12] In some cases, this may lead to selective advantages for the recipient strain (*e.g.,* antibiotic resistance, antigenic variation, mechanisms to evade the antimicrobial effect of defense mechanisms).

In the past few years, there has been an increased occurrence of isolation of moderately penicillin-resistant strains of *Neisseria meningitidis*.[11] This resistance is caused by the emergence of *pen*A genes (encoding penicillin binding protein 2) whose sequence is altered. The PBP2 has lower affinity for β-lactam antibiotics.[7] The sequences of these altered *pen*A genes are highly variable; in contrast, sequences of *pen*A genes from sensitive strains are almost identical to each other.[2] Some authors[10,15] suggested that these alterations were due to genetic exchange between *N. meningitidis* and some of the nonpathogenic commensal naturally resident *Neisseriae*. The genetic distance between all these species is small, and their habitat is the same. Spratt *et al.*[15] proposed transformation to be the mechanism for genetic exchange leading to this type of resistant strain.

Some reports on DNA transfer between *Neisseria* species focus on antibiotic resistance and/or pathogenicity factors.[1,14] All such studies were performed *in vitro* by the use of purified DNA. On the other hand, studies using microcosms have been performed to analyze ecological relationships between microbes.[5] Essentially, they mimic natural conditions and allow co-incubation of microorganisms in the selected conditions. Here we used a microcosm to study the interactions between different *Neisseria* species that could lead to the emergence of new genotypes of a pathogenic species of *Neisseria*. Ultimately, these interactions may be responsible for the failure of antibiotics in the treatment of infectious diseases.

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MATERIALS AND METHODS

Bacterial strains

The bacterial strains used in this study were *N. meningitidis* C311, a penicillin-susceptible, serogroup B strain, and *N. meningitidis* 5179V, a penicillin-resistant serogroup B strain. Several commensal *Neisseriae* were used: *N. polysaccharea* 3008, *N. perflava* 1659, and *N. sicca-subflava* 95-1. The strains were maintained at −40°C in Trypticase Soy Broth with 20% glycerol. For routine cultures, brain heart infusion and Mueller-Hinton supplemented with 0.5% yeast extract were used.

Microcosm

The simple microcosm used consisted of Castaneda's flasks with a brain-heart infusion (BHI) agar layer covering one of the walls and 25 mL of BHI broth at 37°C. This allowed bacterial populations to grow both as plancktonic microorganisms and also as biofilms. The selective medium used to reisolate *N. meningitidis* was meningococcal defined agar (MCDA)[3] with no cysteine added. Mueller-Hinton agar with 0.5% (w/v) of yeast extract and 1.5% (w/v) of sucrose was used as differential medium to distinguish between meningococcus and nonpathogenic species.

Chromosomal DNA extraction

DNA from donor strains were prepared as follows: Briefly, a volume of 100 mL of culture was centrifuged, and bacteria were resuspended in 2 mL of TRIS-HC1 25 mM, EDTA 10 mM (TE), glucose 50 mM, and 4 μg/mL lysozyme. After incubation of 20 min at 4°C, 100 μL of 2% SDS was added, and the mixture was incubated at 37°C for 30 min. Finally, extraction with phenol-chloroform, precipitation with NaCl 5M isopentanol, and resuspension in TE buffer were performed. The concentration and purity of DNA were determined spectrophotometrically at 260- and 280-nm wavelengths.

Transformation experiments

In the microcosm experiments, pairwise species were co-cultured in three different growth conditions: during a lag phase preceding growth, during logarithmic growth, and during stationary phase. Cultures were either stationary or were shaken. When necessary, DNase I (Boehringer Manheim) was added at a final concentration of 100 μg/mL at the beginning of the experiment followed by the addition of 4 μg/mL of at 2-h intervals.[8] In experiments with purified DNA, the DNA was added to the microcosm at a saturating concentration (>1 μg/mL). Selection of transformants was done by plating on MCDA (for transformants) and on Mueller-Hinton agar (for total survivors). Plates were incubated at 37°C for 6 h and then 5 mL of soft overlay agar with the selected antibiotic concentration was poured onto the plates. Incubation was followed at 37°C for 48 h and colonies scored. In all cases, an identical number of control plates containing identical numbers of recipient bacteria were also prepared.

