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Topology of porin, the major outer membrane protein of *Haemophilus influenzae* type b

By

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November 1995

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Doctor of Philosophy

ISBN 0-612-12490-8

This thesis is dedicated to the memory of

my father

Srinivasa Iyer Ramakrishnan

ABSTRACT

Ph.D. Ramakrishnan Srikumar Microbiology and Immunology

Haemophilus influenzae type b (Hib) is a Gram-negative bacterium that causes meningitis in infants. A protein called porin of 341 amino acids, M_r 37,782 daltons, is located in the outer membrane of Hib and allows for the diffusion into the periplasmic space of small solutes up to a molecular mass of 1400 daltons.

Based on parameters of hydrophilicity and amphiphilicity a model for Hib porin was generated. The model proposed an organization of sixteen anti-parallel β-strands that traverse the outer membrane, eight long loops that connect the β-strands on one side and short turns on the other side.

By flow cytometry, six out of a panel of nine monoclonal antibodies against Hib porin recognized amino acid sequences at the cell surface. Hib porin was purified and subjected to chemical and enzymatic digestions. The fragments were immunoblotted; N-terminal sequencing identified boundaries of fragments. C-terminal deletions of Hib porin generated in the baculovirus expression system identified C-terminal boundaries of monoclonal antibody reactivities.

To map precisely the primary sequences to which these monoclonal antibodies bound, overlapping hexapeptides for the entire sequence of Hib porin were synthesized. These studies identified two surface-exposed regions in the mature sequence

of Hib porin, amino acid residues 162-172 and 318-325. In the Hib porin model, these regions correspond to loops 4 and 8, respectively. Two regions between residues 112-126 (loop 3) and residues 148-153 were buried or inaccessible at the surface of the outer membrane.

Recombinant Hib porin was expressed in *Bacillus subtilis*. The biophysical and immunological properties of this lipooligosaccharide-free recombinant Hib porin were compared with those of native Hib porin.

In order to examine the role of loop 3, site-directed mutagenesis of the cloned Hib porin gene was undertaken. Six or twelve amino acid deletions in loop 3, expressed in a porin deletion strain, showed significant increase in sensitivities to several anti-microbial agents as compared to wild-type Hib porin. Deletion of twelve amino acids showed more pronounced phenotypes than deletion of six amino acids. Such mutagenesis experiments provided support to the notion that loop 3 in Hib porin folds back into the pore and produces a constriction of the channel.

ABRÉGÉ

Ph.D. Ramakrishnan Srikumar Microbiologie et Immunologie

Haemophilus influenzae type b (Hib) est une bactérie Gram-négative qui s'avère une des cause des méningites chez les enfants en bas âge. La porine, protéine de 341 acides aminés et de 37,782 daltons, est localisée dans la membrane externe de Hib et permet la diffusion dans l'espace périplasmique de petits solutés de masse moléculaire jusqu'à 1400 daltons.

Un modèle pour la porine de Hib fut élaboré en fonction des paramètres d'hydrophilicité et d'amphiphilicité. Le modèle proposé consiste en une organisation de seize brins β antiparallèles traversant la membrane extérieure, de huit longues boucles qui relient les brins β d'un côté, et des petites boucles à l'autre côté.

À l'aide du cytométrie de flux, six sur un total de neuf anticorps monoclonaux dirigés contre la porine de Hib ont reconnu la séquence des acides aminés à la surface cellulaire. La porine de Hib a été purifiée avant de subir des digestions chimiques et enzymatiques. Les fragments ont été immunodétectés; le séquençage par le N-terminal a identifié les extrémités des fragments. Les délétions C-terminales de la porine de Hib générées dans le système d'expression de baculovirus, ont identifié la réactivité des extrémités C-terminales des anticorps monoclonaux.

Pour identifier avec précision les séquences primaires sur lesquelles se lient ces anticorps monoclonaux, des hexapeptides se chevauchant ont été synthétisés pour la séquence complète de la porine de Hib. Ces études ont permis d'identifier dans la séquence mature de la porine de Hib, deux régions dont la surface est exposée, soient les résidus d'acides aminés 162-172 et 318-325. Dans le modèle de la porine Hib, ces régions correspondent aux boucles 4 et 8 respectivement. Deux régions, entre les résidus 112-126 (boucle 3) et les résidus 148-153 étaient cachées ou inaccessibles à la surface de la membrane extérieure.

La porine recombinante de Hib a été exprimée dans *Bacillus subtilis*. Les propriétés biophysiques et immunologiques de cette porine recombinante de Hib, dépourvue de lipooligosaccharides, ont été comparées avec celles de la porine native de Hib.

