Altered Lipopolysaccharide Characteristic of the I69 Phenotype in Haemophilus influenzae Results from Mutations in a Novel Gene, isn

ANDREW PRESTON,^{1*} DUNCAN MASKELL,² ALAN JOHNSON,¹[†] AND E. RICHARD MOXON¹

Molecular Infectious Diseases Group, Institute of Molecular Medicine, John Radcliffe Hospital, Oxford OX3 9DU,¹ and Department of Biochemistry, Imperial College of Science, Technology and Medicine, Exhibition Road, London SW7 2AY,² United Kingdom

Received 17 July 1995/Accepted 2 November 1995

The I69 phenotype of *Haemophilus influenzae* results from a mutation leading to a lipopolysaccharide molecule consisting only of lipid A and a single phosphorylated 2-keto-3-deoxyoctulosonic acid residue. In this paper we describe the identification of a gene which, when mutated, results in the I69 phenotype. We have named the gene *isn*. The predicted amino acid sequence of Isn is homologous to the product of the *lmbN* gene involved in the biosynthesis of the sugar-containing antibiotic lincomycin by *Streptomyces lincolnensis. isn* is situated between two loci that are homologous to the *dpp* and *art* periplasmic permease systems in *Escherichia coli*. Northern (RNA) blot and primer extension analyses reveal that *isn* is transcribed as a monocistronic mRNA. Potential functions of Isn protein are discussed.

Haemophilus influenzae is a gram-negative bacterium often found as a commensal of the human upper respiratory tract. In infants, serotype b (Hib) strains are a major cause of invasive diseases such as meningitis, cellulitis, epiglottitis, and pneumonia, whilst nontypeable strains are a common cause of otitis media and lower respiratory tract infections, particularly in the developing world (36). Surface molecules such as capsular polysaccharide and lipopolysaccharide (LPS) are important contributors to the virulence of the bacterium (26, 27).

H. influenzae LPS consists of a toxic lipid A molecule linked via a single phosphorylated 2-keto-3-deoxyoctulosonic acid (KDO) residue to a branched-chain oligosaccharide (26). Microheterogeneity and phase-variable expression of oligosaccharide epitopes have led to difficulty in solving the fine structure of the oligosaccharide, but recently partial structures have been deduced for several strains (29, 30).

Molecular genetic studies have identified a number of loci necessary for the biosynthesis of H. influenzae LPS. Among these, the lic and lsg loci have been cloned and sequenced, and these data have led to hypotheses about LPS biosynthesis, structure, and mechanisms of phase variation (2, 3, 10, 15, 21, 22, 35, 40-42). Of the loci identified, one of the least studied at the DNA level is that defined by the I69 mutation. During the construction of a genomic library of chromosomal DNA from an *H. influenzae* type b strain in a λ vector, it was observed that transformation of the DNA from one of the recombinant clones back into wild-type H. influenzae produced an opaque colony phenotype. This clone was called I69 and contained a 9.4-kb EcoRI restriction fragment (44). Subsequent analysis of the I69 transformants revealed that their LPS consisted of only lipid A and a single molecule of phosphorylated KDO (13). H. influenzae transformants displaying the I69 phenotype are serum sensitive and avirulent, failing to colonize infant rats after intranasal, intraperitoneal, or intravascular challenge (44).

In this report, we show that the I69 phenotype results from the mutation of a previously unidentified gene situated between homologs of the *Escherichia coli dpp* and *art* loci, which encode dipeptide and arginine periplasmic permeases, respectively. We have named the gene *isn* (i-sixty-nine) and speculate that it represents a novel LPS biosynthetic function.

MATERIALS AND METHODS

Bacterial strains and plasmids. Wild-type Hib strains RM7004 (38), Eagan (5), RM142 (27), RM156 (24), wild-type type d *H. influenzae* strain RM132 (8), the 169 *H. influenzae* strain RM4066 (44), and the *E. coli* cloning strain DH5 α (16) have all been described previously. *H. influenzae* growth was supported by BHI medium (Oxoid Ltd.) supplemented with hemin (10 μ g μ l⁻¹ of medium) and NAD (2 μ g μ l⁻¹). Hemin (0.1%, wt/vol), 0.1% (wt/vol) L-histidine, and 0.4% triethanolamine were dissolved in distilled water, sterilized by heating at 70°C for 20 min and stored at 4°C.

BHI agar was supplemented with 10% Levinthal's base (4). *E. coli* DH5 α was used to maintain plasmids. *E. coli* growth was supported by 2xLB medium. Media were supplemented with antibiotics as required. For *E. coli* growth, ampicillin was used at 100 μ g ml⁻¹, and kanamycin was used at 50 μ g ml⁻¹. For *H. influenzae* growth, kanamycin was used at 8 μ g ml⁻¹.

pBluescript (Stratagene) and pUC18 (Pharmacia) were used as cloning vectors. pGex3X (Pharmacia) is an expression vector that was used here as a cloning vector. pUC4K (Pharmacia) was used as a source of kanamycin resistance cassette.

Chemicals and enzymes. Chemicals were purchased from BDH (Atherstone, Warwickshire, U.K.) Aldrich (Gillingham, Dorset, U.K.), Pharmacia Biotech Ltd. (St. Albans, Hertfordshire, U.K.), and Sigma Chemical Company (Poole, Dorset, U.K.) as Analar or technical grade.

Radiochemicals were purchased as the triethylammonium salt in an aqueous solution at an activity of 10 μ Ci μ l⁻¹ from Amersham International (Aylesbury, Buckinghamshire, U.K.). Deoxyadenosine 5'-(α -1³⁵S]thio)triphosphate was used for labelling DNA during DNA sequencing, and deoxycytidine 5'-[α -3²P]triphosphate was used to label DNA probes. [γ -3²P]ATP was used for radiolabelling oligonucleotide primers.

DNA modification and other enzymes were bought from a variety of sources: Amersham International, Boehringer Mannheim (Lewes, Sussex, U.K.), New England Biolabs (Bishops Stortford, Hertfordshire, U.K.), and Northumbria Biologicals (Cramlington, Northumberland, U.K.).

^{*} Corresponding author. Mailing address: Department of Microbiology, Bowen Science Building, University of Iowa, Iowa City, IA 52242. Phone: (319) 353-5654. Fax: (319) 335-9006. Electronic mail: apreston@vaxa.weeg.uiowa.edu.

[†] Present address: Antibiotic Reference Laboratory Unit, Laboratory of Hospital Infection, Central Public Health Laboratory, London NW9 5HT, U.K.

