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Carried Meningococci in the Czech Republic: a Diverse Recombining Population

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Population and evolutionary analyses of pathogenic bacteria are frequently hindered by sampling strategies that concentrate on isolates from patients with invasive disease. This is especially so for the gram-negative diplococcus *Neisseria meningitidis*, a cause of septicemia and meningitis worldwide. Meningococcal isolate collections almost exclusively comprise organisms originating from patients with invasive meningococcal disease, although this bacterium is a commensal inhabitant of the human nasopharynx and very rarely causes pathological effects. In the present study, molecular biology-based techniques were used to establish the genetic relationships of 156 meningococci isolated from healthy young adults in the Czech Republic during 1993. None of the individuals sampled had known links to patients with invasive disease. Multilocus sequence typing (MLST) showed that the bacterial population was highly diverse, comprising 71 different sequence types (STs) which were assigned to 34 distinct complexes or lineages. Three previously identified hyperinvasive lineages were present: 26 isolates (17%) belonged to the ST-41 complex (lineage 3); 4 (2.6%) belonged to the ST-11 (electrophoretic type [ET-37]) complex, and 1 (0.6%) belonged to the ST-32 (ET-5) complex. The data were consistent with the view that most nucleotide sequence diversity resulted from the reassortment of alleles by horizontal genetic exchange.

Despite its reputation as a pathogen of global significance (9, 34), the gram-negative bacterium *Neisseria meningitidis* is routinely present in the nasopharynges of approximately 10% of healthy individuals in Europe and the United States (6, 8, 27). The severity of meningococcal disease, together with its propensity to affect infants and young adults, has resulted in a concentration of research efforts on those meningococci isolated from patients with meningococcal septicemia or meningitis. Consequently, comparatively little work has been directed at carried meningococci isolated from healthy subjects. As carriage of meningococci is common and meningococcal disease is rare, carriage strains are very much underrepresented in isolate collections, perhaps by several hundred- or even thousand-fold (30). This is a serious obstacle to a full understanding of the biology of this organism.

Current models envisage that populations of the meningococcus are highly diverse (15), comprising many different genotypes which are rarely isolated from patients with invasive disease (14). This is consistent with the fact that many patients with meningococcal disease have no direct contact with other patients, indicating that carriage in asymptomatic individuals represents the major route for the transmission of meningococci. It is thought that lineages of meningococci with an elevated capacity to cause invasive disease arise periodically from this population and spread, sometimes globally (2). Relatively few of these hyperinvasive lineages, defined on the basis of their frequency of isolation from patients with disease relative to a low isolation rate from healthy carriers (29), are responsible for most cases of invasive disease worldwide (10). Meningococcal lineages diversify during spread (11, 12), and much of this diversification is generated by horizontal genetic exchange in this transformable organism (7, 16, 23).

Of the many carriage studies that have been performed over the last 90 years, few have been directed solely to the study of meningococci isolated from the general population. Isolates have usually been obtained from individuals with meningococcal disease, contacts of individuals with invasive disease, healthy carriers during disease outbreaks, or members of closed communities, particularly military recruit camps, which are prone to elevated levels of both carriage and disease (3, 4, 21, 22, 35). The results of those carriage studies that have included the population at large and that have used appropriate isolate characterization techniques are consistent with the view that meningococci isolated from carriage are highly diverse, with hyperinvasive lineages representing a minority of the population of meningococci (13, 14).

The present study applied nucleotide sequence-based characterization techniques (29) to a collection of 156 carried meningococci isolated in the Czech Republic in a 4-month period (March to June) of 1993 from young adults with no association with patients with meningococcal disease. Serological analyses of carriage isolates from the Czech Republic have indicated that carriage is dynamic, with carriage episodes lasting from a few days to several weeks, and that the serological composition of carriage isolates differs from that of isolates from patients with invasive disease (27); however, these isolates had not been genetically characterized. The data presented here demonstrated that the meningococcal population was highly diverse and that hypervirulent meningococci were a minority of the population. The diversity observed was consistent with the view that high levels of recombination among meningococci continually generate new genetic types.

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TABLE 1. Genetic variation in MLST loci

Locus	Size (bp)	156 Czech carriage isolates			107 isolates isolated worldwide			No. (%) of alleles shared	No. (%) of polymorphic sites shared
		No. of alleles (no./100 isolates)	No. (%) of polymorphic sites	d_N/d_S	No. of alleles (no./100 isolates)	No. (%) of polymorphic sites	d_N/d_S		
abcZ	432	20 (12.8)	74 (17.1)	0.071	15 (14)	75 (17.4)	0.05	10 (50)	64 (86.5)
adk	465	15 (9.6)	18 (3.9)	0.008	10 (9.4)	17 (3.7)	0.02	8 (53.3)	15 (83.3)
aroE	489	17 (10.9)	133 (27.2)	0.293	18 (16.8)	166 (34)	0.293	10 (58.8)	126 (94.7)
fumC	465	25 (16)	42 (9.0)	0.002	19 (17.8)	38 (8.2)	0.024	10 (40.0)	32 (76.2)
gdh	501	18 (11.5)	26 (5.2)	0.047	16 (15)	28 (5.6)	0.05	8 (44.4)	24 (92.3)
pdhC	480	23 (14.7)	82 (17)	0.066	24 (22.4)	80 (16.7)	0.07	14 (60.9)	76 (92.7)
pgm	450	24 (15.4)	80 (17.8)	0.112	21 (19.6)	77 (17)	0.121	14 (58.3)	78 (97.5)

MATERIALS AND METHODS

Meningococcal isolates. The study sample comprised 156 meningococci isolated from throat swab specimens obtained during the period from March to June 1993 from 1,400 individuals aged 15 to 24 years, a carriage rate of 11.1%. There were nine main sampling sites, which included school and workplace settings at five locations in the Czech Republic (Prague, Ceske Budejovice, Plzen, Olomouc, and Opava). Four isolates were from individuals not related to any of these sites. All of the individuals sampled were healthy, with no known contact with patients with invasive meningococcal disease.

