Evolution of Sequence Type 4821 Clonal Complex Hyperinvasive and Quinolone-Resistant Meningococci

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Expansion of quinolone-resistant *Neisseria meningitidis* clone China$^{CC4821-R1-C/B}$ from sequence type (ST) 4821 clonal complex (CC4821) caused a serogroup shift from serogroup A to serogroup C invasive meningococcal disease (IMD) in China. To determine the relationship among globally distributed CC4821 meningococci, we analyzed whole-genome sequence data from 173 CC4821 meningococci isolated from 4 continents during 1972–2019. These meningococci clustered into 4 sublineages (1–4); sublineage 1 primarily comprised of IMD isolates (41/50, 82%). Most isolates from outside China (40/49, 81.6%) formed a distinct sublineage, the Europe–USA cluster, with the typical strain designation B:P1.17-6,23:F3-36:ST-3200(CC4821), harboring mutations in penicillin-binding protein 2. These data show that the quinolone-resistant clone China$^{CC4821-R1-C/B}$ has expanded to other countries. The increasing distribution worldwide of serogroup B CC4821 raises the concern that CC4821 has the potential to cause a pandemic that would be challenging to control, despite indirect evidence that the Trumenba vaccine might afford some protection.

Neisseria meningitidis, a leading cause of bacterial meningitis and septicemia globally, causes ≈1.2 million invasive meningococcal disease (IMD) cases annually and a case-fatality rate of 11% (*1*). Meningococci are classified into 12 serogroups based on capsular polysaccharides (*1*); genetic relationships among isolates are defined by clonal complexes (CCs) identified by multilocus sequence typing (MLST), which are surrogates for lineages (*2*). The relationship among serogroups, CCs (lineages), and IMD fluctuates over time and by location, but IMD isolates are dominated by CCs known as hyperinvasive lineages, usually associated with one of the 6 disease-causing serogroups (MenA, MenB, MenC, MenW, MenX, and MenY).

In China, the national dissemination of hyperinvasive sequence type (ST) 4821 clonal complex (CC4821) meningococci led to a shift in IMD epidemiology from mostly MenA to predominantly MenC (*3*,*4*). Although no quinolone resistance was identified in CC4821 in China during 1965–1985, high-frequency resistance (79%) occurred from 2005 onward due to expansion of the quinolone-resistant clone China$^{CC4821-R1-C/B}$ (*5*). Previous studies discovered that CC4821 can be divided into 2 groups, with group 1 associated with IMD (*6*,*7*). Peng et al. identified 6 strain-specific genome regions resulting from horizontal gene transfer (HGT) in isolate 053442 (*8*); this finding was consistent with the emergence of the China$^{CC4821-R1-C/B}$ clone associated with multiple HGT events within genes encoding surface antigens (*6*), although the donors of these events were not identified.

Globally, the number of CC4821 IMD isolates has increased. At the time CC4821 was identified, isolates were confined to China (*4*,*9*); however, by June 2020, a total of 59 CC4821 isolates had been identified in 19 countries worldwide (Figure 1). Moreover, 3 IMD cases caused by quinolone-resistant CC4821 isolates were reported in Canada (n = 2) and Japan (n = 1) after 2013 (*10*,*11*); 3 other CC4821 isolates were found to colonize the anorectal tract of men who have sex with men (MSM) (*12*). We investigated the genomic events leading to the emergence and expansion of hyperinvasive CC4821 meningococci by describing the phylogenetic relationships among meningococci with different serogroups (MenC, MenB, MenW, and nongroupable), sources (IMD, carriage, and MSM), locations (China or other countries),

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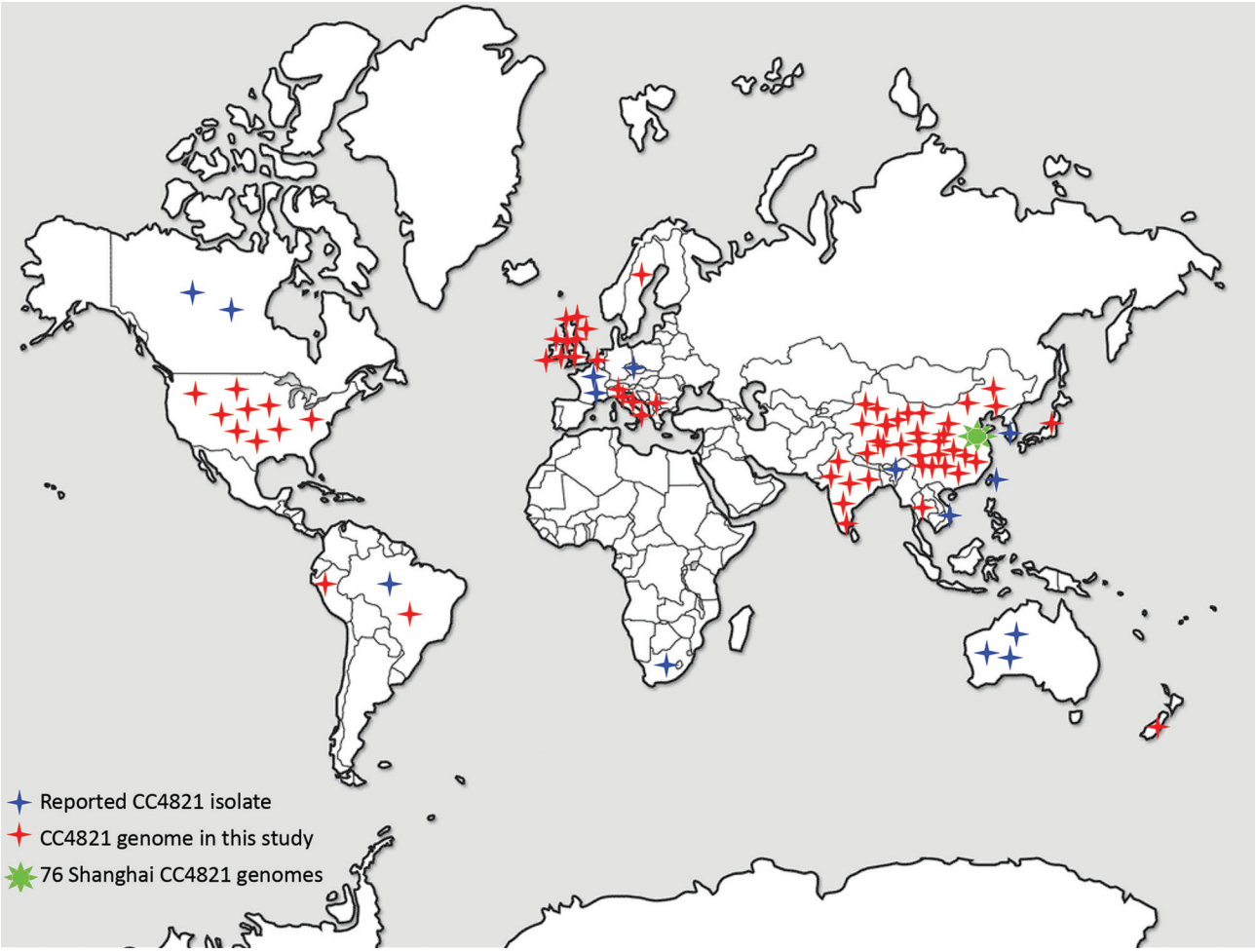


Figure 1. Distribution of CC4821 *Neisseria meningitidis* isolates worldwide. CC4821 isolates were identified in China and in 19 countries of Europe, Africa, North America, South America, Oceania, and Asia. CC, clonal complex.

and dates of isolation (1972–1978 vs. 2004–2019). We assessed genes encoding key antigens and antimicrobial resistance phenotypes, identified putative donors of HGT events unique to the epidemic and quinolone-resistant clone China$^{CC4821\text{-}R1\text{-}C/B}$, and characterized isolates outside of China.

Materials and Methods

Isolate Collection and Whole-Genome Sequencing

A total of 173 CC4821 genomes were collected dating from 1972–1978 (n = 19) and 2004–2019 (n = 154), including isolates from IMD (66/173, 38.2%), genitourinary sites (6/173, 3.5%), asymptomatic carriage (86/173, 49.7%), and unknown sources (15/173, 8.7%) (Appendix 1 Table 1, https://wwwnc.cdc.gov/EID/article/27/4/20-3612-App1.xlsx). Shanghai CDC sequenced 76 CC4821 isolates with Illumina HiSeq (Illumina, https://www.illumina.com) using paired-end 150 base reads as previously described (*13*). An additional 97 publicly available CC4821 genomes consisted of 48 genomes from 14 provinces of China, including the reference strain 053442 (*6–8*) and 49 genomes from countries outside of China, including the United Kingdom (n = 20), United States (n = 8), and 11 other countries (n = 21) (Figure 1; Appendix 1 Table 1) (*10,12,14–17*). The completeness and contamination of the genomes were evaluated using CheckM (*18*).

Antigenic and Antimicrobial Resistance Characteristics of CC4821 Genomes

To describe the antigenic and antimicrobial resistance characteristics of CC4821 genomes, we extracted from genomes nucleotides of 9 antigen coding genes (*porA*, *fHbp*, *nhba*, *porB*, *fetA*, *opcA*, *nspA*, *tbpA*, and *NMB0315*) (*19–22*) and 5 resistance-associated genes (*gyrA*, *parC*, *penA*, *ponA*, and *rpoB*) (*23,24*) for analysis. We annotated and analyzed deduced

encoding factor H–binding protein (fHbp), *Neisseria* heparin-binding antigen (NHBA), *Neisseria* adhesion antigen (NadA), and outer membrane protein (PorA) peptides and deduced meningococcal vaccine antigen reactivity (MenDeVAR) index from the PubMLST *Neisseria* database (*25*).

Identifying CC4821 (L44) Sublineages

In the *Neisseria* PubMLST database, a lineage-specific core genome MLST typing scheme containing loci found in 95% of CC4821 isolates was established and designated L44 cgMLST consistent with the previously described CC4821 lineage 44 (*26*). We compared the 173 CC4821 genomes using Genome Comparator (*27*) and the L44 cgMLST scheme, identifying distinct sublineages. To characterize each sublineage, we visualized a FASTA output from the Genome Comparator Tool using all 2,860 defined loci (NEIS0001–NEIS3173, not contiguous) using MEGA version 5 (*28*). We used Z2491 (GenBank accession no. NC_003116) as outgroup in accordance with previous studies (*6*,*8*). Assembled contigs and annotation information of 173 genomes in this study can be accessed at https://pubMLST.org/neisseria (Appendix 1 Table 1).

Identifying and Characterizing Unique Alleles in Sublineages

We determined shared and unique alleles using outputs from Genome Comparator. An allele was defined as unique to a sublineage if it was present in >90% of the genomes in that sublineage but absent in other sublineages. Genes with unique alleles were functionally characterized according to the Kyoto Encyclopedia of Genes and Genomes Orthology groupings of its database (*29*).

Identifying HGT Events and Putative Donors

Inputting the aligned sequences generated from Parsnp (*30*), we predicted putative HGT events using Gubbins (*31*). To search for potential donors, we blasted alleles and sequences of contiguous loci that were predicted to originate from HGT against the PubMLST database. We identified potential donors as previously described (*32*). We labeled recombination areas with unique loci on the circular genome map of genome 053442 by BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) comparisons to strains of other sublineages, as generated using BRIG (*33*).

Screening Molecular Markers of MSM Infection Strains from Europe

In addition to the lineage of 11.2 possessing PorA P1.5–1,10–8, 3 other molecular features have been identified in meningococci causing infections among MSM in Europe during 2012–2014; these features were functional nitrite reductase (AniA); frameshifted *fHbp* allele found mostly in urethritis and proctitis isolates; and *penA327* that had reduced susceptibility to penicillin and third-generation cephalosporins (*34*). These 3 molecular markers were screened among all the 173 CC4821 genomes.

Results

Isolate Characterization

The 173 CC4821 isolates represented 46 different STs; ST4821 (n = 41, 23.7%) and ST3200 (n = 30, 17.3%) were the most prevalent. We identified 43 PorA subtypes, of which P1.7-2,14 (n = 25, 14.5%) and P1.17-6,23 (n = 18, 10.4%) were the most frequent. We identified 27 FetA variants; F3-3 (n = 47, 27.2%) and F3-36 (n = 37, 21.4%) were the most prevalent (Appendix 1 Table 1).

Identifying 4 Sublineages

We identified 2,161 loci in reference genome 053442, including 1,699 core genes. Most (1,527/1,699, 89.9%) of the core loci had *p*-distance values of 0–0.1; 0.8% (14/1,699) showed high *p*-distance values of 0.50–0.68. On the basis of the L44 cgMLST scheme, we divided the CC4821 isolates into 4 sublineages (Figure 2): L44.1, identical to the China$^{CC4821\text{-}R1\text{-}C/B}$ clone (n = 50, 28.9%), composed of isolates from China (n = 44) and other countries (n = 6) during 2004–2019 that were very closely related (Figure 3); L44.2 (n = 29, 16.8%), composed of isolates from China (n = 28) and the United Kingdom (n = 1) during 2005–2019; L44.3 (n = 58, 33.5%), composed of isolates from China (n = 18) and countries outside China (n = 40) during 1977–2019; and L44.4 (n = 32, 18.5%), composed of isolates from China (n = 30) and India (n = 2) during 1972–2017. Four additional isolates from China were not assigned to any sublineages.

Features of the 4 Sublineages

The percentage of IMD isolates was significantly higher in L44.1 (41/50, 82%) than the other 3 sublineages (17.2%–22.4%; p<0.001) (Figure 2). L44.1, containing the reference strain 053442, was mainly composed of MenC isolates (44/50, 88%) and had ST4821 as its central ST. L44.2, was mainly composed of MenB isolates (27/29, 93.1%) and its central ST was ST5664. L44.3 was mainly composed of MenB (55/58, 94.8%) with ST3200 as its central ST. L44.4 was mainly composed of MenC (14/32, 43.8%) and MenW (11/32, 34.4%) with its central ST3436

(Appendix 2 Figure 1, https://wwwnc.cdc.gov/EID/article/27/4/20-3612-App2.pdf).

Analysis of the 5 antimicrobial resistance genes revealed that both *gyrA*-71 (with T91I) and *parC*-12 were specific to L44.1; *parC*-275 and *penA*-9 (with 5 mutations) were both specific to L44.3, and *gyrA*-294 (with T91I) was discovered only in L44.4 (Table 1; Appendix 2 Figures 2–6). In L44.1, all of the isolates possessed the quinolone resistance–associated mutation T91I in GyrA (Figure 4). In L44.3, 40/58 (69.0%) harbored PBP2 mutations, almost always from countries outside of China (38/40, 95%) (Figures 5, 6).

Vaccine Antigens among the 4 Sublineages

Analysis of 9 antigenic genes identified several alleles unique to a certain sublineage (Table 2; Appendix 2 Figures 7–17). For example, FetA-VR F3-3 was found in L44.1, F1-91 in L44.2, F3-36 and F3-9 in L44.3, and F1-7 in L44.4 isolates (Appendix 2 Figure 11). In L44.1, most isolates had the same antigenic gene profile (*nhba*-124, *porB*-29, *fetA*-64, *opcA*-4, *nspA*-4, *tbpA*-7, and *NMB0315*-21) (Figure 4), and 25/50 (50%) had the PorA subtype of P1.7-2,14 (Figure 3). In L44.3, most had the same gene profile (*fHbp*-16, *nhba*-553, *porB*-265, *fetA*-1069, *opcA*-100, *nspA*-26, and *NMB0315*-194), with *porA* and *tbpA* showing high genetic diversity (Figure 5).

We analyzed deduced peptide sequences for vaccine antigen constituents among MenB isolates (n = 97). We identified 16 fHbp peptides, of which peptide 16 (variant 2/subfamily A) was present in 70/97 (72.2%) isolates, including 31/70 isolates from China. There were 20 NHBA peptides, of which alleles 669 (46/95, 48.4%), 901 (11/95, 11.6%), and 668 (10/95, 10.5%) occurred most frequently. The *nadA* gene was absent in all isolates (including other serogroups). Of 31 PorA VR1/VR2 combinations, the most frequently occurring was P1.20,23 (11/97, 11.3%).