Preparation of DNA. Small-scale preparations of plasmid DNA were made by a modified method of Birnboim and Doly (7). Larger-scale plasmid preparations (100 to 200 μ g) were performed with the Qiagen plasmid preparation kit. Chromosomal DNA was prepared by pelleting 3 ml of an overnight bacterial culture. The cell pellet was washed in phosphate-buffered saline (PBS) before resuspension in 250 μ l of TNE (10 mM Tris-HCl [pH 8], 100 mM NaCl, 1 mM EDTA);

25 μ l of 10% sodium dodecyl sulfate (SDS) was added, followed by 25 μ l of proteinase K (25 mg ml⁻¹). The reaction was incubated for 4 to 5 h or overnight at 55°C. The reaction was phenol extracted twice and then extracted twice with phenol-chloroform. DNA was precipitated by addition of 10 μ l of 3 M sodium acetate and 1 ml of 100% ethanol. Chromosomal DNA was then lifted from the tube on the end of a pipette tip and washed by repeated transfer to tubes containing 500 μ l of 70% (vol/vol) ethanol. Washed DNA samples were then air dried before resuspension in 100 μ l of TE with RNase.

DNA sequencing and sequence analysis. DNA was sequenced by the dideoxynucleotide chain termination method of Sanger et al. (31) with the Sequenase Version II DNA sequencing kit (United States Biochemical Corporation). Several sequencing strategies were employed. Subclones constructed to map the I69 mutation within p700 (Fig. 1AI) provided convenient sequencing templates. Additional subclones were constructed by using restriction sites identified from initial sequence data (data not shown). p719, p715, and p714 were further subcloned by digestion with the frequently cutting enzymes *Sau3A*, *RsaI*, *SspI*, and *DraI*. Oligonucleotide primers were used to extend sequence data and to confirm the DNA sequence at subclone junctions. Analysis of the DNA sequence was performed with the Staden and Genetics Computer Group packages (Staden DNA and RNA Analysis software, Cambridge, U.K., 1991; Genetics Computer Group, version 7, Madison, Wis., 1991) run on the Molecular Biology Users Group VAX at the University of Oxford and at the National Center for Biotechnology Information (NCBI) with the BLAST network service.

PCR products were directly sequenced, without cloning, with the Δ Taq Cycle-Sequencing Kit (US Biochemicals). Genomic DNA PCR templates were prepared from single colonies by picking single colonies into 50 µl of sterile distilled water. The dispersed colonies were heated in a boiling water bath for 5 min. Samples were centrifuged at 12,000 rpm for 2 min and the supernatant was collected; 10 µl of this supernatant was used directly as the template in a PCR.

Southern hybridizations. Non-radioactively-labelled probes were used in Southern blot analyses. Probes were labelled with digoxygenin with the DIG labelling kit (Boehringer Mannheim). Hybridizing DNA fragments were detected with the DIG detection kit (Boehringer Mannheim). Southern blots were performed as described previously (20).

RNA analysis. RNA was prepared from bacterial cultures by hot phenol extraction as described previously (23). The protocol was scaled down to accommodate culture volumes of 80 ml. Resultant RNA pellets were resuspended in 50 to 100 μ l of diethylpyrocarbonate (DEPC)-treated sterile distilled water. One-tenth volume of nick translation buffer (DEPC treated) was added, and DNA in the samples was degraded by addition of 10 U of RNase-free DNase I and incubation of the samples at room temperature for 10 min.

Northern (RNA blot) analysis. Northern blots were performed as described previously (20). Radiolabelled DNA probes were hybridized with filters in hybridization solution ($6 \times SSC$, $2 \times$ Denhardt's solution, 0.1% sodium dodecyl sulfate (SDS), 100 µg of salmon sperm DNA per ml) for 12 to 16 h at $68^{\circ}C$ ($1 \times SSC$ is 0.15 M NaCl plus 0.015 M sodium citrate). Filters were washed first in $1 \times SSC-0.1\%$ SDS at room temperature for 20 min and then in $1 \times SSC-0.1\%$ SDS at the hybridization temperature for 20 min.

Primer extension. Oligonucleotide primers were 5' end labelled by the action of polynucleotide kinase. Approximately 50 µg of RNA was annealed with approximately 1 pmol of labelled primer. Annealing was carried out in the presence of annealing buffer (0.5 M KCl, 0.25 M Tris-HCl [pH 8.3]) in a total volume of 10 µl. The reaction was incubated at 90°C for 1 min and then immediately at 65°C for 2 min. The reaction was then allowed to cool to room temperature, and 3 µl of the annealing reaction was added to 1 µl of reverse transcriptase buffer (0.25 M Tris-HCl [pH 8.3], 0.2 M KCl, 0.067 M magnesium acetate, 0.01 M dithiothreitol, 1 mM dATP, 1 mM dGTP, 1 mM dCTP, 1 mM dTTP, 230 U of RNAguard [Pharmacia]) and 1 µl (5 U) of reverse transcriptase. This reaction was incubated at 37°C for 5 min and then at 47°C for 2 h. The reaction was terminated by addition of 5 µl of Sequenase stop solution. Reactions were denatured by heating at 80°C for 2 min immediately prior to agarose electrophoresis. Primer extension reactions were loaded next to DNA sequence reactions that had been primed with the same primer as used in the primer extension reaction to allow accurate identification of the transcriptional start site in the DNA sequence.

Transformation of *H. influenzae. H. influenzae* was made competent by the method of Herriott et al. (14). Briefly, a 10-ml culture of *H. influenzae* was grown to an optical density at 650 nm of 0.2 to 0.25. Cells were pelleted by centrifugation at 3,000 × g for 5 min. The cell pellet was washed once with M_{IV} medium (14) that had been warmed to 37°C. The pellet was then resuspended in 10 ml of warmed M_{IV} medium and incubated at 37°C with very gentle aeration for 100 min. Cells were then competent. Aliquots (250 µl) of competent cells (approximately 10⁹ cells) were added to the transforming DNA (1 to 2 µg) and incubated at 37°C for 30 min; 1 ml of supplemented BHI medium was then added, and the cells were incubated at 37°C for a further 1 h before plating onto the appropriate medium. p700, which was known to contain the I69 mutation, was used as a positive control in transformation experiments. Competent cells were incubated in the absence of exogenous DNA as a negative control in these experiments.

