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Heterogeneity of *tbpB*, the Transferrin-Binding Protein B Gene, among Serogroup B *Neisseria meningitidis* Strains of the ET-5 Complex

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ET-5 complex strains of *Neisseria meningitidis* were traced intercontinentally and have been causing hyperendemic meningitis on a worldwide scale. In an attempt to develop a fully broad cross-reactive transferrin-binding protein B (TbpB)-based vaccine, we undertook to assess the extent of variability of TbpB proteins among strains of this epidemiological complex. For this purpose, a PCR-based method was developed to study the heterogeneity of the *tbpB* genes from 31 serogroup B *N. meningitidis* strains belonging to the ET-5 complex. To define adequate primers, the *tbpB* gene from an ET-5 complex strain, 8680 (B:15:P1.3; isolated in Chile in 1987), was cloned and the nucleotide sequence was determined and compared to two other previously published *tbpB* sequences. A *tbpB* fragment was amplified from genomic DNA from each of the 31 strains. By this method, heterogeneity in size was observed and further characterized by restriction pattern analysis with four restriction enzymes and by sequencing *tbpB* genes from three other ET-5 complex strains. Four distinct *tbpB* gene types were identified. Fifty-five percent of the strains studied (17/31) harbored *tbpB* genes similar to that of strain BZ83 (B:15:-) isolated in The Netherlands in 1984. Ten of the 31 strains (32.2%) had *tbpB* genes close to that of strain M982. Only 3 of the 31 (9.6%) were found to harbor *tbpB* genes close to that of strain 8680, and finally one strain, 8710 (B:15:P1.3; isolated in Chile in 1987), was found to harbor a *tbpB* gene different from all the others. These results demonstrated a pronounced variability among *tbpB* alleles within a limited number of ET-5 complex strains collected over a 19-year period. Despite the genetic heterogeneity observed, specific antisera raised to purified Tbps from ET-5 complex strains showed broad cross-reactivity between different TbpBs both by Western blot analysis and bactericidal assay, confirming that a limited number of TbpB molecules included in a vaccine are likely to induce broadly cross-reactive antibodies against the different strains.

Meningococcal disease is a significant cause of mortality and morbidity throughout the world (18). *Neisseria meningitidis* strains of serogroup B are the most common cause of sporadic meningococcal disease in developed countries. In Oregon and parts of Washington State the incidence of serogroup B meningococcal disease increased substantially in 1994 (8). Multilocus enzyme electrophoresis of *N. meningitidis* serogroup B strains collected in these areas during 1993 and 1994 suggested that this increase was due to a group of genetically related strains of the electrophoretic type 5 (ET-5) complex (28). These strains were first identified as the cause of the serogroup B meningococcal epidemic in Norway which began in 1974 (6). After their identification in 1974, serogroup B meningococci belonging to the ET-5 complex subsequently caused epidemics in Europe, Cuba, and Chile (7, 10). In Brazil, a retrospective analysis of isolates recovered from patients in the state of Sao Paulo between 1977 and 1987 showed that clones of the ET-5 complex were already causing disease in 1979 (32). The ET-5 complex has been associated with different serological markers: most strains from Norway were B:15:P1.16, those from Spain and Brazil were B:4:P1.15, and those from Iquique (Chile) were B:5:P1.3 (7). All these epidemics and outbreaks caused by the ET-5 complex increased the disease rate for many years and led to sustained efforts for vaccine development.

Among antigen candidates considered for inclusion in future meningococcal vaccines are transferrin-binding proteins A and B (TbpA and TbpB) which are molecules involved in iron acquisition in meningococci (34). The pronounced variability of TbpB that exists among different strains (13, 14, 16) could be considered a problem for the use of TbpB as a broadly cross-reactive antigen. However, we showed previously that while TbpB varied among strains, antigenic features of TbpB and genomic features of *tbpB* genes allowed the meningococcal strains to be classified into two major families: B16B6-like strains and M982-like strains, harboring 1.8- and 2.1-kb *tbpB* genes, respectively (30). Antisera raised to B16B6 Tbps have been shown to be bactericidal for strains harboring 1.8-kb *tbpB* genes (11), and recently we showed that a full-length recombinant TbpB (rTbpB) from strain M982 was able to induce bactericidal antibodies active against 81% of the strains tested (31). However, we noted in this previous study that among 58 strains harboring 2.1-kb genes, only two of the four ET-5 complex strains analyzed were killed with the antiserum raised to TbpB from strain M982. This indicated that TbpB proteins may be heterogeneous within this complex. Notably, ET-5 complex strain 8680 (B:15:P1.3) isolated in Chile in 1987, unlike strain BZ83 isolated in The Netherlands in 1984, failed to react in both dot blot and bactericidal assays with any of the antisera tested (31).

In the present study we assessed the extent of variability of *tbpB* genes among a representative collection of 31 ET-5 complex strains chosen to represent different serotypes and subtypes and 10 different geographic origins. For this purpose the *tbpB* gene from strain 8680 was cloned and sequenced and a rapid PCR-based technique was developed that allowed the typing of *tbpB* genes. This typing was confirmed by a restriction

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TABLE 1. Characteristics of the 31 *N. meningitidis* strains of the ET-5 complex used in this study and of their *tbpB* genes

