INFECTION AND IMMUNITY, Aug. 1993, p. 3334–3341
0019-9567/93/083334-08$02.00/0

Vol. 61, No. 8

Immunological Properties of Recombinant Porin of *Haemophilus influenzae* Type b Expressed in *Bacillus subtilis*

RAMAKRISHNAN SRIKUMAR,[1] DAVID DAHAN,[1] MARIE FRANCE GRAS,[1] LEENA SAARINEN,[2] HELENA KÄYHTY,[2] MATTI SARVAS,[2] LIESBETH VOGEL,[3] AND JAMES W. COULTON[1,2]*

Department of Microbiology and Immunology, McGill University, Montreal, Quebec, Canada H3A 2B4[1]; National Public Health Institute, SF-00300 Helsinki, Finland[2]; and Department of Medical Microbiology, University of Amsterdam, NL-1105 AZ Amsterdam, The Netherlands[3]

Received 11 March 1993/Accepted 11 May 1993

The major surface-located, channel-forming protein in the outer membrane of *Haemophilus influenzae* type b (Hib) is porin (341 amino acids; M_r, 37,782). In order to generate Hib porin that is devoid of lipooligosaccharides and capsular polysaccharide, the Hib porin gene *ompP2* was subcloned into a plasmid vector and recombinant Hib porin was expressed in *Bacillus subtilis*. Recombinant porin was produced in large quantities in *B. subtilis* and formed intracellular inclusion bodies. Recombinant porin was extracted from inclusion bodies and shown to be active in forming pores in synthetic black lipid membranes. However, these pores demonstrated different pore characteristics than wild-type Hib porin. Mouse hyperimmune sera against recombinant porin were generated and subjected to epitope scanning with a library of 336 overlapping synthetic hexapeptides that corresponded to the entire sequence of Hib porin. The epitope specificities of the anti-recombinant porin antibodies were similar to those of antibodies against Hib porin: selected regions near the amino terminus which include a buried loop in the native structure of Hib porin were more immunogenic than regions at the carboxy terminus. Although some mouse anti-recombinant porin antibodies mediated complement-dependent binding to Hib by polymorphonuclear leucocytes in opsonophagocytosis assays, the antibodies were not bactericidal, nor did they abrogate bacteremia in the infant rat model of infection. It was concluded that the native state of Hib porin is required for the generation of a protective immune response against the bacterium.

The outer membrane of gram-negative bacteria, including *Haemophilus influenzae* type b (Hib), contains proteinaceous water-filled pores, termed porins, that allow the diffusion of solutes up to a defined molecular mass (17, 19). For the well-characterized OmpF porin of *Escherichia coli* K-12, the molecular mass exclusion limit is about 600 Da (18). By comparison, the value for the nonspecific porin of Hib is 1,400 Da (34), suggestive of some difference in the molecular architecture and perhaps the lumen of the pore. The structural features which contribute to this difference are unknown.

By calculating the hydrophobicity, amphiphilicity, and turn propensity of the Hib porin sequence (8, 16), a model for the topological organization of Hib porin was proposed (30). In the absence of spectroscopic or X-ray crystallographic data for Hib porin, we adopted other methods to provide direct experimental evidence for the topological organization of Hib porin. We generated monoclonal antibodies (MAbs) against Hib porin and characterized their ability to bind to whole cells (29). To define the epitope specificities of the MAbs, overlapping hexapeptides of the entire Hib porin sequence were synthesized and subjected to epitope scanning. The molecular reactivities of binding of the MAbs provided support for the orientation of some loops in the structural model (30). Verification of our structural model for Hib porin was recently provided by the molecular analysis of porin sequences from three nontypeable *H. influenzae* strains (26).

The immunological responses elicited against surface macromolecules of gram-negative bacteria constitute important determinants of the host's defenses (3). Because porins are major surface-located proteins of gram-negative bacteria, they are considered primary targets for immune recognition (15). Antibodies specific for noncapsular surface components of Hib apparently contribute to humoral defense mechanisms. An often-cited observation (6) is that antibodies against noncapsular antigens were able to protect against experimental infection, as assessed by the infant rat model of bacteremia. One of these protective noncapsular antigens was reported to be porin, 38 kDa.

To extend our investigations on the immunological properties of Hib porin, we wished to address the following questions. (i) Does recombinant Hib porin elicit antibodies which show specificities that are similar or identical to those of antibodies against native Hib porin? (ii) Are anti-recombinant Hib porin antibodies bactericidal and protective against bacteremia? The gram-positive bacterium *Bacillus subtilis* was chosen to generate recombinant Hib porin (also called Bac porin). This organism was attractive for the expression of the *ompP2* (porin) gene for the following reasons. Recombinant porin was produced in an environment devoid of lipooligosaccharides (LOS), and therefore we were able to assess porin's function independent of these gram-negative polymers. Recombinant porin was expressed in an environment devoid of the capsular polysaccharide polyribosyl ribitol phosphate (PRP). We assayed the immune response against recombinant Hib porin and the immunological value of anti-recombinant porin (Bac porin) antibodies in three tests: opsonophagocytosis, complement-mediated Hib lysis, and immunoprotection in the infant rat model of bacteremia.

MATERIALS AND METHODS

Molecular cloning of *ompP2*. The *ompP2* gene from pACC03, 5,523 bp (29), was used as the template for polymerase chain reaction (PCR) with the following two

* Corresponding author.

synthetic 30-mer oligonucleotide primers: 5'-AAG CTT AAG CTT GCT GTT GTT TAT AAC AAC-3', corresponding to two tandem *Hin*dIII sites plus codons for the first six amino acids of the sequence (8, 16) of the mature form of Hib porin; and 5'-AAG CTT AAG CTT TTA GAA GTA AAC GCG TAA-3', corresponding to two tandem *Hin*dIII sites, the termination codon, and the penultimate five amino acids of Hib porin. The oligonucleotides were synthesized on an Applied Biosystems (Foster City, Calif.) 392 DNA Synthesizer and gel purified to remove contaminating species. PCR was conducted with AmpliTaq DNA polymerase (1.25 U), commercially prepared reagents from Perkin-Elmer Cetus, 1.0 ng of template DNA, and primers at a final concentration of 1 μM. The PCR reaction mixture was subjected to 25 cycles of denaturation (95°C, 1 min), annealing (55°C, 2 min), and extension (72°C, 3 min) with a Techne PHC-2 thermocycler (Techne Ltd., Duxford, Cambridge, United Kingdom). The PCR-generated fragment was trimmed with *Hin*dIII and then ligated at a threefold molar excess into the expression vector pKTH288 (4.5 kbp) that had also been restricted with *Hin*dIII and dephosphorylated. Plasmid pKTH288 was derived from pKTH39 (23) by insertion of a linker (5'-AAT TCG AAG CTT CG-3') at its *Eco*RI site.

