

Environmentally regulated proteins and metabolism

Transferrin-binding proteins of *Neisseria meningitidis*

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Neisseria meningitidis is a Gram negative bacterium which does not produce siderophores and is able to acquire iron directly from transferrin and lactoferrin (6). This acquisition is mediated by specific receptors identified as transferrin-binding proteins (Tbps) and lactoferrin binding proteins (Lbps) (8). Because meningococcal disease starts with active multiplication of the bacteria in the blood stream, more emphasis has been directed towards the understanding of the structure, function and immunological properties of Tbps in view of an eventual use in a meningococcal vaccine.

The transferrin-binding proteins also referred to as the transferrin receptor are comprised of two polypeptides named Tbp1 and Tbp2 whose precise interaction and organisation in the membrane remain unclear. Both polypeptides are able to bind human transferrin under different experimental conditions. The transferrin clearly binds to the cell surface as evidenced by immunogold labelling studies (1) and both polypeptides are required for iron acquisition as demonstrated by iron uptake studies with isogenic mutants (3). Furthermore, purified transferrin-binding proteins induce bactericidal antibodies in rabbits and protect immunized mice from a lethal challenge (2). Two distinct strains B16B6 (B: 2a: P1.2) and M982 (B: 9:P1.9) have been identified as "prototype" strains on the basis of distinct immunological and genetic features of the Tbp2 protein and the corresponding gene (7). Alignment of the amino acid sequences deduced from the nucleotide sequences of *tbp* genes from the two strains showed that while Tbp1 shared 76% of homology, Tbp2 were more divergent and only shared 47% of homology (4). The variability observed for Tbp2 has been described several times by different authors and may result from strong immune pressure exerted by the host during infection. It can be hypothesized that Tbp1 which is less variable, is less exposed to this immune pressure implying that it is less exposed on the bacteria. This is in part confirmed by the work of Lissolo *et al* in which it has been described that only antibodies to Tbp2 were bactericidal (5), indicating that the polypeptide which conferred to the Tbp1-Tbp2 complex a protective activity (2) is likely to be Tbp2.

Tbp2 being a lipoprotein (4), we sought to determine whether the apparent predominant role of Tbp2 within the complex may not be due to the strong

immunogenicity described previously for lipoproteins. To study this, mice were immunized either with the Tbp1-Tbp2 complex or with the each polypeptide purified separately from *Neisseria meningitidis* strain B16B6. The immune response to each polypeptide was determined by ELISA. Although the ELISA titers are expressed in arbitrary units, no significant difference in immunogenicity could be observed, both peptides being highly immunogenic. To assess the influence of the presence of fatty acids on the immunogenicity and biological activities of Tbp2, recombinant lipidated and non-lipidated Tbp2s were produced in *E. coli*. The lipidated and nonlipidated recombinant proteins were purified and tested for their ability to induce bactericidal antibodies in rabbits and to protect mice against a lethal challenge dose of meningococci. Recombinant proteins produced in *E. coli* displayed similar biological activities than proteins purified from meningococci. Under our experimental conditions recombinant proteins with fatty acids did not display significantly different immunological and protective activities as compared to proteins without fatty acid. In order to study recombinant proteins which resembled as closely as possible the meningococcal protein we decided to produce lipidated Tbp2s for further studies notably to investigate whether the entire polypeptide was required for the induction of protective antibodies. Lipidated recombinant truncated Tbp2 proteins were produced and purified and their biological properties will be discussed.

References

1. **Ala'Aldeen, D., N. B. Powell, R. A. Wall, and S. P. Boriello.** 1993. Localization of the meningococcal receptors for human transferrin. *Infect. Immun.* **61**: 751-759.
2. **Danve, B., L. Lissolo, M. Mignon, P. Dumas, S. Colombani, A. B. Schryvers, and M.-J. Quentin-Millet.** 1993. Transferrin-binding proteins isolated from *Neisseria meningitidis* elicit protective and bactericidal antibodies in laboratory animals. *Vaccine*, **11**: 1214-1220.
3. **Irwin, S. W., N. Averil, C. Y. Cheng, and A. B. Schryvers.** 1993. Preparation and analysis of isogenic mutants in the transferrin receptor protein genes, *tbpA* and *tbpB*, from *Neisseria meningitidis*. *Mol. Microbiol.* **8**: 1125-1133.
4. **Legrain, M., V. Mazarin, S. W. Irwin, B. Bouchon, M.-J. Quentin-Millet, E. Jacobs and A. B. Schryvers.** 1993. Cloning and characterisation of *Neisseria meningitidis* genes encoding the transferrin-binding proteins Tbp1 and Tbp2. *Gene* **130**: 73-80.
5. **Lissolo, L., G. Maitre-Wilmotte, P. Dumas, M. Mignon, B. Danve and M.-J. Quentin-Millet.** Submitted for publication.
6. **Mickelsen, P. A., and P. F. Sparling.** 1981. Ability of *Neisseria gonorrhoeae*, *Neisseria meningitidis* and commensal *Neisseria* species to obtain iron from transferrin and iron compounds. *Infect. Immun.* **33**: 555-564.
7. **Rokbi, B., V. Mazarin, G. Maitre-Wilmotte, and M.-J. Quentin-Millet.**

1993. Identification of two major families of transferrin receptors among *Neisseria meningitidis* strains based on antigenic and genomic features. FEMS Microbiol. Lett. **110**: 51-58.
8. **Schryvers, A. B., and L. J. Morris.** 1988. Identification and characterization of the transferrin receptor from *Neisseria meningitidis*. Mol. Microbiol. **2**: 281-288.

Purification of *Neisseria meningitidis* transferrin binding proteins and characterisation by epitope mapping and iron release studies

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Neisseria meningitidis produces two iron-regulated proteins that are associated with binding to human transferrin. Transferrin binding protein 1 (TBP1) has a molecular weight of approximately 95kDa and transferrin binding protein 2 (TBP2) has a molecular weight ranging from 68-85 kDa depending on bacterial strain. TBP2 can bind transferrin on a blot after transfer from SDS-PAGE, but TBP1 does not. There is a growing interest in these proteins as candidate vaccine antigens and a method to purify separate TBP1 and TBP2 is essential for their immunological characterisation and to determine their roles in iron uptake from transferrin.

1. Purification and separation of TBP1 and TBP2. We have developed a method for purification of separate TBP1 and TBP2. Firstly the TBPs were solubilised from whole cells with either octyl β -D-glucopyranoside or Eluent (Calbiochem), and purified using affinity chromatography on transferrin-Sepharose 4B (5). Elution with low pH released two proteins, identified as TBP1 and TBP2. Yields of 15mg TBPs were obtained from 40g (wet weight) cells. The TBPs were then separated by Chromatofocussing using Polybuffer exchanger (PBE 94, Pharmacia). Affinity purified TBP 1+2 were applied directly to the column and eluted with Polybuffer 74 (Pharmacia), containing 0.1% (v/v) Triton X-100 to create a pH 7.0 to 3.5 elution gradient. TBP1 was eluted at the start of the pH gradient and TBP2 at low pH. Both separate TBP1 and TBP2 bound transferrin on dot blots but stronger binding was shown by TBP2. This demonstrated that both proteins bind transferrin and retain biological activity following this purification.

2. Production of monoclonal antibodies against TBPs and characterisation of TBP2 by epitope mapping. Monoclonal antibodies (MABs) were raised against affinity purified TBP 1+2 from strain SD (described by Ala'Aldeen(2), high molecular weight TBP2). All positive clones reacted with separate TBP2, although some also showed cross-reaction with TBP1, indicating that these proteins share common epitopes. From 18 positive clones, 3 MABs were found that inhibited transferrin binding to purified TBP1+2 on dot blots. Using separate TBP1 and TBP2 it was demonstrated that these MABs inhibited transferrin binding to TBP2 and not to

TBP1. The MABs that inhibited transferrin binding were added to iron-limited *N. meningitidis* cultures where sufficient diferric transferrin was added to relieve the iron-limitation. One MAB (103) was seen to inhibit transferrin-mediated growth, whereas the other two MABs did not. None of the MABs showed any bactericidal activity in the presence human complement.

To characterise the epitopes on TBP2 defined by these MABs, multiple overlapping peptides were synthesised on pins and screened for binding by the MABs (4) The complete TBP2 sequence of strain M982 (6) was synthesised as 10 amino acid peptides, overlapping by 8. Initially, the peptides were screened for binding of transferrin but no reproducible regions of binding were seen. Of the MABs that inhibited transferrin binding, MAB103 bound strongly to one peptide and MAB 112 bound weakly to the same peptide (amino acids 45-54 of the M982 TBP2 sequence) (6).

The inhibition of transferrin binding by two MABs that bind to the same site on the TBP2 sequence, indicates that either this region is important for transferrin binding or that antibodies attached to this region sterically hinder transferrin binding. To assess the importance of this antigenic site on TBP2, free peptide (amino acids 43-56) was synthesised and coupled to keyhole limpet haemocyanin and used to raise rabbit antiserum. Following 5 immunisations, the anti-peptide serum was of sufficient titre to give clear inhibition of transferrin binding on dot blots and ELISA. This anti-peptide serum also reacted with whole cells from a range of meningococcal strains and with TBP2 on immunoblots following transfer from SDS-PAGE. The antiserum also showed cross reaction with affinity purified TBPs from other strains, including B16B6 which has a low molecular weight TBP2.

3. Iron release studies. The mammalian transferrin receptor has been extensively studied and a cycle of receptor-mediated endocytosis of transferrin and release of iron has been described. At extracellular pH, 7.4, binding of transferrin to the receptor retards the release of iron, but at the pH of the endosome within the mammalian cell, 5.6, the receptor accelerates the release of iron (1). Preliminary studies have been made on the effect of binding of transferrin to the bacterial receptor on iron release. Diferric transferrin was added to affinity purified TBP 1+2 from strain B16B6 and the TBP-transferrin complex isolated from free transferrin by gel filtration. The rate of iron release to pyrophosphate was then determined using a spectrofluorimetric method (3). At pH 7.8 the rate of iron release from the complex was greater than twice that measured for transferrin alone. Thus the *N. meningitidis* TBPs may function not only to bind transferrin from the host but also to accelerate iron release from transferrin when it is bound to the bacterial membrane.

References

1. **Aisen, P.** 1993. Entry of iron into cells: a new role for the transferrin receptor in modulating iron release from transferrin. *Ann. Neurol.* **32**:S62-68.
2. **Ala'Aldeen, D.A., Wall, R.A. and S.P. Borriello.** 1990. Immunogenicity and cross-reactivity of the 70-kDa iron-regulated protein of *Neisseria meningitidis* in man and animals. *J. Med. Microbiol.* **32**: 275-281.
3. **Egan, T.J., Zak, O. and P. Aisen.** 1993. The anion requirement for iron release from transferrin is preserved in the receptor-transferrin complex. *Biochemistry*, **32**: 8162-8167.
4. **Geysen, M.H., Rodda, S.J., Mason, T.J., Tribbick, G. and P.G. Schoofs.** 1987. Strategies for epitope analysis using peptide synthesis. *J. Immunol. Meth.* **102**:259-274.
5. **Gorringe, A.R., Irons, L.I. and A. Robinson.** 1992. Purification and characterisation of transferrin binding proteins from *Neisseria meningitidis*. 8th International Pathogenic Neisseria Conference, Cuernavaca, Mexico.
6. **Legrain, M., Mazarain, V., Irwin, S.W., Bouchon, B., Quentin-Millet, M-J., Jacobs, E. and Schryvers A.B.** 1993. Cloning and characterisation of *Neisseria meningitidis* genes encoding the transferrin-binding proteins Tbp1 and Tbp2. *Gene* **130**:73-80.

Characterization of the gonococcal transferrin receptor

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Gonococcal strain FA19 makes two iron-repressible Tf-binding proteins, a 100 kDa protein designated Tbp1 and an 85 kDa protein designated Tbp2. Tbp1 is homologous to TonB-dependent outer membrane receptors, is necessary for iron utilization from Tf (3), and can confer upon *E. coli* the ability to bind but not utilize Tf (2). Tbp2 is lipid-modified and although not essential for utilization of Tf, it appears to make the process of Fe-uptake from Tf more efficient (1). Transposon insertions into the gene encoding Tbp2, *tbpB*, have a strong polar effect on expression of Tbp1, encoded by *tbpA* which is located immediately downstream. This observation indicates that *tbpB* and *tbpA* are cotranscribed and coregulated from a common upstream promoter, which includes a consensus Fur-binding site (1). To determine the relative contributions of Tbp1 and Tbp2 to Tf binding, we have used a solid-phase dot blot Tf-binding assay as well as an equilibrium liquid-phase Tf-binding assay. In the solid-phase assay, Tbp1 appears to play the major role in Tf binding while in the liquid-phase assay, Tbp2 seems to contribute most to surface binding. Tbp2 may also discriminate slightly between saturated and apoTf but Tbp1 does not appear to have this capability. To assess the diversity of Tbp1 and Tbp2, we are sequencing *tbpA* and *tbpB* from various gonococcal strains. Antibodies against predicted surface exposed portions of Tbp1 and Tbp2 (from strain FA19) will be used to determine antigenic conservation among these domains and to test models of protein structure. A *tbpA/tbpB* deletion mutant is being constructed without a selectable marker to determine if these proteins are important for gonococcal virulence in an experimental infection of human volunteers.

References

1. **Anderson, J. E., P. F. Sparling, and C. N. Cornelissen.** 1994. Gonococcal transferrin binding protein 2 facilitates but is not essential for transferrin utilization. *J. Bacteriol.* **176**:3162-3170.
2. **Cornelissen, C. N., G. D. Biswas, and P. F. Sparling.** 1993. Expression of gonococcal transferrin-binding protein 1 causes *Escherichia coli* to bind human transferrin. *J. Bacteriol.* **175**:2448-2450.
3. **Cornelissen, C.N., G. D. Biswas, J. Tsai, D.K. Paruchuri, S.A. Thompson, and P.F. Sparling.** 1992. Gonococcal transferrin-binding protein 1 is required for transferrin utilization and is homologous to TonB-dependent outer membrane receptors. *J. Bacteriol.* **174**:5788-5797.

Iron-acquisition and disease produced by pathogenic *Neisseria*

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An intriguing aspect of gonococcal iron acquisition is that while all gonococci obtain iron from human transferrin, the ability to utilize iron from human lactoferrin and vertebrate haemoglobin is variable (1,2). We are actively analysing strains obtained from the STD population presenting at the Public Health Clinic in Pittsburgh, Pennsylvania. Currently, 29% of all strains collected can utilize iron from human lactoferrin and 57% can utilize iron from human haemoglobin. These data are being collected and evaluated in order to correlate these iron-utilization phenotypes with the transmission and severity of gonococcal disease.

The molecular events involved in iron-acquisition by pathogenic *Neisseria* include (i) up-regulation of iron transport proteins when organisms are cultivated under conditions in which a host iron-binding protein (eg., transferrin, lactoferrin, haemoglobin, *etc.*) is the sole source of iron, (ii) sequestration of the iron-binding protein at the cell surface, (iii) removal of iron from the protein source and transport across the outer membrane, and (iv) transport through the periplasmic space and across the cytoplasmic membrane. Various aspects of this process have been studied by our laboratory as reported below.

Iron-regulation may occur at the level of DNA supercoiling. We have shown that iron-source stress in gonococci leads to an increase in the negative supercoiling of the Neisserial cryptic plasmid. In addition, the ATP/ADP ratio, a key regulatory signal for gyrase activity, is altered in response to iron-source stress. Furthermore, a known *E. coli* supercoiling sensitive promoter, *lac*, has been positioned upstream of the luciferase reporter gene and expressed in a gonococcal background. Luciferase activity from this construct is sensitive to growth under iron-source stress conditions suggesting that iron-regulation may proceed by a Fur-independent mechanism.

Chen *et al* (3) have previously reported that the periplasmic iron-binding protein Fbp represents the 'nodal point' for acquisition of iron from transferrin, lactoferrin, and citrate. Biochemical studies of purified Fbp have shown that it is capable of binding a wide range of metals. Therefore, *Neisseria* spp. expressing this protein should be particularly susceptible to growth inhibition by heavy metal analogue of iron. We observe that gonococci are particularly sensitive to copper and gallium imposed

growth inhibition. These results are significant since heavy metals may be used to selectively inhibit gonococci over meningococci.

The mechanism by which Fbp binds iron has been extensively investigated by this laboratory. This protein possesses common structural and functional features with two well-characterized families of proteins, the Transferrins and the Periplasmic Binding Proteins. We have demonstrated that as a periplasmic binding protein, Fbp binds iron by a mechanism that is remarkably similar to that employed by the iron-binding sites of the Transferrins. Specifically, two Tyr, one His and a bicarbonate anion are implicated in iron-binding by both Fbp and the Transferrins. Structural modelling of Fbp predicts specific aromatic residues that occur within the metal binding site. Using site-directed mutagenesis in conjunction with an assay employing the luminescent lanthanide, terbium, the metal-binding environment of Fbp is currently being probed. We are attempting to introduce site-directed Fbp mutants into a gonococcal background. These gonococcal variants will be evaluated for their ability to obtain iron from their growth environment.

References

1. **Mickelson, P.A., E. Blackman and P. F. Sparling.** 1982. Ability of *Neisseria gonorrhoeae*, *Neisseria meningitidis*, and other commensal *Neisseria* spp. to obtain iron from lactoferrin. *Infect. Immun.* **35**: 915-20.
2. **Mickelson, P. A. and P. F. Sparling.** 1981. Ability of *Neisseria gonorrhoeae*, *Neisseria meningitidis* and commensal *Neisseria* spp. to obtain iron from transferrin and other compounds. *Infect. Immun.* **33**:555-64.
3. **Chen, C.-Y., Berish, S.A., Morse, S.A., and Mietzner, T.A.** 1993. The iron-binding protein (Fbp) of pathogenic *Neisseria* functions as a periplasmic transport protein in iron-acquisition from human transferrin. *Mol. Microbiol.* **10**: 311-318.

***Neisseria* Cytotoxins**

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Gonococci and meningococci are recognized to produce only two cytotoxins: LOS (1) and peptidoglycan fragments (2). No secreted protein toxins have been identified in either organism. Various cytokines, including TNF, may well be associated with local inflammation and tissue damage, but these are not toxins in the ordinary sense. Recently, we discovered that many meningococci produce two iron-regulated proteins that are clearly related to the RTX family of cytotoxins. We raised a monoclonal antibody (Mab) designated A4.85 that recognized a smear of iron-regulated high molecular weight outer membrane protein in meningococcal strain FAM20, but not in gonococcal strain FA19. A4.85 was used to clone a meningococcal gene encoding the relevant epitope, and subsequently two different, but related meningococcal genes that encoded the same epitope were completely sequenced and characterized. The first of these, *frpA*, encodes a protein of 122kDa. The second, designated *frpC*, encodes a protein predicted to be 198kDa. The predicted FrpA protein contained 13 copies of a directly repeated peptide with the consensus LXGGXGNDX. The predicted FrpC protein contained 43 copies of this same peptide repeat. The two proteins are very similar in their mid- and C-terminal domains, but differ entirely at their N-termini. Their structures are related most closely to those of the *E. coli* haemolysin (HlyA) and to the *B. pertussis* adenylate cyclase (CyaA). The meningococcal FrpA and FrpC proteins react with Mabs against HlyA and CyaA, and A4.85 cross reacts with both HlyA and CyaA. The reassembled *frpA* gene product is secreted in *E. coli* through a *hlyBD*-dependent pathway that is unique to the RTX family of exotoxins. Five of eight tested meningococcal strains contained similar proteins in immunoblots, but only in 1 of 14 other tested *Neisseria*. Despite repeated attempts, we did not demonstrate reproducible haemolytic or leukocytic activity attributed to FrpA and/or FrpC. Preliminary experiments failed to show specific toxicity for endothelial cells. In other preliminary experiments, Dr. Xavier Nassif and his colleagues apparently have demonstrated that a double *frpA frpC* mutant is impaired in its ability to cause a sustained bacteraemia in the infant rat model. It is possible that meningococcal RTX homologues are related to meningococcal virulence.

