INFECTION AND IMMUNITY, Apr. 2005, p. 2424–2432 Vol. 73, No. 4
0019-9567/05/$08.00+0 doi:10.1128/IAI.73.4.2424–2432.2005

Genetic Diversity and Carriage Dynamics of *Neisseria lactamica* in Infants

Julia S. Bennett,[1] David T. Griffiths,[2] Noel D. McCarthy,[1] Karen L. Sleeman,[3] Keith A. Jolley,[1] Derrick W. Crook,[2] and Martin C. J. Maiden[1]*

The Peter Medawar Building for Pathogen Research and Department of Zoology, Oxford University,[1] and Nuffield Department of Clinical Microbiology[2] and Oxford Vaccine Group, Department of Paediatrics,[3] Oxford University, John Radcliffe Hospital, Oxford, United Kingdom

Received 17 September 2004/Returned for modification 28 October 2004/Accepted 20 December 2004

***Neisseria lactamica*, a harmless human commensal found predominantly in the upper respiratory tracts of infants, is closely related to *Neisseria meningitidis*, a pathogen of global significance. Colonization with *N. lactamica* may be responsible for the increase in immunity to meningococcal disease that occurs during childhood, when rates of meningococcal carriage are low. This observation has led to the suggestion that *N. lactamica* whole cells or components are potential constituents of novel meningococcal vaccines. However, the dynamics of carriage and population diversity of *N. lactamica* in children are poorly understood, presenting difficulties for the choice of representative isolates for use in vaccine development. This problem was addressed by the multilocus sequence typing of *N. lactamica* isolates from two longitudinal studies of bacterial carriage in infants. The studies comprised 100 and 216 subjects, with *N. lactamica* carriage monitored from age 4 weeks until age 96 weeks and from age 2 weeks until age 24 weeks, respectively. The maximum observed carriage rate was 44% at 56 weeks of age, with isolates obtained on multiple visits for the majority (54 of 75, 72%) of carriers. The *N. lactamica* isolates were genetically diverse, with 69 distinct genotypes recovered from the 75 infants. Carriage was generally long-lived, with an average rate of loss of under 1% per week during the 28 weeks following acquisition. Only 11 of the 75 infants carried more than one genotypically unique isolate during the course of the study. Some participants shared identical isolates with siblings, but none shared identical isolates with their parents. These findings have implications for the design of vaccines based on this organism.**

The genus *Neisseria* comprises gram-negative, oxidase-positive diplococci that are frequently isolated from the mucosal surfaces of humans and animals (39). Two members of the genus cause distinct human diseases: *Neisseria meningitidis*, which can be responsible for septicemia and meningitis (8), and *Neisseria gonorrhoeae*, the etiological agent of gonorrhea (44). *Neisseria lactamica* is closely related to both of these species (24) but is associated with invasive disease only in exceptional circumstances (13, 35, 42). Carriage of *N. lactamica* throughout the world is high in infants and young children but declines with age. This is the converse of the carriage of the meningococcus, which is low during infancy and rises to high levels in adolescents and young adults (2, 9, 21, 26, 41, 43).

The observation that antimeningococcal bactericidal titers rise steadily in infants, despite low rates of meningococcal carriage, has provided indirect support for the concept that carriage of *N. lactamica* by children (9) may confer protection against disease caused by *N. meningitidis* (7). This suggestion has in turn led to the proposal that *N. lactamica* could be used to induce protection against meningococcal infection, by using killed whole cells, outer membrane vesicles, or outer membrane proteins, as demonstrated in a murine model (40), or by the deliberate inoculation of infants with live *N. lactamica* cultures (23).

One of the factors that has proved to be a hindrance to the development of vaccines against the meningococcus is its genetic and antigenic diversity (11, 12, 15). Although progress has been made in the development of vaccines to serogroup A and C meningococci, with a polysaccharide-protein conjugate vaccine to serogroup C meningococci found to be effective in young children (3), there is still no comprehensive vaccine for *N. meningitidis*. Of particular concern are serogroup B organisms, as the capsular polysaccharide is not considered to be suitable for inclusion in a vaccine due to its structural similarity with human embryonic carbohydrates (20) and poor immunogenicity (55).

To date the variation present in natural populations of *N. lactamica* has not been fully investigated, and the dynamics of carriage in individuals and populations are unknown. This makes an informed choice of isolates for investigation as potential vaccine candidates and the planning of any intervention strategy difficult. High-throughput nucleotide sequence determination provides a means for the rapid and comprehensive investigation of bacterial population diversity and structure (37). These techniques have been exploited in the development of isolate characterization techniques such as multilocus sequence typing (MLST) for a number of bacterial pathogens, including the meningococcus (38). Data from MLST studies have measured the high diversity of meningococcal populations and established the role of horizontal genetic exchange in the generation of this diversity (5, 16, 19, 25, 30). Although there have been no previous population studies of *N. lactamica* that used MLST or multilocus enzyme electrophoresis, it is plausible to suggest that this population is also diverse and

* Corresponding author. Mailing address: The Peter Medawar Building for Pathogen Research and Department of Zoology, University of Oxford, South Parks Rd., Oxford OX1 3SY, United Kingdom. Phone: 44 (1865) 271284. Fax: 44 (1865) 271284. E-mail: martin.maiden@zoo.ox.ac.uk.

TABLE 1. Oligonucleotide primers used for *N. lactamica* MLST

Locus	Primer	Sequence (5′→3′)	Function
abcZ	abcZ-P1	AATCGTTTATGTACCGCAGG	Amplification and sequencing
	abcZ-S2	GAGAACGAGCCGGGATAGGA	Amplification and sequencing
adk	C-adk-P1	CCKCAGATYTCYACAGGCGA	Amplification and sequencing
	C-adk-P2	AATAYTTCKGCTTTCACGGC	Amplification and sequencing
aroE	aroE-P1	ACGCATTTGCGCCGACATC	Amplification
	aroE-P2	ATCAGGGCTTTTTTCAGGTT	Amplification
	aroE-S1	GCGGTCAAYACGCTGATT	Sequencing
	aroE-S2	ATGATGTTGCCGTACACATA	Sequencing
fumC	fumC-S1	TCCGGCTTGCCGTTTGTCAG	Amplification and sequencing
	fumC-S2	TTGTAGGCGGTTTTGGCGAC	Amplification and sequencing
gdh	L-gdh-P3	GTTTTGCCAACGTGATGTTC	Amplification and sequencing
	L-gdh-P4	TATAGAGGCGGACGGATTCGGT	Amplification and sequencing
pdhC	pdhC-P1	GGTTTCCAACGTATCGGCGAC	Amplification
	pdhC-P2	ATCGGCTTTGATGCCGTATTT	Amplification and sequencing
	pdhC-S1	TCTACTACATCACCCTGATG	Sequencing
pgm	pgm-S1	CGGCGATGCCGACCGCTTGG	Amplification and sequencing
	pgm-S2	GGTGATGATTTCGGTTGCGCC	Amplification and sequencing

recombining, on the basis of gene sequences obtained from a number of *N. lactamica* isolates (4, 14, 17, 36, 46, 47, 50, 54) and a previous study of *N. lactamica* diversity (1). An improved understanding of the population biology of *N. lactamica* will provide valuable insights for investigating the role that *N. lactamica* plays in inducing immunity against the meningococcus and in choosing isolates and subcellular components that might be suitable for inclusion in meningococcal vaccines.