The host bacterial strain was *B. subtilis* IH6140 (23), a prototrophic derivative of *B. subtilis* Marburg strain 1A298 from the Bacillus Genetic Stock Center, Department of Biochemistry, Ohio State University, Columbus. Strain IH6140 is sporulation deficient and has reduced exoprotease activity. Bacterial cells were made competent by the method of Gryczan et al. (7), transformed with 700 ng of the postligation species, and selected on plates of Luria broth containing kanamycin (10 μg/ml). The transformation frequency was 350 colonies per μg of DNA. Candidate recombinant colonies were readily identified after visual inspection of the plates by their obviously different color and morphology. These candidates were transferred to plates containing a higher kanamycin concentration (30 μg/ml) to maintain the plasmid and screened for the presence of plasmids of the anticipated size, 5.6 kbp. The methods used for manipulation of recombinant DNA were those described by Sambrook et al. (25).

Isolation of inclusion bodies. The following small-scale protocol was devised to isolate inclusion bodies which contained Bac porin. Recombinant bacteria (1.5 ml) were grown at 37°C and with strong aeration to saturation (optical density at 578 nm of 2.5) in 2× L broth containing kanamycin (30 μg/ml). Cells were harvested by centrifugation, washed twice with distilled water, suspended in 0.2 volume of 10 mM Tris-HCl (pH 8.0), and converted to protoplasts by lysozyme (0.5 mg/ml) digestion at 37°C for 30 min. The addition of DNase (10 μg/ml) and RNase (10 μg/ml) served to reduce the viscosity of the lysing protoplast suspension; phenylmethylsulfonyl fluoride (10 μM) was added to minimize proteolysis of Bac porin. The material was centrifuged (13,000 × *g*) to recover membranes plus inclusion bodies. To solubilize membranes, the pellet was extracted three times at ambient temperature for 30 min each with buffer containing a nonionic detergent and high salt: 40 mM Tris-HCl (pH 8.0), 1% Nonidet P-40, 1.0 M NaCl, and 5 mM EDTA.

Identification of proteins. Proteins were quantitated by the bicinchoninic acid assay (28) and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in gels containing 12 or 15% acrylamide (12). Proteins on gels were detected either with Coomassie brilliant blue or by silver staining (13). Alternatively, proteins in the polyacrylamide gels were transferred electrophoretically to nitrocellulose, blocked with 1% bovine serum albumin (Boehringer Mannheim Canada) in Tris-buffered saline, and reacted for 2 h at room temperature with primary antibody. After the nitrocellulose was washed free of primary antibody, the secondary antibody used was a rat monoclonal anti-mouse κ light chain (36) coupled to alkaline phosphatase. The immunoreactive conjugates bound to Hib porin were identified with the enzyme substrates 5-bromo-4-chloro-3-indolylphosphate toluidinium and nitroblue tetrazolium (Bio-Rad Laboratories).

Biophysical assays. Planar bilayer studies were completed by a modification (33) of the technique of Mueller et al. (14). Two Teflon chambers were separated by a Teflon foil having a thickness of 100 μm and a small circular aperture 600 μm in diameter. Lipid bilayers were formed across this aperture with a solution of 2.5% glyceryl monooleate (Sigma) dissolved in *n*-decane. Formation of the bilayer was monitored through a glass window on the end of one of the compartments with a light source and a microscope. Bilayer formation was indicated by the membrane's turning optically black to incident light. The porin sample was added to the aqueous phase either before membrane formation or after the membrane had turned optically black. Conductance across the membrane was measured by a fixed transmembrane potential. A pair of Ag-AgCl electrodes were inserted into solutions of 1 M KCl on both sides of the membrane. An operational amplifier (Analog Devices, Norwood, Mass.; type AD 40K) was used in a current amplifier configuration so that the flow of Cl^- ions could be recorded on a strip chart recorder.

Preparation and analysis of antibodies. Antibodies against recombinant porin were raised in the NIH strain of mice (males, 6 to 8 weeks old) by immunization of five groups of 10 animals. Each animal received a primary immunization with 20 μg of recombinant porin from inclusion bodies and then, 6 weeks later, a second immunization, also with 20 μg of recombinant porin from inclusion bodies. The materials administered to the five groups of animals differed according to whether or not Freund's complete adjuvant (FCA) was used; lipopolysaccharide (LPS) O-6,7 from *Salmonella enterica*, serovar typhimurium (21), or LOS from Hib was included with some samples.

To produce antibodies against Hib porin, New Zealand White rabbits were immunized with protein (100 μg) that was purified by the method of Vachon et al. (34). The primary immunization was done with FCA; secondary immunization was done with protein but no adjuvant. The protocols for raising antibodies were those of Harlow and Lane (9) and conformed to the guidelines of the Canadian Council on Animal Care.

The reactivity of antibodies to intact Hib or isolated porin on a solid phase was assayed by enzyme-linked immunosorbent assay (ELISA). Hib cells or purified Hib porin was adsorbed to microtiter plates (Maxisorp F96; Nunc) in carbonate buffer (50 mM sodium bicarbonate, pH 9.6) overnight at 37°C. Blocking or antibody incubation steps were done in 5% milk and 0.5% milk, respectively. The secondary antibody was an MAb (rat anti-mouse κ light chain) conjugated to alkaline phosphatase, and the substrate was 2 mg of *p*-nitrophenyl phosphate (GIBCO BRL) per ml in 10 mM diethanolamine buffer, pH 9.5.

The synthesis of overlapping hexapeptides (5) corresponding to the entire sequence of Hib porin (outer membrane protein [OMP] subtype 1H) on solid-phase supports was described previously (30). The immunoreactivity of poly-

clonal sera to the solid-phase peptides was assayed by ELISA (30).

