Distribution of transferrin binding protein B gene (tbpB) variants among Neisseria species
Odile B Harrison*1, Martin CJ Maiden1 and Bachra Rokbi2

Address: 1The Peter Medawar Building for Pathogen Research and Department of Zoology, University of Oxford, South Parks Road, Oxford, OX1 3SY, UK and 2Sanofi Pasteur, 69280 Marcy l’Etoile, France
Email: Odile B Harrison* - odile.harrison@zoo.ox.ac.uk; Martin CJ Maiden - martin.maiden@zoo.ox.ac.uk; Bachra Rokbi - bachra.rokbi@sanofi.com
* Corresponding author

Abstract

Background: Transferrin binding protein B (tbpB), an outer membrane lipoprotein, is required for the acquisition of iron from human transferrin. Two tbpB families have been documented in Neisseria meningitidis: an isotype I tbpB gene of 1.8 kb and an isotype II tbpB gene of 2.1 kb, the former expressed by meningococci in the disease-associated ST-11 clonal complex and the latter found among meningococci belonging to the hyper-invasive clonal complexes including ST-8, ST-18, ST-32, ST-41/44 as well as N. gonorrhoeae isolates. The origin of the isotype I tbpB gene is unknown, however several features in common with non-pathogenic Neisseria and the ST-11 clonal complex N. meningitidis isolate FAM18 have been documented leading to the hypothesis that the isotype I tbpB gene may also be shared between non-pathogenic Neisseria and ST-11 meningococci. As a result, the diversity of the tbpB gene was investigated in a defined collection of Neisseria species.

Results: Two families of isotype I tbpB were identified: family A containing conserved genes belonging to ST-11 meningococci, N. polysaccharea and N. lactamica isolates and family B including more diverse isotype I tbpB genes from N. sicca, N. mucosa, N. flava, N. subflava as well as N. cinerea, N. flavescens and N. polysaccharea isolates. Three isotype II tbpB families were identified with: family C containing diverse tbpB genes belonging to N. polysaccharea, N. lactamica, N. gonorrhoeae and N. meningitidis isolates, family D including another subset of isotype II tbpB genes from N. lactamica isolates and family E solely composed of N. gonorrhoeae tbpB genes.

Conclusion: This study reveals another instance of similarity between meningococci of the ST-11 clonal complex and non-pathogenic Neisseria with the origin of the isotype I tbpB gene resulting from a horizontal genetic transfer event occurring between these two populations.

Background

The genus Neisseria contains 12 species and biovars colonising humans most of which are non-pathogenic colonisers of the upper respiratory tract [1,2]. Only two species, Neisseria gonorrhoeae, the etiological agent of gonorrhoea and Neisseria meningitidis, a major cause of meningitis and septicaemia worldwide, regularly cause disease in humans [3,4].
A major determinant in the survival of *Neisseria* within the human host is the ability to acquire iron, the majority of which is not circulating freely in the human body but is stored in ferritin and haemoglobin or is complexed with the glycoproteins transferrin and lactoferrin [5]. *Neisseria* have devised ways to counteract this iron limitation through the evolution of several high-affinity receptor systems including the lactoferrin binding proteins A and B, the transferrin binding proteins A and B, and the haptoglobin-haemoglobin receptor HpuAB, each composed of an accessory lipoprotein subunit and a TonB-dependent gated porin [6-11]. In addition, *Neisseria* can obtain iron through the expression of the surface-exposed hae-moglobin receptor HmbR [12,13].

Based on antigenic and genomic features of TbpB and *tbpB*, *N. meningitidis* isolates can be classified into two major families: isotype I (*tbpB* gene of 1.8 kb and TbpB protein with a mass of approximately 68 kDa) and isotype II (*tbpB* gene of 2.1 kb and TbpB protein with a mass of approximately 80 to 90 kDa) [14]. Isotype II *tbpB* genes have been documented in several *N. meningitidis* clonal complexes including ST-8, ST-32, ST-18 and ST-41/44 as well as non-pathogenic *Neisseria* [14-17]. Isotype I *tbpB* genes, on the other hand, have solely been identified among *N. meningitidis* isolates belonging to the ST-11 clonal complex and have not been detected among other *Neisseria* species. Four *tbpB* families were recently described based on partial nucleotide sequences from serogroup A clonal complex ST-4 *N. meningitidis* and *N. lactamica* isolates collected in The Gambia [16,17]. Families one and four contained diverse isotype II *tbpB* alleles from *N. meningitidis* and *N. lactamica* isolates and families two and three included isotype I *tbpB* alleles. Importantly, among the 138 isolates analysed (98 serogroup A ST-4 meningococci, 12 unrelated meningococci, 22 *N. lactamica* isolates, and 6 unidentified *Neisseria* spp.) only three isotype I *tbpB* alleles were found, all of which belong to meningococci [16,17].