Collection of throat swab specimens and microbiology. Nasopharyngeal and laryngeal swab specimens were collected in the morning, before individuals had breakfasted, or 2 h after a previous meal. The swabs were immediately inoculated onto Thayer-Martin selective medium, and the inoculated petri dishes were immediately transported into the laboratory in thermally protected boxes, where they were incubated at 37°C in an atmosphere containing 5% CO_2. The petri dishes were examined after 18 to 24 and 48 h of incubation. Presumptive meningococcal colonies were subcultured onto heated blood Mueller-Hinton agar, and species identification was done by Gram staining, by the oxidase reaction, and with the following commercial panels of biochemical tests: the Neisseria 4H system (Sanofi Diagnostics Pasteur, Paris, France) or the API NH system (bio Mérieux, Marcy l'Etoile, France). Serogroups were determined by slide agglutination with commercial antisera (Sanofi Diagnostics Pasteur; Murex, Dartford, United Kingdom; ITEST, Hradec Králové, Czech Republic) or monoclonal antibodies (National Institute of Biological Standards and Control, Potters Bar, United Kingdom). Serotypes and subtypes were determined by standard whole-cell enzyme-linked immunosorbent assay (1) with monoclonal antibodies (National Institute for Biological Standards and Control).

Preparation of chromosomal DNA. Meningococcal isolates were revived from storage in brain heart infusion broth with 10% glycerol by plating on heated-blood Mueller-Hinton agar. For each isolate, the growth obtained from the surface of a single petri dish after overnight incubation in an atmosphere of 5% CO_2 was used to make an opaque cell suspension in 1 ml of deionized water. Meningococcal DNA was extracted from 100 μl of these cell suspensions with the Isoquick Nucleic Acid Extraction kit (Orca Research Inc.) by following the manufacturer's instructions.

Nucleotide sequence determination. All nucleotide sequences were determined directly from the PCR products. Briefly, amplification primers were used to generate a sequence template by the PCR, the resultant templates were purified by precipitation with polyethylene glycol and sodium chloride, termination products were generated by cycle sequencing with appropriate primers and BigDye terminators (Applied Biosystems), and the products were separated with an ABI Prism 377 XL automated DNA sequencer. The sequence of each strand was determined at least once, and the DNA sequences were assembled with the STADEN suite of computer programs (37).

MLST. The primers used for amplification of the loci used for multilocus sequence typing (MLST) (7, 19, 29) were abcZ-P1 (5′-AAT CGT TTA TGT ACC GCA GG-3′) and abcZ-P2 (5′-GTT GAT TTC TGC CTG TTC GG-3′), adk-P1 (5′-ATG GCA GTT TGT GCA GTT GG-3′) and adk-P2 (5′-GAT TTA AAC AGC GAT TGC CC-3′), aroE-P1 (5′-ACG CAT TTG CGC CGA CAT C-3′) and aroE-P2 (5′-ATC AGG GCT TTT TTC AGG TT-3′), fumC-A1 (5′-CAC CGA ACA CGA CAC GAT GG-3′) and fumC-A2 (5′-ACG ACC AGT TCG TCA AAC TC-3′), gdh-P1 (5′-ATC AAT ACC GAT GTG GCG CGT-3′) and gdh-P2 (5′-GGT TTT CAT CTG CGT ATA GAG-3′), pdhC-P1 (5′-GGT TTC CAA CGT ATC GGC GAC-3′) and pdhC-P2 (5′-ATC GGC TTT GAT GCC GTA TTT-3′), and pgm-P1 (5′-CTT CAA AGC CTA CGA CAT CCG-3′) and pgm-P2 (5′-CGG ATT GCT TTC GAT GAC GGC-3′). For sequencing of these amplification products the following primers were used: abcZ-S1 (5′-AAT CGT TTA TGT ACC GCA GG-3′) and abcZ-S2 (5′-GAG AAC GAG CCG GGA TAG GA-3′), adk-S1 (5′-AGG CTG GCA CGC CCT TGG-3′) and adk-S2 (5′-CAA TAC TTC GGC TTT CAC GG-3′), aroE-S1 (5′-GCG GTC AAY ACG CTG ATT-3′) and aroE-S2 (5′-ATG ATG TTG CCG TAC ACA TA-3′), fumC-S1 (5′-TCG GCA CGG GTT TGA ACA GC-3′) and fumC-S2 (5′-CAA CGG CGG TTT CGC GCA AC-3′), gdh-S3 (5′-CCT TGG CAA AGA AAG CCT GC-3′) and gdh-S4 (5′-GCG CAC GGA TTC ATA TGG-3′), pdhC-S1 (5′-TCT ACT ACA TCA CCC TGA TG-3′) and pdhC-S2 (5′-ATC GGC TTT GAT GCC GTA TTT-3′), and pgm-S1 (5′-CGG CGA TGC CGA CCG CTT GG-3′) and pgm-S2 (5′-GGT GAT GAT TTC GGT TGC GCC-3′). Housekeeping alleles and sequence types were assigned by interrogating the MLST database (http://mlst.zoo.ox.ac.uk).

Characterization of the *siaD* gene. The *siaD* gene, part of the capsular operon responsible for synthesis of the polysaccharides conferring serogroup B and C polysaccharides on meningococcal isolates, were amplified and sequenced with primers siaD-P1 (5′-AYA TWT TGC ATG TMS CYT TYC CTG-3′) and siaD-P2 (5′-AGA CAT TGG GTW GWR GGK GAR AGT AA-3′) (5).

Data analysis. The relationships among the sequence types (STs) were determined by constructing a distance matrix of allelic mismatches. Each locus difference was treated identically in that no relationships were assumed among the different alleles. The different lineages in the sample were then resolved from the clusters obtained when this distance matrix was visualized by Split decomposition analysis with the program SPLITSTREE, version 3.1 (20). The STs were also assigned to lineages with the program BURST (written by E. J. Feil and Man-S. Chan), which resolved lineages, defined as groups of strains in which each member shares at least four alleles with at least one other member of the lineage. Lineages were named after the central ST, as defined by the BURST program, followed by the word complex; for example, the ST-92 complex. If the lineage had previously been identified then the previously associated ST was used for the name. For example, ST-118 was a member of the ST-32 (electrophoretic type 5 [ET-5]) complex, although no examples of ST-32 were present in this sample, and ST-44 was a member of the ST-41 complex (lineage 3). Once the different lineages were identified, the relationships within the lineages were represented by using annotated splits graphs (7, 19). An estimate of the relative contributions of recombination and mutation to allelic change was made by the method recently described by Feil et al. (17, 18). Briefly, this method assigns the variant alleles between STs which are identical at six loci but which differ at the seventh locus as having arisen by recombination or mutation on the basis of the number of nucleotide sites at which the two alleles differ. The index of association (I_A) (33) was calculated by using a program written by J. Maynard Smith. Other data analyses were performed by using programs written by K. A. Jolley and the MEGA suite of programs (26). All of the programs are available for electronic download (http://mlst.zoo.ox.ac.uk, http://bibiserv.techfak.uni-bielefeld.de/splits, http://evolgen.biol.metro-u.ac.jp/MEGA/).

