Genetic structure of *Neisseria gonorrhoeae* populations: a non-clonal pathogen

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Reproduction by binary fission generates a clonal genetic structure in bacterial populations in the absence of a high rate of recombination. The extent of recombination in natural populations of *Neisseria gonorrhoeae* was determined from an analysis of electrophoretically demonstrable allelic variation at structural genes encoding nine enzyme loci in 227 worldwide isolates. No significant linkage disequilibrium was evident in the population, indicating that recombination must be frequent, relative to binary fission. The genetic structure of *N. gonorrhoeae* was compared with that of *Bacillus subtilis* from an earlier study. Linkage disequilibrium was less extreme in the *N. gonorrhoeae* population than in the local population of *B. subtilis*, in which only modest clonal structure was evident. Thus, *N. gonorrhoeae*, unlike pathogens so far examined, has a non-clonal population structure. As expected in a freely recombining population, no correlation was found between electrophoretic genotype and serovar or auxotype.

Introduction

Neisseria gonorrhoeae is the causative agent of gonorrhoea, a disease which has been recognized since antiquity and is still a common bacterial infection (Centers for Disease Control, 1992). Auxotyping and serovar determination have been accepted as the two major phenotypic systems for the typing of *N. gonorrhoeae* and the combination of both techniques has enabled temporal and geographic changes in gonococcal populations to be monitored (Sarafian & Knapp, 1989). Information on genetic variation in *N. gonorrhoeae* has been determined from restriction endonuclease analysis (Falk *et al.*, 1984; Poh *et al.*, 1989) and more recently from RFLP analysis using rRNA probes (Poh *et al.*, 1992), while several studies have documented both phase and antigenic variation in gonococcal surface antigens (Seifert & So, 1988). Although most of these methods have revealed extensive variation in *N. gonorrhoeae*, none has yielded reliable estimates of the extent of variation in the genome as a whole.

The technique of multilocus enzyme electrophoresis

(MLEE), which indexes allelic variation in multiple chromosomal genes, has been used extensively to determine the genetic structure of natural populations. Such electrophoretic studies have suggested that the population structures of many species, including *Escherichia coli* (Selander & Levin, 1980; Selander *et al.*, 1987), *Legionella pneumophila* (Selander *et al.*, 1985), *Listeria monocytogenes* (Piffaretti *et al.*, 1989), *Haemophilus influenzae* (Musser *et al.*, 1988), *Serratia marcescens* (Gargallo-Viola, 1989) and *Bordetella bronchiseptica* (Musser *et al.*, 1987) are basically clonal. Clonal structure is characterized by the existence of strong linkage disequilibrium (non-random association of alleles), indicating restricted recombination among alleles at different loci (Selander & Musser, 1990). As a consequence of infrequent recombination and periodic selection, only a fraction of all possible genotypes are detected and isolates of identical multilocus genotype are recovered from diverse geographic areas over long periods of time.

Although bacterial reproduction is asexual, the transfer of chromosomal genes can be achieved through the processes of transduction, conjugation and transformation. Among those species which exhibit strong linkage disequilibrium, many possess the ability to exchange genetic material by these parasexual methods. The extent of gene transfer by transduction and conjugation has been considered insufficient to disrupt the clonal framework of *E. coli* populations (Levin, 1981; Whittam *et al.*, 1983*b*), although nucleotide sequence evidence suggests

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Abbreviations: ET, electrophoretic type; MLEE, multilocus enzyme electrophoresis; RFLP, restriction fragment length polymorphism.