Susceptibility determination

Minimal inhibitory concentrations (MIC) of penicillin for donor, recipient, and transformants were determined by the plate dilution method using inocula of 10^3 to 10^4 cfu/mL of early logarithmic phase bacteria. Measurements were made af-

TABLE 1. MICs OF PENICILLIN G FOR THE DIFFERENT STRAINS USED IN THIS WORK

	MIC of penicillin G (μg/mL)				
Bacterial strains					
N. meningitidis C311	0.025				
N. polysaccharea 3008	0.4				
N. meningitidis 5179V	1.0				
N. perflava 1659	2.5				
N. sicca-subflava 95-1	1.8				
		Selected phenotype			
Transformants in microcosm co-incubating C311-3008					
1st step of transformation		0.05 μg/mL	0.1		
2nd step of transformation		0.2 μg/mL	0.3		
3rd step of transformation		0.4 μg/mL	0.5		
4th step of transformation		0.6 μg/mL	0.7		
5th step of transformation		0.8 μg/mL	1.1		
	Selected phenotype	*3008*	*5179V*	*1659*	*95-1*
Transformants in microcosm C311-purified DNA from 3008, 5179V, 1659, or 95-1, as indicated					
1st step of transformation	0.05 μg/mL	0.1	0.1	0.1	0.1
2nd step of transformation	0.2 μg/mL	0.3	0.3	0.3	0.3
3rd step of transformation	0.4 μg/mL	0.5	0.5	0.5	0.5
4th step of transformation	0.6 μg/mL	0.7	0.7	0.7	0.8
5th step of transformation	0.8 μg/mL	1.1	0.9	0.9	0.9

TABLE 2. FREQUENCIES OF TRANSFORMATION OF *N. meningitidis* C311 WITH *N. polysaccharea* 3008 AS DONOR STRAIN IN DIFFERENT TYPES OF MICROCOSMS ($\times 10^{-5}$)

Microcosm	*Transformation frequency (mean ± SD; log phase)*
Static	130 ± 38
Shaken	1.5 ± 0.35

ter 18 h at 37°C. The end point was fixed as the lowest concentration at which there was no visible growth or fewer than 10 colonies.

Polymerase chain reaction

Chromosomal DNA, prepared as indicated before, was subjected to PCR. The region of the *penA* gene known to encode for the transpeptidase domain of PBP2 was amplified using primers GCup2 (5′-TTTGCACACGTCATCGGATTTAC) and GCdown3 (5′-TCGTGAATTCGGGATATAACTGCGGC-CGTC).[17] A region of 1.4-Kb was sequenced by the ABI PRISM dRhodamine Terminator Cycle sequencing kit and subsequently analyzed by capillary electrophoresis in an ABI 310 Analyzer (Perkin Elmer). Regions from transformants MC2 and MC2-26 (first and second step transformants respectively) as well as the *N. polysaccharea* 3008 were sequenced.

RESULTS

Table 1 shows the MICs values for both original strains and presumptive transformants obtained in the experiments after co-incubation of fully susceptible strains of *N. meningitidis* and different commensal *Neisseria*. The effect of the phase of growth was also analyzed by pairwise co-incubation of strains harvested at different phases of growth. No penicillin-resistant meningococci were found in control experiments. Table 2 summarizes the results obtained in shaking and static conditions, demonstrating that static conditions favor genetic exchange. Table 3 shows the results concerning experiments done with purified DNA and by co-incubation in the microcosms. The presence of DNase did not greatly affect the genetic exchange in microcosm (Table 3). All results are based on at least three independent assays. No spontaneous penicillin-resistant mutants were detected in any experiment.

In experiments done with purified DNA, the transformation efficiencies were similar in the lag and logarithmic phases, but decreased dramatically in the stationary phase (Table 3). The efficiency of transformation in these sets of experiments depended upon the origin of the DNA: while DNA from the resistant strain *N. meningitidis* 5179V showed the highest frequency of transformation, DNA from other neisserial species gave much lower efficiencies (data not shown).