Recently, we have attempted to clone other RTX-related meningococcal genes, and isolated a meningococcal clone that appeared to complement *hlyC* in *E. coli* (*hlyC* acylates and therefore activates HlyA.) The DNA sequence of the clone revealed, however, that it is a glutathione peroxidase. In Southern blots, each of six meningococci, but none of seven gonococci reacted with the meningococcal glutathione peroxidase probe. Thus, we have discovered another gene that appears to differentiate meningococci from gonococci, although the importance of glutathione peroxidase, as well as FrpA and FrpC, to meningococcal pathogenesis remains to be proven.

References

1. **McGee, ZA, AP Johnson, & D Taylor-Robinson.** 1983. Pathogenic mechanisms of *Neisseria gonorrhoeae*: observations on damage to human fallopian tubes in organ culture by gonococci of colony type 1 or type 4. *J Infect Dis* **143**:413-422.
2. **Melly, MA, ZA McGee, & RS Rosenthal.** 1984. Ability of monomeric peptidoglycan fragments from *Neisseria gonorrhoeae* to damage human fallopian-tube muocsa. *J Infect Dis* **149**:378-386.
3. **Thompson, SA, LL Wang, A West, & PF Sparling.** 1993. *Neisseria meningitidis* produces iron-regulated proteins related to the RTX family of exoproteins. *J Bacteriol* **175**:811-818.
4. **Thompson, SA, LL Wang & PF Sparling.** 1993. Cloning and nucleotide sequence of *frpC*, a second gene from *Neisseria meningitidis* encoding a protein similar to RTX Cytotoxins. *Molec Microbiol* **9**:85-96.
5. **Thompson, SA & PF Sparling.** 1993. The RTX cytotoxin-related FrpA protein of *Neisseria meningitidis* is secreted extracellularly by meningococci and by HlyBD + *Escherichia coli*. *Infect Immun* **61**:2906-2911.

Regulation of *aniA* expression by oxygen availability in *Neisseria gonorrhoeae* and *N. meningitidis*

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Neisseria gonorrhoeae is a facultative anaerobe that grows in the absence of oxygen by anaerobic respiration using nitrite as a terminal electron acceptor. We have previously demonstrated that some outer membrane proteins are induced and others are repressed when gonococci are grown anaerobically. Our studies have focused on the major anaerobically expressed outer membrane protein. Pan1, as it appears to be a major antigen recognized by antibodies in sera from women with gonococcal infections and because it is expressed at significantly reduced levels in *N. meningitidis*.

We cloned and sequenced the gene (*aniA*) that encodes the Pan1 protein and used this data to insertionally inactivate *aniA* with a *cat* gene under the control of the *tac* promoter. The gonococcal mutant that was generated was unable to grow anaerobically and was devoid of detectable nitrite reductase activity. This suggests that Pan1 either is part of a nitrite reductase system or that it may be required for expression of nitrite reductase.

To study regulation of *aniA*, we constructed a fusion of 350 base pairs of *aniA* upstream sequence, plus the first 2 codons of the open reading frame, to a promoterless *lacZ* gene. Since the *aniA* mutant was unable to grow anaerobically, we inserted the *aniA::lacZ* fusion construct, plus a *cat* gene under the control of the *tac* promoter, into the proline gene. When gonococcal strain F62 was transformed with this construct, the *aniA::lacZ* fusion recombined into the chromosomal proline gene rather than into the parental *aniA* gene.

The *aniA::lacZ* fusion strain was grown aerobically in broth and was found to express ~1 Miller unit of β -galactosidase, while anaerobically grown cells expressed ~1500 units. This 1500-fold induction of *aniA* corresponds to western blot data, in which no detectable Pan1 is expressed by aerobically grown cells. In contrast, considerable nitrite reductase activity was detectable in aerobically grown cells, and this activity was induced only 10-15-fold by anaerobic growth. Additionally, nitrite reductase, but not β -galactosidase, was induced aerobically by nitrite. These data suggest that Pan1 is not the gonococcal nitrite reductase.

We constructed a double mutant, with the parental *aniA* replaced by an *aniA* with a deletion in the coding region and the *aniA::lacZ* fusion inserted into the proline gene. When cells were shifted from aerobic to anaerobic conditions, the fusion was induced but to a lesser level than was observed in the strain with a functional *aniA* gene (400 units vs. 1500 units). However, this difference in induction of *aniA* was observed only in the presence of nitrite; the F62 *aniA*⁺*pro::aniA::lacZ* strain expressed ~400 units of β-galactosidase when it was incubated anaerobically in the absence of nitrite.

In addition to examining the regulation of *aniA* expression in gonococci, we have begun to determine why this gene is expressed at significantly lower levels in meningococci, despite Southern blot data that suggests that the gene is present in *N. meningitidis*. We transformed the gonococcal *pro::aniA::lacZ* fusion described above into a strain of *N. meningitidis*. When meningococci were grown aerobically and anaerobically, the anaerobic induction of β-galactosidase was only 250-300-fold, with the same aerobic expression of ~1 unit. In contrast to results from *N. gonorrhoeae*, only ~10 units of β-galactosidase were found when *N. meningitidis* was incubated anaerobically in the absence of nitrite. These data suggest that *N. meningitidis* lacks a factor that acts in *trans* that is necessary for full induction of *aniA*. We also sequenced the region upstream of the *aniA* gene from 3 meningococcal strains; in all cases there were alterations in the sequence of the -35, and in 2 cases a deletion in the -10 region of the gearbox promoter. This is the major promoter used for anaerobic expression of *aniA* in gonococci, and the data suggest that *N. meningitidis* may only be capable of expressing *aniA* from the minor F⁷⁰ promoter.

In summary, our present model for *aniA* regulation is that transcription occurs from two promoters, the major gearbox promoter and a minor F⁷⁰ promoter. The gearbox promoter would be responsible for expression in response to anaerobiosis, regardless of whether the cells are able to grow under these conditions, while the F⁷⁰ promoter would be responsible for expression under conditions in which anaerobic growth also occurs.

Electron microscopic localisation of iron-regulated proteins in *N. meningitidis* with particular reference to the lactoferrin and transferrin receptors

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Gold-labelled lactoferrin (Au-LF) was used to localise and quantify the distribution of lactoferrin binding receptors on live meningococci *in vitro* by electron microscopy. On most cells there was a uniform distribution of lactoferrin receptors, although the occasional cell had localised clusters of gold particles on the cell surface. A few cells also exhibited significant labelling of outer membrane blebs. Double labelling experiments with 5nm Au-LF and 15nm gold-labelled human transferrin (Au-HTF), indicated that lactoferrin and transferrin receptors in meningococci are separate and not closely situated on the cell surface. The lactoferrin receptor density in *N. meningitidis* strain SD (B15P1.16) when labelled with 5nm Au-LF was 39 particles per cell. This compared with a transferrin density of 113 per cell when labelled with 5nm Au-HTF.

Isogenic transferrin binding protein (TBP) mutants of *N. meningitidis* B16B6 (B2aP1.2) bound less Au-HTF and Au-LF than strain SD. The lower level of lactoferrin receptors was a surprise. Strain N97 (TBP-1 mutant) bound as much gold-labelled transferrin as the parent strain, whereas strains N91 (TBP-2 mutant) and N96 (TBP-1, TBP-2 mutant) were less effective at binding gold-labelled transferrin. The implication was that transferrin binding in this strain could be accounted for by the activity of TBP-2 alone. Strain SD bound 74% more 15nm gold-labelled holo-transferrin than 15nm gold-labelled apo-transferrin, whereas strain B16B6 showed no such increase.

Pre-embed labelling of the transferrin receptors of strain SD with 15nm gold-labelled transferrin, followed by post-embed labelling of the 37 KDa iron-regulated protein (Fbp) with a polyclonal antiserum and 5nm protein A gold, failed to show any close spatial relationship between the two. Fbp was mainly periplasmic, as expected, although it was also present in outer membrane blebs.

Genetic heterogeneity of gonococcal transferrin-binding proteins

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Pathogenic *Neisseria* species can obtain iron from human transferrin by a receptor-mediated mechanism which is not fully understood. Two iron-repressible transferrin-binding proteins have been identified and their genes cloned. These proteins, Tbp1 and Tbp2, are thought to be outer membrane associated and thus are potential targets for vaccine development. In this study, we address the heterogeneity of gonococcal *tbpA*, and *tbpB* and their respective gene products by DNA sequence and western blot analysis.

Gonococcal Tbp1 has an apparent molecular mass of 95-100 kDa in the strains thus far examined and the *tbpA* gene appears to be highly conserved. In a western blot, the proteins encoded by gonococcal *tbpB* migrate within a molecular weight range of 80-90 kDa. The *tbpB* gene shows significant sequence divergence among the strains analyzed. While the 5' and 3' ends of *tbpB* appear to be conserved, the gene does not appear to contain cassettes of variable and conserved regions as described in other *Neisseria* genes. Preliminary data suggest that unlike the meningococcus, the gonococcus could have only one class of Tbp2. Comparison of the genes encoding gonococcal and meningococcal transferrin-binding proteins suggests that they may have resulted from inter-species genetic exchange.

Production of meningococcal transferrin binding protein 2 in *E.coli*

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Neisseria meningitidis strains grown under iron starvation conditions produce transferrin binding proteins (Tbp1 and Tbp2) which have been shown to play a major role in iron acquisition. Transferrin binding proteins isolated from serogroup B *Neisseria meningitidis* (strain B16B6) elicit protective and bactericidal antibodies in laboratory animals (1). Several lines of evidence show that Tbp2 may constitute a good vaccine candidate (3). Low amounts of Tbp2 are produced by *Neisseria* and a heterologous expression system is therefore needed to prepare sufficient quantity of this protein. Expression of *tbp2* has been achieved in *E.coli* using an arabinose inducible promoter.

Tbp2 is produced in *Neisseria* as a precursor with a signal peptide whose cleavage follows a lipidation step on a cysteine residue which is the first amino acid in the mature protein (2). When produced in *E.coli* with its natural signal peptide high amount of rTbp2 (about 10% of total cell proteins) was detected. However, most of the protein was non-lipidated precursor and only a small fraction was mature rTbp2. In order to optimize the maturation of the precursor, the natural signal sequence was replaced by several *E.coli* lipoprotein signal peptides. Expression levels and maturation of the precursor were highly variable depending on the signal peptide used. With one of these, an efficient maturation and a high amount of mature lipidated rTbp2 were obtained (about 3% of total cell proteins). A large scale production process was established for this *E.coli* -produced rTbp2, and immunogenicity in animal models is currently being evaluated.

References

1. **Danve, B., L. Lissolo, M. Mignon, P. Dumas, S. Colombani, A.B. Schryvers and M.-J. Quentin-Millet.** 1993. Transferrin-binding proteins isolated from *Neisseria meningitidis* elicit protective and bactericidal antibodies in laboratory animals . *Vaccine* **11**: 1214-1220.
2. **Legrain, M., V. Mazarin, S.W. Irwin, B. Bouchon, M.-J. Quentin-Millet, E. Jacobs and A.B. Schryvers.** 1993. Cloning and characterization of *Neisseria meningitidis* genes encoding the transferrin-binding proteins Tbp1 and Tbp2. *Gene* **130**: 73-80.
3. **Lissolo, L., G. Maitre-Willmotte, P. Dumas, M. Mignon, B. Danve and M.-J. Quentin-Millet .** Submitted for publication.

Development and optimization of a gene replacement system for the *tbp* genes of *N. meningitidis*

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Neisseria meningitidis, an important human pathogen, utilizes a mechanism of iron acquisition that involves direct binding of the host iron-binding glycoprotein, transferrin, by receptor proteins at the bacterial surface. Recently, the genes encoding the transferrin binding proteins (Tbp1 and Tbp2) of *N. meningitidis* and *N. gonorrhoeae* have been cloned and sequenced, and isogenic mutants lacking expression of these proteins have been characterized (1,3,5,6). Although Tbp1 alone is sufficient to mediate transferrin iron acquisition in *N. gonorrhoeae* (1), its expression at the surface of *Escherichia coli* did not confer the ability to acquire iron from transferrin in spite of effective binding of this glycoprotein (2). These results suggest that either the conformation of Tbp1 within the membrane is not correct or that other component(s) of this pathway are not present and/or functional in *E. coli*. In addition, the Tbp2 protein was required for iron acquisition in *N. meningitidis* (5) and is not exported to the bacterial surface when expressed in *E. coli* (unpublished observations). These results indicate that structure/function analysis of these fascinating proteins will have to be performed in the host species.

Although *N. meningitidis* possesses a natural transformation system, which allows it to efficiently recognize and specifically take up meningococcal DNA, there is a dearth of genetic tools available for use in this species and biosafety concerns related to genetic manipulation in this organism. The potential need for a large number of *tbpA* and/or *tbpB* constructs to be generated and reintroduced into *N. meningitidis* has therefore prompted us to look into the development of a system which would allow this to be done simply and efficiently.

Due both to the limited number of available markers and to the biosafety concerns of introducing heterologous sequences into *N. meningitidis*, the generation of a biosafe strain to provide the background in which such manipulations could occur is appealing. Recently, our laboratory has cloned the meningococcal *asd* gene encoding aspartate b-semialdehyde dehydrogenase (Asd; 4). The Asd enzyme is key for the synthesis of diaminopimelic acid (DAP), a component of the cell wall of Gram negative bacteria. Bacteria unable to synthesize DAP undergo lysis when an exogenous source is not supplied. Due to the absence of DAP in mammalian tissues,

asd mutants lack the ability to proliferate *in vivo* and may therefore be considered innocuous. Preliminary attempts to develop an *asd* meningococcal strain have, however, proven unsuccessful (4).

In the present study, the intact *asd* gene and flanking regions of *N. meningitidis* has been sequenced. Sequence data obtained from this region has been used to develop a series of constructs designed such that the heterologous constructs are flanked by the native sequences adjacent to *asd*. In order to reduce the possibility that polar effects would affect the likelihood of successful insertion into this locus, inserted sequences were cloned using site directed mutagenesis such that they replace the entire *asd* open reading frame and lie in frame with the native *asd* promoter. Coding sequences inserted in this manner will therefore be transcribed from the meningococcal *asd* promoter, allowing constitutive expression of the gene without confounding problems associated with heterologous promoter recognition and expression levels. Analogous constructs have also been prepared within the *N. meningitidis* *tbp* locus, and a comparison of these systems will be discussed.

A number of insertion cassettes were generated and employed in order to directly compare the suitability of these systems for genetic replacement. Insertion of the Tn903-derived aminoglycoside 3'-phosphotransferase allows simple detection of the replacement of the *asd* gene by its ability to confer resistance to kanamycin. Similarly, the introduction of the *E. coli* beta-galactosidase gene (*lacZ*) into this system allows blue/white colour selection and quantitation of expression levels, since this enzyme is absent from wild type meningococci. Finally, the utilization in *N. meningitidis* of a cassette consisting of the *Bacillus subtilis* *sacB* gene followed by gentamycin acetyltransferase-3-1 gene (*GmR*) will be described. With this cassette, resistance to gentamycin (*GmR*) allows the *asd*⁻ phenotype to be easily screened and stably maintained, while the sensitivity to sucrose conferred by the *sacB* gene will allow subsequent replacement events to be selected for by growth on media containing sucrose.

References

1. **Anderson, J.A., P.F. Sparling and C.N. Cornelissen** 1994. Gonococcal transferrin-binding protein 2 facilitates but is not essential for transferrin utilization.. J. Bact. **176**: 3162-3170.
2. **Cornelissen, C.N., G.D. Biswas and P.F. Sparling**. 1993. Expression of gonococcal transferrin-binding protein 1 causes *Escherichia coli* to bind human transferrin. J.Bacteriol. **175**: 2448-2450.
3. **Cornelissen, C.N., G.D. Biswas, J. Tsai, D.K. Paruchuri, S.A. Thompson and P.F. Sparling**. 1992. Gonococcal transferrin-binding protein 1 is required for transferrin utilization and is homologous to TonB-dependant outer membrane receptors. J.Bacteriol. **174**: 5788-5797.

4. **Hatten, L.A., H.P. Schweizer, N. Averill, L. Wang and A.B. Schryvers.** 1993. Cloning and characterization of the *Neisseria meningitidis* *asd* gene. *Gene* **129**: 123-128.
5. **Irwin, S.W., N. Averill, C.Y. Cheng and A.B. Schryvers.** 1993. Preparation and analysis of isogenic mutants in the transferrin receptor protein genes, *tbp1* and *tbp2*, from *Neisseria meningitidis*. *Mol.Microbiol.* **8**: 1125-1133.
6. **Legrain, M., E. Jacobs, S.W. Irwin, A.B. Schryvers and M.J. Quentin-Millet.** 1993. Molecular cloning and characterization of *Neisseria meningitidis* genes encoding the transferrin binding proteins Tbp1 and Tbp2. *Gene* **130**: 73-80.

Characterization of a highly structured domain in Tbp2 from *N. meningitidis* involved in the binding to human transferrin

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The binding of iron-loaded human transferrin at the surface of *Neisseria meningitidis* is mediated by two polypeptides, Tbp1 and Tbp2. Predicted Tbp amino acid sequences from *N. meningitidis* strains are highly divergent (1). This variability is particularly pronounced for Tbp2s, throughout the polypeptide. In this study, a highly structured and extremely stable domain of about 270 to 290 amino acids has been characterized, which is involved in the binding to transferrin and whose position is well conserved in very divergent Tbp2 polypeptides. Only strong denaturing treatments are able to disorganize this protein domain.

The conservation of such a remarkable structure in very divergent Tbp2s suggests that it plays an essential biological role. These observations as well as other experimental data raising a number of questions regarding *tbp2* evolution will be discussed.

References

1. **Legrain, M., V. Mazarin, S.W. Irwin, B. Bouchon, M.-J. Quentin-Millet, E. Jacobs and A.B. Schryvers**. 1993. Cloning and characterization of *Neisseria meningitidis* genes encoding the transferrin-binding proteins Tbp1 and Tbp2. *Gene* **130**: 73-80.

Identification of transferrin binding regions of the *Neisseria meningitidis* transferrin receptor proteins by the use of chimeric Tbp's

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Neisseria meningitidis is an important human pathogen which acquires transferrin bound iron via host species-specific transferrin binding proteins designated transferrin binding protein 1 (Tbp1) and transferrin binding protein 2 (Tbp2; 3). In conjunction with biochemical data, the protein sequences of the Tbp1 and Tbp2 proteins, predicted from the nucleotide sequence of the *tbpA* and *tbpB* genes, suggested that Tbp1 is a tonB-dependant transmembrane receptor and that Tbp2 is lipoprotein anchored to the outer membrane surface by its lipid tail (2). Insertional mutagenesis of either or both of the *tbpA* and *tbpB* genes in *N. meningitidis* eliminated utilization of transferrin iron indicating that both proteins are essential for iron acquisition (1). However, the process by which the receptor proteins interact with human transferrin (hTf) has not been characterized.