RESULTS

Diversity of housekeeping genes and STs. The total number of alleles present at each locus for the set of 156 isolates, which ranged between 15 for *adk* and 25 for *fumC*, are shown in Table 1, along with the number of polymorphic sites present at each locus, which was between 18 (4% of sites for *adk*) and 133 (27% of sites for *aroE*). In Table 1 these data are compared with those obtained from 107 isolates, mainly from patients with disease, isolated worldwide from 1937 to 1996 (29). The data were comparable, with some differences in the proportion of nonsynonymous to synonymous nucleotide substitutions (d_N/d_S) due to the small number of nonsynonymous sites present at some loci. Figures 1a to g show the number of alleles as a function of the number of isolates examined. The frequency of alleles in the data set ranged from 1 to 43 occurrences (with *aroE*, allele 4 the most prevalent). The allelic sequences for each locus gave results very similar to those obtained previously for the 107 isolates isolated worldwide (19) when examined by split decomposition (data not shown).

STs and lineages. There were 71 STs, and the number of STs against the number of isolates examined is given in Fig. 1h. The

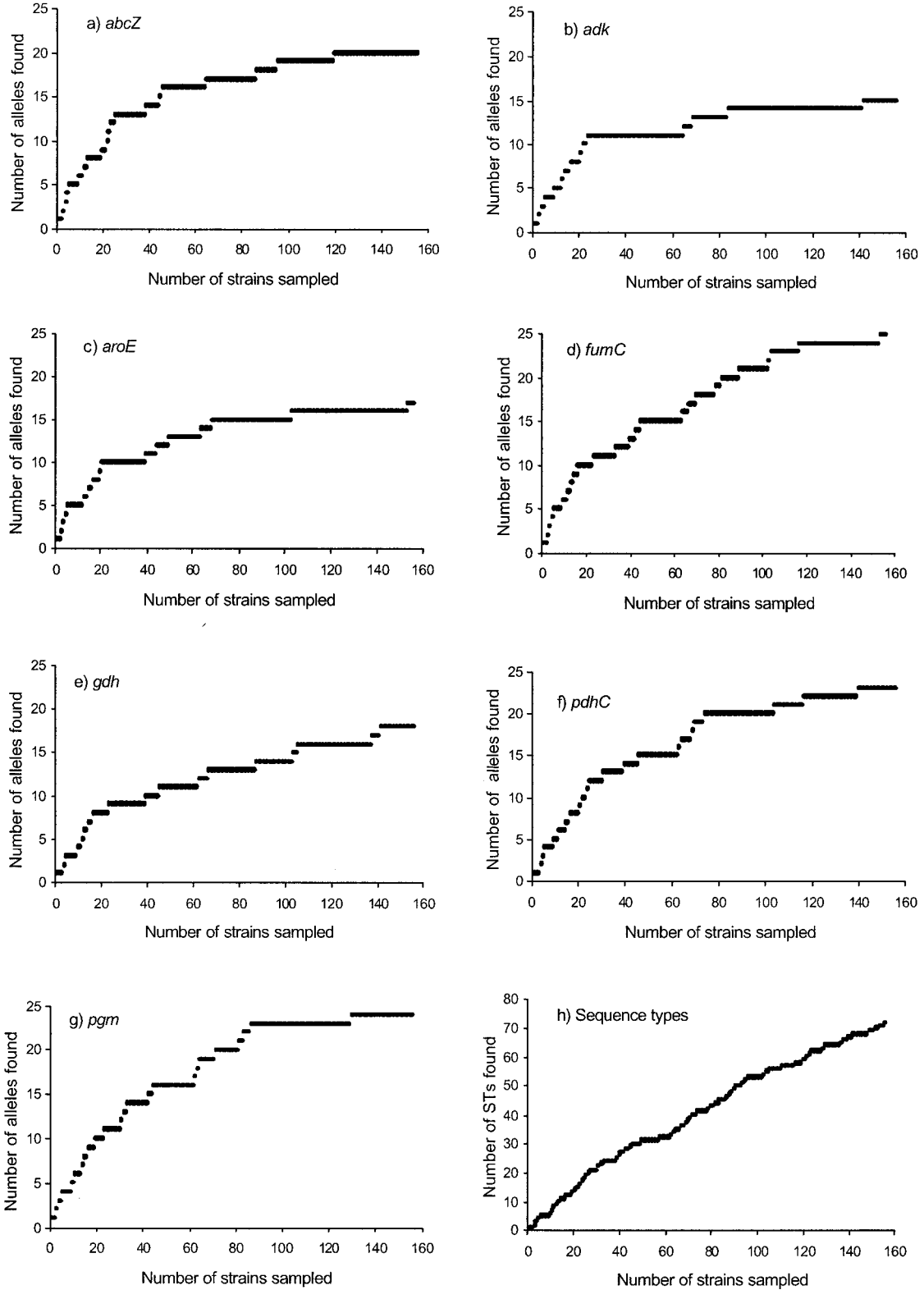

FIG. 1. Number of alleles present at each locus (a to g) and number of STs (h) plotted against the number of isolates sampled, given in numerical order of isolation.

two approaches used to assign the STs to lineages gave the same assignments. The 71 STs were resolved into 34 distinct lineages which occurred between 1 (0.64%) and 26 (16.5%) times in the collection of 156 isolates (Table 2). Fourteen lineages were represented by a single ST, 4 lineages were represented twice (1.3%), 2 lineages were represented three times (1.9%), and 14 lineages were represented four or more times. The two most common lineages were also the most diverse in