Meningococci from the ST-11 clonal complex have been a major cause of meningococcal disease worldwide throughout the last century and despite low carriage rates continue to be associated with disease [18]. In addition to the isotype I *tbpB* gene, ST-11 meningococci can be distinguished from other hyper-virulent clonal complexes by the absence of an *opCA* gene and the possession of a class 2 *porB* gene [19-21]. Furthermore, similarities between the ST-11 clonal complex isolate FAM18 and non-pathogenic *Neisseria* spp. have been reported including the clustering of *pile* sequences [22] and the comparable genetic organisation of the *opCA* negative locus in two *N. polysaccharea* isolates [23]. Taken together, these observations indicate the occurrence of specific horizontal genetic exchange events between commensal *Neisseria* and ST-11 meningococci which may have contributed to the described genetic isolation of this clonal complex [24]. The origin of the isotype I *tbpB* gene is unknown. Consequently, the distribution of the gene in a defined collection of *Neisseria* spp. was investigated with the hypothesis that the isotype I *tbpB* gene was present in other *Neisseria* spp.

### Results

#### Identification of the *tbpB* gene

Isotype I *tbpB* genes were isolated from the non-pathogenic *Neisseria* spp. Two families of the gene became apparent. The first contained sequences closely related to meningococcal ST-11 *tbpB* genes belonging to three *N. polysaccharea* and two *N. lactamica* isolates. The second included more divergent isotype I *tbpB* genes from the non-pathogenic *Neisseria* spp. *N. sicca*, *N. mucosa*, *N. flava*, *N. subflava*, *N. cinerea*, *N. flavescens* as well as from another three *N. polysaccharea* isolates (Table 1). Isotype II *tbpB* genes were obtained from the remaining six *N. lactamica* and two *N. polysaccharea* isolates, while in agreement with previous studies, *N. gonorrhoeae* isolates contained isotype II *tbpB* genes (Table 1) [25]. A further 23 non-pathogenic *Neisseria* isolates were analysed and found to be negative for the *tbpB* gene. Among these were *N. polysaccharea*, *N. perflava*, *N. sicca*, *N. subflava*, *N. flava* and *N. mucosa* isolates. These isolates may contain divergent or truncated *tbpB* genes unable to be amplified with the primers used, however the remainder of this study will focus on the *tbpB* genes that were sequenced.

### Table 1: Summary of *tbpB* families and nomenclature used

| *tbpB* Family | Size (kb) | *tbpB* isotype | Previous designation [16,17] | Neisseria species |
|--------------|-----------|----------------|-------------------------------|------------------|
| *tbpB*<sub>A</sub> | 1.8 | I | | *N. meningitidis* clonal complex ST-11, *N. polysaccharea* and *N. lactamica* |
| *tbpB*<sub>B</sub> | 1.8 | I | | |
| *tbpB*<sub>C</sub> | 2.1 | II | | *N. meningitidis* belonging to the clonal complexes ST-32, ST-41/44, ST-8, ST-18, *N. lactamica*, *N. polysaccharea* and *N. gonorrhoeae* |
| *tbpB*<sub>D</sub> | 2.1 | II | | *N. meningitidis* and *N. lactamica* |
| *tbpB*<sub>E</sub> | 2.1 | II | | *N. gonorrhoeae* |
Functional assessment of the protein was beyond the scope of the present study. Nevertheless, previously documented putative transferrin binding sites were observed based on predicted translations of the nucleotide sequences [26,27]. In particular, the highly conserved domains N3, N4 and C3, critical for efficient iron uptake and located in both the N- and C- terminal segments among isolates N. gonorrhoeae FA19, N. meningitidis M982, Moraxella catarrhalis 4223 and Acinetobacter pleuropneumoniae serotype 7, were also identified [27]. Domain N3 (residues 377 to 388 in N. gonorrhoeae FA19) displayed 100% sequence identity in both isotype I TbpB families, whereas six non-synonymous changes were observed among isotype II TbpB. Domain N4 (residues 409 to 422 in N. gonorrhoeae FA19) was also highly conserved among isotype I TbpB with the occurrence of three non-synonymous substitutions. Five variable sites were present among isotype II TbpB. Domain C3 (residues 703 to 713 in N. gonorrhoeae FA19) showed the most diversity with the occurrence of four non-synonymous substitutions among both TbpB isotypes.

**Phylogenetic relationships inferred from novel Neisseria tbpB sequences**

All of the sequences were aligned manually with sequences starting from and ending at the same amino acid residue in each isolate. Published isotype I and II tbpB sequences from isolates B16B6, M982, 8680, 8726, 2713, 2717 and FA19, used in previous analyses [14,16,17,27], were also included in the alignment as well as those from the sequenced genomes of N. meningitidis isolates FAM18, Z2491, MC58 and N. gonorrhoeae FA1090.

Phylogenetic analysis was undertaken using the software package ClonalFrame version 1.1, which is a statistical model-based method initially described for inferring bacterial clonal relationships using multilocus sequence data [28]. Inference is performed in a Bayesian framework and a neutral coalescent model is assumed based on the hypothesis that the bacteria in the sample come from a constant-sized population in which each bacterium is equally likely to reproduce, irrespective of its previous history. The key assumption of ClonalFrame is that recombination events introduce a constant rate of substitutions to a contiguous region of sequence with the end result that a clonal frame can be inferred. In the present study, over 50,000 iterations were performed with every hundredth tree sampled after which a 95% majority-rule consensus tree was derived. Annotation was then undertaken by importing the tree into the Molecular Evolutionary Genetics Analysis software package (MEGA version 4.0) [29].