The goal of the study outlined in this abstract was to localize the domains of the receptor proteins involved in binding to hTF. The overall strategy was to prepare hybrid receptor genes and analyze the binding properties of the resultant recombinant chimeric receptor proteins. The *tbpA* and *tbpB* genes from the porcine pathogen, *Actinobacillus pleuropneumoniae*, were cloned and sequenced in our laboratory and compared to the receptor genes from *N. meningitidis* to identify regions of homology. This analysis enabled us to select sites at which the *N. meningitidis* and *A. pleuropneumoniae* genes were spliced together by the PCR-based splicing by overlap extension (SOEing) technique. The hybrid genes were cloned into a pT7-7 expression system and the properties of the recombinant chimeric proteins were evaluated by a combination of binding assays and affinity isolation procedures. The preliminary results have enabled us to tentatively localize regions involved in binding to hTf and/or interactions between the two receptor proteins.

References

1. **Irwin, S.W., N. Averill, C.Y. Cheng and A.B. Schryvers.** 1993. Preparation and analysis of isogenic mutants in the transferrin receptor protein genes, *tbp1* and *tbp2*, from *Neisseria meningitidis*. *Mol. Microbiol.* **8**: 1125-1133.
2. **Legrain, M., E. Jacobs, S.W. Irwin, A.B. Schryvers and M.J. Quentin-**

- Millet.** 1993. Molecular cloning and characterization of *Neisseria meningitidis* genes encoding the transferrin binding proteins Tbp1 and Tbp2. *Gene* **130**: 73-80.
3. **Schryvers, A.B., S.W. Irwin, M.J. Middelveen, J.A. Ogunnariwo and J. Alcantara.** 1991. Iron acquisition in *Neisseria*: Bacterial receptors for human transferrin and human lactoferrin in *Neisseria meningitidis* p. 481-486. In M. Achtman, P. Kohl, C. Marchal, G. Morelli, A. Seiler and B. Thiesen *Neisseriae* 1990. Walter de Gruyter, Berlin.

Identification of the domains of human transferrin involved in binding to transferrin receptors of human pathogens by the use of chimeric recombinant transferrins expressed in a baculovirus expression system

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Pathogenic bacteria of the Neisseriaceae and Pasteurellaceae families possess iron repressible outer membrane proteins (designated Tbp1 and Tbp2) which are involved in iron acquisition from transferrin and are specific for their respective hosts' transferrin (4). This receptor-mediated iron acquisition mechanism enables the pathogens to acquire iron *in vivo* from the iron restricted environment of the host. The N-linked oligosaccharide side chains of human transferrin (hTf) are not required for binding to the bacterial receptor proteins indicating that the amino acids at the surface of hTf mediate receptor binding (3). The observation that the human pathogens *Neisseria meningitidis*, *Haemophilus influenzae* and *Moraxella catarrhalis* recognize the same spectrum of primate transferrins and that transferrin binding in these species is inhibited by the same monoclonal antibody suggests that the transferrin-receptor interaction involves a similar region of transferrin in these species (2). The primary region of interaction has been localized to the hTf C-lobe by binding and affinity isolation experiments with proteolytically-derived C-lobe and N-lobe fragments (1). This approach was unable to localize the region recognized by the *M. catarrhalis* Tbp2 (5) and has several inherent limitations for further delineating the regions involved in receptor binding.

In this study we describe an approach involving production and testing of recombinant chimeric molecules of human and bovine transferrin to further identify the regions of interaction. In order to prepare bovine/human hybrid genes, the bovine transferrin gene was cloned and sequenced. Bovine transferrin cDNA was isolated by plaque hybridization of a lambda library using human transferrin cDNA as the probe. The hybrid transferrin genes were subsequently constructed using the PCR-based splicing by overlap extension (SOEing) technique. Five hybrid genes containing sequences from both bovine and human transferrin were constructed. The hybrids and recombinant human and bovine transferrin genes, were subsequently expressed in a baculovirus expression system. Expressed proteins were detected by SDS PAGE and Coomassie blue staining and confirmed using electroblotting followed by incubation with antibodies directed against human or bovine transferrin. The concentrations of the isolated molecules were approximately 50-100: g per : l.

The expressed proteins were subsequently iron loaded and utilized in a series of competitive dot binding assays. This enabled us to localize the region of hTf involved in binding to the *N.meningitidis* receptors to amino acid residues 365 to 607.

References

1. **Alcantara, J., R.-H. Yu and A.B. Schryvers**. 1993. The region of human transferrin involved in binding to bacterial transferrin receptors is localized in the C-lobe. *Mol.Microbiol.* **8**: 1135-1143.
2. **Gray-Owen, S.D. and A.B. Schryvers**. 1993. The interaction of primate transferrins with receptors on bacteria pathogenic to humans. *Microb.Pathog.* **14** :389-398.
3. **Padda, J.S. and A.B. Schryvers**. 1990. N-linked oligosaccharides of human transferrin are not required for binding to bacterial transferrin receptors. *Infect.Immun.* **58**: 2972-2976.
4. **Schryvers, A.B. and G.C. Gonzalez** 1990. Receptors for transferrin in pathogenic bacteria are specific for the host's protein. *Can.J.Microbiol.* **36**: 145-147.
5. **Yu, R.-H. and A.B. Schryvers**. 1993. The interaction between human transferrin and transferrin binding protein 2 from *Moraxella (Branhamella) catarrhalis* differs from that of other human pathogens. *Microbial Pathogenesis* **15**: 443-445.

Transferrin-transferrin receptor interactions in *Neisseria meningitidis*

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It is now firmly established that receptors for serum transferrin are present on the surface of a number of bacterial pathogens including *Neisseria meningitidis*. As part of a study to identify regions on serum transferrins important for transferrin-receptor interactions, we carried out receptor-binding studies on a number of native and deglycosylated transferrins using both bacterial and the already well-characterized mammalian systems.

To investigate the role of the glycan on transferrin-receptor interactions we investigated the properties of native and enzymatically-deglycosylated pig serum transferrin. Although pig transferrin has 70% sequence homology with human transferrin, we found that it does not bind to the receptor for human transferrin on *Neisseria meningitidis*. This failure to bind is not a consequence of steric hindrance by the glycan as deglycosylated pig transferrin is also not recognised by the bacterial transferrin receptor. In contrast, we found that the native, iron-saturated pig transferrin binds to K562 cells with an affinity comparable to that of human transferrin and that deglycosylation of the protein has no effect on affinity.

From these results with K562 cells we conclude that human and pig serum transferrins share common receptor binding regions and that the glycan chains are not involved in receptor binding. However, our results also suggest that the meningococcal receptor recognizes regions of the transferrin molecule which differ from those involved in binding to the human receptor. We have identified several amino acids located on the surface of the C-lobe which could be involved in binding interactions.

As has been previously observed with mammalian transferrin receptors, we found that apo human serum transferrin has a reduced affinity for the receptor on *Neisseria meningitidis*. We also found that a variant form of human diferric transferrin, with a single amino acid substitution in the C-lobe at position 394, has a reduced affinity for both mammalian and meningococcal receptors.

Antigenicity and cross-reactivity of purified *Neisseria meningitidis* transferrin binding protein 2 (TBP2)

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The strains used in this study were *Neisseria meningitidis* P000 (serogroup B; serotype 15; carrier strain; TBP2 . 80kDa), P391 (autoagglutinable; serotype 15; carrier strain; TBP2 . 80 kDa), P636 (autoagglutinable; serotype 8, 15; carrier strain; TBP2 . 85 kDa), V002 (serogroup B; serotype 15; case strain; TBP2 . 70 kDa), V021 (serogroup B; nontypeable; case strain; TBP2 . 80 kDa) and HG7 (serogroup C; serotype 2b; case strain; TBP2 . 65 kDa).

The TBP2s from strains P000, P391 and HG7 were purified to homogeneity by non denaturing procedures using affinity chromatography on transferrin-Sepharose followed by ion-exchange chromatography on a Pharmacia mono-Q column and used for hyperimmunization of mice to obtain polyclonal anti-TBP2 specific antisera. Reactivity was assayed after SDS-PAGE and electroblotting of homologous outer membrane vesicles (OMV's) extracted from cultures in iron-restricted conditions (3). Cross-reactivities of the anti-TBP2 antibodies were determined in the same way for five heterologous *N. meningitidis* strains.

The three purified TBP2's were fully antigenic in mice, with no antibodies against other OMV antigens detected. Cross-reactivity patterns of the anti-TBP2 sera were as follows: 1) anti-TBP2 from strain P000 did not cross-react with any heterologous strain; 2) anti-TBP2 from strain P391 cross-reacted with strain P636; 3) anti-TBP2 from strain HG7 cross-reacted with strain V002.

Cross-reactivity patterns for anti-TBP2 sera from strains P000 and HG7 were coincident with those observed for the TBP2 bands in previous studies using anti-OMV's sera (2, 3), but cross-reactivity of the anti-TBP2 serum from strain P391 was more restricted than that showed by the anti-OMV's serum (which cross-reacted with strains P000, P636 and V021). A mouse antiserum raised against TBP1/2 complexes from this last strain (obtained after the first step of our purification protocol), regained the cross-reactivity pattern showed by the anti-OMV's serum.

Results obtained by Ala'Aldeen (1) showed that anti-TBP2 antibodies in mouse sera showed raised against TBP1/2 complexes cross-reacted with only some

heterologous, agreeing with our results. In contrast, rabbit sera against TBP1/2 complexes (1) or TBP2 purified by SDS-PAGE (4) cross-reacted with the TBP2 of all heterologous strains tested, suggesting that the response can differ depending on the animal model chosen, which is also supported by the results obtained with human sera (1, 2).

Our results show that TBP2's purified by non denaturing procedures maintain their antigenicity in mouse, although, as in strain P391, cross-reactivity can differ from those obtained for the same protein when immunization is made with whole OMV's (in which TBP's are in native natural conformation), implying some epitope variation. Regained cross-reactivity of the P391 strain anti-TBP1/2 serum suggests that possibly TBP1 and TBP2 can be associated in such a way that conformational epitopes, perhaps with domains from each TBP, can be relevant for antigenicity.

References

1. **Ala'Aldeen, D.** 1994. Personal communication.
2. **Ferreirós, C.M., Ferrón, L. and Criado, M.T.** 1994. *In vivo* human immune response to transferrin-binding protein 2 and other iron-regulated proteins of *Neisseria meningitidis*. FEMS Immunol. Med. Microbiol. **8**: 63-68.
3. **Ferrón, L., Ferreirós, C.M., Criado, M.T. and Pintor, M.** 1992. Immunogenicity and antigenic Heterogeneity of a human transferrin-binding protein in *Neisseria meningitidis*. Infect. Immun. **60**: 2887-2892.
4. **Stevenson, P., Williams, P. and Griffiths, E.** 1992. Common antigenic domains in transferrin-binding protein 2 of *Neisseria meningitidis*, *Neisseria gonorrhoeae*, and *Haemophilus influenzae* type b. Infect. Immun. **60**: 2391-2396.

Human and animal immune response to meningococcal transferrin-binding proteins: Implications for vaccine design

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The results reported here show that the two meningococcal transferrin-binding proteins (TBP1 and TBP2) generate different immune responses in different host species and that there is variation in response dependent on the method of antigen preparation, and possibly the route administration.

Mice immunised with either whole cells of *N. meningitidis* strain SD (B:15:P1.16) or the isolated TBP1/TBP2 complex from the same strain produced antisera which, when tested against a representative panel of meningococcal isolates by Western blotting, recognised some but not all heterologous TBP2 molecules. In contrast, rabbit antisera raised to the same preparations were cross-reactive with almost all the TBP2 molecules. The immune response to TBP1 was also host species dependent. Western blot analysis using denatured TBP1 failed to detect antibodies in antisera raised in mice to whole cells or in a rabbit to TBP1/TBP2 complex, but detected broadly cross-reactive antibodies in mouse anti-TBP1/TBP2 complex sera and strain-specific antibodies in rabbit anti-whole cell serum. Human convalescent sera obtained from five patients infected with meningococci of different serogroups and serotypes, contained fully cross-reactive antibodies to TBP2 but no anti-TBP1 antibodies, when examined on Western blots. However, on dot immunoblots, the same patients sera, as well as the mouse anti-whole cell and the rabbit anti-TBP1/TBP2 complex sera, reacted with purified biologically active TBP1 of strain SD.

This indicates that native TBP1, a protein which loses its biological and part of its immunological activities when denatured, is immunogenic and that humans generate cross-reactive antibodies to native epitopes. These observations have important implications for assessing the vaccine potential of TBPs and other meningococcal antigens. Conclusions regarding the usefulness of TBPs as candidate components of meningococcal serogroup B vaccines based on results from certain animal species

such as mice, or using methods such as Western blotting, may have little bearing on the situation in man and may lead to some potentially useful antigens being disregarded.

References

1. **Ala'Aldeen, D.A.A., P. Stevenson, E. Griffiths, A.R. Gorringer, L.I. Irons, A. Robinson, S. Hyde and S.P. Borriello.** 1994. Immune responses in humans and animals to meningococcal transferrin-binding proteins: implications for vaccine design. *Infect Immun.* **62**: in press.

Blocking of iron uptake from transferrin by antibodies against the transferrin-binding system of *Neisseria meningitidis*

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In order to analyse the importance of the proteins of the transferrin-iron uptake system of *Neisseria meningitidis* as potential targets for the development of effective vaccines, in this work we evaluated the effect of antibodies raised in mouse against those proteins on their metabolic functions.

Three *Neisseria meningitidis* strains used in this study were obtained from healthy carriers: P000 (serogroup B; serotype 15; TBP2 . 80kDa), P391 (autoagglutinable; serotype 15; TBP2 . 80kDa), P636 (autoagglutinable; serotype 8, 15; TBP2 . 85 kDa), and the other three were case strains: V002 (serogroup B; serotype 15; TBP2 . 70 kDa), V021 (serogroup B; nontypeable; TBP2 . 80 kDa) and HG7 (serogroup C; serotype 2b; TBP2 . 65 kDa). Immune sera were produced in Balb/c mice hyperimmunized with outer membrane vesicles (OMV's) obtained from cultures grown in iron sufficiency or iron restriction (1) or with transferrin binding protein 2 (TBP2) or TBP1/2 complex purified by non-denaturing procedures from the strains P000, P391 and HG7. Iron internalization was determined in an uptake assay using either [⁵⁵Fe](Fe₂) human transferrin or [⁵⁵Fe] ferric citrate as the only iron source (2,3).

Our results demonstrate that immune sera against OMV's obtained from iron-restricted cultures, but not those against OMV's from iron-sufficient cultures, blocked iron uptake from transferrin. Blocking was about 90% for the homologous strain, with variable degrees of cross-blocking for heterologous strains (from 0 to 70%), blocking being in agreement with anti-TBP2 cross-reactivity determined in immunoblotting assays after SDS-PAGE and electroblotting of OMV's. Both immune cross-reaction and cross-blocking of iron uptake occurred preferentially between strains possessing similar molecular weight TBP2, in a way according to the two-group classification suggested by Rokbi *et al.* (4). Similar blocking results were observed using anti-TBP2 and anti-TBP1/2 antisera, although cross-reactivities were more restricted with the latter.

Iron uptake from ferric citrate (determined in strains P391 and HG7) was unaffected by any of the immune sera, which demonstrates that blocking was due to antibodies specific for the *N. meningitidis* transferrin binding system (in a previous study (3)

we demonstrated the existence of specific meningococcal receptors for ferric citrate). Finally, we have found that meningococcal growth is affected by the immune sera in parallel with blocking of iron uptake, which demonstrates that our antibodies, by blocking the uptake of this metal, impede the subsequent growth of the bacteria.

Our results show that the protein component of the neisserial transferrin binding system can be considered as potentially valuable antigens, and that culture in iron restriction could be essential for the development of effective meningococcal vaccines.

References

1. **Ferrón, L., Ferreirós, C.M., Criado, M.T. and Pintor, M.** 1992. Immunogenicity and antigenic heterogeneity of a human transferrin-binding protein in *Neisseria meningitidis*. *Infect. Immun.* **60**: 2887-2892.
2. **Pintor, M., Ferreirós, C.M. and Criado, M.T.** 1993. Characterization of the transferrin-iron uptake system in *Neisseria meningitidis*. *FEMS Microbiol. Lett.* **112**: 159-166.
3. **Pintor, M., Ferreirós, C.M. and Criado, M.T.** 1994. Energy-independent binding of iron complexed to small organic chelants by *Neisseria meningitidis*. *J. Gen. Appl. Microbiol.* **40**: 23-34.
4. **Rokbi, B., Mazarin, V., Maitre-Wilmotte, G. and Quentin-Millet, M.J.** 1993. Identification of two major families of transferrin receptors among *Neisseria meningitidis* strains based on antigenic and genomic features. *FEMS Microbiol. Lett.* **110**: 51-58.

Production of monoclonal antibodies to meningococcal transferrin binding protein 2

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To gain insight on the structure and topology of meningococcal transferrin binding proteins and to have well defined tools for further studies, we undertook to produce monoclonal antibodies. Because polyclonal antibodies to the Tbp1-Tbp2 complex are bactericidal (1), we sought to drive the immune response to have a majority of IgG2a and IgG2b which are known to be the isotypes of immunoglobulin in mice which bind most efficiently complement. Therefore if the monoclonal reacted with surface exposed epitopes, it should bind to the surface of the bacteria and induce killing in the presence of complement. To obtain a majority of IgG2a and IgG2b, MRL/Lpr.Lpr and CD1 mice were used.

To obtain antibodies to Tbps in a conformation as close as possible to that of the native protein, mice were primed with an outer membrane fraction prepared from *Neisseria meningitidis* strain B16B6 (B:2a;P1.2) grown under iron limiting conditions and then boosted several times with purified Tbp2. After fusion, clones were screened by ELISA and the reactivity of the selected clones was further determined by Western blot analysis. Out of an initial number of approximately 700 clones, 18 were selected. Each monoclonal antibody was produced and purified. 16 were specific of Tbp2 and 2 reacted with Tbp1. The isotype of the Mabs was determined, the ELISA titers to transferrin-binding proteins were measured and their bactericidal activity titered. All Mabs produced were IgG2a and IgG2b, but only half displayed a bactericidal activity.

References

1. **Danve, B., L. Lissolo, M. Mignon, P. Dumas, S. Colombani, A. B. Schryvers and M.-J. Quentin-Millet.** 1993. Transferrin-binding proteins isolated from *Neisseria meningitidis* elicit protective and bactericidal antibodies in laboratory animals. *Vaccine* **11**: 1214-1220.

Mapping monoclonal antibodies directed against meningococcal transferrin-binding protein 2

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Monoclonal antibodies (Mabs) to meningococcal transferrin-binding protein 2 from strain B16B6 (B:2a:P1.2) have been produced and purified. Some Mabs displayed bactericidal activity while others were devoid of it. To define the specificity of the Mabs in order to attempt to localise precisely surface exposed epitopes, two different techniques were used.

