INFECTION AND IMMUNITY, Feb. 1997, p. 822–828
0019-9567/97/$04.00+0
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Vol. 65, No. 2

Characterization of the Diversity and the Transferrin-Binding Domain of Gonococcal Transferrin-Binding Protein 2

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Received 21 August 1996/Returned for modification 2 October 1996/Accepted 5 November 1996

The molecular weight heterogeneities of Tbp1 and Tbp2 among a panel of 45 gonococcal isolates were assessed. The *tbpB* genes from four of these strains were sequenced to characterize the Tbp2 sequence diversity among gonococci. By expressing truncated versions of gonococcal Tbp2, we delimited the extent of Tbp2 necessary for transferrin binding in a Western blot.

The pathogenic *Neisseria* spp. are capable of utilizing iron bound to human transferrin as their sole source of iron (24). Two iron-repressed proteins that are thought to contribute to transferrin receptor function have been identified in these bacterial pathogens (2, 5, 16, 20, 31). These proteins, transferrin-binding proteins 1 and 2 (Tbp1 and Tbp2), are surface exposed (6, 21) and thus potential vaccine candidates. Tbp1, which has a molecular weight of approximately 100,000, has homology with the family of integral outer membrane receptors (5, 20), members of which are energized by a complex of proteins including TonB, ExbB, and ExbD (17, 26). Mutants that do not express Tbp1 cannot grow on transferrin as a sole iron source, although they remain capable of some transferrin binding (5, 6). Tbp2 does not share identity with TonB-dependent outer membrane receptors but is lipid modified (2). Gonococcal Tbp2$^-$ mutants internalize iron from transferrin less efficiently than do wild-type gonococci; however, the mutants remain capable of growth on transferrin-bound iron (2). Tbp2s range in molecular weight from 64,000 to 85,000 among clinical isolates of *Neisseria meningitidis* (29) and have been shown to fall into two classes based upon molecular weight and genomic characteristics (29). Meningococcal Tbp2 is expressed during infection, elicits bactericidal antibodies that block transferrin binding, and elicits an antibody response in humans that is reactive against multiple meningococcal strains (1, 7, 14, 21). These observations have engendered optimism about the vaccine potential of meningococcal Tbp2. Because gonococcal Tbp2 is similarly modified (2) and functionally related, we are equally optimistic about the vaccine potential of this antigen, although this communication represents the first characterization of the antigenic heterogeneity of this protein among gonococci.

Tbp1 and Tbp2 exhibit distinct transferrin-binding characteristics as well as distinct physical characteristics. Tbp1 binds to transferrin in affinity purification assays as well as in solid- and liquid-phase transferrin-binding assays (6). Tbp1 also binds transferrin when expressed alone by recombinant *Escherichia coli* (4). Tbp1, however, does not maintain its transferrin-binding capability after boiling and sodium dodecyl sulfate-polyacrylamide gel electrophoresis; this characteristic is specific to Tbp2 (2, 5). Tbp2 binds to transferrin in affinity purifications but cannot be affinity purified from a Tbp1$^-$ mutant in the presence of the detergent Sarkosyl (6, 16). This observation has been interpreted as indicative of a physical association between Tbp1 and Tbp2. Another transferrin-binding characteristic that is particular to Tbp2 is its ability to discriminate between apo- and ferrated forms of transferrin in both solid- and liquid-phase transferrin-binding assays (6).

Determination of the utility of Tbp2 as a gonococcal-vaccine component requires assessment of the heterogeneity of this protein in nature. This report represents the first characterization of the extent of molecular weight, sequence, and antigenic heterogeneity among Tbp2 proteins expressed by gonococci. We have delimited a minimal domain, which extends through the amino-terminal half of the protein, that is necessary for transferrin binding to gonococcal Tbp2 in a Western blot. This transferrin-binding domain is presumably important for the function of this protein and, since it contains conserved sequences, represents a potential target for immunoprophylaxis. Conserved and variable domains of gonococcal Tbp2s are discussed in the context of observations of functional constraints made for Tbp2s from other pathogenic bacteria.

Molecular weight heterogeneity of gonococcal Tbp2s. We obtained a set of 40 gonococcal strains from R. Brunham (University of Manitoba, Winnipeg, Canada), which were isolated over a period of 1 year from prostitutes in Nairobi. These strains represent a broad range of serotypes and genotypes (3). These strains were grown from frozen stocks on chemically defined medium (CDM)-agarose plates (10) containing 2.5 μM transferrin (approximately 30% saturated with iron) as an iron source. Whole-cell lysates of these iron-stressed gonococcal strains were prepared by swabbing overnight growth from CDM-transferrin plates grown at 37°C in a 5% CO_2 atmosphere into CDM broth to a density of 100 Klett units, followed by pelleting of the organisms and resuspension of the pellet in Laemmli solubilizing buffer (19). β-Mercaptoethanol was added to 5%, and the lysates were boiled for 2 min prior to loading onto 7.5% polyacrylamide gels (19). The proteins were transferred to nitrocellulose as described previously (6). Figure 1 shows a representative sample of the 45 gonococcal strains tested for molecular weight heterogeneity of Tbp1 and Tbp2. Tbp1 was detected (Fig. 1A) with a polyclonal antiserum raised against Tbp1, an antiserum which has been described previously (5). Tbp2 was detected (Fig. 1B) with a horseradish peroxidase (HRP)-human transferrin conjugate (Jackson ImmunoResearch, West Grove, Pa.) by methods that have been previously described (2, 6). Among the gonococcal strains screened, Tbp1s ranged in molecular weight from 100,000 to 103,000, and gonococcal Tbp2s ranged from 78,000 to 86,000.

