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Genetics

Electrophoretic variation in adenylate kinase of *Neisseria meningitidis* is due to inter- and intraspecies recombination

(natural transformation/linkage disequilibrium/mosaic genes/multilocus enzyme electrophoresis/nonclonal population structure)

EDWARD FEIL, GILL CARPENTER, AND BRIAN G. SPRATT*

Molecular Microbiology Group, School of Biological Sciences, University of Sussex, Falmer, Brighton BN1 9QG, United Kingdom

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ABSTRACT **In prokaryotic and eukaryotic organisms, the electrophoretic variation in housekeeping enzymes from natural populations is assumed to have arisen by the accumulation of stochastic predominantly neutral mutations. In the naturally transformable bacterium *Neisseria meningitidis*, we show that variation in the electrophoretic mobility of adenylate kinase is due to inter- and intraspecies recombination rather than mutation. The nucleotide sequences of the adenylate kinase gene (*adk*) from isolates that express the predominant slow electrophoretic variant were rather uniform, differing in sequence at an average of 1.1% of nucleotide sites. The *adk* sequences of rare isolates expressing the fast migrating variant were identical to each other but had a striking mosaic structure when compared to the *adk* genes from strains expressing the predominant variant. Thus the sequence from the fast variants was identical to those of typical slow variants in the first 158 bp of the gene but differed by 8.4% in the rest of the gene (nt 159–636). The fast electrophoretic variant appears to have arisen by the replacement of most of the meningococcal gene with the corresponding region from the *adk* gene of a closely related *Neisseria* species. The *adk* genes expressing the electrophoretic variant with intermediate mobility were perfect, or almost perfect, recombinants between the *adk* genes expressing the fast and slow variants. Recombination may, therefore, play a major role in the generation of electrophoretically detectable variation in housekeeping enzymes of some bacterial species.**

In recent years there has been considerable interest in the significance of genetic recombination within and between populations of bacteria (1–4). Although mechanisms that promote the exchange of segments of chromosomes between different lineages exist in most (perhaps all) bacterial species, it has been unclear to what extent these recombinational mechanisms are utilized in natural populations. Recombination is believed to be rare in *Escherichia coli* and *Salmonella enterica* populations as multilocus enzyme electrophoresis (MLEE) surveys have revealed high levels of linkage disequilibrium between alleles (4–6). There is molecular evidence that recombination does occur within these clonal populations, but it is thought to be too rare and too localized to dissipate linkage disequilibrium (7–10). It is now realized that the extent of recombination within bacterial species varies considerably, such that their population structures range from strictly clonal to panmictic (3). High levels of recombination have been inferred to occur in species that are naturally transformable (3, 11) but also in species that are not known to be naturally transformable (12). Some of the most persuasive evidence for extensive recombination in bacterial populations comes from studies on *Neisseria meningitidis* (the meningococcus) and *Neisseria gonorrhoeae* (the gonococcus). These bacteria are naturally competent for genetic transformation throughout their growth cycles and the availability of this mechanism of genetic exchange appears to have a profound effect on their evolution and population biology (3, 13–17).

Caugant *et al.* (18) carried out an extensive MLEE survey of meningococci, analyzing variation at 15 enzyme loci in 688 isolates recovered predominantly from patients with invasive disease. Although the results from this survey appeared to imply relatively high levels of linkage disequilibrium, it has been pointed out (3, 17) that artificially high levels of linkage disequilibrium may result from sampling bias, in this case through the disproportionately high frequency in the data set of adaptive disease-causing genotypes. Once the data set is subdivided to account for this bias, measures of the association between loci approach zero, implying a high rate of recombination within the general meningococcal population (3). Similar MLEE surveys of *N. gonorrhoeae* suggest that populations of this species have been recombining with sufficient frequency to reach linkage equilibrium (3, 13, 14). As expected of species in which alleles in the population are being shuffled by recombination, there is a poor correlation between the level of relatedness of isolates inferred from MLEE data and those from analysis of serological or immunological characters (13, 18, 19). Serogroup A meningococci are, however, an exception as a much higher level of congruence between phylogenies derived from MLEE and from other characters has been observed in this group (20). This has led to the view that recombination is less frequent in serogroup A isolates than in other meningococci and that the serogroup A subpopulation is essentially clonal (17, 20). This idea is supported by the high levels of linkage disequilibrium between alleles observed in MLEE studies of serogroup A isolates (20).