TABLE 2. Meningococcal lineages present in the sampled population

Lineage name	Other name(s)	Members recovered in sample		Total no. (%) of sample of members
		ST	Frequency (no. of isolates)	
ST-41 complex	Lineage 3	44	7	
		108	1	
		109	1	
		110	6	
		111	2	
		112	1	
		136	6	
		137	1	
		142	1	26 (16.67)
ST-92 complex		84	1	
		91	1	
		92	12	
		93	1	
		94	1	
		95	1	
		129	1	18 (11.54)
ST-106 complex		106	14	
		119	1	
		216	1	16 (10.26)
ST-116 complex		116	11	
		133	1	12 (7.69)
ST-53 complex		53	8	
		122	1	
		123	1	
		124	1	11 (7.05)
ST-104 complex		103	2	
		104	4	
		105	1	7 (4.49)
ST-125 complex		125	6	6 (3.85)
ST-101 complex		100	2	
		101	4	6 (3.85)
ST-87 complex		87	1	
		88	1	
		89	2	
		90	1	5 (3.21)
ST-85 complex		85	5	5 (3.21)
ST-18 complex		18	1	
		102	1	
		117	1	
		145	1	4 (2.56)
ST-11 complex	ET-37 complex, ET-15	11	4	4 (2.56)
ST-132 complex		131	1	
		132	3	4 (2.56)
ST-36 complex		36	1	
		83	1	
		115	1	
		139	1	4 (2.56)
ST-127 complex		127	2	
		140	1	3 (1.92)
ST-114		113	1	
		114	2	3 (1.92)
ST-97 complex		97	1	
		98	1	2 (1.28)
ST-130		130	2	2 (1.28)
ST-121		121	2	2 (1.28)
ST-135 complex		135	1	
		143	1	2 (1.28)
ST-32 complex		118	1	1 (0.64)
ST-99		99	1	1 (0.64)
ST-86		86	1	1 (0.64)
ST-96		96	1	1 (0.64)
ST-120		120	1	1 (0.64)
ST-128		128	1	1 (0.64)
ST-107		107	1	1 (0.64)
ST-134		134	1	1 (0.64)
ST-82		82	1	1 (0.64)
ST-138		138	1	1 (0.64)
ST-141		141	1	1 (0.64)
ST-144		144	1	1 (0.64)
ST-126		126	1	1 (0.64)
ST-81		81	1	1 (0.64)

terms of numbers of the STs present (Table 2). Reference to the MLST website showed that 27 of the 34 lineages and 65 STs were first identified in this data set. Isolates related to three previously described hyperinvasive meningococcal lineages were present: 26 isolates (17%) (ST-41 complex) were related to lineage 3, 4 isolates (2.6%) (ST-11) were related to the ET-37 complex, and 1 isolate (0.6%) (ST-118) was a novel variant of the ET-5 (ST-32) complex (Table 1).

Within-lineage variability. The relationships of the 26 members of the ST-41 complex (lineage 3) present in the sample are illustrated by the splits graph in Fig. 2a. This analysis placed the most common ST (ST-44; seven isolates) at the center of the graph, indicating that this ST is a possible ancestor of at least some of the remaining STs present in this sample: it is a double-locus variant of ST-41 at *abcZ* and *fumC* (19, 29). Four STs (ST-110, six isolates; ST-137, one isolate; and ST-142, one isolate) were single-locus variants of ST-44, and there were two distinct two-locus variants (ST-109, one isolate; ST-111, two isolates). Three STs (ST-108, ST-112, and ST-136) differed from ST-44 at three loci. The networking at the center of Fig. 2a illustrates that ST-110 and ST-111 are double-locus variants of STs 108 and 109 and that there was a parsimonious evolutionary path among these STs that did not involve ST-44.

The next most common lineage, the ST-92 complex, was previously unreported and had fewer members with less complicated relationships than those for the ST-41 complex (Fig. 2b). In this case ST-92 (12 isolates) occupied the central position with two single-locus variants (ST-91, one isolate; ST-129, 1 isolate), two two-locus variants (ST-93, one isolate; ST-94, one isolate), and two three-locus variants (ST-84, a double-

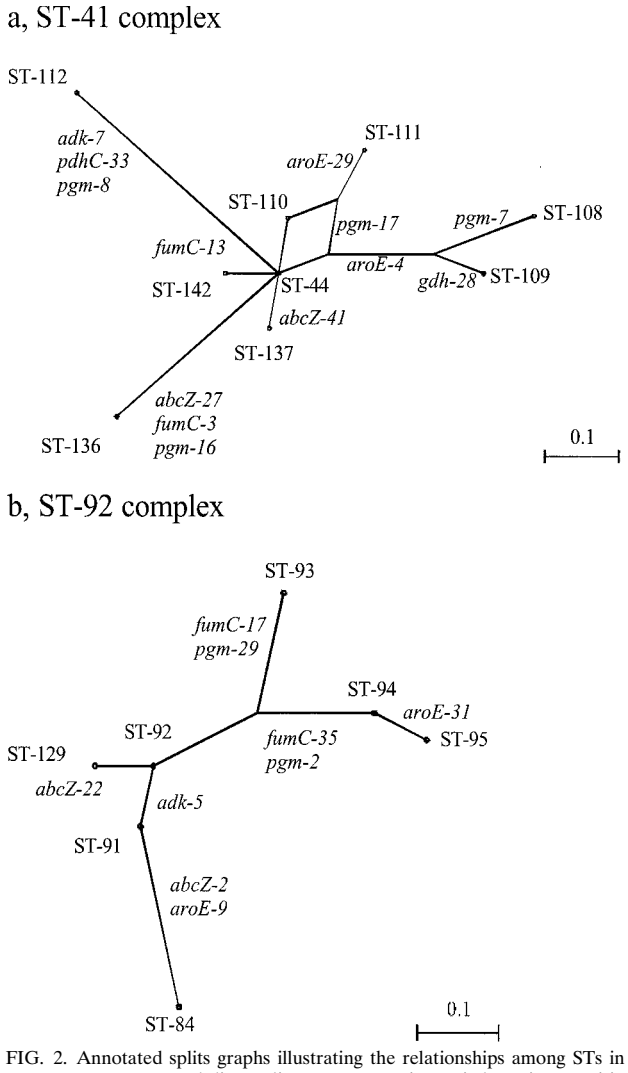

FIG. 2. Annotated splits graphs illustrating the relationships among STs in the two most common and diverse lineages present in carried meningococci in the Czech Republic during 1993. (a) ST-41 complex; (b) ST-92 complex. Each of the vertices has been annotated with the allelic differences that define STs relative to the central STs, ST-44 and ST-92.

locus variant of ST-91, one isolate; ST-95, a single-locus variant of ST-94, one isolate).