Table 1. *Allele profiles, auxotypes and serovars for 89 ETs of N. gonorrhoeae*

ET	Strain	Allele at indicated enzyme locus:*									No. of isolates	Source†	Year	Auxotype	Serovar‡
		GD2	IDH	GOT	PGI	MAE	G6P	ALP	LAP	ADK					
1	31423	2	3	4	3	1	3	2	2	2	(35)	Denmark	1963	P ⁻	1A-1
1	56567	2	3	4	3	1	3	2	2	2		Denmark	1963	NR	1B-3
1	1/86	2	3	4	3	1	3	2	2	2		Botswana	1986	NR	1B-3
1	3091	2	3	4	3	1	3	2	2	2		Dakar	1982	P ⁻	1B-3
1	3096	2	3	4	3	1	3	2	2	2		Dakar	1982	NR	-
1	10 Godhab	2	3	4	3	1	3	2	2	2		Greenland	1987	P ⁻	1A-14
1	16 Godhab	2	3	4	3	1	3	2	2	2		Greenland	1987	NR	1B-20
1	A9446/88	2	3	4	3	1	3	2	2	2		Norway	1988	-	-
1	A9972/88	2	3	4	3	1	3	2	2	2		Norway	1988	A ⁻ H ⁻ U ⁻	1B-3
1	024483/87	2	3	4	3	1	3	2	2	2		Scotland	1987	A ⁻	-
1	34083/87	2	3	4	3	1	3	2	2	2		Scotland	1987	P ⁻	1B-3
1	3551/87	2	3	4	3	1	3	2	2	2		Scotland	1987	NR	1B-3
1	J317B	2	3	4	3	1	3	2	2	2		Jamaica	-	P ⁻	-
1	J326B	2	3	4	3	1	3	2	2	2		Jamaica	-	NR	1B-3
1	J411B-2	2	3	4	3	1	3	2	2	2		Jamaica	-	P ⁻ A ⁻	1A-8
1	SB3270	2	3	4	3	1	3	2	2	2		Thailand	1988	P ⁻	1B-7
1	SB2961	2	3	4	3	1	3	2	2	2		W. Africa	1988	NR	1B-6
1	SB2962	2	3	4	3	1	3	2	2	2		W. Africa	1988	NR	1B-6
1	Medan 7	2	3	4	3	1	3	2	2	2		Indonesia	1983	P ⁻	1A-6
1	Medan 11	2	3	4	3	1	3	2	2	2		Indonesia	1983	NR	1A-4
1	Medan 21	2	3	4	3	1	3	2	2	2		Indonesia	1983	P ⁻ A ⁻	1B-13
1	Medan 26	2	3	4	3	1	3	2	2	2		Indonesia	1983	P ⁻	-
1	Medan 56	2	3	4	3	1	3	2	2	2		Indonesia	1983	P ⁻	1B-6
1	Medan 60	2	3	4	3	1	3	2	2	2		Indonesia	1983	NR	1A-1
1	JKD179	2	3	4	3	1	3	2	2	2		Australia	-	-	-
1	LDJ164	2	3	4	3	1	3	2	2	2		US	1989	P ⁻ H ⁻	1B-7
1	30991	2	3	4	3	1	3	2	2	2		US	1989	NR	1B-18
1	36151	2	3	4	3	1	3	2	2	2		US	1989	NR	-
1	5615	2	3	4	3	1	3	2	2	2		Ireland	1986	NR	1B-3
1	5188	2	3	4	3	1	3	2	2	2		Ireland	1985	A ⁻	1B-3
1	5936	2	3	4	3	1	3	2	2	2		Ireland	1985	P ⁻	1A-7
1	8030	2	3	4	3	1	3	2	2	2		Ireland	1984	NR	1B-16
1	4513	2	3	4	3	1	3	2	2	2		Ireland	-	NR	1A-5
1	4539	2	3	4	3	1	3	2	2	2		Ireland	1985	P ⁻	1B-3
1	6	2	3	4	3	1	3	2	2	2		Ireland	1988	NR	-
2	537	2	3	4	5	1	3	2	2	2	(3)	Ireland	1987	NR	1B-2
3	36736/87	2	3	4	2	1	3	2	2	2		Scotland	1987	A ⁻	1B-3
4	SB2701	2	3	4	4	1	3	2	2	2		Thailand	-	NR	-
5	Godhab/17	2	3	4	1	1	3	2	2	2	(1)	Greenland	1987	P ⁻	1B-30
6	HNL890102	4	3	4	2	1	3	2	2	2		US	1989	P ⁻ H ⁻	1B-11
7	JKD174	2	1	4	1	1	3	2	2	2		Australia	-	NR	1A-8
8	SB2629	2	3	1	4	1	3	2	2	2	(1)	Thailand	-	NR	1B-7
9	56914/15	2	3	3	4	1	3	2	2	2		Denmark	1963	NR	-
10	30990	1	3	2	4	1	3	2	2	2		US	1989	-	1A-2
11	Godhab 7	2	2	4	3	1	2	2	2	2	(3)	Greenland	1987	P ⁻	-
12	J244A	2	3	4	3	1	2	2	2	2		Jamaica	-	NR	1B-3
13	608	2	3	4	2	1	2	2	2	1		Ireland	1984	P ⁻	1B-3
14	33758	2	3	4	2	1	2	2	2	2	(2)	US	1989	P ⁻	1A-4
15	JKD121	2	3	4	2	1	4	2	2	2		Australia	-	P ⁻	1B-2
16	3081	2	3	4	3	1	3	2	2	3		Dakar	1982	P ⁻	1B-4
17	35870	2	3	2	3	1	3	2	2	3	(4)	US	1989	P ⁻ H ⁻	1B-1
18	A9122/88	2	3	4	5	1	3	2	2	3		Norway	1988	P ⁻ A ⁻	1A-4
19	G3	2	1	4	3	1	3	2	2	3		Ireland	1988	A ⁻ H ⁻	1A-1
20	LDJ 244	2	3	2	2	1	3	2	2	1	(1)	US	1989	P ⁻ H ⁻	1A-4
21	3090	4	3	1	3	1	3	2	2	2		Dakar	1982	NR	1A-19
22	57524	2	3	2	3	1	3	2	2	2		Denmark	1963	NR	-
23	Medan 14	2	3	2	3	1	3	2	3	2	(1)	Indonesia	1983	P ⁻	1B-2
24	SB3077	2	3	2	3	1	3	2	1	2		Nigeria	-	P ⁻	-
25	5330	3	3	2	3	1	3	2	3	2		Ireland	-	NR	1A-15
26	JKD185	2	3	3	3	1	3	2	2	2	(7)	Australia	-	P ⁻	-
27	37178/87	2	2	3	3	1	3	2	2	2		Scotland	1987	NR	1B-14
28	SB3326	2	2	2	3	1	3	2	2	2		Grenada	-	NR	-
29	3097	2	4	4	3	1	3	2	2	2	(3)	Dakar	1982	NR	1B-6
30	Sb	2	4	4	3	1	3	3	2	2		Unknown	-	P ⁻	1B-6
31	J186B	2	4	4	3	1	3	2	3	2		Jamaica	-	NR	1B-4