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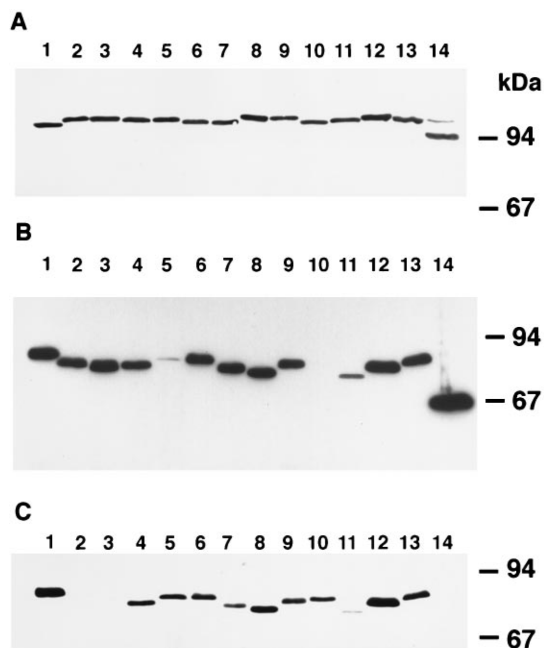

FIG. 1. Heterogeneity of Tbp1 and Tbp2 among gonococcal strains. Western blots were probed with anti-Tbp1 serum (A), HRP-transferrin, which detects Tbp2 (B), or an antiserum raised against a peptide of gonococcal Tbp2 (shown in Fig. 2) (C). Lanes 1 to 5 contain whole-cell lysates of the gonococcal strains FA19, FA1090, UU1008, Pgh3-2, and FA6642, respectively. Lanes 6 to 13 contain lysates of gonococcal strains obtained from R. Brunham, as follows: 6, 4102; 7, 4121; 8, 4125; 9, 4136; 10, 4141; 11, 4146; 12, 4178; 13, 4196. Lane 14 contains a whole-cell lysate from meningococcal strain FAM20. Approximate positions of molecular mass markers are on the right. Blots were scanned with a Relisys 2412 scanner, and images were annotated with Adobe Photoshop software.

Meningococcal Tbp1s and Tbp2s have been described as a heterogeneous group of proteins that fall into two broad molecular weight classes, a high-molecular-weight class and a low-molecular-weight class (29). We detected no evidence of the low-molecular-weight class (approximately 63,000 to 65,000) of Tbp2 among the gonococcal strains that we tested. An example of the low-molecular-weight class of Tbp2 expressed by the minority of meningococcal strains is that expressed by meningococcal strain FAM20 (9) (Fig. 1, lane 14).

We assessed the localized antigenic heterogeneity of Tbp2 among a selected subset of the 45 gonococcal strains by reacting solubilized whole-cell proteins with an antiserum raised against a 16-mer peptide predicted from the sequence of Tbp2 from gonococcal strain FA19. The sequence of this 16-mer peptide is shown in Fig. 2. The antiserum was raised in mice against a conjugate of the 16-mer peptide with Pierce's SuperCarrier (cationized bovine serum albumin) according to manufacturer recommendations (Pierce, Rockford, Ill.). Mice were immunized subcutaneously, initially with the peptide conjugate in Freund's complete adjuvant and twice subsequently with the conjugate in Freund's incomplete adjuvant. A Western blot demonstrating the reactivity of this antiserum with Tbp2s from 13 gonococcal strains is shown in Fig. 1C. Although gonococcal Tbp2 proteins reacted poorly with HRP-transferrin (Fig. 1B, lanes 5 and 10), these proteins reacted strongly with the anti-Tbp2 peptide serum (Fig. 1C, lanes 5 and 10). Surprisingly, two gonococcal strains (FA1090 and UU1008) that are closely related to strain FA19 at the predicted-protein-sequence level (see next section) did not express Tbp2 proteins to which the antipeptide serum bound (Fig. 1C, lanes 2 [FA1090] and 3 [UU1008]). The Tbp2 expressed by meningococcal strain FAM20, a representative of the low-molecular-weight class of Tbp2s, also was not recognized by this antipeptide serum.

Sequence diversity among gonococcal and meningococcal Tbp2s. We sequenced genes encoding Tbp2 (*tbpB*) from four gonococcal strains among the panel tested for molecular weight and antigenic heterogeneity. The *tbpB* genes were amplified by PCR from chromosomal DNA of the following gonococcal strains: FA6642 (a disseminated strain isolated from a patient in North Carolina), FA1090 (obtained from Janne Cannon, University of North Carolina), Pgh3-2 (obtained from Lederle-Praxis Biologicals, Rochester, N.Y.), and UU1008 (obtained from Z. McGee, University of Utah). (For the positions of primers used to amplify the *tbpB* genes, see Fig. 4.) The 5′ amplimer sequence was GGGGTACCTCATTCTTTA ATTGAATCGGGTTTGTTATG, and the 3′ amplimer sequence was CGGATCCGGAACGATGCCGTCTGAAGCCT TATTCTCG. PCR products were purified by agarose gel electrophoresis followed by use of Magic PCR columns (Promega, Madison, Wis.). PCR products were then cloned into the pCRII vector (Invitrogen, San Diego, Calif.), and their size was confirmed by restriction digestion with *Eco*RI, which released the insert. The *E. coli* strain used for cloning was DH5αMCR (Gibco BRL). Plasmid DNA was purified by alkaline lysis, followed by CsCl gradient ultracentrifugation (22). Purified DNA from five to six individual PCR clones was pooled to provide a sequencing template preparation for dideoxy chain termination sequencing with Sequenase (30). Both strands of the pooled PCR templates were sequenced in both directions with internal primers designed from the *tbpB* sequence of strain FA19. In some cases, specific primers were synthesized to sequence the *tbpB* genes from the other gonococcal strains. Sequence analysis was performed with the University of Wisconsin Genetics Computer Group software package (8).