MenDeVAR Index values were assigned for MenB disease isolates (n = 29, including the 6 isolates from genitourinary sites), but 27/29 (93.1%) isolates had insufficient data from experimental studies to estimate the coverage of the MenB vaccine Bexsero (Appendix 2 Table 2). We predicted cross-reactivity to the MenB vaccine Trumenba for 18/29 (62.1%) isolates. For the MenB disease isolates from China, 7/17 (41.2%) were deemed cross-reactive with Trumenba;

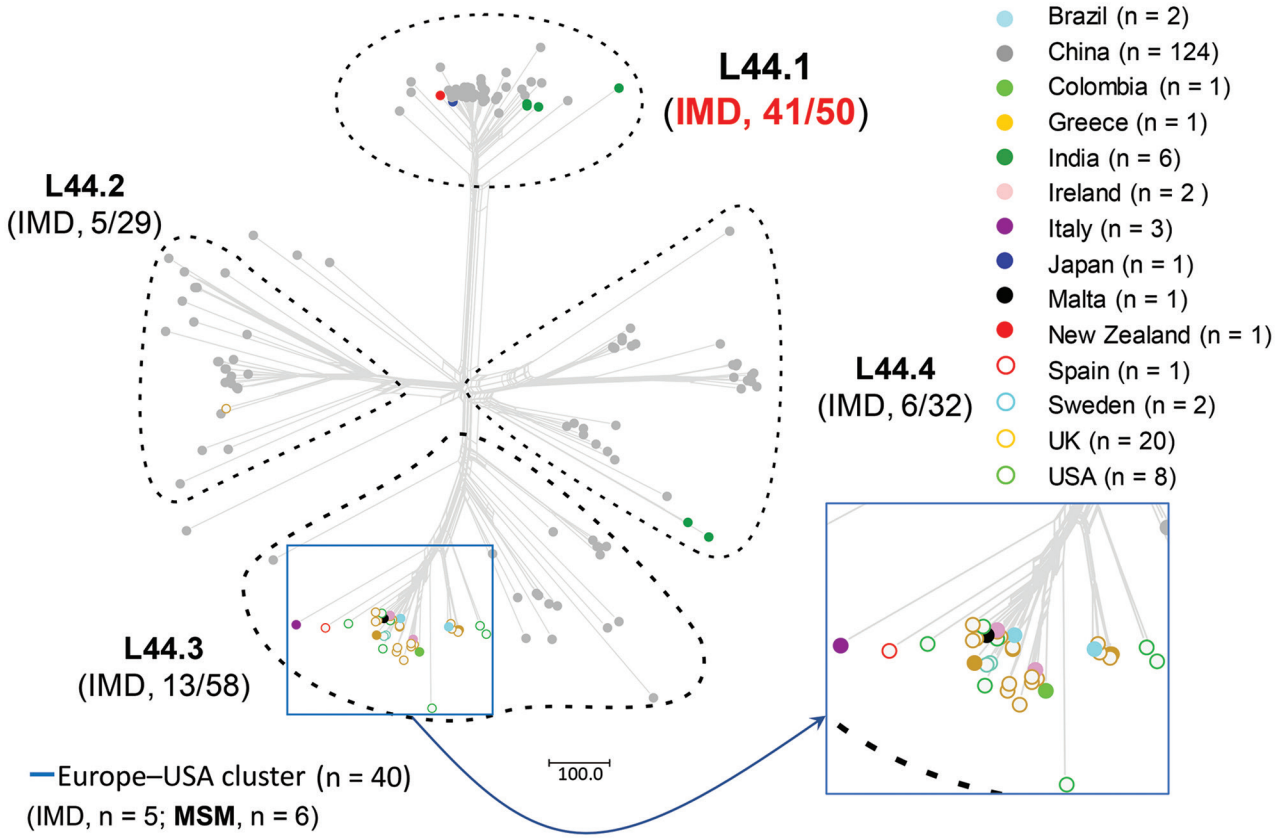
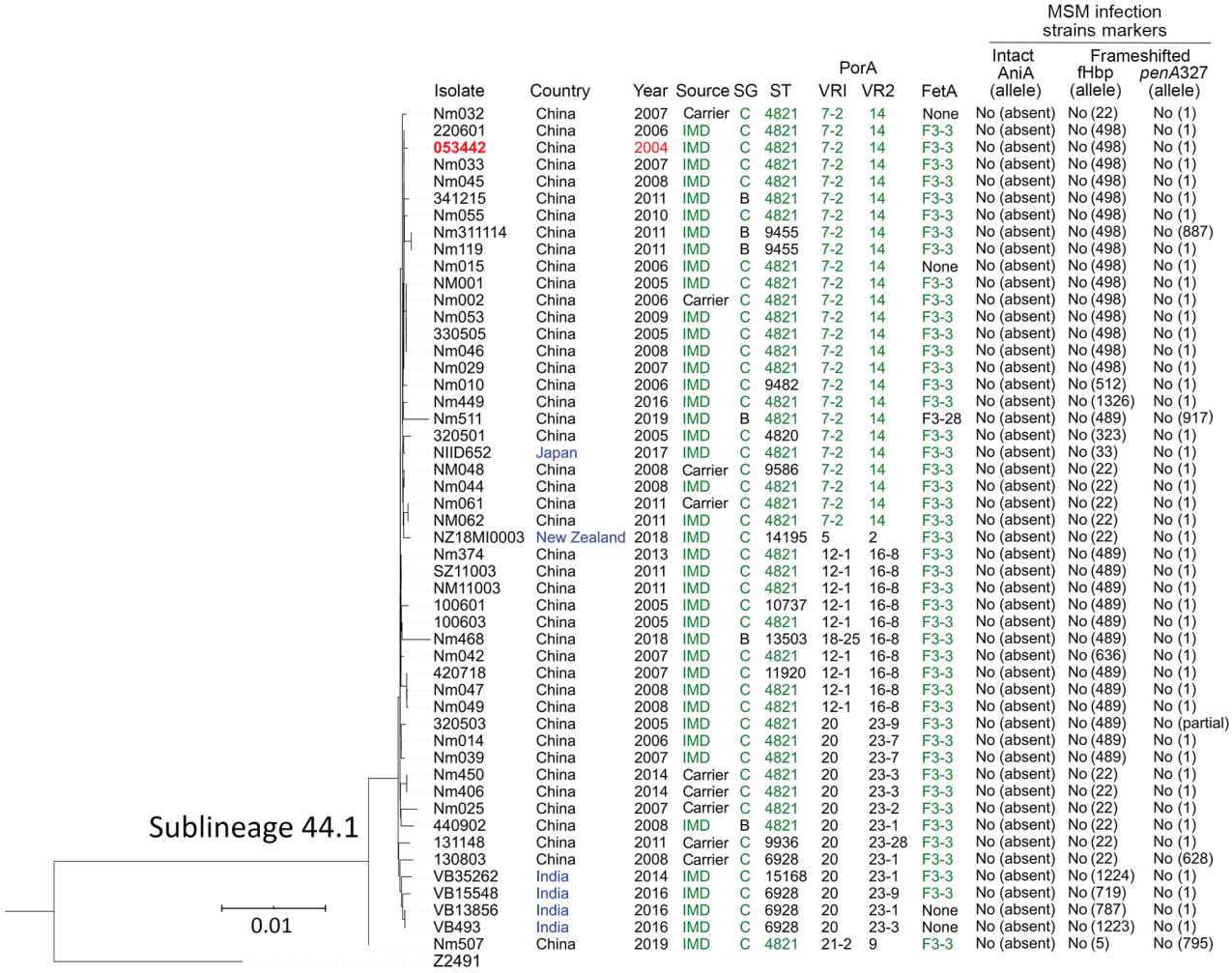


Figure 2. Allele-based sublineages of clonal complex 4821 *Neisseria meningitidis* identified using lineage 44 core genome multilocus sequence typing scheme. The inset shows the country distribution of the 40 genomes constituting the Europe–USA cluster. IMD, invasive meningococcal disease; MSM, men who have sex with men.



Isolate	Country	Year	Source	SG	ST	PorA VRI	PorA VR2	FetA	MSM infection strains markers: Intact AniA (allele)	Frameshifted fHbp (allele)	Frameshifted *penA*327 (allele)
Nm032	China	2007	Carrier	C	4821	7-2	14	None	No (absent)	No (22)	No (1)
220601	China	2006	IMD	C	4821	7-2	14	F3-3	No (absent)	No (498)	No (1)
053442	China	2004	IMD	C	4821	7-2	14	F3-3	No (absent)	No (498)	No (1)
Nm033	China	2007	IMD	C	4821	7-2	14	F3-3	No (absent)	No (498)	No (1)
Nm045	China	2008	IMD	C	4821	7-2	14	F3-3	No (absent)	No (498)	No (1)
341215	China	2011	IMD	B	4821	7-2	14	F3-3	No (absent)	No (498)	No (1)
Nm055	China	2010	IMD	C	4821	7-2	14	F3-3	No (absent)	No (498)	No (1)
Nm311114	China	2011	IMD	B	9455	7-2	14	F3-3	No (absent)	No (498)	No (887)
Nm119	China	2011	IMD	B	9455	7-2	14	F3-3	No (absent)	No (498)	No (1)
Nm015	China	2006	IMD	C	4821	7-2	14	None	No (absent)	No (498)	No (1)
NM001	China	2005	IMD	C	4821	7-2	14	F3-3	No (absent)	No (498)	No (1)
Nm002	China	2006	Carrier	C	4821	7-2	14	F3-3	No (absent)	No (498)	No (1)
Nm053	China	2009	IMD	C	4821	7-2	14	F3-3	No (absent)	No (498)	No (1)
330505	China	2005	IMD	C	4821	7-2	14	F3-3	No (absent)	No (498)	No (1)
Nm046	China	2008	IMD	C	4821	7-2	14	F3-3	No (absent)	No (498)	No (1)
Nm029	China	2007	IMD	C	4821	7-2	14	F3-3	No (absent)	No (498)	No (1)
Nm010	China	2006	IMD	C	9482	7-2	14	F3-3	No (absent)	No (512)	No (1)
Nm449	China	2016	IMD	C	4821	7-2	14	F3-3	No (absent)	No (1326)	No (1)
Nm511	China	2019	IMD	B	4821	7-2	14	F3-28	No (absent)	No (489)	No (917)
320501	China	2005	IMD	C	4820	7-2	14	F3-3	No (absent)	No (323)	No (1)
NIID652	Japan	2017	IMD	C	4821	7-2	14	F3-3	No (absent)	No (33)	No (1)
NM048	China	2008	Carrier	C	9586	7-2	14	F3-3	No (absent)	No (22)	No (1)
Nm044	China	2008	IMD	C	4821	7-2	14	F3-3	No (absent)	No (22)	No (1)
Nm061	China	2011	Carrier	C	4821	7-2	14	F3-3	No (absent)	No (22)	No (1)
NM062	China	2011	IMD	C	4821	7-2	14	F3-3	No (absent)	No (22)	No (1)
NZ18MI0003	New Zealand	2018	IMD	C	14195	5	2	F3-3	No (absent)	No (22)	No (1)
Nm374	China	2013	IMD	C	4821	12-1	16-8	F3-3	No (absent)	No (489)	No (1)
SZ11003	China	2011	IMD	C	4821	12-1	16-8	F3-3	No (absent)	No (489)	No (1)
NM11003	China	2011	IMD	C	4821	12-1	16-8	F3-3	No (absent)	No (489)	No (1)
100601	China	2005	IMD	C	10737	12-1	16-8	F3-3	No (absent)	No (489)	No (1)
100603	China	2005	IMD	C	4821	12-1	16-8	F3-3	No (absent)	No (489)	No (1)
Nm468	China	2018	IMD	B	13503	18-25	16-8	F3-3	No (absent)	No (489)	No (1)
Nm042	China	2007	IMD	C	4821	12-1	16-8	F3-3	No (absent)	No (636)	No (1)
420718	China	2007	IMD	C	11920	12-1	16-8	F3-3	No (absent)	No (489)	No (1)
Nm047	China	2008	IMD	C	4821	12-1	16-8	F3-3	No (absent)	No (489)	No (1)
Nm049	China	2008	IMD	C	4821	12-1	16-8	F3-3	No (absent)	No (489)	No (1)
320503	China	2005	IMD	C	4821	20	23-9	F3-3	No (absent)	No (489)	No (partial)
Nm014	China	2006	IMD	C	4821	20	23-7	F3-3	No (absent)	No (489)	No (1)
Nm039	China	2007	IMD	C	4821	20	23-7	F3-3	No (absent)	No (489)	No (1)
Nm450	China	2014	Carrier	C	4821	20	23-3	F3-3	No (absent)	No (22)	No (1)
Nm406	China	2014	Carrier	C	4821	20	23-3	F3-3	No (absent)	No (22)	No (1)
Nm025	China	2007	Carrier	C	4821	20	23-2	F3-3	No (absent)	No (22)	No (1)
440902	China	2008	IMD	B	4821	20	23-1	F3-3	No (absent)	No (22)	No (1)
131148	China	2011	Carrier	C	9936	20	23-28	F3-3	No (absent)	No (22)	No (1)
130803	China	2008	Carrier	C	6928	20	23-1	F3-3	No (absent)	No (22)	No (628)
VB35262	India	2014	IMD	C	15168	20	23-1	F3-3	No (absent)	No (1224)	No (1)
VB15548	India	2016	IMD	C	6928	20	23-9	F3-3	No (absent)	No (719)	No (1)
VB13856	India	2016	IMD	C	6928	20	23-1	None	No (absent)	No (787)	No (1)
VB493	India	2016	IMD	C	6928	20	23-3	None	No (absent)	No (1223)	No (1)
Nm507	China	2019	IMD	C	4821	21-2	9	F3-3	No (absent)	No (5)	No (795)
Z2491											

Figure 3. Phylogenetic tree and data of clonal complex 4821 *Neisseria meningitidis* sublineage L44.1 (China$^{CC4821\text{-}R1\text{-}C/B}$) isolates. Red text indicates the oldest isolate of the sublineage; blue text, the isolates from countries outside of China; and green text, the dominant type or allele. Scale bar indicates substitutions per site. IMD, invasive meningococcal disease; MSM, men who have sex with men; SG, serogroup; ST, sequence type; VR, variable region.

however, we had insufficient data for the remaining 10/17 (58.8%) to determine reactivity.

Molecular Markers of Strains from Europe Infecting MSM

None of the CC4821 isolates harbored frameshifted *fHbp* allele or *penA327*, but the distribution of putatively functional AniA proteins was diverse. The *aniA* gene was absent in all L44.1 isolates (Figure 3) but was present in all of the other 123 CC4821 isolates, of which 96.7% (119/123) isolates harbored putatively functional AniA proteins (Figure 5; Appendix 2 Figures 16–17).

Evolution of Sublineage L44.1 (China$^{CC4821\text{-}R1\text{-}C/B}$ Clone)

Five specific loci were present in >90% of L44.1 but in <10% of other sublineages. These loci were involved in signaling and cellular processes (n = 2), metabolism (n = 1), and genetic information processing (n = 1) (Table 3). No loci were specific to any of other 3 sublineages.