Strain[a]	Serogroup	Year	Origin	Size of PCR product (nt)[b]	Restriction pattern of *tbpB* gene[c] with:			
					*Ava*II	*Vsp*I	*Xho*I	*Hin*cII
BZ83 (1)*	B:15:-	1984	The Netherlands	772	A	B	N	C
BZ169	B:15:P1.16	1985	The Netherlands	772	A	B	N	C
NG080	B:15:P1.16	1981	Norway	772	A	B	N	C
NG1/84	B:15:P1.16	1985	Norway	772	A	B	N	C
NGP355	B:15:P1.15	1975	Norway	772	A	B	N	C
NGPB24	B:15:P1.7,16	1984	Norway	772	A	B	N	C
NGPB37	B:15:P1.7,16	1987	Norway	772	A	B	N	C
32/94 (e)*	B:15:P1.7,16	1994	Norway	772	A	B	N	C
44 (g)	B:15:P1.7,16	1993	Finland	772	A	B	N	C
52 (f)	B:15:P1.7,16	1993	Finland	772	A	B	N	C
G111/91 (c)	B:15:P1.3,15	1991	Iceland	772	A	B	N	C
M359/91 (d)	B:15:P1.3,15	1991	Iceland	772	A	B	N	C
MA-5850 (n)	B:4:P1.15	1985	Spain	772	A	B	N	C
28I (l)	B:4:P1.15	1992	Spain	772	A	B	N	C
8679 (q)	B:15:P1.3	1987	Chile	772	A	B	N	C
AO15 (k)	B:4:P1.12	1988	South Africa	772	A	B	N	C
AO20 (j)	B:4:P1.15	1989	South Africa	772	A	B	N	C
8680 (2)*	B:15:P1.3	1987	Chile	805	D	E	F	G
8726 (s)*	B:4:P1.3	1987	Chile	805	D	E	F	G
NG3/83	B:15:P1.16	1984	Norway	805	D	E	F	G
M982 (3)*	**B:9:P1.9**		**United States**	**844**	**N**	**H**	**N**	**J**
NG144/82	B:15:P1.16	1982	Norway	844	N	H	N	I
58/94 (b)	B:15:12,13a	1994	Norway	844	N	H	N	J
92/94 (a)	B:15:7,16	1994	Norway	844	N	H	N	J
504/91 (i)	B:4:-	1991	Argentina	844	N	H	N	K
M871 (h)	B:15:P1.7,16	1992	Israel	844	N	H	N	G
230/89 (m)	B:4:P1.15	1989	Cuba	844	N	N	N	G
BB393 (u)	B:15:P1.3	1986	Chile	844	N	N	N	G
BB396 (t)	B:15:P1.3	1986	Chile	844	N	N	N	G
8694 (r)	B:15:-	1987	Chile	844	N	N	N	G
8696 (p)	B:15:P1.3	1987	Chile	844	N	N	N	G
8710 (o)*	B:15:P1.3	1987	Chile	832	N	N	N	H

[a] Strains are classified according to the size of the PCR product amplified (from the smallest to the largest). Numbers and letters in parentheses after the strain names correspond to those in the legend of Fig. 2. Nucleotide sequences are available for the *tbpB* genes of strains indicated by an asterisk. Strain M982 (a non-ET-5 complex strain) is in boldface because it represents the prototype of strains for which the TbpB protein is encoded by a 2.1-kb gene (22, 30, 31).

[b] The fragment sizes indicated are those of the *tbpB* fragments amplified on genomic DNA with primers P3 and P4. The strains for which *tbpB* nucleotide sequences were available have the exact size given. For the other strains, the sizes were determined after migration on a 3% agarose gel by comparison to the fragment of a strain for which the nucleotide sequence is known (M982, 8680, or BZ83).

[c] Restriction patterns of *tbpB* genes amplified by PCR on genomic DNA with primers P1 and P2 were determined with *Ava*II, *Vsp*I, *Xho*I, and *Hin*cII in separate reactions. Letters A to K designate profiles; letters B and N designate a nondigested fragment.

analysis of *tbpB* genes and by sequencing *tbpB* genes from three other ET-5 complex strains. Tbp complex was affinity purified from two ET-5 complex strains, and specific antisera were produced. The bactericidal activities of these sera were evaluated against those of the homologous strain and nine heterologous ET-5 complex strains.

Overall, this study demonstrates heterogeneity among *tbpB* genes from ET-5 complex strains but reinforces the fact that, despite heterogeneity, cross-reactive epitopes were present in TbpBs from ET-5 complex strains.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Thirty-one *N. meningitidis* serogroup B strains from the ET-5 complex were studied; they were characterized by their combinations of alleles at 14 enzyme loci as described previously (5). They were then serotyped and subtyped with monoclonal antibodies (27). The characteristics of the strains are reported in Table 1. Prototype strain M982 was added (22, 25). For DNA extraction, *N. meningitidis* strains were grown on Mueller-Hinton agar plates (MHA; Difco). To detect Tbps, strains were grown in flasks containing 50 ml of Mueller-Hinton broth (MHB; Difco) supplemented with 30 μM EDDA [ethylenediamine di(*o*-hydroxyphenylacetic acid); Sigma] for 5 h at 37°C. For bactericidal assays the same iron-chelated medium was used but the time of incubation was reduced to 4 h.

Escherichia coli XL1-Blue {*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1* lac [F′ *proAB lacZ*^{q}ΔM15 Tn*10* (Tet^r)]} was obtained from Stratagene Cloning Systems and was routinely cultured on Luria-Bertani medium (Difco) supplemented with ampicillin (100 μg/ml) for maintenance of plasmids.