Table 1 (cont.)

ET	Strain	Allele at indicated enzyme locus:*									No. of isolates	Source†	Year	Auxotype	Serovar‡
		GD2	IDH	GOT	PGI	MAE	G6P	ALP	LAP	ADK					
32	SB3268	2	4	2	3	1	1	2	2	2	(1)	Thailand	—	NR	1B-30
33	16328/87	2	3	4	3	1	3	3	2	2	(9)	Scotland	1987	NR	1A-4
33	16301/87	2	3	4	3	1	3	3	2	2		Scotland	1987	NR	1A-4
34	3075	2	3	4	3	1	3	1	2	2	(6)	Dakar	1982	NR	1A-11
35	Medan 23	4	3	4	3	1	3	1	2	2	(4)	Indonesia	1983	NR	1B-1
36	3084	3	3	4	3	1	3	1	2	2	(3)	Dakar	1982	NR	—
37	SB3038	3	3	4	3	1	4	1	2	2	(2)	Thailand	—	NR	1B-1
38	5793	3	3	2	3	1	3	1	2	2	(1)	Ireland	—	A ⁻	1A-6
39	3094	2	3	4	3	1	3	1	1	2	(2)	Dakar	1982	NR	—
40	JKD213	2	3	4	3	1	3	1	3	2	(11)	Australia	—	P ⁻ A ⁻	1B-2
41	3078	2	3	4	3	1	3	1	1	2	(1)	Dakar	1982	P ⁻	—
42	4354	3	3	4	3	1	3	2	2	2	(4)	Ireland	1985	NR	—
43	C5	3	3	4	3	1	3	2	1	2	(1)	Canada	—	A ⁻ H ⁻ U ⁻	—
44	17/86	3	3	4	3	1	3	2	1	3	(1)	Botswana	1986	P ⁻	—
45	3086	3	3	4	3	1	2	2	3	2	(1)	Dakar	1982	NR	1A-1
46	4370	3	3	4	2	1	3	3	2	2	(1)	Ireland	—	NR	1B-13
47	415	3	3	4	3	1	3	3	2	2	(2)	Ireland	1986	NR	1B-6
48	J367B-2	4	3	4	1	1	3	3	2	2	(1)	Jamaica	—	P ⁻	1A-2
49	5050	4	3	4	2	1	3	3	2	3	(1)	Ireland	1986	NR	—
50	15/86	2	3	4	2	1	3	3	4	2	(3)	Botswana	1986	P ⁻ A ⁻	1B-6
51	Godhab 8	2	3	4	3	1	3	2	4	2	(1)	Greenland	1987	P ⁻	—
52	2329	3	3	4	2	1	3	3	1	2	(1)	Ireland	1987	P ⁻	1B-1
53	14/86	2	3	4	2	1	3	3	1	2	(1)	Botswana	1986	P ⁻	1A-1
54	7993	2	3	4	2	1	3	2	1	2	(1)	Ireland	1985	NR	1B-3
55	32211/87	2	3	4	2	1	3	1	2	2	(1)	Scotland	1987	NR	—
56	HNL890105	2	3	4	2	1	3	3	2	2	(2)	US	1989	P ⁻ A ⁻ U ⁻	1B-16
57	J49B	2	3	4	2	1	1	3	2	3	(1)	Jamaica	—	P ⁻	1B-2
58	28517/87	2	3	3	2	1	3	3	2	2	(2)	Scotland	1987	—	1A-15
59	J140A	2	2	3	2	1	3	3	2	2	(1)	Jamaica	—	NR	—
60	6/86	2	2	3	3	1	3	3	2	2	(1)	Botswana	1986	P ⁻	1B-3
61	35871	2	2	4	3	1	3	3	2	2	(2)	US	1989	P ⁻ A ⁻	1B-1
62	LDJ 160	2	2	4	2	1	3	3	2	2	(1)	US	1989	P ⁻	1B-7
63	Godhab 24	2	2	4	3	1	3	1	2	2	(2)	Greenland	1987	NR	1B-3
64	18/86	2	3	2	3	1	3	3	2	2	(3)	Botswana	1986	NR	1A-6
65	A9706/88	2	3	2	4	1	3	3	2	2	(1)	Norway	1988	P ⁻	1B-3
66	J198A-1	2	3	4	4	1	3	3	2	2	(1)	Jamaica	—	P ⁻	1B-16
67	Medan 2	2	4	4	1	1	3	3	3	2	(1)	Indonesia	1983	P ⁻	1A-6
68	26778/87	2	3	4	1	1	3	3	3	2	(1)	Scotland	1987	NR	—
69	Medan 32	2	3	4	3	1	3	3	3	2	(8)	Indonesia	1983	P ⁻	1A-6
70	8234	2	3	3	3	1	3	3	3	2	(2)	Ireland	—	A ⁻ H ⁻ U ⁻	—
71	5865	2	3	2	2	1	3	3	3	2	(3)	Ireland	1985	P ⁻	1B-2
72	3076	2	3	4	2	1	3	1	3	2	(1)	Dakar	1982	P ⁻	1B-1
73	8187	2	3	4	5	1	3	1	3	2	(1)	Ireland	1984	NR	1B-4
74	19/86	2	3	4	5	1	3	2	3	2	(1)	Botswana	1986	P ⁻ H ⁻	1B-5
75	9294	4	2	4	2	1	3	1	2	2	(1)	Ireland	—	NR	1B-6
76	JKD192	2	2	4	2	1	3	2	3	2	(2)	Australia	—	NR	1B-6
77	31830	2	2	4	2	1	3	2	3	1	(1)	Scotland	1987	H ⁻	1B-29
78	34732/87	2	2	4	2	1	3	2	2	2	(1)	Scotland	1987	P ⁻	1A-4
79	12/86	2	2	4	2	1	3	4	4	2	(1)	Botswana	1986	NR	1B-1
80	34583/87	2	2	2	5	1	3	2	3	2	(1)	Scotland	1987	NR	1B-13
81	27422-88	2	3	4	3	1	4	2	3	1	(3)	Sweden	1988	—	1A-1
82	9/86	2	3	4	3	1	2	2	3	1	(1)	Botswana	1986	NR	—
83	54777/78	2	3	4	3	1	3	2	4	1	(2)	Denmark	1963	P ⁻	1B-3
84	C1	2	3	4	1	1	2	4	3	1	(1)	Canada	—	A ⁻	1A-2
85	Unknown	2	3	4	4	1	1	3	2	1	(1)	Ireland	1986	NR	1A-1
86	SB2980	4	3	2	1	1	4	2	2	2	(1)	Zambia	—	P ⁻	1A-1
87	SFO890202	3	3	3	5	1	4	2	2	2	(1)	US	1989	—	—
88	POR890201	4	3	3	3	1	3	1	3	2	(1)	US	1989	P ⁻	1B-1
89	30993	3	4	3	3	1	4	2	3	2	(1)	US	1989	P ⁻	1B-1