Preparation of LPS. Bacteria were pelleted from 3 ml of an overnight culture by centrifugation at 12,000 rpm. Cell pellets were washed once with PBS and then resuspended in 500 µl of PBS; 250 µl of LPS buffer I (0.1875 M Tris-HCI [pH 6.8], 6% [wt/vol] SDS, 30% [wt/vol] glycerol) was added, and the mixture was



FIG. 1. (AI) Restriction map of p700. E, *Eco*RI; P, *Pvul*; C, *ClaI*; N, *Nvul*; B, *Nsil*; S, *SnaBI*; A, *ApaI*. (AII) Subclones derived from p700. p720, *Eco*RI-*ClaI*; p719, *ClaI*-*ClaI*; p718, *ClaI*-*NvuI*; p717, *NvuI*-*PvuI*; p714, *PvuI*-*Eco*RI; p716, *ClaI*-*Eco*RI; p715, *ClaI*-*PvuI*. (B) SDS-PAGE of LPS prepared from *H. influenzae* RM132 (wild type) (lane 2) and RM4066 (I69) (lane 3) and from opaque colonies produced by using p715 (lane 1) and p717 (lane 4). The LPS of the p715and p717-derived transformants comigrated with that of the I69 strain RM4066 and consisted of the fast-migrating band characteristic of I69 LPS, confirming that plasmids p715 and p717 could confer the I69 phenotype on wild-type *H. influenzae*.

heated in a boiling water bath for 5 min. Then 10 μ l of the cell lysate was added to 35 μ l of LPS buffer II (0.0625 M Tris-HCl [pH 6.8], 0.1% [wt/vol] SDS, 10% [wt/vol] glycerol, 0.1% [wt/vol] bromophenol blue) along with 10 μ l of proteinase K (25 mg ml⁻¹). The sample was incubated at 55°C for 12 to 16 h. Samples were heated in a boiling water bath for 5 min prior to SDS-PAGE. Between 15 and 30 μ l of sample was loaded onto SDS-PAGE gels.

SDS-PAGE of LPS. LPS samples were analyzed by SDS-PAGE in the tricinebuffered gel system originally described by Schagger and Von Jagow (32) with the modifications described by Lesse and colleagues to optimize resolution of LPS (18). Gels were stained with the Ouicksilver (Amersham) silver stain kit.

Nucleotide sequence accession number. The sequence reported here has Gen-Bank accession number U17295.

RESULTS

Mapping the I69 mutation. The 9.4-kb EcoRI insert of the original I69 clone was used as a source of DNA to generate subclones spanning the entire locus (Fig. 1A). The subclones were in pBluescript replicons and would thus not replicate in H. influenzae. Transformation of H. influenzae with these subclones will result, in some cases, in homologous recombination between the H. influenzae DNA in the subclone and the wildtype copy of the gene in the recipient. After plating out the transformation mixture followed by overnight growth, a proportion of the colonies will have the opaque phenotype characteristic of the I69 mutation only if the subclone used in the transformation contains the I69 mutation. By this strategy, subclones p715, p716, and p717 conferred the I69 colony phenotype on the wild-type H. influenzae RM132, whereas p714, p718, p719, and p720 did not (Fig. 1A). That the opaque phenotype correlated with an altered LPS molecule was confirmed when the LPS isolated from the opaque colonies produced with p715 and p717 comigrated with that of the prototype I69 strain, H. influenzae RM4066, on SDS-PAGE (Fig.

