

Prevalence and genetic diversity of two adhesion-related genes, *pilE* and *nadA*, in *Neisseria meningitidis* in China

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SUMMARY

The main *Neisseria meningitidis* adhesion molecules, type IV pili (Tfp) and *Neisseria* adhesion A (NadA), play important roles in the pathogenesis of invasive meningococcal disease. PilE is the major Tfp subunit. In this study, the prevalence and genetic diversity of *pilE* and *nadA* were investigated in the prevalent serogroups and clonal complexes (CC) of *N. meningitidis* isolated in China. All serogroup A strains belonging to CC1 and CC5 and all CC11 serogroup W135 strains were clustered into class II PilE clades. All serogroup C and most of serogroup B isolates except CC8 and ST5642 were class I PilE clades. Class II *pilE* sequences were highly conserved. All isolates belonging to class I PilE isolates were *nadA* negative. However, *nadA*-positive strains were exclusively found in CC5 and CC11 isolates (class II PilE). This study showed that PilE and NadA may be related to epidemic or endemic meningococcal disease.

Key words: Infectious disease epidemiology, meningococcal disease, molecular epidemiology, *Neisseria meningitidis*.

INTRODUCTION

Neisseria meningitidis is a leading cause of invasive bacterial infections globally, with devastating morbidity and mortality [1, 2]. The incidence rates of infections with meningococcal disease vary from 1 to 1000 cases per 100 000 in different regions worldwide with a constant fatality rate of about 10% [2, 3]. There are 12 serogroups of meningococci based on the biochemical composition of the polysaccharide capsule. Serogroups A, B, C, W135 and Y are responsible

for more than 90% of the invasive meningococcal disease [4]. The epidemic of meningococcal disease shows regional characteristics. Serogroup A caused two global pandemics in the last century and was still associated with the highest morbidity in the meningitis belt of sub-Saharan Africa and Asia [2]. Serogroup B and C meningococci only caused regional epidemics of invasive meningococcal disease, in North American, Europe and China. Serogroup W135 strains caused an outbreak in 2000 and have subsequently caused sporadic disease and epidemics worldwide [2]. Multilocus sequence typing (MLST) is a commonly used tool for molecular characterization of *N. meningitidis* [5]. Based on MLST, hyperinvasive lineages can be recognized as different clonal complexes (CCs). Hyperinvasive CC8, CC11, CC32 and

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CC41/44 meningococci have been responsible for endemic invasive meningococcal disease in different regions. Serogroup A meningococci belonging to CC5 and CC1 were associated with two pandemics globally [6]. Serogroup W135 meningococci belonging to CC11 led to several outbreaks and were reported in different countries [7]. In China, CC1 and CC5 serogroup A were the main causes of meningococcal disease before outbreaks associated with CC4821 serogroup C occurred in 2003 [8, 9].

Colonization, which is indispensable for bacterial propagation and dissemination, is the first step in the pathogenesis of most infectious diseases [10]. Type IV pili (Tfp), the major adhesion molecules of *N. meningitidis*, are composed of a multimer of the major pilin subunit, Pile [11]. *N. meningitidis* expresses one of two structurally distinct types of pili according to their reaction (class I) or lack of reaction (class II) with the monoclonal antibody SM1 [12]. Not only do Tfp function as an important virulence factor involved in mediating bacteria adhesion to human cell receptors, they are also involved in twitching motility [13], biofilm formation [14] and natural competence for DNA uptake [15].

In addition to Tfp, the outer membrane protein and potential virulence factor *Neisseria* adhesion A (NadA) also plays an important role in adhesion and invasion of human cells. It is reported that *nadA* is present in about 50% of invasive meningococcal isolates [16] and is more frequently associated with strains belonging to hypervirulent lineages, such as CC32, CC11 and CC8. However, this gene is absent from CC41/44 [16, 17] and CC269 [18]. NadA is poorly represented in carriage isolates [16, 17], supporting the hypothesis that this is a disease-related gene essential for full meningococcal virulence. Currently, five NadA variants (NadA-1 to NadA-5) have been described, of which NadA-1, -2 and -3 are mainly represented in the CC32, CC11 and CC8 hypervirulent lineages and are highly cross-reactive. NadA-4 is mainly represented in carriage isolates [17]. NadA-5 is associated with sequence type (ST) 213 CC isolates [19, 20].