The per-site recombination:mutation ratio was estimated from the data set by the method of Feil et al. (17, 18) to be 275:1 on the basis of the fact that 16 of the allelic changes observed within lineages, which resulted in 275 nucleotide changes, were likely to be a consequence of recombinational replacement and that only 1 within-lineage change was likely to be due to mutation. However, the number of allelic comparisons in both cases is small, and this number cannot be considered precise. The I_A calculated for the whole set of 156 STs was 2.47, which decreased to 0.132 when one representative of each lineage was included. There was no evidence of a geographical localization of lineages.

Serogroup diversity. Serologically, 48 of the 156 isolates were serogroup B, 12 were serogroup C, 9 were serogroup 29E, 6 were serogroup X, 5 were serogroup Y, and 2 were serogroup Z, with 74 (47%) being nongroupable. Sequencing of the *siaD* genes of the 74 nongroupable isolates with primers specific for serogroups B and C showed that 21 (28%) had the serogroup B gene, while 6 (8%) had the serogroup C gene. The *siaD* genes of the remaining 47 (64%) isolates could not be sequenced with these primers. The nucleotide sequence and serogrouping data were consistent. In combination, the serogrouping and *siaD* sequence data confirmed that while some lineages, notably, lineage 3 (ST-41 complex), were uniform for capsular group, several exhibited several serogroups, for example, the ST-92 complex, which contained isolates belonging to serogroups B, C, Y, and Z (Table 3).

DISCUSSION

The majority of population studies of *N. meningitidis* have been performed with collections of disease-associated meningococci. The characterization of carried isolates by MLST permitted direct comparison of the data with those stored on the MLST website, which included data for the collection of 107 mainly disease-associated invasive meningococci used to develop MLST (29). While the diversity of the alleles present at each locus was similar for both invasive and carried meningococci, they did not represent the same population, as there were multiple alleles unique to each data set (Table 1). This may represent genuine differences among the meningococci isolated from patients with invasive disease and carriers but is perhaps more likely to be the consequence of the different sampling frames of these collections: the 107 disease-associated isolates were collected globally between 1937 and 1996 (29). Studies that include disease and carriage isolates from equivalent temporal and geographical sampling frames are required for detailed genetic comparisons of disease and carried meningococci.

The data were consistent with models of meningococcal population structure which envisage recombination as the predominant mechanism for genetic variation (33) and no deep tree-like phylogeny (19). While between 40 and 60% of the alleles were shared between the isolates from carriage and the 107 disease-associated isolates, a higher proportion of polymorphisms were shared (76 to 95%) (Table 1), supporting the ideas that the polymorphisms were much older than the alleles and that new alleles were being generated by recombinational reassortment of polymorphisms. The reduction of the I_A value from 2.47 for all samples to 0.132 when only one example of each lineage was included was further evidence for a weakly clonal population structure. The number of alleles present at

TABLE 3. Serogroup diversity of lineages

Lineage	No. of isolates of serogroup:								No. of isolates with *siaD* allele:		
	B	C	29E	X	Y	W-135	Z	NG[a]	B	C	NR[b]
ST-41 complex	20	0	0	0	0	0	0	6	26	0	0
ST-92 complex	1	1	0	0	5	0	1	10	4	1	13
ST-106 complex	2	0	6	0	0	0	0	8	3	0	13
ST-116 complex	2	0	0	0	0	0	1	9	4	0	8
ST-53 complex	0	0	0	0	0	0	0	11	4	0	7
ST-104 complex	0	0	1	0	0	0	0	6	0	0	7
ST-125 complex	0	0	0	0	0	0	0	6	1	0	5
ST-101 complex	0	5	0	0	0	0	0	1	0	6	0
ST-87 complex	0	0	0	2	0	0	0	3	1	0	4
ST-85 complex	5	0	0	0	0	0	0	0	5	0	0
ST-18 complex	2	0	0	1	0	0	0	1	3	0	1
ST-11 complex	0	4	0	0	0	0	0	0	0	4	0
ST-132 complex	4	0	0	0	0	0	0	0	4	0	0
ST-21 complex	2	0	0	0	0	0	0	2	2	2	0

[a] NG, nongroupable.
[b] NR, no result.

each locus as a function of the number of isolates examined followed an approximately logarithmic relationship (Fig. 1a to g), while the number of STs increased linearly, providing further evidence for generation of STs by recombination and indicating an average recombinational replacement size larger than the size of MLST alleles. Furthermore, this observation suggested that the sample of 156 carriage isolates, while sufficiently large to identify most of the housekeeping alleles circulating in the meningococcal population examined, was not large enough to identify all of the STs present. Nearly half (15 of 34) of the lineages observed were isolated only once, with 10 lineages represented five or more times. It is therefore likely that the generation of new meningococcal STs by recombination is sufficiently rapid that it will be difficult or impossible to sample exhaustively the genotypes present in a given meningococcal population. Further evidence for the role of recombination in the diversification of meningococcal lineages came from the allele sequences. First, identical alleles were distributed among otherwise unrelated lineages. Second, examination of allele sequences by split decomposition analysis indicated a phylogenetic signal consistent with recombination (19). Third, the majority of single genetic changes within identified lineages were likely to be the result of the importation of alleles by recombination rather than by the accumulation of mutations, which was consistent with the high probability of recombinational changes reported elsewhere for this bacterium (17, 18).

During 1993 an increased incidence of meningococcal disease in the Czech Republic was caused by the ET-15 variant of the ET-37 (ST-11) complex (25), which is distinguished by multilocus enzyme electrophoresis but not by MLST studies. In that year, ET-15 meningococci caused 10 of 44 (22.7%) cases of invasive disease in Czech 15- to 19-year-olds. Three of the 26 meningococci recovered from the 200 members of this age group sampled were ST-11, a carriage rate of 1.5% for the human population or 12% for the meningococcal population. Therefore, ET-37 (ST-11) complex meningococci were approximately twofold overrepresented among disease-causing meningococci. Only 1 of 130 carriage meningococci recovered from 1,200 individuals aged 20 to 24 years was ST-11, and, together with the single case of invasive disease caused by an ET-15 meningococcus in this age group, this gave an overrepresentation of 16-fold. These data were consistent with the hyperinvasive status of ET-37 complex meningococci, but a potential problem of this definition of hyperinvasive is that it assumes a similar average duration of carriage for all meningococci. If the duration of carriage for distinct meningococcal lineages is uneven, with members of the ET-37 (ST-11) complex being carried for shorter periods of time, then the number of acquisitions per year would be higher for ET-37 (ST-11) meningococci than for other lineages and their invasive potential per acquisition might be similar to or lower than that for other meningococci. Comparative information on the duration of carriage for different meningococcal lineages is necessary to investigate this possibility. Members of the ET-37 (ST-11) complex can be regarded as hypervirulent, in that they are associated with especially severe disease and high rates of mortality (24, 38).