PCR amplification of *tbpB* genes and restriction analysis of the amplified fragments. Extraction of DNA from each strain was performed by a rapid method using guanidium isothiocyanate (26). PCRs to amplify *tbpB* genes were performed on genomic DNA by using primers P1 (5′-TGCTATGGTGCTGC CTGTG-3′) and P2 (5′-TGCCGTCGAAGCCTTATTC-3′). P1 corresponds to positions 115 to 133 of the *N. meningitidis* M982 *tbpB* gene (EMBL accession no., Z15130) and P2 is the complement to positions 2264 to 2244 of this same gene (25). These primers allowed the amplification of a unique fragment for all the strains tested under the following conditions: 25 cycles, each cycle consisting of denaturation of DNA at 94°C for 1 min, annealing at 58°C for 2 min, and extension at 72°C for 3 min. Positive and negative controls were introduced in each PCR experiment: the positive control corresponds to the amplification of a 2.1-kb fragment on genomic DNA of prototype strain M982, and the negative control was made by replacing genomic DNA by water. Each PCR was analyzed on 1% agarose gel, and the size of each *tbpB* gene was determined with molecular weight marker λ*Hin*dIII (New England Biolabs). The amplified fragment was purified on a Qiaquick column (Qiagen) and then digested by the following enzymes: *Hin*cII, *Ava*II, *Vsp*I, and *Xho*I in four separate reactions in accordance with the protocols specified by the manufacturer (New England Biolabs). The restriction products were separated by electrophoresis on 2% agarose gels. The gels were stained with ethidium bromide and photographed, and the patterns were visually compared.

A second PCR was carried out on genomic DNA with primers P3 (5′-AAGA CCAAGGCGGATACGGTTTTGC-3′) and P4 (5′-GAAGACGAGTCGGAA ACAAAGGGATG-3′) (Fig. 1). Reactions were performed in a volume of 100 μl containing 200 μM (each) dCTP, dGTP, dATP, and dTTP (Pharmacia-LKB), 0.2 μM (each) primer, and 2.5 U of *Taq* polymerase (Appligene). Amplifications were performed in a DNA thermocycler (Biometra; Trio-thermobloc) programmed as follows: initial denaturation at 95°C for 5 min and then 25 cycles comprising consecutive denaturation (30 s at 95°C), annealing (30 s at 58°C), and DNA chain extension (1 min at 72°C). The size of the amplified fragment was determined after electrophoresis of PCR product on 3% agarose gels.

Cloning and sequencing *tbpB* genes from four ET-5 complex strains. *tbpB* genes were PCR amplified with primers P1 and P2 and cloned in the pBluescript plasmid (Stratagene). Standard methods were used for plasmid DNA preparation, restriction endonuclease analysis, and ligations (33). For the *tbpB* gene of strain 8680, DNA sequencing was performed by using the dideoxy chain termination method using the Sequenase 2.0 kit U.S. Biochemicals. When results were questionable or unexpected, the nucleotide sequence was confirmed by sequencing another clone obtained from an independent PCR. The nucleotide sequencing of *tbpB* genes from strains 32/94, 8710, and 8726 was by Genome Express (Grenoble, France), according to the Applied Biosystems protocol. Sequence analyses were performed by using the Clustal multialignment program (Infobiogen; Bisance) (12).

Purification of meningococcal Tbp complex and preparation and analyses of rabbit antisera. The Tbp complexes (TbpA-TbpB) from meningococcal strains BZ83, 8680, and M982 were affinity purified as described previously (11, 31), and the purified proteins were administered to rabbits in the presence of Freund's adjuvant on days 0, 21, and 42. The antisera were collected on day 57 as described previously (11). From the hyperimmune sera obtained, immunoglobulin G (IgG) was purified over a protein A-Sepharose column (17). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the purified proteins was performed as described by Laemmli (20) on a 10% agarose gel, and the polypeptides resolved on gels were electrotransferred to nitrocellulose membranes (37). The strips were incubated with either human transferrin (hTf) conjugated to peroxidase (100 μg/ml) (Jackson Immunoresearch) or with specific antisera diluted 1:1,000, followed by incubation with the conjugate (goat anti-rabbit IgG conjugated to peroxidase; Zymed). The reaction was developed by incubating the membrane in a substrate solution containing 4-chloro-1-naphthol and H_2O_2 as described previously (30).

Bactericidal activity was determined as described previously (31). Briefly, 50 μl of serial twofold dilutions of IgG solutions was added to 96-well microplates (Nunc) and incubated with 25 μl of an iron-starved meningococcal suspension adjusted to 9×10^4 CFU/ml and containing 25 μl of baby rabbit complement. After 1 h of incubation at 37°C, 20 μl of the mixture of each well was plated onto Mueller-Hinton agar plates. The plates were incubated overnight at 37°C in 10% CO_2. The bactericidal titer of each serum was expressed as the last dilution of serum at which 50% or greater killing was observed compared to the complement control. In the case of strains that were directly killed by the complement without addition of purified IgG, the complement was first adsorbed on formaldehyde-fixed bacteria (10^{10} CFU/3 ml of complement) before being used for the bactericidal assay. All bactericidal assays were performed in duplicate in two independent experiments.

Nucleotide sequence accession numbers. The nucleotide sequences of *tbpB* genes will appear in the EMBL data library under accession numbers YO9617 (strain 32/94), YO9618 (strain 8710), YO9619 (strain 8726), and Y09977 (strain 8680). Nucleotide sequences of *tbpB* genes from strains M982 and BZ83 have been reported previously (21, 22) and are available in the EMBL data library under accession numbers Z15130 and Z50732, respectively.

