Fit genotypes and escape variants of subgroup III *Neisseria meningitidis* during three pandemics of epidemic meningitis

Peixuan Zhu*[†], Arie van der Ende[‡], Daniel Falush*, Norbert Brieske*, Giovanna Morelli*, Bodo Linz*, Tanja Popovic[§], Ilse G. A. Schuurman[‡], Richard A. Adegbola[¶], Kerstin Zurth*, Sébastien Gagneux[∥], Alexander E. Platonov**, Jean-Yves Riou[††‡‡], Dominique A. Caugant[§§], Pierre Nicolas[¶¶], and Mark Achtman*[∥∥]

*Max-Planck Institut für Molekulare Genetik, 14195 Berlin, Germany; [‡]Department of Medical Microbiology, Academic Medical Center, 1105 AZ Amsterdam, The Netherlands; [§]Meningitis and Special Pathogens Branch, Centers for Disease Control and Prevention, Atlanta, GA 30333; [¶]Medical Research Council Laboratories, Fajara, The Gambia; [∥]Swiss Tropical Institute, 4002 Basel, Switzerland; **Central Research Institute of Epidemiology, Moscow 111 123, Russia; [††]Institut Pasteur, 75724 Paris Cedex 15, France; [§§]World Health Organization, Collaborating Centre for Reference and Research on Meningococci, National Institute of Public Health, N-0403 Oslo, Norway; and [¶¶]Institut de Médecine Tropicale du Service de Santé des Armées, World Health Organization Collaborating Center for Reference and Research on Meningococci, Parc du Pharo, BP 46, 13998 Marseilles Armées, France

The genetic variability at six polymorphic loci was examined within a global collection of 502 isolates of subgroup III, serogroup A *Neisseria meningitidis.* Nine ''genoclouds'' were identified, consisting of genotypes that were isolated repeatedly plus 48 descendent genotypes that were isolated rarely. These genoclouds have caused three pandemic waves of disease since the mid-1960s, the most recent of which was imported from East Asia to Europe and Africa in the mid-1990s. Many of the genotypes are escape variants, resulting from positive selection that we attribute to herd immunity. Despite positive selection, most escape variants are less fit than their parents and are lost because of competition and bottlenecks during spread from country to country. Competition between fit genotypes results in dramatic changes in population composition over short time periods.

genetic variation | clonal descent | intercontinental spread | population structure | microevolution

Epidemic meningitis has posed a major health problem since it was first recognized in 1805 (1). Epidemics have recurred approximately every 10 years in this century within the African ''meningitis belt'' of sub-Saharan Africa (2–4) and until the mid-1980s within China (5). Epidemics in other geographical areas are more sporadic, and, in recent decades, only a few industrialized countries have suffered from large epidemics (3), with the notable exceptions of Norway and New Zealand.

Historically, most large epidemics of meningitis were caused by *Neisseria meningitidis* that express the serogroup A capsular polysaccharide. Most recent epidemics have been caused by two pandemic waves (6) of the clonal grouping of serogroup A bacteria that is called subgroup III (5, 7). The first pandemic wave affected China (mid-1960s), northern Europe (Russia and the Scandinavian countries; 1969-mid 1970s), and Brazil (mid-1970s). A second subgroup III pandemic wave began in China and Nepal in the early 1980s, followed by an epidemic during the annual Hajj pilgrimage to Mecca, Saudi Arabia of 1987. Pilgrims returning from Mecca distributed subgroup III meningococci to most countries of the globe (6, 8). Subgroup III has subsequently caused numerous epidemics throughout Africa (3, 9); in 1996, 150,000 cases of meningococcal disease were reported from Africa (10), most caused by subgroup III (3).

Subgroup III meningococci are so uniform that only a few genetic variants have been revealed by MLEE (multilocus enzyme electrophoresis) (5, 6), RAPD (random amplified polymorphic DNA) (11, 12), or MLST (multilocus sequence typing) (7, 12). Such variants can arise during cocolonization of the nasopharynx with other *N. meningitidis* and related species because of the import of genes by DNA transformation (13, 14).