The MLST scheme for *N. meningitidis* was adapted and optimized in order to analyze the genotypic diversity and carriage dynamics of *N. lactamica* isolates obtained from two longitudinal studies of bacterial carriage in infants. These data established that the population of *N. lactamica* was diverse and that individual infants carried single genotypes for prolonged periods.

MATERIALS AND METHODS

Bacterial isolates. (i) Study 1. Throat and nasopharyngeal swab samples were collected from a cohort of 100 infants from Oxfordshire, United Kingdom, between December 1996 and February 1999. Throat swab samples were taken at 4-week intervals from age 4 weeks during the first 24 weeks of life and at 8-week intervals for the subsequent 24 weeks. Throat swab samples were also taken from 15 infants at 56 weeks of age and from 78 infants at age 96 weeks. In addition, nasopharyngeal swab samples were taken from the majority of children at 4-week intervals from age 4 weeks until age 24 weeks and then at 8-week intervals until 96 weeks of age. Each swab collected was vortex mixed briefly in brain heart infusion (BHI) broth with 20% (vol/vol) glycerol, and the resultant suspension was stored at −80°C. These stored samples were used as a source of *N. lactamica* isolates.

(ii) Study 2. *N. lactamica* isolates were obtained from a longitudinal study of bacterial carriage in infants conducted between August 1999 and May 2001 in the same geographic area as study 1. In this survey of 216 infants, throat swab samples were taken at 2-week intervals from age 2 weeks until age 12 weeks and then at 4-week intervals until age 24 weeks. Throat swab samples were also obtained at week 2 from 166 mothers, 55 fathers, and 50 siblings of the infant participants.

Appropriate consent was obtained for all of the individuals sampled, and the studies conducted with ethical approval from the Oxford Ethics Committee, with study reference numbers C99.044 and 96.152.

Isolation and identification of *N. lactamica*. Samples from study 1 were plated onto a medium containing 0.04 to 0.08 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) per liter dissolved in *N,N*-dimethylformamide (Sigma)–41 g of Columbia agar per liter–5 g of yeast extract per liter–10% horse serum–3 mg of vancomycin per liter–25,000 U of polymixin B per liter. X-Gal, a lactose analogue, was included in the growth medium to enable rapid identification of the lactose-fermenting *N. lactamica* species without the necessity of a further step to detect β-galactosidase activity. Samples were also plated on meningitidis nutrient agar (MNA) containing 40 g of blood agar base (Oxoid) per liter, 10% horse blood, 3 mg of vancomycin per liter, and 25,000 U of polymixin B per liter so that direct comparisons could be made between the two media. The inoculated samples were incubated for 48 h at 37°C in a 5% CO_2 atmosphere. Potential *N. lactamica* colonies were subcultured onto blood agar and further identified by oxidase reaction, Gram staining, and microscopic morphology. For colonies that grew only on MNA plates, β-galactosidase activity was tested with *ortho*-nitrophenyl-β-D-galactopyranoside as the substrate. Samples from study 2 were directly inoculated onto MNA plates and incubated for 48 h at 37°C in a 5% CO_2 atmosphere. Potential *N. lactamica* colonies were subcultured and identified by oxidase reaction, Gram staining, microscopic morphology, and *ortho*-nitrophenyl-β-D-galactopyranoside reaction. Axenic cultures of *N. lactamica* were stored in brain heart infusion broth with 20% (vol/vol) glycerol at −80°C.

DNA preparation and MLST. Frozen isolates were revived on blood agar, and opaque suspensions of *N. lactamica* cells were made in deionized water (1 ml) and immediately boiled for 15 min to lyse the cells and inactivate nucleases. Amplification of the seven MLST housekeeping gene fragments was carried out directly from the boiled cell suspensions by PCR with the oligonucleotide primers detailed in Table 1. Amplification reaction mixtures contained reaction buffer (10 mM Tris-Cl [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% [wt/vol] gelatin); 200 μM (each) dATP, dCTP, dGTP, and dTTP; a 1 μM concentration of each primer; 1.25 U of *Taq* polymerase (Amplitaq; Applied Biosystems) per 50 μl; and 0.5 μl of template DNA (from the boiled cell suspensions) per 50 μl. The PCR conditions consisted of an initial denaturation step at 94°C for 2 min, followed by 35 to 40 cycles of denaturation (94°C for 1 min), annealing (55 to 70°C for 1 min), and extension (72°C for 2 min) and then a final extension step at 72°C for 2 min. The PCR products were precipitated by incubation at room temperature for 30 min with 20% (wt/vol) polyethylene glycol 8000–2.5 M NaCl. After centrifugation for 60 min at 2,750 × *g*, the precipitates were washed twice in 70% (vol/vol) ethanol, dried, and resuspended in 5 to 10 μl of sterile distilled water.

The nucleotide sequences of the amplified gene fragments were determined at least once on each DNA strand by cycle sequencing with BigDye Ready Reaction Mix (Applied Biosystems), used in accordance with the manufacturer's instructions. Sequencing reactions were performed with the oligonucleotide primers listed in Table 1. Unincorporated dye terminators were removed by precipitation

of the termination products with 95% (vol/vol) ethanol, and the labeled extension products were separated and detected with either a Prism 3700 DNA analyzer or a Prism 377 XL DNA analyzer (Applied Biosystems). Sequences were assembled from the resultant chromatograms with the Staden suite of computer programs (49). Allele numbers and sequence types (STs) were assigned by using the *Neisseria* MLST database (http://pubmlst.org/neisseria/) (28).

Genetic analyses. The number of alleles, percentage of polymorphic sites, and ratio of nonsynonymous to synonymous nucleotide substitutions (d_N/d_S) were calculated by using the program START (29). Presumptive mosaic gene structures were visualized by using MEGA version 2.1 (34). The STs were assigned to clonal complexes by using a combination of analysis techniques. Central genotypes were identified by using the BURST algorithm (18) implemented in the START package (29), and split decomposition was identified by using SplitsTree version 3.1 (27). Members of a clonal complex were defined as isolates with STs that shared identical alleles at four or more loci with the central genotype.

Sensitivity of detection on each visit. The sensitivity of swabbing and culture to carriage was estimated among individuals who exhibited at least two isolates sharing the same ST with one or more intermediate sampling episodes. The proportion of intermediate swabbings which were positive between the first and last positive isolations was the sensitivity rate. The 95% confidence intervals were estimated, allowing for the effect of clustering within individual children, by using the STATA Svy command suite (StataCorp).