Lineage 3 (or ST-41 complex), a hyperinvasive lineage, was the most common single lineage in the collection (26 of 156 isolates, or 17% of all isolates, belonged to lineage 3). The comparable number of cases of disease caused by lineage 3 was unknown, but it is possible that, in common with other European countries (36), the Czech Republic experienced a lineage 3-associated hyperendemic outbreak during the 1990s. The serological characteristics of these meningococci were diverse (http://mlst.zoo.ox.ac.uk), and routine isolate characterization would not have detected such an outbreak. Alternatively, as the carried lineage 3 STs were underrepresented among disease-associated isolates (http://mlst.zoo.ox.ac.uk), it is possible that these variants were of low invasive potential. One member of the ET-5 (ST-32) complex, a previously unreported variant, ST-118, was present.

These data confirm that carried meningococci represent a highly diverse recombining population, carriage of hyperinvasive meningococci is rare, and a given lineage may exhibit several serogroups. As *N. meningitidis* does not cause disease as part of its transmission cycle (28), carriage studies are essential to understand meningococcoal spread and develop public health policy. Meningococcal diversity presents problems for vaccine design by enabling hyperinvasive meningococci to change their antigens rapidly, perhaps in response to vaccine pressure (32). In this context, carried meningococci provide a diverse, continually reassorted gene pool (31) from which new genotypes and antigenic types arise. Occasionally, new hyperinvasive lineages emerge which are detected by epidemiological monitoring; however, these data show that most novel meningococcal variants remain unidentified in the absence of large-scale carriage studies.

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REFERENCES

1. **Abdillahi, H., and J. T. Poolman.** 1988. Definition of meningococcal class 1 OMP subtyping antigens by monoclonal antibodies. FEMS Microbiol. Immunol. **1:**139–144.
2. **Achtman, M.** 1995. Global epidemiology of meningococcal disease, p. 159–175. *In* K. A. V. Cartwright (ed.), Meningococcal disease. John Wiley & Sons Ltd., Chichester, United Kingdom.
3. **Andersen, J., L. Berthelsen, B. Bech Jensen, and I. Lind.** 1998. Dynamics of the meningococcal carrier state and characteristics of the carrier strains: a longitudinal study within three cohorts of military recruits. Epidemiol. Infect. **121:**85–94.
4. **Block, C., M. Gdalevich, R. Buber, I. Ashkenazi, S. Ashkenazi, and N. Keller.** 1999. Factors associated with pharyngeal carriage of *Neisseria meningitidis* among Israel Defense Force personnel at the end of their compulsory service. Epidemiol. Infect. **122:**51–57.
5. **Borrow, R., H. Claus, M. Guiver, L. Smart, D. M. Jones, E. B. Kaczmarski, M. Frosch, and A. J. Fox.** 1997. Non-culture diagnosis and serogroup determination of meningococcal B and C infection by a sialyltransferase (*siaD*) PCR ELISA. Epidemiol. Infect. **118:**111–117.
6. **Broome, C. V.** 1986. The carrier state: *Neisseria meningitidis*. J. Antimicrob. Chemother. **18**(Suppl. A)**:**25–34.
7. **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.
8. **Cartwright, K. A. V.** 1995. Meningococcal carriage and disease, p. 115–146. *In* K. A. V. Cartwright (ed.), Meningococcal disease. John Wiley & Sons Ltd., Chichester, United Kingdom.
9. **Cartwright, K. A. V. (ed.).** 1995. Meningococcal disease. John Wiley & Sons Ltd., Chichester, United Kingdom.
10. **Caugant, D. A.** 1998. Population genetics and molecular epidemiology of *Neisseria meningitidis*. APMIS **106:**505–525.
11. **Caugant, D. A., L. O. Froholm, K. Bovre, E. Holten, C. E. Frasch, L. F. Mocca, W. D. Zollinger, and R. K. Selander.** 1986. Intercontinental spread of a genetically distinctive complex of clones of *Neisseria meningitidis* causing epidemic disease. Proc. Natl. Acad. Sci. USA **83:**4927–4931.
12. **Caugant, D. A., L. O. Froholm, K. Bovre, E. Holten, C. F. Frasch, L. F. Mocca, W. D. Zollinger, and R. K. Selander.** 1987. Intercontinental spread of *Neisseria meningitidis* clones of the ET-5 complex. Antonie Leeuwenhoek J. Microbiol. **53:**389–394.
13. **Caugant, D. A., E. A. Hoiby, E. Rosenqvist, L. O. Froholm, and R. K. Selander.** 1992. Transmission of *Neisseria meningitidis* among asymptomatic military recruits and antibody analysis. Epidemiol. Infect. **109:**241–253.