Adhesion is the first and most important step in disease generation, dissemination and epidemic [10]. Molecular and epidemiological characterization of the diverse serogroups and clonal complexes of adhesion-related proteins of *N. meningitidis* is crucial for the elucidation of the mechanism underlying the epidemiology of meningococcal disease. Therefore, in this study, the prevalence and sequence variation of

the genes encoding the major pilin and NadA were investigated in disease-causing and carrier isolates of *N. meningitidis*.

METHODS

Meningococcal strains and DNA preparation

A total of 144 *N. meningitidis* strains, isolated from 25 provinces of China between 1963 and 2011, were selected for this study. This panel of strains was selected from our *N. meningitidis* strain database with 1433 *N. meningitidis* strains isolated from China with accurate data on serogroup and clonal complex, as well as epidemiological information such as isolation date, isolation province and source. We selected the panel of strains tested in this study based on both experimental data and epidemiological information. Almost 10% of strains of our database were used in this study. Details of these strains including year of isolation, sequence type (ST), clonal complex (CC) and serogroup are shown in the Supplementary online material (Supplementary Table S1). Isolates were propagated on single plates of Columbia agar in a 5% CO₂ atmosphere at 37 °C for 18 h. Genomic DNA was extracted using a Qiagen DNA mini-kit (Qiagen, Germany) according to the manufacturer's instructions. The strains were isolated from 70 (48.6%) throat swab samples from healthy carriers and 74 (51.4%) blood or cerebrospinal fluid samples from patients. The following 10 clonal complexes were represented among these strains: ST1 ($n=11$), ST103 ($n=1$), ST11 ($n=10$), ST174 ($n=3$), ST198 ($n=3$), ST32 ($n=2$), ST41/44 ($n=2$), ST4821 ($n=40$), ST5 ($n=44$) and ST8 ($n=1$). Twenty-seven other strains had unassigned (UA) complex sequence types. The serogroups were A ($n=48$), B ($n=35$), C ($n=43$), W135 ($n=10$), X ($n=2$) and non-groupable (NG, $n=6$).

PCR amplification and sequencing

The major pilin genes *pilE* and *nadA* were amplified and sequenced using primers described previously [17, 20, 21]. The PCR conditions used were as described previously [20, 21]. Each locus sequence was analysed on an ABI Prism 3130 or 3730 DNA sequencer (Applied Biosystems, USA).

Nucleotide sequence determination, sequence alignment and analysis

The nucleotide sequence of each gene was analysed using NCBI nucleotide BLAST and by comparison

Table 1. Prevalence and distribution of class I and class II *pilE* genes of *N. meningitidis* strains isolated in China

<i>pilE</i>	No. of isolates	Clonal complex (CC)	Serogroup
Class I	60	CC4821 ($n=34$)	B ($n=6$), C ($n=26$), W135 ($n=1$), X ($n=1$)
		UA ($n=16$)*	B ($n=9$), C ($n=5$), NG ($n=2$)
		Five other clonal complexes ($n=10$)†	B ($n=5$), C ($n=1$), W135 ($n=3$), X ($n=1$)
Class II	71	CC1 ($n=11$)	A ($n=11$)
		CC5 ($n=44$)	A ($n=37$), C ($n=7$)‡
		CC11 ($n=10$)	B ($n=4$), C ($n=1$), W135 ($n=5$)
		CC8 ($n=1$) and ST5642 ($n=5$)§	B ($n=6$)

* UA, Unassigned to any currently known clonal complex.

† Five other clonal complexes contain CC103 ($n=1$), CC174 ($n=3$), CC198 ($n=2$), CC32 ($n=2$) and CC41/44 ($n=2$).

‡ Seven ST7 serogroup C strains were confirmed to have undergone capsular switching from serogroup A ST7 strains [27].

§ Five ST5642 strains exhibited MLST allele profiles similar to CC8, and the sequences of class II *pilE* genes were identical to the strain of CC8.

with previously published data. Published whole-genome sequences were used in this study including 8013 [22], Z2491 [23] and MC58 [24], with *pilE* belonging to class I, and FAM18 [25], and WUE-2594 [26] belonging to class II *pilE*. The coding sequences of the *pilE* genes were identified by manual alignment with either 8013 or FAM18 *pilE* sequences using the DNASTAR program. The NadA amino-acid (aa) sequence was assigned to one of the five main protein forms; NadA-1 to NadA-5. The published NadA GenBank accession numbers used in this study for determination and alignment were FJ619641, FJ619642, AF452482, FJ619644 and FJ619645, corresponding to variants 1–5, respectively [16, 20]. The subvariants of NadA were classified in terms of their main variant group, i.e. variants 1, 2, 3, 4, or 5 [16, 17, 19]. Nucleotide and aa sequences were aligned using the Clustal W program of BioEdit software and manual adjustment, and presented using Boxshade (http://www.ch.embnet.org/software/BOX_form.html). Phylogenetic analyses of nucleotide and deduced aa sequences were performed with the MEGA 4 software package using the neighbour-joining method and Kimura's two-parameter and p-distance models. The positions containing alignment gaps and missing data were eliminated in pairwise sequence comparisons (pairwise deletion option).