An alignment of gonococcal Tbp2 sequences is shown in Fig. 2 along with the published sequences for several meningococcal Tbp2 sequences, four of which are members of the high-molecular-weight class (M982, M987, 6940, and S3032) (20, 23) and one of which represents the low-molecular-weight class (B16B6) (20). The three meningococcal Tbp2 sequences most recently added to the database (23) did not include the signal sequences, which are omitted in Fig. 2. A diversity plot is shown in Fig. 3, in which the number of different residues found at any one position in the alignment is plotted against the residue number. Such plots have been utilized to identify conserved membrane-spanning stretches of residues in outer membrane proteins (11). Several regions of relative conservation were detected by this method and are indicated in Fig. 2 and 3. These regions do not have the characteristics of membrane-spanning segments of other outer membrane proteins, including amphipathic stretches of residues predicted to form β-strands (18). The cross-hatched bars in Fig. 2 highlight approximate extents of sequence conservation, which are identified as valleys in the diversity plot presented in Fig. 3. Conserved domains 1 and 2 (Fig. 2) encompass residue positions at which there are no more than three amino acid differences among 10 aligned Tbp2 sequences. The end points of these domains were determined by the first position in the alignment at which more than three amino acids exist. Position 375, at which there are four different amino acid residues, is included in conserved domain 2 since it is flanked on either side by identities. The amino terminus beyond the signal sequence is conserved for approximately 60 residues. Conserved region 2, encompassing positions 345 to 388 in the alignment, exhibits 61% identity among all 10 strains aligned. Of the 17 nonidentical residues, 9 are identical among all sequences in the alignment except for B16B6. Four shorter regions in the carboxy-

```
          1                                                                                                   100
g-FA1090 MNNPLVNQAA MVLPVFLLSA CLGGGGSFDL DSVDTEAP RPAPKYQDVP SKKPEARKD. ....QGGYGF AMRFKRRNWY PPSNPKENEI KLSEGDWEQT
g-UU1008 MNNPLVNQAA MVLPVFLLSA CLGGGGSFDL DSVDTEAP RAAPKYQDVP SKKPEARKD. ....QGGYGF AMRFKRRNWY RAAN..ENEV KLKESDWEQT
g-FA6642 MNNPLVNQAA MVLPVFLLSA CLGGGGSFDL DSVDTEAP RPAPKYQDVP SKKPEARKH. ....QGGYGF AMRFKRRNWN RAAN..ENEV KLNESNWKRT
  g-FA19 MNNPLVNQAA MVLPVFLLSA CLGGGGSFDL DSVDTEAP RPAPKYQDVP SKKPEARKD. ....QGGYGF AMRFKRRNWH PSANPKEDEV KLKNDDWEAT
g-Pgh3-2 MNNPLVSQAA MVLPVFLLSA CLGGGGSFDL DSVDTEAP RPAPKYQDVP SKKPEARKD. ....QGGYGF AMRFKRRNWH PMAIPKENEV KLKDDDWEAT
  m-6940 .......... .......... CLGGGGSFDL DSVDTEAP RPDPKYQDVS SEKPQAQKD. ....QGGYGF AMRLKRRNWY SAA..KEDEV KLNESDWETT
  m-M982 MNNPLVNQAA MVLPVFLLSA CLGGGGSFDL DSVDTEAP RPAPKYQDVS SEKPQAQKD. ....QGGYGF AMRLKRRNWY PGA..EESEV KLNESDWEAT
 m-S3032 .......... .......... CLGGGGSFDL DSVDTEAP RPAPKYQDVS SEKPQAQKD. ....QGGYGF AMRLKRRNWY PSA..KENEV KLNESDWETT
  m-M987 .......... .......... CLGGGGSFDL DSVDTEAP RPAPKYQDVS SEKPQAQKD. ....QGGYGF AMRLKRRNWH PQANPKEDEI KLSENDWEAT
 m-B16B6 MNNPLVNQAA MVLPVFLLSA CLGGGGSFDL DSVETVQDM HSKPKYEDEK SQPESQQDV SENSGAAYGF AVKLPRRNAH FNPKYKEKHK PLGSMDWKKL
                                                              1

          101                                                                                                 200
g-FA1090 G..NGNIKNP SKQKNIIDAL SGNGEAP... ......LQDS SQQGRGIS.. ......KVTD YHDFKYVWSG FFYKQIGNTI KKDDSSSKII EARNGPDGYI
g-UU1008 D..DDEIKNP FKQKNIIKAL PGNEGEL... ......LQDS SQQGKGTS.. ......KVRD KHDFKYVWSG FFYKRIKITT KKDE.SHKII EARSGPDGYI
g-FA6642 D..DGDIKKP SKQQNIIDAL TGNDGET... ......LQDS NQEGQSIS.. ......KVEG EHDFKYVWSG FFYKHIQ... TKHETIDGKI TVRSGPDGYI
  g-FA19 GLPTEPKKLP LKQQSVISEV ETNG...NSK MYTSPYL..S QDADSSHANG ANQPKNEVTD YKKFKYVYSG WFYKHAKSEV KNENGLV... SAKRGDDGYI
g-Pgh3-2 GLPGDPKDLP GRQKSVIDEV SAND...NND IYFSPYLKPS NHQNSSINGS ANQPKNEVKD YKNFKYVYSG WFYKHAKFII DGIQNKL... ..QGGDDGYI
  m-6940 GLPTEPKKLP LKQESVISKV QANNG..DNN IYTSPYLTQS NHQNSSINGG ANLPKNKVTN YKDFKYVYSG WFYKHAKNEI IRENSSIK.. GAKNGDDGYI
  m-M982 GLPTKPKELP KRQKSVIEKV ET.DG..DSD IYSSPYLTPS NHQNGSAGNG VNQPKNQATG HENFKYVYSG WFYKHAASE. ..KDFSNK.. KIKSGDDGYI
 m-S3032 GLPSNPKNLP ERQKSVIDQV ETDGDSNNSN IYSSPYLTQS NHQNGNTGNG VNQPKNEVTD YKNFKYVYSG WFYKHAKRE. VNLAVEPK.. IAKNGDDGYI
  m-M987 GLPGNPKNLP ERQKSVIEKV KTGS...DSN IYSSPYLTQS NHQKGS.... ANQPKNEVKD YKEFKYVYSG WFYKHAKLEI IKENNLIK.. GAKSGDDGYI
 m-B16B6 Q.RGEPNSFS ERDE...... .......... ......LEKK RGSSELIESK WEDGQSRVVG YTNFTYVRSG YVY.....L NKNNIDIKNN IVLFGPDGYL

          N1
          201                                                                                                 300
g-FA1090 FYKGTDPSRK LPVSGEVMYK GTWDFLTDVQ ANQKFTDLGS AFTKSGDRYS AFSGELD..Y IVRKEEDKKD GEVGLALTTE ITVNFEKKTL SGKLIKNNKV
g-UU1008 FYKGRNPSRK LPVSGEVTYK GTWDFLTDVK ANQKFTDLGN ASTKSGDQYS AFSGELD..Y IVKKEEDKKE KHKGLGLTTE ITVDFEKKTL IGKLIKNNKL
g-FA6642 FYKGKDPSRK LPVSGKVMYK GTWDFLTDVK ANQKFTDLGN TSARSGDRYS AFSGELD..Y IVRKEEDKKD GHVGWGLTTE ITVDFEKKTL NGKLIKNNRV
  g-FA19 FYHGDKPSRQ LPASEAVTYK GVWHFVTDTK QGQKFNDILE TSKGQGDKYS GFSGDDGETT SNRTDSNLND KHEGYGFTSN FKVDFNNKKL TGKLIRNNKV
g-Pgh3-2 FYHGNKPSRQ LPASEAVTYK GVWHFVTDTK QGQKFNDILE ASKGQGDKYS GFSGDEGETT SNRTDSNLND KHEGYGFTSN FKVDFGNKKL TGKLIRNNKS
  m-6940 FYHGKEPSRQ LPASGTVTYK GVWHFATDVK KSQNFRDIIQ PSKKQGDRYS GFSGDDDEQY SNKNESMLKD GQEGYGFTSN LEVDFGSKKL TGKLIRNNKR
  m-M982 FYHGEKPSRQ LPASGKVIYK GVWHFVTDTK KGQDFREIIQ PSKKQGDRYS GFSGDGSEEY SNKNESTLKD DHEGYGFTSN LSVDFGNKKL TGKLIRNNAS
 m-S3032 FYHGKDPSRQ LPASGKITYK GVWHFATDTK RGQKFREIIQ PSKNQGDRYS GFSGDDDEQY SNKNESMLKD GHEGYGFASN LEVDFDNKKL TGKLIRNNAN
  m-M987 FYHGEKPSRQ LPASGEVTYK GVWHFVTDTK QGQKFNDILG TSKKQGDRYS GFPGDDGEEY SNKNEATLQG SQEGYGFTSN LKVDFNKKKL TGELIRNNKR
 m-B16B6 YYKGKEPSKE LPSEKITYK GTWDYVTDAM EKQRFEGL.. GSAAGGDKSG ALSALEEG.. VLRNQAEASS GHTDFGMTSE FEVDFSDKTI KGTLYRNNRI
                                                                                            C4

          301                                                                                                 400
