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Neisseria meningitidis encodes an FK506-inhibitable rotamase

(peptidyl-prolyl cis–trans isomerase/FK506-binding protein/enzyme evolution)

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ABSTRACT **Eukaryotic peptidyl-prolyl cis–trans isomerases (rotamases) fall into two classes, the cyclophilins inhibited by cyclosporin A and the FK506-binding proteins inhibited by the macrolide antibiotic FK506. In prokaryotes homologs of cyclophilins have been identified and found to have rotamase activity. Sequence similarities have been noted between FK506-binding proteins and gene products in a number of bacterial species, but whether these bacterial proteins have rotamase activity is not known. Using the polymerase chain reaction, we have cloned and sequenced a homolog of an FK506-binding protein from *Neisseria meningitidis* and expressed the gene product as a fusion protein with maltose-binding protein. The fusion protein was purified by affinity chromatography. By measuring the rate of chymotrypsin cleavage of the substrate succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide, we found that the fusion protein had rotamase activity comparable to that of human FK506-binding protein. This rotamase activity was inhibited by FK506.**

The study of the immunosuppressive action of cyclosporin A has demonstrated that this molecule is bound by a class of eukaryotic proteins, the cyclophilins, which have been characterized in a large number of species (1). In 1989 it was discovered that cyclophilins have peptidyl-prolyl cis–trans isomerase (rotamase) activity (2, 3). Sawada *et al.* (4) reported that the macrolide antibiotic FK506 was nearly 100-fold more immunosuppressive than cyclosporin A. Proteins binding FK506 were characterized and found also to have rotamase activity (5, 6). However, the cyclophilins and the FK506-binding proteins (FKBPs) are unrelated and show very little similarity (7).

Recently two important findings have shed light on the actions of these immunosuppressants and the immunosuppressant–receptor complex. Flanagan *et al.* (8) have found that cyclosporin A and FK506 block the calcium-dependent translocation of the cytoplasmic component of NF-AT (nuclear factor of activated T cells) to the nucleus. Liu *et al.* (9) showed that the drug–immunophilin complexes can bind to and inhibit calcineurin, a calcium/calmodulin-dependent phosphatase.

There is less information on the presence and role of rotamases in bacteria. Kawamukai *et al.* (10), while studying a locus responsible for filamentous growth of *Escherichia coli*, noted that the predicted protein sequence of an uncharacterized open reading frame had similarity to cyclophilin. Liu and Walsh (11), using PCR methodology, cloned this gene in *E. coli* and found that it expressed a rotamase that was exported to the periplasm. Hayano *et al.* (12), using protein chemical methods, identified a periplasmic and a cytoplasmic rotamase in *E. coli*. When these were characterized on a molecular level, the former was found to be identical to the rotamase previously described, while the cytoplasmic protein was the product of a closely related but distinct locus. The cyclophilin-homologous rotamases of *E. coli* are not inhibited by cyclosporin A.

Similarly, investigators studying FKBPs have noted similarities to DNA sequences of bacterial gene products from *Legionella pneumophila* (13), *Neisseria meningitidis* (14), and *Pseudomonas aeruginosa* (7). The *Legionella* product, Mip (macrophage infectivity potentiator), had previously been described (15) and is an outer membrane antigen that enhances both the ability of this organism to invade macrophages (16) and the lethality of the infection in guinea pigs (17). Lundemose *et al.* (18) have determined part of the primary sequence of a surface protein of *Chlamydia trachomatis* and found it to be similar to the *Legionella* Mip protein. Standaert *et al.* (14) noted the striking similarity of human FKBP and the protein sequence predicted by a region of *N. meningitidis* DNA immediately preceding two incomplete pilin genes that had been cloned by Perry *et al.* (19). Aho and Cannon (20) have studied the homologous region in another strain of meningococcus, and the DNA sequence of the region preceding the incomplete pilin genes is almost identical. Both groups noted that the cloned meningococcal DNA expressed a M_r 13,000 product.

To date, none of the bacterial proteins resembling FKBP have been analyzed to determine whether they have rotamase activity or bind FK506. We report the cloning, expression, and purification of the meningococcal protein as a fusion protein with maltose-binding protein (MBP). It is an active rotamase that is inhibited by FK506.