MLEE has been used extensively in studies of population biology, taxonomy, and phylogenetics in both eukaryotic and prokaryotic species (3–6, 21, 22). Phylogenetic inferences from MLEE data sets are only validated by the assumption that starch gel allozymes (electrophoretic variants) are primarily generated through the accumulation of largely neutral stochastic mutations over time. By working under this assumption, the genetic distances between pairs of isolates can be calculated from the number of allelic mismatches. If, however, polymorphisms detected by MLEE have arisen through recombinational replacements, then phylogenetic inferences based on MLEE data will be meaningless. There is convincing evidence that the polymorphisms detected by MLEE in the enterobacteria *E. coli* and *S. enterica* arose predominantly through mutation. Dendrograms constructed from MLEE data are largely congruent with those drawn from sequence data for the *mdh*, *putP*, and *gapA* genes (9, 23–25). This not only suggests that recombination within these genes has been relatively infrequent but also that the dendrograms constructed with MLEE data reflect the long-term evolutionary histories of the isolates. More direct evidence for the predom-

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Abbreviations: MLEE, multilocus enzyme electrophoresis; ADK, adenylate kinase.

*To whom reprint requests should be addressed.

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                    1111112222222222233333334444445555556666666666
                   1260256670111244666711135561122551333790002333333
                   3468392314069849178625804764536060127370131123 56
P63                CGCCTTCGCCACGCCCGATATACCCTCCCATCTTTTCTTGCGCGGCCA ALLOZYME 2
HF46               ..........................................       "
HF130              ........................T..................       "
S3446              T...A.T........T.......T.C.....................    "
M470               ..............T.GCG.....C.....A....T...........    "
44/76              ..ATA.........TT.......T.C.............CAGA......    "
N9411              .AATA..........T.......T.C.....................    "
HF113,HF147        ..........................T.GC.CGGC.GCAGAAAAAGT ALLOZYME 3
HF79,HF200         ........................TT.............CAGAAAAAGT    "
HF4,HF8,HF16,HF85  .....CTATTGTCTTTAGCGCGTTTCTT.GC.CGGC.GCAGAAAAAGT ALLOZYME 4
HF110,HF116,HF175
                   1333333133333313331333333333133333313333313312323
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FIG. 1. Polymorphic sites within the *adk* genes of meningococci. The *adk* sequence of strain P63 is used as the master sequence and only those nucleotides in the *adk* genes of the other meningococci that differ from the P63 sequence are shown. The position of each of these polymorphic sites within the *adk* sequence is shown above in vertical format, and the position within the codon of each polymorphism is shown below. Nucleotide changes that alter the charge of ADK allozymes 3 and 4 are double-underlined.

inant role of mutation in the generation of electrophoretic variation has been provided by sequencing *mdh* in a sample of *E. coli* and *S. enterica* isolates expressing malate dehydrogenase allozymes of differing electrophoretic mobilities (9).

We have investigated whether the gradual accumulation of mutations also accounts for the electrophoretic variation of housekeeping enzymes in species that appear to have a higher frequency of recombination than enterobacteria. In contrast to the conclusions drawn from studies with the enterobacteria, our results demonstrate that electrophoretic variation in adenylate kinase (ADK)† of meningococci is predominantly the result of inter- and intraspecific recombination events rather than mutation.