Biological assays. Opsonophagocytosis was performed with Hib strain 3527, OMP subtype 3L (35). Bacteria were cultured overnight on chocolate agar plates in a humid atmosphere with 5% CO_2 at 37°C. The Hib strain was inoculated in brain heart infusion broth and incubated for 2 h at 37°C with vigorous shaking to obtain late-exponential-phase cultures. Bacteria were washed twice in 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.4) containing 0.1% glucose (HEPES-glucose) and incubated with 0.015 mg of fluorescein isothiocyanate per ml for 15 min at 37°C. Bacteria were washed three times in HEPES-glucose to remove unbound fluorescein isothiocyanate. The concentration of bacteria was adjusted to 10^8 CFU/ml in opsonization buffer (20 mM HEPES buffer [pH 7.4], 5 mM $CaCl_2$, 0.1% gelatin, 0.1% glucose, 0.1% human serum albumin). Antisera were added to the bacterial suspension at a final concentration of 15% (vol/vol). Serum from an agammaglobulinemic patient at a concentration of 1% (vol/vol) was used as a source of complement. The bacterial suspension (20 μl, 10^8 CFU/ml) was added to 100 μl of a suspension of human polymorphonuclear leukocytes (PMNs; 10^7/ml) isolated as described by Kuijpers et al. (11). Bacteria which were not opsonized were used as a control. The mixtures were incubated at 37°C and shaken gently. After 30 min, samples (100 μl) were diluted in 400 μl of ice-cold opsonization buffer containing 1% paraformaldehyde to stop phagocytosis. The samples were analyzed with a FACScan (Becton Dickinson, Heidelberg, Germany). Opsonophagocytosis was defined as the percentage of the PMNs that became fluorescent after association with fluorescein isothiocyanate-labeled bacteria.

The bactericidal activities of pooled mouse sera were tested as described by Käyhty et al. (10). Bacteria were incubated for 60 min at 37°C in microwells with dilutions of mouse sera plus 25% human serum as the complement source. The complement alone did not kill the bacteria. Following incubation, aliquots of bacterial suspensions were plated in triplicate on chocolate agar plates containing 15 U of bacitracin per ml and incubated overnight at 37°C in a CO_2 incubator. The highest dilution that showed 50% killing was the bactericidal antibody titer.

To assess whether passively transferred polyclonal antibodies were protective in vivo, the infant rat model of bacteremia was used (27). The antisera were given intraperitoneally 2 h before the challenge dose of 4,000 CFU of Hib. Bacteria were also administered intraperitoneally. Bacteremia was detected 24 h later by plating 20 μl of undiluted or 100 μl of diluted (1:10 or 1:100) blood.

RESULTS

Expression of recombinant Hib porin. The expression vector pKTH288 of *B. subtilis* was selected for the cloning of the *ompP2* gene under the control of the promoter for α-amylase of *Bacillus amyloliquefaciens* (22). By PCR amplification of the *ompP2* gene, the DNA sequence of the mature form of Hib porin was generated as a *Hin*dIII fragment. This fragment (1,032 bp) was ligated into pKTH288 restricted with *Hin*dIII so that the codons for the first seven amino acids of the signal sequence of α-amylase plus four amino acids from a linker were fused in-frame to codons for the 341 amino acids of Hib porin. *B. subtilis* IH6140 was transformed with the ligated plasmid species, selected on kanamycin-containing plates, and screened initially for colony variants that differed from a background of antibiotic-resistant transformants. Candidate colonies were shown to harbor the desired recombinant gene in pKTH288 and in the correct orientation by their size and by the pattern obtained after double digestions with restriction enzymes.

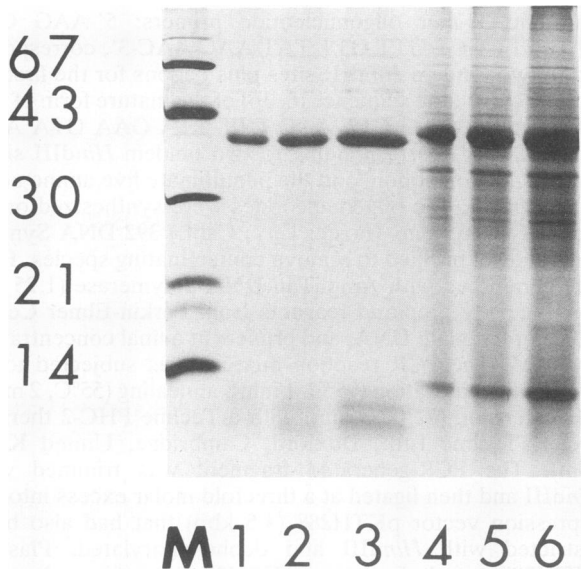

FIG. 1. Identification of recombinant Bac porin. Protein samples were loaded onto SDS–15% PAGE gels and revealed on the gel by silver staining. Lane M, size marker proteins (in kilodaltons); lanes 1 to 3, porin purified from the outer membrane of Hib (250, 500, and 1,000 ng, respectively); lanes 4 to 6, proteins from inclusion bodies from *B. subtilis* containing the recombinant plasmid that encodes Bac porin (500, 1,000, and 2,000 ng, respectively).

The protein created by this fusion (352 amino acids) was predicted to have the following sequence at the amino terminus: Met-1 Ile Gln Lys Arg Lys Arg Asn Ser Lys Leu Ala-12 Val-13 Val-14 Tyr-15 Asn-16 Asn-17; Ala-12 to Asn-17 correspond to the amino terminus of the mature form of Hib porin. Bac porin was expressed to such high levels that it aggregated within the cell and formed inclusion bodies (20, 24). To identify Bac porin, the cells were first converted to protoplasts; inclusion bodies were collected and washed with buffer containing nonionic detergent plus a high salt concentration. As assessed by SDS-PAGE, this buffer solubilized proteins from the bacterial cytoplasmic membranes. Because inclusion bodies were refractory to this treatment, they were collected by centrifugation and suspended in 10 mM Tris-HCl (pH 8.0)–1 mM EDTA to a protein concentration of about 1 mg/ml.

Identification by SDS-PAGE of the proteins at each step of the above protocol showed that almost no Bac porin was lost by detergent extraction, whereas the inclusion bodies were enriched for Bac porin (Fig. 1, lanes 4 to 6). A persistent, contaminating species of approximately 14 kDa was always noted with Bac porin in inclusion bodies. Several minor protein species of both higher and lower molecular mass than Bac porin were also routinely observed in preparations of inclusion bodies. The identity of Bac porin as the desired recombinant protein was confirmed by Western immunoblotting onto nitrocellulose of an SDS-PAGE gel that displayed the inclusion body proteins and by probing with two primary antibodies: (i) mouse MAb POR.1, which is specific to Hib porin (29) and (ii) a rabbit polyclonal antiserum which showed specificity towards the first 11