RESULTS

Prevalence and distribution of *pilE* in Chinese strains

In all the strains ($n=144$) analysed in this study, the *pilE* gene was detected in 91% (131/144) of strains with primers specific for either class I or class II alleles. We used PCR and dot blotting methods to detect the *pilE* gene in 144 strains. The *pilE* gene was not amplified from 13 strains, from CC4821 ($n=6$), CC198 ($n=1$) and UA ($n=6$). Of the 13 strains, nine were carriage isolates and four were isolated from patients. Results from this primary analysis (Table 1) demonstrated that class I *pilE* alleles were present in 41.7% (60/144) of tested isolates in this study, containing strains belonging to seven different clonal complexes and five divergent serogroups. All 34 CC4821 *pilE*-positive strains contained class I *pilE* alleles. However, class II *pilE* alleles (49.3%, 71/144) were only present in isolates belonging to CC1, CC5, CC11 CC8 and ST5642 (UA). Of the 71 class II *pilE* isolates, 62% (44/71) of isolates belonged to CC5 and all of serogroup A were represented in class II. Of the 60 class I *pilE* isolates, 33 were carriage strains and 27 were disease strains. Carriage strains were from CC174, CC198, CC32, CC41/44, CC4821, UA, and disease strains were from CC103, CC32, CC41/44, CC4821, UA. In a total of 71 class II *pilE* isolates,

Table 2. Genetic diversity of the major pilin (*PilE*) in *N. meningitidis*. Alignment length, number of isolates, alleles, amino-acid sequence types and overall mean distance between nucleotide sequences

<i>pilE</i> class	Length (bp)	No. of isolates	No. of alleles	Mean distance between nucleotide sequences*	No. of peptide sequences
Class I	498–528	63†	55	0.122	55
Class II (CC1)	432	11	2	0.000	2
Class II (CC5)	444	45‡	4	0.000	4
Class II (CC11)	447–459	11§	6	0.094	6
Class II (CC8, ST5642)	447	6	1	0.000	1

* The number of base differences per nucleotide site from averaging all sequences pairs using Kimura's two-parameter model.

† Corresponds to 60 isolates described in Supplementary Table S1, and three sequenced *N. meningitidis* strains (8013, Z2491, MC58).

‡ Corresponds to 44 isolates described in Supplementary Table S1, and one sequenced *N. meningitidis* strain (WUE-2594).

§ Corresponds to ten isolates described in Supplementary Table S1, and one sequenced *N. meningitidis* strain (FAM18).

28 carriage strains were from CC1, CC5, CC11, ST5642, and 43 disease strains were from CC1, CC5, CC8, CC11, ST5642.

Diversity of *pilE* in Chinese strains

The major pilin genes ($n = 131$) were sequenced, and the nucleotide and the deduced aa sequences of the corresponding mature proteins were aligned. The *pilE* gene from five sequenced meningococcal genomes was also included for comparison. The extent of sequence variation of class I and class II *pilE* genes was calculated as the overall mean distance between nucleotide sequences using Kimura's two-parameter model. As previously reported [21, 28], there was considerable variation in *pilE* between the strains harbouring class I pilins, as shown by the substantial overall mean distance between nucleotide sequences (0.122) (Table 2) and the alignment of *pilE* sequences (Fig. 1).

In contrast, the class II *pilE* genes exhibited a high degree of sequence conservation. Pilins within the CC1, CC5, CC11, CC8 (and five of ST5642) were almost identical, as indicated by the very small mean distances between nucleotide sequences (0.000, 0.000, 0.094, 0.000, respectively) in these strains (Table 2). The mature pilins from the isolates belonging to CC1 differed only by a single aa at position seven (Supplementary Fig. S1). The majority (42/45) of CC5 strains were found to encode a PilE that was identical with the exception of one or two aa changes at positions 30, 102, 110 and 146 (Supplementary Fig. S2). Only one strain (ST5655) belonged to CC8, while five ST5642 strains contained three alleles shown by

MLST to be identical to CC8 (ST8). Furthermore, the aligned aa sequences of class II PilE were identical to the CC8 strains (Supplementary Fig. S3). Of the CC11 isolates, six strains differed by only four aa residues (Supplementary Fig. S4). The remaining isolates, encoded a slightly different PilE compared to these six strains, although at least 99.5% identity was observed in the remaining five strains (Supplementary Fig. S5). The phylogenetic analysis of the major pilins revealed that only 13 aa sequence types were identified in all 73 isolates expressing class II pilins, whereas as many as 55 different aa sequence types were found for class I pilins ($n = 63$). The level of prevalence, distribution and diversity of *pilE* genes in Chinese strains was further highlighted by phylogenetic analysis of all the PilE aa sequences (Fig. 2).