MATERIALS AND METHODS

Bacterial Isolates. The ADK gene (*adk*) was sequenced in 29 strains of *N. meningitidis.* The following isolates, which include strains expressing each of the four allozymes of ADK detected by Caugant *et al.* (18), were studied: allozyme 1, 020; allozyme 2, S3446, HF130, P63, N94II, HF46, 44/76, and M470; allozyme 3, HF79, HF113, HF147, and HF200; allozyme 4, HF4, HF8, HF16, HF85, HF110, HF116, and HF175. The following *adk* genes of 10 strains of *N. meningitidis* serogroup A from the collection of Wang *et al.* (20) were sequenced: 1001, 3910, 4100, 4146, 5005, 5010, 5013, 5035, 5037, and 5043.

Cloning and Sequencing of *adk*. The amino acid sequences of ADK from *E. coli* and *Haemophilus influenzae* were aligned and degenerate primers were synthesized for two conserved amino acid motifs. Primer ADK-11 [5′-d(GGCGCCGGCAAAGGNACNCAG/AGC)-3′] corresponds to the conserved Gly loop (residues 10–17) (26), and primer ADK-12 [5′-d(GGTGCGG/CGGG/AAANCCG/ATC)-3′] corresponds to residues involved in AMP binding situated at positions 84–89 (26). These primers were used in the PCR to amplify a 240-bp fragment of *adk* from chromosomal DNA of *N. gonorrhoeae* CH95. The sequence of the PCR product confirmed that this fragment originated from the gonococcal *adk* gene and it was used as a probe to identify a plasmid carrying the complete gene from a gonococcal gene library (27).

The complete coding region of the gonococcal *adk* gene and flanking regions was obtained and primers were synthesized, corresponding to regions of 82–101 bp upstream of the initiation codon [ADK-1 5′-d(ATGGCAGTTTGTGCAGTTGG)-3′] and 148–167 bp downstream of the termination codon [ADK-18 5′-d(GATTTAAACAGCGATTGCCC)-3′]. These primers were used in the PCR to amplify *adk* from the *N. meningitidis* isolates. Single-stranded template DNA for sequencing was prepared by treatment with λ exonuclease as described by Higuchi and Ochman (28), and sequencing was carried out on both strands by using Sequenase (United States Biochemical).

Electrophoretic Mobility of ADK Allozymes. Cell lysates of *N. meningitidis* were prepared, fractionated by starch gel electrophoresis, and stained for ADK as described (29). Allozymes of ADK are numbered according to their rate of anodal migration from 2 (slowest) to 4 (fastest).

RESULTS

Sequences of the *adk* Genes of Meningococci Expressing Different Allozymes. ADK catalyzes the transfer of a phosphoryl group from ATP to AMP and plays a major role in energy metabolism (30). The enzyme is ubiquitous, monomeric, and relatively small (20–26 kDa) (26, 30). Caugant *et al.* (18) reported four allozymes of ADK in the meningococcus. Allozyme 2 was expressed by 97.6% of 688 isolates, allozyme 4 was expressed by 1.6%, allozyme 3 was expressed by 0.8%, and allozyme 1 was unique to strain 020. The entire coding region of *adk* (648 bp) was sequenced from 19 *N. meningitidis* strains expressing examples of each of these allozymes. The 48 polymorphic nucleotide sites within the coding region are given in Fig. 1. A total of six ADK amino acid sequences were found among these 19 isolates.

Two of the six amino acid polymorphisms alter the charge of ADK. These electrophoretically detectable amino acid substitutions are Gly-211 → Lys, which is present in strains exhibiting allozymes 3 and 4, and Glu-55 → Lys, which is found only in strains expressing allozyme 4 (Fig. 2). In all cases but one, the deduced amino acid sequences of ADK were consistent with the reported electrophoretic mobilities. The one exception was strain 020, which was the only isolate in the collection of Caugant *et al.* (18) that expressed allozyme 1. The sequence of *adk* from strain 020 was identical to that of strain S3446 (an allozyme 2 strain), and when the mobility of this ADK variant was checked on a starch gel against reference meningococcal strains expressing allozymes 2, 3, and 4, it was found to be indistinguishable from allozyme 2. It therefore seems likely that strain 020 has been wrongly assigned as allozyme 1, and we assume that there are only three electrophoretic variants of ADK in the meningococcal population studied by Caugant *et al.* (18).

