Cloning and Characterization of the Meningococcal Polyphosphate Kinase Gene: Production of Polyphosphate Synthesis Mutants

C. R. TINSLEY* AND E. C. GOTSCHLICH

Laboratory of Bacterial Pathogenesis and Immunology,
The Rockefeller University, New York, New York

Received 31 October 1994/Accepted 31 January 1995

The pathogenic *Neisseria* species accumulate polyphosphate to levels between 10 and 20% of their total phosphate content. However, the significance of this compound for the growth and pathogenicity of these species is not understood. A previous report (C. R. Tinsley, B. N. Manjula, and E. C. Gotschlich, *Infect. Immun.* 61:3703–3710, 1993) describes the purification of polyphosphate kinase, the enzyme responsible for synthesis of polyphosphate, from *Neisseria meningitidis* BNCV. By use of probes based on the amino acid sequence of the purified enzyme, the structural gene *ppk* has been cloned and sequenced. The coding sequence is 2,055 bp long and codes for a protein of 77.2 kDa. The open reading frame of the cloned gene was interrupted by the insertion of a kanamycin resistance cassette, and *ppk* mutants were obtained in both *Neisseria gonorrhoeae* and *N. meningitidis* by transformation with the recombinant plasmid. Amounts of polyphosphate in the *ppk* mutants were reduced to between 2 and 10% of wild-type levels. The mutants grew less vigorously than wild-type organisms *in vitro* and showed a striking increase in sensitivity to killing by human serum.

Polyphosphate (polyP) is a long-chain polymer of phosphate residues synthesized in bacteria from ATP by the enzyme polyphosphate kinase (PPK). Several possible functions of polyP in microorganisms have been suggested or demonstrated: polyP might serve as an energy or phosphate store (12, 18), as a substitute for ATP in some reactions (27), as a buffer for the maintenance of constant intracellular phosphate concentration (12, 16), or as a component of a hydrophilic membrane pore involved in DNA uptake (28). The pathogenic species *Neisseria meningitidis* and *Neisseria gonorrhoeae* accumulate a large proportion of their phosphate as polyP, but the function of the molecule has not been defined in these bacteria. The polyanionic nature of the polyP has suggested that it may form a protective layer on the exterior of the cells, which constitutes the gonococcal “capsule” observed microscopically (13–15, 34).

To try to elucidate the function of polyP in the pathogenic neisseriae, the enzyme PPK was purified from *N. meningitidis* BNCV (36). Amino acid sequence data have been used to design oligonucleotide probes specific for the structural gene for PPK. This report describes the cloning of the *ppk* gene and the production of mutants in *N. meningitidis* and *N. gonorrhoeae* lacking PPK.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *N. gonorrhoeae* F62 and MS11 were isolated originally by Kellogg et al. (17) and Swanson et al. (33), respectively. *N. meningitidis* M1080 and BNCV (a nonencapsulated variant of M986) have been described (7, 8). Neisseriae were grown in proteose peptone no. 3 (Difco) medium containing 1% IsoVitalEx (GC medium) (2) or on GC agar plates (32). For some experiments, neisseriae were grown in the defined medium described by Wong et al. (39) (Wong medium) or on plates with Wong medium containing 0.7% agarose (Seakem; FMC Bioproducts). All media were adjusted to pH 7.5 before autoclaving or sterile filtering. Plates were incubated at 37°C in a moist atmosphere containing 5% carbon dioxide. Liquid media for the growth of

Neisseria spp. were equilibrated at 37°C, under 5% CO₂, before inoculation with bacteria.

Molecular genetic techniques. Unless noted otherwise, molecular genetic procedures were performed as described by Sambrook et al. (30). Cloning into the mp series of M13 vectors was performed as described by Messing (20). PCR was carried out with the GeneAmp kit from Perkin-Elmer Cetus.

Restriction endonucleases and buffers were obtained from New England Biolabs, with the exception of *Sfi*I, which was from Boehringer Mannheim. Other DNA-modifying enzymes were purchased from Pharmacia.

Gels for separation of DNA restriction fragments consisted of between 0.6 and 1.0% agarose (SeaKem; FMC Bioproducts) in taurine buffer (89 mM Tris, 28.5 mM taurine, 1 mM EDTA). Gels for purification of DNA fragments consisted of between 0.6 and 1.0% low-melting-point agarose (SeaPlaque; FMC Bioproducts) in Tris-acetate-EDTA (TAE) buffer (30).

Oligonucleotide probes for Southern blotting were labelled at their 5' ends with [γ -³²P]ATP by use of T4 polynucleotide kinase. Other probes were produced by random-primed labelling of cloned DNA fragments with [α -³²P]dCTP by use of a kit obtained from Boehringer Mannheim. Southern blotting was performed by capillary transfer of DNA fragments to positively charged nylon membranes (Hybond-N⁺) and hybridization under conditions described by Church and Gilbert (4). Excess binding capacity was blocked by incubating the membranes at 65°C for 1 h in 0.5 M sodium phosphate (pH 7.2)–1 mM EDTA–1% bovine serum albumin–7% sodium dodecyl sulfate (SDS) (4). Hybridization was performed in the buffer described above for 18 h at 65°C (random-primed labelled probes) or at 5°C below the melting temperature (radio-labelled oligonucleotides). Membranes were washed at room temperature once with blocking-hybridization buffer and four times in 40 mM sodium phosphate (pH 7.2)–1 mM EDTA–1% SDS. The membranes were blotted dry and subjected to autoradiography.

In some experiments, detection of specific DNA fragments was achieved by probing with fluorescein-labelled probes. An image was produced on photographic film after reaction with horseradish peroxidase-conjugated anti-fluorescein antibodies and luminol plus H₂O₂ (Enhanced Chemiluminescence kit; Amersham).

DNA sequencing techniques. DNA sequencing was performed with Sequenase II T7 DNA polymerase from United States Biochemical with reagents from the 7-deaza-dGTP sequencing kit and α -³⁵S-dATP (New England Nuclear). Single-stranded DNA was produced for sequencing by utilizing the f origin of replication in pBluescript, coinfecting transformants of *Escherichia coli* XL1-Blue with the defective f1 phage R408 (29). DNA was prepared from the supernatants by polyethylene glycol precipitation of phage particles, phenol extraction, and precipitation with ethanol-sodium acetate. Single-stranded DNA was redissolved in water and stored in 80% ethanol at –20°C.