The two major isotype families were evident with each family containing a distinct cluster of genes (Fig. 1). The shortness of the branches for isotype I tbpB genes indicated that these were a closely related group of sequences compared with the depth of the branches seen for isotype II tbpB genes where greater diversity is known to occur [30]. Closer inspection of the tree revealed the two families of isotype I tbpB genes observed by sequence analysis as well as three clusters of isotype II tbpB genes. For ease of interpretation, the two isotype I tbpB families have been named tbpB_A and tbpB_B with the isotype II clusters named tbpB_C through to tbpB_E (Fig. 1 and Table 1). This nomenclature is proposed according to published guidelines in bacterial genetics [31] and is recommended in light of the emergence of the new families revealed in this study. Hitherto, studies in tbpB genetic diversity have focussed on a specific Neisseria spp. or meningococcal clonal complexes and have not encompassed all of the Neisseria species included in the present work. This inclusion has provided a more detailed analysis of tbpB diversity and will allow a more flexible nomenclature for tbpB genes.

Family tbpB_A was comprised of genes most closely related to those of clonal complex ST-11 meningococci with four of these belonging to N. lactamica and N. polysaccharea isolates. Family tbpB_B included a divergent cluster of isotype I genes (75% identity) belonging to a variety of Neisseria spp. as well as containing a subset of N. polysaccharea isolates (Fig. 1 and Tables 1 &2).

Three distinct isotype II tbpB families were apparent (Fig 1 and Tables 1 &2). Several gonococcal genes have clustered together and can be found in family tbpB_C with families tbpB_C and tbpB_D containing genes belonging to N. lactamica, N. polysaccharea, N. gonorrhoeae and N. meningitidis isolates. Throughout the tree isolates have clustered by Neisseria species indicative of within species conservation of tbpB genes. The Genbank accession numbers for new tbpB genes sequenced in this study are listed in Table 2 as well as those belonging to previously submitted tbpB sequences.

**Genetic diversity of the tbpB genes**

Genes belonging to family tbpB_A were the least diverse (mean p-distance ranging from 0.001 to 0.040) with 85 polymorphic sites, the majority of which occur among N. polysaccharea and N. lactamica isolates. Overall, six fixed differences were observed between the genes of ST-11 meningococci and those of N. lactamica and N. polysaccharea with no shared polymorphisms between the two populations. Family B tbpB genes were more diverse (mean p-distance value 0.117) with 415 polymorphic sites. In a comparison of both gene families, there were 210 fixed differences and 54 shared mutations.

As expected, families tbpB_C, D and E were more diverse (mean p-distances = 0.187, 0.140 and 0.112 respectively). Genes belonging to the N. polysaccharea isolates shared
Figure 1

**Phylogenetic tree inferred from aligned tbpB genes.** Over 500 trees were generated using Clonalframe from which a 95% majority-rule consensus tree was derived and imported into MEGA version 4.0 for further annotation. Meningococcal reference tbpB genes (accession numbers in brackets) belonging to isolates B1686, M982, 2713, 2717, 8710, 8680, FA19, FA1090, FAM18, Z2491 and MC58 [Genbank: Z15129, Z15130, AJ223044, AJ279554, Y09618, Y09977, U05205, U65219, AM421808, AL157959 and AE002098, respectively] were also included in the phylogenetic analysis. The proposed nomenclature for each tbpB family is indicated by large encircled letters. The nodes 1, 2 and 3 depicted with a diamond correspond to the recombination events presented in Figure 2. Open squares denote *N. gonorrhoeae* tbpB sequences, open circles *N. lactamica*, open triangles all of the other *Neisseria* spp. excluding *N. polysaccharea*, which are depicted with black circles and *N. meningitidis* which are represented by black squares.
Very little recombination was noticeable among indicative of recombination, while only six were apparent indicating that recombination occurred frequently among mosaic gene structure was present among the latter indic-

genes or between these and family encoding non-synonymous changes. 99% identity with 16 segregating sites, seven of which encoded non-synonymous changes. N. lactamica tbpB genes were more diverse with 696 polymorphic sites while 829 polymorphisms were observed among N. gonorrhoeae tbpB genes. A total of 445 shared mutations were observed between N. lactamica and N. meningitidis tbpB genes indicative of recombination, while only six were apparent between N. polysaccharea and N. meningitidis.

Very little recombination was noticeable among tbpB\(_3\) genes or between these and family tbpB\(_6\), however a mosaic gene structure was present among the latter indicating that recombination occurred frequently among tbpB\(_6\) genes from N. sicca, N. flava, N. subflava, N. mucosa, N. flavescens, N. cinerea and N. polysaccharea (Fig. 2a). As expected, tbpB genes from families tbpB\(_{C, D}\) and \(_E\) recombined often (Fig. 2b &2c) with most of this occurring from bases 200 to 800.

**Discussion**

The aim of this study was to identify the origin of the isotype I tbpB gene. Previous observations have determined that these were confined to meningococci belonging to the ST-11 clonal complex [14,32]. In contrast, isotype II genes were widely distributed among N. meningitidis clonal complexes and N. lactamica isolates [14,16,17]. The results presented here reveal the existence of isotype I tbpB genes among diverse Neisseria spp. Based on phylogenetic analysis these could be divided into two families: tbpB\(_3\) containing genes homologous to ST-11 meningococci