The *adk* genes from strains exhibiting allozyme 2 are rather uniform, pairwise comparisons ranging from 0% to 2.2% in nucleotide diversity (average of 1.1%). All seven strains exhibiting allozyme 4 are identical in nucleotide sequence for *adk.* The allozyme 4 sequence differed from those expressing allozyme 2 at between 5.4% and 6.3% of nucleotide sites

†The sequences reported in this paper have been deposited in the GenBank data base (accession nos. L36469 and L47130–L47158).

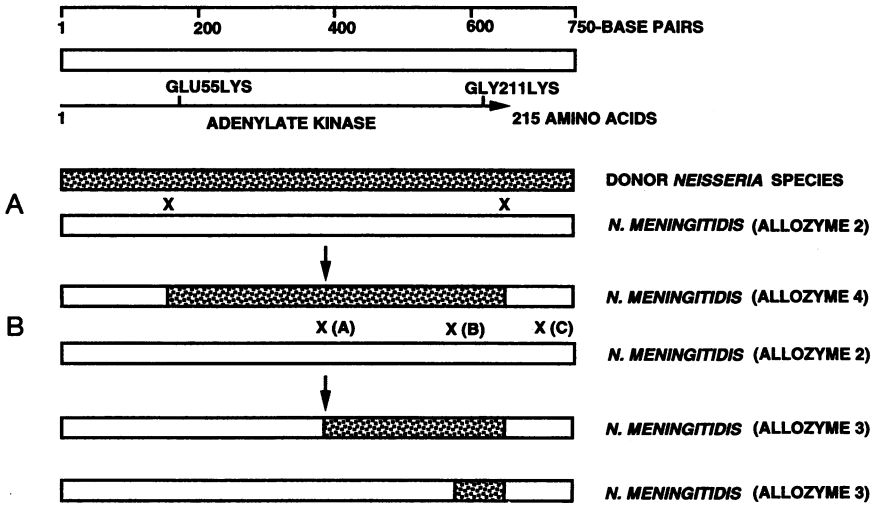

FIG. 2. Mosaic structure and recombinational events in the *adk* gene. The upper open rectangle represents the *adk* gene and the region immediately downstream of *adk*. The region encoding ADK is shown by the horizontal arrow. The amino acid substitutions that alter the charge of ADK in the allozyme 3 and 4 strains are shown. The stippled regions represented the diverged regions of *adk* present in strains expressing allozymes 3 and 4. (*A*) The putative interspecies recombinational event that generated the allozyme 4 sequence. Positions of recombinational crossovers are marked X. (*B*) The putative intraspecies recombinational events that generated the allozyme 3 sequence of strains HF113 and HF147 (crossovers A and C) and of strains HF79 and HF200 (crossovers B and C).

(average of 6.0%). The *adk* gene of strains expressing allozyme 4 differed at 40 sites within the 648-bp coding region from that of strain HF46, which has an *adk* gene typical of allozyme 2 strains. These sequences differed by 8.4% within nt 159–636, whereas there were no differences within the first 158 nt or from nt 637 to 122 downstream of the *adk* termination codon (Fig. 2). The maximum χ^2 procedure (31) was used to show that the highly nonrandom distribution of nucleotide differences in the allozyme 4 *adk* gene would occur by chance with a probability of <0.001. The allozyme 4 *adk* gene, therefore, has a mosaic structure, implying that the diverged block between nt 159 and 636 does not share the same evolutionary history as the rest of the gene.

The *adk* sequences from the four strains expressing allozyme 3 are grouped into two pairs; HF79 and HF200 have identical *adk* sequences, as do HF113 and HF147. Both sequences are striking mosaics ($P < 0.001$) between the *adk* genes of strains expressing allozyme 4 and those expressing allozyme 2 (Fig. 2). The putative upstream recombinational crossover point between the two sequences is between nt 573 and 597 in the *adk* genes of HF79 and HF200 and between nt 366 and 414 in those of HF113 and HF147 (Fig. 1). The sequences for 91 bp downstream of the *adk* gene were identical in these meningococcal strains and the putative downstream crossover point in the two classes of allozyme 3 *adk* genes was indistinguishable from that in the allozyme 4 genes (Fig. 2).