Sensitivity of studies to acquisition. Acquisition of *N. lactamica* might not be detected if either (i) carriage was very short, with acquisition and loss occurring between successive sampling visits, or (ii) carriage was present at a swabbing but was not detected due to imperfect sensitivity of swabbing and culture for prevalent carriage as outlined above. Using the sampling attendance and culture results for each individual, the proportion of incident infections likely to be detected in these studies was calculated by using the sensitivity of detection on each visit as described above and the rate of carriage loss estimated as described below. Assumptions were that the rate of acquisition was constant during the generally short intervals between successive swab samples and that any variation in an individual's probability of incident infection was unrelated to the completeness of his or her attendance for sampling. The overall estimates were a pooled average of the sensitivity for each participating child weighted by the duration of follow-up time in the study. Only the first 48 weeks of study 1 were considered, since sampling became infrequent during the second year.

Calculations of carriage dynamics. Acquisition rates among those without current or previous *N. lactamica* carriage were estimated by using survival analysis and Poisson regression. Study groups were compared graphically by using Kaplan-Meier survival curves and statistically by estimation of study effect in a Poisson regression controlling for age at acquisition. The rate of loss of carriage was estimated in the same way. Following detection, carriage was considered to extend until the last positive swab for that genetic type. For individuals with a positive swab on their last visit, the time of loss of carriage was not available. These individuals were included in the survival analysis up to this time, with censoring at the date of their last swabbing. All estimates were made both from the first detected carriage and in a corrected form to allow for the estimated delay in initial detection based on an assumption that the acquisition rate was constant in the interval prior to detection. The extent to which strains carried by parents and siblings of the study individuals overlapped with the strains carried by the study individuals was assessed.

RESULTS

Isolation of *N. lactamica*. In study 1, a total of 1,353 nasopharyngeal and 938 throat swab samples were analyzed. Where both throat and nasopharyngeal swab samples were taken, there were no nasopharyngeal swab samples positive for *N. lactamica* when the throat swab samples were negative, and only 4 (0.3%) of the nasopharyngeal swab samples were culture positive for *N. lactamica*, compared to 145 (15.5%) of the throat swab samples.

No meningococci were isolated from the children included in either of these two studies. A total of 250 *N. lactamica* isolates were obtained from the 316 infants, and 21 isolates were obtained from the 271 family members sampled. The sensitivity of each swabbing between the first and last isolations was 84% (95% confidence interval, 74 to 94%). Growth of *N. lactamica* appeared to be inhibited when it was grown on X-Gal medium, compared with colonies grown in parallel on MNA. In each case growth was more robust on the MNA plates, and on occasion growth was absent on X-Gal plates when a few colonies were observed on MNA. An X-Gal concentration of 0.06 mg/liter in the isolation medium gave optimal identification of *N. lactamica*, with colonies recognizable by a light blue coloration.

TABLE 2. Genetic variation in *N. lactamica* MLST gene fragments

Locus	Size (bp)	No. of alleles	No. (%) of polymorphic sites	d_N/d_S
abcZ	433	12	45 (10.4)	0.154
adk	465	18	43 (9.2)	0.0139
aroE	489	16	45 (9.2)	0.4643
fumC	465	19	44 (9.5)	0.042
gdh	501	27	46 (9.2)	0.0462
pdhC	480	12	16 (3.3)	0.0229
pgm	450	22	96 (21.3)	0.0949

Optimization of *Neisseria* MLST for *N. lactamica*. For the *aroE* and *pdhC* gene fragments, the previously published *N. meningitidis* oligonucleotide primers (30) were employed for amplification and sequencing. The amplification and sequencing of the *abcZ*, *fumC*, and *pgm* gene fragments were performed with the published *N. meningitidis* sequencing primers, and additional primers were designed for the amplification and sequencing of the *adk* and *gdh* gene fragments (Table 1). The annealing temperature for PCR amplifications was 55°C, except for the *pgm* locus, for which an annealing temperature of 70°C gave optimal results in terms of sensitivity and specificity.

Genetic diversity of isolates. The total number of alleles present at each locus varied between 12 for *abcZ* and *pdhC* and 27 for *gdh*, and the percentage of polymorphic sites ranged from 3.3 for *pdhC* to 21.3 for *pgm*. The ratio of nonsynonymous to synonymous substitutions (d_N/d_S) in each case was less than one, ranging between 0.0229 (*pdhC*) and 0.4643 (*aroE*) (Table 2). Inspection of the polymorphic sites in the nucleotide sequences of the alleles at each locus identified possible mosaic structures (data not shown).

A total of 72 unique allelic profiles were identified among the 96 *N. lactamica* carriers (75 infants and 21 family members), 69 of which were obtained from infants. Of these, 54 unique allelic profiles were found in single individuals. Each of these allelic profiles was assigned a unique ST, and related STs were assigned to four clonal complexes (Table 3), with the ST-624 complex being the largest, comprising 18 STs, with 22 independent isolations. The remaining clonal complexes contained fewer STs, with the ST-613 complex consisting of five STs and the ST-595 and ST-640 complexes having three each. There were 43 STs which could not be assigned to complexes on the basis of these data alone. Given the diversity of this organism and the sample size analyzed, these clonal complex definitions must be regarded as preliminary.

Genotypic differences in carried isolates. Isolates with a single genotype were obtained from the majority (64 of 75, 85%) of carriers (Fig. 1). In study 1, most of the genotypic differences within isolates obtained from single carriers were due to multiple changes. A single child, infant 1-058, carried isolates with three unrelated genotypes; the second genotype

TABLE 3. Predominant *N. lactamica* genotypes

Clonal complex	Sequence type	No. of independent isolations	Allele						
			abcZ	*adk*	*aroE*	*fumC*	*gdh*	*pdhC*	*pgm*
ST-595	595	3	*40*	*46*	*102*	*89*	*91*	*82*	*82*
	599	2	*40*	*54*	*102*	*89*	*91*	*82*	*82*
	645	2	*91*	*46*	*102*	*89*	*91*	*82*	*82*
ST-613	588	1	*33*	*45*	*48*	*96*	*94*	*46*	*56*
	606	1	*80*	*44*	*48*	*96*	*94*	*46*	*56*
	607	1	*80*	*45*	*99*	*97*	*94*	*44*	*56*
	613	4	*80*	*45*	*48*	*96*	*94*	*46*	*56*
	614	1	*80*	*45*	*97*	*100*	*94*	*46*	*56*
ST-624	587	1	*32*	*57*	*100*	*98*	*81*	*46*	*88*
	617	1	*81*	*37*	*100*	*98*	*81*	*46*	*88*
	618	1	*81*	*47*	*100*	*99*	*81*	*46*	*89*
	619	1	*81*	*47*	*100*	*103*	*81*	*46*	*89*
	620	1	*81*	*47*	*100*	*100*	*81*	*46*	*88*
	621	1	*81*	*47*	*103*	*100*	*81*	*46*	*88*
	622	1	*81*	*47*	*100*	*45*	*81*	*46*	*88*
	623	1	*81*	*47*	*103*	*98*	*81*	*46*	*88*
	624	2	*81*	*47*	*100*	*98*	*81*	*46*	*88*
	625	2	*81*	*47*	*100*	*98*	*81*	*83*	*88*
	626	1	*81*	*49*	*100*	*97*	*81*	*83*	*88*
	628	2	*82*	*47*	*96*	*102*	*81*	*46*	*88*
	635	1	*83*	*47*	*97*	*94*	*81*	*46*	*88*
	636	2	*83*	*47*	*100*	*94*	*81*	*46*	*88*
	637	1	*83*	*47*	*54*	*94*	*81*	*46*	*88*
	1191	1	*81*	*47*	*103*	*98*	*133*	*46*	*88*
	1201	1	*82*	*47*	*96*	*98*	*81*	*123*	*88*
	1208	1	*81*	*47*	*98*	*98*	*81*	*46*	*88*
ST-640	640	2	*84*	*49*	*48*	*50*	*92*	*46*	*45*
	641	3	*84*	*49*	*48*	*50*	*85*	*46*	*90*
	646	1	*84*	*43*	*48*	*50*	*92*	*46*	*45*

