Further evidence for the non-clonal population structure of *Neisseria gonorrhoeae*: extensive genetic diversity within isolates of the same electrophoretic type

Maria O'Rourke and Brian G. Spratt

Author for correspondence: Brian G. Spratt. Tel: +44 273 678309. Fax: +44 273 678433.

Microbial Genetics Group,
School of Biological
Sciences, University of
Sussex, Falmer, Brighton
BN1 9QG, UK

Analysis of data from multilocus enzyme electrophoresis has revealed that *Neisseria gonorrhoeae* populations are non-clonal. Fifteen percent of 227 isolates of *N. gonorrhoeae* had an identical multilocus genotype (ET1) and were recovered world-wide over a 26 year period. The recovery of isolates of identical multilocus genotype from geographically and temporally unassociated hosts is a common criterion of a clonal population structure. However, in a recombining (non-clonal) population, isolates with the same multilocus genotype can arise by the random association of the most common alleles in the population. Analysis of the variation in two further enzymes, in the restriction patterns obtained from the glutamine synthetase gene, and in the DNA fragments obtained using an arbitrarily primed polymerase chain reaction, was used to show that members of ET1 were almost as variable as randomly selected *N. gonorrhoeae* isolates. Unlike the situation in a strongly clonal species, the 26 ET1 isolates examined were increasingly sub-divided to give 19 distinguishable groups as variation at further loci was examined, and 24 distinguishable groups when auxotypes were also considered. We conclude that, as expected of a non-clonal population, the most commonly encountered *N. gonorrhoeae* multilocus genotype does not define a clone.

Keywords: *Neisseria gonorrhoeae*, electrophoretic type, recombination, non-clonal population structure

INTRODUCTION

Electrophoretic studies of bacterial populations have revealed that most bacterial species are clonal in structure, implying low rates of recombination between chromosomal genes in nature (Musser *et al.*, 1987, 1988; Ochman *et al.*, 1983; Piñero *et al.*, 1988; Denny *et al.*, 1988; Gargallo-Viola, 1989). Clonal structure is characterized by the existence of strong linkage disequilibrium between alleles, and by the repeated recovery of isolates of the same multilocus genotype (or electrophoretic type, ET) from temporally and geographically unassociated hosts (Selander & Musser, 1990). Isolates that have the same multilocus genotype are considered to be members of the same clone and, as expected, typically exhibit identity in other characters including ribotypes (Eardly *et al.*, 1990;

Arthur *et al.*, 1990), RFLP profiles (Denny *et al.*, 1988; Musser *et al.*, 1988) and metabolic characters (Selander *et al.*, 1990; Miller & Hartl, 1986).

Recent studies suggest that the clonal paradigm for bacterial population structures may be an oversimplification, and that in nature, rates of recombination may be highly variable, resulting in population structures that range from strongly clonal to non-clonal (Istock *et al.*, 1992; Souza *et al.*, 1992; Maynard Smith *et al.*, 1993; O'Rourke & Stevens, 1993; Vázquez *et al.*, 1993). We have recently shown that the naturally-transformable species *Neisseria gonorrhoeae* has a non-clonal population structure, implying extensive recombination in nature (O'Rourke & Stevens, 1993; Vázquez *et al.*, 1993). Although the alleles in a population of gonococci isolated from world-wide locations were at, or close to, linkage equilibrium, 35/227 (15%) of the isolates were indistinguishable by multilocus enzyme electrophoresis using nine enzymes, and were thus assigned to a single ET

Abbreviations: AP-PCR, arbitrarily primed polymerase chain reaction; ET, electrophoretic type.

(ET1) (O'Rourke & Stevens, 1993). Similarly, eight other ETs contained more than four isolates.

The existence of apparent 'clones' in a non-clonal population is at first sight contradictory. However, in contrast to a clonal population, in which identity of multilocus genotype implies recent common ancestry, in a recombining (non-clonal) population, isolates of identical multilocus genotype can arise by the random association of the most common alleles at each locus. Thus, in a non-clonal population, isolates with the same multilocus genotype are not the result of recent common ancestry, but represent a collection of genetically diverse isolates that, as a result of frequent recombination, possess the same alleles at each of those loci that are examined.

We have suggested that the *N. gonorrhoeae* ETs that are represented by multiple isolates do not define clones since the numbers of isolates in these ETs are not significantly different from those expected to arise by assortative recombination, given the observed allele frequencies (Maynard Smith *et al.*, 1993; O'Rourke & Stevens, 1993). In this paper we establish that the *N. gonorrhoeae* isolates of ET1 are not members of a clone.