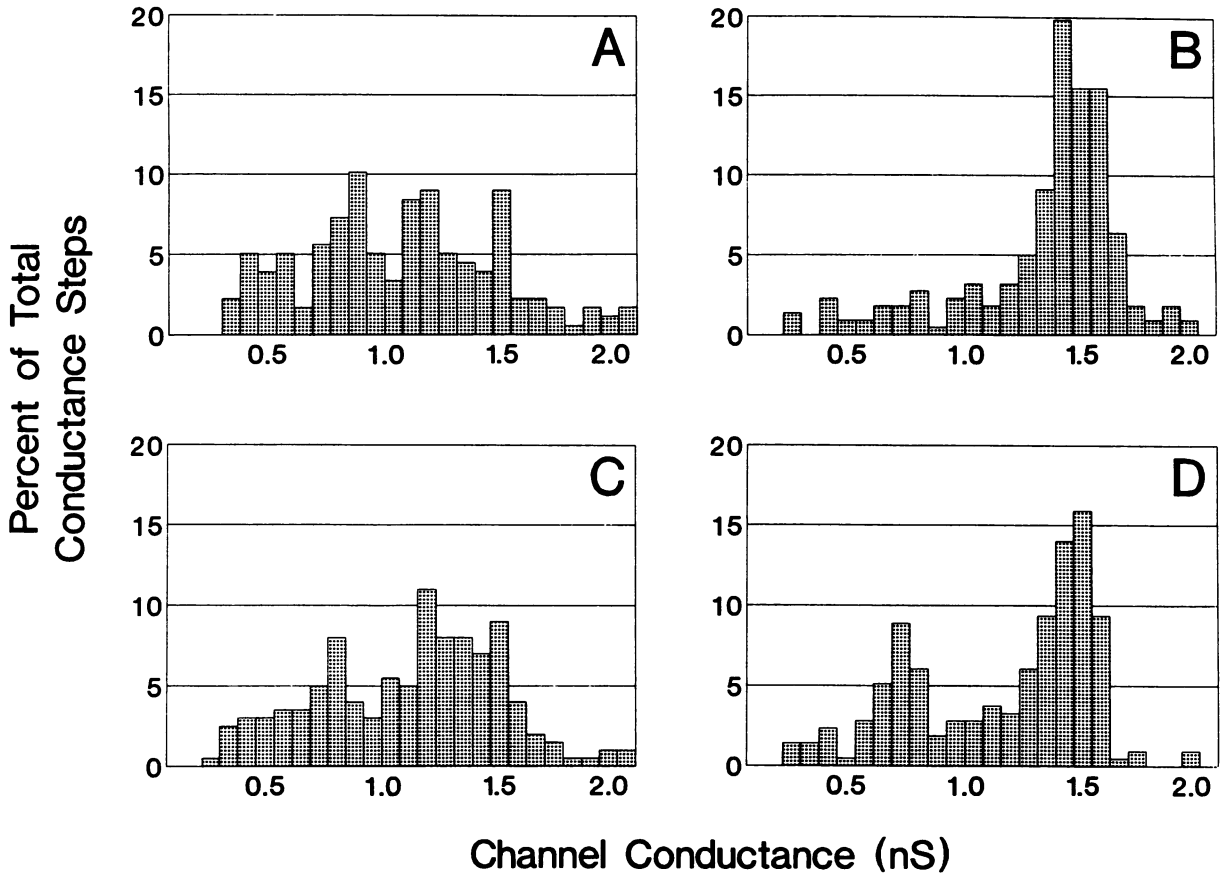

FIG. 2. Comparison of channel conductances of Hib porin and Bac porin in planar bilayers. Conductance steps were recorded at a transmembrane potential of 10 mV in 1 M KCl. (A) FPLC-purified Hib porin at 2 μg/μl in 50 mM Tris-HCl (pH 8.0)–0.1% Zwittergent Z-3,14 was diluted 500-fold into 50 mM Tris-HCl (pH 8.0); 1 to 5 μl of this material was then added to the Teflon chamber. (B) *Bacillus* inclusion bodies were suspended at 50 ng/μl in 50 mM Tris-HCl (pH 8.0)–1% Zwittergent Z-3,14 and then sonicated. Insoluble material was pelleted by centrifugation. The supernatant, which contained Bac porin at approximately 1 ng/μl, was diluted fivefold into 50 mM Tris-HCl (pH 8.0); 5 to 10 μl of this material was added to the Teflon chamber. (C) Bac porin extracted from inclusion bodies with 1% Zwittergent Z-3,14 as above and diluted fivefold into 10 μg Hib LOS per μl–50 mM Tris-HCl (pH 8.0); 5 to 10 μl of this material was added to the Teflon chamber. (D) FPLC-purified Hib porin at 2 μg/μl was diluted 500-fold into 50 mM Tris-HCl (pH 8.0)–5% Zwittergent Z-3,14; 1 to 5 μl of this material was added to the Teflon chamber. The total number of conductance steps analyzed was as follows: panel A, 178; panel B, 219; panel C, 200; and panel D, 214. Conductance steps of greater than 2.08 nS accounted for less than 5% of the total number of events and were not included in this analysis.

amino acids that are characteristic of recombinant proteins generated from pKTH288-related vectors. The calculated molecular mass of 39,183 Da for Bac porin, 352 amino acids, matched the estimate of 39 kDa derived by comparison with Hib porin (37 kDa; Fig. 1, lanes 1 to 3) and with standard proteins on SDS-PAGE (Fig. 1, lane M).

Channel-forming properties of Hib porin and Bac porin. Fast protein liquid chromatography (FPLC)-purified Hib porin was reconstituted into black lipid membranes and assayed for channel-forming activity. On SDS-PAGE gels (Fig. 1, lanes 1 to 3), FPLC-purified Hib porin appeared as a single protein band when silver stained. LOS migrated as a low-molecular-weight, minor contaminant. To the 1 M KCl solution bathing the planar bilayer was added Hib porin at a final concentration of 1 to 5 ng/ml. Stepwise increases in membrane conductance of Cl^- ions were attributed to the spontaneous insertion of porin into the bilayer. With the transmembrane potential held at 10 mV, the changes in membrane conductance were recorded until the conductivity was beyond the range of the apparatus. Histograms of the amplitude of the conductance steps for Hib porin are shown in Fig. 2A. Hib porin showed the usual wide distribution of conductance steps, so that for any interval of 0.2 nS, the percentage of total conductance steps was less than 25%.