g-FA1090 INN...GDKP TTQYYSLEAQ VTGNRFNGKA MVTDKPENS KSKQHPFVSD SSSLSGGFFG PQGEELGFRF LSDDQKVAVV GSAKTKDETA SS..GGTSGG
g-UU1008 INN...NTKP TTQYYSLEAQ VTGNRFSGKA MATDKGEK. ..KQHPFVSD SSSLSGGFFG PQGEELGFRF LSDDQKVAVV GSAKTKDETA SS..GGTSGG
g-FA6642 IND...NDEH TTQYYSLEAQ VTGNRFNGKA IATDKPKAN ETKEHPFVSD SPSLSGGFFG PKGEELVFRF LSDDQKVAVV GSAKTRDKAA N......GNT
  g-FA19 VINTAASDGY TTEYYSLDAT LRGNRFSGKA IATDKPNTG GTKLHPFVSD SSSLSGGFFG PKGEELGFRF LSDDQKVAVV GSAKTKDETA NGNAPAASSG
g-Pgh3-2 VINTAASDGY TTEYYSLDAT LRGNRFSGKA MATDKPNTG GTKLHPFVSD SSSLSGGFFG PQGEELGFRF LSDDQKVAVV GSAKTRDKAK NGNTASASGD
  m-6940 VTNAPTNDKY TTQYYSLDAQ ITGNRFNGTA IRTDKPDTG GTKLHPFVSD SSSLSGGFFG PKGEELGFRF LSDDQKVAVV GSAKTKDKTE NG..AVASGG
  m-M982 LNNNTNNDKH TTQYYSLDAQ ITGNRFNGTA TATDKKE.N ETKLHPFVSD SSSLSGGFFG PKGEELGFRF LSDDQKVAVV GSAKTKDKLE NG..AAASGS
 m-S3032 QNNNTNNDKH TTQYYSLDAT LKGNRFSGKA EATDKPKNDG ETKEHPFVSD SSSLSGGFFG PQGEELGFRF LSDDQKVAVV GSAKTKDKPA NGNTAEASGG
  m-M987 VTNATANDKY TTQYYSLEAQ VTGNRFNGKA TATDKPGTG ETKQHPFVSD SSSLSGGFFG PKGEELGFRF LSDDQKVAVV GSAKTQDKAA NGNTAAASGG
 m-B16B6 TQNNSENKQI KTTRYTIQAT LHGNRFKGKA LAADKGATNG S...HPFISD SDSLEGGFYG PKGEELAGKF LSNDNKVAAV FGAKQKDKKD GENAAGPATE
                                                                          2

          401                                                                                                 500
g-FA1090 ASVSASNGAT GTSSGNSNLT TVLDAVELTP DGKEIKDLDN FSNAAQLVVD GIMIPLLPS.. TESGNGQADK ......GKNG GTDFTYTTTY MPESDKKDTK
g-UU1008 ASVSTSNGAA GTSSEN.KLT TVLDAVELTP NGKKIKDLDN FSNAAQLVVD GIMIPLLPKD SESGNGQADK ......GKNG KTAFIYETTY TPESDKEDAQ
g-FA6642 ASASGDTGAA RMPSETG.LT TVLDAVELTL DGKEIKNLDN FSDATRLVVD GIMIPLLPS.. TESGNGQADK ......GKNG GTDFIYETTY MPESDKKDTQ
  g-FA19 PGAATMPS.. .....ETRLT TVLDAVELTP DGKEIKNLDN FSNATRLVVD GIMIPLLP.. TESGNGQADK ......GKNG GTDFTYETTY TPESDKKDTK
g-Pgh3-2 TGAAAMPS.. .....ETRLT TVLDAVELTP NGKEIKNLDN FSNAARLVVD GIMIPLLP.. TESGNGQADK ......GKNG GTAFTYTTTY TPESDKKDTK
  m-6940 TDAAASNGAA GTSSENSKLT TVLDAVELKL GDKEVQKLDN FSNAAQLVVD GIMIPLLPEA SESGNNQANQ ......GTNG GTAFTRKFDH TPESDKKDAQ
  m-M982 TCAAASGGAA GTSSENSKLT TVLDAVELTL NDKKIKNLDN FSNAAQLVVD GIMIPLLPKD SESGNTQADK ......GKNG GTEFTRKFEH TPESDKKDAQ
 m-S3032 TDAAASGGAA GTSSENSKLT TVLDAVELTH GGTAIKNLDN FSNAAQLVVD GIMIPLLPQN STGKNNQPDQ ......GKNG GTAFIYKTTY TPKNDDKDTK
  m-M987 TDAAASNGAA GTSSENSKLT TVLDAVELCL NDKKIKNLDN FSNAAQLVVD GIMIPLLPET SESGSNQADK GKKGKNGKNG GTDFTYKTTY TPKNDDKDTK
 m-B16B6 .......... .......... TVIDPYRITG EEFKKEQIDS FGDVKKLLVD GVELSLLPSE GN........ .......... ......KAAF QHEIEQNGVK

          501                                                                                                 600
g-FA1090 AQTGA...GG MQTASDAAGV NGGQAGTKTY KVEACCSNLN YLKYGLLTRE NSNSVMQTVR NSSQAAARTA QGAQSMFLQG ERTDEKEIP. .KEQKVVYLG
g-UU1008 TGMAT...NG VQTVSNTAGG TSGKTKTH.Y KVQACCSNLN YLKYGLLTR. ........K NSSQADAKMG QVEQSMFLQG ERTDEKEIP. .QEQNVVYSG
g-FA6642 TGMAA...DG VQTVSNAAGG TSGKTKT..Y KVQVCCSNLN YLKYGLLTRE NNNSVMQAGE SSSRTAAQTA QGAQSMFLQG ERTDEKEIP. .KDENVVYLG
  g-FA19 AQTGA...GG MQTASGTAGV NGGQVGTKTY KVQVCCSNLN YLKYGLLTRE NNNSVMQAVK NSSQADAKTK QIEQSMFLQG ERTDENKIP. .QEQGIVYLG
g-Pgh3-2 AQTGA...GG MQTASDAAGV NGGQAGTKTY EVKACCSNLN YLKYGLLTRK TAGNTGEGGK .GSQAAAQTA QGAQSMFLQG ERTDEKEIP. .KDGDVVYLG
  m-6940 AGTQT...NG AQTASNTAGD TNGK..TKTY EVEVCCSNLN YLKYGMLTRK NSKSAMQAGE SSSQADAKTE QVEQSMFLQG ERTDEKEIP. .SEQNIVYRG
  m-M982 AGTQT...NG AQTASNTAGD TNGK..TKTY EVEVCCSNLN YLKYGMLTRK NSKSAMQAGG NSSQADAKTE QVEQSMFLQG ERTDEKEIP. .TDQNVVYRG
 m-S3032 AQTVT...GG TQTASNTAGD ANGK..TKTY EVEVCCSNLN YLKYGLLTRK TAGNTVGSGN SSPTAAAQTD ...AQSMFLQG ERTDENKIP. .SEQNVVYRG
  m-M987 AQTGAAGSSG AQTDLGKADV NGGKAETKTY EVEVCCSNLN YLKYGMLTRK KSKSAMQAGG NSSQADAKTE QVEQSMFLQG ERTDEKEIP. .KDQNVVYRG
 m-B16B6 AT........ .......... .......... ...VCCSNLD YMSFGKLSKE NK........ .......... ...DDMFLQG VRTPVSDVAA RTEANAKYRG
                                           3                                    4

          601                                                                                                 700
g-FA1090 TWYGHIAAN. GTSWSGNASD QQSGNRAKFD VNFKDKKITG TLTAHNRQEA TFTIDAMIDD NGFKGTAKTG NDGFAPDQNS STSTYKVHIA NAEVQGGFYG
g-UU1008 TWYGHIATN. GTSWTREASD QENGNRANFD VNFKDKKITG TLTASNRSEA TFTIEAMIEG NGFKGTAKTG NGGFAPDQNS STGTHKVHIT NAAVQGGFYG
g-FA6642 SWYGYIA.N. GTSWSGNASN TTSGNKAEFT VNFKDKKITG MLTARNRQEA TFTIDAMIES NGFKGMAKTG NGGFAPDQNS STGTHKVHIA EAEVQGGFYG
  g-FA19 FWYGRIA.N. GTSWSGKASN ATDGNRAKFT VNFDRKKITG TLTAENRQEA TFTIDAMIEG NGFKGTAKTG NDGFAPDQNK STVTHKVHIA NAEVQGGFYG
g-Pgh3-2 TWYGHIAIN. GTSWTREASN QENGNRAKFD VNFKDKKITG TLTAENRSEA TFTIDGEIEG NGFKGTAKTG NEGFAPDQNN SIVTHKVRIA NAEVQGGFYG
  m-6940 SWYGYIANDK STSWSGNASN ATSGNRAEFT VNFADKKITG TLTADNRQEA TFTIDGNIKD NGFEGTAKTA ESGFDLDQSN TTRTPKAYIT DAKVKGGFYG
  m-M982 SWYGHIANG. .TSWSGNASD KEGGNRAEFT VNFADKKITG KLTAENRQAQ TFTIEGMIQG NGFEGTAKTA ESGFDLDQKN TTRTPKAYIT DAKVKGGFYG
 m-S3032 SWYGHIAS.. STSWSGNASD KEGGNRAEFT VNFGEKKITG TLTAENRQEA TFTIDGKIEG NGFSGTAKTA ELGFDLDQKN TTRTPKAYIT DAKVKGGFYG
  m-M987 SWYGHIAS.. STSWSGNASN ATSGNRAEFT VNFADKKITG TLTAENRQEA TFTIDGKIEG NGFSGTAKTA DLGFDLDQSN TTGTPKAYIT DAKVQGGFYG
 m-B16B6 TWYGYIAN.. GTSWSGEASN QEGGNRAEFD VDFSTKKISG TLTAKDRTSP AFTITAMIKD NGFSGVAKTG ENGFALDPQN TGNSHYTHI. EATVSGGFYG
                                                                                                             5

          701                                        751
g-FA1090 PNAEELGGWF AYPGNGQTKN AQENAQASSG NGNSAVSATV VFGAKRQQLV K
g-UU1008 PNAEELGGWF AYPGNGQTK. ...NAQTSSG NGNSAGSATV VFGAKRQQLV K
g-FA6642 PNAEELGGWF AYPGNGQTKN AQENAQASSG NGNSAGSATV VFGAKRQELV K
  g-FA19 PNAEELGGWF AYPGNEQTK. ...NATVESG NGNSASSATV VFGAKRQKLV K
g-Pgh3-2 PNAEELGGWF AYPGNEQTK. ...NAQASSG NGNSAGSATV VFGAKRQKLV Q
  m-6940 PKAEELGGWF AYPGDKQTK. ...NATNASG N....SSATV VFGAKRQQPV R
  m-M982 PKAEELGGWF AYPGDKQTK. ...KATATSS DGNSASSATV VFGAKRQQPV Q
 m-S3032 PKAEELGGWF AYSDDKQTK. ...KATDASS NGNSASSATV VFGAKRQQPV Q
  m-M987 PKAEELGGWF AYPGDKQTE. ...KATVASS DGNSASSATV VFGAKRQQPV Q
 m-B16B6 KNAIEMGGSF SFPGN..... .........A PEGKQEKASV VFGAKRQQLV Q
          5                             6
```