RESULTS

Sequence analysis of the *tbpB* gene from *N. meningitidis* 8680. In a previous study, we showed that among 58 strains harboring a 2.1-kb gene, only one strain was neither recognized nor killed by any tested anti-rTbpB from strain M982 (31). Strain 8680 corresponded to an ET-5 complex strain isolated in Chile in 1987 (31). To further characterize the TbpB protein from this strain, its *tbpB* gene was amplified by PCR from genomic DNA, cloned, and sequenced. The amino acid sequence deduced from the nucleotide sequence showed that TbpB from strain 8680 was composed of 688 amino acids (aa), which made it intermediate in length among the known TbpB amino acid sequences (22, 25), where the smallest contained 579 aa (that of strain B16B6) and the largest contained 705 aa (that of strain M978). The alignment of deduced amino acid sequences of TbpB proteins from strains 8680 and M982 showed that TbpB from 8680 contained a hinge domain (aa 345 to 543) delimited by two conserved sequences (VAVVG SAK and VVYRGSWY) similar to those found in the TbpB protein from strain M982 (29). These conserved sequences were the same as those described for all TbpB proteins previously characterized, which have molecular weights of approximately 85 kDa and which are encoded by 2.1-kb genes (29). This result confirmed that *N. meningitidis* 8680 was related to strains displaying a large TbpB in contrast to strains which harbor a 1.8-kb *tbpB* gene and in which the deduced TbpB amino acid sequence lacked this particular region (22). However, the similarity of the TbpB proteins from strain 8680 and strain M982 was only 65.5% according to the Kanehisha program of Infobiogen (12). Among all known TbpB amino acid sequences encoded by a 2.1-kb gene, TbpB from strain S3032 presented the highest level of homology with the TbpB of prototype strain M982 (81.2%) (25). The percentage of homology between the TbpB proteins from strain 8680 and strain M982 was the lowest described so far (21, 25). The level of homology obtained by the Kanehisha program between the N-terminal parts of the TbpB proteins (aa 1 to 345) from strains 8680 and M982 was 59.3%; corresponding values were 69.2% between the hinge domains (aa 346 to 543) and 79.4% between the C-terminal parts (aa 544 to 691).

Because of the low degree of homology observed between TbpB molecules from strains 8680 and M982, we undertook to determine whether *tbpB* genes related to the *tbpB* gene of 8680 were frequent among ET-5 complex strains or whether strain 8680 was an exception. To address the question we developed a rapid technique to detect this type of strain among different ET-5 complex strains. A multialignment was performed on the first 1,000 nucleotides (nt) of *tbpB* genes from strains 8680 and M982 and from BZ83, another ET-5 complex strain (Fig. 1). The alignment allowed us to identify, in *tbpB* nucleotide sequences from ET-5 strains (lanes 2 and 3), deletions (boxed areas) absent in the *tbpB* gene of strain M982. Based upon this alignment, it was possible to define two primers, P3 and P4 (Fig. 1), designed to amplify fragments of the *tbpB* genes whose expected sizes are specific to the strain: 844, 805, and 772 nt for strains M982, 8680, and BZ83, respectively.

Characteristics of *tbpB* genes from 31 strains of the ET-5 complex by PCR analysis. Thirty-one ET-5 complex strains corresponding to different types, subtypes, and geographic locations were studied (Table 1). The *tbpB* gene of each strain was amplified on genomic DNA with primers P1 and P2, and the size of the amplified fragment was determined. The results for 21 of the 31 strains studied are shown in Fig. 2A. A unique PCR product with an apparent size of approximately 2.1 kb was observed. However, it can be noted that there is variability in the size of the gene (between 2.1 and 2.3 kb), indicating that the *tbpB* gene is heterogeneous among the strains studied. To determine the presence of 8680-like strains, a PCR with primers P3 and P4 was performed. The size of the amplified fragment from each strain was determined and compared with those of fragments amplified for control strains BZ83 (lane 1), 8680 (lane 2), and M982 (lane 3) (Fig. 2B). It was then possible to group the *tbpB* genes of the 31 strains into four genetic types: type M982, type BZ83, type 8680, and a fourth type different from all others. For example, strains 32/94 (lane c) and M359/91 (lane d) displayed *tbpB* fragments similar in size to the fragment amplified for strain BZ83; strains M871 (lane h) and 504/91 (lane i) displayed *tbpB* fragments similar in size to the fragment amplified for strain M982.

To confirm this grouping, a restriction enzyme analysis of *tbpB* genes from the 31 strains with four enzymes (*Ava*II, *Xho*I, *Vsp*I, and *Hin*cII) was performed. The enzymes were chosen because they produced specific patterns for the *tbpB* genes of the three control strains (BZ83, 8680, and M982). The restric-