Prevalence and distribution of *nadA* in Chinese strains

The *nadA* gene was detected in 54/144 (37.5%) *N. meningitidis* isolates, and the distribution of clonal complexes in the 23 carriage and 31 disease strains was the same, with only CC5 and CC11 present in those isolates. The prevalence of *nadA* in different serogroups and its correlation with the *pilE* gene class is shown in Table 3. The *nadA* gene was more frequently detected in serogroup A strains (77.1%) than in other serogroups, with none detected in class I *pilE* isolates. All the strains ($n = 44$) belonged to CC5 and consisted of 37 serogroup A *nadA* PCR⁺ isolates and seven serogroup C strains, while the remaining isolates ($n = 10$) belonged to CC11 and consisted of four serogroup B *nadA* PCR⁺ isolates, one serogroup

Strain ID	1	20	40	60	80
40-ST4821-C	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
22491-ST4-A	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
MC58-ST32-B	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
053442-ST4821-C	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
8013-ST18-C	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
121-ST4821-X	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
115-ST4821-C	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
114-ST4821-C	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
113-ST4821-C	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
111-ST4821-C	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
110-ST4821-C	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
109-ST4821-C	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
108-ST4821-C	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
106-ST4821-C	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
105-ST4821-C	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
104-ST4821-C	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
103-ST4821-C	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
102-ST4821-C	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
101-ST4821-C	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
99-ST4821-C	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
98-ST4821-C	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
97-ST4821-C	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
94-ST4821-C	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
93-ST4821-C	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
85-UA-C	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
83-UA-C	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
82-UA-C	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
81-UA-C	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
80-UA-C	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
78-ST4821-B	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
77-ST4821-C	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
75-ST198-C	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
74-ST174-W135	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
70-ST4821-C	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
69-ST4821-C	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
67-ST4821-C	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
65-ST198-B	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
64-UA-NG	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
61-UA-B	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
59-UA-B	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
58-UA-B	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
57-ST4821-B	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
56-ST4821-C	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
54-ST4821-B	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
52-ST4821-C	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
49-ST3615-B	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
48-ST32-B	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
38-ST4821-C	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
30-UA-NG	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
25-ST103-X	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
22-ST5863-B	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
19-ST4821-B	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
18-ST41/44-B	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
14-UA-B	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
8-ST4821-B	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
5-ST41/44-B	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
4-ST32-B	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
3-UA-B	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
2-UA-B	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV
1-UA-B-ST5566 consensus	1	MNTLQKGFLLIEMLVIAIVGILAAVALPAYQD	TARAQVSEAILLAEGQKSAVTEYLLNHG	WPGNNSAGVAVS	PSDIKGGKVKVEV

Fig. 1. For legend see next page.

C and five W135 isolates. Of the *nadA*⁻ isolates, 100% and 24% contained class I *pilE* and class II *pilE* genes, respectively.

Diversity of *nadA* in Chinese strains

The *nadA* genes of these 54 isolates were sequenced. The nucleotide and deduced aa sequences of the corresponding mature proteins were aligned with the published data as described above. Six unique NadA subvariants were identified (Fig. 3), belonging to two main variants, NadA-3 (including five different subvariants) and NadA-4 (only one invasive isolate which belonged to CC5). The overall mean distance of five subvariants in NadA-3 was 0.003. However, only one isolate of NadA-4 was detected and therefore, it was not possible to determine mean distances for this subvariant. The level of conservation of NadA was shown by the result of phylogenetic analysis of all the NadA aa sequences (Supplementary Fig. S6). All the NadA-3 isolates (*n*=53) were assigned to five subvariants belonging to CC5 and CC11. Compared to the published *nadA* sequence (GenBank accession number AF452482, NadA-3), 40 CC5 isolates were

found to encode a completely identical NadA-3 variant. This was therefore designated subvariant NadA-3.1 in this study. The remaining three isolates of CC5 encoded three slightly different NadA-3 proteins (designated NadA-3.2 to NadA-3.4), which differed by a single aa at positions 115, 137 and 343, respectively (Fig. 3). Five out of ten isolates belonging to CC11 contained one copy of the insertion sequence IS1301. The insertion sites were identical and the whole sequence of IS1301 substituted part of the *nadA* sequence for 538 bp. The final 747 bp of the *nadA* sequence was consistent with AF452482 (NadA-3). The remaining five isolates of CC11 had four aa point mutations at the same positions (311, 312, 314, 322) in each sequence (designated NadA-3.5) (Fig. 3).