Single-stranded DNA from M13 vectors (20) was obtained from the supernatants of cultures of *E. coli* JM103 infected in the early exponential phase with the recombinant M13.

Preparation of competent *E. coli*. *E. coli* XL1-Blue was grown at 30°C with good aeration in 100 ml of medium (pH 7.2) containing 2% Bacto Tryptone, 1%

* Corresponding author. Mailing address: Laboratory of Bacterial Pathogenesis and Immunology, The Rockefeller University, 1230 York Ave., New York, NY 10021-6399. Phone: (212) 327-8157. Fax: (212) 327-8960.

yeast extract powder (Difco), 0.1% sodium chloride, and 0.2% glucose. Cultures which had reached an optical density at 600 nm (OD_{600}) of 0.5 were chilled rapidly on ice and left for 2 h, and the cells were sedimented at $2,500 \times g$ for 15 min at 0°C. The bacteria were resuspended in 2 ml of ice-cold 100 mM calcium chloride–70 mM manganese chloride–40 mM sodium acetate (pH 5.5) and left on ice for 45 min. They were then pelleted by centrifugation at $1,800 \times g$ for 10 min at 0°C and resuspended in 5 ml of the same solution. Glycerol was added to give a final concentration of 15%, and the competent bacteria were stored as aliquots of 200 μ l at –80°C.

Preparation of chromosomal DNA from neisseriae. The procedure for preparation of chromosomal DNA was based on that of Moxon et al. (23). Bacteria were harvested by centrifugation from late-logarithmic-phase cultures in 4 ml of GC medium. They were resuspended in 1 ml of 10 mM Tris-HCl (pH 8.0) containing 100 mM NaCl, 10 mM EDTA, and 10 μ g of RNase A and lysed by the addition of 0.1 ml of 10% SDS and heating at 65°C for 30 min. The suspension was cooled to 37°C and digested for 2 h with proteinase K (20 μ l of a 20-mg/ml solution). Extraction was performed twice with phenol-chloroform-isopentanol (25:24:1) and once with water-saturated *n*-butanol. DNA was precipitated with ethanol-sodium acetate, washed with 70% ethanol, and redissolved in 100 μ l of 10 mM Tris–1 mM EDTA (pH 8.0). Yields of DNA were approximately 0.2 mg.

Isolation of cloned *ppk* DNA from a λ gt11 library. A library of DNA from strain BNCV in λ gt11 (24) was screened with end-labelled oligonucleotides, corresponding to the partial amino acid sequence of purified PPK (see Fig. 1), essentially as described previously (30). Ten plates, each with 5×10^4 CFU in a lawn of *E. coli* Y1090 bacteria, were screened. A replica of the plaques bound to Hybond-N⁺ membranes was produced and screened with labelled oligonucleotides as described for Southern blotting. Reactive plaques were picked from the original plates and purified by repeated screenings.

Subcloning of the insert DNA into the phagemid vector pBluescript. Phage DNA was prepared by adsorption of phage lysates to anti-lambda phage antibodies attached to fixed cells of *Staphylococcus aureus* (LambdaSorb; Promega) followed by purification of the DNA by phenol extraction. The insert DNA was cleaved from the phage arms by digestion with *Eco*RI, purified by agarose gel electrophoresis, and ligated into pBluescript SK[–]. The plasmid used for subsequent investigations was named pBluePPK6001 (2.8-kb insert size) (see Fig. 2).

Cloning of restriction endonuclease fragments of chromosomal DNA from BNCV and identification of a fragment containing a portion of the *ppk* gene. DNA from *N. meningitidis* BNCV was digested with the enzymes *Sfu*I and *Pst*I. An *Sfu*I-*Pst*I fragment (3 kb) containing the *ppk* sequence was identified by Southern blotting. The digested DNA was subjected to agarose gel electrophoresis, and the portion of the gel around the position of the reactive fragment was excised. DNA was purified by adsorption to glass milk (Geneclean; Bio101) and ligated into pBluescript SK[–] which had been cut with *Cla*I and *Pst*I. Transformants of *E. coli* XL1-Blue were grown on Luria-Bertani agar. Circles of Hybond-N⁺ were placed onto the plates for 5 min. After removal of the membranes, the plates were stored at 4°C. The colony blots were probed with a ³²P-end-labelled oligonucleotide, as described for screening of the λ gt11 library, to identify recombinants containing the *ppk*-reactive *Sfu*I-*Pst*I fragment. The plasmid chosen for subsequent investigation was named pBluePPK7001 (see Fig. 2).

Sequencing of the *ppk* gene. The plasmid pBluePPK6001 was cut with restriction enzymes *Sfu*I (unique site in insert DNA) and *Cla*I (unique site in pBluescript polylinker), which produce compatible cohesive ends. Removal of the *Cla*I-*Sfu*I fragment and religation produced pBluePPK6019 (1.5-kb insert size). Subcloning of the *Cla*I-*Sfu*I fragment into pBluescript produced pBluePPK6025 (1.3-kb insert). These two plasmids were sequenced in the 5' to 3' direction, relative to the *ppk* gene, after production of progressive deletions with exonuclease III and S1 nuclease (Pharmacia).

The parental plasmid pBluePPK6001 was cut with restriction endonucleases *Eco*RI and *Sfu*I, and the two fragments were both subcloned into M13mp10 and M13mp11 which had been cut with *Eco*RI and *Acc*I. The appropriate recombinant phages (single-stranded DNA) were used to obtain sequence in the 3' to 5' direction relative to *ppk* with primers based on the sequence obtained previously in the 5' to 3' direction from the deletions of plasmids pBluePPK6019 and -6025.

To obtain sequence from the 3' end of the *ppk* gene, an appropriate clone was isolated from a library of *Sfu*I-*Pst*I fragments cloned into pBluescript as described above. Plasmid pBluePPK7001 was digested with enzymes *Eco*RI and *Pst*I, and the 1.5-kb fragment was subcloned into the vectors M13mp18 and M13mp19 for sequencing of single-stranded DNA.

The positions of the sites for restriction enzymes *Sfu*I and *Eco*RI were confirmed by sequencing of the appropriate regions of double-stranded DNA from the plasmids pBluePPK6001 and pBluePPK7001, respectively.

DNA sequence information was analyzed by the computer program package of R. Staden (the DB system [31]). The sequence of the *ppk* gene and surrounding regions has been submitted to GenBank under accession number U16262.