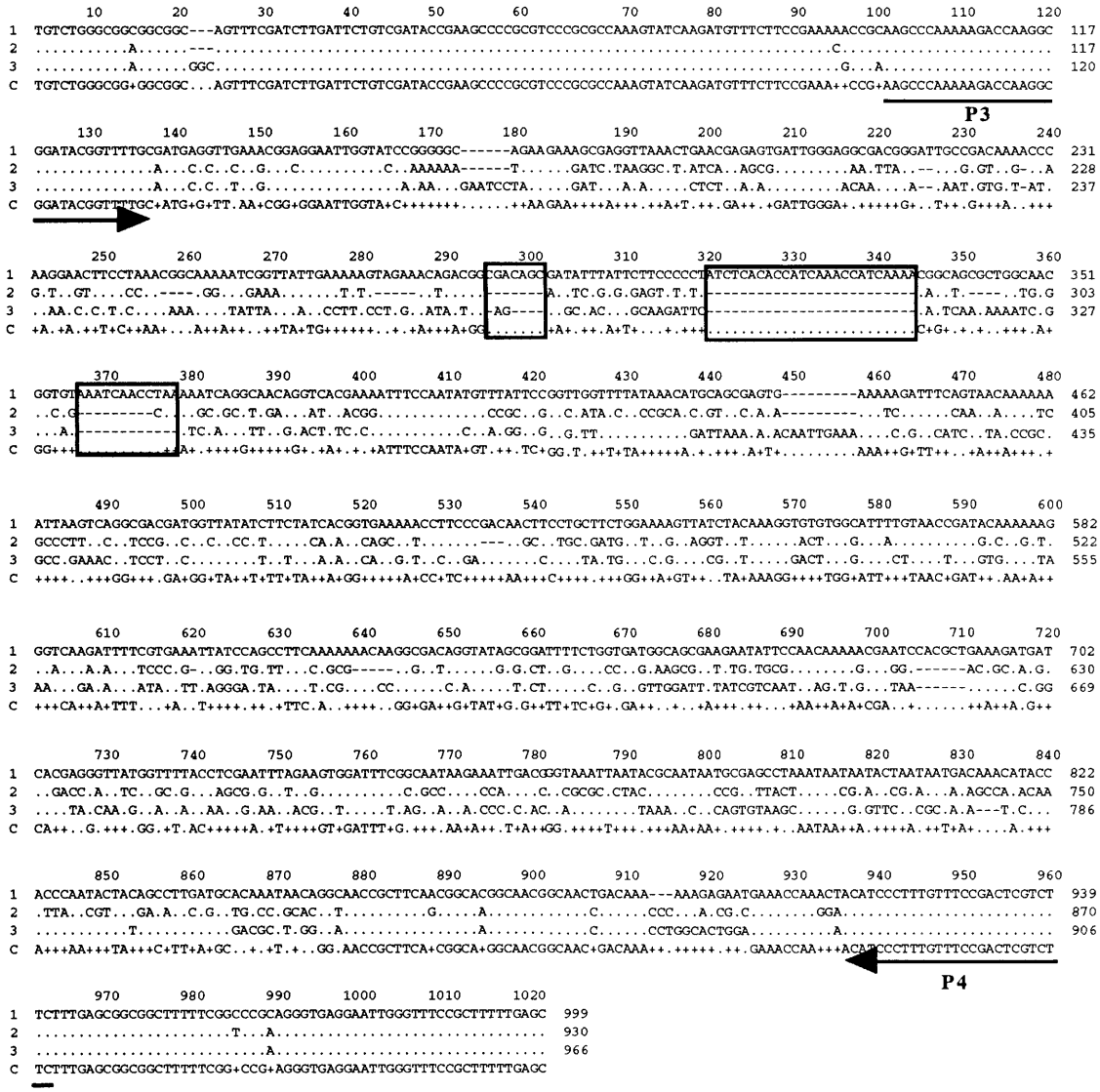

FIG. 1. Nucleotide sequence comparison of the 5′ regions of *tbpB* genes from two ET-5 complex strains (8680 and BZ83) and strain M982. The comparison of the first 1,000 nt was performed with the Clustal program from Bisance (12). Dots indicate identical bases, and spaces have been added to adjust for the best alignment. Boxed regions represent major deletions in the sequences of the *tbpB* genes of strain 8680 (lane 3) and strain BZ83 (lane 2), in comparison to the sequence of the *tbpB* gene from reference strain M982 (lane 1). P3 and P4 are primers designed to amplify a fragment of 844, 805, or 772 nt from the *tbpB* gene of strain M982, 8680, or BZ83, respectively. C, consensus sequence. +, difference between sequences.

tion pattern obtained with *Ava*II is given for the *tbpB* genes of some of the strains studied (Fig. 2C) and allowed the subgrouping of the *tbpB* genes of ET-5 complex strains into 8680 type (for example, strain 8726; lane s), BZ83 type (for example, strain AO15; lane k), or M982 type (for example, strain BB393; lane u).

The results obtained with the three other enzymes on all 31 strains are summarized in Table 1. The *tbpB* gene of each ET-5 complex strain was characterized by its "PCR type" according to the size of the PCR product obtained with primers P3 and P4 and by the restriction profile with four restriction enzymes. Among 31 strains we found 17 strains of the BZ83 type: for all these strains the restriction patterns of the *tbpB* genes with the four enzymes were identical to the *tbpB* profiles of BZ83 strain (patterns A, B, N, and C; Table 1). Based upon the criteria used in this study, *tbpB* genes of the BZ83 type seemed to be highly conserved. Only two strains were found to be of the 8680 type (strains 8726 and NG3/83): these strains displayed the same restriction profiles (patterns D, E, F, and G; Table 1). Ten strains were found to be of the M982 type: among these strains, 2 (strains 58/94 and 92/94) were found to be strictly of the M982 type; i.e., the restriction patterns were all identical to those of the *tbpB* gene of prototype strain M982 (restriction profiles N, H, N, and J; Table 1). The others were defined as M982-like, as one or two enzymes gave profiles that were not identical to the reference profile. One strain (8710) of the 31 could not be classified by the PCR size of the *tbpB* product obtained with primers P3 and P4, and the four restriction patterns did not correspond to any of the previous types; it was referred to as being of the 8710 type. However, the size of the PCR fragment and its profiles with *Ava*II and *Xho*I tend to indicate that this strain is closer to the M982 type than to the BZ83 or 8680 types.