The availability of hundreds of subgroup III strains isolated in different countries since the mid-1960s provided an opportunity to elucidate important features of bacterial microevolution. We have used a multilocus approach involving sequence variability at six polymorphic loci to elucidate the molecular epidemiology of subgroup III bacteria. These six loci encode three outer membrane proteins (*opaB*, *opaD*, *tbpB*), one secreted protein (*iga*), one housekeeping protein (*pgm*), and one defective insertion sequence (IS) element (IS*1106*A) and were chosen because of their relatively high degree of polymorphism in subgroup III. Previous work has shown that differences at the *opaB*, *opaD*, and *iga* loci (which encode opacity adhesins and IgA1 protease) distinguish bacteria isolated during the first pandemic wave or at the beginning of the second pandemic wave from those isolated since 1987 from Mecca pilgrims and in Africa (15). The *pgm* gene encoding phosphoglucomutase differentiates subgroup III isolates of MLST sequence types 5 through 7 (7). *tbpB* (transferrin-binding protein B) differed between older and newer isolates from Moscow (12). IS*1106*A is a defective IS element that is inserted upstream of the *opcA* locus (16) and was shown by preliminary experiments to be polymorphic in subgroup III.

The results show that successive waves of pandemic spread are associated with genoclouds of genetic variants, each containing a particularly fit, dominant genotype. Furthermore, the recent evolution of a new genocloud has now resulted in a third pandemic wave of disease.

Materials and Methods

Bacterial Strains. Five hundred two subgroup III strains isolated between 1966 and 2000 were tested. The countries and detailed dates of isolation have been described for 314 strains (1966–1996) from diverse global sources (15) and 29 ST7 strains (1995–1999) from Africa (9). One hundred fifty-nine additional

Table 1. Geographic distribution of frequent and rare genotypes of subgroup III

| | | | | Frequent genotypes, | Rare genotypes | |
| | | | | | No. of | No. of |
Genocloud	Pandemic	Sources	Years	no. of isolates	genotypes	isolates
1	1	China	1966	2	3	5
2	1	Moscow	1969–1971	14	3	3
3	1, 2	Europe, Brazil, China	1969–1984	43	23	31
4	2	China	1984–1987	26	4	5
5	2	Pilgrims, Africa	1987–1998	236	7	8
6	2	Africa	1991–1993	27	0	
7	2	Africa	1991–1993	15	1	1
9	2	The Gambia	1997	8	0	
8	3	China, Mongolia, Moscow, Africa, U.K.	1993–2000	66	3	3
Intermediate					4	9
Total			1966–2000	437	48	65

isolates were from epidemics in Chad (1988, 9 strains), The Gambia (1997, 54), Ghana (1997–1998, 39; ref. 17), Mongolia (1994–1995, 10; ref. 18), Moscow (1969–1977, 8; 1994–1997, 21; ref. 12), and Sudan (1988, 14), and from endemic disease in the United Kingdom (1997–2000, 4). The assignment of these additional isolates to subgroup III is based on multilocus enzyme electrophoresis for isolates from Chad, Sudan, and Mongolia, random amplified polymorphic DNA for isolates from Moscow (12), and by the possession of strongly related pulse-field gel electrophoresis (PFGE) patterns with two restriction endonucleases (17) for isolates from The Gambia, Ghana, and the United Kingdom. Of the 502 isolates, 60 have been shown to belong to the MLST sequence types ST5 to ST7 that are characteristic of subgroup III (9, 12). Most of the strains have been serogrouped, serotyped, and serosubtyped by ELISA using monoclonal antibodies and are A:4,21:P1.9,20, as is typical of subgroup III (5).