MATERIALS AND METHODS

Reagents and Chemicals. Restriction enzymes and the MBP purification system were purchased from New England Biolabs. Media were obtained from Baltimore Biological Laboratories. Prestained protein molecular weight markers were from GIBCO/BRL. Other reagents were obtained from Sigma.

Molecular Biological Methods. Genomic DNA was isolated from a nonencapsulated variant (BNCV) of strain M986 group B *N. meningitidis* by lysing the organisms with EDTA, lysozyme, and 1% SDS. The lysate was digested first with pancreatic ribonuclease and then with proteinase K. The preparation was gently extracted with phenol, phenol/chloroform, and chloroform and then dialyzed extensively against Tris/EDTA/saline (TES) (21). PCR primers were designed to allow cloning of the product into plasmid pMAL-cRI (New England Biolabs), to result in isopropyl β-D-thiogalactopyranoside (IPTG)-inducible expression of the meningococcal gene product as a MBP fusion protein. The sequence of the 5′ PCR primer is shown in Fig. 1. The 3′ PCR primer included a *Bam*HI recognition site and had the sequence TTGGATCCCAGGCAGCTTTATTCGTACAC. PCR amplification (22) was performed with 1 μg of meningococcal DNA as template with the instructions and reagents

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Abbreviations: MBP, maltose-binding protein; IPTG, isopropyl β-D-thiogalactopyranoside; FKBP, FK506-binding protein.

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      M  G  S  L  I  I  E  D  L  Q  E  S        Predicted Protein Sequence
...ATGGGCAGCCTGATTATTGAAGATTTGCAGGAAAGC...     Genomic DNA Sequence
     agaattcATTATTGAAGATTTGCAGGAAAGC           PCR Primer

          EcoRI

...ATCGAGGGAAGGATTTCAGAATTC...                 pMAL-cRI Vector Sequence

...ATCGAGGGAAGGATTTCAGAATTCATTATTGAAGATTTGCAGGAAAGC   Predicted DNA Sequence
   I  E  G  R  I  S  E  F  I  I  E  D  L  Q  E  S     Predicted Fusion Protein
            ↑
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FIG. 1. Design of PCR primer to create fusion protein. The genomic DNA sequence (19) and the predicted native protein sequence of rotamase are shown in the top two lines. The next line shows the sequence of the PCR primer with the lowercase letters indicating the part of the primer not matching the genomic sequence. Below is the sequence of the plasmid vector pMAL-cRI with the *Eco*RI site underlined. The last two lines indicate, respectively, the predicted DNA and fusion protein sequences resulting from cloning the PCR insert into the vector. The arrow points to the factor X_a cleavage site.

provided in the GeneAmp kit (Perkin–Elmer/Cetus). The PCR product was digested with *Eco*RI and *Bam*HI, ligated into similarly digested plasmid pUC19, and transformed into *E. coli* XL1-Blue (Stratagene). The insert in pUC19 was sequenced by the chain-termination method (23) using deaza ^{T7}Sequencing mixes (Pharmacia) and Sequenase enzyme (United States Biochemical). The sequence of both strands was determined by using universal primers and two additional primers based on the available sequence. The PCR and sequencing primers were synthesized by the Rockefeller University Biotechnology Facility.

Production and Purification of MBP Fusion Protein. The *Eco*RI–*Bam*HI insert was recloned from pUC19 into plasmid pMAL-cRI and transformed into strain XL1-Blue. A characterized transformant was grown overnight in LB medium, diluted 1:100 into fresh LB medium, grown for 90 min, induced with 0.3 mM IPTG, and incubated an additional 120 min. The cells were harvested, suspended in 5 ml of lysis buffer (10 mM Tris·HCl, pH 7.2/10 mM EDTA/30 mM NaCl), and lysed by sonication at 0°C. The lysate was centrifuged at 49,000 × *g* for 10 min. The supernatant was applied to a 4-ml column of amylose resin (New England Biolabs). The column was washed with 12 column volumes of lysis buffer, and the fusion protein was eluted with 5 ml of lysis buffer containing 10 mM maltose. Cleavage was attempted at factor X_a/fusion protein weight ratios of 1%, 5%, and 20% at room temperature in 10 mM Tris·HCl, pH 7.2/10 mM EDTA/100 mM NaCl and was monitored by SDS/PAGE at 2, 4, 8, and 24 hr of incubation. MBP was expressed from *E. coli* harboring pMAL-cRI and was purified as described above.