DISCUSSION

Adhesion of *N. meningitidis* to the mucosa of the human nasopharynx is the first step in host colonization and, consequently, the primary event leading to disease manifestation. Adhesion to the host is a dynamic process resulting from a balance of interactions between bacterial surface structures and host receptors.

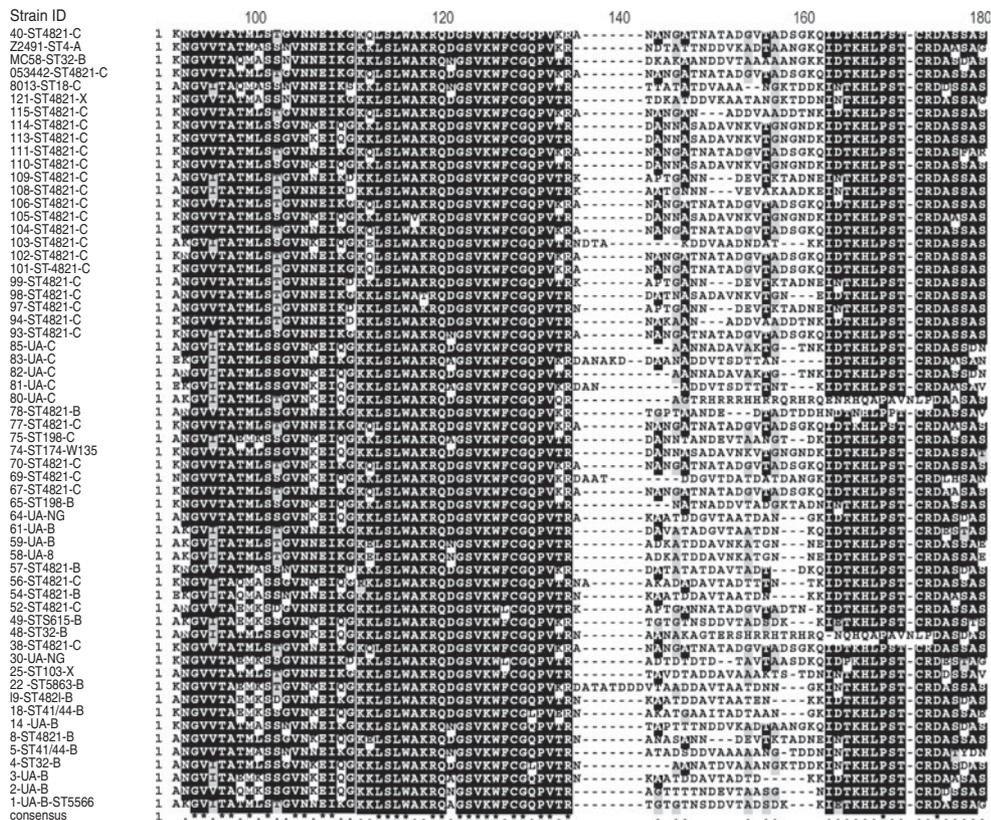


Fig. 1. Alignment of class I pilins Pile sequences. Isolate identification number or name, clonal complex (CC) and serogroup are shown for each sequence. Conserved residues are white, non-identical residues are black on white background, similar residues are grey and gaps are indicated by hyphens. The mature full-length pilin sequences deduced from 60 *N. meningitidis* isolates and three sequenced strains Z2491, MC58 and 8013 were aligned using Clustal W. The sequenced strain 053442 (ST4821 CC) was isolate number 40 in our study.

The success of such interactions is determined by the cooperation of multiple adhesins during the different stages of infection. Tfp is essential for the dynamic balance process of bacterial colonization and detachment, which is a prerequisite for the generation and dissemination of invasive disease [29]. At the same time, as a newly highlighted adhesin, NadA is regarded as an important co-factor in the pathogenesis of *Neisseria* and an encouraging candidate for the development of vaccines. In this study, the prevalence and genetic diversity of two adhesion-related genes, *pilE* and *nadA*, were determined. This formed the basis of a preliminary investigation into the potential relationship between the presence and distribution of the multiple adhesins detected in carriage and disease isolates with the presence of distinct epidemic characteristics of different clonal complexes.