Inactivation of the *ppk* gene. The *ppk* gene was interrupted by insertion of the aminoglycoside phosphotransferase gene from the plasmid pUC-4K (Pharmacia). The kanamycin resistance gene was excised with restriction endonuclease *Acc*I and ligated into the unique *Sfu*I site of pBluePPK6001, within the coding region of the *ppk* gene.

N. meningitidis M1080 and *N. gonorrhoeae* MS11 and F62 were transformed to kanamycin resistance with the plasmid containing the interrupted *ppk* gene

essentially as described by Wetzler et al. (37). Piliated neisseriae from GC agar plates were suspended to give an OD_{600} of 0.2 in 0.5 ml of GC-HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (37), and 10 μ g of transforming DNA was added. After incubation for 30 min at 37°C, the suspensions were diluted to 5 ml with GC-HEPES and incubated with good aeration for a further 2 h. Portions (100 μ l) were spread onto GC agar plates containing 50 μ g of kanamycin per ml for selection of transformed gonococci.

Measurement of PPK activity and of polyP. The activity of PPK was measured as described previously (36) by the incorporation of label from [γ -³²P]ATP into polyP, which was precipitated from reaction mixtures with the cationic detergent cetyltrimethylammonium bromide.

For the polyP assay, polyP was isolated by a procedure based on that of Noegel and Gotschlich (25). Bacteria were harvested and then reacted for 45 min at room temperature in 5.3% sodium hypochlorite (7.5 ml/g [wet weight] of cells, but a minimum of 0.5 ml). The pellet was washed twice at 0°C with 1 M NaCl containing 1 mM EDTA, and the polyP was extracted twice with water at 0°C (the volume per gram of cells was the same as that for sodium hypochlorite). PolyP was precipitated by the addition of 1/50 volume of 5 M sodium chloride and 1 volume of ethanol, washed with 70% ethanol, and redissolved in water. Acid-labile phosphate was released by hydrolysis in 1 M HCl at 100°C for 15 min. PolyP was measured as acid-labile phosphate by the method of Chen et al. (3).

Experiments with iron limitation and phosphate limitation or excess. For experiments involving iron limitation, cells of MS11, transparent and nonpilated, parental or *ppk* mutant, were inoculated into a soft agar overlay on plates of GC agar, and wells in the agar were filled with 3, 10, or 30 nmol of the iron chelator desferoxamine mesylate (Desferal). Zones of inhibition of growth were examined after 24 and 48 h. In the converse experiment, zones of stimulation of growth were observed around wells containing $Fe(NO_3)_3$ on plates of GC agar deferrated by the addition of 200 μ g of Desferal per ml.

For experiments involving phosphate limitation transparent, nonpilated variants of MS11 and M1080 were inoculated into Wong medium containing either 0, 0.03, 0.1, 0.3, 1, 3, 10, or 30 mM potassium phosphate and supplemented with HEPES-KOH to give a final buffer concentration of 30 mM. Growth was measured by monitoring the increase in OD_{600} . In experiments with increased phosphate concentrations, transparent nonpilated variants were grown on Wong medium-agarose plates buffered with 1, 10, 30, 100, or 250 mM potassium phosphate.

Assay for transformation of *N. gonorrhoeae*. Experiments were performed as described above in "Inactivation of the *ppk* gene" with chromosomal DNA (1 μ g) from a rifampin-resistant variant of MS11 as the transforming DNA. After the transformation, 100- μ l aliquots were spread onto GC agar plates containing 25 μ g of rifampin per ml for enumeration of transformed gonococci.

Bactericidal assays on meningococci. Bacteria were grown to the mid-log phase (OD_{600} between 0.3 and 0.5) in GC medium and diluted to give around 10^4 CFU/ml in Wong medium lacking glucose and containing 0.25 mM $CaCl_2$. Killing assays contained 100 μ l of the bacterial suspensions and 100 μ l of freshly thawed human serum (or serum de complemented by heating at 56°C for 30 min) in a volume of 1 ml. Aliquots (100 μ l) were taken in duplicate at the beginning of the experiment and spread onto GC agar plates for enumeration of CFU and then in triplicate after 30 min at 37°C. The percentage of bacteria surviving was expressed relative to that of de complemented controls. Growth of meningococci during the course of the experiment was around 20%.

RESULTS

Cloning and sequencing of the *ppk* gene. The purification and partial amino acid sequence analysis of PPK from *N. meningitidis* BNCV have been described previously (36). For screening of a λ gt11 library of DNA from BNCV (24), oligonucleotides were designed on the basis of amino acid sequences, with multiple bases or inosine at positions of ambiguity (Fig. 1). Oligonucleotide 1 (oligo 1) corresponded to a region near the N terminus of the protein and was of 5' to 3' polarity with respect to the gene. Oligo 2 corresponded to a region further toward the C terminus of the protein and was of reverse polarity with respect to the gene. Several clones reactive with oligo 1 were isolated from the λ gt11 library, and one was chosen for further study. This clone, containing a 2.8-kb insert, was also reactive with oligo 2 on Southern blotting. The insert was cleaved from the lambda vector and ligated into the phagemid vector pBluescript SK[–], producing the plasmid pBluePPK6001 (Fig. 2).

PCR analysis performed on plasmid pBluePPK6001, with the degenerate oligonucleotides oligo 1 and oligo 2 and Stratagene's primers SK and KS (which bind to regions of pBluescript on opposite sides of the polylinker cloning site), showed

N-terminal sequence and oligo 1

X P E Q N R I L X R E L S L L A F N R R V L A Q A E D K N
 GCICARGCIGARGAYAARAA

Internal sequence and oligo 2

X P S E T I A D V T E A A R S L I R H Q Y D L F N N V L Q P
 CTYTGTADCGICTRCAITGICTYCG
 E L A R E S I H F Y R R R N

FIG. 1. Amino acid sequences derived from purified PPK and corresponding sequences of synthetic oligonucleotides. Amino acid sequences use standard single-letter designations. X represents amino acids whose identity was not determined. The nucleotide sequences use codes recommended by the International Union of Biochemistry for designation of incompletely specified bases (26). The nucleotide sequence of oligo 1 corresponds to the sense strand of the gene. That of oligo 2 is the anti-sense strand and is therefore shown in the 3' to 5' direction.

that oligo 1 and oligo 2 bound approximately 150 bp apart and near the middle of the insert DNA (data not shown). DNA sequence information, obtained by using oligo 1 and oligo 2 as primers and the plasmid as template, confirmed the distance separating the oligonucleotide sequences and also identified a cleavage site for restriction endonuclease *SfuI* at around base 150 of the gene. *SfuI* was used to divide the insert DNA into two fragments with lengths of about 1.3 and 1.5 kb to facilitate sequence determination.