* For abbreviations, see Methods.

† Strains from Botswana, Denmark, Indonesia, Greenland and Dakar were provided by I. Lind. Strains from Thailand, Nigeria and Zambia were supplied by the PHLS, Bristol, UK. Strains from Australia were supplied by L. Muir, strains from Scotland by H. Young and strains from the US by J. Schwebke. The isolates from Norway were provided by E. Falk, those from Jamaica were provided by J. Dillon and the remaining isolates from Ireland were provided by A. Rosney.

‡ Serovars were determined from reaction patterns with monoclonal antibodies according to the scheme of Knapp *et al.* (1984). —, Serovar not determined.

that localized recombination does occur in *E. coli* (Dykhuizen & Green, 1991; Barcak & Wolf, 1988; Stoltzfus *et al.*, 1988; Milkman & Bridges, 1990) and in other bacteria (Maynard Smith *et al.*, 1991).

Values of diversity from electrophoretic data indicate that bacteria which are naturally competent for transformation (such as *Bacillus*, *Streptococcus*, *Neisseria* and *Haemophilus*) are genetically more variable than non-transformable bacteria (Selander *et al.*, 1987). Levels of diversity within *Neisseria meningitidis* populations are greater than those reported for other species examined (Caugant *et al.*, 1987), and both sequence data and electrophoretic data have shown evidence of recombination in *N. meningitidis* populations (Zhou & Spratt, 1992; Caugant *et al.*, 1986, 1990).

Recent studies suggest that recombination may be more frequent in some bacterial species; tests of linkage disequilibrium reveal that recombination is more frequent in both *Bacillus subtilis* (Istock *et al.*, 1992) and *Rhizobium leguminosarum* (Souza *et al.*, 1992) than in *E. coli*. Our results indicate that *N. gonorrhoeae*, unlike all pathogens so far examined, has a non-clonal population structure, implying extensive recombination in nature.