Assay for Rotamase Activity. Rotamase activity was measured and the kinetic parameters were calculated according to the methods outlined by Standaert *et al.* (14) and Harrison and Stein (24). The concentration of the fusion protein was measured by absorbance at 280 nm with an extinction coefficient of 86,100 $M^{-1}\cdot cm^{-1}$, which was calculated according to Gill and von Hippel (25) on the basis of the predicted aromatic amino acid content. All reagents were used at room temperature. The hydrolysis of *N*-succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide by chymotrypsin in the presence of various concentrations of the purified fusion protein was monitored at 0.5-sec intervals at 390 nm for 90 sec in a Milton Roy Spectronic 3000 spectrophotometer. The reaction volume of 1 ml (100 mM Tris·HCl, pH 7.8) contained 300 μg of chymotrypsin, 0.051 mM substrate, and various concentrations of

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 MGSL
 E  F  I  I  E  D  L  Q  E  S  F  G  K  E  A  V  K  G  K  E

GAATTCATTATTGAAGATTTGCAGGAAAGCTTCGGAAAAGAAGCAGTTAAAGGCAAAGAG
       10        20        30        40        50        60

 I  T  V  H  Y  T  G  W  L  E  D  G  T  K  F  D  S  S  L  D

ATTACCGTGCATTACACAGGTTGGCTGGAAGACGGCACCAAATTCGACTCCAGCCTCGAC
       70        80        90       100       110       120

 R  R  Q  P  L  T  I  T  L  G  V  G  Q  V  I  K  G  W  D  E

CGCCGCCAGCCGCTGACCATCACGCTCGGCGTCGGACAAGTCATCAAAGGCTGGGACGAA
      130       140       150       160       170       180

 G  F  G  G  M  K  E  G  G  K  R  K  L  T  I  P  S  E  M  G

GGCTTCGGCGGAATGAAGGAGGGCGGCAAACGCAAGCTGACCATCCCTTCGGAAATGGGC
      190       200       210       220       230       240

 Y  G  A  H  G  A  G  G  V  I  P  P  H  A  T  L  I  F  E  V

TACGGCGCACACGGCGCCGGCGGCGTGATTCCCCCGCACGCCACTTTGATATTTGAAGTC
      250      *260       270       280       290       300

 E  L  L  K  V  Y  E
GAGCTGCTGAAAGTGTACGAATAAAGCTGCCTGGGATCC
      310       320       330
```

FIG. 2. DNA sequence of the PCR product. The PCR product cloned in pUC19 was sequenced as described. The sequence was compared with that determined by Perry *et al.* (19). Other than the differences introduced by the 5′ PCR primer, there was a single difference. An additional C at position 257 is indicated with an asterisk. This corresponds to residue 347 in the previously published sequence. Note that the amino-terminal amino acids (MGSL) of the protein predicted from the genomic sequence of Perry *et al.* (19) are indicated in italics and replace the amino acids encoded by the *Eco*RI site. This difference is due to the 5′ PCR primer used (see Fig. 1).

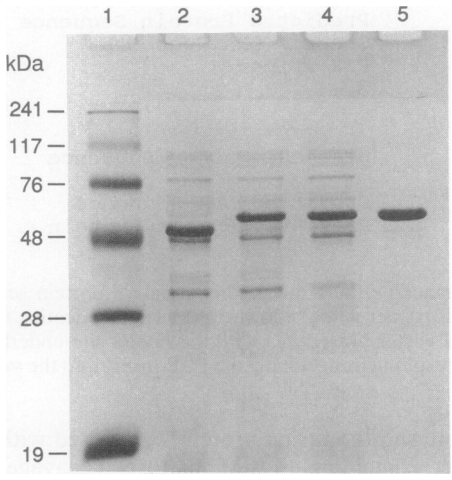

FIG. 3. Purification of rotamase fusion protein. SDS/PAGE was performed in a 12.5% polyacrylamide gel (27). Lane 1, prestained molecular weight markers (myosin heavy chain, phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, β-lactoglobulin); lane 2, whole cell lysates of *E. coli* harboring pMAL-cRI, the vector without insert; lane 3, whole cell lysates of *E. coli* harboring pMAL/ROT; lane 4, extract obtained by sonication of *E. coli* with pMAL/ROT; lane 5, fusion protein purified by affinity chromatography on amylose resin.