To test whether Bac porin obtained from inclusion bodies could form channels, the total protein fraction from inclusion bodies was extracted with a solution of 50 mM Tris-HCl (pH 8.0) and 1% Zwittergent Z-3,14. When Zwittergent-solubilized Bac porin was added to the Teflon chamber at a final concentration of 0.5 ng/ml and analyzed as above, stepwise increases in membrane conductance were observed. Bac porin showed a surprisingly narrow distribution in channel conductance, with 50% of the current increment events having a conductance of 1.4 to 1.6 nS (Fig. 2B). The same narrow distribution was also seen after extraction of inclusion bodies with either 1% octyl-pentaoxyethylene or 1.6% cetyltrimethyl ammonium bromide (CTB) (data not shown). When Zwittergent-extracted Bac porin was mixed with a solution of Hib LOS (10 μg/μl in 50 mM Tris-HCl [pH 8.0]) (34), a different distribution of conductance steps was ob-

TABLE 1. Antibodies against recombinant Hib porin

Immunogen combination[a]	Reciprocal ELISA titer[b] against:		Opsonophagocytosis assay (% positive PMNs)
	Intact Hib	Hib porin	
i	1,194	25	12 ± 4
ii	2,008	25	22 ± 4
iii	10,601	125	72 ± 7
iv	19,109	125	26 ± 8
v	2,560	25	23 ± 0
Control			2 ± 3

[a] Sera are numbered to correspond to the combinations of materials used for immunization of groups of 10 mice, as described in Results. The control was complement alone, without antibody.

[b] The reciprocal titer versus intact Hib is the dilution of pooled mouse hyperimmune sera that corresponds through extrapolation to 50% of the maximum absorbance value for the colorimetric assay of enzymatic activity. The reciprocal titer versus FPLC-purified Hib porin (10 μg/ml) is expressed as the dilution of affinity-purified antibodies (already a sixfold dilution of pooled mouse hyperimmune sera) that gave an absorbance of at least 0.2 over background.

served (Fig. 2C). This distribution matched the distribution of conductance steps seen for FPLC-purified Hib porin (Fig. 2A). Furthermore, when FPLC-purified Hib porin was diluted into 50 mM Tris-HCl (pH 8.0)–5% Zwittergent Z-3,14, the histogram (Fig. 2D) became similar to that of Zwittergent-extracted Bac porin (Fig. 2B).

Antibodies against Bac porin. Polyclonal antisera specific for Bac porin from inclusion bodies were raised in mice. All proteins from inclusion bodies were readily solubilized in 100 mM Tris-HCl (pH 8)–2% CTB and then diluted to reduce the CTB concentration to less than 0.4%. For immunization of groups of 10 mice, proteins from inclusion bodies (200 μg) were combined with the following materials: (i) 50 μg of *Salmonella* O-6,7 LPS (21); (ii) 50 μg of LOS from Hib; (iii) 50 μg of *Salmonella* LPS plus FCA; (iv) 50 μg of Hib LOS plus FCA; or (v) FCA alone. FCA was used only in the primary immunization, and no FCA was used in the secondary immunizations. The pooled hyperimmune sera were numbered i to v to correspond to the above combinations. For the three biological assays, undiluted pooled sera or dilutions of pooled sera were used as indicated in the relevant sections. The pooled sera were purified by passage over an anti-κ light-chain immunoaffinity column, which also diluted the antibodies in the pooled sera by sixfold. These purified mouse sera were the source of the antibodies used for epitope scanning. As evaluated by ELISA of pooled mouse sera against intact Hib cells or ELISA of purified mouse sera against purified Hib porin, the highest titers of antiporin antibodies were found in sera iii and iv (Table 1).

Epitope scanning. We previously synthesized 336 sequential overlapping hexapeptides that correspond to the complete 341-amino-acid sequence of Hib porin, OMP subtype 1H. These hexapeptides were used to define the molecular reactivities of nine mouse MAbs raised against Hib porin. Seven MAbs reacted to the region between Thr-112 and Gly-172, and two MAbs recognized the region between Thr-318 and Val-325 (Fig. 3A) (30).

In this study, we used the overlapping hexapeptides to test the reactivities of polyclonal sera raised against Hib porin from a rabbit. Two regions of positive reactivity were identified: between Ile-128 and Asn-133 and between Gly-141 and Leu-147 (Fig. 3B). Thus, the immunological reactivities of antibodies against Hib porin that reacted to the synthetic hexapeptides and therefore recognized linear

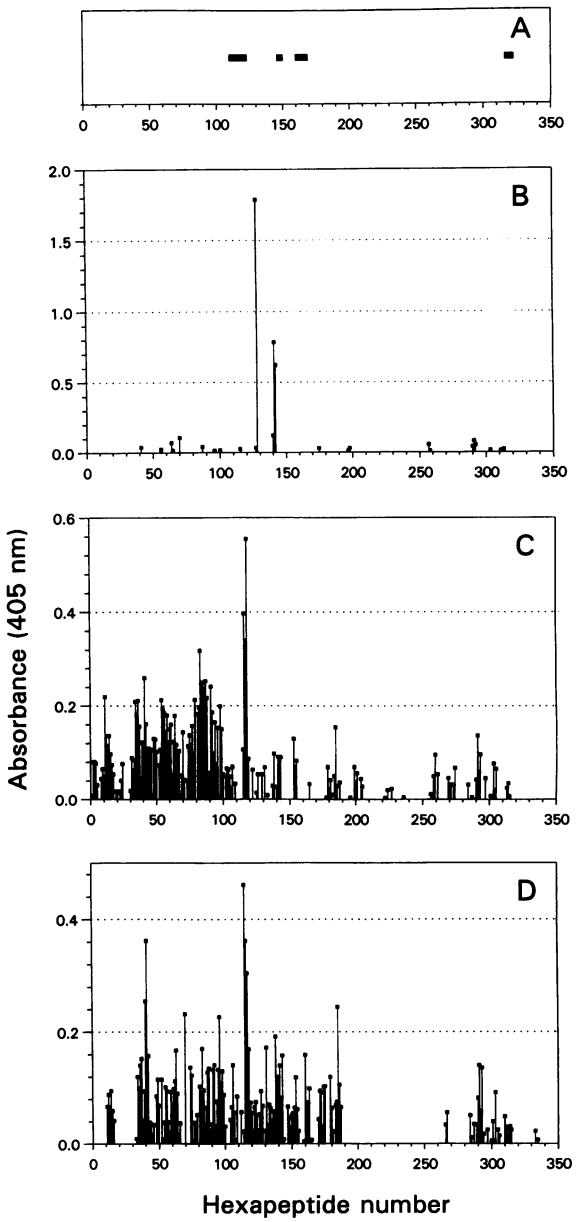

FIG. 3. Epitope scanning with 336 overlapping hexapeptides corresponding to the sequence of Hib porin, OMP subtype 1H, with purified antibodies raised against Hib porin or Bac porin. (A) Regions of the hexapeptides recognized by four different groups of mouse anti-Hib porin MAbs as described previously (30). (B) ELISA reactivities to the hexapeptides of polyclonal antibodies against Hib porin obtained from a rabbit. (C) ELISA reactivities to the hexapeptides of pooled mouse antisera against Bac porin preincubated with *Salmonella* O-6,7 LPS. (D) ELISA reactivities to the hexapeptides of pooled mouse antisera against Bac porin preincubated with Hib LOS. The hexapeptides were incubated with the anti-Hib porin antibodies or anti-Bac porin antibodies, washed free of unbound antibodies, and reacted with a secondary antibody. ELISA values are expressed as A_{405} units measured after 30 min of incubation with substrate and after substraction of background reactivity. The value of the background was an average of three reactivities against pins not containing any peptides and subjected to scanning under the same conditions. Negative absorbance values for the hexapeptides were considered to be zero.