FIG. 2. Alignment of gonococcal and meningococcal Tbp2 sequences. Gonococcal sequences in the alignment are preceded by the letter g; meningococcal sequences are preceded by the letter m. Residues identical in all 10 sequences are indicated by shading. Six regions of conservation are highlighted by cross-hatched boxes numbered 1 to 6. The position of the FA19 Tbp2 peptide to which antiserum was raised is indicated by the double box. The single-boxed region indicates the position of the conserved *A. pleuropneumoniae* Tbp2 peptide to which transferrin bound in a solid-phase assay (32). The transferrin-binding domain of Tbp2 from gonococcal strain FA19 was determined to be between the 5′ end point of pUNCH750 and the 3′ end point of deletion construct C3. This domain is located within the sequence flanked by the solid arrows. The end point of the first amino-terminal deletion that prevented transferrin binding to FA19 Tbp2 (N1) and the end point of the first carboxy-terminal deletion that prevented transferrin binding to FA19 Tbp2 (C4) are indicated. The transferrin-binding domain of Tbp2 from meningococcal strain M982 (34) is within the sequence flanked by the open arrows. The first amino- and carboxy-terminal deletions that prevent transferrin binding to M982 Tbp2 (34) are indicated by open triangles. The transferrin-binding domain of Tbp2 from meningococcal strain B16B6 (34) is within the sequence flanked by small solid circles. The first amino- and carboxy-terminal deletions that prevent transferrin binding to B16B6 Tbp2 (34) are indicated by large solid circles. The carboxy-terminal end point of the deletion in FA6819 is indicated by an open circle. Dots prior to the conserved cysteine residues indicate signal sequences not included in the published sequences.