identified shared no alleles with the first, and the third genotype identified shared one allele (*abcZ40*) with the first genotype but none with the second. Two different genotypes were identified from isolates obtained from throat swab samples from each of eight infants in study 1. Infant 1-051 carried isolates of ST-622 and ST-625, which are members of the ST-624 complex. ST-625 is a double-locus variant of ST-622, having 11 nucleotide substitutions in the *fumC* gene fragment and a single nucleotide substitution in the *pdhC* gene fragment. Infant 1-081 carried isolates characterized as ST-633 and ST-634. These genotypes differed by a single nucleotide substitution in the *gdh* gene fragment. The genotypes isolated from the other six infants (1-005, 1-014, 1-032, 1-044, 1-066, and 1-094) were unrelated, sharing between no and two alleles. One child, infant 1-097, carried two related isolates at age 40 weeks, with ST-623 obtained from the throat and ST-1191 obtained from the nasopharynx, which differed by two nucleotides in *gdh*. In study 2, infant 2-044 carried isolates with identical genotypes except for the isolate obtained at age 16 weeks, which had a single synonymous nucleotide substitution in the *pdhC* gene fragment. Infant 2-110 appeared to carry isolates with only one genotype, but due to the presence of a second *abcZ* gene with a deletion, this could not be confirmed.

Carriage prevalence and rate of carriage loss. Of the 316 infants (study 1 and study 2), 75 (23%) carried *N. lactamica* during the study period, with the majority (54, 72%) carrying the organism on multiple, usually successive, visits (Fig. 1). Carriage rates at a given point in time ranged from 1.8% (children age 2 weeks in study 2) to 43.8% (children age 56 weeks in study 1), and the age-specific carriage rates were consistent between the two studies (Table 4). Of the 54 individuals from whom the bacterium was isolated more than once, the largest number of identical isolates (nine), as defined by MLST, were obtained from infant 2-065 (study 2), who carried *N. lactamica* for the duration of the study. Infant 1-017 (study 1) was culture positive for identical isolates of *N. lactamica* for the longest recorded duration, from 8 to 96 weeks of age. Of the 21 individuals from whom *N. lactamica* was isolated only once, 12 were culture positive on the last visit of the study (age 96 weeks for study 1 and 24 weeks for study 2).

The rates of loss of carriage during the first 28 weeks following acquisition are summarized in Fig. 2; later loss was not estimated because the data became increasingly sparse. Carriage was persistent, with overall loss rates of 0.77% (95% confidence interval, 0.32 to 1.85%) per week during the 28 weeks following acquisition. Loss of carriage was estimated to be threefold higher in the second 14-week period than in the first, but given the relatively small numbers, there was statistical uncertainty in this estimate, with a 95% confidence interval extending from 0.5- to 18-fold.

Acquisition rates. The acquisition rates were similar for each study for the age range covered by both studies, and statistical testing did not indicate any evidence for a difference between studies (log rank $P = 0.46$). Modeling of acquisition rates by

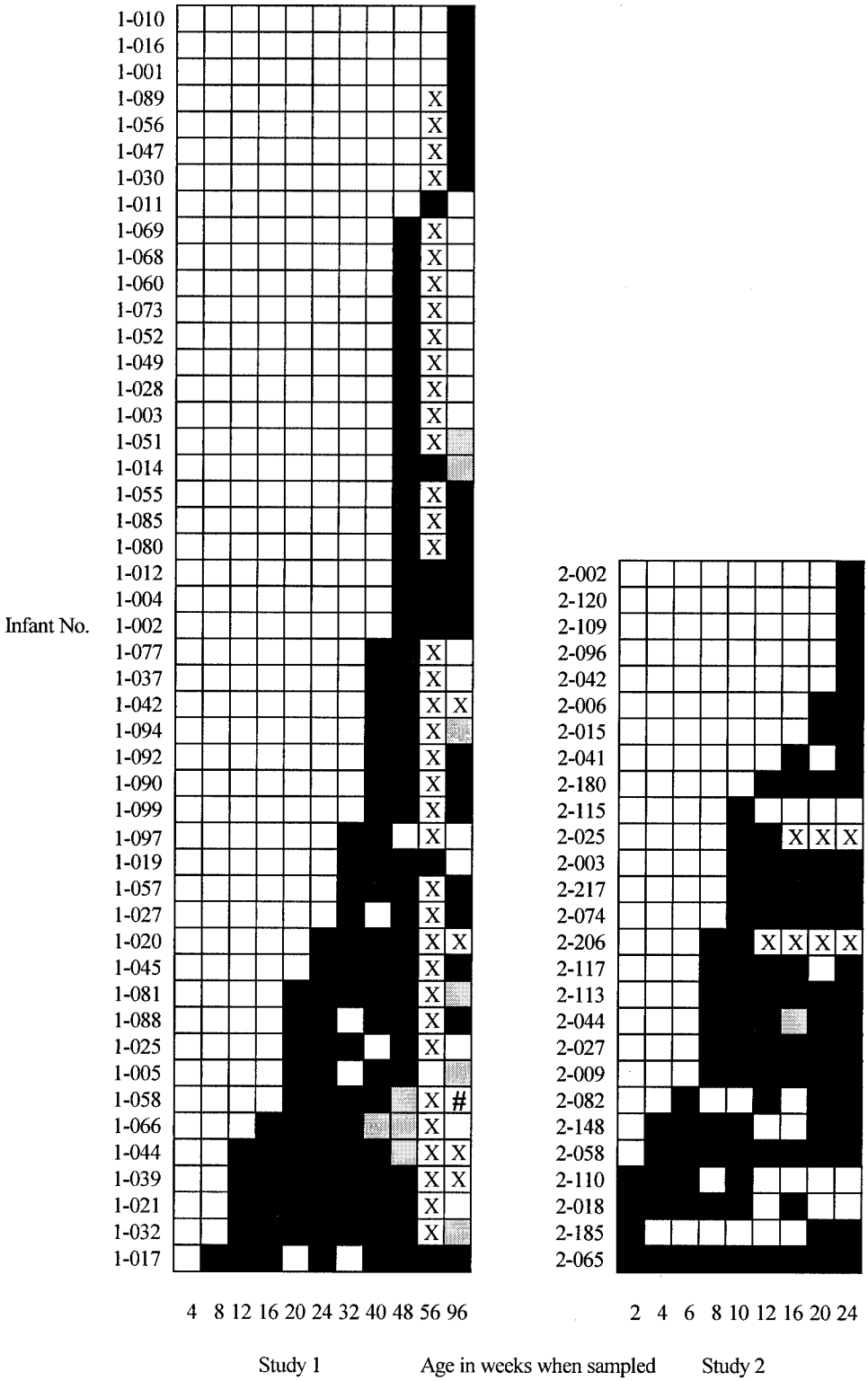

FIG. 1. Longitudinal carriage of *N. lactamica*. White boxes, negative throat swab; black boxes, first genotype; gray boxes, second genotype; #, third genotype; X, no throat swab taken. A nasopharyngeal isolate from individual 1-097 had a genotype different from that of the isolate obtained from the throat when both swabs were taken.