The four different types described (8680, BZ83, M982, and

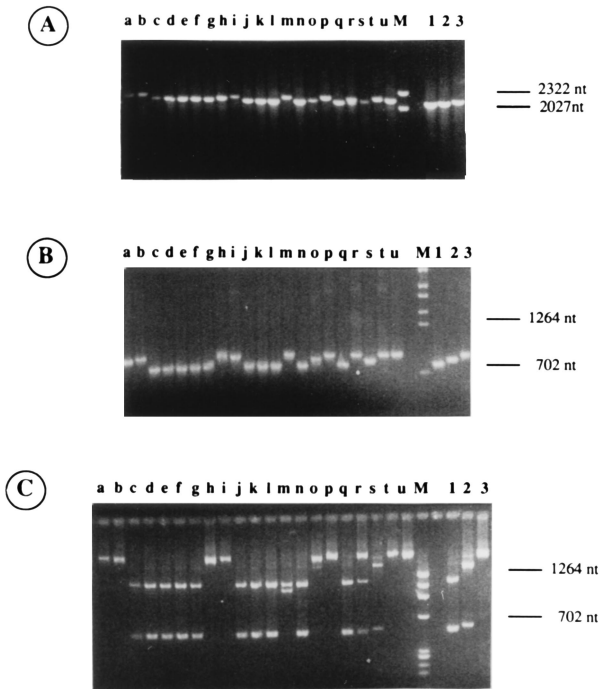

FIG. 2. PCR amplification and *Ava*II restriction patterns of *tbpB* genes from ET-5 complex strains. *tbpB* genes from strains 92/94 (lanes a), 58/94 (lanes b), G111/91 (lanes c), M359/91 (lanes d), 32/94 (lanes e), 52 (lanes f), 44 (lanes g), M871 (lanes h), 504/91 (lanes i), AO20 (lanes j), AO15 (lanes k), 28I (lanes l), 230/89 (lanes m), MA-5850 (lanes n), 8710 (lanes o), 8696 (lanes p), 8679 (lanes q), 8694 (lanes r), 8726 (lanes s), BB396 (lanes t), and BB393 (lanes u) and from control strains BZ83 (lanes 1), 8680 (lanes 2), and M982 (lanes 3) were amplified by PCR on genomic DNA with primers P1 and P2 (A) or were digested with *Ava*II enzyme (C). A 772- to 844-nt fragment was amplified from the *tbpB* genes with primers P3 and P4 (B). M corresponds to size marker λ*Hin*dIII or ϕX 174.

8710) were found among Chilean strains. In spite of the fact that all the strains were isolated between 1986 and 1987 and correspond to serotype and subtype B:15:P1.3 (except 8726), they seemed to be heterogeneous for the *tbpB* gene. Among the 31 strains studied, the BZ83 type (54.8%) and the M982 type (32.2%) were more common than the 8680 type (9.6%).

Sequence analysis of *tbpB* genes from ET-5 complex strains. To further assess the predictive value of the PCR-based classification of *tbpB* genes from ET-5 complex strains, the *tbpB* genes of three strains were cloned and sequenced: an 8680-like strain, 8726 (Chile, 1987; B:4:P1.3); a BZ83-like strain, 32/94 (Norway, 1994; B:15:P7.16); and the 8710 type, 8710 (Chile, 1987; B:15:P1.3). Nucleotide sequences of these three *tbpB* genes were multialigned with *tbpB* sequences from prototype strain M982 and from ET-5 complex strains BZ83 and 8680 with the Clustal program (12). The results (Table 2) showed that the *tbpB* nucleotide sequences from BZ83 and 32/94 had 99% homology. This confirmed the classification based on PCR analysis and the observation that BZ83-like genes seemed to be highly conserved. Similar findings were made for 8680 and 8726 *tbpB* genes: their nucleotide sequences had 90% homology. As far as the *tbpB* gene of strain 8710 is concerned, we found 86% homology with the M982 *tbpB* gene and only 77 to 80% homology with the other genes analyzed.

Presence of cross-reactive epitopes in divergent TbpB molecules. Tbp complexes were affinity purified from the two ET-5 complex strains 8680 and BZ83 and from prototype strain M982. The purified proteins were apparently devoid of contaminating proteins, as illustrated in Fig. 3A, where SDS-PAGE analysis of Coomassie blue-stained Tbps showed no contaminating bands. The TbpB proteins of strains 8680 and BZ83 displayed an apparent molecular mass of 73 kDa (lanes 2 and 3), while that of TbpB of strain M982 was 88 kDa (lane 1). Under our experimental conditions only TbpBs from outer membrane protein (OMP) bound hTf on Western blots (Fig. 3B). Rabbit sera were produced against each purified complex. Their reactivities were assessed on OMPs purified from iron-starved cultures of strains M982, 8680, and BZ83 by Western blotting (Fig. 3C, D, and E). The sera reacted specifically with Tbps in outer membranes, and they all reacted with TbpAs and TbpBs of the three strains. This result indicated that in spite of the low level of homology between the amino acid sequences of M982 and 8680 TbpBs (65.5%), cross-reactive epitopes were detected by Western blotting, while no cross-reactivity was observed by dot blot analysis in our previous study (31). Western blotting and dot blot analysis are different techniques, and both have their limitations. In the dot blot assay the bacterial cells are merely spotted on nitrocellulose, so the exposure of TbpB may be closer to that of intact bacteria than in the Western blot assay. However, it has been suggested that the drying process used in the dot blot assay may lead to modification in the TbpB molecule (9). Therefore, whenever possible, when the strains are not fully sensitive to complement killing alone in the absence of Igs, the bactericidal activity of the antiserum was tested. The bactericidal activity of antibodies induced by immunization with the Tbp complex was further studied with nine ET-5 complex strains belonging to the four

TABLE 2. Percentages of homology[a] among *tbpB* nucleotide sequences

Strain	% Homology with *tbpB* sequence of strain:					
	32/94	8680	8710	8726	BZ83	M982
32/94	100	74	77	75	99	78
8680		100	79	90	74	75
8710			100	80	77	86
8726				100	76	74
BZ83					100	79
M982						100

[a] Percentages of homology were calculated according to the Clustal multialignment program of Infobiogen (12). For accession numbers, see Materials and Methods.