The sequence information showed an open reading frame extending from position 1134 to the end of the cloned sequence, indicating that the entire *ppk* gene was not present in

the cloned DNA. To find a combination of enzymes which would produce a fragment from chromosomal DNA encompassing the 3' end of the *ppk* gene, Southern blots of restriction digestions of DNA from BNCV were probed with an oligonucleotide (oligo 3) corresponding to the 3' end of the cloned portion of the *ppk* gene in pBluePPK6001 (Fig. 2). A fragment (3 kb) produced by *SfuI* and *PstI* was cloned into pBluescript to produce plasmid pBluePPK7001, and the sequence was determined to a position around 100 bp downstream of the *ppk* stop codon.

The *ppk* coding sequence (Fig. 3) is 2,055 bp in length, coding for a protein with a predicted size of 77.2 kDa, and is preceded by a purine-rich sequence which is probably the ribosome binding site. The open reading frame is followed by an inverted repeat and short stretch of T residues which could serve as a signal for termination of transcription. Inspection of the DNA sequence revealed no signals with homology to *E. coli* consensus sequences for the initiation of transcription.

The presence of the *ppk* gene in other *Neisseria* species. Radio-labelled probe was prepared from the plasmid pBluePPK6019 (Fig. 2) and used to probe Southern blots of *EcoRI*-cut genomic DNA from two meningococcal and three gonococcal strains and one strain of *Neisseria flava* (Fig. 4). The DNA of each of the pathogenic *Neisseria* strains contained a 6.5-kb fragment reactive with the *ppk* probe, suggesting that the gene is conserved among the strains. The digest of *N. flava* DNA contained a reactive band with an approximate size of 4 kb. No reactive fragment was seen in the two strains of *E. coli* and the one of *Haemophilus influenzae* which were tested (data not shown).

Production of mutants lacking PPK activity. The apparent similarity between the meningococcal and gonococcal *ppk* genes suggested that it might be possible to produce *ppk* mutants of both species by transformation with the mutated gene from BNCV. The cloned portion of the *ppk* gene in plasmid pBluePPK6001 was interrupted by the insertion of a kanamycin resistance cassette at the unique *SfuI* site. One of the recombinant plasmids in which the kanamycin resistance gene was transcribed in the same direction as that of the *ppk* gene was used to transform meningococcus strain M1080 and gonococcus strains MS11 and F62 to kanamycin resistance. Southern blotting of chromosomal DNA from Km^r transformants of each strain, digested with *EcoRI*, showed an increase in size of the reactive fragment from 6.5 to about 8 kb, consistent with the insertion of a Km^r cassette (Fig. 4).

PPK activity and polyP production in the parental and mutant *Neisseria* strains. The parental strain and one kanamycin-resistant transformant of gonococcus strain MS11 and meningococcus strain M1080 were grown for 18 h on Wong medium-

FIG. 2. Plasmids used in the cloning and sequencing of the *ppk* gene from BNCV. The orientation of the inserts relative to the plasmid pBluescript is shown by the letters K and S, representing the *KpnI* and *SacI* sites, respectively, of the multiple cloning site. The coding sequences of the *ppk* gene are shown as shaded boxes; the flanking regions are shown as open boxes. Plasmid pBluePPK 6001 was derived from a library in λ gt11 of sheared DNA from *N. meningitidis* BNCV. Subsequent sequencing of the cloned DNA and Southern blotting of *EcoRI*-digested chromosomal DNA revealed that the *EcoRI* site within the coding region existed in the chromosomal DNA, while the upstream *EcoRI* site was introduced in the creation of the λ gt11 library. The approximate positions of oligo 1 and oligo 2 (used to identify the λ gt11 clone from which pBluePPK6001 was derived) and of oligo 3 (used to screen for a restriction fragment of chromosomal DNA containing the 3' end of the *ppk* gene) are shown. pBluePPK6001 was cleaved with *SfuI* (in the coding sequence of the *ppk* gene) and *ClaI* (in the multiple cloning site of pBluescript). Religation of the plasmid produced pBluePPK6019. The *SfuI*-*ClaI* fragment was ligated into *ClaI*-cut pBluescript, giving pBluePPK6025. Plasmid pBluePPK7001 contains the restriction fragment of chromosomal DNA corresponding to the 3' end of the *ppk* gene. Chromosomal DNA was digested with *SfuI* and *PstI* and ligated between the *ClaI* and *PstI* sites of pBluescript.