DDDB M F GGAATGTTTAAAT D P V E I F V F K R I L M V I F T F I A I T L I T F A L V H F I P G D F V E I 101 TTGTATTCAAACGAATCTTAATGGTGATCCCTACTTTTATTGCCATTATTACCTTTGCCCTTGTGCATTTTATTCCCGGTGATCCTGGGAAAT M M G E R G L T A E V H Q Q M M H Q L G L D L P L Y Q Q Y F H Y I 201 TATGATGGGGGAACGTGGCTTAACGGCAGAAGTACATCAACAAATGATGGCCAAATGGGGTTTAGATTTACCTCTTTATCAGCAATATTTCCACTAATATT F А T. F WSLLGGT TLGT ТΑ А v К ĸ р SW - **T** S н T 37 E F W I D T P T G F M L I D S W L S G V S G A F E N A V K S L I L 601 GAATTTTGGATGATGATAGCAACAGAATTATGGTTATGATAGCTGGCTTTCAGGGGGTTTCTGGGGCTCTGAAAATGGGGGGAAAATCCGTGGAAATCCTTAATTGATAGCTGGTATGGACGGGGAGGTTCCTGGGAGGGGAGGATTACGTCGCACGGCAAA 701 CTGCAATAGTCCTAGGCACGGTCCCTCCGCCCATTATTACGCGTATGGCAGGAGTCTGCAATGCCTGGAATGCCTGGAATGCCTGGGAAGTTACGTCGCACGGCAAA A K G L S Y T R I V I V H A L R N A L I P V V T V V G L I V A Q L 801 AGCAAAAGGTTTAAGCTATACTCGAATTGTTATTGTTGTCACGCATTGCGTAATGCGTTAATTCCTGTTGTCACTGTGGTCGGGTTAATTGTAGCTCAATTG 1001 1101 GAAATTATGTCTGACACGCCTTTAACTTCGCGCCAAAAACACCCTTACAAGAATTTGGTTTACTTTAAACAAAATCGTGGTGGGCGCTAATTGGGTTAA I F I L I V A L I S I L A P Y I A P F D P T E Q N R T A L L L P P A 1201 TTTTTATTTGATGTTGTCGCTTAATCAGCATTCTCGCGCCTTTATATGCACCTTTTGATCCAACAGAACAAAATCGTACCGCACCTTTTACTGCCACCGCG A G F I I V L L S C A F G T S L G L I S G Y Y G G V L D T I I I R 1401 GCGGGATTTACCATCGTATTACTATCTTGCGCCTTTGGTACCAGCCTTGGCTTGATTTCTGGCGCGCGGCGTGCTAGATCAATTATCATCGCC L I D I M L A I P N L L T I V V V S I L E P S L A N A T L A I A V 1501 TAATTGATATTATGCTCGGAATTCGAAACTTACTTATTGACGATGCTCTATTTTAGGGCCATCGCCATGCGACTTAGGGATGCGCAT V S I P S Y V R L T R A M M N E K N R D Y V T S S K V A G A G I 1601 GGTATCGATTCCTAGCTATGTGGGTTTAACTCGAGGAGGATGAAAAAAATGTGGATTATGTGACTTCATCTAAAGTGGCAGGGGGGAGGATAT L R L M F I V I L P N C L A P L I V Q M T M G I S N A I L E L A T 1701 TTACGCCTCATGTTTATTGTGATTTTACCAAACTGTTTAGCCCCTTTAATTGTGCAAATGACTATGGGAATATCCAATGCTATCTTAGAACTCGCGACAC 2201 ATCCTTGCAATTTGAAAATACTGATTTACTCACTTTAGAGAGTAAAGCTAAACGCCAGCTTATTGGTGCTGATGGTAGCAATGATTTTCCAAGAATCCCGAGG T S L N P A Y T V G F Q I M E A L K T H E G G T K K A R K D R T L 2301 ACAAGTTTGGATCCTGCTTATACTGTGGGCTTCCAAATTATGGAAGCGTTAAAAACTCACGAAGGCGGCACAAAAAAGGCGAGAAAAAGGCCGCACTTTAG 2010 GUARTIGETECTRACTIGETECTRACTIGETECTRACTIC TO CONTROL CONTRUCTURA CONTROL CONTROL CONTROL CONTROL CONTRUCTURA CONTROL CONT L E S L Q G V V P G K Y D R P T G C L L N P R C P Y A T E Y C R Q 2801 CTTAGAATCATTACAAGGGGTCGTCCCTGGAAAATACGATCGCCCAACAGGCTGTTTATTAAATCCTCGCTGCCCTATGCCACCGAATATTGTCGCCAA DppF Е нн IGSRKVKCHTPLNEO GN Р VE G $\begin{array}{cccccccc} 1 & V & F & Q & N & P & X & S & L & N & P & K & K & I & G & S & I & L & E & P & L & I & I & N & K & L & S & K \\ 3301 & AATAGOTITTCAAAAATCCTTACGCATCGTTAAATCCACGCCAAAAAGATTGGTTCAATTTTGGAAGAACCATTAATCATCAATAACTTTCCGCCAAA & F & F & Y & D & R & K & V & S & M & E & K & V & G & L & R & A & E & F & Y & D & R & Y & P & M & F & S & G & Q & R \\ 3401 & GAACGTCGAGAAAAAAAAGTTTTATCGATGATGAAAAAAGTGGGGCTTGCGCCCTGAATTTTTATGATCGTTATCCTCAAGGCGGACAACGCAACGCCAAC$ S P N L R R E R I K L T G E L P S P I N P P K G C A F N P R C W K A 3801 CGCCAAACTTCGCCGTGAGCGTATAACGAGGGGAATAACCAAGCGGATGATCCTCCAAAGGGTGTGCATTAATCCTCGTTGTGGAAAGC T E K C R E N Q P H L E Q H T D G K L I A C F H I D * 3901 GACAGAAAAATGCGTGGAAAATCAACCGCACTTGGAGGCAACATACTGATGGAAAATTAATCGCTTGTTTCCATATTGATTAAATTCATTTGTCAGATTT 4001 ATAAATAGGCTCATTTTTTTCTCTCGCCAATAGGTTGCAAAATCGAGGTTTTCCACTATTCGTAATGCCTCGATATTTATAAGTAATCACAGATCCTATCGG URF1 * K K E R W Y T A F R P K G S N T I G R Y K Y T I V S G I P 4101 AGGGGGATTTTCTCGTTCGTTTAAATTAAAACCCGAACCGATTTTAAAATCTCCCCCGATGATTTTTACAAGTTAAAGCACCCCATCACATTTTCAAATTGT $\begin{array}{c} \text{ FP } \text{ N } \text{ F } \text{ R } \text{ E } \text{ N } \text{ L } \text{ N } \text{ F } \text{ G } \text{ S } \text{ G } \text{ I } \text{ K } \text{ F } \text{ E } \text{ G } \text{ R } \text{ H } \text{ N } \text{ K } \text{ C } \text{ T } \text{ L } \text{ A } \text{ G } \text{ M } \text{ V } \text{ N } \text{ E } \text{ F } \text{ Q } \\ \text{ 4201 CCTTTGCCTTTATGGTGCAATAACCGTACATTCTTCACCTCGTGCTGTTTTCAATTTTTAAATTTTGTGAGCTTCGCTTACGTTCATAAGGTGCATTAG \\ \text{ G } \text{ K } \text{ G } \text{ K } \text{ H } \text{ A } \text{ I } \text{ V } \text{ T } \text{ C } \text{ E } \text{ G } \text{ R } \text{ A } \text{ T } \text{ K } \text{ L } \text{ K } \text{ L } \text{ I } \text{ Q } \text{ S } \text{ S } \text{ R } \text{ K } \text{ R } \text{ E } \text{ Y } \text{ P } \text{ N } \text{ P } \\ \text{ 4301 GCTTTGCCACACACCGCCTCGCCTTGCGAATTTCTGCACTTGTCGAAGGAAATTGATATAATGGTGCATTAGTTTTAACTTTAACAGAGTGCATTAGTATATTCGACTTGCGACAGAGAAATTGATATAATAGTTTTAACAGGTGCATTAGCTAAAGTTGCTACAAGCAGCAATTGGTACGAACAAGCGCCTTAACGTTTAACCAGGAATTGGTACAATTGTTCGACTTGCGACGAGGAAATTGATATAATGGTTTAACTTTAACAGGAATTGGTCAATATTCC \\ \text{ N } \text{ R } \text{ V } \text{ V } \text{ V } \text{ G } \text{ E } \text{ G } \text{ Q } \text{ L } \text{ N } \text{ E } \text{ V } \text{ Q } \text{ A } \text{ L } \text{ F } \text{ Q } \text{ Y } \text{ L } \text{ H } \text{ T } \text{ K } \text{ D } \text{ K } \text{ V } \text{ P } \text{ I } \text{ Q } \text{ E } \text{ I } \text{ I } \\ \end{array}{}$ N R V V V G E G Q L N E V Q A L F Q Y L H T K D K V P I Q E I I E 4401 AATATAAGTIGTAGAATGTCTCAAGTAAAAGGGCITTTAATTAAGTAAACGCACATCAAAAGCATGCACACGACAACAAAAGACATACAATTC I Y T T P H E L L H A K L K A L R E F L N G E A D P V D F V Y L 4501 AGTITTTCCCAACCATCGCCTTTAAAAGATTTGTGATGGTCGAAATTCCTCAAAAGATTTCGCCACAAAAAGACGC L K E W G D G K F S K T I T S I E E F H N R E S F L E G D I A F P P 4601 GAAAATCCTTAATGAAATAAGCTGGCGGCGATAAACGTTGCCCTTGTCGGGGTTAATAATTGCTTTCCAATAACCGCGTACACCATCTAACTTTTC F D K I F Y A P P S L R Q G Q R T L L Q K G N W Y G R V G D L K E F D K I F Y A P P S L R Q G Q R T L L Q K G N W Y G R V G D L K E 4701 AGACATTACCCAACCCTCTATGGGTTGATTATATGGTATGAAGAAGCATTAAATCTGAGTTAGCAAAAGCAAAAGAACTAGCAAAAGAAAAGTAACAAA S M V W G E I P Q N N Y T H L L M L D S N A F A F S S A F F L L L 4801 GTGCGGTAAAATTTCATTGTGTTTTTGAAAATTAAAGGGCTAAGAATTTACGTAAGATAACATATCTTGAAAAATCCCCCAACTACTCATATTTTT -35 -10 +1 Isn Wild-type M Y L D Q I K S E L V E A Q D V L N K F I S D E N N (ATGTATTTAGATCAAATCTGAACTTGTGGAAGCACAAGATGTGTTAAACAAATTTATTCTGACGAAAATAAC)