**Table 2: N. gonorrhoeae and commensal Neisseria isolates used in this study**

| Isolate          | Site of isolation | Country of origin | tbpB family | Genbank Accession No | Reference |
|------------------|-------------------|-------------------|-------------|----------------------|-----------|
| N. gonorrhoeae 22584 | genitourinary     | USA               | tbpB\(_E\)  | AM849572             | This study|
| N. gonorrhoeae 25362 | DGI               | unknown           | tbpB\(_E\)  | AM849573             | This study|
| N. gonorrhoeae 26034 | DGI               | unknown           | tbpB\(_E\)  | AM849574             | This study|
| N. gonorrhoeae 26399 | DGI               | unknown           | tbpB\(_E\)  | AM849575             | This study|
| N. gonorrhoeae 26593 | DGI               | unknown           | tbpB\(_E\)  | AM849576             | This study|
| N. gonorrhoeae 27006 | DGI               | UK                | tbpB\(_E\)  | AM849577             | This study|
| N. gonorrhoeae 27886 | genitourinary     | Bangladesh        | tbpB\(_E\)  | AM849578             | This study|
| N. gonorrhoeae 27921 | genitourinary     | Uzbekistan        | tbpB\(_E\)  | AM849579             | This study|
| N. gonorrhoeae 28197 | genitourinary     | Russia            | tbpB\(_E\)  | AM849580             | This study|
| N. gonorrhoeae 28622 | genitourinary     | UK                | tbpB\(_C\)  | AM849581             | This study|
| N. gonorrhoeae 29528 | genitourinary     | UK                | tbpB\(_E\)  | AM849582             | This study|
| N. gonorrhoeae F62  | genitourinary     | USA               | tbpB\(_E\)  | AM849571             | This study|
| N. gonorrhoeae F41  | DGI               | USA               | tbpB\(_E\)  | U05205               | [35]      |
| N. gonorrhoeae F41090 | DGI              | USA               | tbpB\(_E\)  | U65219               | [25]      |
| N. lactamica 8064 | nasopharynx       | France            | tbpB\(_C\)  | AM849588             | [40, 41]  |
| N. lactamica 241 | nasopharynx       | Oman              | tbpB\(_D\)  | AJ704747             | This study|
| N. lactamica 2494 | nasopharynx       | Oman              | tbpB\(_A\)  | AJ704737             | This study|
| N. lactamica 24223  | nasopharynx       | Oman              | tbpB\(_D\)  | AM849585             | This study|
| N. lactamica 24290  | nasopharynx       | Oman              | tbpB\(_C\)  | AJ704748             | This study|
| N. lactamica 24291  | nasopharynx       | Oman              | tbpB\(_C\)  | AM849586             | This study|
| N. lactamica 24292  | nasopharynx       | Oman              | tbpB\(_C\)  | AM849587             | This study|
| N. lactamica 243170 | nasopharynx       | Oman              | tbpB\(_A\)  | AJ704746             | This study|
| N. flav 30008      | nasopharynx       | USA               | tbpB\(_B\)  | AJ704732             | This study|
| N. subflava 9992   | nasopharynx       | USA               | tbpB\(_B\)  | AJ704745             | This study|
| N. mucosa ATCC 19696 | sputum           | unknown           | tbpB\(_B\)  | AJ704738             | [42, 43]  |
| N. sicca ATCC 9913  | unknown           | unknown           | tbpB\(_B\)  | AJ704730             | [44]      |
| N. flavescens ATCC 13120 | CSF meningitis  | USA               | tbpB\(_B\)  | AJ704733             | [45, 46]  |
| N. flavescens 414   | unknown           | France            | tbpB\(_B\)  | AJ704736             | [47]      |
| N. flavescens ATCC 13119 | CSF meningitis  | USA               | tbpB\(_B\)  | AJ704734             | [48]      |
| N. flavescens 3536  | CSF meningitis    | USA               | tbpB\(_B\)  | AJ704735             | [49]      |
| N. cinerea ATCC 14685 | nasopharynx     | Germany           | tbpB\(_B\)  | AJ704731             | [47, 49]  |
| N. polysaccharea ATCC 43768 | nasopharynx  | France            | tbpB\(_B\)  | AJ704740             | [47, 49, 50]|
| N. polysaccharea 90400 | nasopharynx     | Canada            | tbpB\(_B\)  | AJ704743             | [23, 51]  |
| N. polysaccharea 89536 | nasopharynx     | Canada            | tbpB\(_C\)  | AJ704762             | [52]      |
| N. polysaccharea 85322 | nasopharynx     | Germany           | tbpB\(_C\)  | AJ704761             | [23, 53]  |
| N. polysaccharea 87043 | nasopharynx     | Canada            | tbpB\(_B\)  | AJ704742             | [23, 44, 51, 52]|
| N. polysaccharea P4-A | nasopharynx     | UK                | tbpB\(_B\)  | AJ704739             | [48]      |
| N. polysaccharea P7-A | nasopharynx     | UK                | tbpB\(_B\)  | AJ704741             | [48]      |
| N. polysaccharea P8-A | nasopharynx     | UK                | tbpB\(_B\)  | AJ704744             | [48]      |
ClonalFrame representation of \textit{tbpB} recombination events. The nucleotide sequence of \textit{tbpB} genes is represented on the x axis with the red line indicating at each locus the probability for an import on a scale from 0 (bottom of the y axis) to 1 (top of the y axis). Each inferred substitution in the graph is represented by a cross, the intensity of which indicating the posterior probability for that substitution. In panel A, recombination events occurring at node 1 in the phylogenetic tree (fig. 1) are represented. A mosaic gene structure is evident with fragments present between bases 150 and 300, 800 and 1000 and 1400 and 1800. In panel B, horizontal genetic exchange at node 2 are depicted occurring from bases 200 to 800 and, in panel C node 3 is represented.
and \textit{tbpB}_p including more distantly related isotype I genes belonging to diverse non-pathogenic \textit{Neisseria} spp. (Table 1 and Fig. 1). \textit{N. lactamica} and \textit{N. polysaccharea} isolates were found with both \textit{tbpB} isotypes while, in agreement with previous studies, \textit{N. gonorrhoeae} isolates solely contained isotype II \textit{tbpB} genes [25]. Phylogenetic analysis demonstrated the presence of three isotype II families, named \textit{tbpB}_C through to \textit{tbpB}_E. Family C contained genes belonging to \textit{N. lactamica}, \textit{N. polysaccharea}, \textit{N. meningitidis} and \textit{N. gonorrhoeae} isolates, family D included another subset of isotype II \textit{tbpB} genes belonging to \textit{N. lactamica} and \textit{N. meningitidis} isolates and finally, family E comprised \textit{N. gonorrhoeae} genes (Table 1 and Fig. 1). In light of the \textit{tbpB} families now present a new nomenclature is proposed according to published guidelines in bacterial genetics [31]. Previously, studies in \textit{tbpB} genetic diversity focussed on a specific \textit{Neisseria} spp. or meningococcal clonal complex and did not encompass all of the \textit{Neisseria} spp. included in the present work [14-17,25]. This inclusion has provided a more detailed analysis of \textit{tbpB} diversity with the proposed nomenclature allowing more flexibility for future \textit{tbpB} genes. Using this scheme genes can be grouped according to the family they belong to followed by an allele number.