TABLE 4. Carriage rates of *N. lactamica*

Age (wk)	Study 1			Study 2		
	No. sampled[a]	No. of isolates	%	No. sampled	No. of isolates	%
2	0			216	4	1.9
4	100	0	0	214	5	2.3
6	0			214	6	2.8
8	99	1	1	214	10	4.7
10	0			213	16	7.5
12	99	5	5.1	211	13	6.2
16	97	6	6.2	208	13	6.3
20	98	10	10.2	209	14	6.7
24	96	13	13.5	209	22	10.5
32	95	14	14.7	0		
40	95	22	23.2	0		
48	95	39	41	0		
56	16	7	43.8	0		
96	77	28	36.8	0		

[a] Number of throat swabs taken.

Poisson regression adjusted for age group again indicated no evidence for a study effect. The detected acquisition rate of 41 acquisitions per 100 infants per year over the first 48 weeks (95% confidence interval, 32 to 52) was little changed by adjustment for imperfect sensitivity of the study design to incident acquisition (adjusted result, 42 [95% confidence interval, 33 to 54]). The acquisition rate was relatively constant up to age 32 weeks, with detected acquisition rates per 100 infants per year of 34 (95% confidence interval, 19 to 61) up to age 16 weeks and 36 (21 to 63) between 16 and 32 weeks, which then increased to 102 (68 to 153) between 33 and 48 weeks of age. The estimated underlying acquisition rate was 3.33 (1.62 to 6.83) times higher in the period from 33 to 48 weeks compared to pre-16 weeks. The rate of acquisition of a second strain, measured from the time of acquisition of the first, was 28% per year (95% confidence interval, 11 to 76%). This was similar to the rate of acquisition for a first strain, but the estimate was imprecise and consistent with identical or substantially different acquisition rates for first and second strains.

Carriage by family members. Isolates of *N. lactamica* were obtained, at week two of study 2, from throat swab samples from one (1 of 55, 1.8%) of the fathers, six (6 of 166, 3.6%) of the mothers, and 14 (14 of 50, 28%) of the siblings of 12 infant participants. None of the strains isolated from the parents were shared with the infants; 7 of the 12 infants shared a strain with a sibling ($P < 0.02$ by Fisher's exact test).

DISCUSSION

The dynamics of carriage and population diversity of *N. lactamica* will influence any immunity to meningococcal disease that is generated by the asymptomatic carriage of this commensal during infancy (6, 21, 22). As *N. lactamica* is acapsulate (22), this immunity would have to be active against subcapsular components of the meningococcus, and given the antigenic and genetic diversity of the meningococcal populations (10), any active protection induced by *N. lactamica* would also have to be cross-protective against many meningococcal strains. Such broad protection could, for example, be a consequence of exposure to many different *N. lactamica* strains, resulting in an immune response against many variants of a diverse *Neisseria* cell component, such as one of the porins (14). Alternatively, exposure to a single *N. lactamica* strain could result in immunity to cell components that are highly conserved among members of the genus *Neisseria*. These alternative scenarios have different implications for vaccine de-

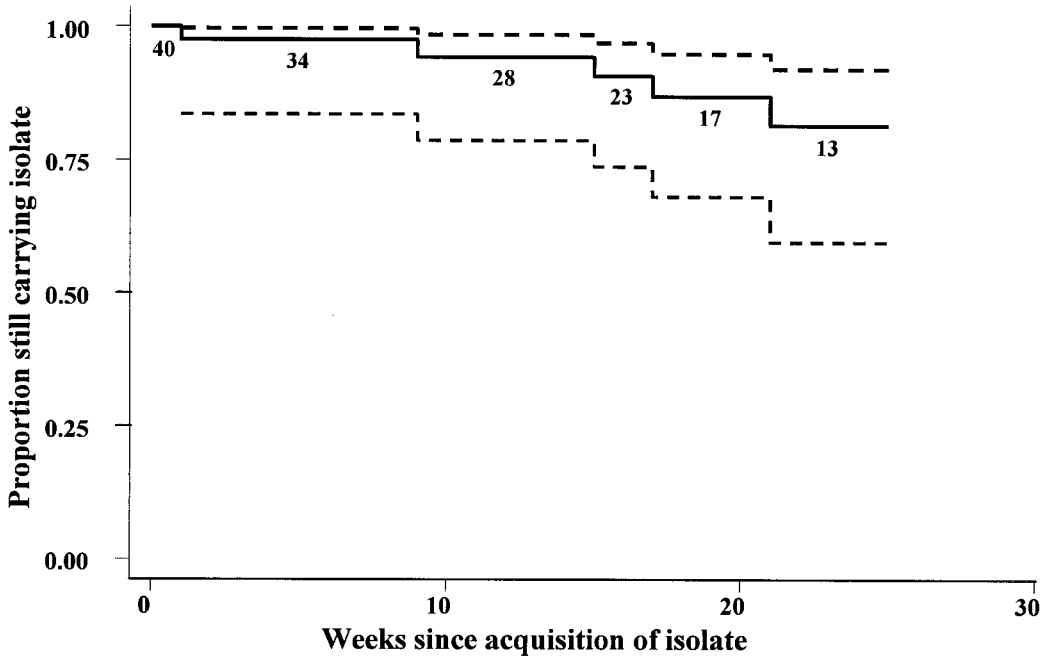

FIG. 2. Kaplan-Meier graph of loss of carriage following acquisition. Numbers indicate the number of individuals contributing follow-up time to each estimate. The dotted line indicates 95% confidence limits of survival.

velopment; the former implies that a multicomponent vaccine, based on many different *N. lactamica* strains, would be required to mimic natural immunity, while the latter suggests that a vaccine based on a single strain could be effective. The molecular genetic typing of the *N. lactamica* isolates obtained from the longitudinal sampling of infants reported here suggests that *N. lactamica* populations are highly diverse and that the prevalence of carriage among infants is high. As most children were colonized by a single *N. lactamica* strain for a prolonged period of time, these data provide support for the concept of antimeningococcal immunity arising from exposure to a single *N. lactamica* isolate. This raises the question of whether the long duration of carriage is important in conferring this immunity.