Prediction of HGT events contributing to the emergence of L44.1 using Gubbins discovered 126 events involving 686 loci shared by the 50 L44.1 isolates (Appendix 2 Figure 18). These events included 216 loci with alleles specific to L44.1. We discovered an additional 83 unique loci based on analysis of the accessory genome. Therefore, a total of 299 unique loci were identified in L44.1; of those, 139 (46.5%) were involved in metabolic function (Appendix 1 Table 3). These 299 unique loci were distributed across the chromosome; we observed 44 areas (216 loci) harboring contiguous loci with unique alleles (Figure 7), among which the exact donors of 36 areas across 149

Table 1. Specific alleles of antimicrobial resistance genes in 4 sublineages of clonal complex 4821 of *Neisseria meningitidis*

	Resistant, allele no. (no. isolates)				
Sublineage	*gyrA*	*parC*	*penA*	*ponA*	*rpoB*
L44.1, n = 50	71 (50)	12 (43)	None	None	None
L44.2, n = 29	None	None	None	None	None
L44.3, n = 58	None	275 (41)	9 (35)	None	None
L44.4, n = 32	294 (11)	None	None	None	None

loci were identified in 46 putative HGT events. The total length of these putative recombination fragments was ≈225 kb, including 87 kb (38.7%) originating from the C:ST-9514 cluster isolates in China during 1966–1977, followed by 25 kb (11.1%) from MenA isolates (CC5 and CC1) in China during 1966–1984 (Table 4, https://wwwnc.cdc.gov/EID/article/27/4/20-3612-T4.htm).

Evolution of CC4821 Isolates from Outside China

We identified 49 CC4821 isolates from countries outside of China, and most (40/49, 81.6%) were assigned to L44.3, of which there were 39 MenB and 1 MenC, constituting the distinct Europe–USA cluster (Figures 2, 5). The representative molecular characteristics of the Europe–USA cluster was B:P1.17-6,23:F3-36:ST-3200(CC4821); its antigen gene profile was *porA*-423, *fHbp*-16, *nhba*-553, *porB*-265, *fetA*-1069, *opcA*-100, *nspA*-26, *tbpA*-1333, and *NMB0315*-194 and antimicrobial resistance profile *gyrA*-12, *parC*-275, *penA*-9 with PBP2 mutations, *ponA*-7, and *rpoB*-85 (Figure 6). In Gubbins analysis, 33 events involving 193 loci were shared by all the Europe–USA cluster isolates (Appendix 2 Figure 18); we discovered 60 unique loci for which we could not identify their potential donors. These unique loci were involved in functions mainly associated with metabolism (23/60, 38.3%) and genetic information processing (18/60, 30%) (Appendix 1 Table 4).

In addition to the 40 Europe–USA cluster isolates, there were 6 MenC invasive isolates from India (n = 4, identified 2014–2016), Japan (n = 1, identified in 2017),

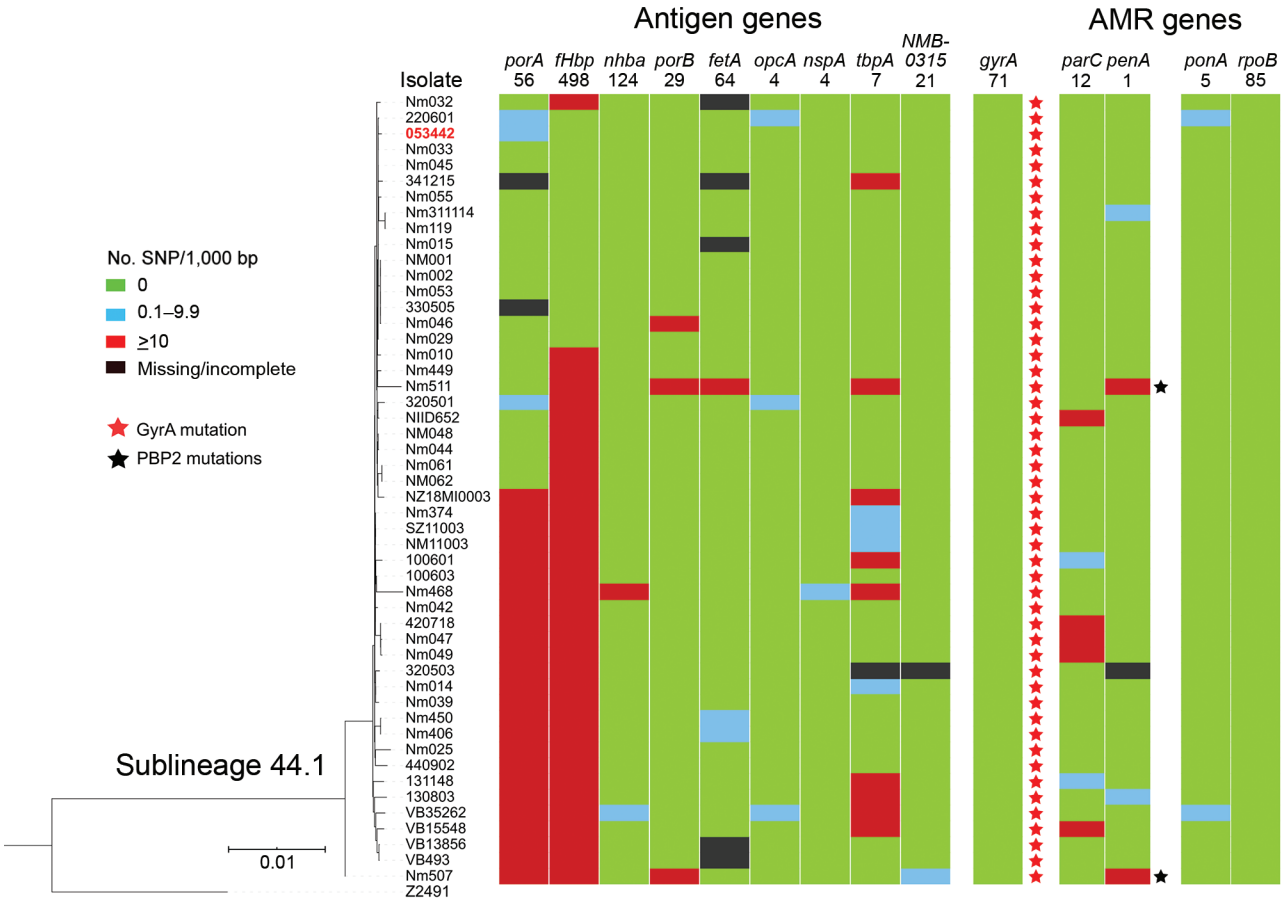


Figure 4. Genomic diversity of clonal complex 4821 *Neisseria meningitidis* sublineage L44.1 (China$^{CC4821\text{-}R1\text{-}C/B}$) isolates. The numbers underneath the antigen genes and AMR genes are the dominant alleles for that particular gene, and the colored blocks for SNPs/1,000 bp were determined using the allele number labeled above each column as the reference allele. AMR, antimicrobial resistance; SNP, single-nucleotide polymorphism.

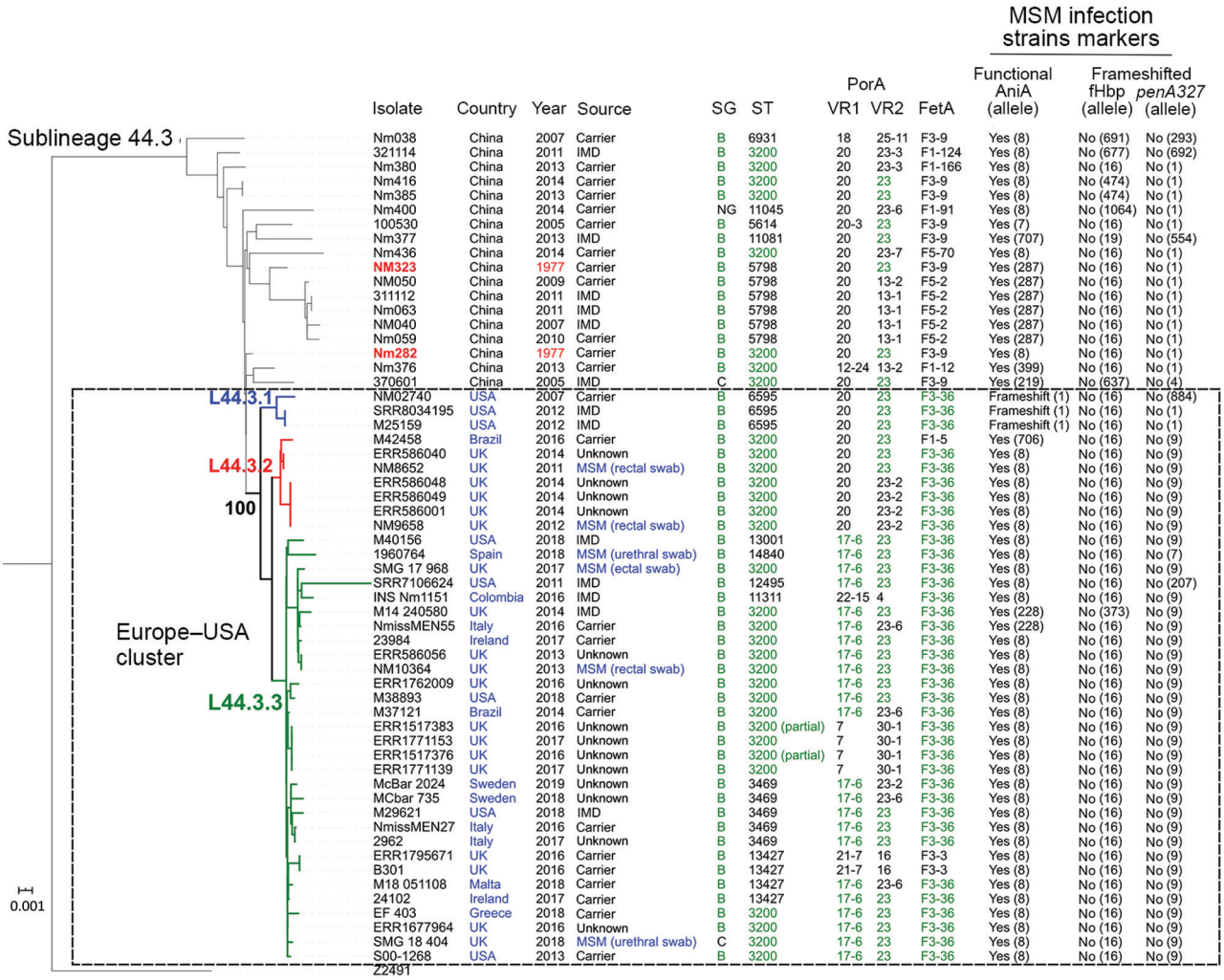


Figure 5. Phylogenetic tree and data of clonal complex 4821 *Neisseria meningitidis* sublineage L44.3 isolates. Red text indicates the oldest isolates of the sublineage; blue text, the isolates from countries outside of China and the isolates from genitourinary swabs from MSM; and green text, the dominant type or allele. The Europe–USA cluster can be further divided into 3 subclusters: subcluster L44.3.1, composed of 3 ST6595 isolates from the United States, all of which contained putatively nonfunctional AniA; L44.3.2, composed of 7 ST3200 isolates from the United Kingdom (n = 6) and Brazil (n = 1); and L44.3.3, composed of 30 isolates with multiple geographic locations. All the isolates from urethral (n = 2) and rectal (n = 4) swabs were assigned to L44.3.2 and L44.3.3, both of which comprised isolates with putatively functional AniA. Scale bar indicates substitutions per site. IMD, invasive meningococcal disease; MSM, men who have sex with men; NG, nongroupable; SG, serogroup; ST, sequence type; VR, variable region.

and New Zealand (n = 1, identified in 2018). These 6 isolates were clustered together and were closely related with 44 isolates from China within sublineage L44.1 (Figures 2–3). Only the isolate from Japan showed the typical molecular feature of Anhui outbreak strain (C:P1.7-2,14:F3-3:ST-4821[CC4821]).

Features and Evolution of Serogroup W CC4821 Isolates

A total of 11 MenW isolates from China were identified; the representative strain designation was W:P1.5-3,10-2:F1-7:ST-8491(CC4821), with similar gene profiles of antigen-encoding loci (*porA*-1804, *fHbp*-474, *nhba*-966, *fetA*-37, *opcA*-4, *nspA*-117, and *NMB0315*-21) and antimicrobial resistance loci (*gyrA*-294 with T91I, *parC*-779, *ponA*-7, and *rpoB*-85). These MenW isolates constituted a distinct cluster in L44.4; they were more closely related to NM193 (C:P1.20-3,23-1:F1-5:ST-3436[CC4821], dating from 1972) than to NM205 (C:P1.20,23-2:F5-135:ST-4821[CC4821], dating from 1973) (Appendix 2 Figure 17).

Discussion

The meningococci can cause IMD, leading to endemic disease in most if not all human populations. Several

genotypes belonging to hyperinvasive lineages, in combination with the disease-associated capsular serogroups, can cause elevated levels of disease; some of which also possess epidemic and pandemic potential. In the past 100 years, notable epidemics and pandemics have included meningococci such as A:CC1, A:CC5, B:CC41/44, C:CC11, and W:CC11 (*35*). Here, we employed a genomic analysis of MenB, MenC, and MenW CC4821 isolates dating from 1972–2019 to assess their epidemic and pandemic potential. Of special concern are the expansion of the quinolone-resistant clone China$^{CC4821\text{-}R1\text{-}C/B}$ from China to other countries; the potential possession of universal resistance to penicillin in Europe–USA cluster isolates; and the uncertainty over the potential efficacy of existing vaccines to prevent B:CC4821 diseases.

CC4821, which corresponds to lineage 44, shares several properties in common with the hyperinvasive CC11 meningococci (lineage 11): its ability to express several serogroups, global distribution, colonization of urogenital and anorectal tracts, and separation into distinct sublineages. CC11 has caused well-documented epidemics and pandemics on several occasions, including US military outbreaks in the 1960s; Hajj-associated outbreaks in 2000s; and the global epidemics from 2010, especially outbreaks among MSM (*34*–*38*). These similar characteristics raise the concern that the CC4821 may have the potential to cause similar global pandemics.

Consistent with the presence of the epidemic CC4821 clone in countries outside of China, 6 CC4821 IMD meningococci from India, Japan, and New Zealand, isolated during 2014–2018, clustered with China$^{CC4821\text{-}R1\text{-}C/B}$ meningococci in L44.1 (Figure 3). IMD cases caused by these 6 isolates were all found in native inhabitants (*10*,*15*,*39*); all 6 isolates shared

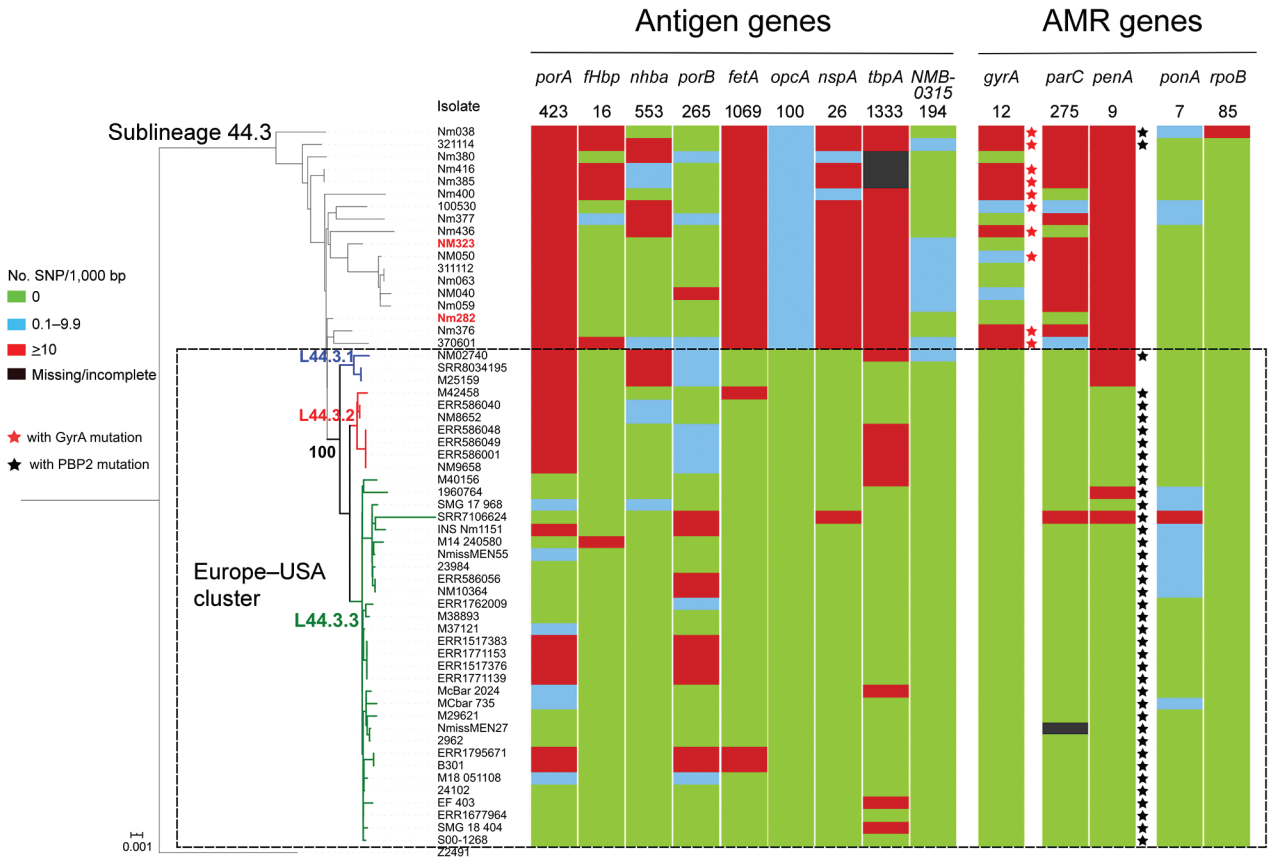


Figure 6. Genomic diversity of clonal complex 4821 *Neisseria meningitidis* sublineage L44.3 isolates. The numbers underneath the antigen genes and AMR genes are the dominant alleles for that particular gene, and the color blocks for SNPs/1,000 bp were determined using the allele number labeled above each column as the reference allele. The Europe–USA cluster can be further divided into 3 subclusters: subcluster L44.3.1, composed of 3 ST6595 isolates from the United States, all of which contained putatively nonfunctional AniA; L44.3.2, composed of 7 ST3200 isolates from the United Kingdom (n = 6) and Brazil (n = 1); and L44.3.3, composed of 30 isolates with multiple geographic locations. All the isolates from urethral (n = 2) and rectal (n = 4) swabs were assigned to L44.3.2 and L44.3.3, both of which comprised isolates with putatively functional AniA. Scale bar indicates substitutions per site. AMR, antimicrobial resistance; SNP, single-nucleotide polymorphism.