14. **Caugant, D. A., B. E. Kristiansen, L. O. Froholm, K. Bovre, and R. K. Selander.** 1988. Clonal diversity of *Neisseria meningitidis* from a population of asymptomatic carriers. Infect. Immun. **56:**2060–2068.
15. **Caugant, D. A., L. F. Mocca, C. E. Frasch, L. O. Froholm, 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.
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., M. C. J. Maiden, M. Achtman, and B. G. Spratt.** 1999. The relative contribution of recombination and mutation to the divergence of clones of *Neisseria meningitidis.* Mol. Biol. Evol. **16:**1496–1502.
18. **Feil, E. J., J. Maynard Smith, M. C. Enright, and B. G. Spratt.** 2000. Estimating recombinational parameters in *Streptococcus pneumoniae* from multilocus sequence typing data. Genetics **154:**1439–1450.
19. **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.
20. **Huson, D. H.** 1998. SplitsTree: a program for analysing and visualising evolutionary data. Bioinformatics **14:**68–73.
21. **Jones, G. R., M. Christodoulides, J. L. Brooks, A. R. Miller, K. A. Cartwright, and J. E. Heckels.** 1998. Dynamics of carriage of *Neisseria meningitidis* in a group of military recruits: subtype stability and specificity of the immune response following colonization. J. Infect. Dis. **178:**451–459.
22. **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.
23. **Kriz, P., D. Giorgini, M. Musilek, M. Larribe, and M. K. Taha.** 1999. Microevolution through DNA exchange among strains of *Neisseria meningitidis* isolated during an outbreak in the Czech Republic. Res. Microbiol. **150:** 273–280.
24. **Krizova, P., and M. Musilek.** 1995. Changing epidemiology of meningococcal invasive disease in the Czech Republic caused by new clone *Neisseria meningitidis* C:2a:P1.2(P1.5), ET-15/37. Cent. Eur. J. Public Health **3:**189–194.
25. **Krizova, P., M. Musilek, and J. Kalmusova.** 1997. Development of the epidemiological situation in invasive meningococcal disease in the Czech Republic caused by emerging *Neisseria meningitidis* clone ET-15/37. Cent. Eur. J. Public Health **5:**214–218.
26. **Kumar, S., K. Tamura, and M. Nei.** 1994. MEGA: molecular evolutionary genetics analysis software for microcomputers. Comput. Appl. Biosci. **10:** 189–191.
27. **Kuzemenska, P., and B. Kriz.** 1987. Epidemiology of meningococcal disease in central and eastern Europe, p. 103–137. *In* N. A. Vedros (ed.), Evolution of meningococcal disease, vol. I. CRC Press, Inc., Boca Raton, Fla.
28. **Maiden, M. C. J.** 2000. High-throughput sequencing in the population analysis of bacterial pathogens of humans. Int. J. Med. Microbiol. **290:**183–190.
29. **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.
30. **Maiden, M. C. J., and I. M. Feavers.** 1995. Population genetics and global epidemiology of the human pathogen *Neisseria meningitidis,* p. 269–293. *In* S. Baumberg, J. P. W. Young, E. M. H. Wellington, and J. R. Saunders (ed.), Population genetics of bacteria. Cambridge University Press, Cambridge, United Kingdom.
31. **Maiden, M. C. J., B. Malorny, and M. Achtman.** 1996. A global gene pool in the neisseriae. Mol. Microbiol. **21:**1297–1298.
32. **Maiden, M. C. J., and B. G. Spratt.** 1999. Meningococcal conjugate vaccines: new opportunities and new challenges. Lancet **354:**615–616.
33. **Maynard Smith, J., N. H. Smith, M. O'Rourke, and B. G. Spratt.** 1993. How clonal are bacteria? Proc. Natl. Acad. Sci. USA **90:**4384–4388.
34. **Peltola, H.** 1983. Meningococcal disease: still with us. Rev. Infect. Dis. **5:**71–91.
35. **Riordan, T., K. Cartwright, N. Andrews, J. Stuart, A. Burris, A. Fox, R. Borrow, T. Douglas-Riley, J. Gabb, and A. Miller.** 1998. Acquisition and carriage of meningococci in marine commando recruits. Epidemiol. Infect. **121:**495–505.
36. **Scholten, R. J. P. M., J. T. Poolman, H. A. Valkenburg, H. A. Bijlmer, J. Dankert, and D. A. Caugant.** 1994. Phenotypic and genotypic changes in a new clone complex of *Neisseria meningitidis* causing disease in The Netherlands, 1958–1990. J. Infect. Dis. **169:**673–676.
37. **Staden, R.** 1996. The Staden sequence analysis package. Mol. Biotechnol. **5:** 233–241.
38. **Whalen, C. M., J. C. Hockin, A. Ryan, and F. Ashton.** 1995. The changing epidemiology of invasive meningococcal disease in Canada, 1985 through 1992. Emergence of a virulent clone of *Neisseria meningitidis*. JAMA **273:** 390–394.

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AUTHOR'S CORRECTION

Carried Meningococci in the Czech Republic: a Diverse Recombining Population

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Volume 38, no. 12, p. 4492–4498, 2000. We described the genetic characterization of 156 *Neisseria meningitidis* isolates obtained from healthy young adults in the Czech Republic during 1993. Subsequent work has established that a further 61 isolates collected during that year had been stored separately and had been overlooked. These isolates were not a random sample of those collected, as isolates with a phenotype resembling the strain responsible for a disease outbreak that year were overrepresented. All but one of the additional meningococci were isolated from individuals who were 20 to 24 years old, giving a total of 190 isolates from this age group, rather than the 130 isolates originally reported; the other isolate was from the younger cohort (age range, 15 to 19 years).

The revised multilocus sequence typing (MLST) data are available at the *Neisseria* MLST website (http://neisseria.mlst.net/links.htm). These data show a total of 88 sequence types (STs), which were resolved into 16 clonal complexes (lineages), with the remaining STs not presently assigned to clonal complexes. The six most prevalent clonal complexes were the ST-11 complex (33 isolates [15.2%]), the ST-44 complex (31 isolates [14.3%]), the ST-92 complex (21 isolates [9.7%]), the ST-106 complex (20 isolates [9.2%]), the ST-116 complex, (13 isolates [6%]), and the ST-53 complex (12 isolates [5.5%]). Since the original publication, some minor changes have been made to the assignment and names of the clonal complexes; for example, the ST-41 complex has been renamed the ST-44 complex. The present assignments are available at the PubMLST isolate database website (http://neisseria.mlst.net).

The principal conclusions of the paper, that the population was diverse and that this diversity was principally generated by recombination, are unaltered (Table 1; Fig. 1). However, the revised data show that the prevalence of meningococci belonging to the ST-11 (ET-37) complex was almost six times higher than that calculated on the basis of the data from the 156 original isolates and much greater than any previously measured prevalence of this complex among carriage isolates. This affects estimates of the overrepresentation of ST-11 complex meningococci among isolates from invasive disease. Of the 27 meningococcal isolates carried by members of the younger cohort (age range, 15 to 19 years), 3 (11.1%) belonged to the ST-11 complex. As 10 (22.7%) of the 44 cases of invasive disease in this age group were caused by ST-11 complex organisms, this clonal complex was overrepresented approximately twofold among disease-causing meningococci, as originally reported. However, meningococcus carriage data for individuals in the older cohort (age range, 20 to 24 years) indicated that a total of 30 (15.8%) of the 190 isolates belonged to the ST-11 complex, with 2 out of 9 cases of disease having been caused by ST-11 complex organisms (22.2%). This clonal complex was therefore overrepresented by approximately 1.4-fold (not the originally reported 16-fold) among the disease-associated meningococci isolated from this age group. The ST-11 complex meningococci were found in five of the geographical regions sampled, suggesting that the distribution of these meningococci was widespread. At present, it is unclear why the levels of carriage of the ST-11 complex meningococci (all but two of the isolates expressed serogroup C capsular polysaccharide) were so high in the Czech Republic during 1993, although it could have been a consequence of this clonal complex spreading through the Czech population after a period of absence.