the fusion protein. Inhibition of activity by FK506 was measured by adding various concentrations of FK506 (in a methanol solution) to a standard reaction mixture containing 0.47 μM fusion protein.

RESULTS

Cloning of Meningococcal Rotamase. PCR primers were designed to allow the cloning of the putative meningococcal rotamase as a fusion protein with MBP in plasmid pMAL-cRI. This vector permits cleavage of the fusion protein by treatment with factor X_a protease, releasing MBP and the protein of interest (see Fig. 1 for details). After proteolysis the predicted product would be native rotamase with the exception of the first four amino acids (Fig. 1). The primers were used to amplify the desired DNA segment from genomic DNA of a nonencapsulated group B meningococcal strain. The product was digested with the restriction enzymes *Eco*RI and *Bam*HI and ligated to plasmid pUC19 that had been digested with the same restriction enzymes. The insert in pUC19 was sequenced (Fig. 2). The *Eco*RI–*Bam*HI insert fragment was excised from pUC19 and ligated into pMAL-cRI to produce a plasmid named pMAL/ROT.

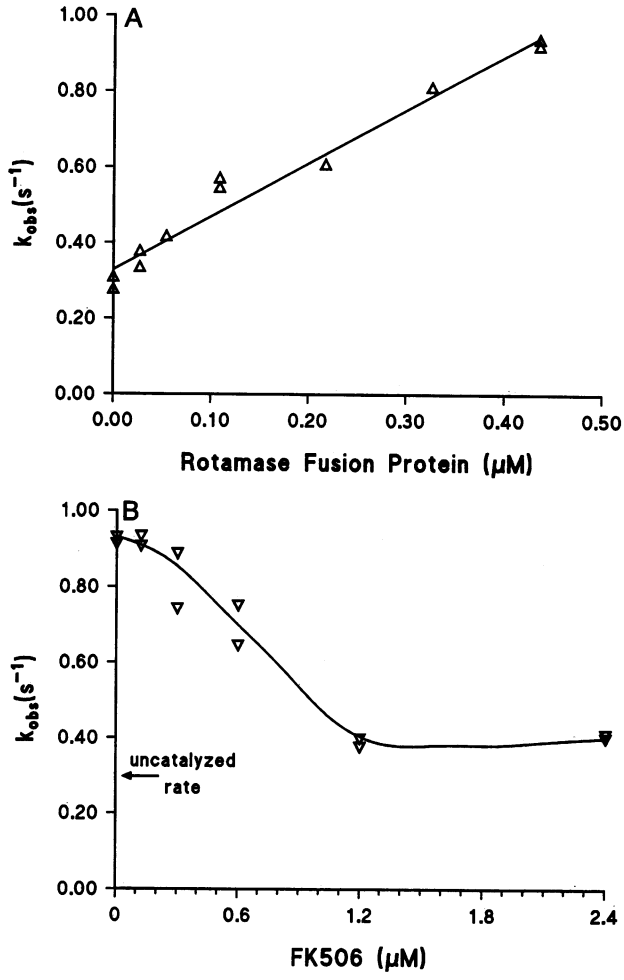

FIG. 4. Rotamase activity of the fusion protein. (*A*) Rotamase activity of the fusion protein at various concentrations. (*B*) Inhibition of fusion protein (0.47 μM) with various concentrations of FK506. Arrow indicates the uncatalyzed rate of hydrolysis.

Purification of the Fusion Protein. *E. coli* harboring pMAL/ROT, when induced with IPTG and analyzed by SDS/PAGE (27), gave rise to a preponderant product with an apparent molecular mass of 54 kDa (Fig. 3). The purification of the fusion protein is described in *Materials and Methods*. Briefly, cells from 100 ml of culture were collected by centrifugation, suspended in 5 ml of lysis buffer, and disrupted by sonication. The suspension was cleared by centrifugation and the supernatant was applied to a column of amylose resin. Upon elution with 5-ml aliquots of lysis buffer

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          1        10        20        30        40        50        60
hFKBP     MGVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKFDSSRDRNKPFKFMLGKQEVIRGW
          ::  . .  ...  . . .:.. .:::::: ::::.:::::: ::..:... ::  .::.::
NmFKBP    MGSLIIEDLQAEFGKEAVKGKEITVHYTGWLEDGTKFDSSLDRRQPLTITLGVGQVIKGW
           ..:  .:.... : .. :....::.::::.: ::: :::. .  .:   :. :.::: ::
LpMip     PSGLQYKDINAGNGVKPGKSDTVTVEYTGRLIDGTVFDSTEKTGKP--ATFQVSQVIPGW
             130       140       150       160       170       180

                   70        80        90       100
hFKBP     EEGVAQMSVGQRAKLTISPDYAYGATGHPGIIPPHATLVFDVELLKLE
          .:: . :. : . :::::.. .::: :  :.::::::::.:.::::::.
NmFKBP    DEGFGGMKEGGKRKLTIPSEMGYGAHGAGGVIPPHATLIFEVELLKVYE
          .:..  : .:..... .::...::. . :: : :..:::::...:..:
LpMip     TEALQLMPAGSTWEIYVPSGLAYGPRSVGGPIGPNETLIFKIHLISV
               190       200       210       220
```