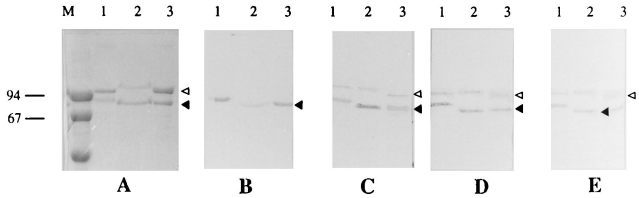

FIG. 3. SDS-PAGE and Western blot characterization of Tbp complexes from ET-5 complex strains BZ83 and 8680. Five micrograms of purified Tbps (TbpA-TbpB) from strains M982 (lane 1), 8680 (lane 2), and BZ83 (lane 3) was separated on a 10% acrylamide gel and stained with Coomassie blue (A). Twenty-five micrograms of OMPs from iron-starved *N. meningitidis* strains M982 (lane 1), 8680 (lane 2), and BZ83 (lane 3) were separated on homogeneous 7.5% polyacrylamide gel, transferred onto nitrocellulose, and then incubated with hTf conjugated to peroxidase at 100 μg/ml (B) or with polyclonal rabbit antiserum (dilution: 1/1,000) raised either to the Tbp complex from strain M982 (C), to the Tbp complex from strain 8680 (D), or to the Tbp complex from strain BZ83 (E). Nitrocellulose strips used for panels C, D, and E were incubated with a goat anti-rabbit horseradish peroxidase-conjugated serum (1/1,000; Zymed). Solid and open arrowheads indicate, respectively, the positions of TbpA and TbpB for each strain. Molecular mass markers (M; LMW; Pharmacia) are in kilodaltons.

TABLE 3. Bactericidal activities of anti-Tbp complexes from strains M982, 8680, and BZ83 versus nine ET-5 complex *N. meningitidis* strains

Strain[a]	Type of *tbpB* gene[b]	Bactericidal titer of anti-Tbps complex[c] from strain:		
		M982	8680	BZ83
BZ83	BZ83-like	128	32	8,192
44	BZ83-like	256	64	16,384
52	BZ83-like	128	16	4,096
8680	8680-like	<4	1,024	<4
8726	8680-like	<4	1,024	128
8710	8710	<4	256	<4
M982	M982-like	512	<4	<8
92/94	M982-like	64	128	<4
504/91	M982-like	8	512	128
M871	M982-like	8	256	256

[a] Strains are classified as in Table 1.

[b] The *tbpB* gene of each strain was typed on the basis of the length of the product amplified by PCR with primers P3 and P4 and on the basis of restriction profiles of the *tbpB* gene with *Ava*II, *Vsp*I, *Hin*cII, and *Xho*I.

[c] Bactericidal titers are expressed as the last dilution of IgG in the presence of which 50% of the initial inoculum is killed. A non-cross-reactive titer is defined as being <4.

PCR types described (Table 3). None of the sera killed the B16B6 strain as expected (30, 31). This strain was used as a negative control representative of strains that displayed a small TbpB (68 kDa) molecule encoded by a 1.8-kb gene. The Tbp molecules of non-ET-5 complex strain M982 induced antibodies that were bactericidal for the homologous M982- and BZ83-like strains but that did not kill the strains from other groups. Tbps from ET-5 complex strain 8680 induced antibodies that were not bactericidal for strain M982 but that were fully cross-bactericidal with other ET-5 complex strains. Tbps from ET-5 complex strain BZ83 induced antibodies that killed six ET-5 complex strains of the nine studied. Overall, the cross-bactericidal activity observed among ET-5 complex strains, was high, ranging from 100 to 67% for anti-Tbp 8680 and anti-Tbp BZ83 sera, respectively.

DISCUSSION

Tbps from meningococci have been proposed as good vaccine candidates (2, 3, 11, 15, 23, 31). Among the two components of the Tbp complex, TbpB has been shown to induce broadly cross-reactive antibodies to many different isolates in spite of its molecular variability (31). Most serogroup B disease isolates from outbreaks have been assigned to four genetically distinct lineages: ET-5 complex, cluster A4, lineage III, and ET-37, based on multilocus enzyme electrophoresis (5, 6, 42). Because serogroup B, ET-5 complex meningococci have been responsible for epidemics and outbreaks all over the world since 1970 (7, 10, 28, 32), a successful vaccine based on Tbps should be able to induce antibodies able to recognize and kill strains from this complex. In the present study, the TbpB protein from strain 8680, an ET-5 complex strain which was neither recognized in a dot blot assay nor killed by any of the previously tested sera raised against rTbpB from strain M982 (31), was characterized, and we determined whether 8680-like strains were widely represented among ET-5 complex strains. The *tbpB* gene from strain 8680 was cloned and sequenced, and the deduced amino acid sequence was found to be consistent with other previously published sequences of 2.1-kb *tbpB* genes (21, 25). This protein contained a hinge domain delimited by two conserved boxes (29), but the deduced amino acid sequence was the most divergent described so far in the family of strains in which TbpB is encoded by a 2.1-kb gene and has only 65.5% homology with TbpB of reference strain M982 and only 56.5% homology in the N-terminal half (aa 1 to 350), which is referred to as the hTf domain (41).