Sequencing and Accession Numbers. Independent PCR products from each locus were sequenced from both DNA strands by using automated cycle sequencing (ABI 377) with dRhodamine-labeled terminators (Perkin–Elmer). Sequences of IS*1106*A elements have been deposited under AJ276902-AJ276908 and AJ292239. Sequences of *tbpB* alleles have been deposited under AJ276909-AJ276937. Other sequences have been described elsewhere (7, 15, 19) (GenBank AJ292235–AJ292238).

Restriction Fragment Length Polymorphism (RFLP) Typing. All 502 isolates were tested for RFLP at six loci (*pgm, iga, opaB, opaD,* IS*1106*A, and *tbpB*) as described (12, 15). In addition, all variant alleles were sequenced from representative strains (*pgm*, 60 strains; *iga*, 18; *opaB*, 43; *opaD*, 50; IS*1106*A, 21; and *tbpB,* 35). The sequence variability at *iga, opaB,* and *opaD* has been described elsewhere (15). Details of the sequence variability at IS*1106*A are presented in the supplemental data (which are published on the PNAS web site, www.pnas.org). The RFLP analysis of *pgm* distinguishes *pgm3* from *pgm19*, but not from *pgm11*, which differs by only one nucleotide (7). *tbpB* alleles that are identical to previously described sequences (*tbpB1, 10, 11,* and *101*; ref. 13) were assigned the same numbers, and other alleles were assigned arbitrary numbers from *tbpB38* to *tbpB66*. These allele assignments were based on an aligned stretch of approximately 660 bp that has been sequenced from all isolates. [We note that the *tbpB1* allele is assigned to both subgroups III and IV-1 although the complete 2057-bp *tbpB* sequences from subgroups III (accession number AJ276909) and IV-1 (AF058689) differ by two nucleotides outside this aligned region.]

Phylogenetic Analyses. *tbpB* sequences were aligned manually to ensure that codons were not split. The aligned sequences were analyzed by using SPLITSTREE 3.1 (20), as described (13). χ^2 analysis of significance between success and failure was performed by using tests 3 and 4 of CLUMP (21), which tests the significance of data in sparse matrices by a Monte Carlo method.

Results

Genocloud Structure of Subgroup III. The genetic fine structure of subgroup III was investigated among 502 subgroup III isolates by using RFLP at *opaB, opaD, tbpB, pgm, iga,* and IS*1106*A. The isolates tested include 314 subgroup III meningococci from diverse global sources that have been previously described (15) plus 188 recent isolates from Mongolia, Moscow, Africa, and the United Kingdom. Two hundred twenty-seven variant alleles were sequenced from representative strains, and at least two alleles were found for each of the six loci.

Each unique combination of alleles is referred to as a genotype, and a total of 57 genotypes were found. Most (87%) of the 502 isolates possess one of nine frequent genotypes (Table 1). The nine frequent genotypes differed from each other by one to six of the six loci (Fig. 1; see Table 5 in supplemental data for details). The other 48 genotypes were isolated only rarely (one to four isolates). They were isolated concurrently with a frequent genotype from the same geographical area and differed from that frequent genotype at only one, or rarely two, of the loci (Fig. 1; Fig. 5). All three criteria (minimal genetic, physical, and temporal distance) were used to assign rare genotypes to groups consisting of one frequent genotype plus its close relatives (Fig. 1). These groups will be referred to as "genoclouds."

Six of the genoclouds contain a frequent genotype plus descendent genotypes, but genoclouds 1, 6, and 9 do not fulfill both requirements (Fig. 1). Genocloud 1 contains only a few isolates (Table 1). It was designated as a separate genocloud because it includes all of the available subgroup III strains that were isolated from several million patients with meningococcal disease in China during the mid-1960s, contains distinct genotypes, and is closely related to the deduced ancestral genotype (see below). Genoclouds 6 and 9 possess only one frequent genotype and no rare genotypes. Their designation as genoclouds is somewhat arbitrary, and they might equally well represent the transient expansion of rare genotypes.