FIG. 5. Comparison of meningococcal and human FKBP homologs with the *Legionella* Mip protein. The predicted protein sequence of the meningococcal FKBP (NmFKBP) is compared with that of the human FKBP (hFKBP) (14) and the *Legionella* Mip protein (LpMip) (15). Numbers above the top line refer to the human and *Neisseria* sequence, while those below the bottom line refer to the *Legionella* sequence.

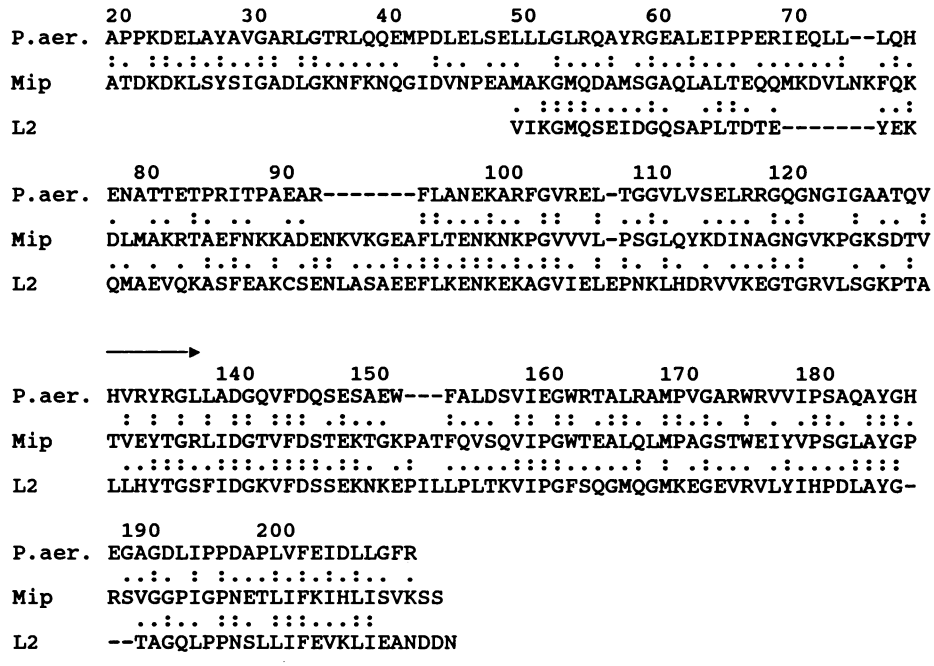

FIG. 6. Comparison of bacterial FKBP homologs. The predicted protein sequence of the *Legionella* Mip protein (Mip) (15) is compared with the *P. aeruginosa* (P. aer.) open reading frame described by Kato *et al.* (26), and with the L2 antigen of *C. trachomatis* (18). Numbers refer to the *Pseudomonas* open reading frame. Arrow indicates the point where homology to FKBP begins, and the homology continues to the end of the proteins. Notable are the similarities of these proteins in the region preceding the FKBP-homologous carboxyl-terminal portion.