METHODS

Enzyme electrophoresis. Each of the gonococcal strains was grown in GC broth plus supplements and harvested and lysed using the method described previously (O'Rourke & Stevens, 1993). Lysates were electrophoresed on 11% (w/v) starch gels and stained for NADH-dependent diaphorase and NADPH-dependent diaphorase as described elsewhere (Selander *et al.*, 1986; Chun *et al.*, 1985).

Polymorphisms within the *glnA* gene. DNA was purified from lysates by RNase A treatment, followed by phenol/chloroform extraction, and ethanol precipitation. The region encoding the glutamine synthetase gene (*glnA*) was amplified by PCR using the oligonucleotide primers *glnA*-up (5'-GTCGATTTGCG-(GATC)TT(TC)AC(GATC)GA-3') and *glnA*-down (5'-GACGTTTACAG(GATC)CT(AG)TA(AG)TA-3'). These primers correspond to nucleotides 174–193 and 1547–1527 in the sequence of the *N. gonorrhoeae glnA* gene (GenBank accession number M84117). The PCR was performed for 22 cycles, as described previously (Zhang *et al.*, 1990), using a cycle of 95 °C for 1 min, 55 °C for 2 min, and 72 °C for 2 min. The amplified 1.36 kb *glnA* fragments were purified by GeneClean (Bio101), digested with *HpaII*, labelled with [α -³²P]dCTP, and electrophoresed on a 6% (w/v) non-denaturing polyacrylamide gel as described previously (Zhang *et al.*, 1990).

Arbitrarily primed PCR (AP-PCR). This was performed using the oligonucleotide Pn-2X-1 (5'-AGGACT(TC)TGTTTGGCGT-GATAT-3'). Reaction mixtures were prepared in 50 µl volumes containing 100 ng genomic DNA, 1 µg Pn-2X-1, 2 mM of each dNTP, 3.5 mM magnesium chloride, 1% (v/v) DMSO, 15 mM ammonium sulphate, 60 mM Tris/HCl, pH 8.5). Amplification was carried out for 10 cycles of 95 °C for 1 min, 33 °C for 30 s, and 67 °C for 2 min, followed by a further 25 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min. AP-PCR was performed on each strain seven times.

Auxotyping and serotyping. Auxotyping and serotyping were performed as previously described (O'Rourke & Stevens, 1993). Strains were tested for single or multiple requirements for arginine, hypoxanthine, uracil and proline.

RESULTS

Isolates with identical multilocus genotypes that are members of a clone can readily be distinguished from those that are due to random associations of common alleles by an examination of variation at further loci. If the isolates of ET1 are members of a clone there should be essentially no variation at other loci, whereas if they are the result of recombination there should be as much variation within ET1 as in a random collection of gonococcal isolates. We therefore analysed electrophoretic variation in two further enzymes, in the restriction fragments from the glutamine synthetase gene (*glnA*), and in the DNA fragments amplified by AP-PCR (Fekete *et al.*, 1992), in 26 isolates of ET1 and in 26 control gonococci representing isolates of 21 different ETs (Table 1).

Variation at the diaphorase loci

NADH- and NADPH-dependent diaphorase were both polymorphic for three alleles in the strains analysed (Table 1). The NADH-dependent enzyme was not discriminatory, as allele 2 was present in almost all of the 52 isolates (allele frequency 0.92). The NADPH-dependent enzyme was more variable, with each of the three alleles being found in both the ET1 strains and the control strains.

Variation within the *glnA* gene

The *glnA* gene of both *N. gonorrhoeae* and *N. meningitidis* has been shown to be highly variable (J. Zhou, N. H. Smith & B. G. Spratt, unpublished). Four different patterns of *HpaII* fragments were obtained from the *glnA* genes of the 52 *N. gonorrhoeae* isolates (Fig. 1). Each of these patterns was found among both the ET1 strains and the control strains (Table 1).