Phylogenetic Relationships and Pandemic Spread. By minimizing genetic and temporal distances between the genoclouds, we constructed a fully parsimonious tree in which each allele arises only once. Four rare genotypes seem to represent intermediates during the evolution from one genocloud to a second and were therefore not assigned to either genocloud (Fig. 1).
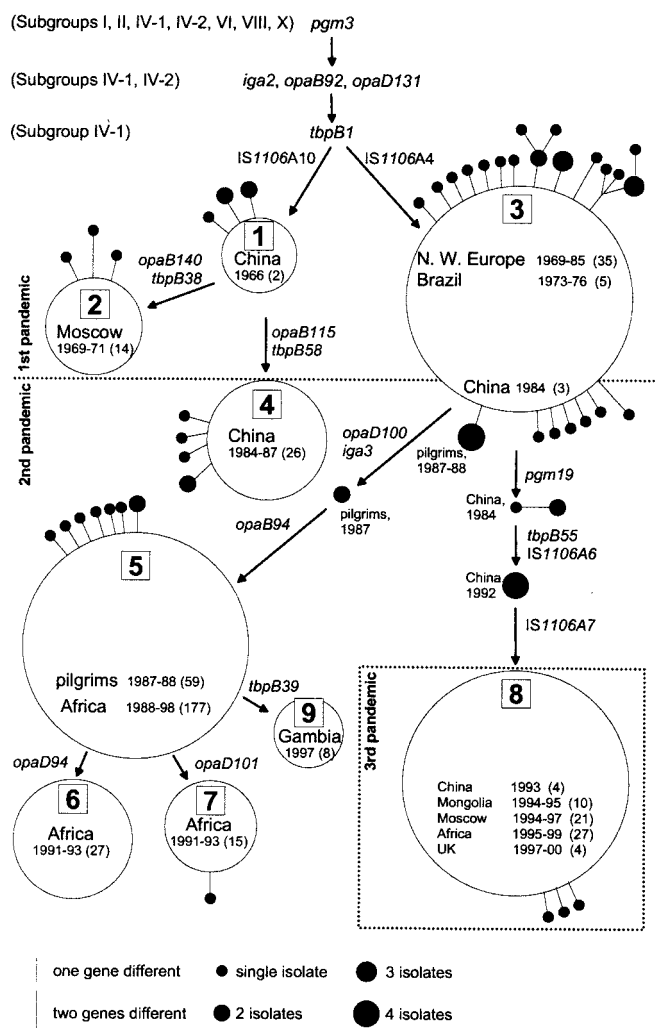
Fig. 1. Parsimonious relationships among 57 genotypes in nine genoclouds of subgroup III. Large boxed numbers from 1 to 9 indicate the genocloud designations. Alleles inherited from common ancestors with other serogroup A subgroups are shown at the top, and subsequent changes are shown next to the arrows that indicate lines of descent. Numbers in parentheses indicate numbers of isolates of the frequent genotypes, which are also reflected to a limited extent by the sizes of the circles. Rare genotypes were parsimoniously grouped in genoclouds after consideration of genetic relationships, sources, and dates of isolation. They are indicated by filled dots whose sizes indicate the numbers of isolates. The lengths of the lines connecting the dots to the genocloud circles indicate the numbers of genetic changes from the frequent genotype. Intermediate strains that were not assigned to genoclouds are shown along the arrows leading from genocloud 3.

The tree is rooted at genoclouds 1 and 3. These genoclouds share *pgm3, iga2, opaB92*, and *opaD131* alleles with older strains of the related subgroups IV-1 and IV-2 (15) and the *tbpB1* allele with older strains of subgroup IV-1 (ref. 13; Fig. 1). The other genoclouds possess variant alleles at these loci. Genoclouds 1 and 3 differ from subgroup IV-1 at the IS*1106*A locus. Genocloud 1 possesses one IS*1106*A element inserted into a second, defective IS*1106*A element whereas genocloud 3 possesses only the defective IS*1106*A element (see supplemental data). Possibly, genocloud 1 is older and was the direct ancestor of genocloud 3 because an IS*1106*A element inserted into a second, defective IS*1106*A element was also found in subgroup IV-1 (16). Genoclouds 2 and 4 are descended from genocloud 1 because they possess the same double IS*1106*A elements, and genoclouds 5–7

and 9 are derived from genocloud 3 because they possess the same single IS*1106*A element.