Firstly overlapping peptides of seven amino acids spanning the entire Tbp2 molecule were synthesized by the pin technology (Cambridge Research Biochemicals, UK.). The reactivity of each purified Mab was assayed versus the 188 peptides. Some of the Mabs showed a strong reactivity towards the linear peptides while others could not be mapped to a single site. Secondly to confirm the specificity determined by the pin technology, Mabs were tested for their ability to react with various truncated Tbp2's produced in *E. coli*. Finally short Tbp2 domains fused to MalE were produced in *E. coli* and the reactivity of the Mabs with the fusion proteins was determined.

The different methods were complementary and allowed the determination of the specificity of the Mabs produced. Out of the 15 Mabs studied there was an even distribution of conformational Mabs and those which could be mapped to a linear epitope. This set of well characterized Mabs should provide useful tools for further studies.

Identification of the protective component within the meningococcal transferrin-binding protein complex

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Neisseria meningitidis is capable of acquiring iron via surface accessible transferrin receptors expressed in the outer membrane (2). The transferrin receptor is composed of two polypeptides, Tbp1 and Tbp2 (transferrin-binding proteins 1 and 2). Previous studies have demonstrated that the complex Tbp1-Tbp2 elicited bactericidal and protective antibodies in laboratory animals (1). Two main questions remained: could both polypeptides independently confer protection and if not which of the two induced protective antibodies? Which mechanism(s) could be involved in the protection, simple clearance by common opsonophagocytosis along with complement mediated killing? Or, whether antibodies to the transferrin receptor could interfere with iron acquisition therefore inhibiting or limiting bacterial growth?

To address these questions, milligram quantities of individual polypeptides were purified by a chromatographic method based on their different hydrophobic characters. Monospecific antisera were raised against independent polypeptides allowing the evaluation of their biological properties. The antibodies were tested for their ability to inhibit transferrin-binding to the receptor as measured by a solid-phase binding assay and to inhibit growth of meningococci when given human transferrin as the sole iron source. Finally, the protective activities of the isolated protein were evaluated in mice in comparison to that of the Tbp1-Tbp2 complex. Taken together these results will be discussed in the scope of vaccine design.

References

1. **Danve, B., L. Lissolo, M. Mignon, P. Dumas, S. Colombani, A.B. Schryvers and M.-J. Quentin-Millet.** 1993. Transferrin-binding proteins isolated from *Neisseria meningitidis* elicit protective and bactericidal antibodies in laboratory animals. *Vaccine* **11**: 1214-1220.
2. **Schryvers, A.B. and L.J. Morris.** 1988. Identification and characterization of the transferrin receptor from *Neisseria meningitidis*. *Mol. Microbiol.* **2**: 281-288.

Biochemical and genetic analysis of the lactoferrin binding protein and *lbpA* gene of *Neisseria* spp.

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Iron is an essential element for sustained growth of most organisms and thus pathogenic bacteria require mechanisms of iron-acquisition that are effective in the iron-limited environment of the host. Pathogenic *Neisseria* species such as *N. meningitidis* and *N. gonorrhoeae* and some commensal *Neisseria* species possess surface receptors that mediate iron acquisition from human lactoferrin (hLf) on mucosal surfaces and human transferrin (hTf) in serum. These receptors are specific for the human forms of these glycoproteins, and the interaction does not involve the N-linked oligosaccharides moiety (2). Affinity isolation experiments using immobilized hLf or hTf have identified a single Lf-binding protein (Lbp), whereas two Tf-binding proteins or Tbps (Tbp1 and Tbp2) have been identified. The gene encoding the Lbp of *N. meningitidis* has been recently cloned and sequenced (6) and the predicted protein sequence of Lbp was found to have greater than 43% identity at the amino acid level to the gonococcal and meningococcal Tbp1s (1,3). The cloned *N. meningitidis* Lbp can be expressed in a heterologous genetic background (*Escherichia coli*) and is directed to the cell surface in a functional form that is able to bind native hLf (5).

Previous studies examining different *Neisseria* species for their ability to use Lf as a sole source of iron (4) or for binding hLf and affinity isolation of Lbp (4) have provided somewhat different indications of the extent of this mechanism of iron acquisition within the Neisseriaceae. In this study, we have specifically amplified regions of the gene encoding Lbp (*lbpA*) from various pathogenic and commensal *Neisseria* sp. by the polymerase chain reaction (PCR) and found evidence for the presence of this gene in almost all isolates tested. In addition, restriction endonuclease digestion analysis was performed to provide an indication of the extent of genetic variation of this gene within the Neisseriaceae.

In a second component of our study, various characteristics of heterologously-expressed Lbp were examined to better characterize the hLf-Lbp interaction. Various altered derivatives (either by limited proteolysis or by specific *lbpA* genetic recombinational events) of the meningococcal Lbp were evaluated for their ability to bind enzymatically-derived preparations of C-lobe and N-lobe of iron-loaded

human lactoferrin. Prior studies demonstrated that the both isolated C- and N-lobe preparations are capable of binding to native Lbp from iron-deficient *N. meningitidis* and *N. gonorrhoeae* (7). The purpose of these studies is to examine those domains of the meningococcal Lbp involved in binding to hLf.

References

1. **Cornelissen, C.N., G.D. Biswas, J. Tsai, D.K. Paruchuri, S.A. Thompson and P.F. Sparling.** 1992. Gonococcal transferrin-binding protein 1 is required for transferrin utilization and is homologous to TonB-dependant outer membrane receptors. *J.Bacteriol.* **174**: 5788-5797.
2. **Irwin, S.W., R.-H. Yu, J. Alcantara and A.B. Schryvers.** 1993. Transferrin and lactoferrin receptor proteins of *Neisseria meningitidis*. *Neisseriae* 1992.
3. **Legrain, M., E. Jacobs, S.W. Irwin, A.B. Schryvers and M.J. Quentin-Millet.** 1993. Molecular cloning and characterization of *Neisseria meningitidis* genes encoding the transferrin binding proteins Tbp1 and Tbp2. *Gene* **130**: 73-80.
4. **Mickelsen, P.A., E. Blackman and P.F. Sparling.** 1982. Ability of *Neisseria gonorrhoeae*, *Neisseria meningitidis*, and commensal *Neisseria* species to obtain iron from lactoferrin. *Infect.Immun.* **35**: 915-920.
5. **Pettersson, A., A. Maas and J. Tommassen.** 1994. Identification of the *iroA* gene product of *Neisseria meningitidis* as a lactoferrin receptor. *J.Bacteriol.* **176**: 1764-1766.
6. **Pettersson, A., P. VanderLey, J.T. Poolman and J. Tommassen.** 1993. Molecular characterization of the 98-kilodalton iron-regulated outer membrane protein of *Neisseria meningitidis*. *Infect.Immun.* **61**:4724-4733.
7. **Yu, R.-H. and A.B. Schryvers.** 1993. Regions located in both the N-lobe and C-lobe of human lactoferrin participate in the binding interaction with bacterial lactoferrin receptors. *Microb.Pathog.* **14**: 343-353.

Cloning a lactoferrin (Lf) receptor gene from *Neisseria gonorrhoeae* and characterization of strains unable to use Lf-bound iron

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Many but not all gonococcal isolates are able to obtain Fe from Lf. The Lf-Fe uptake system in *Neisseria* is mediated by a Lf-specific receptor. We report here the isolation of a Lf-receptor gene from the gonococcus.

The method consisted of transformation of a gonococcal Lf⁻ mutant unable to utilize Lf as an Fe source with a wild type genomic library constructed in modified pBluescript, selecting for Lf⁺ transformants. The transforming DNA was initially located on a 2 kb fragment. Further probing genomic DNA with insert specific primers yielded overlapping fragments. DNA sequencing revealed an open reading frame of 2832 bases with a predicted molecular weight of 103,077. A comparison of the DNA sequence with the Lf receptor *lbpA* gene from meningococcus showed that they are 94% identical (1,2). Insertion mutagenesis in the gonococcal *lbpA* followed by introduction of the mutation into the chromosome generated mutants, unable to support growth in the presence of Lf, lacking the 103 kD protein by SDS-PAGE, and showing no binding of Lf in a dot blot assay. A monoclonal antibody (MAb) to lactoferrin receptor protein from the meningococcus (kindly provided by Jan Poolman) recognized the 103 kD protein from the wild type strains of gonococci, but failed to detect a similar protein in the mutant.

An examination of several Lf⁻ clinical isolates revealed that they were unable to bind Lf, and failed to recognize the MAb directed against meningococcal Lbp. However, Lf⁺ transformants obtained by transforming Lf⁻ strains with the cloned *lbpA* DNA were able to bind Lf, and recognized the anti-Lbp MAb, suggesting that naturally occurring Lf⁻ strains carry defects in the *lbpA* gene.

References

1. **Pettersson, A., P. van der Ley, J. T. Poolman and J. Tommassen** 1993. *Infect. Immun.* **61**: 4724-4733.
2. **Pettersson, A., A. Maas and J. Tommassen**. 1994. *J. Bacteriol.* **76**: 1764-1766.

Characterization of FrpB, the 74kD iron-regulated, outer-membrane protein of *N. gonorrhoeae*

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FrpB (for Fe-regulated protein B) is a 74kD outer membrane protein that is part of the iron regulon of *Neisseria gonorrhoeae* and *Neisseria meningitidis*. The *frpB* gene from gonococcal strain FA19 was cloned and sequenced. FrpB was homologous to several TonB-dependent outer membrane receptors of *E.coli* as well as HemR of *Yersinia enterocolitica* and CopB of *Moraxella catarrhalis*.

An omega insertion into the *frpB* coding sequence abolished expression of FrpB. This mutant grew on haem as an iron source but was reduced in ⁵⁵Fe-haem uptake. Expression of FrpB from its own promoter in pACYC184 was iron repressible, and allowed *E.coli* RK1065 *hemA* to grow on haem as a source of porphyrin and iron. These data suggest that FrpB may play a role in haem utilization by *N. gonorrhoeae*.

Cloning and DNA sequence of the gene coding for a DnaK homologue from *Neisseria meningitidis*

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A rabbit antiserum, raised against a 70 kD iron-regulated protein (70 kD FeRP) present in lithium acetate-chloride extracted outer membrane vesicles of *N. meningitidis*, was used to screen a λ gt11 genomic library. Y1089 lysogens were prepared from positive clones and an individual expressing a full-sized reactive polypeptide was chosen for further study.

Subcloning and sequence analysis revealed the presence of an open reading frame coding for a 69 kD DnaK homologue. It was flanked by consensus heat shock promoter and transcriptional terminator sequences, and associated with a neisserial 152 bp repeat (4). This open reading frame was subcloned and over-expressed under a p *trp* promoter, yielding a 70 kD protein reactive with the rabbit hyperimmune serum.

The 70 kD FeRP has been described as either a conserved, widely cross-reactive and strongly immunogenic (1,2,3) or an antigenically variable, strain-specific antigen (5). Since the fractionation techniques employed in these reports differ, this contradiction might have arisen from the presence of co-migrating iron-regulated 70 kD proteins in some of these preparations. In fact, the immunological properties of stress proteins fit well into those ascribed to the cross-reactive 70 kD FeRP (6). Additional work is currently under way to verify this hypothesis.

References

1. **Ala'Aldeen, D., R.A. Wall and S.P. Borriello.** 1990. Immunogenicity and cross-reactivity of the 70-kDa iron-regulated protein of *Neisseria meningitidis* in man and animals. *J.Med.Microbiol.* **32**: 275-281.
2. **Banerjee-Bhatnagar, N. and C.E. Frasch.** 1990. Expression of *Neisseria meningitidis* iron-regulated outer membrane proteins, including a 70-Kilodalton transferrin receptor, and their potential for use as vaccines. *Infect.Immun.* **58**: 2875-2881.
3. **Black, J.R. D.W. Dyer, M.K. Thompson and P.F. Sparling.** 1986. Human immune response to iron-repressible outer membrane proteins of

Neisseria meningitidis. Infect.Immun. **54**: 710-713.

4. **Correia, F.F., S. Inouye and M. Inouye.** 1988. A family of small repeated elements with some transposon-like properties in the genome of *Neisseria gonorrhoeae*. J. Biol. Chem. **263**: 13194-12198.
5. **Pettersson, A., B. Kuipers, M. Pelzer E. Verhagen, R. Tiesjema, J. Tommansen and J. Poolman.** 1990. Monoclonal antibodies against the 70kD iron-regulated protein of *Neisseria meningitidis* are bactericidal and strain specific. Infect. Immun. **58**: 3036-3041.
6. **Young, R.A.** 1990. Stress proteins and immunology. Ann. Rev. Immunol. **8**: 401-420.

Homology models of *N. gonorrhoeae* ferric binding protein

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Three dimensional models of the ferric binding protein (Fbp) from *Neisseria gonorrhoeae* have been constructed based on the crystallographically determined structures of human lactoferrin and the sulphate-binding protein from *Salmonella typhimurium*. The sulphate-binding protein belongs to a class of bacterial nutrient transport proteins which are all approximately 300 amino acids and share a common fold. Each of the two identical domains of the mammalian transferrins are structurally homologous to these bacterial proteins and the transferrins share many iron-binding characteristics with Fbp. The energy-minimized homology models of Fbp contain an iron-binding site which is chemically similar to that of lactoferrin and is consistent with biochemical data implicating functionally important residues in Fbp.

Crystallographic studies of Fbp have been initiated and crystals of apo and ferric Fbp have been obtained. The homology models of Fbp are being used with the diffraction data to obtain a crystallographic structure of Fbp.

High affinity iron-binding by Fbp is homologous to the transferrins at both the structural and functional level

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The ferric iron-binding protein, Fbp, functions in the high-affinity active transport of growth-essential iron by pathogenic *Neisseria* from human transferrin (hTf), lactoferrin and citrate (1). Fbp participates in this process as a periplasmic binding protein, shuttling iron across the periplasmic space from hTf bound on the surface of the bacterium. Previous studies have demonstrated that Fbp binds a single molecule of iron per molecule of protein with an affinity approximating that of hTf, and is approximately half the size of hTf (2). The transferrins, of which hTf is a member, are a highly conserved family of vertebrate iron-binding proteins which (i) reversibly bind two molecules of ferric iron per molecule of protein with high affinity, (ii) have a molecular mass of *ca.* 80kD, and (iii) function in extracellular iron metabolism and bacteriostasis. Similarities between the N- and C-terminal iron-binding lobes of hTf, especially as they relate to critical active site binding residues, provide compelling evidence that these molecules evolved by gene duplication, implying the existence of a primordial mono-sited transferrin. We have demonstrated that the spectral properties of purified Fbp and hTf are similar in the visible range. Chemical modification of purified Fbp in the presence and absence of iron demonstrates that, similar to each iron-binding site of the transferrins, two Tyr residues and one His residue are implicated in iron binding. In addition, like the transferrins, a bicarbonate anion is required for the efficient co-ordination of iron by Fbp. The range of metals bound by Fbp and hTf, including the fluorescent lanthanide terbium, is identical. Finally, terbium derivatives of Fbp and hTf yield virtually identical luminescence excitation spectra, implying a highly similar binding site environment. These studies suggest that the prokaryotic Fbp is a mono-sited analogue for iron-binding by the eukaryotic transferrins (3).

The structure of Fbp has been further probed by partial X-ray diffraction analysis and homology modelling with human lactoferrin. These results have allowed the prediction of specific residues involved in iron-binding. Site-directed mutants of aromatic residues predicted to be proximal to the binding site have been constructed and probed in the terbium luminescence assay described above. Preliminary experiments in which a mutant Fbp was created that converted Tyr at position 265 to a Phe, indicated that mutation of this residue caused a decrease of two orders of

magnitude in affinity for iron; however, terbium derivatives of this mutant showed no difference in excitation spectra, implying that the residue is more than 10 Å distant from the metal-binding site. Conversion of Trp at position 196 to tyrosine altered the terbium derivative spectra, implying that this residue is more proximal to the metal-binding site. Further experiments to analyze the position of key aromatic residues and the effect of mutation on iron-binding affinity are in progress. We hope to define the structural and functional anatomy of the metal-binding site of Fbp with these mutants.

References

1. **Chen, C.-Y., Berish, S.A., Morse, S.A. and Mietzner, T.A.** 1993. The iron-binding protein (Fbp) of pathogenic *Neisseria* functions as a periplasmic transport protein in iron-acquisition from human transferrin. *Molec. Microbiol.* **10**: 311-318.
2. **Mietzner, T.A., Bolan, G., Schoolnik, G.K. and Morse, S.A.** 1987. Purification and characterization of the major iron-regulated protein expressed by pathogenic *Neisseriae*. *J. Exp. Med.* **165**: 1041-1057.
3. **Nowalk, A.J., Tencza, S.B. and Mietzner, T.A.** 1994. Co-ordination of iron by the ferric iron binding protein of pathogenic *Neisseria* is homologous to the transferrins. Submitted for publication.

Heavy metal inhibition of pathogenic *Neisseria*

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Fbp is the major iron-regulated protein that is conserved among all pathogenic *Neisseria*. Previous studies from our laboratory and others have demonstrated that the iron-binding protein, Fbp, functions in the transport of growth-essential iron in the capacity of a periplasmic binding protein. It has been suggested that the existence of pathogenic *Neisseria* lacking the gene has not been described because it may represent a lethal condition. The current hypothesis is that Fbp is the 'nodal point' for the transport of growth-essential iron obtained from a variety of sources such as transferrin, lactoferrin and citrate. Additional studies on the biochemistry of purified Fbp indicates that this protein is quite promiscuous with regard to its binding of a variety of metals, including copper and gallium. It was reasoned that pathogenic *Neisseria* would be particularly sensitive to these metals based on the premise that they may act as competitive antagonists of iron.

This reasoning was tested by the following experiment. A lawn growth of gonococci or meningococci cultivated on GC Agar medium with IsoVitaleX was exposed to a sterile disc containing 25 : 1 of a 1 M solution of either cupric citrate or gallium citrate. After 20 h cultivation, a significant zone of inhibition (25-30cm) around both the Ga³⁺ and Cu²⁺ discs were observed. In contrast, an *Escherichia coli* strain cultivated on Luria-Bertani Agar medium was only minimally inhibited by Ga³⁺ and Cu²⁺ (zone size 2cm). Addition of ferric citrate rescued the pathogenic *Neisseria* from Ga³⁺ inhibition but not Cu²⁺ inhibition, suggesting that Ga³⁺ acts as a competitive antagonist for iron and that Cu²⁺-dependent inhibition acts by a method independent of iron competition.

Inspection of the growth characteristics around the copper-induced zone of inhibition revealed a surprising finding. Gonococci were more sensitive to copper inhibition than meningococci and exhibited a characteristic sterile zone of inhibition. Meningococci were less sensitive to copper inhibition, having a correspondingly smaller sterile zone of inhibition. However, proximal to this zone was a brown metallic ring typical of the reduction of Cu²⁺ to elemental copper (Cu¹⁺). These phenotypes are characteristic of all gonococci and meningococci tested to date.