Sequencing experiments gave identical results for transformants MC2 and MC2-26 (100% identity). There were five nucleotide differences in a region of 110 base pairs (compared with *penA* sequence of strain C311) localized between nucleotides 1152 and 1260 (Fig. 1 and 2). A total of five substitutions were detected, although only the substitution of nucleotide 1259 produces an amino acid substitution (proline → leucine). An amino acid substitution at this position (proline → lysine) was described in *N. flavescens* and proposed to contribute to decreased affinity of PBP2 for penicillin.[14] All nucleotide substitutions were coincident with the sequence determined in donor strain *N. polysaccharea* 3008. The sequence of strain 3008 was 11.5% divergent with respect to that of *N. meningitidis* C311,[17] with 140 nucleotide substitutions leading to 37 amino acid substitutions. Neither deletions nor insertions were detected. The determination of the sequence of strain *N. polysaccharea* 3008 was 90% identical to the sequence of *N. polysaccharea* NCTC11858. Such a difference (10%) can probably be explained by the fact that the *penA* gene of strain 3008 already has alterations compared to a *penA* of a truly penicillin-sensitive *N. polysaccharea* strain (if such a strain exists). Strain NCTC 11858 has also been proposed to have an altered *penA* gene.[15]

DISCUSSION

The MIC values of penicillin for transformants (Table 1) indicate that genetic exchange occurred between commensal-resistant *Neisseria* and the truly susceptible meningococcus. The lack of resistant strains in control experiments indicates that the appearance of such genetic variants was not due to mutational events. This mimics the phenomena suggested to explain the origin and spread of penicillin-resistant meningococci in Spain.[6,9,11] This exchange was also demonstrated in the case of co-incubation of both fully susceptible and resistant meningococci. Phenotypically, no differences were detected irrespective of the origin of DNA. The differences observed in the efficiency of transformation which depended on the source of transform-

TABLE 3. FREQUENCIES OF TRANSFORMATION OF *N. meningitidis* C311 WITH *N. polysaccharea* 3008 AS DONOR STRAIN WITH RESPECT TO DNASE PRESENCE AND TO PHASE OF GROWTH ($\times 10^{-5}$)

Culture conditions	*DNase?*	*Transformation frequency (mean ± SD)*		
		Lag	*Logarithmic (max)*	*Stationary*
Microcosm (co-incubating C311-3008)	No	39 ± 9.3	130 ± 38	14 ± 3.9
Microcosm (co-incubating C311-3008)	Yes	5.6 ± 0.1	1.02 ± 0.3	ND
Microcosm (C311-purified DNA from 3008)	No	24 ± 6.2	27 ± 7.1	0.3 ± 0.08
Microcosm (C311-purified DNA from 3008)	Yes	2.9 ± 0.8	1.2 ± 0.1	ND
Normal culture (C311-purified DNA from 3008)	Yes	<0.01	<0.01	ND

ND, not determined.