epitopes were directed primarily to a region between Thr-112 and Gly-172 in the amino-terminal portion of the protein.

We also tested the reactivities to the hexapeptides of pools of mouse polyclonal antisera raised against Bac porin from inclusion bodies. Affinity-purified antibodies from two pooled sera that provided the highest titers of anti-Hib porin antibodies were tested: that raised against Bac porin plus *Salmonella* O-6,7 LPS plus FCA (immunogen combination iii, above), and the other against Bac porin plus Hib LOS plus FCA (immunogen combination iv, above). There were no significant differences in the patterns of reactivities to the hexapeptides of antibodies raised against Bac porin in the presence of *S. enterica* O-6,7 LPS (Fig. 3C) and antibodies raised against Bac porin in the presence of Hib LOS (Fig. 3D). Moreover, the overall specificity of antibodies against Bac porin (Fig. 3C and D) was similar to the specificity of antibodies against Hib porin (Fig. 3A and B), because selected regions in the amino-terminal portion of the protein were clearly more immunogenic than regions in the carboxy-terminal portion of the protein.

Biological activities of antibodies against Bac porin. Each of the five pools of antibodies against Bac porin from inclusion bodies was tested in duplicate for opsonophagocytosis of Hib strain RH3527. The results (Table 1) indicated that sera ii, iii, iv, and v were opsonic for Hib, with the highest value shown by serum iii.

Bactericidal assays provided an assessment of the ability of the anti-Bac porin antibodies to bind complement and to direct complement-mediated lysis. Hib strain RH3527 was resistant to 25% human complement. In the absence of complement, this test strain was not lysed by any of the five pooled hyperimmune sera (i, ii, iii, iv, or v) raised against Bac porin. With 25% human complement plus anti-Bac porin antibodies (the same five pooled sera), no bacteriolysis of Hib strain RH3527 was observed, even at the lowest dilution (1:4) of pooled sera in the standard assay. For controls, a human pool of anti-PRP antibodies up to a dilution of 1:128 (32 ng of anti-PRP antibodies per ml) plus 25% human complement gave 50% killing of the input number of CFU (data not shown).

Finally, the infant rat model of bacteremia was used to determine whether passive transfer of anti-Bac porin antibodies prior to intraperitoneal challenge with live Hib might be able to abrogate bacteremia. Four groups of infant Wistar rats were injected with saline or anti-PRP antibodies (400 ng per animal) or with 1:10-diluted pooled mouse sera, either anti-Bac porin antibodies (serum iii) or anti-Hib porin antibodies. After administration of a challenge dose of 4,000 CFU of Hib strain RH3527 followed by an interval of 18 h, the geometric mean titer of CFU per ml of blood for the control group indicated high-level bacteremia. No rats were bacteremic when anti-PRP antibodies had been passively transferred before challenge. The geometric mean titers for rats receiving anti-Bac porin antibodies or anti-Hib porin antibodies indicated that neither of these pools of antisera conferred protection against bacteremia (Table 2).

TABLE 2. Passive protection of infant rats by antibodies against PRP, Bac porin, or Hib porin

Antibody	No. of rats/no. in group (%)[a] with:		Geometric mean titer (10^3 CFU/ml of blood)
	Bacteremia	High-level bacteremia	
Saline	11/11 (100)	11/11 (100)	60.2
Anti-PRP	0/11 (0)	0/11 (0)	0.05
Anti-Bac porin[b]	9/9 (100)	7/9 (78)	14.6
Anti-Hib porin	9/9 (100)	7/9 (78)	4.1

[a] Number of rats showing viable CFU of Hib in blood samples at 18 h postchallenge; high-level bacteremia is defined as greater than 2.5×10^3 CFU/ml of blood.

[b] Serum iii (Table 1) was the source of anti-Bac porin antibody.

DISCUSSION

Our first report of the expression of the *ompP2* gene of Hib in a non-gram-negative expression system described the production of recombinant Hib porin in Sf9 insect cells (29). Recombinant baculoviruses were isolated and shown to express the *ompP2* gene. In spite of the low levels of expression, the recombinant Hib porin was shown to be functionally active in its channel-forming behavior (2). To obtain markedly higher amounts of LOS-free recombinant Hib porin for immunological studies, the *ompP2* gene was expressed in *B. subtilis*.

Bac porin obtained by Zwittergent extraction of inclusion bodies was shown to form pores in black lipid membranes, demonstrating that this protein is capable of folding into a functional form in the absence of LOS. However, these pores displayed electrical properties that were different from those of the channels formed by Hib porin in planar bilayers. FPLC-purified Hib porin in planar bilayers showed a wide distribution of conductance steps, a distribution that reproduced our previous results (33) and that is typical of porins analyzed in black lipid membranes (1). The differences in electrical properties of Bac porin from inclusion bodies were a higher average single-channel conductance (1.4 versus 1.1 nS) as well as a narrower distribution of single-channel conductance steps (Fig. 2B). This altered biophysical behavior was also seen with Bac porin obtained after treatment of inclusion bodies with either a nonionic detergent (octylpentaoxyethylene) or a cationic detergent (CTB), demonstrating that the observed differences in biophysical behavior were not detergent dependent. When FPLC-purified Hib porin was subjected to 5% Zwittergent and tested in planar bilayers, it showed a narrow distribution of conductance steps very similar to what was seen for Bac porin from inclusion bodies. The treatment with 5% Zwittergent may have caused some perturbation of the native conformation of Hib porin. Bac porin isolated from inclusion bodies may be in a similar partially denatured state and able to assume the native conformation only by forming a complex with LOS. In support of this idea, Bac porin extracted from inclusion bodies was reconstituted with a solution of LOS; the Bac porin-LOS complex showed a distribution of conductance steps (Fig. 2C) that matched the profile seen for FPLC-purified Hib porin.