The basis for this cupric reductase activity is dependent upon the cysteine/cystine

supplement within the IsoVitaleX component of the GC Agar medium and the oxygen tension of the environment. We are currently attempting to define the molecular basis for the meningococcal cupric reductase activity and the lack of gonococcal cupric reductase. Two possibilities are (a) the production of a cysteine oxidase by meningococci but not by gonococci or (b) the consumption of cysteine by gonococci but not by meningococci.

This study demonstrates that gonococci may be selectively inhibited by the heavy metal gallium, and that a fundamental differentiation between gonococci and meningococci can be made by a simple visual assay involving the observation of a cupric reductase activity or lack thereof.

Promoter mapping and transcriptional regulation of the iron-regulated *Neisseria gonorrhoeae* Fbp gene

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Introduction: The iron-regulated *Neisseria gonorrhoeae* ferric binding protein (Fbp), is highly conserved among gonococcal strains, and is synthesized in greater amounts than any other iron-stress induced protein. We have reported previously on the cloning and sequencing of the structural gene for Fbp (1). We have also shown that the *Escherichia coli* ferric uptake regulator protein (Fur) binds to the Fbp promoter (2). In order to gain a more complete understanding of the role of iron in the genetic control of Fbp, we have constructed *N. gonorrhoeae* Fbp promoter-CAT fusions in *E. coli*. We have also mapped the promoter region of *fbp*, and studied the accumulation and stability of the Fbp mRNA and the regulation of Fbp transcription by iron.

Experimental Design: The Fbp promoter sequence was cloned upstream of the structural gene for chloramphenicol acetyltransferase (CAT) in the *E. coli* / *N. gonorrhoeae* shuttle vector pLES-2. This Fbp-CAT construct was transformed into *E. coli* and cells were grown to a semi-logarithmic phase in a chemically defined medium (M9) under iron replete and iron deplete conditions. The expression of CAT activity was assayed using a simple phase extraction technique (3). For Fbp mRNA studies, *N. gonorrhoeae* F62 was grown in either GCB (iron replete) or GCB with 50: M desferal (iron deplete) media, and samples removed at one hour intervals. Total cellular RNA was isolated (4) and analyzed by Northern analysis using a radiolabelled 200 bp *fbp* specific structural gene probe. Stability of the Fbp transcript was determined by treatment of exponentially growing cells with 200 : g/ml rifampicin just prior to RNA isolation; quantitative results were obtained. To correlate the production of Fbp with the *fbp* mRNA, whole cell lysates were also analyzed by SDS-PAGE and western blot analysis using antisera to *N. gonorrhoeae* Fbp.

For primer extension analysis, total RNA was isolated (4) from *Neisseria gonorrhoeae* F62 grown at 35°C both in gonococcal broth (GCB) and chemically defined medium (CDM) (5) under condition of either iron limitation (25 : M desferal) or iron excess (50 : M ferric nitrate). Annealing of an 18 bp oligonucleotide complementary to the 5' end of the noncoding strand of *fbp* and primer extension reactions using reverse transcriptase were carried out (5) using 45 : g RNA, and the samples were analysed on a 6% sequencing gel alongside the M13mp18 sequence

which was generated using a standard M13 primer.

Results: A statistically significant increase in the expression of CAT activity was observed when *E. coli* containing the Fbp-CAT fusion was grown under iron-depleted conditions. In contrast, CAT activity decreased substantially when cells were grown under iron-replete conditions. Northern blot analysis using a 200 bp *fbp* structural gene probe detected a single Fbp transcript of approximately 1 kb. In cultures grown in iron-replete conditions, the Fbp transcript was found to increase over time, closely reflecting the specific stage of growth. In contrast, Fbp mRNA isolated from cultures grown in iron-depleted medium accumulated at a steady state throughout the growth cycle. Under these conditions, we observed a three-fold increase in Fbp mRNA accumulation during exponential growth relative to that present during early logarithmic growth phase. In order to determine the half-life of Fbp mRNA, cells were treated with 200 µg/ml rifampicin just prior to RNA isolation. Northern blots indicated that the half-life of Fbp RNA was approximately one minute. Simultaneous determination of Fbp by immunoblotting with anti-Fbp sera confirmed that Fbp expression was also regulated by iron. Primer extension analysis of the *fbp* promoter region indicated a single transcriptional start site located 51 bp upstream of the ATG translational start site. There appeared to be a greater amount of *fbp* transcript in RNA from cells grown in CDM under conditions of iron limitation compared to iron excess.

Conclusions: Results obtained from our *in vivo* Fbp mRNA studies showed that the production of the Fbp transcript paralleled the expression of Fbp, indicating that the transcription and translation of the gonococcal Fbp were closely linked. Primer extension analysis indicated that there was a single transcriptional start site located between one of the potential Fur binding sites and the Shine-Dalgarno. In addition, our studies in *E. coli* confirmed that the Fbp promoter was regulated by iron; this regulation most likely was in response to the regulatory protein, Fur. We are currently examining the regulation of the Fbp promoter-CAT construct in *fur*⁻ strains of *E. coli* to study the expression of the Fbp promoter as it relates to Fur.

References

1. **Berish, S.A., Mietzner, T.A., Mayer, L.W., Genco, C.A., Holloway, B.P. and Morse, S.A.** 1990. Molecular cloning and characterization of the structural gene for the major iron-regulate protein expressed by *Nisseria gonorrhoeae*. *J. Exp. Med.* **171**: 1535 - 1546.
2. **Berish, S.A., Subbarao, S., Chen, C-Y., Trees, D.L. and Morse, S.A.** 1993. Identification and cloning of a *fur* homolog from *Neisseria gonorrhoeae*. *Infect. Immun.* **61**: 4599 - 4606.
3. **Seed, B. and Sheen, J-Y.** 1988. A simple phase-extraction assay for chloramphenicol acyltransferase activity. *Gene* **67**: 271 - 277.
4. **Ansubel, F.M., R. Brent, R.E. Kingston, D. Moore., J.G. Seidman, J.A. Smith and K. Struhl (ed.).** 1987. Current protocols in molecular biology, vol. 2, p.4.4.2 John Wiley & Sons, New York.
5. **Moran, C.P.** 1990. Measuring gene expression in *Bacillus*, p. 267-293. In C.R. Harwood and S.M. Cutting, (ed.), *Molecular biology methods for Bacillus*. John Wiley & Sons, Ltd., London.

Use of operon fusions to study the effect of gonococcal Fur on iron-regulated gonococcal genes

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Background and objectives: *Neisseria gonorrhoeae* synthesizes a unique set of proteins when confronted with an iron restricted environment. Some, like the periplasmic iron binding protein Fbp, which is synthesized by all strains of *N. gonorrhoeae*, are thought to play an essential role in iron acquisition and utilization (4,5). The transcription of iron-regulated genes in *Salmonella typhimurium* and *Escherichia coli* is regulated by Fur (1), and homologues of *fur* have been shown to exist in genetically diverse microorganisms including *N. gonorrhoeae* (2) and *N. meningitidis* (8). The promoter regions of gonococcal *fur* and *fbp* contain sequences with a high degree of similarity to the consensus Fur binding sequence (3) suggesting that Fur may be involved in the regulation of these genes. Attempts to obtain a *fur* mutant of *N. gonorrhoeae* to study the regulation of the *fur* and *fbp* genes have been unsuccessful. Therefore, gonococcal promoter fusions were studied in a Fur⁻ strain of *E. coli*, in the presence of a high copy number plasmid on which gonococcal *fur* was under the control of the *lac* promoter, in order to quantitatively determine the effect of gonococcal Fur on the *fbp* and *fur* promoters under both iron-deficient and iron-sufficient conditions.

Experimental design: The 235 bp region of DNA containing the *fbp* promoter was cloned upstream of a promoterless chloramphenicol acetyl transferase (*cat*) gene; a promoterless *lacZ* gene was cloned in the opposite orientation to the *fbp* promoter. This double fusion was transferred onto a derivative of phage lambda by homologous recombination (7) and subsequently, a lysogen containing a single copy of the fusion was selected in *E. coli* strain W3110(*fur*⁻) *lac*. Using a similar strategy, the gonococcal *fur* promoter region was cloned into plasmid pRS551 upstream of the promoterless *lacZ* gene (7) and a single copy of the fusion was obtained in *E. coli* strain W3110(*fur*⁻) *lac*. Expression of the reporter genes was examined under iron-sufficient (50 : M ferric chloride) and iron-deficient (200 : M 2,2'-dipyridyl) conditions in the presence and absence of gonococcal Fur, which was expressed from a high copy number plasmid under *lac* promoter control. Genome walking by single-specific-primer polymerase chain reaction (SSP-PCR) (6) of gonococcal DNA was used to clone and sequence the region upstream of *fbp*.

Results: The *fur'*-*lacZ* fusion exhibited a relatively high basal level of β -galactosidase expression either in the presence or absence of gonococcal Fur. Under iron-sufficient conditions, the level of β -galactosidase was repressed by 50% in the presence of gonococcal Fur; however under iron-deficient conditions, enzyme activity increased only by 5%. When Fur was maximally induced using IPTG, a higher level of β -galactosidase activity was observed under iron-deficient conditions (50%) than was observed under iron-sufficient conditions.

Analysis of the *lacZ'*-*fbp'*-*cat* fusion construct in the presence of gonococcal Fur indicated a significant (50-60%) increase in CAT activity under iron-deficient conditions compared to that observed under iron-sufficient conditions. In addition, there was also a significant increase in β -galactosidase activity observed under iron-restricted conditions. These results together with the presence of potential Fur binding sequences in both orientations suggested that there was also a Fur-regulated promoter in the opposite orientation to *fbp*.

Using the technique of SSP-PCR with an *fbp* specific primer, a clone containing the region upstream of *fbp* was obtained and sequenced. The results indicated the presence of a 537 bp open reading frame (ORF).

Conclusions: We were interested in ascertaining whether the gonococcal *fur* gene, like other repressor-encoding genes, was self-regulated and whether Fur was involved in the regulation of the major gonococcal iron-regulated gene, *fbp*. The observation that both the *fur* and *fbp* promoters contained Fur binding sequences suggested that both of these genes were regulated by the product of the *fur* gene. However, the gonococcal *fur* gene was only moderately auto-regulated by its product, Fur, and even under iron-sufficient conditions the expression of the *fur* promoter was never completely repressed. It is possible that other regulatory factors may have a role in the regulation of *fur*. A putative CAP binding site has been identified in the gonococcal *fur* promoter region (2). The role of the catabolite-activator-protein (CAP) in the regulation of *fur* remains unknown; however, experiments are underway to study the effect of a mutagenized CAP site on *fur* expression. The *fbp* promoter and the ORF promoter were shown to be negatively regulated by gonococcal Fur under conditions of iron limitation. The role of this iron-regulated ORF is currently under investigation.

References

1. **Bagg, A. and J.B. Neilands.** 1987. Molecular mechanisms of regulation of siderophore-mediated iron assimilation. *Microbiol. Rev.* **51**: 509-518.
2. **Berish, S.A., S. Subbarao, C.Y. Chen, D.L. Trees and S.A. Morse.** 1993. Identification and cloning of a *fur* homolog from *Neisseria*

- gonorrhoeae*. Infect. Immun. **61**: 4599-4606.
3. **Calderwood, S.B. and J.J. Mekalanos**. 1988. Confirmation of the *fur* operator site by insertion of a synthetic oligonucleotide into an operon fusion plasmid. J. Bacteriol. **170**: 1015-1017.
 4. **Mietzner, T.A., R.C. Barnes, Y.A. JeanLouis, W.M. Shafer and S.A. Morse**. 1986. Distribution of an antigenically related iron-regulated protein among the *Neisseria spp.* Infect. Immun. **51**: 60-68.
 5. **Meitzner, T.A., G.H. Luginbuhl, E.C. Sandstrom and S.A. Morse**. 1984. Identification of an iron-regulated 37,000-dalton protein in the cell envelope of *Neisseria gonorrhoeae*. Infect. Immun. **45**: 410-416.
 6. **Shyamala, V. and G. Ferro-Luzzi Ames**. 1987. Genome walking by single specific-primer polymerase chain reaction. SSP-PCR. Gene. **84**: 1-8.
 7. **Simmons, R.W., F. Houtman and N. Kleckner**. 1987. Improved single and multicopy *lac*-based cloning vectors for protein and operon fusions. Gene. **53**: 85-96.
 8. **Thomas, C.E., and P.F. Sparling**. 1994. Identification and cloning of a *fur* homologue from *Neisseria meningitidis*. Molec. Microbiol. **11**: 725-737.

Isolation of a *Neisseria gonorrhoeae fur* mutant and its analysis by 2-D protein gel electrophoresis

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Recently *fur* homologues have been identified and characterized in both *Neisseria gonorrhoeae* (GC) (1) and *Neisseria meningitidis* (Nm) (3). Fur is responsible for transcriptional repression in the presence of iron in many Gram-negative bacteria. Since the pathogenic *Neisseriae* produce a number of iron-regulated gene products that are thought to be important in virulence we have sought to more completely define the Fur regulon in these species. One of the standard methods of defining a regulon is by analysis of a mutation in the regulon's central regulatory factor. Unfortunately, null mutations of *fur* in GC or Nm are apparently non-viable (1,3). To circumvent this problem, we have used manganese (Mn) selection on chemically defined media (CDM) to isolate a missense mutations of *fur* in GC (2).

Mn-resistant strains were isolated on Mn-CDM-transferrin plates and screened for loss of the ability to properly repress a transcriptional fusion of an iron-regulated neisserial promoter (5' fbp) with an enzymatic reporter gene (*phoA*). Mutations were shown to be genetically linked to *fur* by co-transformation with a selectable marker insertion downstream of *fur*. The linked mutation with the most profound defect was PCR amplified, cloned and sequenced. The defect was found to be the result of a single base change converting tyrosine 82 to a cysteine and reduced regulation of the reporter fusion from 7- to 10-fold down to approximately 2-fold. This mutation was analyzed along with the parent by 2-D protein gel electrophoresis. Bacterial cultures grown in presence or absence of inorganic iron were intrinsically labelled with a ¹⁴C amino acid mixture in CDM. Whole cell lysates were subjected isoelectric focusing (IEF) (1st dimension) and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) (2nd dimension). Preliminary analysis indicates that the majority of Fe-repressible proteins (frps) were expressed semi-constitutively in this *fur* mutant, however, Fe-inducible proteins (fips) seem to be unaffected by this mutation in *fur*. A detailed analysis of the protein expression patterns of this *fur* mutant and its parent will be presented.

References

1. **Berish, S.A., S. Subbarao, C.Y. Chen, D.L. Trees and S.A. Morse.** 1993. Identification and cloning of a *fur* homolog from *Neisseria gonorrhoeae*. *Infect Immun.* **61**: 4599-606.
2. **Hantke, K.** 1987. Selection procedure for deregulated iron transport mutants (*fur*) in *Escherichia coli* K 12: *fur* not only affects iron metabolism. *Mol Gen Genet.* **210**: 135-139.
3. **Thomas, C.E. and P.F. Sparling.** 1994. Identification and cloning of a *fur* homologue from *Neisseria meningitidis*. *Mol Microbiol.* **11**: 725-737.

Analysis of the complexity of iron regulation by *Neisseria gonorrhoeae*

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Introduction: In most gram-negative bacteria, iron transport is typically characterized by a specific transport system that involves outer membrane protein receptors, periplasmic binding proteins and cytoplasmic membrane proteins. The precise mechanisms by which *Neisseria gonorrhoeae* transports iron are not well defined; however, previous studies indicate that the ferric binding protein (Fbp), plays an important role (1). Fbp appears to function within the periplasm of *N. gonorrhoeae* to transport iron from the outer membrane to the cytoplasmic membrane. As iron is shuttled through the cell, it is highly probable that Fbp associates with the protein components in the outer and inner membranes. Although a number of other iron-regulated proteins have been described in *N. gonorrhoeae*, their potential interactions with Fbp have not been defined. We describe here, studies designed to identify additional proteins involved in iron transport and to further examine the regulation of expression of *N. gonorrhoeae* proteins by iron.

Experimental Design: *N. gonorrhoeae* F62 was grown under iron replete (GCB) or deplete (GCB with 25: M desferal) conditions and cells fractionated into outer membrane, cytoplasmic membrane, and periplasmic fractions as previously described (2). To examine the association of Fbp with specific protein components, we performed similar studies using the cross-linking agent formaldehyde prior to fractionation of cells (3). All samples were analyzed by two-dimensional gel electrophoresis, and data assessed by computer aided analysis.

Results: When grown under iron replete conditions, a total of 505 protein spots were identified in the outer membrane, periplasm and cytoplasmic membrane fractions as compared to 432 spots under iron deplete conditions. 54% of protein spots were present in the periplasmic fraction, 24% in the outer membrane and 22% in the cytoplasmic membrane under iron replete conditions. Under iron deplete conditions, 84% of the proteins were found in the periplasmic fraction, 11% in the outer membrane and 5% in the cytoplasmic membrane fraction. Fbp was found primarily associated in the periplasmic fraction as determined by molecular weight, isoelectric pH, and reactivity with Fbp specific antibody. We also identified protein spots corresponding to the conserved gonococcal proteins pI, and pIII in outer membrane fractions from both iron replete and deplete samples.

When the crosslinking agent was added to cultures grown under iron deplete

conditions, 56% of the total proteins were associated with the cytoplasmic membrane, 23% were associated with the outer membrane, and 22% were associated with the periplasmic fraction. Under iron replete conditions, 1%, 5%, and 94% of the proteins were associated with the cytoplasmic, outer membrane and periplasmic fractions respectively. Taken together, these results indicate that crosslinking results in the association of proteins in the periplasm with proteins in the cytoplasmic and outer membranes. Under these conditions, Fbp was found associated primarily with the cytoplasmic membrane supporting its role as a periplasmic binding protein that interacts with potential transport proteins present in the cytoplasmic membrane.

Conclusion: A crucial aspect of the elucidation of the process involved in the active transport of iron across the cytoplasmic membrane is the determination of the protein-protein interactions that take place and the mode of interaction between the membrane components and the periplasmic binding proteins. The results presented here indicate that Fbp interacts with cytoplasmic membrane proteins, consistent with its role as a bacterial periplasmic transport protein. In addition, our studies indicate that in *N. gonorrhoeae*, regulation of protein expression by iron is much more complex than previously thought, and may be similar to that described in other pathogenic bacteria such as *Vibrio cholerae* and *Salmonella typhimurium* (4).

References

1. **Morse, S.A., Berish, S.A., Chen, C.Y., Trees, D.L., Mietzner, T.A., Genco, C.A. and D.R. Kapczynski.** 1991. Structure function and regulation of the iron-binding protein, Fbp. pp. 453-458. In: *Neisseriae 1990*. M. Achtman, P. Kohl, C. Marchat, G. Morelli, A. S. Seiler, and B. Thiesen, ed. Walter de Gruyter & Co., Berlin, Germany.
2. **Johnson, K.H. and E.C. Gotschlich.** 1974. Isolation and characterization of the outer membrane of *Neisseria gonorrhoeae*. *J. Bacteriol.* **119**: 250-257.
3. **Prossnitz, E., Nikaido, K., Ulbrich, S.J. and G.F.L. Ames.** 1988. Formaldehyde and photoactivatable cross-linking of the periplasmic binding protein to a membrane component of the histidine transport system of *Salmonella typhimurium*. *J. Biol. Chem.* **263**: 17917-17920.
4. **Litwin, C.M. and S.B. Calderwood.** 1994. Analysis of the Complexity of gene regulation by Fur in *Vibrio cholerae*. *J. Bacteriol.* **176**: 240-248.