terminal half can be identified (regions 3 to 6 in Fig. 2 and 3) as containing at least 5 consecutive identities. Positions that flank consecutive identities and that contain at least 9 of 10 identical residues are included in the conserved regions. The first of these short regions (cross-hatched bar 3) contains a pair of cysteine residues that are also conserved in the Tbp2 sequences from *Actinobacillus pleuropneumoniae* (12, 13) and from *Haemophilus influenzae* (15). The boxed region of aligned Tbp2s in Fig. 2 indicates the position of a conserved peptide identified in *A. pleuropneumoniae* that bound to transferrin in a solid-phase assay and thus may be important for ligand interaction (32). Between these short homologous stretches are quite divergent sequences. The amino-terminal half of Tbp2 is particularly high in sequence diversity in that at some positions as many as 8 of 10 sequences contain different amino acids. This is somewhat in contrast to the observations of Rokbi et al. (28), who concluded that the region of highest sequence diversity among meningococcal strains resided in the carboxy-terminal half of Tbp2 just downstream of the meningococcal transferrin-binding domain. This so-called "hypervariable" domain does, however, contain the largest proportion of deletions found in B16B6 Tbp2 when it is aligned with other Tbp2s of the high-molecular-weight class.

Identification of a domain of gonococcal Tbp2 required for transferrin binding. We utilized the ability of Tbp2 to bind transferrin in a Western blot to delimit a minimal domain necessary for transferrin binding. The identified domain is necessary for interaction with transferrin under these in vitro conditions, but this analysis does not preclude the possibility that other binding domains that facilitate ligand interaction in other assay formats exist within Tbp2. A fragment of the *tbpB* gene from FA19 was cloned into the *Bam*HI site of pBluescript SKII(+) behind the inducible *lac* promoter (Stratagene, La Jolla, Calif.). This clone (pUNCH750) expressed a portion of Tbp2 translationally fused to the α-peptide of β-galactosidase (Fig. 4). This fusion product retained transferrin-binding capability following denaturation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 5B, lane 2). One amino-terminal deletion product of this clone was constructed such that the Tbp2 sequence was translationally fused to the α-peptide. This clone was created by unidirectional exonuclease III digestion (25) and was used previously for sequencing