Variation detected by AP-PCR

AP-PCR was carried out using a number of oligonucleotides designed for other purposes. Pn-2X-1, corresponding to a sequence within the *Streptococcus pneumoniae* penicillin-binding protein 2X gene, gave the largest number of fragments using *N. gonorrhoeae* FA19 chromosomal DNA and was chosen for further work. Since the AP-PCR technique uses a single primer, the intensity of bands may differ in different PCR reactions of the same strain. Hence, the technique was performed on each strain seven times using standard reaction conditions and a standard reaction mixture. The fragments produced using Pn-2X-1 were reproducible since the same pattern of fragments was obtained using DNA prepared from six independent cultures of *N. gonorrhoeae* strain 8030. The same pattern of fragments was obtained from DNA prepared from this isolate following serial subculture each day for 10 d. A similar stability of fragment pattern was found for a second isolate (4513) following 10 serial subcultures. Thirteen different AP-PCR fragment patterns

Table 1. Variation among 26 isolates of *N. gonorrhoeae* ET 1 and 26 isolates of varied ET

Strain	Location	Year	ET†	<i>glnA</i> fingerprint pattern	Diaphorase allele		AP-PCR	Serovar	Auxo- type‡
					NADH	NADPH			
Medan 11	Indonesia	1983	1	4	2	2	7	1A-4	NR
Medan 21	Indonesia	1983	1	4	2	2	7	1B-13	PA ⁻
A9446/88	Norway	1988	1	4	2	2	7	—	A ⁻
*J326B	Jamaica	—	1	1	2	2	1	1B-3	NR
*3551/87	Scotland	1987	1	1	2	2	1	1B-3	NR
*SB2962	Thailand	1988	1	1	2	2	1	1B-6	NR
J411B-2	Jamaica	—	1	1	2	2	1	1A-8	PAU ⁻
Medan 56	Indonesia	1983	1	1	2	1	1	1B-6	P ⁻
5615	Ireland	1986	1	1	2	1	1	1B-3	NR
56567	Denmark	1963	1	1	2	2	7	1B-3	NR
J317B	Jamaica	—	1	1	2	2	7	—	P ⁻
Medan 26	Indonesia	1983	1	3	2	2	7	—	P ⁻
Medan 60	Indonesia	1983	1	1	2	2	6	1A-1	NR
8030	Ireland	1984	1	1	1	2	7	1B-16	NR
5188	Ireland	1985	1	1	2	1	7	1B-3	A ⁻
5936	Ireland	1985	1	1	2	2	8	1A-7	P ⁻
6	Ireland	1988	1	1	2	1	8	—	NR
4513	Ireland	—	1	2	2	2	6	1A-5	NR
SB3270	Thailand	1988	1	1	2	3	6	1B-7	P ⁻
LDJ164	USA	1989	1	4	2	1	7	1B-7	PH ⁻
30991	USA	1989	1	4	2	2	12	1B-18	NR
36151	USA	1989	1	1	2	2	13	—	NR
31423	Denmark	1963	1	3	2	1	7	1A-1	P ⁻
34083/87	Scotland	1987	1	2	2	2	12	1B-3	P ⁻
Godhab 10	Greenland	1986	1	1	2	1	10	1A-14	P ⁻
A9972/88	Norway	1988	1	4	2	2	1	1B-3	AHU ⁻
9/86	Botswana	1986	82	4	2	2	1	—	NR
2162	Ireland	1987	3	4	2	2	1	1B-3	P ⁻
5/86	Botswana	1986	3	2	2	2	1	1B-24	P ⁻
34732/87	Scotland	1987	78	2	2	2	1	1A-4	P ⁻
56917	Denmark	1963	51	2	2	2	1	1B-1	A ⁻
8	Ireland	1988	6	1	1	2	6	1B-2	NR
HNL890102	USA	1989	6	1	2	3	10	1B-11	PH ⁻
16328/87	Scotland	1987	33	3	2	1	7	1A-4	NR
30995	USA	1989	33	3	2	2	9	1B-3	P ⁻
Medan 32	Indonesia	1983	69	4	2	3	7	1A-6	P ⁻
Medan 63	Indonesia	1983	69	1	2	1	8	—	NR
SB3327	Thailand	1988	69	1	2	2	7	—	NR
5465	Ireland	1986	42	1	1	2	1	—	A ⁻
37178/87	Scotland	1987	27	1	2	2	3	1B-14	NR
57524	Denmark	1963	22	4	2	2	11	—	NR
3081	Dakar	1982	16	2	2	1	1	1B-4	P ⁻
3084	Dakar	1982	36	1	2	2	4	—	NR
DA13	Dakar	1982	40	1	2	2	2	—	NR
Godhab 25	Greenland	1987	31	1	2	1	7	1B-4	P ⁻
12/86	Botswana	1986	79	1	3	1	1	1B-1	NR
19/86	Botswana	1986	74	3	2	1	5	1B-5	PH ⁻
SB2629	Thailand	1988	8	1	2	2	10	1B-7	NR
J140A	Jamaica	—	59	1	2	3	2	—	NR
J319A	Jamaica	—	14	1	2	3	1	—	—
JKD170	Australia	—	4	1	2	2	1	1B-3	NR
HNL890105	USA	1989	56	3	2	1	1	1B-16	PAU ⁻

* These three isolates are indistinguishable, except by serovar (see text).