Characterization of haemin transport in *Neisseria gonorrhoeae*

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Introduction: *Neisseria gonorrhoeae* is capable of utilizing a wide variety of iron sources, including transferrin, lactoferrin, and haemin containing compounds (1,2). Acquisition of iron from these host-iron binding proteins by the gonococcus involves a family of distinct iron-regulated outer membrane receptors (1). Previous studies have established that the ferric binding protein (Fbp), functions as a periplasmic binding protein, shuttling iron from transferrin through the periplasm (3). In this study, we have characterized the transport of haemin by *N. gonorrhoeae* and determined the role of Fbp in haemin transport. In addition, we have identified and characterized several *N. gonorrhoeae* Tn1545) 3 insertional mutants that are defective in the ability to utilize haemin for growth.

Experimental Design: *N. gonorrhoeae* F62 was grown in a chemically defined media (CDM) containing the iron chelator desferal; haemin transport initiated by the addition of ¹⁴C-haemin (0.5: M, specific activity 112Ci/mole), ⁵⁵Fe-haemin or ⁵⁹Fe-haemin (16: M, specific activity 1.5 Ci/mole). To confirm the active transport of haemin, KCN, an inhibitor of energy transduction, was used. For Fbp extraction studies, cells were grown with ⁵⁵Fe-haemin and the Fbp extracted as previously described (4). Tn1545) 3 was introduced by conjugation into *N. gonorrhoeae* F62 (5), and transconjugants screened for the ability to utilize haemin for growth by plating on CDM with haemin as a sole source of iron.

Results: Our results indicated that the Fe from haemin was transported into the cell in an energy dependent fashion; cultures sampled at 1 hr transported 7323 pmole haemin/mg protein. The uptake of Fe was proportional to both the amount of ⁵⁹Fe-haemin added as well as the stimulation of growth. In addition, we did not observe the uptake of ⁵⁹Fe-haemin if inorganic iron or if cold haemin were present. We found that the majority of the ⁵⁵Fe-haemin was associated with the gonococcal Fbp indicating that iron bound haemin associates with Fbp during the course of haemin uptake. Although ¹⁴C-haemin was found to stimulate the growth of *N. gonorrhoeae* in CDM, we were unable to detect the transport of ¹⁴C-haemin into the cell.

We have also isolated two haemin uptake mutants of *N. gonorrhoeae* F62 by Tn1545) 3 mutagenesis (GCMT-6 and GCMT-10). We have demonstrated that

0.1- 0.2 : M haemin is sufficient to support the growth of the parent strain F62; however GCMT-6 and GCMT-10 are not capable of growth even with 4.0 : M haemin. Although GCMT-6 and GCMT-10 can utilize ferric citrate for growth, they do not utilize haemoglobin or normal human serum as sole iron sources for growth *in vitro*. These results indicate that the utilization of haemin and haemoglobin may occur by similar mechanisms in the gonococcus.

Conclusions: The results presented here indicate that *N. gonorrhoeae* can remove Fe from haemin prior to internalization and utilization, but does not appear to transport the porphyrin ring. In addition to its role in the transport of Fe from transferrin, Fbp also appears to function in the transport of Fe from haemin. Initial characterization of *N. gonorrhoeae* mutants supports the existence of similar pathways for the acquisition of iron from haemin and haemoglobin. Competition assays should allow us to delineate the specific haemin / haemoglobin acquisition pathway in the gonococcus.

References

1. **Chen, C.Y, Genco, C.A., Rock, J.P. and S.A. Morse.** 1989. Physiology and metabolism of *Neisseria gonorrhoeae* and *Neisseria meningitidis*. Clin. Microbiol. Rev. **2S**: S35-40.
2. **Dyer, D.W., West, E.P. and P.F. Sparling.** 1987. Effects of serum carrier proteins on the growth of pathogenic *Neisseria* with heme-bound iron. Infect. Immun. **55**: 2171 - 2175.
3. **Morse, S.A., Berish, S.A., Chen, C.-Y., Trees, D.L., Mietzner, T.A., Genco, C.A. and D.R. Kapczynski.** 1991 Structure, function, and regulation of the iron-binding protein, Fbp, pp.453- 458. In: *Neisseriae* 1990. M. Achtman, P. Kohl, C. Marchal, G. Morelli, A. Seiler, and B. Thiesen, ed. Walter de Gruyter & Co., Berlin, Germany.
4. **Mietzner, T.A., Bolan, G., Schoolnik, G.K. and S.A. Morse .** 1987. Purification and characterization of the major iron-regulated protein expressed by pathogenic *Neisseria*. J. Exp. Med. **165**: 104 -1057.
5. **Nassif, X., Puaoli, D. and M. So.** 1991. Transposition of Tn1545) 3 in the pathogenic *Neisseriae*: a genetic tool for mutagenesis. J. Bacteriol. **173**: 2147-2154.

Identification of an iron-regulated outer membrane protein of *Neisseria meningitidis* involved in the utilization of haemoglobin complexed to haptoglobin

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Haemoglobin complexed to the plasma protein haptoglobin can be used by *Neisseria meningitidis* as a source of iron to support growth *in vitro*. A *N. meningitidis* mutant, DNM2E4, was generated by insertion of the mTn3erm transposon into the gene coding for an 85 kilodalton (kDa) iron regulated outer membrane protein. Membrane proteins prepared from DNM2E4 were identical to the wild-type strain except that the 85 kDa protein was not produced. This mutant was unable to use haemoglobin-haptoglobin complexes as an iron source to support growth and was also impaired in the utilization of free haemoglobin. The mutant failed to bind both haemoglobin-haptoglobin complexes and apo-haptoglobin on a solid-phase dot blot assay. We hypothesize that the 85 kDa iron regulated protein is the haemoglobin-haptoglobin receptor and tentatively designate this protein Hpu (haptoglobin utilization).

Lactoferrin and haemoglobin iron-utilization does not influence the transmission of *Neisseria gonorrhoeae*

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The frequency of male to female transmission of gonorrhoea is estimated to be 50-80%. The factors affecting the transmission rate have not been well-characterized but are likely to include properties of both the host and the infecting organism. Iron acquisition is an important factor in the pathogenesis of infection by *Neisseria gonorrhoeae*. While all gonococci utilize iron from transferrin, lactoferrin (Lf) and Haemoglobin (Hb) iron-utilization is variable. One study reported that 53% of strains from a laboratory collection utilized iron from Lf (1). Similarly, 62% of these strains used Hb as an iron source (2). Infecting gonococci encounter different levels of iron sources (*e.g.* Lf or Hb) at various times during the menstrual cycle. Lf concentration in vaginal secretion is high in the proliferative (early) phase of the menstrual cycle, and is low during the secretory (late) phase (3). Similarly, Hb concentrations in these secretions are suspected to be low prior to menses, and high during and following the onset of the menstrual period. Therefore, the ability to utilize these alternative iron sources may confer a selective advantage for transmission of *N. gonorrhoeae* at various times in the menstrual cycle.

We are examining these issues by determining the iron-utilization phenotypes of *N. gonorrhoeae* from a well-characterized STD population in Pittsburgh, Pennsylvania. Iron-utilization phenotype, serovar/auxotype and clinical history were determined in an attempt to correlate these characteristics with given clones of *N. gonorrhoeae*. We are investigating whether the ability to utilize iron from Lf or Hb affects the male-to-female transmission of *N. gonorrhoeae* at different stages of the menstrual cycle.

Currently, the prevalent serovar within the Pittsburgh population is IB-3 (66.6%). Iron-utilization phenotype could not be correlated with a given serovar. The prevalence of human Lf utilization (Lf⁺) in our population was 29.5% and 70.5% of these isolates utilized iron from Hb (Hb⁺). Only one IB-3 isolate could use iron from Lf, suggesting that Lf utilization may not be important for the maintenance of a clone within a population. Iron-utilization phenotypes of the paired transmitted strains were identical, indicating that this phenotype appears to be stably maintained by an infectious clone.

Transmission of infection to the female sexual partner did not correlate with the Lf utilization phenotype; 66.7% Lf⁺ isolates and 68.4% of Lf⁻ isolates were transmitted. Transmission occurred in 58.8% of Hb⁺ isolates and 87.5% of Hb⁻ isolates. In the proliferative phase of the menstrual cycle, when high levels of Lf are present in vaginal secretions, there was no difference in transmission rates of Lf utilizing and non-utilizing phenotypes. Similarly, no difference in transmission rates was found in the secretory phase, when low levels of Lf are present. Utilization of iron from Hb did not affect the transmission rates of *N. gonorrhoeae* in either phase of the menstrual cycle.

Our study documents the variability of iron utilization from Lf and Hb among strains of *N. gonorrhoeae* in an active clinical population. We have shown that there are differences in iron-utilization phenotypes within each serovar/auxotype, a finding that may prove to be useful in further characterizing and classifying *N. gonorrhoeae* isolates. In addition, the phenotype of each transmitted strain was conserved within each male and female sexual partner, indicating that this iron-utilization phenotype is stably maintained in the host. While the ability to utilize iron from Lf or Hb do not appear to be important factors in the male-to-female transmission of *N. gonorrhoeae*, it may be relevant to other aspects of gonococcal pathobiology. For instance, it is postulated that gonococci ascend to the upper genital tract during menses as the initiating step in pelvic inflammatory disease. Growth of Hb⁺ phenotypes may be enhanced in this haemoglobin-enriched environment. We are currently investigating iron-acquisition phenotypes from strains derived from upper genital tract infections to address this possibility.

References

1. **Mickelson, P.A., E. Blackman and P. F. Sparling.** 1982. Ability of *Neisseria gonorrhoeae*, *Neisseria meningitidis*, and other commensal *Neisseria* spp. to obtain iron from lactoferrin. *Infect. Immun.* **35**: 915-20.
2. **Mickelson, P. A. and P. F. Sparling.** 1981. Ability of *Neisseria gonorrhoeae*, *Neisseria meningitidis* and commensal *Neisseria* spp. to obtain iron from transferrin and other compounds. *Infect. Immun.* **33**:555-64.
3. **Cohen, M.S., Britigan, B.E., French, M. and Bean, K.** 1987. Preliminary observations on lactoferrin secretions in human vaginal mucus: Variation during the menstrual cycle, evidence of hormonal regulation, and implications for infection with *Neisseria gonorrhoeae*. *Am. J. Obstet. Gynecol.* **157**: 1122-25.

Identification and characterization of a gene in *Neisseria meningitidis* and *Neisseria gonorrhoeae* that enables an *Escherichia coli* TonB mutant to grow with ferrichrome A as the sole iron source

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Introduction: *Neisseria meningitidis* and *N. gonorrhoeae* are capable of synthesizing a unique set of proteins when confronted with an iron-limited environment. Several of these iron-regulated proteins have been characterized and one, the transferrin receptor (TBP1), has been shown to have a TonB box (2). The TonB protein couples cytoplasmic membrane electrochemical potential to active transport of iron-siderophore complexes and other substrates via high-affinity outer-membrane receptors in Gram-negative bacteria. It was postulated that the presence of a TonB box on an outer membrane receptor may indicate that a TonB homologue is present in the pathogenic *Neisseria* species.

Experimental Design: Dipeptide repeats of the TonB protein were amplified by PCR from the following organisms: *Escherichia coli* (7), *Salmonella typhimurium* (6), and *Serratia marcescens* (4). The PCR products were nick translated and hybridized to Southern blots of DNA from *N. meningitidis* (strain 80084313, serogroup A) digested with various restriction endonucleases. All three labelled products hybridized to a 1.6 Kb *Sau3A* fragment. Purified *N. meningitidis* DNA was digested with *Sau3A* and size fractionated to obtain fragments between 1.5 Kb and 1.7 Kb. The size fractionated DNA was ligated into pACYC177, pgB2 and pUC18 and transformed into *E. coli* DH5". DNA from the transformants was pooled and transformed into *E. coli* strain AB001 (TonB⁻). Transformants were screened for growth on NBD plates (2) that contained 3: mferrichrome A as the sole source of iron. A single positive transformant was obtained with the pUC18 vector (clone SBL 149). Additional sequence data upstream of SBL 149 was obtained by using SSP-PCR (8). All sequencing was done directly on PCR products using the 373 Automated DNA sequencing system (Applied Biosystems).

Results: Clone SBL 149 was sequenced directly as a PCR product in both directions and found to be 1702 bp in length. Sequence analysis and searches were done by using Genetics Computer Group Software (1992, version 7.2, Madison, Wisconsin). A FASTA search revealed that there were sequences highly homologous

to ATP-binding proteins at the left end of the clone. At the left *Sau*3A junction of clone SBL 149 there was a 243 amino acid open reading frame designated *nucB* which exhibited homology to the *E. coli* ATP-binding proteins HisP (62% identity) MalK (60% identity), PstB (59% identity), and CysA (63% identity). The DNA sequence predicted a hydrophilic protein of 27.1 Kd. A strong inverted repeat was located 2bp downstream from the stop codon and the promoter was devoid of a Fur box or conventional -10 or -35 sequences. The same gene was found to be present in *N. gonorrhoeae* (strain F62) with minor changes in the wobble position.

To investigate the possibility that *nucB* was part of an operon, sequences 5' to *nucB* were determined in both *N. meningitidis* and *N. gonorrhoeae*. A 220 amino acid open reading frame was found to be present upstream of *nucB* in both pathogenic *Neisseria* species (designated RMT). A FASTA analysis showed homology to the following RNA methyltransferases: *ksgA* from *E. coli* (60.5% identity), *ermD* from *Bacillus licheniformis*, *ermC* from *Staphylococcus aureus* and the methylase structural gene on plasmid pAM77 from *Streptococcus sanguis*.

Conclusions : Bacterial periplasmic transport systems are involved in the uptake of a variety of substrates and are characterized by a common global organization. Typically they consist of a periplasmic protein which binds the substrate with a high affinity and delivers it to a complex of membrane associated proteins. Usually two very hydrophobic integral membrane proteins and one hydrophilic peripheral protein are involved. These peripheral membrane proteins contain sequences homologous to the ATP-binding motifs found in ATPases.

Here we report the nucleotide sequence of *nucB* and homology of the deduced amino acid sequence with a family of the transport-related ATP-binding proteins. The fact that *nucB* enables a TonB⁻ strain of *E. coli* to grow with Fe³⁺ ferrichrome as the sole source of iron suggests that this gene may be involved in the uptake of iron by the pathogenic *Neisseria* species. The RNA methyltransferase is also of interest as several investigators observed that unique methylated tRNAs were synthesized by *E. coli* (5) and *N. meningitidis* (1) under iron-restricted conditions. Ongoing experiments will attempt to characterize *nucB* and the potential RNA methylase further and define their role in the uptake of iron by the pathogenic *Neisseria* species.

References

1. **Ala'Aldeen, D.A., Borriello, S.P. and E. Griffiths.** 1993. Alteration of transfer ribonucleic acid of *Neisseria meningitidis* during growth in the presence of human transferrin. *J. Med. Microbiol.* **38**: 442-448.
2. **Cornelissen, C.N., Biswas, G.D., Tsai, J., Parachuri, D.K. Thompson, S.A. and P.F. Sparling.** 1992. Gonococcal transferrin-binding protein 1 is required for transferrin utilization and is homologous to TonB⁻ dependent

- outer membrane receptors. J. Bacteriol. **174**: 5788-5797.
3. **Eick-Helmerich, K. and V. Braun.** 1989. Import of biopolymers into *Escherichia coli*: Nucleotide sequences of the *exbB* and *exbD* genes are homologous to those of the *tolQ* and *tolR* Genes, respectively. J. Bacteriol. **171**: 5117-5126.
 4. **Gaisser, S. and V. Braun.** 1991. The *tonB* gene of *Serratia marcescens*; sequence, activity and partial complementation of a *Escherichia coli tonB* mutant. Mol. Microbiol. **5**: 2777-2787.
 5. **Griffiths, E. and J. Humphreys.** 1978. Alterations in tRNA's containing 2-methylthio-N⁶- ()²- isopentenyl)-adenosine during growth of enteropathogenic *Escherichia coli* in the presence of iron-binding proteins. Eur. J. Biochem. **82**: 503-513.
 6. **Hannavy, K., Barr, G.C., Dorman, C.J., Adamson, L.R., Mazengera, L.R., Gallagher, M.P., Evans, J.S., Levine, B.A., Trayer, I.P. and C.F. Higgins.** 1990. TonB Protein of *Salmonella typhimurium*. J. Mol. Biol. **216**: 897-910.
 7. **Postle, K. and R.F. Good.** 1983. DNA sequence of the *Escherichia coli tonB* gene. Proc. Natl. Acad. Sci. USA **80**: 5235-5239.
 8. **Shyamala, V. and G. Ferro-Luzzi Ames.** 1987. Genome walking by single specific-primer polymerase chain reaction, SSP-PCR. Gene. **84**: 1-8.

Environmentally-regulated, periplasmically-located [Cu, Zn]-superoxide dismutase from *N. meningitidis*: Another virulence factor?

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Nearly all bacterial superoxide dismutases (SODs) are Fe- or Mn-containing cytosolic enzymes, necessary for the removal of toxic oxygen free radicals produced during secondary metabolism. We have recently discovered a family of unusual, Cu, Zn-containing, bacterial SODs in respiratory tract colonists and have now found the enzyme in the major human pathogen, *Neisseria meningitidis*.

In non-denaturing SOD gels, whole cell extracts of plate-grown *N. meningitidis* exhibit bands of SOD activity, identified by their sensitivity to H₂O₂ as isoforms of Fe-SOD. No other SOD can be detected. However, following our discovery of inactive [Cu, Zn]-SOD in *Haemophilus influenzae* type b (1), we used degenerate PCR primers designed to amplify genes for [Cu, Zn]-SOD (*sodC*) to examine the meningococcus. We isolated a partial *sodC* from a Group B strain of *N. meningitidis*, which we have now cloned in its entirety. Southern blotting indicates that the gene is also present in Group A and Group C meningococci. The cloned Group B meningococcal *sodC* encodes active SOD when expressed in shaking cultures of *E. coli*. A fusion of the 5'-end of *sodC* to a gene encoding a promoterless, leader-peptide deficient, β -lactamase gene was constructed and expressed in *E. coli*. The resultant strain produced functional β -lactamase, indicating that the fusion protein was exported to the periplasm, and thus providing strong though indirect evidence that meningococcal [Cu, Zn]-SOD is periplasmic.

While [Cu, Zn]-SOD activity could not be detected in extracts of plate-grown *N. meningitidis*, abundant active enzyme was made in shaking liquid culture. This strongly suggests that *sodC* is environmentally regulated, with aerobiosis a strong candidate for the environmental stimulus for gene expression. We speculate that an environmentally-regulated periplasmic SOD, made by an organism exposed intermittently to high levels of oxidative stress (for example, on encountering phagocytic cells in the course of invasive infection), may play a part in enhancing bacterial survival and so act as a determinant of meningococcal virulence.

References

1. **Kroll, J. S., P. R. Langford and B. M. Loynds.** 1991. Copper-zinc superoxide dismutase of *Haemophilus influenzae* and *H. parainfluenzae*. *J. Bacteriol.* **173**: 7449-7457.