The age at which carriage was highest was consistent with previous reports of *N. lactamica* carriage (2, 9, 21, 41, 45), with rates increasing through the first 2 years of life and no detectable carriage of *N. meningitidis*. The efficiency of *N. lactamica* isolation (84%) on repeat sampling was comparable to that obtained in a previous study (41), and the isolation rates in the two studies described here were indistinguishable, notwithstanding the fact that the swab samples from study 1 had been frozen prior to culture for *N. lactamica*, whereas the isolates from study 2 were obtained after direct plating. Although the samples were collected in the context of studies on the carriage of other respiratory pathogens and as a consequence did not fully cover the ages at which maximum carriage of *N. lactamica* have been reported (2, 41, 45), the observed isolation rates support the contention that swab samples taken from the throat are more efficient for the isolation of this organism than those taken from the nasopharynx of young children.

The *N. meningitidis* MLST scheme (25, 38), was easily adapted to *N. lactamica*, providing the first isolate characterization scheme for this organism. Given the close genetic relationship between these two bacteria (24) and the reported sharing of gene sequences between them (17), the *N. lactamica* data were deposited in the same database as the meningococcal MLST data (http://pubmlst.org/neisseria) (28, 53). This avoided the possibility of the same allele sequence being assigned to different MLST allele numbers in different schemes. Further, *N. lactamica* isolates, especially those from carriage, are occasionally misidentified by microbiological techniques (52), and inclusion of both species in the same database has the advantage that such misclassifications are immediately apparent. MLST data may assist in future sequence-based species definitions of closely related organisms such as these (48).

The MLST analysis revealed patterns of genetic diversity largely similar to those observed in meningococci (10, 38) with respect to numbers of polymorphic sites, d_N/d_S ratios, numbers of alleles at each MLST locus, and numbers of STs (25, 30, 38), although there were some differences in locus-by-locus comparisons. The high levels of diversity seen were consistent with previous studies using phenotypic analysis (21), macrorestriction analysis (45), and nucleotide sequence-based methods (1). Visual inspection of the *N. lactamica* gene sequences identified possible mosaic structures at many of the loci, and these observations, together with the sharing of identical alleles in otherwise unrelated sequence types (e.g., the *fumC100* allele, which was shared between ST-614 and ST-620 [Table 3]), were consistent with horizontal genetic exchange playing a major role in generating population structure in *N. lactamica*, as seen in meningococcal populations (25). It is also likely that *N. lactamica* populations contain clonal complexes, although, as a consequence of the levels of diversity present in these isolate collections, few of these complexes could be defined with confidence from these data alone.

The availability of reliable molecular typing enabled accurate estimates for acquisition rates and the duration of carriage. The data show that the first 2 years of life are characterized by rates of acquisition that exceed rates of loss of carriage, resulting in rising carriage prevalence within individuals. Once an individual had acquired a given genotype, this could be recovered for prolonged periods, and there was no evidence for a rapid acquisition and loss of multiple genotypes. A previous study (21), which did not have the benefit of molecular isolate characterization, reported median carriage durations of 3.8 months in infants 3 to 13 months of age and of 3 months in 6- to 8-year-olds as determined by using a life table method; the persistence of carriage described here is inconsistent with a carriage duration as short as this. Possible explanations for this include inherent differences in the populations studied or differences in the sensitivities of the sampling techniques employed. Persistence of single strains within individuals was reported in the previous study (21), with two-thirds of infants whose cultures were positive more than once in the first year of life appearing to carry the same organism on each occasion when tested with meningococcal typing antisera. The increase in the acquisition rate observed between the ages of 32 weeks and 1 year in the present study might be a consequence of the decline in maternally derived antibodies and/or the increased opportunity for exposure to other children carrying *N. lactamica* at this age.

Study 2 provided some information on within-family transmission, suggesting that infants are more likely to acquire *N. lactamica* from siblings and other children than from their parents. The high prevalence of *N. lactamica* carriage in young children reported in this study and others may be a consequence of their high milk consumption (22) and the ability of *N. lactamica* to ferment lactose. Carriage of *N. lactamica* is low in young adult males (33) and in the elderly, both male and female (32), who are less likely to have prolonged contact with children. In adults age 21 to 59 years, the majority of *N. lactamica* was isolated from women (32), possibly reflecting their longer or closer contact with children, but as *N. lactamica* has evolved in a niche where lactose is prevalent, it is possible that carriage in adults may be lower as a consequence of differences in adult and infant diets.

Despite the interest in the exploitation of this bacterium in vaccines, the mechanisms responsible for the age-related variation in carriage of *N. lactamica* and *N. meningitidis* are not well understood. Further, the mechanisms for the induction of immunity to meningococcal disease by *N. lactamica* carriage have not been established. It has been documented that antibodies that are cross-reactive against meningococci are present in infants after carriage of *N. lactamica* (6, 21, 22), and it has been suggested that bacterial interference is a protective factor, with the more predominant *N. lactamica* inhibiting colonization by *N. meningitidis* (21). If this is correct, then the widespread intentional colonization by *N. lactamica* strains could enhance natural protection (23) and inhibit colonization

by *N. meningitidis*. It has been suggested that live attenuated meningococci could be used in a vaccine (51), but as the virulence factors associated with *N. meningitidis* are poorly understood, a less hazardous approach would be to employ *N. lactamica*, which has been shown to have cross-reactive surface antigens (22, 31), in vaccines. Whatever the mechanisms or extent of immunity induced by *N. lactamica* against meningococcal disease, this study demonstrates that such immunity is likely to be induced by the prolonged carriage of a single genotype rather than from the short-term carriage of multiple genotypes of this highly diverse commensal bacterium.

ACKNOWLEDGMENTS

We are grateful to the staff of the Oxford Vaccine Group, and Linda Diggle in particular, for supporting the collection of samples. This publication made use of the *Neisseria* multilocus sequence typing website (http://pubmlst.org/neisseria/) developed by Keith Jolley and Man-Suen Chan and sited at the University of Oxford. We thank all of the families and children who participated in this study.

This study was funded by the Meningitis Research Foundation. M.C.J.M. is a Wellcome Trust Senior Research Fellow, N.M.C. is a Wellcome Trust Clinical Training Fellow, and D.W.C. was a Wellcome Leave Fellow at the time of the study. The nasopharyngeal and throat swab samples were obtained from studies carried out by the Oxford Vaccine Group, supported by the Wellcome Trust (reference number 056886/2/994/Z). The development of the *Neisseria* multilocus sequence typing website has been funded by the Wellcome Trust and the European Union.