† ETs are as designated previously (O'Rourke & Stevens, 1993).

‡ Requirements for arginine (A⁻), proline (P⁻), proline + arginine (PA⁻), proline + arginine + uracil (PAU⁻), proline + hypoxanthine (PH⁻), arginine + hypoxanthine + uracil (AHU⁻). NR, no requirements.

Fig. 1. *HpaII* fingerprints of the *glnA* genes of *N. gonorrhoeae*. The ^{32}P -labelled *HpaII* fragments from the *glnA* genes of gonococci were fractionated on a 6% (w/v) polyacrylamide gel and autoradiographed as described in the text. Lanes A–H correspond to fingerprints of the *N. gonorrhoeae* strains 4513 (2), 31423 (3), 5936 (1), 30991 (4), 2162 (4), 30995 (3), 3081 (2) and J140A (1) respectively; the numbers in parentheses correspond to fingerprint alleles listed in Table 1. Lanes marked m contain size markers (pBR322 digested with *HpaII*), and the sizes of some of the fragments (in bp) are shown to the right of lane H. The sizes of the visible *HpaII* fragments of pBR322 are 622, 527, 404, 309, 242, 238, 217, 201, 190, 180, 160, 147, 123, 110, 90, 76, 67, 34 and 26 bp.

were found among the 52 isolates (Fig. 2). Extensive variation was found among both the ET1 isolates and the control isolates (Table 1).

Variation within ET1 isolates and non-ET1 isolates

Examination of the variation in the 26 ET1 isolates using the two diaphorase enzymes, *glnA* fingerprinting and AP-PCR resulted in the splitting of these previously indistinguishable isolates into 19 groups. If the variation in auxotype was also included, the ET1 strains were divided

Fig. 2. Electrophoretic analysis of AP-PCR patterns of 12 different *N. gonorrhoeae* strains amplified by primer Pn-2X-1. Patterns depicted in lanes A–L correspond to *N. gonorrhoeae* strains Medan 32, 36151, 34083/87, SB2629, 5936, 30995, Godhab 10, 30991, 8, 3084, 5615 and 57524 respectively. Lanes A–L correspond to AP-PCR types 7, 13, 12, 10, 8, 9, 10, 12, 6, 4, 1 and 11 respectively. Lanes marked m contain size markers (1 kb DNA ladder) and the sizes of some of the fragments are shown (in bp) to the right of lane L. Three AP-PCR types are not depicted: type 2 consists of a single 370 bp fragment, type 3 resembles type 8 with an additional 750 bp fragment, and type 5 consists of three fragments of 370, 390 and 650 bp.

further to give 24 groups; and when serovars were considered, only a single pair of isolates were identical. The 26 control gonococci included three isolates from ET69, and two isolates from ETs 3, 6 and 33. Examination of the additional loci showed in each case that these previously indistinguishable isolates could also now be distinguished (Table 1).

If the ET1 isolates are not members of a clone, but are genetically diverse isolates, there should be as much variation within these 26 isolates as in the 26 control isolates. The distribution of alleles, or characters, among ET1 and non-ET1 strains was determined and is illustrated in Fig. 3. The frequency at which the different electrophoretic alleles, *glnA* fingerprints and auxotypes were observed among ET1 strains was almost identical to the frequency at which the characters were observed in non-ET1 strains (Fig. 3). However, particular AP-PCR patterns were encountered at different frequencies in the two groups of strains. Considering the nature of AP-PCR, the number of possible patterns generated could be quite large. To determine whether the differences in the two groups were statistically significant, the variation of this character within the two groups of isolates was estimated as the conditional probability that a pair of isolates drawn randomly from the ET1 group or the control group would match in character. Each isolate was compared with every other isolate in the group and the number of pairs of isolates that matched in character state was recorded. The conditional probability that a pair of

Fig. 3. (a) Distribution of auxotype and *glnA*, NADH and NADPH alleles among 26 ET 1 (■) and 26 non-ET 1 (▒) isolates. (b) Distribution of AP-PCR patterns among 26 ET 1 (■) and 26 non-ET 1 (▒) isolates. Auxotypes and allele/character numbers correspond to those listed in Table 1.

isolates drawn from the ET1 group would match in character state was 21.8%, while the probability that a pair of isolates from the control group would match in character state was 19.4%. The variation in the AP-PCR patterns, and in each of the other characters, was therefore as great or almost as great in the members of ET1 as that in the control group of gonococcal isolates.