Environmental control of gonococcal sialyltransferase activity, LOS biosynthesis, sialylation and sensitivity to serum killing

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When grown in the presence of cytidine 5' monophospho-N-acetylneuraminic acid (CMP-NANA, or CMP-sialic acid), gonococci (gc) covalently coat themselves with sialic acid. In so doing, they become gradually more resistant to killing by normal and immune human serum. Gonococci possess a membrane-associated enzyme activity, sialyltransferase (stase), that transfers sialic acid from CMP-NANA to the terminal galactose of gonococcal lipooligosaccharide (LOS). We have previously observed that: 1) gc grown anaerobically express about 4 times more stase activity than do those grown aerobically; 2) that anaerobic gc have altered (more easily sialylated) LOS, and; 3) that, regardless of the presence of CMP-NANA, anaerobic gc are less sensitive to killing by normal human serum than are aerobic gc. In the presence of limiting concentrations of CMP-NANA, anaerobic gc are substantially more resistant to serum killing than are aerobic gc. We have expanded on these observations by investigating how additional environmental stimuli, i.e., those expected to be found *in vivo* during infection and disease, affect gonococcal sialyltransferase activity, LOS phenotype, DNA supercoiling and serum sensitivity.

Our initial investigations indicated that negative supercoiling of the gonococcal 24 kb cryptic plasmid increased under strict anaerobic conditions. Since stase activity also increased under these conditions, we set out to determine whether DNA supercoiling and stase activity co-varied under other conditions. We first compared gc grown on plates and in broth to log phase. Broth-grown gc expressed about twice as much stase activity, and had more negatively supercoiled plasmid DNA than did gc grown on plates. Thus, DNA supercoiling and stase activity again changed in parallel.

In another series of experiments, gonococcal stase activity decreased incrementally with decreasing growth medium osmolarity, from 0.17 to 0.025 M NaCl; stase activity did not increase with increasing osmolarity (> 0.17 M). Interestingly, whereas stase activity was dramatically affected by changes in osmolarity of the growth medium, no significant effect on plasmid supercoiling was observed. Thus, these experiments indicated that there was no correlation between plasmid DNA supercoiling and increased stase activity. Finally in this regard, whereas we have observed that anaerobic conditions alter LOS biosynthesis, no obvious differences in

LOS phenotype were observed under the various osmotic conditions. Thus, stase activity and LOS biosynthesis appear to be controlled by overlapping, but separate regulatory systems. In all cases, as stase activity decreased, the ability of gc to utilize CMP-NANA, and to gain serum resistance also decreased. We are presently investigating the role of other osmolites and beginning to measure chromosomal DNA supercoiling.

Similar to those affects we saw with NaCl, as iron concentration in the growth medium decreased from the "normal" concentration of 50 : M, gonococcal stase activity decreased; in the absence of added iron, with added desferrioxamine (an iron chelator), stase activity was approximately 5-fold lower than with 50 : M iron. We are presently investigating the effects of physiologic iron sources (lactoferrin and transferrin), and growth medium pH on regulation of stase activity, LOS biosynthesis and serum resistance.

At this early stage in the investigations, it is certainly not clear how, or even if, our observations bear on gonococcal virulence. They do, however, indicate an incredibly intricate environmental control of suspected gonococcal virulence factors, specifically stase activity and its consequent activities. Future research should be very exciting and rich with discoveries.

Novel gene organization for *carA* and *carB* in *Neisseria gonorrhoeae*

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Carbamoylphosphate synthetase (CPS) catalyses the formation of carbamoylphosphate from CO₂, ATP, and ammonia; the first committed step in the arginine and pyrimidine biosynthetic pathways. Initial interest in the CPS enzyme of *Neisseria gonorrhoeae* stemmed from the natural occurrence of CPS deficiencies in 10-20% of clinical isolates (5, Dillon *et al.*, unpublished data). This property, causing a concurrent citrulline and uracil auxotrophy, is employed in a typing scheme (1). Prokaryotic CPS is a heterodimer, consisting of two subunits encoded by genes commonly called *carA* and *carB*. In *Escherichia coli*, *Salmonella typhimurium* and *Pseudomonas stutzeri* these genes are separated by 17, 18 and 24 bp respectively, and are co-transcribed (4,6,7). Recently, it was reported that *Pseudomonas aeruginosa carA* and *carB* are separated by 682 bp, though they are still co-transcribed (6). We now report the complete sequence of the *carA* and *carB* genes of *N. gonorrhoeae* CH811.

CarA (1125 bp) and *carB* (3237 bp) are comparable sizes with other prokaryotic CPS genes, and deduced protein sequences from these genes indicate the gonococcal CPS is similar in function to other CPS's. However, these *carA* and *carB* genes in strain CH811 are separated by 3287 bp and putative transcription terminators are found downstream of both genes, suggesting they may be separately transcribed. Prokaryotic CPS gene organization, therefore, seems more variable than previously assumed. The sequence between the gonococcal CPS genes has also been investigated in other strains of *N. gonorrhoeae*, through use of the polymerase chain reaction. We report that the intervening sequence varies in size from approximately 2.2 to 3.7 kb. This variation occurs both between gonococcal isolates of different auxotype/serotype/plasmid class, and even within some classes, a property which may be useful in a molecular-based typing scheme. Though this intervening sequence is variable, PCR amplification of the *carA* and *carB* genes from different isolates with functional CPS shows the genes themselves do not vary in size. Previously characterized repeats commonly found around gonococcal *pil* genes (3) are found upstream of *carA* as well as a 152 bp repeat first reported by Correia *et al.* (2). Two novel 120 and 150 bp repeats, which we have named RS6 and RS7, are found in the intervening sequence between *carA* and *carB*. These RS6 and RS7 sequences contain GC rich inverted repeats, and are flanked by gonococcal uptake sequences.

These repeats are likely facilitators of the variation in sequence seen in this region. This may in part explain why deficiencies in CPS are frequently found in *Neisseria gonorrhoeae*.

References

1. **Carifo K. and B.W. Catlin.** 1973. *Neisseria gonorrhoeae* auxotyping: differentiation of clinical isolates based on growth responses on chemically defined media. *Appl. Microbiol.* **26**: 233-230.
2. **Correia F.F., S. Inouye and M. Inouye.** 1988. A family of small repeated elements with some transposon-like properties in the genome of *Neisseria gonorrhoeae*. *J. Biol. Chem.* **263**: 12194-12198.
3. **Haas R. and T.F. Meyer.** 1986. The repertoire of silent pilus genes in *Neisseria gonorrhoeae*: evidence for gene conversion. *Cell.* **44**: 107-115.
4. **Kilstrup M., C. Lu, A.T. Abdelal and J. Neuhard.** 1988. Nucleotide sequence of the *carA* gene and regulation of the *carAB* operon in *Salmonella typhimurium*. *Eur. J. Biochem.* **176**: 421-429.
5. **Li, H.** 1994. M.Sc. thesis. University of Ottawa, Ottawa, Canada.
6. **Kwon D.-H., C.-D. Lu, D.A. Walthall, T.M. Brown, J.E. Houghton and A.T. Abdelal.** 1994. Structure and regulation of the *carAB* operon in *Pseudomonas aeruginosa* and *Pseudomonas stutzeri*: no untranslated region exists. *J. Bacteriol.* **176**: 2532-2542.
7. **Piette J., H. Nyunoya, C.J. Lusty, R. Cunin, G. Weyens, M. Crabeel, D. Charlier, N. Glansdorff and A. Piérard.** 1984. DNA sequence of the *carA* gene and the control region of *carAB*: Tandem promoters, respectively controlled by arginine and the pyrimidines, regulate the synthesis of carbamoyl-phosphate synthetase in *Escherichia coli* K12. *Proc. Natl. Acad. Sci. USA.* **81**: 4134-4138.

Cloning and sequencing of the meningococcal polyphosphate kinase gene: Production of mutants in polyphosphate synthesis

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Polyphosphate is a long-chain polymer of phosphate residues. It is synthesized, in bacteria, from ATP by the enzyme polyphosphate kinase (PPK). It has been suggested that polyphosphate in microorganisms might serve as an energy or as a phosphate store, as a substitute for ATP in some reactions, as a buffer for the maintenance of constant intracellular phosphate concentration or as a component of a hydrophilic membrane pore involved in DNA uptake. The purification and partial amino acid sequence analysis of PPK from *N. meningitidis* strain BNCV has been described previously (6). This report describes the cloning of the *ppk* gene and the production of mutants lacking polyphosphate kinase in order to investigate the function of polyphosphate in the pathogenic *Neisseria*.

Oligonucleotide probes, based on the partial amino acid sequence of the purified PPK, were used to screen a library of meningococcal DNA in λ gt11 (3). The sequence of the 5' portion of the *ppk* gene was determined from one λ gt11 clone, subcloned into the vector pBluescript. Its 3' end was sequenced from a restriction fragment of the chromosomal DNA cloned into an M13mp vector. The *ppk* gene was an open reading frame of length 2047 bp, coding for a protein of predicted size 77 kDa, and followed by a terminator structure consisting of an inverted repeat of the neisserial uptake sequence (1,2).

Southern blotting of chromosomal DNA from two meningococcal, three gonococcal and one *N. flava* strain demonstrated a reactive 6.6 kbp *Eco*RI fragment in the gonococci and meningococci and a 4 kbp band in the commensal species, suggesting that the *ppk* gene is very similar in the pathogenic *Neisseria* species. No reactive fragment was seen in two strains of *E. coli* and one of *H. influenzae* which were tested.

The *ppk* open reading frame in the Bluescript vector was interrupted at a unique *Sfu*I site with a selectable kanamycin resistance marker, and was used to transform gonococcus strains F62 and MS11 and meningococcus strain M1080 to kanamycin resistance. The success of the transformations was further evidence for the similarity of the genes.

The mutants lacked PPK activity and did not produce polyphosphate. The growth of the *ppk* mutants is somewhat slower than that of the parental strains, and survival after the cessation of growth in liquid media is diminished, especially in the case of the gonococcus. Suggested functions of polyphosphate include scavenging of metal ions (4) and uptake of transforming DNA (5). The ability to grow in conditions of low phosphate or of iron deprivation was not affected, nor was the efficiency of transformation by neisserial DNA affected.

References

1. **Goodman, S.D. and J.J. Scocca.** 1988. Identification and arrangement of the DNA sequence recognized in specific transformation of *Neisseria gonorrhoeae*. Proc.Natl.Acad.Sci.USA **85**: 6982-6986.
2. **Goodman, S.D. and J.J. Scocca.** 1991. Factors influencing the specific interaction of *Neisseria gonorrhoeae* with transforming DNA. J.Bacteriol. **173**: 5921-5923.
3. **Murakami, K., E.C. Gotschlich and M.E. Seiff.** 1989. Cloning and characterization of the structural gene for the class 2 protein of *Neisseria meningitidis*. Infect.Immun. **57**: 2318-2323.
4. **Noegel, A. and E.C. Gotschlich.** 1983. Isolation of a high molecular weight polyphosphate from *Neisseria gonorrhoeae*. J.Exp.Med. **157**: 2049-2060.
5. **Reusch, R.N. and H.L. Sadoff.** 1988. Putative structure and functions of a poly- γ -hydroxybutyrate/calcium polyphosphate channel in bacterial plasma membranes. Proc.Natl.Acad.Sci.USA. **85**: 4176-4180.
6. **Tinsley, C.R., B.N. Manjula and E.C. Gotschlich.** 1993. Purification and characterization of polyphosphate kinase from *Neisseria meningitidis*. Infect.Immun. **61**: 3703-3710.

Novel lipoprotein found in meningococci

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The *ppk* gene, coding for the enzyme polyphosphate kinase (Tinsley and Gotschlich, unpublished) is preceded by an open reading frame coding for a protein of predicted size 19 kDa, with a typical lipoprotein signal sequence of 21 amino acids. The protein has significant homology to the N-terminal portion of an outer membrane protein from *Haemophilus somnus* (2). This region of the chromosomes of several strains of *N. meningitidis* and *N. gonorrhoeae* was found to be very similar by Southern blotting.

Using PCR technology the meningococcal gene from strain BNCV was cloned into a pGEX expression vector, producing a GST-hybrid protein (Pharmacia). The protein was purified by glutathione-affinity chromatography, was enzymatically cleaved to liberate the neisserial protein and was used to prepare rabbit antisera. The rabbit antisera did not have significant bactericidal activity. Western blots demonstrated that the protein was present in the membrane fraction of meningococci, but not in gonococci. This was surprising in view of the apparent similarity of this region of the chromosome between the species.

In order to try to explain this discrepancy, sequencing of the gene from several strains of *Neisseria* was performed, directly on DNA fragments produced by PCR from chromosomal DNA (1). The strains used, meningococcus strains BNCV and M1080 and gonococcus strains MS11, UU1, R10 and F62 were isolates from different times and places in the USA. The meningococcal sequences were identical. The gonococcal sequences were similar to those of the meningococci. However, the insertion of one base pair at position 73 of the gene results in a frameshift relative to the meningococcal reading frame, and the open reading frames of all the gonococcal sequences terminate at the same point approximately 100 bp later.

The function of the neisserial protein is as yet open to speculation. The amino-terminal domain of the antigen from *Haemophilus somnus* has been implicated in the haemolytic activity of this protein, but the authors note the possibility that the observed property of the antigen may be artifactual. The possibility that the neisserial protein is in some way linked to polyphosphate metabolism or transport is presently being addressed. The disparity of the single base pair insertion in the gene, and consequent absence of the protein from *N. gonorrhoeae*, in the face of the conservation of the

remainder of the coding sequence deserves further investigation.

References

1. **Hultman, T., S. Stahl, E. Hornes and M. Uhlen.** 1989. Direct solid phase sequencing of genomic and plasmid DNA using magnetic beads as solid support. *Nuc.Acids Res.* **17**: 4937-4946.
2. **Won, J. and R.W. Griffith.** 1993. Cloning and sequencing of the gene encoding a 31-kilodalton antigen of *Haemophilus somnus*. *Infect.Immun.* **61**: 2813-2821.

Characterization of the Phosphoglucose isomerases of *Neisseria gonorrhoeae* and *Neisseria meningitidis*

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Phosphoglucose isomerase (PGI) is a dimeric enzyme that catalyzes the reversible isomerization of glucose-6-phosphate and fructose-6-phosphate in glycolysis (2). In the course of studies on gonococcal pilus assembly genes, immediately upstream of *pilG* (5) we located another gene. DNA sequence analysis showed that this gene consisted of an open reading frame of 1647 bp that was read in the opposite direction of *pilG*. The deduced amino acid sequence of the gonococcal gene was found to be highly homologous to the gene for PGI (*pgi*) in *E. coli*. *In vitro* transcription-translation of the *pgi*-containing plasmid DNA predicted the PGI homologue in *N. gonorrhoeae* to be a protein of approximately 61 kD.

Transposon mutagenesis of the plasmid containing the *Neisseria gonorrhoeae pgi* was performed (3), and the linearized plasmid was transformed into wildtype gonococcal and meningococcal strains. The mutants displayed no growth defect on standard gonococcal medium. In *Escherichia coli* there is a single *pgi* gene and PGI appears to be dispensable because strains lacking it are also able to grow on glucose minimal media, apparently utilizing glucose primarily by the pentose phosphate shunt (1,7).

PGI enzyme activity of *E. coli* with and without *pgi*-containing plasmids and of gonococcal and meningococcal wild-type and *pgi*-mutant transformants were assayed by starch gel electrophoresis and isoenzyme analysis (6). Wildtype *E. coli* displayed one band, while *E. coli* with the *pgi*-containing plasmid revealed an additional strong band. Gonococcal and meningococcal wildtype strains expressed two bands, but the *pgi*-mutant transformants lacked the lower and weaker band. This finding suggested the existence of two distinct isoforms of PGI in *N. gonorrhoeae* and *N. meningitidis*. Indeed, Southern blot analysis of *N. meningitidis* and *N. gonorrhoeae* chromosomal DNAs with the PCR product of the gonococcal *pgi* as a hybridization probe indicated that there are two *pgi* genes in the genomes of pathogenic *Neisseria*.

Electron microscopy of *N. meningitidis* and *N. gonorrhoeae* wildtype and *pgi*- and *pilG*-mutants demonstrated abundant piliation in the wildtype and *pgi*-mutants, while

the *pilG*-mutant was non-piliated, indicating that PGI activity of this *pgi* gene product is not essential for the expression of the piliated state. Further characterization of both *pgi* gene products may contribute to an understanding of their relative roles in glycolytic metabolism and other potentially related processes.

References

1. **Fraenkl, D.G. and S.R. Levisohn.** 1967. Glucose and gluconate metabolism in an *Escherichia coli* mutant lacking phosphoglucose isomerase. *J. Bacteriol.* **93**: 1571-1578.
2. **Froman, B.E., R.C. Tait and L.D. Gottlieb.** 1989. Isolation and characterization of the phosphoglucose isomerase gene from *Escherichia coli*. *Mol. Gen. Genet.* **217**: 126-131.
3. **Seifert, H.S., E.Y. Chen, M. So and F. Heffron.** 1986. Shuttle mutagenesis: A method for transposon mutagenesis for *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA.* **83**: 735-739.
4. **Thomson, J., P.D. Gerstenberger, D.E. Goldberg, E. Gociar, A.O. deSilva and D. G. Fraenkl.** 1979. ColE1 hybrid plasmids for *Escherichia coli* genes of glycolysis and the hexose monophosphate shunt. *J. Bacteriol.* **137**: 502-506.
5. **Tønjum, T., N.E. Freitag, E. Namork and M. Koomey.** Characterization of the PilG protein of *Neisseria gonorrhoeae* and *Neisseria meningitidis* and its role in pilus biogenesis. (Manuscript in preparation)
6. **Selander, R.K., D.A. Caugant, H. Ochman, J.M. Musser, M.N. Gilmour and T.S. Whittam.** 1986. Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics. *Appl. Environ. Microbiol.* **51**: 873-884.
7. **Vinopal, R.T., J.D. Hillman, H. Schulman, W.S. Reznikoff and D.G. Fraenkl.** 1975. New phosphoglucose isomerase mutants of *Escherichia coli*. *J. Bacteriol.* **122**: 1172-1174.

44,000 dalton proteins of *Neisseria gonorrhoeae*

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A variety of observations have been made regarding proteins of *Neisseria gonorrhoeae* having molecular masses in the range of 44,000 daltons (44kDa). These include surface-exposure, based on immunoprecipitation (7), susceptibility to *in situ* enzymatic cleavage (5) and surface-radioiodination (4), reactivity with patient serum (8), lipidation and association with the multiple transferrable resistance phenotype (MTR⁺) (1), presence in shock extracts of the gonococcal periplasm (3), association with peptidoglycan (2) and penicillin-binding (6). To sort out these many observations we used various immunological reagents to identify clones expressing gonococcal 44kDa genes.

We have identified metabolic proteins, elongation factor Tu (EFTu; data presented in an accompanying abstract by Porcella, *et al.*) and what appears to be the MTR⁺-associated protein (Hageman, *et al.*) all having molecular masses of about 44kDa. Two-dimensional isoelectric focusing SDS-PAGE revealed as many as thirty distinct proteins having a molecular masses between 43kDa and 45kDa, making this the most common protein size in the gonococcus. The most prevalent gonococcal protein (believed to be EFTu) had a molecular mass of 44kDa. At least eight 44kDa proteins reacted with antisera generated against proteins isolated from purified outer membranes (1). Six of twenty dominant surface-radiolabeled proteins resided in this molecular weight range as well. The large number of proteins in the 44kDa region supports the view that the manifold characteristics previously ascribed to a single "44kDa" protein actually reflect the contributions of many different 44kDa proteins. Efforts are underway to identify and characterize other 44kDa proteins that may be important to the survival of this canny pathogen.