TGAAATCAAAATCGTCAACCGGGCTTGGCAGTATTGGCGCGGGCTTCTGTCTGCTGCGCAACCAAGCAACGTCAAAGCCGACGGAACGACGACATCCGGTTTTCCCGA	120
AACCCATTTCGTACCGCTCGACAACATCGCGGTACATCCCGACCTAGACGAATGGACTGATGCGTCCGCGTGTGACCAAGACGACATCTACAAAATCTGGGTGTCGCGCATT	240
ACGACGAAGTATGACGCGTGCAGGAATGGGATATCTGTTCCACTTCCACACCCCGGGGTAGGCATCGACCTGAAACACTTCCGCGTGAAGGCATTACCACCTGTCAATACA	360
AAATTATTTTCGATAAAGCAAAATTTGCCCGAGCTTCTACTGGAACCCCGCTTCCCGAAGATGCCGCTGTCCGCGCCGCAACCAAGCCGAGCCGAAGTCATCCGCGAAA	480
TCGTGCGCGCAAAACCGAAACGTATCCGCCAATATCCGACATGCCGTTCGCGCTGTTTTAGGGATATTATGCGGCTGTCAATGGTGGCCCGTATATGCACAGGGGCAACCGGATG	600
CGGTCAACGCTTATATTCAAAGAAAAAGTCATCGTAACACATCCAAAGCGGAACCTGTGTTTGGCGATGACAGGCGAGTCCACCCGCTCATCGGTGTGCCACGCCAAGGGGA	720
CGTTCCGGCTGACGCTGAACAGTACCGACAAGCGTGGATACGCGCGGCAAGTCATCGGTTTCAAGCAGGAGGGTATTCTTTTCGCCCTGCACCGGCTTGAATCAGATACCGTCGG	840
AAAGCGGAACGAACGATCGCTCCCGTCCGTCGCGACAGGATTATGACCAACGGCTGCATCAACGTCAGCGATGCGGTGTACGAAAACTGCGTCATTATTTGTGTTGAAGTGA	960
TTTGAACAGACGATACCGCAGCGCGGTATCTGTTTTCACATTGCCCGATGCTGAAACAGACTGTCCGCGACGTATGCCGCTGAAACCGGCGCAGATGCCCGCAAGCCTTCAG	1080
ACGCGATTGCGTCCCGCTCCGACGGAACAACACCATCTTTGGGACAACTTATGCCGGAACAAACCGCATCTCTGCCGGAACCTAGCCCTGTGCGATTCAACCCGCGCTGTTGG	1200
M P E Q N R I L C R E L S L L A F N N R V L Q	
CACAGCGGAACAAAAAGTCCCTTTTGAACGCTCGCTTCTGTGATCGTGTGCTGCTCAATCTTGACGAGTTTTTGAAGTCCGATGGCGTCAAGCGTGAAACCAAGC	1320
A Q A E D K N V P L L E R L R F L C I V S S N L D E F F E V R M A W L K R E N K	
TGACCCCGCGCGAGGCTGGACAACGCAAAATGCCGCTGAAACCATCGCGCAGTACCGAAGCGCGCGCTCCCTGATACGGCATCAGTACGACCTGTTCAACAACGCTCTTCAGC	1440
L H P R R R L D N G K M P S E T I A D V T E A A R S L I R H Q Y D L F N N V L Q	
CCGAGTTGGCGCGGAAGCATCCATTTTACCGCGCGCAACTGGACGGGACGACGAAAAATGGATCGAAGACTATTTGACCGGCAACTGCTGCCGATCTGACCCCATCGGAC	1560
P E L A R E S I H F Y R R R N W T G T Q K K W I E D Y F D R E L L P I L T P I G	
TGACCCCGTCCACCCCTTCCCGCGACGCTGAACAAATCGCTCAACTTCCGCGTGAACCTGACGCGACGCGCATCGCGAGGCTTGGGGATGGCGATTGTGACGACCGCGCA	1680
L D P S H P F R P L N K S L F A V E L D G T D A F G R P S G M A I V N R A V R	
TCCTCCGCGCGTCTTCCCTGCCGTCCGAACGTGTGGCGCGGACACGGCTTCTGCTTCTGCTGCTCCCTCCACGCCACGTCGGCAAACTTCCCGGGCATGAACGTCAAAG	1800
I L P R V V P L P S E L C G G G H G F V F L S S I L H A H V G K L F P G M N V K	
GCTGCCACGATTCGCGTACGCGCGACGCGACTTGACCGTTGACGAAGAAGACCTGCAAAACCTCCGCGCGCATCCAAACGAACTGCACGACCGGAATACGCGACGCGCTGC	1920
G C H Q F R L T R D S D L T V D E E D L Q N L R A A I Q N E L H D R E Y G D G V	
GGCTCGAAGTCGCGACACCTGTCCCGCTACATCCGCGACTTCTGCTCGCGCAATTCAAACTGACCGCGCGCAACTTATCAGGTCAAAGGCCGCTCAACCTCGTCCGCTCAACG	2040
R L E V A D T C P A Y I R D F L L A Q F K L T A A E L Y Q V K G P V N L V R L N	
CGTCCCGACCTAGTCAACGCCCGGATTGAAATTTCCACACACGCGCGGACAGTGAAGCCTTGGGCAAAACCGCGCTCATATTGATTGTCGCGCAATCGCCCATCTGC	2160
A V P D L V N R P D L K F P T H T P G R L K A L G K T A S I F D L V R Q S P I L	
TGCACCCCTACCAATCGTTGACCCCGTGTGCAAAATGATGCGCGAACGTCGCGCGACCCGACGCTGCTTCCGCTCAAAATGACGATTACCGCACCGGACGCGTCCGACCTCG	2280
L H P Y Y Q V D P V V E M M R E A A D P D V L A V K M T I Y R T G T R S E L	
TCCGCGCGCTGATGAAGCGGCGCATCGCGGCAACAAAGTAAACGTCGTCTGCAACTGATGCGCGTTTTGACGAAGCAACCAACGTCAACTGGCGGAAGCAGCTCGAAGGCGGCG	2400
V R A L M K A A L A G K Q V T V V V E L M A R F D E A N N V N W A K Q L E E A G	
CGCACGTCGTGTACGCGCTGTTCCGCTACAAAGTCCACGCCAAGATGGCATGGTCATCCGCGCGAAGACGCGCTACTCAAGCGTTACGCCCACTCGGCACGGGTAACACCAAG	2520
A H V V Y G V F G Y K V H A K M A L V I R R E D G V L K R Y A H L G T G N Y H Q	
GCACATCGCGCATCTACCGACTTCGGCATCATTACCGCGCGAACAATCACCGCCGATGTGAACACATTGTTATGAAATCACAGGCTTGGGCAACCCGGGCGCTGAACAAAC	2640
G T S R I Y T D F G I I T A D E Q I T A D V N T L F M E I T G L G K P G R L N K	
TCTACCAAGCCGTTTACCTCGCAAAATGGTATCGACCGCATCGACGCGAAGCAACGCAACGCAAAAGCCGCGCAACCGCGCGGATTACCGCAAGATGAATTCGTCATCGAAC	2760
L Y Q S P F T L H K M V I D R I A R E T E H A K A G K P A R I T A K M N S L I E	
CGACCGTCATCGAAGCCCTGTATCGGCAAGCGCGCGAGCGTACAAATCGATTGATTGTGCGCGGTATGTGACCTTGGCGCGGGTGTAAAGGCTTGTCCGAAACATCCGCGTC	2880
P T V I E A L Y R A S A A G V I D L I V R G M C T L R P G V K G L S E N Y A C	
GCTCCATCATCGGCGAGCTCGAACACGCGCGGTATTGCTTCCACAAACAGCGCGGACGATACCTTTATCTCCAGCGCGGATTGGATGGGCGCAACTTCTCCGCGCATCG	3000
R S I I G R Q L E H A R V Y C F H N N G A D D T F I S S A D W M G R N F F R R I	
AAACCGCGCGCGATTGCCGCGCGCAACTCAAAACACGCGTCATCCGCGAAGGTTTGAATGGCACTGGCAGCAACATACGACGCGTGGCTGATGACGCGCGCGCGGTATATCC	3120
E A A P I A P E L K R V I R E G L E M A L A D N T H A W L M Q P D G Y I	
GCGCGCGCTGCCGAGGCGGAAGCGAAGCGACCTGCAAAACGATTGTGGACACTGCTCGGAGGCTGACCGCGACCGCCCAATCAAAACCATGCCGCTGAAACCTTCCGTTTC	3240
R A A P A E G E S E A D L Q N D L W T L L G G *	
AGACGGCATGGTTTTACAGCAATCTAAACAGGGCGGACTGGAATCAAAACACACCTTCGC	3301