The first subgroup III pandemic (mid-1960s to late 1970s) was associated with genoclouds 1 to 3 (Table 1; Fig. 1). The second subgroup III pandemic (early 1980s to present) was associated with genoclouds 3 to 7 and 9. A third pandemic caused by genocloud 8 seems to have started in China in 1993. The progressive evolution of genocloud 8 from genocloud 3 is documented by the isolation in China between 1984 and 1992 of intermediate genotypes with progressive genetic changes. Genocloud 8 has now been isolated from epidemics in Mongolia (1994–1995; ref. 18), Moscow (1994–1997; ref. 12), and Africa (1995–1999; ref. 9), and from endemic disease in the United Kingdom (1997–2000).

Mechanisms of Genetic Change. Alleles that differed by at least 2 bp from parental alleles were considered to represent import by DNA transformation. This interpretation applies to all novel alleles at *tbpB* (28 alleles) and to one allele each at *iga* and *pgm* (Table 2). Similarly, all eight sequenced IS*1106*A alleles also resulted from import (supplemental data). Eighteen alleles at the *opaB* and *opaD* loci are mosaic genes resulting from gene conversion/translocation with segments of orthologous *opa* genes (most frequently *opaA* and *opaJ*) that are present elsewhere in the genome (15). Four alleles differed from their parental alleles by only one nucleotide or by a 12-bp deletion and possibly represent single-step mutations (Table 2).

Fitness of Individual Genetic Changes. It is striking that so many novel alleles were observed for the loci investigated here although only 2 novel alleles were detected for six other housekeeping gene fragments among 137 subgroup III isolates (9, 12). Possibly sequence polymorphism was particularly high for the loci tested here because some of them encode gene products that are under selection. Indeed, the *tbpB, opaB,* and *opaD* loci encode immunogenic outer membrane proteins, and variants at these loci might enable the bacteria to escape herd immunity.

A conservative test for differences in fitness is to compare the number of descendent genotypes associated with different classes of genetic change. Changes at the *tbpB* locus reflect the import of novel *tbpB* alleles belonging to one of four very distinct allele families (13). The 28 alleles described here fell into three of the same four families (Fig. 2). The ancestral allele was in family 4, and most of the imported alleles were in family 1. Of the 19 occasions where a *tbpB* allele in family 1 was imported, only one of these alleles was inherited by descendent genotypes, a significantly lower rate of dissemination than for all other observed changes (Table 3). These results suggest that the fitness of subgroup III is impaired by the acquisition of *tbpB* alleles of family 1. Acquisition of *tbpB* of family 3 or translocation of alleles from either the *opaJ* or *opaA* loci to the *opaB* or *opaD* loci may also have resulted in reduced fitness (Table 3), but the numbers of such events were too small to be statistically significant. In contrast, three of the six novel *tbpB* alleles in family 4, the ancestral family in subgroup III, disseminated to descendent genotypes.

Discussion

We analyzed microevolution within an epidemiological framework for a globally representative collection of subgroup III meningococci. Fifty-seven distinct genotypes in nine genoclouds were detected among 502 strains collected during pandemic spread between 1966 and 2000.

The Genocloud Concept. We designate a frequent genotype plus its epidemiologically associated descendents as a genocloud. The nine subgroup III genoclouds contained rare genotypes that differed by one or occasionally two of the six polymorphic loci.