A number of features are shared between clonal complex ST-11 \textit{N. meningitidis} isolates and non-pathogenic \textit{Neisseria}. Sequences upstream of the \textit{pilE} gene from the class II pilin-producing \textit{N. meningitidis} strain FAM18 were identical to the short region characterised upstream from \textit{N. polysaccharea} \textit{pilE} [22]. The \textit{N. polysaccharea} isolate analysed (ATCC 43768) was included in the present study and harboured a \textit{tbpB} gene similar to that of \textit{N. meningitidis} isolate FAM18. Furthermore, \textit{opcA} genes are absent among meningococci belonging to the ST-11 clonal complex and were also undetectable among \textit{N. polysaccharea} isolates 87043 and 90400 [19,20], which were found in this study with isotype I \textit{tbpB} genes. The identification of isotype I family A and B genes among \textit{Neisseria} spp. is another characteristic shared with \textit{N. meningitidis} isolates belonging to clonal complex ST-11 and is indicative of the occurrence of several horizontal genetic transfer events between non-pathogenic \textit{Neisseria}, in particular \textit{N. polysaccharea} and meningococci belonging to this clonal complex.

The evolutionary reasons leading to the existence of two \textit{tbpB} isotypes among \textit{Neisseria} are unknown. However, seclusion of isotype I \textit{tbpB} to ST-11 clonal complex meningococci may be due to clonal expansion or selection for this isotype. Indeed, the isotype I TbpB protein has been shown to play an essential role in iron acquisition from human transferrin with isogenic mutants deficient in TbpB failing to grow on hTf as a sole iron source [33,34]. Thus, both the TbpA and TbpB parts of the transferrin complex are critical. This was reflected in the lower diversity observed among \textit{tbpB} genes belonging to families A (mean p-distance ranging from 0.001 to 0.040) and B (mean p-distance 0.117), highlighting the importance of this gene in contributing to the fitness of the organism. There has been selection for isotype I \textit{tbpB} among meningococci belonging to the ST-11 clonal complex such that it has become restricted to this clonal complex. In contrast, isotype II \textit{tbpB} genes have been found to provide a purely facilitating role such that TbpB-deficient mutants were only incapacitated with slower growth [34]. This has been confirmed in isogenic mutagenesis studies of both TbpA and TbpB in \textit{N. gonorrhoeae}, \textit{H. influenzae} and \textit{M. catarrhalis} (which all contain isotype II-like \textit{tbpB} genes) [35-37]. The non-essential role the isotype II \textit{tbpB} gene has in iron acquisition may contribute to its hyper-variability. Indeed, Zhu et al found that the high rate of import among isotype II \textit{tbpB} genes, although providing a temporary advantage because of antigenic composition, resulted in reduced fitness of the isolates [16,17]. The higher recombination patterns observed in the present study among isotype II \textit{tbpB} genes (Fig. 2b &2C) combined with the deeper phylogeny seen (Fig. 1) support this.

**Conclusion**

This work investigated the distribution of the two \textit{tbpB} variants among \textit{Neisseria} spp. and aimed to discover the origin of the isotype I \textit{tbpB} gene. Results revealed this gene was found among diverse \textit{Neisseria} spp. indicating the occurrence of a horizontal genetic transfer event between \textit{N. meningitidis} and non-pathogenic \textit{Neisseria}. Three features shared between ST-11 meningococci and non-pathogenic \textit{Neisseria} have now been described: (i) the presence of isotype I \textit{tbpB} genes (ii) the identical sequences upstream of the \textit{pilE} gene and (iii) the analogous genetic organisation of the \textit{opcA} negative locus.