Methods

Bacterial isolates. The 227 strains of *N. gonorrhoeae* studied were isolated between 1963 and 1989. The collection includes 24 strains from various locations in the United States isolated in 1989, and 53 strains from Ireland, isolated between 1984 and 1988. Thirty of the strains were isolated between 1982 and 1986 from various locations in Africa. The collection also includes 12 strains from Greenland isolated in 1987, 13 strains from Indonesia isolated in 1983, 23 Scottish strains isolated in 1987 and 21 strains from Thailand isolated in 1988. A total of 15 strains were obtained from Scandinavia, 9 of which were isolated in Denmark in 1963. The remaining 34 strains were from Jamaica and Australia (year of isolation unknown) and a pair of strains from Canada (1988) were also examined. There is little epidemiological information available for the strains. The isolates from Denmark, Botswana, Greenland and Dakar comprise non-consecutive patient isolates and the strains from Indonesia were isolated from female prostitutes. Two pairs of strains were epidemiologically related: strains 16328/87 and 16301/87 from Scotland, and strains SB2961 and SB2962 from W. Africa (Table 1).

Electrophoresis of enzymes. Each isolate was grown in GC broth, whose constituents were the same as GC agar (Oxoid), with the omission of agar and the inclusion of 0.03 M-NaHCO₃, 0.03 M-glucose and yeast extract (4 g l⁻¹). Broth was dispensed into hypovials (Pierce Warner) into which CO₂ was injected at a concentration of 5% (v/v). Cells were harvested by centrifugation at 5000 g for 5 min at 4 °C. After suspension in 4 ml 0.5 M-Tris (pH 7.5)/20% (w/v) sucrose, the bacteria were lysed by storage at -20 °C overnight. The extracts were then centrifuged at 13 000 g for 10 min to remove cellular debris and stored at -20 °C in 20 µl aliquots.

Soluble enzyme extracts of each isolate were separated on 11% (w/v) starch (Sigma) gels using the method described by Selander *et al.* (1986). The electrophoretic buffer systems and the enzymes assayed were as follows: (i) Tris/citrate, pH 8.0 [glutamate dehydrogenase (GDH), isocitrate dehydrogenase (IDH), glutamic-oxaloacetic trans-

aminase (GOT)]; (ii) Tris/maleate, pH 7.4 [alkaline phosphatase (ALP), leucine aminopeptidase (LAP), adenylate kinase (ADK)]; (iii) potassium phosphate, pH 7.0 [glucose-6-phosphate dehydrogenase (G6P), phosphoglucose isomerase (PGI), malic enzyme (MAE)].

Distinctive mobility variants of each enzyme were numbered in order of increasing rate of anodal migration and were equated with alleles of the corresponding structural genes. Each isolate was characterized by its combination of alleles at the nine enzyme loci (Table 1). Distinctive combinations of alleles, corresponding to unique multilocus genotypes, were designated as electrophoretic types (ETs). A table which lists the genotype, auxotype, serotype, date of isolation and geographic origin of each of the 227 isolates is available from the corresponding author.

Statistical procedures. Statistical analyses were performed using SPSS/PC+ software (Norusis, 1986). Chi-square tests were performed as described by Weir (1990). An index to describe multilocus linkage disequilibrium in haploid populations based on the distribution of allelic mismatches between pairs of isolates was also calculated (Maynard Smith *et al.*, 1993). This index, termed the Index of association (I_A) was calculated from the formula $I_A = V_o/V_e - 1$, where V_o is the variance in mismatches observed for the sample and V_e is the variance in mismatches expected in a population at linkage equilibrium. For populations in linkage equilibrium $V_o = V_e$ and I_A is not significantly different from zero.

Serological classification. One hundred and seventy-one isolates were serologically characterized by coagglutination using monoclonal antibodies to protein I (PI) as described by Tam *et al.* (1982). Isolates were classified as being PI class A (WI) or B (WII/WIII) and further subdivided into different serovars by reaction patterns with monoclonal antibodies specific for protein IA and IB (Knapp *et al.*, 1984). The isolates were serotyped at the *Neisseria* Reference Laboratory, Seattle, Washington, USA.

Auxotypes. The isolates were auxotyped using the system described by Copley & Egglestone (1983). The strains were tested for their requirements for proline (P⁻), arginine (A⁻), hypoxanthine (H⁻) and uracil (U⁻) or combinations of these requirements. Isolates with no requirements for these nutrients were classified as prototrophic (NR). A total of 202 isolates were auxotyped (89%).

Results

Electrophoretic types and genetic diversity

The 227 isolates of *N. gonorrhoeae* comprised 89 distinct nine-locus electrophoretic genotypes (ETs). One of the enzymes (MAE) was monomorphic; the number of alleles for the remaining eight enzymes ranged from three to five. Of the 89 ETs, 47 were represented by single isolates and 42 by multiple isolates (range, 2 to 35 isolates; Table 1). The mean genetic diversity per locus was 0.41.

Tests of linkage disequilibrium

The 28 two-locus comparisons were tested for linkage disequilibrium by Fisher's exact test; none of them were significant ($0.01 < P$). The 28 comparisons were also tested for linkage disequilibrium by chi-squared analysis; 92.6% of them did not significantly depart from linkage equilibrium ($0.05 < P < 0.9$).