Table 2. Specific alleles of antigenic genes in 4 sublineages of clonal complex 4821 of *Neisseria meningitidis*

Sublineage	Antigen allele no. (no. isolates)								
	PorA	*fHbp*	*nhba*	PorB	FetA-VR	*opcA*	*nspA*	*tbpA*	*NMB-0315*
L44.1, n = 50	P1.7–2,14 (25) P1.12–1,16–8 (9)	22 (12) 489 (13) 498 (15)	124 (48)	3–48 (47)	F3–3 (45)	None	4 (49)	7 (36)	None
L44.2, n = 29	None	None	965 (10) 967 (12)	3–81 (15)	F1–91 (20)	None	None	None	335 (26)
L44.3, n = 58	P1.17–6,23-x* (23)	None	None	3–229 (35)	F3–9 (8) F3–36 (37)	100 (40)	26 (39)	1,333 (31)	194 (49)
L44.4, n = 32	P1.5–3,10–2 (8)	None	None	3–460 (7)	F1–7 (11)	None	117 (20)	None	None

*23-x refers to 23, 23–2, and 23–6.

similar serogroup, antibiotic, and antigen (except *porA* and *fHbp*) gene characteristics with isolates from China in this sublineage. In particular, all 6 isolates harbored the T91I mutation in GyrA, the molecular marker of quinolone resistance, compatible with their quinolone-resistant phenotype (*10*,*15*,*39*). These isolates also had strain-specific features, which suggested that they resulted from transmission from the China$^{CC4821\text{-}R1\text{-}C/B}$ clone. The isolate from Japan, which had the typical molecular features of the Anhui outbreak strain (C:P1.7-2,14:F3-3:ST-4821[CC4821]) (*4*), became the earliest-reported quinolone-resistant meningococcus harboring ParC mutation (S87I, allele 1538) to cause IMD worldwide (*10*). In contrast, none of the China$^{CC4821\text{-}R1\text{-}C/B}$ clone isolates from China had ParC mutations conferring antimicrobial resistance. The isolate from Japan was more closely related to the reference strain 053442 than were the 4 India and 1 New Zealand isolates, which had different STs, *porA*, *fHbp,* and *tbpA* alleles (Figure 4).

Although we did not identify a putative ancestor of the quinolone-resistant clone China$^{CC4821\text{-}R1\text{-}C/B}$ in this study, we found 299 loci with alleles unique to this sublineage. Approximately half of these loci were associated with metabolic pathways, suggesting that divergence in metabolic genes may play a role in the emergence of epidemic meningococci. Several studies have indicated that metabolic genes can influence the pathogenesis and virulence of the meningococcus, for example by allowing alternative host resources to be exploited in invasive disseminated infections (*40*). Changes in the hyperinvasive A:CC5 meningococci circulating in Africa have been associated with HGT of core genes involved in metabolic processes (*41*). The putative donors of these unique alleles included lineages from different serogroups and dates of isolation, such as C:ST-9514 cluster, 1960s–1970s; A:CC5 and A:CC1, 1960s–1980s; B:CC32, 1960s; B:CC41/44, 1970s; and E:CC178, 1980s (Table 4). The C:ST-9514 cluster, STs that do not presently form part of a clonal complex documented in PubMLST, has ST9514 as the central ST and was predominant in MenC carriage isolates during 1965–1980 in Shanghai, China (*42*). Therefore, the emergence of China$^{CC4821\text{-}R1\text{-}C/B}$ clone was perhaps associated with accumulation of these unique alleles, which accounted for the separation from other sublineages in the allele-based phylogeny (Figure 2).

In the PubMLST database, >60% of the CC4821 isolates from outside China were MenB. Of these, 49 genomes were available in this study, including isolates from IMD (n = 15) and urogenital and rectal tracts (n = 6). Most of these genomes clustered in sublineage L44.3 and constituted a distinct cluster, the Europe–USA cluster, showing the typical strain designation: B:P1.17-6,23-x:F3-36:ST-3200(CC4821), wherein 23-x refers to 23, 23-2, and 23-6. The PorA and FetA types P1.17-6,23-x and F3-36 were only found in this cluster. The *Neisseria* PubMLST database had no genome data for 24 CC4821 isolates from other countries (United States, Brazil, France, Czech Republic, Spain, Italy, Australia, and Vietnam), but included PorA or FetA variants for the 24 isolates (Appendix 1 Table 5). Of these, 19 (79.2%) exhibited P1.17-6,23-x or F3-36, suggesting they might belong to the Europe–USA cluster. This cluster was distinct from the epidemic clone China$^{CC4821\text{-}R1\text{-}C/B}$. For example, the antigen profile characteristic of the Europe–USA cluster was P1.17-6,23-x, F3-36, PorB-3-229, *fHbp* -16, *nhba*-553, *opcA*-100, *nspA*-26, and *tbpA*-1333, compared with P1.7-2,14, F3-3, PorB-3-48, *fHbp*-498, 22 and 489, *nhba*-124, *opcA*-4, *nspA*-4, and *tbpA*-7 in the China$^{CC4821\text{-}R1\text{-}C/B}$ clone. In addition, all the China$^{CC4821\text{-}R1\text{-}C/B}$ isolates harbored the mutation T91I in GyrA, whereas almost all of the Europe–USA cluster isolates possessed mutations in PBP2 (F504L, A510V, I515V, H541N, and I566V). This may reflect different antibiotic selective pressures experienced by the Europe–USA and the China$^{CC4821\text{-}R1\text{-}C/B}$ meningococci. Penicillins were the most-used antimicrobial drugs in outpatients in Europe, whereas China has the second largest global increases of fluoroquinolone consumption (*43*,*44*). A high frequency (>70%) of quinolone resistance has been reported in China since 2005 (*5*), whereas 65% of meningococci

in Europe showed reduced susceptibility to penicillin G during 1945–2006 (*45*). In the 2 oldest isolates of the sublineage L44.3, Nm282 (B:P1.20,23:F3-36:ST-3200[CC4812]) was much closer to the Europe–USA cluster isolates than Nm323 (B:P1.20,23:F3-36:ST-5798[CC4821]) (Figure 5), and it seemed more likely to be the ancestor of the Europe–USA cluster isolates.

Urogenital and rectal meningococci have raised increasing public health concerns (*34*). In 2017, CC4821 anorectal isolates were identified in the United Kingdom (*12*). In this study, we identified CC4821 isolates from urethral and rectal tracts that clustered with isolates from IMD specimens and oropharyngeal carriage (Figure 5). With the exception of L44.1 isolates, most of the CC4821 isolates contained a putatively functional nitrite reductase (AniA), required for growth in anaerobic environments. The CC4821 isolates acquired quinolone resistance alleles from *N. lactamica* and *N. subflava* (*46*); the ability to grow in anaerobic environments will facilitate acquisition of gonococcal alleles, including antimicrobial resistance alleles. Such events seem to have already occurred in a sublineage of CC11, which was responsible for several IMD outbreaks and urethritis among MSM (*34*). They shared the same *penA* allele (*penA327/penAXXX-IV*) with gonococcal bacteria and showed decreased susceptibility to third-generation cephalosporins (*47*). Although PubMLST is the largest global repository of meningococcal genomes (>22,000), a paucity of genomic data were available from isolates originating from the genitourinary or respiratory tract, suggesting an underestimation of the global dissemination of CC4821. Therefore, we recommend WGS for urogenital-, rectal-, and respiratory-derived meningococci if they are exhibiting antimicrobial resistance.

CC4821 lineage 44 includes isolates from different serogroups, including MenB, MenC, and MenW. In China, MenC and MenW isolates can be prevented

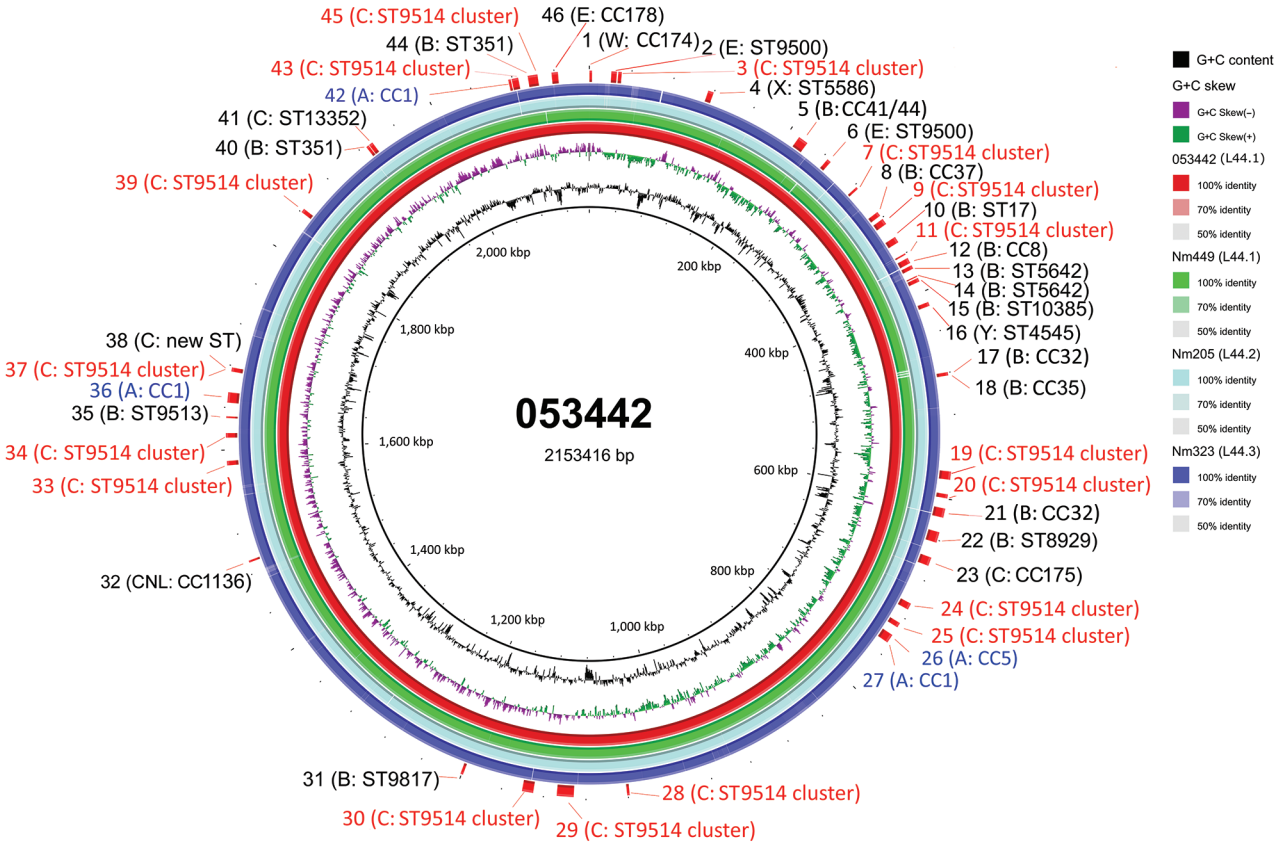


Figure 7. Circular genome map of CC4821 *Neisseria meningitidis* genome 053442 with BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) comparisons to the genomes of other sublineages. The innermost rings show guanine and cytosine (G+C) content (black) and G+C skew (negative in purple, positive in green) of genome 053442. The 4 outer rings show BLAST comparisons (using BLASTn and an E-value cutoff of 10.0) to the complete genome sequence of 053442 (red), Nm449 (green), Nm205 (pale blue), and Nm323 (blue); shading on rings indicates percentage identity as indicated in the key. Labels around the outer ring refer to the 46 HGT events involving 149 unique loci that are labeled with their possible donor strain. Red text indicates loci related to most common donors; blue text indicates those with serogroup A hyperinvasive lineage donors. CC, clonal complex; HGT, horizontal gene transfer; Nm, *N. meningitidis*; ST, sequence type.

Table 3. Loci present only in sublineage L44.1 of clonal complex 4821 of *Neisseria meningitidis**

Locus	Gene ID	Product	KEGG pathway
NEIS0364	NMCC_0368	Conserved hypothetical protein	Genetic information processing
NEIS0365	NMCC_0369	Competence protein ComFC	Signaling and cellular processes
NEIS0632	NMCC_0638	Lipoprotein	Signaling and cellular processes
NEIS2493	NMBG2136_1300	Hypothetical integral membrane protein	Not included
NEIS3165	NMCC_2037	Histone macro-H2A1-related protein	Metabolism

*KEGG, Kyoto Encyclopedia of Genes and Genomes.

by vaccines, such as group A and C meningococcal polysaccharide vaccine (MPV-AC) and MPV-ACYW, but no routinely administered vaccine is available to prevent MenB IMD (*48*). Two protein-based vaccines, targeting MenB meningococci 4CMenB (Bexsero) and rLP2086 (Trumenba), have been licensed in several countries (*49*–*50*; reference *51* in Appendix 2), but limited data are available on the bacterial coverage of these vaccines to CC4821 isolates directly from serum bactericidal activity assays, the Meningococcal Antigen Typing System (MATS) for Bexsero, or meningococcal antigen surface expression for Trumenba. One B:CC4821 isolate (M14-240580, UK) was reported to be tested using the MATS assay and showed no potential protection (reference *52* in Appendix 2). Using systems to index complex genotypic and phenotypic data, such as the MenDeVAR Index, we predicted that ≈60% of B:CC4821 disease-causing isolates might be prevented through vaccination with Trumenba; data are insufficient to infer Bexsero reactivity. Further testing of globally diverse meningococci is needed with these experimental assays to analyze potential vaccine impact in settings outside Europe.