Mutant М

I R V P H F R Y A D R I Q E I H I K V I H I L M M L I E F E M A K Q 5501 TTCGCGTACCCCATTTCCGTTATGCAGATCGTATTCAAGAAATCCATATCAAAGTTATTCAATTTTAATGAGTTTGAAATGGCAAAACA од * 5601 адосталадтяссадттататаладатттта<u>алатоссаалатттоттаддаттт</u>тттатталадоасттвоаталаттатосаталадатвтал

Terminator ArtP M A I R V K N L N F F Y G S S Q T L FD N G E LSIANNEFNLSNAMANPK А т 0 0 L R D 5901 TGGTGAATTAAGTATTGGAAATAATGAATTTAATTTATCCAATGGCAATGGCAAATCCCAAAGCAATCCAACAATTACGCCAAAGATGTAGGAATGGTGTTT Q Q Y H L W P H L T V I E N L I E A P M K V R G V S E N E A K T D 6001 CAACAATATCATCTTTGGCCGCACTTTAACGGTAATTGAAAACCTAATTGAAGCACCAATGAAAGTGCGTGGTGTGAGGAAAATGAAGCGAAAACCGATG E T G I T Q V I V T H E V N V A Q K V A T K V V Y M E Q G K I V E 6301 GAAACTGGCATTACGCAAGTGATTGTGACCCACGAAGTCAATGTGGCGCAAAAAGTGGCGACAAAAGTCGTCTATATGGAACAAGGTAAAATCGTCGAAA M G S A D C F E N P K T E Q F K H Y L S H * **Arti/j** m K K T L L 6401 TGGGATCGGCTGATTGCTTTGAAAAACCGAAAACGGAACAATTAAACACTATCTTCTCACTAGAAAAAAAGGAGCTTACGATGAAAAAAACATTATTA 7001 GCTCGCCGATATGATTTCCAAAGAGCCAGAAATTCAATTTATAGGCGAAAAAGTGACCAATAAAAAATATTTTGGTAATGGTTTAGGTATTGCAATGCAC K S N K D L A A Q L N K G L A A I K A N G E Y Q K I Y D K W I T K 7101 AAATCAAAGAATTTGGCTGCACAATTAAAGAAAGGTTTAGCTGCAAATAAAGGCAAATGGCGAATACCAAAAAATCTATGATAAATGGATAACAAAAA 7401 GTCTATTTTGGTTCTACTGAATTAGTAGAAATGCTGACAGGAGAATACATTGAATTTGGGGCATTTGGGCGTGGCGTGGCGTTGCGCGTTTCTCTTATTTTTG A A Y A S Q T L R G A I Q A I P K G Q W E S G A A L G L S K S Y T F 7501 CAGCTTATGCTTCACAAACATTACGCGGTGCAATTCAAGCCATACCAAAAGGACAATGGGAATCAGGTGCAGCGTTAGGCTTAAGCAAAAGCTATACGTT ArtM * M F H G A V K A I P K G Q W E S C G A L G L N R I Q T L K I L I P Y A 8201 CACGGTGCAGTGAAAGCCATTCCCAAAGGACAATGGGAAAGTTGTGGGCGCATTAGGATTAAACGGCATACAAACTTTAAAAATTTTAATTCCTTATGCCT L K R A L P S Y S N E I L V F K G T A L A S T I T I M D I M G Y A 8301 TAAAACGGCCTTGCCTTTCTACAGTAAGGAAATTATTTGGAATTATAAGCCACCATTAGGATCAGGATTATCGGATTATGGGTATTATGGGTCGCT C Q L Y G T E Y D A L T I Y G I A G G I Y L I I T G I A T L L R Q L Y G T E Y D A L T I Y G I A G G I Y L I I T G I A T L L L R 8401 ACGCCAACTCTATGGTACAGAATATGATGCACTCACAATTTACGCCATTGCGGGTGGTATCTATTTAATTATCACAGGCATTGCAACATTATTACTACGT K L E K K V L A F E R F E V S K A * 8501 AAATTAGAGAAAAAAGTATTGGCATTTGAACGTTTTGAAGTAAGCAAAAGTAGCGGGTAAAATTTTTAGTGATTTTTAAATCCCCAATCTCCGGTTGG $\begin{array}{c} & * & F & G & Y & G & V & G & V & K \\ & * & F & G & Y & G & V & G & V & K \\ & * & G & Y & N & V & K & T & S & D & F & N & G & L & R & N & Y & E & V & G & G & N & A \\ \hline \\ & 9301 & GCTACAACCCCATATCCGAGCTATACAAAATTACCGAAGAACATTTTAACTCAACCCACCACCAACTATGCGTACCAGGTGCCATCTAACTTATATTTTGCACCT \\ & 9301 & GCTACAACCCCATATCCGAGCTTAATATCCAGAAGAACTTTTAAACTTTTTGTCTGCGCGATTGGTGTATTTAAATTGATTAGTCGCCACCACGAGCACCAA \\ & V & V & G & Y & G & L & K & I & D & S & S & K & F & K & Q & E & A & R & N & T & Y & K & F & Q & N & T & A & V & R & A & G & V \\ & 9401 & CATAGAGGTTGCATTTAGAGCTTTTGAATTG & 9423 & & & & & Y & P & Q & V & K & S & N & OpaH \\ \end{array}$

FIG. 2. DNA sequence of the 9.4-kb EcoRI fragment of p700. Both wild-type and mutant DNA sequences are shown at the site of the I69 mutation (see below). See text for explanation of labels. URF1 lies in the opposite orientation to the other ORFs in the locus, and its translation is shown below the relevant DNA sequence. +1, -10, and -35 refer to the coordinates of the transcriptional start site (+1) and proposed RNA polymerase-binding sites of isn as determined by primer extension (see below). Terminator, putative rho-independent transcriptional terminator as determined by the Terminator program of the GCG computer package.