A revised nomenclature was proposed according to the published guidelines [31]. The scheme now distinguishes isotype I \textit{tbpB} genes into two new families: \textit{tbpB}_A and \textit{tbpB}_B, the former contained \textit{tbpB} genes closely related to ST-11 clonal complex meningococci, the latter included the more distantly related \textit{tbpB} genes belonging to many non-pathogenic \textit{Neisseria} species. The scheme also separates isotype II \textit{tbpB} genes into three new families: \textit{tbpB}_C comprising \textit{tbpB} genes from \textit{N. meningitidis}, \textit{N. lactamica}, \textit{N. polysaccharea} and \textit{N. gonorrhoeae} isolates, \textit{tbpB}_D consisting of \textit{tbpB} genes from \textit{N. lactamica} and \textit{N. meningitidis} isolates and finally, \textit{tbpB}_E containing \textit{N. gonorrhoeae} \textit{tbpB} genes.

**Methods**

**Growth of isolates and DNA preparation**

The non-pathogenic \textit{Neisseria} and \textit{N. gonorrhoeae} isolates used in this study are listed in Table 2. Isolates were cul-
tured overnight on GC agar (Difco) supplemented with 1% isovitalex (Oxoid) and grown at 37 °C in the presence of 10% CO₂. Boiled cell suspensions were prepared for each isolate. Briefly, a PBS solution of overnight GC grown bacteria was boiled for 5 minutes, centrifuged and the supernatant stored at +4 °C before being directly used for PCR.

Nucleotide sequence determination
Amplification and sequencing of tbpB genes were completed using primers listed in Table 3. Degenerate primers were used for some of the sequencing steps. PCR products were PEG purified and either sequenced directly or cloned using the TOPO PCR TA cloning kit for sequencing (Invitrogen). Nucleotide sequence determination was carried out using the Li-Cor Global IR² system along with the Sequitherm Excel DNA sequencing kit (ScienceTec, France). Additional sequencing was carried out by cycle sequencing with BigDye Ready Reaction Mix (Applied Biosystems) according to manufacturer’s instructions and using an ABI 377 automated DNA sequencer.

Data manipulation and analysis
The tbpB nucleotide sequences were assembled using the Staden sequence analysis package [38] and all sequences aligned manually in the Seqlab alignment program (Genetics Computer Group, Madison, Wis.). Phylogenetic analysis was undertaken using the software package ClonalFrame version 1.1, which is a statistical model-based method initially described for inferring bacterial clonal relationships using multilocus sequence data [28]. In the present study, over 50,000 iterations were performed with every hundredth tree sampled after which a 95% majority-rule consensus tree was derived. Annotation was then undertaken by importing the tree into the Molecular Evolutionary Genetics Analysis software package (MEGA version 4.0) [29].

The level of sequence diversity between tbpB genes was assessed by calculating p-distances within each tbpB family revealing the proportion (p) of nucleotide sites at which sequences differed. These analyses were conducted using MEGA. The number of fixed differences and shared polymorphisms were obtained using the software DnaSP [39]. Old and new accession numbers for tbpB genes are listed in table 2.

Authors’ contributions
OBH participated in the planning of this study, performed all experimental work, data analysis and drafted the manuscript. MCJM participated in writing the manuscript. BR participated in the planning of this study, coordinated the study and assisted in writing the manuscript.

Acknowledgements
This work was funded by a Marie Curie fellowship provided by the European Commission and the Human Potential programme. The authors would like to thank D. Caugant (NIPH Norway), P. Kriz (NIPH Czech Republic), C. Ferreiros (Universidad de Santiago, Spain), J.M. Alonso and MK. Taha (The Pasteur Institute, France), Y. Qvarnström (Uppsala University, Sweden), N. Bilek (Imperial College, UK), R. Tsang, (National Microbiology Laboratory, Canada) and N. Saunders (University of Oxford, UK) for the kind donation of strains.

References
1. Knapp JS: Historical perspectives and identification of Neisseria and related species. Clin Microbiol Rev 1988, 1:415-431.
2. Vedros NA: Genus I. Neisseria Trevissan 1885. In Bergey’s Manual of systematic bacteriology Volume I. Edited by: Krieg NR and Holt JG. Baltimore, The Williams & Wilkins Co; 1984:290-296.
3. Gerbase AC, Rowley JT, Mertens TE: Global epidemiology of sexually transmitted diseases. Lancet 1998, 351:2-4.
4. Rosenstein NE, Perkins BA, Stephens DS, Popovic T, Hughes JM: Meningococcal disease. N Engl J Med 2001, 344:1378-1388.
5. Otto BR, Verweij-van Vught AM, MacLaren DM: Transferrins and heme-compounds as iron sources for pathogenic bacteria. Curr Rev Microbiol 1992, 16:217-233.
6. Prinz T, Meyer M, Pettersson A, Tommassen J: Structural characterization of the lactoferrin receptor from Neisseria meningitidis. J Bacteriol 1999, 181:4417-4419.
7. Schryvers AB, Morris LJ: Identification and characterization of the human lactoferrin-binding protein from Neisseria meningitidis. Infect Immun 1988, 56:1144-1149.
8. Ala’Aldeen DA, Borriello SP: The meningococcal transferrin-binding proteins 1 and 2 are both surface exposed and generate bactericidal antibodies capable of killing homologous and heterologous strains. Vaccine 1996, 14:49-53.
9. Schryvers AB, Morris LJ: Identification and characterization of the transferrin receptor from Neisseria meningitidis. Mol Microbiol 1988, 2:281-288.