Two small blocks of clustered polymorphic sites were shared between the allozyme 4 sequence and some of the allozyme 2 sequences. These blocks could represent extremely localized recombinational events but the clustering of polymorphic sites at the CAGA block (between nt 597 and 603 in strain 44/76) and the GCG block (between nt 267 and 276 in strain M470) was not statistically significant by Sawyer's test (32).

Noncongruence of the *adk* and *recA* Gene Trees. Fig. 3 shows the gene trees for *recA* (33) and *adk* for the same meningococcal strains. These trees are noncongruent. For example, strain P63 is relatively divergent at *recA* but is typical for *adk*, whereas HF116 (an allozyme 4 strain) is divergent at *adk* but is typical at *recA*. Similarly, the grouping of strain HF46 with 44/76 is strongly supported by the *recA* data but not by the *adk* data. Conversely, HF46 is grouped with HF130 by using *adk* data but not by using *recA* data.

Sequences of *adk* Within the Serogroup A Meningococci. Recombination appears to be less common within serogroup A meningococci than in the general meningococcal population (17). To compare the level of diversity and extent of recombination within the *adk* genes of serogroup A isolates with that in other meningococci, we sequenced *adk* from 10 serogroup A strains from the collection of Wang *et al.* (20). These were isolated from very wide temporal and spatial ranges and represented each of the nine subgroups defined by MLEE surveys on serogroup A meningococci (20). Seven of these serogroup A isolates had *adk* sequences that were identical to those of isolates HF46 (serogroup A) and P63 (serogroup B). Serogroup A strains 3910, 4100, and 5013 differed from the

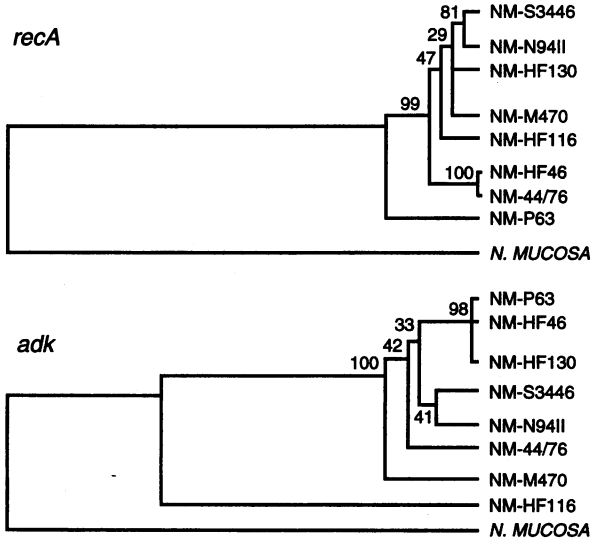

FIG. 3. Trees constructed from the sequences of the *adk* and *recA* genes of the same meningococcal strains. The trees were constructed by the UPGMA method (34) with Jukes–Cantor correction using differences at synonymous sites. The values at the nodes indicate the percentage of the 1000 bootstrap trees that contained the node. The strongly supported nodes (>80% of bootstrap trees) were also strongly supported by using the neighbor-joining method (34). The sequence of *Neisseria mucosa* LNP405 was used as an outgroup. The *recA* sequences are from ref. 33.

other serogroup A strains by a single synonymous C → T substitution at position 579.