1B). Thus, the I69 mutation was mapped to the 1.1-kb NruI-PvuI fragment of p717.

gonorrhoeae) (Table 1). The unidentified reading frame is referred to as URF1.

DNA sequencing of p700. The DNA sequence of the 9.4-kb EcoRI restriction fragment was determined on both strands (Fig. 2) and translated in all six frames. Eleven putative open reading frames (ORFs) were observed, of which 10 were homologous to previously identified protein sequences, namely, DppB, DppC, DppD, DppF, ArtP, ArtI, ArtQ, ArtM (all of E. coli), LmbN (Streptomyces lincolnensis), and OpaH (Neisseria

Identification of the I69 mutation. The region of DNA corresponding to the 1.1-kb NruI-PvuI fragment of p717, previously identified as containing the I69 mutation, was amplified from wild-type H. influenzae RM132, RM7004, RM156, and RM142 by PCR, and the DNA was cloned and sequenced. The wild-type DNA sequences were compared with the mutant DNA sequence (bases 4957 to 6217 [Fig. 2]), and several dif-

TABLE 1. Identity and similarity between ORFs of p700 and protein sequences from the GenBank and EMBL databases^a

ORF	% Identity	% Similarity
DppB	60	80
DppC	62	81
DppD	72	82
DppF	74	81
LmbN	30	51
ArtP	66	78
ArtI/J	48	66
ArtO	58	79
ArtM	56	79
OpaH	31	54

^a Identified by the Blast Network service at NCBI.

ferences were observed. However, only one of these differences was observed in comparisons with all the wild-type strains. A single-base-pair deletion was observed at position 5030 in the mutant DNA within the LmbN-homologous ORF, which would result in the termination of translation of this ORF after the second codon (Fig. 2). The occurrence of this deletion as the only consistent difference between the wild-type strains and the mutant locus in this region of DNA suggested that the deletion was the I69 mutation.

Identification of the deletion in multiple independent 169 phenotype cells. The association of the I69 phenotype with the deletion was tested. DNA spanning the site of the deletion was amplified by PCR from four independent I69 clones and sequenced directly, without cloning. All four clones had the single-base-pair deletion (data not shown). This association of the I69 phenotype and the deletion in four independent transformants supported the hypothesis that the deletion constituted the I69 mutation. We have named the LmbN-homologous ORF *isn* (i-sixty-nine).

Mutational analysis. To confirm that mutation of isn results in the I69 phenotype, a defined insertion mutation was constructed in the isn ORF and introduced into the wild-type H. influenzae chromosome. A kanamycin resistance (kan-R) gene was cloned in both orientations into the NsiI restriction site in the wild-type isn coding region (position 5200 [Fig. 2]). The vector containing these constructs was pGex3X, which is a suicide vector in H. influenzae. Thus, on transformation of these constructs into *H. influenzae* as linear DNA, the vast majority of kanamycin-resistant colonies will result from double homologous recombination events, leading to the replacement of the wild-type isn allele on the chromosome with the mutated allele. In the experiments reported here, Kan^r transformants were recovered when the kan-R gene was in the same orientation as the isn ORF but not when it was in the opposite orientation. The reason for this was unclear.

Thousands of Kan^r colonies were generated on transformation of *H. influenzae* RM132, and all of them displayed the opaque I69 colony phenotype. Of these, two were picked for further analysis. Southern hybridization confirmed that they had the expected chromosomal rearrangement, with the kan-R gene inserted into the *Nsi*I site in *isn* (data not shown). On SDS-PAGE, the LPS from these transformants comigrated with that of *H. influenzae* RM4066 (Fig. 3), confirming that the insertion mutation in *isn* had reproduced the I69 phenotype. This experiment confirms that mutation of *isn* results in the I69 phenotype.

RNA analysis. Northern blot analysis demonstrated that *isn* was transcribed as a monocistronic message of approximately 700 nucleotides in length (Fig. 4). A single transcriptional start



FIG. 3. SDS-PAGE of LPS prepared from two *isn* insertion mutants (lanes 1 and 2) and *H. influenzae* RM132 (lane 3), Eagan (a wild-type strain, lane 4), and RM4066 (I69) (lane 5). The LPS of the *isn* insertion mutants comigrated with that of the I69 strain RM4066 and consisted of the fast-migrating band characteristic of I69 LPS, confirming that mutation of *isn* resulted in the I69 phenotype.

site 28 bp upstream of the initiation codon (Fig. 2) was mapped through primer extension (Fig. 5). A potential rho-independent transcriptional terminator lies 700 bp downstream of the transcriptional start site (Fig. 2). *isn*-specific mRNA was detected in both wild-type and I69 *H. influenzae*, indicating that the I69 mutation does not affect transcription of *isn*. Northern blot analysis of the Art-homologous ORFs and URF1 failed to detect signals from these ORFs.

DISCUSSION

We have cloned a novel gene, *isn*, the mutation of which causes the I69 phenotype. The function of *isn* is unknown. However, the predicted amino acid sequence of the *isn* gene product is homologous to LmbN of *S. lincolnensis*, which is required for the biosynthesis of the antibiotic lincomycin. Lincomycin contains an eight-carbon sugar, methylthiolincosamide, which is formed via a dTDP-6-deoxyhexose pathway. LmbN is probably required for the biosynthesis of this sugar (28). The significance of the homology between the Isn and LmbN amino acid sequences is unclear but may indicate that Isn interacts with the eight-carbon sugar of LPS, namely, KDO.

The LPS of I69 *H. influenzae* consists of lipid A and a single molecule of phosphorylated KDO (unlike the LPS of enteric bacteria, *H. influenzae* LPS contains only a single KDO residue) but lacks the other sugar residues that are present in wildtype *H. influenzae* LPS. Thus, *isn* is required for LPS biosynthesis after completion of the lipid A-KDO unit. *H. influenzae*



FIG. 4. Autoradiograph of Northern blot of RNA prepared from *H. influenzae* RM132 (wild type) (lane 1), an I69 transformant of RM132 produced through transformation with p715 (lane 2), and RM7004 (wild type) (lane 3), hybridized with a probe directed against *isn*.