This study aimed to elucidate the epidemiological characteristics of isolates in China and is complementary to similar analyses of adhesion-related

proteins in *N. meningitidis* isolates conducted internationally. In this study, the majority of isolates belong to CC1, CC5, CC4821 and CC11 (73%, 105/144), which have been identified as the predominant epidemic isolates in China. Isolates belonging to CC32, CC8 and CC41/44 represent the majority of serogroup B and C meningococcal infections in the developed world but are rare in China. During the last century, over 95% of meningococcal disease and four national epidemics in China were due to serogroup A meningococci [30]. Disease due to CC1 was important in the 1970s, but few of these isolates have been detected since 1989. The ST5 clonal complex strains of serogroup A meningococci caused two epidemics in China, as well as a pandemic wave that spread in 1987 via the annual Hajj pilgrimage [30]. More recently, the number of cases of serogroup C disease has increased in China since 2003 due to the emergence of a new hyperinvasive lineage of CC4821 [8]. In addition to CC4821, CC11 also emerged in China during the years from 2003 to 2006, representing a significant

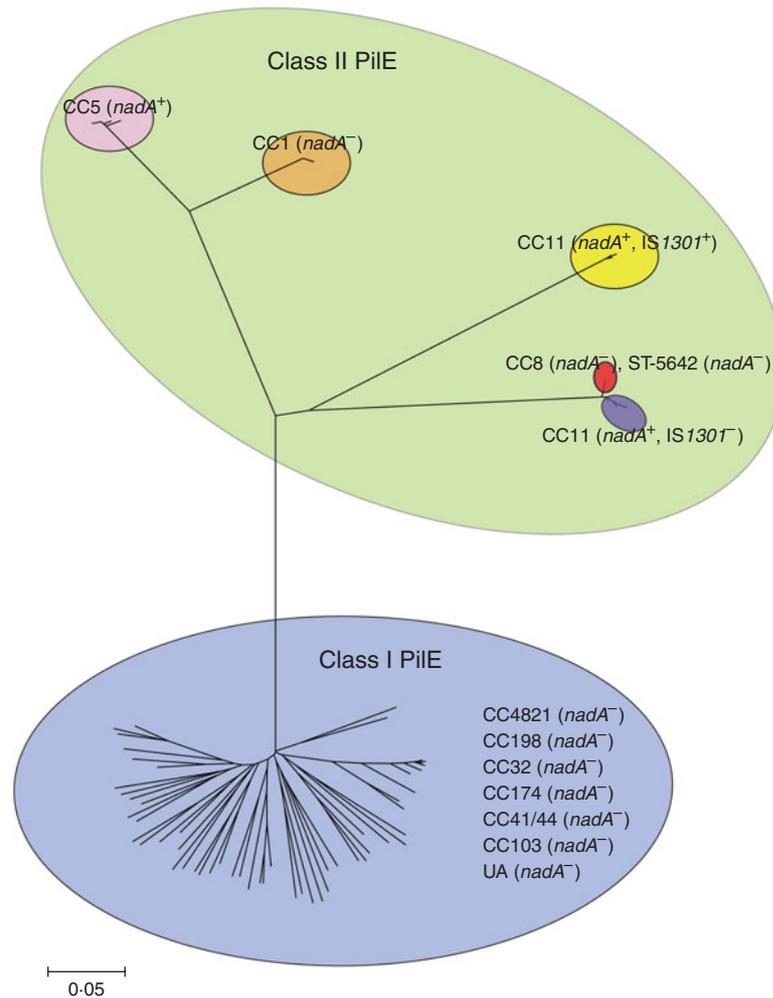


Fig. 2. Phylogenetic analysis of all the *PilE* amino acid (aa) sequences from the meningococcal strains used in this study (Supplementary Table S1) as well as five previously sequenced *N. meningitidis* isolates. All of the *PilE* aa sequences separated into two main parts: class I and class II *PilE*. Isolates ($n = 63$) of class I *PilE* belonging to seven different clonal complexes (CC) are presented in the blue ellipse. All class II *PilE* isolates, which split into five groups, are presented in the green ellipse. The strains of CC5 and CC1 are presented separately in pink and orange ellipses (*nadA* PCR⁺ and PCR⁻, respectively). CC11 strains are presented in yellow and purple ellipses (all *nadA* PCR⁺). However, the isolates in the yellow ellipse expressed *nadA* genes containing a whole insertion sequence *IS1301*. Isolates of CC8 ($n = 1$) and ST5642 strains ($n = 5$) are shown in the red ellipse. These strains harboured the same aa sequence as *PilE* and have no detectable *nadA* gene.

proportion of serogroup C and W135 disease worldwide [31].