Table 3: Primers used in this study

| Primer          | Primer base sequence (5’ – 3’) | Application | Location from 5’ end |
|-----------------|--------------------------------|-------------|----------------------|
| OTG6687         | CAATCCATTGGTAAATCAG           | tbpB forward primer | 6                    |
| OTG6689 [54]    | GCCTCTGAAAGCCTATTCC           | tbpB reverse primer | Intergenic space    |
| seqtbpBII-for1  | CTAYAAAGGSARHRAWCCCTCC        | Isotype I tbpB sequencing | 603                 |
| seqtbpBII-for2  | CCGATTYYGGKMTGACYAG           | Isotype I tbpB sequencing | 817                 |
| seqtbpBII-rev1  | CCRCTTCTCGTGATGAGGG           | Isotype I tbpB sequencing | 1931                |
| seqtbpBII-rev2  | CTGAAATGCCGCTTATTGCC          | Isotype II tbpB sequencing | 1486                |
| seqtbpBII-for1  | GACGGYATATTYTTATMAMGG          | Isotype II tbpB sequencing | 585                 |
| seqtbpBII-for2  | GAACCAARSAACATCCTTGT          | Isotype II tbpB sequencing | 1032                |
| seqtbpBII-rev1  | GAACCCATTGCCGCTCAGC           | Isotype II tbpB sequencing | 1901                |
| seqtbpBII-rev2  | CTGTTCCGCAGCCTTTKACC          | Isotype II tbpB sequencing | 1460                |
10. Lewis LA, Dyer DW: Identification of an iron-regulated outer membrane protein of Neisseria meningitidis involved in the utilization of hemoglobin complexed to haptoglobin. J Bacteriol 1995, 177:1299-1306.

11. Lewis LA, Gray E, Wang YP, Roe BA, Dyer DW: Molecular characterization of hpuAB, the hemoglobin-haptoglobin-utilization operon of Neisseria meningitidis. Mol Microbiol 1997, 23:737-745.

12. Stojilkovic I, Hwa V, de Saint Martin L, O'Gaora P, Fraysse S, Chabanel C, Achtman M: Allelic diversity of the two transferrin binding protein B gene isotypes among a collection of Neisseria meningitidis strains representative of serogroup B disease: implication for the composition of a recombinant TbpB-based vaccine. Infect Immun 2000, 68:4938-4947.

13. Zhu P, Mignon M, Caugant DA, Quentin-Millet MJ: Heterogeneity of tbpB, the transferrin-binding protein B gene, among serogroup B Neisseria meningitidis strains of the ET-5 complex. J Clin Microbiol 1997, 35:522-529.

14. Stojilkovic I, Stojilkovic G, Mignon M, Danve B, Poncet D, Anic S, So M: HmbR outer membrane receptors of pathogenic Neisseria spp.: iron-regulated, hemoglobin-binding proteins with a high level of primary structure conservation. J Bacteriol 1996, 178:4670-4678.

15. Rokbi B, Renauld-Mongenie G, Mignon M, Danve B, Poncet D, Chabanel C, Caugant DA, Quentin-Millet MJ: Variable interspecific genetic exchange between commensal Neisseriae and Neisseria meningitidis. Mol Microbiol 2000, 36:1049-1058.

16. Linz B, Schenker M, Zhu P, Achtman M: Frequent interspecific genetic variation mechanisms viewed through comparative analysis of serogroup C strain FAM18. PLoS Genet 2007, 3:e223.

17. Seiler A, Reinhardt R, Sarkari J, Caugant DA, Achtman M: Allelic polymorphisms and site-specific recombination in the opc locus of Neisseria meningitidis. Mol Microbiol 1996, 19:841-856.

18. Rokbi B, Mignonneau M, Mignon M, Caugant DA, Quentin-Millet MJ: Identification of transferrin-binding protein B, the transferrin-binding protein B gene, among serogroup B Neisseria meningitidis strains of the ET-5 complex. J Clin Microbiol 1997, 35:522-529.

19. Stojilkovic I, Larson J, Hwa V, Anic S, So M: HmbR outer membrane receptors of pathogenic Neisseria spp.: iron-regulated, hemoglobin-binding proteins with a high level of primary structure conservation. J Bacteriol 1996, 178:4670-4678.

20. Lewis LA, Dyer DW: Identification of an iron-regulated outer membrane protein of Neisseria meningitidis involved in the utilization of hemoglobin complexed to haptoglobin. J Bacteriol 1995, 177:1299-1306.

21. Lewis LA, Gray E, Wang YP, Roe BA, Dyer DW: Molecular characterization of hpuAB, the hemoglobin-haptoglobin-utilization operon of Neisseria meningitidis. Mol Microbiol 1997, 23:737-745.

22. Stojilkovic I, Hwa V, de Saint Martin L, O'Gaora P, Fraysse S, Chabanel C, Achtman M: Allelic diversity of the two transferrin binding protein B gene isotypes among a collection of Neisseria meningitidis strains representative of serogroup B disease: implication for the composition of a recombinant TbpB-based vaccine. Infect Immun 2000, 68:4938-4947.