Immunological reactivities were compared for anti-Hib porin antibodies and anti-Bac porin antibodies against hexapeptides corresponding to the complete sequence of Hib porin. In both instances, hexapeptides derived from the amino-terminal portion were clearly more reactive, especially in the region between Thr-112 and Gly-172 (Fig. 3). This effect may be due to the enhanced surface accessibility of this region under nonnative conditions.

Most bacterial porins are organized in their native conformation as stable trimers that are SDS resistant and dissociate into monomers only upon boiling (18). Whereas denatured monomers exist in a random coil or α-helical conformation, native trimers are predominantly β-sheets (4, 31). The secondary structure of a monomer may expose sequences that

might be masked in the trimer (4). Although the native conformation of Hib porin appears to be a trimer (32), the trimers are unstable and migrate on SDS-PAGE as monomers after incubation at room temperature in sample buffer containing 2% SDS. This instability of Hib porin could account for the immunodominance associated with the region from Thr-112 to Gly-172. The region between Thr-112 and Asp-126 in Hib porin, which we assigned to hydrophilic loop number 3, was not surface exposed, as shown by the nonreactivity of intact Hib with MAbs specific to this region (30). We postulated that this loop, in its native conformation, might be folded back into the channel, forming the eyelet of the pore. Under the conditions used for antigen preparation, Hib porin may have undergone denaturation, and this loop may have become immunogenic.

The immunogenicity of Bac porin isolated from inclusion bodies was enhanced in the presence of both FCA and LPS (Table 1). However, this induction of anti-Bac porin activity by LPS was independent of the source of LPS; the use of either *Salmonella* O-6,7 LPS or Hib LOS resulted in anti-Bac porin antibodies at the same titers. LPS from both sources was therefore a general adjuvant. Furthermore, the overall patterns of reactivity of anti-Bac porin antibodies raised in the presence of *Salmonella* O-6,7 LPS (serum iii) or in the presence of Hib LOS (serum iv) to the hexapeptides were similar (Fig. 3C and D). Serum iii was better in bringing about opsonophagocytosis of intact Hib than serum iv (Table 1). Whether this difference in opsonophagocytic response is attributable to the differences in specificities of the two sera is not known. Nevertheless, even serum iii was not bactericidal for Hib.

In the infant rat model of infection, when serum iii was passively transferred into rats, it was able to reduce the bacterial titers in the blood by fourfold compared with the control without antibody. However, it was inefficient in protecting the rats from bacteremia. Pooled antisera from 10 mice raised against FPLC-purified Hib porin were able to reduce the bacterial titers in blood by 15-fold compared with the control without antibody. However, it was also inefficient in abrogating bacteremia in infant rats. Such observations agree with our earlier tests of the biological activities of a panel of MAbs against Hib porin; they were neither bactericidal or protective (29). By comparison, the anti-Hib capsular polysaccharide antibodies were very efficient in preventing bacteremia (Table 2). These results are in contrast with the recently published data for the immunological activities of the class 1 outer membrane protein of *Neisseria meningitidis* produced in *B. subtilis* (BacP1). Antibodies raised against BacP1 complexed with *Salmonella* O-6,7 LPS were bactericidal and protective against *N. meningitidis* (20). The reason for the difference in protective abilities between the two outer membrane proteins will become clearer only through their further characterization.

Antibodies directed against the native determinants of a surface-exposed protein are considered critical for immunoprotection. The following observations indicate that Hib porin tends to lose its native structure when removed from its membrane environment. (i) When incubated at room temperature in sample buffer containing 2% SDS, the monomeric form of the protein predominates on SDS-PAGE. (ii) As seen with Bac porin, the protein devoid of LOS demonstrates altered biophysical behavior. (iii) The antibody response produced against the protein is directed primarily against nonnative epitopes. We are not able to substantiate earlier claims (6, 15) that anti-Hib porin antibodies are protective. We conclude that antibodies to our isolated form of Hib porin or against recombinant Bac porin provide little or no protection against Hib disease.

ACKNOWLEDGMENTS

This project was initiated when J.W.C. was on sabbatical leave at the National Public Health Institute, Helsinki, Finland. Support for the sabbatical was provided by the Medical Research Council, Canada (Visiting Scientist Award), the Natural Sciences and Engineering Research Council, Canada (International Collaborative Research Grant), the Sigrid Juselius Foundation, and the Ministry of Education, Finland. R.S. was the recipient of a fellowship from the Fonds pour la Formation de Chercheurs et l'Aide à la Recherche (FCAR). Research grant MT-6911 to J.W.C. from the Medical Research Council, Canada, is acknowledged. We appreciate the services of the Sheldon Biotechnology Centre, McGill University. The Centre is supported by MRC maintenance (MT-11107) and NSERC infrastructure (INF0103603) grants.