Table 2. Sources of alleles in 502 subgroup III isolates

		No. of variant alleles		
Locus	Ancestral allele	Mutation	Import	Translocation
iga	*iga2*	1 (*iga1*)	1 (*iga3*)	
IS*1106*A	Uncertain		8 (IS*1106*A4-11)*	
pgm	*pgm3*	1 (*pgm11*)	1 (*pgm19*)	
opaB	*opaB92*	1 (*opaB93*)†	2 (*opaB94,5202*)	12 (5× *opaA*; 4× *opaD*; 3× *opaJ*)†
opaD	*opaD131*	1 (*opaD100*)		8 (7× *opaJ*; 1× *opaB*)†
tbpB	*tbpB1*		28 (19× family 1; 6× family 4; 3× family 3)	

*The mechanism by which one additional IS*1106*A allele (IS*1106*A99) arose is unclear because that allele is associated with a 12-kb region that has not been sequenced (see supplemental data).

†The mutation in *opaB93* is a 12-bp deletion. Translocations into *opaB*: from *opaA*: *opaB102, 113, 115, 139, 141*; from *opaD*: *opaB100, 114, 131, 1131*; from *opaJ*: *opaB116, 140, 142*. Translocations into *opaD*: from *opaJ*: *opaD101, 108, 109, 110, 111, 112, 1101*; from *opaB*: *opaD94*.

The frequent genotypes in subgroup III differed from each other by one to six loci (Table 5). All these bacteria are extremely closely related because housekeeping gene fragments were highly uniform among representative isolates (9, 12). Each genocloud persists only transiently (see below), and subgroup III evolution since 1966 has been marked by the successive replacement of individual dominant genoclouds by others.

The genocloud concept may be generally useful for describing sequence variability among clonally related isolates for many bacterial species. Sequence variation is common in most microorganisms, and clonally related bacteria from a common epidemiological source often show some limited genotypic variation (22). Until now, bacteria have been subdivided into clonally related and unrelated groups, with no standard designation for distinct populations within the clonally related groupings. Furthermore, both genetic variability and different frequencies of particular genotypes are integral aspects of the genocloud concept whereas these aspects are lacking in former terminology. Genoclouds are not "quasi-species," a mathematical concept that describes an equilibrium population of small genomes (23). Unlike quasi-species, the genetic changes in a genocloud are probably not reversible, represent unique events that do not

happen repeatedly, and are continuously eliminated during spread from host to host and from country to country.

Immune Selection During Epidemic Spread. In subgroup III, many of the genetic variants are escape variants that can evade the human immune system (Fig. 3). Epidemics and pandemics may begin when particularly fit strains invade a naive human population. Herd immunity develops and selects escape variants for particularly immunogenic proteins such as TbpB. Most TbpB escape variants result from the import of DNA from *Neisseria lactamica* and *Neisseria spp.* (13) that encode TbpBs of family 1. Family 1 differs extensively from family 4 (Fig. 2), which is ancestral in subgroup III, sufficiently that these two families probably share very few epitopes. We have shown previously (13) that the high rate of import of TbpB alleles is due to positive selection rather than to an elevated recombination rate: the only common feature of numerous independent recombination events with different recombination endpoints was the import of *tbpB*. Nevertheless, import of TbpB of family 1 (and possibly of family 3) reduces the long-term fitness of the resulting escape variants. Only one of nineteen genotypes that had imported family 1 alleles succeeded in generating descendent genotypes (Table 3) whereas half of the imports of a family 4 (ancestral) allele were successful. Thus, many of the rare genotypes may represent escape variants that have a temporary advantage because of different antigenic composition even though they are less fit than their parent (Fig. 3).