Five four-allele combinations were then selected

Table 2. Relative frequencies of four-locus allele combinations used in analysing linkage disequilibrium

Comparison	Locus symbol*				Frequency (%) at which comparison was	
	a	b	c	d	Expected†	Observed
I	GD2 '2' (0.744)	IDH '3' (0.744)	PGI '3' (0.50)	G6P '3' (0.822)	22.7	19
II	IDH '4' (0.078)	GOT '2' (0.167)	PGI '3' (0.50)	ALP '2' (0.555)	0.4	1.1
III	GD2 '2' (0.744)	GOT '4' (0.70)	ALP '3' (0.278)	LAP '3' (0.244)	3.5	3.3
IV	GD2 '3' (0.144)	IDH '3' (0.744)	GOT '4' (0.70)	PGI '3' (0.50)	3.7	7.7
V	IDH '2' (0.156)	GOT '4' (0.70)	PGI '2' (0.278)	ALP '3' (0.278)	0.8	1.1

* The relative frequencies of the alleles in the sample of 89 ETs are shown in parentheses.

† The expected frequency of each comparison was determined from the products of allele frequencies.

(Table 2) and the frequency at which these combinations were observed was compared to the frequencies expected under conditions of random assortment. This analysis was similar to that performed on *E. coli* by Whittam *et al.* (1983a); however, the allele combinations selected in this study were not chosen from a principal coordinates analysis, but were selected arbitrarily. Depending on whether the designated allele was present or absent at the locus in question, 16 possible allele combinations resulted. The expected frequency was computed from the products of allele frequencies. The observed frequency of the combinations among each of the 89 ETs was compared to that expected if alleles occurred independently in ETs. Most of the four-allele combinations were at values close to the proportions expected from allele frequencies alone. For example, the four-allele combination GD2 '2' IDH '3' PGI '3' G6P '3' accounted for 19% of the electrophoretic types; however, this combination was slightly less than that predicted from allele frequencies (22.7%). Similarly the combinations IDH '4' GOT '2' PGI '3' ALP '2' and IDH '2' GOT '4' PGI '2' ALP '3' comprised 1.1% of the genotypes; however, both combinations were expected at frequencies of 0.4% and 0.8% respectively (Table 2).

An index to quantify multilocus linkage disequilibrium in bacterial populations, based on the distribution of allelic mismatches, was also calculated. This index, termed the index of association (I_A), measures the ratio of the variance in mismatches observed for the data set, V_o , to the variance expected in a corresponding population at linkage equilibrium, V_e (Maynard Smith *et al.*, 1993). The index of association focuses on the complete data set and not on specific allele combinations, and is,

therefore, an accurate measure of linkage disequilibrium. The value of the index of association is equal to zero for a population in linkage equilibrium, and differs significantly from zero if there is a significant association between alleles. For *N. gonorrhoeae*, the value of this index was not significantly different from zero (0.04 ± 0.09), suggesting a random assortment of alleles within the population. Since *N. gonorrhoeae* populations are in linkage equilibrium, it is not meaningful to construct a dendrogram to reveal genetic relationships from electrophoretic data.

Frequently recovered genotypes

Among the 89 genotypes, four ETs were each recovered more than nine times from strains isolated over a period of years. ET 1, the most common genotype, was represented by 35 strains (15.4%) isolated between 1963 and 1989 from the United States, Indonesia, Ireland, Scotland, Greenland, Jamaica, Thailand, Africa and Scandinavia (Table 1). Recovery of the same ET from different continents over a period of time is generally considered to be an indication of clonality. However, in a panmictic population isolates of the same ET can arise by the random association of the most common alleles in the population. The frequency at which ET 1 was expected in the population was determined from the product of individual allele frequencies. ET 1 possessed the most predominant allele at each locus and this combination of alleles was expected in 32.2 strains if the population was in linkage equilibrium. ET 3, ET 33 and ET 40 were also recovered from diverse geographic locations over a period of years. Similarly, the frequency at which ET 3, ET 33 and ET 40 were recovered is not

Table 3. Mean genetic diversity (H) within localities

Location	No. of isolates	No. of ETs	Mean no. of alleles	$H_{ISO}^\dagger$	$H_{ETs}^\dagger$
US	26	22	3.33	0.356	0.379
Greenland	12	9	2.11	0.217	0.218
Jamaica	22	19	2.67	0.272	0.291
Scotland	23	18	2.33	0.264	0.296
Thailand*	22	16	2.78	0.270	0.275
Indonesia	13	6	1.89	0.148	0.193
Dakar	16	13	2.33	0.199	0.201
Botswana	15	12	2.67	0.278	0.296
Australia	12	10	2.22	0.190	0.203
Scandinavia	14	11	2.44	0.242	0.282
Ireland	53	33	3.22	0.296	0.338

* The 22 strains were isolated in Thailand, Grenada, Nigeria, W. Africa and Zambia.