containing 10 mM maltose, essentially all the adsorbed fusion protein was eluted in the first aliquot and contained only minor protein contaminants (Fig. 3).

Rotamase Activity of the Fusion Protein. The fusion protein was only slightly susceptible to the action of factor X_a protease (data not shown); therefore, this approach was not further pursued. Rotamase activity of the purified fusion protein was assayed by the method of Fischer *et al.* (3) as described in *Materials and Methods*. Purified MBP had no rotamase activity. As shown in Fig. 4*A*, on a molar basis the fusion protein has an activity comparable to the activity reported for purified human FKBP (5, 14, 24). The ability of FK506 to inhibit this rotamase activity was tested (Fig. 4*B*). Inhibition was noted when the concentrations of FK506 and rotamase were equimolar, and with a 2.5 molar excess of inhibitor, k_{obs} approached the uncatalyzed rate. This degree of inhibition is equivalent to the inhibition of human FKBP by FK506 seen by previous workers (6, 14, 24).

DISCUSSION

Standaert *et al.* (14) noted similarity between the protein sequence of human FKBP and a protein predicted by a meningococcal open reading frame in a sequence determined by Perry *et al.* (19). The similarity was particularly striking when a single base was added to the published sequence at position 337 or 338, because this frameshift would predict an additional 24 amino acids at the carboxyl end, with 15 identical to the human FKBP sequence. Crystallographic and NMR studies have shown that this portion of the protein forms the central strand of a five-stranded β-sheet, a crucial structural feature of this protein (28–30). The sequence of the gene segment we obtained by PCR from meningococcal DNA was compared with the sequence published by Perry *et al.* (19) and was found to be identical with the exception noted in Fig. 2. This difference allows for the coding of a protein that does include the strongly homologous carboxyl-terminal portion (Fig. 5). The agreement also indicates that the clone we obtained by the PCR procedure is most likely an accurate copy of the genomic DNA.

To facilitate purification of the product, we designed the primers to produce a fusion of the desired protein with MBP under control of the *tac* promoter, such that the fusion junction contained a recognition site for factor X_a. This strategy was successful insofar as it allowed expression of large amounts of the fusion protein and excellent purification by adsorption to an amylose resin and elution with 10 mM maltose. However, cleavage of the fusion protein with factor X_a was very inefficient. Hence, we characterized the activity of the purified fusion protein. We found that the product had rotamase activity that, on a molar basis, was comparable to that reported for human FKBP. In addition, we determined that the rotamase activity was inhibited by FK506.

While there has been intense interest in eukaryotic rotamases, little is known concerning such enzymes in bacteria. Both a cytoplasmic and a periplasmic rotamase of the cyclophilin family have been described in *E. coli*, and it is presumed that these enzymes are instrumental in protein transport and secretion (11, 12). The meningococcal FKBP shows significant homology with the carboxyl-terminal portion of larger proteins in *L. pneumophila*, *P. aeruginosa*, and *C. trachomatis*. Fig. 5 shows the alignment between human FKBP, meningococcal FKBP, and the carboxyl-terminal portion of the *L. pneumophila* Mip protein. It is of interest that a comparison of the bacterial FKBP homolog sequences demonstrates that these proteins are similar not only in the FKBP homologous carboxyl-terminal domain but also in the amino-terminal portion of the molecule, suggesting that this portion is also homologous (Fig. 6). While no information concerning the *Pseudomonas* protein is available, both the *Legionella* Mip protein and the *Chlamydia* L2 antigen are outer membrane proteins.

Note Added in Proof. A 12-kDa FK506-inhibitable rotamase has been purified from this strain of *N. meningitidis*. It is localized in the cytoplasm.

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