P. H. Mäkelä, R. C. Stewart, and L. van Alphen critically read the manuscript, and J. A. Kashul contributed figures.

REFERENCES

1. **Benz, R., K. Janko, W. Boos, and P. Läuger.** 1978. Formation of large, ion-permeable membrane channels by the matrix protein (porin) of *Escherichia coli*. Biochim. Biophys. Acta **511**:305–319.
2. **Coulton, J. W., A. C. Chin, and V. Vachon.** 1992. Recombinant porin of *Haemophilus influenzae* type b. J. Infect. Dis. **165**: S188–S191.
3. **Dick, W. E., Jr., and M. Beurret.** 1989. Glycoconjugates of bacterial carbohydrate antigens: a survey and consideration of design and preparation factors. Contrib. Microbiol. Immunol. **10**:48–114.
4. **Eisele, J.-L., and J. P. Rosenbusch.** 1990. In vitro folding and oligomerization of a membrane protein. J. Biol. Chem. **265**: 10217–10220.
5. **Geysen, H. M., S. J. Rodha, T. J. Mason, G. Tribbick, and P. G. Schoofs.** 1987. Strategies for epitope analysis using peptide synthesis. J. Immunol. Methods **102**:259–274.
6. **Granoff, D. M., and R. S. Munson, Jr.** 1986. Prospects for prevention of *Haemophilus influenzae* type b disease by immunization. J. Infect. Dis. **153**:448–461.
7. **Gryczan, T. J., S. Contente, and D. Dubnau.** 1978. Characterization of *Staphylococcus aureus* plasmids introduced by transformation into *Bacillus subtilis*. J. Bacteriol. **134**:318–329.
8. **Hansen, E. J., C. Hasemann, A. Clausell, J. D. Capra, K. Orth, C. R. Moomaw, C. A. Slaughter, J. L. Latimer, and E. E. Miller.** 1989. Primary structure of the porin of *Haemophilus influenzae* type b determined by nucleotide sequence analysis. Infect. Immun. **57**:1100–1107.
9. **Harlow, E., and D. Lane.** 1988. Antibodies: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
10. **Käyhty, H., O. Mäkelä, J. Eskola, L. Saarinen, and I. Seppälä.** 1988. Isotype distribution and bactericidal activity of antibodies after immunization with *Haemophilus influenzae* type b vaccines at 18–24 months of age. J. Infect. Dis. **158**:973–982.
11. **Kuijpers, T. W., C. M. Eckmann, R. S. Weening, and D. Roos.** 1989. A rapid turbidimetric assay of phagocytosis and serum opsonizing capacity. J. Immunol. Methods **124**:85–94.
12. **Lugtenberg, B., J. Meijers, R. Peters, and P. van der Hock.** 1975. Electrophoretic resolution of the major outer membrane protein of *Escherichia coli* into four bands. FEBS Lett. **58**:254–258.
13. **Morrissey, J. H.** 1981. Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. Anal. Biochem. **117**:307–310.
14. **Mueller, P., D. O. Rudin, H. T. Tien, and W. C. Westcott.** 1963. Methods for the formation of single bimolecular lipid membranes in aqueous solutions. J. Phys. Chem. **67**:534–535.
15. **Munson, R. S., Jr., J. L. Shenep, S. J. Barenkamp, and D. M. Granoff.** 1983. Purification and comparison of outer membrane protein P2 from *Haemophilus influenzae* type b isolates. J. Clin. Invest. **72**:677–684.
16. **Munson, R. S., Jr., and R. W. Tolan, Jr.** 1989. Molecular

cloning, expression, and primary sequence of outer membrane protein P2 gene of *Haemophilus influenzae* type b. Infect. Immun. **57**:88–94.
17. **Nakae, T.** 1976. Identification of the outer membrane protein of *E. coli* that produces transmembrane channels in reconstituted vesicle membranes. Biochem. Biophys. Res. Commun. **71**:877–884.
18. **Nikaido, H.** 1992. Porins and specific channels of bacterial outer membranes. Mol. Microbiol. **6**:435–442.
19. **Nikaido, H., and M. Vaara.** 1985. Molecular basis of bacterial outer membrane permeability. Microbiol. Rev. **49**:1–32.
20. **Nurminen, M., S. Butcher, I. Idänpään-Heikkilä, E. Wahlström, S. Muttilainen, K. Runeberg-Nyman, M. Sarvas, and P. H. Mäkelä.** 1992. The class 1 outer membrane protein of *Neisseria meningitidis* produced in *Bacillus subtilis* can give rise to protective immunity. Mol. Microbiol. **6**:2499–2506.
21. **Nurminen, M., and R.-M. Ölander.** 1991. The role of O-antigen in adjuvant activity of lipopolysaccharide. FEMS Microbiol. Lett. **83**:51–54.
22. **Palva, I., R. F. Pettersson, N. Kalkkinen, P. Lehtovaara, M. Sarvas, H. Söderlund, K. Takkinen, and L. Kääriäinen.** 1981. Nucleotide sequence of the promoter and NH_2-terminal signal peptide region of α-amylase gene from *Bacillus amyloliquefaciens*. Gene **15**:43–51.
23. **Palva, I., M. Sarvas, P. Lehtovarra, M. Sibakov, and L. Kääriäinen.** 1982. Secretion of *Escherichia coli* β-lactamase from *Bacillus subtilis* by the aid of α-amylase signal sequence. Proc. Natl. Acad. Sci. USA **79**:5582–5586.
24. **Puohiniemi, R., M. Simonen, S. Muttilainen, J.-P. Himanen, and M. Sarvas.** 1992. Secretion of the *Escherichia coli* outer membrane proteins OmpA and OmpF in *Bacillus subtilis* is blocked at an early intracellular step. Mol. Microbiol. **6**:981–990.
25. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. **Sikkema, D. J., and T. F. Murphy.** 1992. Molecular analysis of the P2 porin protein of nontypeable *Haemophilus influenzae*. Infect. Immun. **60**:5204–5211.
27. **Smith, A. L., D. H. Smith, D. R. Averill, J. Marino, and E. R. Moxon.** 1973. Production of *Haemophilus influenzae* type b meningitis in infant rats by intraperitoneal inoculation. Infect. Immun. **8**:278–290.
28. **Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olsen, and D. C. Klenk.** 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. **150**:76–85.
29. **Srikumar, R., A. C. Chin, V. Vachon, C. D. Richardson, M. J. H. Ratcliffe, L. Saarinen, H. Käyhty, P. H. Mäkelä, and J. W. Coulton.** 1992. Monoclonal antibodies specific to porin of *Haemophilus influenzae* type b: localization of their cognate epitopes and tests of their biological activities. Mol. Microbiol. **6**:665–676.
30. **Srikumar, R., D. Dahan, M. F. Gras, M. J. H. Ratcliffe, L. van Alphen, and J. W. Coulton.** 1992. Antigenic sites on porin of *Haemophilus influenzae* type b: mapping with synthetic peptides and evaluation of structure predictions. J. Bacteriol. **174**:4007–4016.
31. **Ulmer, J. B., C. J. Burke, C. Shi, A. Friedman, J. J. Donnelly, and M. A. Liu.** 1992. Pore formation and mitogenicity in blood cells by the class 2 protein of *Neisseria meningitidis*. J. Biol. Chem. **267**:19266–19271.
32. **Vachon, V., D. N. Kristjanson, and J. W. Coulton.** 1988. Outer membrane porin of *Haemophilus influenzae* type b: pore size and subunit structure. Can. J. Microbiol. **34**:134–140.
33. **Vachon, V., R. Laprade, and J. W. Coulton.** 1986. Properties of the porin of *Haemophilus influenzae* type b in planar lipid bilayer membranes. Biochim. Biophys. Acta **861**:74–82.
34. **Vachon, V., D. J. Lyew, and J. W. Coulton.** 1985. Transmembrane permeability channels across the outer membrane of *Haemophilus influenzae* type b. J. Bacteriol. **162**:918–924.
35. **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.
36. **Yelton, D. E., C. Desaymard, and M. D. Scharff.** 1981. Use of monoclonal anti-mouse immunoglobulin to detect mouse antibodies. Hybridoma **1**:5–11.