TABLE 1. Genetic Variation in MLST loci[a]

Locus	Size (bp)	218 Czech carriage isolates			107 isolates isolated worldwide			No. (%) of alleles shared	No. (%) of polymorphic sites shared
		No. of alleles (no./100 isolates)	No. (%) of polymorphic sites	d_N/d_S	No. of alleles (no./100 isolates)	No. (%) of polymorphic sites	d_N/d_S		
abcZ	432	21 (9.6)	75 (17.4)	0.074	15 (14)	75 (17.4)	0.05	10 (47.6)	65 (86.7)
adk	465	19 (8.7)	25 (5.4)	0.011	10 (9.4)	17 (3.7)	0.02	8 (42.1)	15 (60.0)
aroE	489	21 (9.6)	135 (27.6)	0.295	18 (16.8)	166 (34)	0.293	11 (52.4)	126 (93.3)
fumC	465	29 (13.3)	48 (10.3)	0.010	19 (17.8)	38 (8.2)	0.024	13 (44.8)	38 (79.2)
gdh	501	19 (8.7)	26 (5.2)	0.049	16 (15)	28 (5.6)	0.05	9 (47.4)	24 (92.3)
pdhC	480	25 (11.5)	83 (17.3)	0.068	24 (22.4)	80 (16.7)	0.07	15 (60.0)	76 (91.6)

[a] d_N/d_S, the proportion of nonsynonymous to synonymous nucleotide substitutions.

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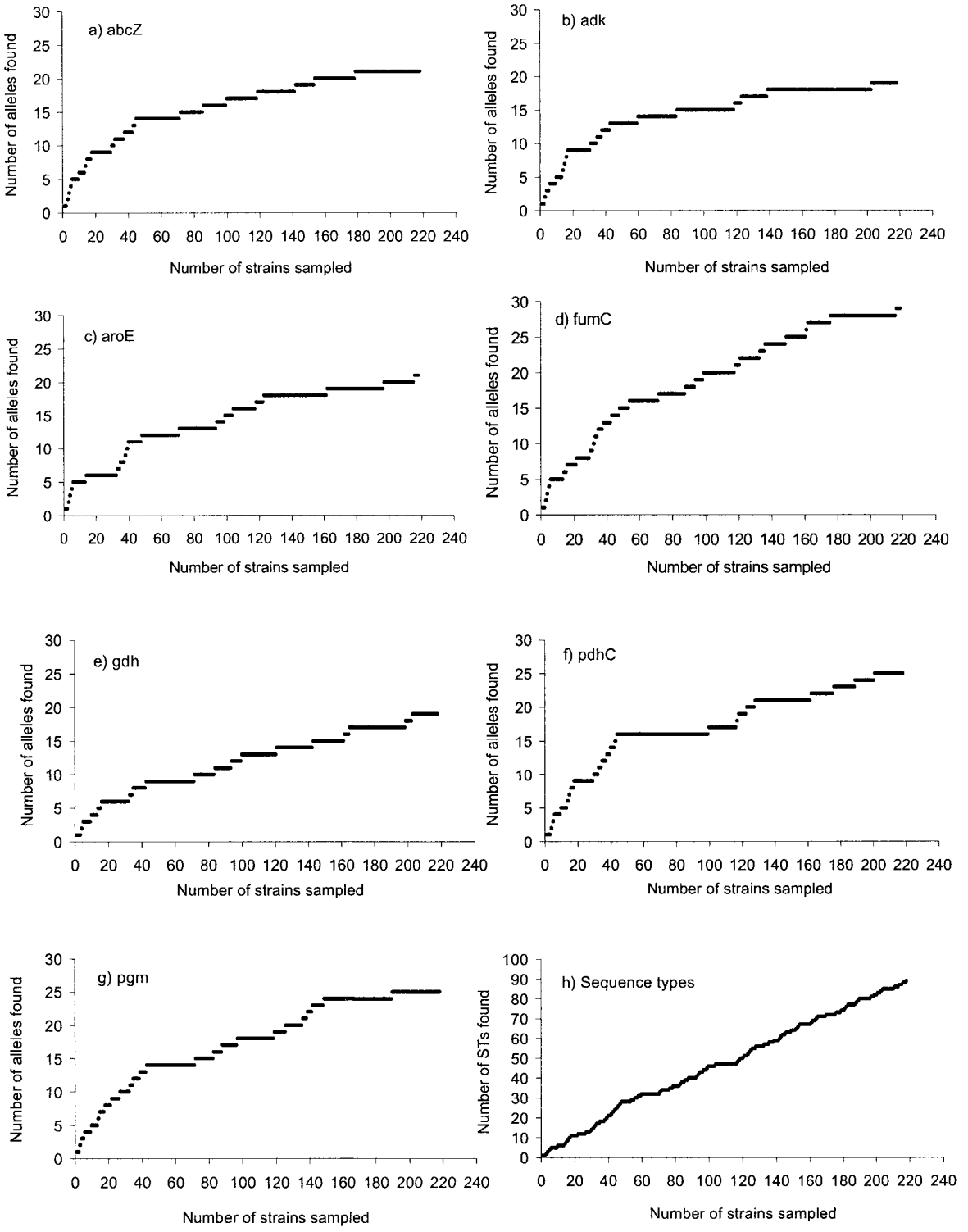

FIG. 1. The number of alleles present at the loci *abcZ* (a), *adk* (b), *aroE* (c), *fumC* (d), *gdh* (e), *pdhC* (f), and *pgm* (g), as well as the number of sequence types (h), plotted against the number of isolates sampled, given in numerical order of isolation.