DISCUSSION

O'Rourke & Stevens (1993) and Vázquez *et al.* (1993) found no evidence of linkage disequilibrium in two different populations of *N. gonorrhoeae*. These results suggest that assortative recombination is frequent in this naturally transformable species and imply that mixed infections are relatively common in gonorrhoea. Even in a non-clonal population, isolates that are indistinguishable will inevitably be recovered from individuals that are epidemiologically linked, but it would be surprising frequently to recover indistinguishable isolates from individuals that are separated both temporally and geographically. The data presented here show that the ET1 isolates, which were indistinguishable by examination of

electrophoretic variation at nine enzyme-encoding loci, were highly diverse and are thus not members of a clone. Isolates from four other ETs that included multiple isolates (ETs 3, 6, 33 and 69) were in each case also differentiated by the examination of additional loci, further revealing that gonococcal isolates of shared ET cannot be assumed to be members of a clone. The analysis of relatively few loci, and the presence of predominant alleles at most of the loci studied by O'Rourke & Stevens (1993), probably accounts for the occurrence of several ETs that were represented by multiple isolates.

Three of the 26 ET1 isolates, recovered at different times in Jamaica, Scotland and Thailand (and indicated by an asterisk in Table 1), could still not be distinguished even when the variation in diaphorase, *glnA* fingerprints, AP-PCR and auxotype were considered (serovars were not considered as they are subject to convergence and rapid mutational change and are thus unsuitable characters for epidemiological studies). Although these isolates appear to be identical, there is little reason to believe that this is the case, since they possessed the most common alleles at the two diaphorase loci, and the commonest *glnA* fingerprint, AP-PCR pattern and auxotype. By chance, 1.5 isolates with this pattern of variation would be expected among the 26 ET1 strains, based on the frequencies of these alleles/patterns in the 52 gonococci that we examined.

The evidence from multilocus enzyme electrophoresis of frequent recombination (linkage equilibrium), and the diversity within strains of a shared ET, suggest that the epidemiology of gonorrhoea is problematic. Epidemiological conclusions based on studies using the nine enzymes examined by O'Rourke & Stevens (1993) would clearly be highly misleading, as this method groups diverse isolates into the same ET. A similar criticism applies to other methods of distinguishing gonococcal isolates that depend on small numbers of characters. The non-clonal nature of gonococcal populations implies that isolates recovered in one decade are distinct from those obtained in another decade, and negates attempts to study the long-term epidemiology of gonorrhoea. Fortunately, most studies on the epidemiology of gonorrhoea are concerned with the transmission of the disease on a very restricted geographical and temporal scale. Presumably, the rates of recombination, and the frequency of mixed infections, are not so high that they invalidate the epidemiological conclusions reached within this restricted setting. Further detailed studies of a local population of *N. gonorrhoeae*, which includes epidemiologically related strains, would be beneficial and could provide a better insight into the frequency of mixed infections and the extent of recombination in nature.

REFERENCES

- Arthur, M., Arbeit, R. D., Cheung, K., Beltran, P., Crowe, H., Steinbach, S., Campanelli, C., Wilson, R. A., Selander, R. K. & Goldstein, R. (1990). Restriction fragment length polymorphisms among uropathogenic *Escherichia coli* isolates: *pap*-related sequences compared with *rrn* operons. *Infect Immun* 58, 471-479.