DISCUSSION

The results of this investigation support the notion that electrophoretic variants of ADK have arisen in meningococci solely via recent recombinational events. Both inter- and intraspecies recombination within the *adk* coding region appear to have played a role in generating novel allozymes. The *adk* sequence from strains expressing allozyme 4 was highly diverged when compared to the relative uniformity of the allozyme 2 sequences. Furthermore, the nonrandom distribution of the polymorphisms between the allozyme 2 and 4 sequences indicates that different parts of the allozyme 4 sequence have different evolutionary histories, which can best be explained by recombination. As the majority of meningococcal *adk* sequences differ on average at ≈1.1% of nucleotide sites, a figure that is comparable to that observed in other meningococcal housekeeping genes (33), it seems implausible that the block responsible for the charge differences in allozyme 4 (which is 8.4% diverged from a typical meningococcal sequence) was donated by a meningococcus. The most likely scenario is the replacement through interspecies recombination of a large portion of the *adk* gene with the corresponding portion from a commensal *Neisseria* species (Fig. 2).

Meningococci coexist in the human nasopharynx with a variety of closely related commensal *Neisseria* species providing opportunities for the exchange of genes, or parts of genes, by homologous recombination mediated through genetic transformation (17). Most of the coding region of *adk* has been sequenced for the following human *Neisseria* species: *N. gonorrhoeae, N. lactamica, N. cinerea, N. polysaccharea, N. flavescens, N. pharyngis* var. *flava*, and *N. mucosa* (E.F. and B.G.S., unpublished data). These *adk* sequences are all markedly diverged when compared to the meningococcal allozyme 4 sequence; thus none of these species can be implicated as the donor. Interspecies recombination between the human pathogenic and commensal *Neisseria* species has been reported previously in genes under intense selection (35, 36). However, it has also been proposed in the meningococcal *argF* and *recA* housekeeping genes (33, 37). The *adk* gene of the allozyme 4 strains appears to provide a further example of interspecies recombination in a housekeeping gene. Interspecific recombination has not been described in typical housekeeping genes of nontransformable species such as *E. coli* and *S. enterica.* These findings therefore suggest that recombination within the *Neisseria* genus is more promiscuous than within the enterobacteria.

The two pairs of allozyme 3 sequences are striking mosaics between the allozyme 4 sequence and allozyme 2 sequences. The allozyme 3 sequence shared by HF113 and HF147 is a base-perfect hybrid between the allozyme 4 sequence and the allozyme 2 sequence from strains P63 and HF46. The recombinational event that generated this sequence must, therefore, have occurred recently, as the mosaicism has not been obscured by subsequent mutation. The allozyme 3 sequence shared by strains HF200 and HF79 is also a perfect hybrid between the allozyme 4 sequence and allozyme 2 sequences of P63 and HF46, except for the presence of the two thymidine residues at positions 330 and 354. These polymorphisms are present in the allozyme 4 sequence and may be the result of the incorporation of two noncontiguous segments of DNA from the donor during the recombinational event that created this allozyme 3 sequence. Alternatively, these two polymorphisms may have been present in an unidentified allozyme 2 strain that was the recipient in the recombinational event.

It is conceivable that the two classes of allozyme 3 sequences arose by independent interspecies recombinational events involving the same DNA donor species as that involved in the formation of the allozyme 4 sequence. However, the complete sequence identity of the diverged regions at the end of the *adk* genes of the allozyme 3 and allozyme 4 sequences and the common putative downstream recombinational crossover point argue strongly that these sequences arose through subsequent intraspecies recombination events between the allozyme 4 mosaic and the predominant allozyme 2 sequences (Fig. 2).

Again, the sequence identity between the diverged regions within the *adk* genes of the allozyme 3 and 4 strains and the ability to identify the donor and recipient *adk* genes in the recombinational events that created the allozyme 3 sequences argue that these recombinational events have occurred recently. This would not be surprising if recombination is frequent among meningococci. In contrast, putative recombinational events in the housekeeping genes of highly clonal species are usually difficult to identify convincingly (but see ref. 10), as they are usually ancient and have been obscured by the accumulation of subsequent mutations (7–9).

The noncongruence of trees constructed from sequence data for *recA* and *adk* from the same meningococcal strains is most easily explained by assuming that recombination events have obscured the phylogeny of one or both of these genes. The divergent *adk* and *recA* genes of strains HF116 and P63, respectively, are most likely due to interspecies recombinational replacements from unidentified donor species (17, 37). Even when the diverged *recA* and *adk* sequences are excluded, the trees remain noncongruent, although the relative uniformity of the remaining sequences reduces the statistical significance of the trees.