23. Zhu P, van der Ende A, Falush D, van der Ende A, Falush D, Brösková N, Nasser G, Linz B, Popovic T, Schuurman IG, Adegbola RA, Zurth K, Gagneux S, Platonov AE, Frosch M, Vogel U: Identification of an iron-regulated outer membrane protein of Neisseria meningitidis: genes, pseudogenes, deletions, insertion elements and DNA islands. Mol Microbiol 1999, 33:635-650.

24. Chillingworth T, Cronin A, Davis PH, Holroyd NE, Jagels K, Maddison M, Moule S, Rabbinowitsch E, Sharp S, Unwin L, Whithead S, Quail MA, Achtman M, Barrett B, Saunders NJ, Parkhill J: Meningococcal genetic variation mechanisms viewed through comparative analysis of serogroup C strain FAM18. PLoS Genet 2007, 3:e223.

25. Seiler A, Reinhardt R, Sarkari J, Caugant DA, Achtman M: Allelic polymorphisms and site-specific recombination in the opc locus of Neisseria meningitidis. Mol Microbiol 1996, 19:841-856.

26. Zhu P, van der Ende A, Falush D, van der Ende A, Falush D, Brösková N, Nasser G, Linz B, Popovic T, Schuurman IG, Adegbola RA, Zurth K, Gagneux S, Platonov AE, Frosch M, Vogel U: Identification of an iron-regulated outer membrane protein of Neisseria meningitidis: genes, pseudogenes, deletions, insertion elements and DNA islands. Mol Microbiol 1999, 33:635-650.

27. Wang JF, Caugant DA, Morelli G, Kouramaire B, Achtman M: Anti- genic and epidemiologic properties of the ET-37 complex of Neisseria meningitidis. Infect Dis Clin North Am 1993, 17:1320-1329.

28. Aho EL, Unwin R, Batcheller AE, Holingren AM, Havig K, Kalukoski AM, Vomhof EE, Longfors NS, Erickson CB, Anderson ZK, Dawlatty JM, Mueller JJ: Neisseria pilin genes display extensive inter species diversity. FEMS Microbiol Lett 2005, 249:327-334.

29. Zhu P, Klutch MJ, Derrick JP, Prince SM, Tsang RS, Tsai CM: Identification of opcA gene in Neisseria polysaccharidica: interspecies diversity of Opc protein family. Gene 2003, 307:31-40.

30. Claus H, Stoevesandt J, Frosch M, Vogel U: Genetic isolation of meningococci of the electrophoretic type 37 complex. J Bacteriol 1993, 175:1225-1235.

31. Cornellissen CN, Anderson JE, Sparling PF: Characterization of the diversity and the transferrin-binding domain of gonococcal transferrin-binding protein 2. Infect Immun 1997, 65:822-828.

32. Zuber-Villeneuve M, Genini G, Lins L, Krell T, Lafly F, Mignon M, Dupuy M, Dangel-Moricville JM, Guinet-Morlot F, Brasseur R, Lissolo L: Transferrin-binding protein B of Neisseria meningitidis: sequence-based identification of the transferrin-Binding site confirmed by site-directed mutagenesis. J Bacteriol 2004, 186:850-857.

33. DeRocco AJ, Cornellissen CN: Identification of transferrin-binding domains in TbpB expressed by Neisseria gonorrhoeae. Infect Immun 2007, 75:3220-3232.

34. Didelot X, Falush D: Inference of bacterial microevolution using multilocus sequence data. Genetics 2007, 175:1251-1266.

35. Tamura K, Dudley J, Nei M, Kumar S: MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) Software Version 4.0. Mol Biol Evol 2007, 24:1596-1599.

36. Zhu P, Mignon M, Caugant DA, Quentin-Millet MJ: Variable interspecific genetic exchange between commensal Neisseriae and Neisseria meningitidis. Mol Microbiol 2000, 36:1049-1058.

37. Stojilkovic I, Hwa V, de Saint Martin L, O'Gaora P, Fraysse S, Chabanel C, Achtman M: Allelic diversity of the two transferrin binding protein B gene isotypes among a collection of Neisseria meningitidis strains representative of serogroup B disease: implication for the composition of a recombinant TbpB-based vaccine. Infect Immun 2000, 68:4938-4947.

38. Stojilkovic I, Stojilkovic G, Mignon M, Danve B, Poncet D, Anic S, So M: HmbR outer membrane receptors of pathogenic Neisseria spp.: iron-regulated, hemoglobin-binding proteins with a high level of primary structure conservation. J Bacteriol 1996, 178:4670-4678.
penicillin resistance in pathogenic and commensal Neisseria species. J Mol Evol 1992, 34:115-125.

51. Zhu P, Tsang RS, Tsai CM: Nonencapsulated Neisseria meningitidis strain produces amylopectin from sucrose: altering the concept for differentiation between N. meningitidis and N. polysaccharea. J Clin Microbiol 2003, 41:273-278.

52. Anand CM, Ashton F, Shaw H, Gordon R: Variability in growth of Neisseria polysaccharea on colistin-containing selective media for Neisseria spp. J Clin Microbiol 1991, 29:2434-2437.

53. Berger U: First isolation of Neisseria polysacchareae species nova in the Federal Republic of Germany. Eur J Clin Microbiol 1985, 4:431-433.

54. Legrain M, Rokbi B, Villeval D, Jacobs E: Characterization of genetic exchanges between various highly divergent tbpBs, having occurred in Neisseria meningitidis. Gene 1998, 208:51-59.