The term "fitness," rather than virulence, is appropriate in this context because, during single epidemics, the same serogroup A
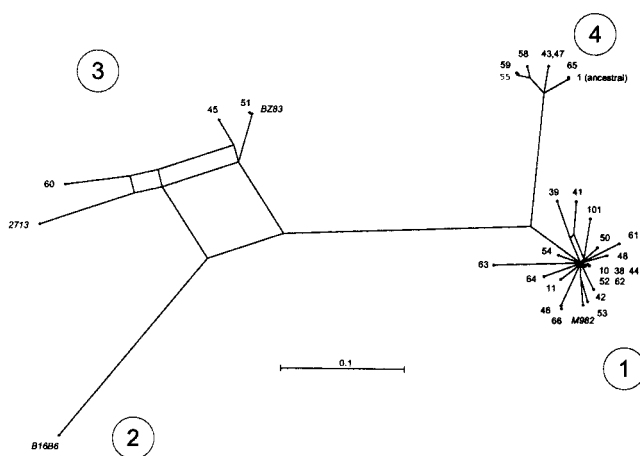

Fig. 2. Splits graph (uncorrected Hamming distances) of relationships of ≈660-bp *tbpB* fragments. Large circled numbers indicate the allele families. The smaller numbers are *tbpB* allele numbers. *tbpB1* is the ancestral allele in subgroups III and IV-1. *tbp10* and *tbp11* were previously found in subgroup IV-1 and commensal neisseriae, and *tbpB101* was found in *N. lactamica* (13). *tbpB38* through *tbpB66* have been found only in subgroup III. Sequences from reference strains of *N. meningitidis* for families 1–3 (B16B6, 2713, BZ83, and M982) are italicized.

Table 3. Fitness analysis of genotypic changes

	No. of genetic events	
No. of descendent genotypes	≥1 (success)	0 (failure)
tbpB family 1	1	18
tbpB family 3	0	3
tbpB family 4	3	3
pgm	1	1
IS*1106*A	5	4
Translocation of *opaJ*	2	8
Translocation of *opaA*	1	4
Other *opa* changes	4	5
iga	1	1

Note: $P = 0.01$ for the null hypothesis of randomness according to a χ^2 test by comparing success of *tbpB* family 1 with all other genotypic changes (CLUMP test 3; 100,000 repeats; ref. 21). The null hypothesis was also rejected when changes at *opa* alleles were categorized into replacement of *opaB* (5 successes; 10 failures) or *opaD* (2 successes; 7 failures).
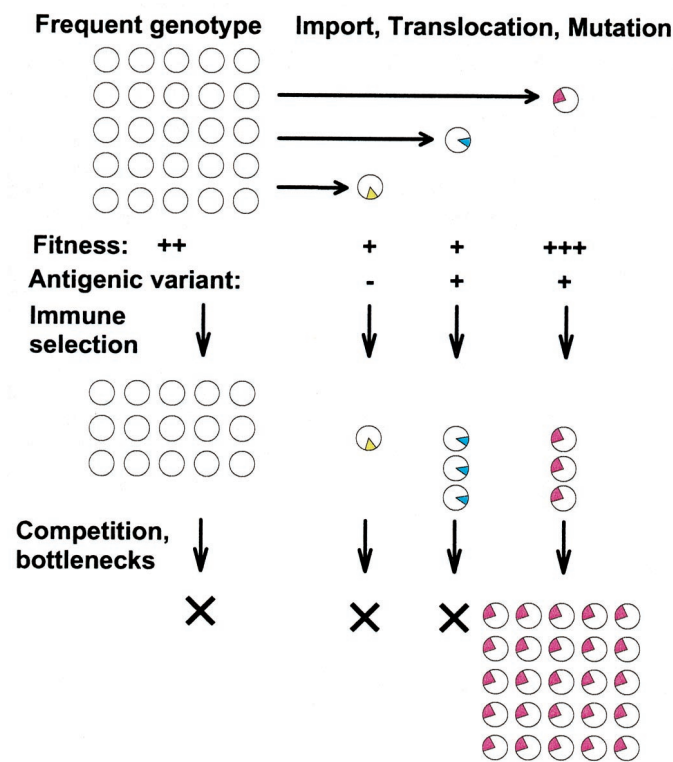
Fig. 3. A model for the formation of rare genotypes and novel genoclouds. Rare genotypes arise by a variety of mechanisms, including import of DNA from unrelated bacteria, translocation of *opa* alleles, and single step mutations. Those variants that affect antigens and result in immune escape will multiply preferentially because of selection pressures by the host immune system. However, many of these variants also result in lessened fitness and are eliminated by competition and bottlenecks during spread from host to host and country to country. On rare occasions, antigenic variants with increased fitness can form new genoclouds and repeat the cycle.

isolates are isolated from both invasive disease and healthy carriers (24). Furthermore, there is no evidence for frequent healthy carriage of serogroup A meningococci without occasional disease. Thus, for serogroup A, the existence of descendent genotypes among disease isolates reflects efficient transmission between individuals and can therefore be used to calculate measures of fitness.