FIG. 3. DNA sequence of the *ppk* gene and flanking regions. The translation of the *ppk* gene is shown below the DNA sequence, in single-letter code. A purine-rich sequence just upstream of the ATG initiation codon, the putative ribosome binding site, is shown in bold type, underlined. Upstream of the *ppk* gene, the same reading frame which contains the *ppk* gene has two stop codons, shown in bold and underlined, which define two other open reading frames. Regions of DNA containing the uptake sequence GCGTCTGAA are shown double-underlined. Four of these motifs are parts of regions, singly underlined, which could base-pair to form stem-loop structures.

agarose plates, and polyP was extracted and measured as described previously (25). In the case of gonococcus strain MS11, the parental strain produced 0.70 ± 0.07 mg of polyP [as $(\text{NaPO}_3)_n$] per g (wet weight) of cells, whereas the Km^r transformant produced only 0.13 ± 0.03 mg of polyP per g of cells. Similarly, in the case of meningococcus strain M1080, the parental strain produced 7.43 ± 0.6 mg of polyP per g of cells and the Km^r transformant produced only 0.21 ± 0.08 mg of polyP per g of cells. (Results are the averages of three determinations.) PPK assays performed on broth-grown F62 showed that the parental strain produced enzymatic activity, while the kanamycin-resistant transformant did not, confirming that the

ppk gene of F62 had been inactivated. However, both the PPK activity and the polyP production under the conditions tested were much less than those of MS11 and M1080, so subsequent experiments did not include strain F62.

To confirm that the difference in polyP production between the parental and mutant strains existed throughout all growth phases, gonococcus strain MS11 and meningococcus strain M1080 were grown in Wong medium. Samples were taken at various time points up to 20 h to test for polyP production and for PPK activity (Fig. 5). In the case of the meningococcus strain M1080, the amount of polyP (per OD unit) produced by the mutant was decreased to less than 10% of the amount

FIG. 4. Southern blot of chromosomal DNA from neisserial strains, with *ppk* probe. Chromosomal DNA extracted from various *Neisseria* strains was digested with restriction endonuclease *Eco*RI. The Southern blot was reacted with a random-primed fluorescein-labelled probe produced from the plasmid pBluePPK 6019. Reactive bands were visualized by immunoperoxidase detection and chemiluminescence as described in Materials and Methods. Size markers were fluorescein-labelled *Hind*III fragments of phage lambda DNA. Strains tested were *N. flava*, *N. gonorrhoeae* R10, *N. meningitidis* BNCV, and the parental and *ppk* mutant strains of *N. meningitidis* M1080 and *N. gonorrhoeae* MS11 and F62.

produced by the parental strain in the exponential phase, while the measured PPK activity was reduced to less than 5% of the levels in the parental strain (Fig. 5a). In the case of the gonococcus, the amount of polyP in the *ppk* mutant was less than 3% and the activity of PPK was less than 10% (in this case, near the limit of detection of the assay) of that of the parental strain (Fig. 5b).

The meningococcus strain M1080 grew exponentially for about 6 h, after which growth slowed and then ceased after 12 to 16 h (Fig. 5a). PPK activity increased with the cell growth during the exponential phase and then remained nearly constant during the period of stagnation of growth. In contrast, the amount of the product polyP, after the exponential increase, decreased markedly once growth had ceased.

The results from the gonococcal culture were similar to those of the meningococci. However, the growth of MS11 was slower than that of M1080 (Fig. 5b). PPK activity and amount of polyP again reached maxima at the end of the exponential growth, and levels of polyP decreased more rapidly than did PPK activity.

In both cases, the *ppk* mutant grew appreciably more slowly than the parental strain, this being more pronounced in the case of the gonococcus, where the ODs suggested that the mutant survived less well after reaching the stationary phase.

Phenotypic differences between *Neisseria* wild-type and mutant strains in polyP synthesis. (i) **Phosphate starvation or excess.** The ability of parental and *ppk* mutant strains of M1080 and of MS11 to grow at various phosphate concentrations was examined. At all phosphate concentrations supporting growth, the parental strains grew better than the mutants. Both the

FIG. 5. Growth, PPK activity, and polyP in *N. meningitidis* M1080 and *N. gonorrhoeae* MS11. Meningococcus strain M1080 (a) and gonococcus strain MS11 (b), parental and *ppk* mutants, were grown in GC medium, subcultured twice into Wong medium, and then inoculated into 100 ml of Wong medium equilibrated to 37°C, under 5% CO₂. Growth was monitored for 20 h by withdrawal of samples for measurement of *A*₆₀₀ (□ and ▴). Samples (6 ml) were taken at the same time points for assay of polyP (△ and ▴) and PPK activity (○ and ●). Solid symbols are data from the parental strains; open symbols are data from the *ppk* mutants. Data for polyP and PPK are averages of three measurements. Typical experiments are shown.

parents and mutants needed at least 0.1 mM phosphate for growth. Optimal growth occurred over a broad range of concentrations between 0.3 and 30 mM phosphate. Growth was reduced at the high phosphate concentrations, being almost absent at 250 mM. However, the inhibition of growth was not greater in the *ppk* mutants than the parental strains.

(ii) **Iron limitation.** One possible function of polyP in *Neisseria* species may be to increase the availability of iron to the organism. Although the organisms possess systems for the uptake of iron from host iron-binding proteins, they are able to use simple iron-chelating compounds to obtain inorganic ferric ions (21). No difference was seen in the sizes of zones of inhibition of growth on agar plates around wells containing desferoxamine mesylate or in zones of stimulation of growth on iron-depleted medium around wells containing iron.