$^\dagger H_{ISO}$, genetic diversity among isolates; H_{ETs} , genetic diversity among ETs.

significantly different ($P < 0.05$) from the frequency at which each was expected assuming a random association of alleles.

Genetic variation in relation to geographic location

The collection of strains was partitioned into separate groups based on geographic origin (Table 3). Among the collection of 89 ETs, 33 (37%) were represented by isolates from two or more countries and in each case the distribution was intercontinental. The genetic variability within isolates from specific geographic regions was as large as that in the whole population. However, two ETs were detected in single locations: ET 11 was exclusive to three isolates from Greenland, while ET 41 was observed only among four isolates from Ireland.

Genetic variation in relation to auxotype

The number of isolates of a given auxotype ranged from 1 (AH⁻) to 109 (NR). Table 1 lists the auxotype for each of the 89 reference strains. Seven of the nine auxotypes were each represented by strains of several multilocus genotypes, many of which differed from one another by alleles at four or more loci. The other two auxotypes were represented by single isolates. Strains which were epidemiologically related were of similar auxotype (Table 1). The four ETs which were encountered repeatedly were each represented by strains of several auxotypes (Table 1, Table 4). The diversity within auxotypes was 58% of the total diversity, while 91% of the total diversity was represented by prototrophic isolates (NR).

Genetic variation in relation to serovar

The 227 isolates comprised 35 different serovars, 11 of which were each represented by 4 or more strains. Table

Table 4. Distribution of auxotypes and serovars within the four predominant ETs

ET	No. of isolates	No. of auxotypes	No. of serovars
ET 1	35	5	10
ET 3	12	4	6
ET 33	9	3	4
ET 40	11	4	5

1 lists the serovar for each of the 89 reference strains. Isolates of shared serovar were represented by a variety of ETs. Strains which were epidemiologically related shared serovar (Table 1). The four most common genotypes were represented by strains of different serovar (Table 1, Table 4). The diversity within serovars was 75% of the total diversity observed.

Discussion

N. gonorrhoeae is constitutively competent at all phases of growth (Biswas *et al.*, 1982) and undergoes intra-specific transformation in the laboratory (Seifert *et al.*, 1988); however, the extent to which transformation occurs in nature is unknown. Actual rates of recombination in natural populations cannot be estimated from presently available data. However, the statistical analysis of electrophoretic data enables a crude estimate of the extent of recombination to be made from the degree of linkage disequilibrium. Our study has focused on electrophoretic variation in nine loci. At present the positions of these nine loci on the gonococcal genomic map are unknown (Bihlmaier *et al.*, 1991), but it is presumed that they are representative of the genome.

Genetics of *N. gonorrhoeae* populations

Our study has revealed linkage equilibrium in natural populations of *N. gonorrhoeae*. There was little evidence of association between pairs of loci, since 92% of two-locus combinations did not depart from linkage equilibrium. Similarly, five four-locus combinations of alleles were found at frequencies close to those expected under conditions of random assortment. The index of association value for *N. gonorrhoeae* did not differ significantly from zero, the value expected for populations in linkage equilibrium.

Since bacterial reproduction is asexual, for *N. gonorrhoeae* to achieve linkage equilibrium, the species must experience a high frequency of recombination relative to binary fission, to reduce the clonal structure certain to arise otherwise. Recombination in *N. gonorrhoeae* is most likely due to transformation, since transformation

is the only known mechanism for the transfer of chromosomal genes in *N. gonorrhoeae* (Biswas *et al.*, 1989) and presumably results in the frequent transfer of relatively small chromosomal segments. A second aspect is that gonococci must encounter other gonococcal genotypes; hence mixed infections with different gonococcal strains must be relatively common. However, the frequency at which mixed gonococcal infections occur, the actual rate of recombination, and the reason for such extensive recombination are, at present, unknown.

Our report of a pathogenic bacterium whose population structure is non-clonal contrasts with the many pathogenic and commensal species studied to date (Selander & Musser, 1990), where evidence of linkage disequilibrium has resulted in clonality being considered the paradigm in bacterial population genetics. However, *B. subtilis* populations reveal only modest clonal structure (Istock *et al.*, 1992), and a recent statistical analysis of electrophoretic data has revealed that population structures can range from strictly clonal to panmictic (Maynard Smith *et al.*, 1993). An intermediate type of population structure has also been recognized in which the population is panmictic in the long term, but occasionally a highly successful ET arises, which spreads rapidly to produce a degree of linkage disequilibrium in the population. *N. meningitidis* populations are an example of this 'epidemic' population structure.