In summary, we have undertaken a comprehensive genomic analysis of a hyperinvasive meningococcal CC4821 expressing MenB, MenC, and MenW with expansion from China to other global geographic locations with currently available genomic data. We identified key genomic factors and putative evolutionary changes that might have led to the emergence and persistence of the epidemic quinolone-resistant clone in China. Vaccine coverage for MenB CC4821 isolates needs further evaluation. Enhanced laboratory surveillance for CC4821 isolates from IMD cases and from oropharyngeal, urethral, and rectal carriage is needed to monitor global trends of expansion, which will be essential for local immunization policies.

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This study made use of *Neisseria* genomic data deposited in the *Neisseria* PubMLST Database (https://pubmlst.org/neisseria/) sited at the University of Oxford (Jolley & Maiden 2018, Wellcome open research, 3:124); the development of this database has been funded by the Wellcome Trust and European Union. This work was supported by National Natural Science Foundation of China under grant 81872909 and 81601801, Shanghai Rising-Star Program from Shanghai Municipal Science and Technology Commission (grant 17QA1403100), a Municipal Human Resources Development Program for Outstanding Young Talents in Medical and Health Sciences in Shanghai (grant 2017YQ039), and the 13th Five-Year Project of National Health and Family Planning Commission of the People's Republic of China (grants 2017ZX10303405004 and 2017ZX10103009-003). The funders had no role in the study design, data collection and interpretation, or the decision to submit the work for publication.

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Appendix 2

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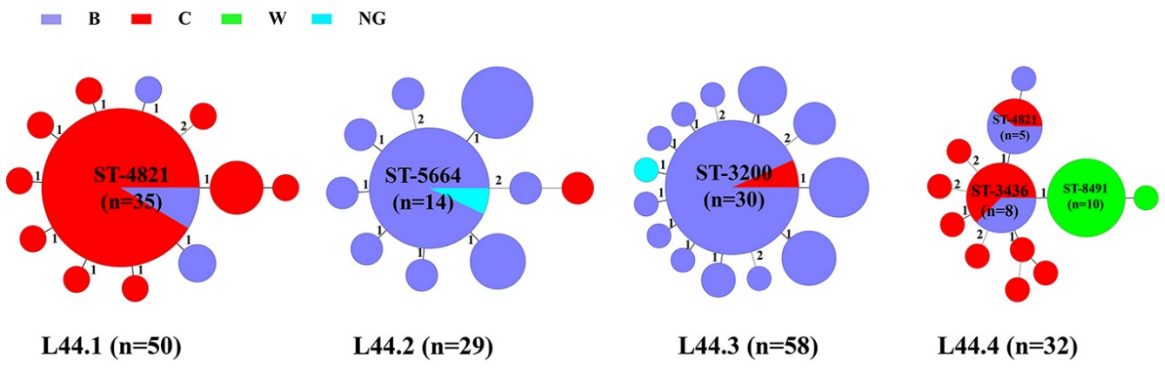
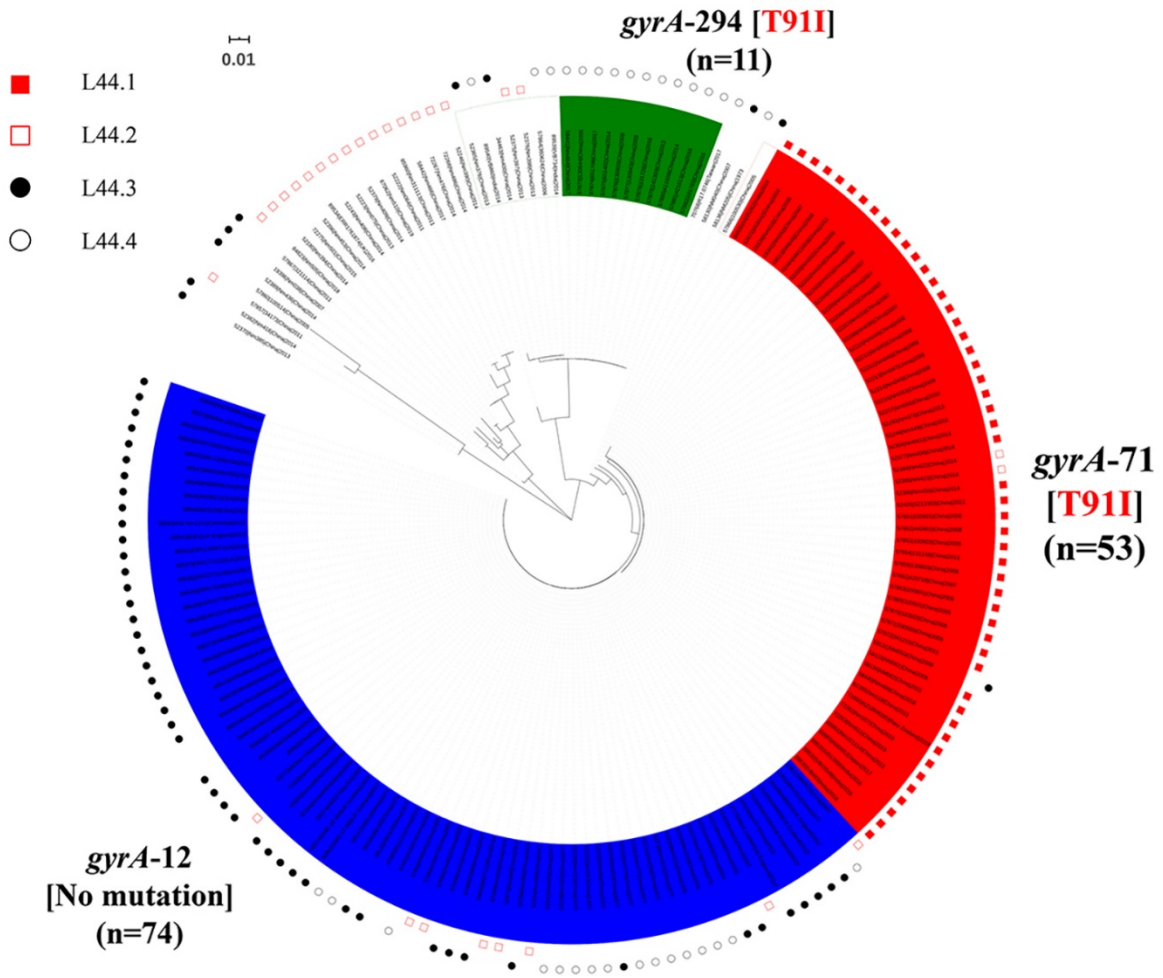
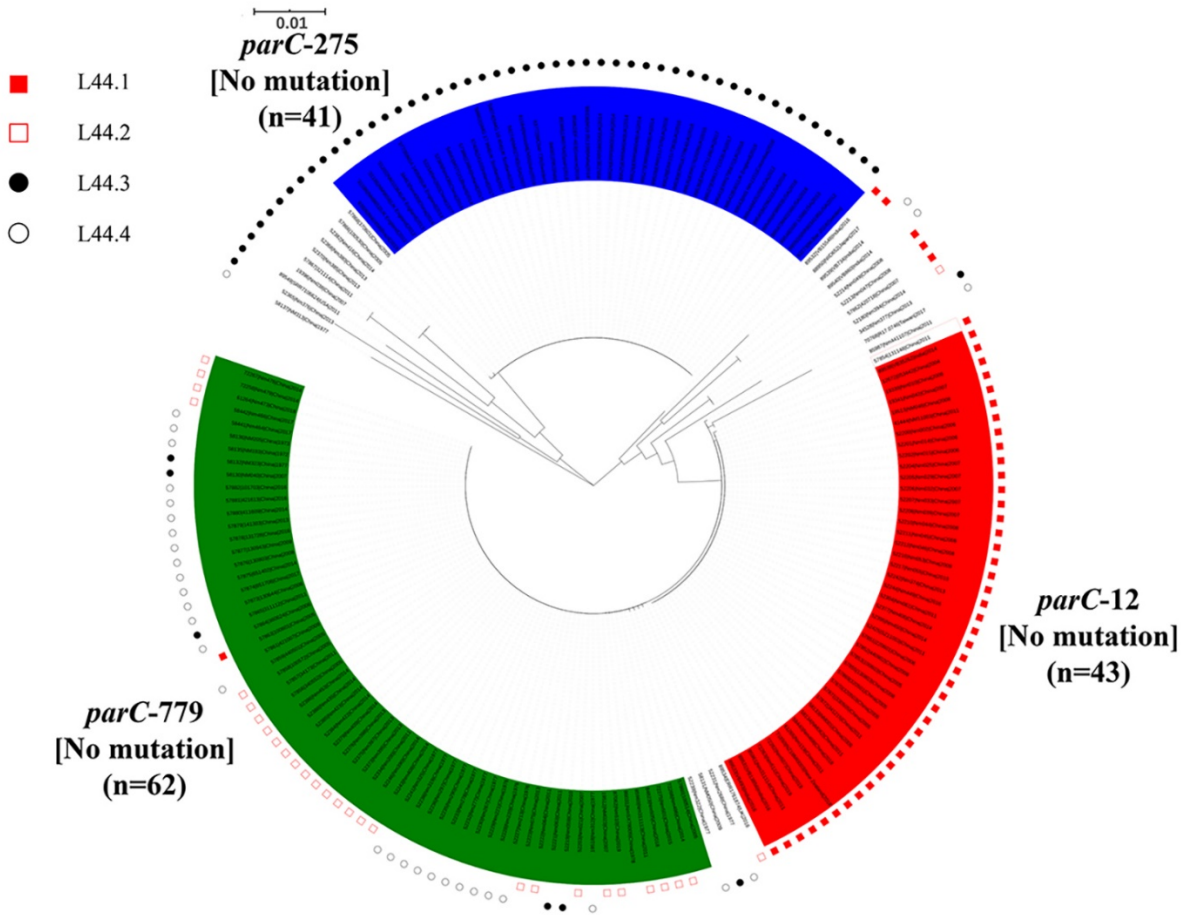


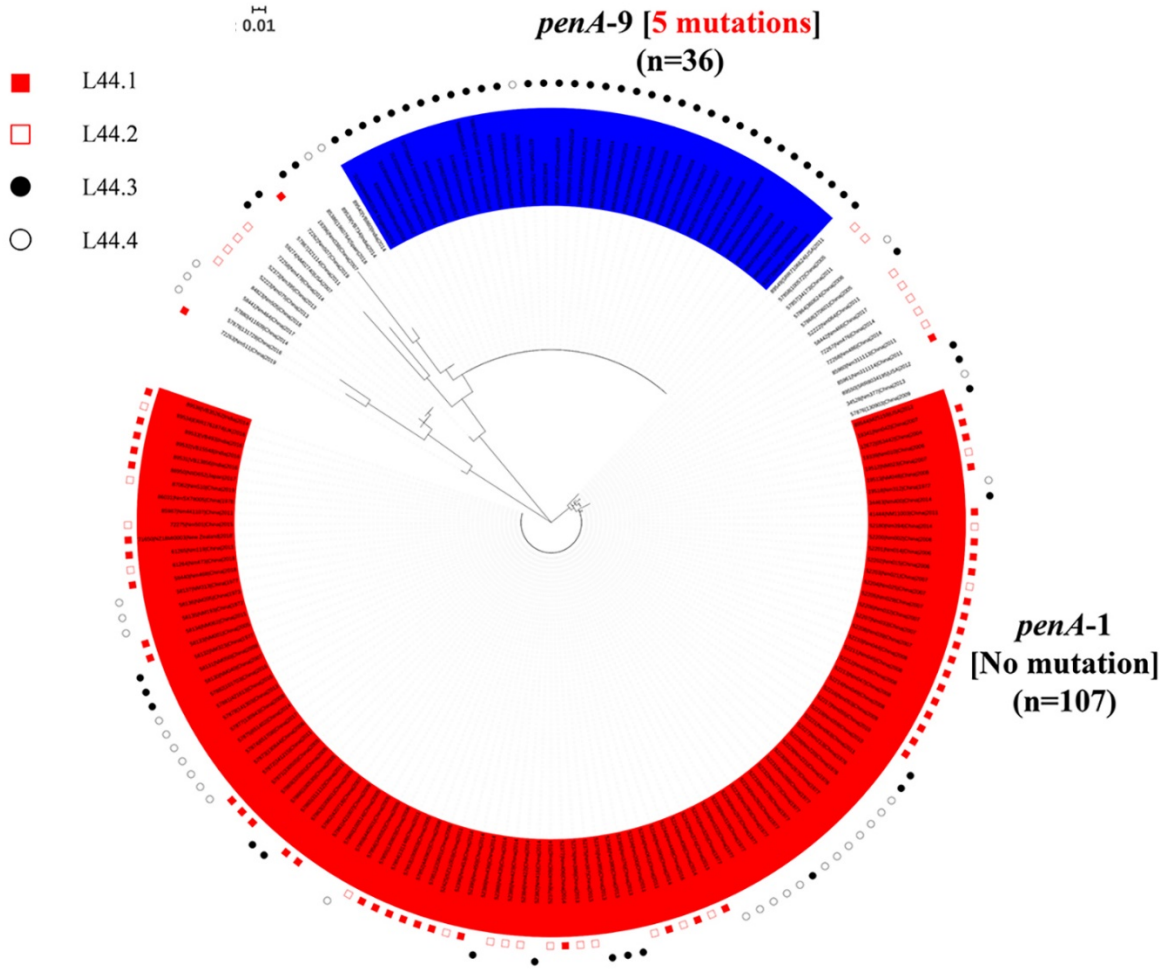
Appendix 2 Figure 1. Central sequence type of each sublineage of *Neisseria meningitidis* ST-4821 clonal complex. The numbers of different loci between the central sequence type (ST) and its neighboring STs are labeled. The ST which differs one locus to the central ST is a single-locus variant (SLV) of the central ST. In sublineage 44.1 (L44.1), ST-4821 (n = 35) and its SLVs (n = 13) account for 96% (48/50); in L44.2, ST-5664 (n = 14) and its SLVs (n = 12) take up for 89.7% (26/29); in L44.3, the percentage of ST-3200 (n = 30) and its SLVs (n = 23) is 91.4% (53/58); in L44.4, the percentage of ST-3436 (n = 8) its SLVs (n = 18) is 81.3% (26/32). B, meningococcus belonging to serogroup B; C, meningococcus belonging to serogroup C; W, meningococcus belonging to serogroup W; NG, nongroupable meningococcus bacteria not identified as any of a serogroup.