(iii) **Transformation.** In *E. coli*, polyP has been found in membrane pores which have been postulated to play a role in DNA uptake (28). Experiments with gonococcus strain MS11 showed no difference in the ability of the parental and *ppk*

FIG. 6. Serum sensitivity of *N. meningitidis* M1080, *ppk* mutants, and transformants back to *ppk*⁺. Bactericidal assays were performed on nonpilated variants of M1080 parental strain, opaque (M1080 O⁺) and transparent (M1080 O⁻), and independently isolated *ppk* mutants K1 and K3. The two independently isolated Km^r PPK⁺ transformants from K1 (K1-1 and K1-2) and from K3 (K3-1 and K3-2) regained the serum resistance of the parental strains. The results shown are the averages of three or more independent experiments. Experimental data were averages of triplicate determinations. All experiments used 10% serum from a single donor.

mutant bacteria to be transformed by homologous gonococcal DNA containing a gene specifying resistance to rifampin.

(iv) **Serum resistance.** Initial experiments with serum from two volunteers suggested that PPK mutant strains of *N. meningitidis* M1080 were considerably more sensitive to killing by 10% serum than was the parental strain. Since the M1080 strain had been transformed to a *ppk* mutant with plasmid DNA from strain BNCV, rather than with homologous DNA, it was possible that the phenotypic difference was due to something other than the interrupted *ppk* gene. The *ppk* mutant bacteria were therefore transformed back to *ppk*⁺ with the original plasmid pBluePPK6001, containing the intact (portion of) *ppk* gene, and Km^r PPK⁺ transformants were chosen for subsequent investigation. In bactericidal assays of all of the strains, the parental strain showed a serum-resistant phenotype, while the PPK mutants were serum sensitive, and upon transformation back to PPK⁺, the serum resistance was regained (Fig. 6).

DISCUSSION

The cloned *ppk* gene codes for a protein with a size of 77.2 kDa, with some homology to the PPK proteins predicted from the genes of *E. coli* (1) (and of *Klebsiella aerogenes* [GenBank accession number D14445], these genes being almost identical). Inspection of the sequence showed the presence of several characteristic sequences (GCCGTCTGAA) which have been shown to participate in the uptake of homologous DNA by *N. gonorrhoeae* (6, 9, 10). The motif ATGCCGTCTGAA occurs five times in the cloned sequence, four of them arranged as inverted repeats which could form stem-loop structures. One such inverted repeat immediately after the *ppk* gene is followed by a short stretch of T residues and therefore may function as a terminator of transcription (9). The possible function of the other pair of motifs, centered about 80 bases

upstream of the gene, is unclear, although such an arrangement is not uncommon, e.g., the *argF* gene from *N. gonorrhoeae* (19) and the *lgtA* and *lgtD* genes from the lipooligosaccharide synthesis locus of *N. gonorrhoeae* (11). The occurrence of single copies of the sequence within the coding frame also has instances in other neisserial genes, e.g., *argF* (19) and *pilA* (35).

The entire sequenced region contained three open reading frames. One containing the *ppk* coding sequence stretched from base 966 to 3191, while two others upstream comprised bases 1 to 515 and 516 to 965 (Fig. 3). The most-upstream open reading frame had significant homology to an antigen from *Haemophilus somnus* of unknown function (38). The middle reading frame showed no homology to sequences in available databases. No signals for initiation of transcription which corresponded to consensus *E. coli* promoter sequences were seen.

The neisserial transformants in which the *ppk* gene had been inactivated insertionally produced much decreased amounts of polyP. The residual amounts of polyP which were measured in the *ppk* mutants may represent a separate pool of polyP produced by some other means. Crooke et al. (5) have found that a small amount of polyP remains in cells of *E. coli* in which the *ppk* gene is inactivated. However, the *E. coli* mutant showed striking deficits in survival in the stationary phase and in tolerance of heat shock and oxidative stress, phenotypes linked to the stringent response. The *ppk* mutant gonococci, in particular, showed an increased tendency towards lysis after growth had ceased, but the basis for this phenomenon was not investigated. Unfortunately, the pathogenic neisseriae do not exhibit a stationary phase as seen in *E. coli* and die within hours of the cessation of growth on normal laboratory media (22). However, it may prove possible to examine further the phenomenon of decreased survival of the *ppk* mutants by subjecting cultures to oxidative stress, hydroxamate, or heat shock treatment as described by Crooke et al. (5).

Other possible functions of the polyP have been suggested. These include energy storage (18), maintenance of a constant intracellular phosphate concentration (16, 18), chelation of metal ions (25), and an involvement in uptake of DNA (28). That energy storage may be one function of the neisserial polyP is suggested by the decrease in polyP after cessation of growth (Fig. 5). Although the *ppk* mutants grew more slowly than the parental strains, they showed no differential decrease in growth rate relative to that of the wild type at growth-limiting low concentrations of phosphate or at high phosphate levels. This supports the idea that polyP is not the main regulator of the levels of free phosphate in the cell. The possible functions of polyP as a scavenger of iron or as a component of a hydrophilic membrane pore involved in uptake of DNA were also not demonstrated by deficits in the *ppk* mutants.

It remains to be determined what importance polyP may have in biological interactions of the pathogenic neisseriae, such as cell invasion, intracellular survival, and resistance to immune lysis. Initial experiments indicate that the resistance of the *ppk* mutants to human serum is reduced significantly compared with that of the parental strain. It is possible that lack of polyP could alter the metabolism of the cells in such a way as to render them more susceptible to killing by complement. If polyP is present on the exterior of the cells, it could chelate the necessary metal ions or interfere in some other way with the action of complement. Certainly, the role of this interesting compound in the metabolism and virulence of the pathogenic *Neisseria* species deserves further investigation.

ACKNOWLEDGMENTS

We thank J. Weiser of the Division of Infectious Diseases, Children's Hospital of Philadelphia, for providing the method for rendering *E. coli* competent for transformation.

This work was supported by Public Health Service grants AI10615 and AI26558.