Obviously the pattern and extent of sampling has an important bearing on the observed population structure. Temporal and geographic isolation of genotypes can give rise to linkage disequilibrium in the presence of recombination (Souza *et al.*, 1992). However, ecological isolation of genotypes can also give rise to linkage disequilibrium despite recombination. For example, if the factors governing infection are such that genotypes do not encounter one another, linkage disequilibrium may be evident since any recombination occurs between genetically similar strains. The difference in population structure between the related species *N. gonorrhoeae* and *N. meningitidis* possibly reflects the fact that mixed infections are more common in cases of gonorrhoea than meningitis; however, it is also possible that gonococci exchange genetic material more readily than meningococci. Alternatively, the difference in population structure between *N. gonorrhoeae* and *N. meningitidis* may be due to differences in sampling, raising the possibility that *N. gonorrhoeae* might have revealed an 'epidemic' population structure if strains from a regional epidemic were examined.

Comparison with the population structure of *B. subtilis*

Recent studies of a *B. subtilis* population taken from a single site have revealed only modest clonal structure (Istock *et al.*, 1992). Statistical tests of association show

that 92% of two-locus combinations of *N. gonorrhoeae* did not depart from linkage equilibrium, a value greater than that reported for *B. subtilis* populations (76%), in which only slight linkage disequilibrium was observed. The shortage of two-locus haploid genotypes in natural populations of *N. gonorrhoeae* (and *B. subtilis*) contrasts markedly with *E. coli* populations, where restricted recombination results in a high association between pairs of loci (Caugant *et al.*, 1981; Whittam & Wilson, 1988).

The genotypic composition of populations varies geographically and temporally; ETs in high frequency in a geographic location at one time may be absent or in undetectable frequencies in other locations. Certain gonococcal genotypes were recovered at high frequency from temporally and geographically unassociated hosts. Four ETs were each recovered more than nine times and a single genotype, ET 1, accounted for 15.4% of the ETs. In *B. subtilis* populations the most frequent ET accounted for only 3% of the types (Istock *et al.*, 1992). Evolutionary convergence to the same ET is generally considered unlikely and ETs are often presumed to mark clones, having resulted from a shared descent from a common ancestral cell (Selander & Levin, 1980). In many species, the global recovery of strains of the same genotype is due primarily to restricted recombination among clonal lineages (Musser *et al.*, 1988; Ochman & Selander, 1984). For *N. gonorrhoeae* the reappearance of strains of the same ET could suggest the existence of specific clones, contrasting with the apparent linkage equilibrium. However, the repeated recovery of identical genotypes of *N. gonorrhoeae* is not at variance with evidence of linkage equilibrium, since each of the four most common genotypes were observed at frequencies close to those predicted from a random assortment of alleles. It is a consequence of linkage disequilibrium that the number of new ETs gradually decreases as the number of loci assayed increases. Hence, it is likely that an examination of the 35 ET 1 isolates by additional enzymes or DNA-based approaches would reveal a lack of genetic identity among isolates of shared genotype. Indeed, 30 of the ET 1 isolates have been further assessed by a combination of MLEE, gene fingerprinting and random-primer DNA typing (RAPD), which has revealed a lack of genetic identity among isolates of shared genotype, as expected from a population in linkage equilibrium (M. O'Rourke & B. G. Spratt, unpublished).

Consequences for epidemiology

Our study has revealed no association between ETs and auxotype or serovar. Isolates of the same auxotype or serovar were of different ETs. Similarly, isolates of the same ET comprised several auxotypes and serovars. Serotypes are concordant with multilocus genotypes in

many clonal species (Selander *et al.*, 1990; Musser *et al.*, 1988), although notable exceptions include *E. coli* (Achtman & Pluschke, 1986) and *L. pneumophila* (Selander *et al.*, 1985). In addition, serogroup, serotype and serosubtype do not correlate with electrophoretic type in serogroup B and serogroup C meningococci (Caugant *et al.*, 1987; Wang *et al.*, 1992); also, nucleotide sequence evidence for recombination in the P1 serosubtyping antigen has been reported (Feavers *et al.*, 1992). Hence, the absence of any correlation between ET and serovar in *N. gonorrhoeae* is not surprising.

Furthermore, surface antigens are likely to be under strong selection and considerable diversity has been reported in many gonococcal surface antigens, including pilin genes (Seifert *et al.*, 1988) and the outer-membrane protein P2 (Stern *et al.*, 1986). There has been little information on the genetic relatedness between gonococcal isolates, and most epidemiological studies have focused on auxotype and serotype data (Sarafian & Knapp, 1989). In this study 75% of the strains were represented by two auxotypes and the combination of auxotype and serovar revealed 79 different auxotype/serovar classes, which provided less discrimination than MLEE.

Previous studies using MLEE have provided useful marker systems for epidemiology and have established population genetic frameworks to study the variation and distribution of typing characters (Selander *et al.*, 1985, 1990; Musser *et al.*, 1988). However, the evidence for extensive recombination in *N. gonorrhoeae* suggests that effective epidemiological typing of these bacteria will prove difficult. Unlike clonal species, genes in *N. gonorrhoeae* are likely to be constantly assorted into new combinations by recombination, with particular genotypes existing only transiently before they are disrupted by recombination. Although typing methods may be useful in the short term (for tracing the spread of a strain between contacts in a localized outbreak), they are likely to be of little use in the long term.

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