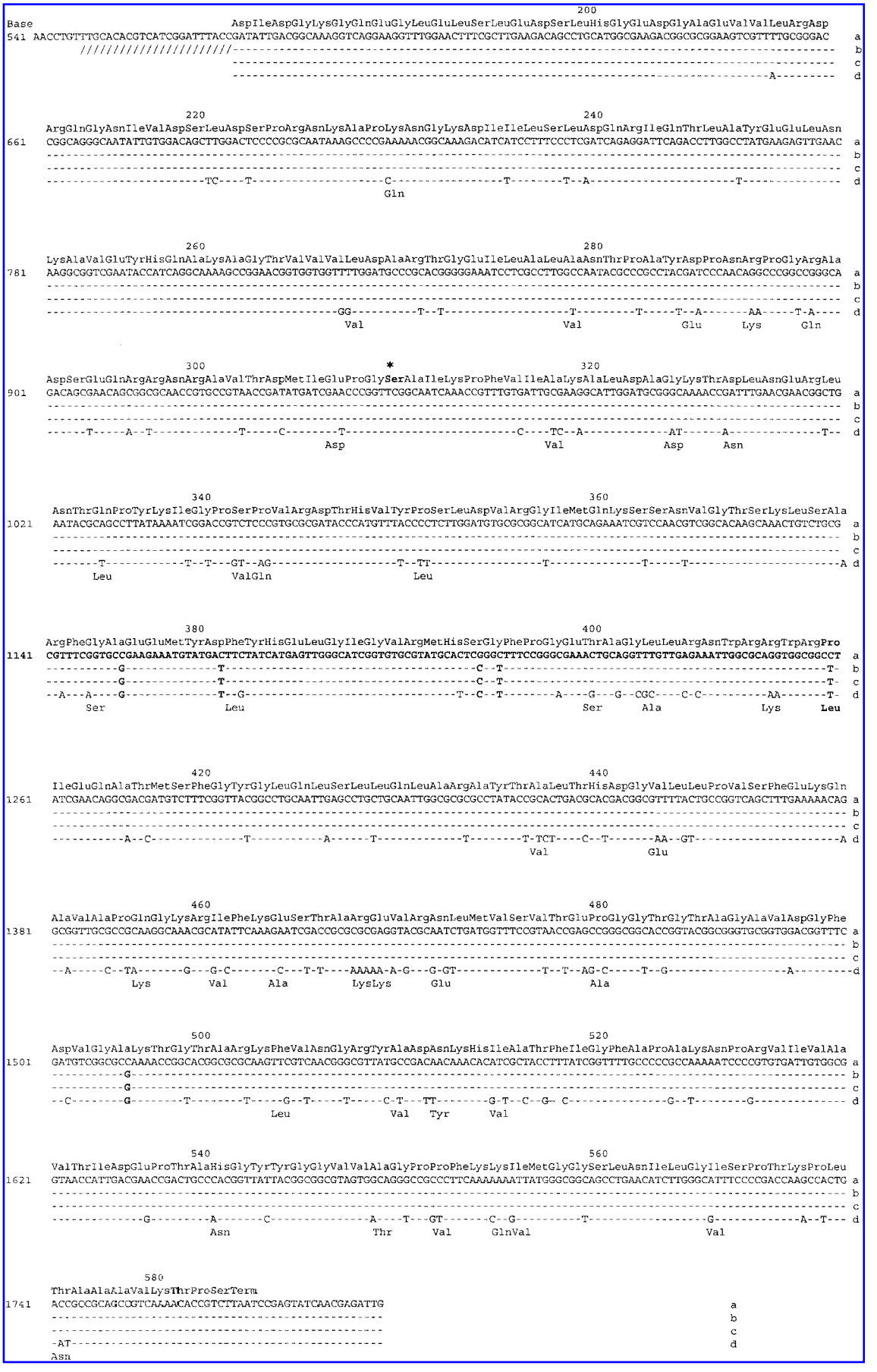

FIG. 1. Transpeptidase domain sequences of PBP2 (*penA* gene; nucleotides 571–1768). (**A**) *N. meningitidis* C311. (**B**) MC2. (**C**) MC2-26. (**D**) *N. polysaccharea* 3008. Recombined region between *N. polysaccharea* and *N. meningitidis* is dotted. //////, GCup primer; *, active site serine residue.

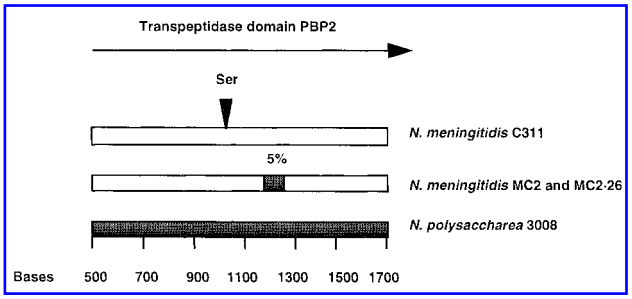

FIG. 2. Schematic diagram of the recombined region of PBP2 transpeptidase domain.

ing DNA can be explained as a consequence of the genetic distance between donor and recipient strains. When the source of DNA was *N. meningitidis*, the efficiency was much higher, whereas the efficiency decreased with heterologous donors. This may be a consequence of the number of sequences necessary to be recognized by the recipient strain. The increases of MIC in recipient strains could be the result of the cumulative effects of different steps in the acquisition of DNA fragments of the *penA* gene from the resistant strains. In some cases the MIC values detected in transformants were even higher than those of the corresponding donor strains. This puzzling results suggest that recombination can generate a wide variety of *penA* sequences, as was observed in natural penicillin-resistant population.[2] This reinforces the hypothesis proposed by Spratt *et al.*[14] based on the mosaic gene structure for *penA* genes. The use of microcosms in which plancktonic and biofilm-forming bacteria can grow, as it happens in the nasopharynx, suggests that this genetic exchange may be responsible of the increasing frequency of antibiotic resistance. However, in their natural habitat, *Neisseria* are subjected to a large number of interactions, either with microorganisms belonging to other groups, or with metabolites produced by the host and human cells that were not included in the microcosm.