- Chun, P. K., Sensabaugh, G. F. & Vedros, N. A. (1985). Genetic relationships among *Neisseria* species assessed by comparative enzyme electrophoresis. *J Gen Microbiol* **131**, 3105–3115.
- Denny, T. P., Gilmour, M. N. & Selander, R. K. (1988). Genetic diversity and relationships of two pathovars of *Pseudomonas syringae*. *J Gen Microbiol* **134**, 1949–1960.
- Eardly, B. D., Materon, L. A., Smith, N. H., Johnson, D. A., Rumbaugh, M. D. & Selander, R. K. (1990). Genetic structure of natural populations of the nitrogen-fixing bacterium *Rhizobium meliloti*. *Appl Environ Microbiol* **56**, 187–194.
- Fekete, A., Bantle, J. A., Halling, S. M. & Stich, R. W. (1992). Amplification fragment length polymorphism in *Brucella* strains by use of polymerase chain reaction with arbitrary primers. *J Bacteriol* **174**, 7778–7783.
- Gargallo-Viola, D. (1989). Enzyme polymorphism, prodigiosin production and plasmid fingerprints in clinical and naturally occurring plasmids of *Serratia marcescens*. *J Clin Microbiol* **27**, 860–868.
- Istock, C. A., Duncan, K. E., Ferguson, N. & Zhou, X. (1992). Sexuality in a natural population of bacteria – *Bacillus subtilis* challenges the clonal paradigm. *Mol Ecol* **1**, 195–203.
- Maynard Smith, J., Smith, N. H., O'Rourke, M. & Spratt, B. G. (1993). How clonal are bacteria? *Proc Natl Acad Sci USA* **90**, 4384–4388.
- Miller, R. D. & Hartl, D. L. (1986). Biotyping confirms a nearly clonal population structure in *Escherichia coli*. *Evolution* **40**, 1–12.
- Musser, J. M., Bemis, D. A., Ishikawa, H. & Selander, R. K. (1987). Clonal diversity and host distribution in *Bordetella bronchiseptica*. *J Bacteriol* **169**, 2793–2803.
- Musser, J. M., Kroll, J. S., Moxon, E. R. & Selander, R. K. (1988). Clonal population structure of encapsulated *Haemophilus influenzae*. *Infect Immun* **56**, 1837–1845.
- Ochman, H., Whittam, T. S., Causant, D. A. & Selander, R. K. (1983). Enzyme polymorphism and genetic population structure in *Escherichia coli* and *Shigella*. *J Gen Microbiol* **129**, 2715–2726.
- O'Rourke, M. & Stevens, E. (1993). Genetic structure of *Neisseria gonorrhoeae* populations: a non-clonal pathogen. *J Gen Microbiol* **139**, 2603–2611.
- Piñero, D., Martinez, E. & Selander, R. K. (1988). Genetic diversity and relationships among isolates of *Rhizobium leguminosarum* biovar. *phaseoli*. *Appl Environ Microbiol* **54**, 2825–2832.
- Selander, R. K. & Musser, J. M. (1990). Population genetics of bacterial pathogenesis. In *Molecular Basis of Bacterial Pathogenesis*, pp. 11–36. Edited by B. H. Iglewski & V. L. Clark. Orlando: Academic Press.
- Selander, R. K., Causant, D. A., Ochman, H., Musser, J. M., Gilmour, M. N. & Whittam, T. S. (1986). Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics. *Appl Environ Microbiol* **51**, 873–884.
- Selander, R. K., Beltran, P., Smith, N. H., Barker, R. M., Crichton, P. B., Old, D. C., Musser, J. M. & Whittam, T. S. (1990). Genetic population structure, clonal phylogeny and pathogenicity of *Salmonella paratyphi* B. *Infect Immun* **58**, 2262–2275.
- Souza, V., Nguyen, T. T., Hudson, R. R., Piñero, D. & Lenski, R. E. (1992). Hierarchical analysis of linkage disequilibrium in *Rhizobium* populations: evidence for sex? *Proc Natl Acad Sci USA* **89**, 8389–8393.
- Vázquez, J. A., de la Fuente, L., Berron, S., O'Rourke, M., Smith, N. H., Zhou, J. & Spratt, B. G. (1993). Ecological separation and genetic isolation of *Neisseria gonorrhoeae* and *Neisseria meningitidis*. *Curr Biol* **3**, 567–572.
- Zhang, Q.-Y., Jones, D. M., Saez Nieto, J. A., Perez Trallero, E. & Spratt, B. G. (1990). Genetic diversity of penicillin binding protein 2 genes of penicillin resistant strains of *Neisseria meningitidis* revealed by fingerprinting of amplified DNA. *Antimicrob Agents Chemother* **34**, 1523–1528.

Received 5 November 1993; revised 6 February 1994; accepted 9 February 1994.