REFERENCES

1. **Alber, D., M. Oberkotter, S. Suerbaum, H. Claus, M. Frosch, and U. Vogel.** 2001. Genetic diversity of *Neisseria lactamica* strains from epidemiologically defined carriers. J. Clin. Microbiol. **39:**1710–1715.
2. **Blakebrough, I. S., B. M. Greenwood, H. C. Whittle, A. K. Bradley, and H. M. Gilles.** 1982. The epidemiology of infections due to *Neisseria meningitidis* and *Neisseria lactamica* in a northern Nigerian community. J. Infect. Dis. **146:**626–637.
3. **Borrow, R., D. Goldblatt, A. Finn, J. Southern, L. Ashton, N. Andrews, G. Lal, C. Riley, R. Rahim, K. Cartwright, G. Allan, and E. Miller.** 2003. Immunogenicity of, and immunologic memory to, a reduced primary schedule of meningococcal C-tetanus toxoid conjugate vaccine in infants in the United kingdom. Infect. Immun. **71:**5549–5555.
4. **Bowler, L. D., Q. Y. Zhang, J. Y. Riou, and B. G. Spratt.** 1994. Interspecies recombination between the *penA* genes of *Neisseria meningitidis* and commensal *Neisseria* species during the emergence of penicillin resistance in *N. meningitidis*: natural events and laboratory simulation. J. Bacteriol. **176:**333–337.
5. **Bygraves, J. A., R. Urwin, A. J. Fox, S. J. Gray, J. E. Russell, I. M. Feavers, and M. C. J. Maiden.** 1999. Population genetic and evolutionary approaches to the analysis of *Neisseria meningitidis* isolates belonging to the ET-5 complex. J. Bacteriol. **181:**5551–5556.
6. **Cann, K. J., and T. R. Rogers.** 1989. Detection of antibodies to common antigens of pathogenic and commensal antigens of pathogenic and commensal *Neisseria* species. J. Med. Microbiol. **30:**23–30.
7. **Cartwright, K.** 1995. Meningococcal carriage and disease, p. 115–146. *In* K. Cartwright (ed.), Meningococcal disease. John Wiley & Sons, Chichester, United Kingdom.
8. **Cartwright, K. (ed.).** 1995. Meningococcal disease. John Wiley & Sons, Chichester, United Kingdom.
9. **Cartwright, K. A. V., J. M. Stuart, D. M. Jones, and N. D. Noah.** 1987. The Stonehouse survey: nasopharyngeal carriage of meningococci and *Neisseria lactamica.* Epidemiol. Infect. **99:**591–601.
10. **Caugant, D. A.** 1998. Population genetics and molecular epidemiology of *Neisseria meningitidis.* APMIS **106:**505–525.
11. **Caugant, D. A., B. E. Kristiansen, L. O. Frøholm, K. Bovre, and R. K. Selander.** 1988. Clonal diversity of *Neisseria meningitidis* from a population of asymptomatic carriers. Infect. Immun. **56:**2060–2068.
12. **Caugant, D. A., L. F. Mocca, C. E. Frasch, L. O. Frøholm, W. D. Zollinger, and R. K. Selander.** 1987. Genetic structure of *Neisseria meningitidis* populations in relation to serogroup, serotype, and outer membrane protein pattern. J. Bacteriol. **169:**2781–2792.
13. **Denning, D. W., and S. S. Gill.** 1991. *Neisseria lactamica* meningitis following skull trauma. Rev. Infect. Dis. **13:**216–218.
14. **Derrick, J. P., R. Urwin, J. Suker, I. M. Feavers, and M. C. J. Maiden.** 1999. Structural and evolutionary inference from molecular variation in *Neisseria* porins. Infect. Immun. **67:**2406–2413.
15. **Feavers, I. M., A. J. Fox, S. Gray, D. M. Jones, and M. C. J. Maiden.** 1996. Antigenic diversity of meningococcal outer membrane protein PorA has implications for epidemiological analysis and vaccine design. Clin. Diagn. Lab. Immunol. **3:**444–450.
16. **Feavers, I. M., S. J. Gray, R. Urwin, J. E. Russell, J. A. Bygraves, E. B. Kaczmarski, and M. C. J. Maiden.** 1999. Multilocus sequence typing and antigen gene sequencing in the investigation of a meningococcal disease outbreak. J. Clin. Microbiol. **37:**3883–3887.
17. **Feil, E., J. Zhou, J. Maynard Smith, and B. G. Spratt.** 1996. A comparison of the nucleotide sequences of the *adk* and *recA* genes of pathogenic and commensal *Neisseria* species: evidence for extensive interspecies recombination within *adk.* J. Mol. Evol. **43:**631–640.
18. **Feil, E. J., B. C. Li, D. M. Aanensen, W. P. Hanage, and B. G. Spratt.** 2004. eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. J. Bacteriol. **186:**1518–1530.
19. **Feil, E. J., M. C. J. Maiden, M. Achtman, and B. G. Spratt.** 1999. The relative contributions of recombination and mutation to the divergence of clones of *Neisseria meningitidis.* Mol. Biol. Evol. **16:**1496–1502.
20. **Finne, J., D. Bitter Suermann, C. Goridis, and U. Finne.** 1987. An IgG monoclonal antibody to group B meningococci cross-reacts with developmentally regulated polysialic acid units of glycoproteins in neural and extraneural tissues. J. Immunol. **138:**4402–4407.
21. **Gold, R., I. Goldschneider, M. L. Lepow, T. F. Draper, and M. Randolph.** 1978. Carriage of *Neisseria meningitidis* and *Neisseria lactamica* in infants and children. J. Infect. Dis. **137:**112–121.
22. **Griffiss, J. M., B. Brandt, and G. A. Jarvis.** 1987. Natural immunity to *Neisseria meningitidis*, p. 99–119. *In* N. A. Vedros (ed.), Evolution of meningococcal disease, vol. II. CRC Press Inc., Boca Raton, Fla.
23. **Griffiss, J. M., R. Yamasaki, M. Estabrook, and J. J. Kim.** 1991. Meningococcal molecular mimicry and the search for an ideal vaccine. Trans. R. Soc. Trop. Med. Hyg. **85**(Suppl. 1)**:**32–36.
24. **Guibourdenche, M., M. Y. Popoff, and J. Y. Riou.** 1986. Deoxyribonucleic acid relatedness among *Neisseria gonorrhoeae*, *N. meningitidis*, *N. lactamica*, *N. cinerea* and "*Neisseria polysaccharea*." Ann. Inst. Pasteur Microbiol. **137B:** 177–185.
25. **Holmes, E. C., R. Urwin, and M. C. J. Maiden.** 1999. The influence of recombination on the population structure and evolution of the human pathogen *Neisseria meningitidis.* Mol. Biol. Evol. **16:**741–749.
26. **Holten, E., D. Bratlid, and K. Bovre.** 1978. Carriage of *Neisseria meningitidis* in a semi-isolated arctic community. Scand. J. Infect. Dis. **10:**36–40.
27. **Huson, D. H.** 1998. SplitsTree: analyzing and visualizing evolutionary data. Bioinformatics **14:**68–73.
28. **Jolley, K. A., M. S. Chan, and M. C. Maiden.** 2004. mlstdbNet—distributed multi-locus sequence typing (MLST) databases. BMC Bioinformatics **5:**86.
29. **Jolley, K. A., E. J. Feil, M. S. Chan, and M. C. Maiden.** 2001. Sequence type analysis and recombinational tests (START). Bioinformatics **17:**1230–1231.
30. **Jolley, K. A., J. Kalmusova, E. J. Feil, S. Gupta, M. Musilek, P. Kriz, and M. C. Maiden.** 2000. Carried meningococci in the Czech Republic: a diverse recombining population. J. Clin. Microbiol. **38:**4492–4498. (Author's correction, 40:3549–3550, 2002.)
31. **Kim, J. J., R. E. Mandrell, and J. M. Griffiss.** 1989. *Neisseria lactamica* and *Neisseria meningitidis* share lipooligosaccharide epitopes but lack common capsular and class 1, 2, and 3 protein epitopes. Infect. Immun. **57:**602–608.
32. **Kremastinou, J., G. Tzanakaki, S. Levidiotou, F. Markou, E. Themeli, A. Voyiatzi, E. Psoma, M. Theodoridou, and C. C. Blackwell.** 2003. Carriage of Neisseria meningitidis and Neisseria lactamica in northern Greece. FEMS Immunol. Med. Microbiol. **39:**23–29.
33. **Kremastinou, J., G. Tzanakaki, E. Velonakis, A. Voyiatzi, A. Nickolaou, R. A. Elton, D. Weir, and C. Blackwell.** 1999. Carriage of *Neisseria meningitidis* and *Neisseria lactamica* among ethnic Greek school children from Russian immigrant families in Athens. FEMS Immunol. Med. Microbiol. **23:**13–20.
34. **Kumar, S., K. Tamura, I. B. Jakobsen, and M. Nei.** 2001. MEGA2: molecular evolutionary genetics analysis software. Bioinformatics **17:**1244–1245.
35. **Lauer, B. A., and C. E. Fisher.** 1976. *Neisseria lactamica* meningitis. Am. J. Dis. Child. **130:**198–199.
36. **Lujan, R., Q.-Y. Zhang, J. A. Sáez Nieto, D. M. Jones, and B. G. Spratt.** 1991. Penicillin-resistant isolates of *Neisseria lactamica* produce altered forms of penicillin-binding protein 2 that arose by interspecies horizontal gene transfer. Antimicrob. Agents Chemother. **35:**300–304.
37. **Maiden, M. C. J.** 2000. High-throughput sequencing in the population analysis of bacterial pathogens. Int. J. Med. Microbiol. **290:**183–190.
38. **Maiden, M. C. J., J. A. Bygraves, E. Feil, G. Morelli, J. E. Russell, R. Urwin, Q. Zhang, J. Zhou, K. Zurth, D. A. Caugant, I. M. Feavers, M. Achtman, and B. G. Spratt.** 1998. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proc. Natl. Acad. Sci. USA **95:**3140–3145.
39. **Morse, S. A., and J. S. Knapp.** 1992. The genus *Neisseria*, p. 2495–2559. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), The prokaryotes, 2nd ed. Springer-Verlag, New York, N.Y.