To determine whether this type of TbpB protein was widely represented among ET-5 complex strains, we designed a set of primers on the basis of a multialignment of the first 1,000 nt of *tbpB* genes from strain 8680, from another ET-5 complex strain (BZ83), and from reference strain M982 characterized in previous studies (21, 22). The use of these primers in PCR led to the amplification of a *tbpB* fragment the size of which allowed the identification of 8680-like strains. By using this rapid technique, 31 different ET-5 complex serogroup B strains representing different types and serosubtypes and 10 different geographic origins were studied. We detected only two other 8680-like strains in this collection (strain 8726 isolated in Chile and strain NG3/83 isolated in Norway). To further characterize *tbpB* genes of ET-5 complex strains, restriction analysis was performed with four different enzymes. Among all the strains studied four different restriction types were found: 8680 type, BZ83 type, M982 type, and a fourth unique type (related to strain 8710). The BZ83 type was the most represented in our collection (54.8%). This type seemed to be well conserved, as the restriction profiles obtained with the four enzymes were always the same. The validity of such a characterization of *tbpB* genes was confirmed by sequencing *tbpB* genes of three other ET-5 complex strains. The nucleotide sequence of the *tbpB* gene of strain 32/94, corresponding to the BZ83-type, was highly homologous with the *tbpB* sequence from strain BZ83 (99%). The level of homology was also high (90%) for *tbpB* genes of strains corresponding to the 8680 type (8680 and 8726). The unique *tbpB* of strain 8710 had a nucleotide sequence that had only 80% homology with the M982 *tbpB* sequence. Thus, the combination of PCR and restriction analysis on *tbpB* genes provided a simple method to identify different *tbpB* types among ET-5 complex strains.

Among strains isolated in Chile during the period from 1986 to 1988 four different *tbpB* types were found. The *tbpB* gene seems to vary more rapidly than those encoding class 1 proteins, as all the strains except 8694 were of type B15:P1.3. This diversity in *tbpB* genes was also detectable in north European strains. For Norwegian strains isolated from 1975 to 1994, three types were found. Two types were found among the three Norwegian strains isolated in 1994. We and others have reported the heterogeneity of *tbpB* genes and TbpB proteins among serogroup B strains (13, 14, 16, 21, 25), but to our knowledge this is the first time that this heterogeneity among strains belonging to the same clonal complex has been described. Some of the north European strains that we have studied here (NG080, NG1/84, NG3/83, NGP355, NGPB24, and NGPB37) have already been analyzed for the polymorphism of *opc* (35), a gene coding for a protein that mediates adhesion to and invasion of endothelial cells (38–40). While these six strains had the same *opc* type (35), we found three restriction types for the *tbpB* genes of these strains. This tends to indicate that the *tbpB* gene is more variable than *opc* gene for these specific strains. The variability of *opa* genes encoding opacity proteins (Opa), which constitute a family of antigenically variable OMPs has been extensively studied for subgroup IV-1 meningococci (1, 19). These studies resulted in the identification of seven electrophoretic and antigenically distinct Opa proteins within this closely related group of strains. Unfortunately, the same kind of data are not available for ET-5 complex meningococci. However, published sequences of the class 1 and class 3 OMP genes from ET-5 complex meningo-

cocci responsible for outbreaks in Norway, the United Kingdom, Brazil, and Cuba showed that among these proteins major antigenic changes have occurred by horizontal genetic exchange on at least four separate occasions. These exchanges involved all or part of the class 1 OMP gene, and one exchange involved the whole class 3 OMP gene (7, 24).

It has been suggested that *tbpB* gene variability can arise from this kind of genetic exchange, as a mosaic-like structure has been described (21, 29). However, the functional significance and the mechanism of recombination among *tbpB* genes of the ET-5 complex remain to be elucidated. TbpB is localized at the bacterial outer membrane surface, in association with TbpA, and its primary function is to bind hTf to allow subsequent iron capture (4, 34). This primary function is fulfilled in spite of extensive divergence throughout the coding sequences. *tbpB* variability may result from direct selective pressure on a surface component involved in the interaction on hTf and/or may serve an immune invasion purpose.

There is currently interest in incorporating Tbps into vaccines against group B meningococcal disease (2, 3, 11, 15, 23, 31). If these proteins are to play a successful role in such vaccines, they have to induce broadly cross-reactive antibodies. To assess if Tbps of the ET-5 complex strain can induce large numbers of cross-reactive antibodies within this clonal group, Tbps from ET-5 complex strains 8680 and BZ83 were purified, and used to produce serum in rabbits. The data presented here provide clear evidence of the existence of common antigenic domains among the molecularly heterogeneous TbpBs of *N. meningitidis*, as cross-reactivity of antisera was detected both by Western blotting on purified Tbps from three different strains and by the bactericidal activities of the antisera. This confirms previous analyses made on other serogroup B strains (30, 36). Up to now, with the goal of developing a vaccine protecting broadly against serogroup B strains, the production of a recombinant TbpB molecule from strain M982 that induced in rabbits antibodies able to kill 80% of the strains tested has been achieved (31). Among the strains resistant to killing was an ET-5 complex strain; this prompted us to focus on this clonal group of strains. In the present study, using the Tbp complex purified from meningococci we have shown that despite the heterogeneity observed, the TbpB proteins from ET-5 complex strains can induce broadly cross-reactive bactericidal antibodies. Additional experiments with purified TbpB from ET-5 complex strains will be needed to assess whether the recombinant protein will induce antibodies that cross-react to a greater extent, as was the case for rTbpB from strain M982 (31).

Overall, this study presents a genetic tool for studying the variation of *tbpB* genes among ET-5 complex strains and indicates that a successful TbpB-based vaccine should include a protein able to induce bactericidal antibodies against strains from the ET-5 complex. Other studies are ongoing to address the same type of questions for the other clonal groups: lineage III, ET-37, and cluster A4.

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