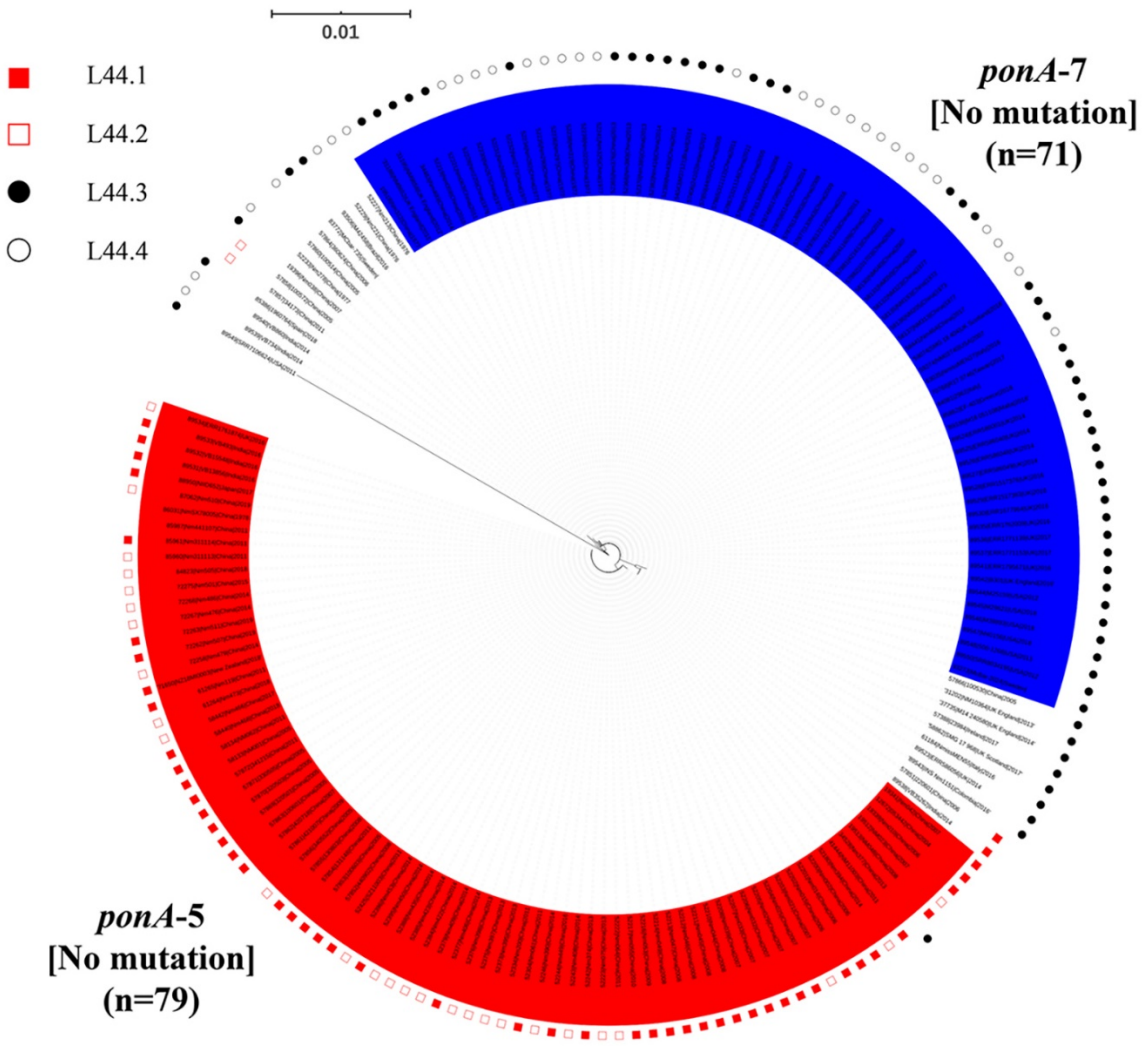
Appendix 2 Figure 2. Phylogenetic analysis of antimicrobial resistance gene sequence variation within the *Neisseria meningitidis* clonal complex 4821 lineage, based on nucleotide sequences of the *gyrA* gene. Scale bar indicates number of base substitutions per site. Blue shading indicates the genomes with *gyrA* allele 12, red shading indicates the genomes with *gyrA* allele 71, green shading, genomes with *gyrA* allele 294.



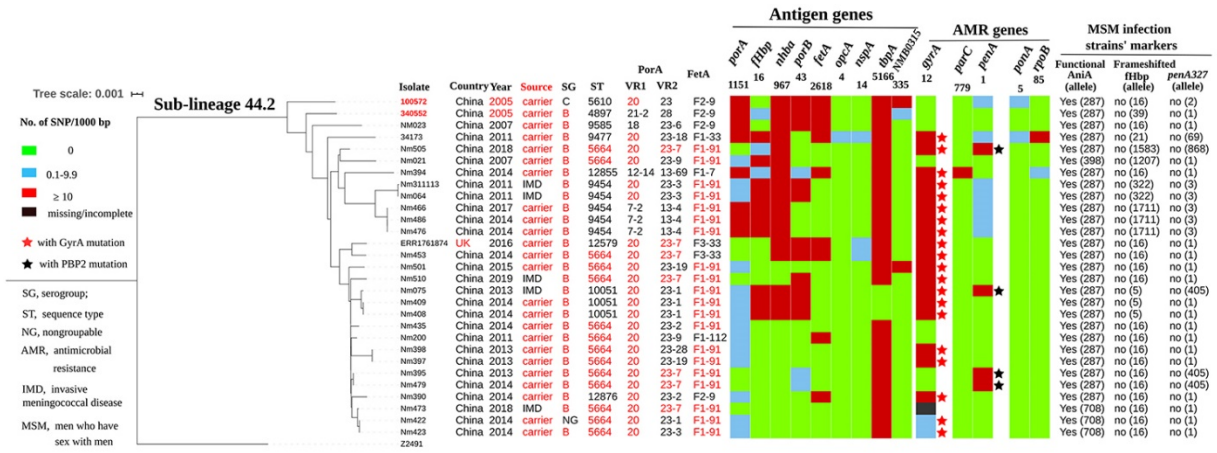
Appendix 2 Figure 3. Phylogenetic analysis of antimicrobial resistance gene sequence variation within the *Neisseria meningitidis* clonal complex 4821 lineage, based on nucleotide sequences of the *parC* gene. Green shading indicates the genomes with *parC* allele 779; red shading indicates genomes with *parC* allele 12; blue shading, genomes with *parC* allele 275.



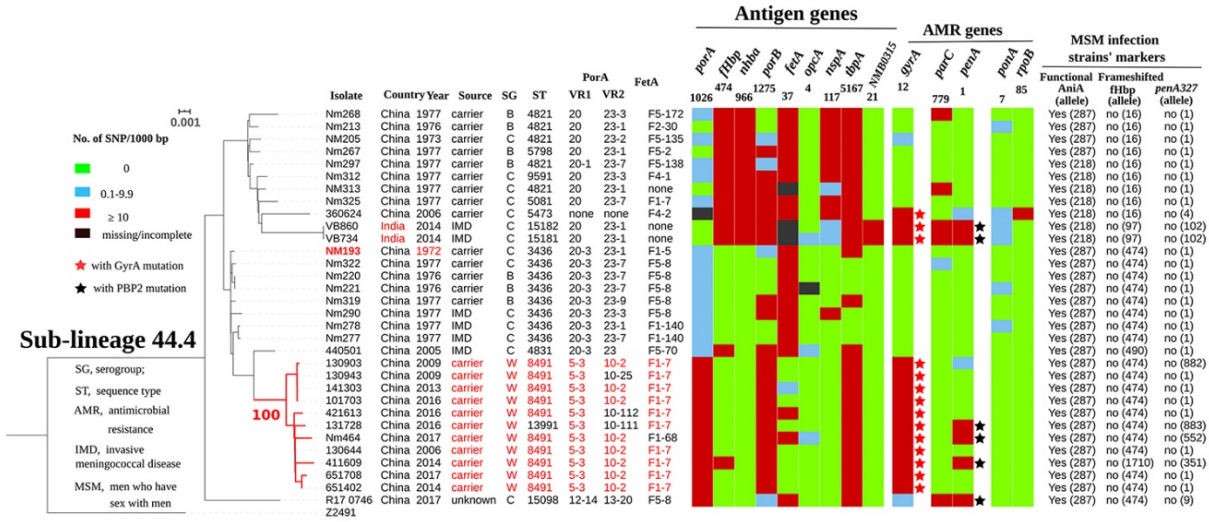
A**ppendix 2 Figure 4.** Phylogenetic analysis of antimicrobial resistance gene sequence variation within the *Neisseria meningitidis* clonal complex 4821 lineage, based on nucleotide sequences of the *penA* gene. The red shading indicates the genomes with *penA* allele 1; blue shading indicates the genomes with *penA* allele 9.



Appendix 2 Figure 5. Phylogenetic analysis of antimicrobial resistance gene sequence variation within the *Neisseria meningitidis* clonal complex 4821 lineage, based on nucleotide sequences of the *ponA* gene. The red shading indicates the genomes with *ponA* allele 5; blue shading indicates the genomes with *ponA* allele 7.

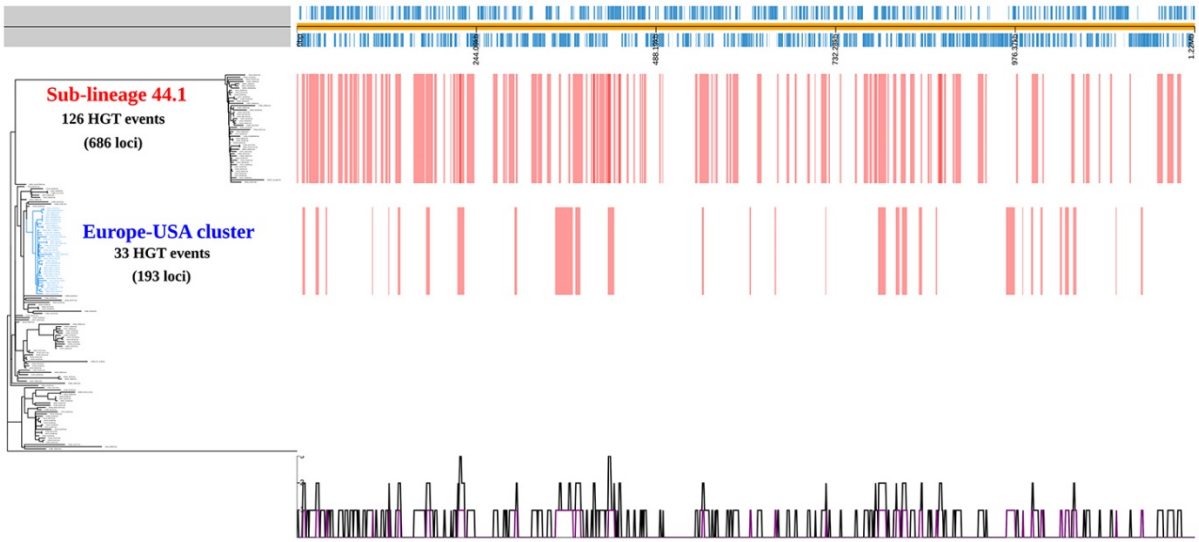


Appendix 2 Figure 6. Phylogenetic analysis of antimicrobial resistance gene sequence variation within the *Neisseria meningitidis* clonal complex 4821 lineage, based on nucleotide sequences of the *rpoB* gene. Gray shading indicates the genomes with *rpoB* allele 85.Scale bar indicates number of substitutions per site.

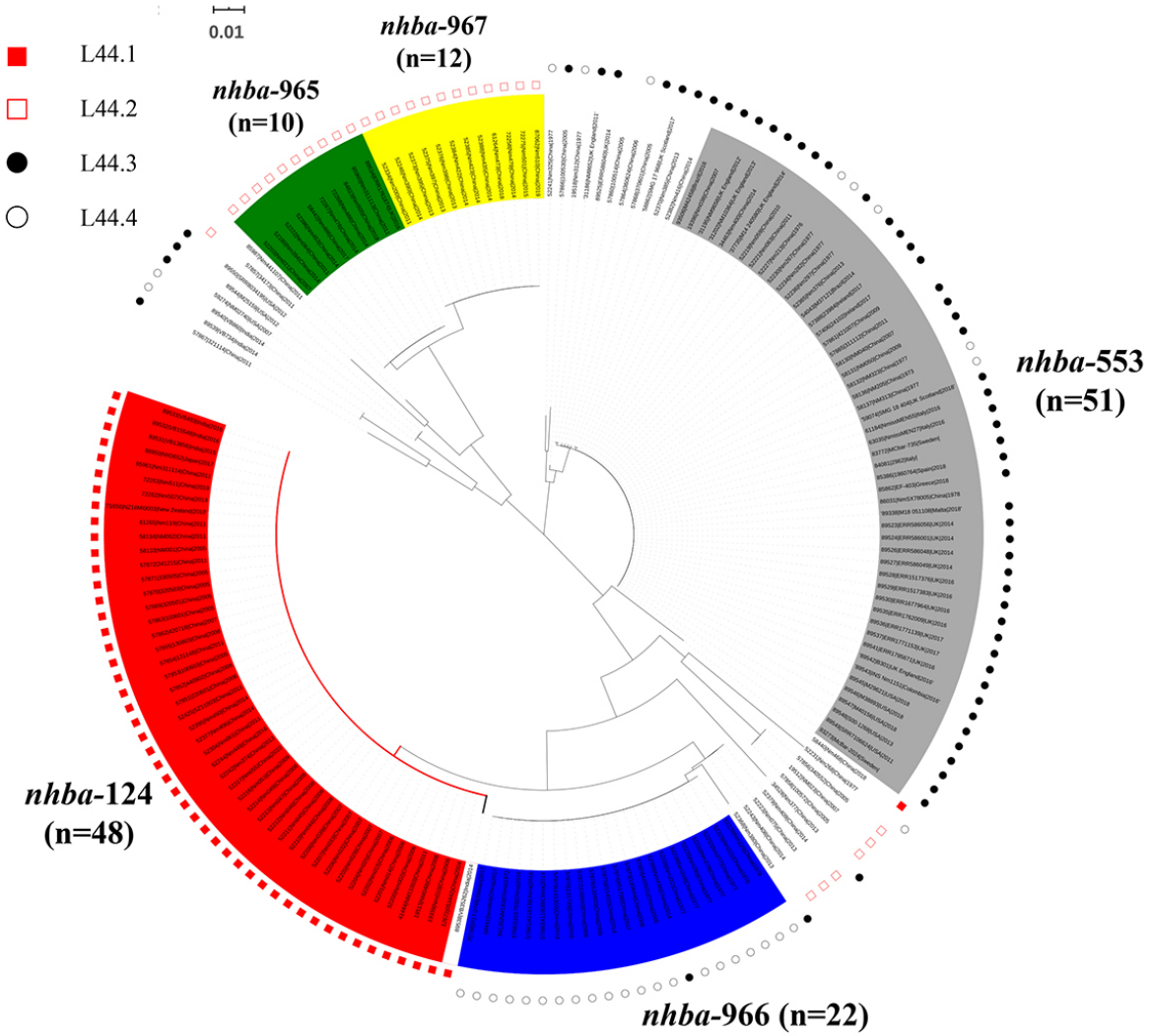


Appendix 2 Figure 7. Phylogenetic analysis of antigen gene sequence variation within the *Neisseria meningitidis* clonal complex 4821 lineage, based on nucleotide sequences of the *porA* gene. A total of 168 *porA* gene sequences were analyzed. Five genome data with incomplete *porA* sequences were excluded, including 57857 (with information of P1.7–2,14 in the PubMLST database), 57864, 57870 (with information of P1.20,23–9 in the PubMLST database), 57871 (with information of P1.7–2,14 in the PubMLST database), and 57872 (with information of P1.7–2,14 in the PubMLST database). 20-x, refers