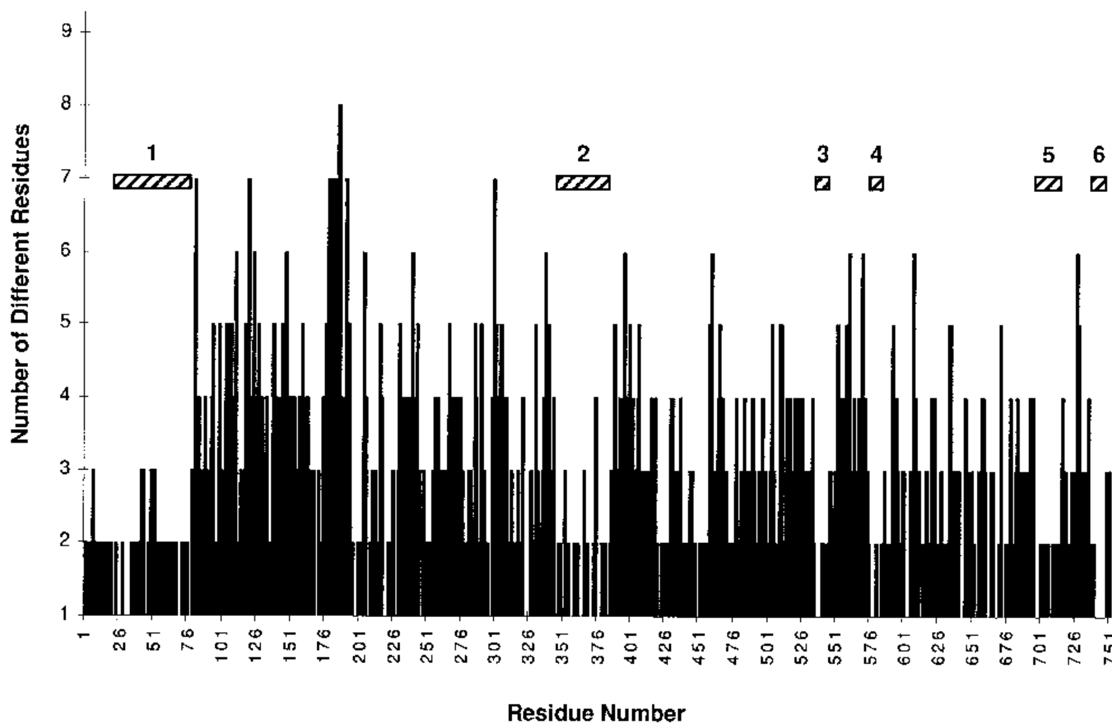

FIG. 3. Diversity plot of five gonococcal and five meningococcal Tbp2 sequences. The number of different residues in the alignment shown in Fig. 2 is plotted as a function of residue number. Regions of sequence conservation are highlighted by cross-hatched bars.

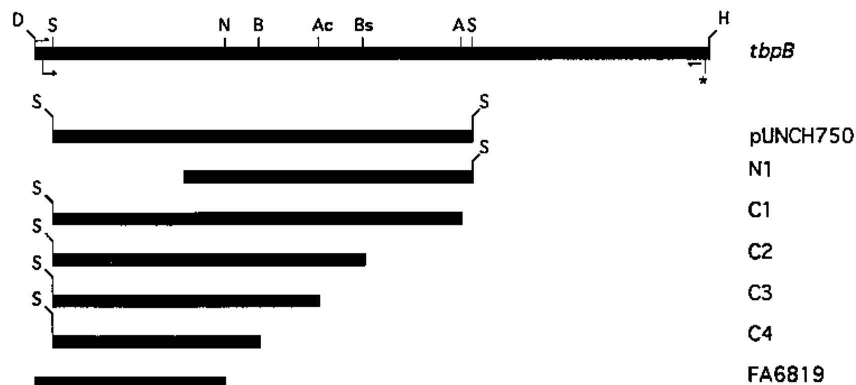

FIG. 4. Schematic diagram of amino- and carboxy-terminal deletions of gonococcal Tbp2. The *tbpB* gene, which encodes Tbp2, is schematically represented at the top. The start of the open reading frame that encodes Tbp2 is indicated by the arrow; the stop codon is indicated by the asterisk. The approximate positions of the amplimers used in PCR to amplify the *tbpB* gene for sequencing are indicated by partial arrows. The extent of gonococcal insert DNA in pUNCH750 is represented below *tbpB*, flanked by *Sau*3AI sites (S). The extent of gonococcal DNA remaining in the amino-terminal deletion construct (N1) and in the carboxy-terminal deletion constructs (C1 to C4) is indicated. The extent of the *tbpB* gene remaining in the chromosome of FA6819 after deletion to the *Nhe*I site (N) is indicated at the bottom. Restriction endonuclease sites: B, *Bbs*I; Ac, *Acc*I; Bs, *Bsp*MI; A, *Age*I; H, *Hinc*II; D, *Dra*I.

of the *tbpB* gene from strain FA19 (2). This deletion construct was cloned in the *E. coli* strain BL21 because it lacks the proteases OmpT and Lon (33). The amino-terminal end point of Tbp2 expressed by this deletion clone (N1) is indicated in Fig. 4. Carboxy-terminal deletions were also constructed by restriction digestion, end repair, and ligation of the repaired ends. Truncated versions of Tbp2 were expressed from these constructs in DH5αMCR (Gibco BRL). End points of these

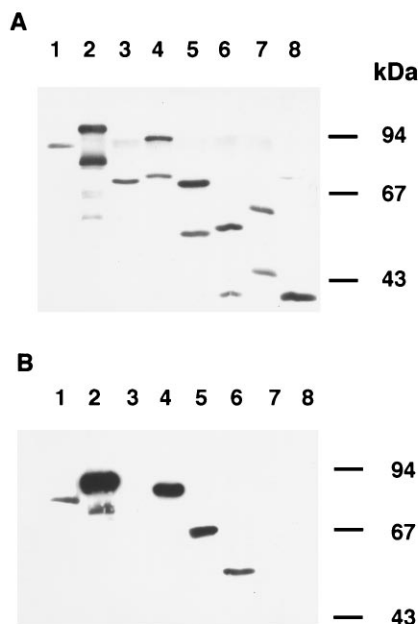

FIG. 5. Transferrin-binding activity of amino- and carboxy-terminal deletions of gonococcal Tbp2. (A) Western blot probed with an antiserum raised against a peptide of gonococcal Tbp2; (B) Western blot probed with HRP-transferrin to detect transferrin binding to Tbp2. Lane 1 contains a whole-cell lysate of gonococcal strain FA19, representing the wild-type size of Tbp2. Lanes 2 to 7 contain whole-cell lysates of *E. coli* strains expressing portions of Tbp2 from the following deletion constructs: 2, pUNCH750; 3, N1; 4, C1; 5, C2; 6, C3; 7, C4. Lane 8 contains a whole-cell lysate of gonococcal strain FA6819, which expresses a truncated version of Tbp2. Approximate positions of molecular mass markers are on the right. Blots were scanned with a Relisys 2412 scanner, and images were annotated with Adobe Photoshop software.