Analysis of the distribution of class I and class II *pilE* genes revealed that class II *pilE* alleles are highly conserved and present in hyperinvasive lineage isolates, which is consistent with previous reports [21]. Class II *pilE* genes have been reported to be present only in ST8 and ST11 clonal complexes [21]. However, in this study, class II *pilE* genes were detected in isolates in the related CC1 and CC5. Of note, according to our study and previous reports, all of the isolates belonging to class II *pilE* have caused epidemics (CC1, CC8) or pandemics (CC5, CC11)

worldwide. In marked contrast, class I *pilE* genes were found in strains belonging to seven different clonal complexes, including some carriage isolates and some local epidemic isolates. For example CC4821 is present only in China while CC32 and CC41/44 are mainly detected in Europe and America.

Sequencing of the class I *pilE* genes confirmed the well-known diversity of the major pilin subunit genes. As many as 55 different aa sequence types were detected in 63 class I pilins. In contrast, class II *pilE* genes exhibited a high degree of sequence conservation. Only 13 different class II pilins were identified in a total of 73 sequences amplified from

Table 3. *nadA* presence in different serogroups of *N. meningitidis* isolates based on correlation with the class of *pilE* gene

Serogroup	Isolates tested (N)	<i>nadA</i> PCR ⁺ (%)	<i>nadA</i> PCR ⁺ in class I <i>pilE</i> (%)	<i>nadA</i> PCR ⁺ in class II <i>pilE</i> (%)
A	48*	77.1 (37/48)	0 (0/0)	77.1 (37/48)†
B	35	11.4 (4/35)	0 (0/20)	40 (4/10)‡§
C	43	18.6 (8/43)	0 (0/32)	100 (8/8)†‡§
W135	10	50 (5/10)	0 (0/4)	100 (5/5)‡
X	2	0 (0/0)	0 (0/2)	0 (0/0)
NG	6	0 (0/0)	0 (0/2)	0 (0/0)

* Consisted of 11 isolates of CC1 and 37 isolates of CC5 (Supplementary Table S1).
 † All of the serogroup A and C isolates, belonging only to the CC5 isolates conserved the *nadA* gene.
 ‡ All of the serogroup B, C and W135 isolates, belonging only to the CC11 isolates conserved the *nadA* gene.
 § Four serogroup B and one serogroup C isolates belonging to CC11 contained one copy of the insertion sequence IS1301.

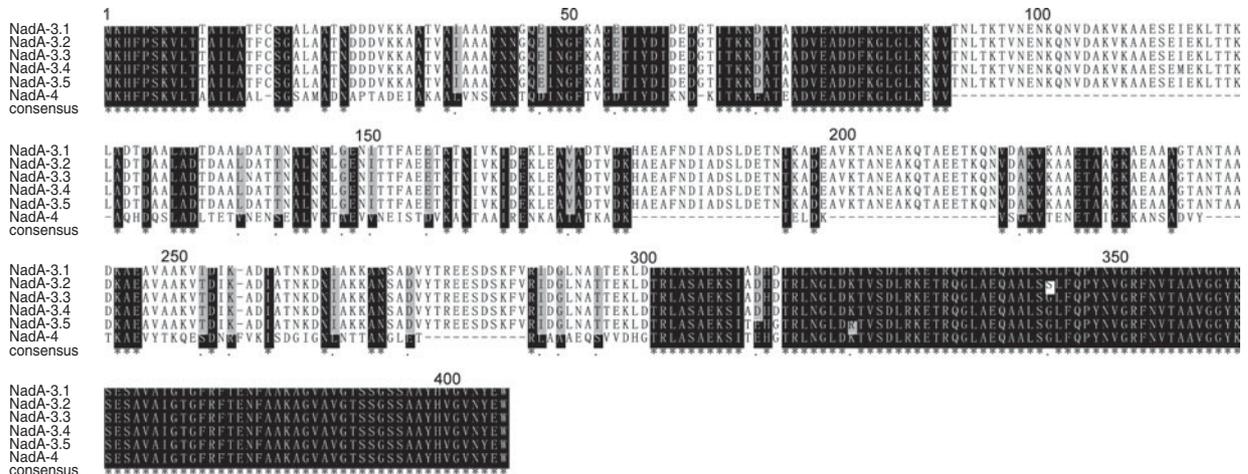


Fig. 3. Alignment of six *NadA* subvariant sequences detected in this study. The designated identifications are shown for each sequence. Conserved residues are white, non-identical residues are black on white background, similar residues are grey and gaps are indicated by hyphens.