The *adk* genes of the serogroup A strains were extremely uniform and were distinguished by only a single synonymous difference at position 579 in the three isolates representing subgroups III and VIII as defined by MLEE studies (20). These subgroups lie adjacent to each other on the dendrogram constructed from the MLEE data. The presence of this single mutation in these three strains, and its absence in all the other serogroup A strains, therefore, is congruent with the dendrogram, the most likely explanation being that it arose once in the ancestor of subgroups III and VIII. This finding, along with the high level of uniformity within the serogroup A *adk* sequences, supports the notion that serogroup A meningococci represent a relatively recent clonal subpopulation in which recombination between lineages is less frequent than within the general meningococcal population (17, 20). Serogroup A meningococci cause epidemic meningitis and have a very different epidemiology than other meningococci. Possible reasons for the different population biology of these isolates have been discussed elsewhere (17, 38).

Recombination has been proposed to occur in the genes of highly clonal bacterial species (7–10). However, in these bacteria the recombinational events are too rare to break down linkage disequilibrium between alleles and mostly involve genes that are unusually diverse (e.g., cell surface genes) and are believed to be under diversifying selection (39–41). It is significant that the meningococcal *adk* gene is not unusually variable. Indeed almost all of the meningococci examined by Caugant *et al.* (18) were electrophoretically monomorphic for ADK (i.e., they expressed allozyme 2). This implies that the *adk* gene has not been subjected to diversifying selection. Similarly, *adk* appears not to have been influenced by diversifying selection acting at a closely linked locus (hitch-hiking). Hitch-hiking drastically inflates the frequency of recombinants at a particular locus, an effect clearly documented in the *gnd* locus of *E. coli*, which is tightly linked to genes (*rfb*) involved in the synthesis of the antigenically variable O antigen (42, 43). The *adk* recombinants are thus not believed to be selectively advantaged, either by diversifying selection at *adk* or at a neighboring locus. Indeed, as for stochastic mutational events, the recombinants are likely to be selectively slightly disadvan-

taged. The implication is, therefore, that nearly neutral stochastic recombinational events, both interspecific and intraspecific, may be influencing the microevolution of this species more profoundly than mutational processes.

It is too early to extrapolate the findings obtained with *adk* to the rest of the meningococcal genome. Although it is likely that intraspecific recombination is very common within the *Neisseria*, most meningococcal and gonococcal housekeeping genes are too uniform to allow the detection of statistically significant recombinational events (14, 17, 33). In the case of *adk*, the introduction of a highly variant sequence into the population via interspecies recombination has allowed the detection of subsequent intraspecific recombination events. The meningococcal glutamine synthetase and shikimate dehydrogenase genes are sufficiently diverse to detect intraspecies recombinational events (J. Zhou and B.G.S., unpublished data). Although extensive recombination can be observed in these neighboring genes, selection for diversity at a locus closely linked to the genes may be influencing the observed rate of recombination. Since putative interspecies recombinational events can be observed by sequencing housekeeping genes (e.g., *argF, recA*, and *adk*) from small samples of the meningococcal population (33, 37), it is likely that intraspecies recombination in these genes is frequent, although mostly difficult to observe.

The MLEE studies and the sequencing of meningococcal genes suggest that the low recombination paradigm that has emerged from studies with enterobacterial species (9) does not apply to *N. meningitidis* and that recombination within this population and between meningococci and closely related human commensal *Neisseria* has a major influence on the evolutionary biology of the species. Recent studies with *E. coli* suggest that recombination may have a more significant role in the diversification of clones than previously considered (10). However, it is unlikely that the rate of recombination in *E. coli* or *S. enterica* approaches that in the pathogenic *Neisseria*, since there is a broad congruence between gene trees and MLEE trees, and significant linkage disequilibrium between alleles, in the former species (9, 10, 24, 25) but not in the latter (3, 17).

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