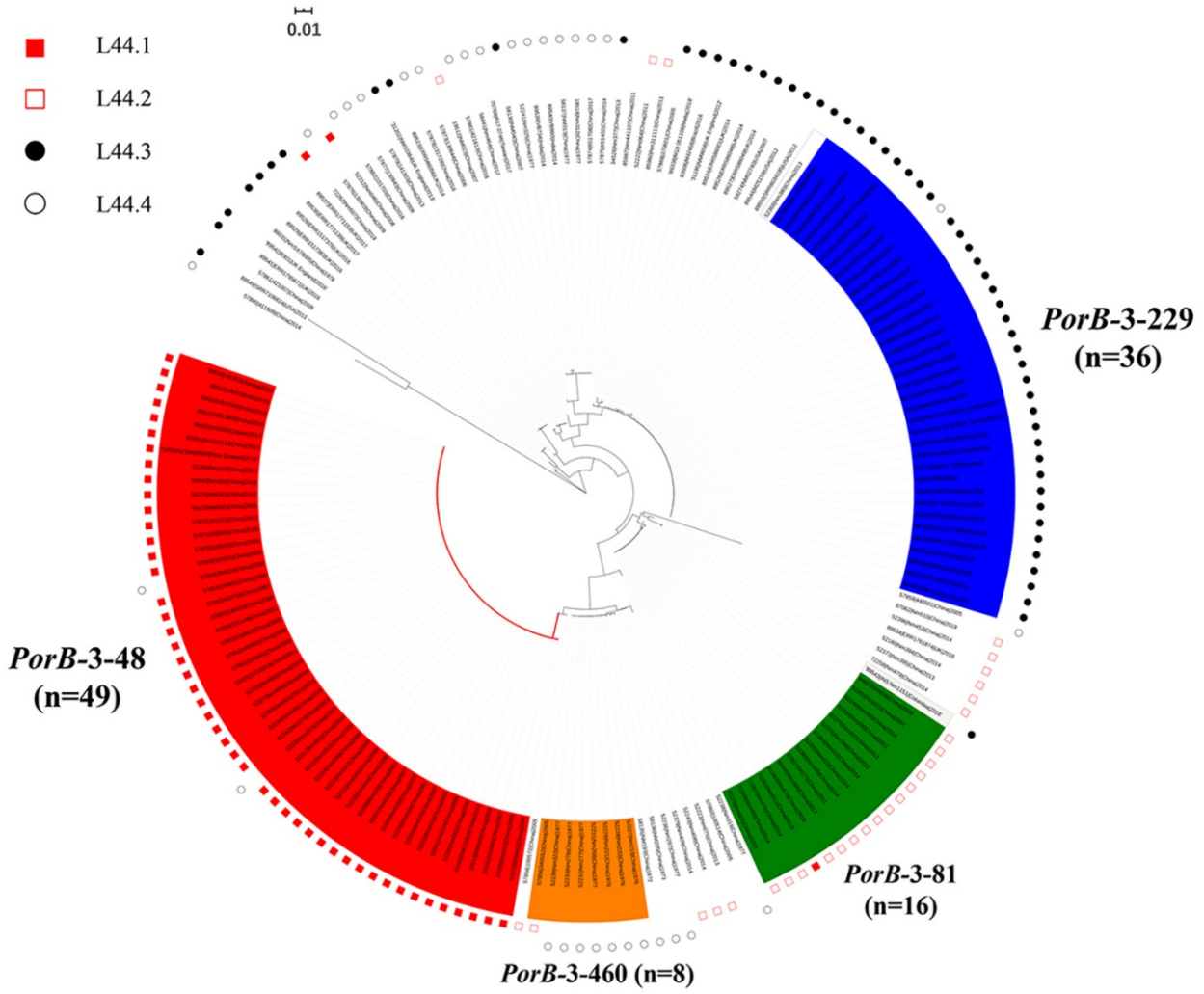
to 20, 20–1, 20–3. 23-x, refers to 23, 23–1, 23–2, 23–3, 23–6, 23–7, 23–9, 23–19, and 23–28. Gray shading indicates the genomes with PorA subtype P1. 20-x–2, 23-x; blue shading indicates the genomes with P1.17–6, 23-x; red shading with P1.7–2,14; the orange shading with P1.12–1,16-8; yellow shading with P1.5–3,10-2.



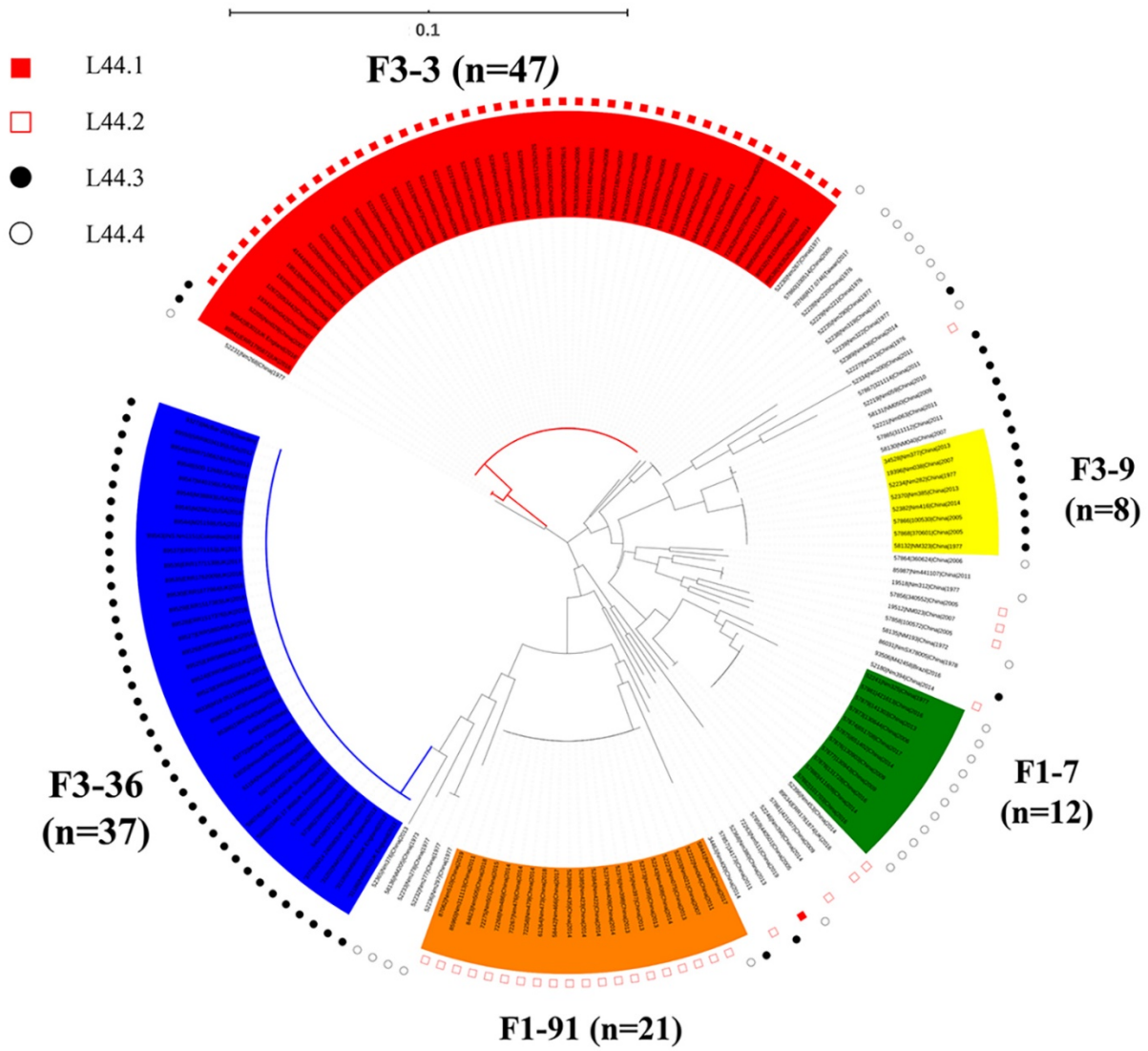
Appendix 2 Figure 8. Phylogenetic analysis of antigen gene sequence variation within the *Neisseria meningitidis* clonal complex 4821 lineage, based on nucleotide sequences of the *fHbp* gene. Gray shading indicates the genomes with *fHbp* allele 16; blue shading indicates the genomes with *fHbp* allele 474;red shading with *fHbp* allele 498; orange shading with *fHbp* allele 489; green shading with *fHbp* allele 22. Scale bar indicates number of base substitutions per site.



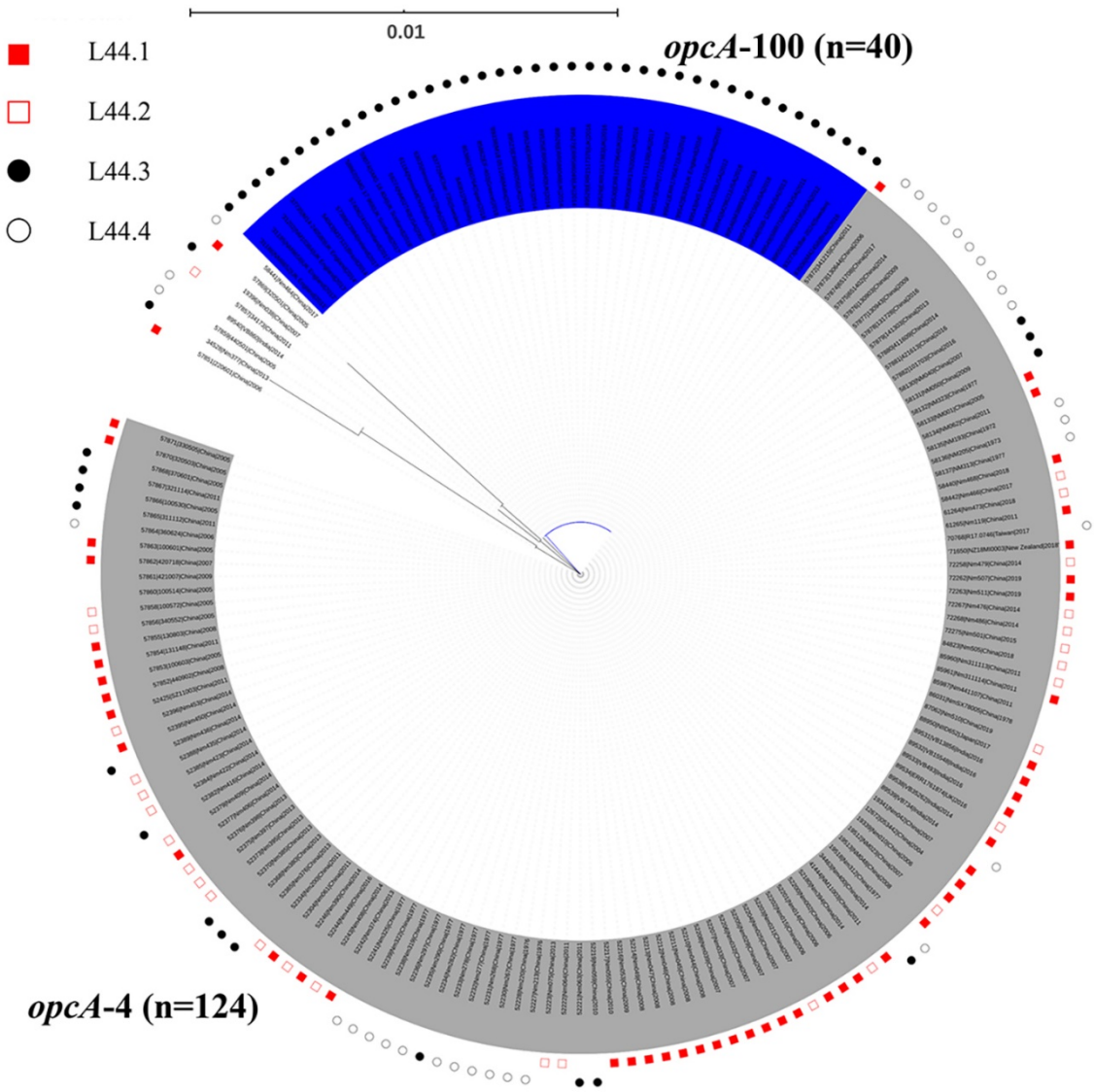
Appendix 2 Figure 9. Phylogenetic analysis of antigen gene sequence variation within the *Neisseria meningitidis* clonal complex 4821 lineage, based on nucleotide sequences of the *nhba* gene. Gray shading indicates the genomes with *nhba* allele 553; red shading with *nhba* allele 124; blue shading indicates the genomes with *nhba* allele 966; orange shading with *nhba* allele 967; green shading with *nhba* allele 965.



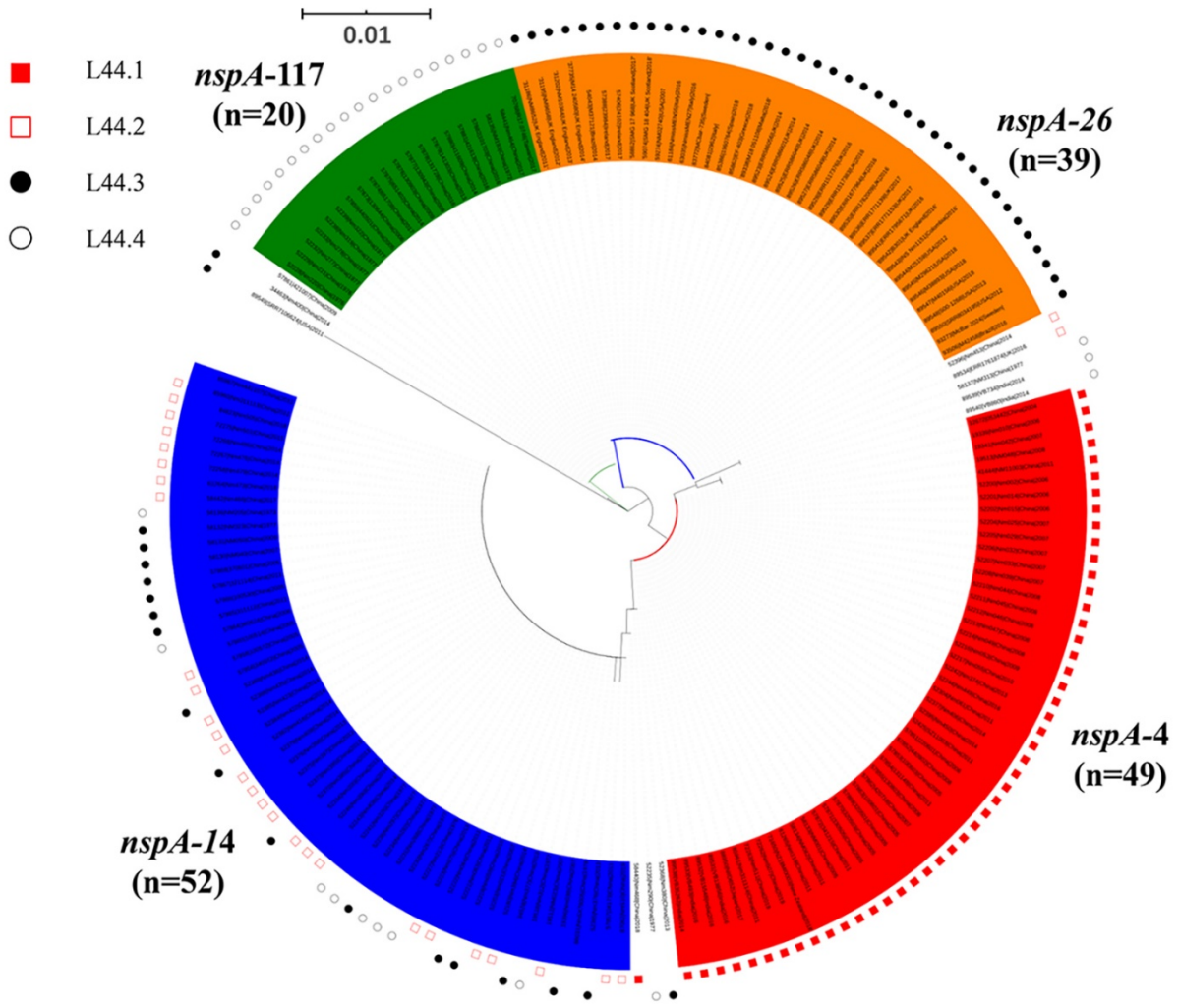
Appendix 2 Figure 10. Phylogenetic analysis of antigen gene sequence variation within the *Neisseria meningitidis* clonal complex 4821 lineage, based on nucleotide sequences of the *porB* gene. Red shading indicates the genomes with PorB peptide 3-48; blue shading indicates the genomes with PorB peptide 3-229; green shading with PorB peptide 3-81; orange shading with PorB peptide 3-460.