deletion constructs (C1 to C4) are also shown in Fig. 4 with respect to the wild-type *tbpB* gene. The junctions of all of these deletion constructs were sequenced to confirm that the expected deletion had occurred. Figure 5 shows that the truncated Tbp2s expressed by the deletion clones reacted in Western blots with the antipeptide serum (Fig. 5A) described above or with HRP-transferrin (Fig. 5B). The amino-terminal deletion (yielding construct N1) removed 171 amino acids from the mature Tbp2 protein and rendered the protein incapable of transferrin binding in a Western blot (Fig. 5B, lane 3). The first three sequential carboxy-terminal deletion constructs of pUNCH750 (C1, C2, and C3) were expressed as truncated Tbps that remained capable of transferrin binding (Fig. 5B, lanes 4 to 6), while deletion to the *Bbs*I site (yielding C4) prevented transferrin binding (Fig. 5B, lane 7). All of the deletion constructs were expressed by *E. coli* since they reacted with the antipeptide antiserum (Fig. 5A). The products of the progressively greater carboxy-terminal deletions do not uniformly decrease in size, since some deletions result in fusion with the α-peptide downstream of the deletion site and others do not. For example, deletion to the *Acc*I site of *tbpB* (C3 in Fig. 4 and lane 6 in Fig. 5) resulted in a protein with the predicted molecular mass of 48 kDa, which was not the result of fusion with the α-peptide downstream of the fusion junction. The next greater deletion, to the *Bbs*I site of *tbpB* (C4 in Fig. 4 and lane 7 in Fig. 5), resulted in a protein with the predicted molecular mass of 55 kDa, which was the result of fusion of Tbp2 with the α-peptide downstream of the fusion junction. The presence of multiple Tbp2-specific bands detected with the antipeptide serum might be the result of proteolytic cleavage of fusion proteins in *E. coli* since expression of the amino-terminal deletion construct (N1) in a protease-deficient strain, BL21, resulted in fewer Tbp2-specific products (Fig. 5, lane 3). Alternatively, the presence of multiple bands might be the result of incomplete denaturation of gonococcal Tbp2, a phenomenon consistent with the observation that transferrin binding by meningococcal Tbp2 requires the presence of a stable, heat-resistant, highly structured domain (34). Also consistent with the presence of a folded, transferrin-binding domain is the observation that all of the deletion species capable of transferrin binding migrate at an apparent molecular mass larger than that predicted by their protein sequence. The minimal extent of a transferrin-binding domain of Tbp2 from gonococ-

cal strain FA19 is indicated in Fig. 2. Deletion of 163 amino acids from the amino terminus and 93 amino acids from the carboxy terminus of this domain prevented transferrin binding in a Western blot.

A deletion mutant of *tbpB* was previously constructed (FA6819 [2]) for analysis of the phenotype of a Tbp2$^-$-Tbp1$^+$ gonococcal mutant. We determined that although this gonococcal mutant made a stable peptide of Tbp2 (Fig. 5A, lane 8), this peptide no longer bound transferrin (Fig. 5B, lane 8). The limits of the truncated Tbp2 synthesized by this mutant gonococcal strain are indicated in Fig. 4. Since this strain and a complete Tbp2 deletion strain react identically in transferrin-binding assays and iron uptake studies (6), we conclude that either this truncated peptide expressed by FA6819 is not processed to the gonococcal cell surface or this peptide does not contribute to transferrin binding and iron uptake from transferrin when expressed by the gonococcus.

Conclusions. This analysis of molecular weight and sequence heterogeneity of Tbp2s among gonococci suggests that the gonococcal proteins are less diverse as a group than are the Tbp2 proteins from meningococcal strains. Gonococcal Tbp2s fell within a narrow molecular weight range, and we found no evidence, among the 45 strains examined, of a low-molecular-weight class of gonococcal Tbp2 proteins analogous to the class of meningococcal Tbp2s with molecular masses of 65 to 68 kDa (29). Pairwise comparisons between predicted Tbp2 sequences of gonococcal strains generated 69 to 84% identity. Pairwise comparisons between the sequenced example of the low-molecular-weight class of meningococcal Tbp2 (from B16B6) and the other Tbp2s of the high-molecular-weight class generated only 46 to 49% identity. Comparisons between gonococcal and meningococcal Tbp2 sequences of the high-molecular-weight class generated identity scores ranging from 64 to 75%.

The diversity plot shown in Fig. 3 points out several regions of conservation that might be important for Tbp2 function, that is, for transferrin binding or for stability and assembly of Tbp2 on the bacterial cell surface. Conserved domain 1 contains the first 53 residues of the mature gonococcal Tbp2 protein. Deletion of eight amino acids of the amino terminus of the mature protein did not affect transferrin binding in a Western blot (Fig. 2); however, this conserved region is probably important for assembly or export of mature Tbp2 to the outer membrane, as is the case for other lipid-modified proteins (27). Deletion of the conserved YGFA residues within domain 1 from meningococcal Tbp2 prevented transferrin binding in a ligand-binding Western blot (34), thus implicating the region containing these residues as important for ligand interaction. Deletion of conserved domain 2 from the carboxy terminus prevented transferrin binding to Tbp2 constructs in a Western blot, which similarly implicates this region in ligand interaction. These regions are not necessarily in direct contact with transferrin but might be required for maintenance of a conformation that facilitates transferrin binding to Tbp2, as has been suggested for meningococcal Tbp2s (34). The short conserved domains numbered 3 to 6 in the carboxy-terminal half of the protein were not required to reconstitute transferrin binding to Tbp2 in a Western blot since in all of the minimal transferrin-binding constructs (Fig. 2) these regions were deleted. Region 3 contains a pair of cysteine residues that are also present in the same relative location in Tbp2s from *A. pleuropneumoniae* and in *H. influenzae*. These residues, while clearly not critical for transferrin interaction, might be necessary for structure maintenance at the cell surface. The importance of the other conserved domains remains unclear but will be addressed by site-specific mutagenesis followed by functional assays for ligand binding, assembly, and export.

Nucleotide sequence accession numbers. Accession numbers for the gonococcal *tbpB* genes presented in this report are as follows: U65219 for *tbpB* from FA1090, U65220 for *tbpB* from FA6642, U65221 for *tbpB* from Pgh3-2, and U65222 for *tbpB* from UU1008.

This work was supported by Public Health Service grants AI26837 and AI31496 from the National Institute of Allergy and Infectious Diseases.

We gratefully acknowledge Chris Thomas for assistance in generating the diversity plot from aligned Tbp2 sequences. The Tbp2-specific peptide was synthesized and conjugated to SuperCarrier at ImClone Systems, Inc., New York, N.Y. Immunizations were carried out at Lederle-Praxis Biologicals in Rochester, N.Y.

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Editor: P. E. Orndorff