REFERENCES

- Akiyama, M., E. Crooke, and A. Kornberg. 1992. The polyphosphate kinase gene of *Escherichia coli*. Isolation and sequence of the *ppk* gene and membrane location of the protein. *J. Biol. Chem.* **267**:22556–22561.
- Blake, M. S., and E. C. Gotschlich. 1982. Purification and partial characterization of the major outer membrane protein of *Neisseria gonorrhoeae*. *Infect. Immun.* **36**:277–283.
- Chen, P. S., Jr., T. Y. Toribara, and H. Warner. 1956. Microdetermination of phosphorus. *Anal. Chem.* **28**:1756–1758.
- Church, G. M., and W. Gilbert. 1984. Genomic sequencing. *Proc. Natl. Acad. Sci. USA* **81**:1991–1995.
- Crooke, E., M. Akiyama, N. N. Rao, and A. Kornberg. 1994. Genetically altered levels of inorganic polyphosphate in *Escherichia coli*. *J. Biol. Chem.* **269**:6290–6295.
- Elkins, C., C. E. Thomas, H. S. Seifert, and P. F. Sparling. 1991. Species specific uptake of DNA by gonococci is mediated by a 10-base-pair sequence. *J. Bacteriol.* **173**:3911–3913.
- Frasch, C. E., and S. S. Chapman. 1972. Classification of *Neisseria meningitidis* group B into distinct serotypes. I. Serological typing by a microbactericidal method. *Infect. Immun.* **5**:98–102.
- Frasch, C. E., and E. C. Gotschlich. 1974. An outer membrane protein of *Neisseria meningitidis* group B responsible for serotype specificity. *J. Exp. Med.* **140**:87–104.
- 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.
- Goodman, S. D., and J. J. Scocca. 1991. Factors influencing the specific interaction of *Neisseria gonorrhoeae* with transforming DNA. *J. Bacteriol.* **173**:5921–5923.
- Gotschlich, E. C. 1994. Genetic locus for the biosynthesis of the variable portion of *Neisseria gonorrhoeae* lipooligosaccharide. *J. Exp. Med.* **180**:2181–2190.
- Harold, F. M. 1966. Inorganic polyphosphates in biology: structure, metabolism, and function. *Bacteriol. Rev.* **30**:772–794.
- Hendley, J. O., K. R. Powell, R. Rodewald, H. H. Holzgreffe, and R. Lyles. 1977. Demonstration of a capsule on *Neisseria gonorrhoeae*. *N. Engl. J. Med.* **296**:608–611.
- Hendley, J. O., K. R. Powell, N. L. Salomonsky, and R. R. Rodewald. 1981. Electron microscopy of the gonococcal capsule. *J. Infect. Dis.* **143**:796–802.
- James, J. F., and J. Swanson. 1977. The capsule of the gonococcus. *J. Exp. Med.* **145**:1082–1086.
- Kaltwasser, H. 1962. Die Rolle der Polyphosphate im Phosphatstoffwechsel eines Knallgasbakteriums (*Hydrogenomonas* Stamm 20). *Arch. Mikrobiol.* **41**:282–306.
- Kellogg, D. S., Jr., W. L. Peacock, W. E. Deacon, L. Brown, and C. I. Pirkle. 1963. *Neisseria gonorrhoeae*. I. Virulence genetically linked to clonal variation. *J. Bacteriol.* **85**:1274–1279.
- Kulaev, I. S., and V. M. Vagabov. 1983. Polyphosphate metabolism in microorganisms. *Adv. Microb. Physiol.* **24**:83–171.
- Martin, P. R., J. W. Cooperider, and M. H. Mulks. 1990. Sequence of the *argF* gene encoding ornithine transcarbamoylase from *Neisseria gonorrhoeae*. *Gene* **94**:139–140.
- Messing, J. 1983. New M13 vectors for cloning. *Methods Enzymol.* **101**:20–78.
- 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**:556–564.
- Morse, S. A. 1979. The biology of the gonococcus. *Crit. Rev. Microbiol.* **7**:93–189.
- Moxon, E. R., R. A. Deich, and C. Connelly. 1984. Cloning of chromosomal DNA from *Haemophilus influenzae*. Its use for studying the expression of type b capsule and virulence. *J. Clin. Invest.* **73**:298–306.
- 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.
- Noegel, A., and E. C. Gotschlich. 1983. Isolation of a high molecular weight polyphosphate from *Neisseria gonorrhoeae*. *J. Exp. Med.* **157**:2049–2060.
- Nomenclature Committee of the International Union of Biochemistry. 1985. Nomenclature for incompletely specified bases in nucleic acid sequences. Recommendations 1984. *Eur. J. Biochem.* **150**:1–5.
- Pepin, C. A., and H. G. Wood. 1986. Polyphosphate glucokinase from *Propionibacterium shermanii*. Kinetics and demonstration that the mechanism involves both processive and nonprocessive type reactions. *J. Biol. Chem.* **261**:4476–4480.
- Reusch, R. N., and H. L. Sadoff. 1988. Putative structure and functions of a poly- β -hydroxybutyrate/calcium polyphosphate channel in bacterial plasma membranes. *Proc. Natl. Acad. Sci. USA* **85**:4176–4180.
- Russel, M., S. Kidd, and M. R. Kelley. 1986. An improved filamentous helper phage for generating single-stranded plasmid DNA. *Gene* **45**:333–338.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Staden, R. 1982. Automation of the computer handling of gel reading data produced by the shotgun method of DNA sequencing. *Nucleic Acids Res.* **10**:4731–4751.
- Swanson, J. 1978. Studies on gonococcus infection. XII. Colony color and opacity variants of gonococci. *Infect. Immun.* **19**:320–331.
- Swanson, J., S. J. Kraus, and E. C. Gotschlich. 1971. Studies on gonococcus infection. I. Pili and zones of adhesion: their relation to gonococcal growth patterns. *J. Exp. Med.* **134**:886–906.
- Szilvasi, J., and P. der Hautabteilung. 1932. Über die Gestaltungsformen des Neisserschen Gonokokkus. *Dermatol. Wochenschr.* **6**:204–207.
- Taha, M. K., M. So, H. S. Seifert, E. Billyard, and C. Marchall. 1988. Pili expression in *Neisseria gonorrhoeae* is under both positive and negative transcriptional control. *EMBO J.* **7**:4367–4378.
- 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.
- Wetzler, L. M., E. C. Gotschlich, M. S. Blake, and J. M. Koomey. 1989. The construction and characterization of *Neisseria gonorrhoeae* lacking protein III in its outer membrane. *J. Exp. Med.* **169**:2199–2209.
- 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.
- Wong, T. P., R. K. Shockley, and K. H. Johnston. 1980. WSJM, a simple chemically defined medium for the growth of *Neisseria gonorrhoeae*. *J. Clin. Microbiol.* **11**:363–369.