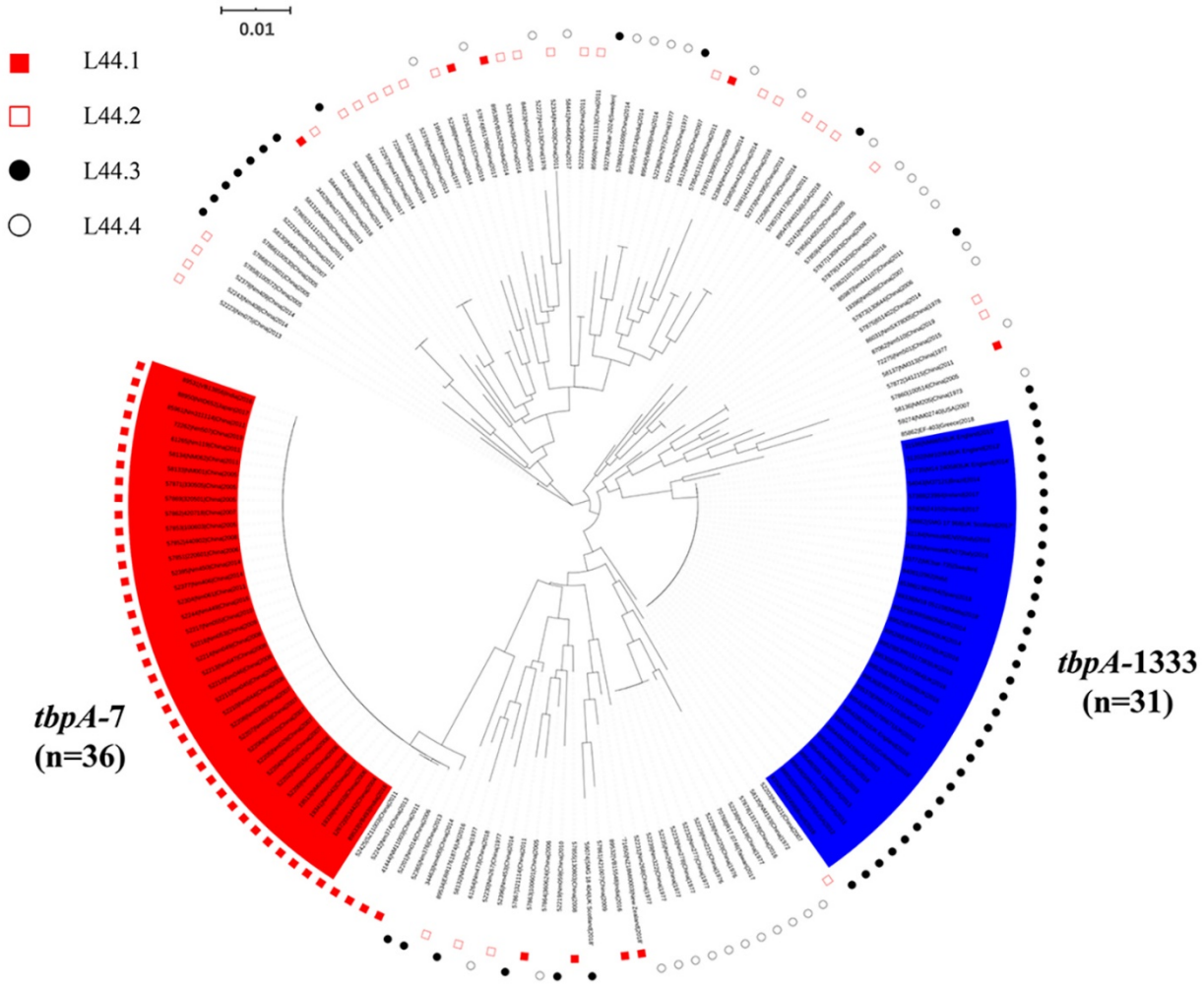
Appendix 2 Figure 11. Phylogenetic analysis of antigen gene sequence variation within the *Neisseria meningitidis* clonal complex 4821 lineage, based on nucleotide sequences of the *fetA* gene. Red shading indicates the genomes with FetA variant 3-3; blue shading indicates the genomes with FetA variant 3-36; the orange shading with FetA variant 1-91; green shading with FetA variant 1-7; yellow shading with FetA variant 3-9.



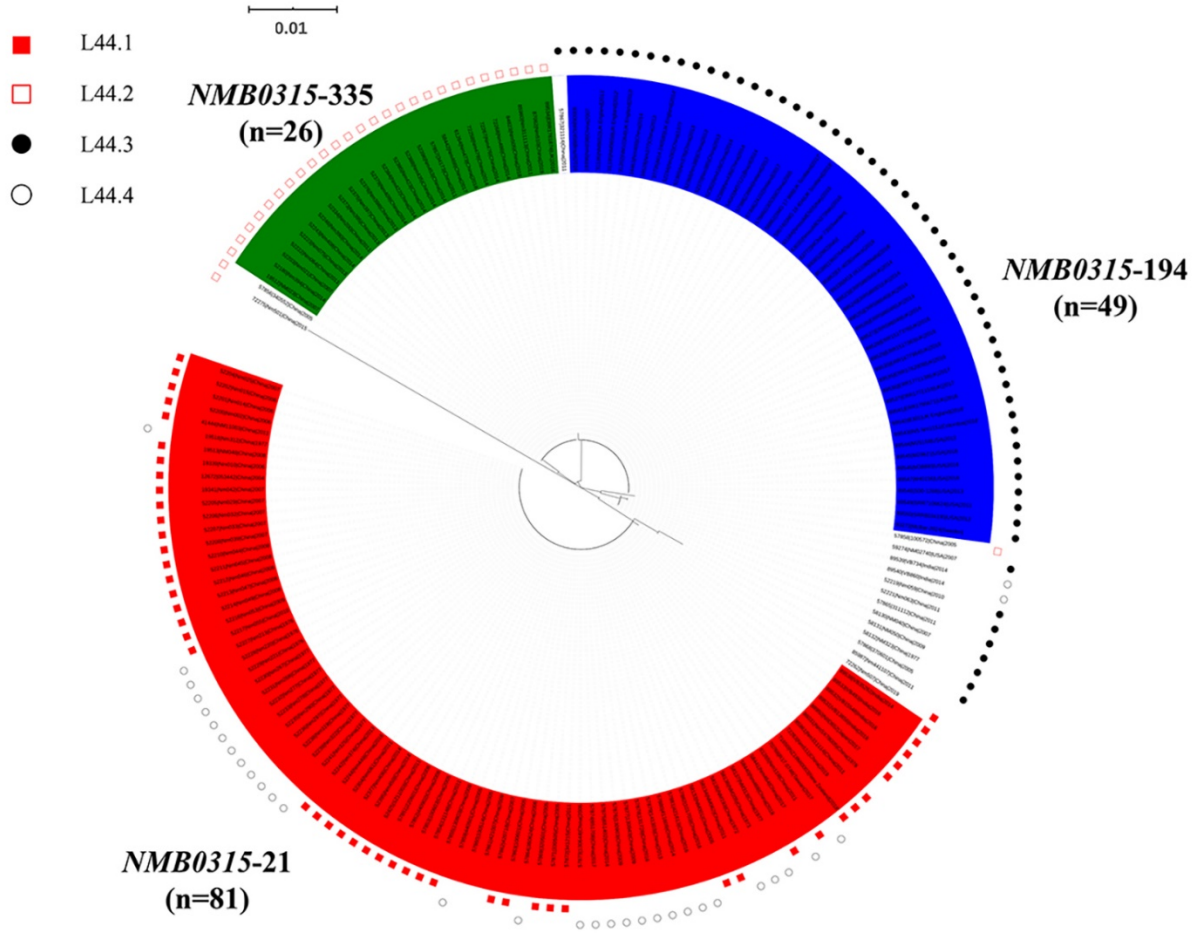
Appendix 2 Figure 12. Phylogenetic analysis of antigen gene sequence variation within the *Neisseria meningitidis* clonal complex 4821 lineage, based on nucleotide sequences of the *opcA* gene. Gray shading indicates the genomes with *opcA* allele 4; blue shading indicates the genomes with *opcA* allele 100.



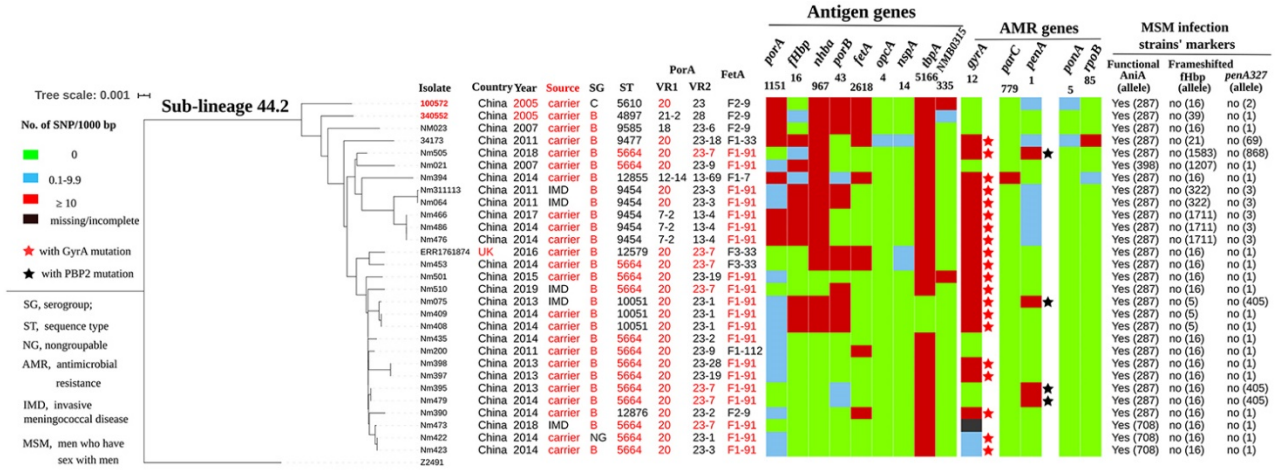
Appendix 2 Figure 13. Phylogenetic analysis of antigen gene sequence variation within the *Neisseria meningitidis* clonal complex 4821 lineage, based on nucleotide sequences of the *nspA* gene. Blue shading indicates the genomes with *nspA* allele 14; red shading with *nspA* allele 4; orange shading with *nspA* allele 26; green shading with *nspA* allele 117.



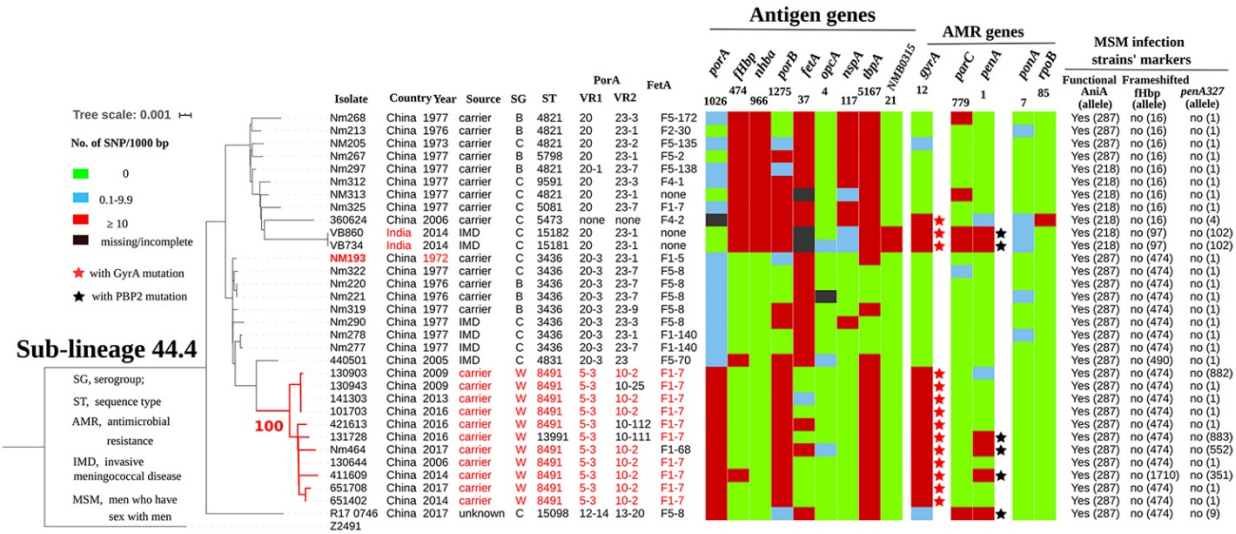
Appendix 2 Figure 14. Phylogenetic analysis of antigen gene sequence variation within the *Neisseria meningitidis* clonal complex 4821 lineage, based on nucleotide sequences of the *tbpA* gene. Red shading indicates the genomes with *tbpA* allele 7; blue shading indicates the genomes with *tbpA* allele 1333.



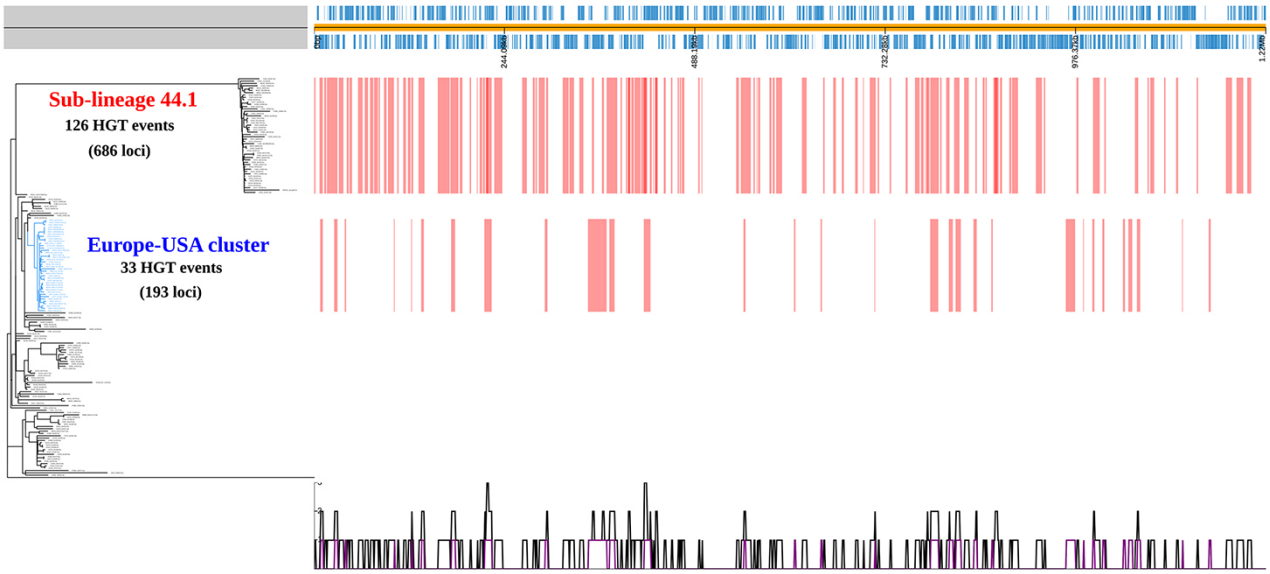
Appendix 2 Figure 15. Phylogenetic analysis of antigen gene sequence variation within the *Neisseria meningitidis* clonal complex 4821 lineage, based on nucleotide sequences of the *NMB0315* gene. Red shading indicates the genomes with *NMB0315* allele 21; blue shading with *NMB0315* allele 194; green shading with *NMB0315* allele 335.



Appendix 2 Figure 16. Genetic diversity of sublineage 44.2 isolates. Red text indicates the oldest isolate of the sublineage in the columns of Isolate and Year, the isolates from countries outside of China in the Country column, and the dominant type/allele in the columns of Source, SG, ST, PorA-VR1, PorA-VR2, and FetA. The numbers underneath the antigen genes and AMR genes are the dominant alleles for that particular gene, and the colored blocks for SNPs/1,000 bp were determined using the allele number labeled above each column as the reference allele.



Appendix 2 Figure 17. Genetic diversity of sublineage 44.4 isolates. The red texts are used to indicate the oldest isolate of the sublineage in the columns of Isolate and Year, the isolates from countries outside of China in the Country column, and the dominant type/allele among serogroup W isolates in the columns of Source, SG, ST, PorA-VR1, PorA-VR2, and FetA. The numbers underneath the antigen genes and AMR genes are the dominant alleles for that particular gene, and the colored blocks for SNPs/1000 bp were determined using the allele number labeled above each column as the reference allele.



Appendix 2 Figure 18. Horizontal gene transfer (HGT) events predicted by Gubbins. Each red vertical line represents a HGT event. A total of 126 HGT events involved with 686 loci in L44.1; 33 events in Europe–USA cluster involved with 193 loci.