FIG. 5. Results of primer extension experiments. Two primers were synthesized to regions of *isn* corresponding to the reverse and complement of positions 5053 to 5070 (primer 1) and 5116 to 5139 (primer 2) (see Fig. 2). These primers were annealed to RNA prepared from an I69 phenotype transformant of *H. influenzae* RM132 produced by transformation with p715 (lanes 1 and 2) and from *H. influenzae* RM132 (lanes 3 and 4). Primer 1, 5'CACATCTTGTGCTTC CAC3' (lanes 1 and 3); primer 2, 5'ATTCGAAATTAATAATGCCGCTTC3' (lanes 2 and 4). The primer extension reactions were then run alongside DNA sequence reactions primed with the corresponding primer. The DNA sequence is loaded in the order GATC. These experiments indicated a single transcriptional start site for *isn* at position 4996 of the sequence shown in Fig. 2, indicated in this figure as the underlined C.

LPS contains L-glycero-D-mannoheptose linked through KDO to lipid A. Thus, an obvious function for *isn* is involvement in either synthesis of the heptose donor molecule, ADP-L-glycero-D-mannoheptose, an area about which little is known (9, 11, 12, 17, 33, 34), or transfer of the heptose residue from the donor molecule to the lipid A-KDO. The *rfaC* and *rfaF* genes, whose products are the heptosyltransferase enzymes of LPS biosynthesis in *H. influenzae*, have been cloned (6), and thus *isn* does not encode the heptosyltransferase function.

We have identified the 169 mutation as a single-base-pair deletion of *isn* which is predicted to cause premature translational termination of *isn*. Allelic replacement mutagenesis of *isn* confirmed that loss of *isn* gene function caused the I69 phenotype.

isn is situated next to and divergent from URF1. Attempts to mutate URF1 in *H. influenzae* were unsuccessful, and assignment of a function to URF1 is not possible.

isn and URF1 lie between two ABC transport systems. Adjacent to URF1 are ORFs which are homologous to those of the dipeptide permease (Dpp) of E. coli, which is involved in peptide transport and chemotaxis (1). In E. coli, a further ORF, dppA, lies upstream of dppB. A dppA homolog may lie upstream of the 9.4-kb clone analyzed here, but this has not been identified. The very high degree of homology between the amino acid sequences of DppB-F of E. coli and the Dpphomologous ORFs identified here (Table 1) suggests that the 9.4-kb EcoRI fragment may in part encode H. influenzae dipeptide permease. Adjacent to isn are ORFs which are homologous to the arginine permease (Art) of E. coli (43). However, E. coli art contains two genes encoding periplasmic components, artI and artJ, whereas the system adjacent to isn in H. influenzae contains only one. In E. coli, ArtJ binds arginine with high affinity, but despite its high homology to ArtJ, ArtI does not bind arginine or any other amino acid (37). It is possible that ArtI binds some other biological amine. Attempts to mutate the H. influenzae Art-homologous system were unsuccessful, and thus assignment of its function was not possible. Identifying the ligand of the periplasmic binding component,

which is highly homologous to both ArtI and ArtJ of *E. coli*, would be very likely to identify the function of this system.

isn is not transcriptionally linked to the ORFs adjacent to it, but this does not preclude the possibility of a functional link between them.

Thus, at present, the precise function of *isn* is unknown.

ACKNOWLEDGMENTS

This work was supported by Medical Research Council grant G8325352. A.P. was supported by Medical Research Council Graduate Studentship 400956.

REFERENCES

- Abouhamad, W. N., M. Manson, M. M. Gibson, and C. F. Higgins. 1991. Peptide transport and chemotaxis in *Escherichia coli* and *Salmonella typhi-murium*: characterization of the dipeptide permease (Dpp) and the dipeptide-binding protein. Mol. Microbiol. 5:1035–1047.
- Abu Kwaik, Y., R. E. McLaughlin, M. A. Apicella, and S. M. Spinola. 1991. Analysis of *Haemophilus influenzae* type b lipooligosaccharide-synthesis genes that assemble or expose a 2-keto-3-deoxyoctulosonic acid epitope. Mol. Microbiol. 5:2475–2480.
- Abu Kwaik, Y., R. E. McLaughlin, M. A. Apicella, and S. M. Spinola. 1992. Cloning and analysis of lipooligosaccharide synthesis genes of *Haemophilus influenzae* type b that assemble or expose a 2-keto-3-deoxyoctulosonic acid epitope in *Escherichia coli*. J. Infect. Dis. 165(Suppl.):S195–S196.
- Alexander, H. E. 1965. The Haemophilus group, p. 724–741. In R. J. Dabos and J. G. Hirschs (ed.), Bacterial and mycotic infections of man. Pitman Medical Publishing Co. Ltd., London.
 Anderson, P., R. B. J. Johnston, and D. H. Smith. 1972. Human serum
- Anderson, P., R. B. J. Johnston, and D. H. Smith. 1972. Human serum activities against *Haemophilus influenzae*, type b. J. Clin. Invest. 51:31–38.
 Apicella, M. A. Personal communication.
- 7. Birnboim, H. C., and J. Doly. 1979. A rapid alkaline extraction procedure for
- Billionin, H. C., and J. Dor, DYN Prapid and an extraction proceeder for screening recombinant plasmid DNA. Nucleic Acids Res. 7:1513.
 Catlin, B. W., J. W. Bendler, and S. H. Goodgal. 1972. The type b capsulation locus of *Haemophilus influenzae*: map location and size. J. Gen. Microbiol. 70:411-422.
- Chen, L., and W. G. J. Coleman. 1993. Cloning and characterization of the Escherichia coli K-12 rfa-2 (rfaC) gene, a gene required for lipopolysaccharide inner core synthesis. J. Bacteriol. 175:2534–2540.
- Cope, L. D., R. Yogev, J. Mertsola, J. L. Latimer, M. S. Hanson, G. J. McCracken, and E. J. Hansen. 1991. Molecular cloning of a gene involved in lipooligosaccharide biosynthesis and virulence expression by *Haemophilus influenzae* type b. Mol. Microbiol. 5:1113–1124.
- Eidels, L., and M. J. Osborn. 1971. Lipopolysaccharide and aldoheptose biosynthesis in transketolase mutants of *Salmonella typhimurium*. Proc. Natl. Acad. Sci. USA 68:1673–1677.
- Eidels, L., and M. J. Osborn. 1974. Phosphoheptose isomerase, first enzyme in the biosynthesis of aldoheptose in *Salmonella typhimurium*. J. Biol. Chem. 249:5642–5648.
- Helander, I. M., B. Lindner, H. Brade, K. Altmann, A. A. Lindberg, E. T. Rietschel, and U. Zahringer. 1988. Chemical structure of the lipopolysaccharide of *Haemophilus influenzae* strain I-69 Rd-/b+: description of a novel deep-rough chemotype. Eur. J. Biochem. 177:483–492.
- Herriott, R. M., E. M. Mayer, and M. Vogt. 1970. Defined non-growth media for stage II development of competence in *Haemophilus influenzae*. J. Bacteriol. 101:517–524.
- High, N. J., M. E. Deadman, and E. R. Moxon. 1993. The role of a repetitive DNA motif (5'-CAAT-3') in the variable expression of the *Haemophilus influenzae* lipopolysaccharide epitope αGal(1-4)βGal. Mol. Microbiol. 9: 1275–1282.
- Jesse, J. 1984. Tools for the molecular biologist. BRL pUC host: *E. coli* DH5α competent cells. Focus 8:9–11.
- Lee, N., M. G. Sunshine, and M. A. Apicella. 1995. Molecular cloning and characterization of the NTHI 2019 *rfaE* gene required for LPS biosynthesis. Infect. Immun. 63:818–824.
- Lesse, A. J., A. A. Campagnari, W. E. Bittner, and M. A. Apicella. 1990. Increased resolution of lipopolysaccharides and lipooligosaccharides utilising tricine-sodium dodecyl sulphate-polyacrylamide gel electrophoresis. J. Immunol. Methods 126:109–117.
- Makino, S., P. J. Van, and T. F. Meyer. 1991. Phase variation of the opacity outer membrane protein controls invasion by *Neisseria gonorrhoeae* into human epithelial cells. EMBO J. 10:1307–1315.
- Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Maskell, D. J., M. J. Szabo, P. D. Butler, A. E. Williams, and E. R. Moxon. 1992. Molecular biology of phase-variable lipopolysaccharide biosynthesis by *Haemophilus influenzae*. J. Infect. Dis. 165(Suppl.):S90–S92.
- 22. McLaughlin, R., S. M. Spinola, and M. A. Apicella. 1992. Generation of