In the experiments in microcosms, the frequency of transformants was strongly dependent on the stage of growth. There was an increase in the frequency at the exponential phase, and a decrease in the stationary phase. These differences in efficiency could be due to bacterial autolysis favored by adverse culture conditions such as lack of shaking. In natural conditions, there should be some effect produced by the lysis induced by host defense mechanisms. Lysis of bacteria is thus an indicator of the efficiency of the immune response, and the DNA released in this process may increase the genetic flexibility of the pathogen. This would enhance its ability to adapt to a range of environmental changes.

Initially, it can be suggested that the best conditions for growth, provided by a good aeration (shaking conditions), prevent bacterial autolysis; this can also be seen in growth curves (data not shown). This could reduce the availability of extracellular DNA and, subsequently, the number of transformants.

Our results show that DNase reduces the efficiency of genetic exchange, but even in the presence of high concentrations of enzyme, a considerable number of transformants were still detected. However, no conjugation is expected in the absence of any kind of plasmids. DNase has been described as a total inhibitor of transformation, however some authors suggest that cell-to-cell contact is necessary for transformation.[5] These processes, despite requiring cell-to-cell contact, should be considered as transformation since no special structures such pili, nor phages are involved. Dorward *et al.*[4] described the role of blebs in genetic exchange in *N. gonorrhoeae*. According to these results and suggestions, not even cell-to-cell contact would be required to protect the DNA against degradation by nucleases. This may also be true for *N. meningitidis*, but further work on bleb formation is required to confirm this idea. On the other hand, a mechanism should be invoked to explain the high level of protection of extracellular DNA. It should be explained how sequences persist in the environment even in the presence of DNase secreted by other microorganisms inhabiting the human nasopharynx, as well as the differences in the transformation frequency obtained in co-cultivation experiments and those carried out with a saturating concentration of DNA (Table 3). It is possible that, under the microcosm conditions, DNA is protected against DNase by the existence of liquid–solid interfaces, since the frequency of transformation was near zero in liquid culture, whereas transformants did appear under the microcosm conditions even when naked DNA was used.

Transformant MC2 with a MIC value of 0.1 μg/mL obtained by co-cultivation of a fully susceptible meningococcus and *N. polysaccharea* 3008 in a single step showed five nucleotide substitutions in the transpeptidase domain of the *penA* gene. All five substitutions were identical to those of the *N. polysaccharea* 3008 sequence. We propose that these alterations are responsible for the increased MIC value of transformant MC2. Similar substitutions have been described in other moderately resistant meningococci.[14] All substitutions in PBP3 of *Escherichia coli* and PBP2 of *N. gonorrhoeae* leading to decreased affinity for penicillin are located near the carboxy-terminal regions of the proteins (between residues 257 and 545).[13] Several reports have described mosaicism of genes in this region.[14] However, in the studies described in the literature, differences ranged in the order of 10–25%, whereas in our case only 5% differences was detected. The MIC increases observed in our experiments were also less extensive.

Transformant MC2-26 was identical in sequence to that of MC2, yet its MIC was three times higher. This suggests that some concomitant mechanism affecting *loci* other than *pbps* may also contribute to antibiotic resistance. This possibility has been postulated by other authors,[1,15] when unaltered *penA* gene has been described in transformants with decreased susceptibility to penicillin.

In summary, this work reports new data that reinforce the idea that penicillin resistance in meningococci is due to the decreased penicillin affinity of their PBP2, which is programmed by the acquisition of DNA fragments originating in the commensal nasopharyngeal flora of nonpathogenic *Neisseriae*, although the possible role of other sources of genetic material cannot be ruled out either.

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