meningococci belonging to four hyperinvasive lineages, and expressing four different capsular groups. However, the reason for this degree of conservation in the major pilin subunits in these clonal complexes and the potential relationships between these clonal complexes and the disease remain to be elucidated. Some studies have confirmed that a vaccine based on pilus preparation is safe and induces the production of specific antibodies that effectively interfere with colonization by inhibiting bacterial attachment to host cells [32, 33]. However, because *PilE* undergoes frequent and extensive antigenic variation in *Neisseria* species, the development of a pilus-based vaccine has been halted [34]. The data obtained in this study

suggest that a class II *PilE*-based vaccine (a previously abandoned idea) might be a suitable approach for these hypervirulent isolates.

Our data indicate that 54 of the 144 *N. meningitidis* isolates harbour the *nadA* gene, which was detected only in CC5 and CC11 of the corresponding class II *PilE* isolates. There was no difference of distribution of the *pilE* and *nadA* genes between the carriage and disease isolates. Hypervirulent lineages such as CC1, CC5, CC11, CC4821 were also found in carriage isolates. Further exploration of carriage and disease isolates from the same hypervirulent clonal complexes would be of interest. *NadA*-1, *NadA*-2 and *NadA*-5 have been reported from CC32 invasive disease

strains from Europe and the USA, CC1 and CC8 invasive disease strains from Europe and USA and CC213 carriage and disease-causing strains from Europe, respectively [17, 19]. These three variants were not detected in this study. Phylogenetic analysis revealed six unique NadA subvariants belonged to two main variants, NadA-3 and NadA-4 (only one invasive isolate of which belonged to CC5), as identified in a previous study [16, 17]. In previous studies, all isolates of CC32 and CC8 harbour the *nadA* gene [16, 17, 19, 20]. However, in this study two isolates from CC32 and one from CC8 did not harbour the *nadA* gene. Furthermore, with the exception of one isolate of variant NadA-4, the isolates from CC5 (the predominant epidemic isolates in China) expressed *nadA* genes belonging to the NadA-3 variant, which forms the recombinant component of the investigational 4CMenB vaccine [35].

Purified surface-exposed capsular polysaccharide and polysaccharide protein conjugate vaccines are currently available to protect against meningococcal disease caused by serogroups A, C, Y and W135 [36]. However, an effective vaccine for prevention of infection with serogroup B meningococci has yet to be developed. The identification of surface-exposed, conserved and widely prevalent meningococcal antigens suitable for use as components of a broadly protective vaccine is a significant challenge [37]. Therefore, our results complement international studies of *N. meningitidis* adhesion-related proteins and vaccine research.

In conclusion, this analysis of the prevalence and genetic diversity of *pilE* and *nadA* in *N. meningitidis* in China provides further elucidation of the molecular characterization of these two adhesion-related genes. More importantly, it is interesting to note a highly significant correlation between clonal complexes and the two adhesion-related genes, although this correlation may not be complete. It can be hypothesized that the isolates from diverse clonal complexes may cluster with distinct epidemic features and virulence due to the different adhesion-related genes. For example, ST5 and ST11 clonal complexes both harbour *pilE* and *nadA* genes and these two hyperinvasive lineages have caused worldwide pandemics [2, 30]. Otherwise, all isolates expressing the class I *pilE* gene lack the *nadA* gene, suggesting that different adhesion-related genes play different roles in the pandemic of *N. meningitidis* disease; moreover, the epidemic of a *N. meningitidis* clone is the result of the combined effect of several adhesion-related genes.

The correlation between pandemic and adhesion-related genes should be studied more in the future. Furthermore, knowledge of the molecular characteristics of adhesion-related genes is beneficial for the design of optimal vaccines. Finally, our results provide further understanding of the prevalence and genetic characterization of *N. meningitidis* adhesion-related genes and direct research into the potential epidemiological mechanism of meningococcal disease and the design of interventions for prevention of carriage, treatment of disease and eradication of infections.

SUPPLEMENTARY MATERIAL

For supplementary material accompanying this paper visit <http://dx.doi.org/10.1017/S0950268812002944>.

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DECLARATION OF INTEREST

None.

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