lipooligosaccharide mutants of *Haemophilus influenzae* type b. J. Bacteriol. **174:**6455–6459.

- 23. Miller, J. H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- 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.
- Moxon, E. R., and J. S. Kroll. 1988. Type b capsular polysaccharide as a virulence factor of *Haemophilus influenzae*. Vaccine 6:113–115.
- Moxon, E. R., and D. J. Maskell. 1992. *Haemophilus influenzae* lipopolysaccharide: the biochemistry and biology of a virulence factor, p. 75–96. *In* C. E. Hormaeche, C. W. Penn, and C. J. Smyths (ed.), Molecular biology of bacterial infection: current status and future perspectives. Cambridge University Press, Cambridge.
- Moxon, E. R., and K. A. Vaughn. 1981. The type b capsular polysaccharide as a virulence determinant of *Haemophilus influenzae*: studies using clinical isolates and laboratory transformants. J. Infect. Dis. 143:517–524.
- Peschke, U., H. Schmidt, H.-Z. Zhang, and W. Piepersberg. 1995. Molecular characterization of the lincomycin-production gene cluster of *Streptomyces lincolnensis* 78-11. Mol. Microbiol. 16:1137–1156.
- Phillips, N. J., M. A. Apicella, J. M. Griffiss, and B. W. Gibson. 1992. Structural characterization of the cell surface lipooligosaccharides from a nontypable strain of *Haemophilus influenzae*. Biochemistry 31:4515–4526.
- Phillips, N. J., M. A. Apicella, J. M. Griffiss, and B. W. Gibson. 1993. Structural studies of the lipooligosaccharides from *Haemophilus influenzae* type b strain A2. Biochemistry 32:2003–2012.
- Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5463–5467.
- Schagger, H., and G. Von Jagow. 1987. Tricine-sodium dodecyl sulphatepolyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100KDa. Anal. Biochem. 166;368–379.
- Schnaitman, C. A., and J. D. Klena. 1993. Genetics of lipopolysaccharide biosynthesis in enteric bacteria. Microbiol. Rev. 57:655–682.
- 34. Sirisena, D. M., K. A. Brozek, P. R. MacLachlan, K. E. Sanderson, and C. R.

Raetz. 1992. The *rfaC* gene of *Salmonella typhimurium*: cloning, sequencing, and enzymatic function in heptose transfer to lipopolysaccharide. J. Biol. Chem. 267:18874–18884.

- 35. Szabo, M., D. Maskell, P. Butler, J. Love, and R. Moxon. 1992. Use of chromosomal gene fusions to investigate the role of repetitive DNA in regulation of genes involved in lipopolysaccharide biosynthesis in *Haemophilus influenzae*. J. Bacteriol. 174:7245–7252.
- Turk, D. C. 1984. The pathogenicity of *Haemophilus influenzae*. J. Med. Microbiol. 18:1–16.
- 37. Unden, G. Personal communication.
- van Alphen, L., T. Riemens, J. Poolman, C. Hopman, and H. C. Zanen. 1983. Homogeneity of cell envelope protein subtypes, lipopolysaccharide serotypes, and biotypes among *Haemophilus influenzae* type b from patients with meningitis in the Netherlands. J. Infect. Dis. 148:75–81.
- Weel, J. F., C. T. Hopman, and J. van Putten. 1991. In situ expression and localization of *Neisseria gonorrhoeae* opacity proteins in infected epithelial cells: apparent role of Opa proteins in cellular invasion. J. Exp. Med. 173: 1395–1405.
- Weiser, J. N., A. A. Lindberg, E. J. Manning, E. J. Hansen, and E. R. Moxon. 1989. Identification of a chromosomal locus for expression of lipopolysaccharide epitopes in *Haemophilus influenzae*. Infect. Immun. 57:3045–3052.
- Weiser, J. N., J. M. Love, and E. R. Moxon. 1989. The molecular mechanism of phase variation of *H. influenzae* lipopolysaccharide. Cell 59:657–665.
- Weiser, J. N., D. J. Maskell, P. D. Butler, A. A. Lindberg, and E. R. Moxon. 1990. Characterization of repetitive sequences controlling phase variation of *Haemophilus influenzae* lipopolysaccharide. J. Bacteriol. 172:3304–3309.
- Wissenbach, U., B. Keck, and G. Unden. 1993. Physical map location of the new artPIQMJ genes of Escherichia coli, encoding a periplasmic arginine transport system. J. Bacteriol. 175:3687–3688.
- 44. Zwahlen, A., L. G. Rubin, C. J. Connelly, T. J. Inzana, and E. R. Moxon. 1985. Alteration of the cell wall of *Haemophilus influenzae* type b by transformation with cloned DNA: association with attenuated virulence. J. Infect. Dis. 152:485–492.