The data are also suggestive of a similar but less strong pattern of selection at the *opa* loci. The ancestral subgroup III genotype possessed distinct alleles at all four *opa* loci (*opaA, opaB, opaD*, and *opaJ*). The *opa101* allele at the *opaJ* locus is not normally expressed because of a stretch of four CTCTT repeats in the signal peptide region, which results in a nonfunctional translational reading frame. The *opa101* allele was repeatedly translocated to the *opaB* and *opaD* loci whereas translocation from the other *opa* loci to *opaJ* is very rare (15). This asymmetry is suggestive of positive selection for replacement of OpaB or OpaD by the immunologically distinct Opa101 protein. Transposition of the *opa101* allele may also be detrimental to long-term fitness (Table 3), but more observations

are needed to test whether this pattern is statistically significant. It is also likely that genetic variation does not always reflect selection, either positive or negative. For example, IS*1106*A sequence variants are also frequent although it is hard to imagine how different alleles of IS*1106*A could affect fitness.

Transience of Genoclouds. The half-life of nasopharyngeal colonization is only a few months (25), and genoclouds disappear from individual countries and areas after several years, even in the absence of vaccination (26–28). Genocloud 1 disappeared from China in the 1960s, and epidemic disease during the 1970s was caused by subgroup V meningococci (5). Genocloud 2 disappeared from Moscow in the 1970s and was replaced by subgroups VI and X (12). Genoclouds 1–4 have not been isolated since 1985. Thus, meningococcal genoclouds survive only if they compete successfully with their parent and other variant genotypes for transmission to other countries and geographic areas.

Genoclouds persist by migrating from country to country or by generating new variants that escape the immune response against previous infections. Our analysis of the *tbpB* locus suggests that variants that achieve both significant immune escape and high fitness are unusual, possibly because of the rarity of appropriate DNA in the available gene pool. The genocloud structure that we have observed probably reflects how rarely novel genotypes are generated that combine fitness with immune escape at key antigenic sites.

The Third Subgroup III Pandemic. A third subgroup III pandemic associated with genocloud 8 began in China in 1993 after a decade of slow, progressive microevolution from genocloud 3. Since 1994, genocloud 8 has caused large epidemics in Mongolia (18), Moscow (12), and Africa (9). Genocloud 8 has now reached Western Europe and caused several cases of endemic disease in the United Kingdom between 1997 and 2000.

Based on historical experience (4), it might have been expected that the recent subgroup III epidemics in diverse African countries had surpassed their zenith. In the past, epidemic waves have not extended much longer than 10 years, and most African countries in the "meningitis belt" have now experienced major epidemics caused by genocloud 5 (Table 4 in supplemental data). However, these epidemics do not seem to have generated long-lasting herd immunity against genocloud 8. Genocloud 8 has recently caused epidemics in Sudan, Chad, and Niger (Table 4), where epidemics had been caused by subgroup III bacteria with *pgm3* (presumably genocloud 5) only a few years earlier (9). These observations bode poorly for the rapid disappearance of epidemic meningitis from Africa and raise the possibility of renewed epidemic serogroup A disease in Europe and the Americas. Before epidemics in Norway and Finland in the early 1970s, serogroup A disease had long been absent, similarly to the current situation. The availability of vaccines based on the A polysaccharide can help control epidemics, but new generation A-conjugate vaccines are necessary to ensure the prevention of epidemic disease on a global basis.

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