References

1. **Chen, C.Y., C.S. Parsons and S.A. Morse.** 1985. Membrane proteolipids of *Neisseria gonorrhoeae*, pp. 360-365, In: Schoolnik, G.K., (ed.), *The Pathogenic Neisseriae*, American Society for Microbiology, Washington, D.C.
2. **Hill, S.A. and R.C. Judd.** 1989. Identification and characterization of peptidoglycan-associated proteins in *Neisseria gonorrhoeae*. *Infect. Immun.* **57**: 3612-3618
3. **Judd, R.C. and S.F. Porcella.** 1993. Isolation of the periplasm of *Neisseria*

gonorrhoeae. Mol. Microbiol. **10**: 567-574

4. **Judd, R.C., J.C. Strange, R.K. Pettit and W.M. Shafer.** 1991. Identification and characterization of a conserved outer-membrane protein of *Neisseria gonorrhoeae*. Mol. Microbiol. **5**: 1091-1096
5. **Shafer, W.M.** 1988. Lipopolysaccharide masking of gonococcal outer-membrane proteins modulates binding of bactericidal cathepsin G to gonococci. J. Gen Microbiol. **134**: 539-545
6. **Shafer, W.M. and R.C. Judd.** 1991. Gonococcal penicillin-binding protein 3 and the surface-exposed 44kDa peptidoglycan-binding protein appear to be the same molecule. Mol. Microbiol. **5**: 1097-1103
7. **Swanson, J.** 1981. Surface-exposed protein antigens of the gonococcal outer membrane. Infect. Immun. **34**: 804-816
8. **Zak, K., J.L. Diaz, D. Jackson and J.E. Heckels.** 1984. Antigenic variation during infection with *Neisseria gonorrhoeae*: detection of antibodies to surface proteins in sera of patients with gonorrhea. J. Infect. Dis. **149**: 166-174

Genetic characterization of the *str* and *sucAB-lpd* operons of *Neisseria gonorrhoeae*

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Antibodies to two 44,000 dalton (44kDa) proteins (one periplasmic, the other non-periplasmic) were used to screen a genomic library of *Neisseria gonorrhoeae* in order to locate the genes coding for the 44kDa protein. The *tufA* gene of the gonococcal *str* operon and the *sucB* and *lpd* genes of the gonococcal *sucAB-lpd* operon were thus cloned and sequenced.

These genes code for proteins that have a potential molecular mass in the range of 44kDa. In *E. coli*, *tufA* codes for elongation factor-TuA (EFTuA) which is found in periplasmic shock extracts. EFTuA plays a major role in protein synthesis and may be intricately involved in nutrient sensing and growth regulation of the bacterium. *sucB* codes for dihydrolipoamide succinyltransferase (SucB) and forms the central core region of the 2-oxoglutarate dehydrogenase complex. SucB contains an inverted AAEAP repeat motif, similar to that found in H8-antigen (Lip), and a lipoyl group which may explain previous lipidation observations related to the 44kDa protein. *lpd* codes for lipoamide dehydrogenase; a ubiquitous enzyme able to interact equally well with two different 2-oxo acid dehydrogenase complexes and thought to be involved with membrane-bound ribosomes and protein binding-dependent transport processes.

The *lpd* gene in the cloned fragment was truncated by a gonococcal compound transposable-like element (1) and lacked the coding region for the carboxy-terminal portion of the protein involved in E1_o and E2_o subunit binding and interaction. Genetic deletion experiments, employing the gonococcal Tn shuttle mutagenesis protocol developed by Seifert, were performed in an effort to delete the genes and thereby assign function to the resultant mutant phenotypes. An apparent duplication of the *tuf* gene, or the essential nature of the *tufA* gene product, precluded deletion of this gene. The *lpd* gene was shown to exist in three separate copies, thwarting efforts to inactivate the gene. Deletion of the *sucB* gene was successful and efforts to understand this interesting mutant are under way.

References

1. **Correia, F.F., S. Inouye, and M. Inouye.** 1988. A family of small repeated elements with some transposon-like properties in the genome of *Neisseria gonorrhoeae*. J. Biol. Chem. **263**: 12194-12198.

Genetics of gonococcal resistance to toxic hydrophobic antimicrobial agents

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The gonococcus often infects sites rich in toxic fatty acids and/or bile salts. These hydrophobic agents (HA) display potent antimicrobial action against numerous Gram-negative bacteria, including *N. gonorrhoeae*. Thus, successful infection of mucosal sites rich in HAs requires that the invading gonococcus resist their lethal action. The capacity of gonococci to resist the antibacterial action of HAs was described in the early 1970's (4) but the molecular genetics of resistance was only recently described (7). The *mtr* (multiple transferable resistance) system of resistance, which imparts high level resistance to hydrophobic drugs, dyes and detergents, has been suggested to be a major mechanism employed by gonococci to resist structurally diverse HA's. This system is expressed by approximately 12.5% of all clinical isolates (5) and HA^R strains are frequently isolated from the rectum, presumably because of the strong selective pressure exerted by faecal fatty acids.

The *mtr* system is composed of four closely linked genes, which are the subject of this report. The *mtrR* gene encodes a transcriptional regulator that resembles numerous repressors of transcription (notably the family of tetracycline repressors [1]) and at least one transcriptional activator, the LuxR protein of *Vibrio harveyi* (10). The MtrR protein possessed by gonococci is analogous to the AcrR and EnvR proteins possessed by *E. coli* that are likely to regulate expression of the *acrAE* (3) and *envCD* (2) operons; these operons are important in mediating resistance of *E. coli* to antimicrobial compounds. In this study we found that two distinct missense mutations, one of which alters the putative DNA-binding domain of the MtrR protein, or null mutations in the *mtrR* gene resulted in intermediate levels of HA^R. However, high level HA^R, such as that expressed by clinical isolates, was correlated with a single bp deletion in a 13 bp inverted repeat that was positioned upstream of the translational start codon of the *mtrR* gene.

A three gene complex (*mtrCDE*) was found downstream and divergent from the *mtrR* gene. These genes encode membrane proteins resembling the MexABOprK membrane proteins of *P. aeruginosa* (8). These proteins regulate levels of bacterial resistance to structurally diverse antimicrobial agents, including HA's, by an

energy-dependent efflux system (9). The MexABOprK proteins are thought to form a continuous channel through the cell envelope that permits efflux of hydrophobic compounds that either damage the integrity of the cell envelope or attack cytoplasmic components (6). The importance of the *mtrCDE* genes in mediating resistance of gonococci to HAs was determined by insertional inactivation of the first gene (*mtrC*) in the operon-like gene complex. Loss of the 44 kDa MtrC membrane lipoprotein resulted in hypersusceptibility of gonococci to a panel of hydrophobic compounds, including crystal violet, erythromycin and Triton X-100. Moreover, overproduction of MtrC due to mutations in the *mtrR* gene resulted in increased resistance of gonococci to these agents.

The *mtrRCDE* gene complex in gonococci represents a hybrid of the *acrRAE/envRCD* operons in *E. coli* and the *mexABOprK* operon of *P. aeruginosa*. By combining homologues of these structural and regulatory genes, gonococci appear to have the unique capacity to modulate levels of resistance to HAs by varying the amount of efflux pumps that serve to actively export toxic agents.

References

1. **Brow, M.A.D., R. Pesin and J.G. Sutcliffe.** 1985. The tetracycline repressor of pS101. *Mol. Biol. Evol.* **2**: 1-12.
2. **Klein, J.R., B.H. Henrich, and R. Plapp.** 1991. Molecular analysis and nucleotide sequence of the *envCD* operon of *Escherichia coli*. *Mol. Gen. Genet.* **230**: 230-240.
3. **Ma, D., D.N. Cook, M. Alberti, N.G. Pon, H. Nikaido and J.E. Hearst.** 1993. Molecular cloning and characterization of *acrA* and *acrE* genes of *Escherichia coli*. *J. Bacteriol.* **175**: 6299-6313.
4. **Maness, M.J., and P.F. Sparling.** 1973. Multiple antibiotic resistance due to a single mutation in *Neisseria gonorrhoeae*. *J. Infect. Dis.* **128**: 321-330.
5. **Morse, S.A., P.G. Lysko, L. McFarland, J.S. Knapp, E. Sandstrom, C. Critchlow and K.K. Holmes.** 1982. Gonococcal strains from homosexual men have outer membranes with reduced permeability to hydrophobic molecules. *Infect. Immun.* **37**: 432-438.
6. **Nikaido, H.** 1994. Prevention of drug access to bacterial targets: permeability barriers and active efflux. *Science* **264**: 382-388.
7. **Pan, W. and B.G. Spratt.** 1994. Regulation of the permeability of the gonococcal cell envelope by the *mtr* system. *Mol. Microbiol.* **11**:769-775.
8. **Poole, K., D.E. Heinrichs and S. Neshat.** 1993. Cloning and sequence analysis of an EnvCD homologue in *Pseudomonas aeruginosa*: regulation by iron and possible involvement in the secretion of siderophore pyoverdine. *Mol. Microbiol.* **10**:529-544.

9. **Poole, K., K. Krebs, C. McNally and S. Neshat.** 1993. Multiple antibiotic resistance in *Pseudomonas aeruginosa*: evidence for involvement of an efflux operon. *J. Bacteriol.* **175**:7363-7372.
10. **Swartzman, E., M. Silverman and E.A. Meighen.** 1992. The *luxR* gene product of *Vibrio harveyi* is a transcriptional activator of the *lux* promoter. *J. Bacteriol.* **174**:7490-7493.

The effects of co-incubation of *Lactobacillus acidophilus* on *Neisseria gonorrhoeae* catalase activity

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Lactobacillus species are found in the vaginas of most women and are believed to contribute to the control of the vaginal bacterial microflora by several mechanisms including lowering of the vaginal pH and the production of bactericidal compounds such as H₂O₂. In the vagina and endocervix *Neisseria gonorrhoea* must interact with a complex microflora including lactobacilli. It has been reported that lactobacilli (especially strains capable of producing H₂O₂) may inhibit the growth of gonococci (1,2). *Lactobacillus acidophilus* that produced H₂O₂ (LB⁺) and *L.acidophilus* and *L. casei* that did not produce H₂O₂ (LB⁻) were co-incubated with both catalase positive and catalase deficient strains of *N. gonorrhoeae*. When the incubation media was maintained at pH 7.3 neither LB⁺ nor LB⁻ organisms affected gonococcal growth. However, LB⁺ caused a significant increase in expression of gonococcal catalase, which could be offset with exposure of the bacteria to exogenous catalase. At lower co-incubation medium pH (4.8-5.0) there was a significant decrease in gonococcal survival and catalase activity, which was only partially reversed by exogenous catalase. Lysates of LB⁺ were also shown to effectively inhibit gonococcal catalase. This inhibition was retained upon heat treatment but lost by proteinase K treatment of the lysate. The results demonstrate that LB⁺ may inhibit growth of gonococci by acidification of the environment, secretion of H₂O₂, and the production of a protein inhibitor(s).

References

1. **Saigh, J.H., C.C. Sanders and W.F. Sanders.** 1978. Inhibition of *Neisseria gonorrhoeae* by aerobic and facultative anaerobic components of the endocervical flora: evidence for a protective effect against infection. *Infect. Immun.* **19**:704-10.
2. **Morin, A., S.A. Saheb J.G. Bisailon, R. Beudet, and M. Sylvester.** 1980 Effect of culture medium composition on inhibition of growth of *Neisseria gonorrhoeae* by lactobacilli. *Curr. Microbiol.* **4**:283-6.

Use of antibiotics to select auxotrophic mutants of *Neisseria meningitidis*

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The ability to select mutants of defined specificity has been extremely useful in elucidating metabolic pathways in *Escherichia coli*. In 1948, Lederberg and Zinder (5) and Davis (2) reported that because penicillin kills growing bacteria only, treatment of mutagenized *E. coli* with penicillin in a defined medium allowed selective survival of auxotrophs. Attempts to adapt this approach to *N. meningitidis* have generally been unsuccessful (6). We describe our efforts to develop a system for selecting meningococcal auxotrophs, by which we have isolated mutants with altered carbon source utilization and with amino acid requirements.

A simple defined medium modified from one described by Catlin and Schloer (1) was used. We used *N. meningitidis* strain M1080-C (3), derived from a group B strain. In some experiments we used Tn916 mutants of the group B strain NMB (4,7).

We tested several bactericidal antibiotics to identify those which killed meningococci only in media which would support growth. Streptomycin (2 : g/ml) reduced viability by 4.9 log units in 5 h in a medium containing L-lactate. Without the lactate, viability was reduced only by 0.9 log unit in either the presence or absence of streptomycin. Thus at this concentration the drug was specific for growing bacteria. At higher concentrations of streptomycin (25 : g/ml), killing was not reduced by omission of the carbon source. For some drugs, the specificity for growing bacteria was strain-dependent. For cefotaxime we were unable to find a concentration at which killing of strain M1080-C was strictly dependent on the presence of a carbon source. In contrast, two Tn916 mutants of strain NMB (one proline-requiring and the other unable to utilize lactate or pyruvate) were killed by cefotaxime only in a permissive medium. We also tested two quinolones. Nalidixic acid (50 : g/ml) was specific for growing cells of either strain. A second quinolone, ciprofloxacin, killed bacteria only slightly more in the presence of L-lactate than in its absence.

Thus nalidixic acid, streptomycin, and for some strains cefotaxime appeared potentially useful for selecting mutants unable to grow in a specific medium. We tested this by adding a small inoculum of one of the Tn916 mutants mentioned above to a suspension of M1080-C and treating with antibiotics in a medium in which M1080-C, but not the mutants, could grow. Nalidixic acid and cefotaxime, but not

streptomycin, increased the proportion of auxotrophs in the mixture by 1000 and 5000 fold respectively.

We next attempted to select mutants of desired specificity from a population of nitrosoguanidine-mutagenized meningococci of strain M1080-C, using three steps: (1) antibiotic treatment in a medium that was nonpermissive for the desired mutations; (2) overnight growth in a permissive defined medium to reduce the proportion of auxotrophic mutants with other phenotypes than that desired; (3) a second antibiotic treatment, in some cases with a different antibiotic. Survivors were plated on GC agar, and isolated colonies were subcultured onto defined media or indicator media for identification of mutants.

Using these methods, we have isolated mutants with four phenotypes: (1) inability to grow on either L-lactate or pyruvate, (2) failure to accumulate acid during growth on medium containing maltose, (3) proline requirement, and (4) methionine requirement.

One problem is that mutagenesis can result in resistance to the antibiotic being used for selection. A second problem is that meningococci (unlike *E. coli*) do not survive well when they are not growing. In these experiments, viability in nonpermissive media declined ½ to 1½ log units during antibiotic treatment.

References

1. **Catlin, B.W. and G.M. Schloer.** 1962. A defined agar medium for genetic transformation of *Neisseria meningitidis*. *J.Bacteriol.* **83**: 470-474.
2. **Davis, B.D.** 1948. Isolation of biochemically deficient mutants of bacteria by penicillin. *J.Amer.Chem.Soc.* **70**: 4267.
3. **Erwin, A.L. and E.C. Gotschlich.** 1993. Oxidation of D-lactate and L-lactate by *Neisseria meningitidis*: purification and cloning of meningococcal D-lactate dehydrogenase. *J.Bacteriol.* **175**: 6382-6391.
4. **Erwin, A.L. and D.S. Stephens.** Unpublished results.
5. **Lederberg, J. and N. Zinder.** 1948. Concentration of biochemical mutants of bacteria with penicillin. *J.Amer.Chem.Soc.* **70**: 4267-4268.
6. **Lie, S.** 1965. On the effect of penicillin in the production of auxotrophic mutants of *Neisseria meningitidis*. *Acta Path.Microbiol.Scand.* **63**: 609-614.
7. **Stephens, D.S., J.S. Swartley, S. Kathariou, and S.A. Morse.** 1991. Insertion of Tn916 in *Neisseria meningitidis* resulting in loss of group B capsular polysaccharide. *Infect.Immun.* **59**: 4097-4102.

Identification and characterization of auxotrophs of *Neisseria meningitidis* produced by Tn916 mutagenesis

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Relatively little is known about the physiology of *N. meningitidis*. In order to identify auxotrophic mutants, we screened a library of Tn916 transformants of the serogroup B strain NMB (2) for mutations resulting in an inability to grow on a simple defined medium, MCDA, with L-lactate as the principal carbon source. Of 1483 isolates tested, 14 had no growth or substantially reduced growth.

To characterize these mutants further we used two defined media, MCDA and Wong medium (3), as well as GC agar base with or without Isovitalex. MCDA was modified from the medium described by Catlin and Schloer (1). In addition to mineral salts and citrate (2.2 mM), the medium contained four amino acids: arginine (1.4 mM), glycine (1.3 mM), glutamate (1 mM), and cysteine (0.06 mM). L-lactate, pyruvate, or glucose (3.8 mg/ml) were used as carbon sources. Wong medium includes all 20 amino acids and several vitamins and cofactors in addition to several compounds that are potential carbon and energy sources.

The 14 mutants fell into four groups. The first group, eight isolates, had wild-type growth on Wong medium but failed to grow on MCDA-L-lactate unless specific amino acids were added. The amino acids required were histidine (2 isolates); leucine (1 isolate); proline (1 isolate); tryptophan (2 isolates); and phenylalanine, tryptophan and tyrosine (all three amino acids required by 2 isolates).

A second group, with three isolates, had wild-type growth on MCDA-glucose, but reduced or no growth on L-lactate or on pyruvate. Of these, one grew well on GC agar base without Isovitalex, while the other two required the addition of either Isovitalex or glucose (10 mg/ml).

A third group contained two isolates which exhibited better growth on MCDA-pyruvate than on MCDA-L-lactate. This was not due to an inability to metabolize L-lactate, since good growth on L-lactate occurred if the amino acid composition of the medium was modified slightly.

The fourth group consisted of one isolate that grew poorly on Wong medium and

poorly on MCDA regardless of carbon source.

In summary, 14 Tn916 transposon mutants with metabolic abnormalities have been identified. Eight of these are amino acid auxotrophs. The remaining isolates have several distinct phenotypes, but the physiologic basis of their mutations has not been determined. These observations provide further evidence that Tn916 can insert into the meningococcal genome at a variety of sites.

References

1. **Catlin, B.W. and G.M. Schloer.** 1962. A defined agar medium for genetic transformation of *Neisseria meningitidis*. J.Bacteriol. **83**: 470-474.
2. **Stephens, D.S., J.S. Swartley, S. Kathariou and S.A. Morse.** 1991. Insertion of Tn916 in *Neisseria meningitidis* resulting in loss of group B capsular polysaccharide. Infect.Immun. **59**: 4097-4102.
3. **Wong, T.P., R.K. Shockley and K.H. Johnston.** 1980. WSJM, a simple chemically defined medium for growth of *Neisseria gonorrhoeae*. J.Clin.Microbiol. **11**: 363-369.