40. **Oliver, K. J., K. M. Reddin, P. Bracegirdle, M. J. Hudson, R. Borrow, I. M. Feavers, A. Robinson, K. Cartwright, and A. R. Gorringe.** 2002. *Neisseria lactamica* protects against experimental meningococcal infection. Infect. Immun. **70:**3621–3626.
41. **Olsen, S. F., B. Djurhuus, K. Rasmussen, H. D. Joensen, S. O. Larsen, H. Zoffman, and I. Lind.** 1991. Pharyngeal carriage of *Neisseria meningitidis* and *Neisseria lactamica* in households with infants within areas with high and low incidences of meningococcal disease. Epidemiol. Infect. **106:**445–457.
42. **Orden, B., and M. A. Amerigo.** 1991. Acute otitis media caused by *Neisseria lactamica.* Eur. J. Clin. Microbiol. Infect. Dis. **10:**986–987.
43. **Saez-Nieto, J. A., J. R. Dominguez, J. L. Monton, P. Cristobal, A. Fenoll, J. Vazquez, J. Casal, and B. Taracena.** 1985. Carriage of *Neisseria meningitidis* and *Neisseria lactamica* in a school population during an epidemic period in Spain. J. Hyg. **94:**279–288.
44. **Sherrard, J. S., and J. S. Bingham.** 1995. Gonorrhoea now. Int. J. Sex. Transm. Dis. AIDS **6:**162–166.
45. **Simmons, G., D. Martin, J. Stewart, and D. Bremner.** 2000. Carriage of *N. lactamica* in a population at high risk of meningococcal disease. Epidemiol. Infect. **125:**99–104.
46. **Smith, N. H., E. C. Holmes, G. M. Donovan, G. A. Carpenter, and B. G. Spratt.** 1999. Networks and groups within the genus *Neisseria*: analysis of *argF*, *recA*, *rho*, and 16S rRNA sequences from human *Neisseria* species. Mol. Biol. Evol. **16:**773–783.
47. **Spratt, B. G., L. D. Bowler, Q. Y. Zhang, J. Zhou, and J. M. Smith.** 1992. Role of interspecies transfer of chromosomal genes in the evolution of penicillin resistance in pathogenic and commensal *Neisseria* species. J. Mol. Evol. **34:**115–125.
48. **Stackebrandt, E., W. Frederiksen, G. M. Garrity, P. A. Grimont, P. Kampfer, M. C. Maiden, X. Nesme, R. Rossello-Mora, J. Swings, H. G. Truper, L. Vauterin, A. C. Ward, and W. B. Whitman.** 2002. Report of the Ad Hoc Committee for the Re-evaluation of the Species Definition in Bacteriology. Int. J. Syst. Bacteriol. **52:**1043–1047.
49. **Staden, R.** 1996. The Staden sequence analysis package. Mol. Biotechnol. **5:**233–241.
50. **Suker, J., I. M. Feavers, and M. C. J. Maiden.** 1993. Structural analysis of the variation in the major outer membrane proteins of *Neisseria meningitidis* and related species. Biochem. Soc. Trans. **21:**304–306.
51. **Tang, C., R. Moxon, and M. M. Levine.** 1999. For discussion: live attenuated vaccines for group B meningococcus. Vaccine **17:**114–117.
52. **Tzanakaki, G., R. Urwin, M. Musilek, P. Kriz, J. Kremastinou, A. Pangalis, C. C. Blackwell, and M. C. Maiden.** 2001. Phenotypic and genotypic approaches to characterization of isolates of *Neisseria meningitidis* from patients and their close family contacts. J. Clin. Microbiol. **39:**1235–1240.
53. **Urwin, R., and M. C. Maiden.** 2003. Multi-locus sequence typing: a tool for global epidemiology. Trends Microbiol. **11:**479–487.
54. **Ward, M. J., P. R. Lambden, and J. E. Heckels.** 1992. Sequence analysis and relationships between meningococcal class 3 serotype proteins and other porins of pathogenic and non-pathogenic *Neisseria* species. FEMS Microbiol. Lett. **94:**283–290.
55. **Wyle, F. A., M. S. Artenstein, B. L. Brandt, E. C. Tramont, D. L. Kasper, P. L. Altieri, S. L. Berman, and J. P. Lowenthal.** 1972. Immunologic response of man to group B meningococcal polysaccharide vaccines. J. Infect. Dis. **126:**514–521.

Editor: J. N. Weiser