Pour déterminer le rôle de la boucle 3, une mutagénèse dirigée de la porine clonée de Hib été entreprise. Des délétions de six ou de douze acides aminés dans la boucle 3, exprimée dans une souche de porine avec délétion, ont montré une augmentation significative de la sensibilité à plusieurs agents anti-microbiens comparativement à la porine native de Hib. La délétion de douze acides aminées a montré des phénotypes plus prononcés que la délétion de six acides aminés. Les expériences de mutagénèse ont appuyé l'idée que la boucle 3 dans la porine de Hib se replie dans le pore et produit une constriction du canal.

ACKNOWLEDGEMENTS

First and foremost I would like to extend my sincere gratitude to my thesis supervisor, Dr. James Coulton, for his time, dynamic guidance, encouragement, and constant support. He has been instrumental in my development as a researcher.

I fully appreciate the assistance and friendship of all the members of the Coulton laboratory whom I have encountered during my studies. Gilles Carmel, Alice Chin, Marie France Gras, David Dahan, Greg Moeck, Dr. Francis Arhin, and Paul Tawa, have contributed more than one way to the progress of my work in the laboratory. Marie France Gras and France Moreau helped to translate the Abstract into French.

I wish to express my thanks to all members of the Department of Microbiology and Immunology, McGill University, for making my tenure here pleasant and memorable. Alain Duclos, who willingly provided computer assistance, deserves special mention. I also thank Drs. Michael Ratcliffe, Rick Stewart, and Teresa Keng for their advice and contributions to my thesis research.

Finally I thank my mother, brothers, and sister for their encouragement, patience, and accommodation.

The financial support from the Medical Research Council (MRC), Canada, (research grant to Dr. Coulton), the Fonds pour la Formation de Chercheurs et l'Aide à la Recherche (FCAR), Québec, the Faculty of Medicine, McGill University, and this department is gratefully acknowledged.

CONTRIBUTION TO ORIGINAL KNOWLEDGE

1. Proposed a model for the folding of the major outer membrane protein, porin, of *Haemophilus influenzae* type b. This model has been tested by our laboratory and by others and was confirmed to be entirely consistent with the experimental evidence.

2. Mapped precisely the epitopes recognized by six monoclonal antibodies against *Haemophilus influenzae* type b porin to stretches of six to eleven amino acids.

3. Provided direct evidence for the surface exposure of two regions in *Haemophilus influenzae* type b porin. These regions correspond to loop 4 and loop 8 in the proposed model for this protein.

4. Demonstrated the exquisite specificity of one monoclonal antibody by its ability to discriminate a single amino acid change, Arg166 to Gln, in the primary sequence of *Haemophilus influenzae* type b porin.

5. Obtained several lines of evidence to indicate that antibodies to the isolated form of *Haemophilus influenzae* type b porin provide no immune protection against this bacterium.

6. Constructed chromosomal deletions of the porin gene *ompP2* in nontypeable *Haemophilus influenzae* and *Haemophilus influenzae* type b.

7. Designed a system for the expression and characterization of mutant *Haemophilus influenzae* type b porins and other heterologous porins in a genetic background in which the porin gene was deleted.

8. Gathered structural and functional evidence to support the proposal that loop 3 in *Haemophilus influenzae* type b porin folds back into the pore and produces a constriction of the channel.

TABLE OF CONTENTS

MANUSCRIPTS AND AUTHORSHIP

In accordance with the guidelines of the Faculty of Graduate Studies and Research, McGill University, concerning thesis preparation, and with the approval of the Department of Microbiology and Immunology, McGill University, I have opted to present the experimental portion of this thesis (Chapters 2, 3, 4, and 5) in the form of manuscripts. A provision in the guidelines concerning thesis preparation reads as follows:

Candidates have the option, **subject to the approval of their Department**, of including, as part of their thesis, copies of the text of a paper(s) submitted for publication, or the clearly-duplicated text of a published paper(s), provided that these copies are bound as an integral part of the thesis.
-If this option is chosen, **connecting texts, providing logical bridges between the different papers, are mandatory.**
-The thesis must still conform to all other requirements of the "Guidelines Concerning Thesis Preparation" and should be in a literary form that is more than a collection of manuscripts published or to be published. **The thesis must include, as separate chapters or sections:** (1) a Table of Contents, (2) a general abstract in English and French, (3) an introduction which clearly states the rationale and objectives of the study, (4) a comprehensive general review of the background literature to the subject of the thesis, when this review is appropriate, and (5) a final overall conclusion

and/or summary.

-Additional material (procedural and design data, as well as descriptions of equipment used) must be provided where appropriate and in sufficient detail (eg. in appendices) to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

-In the case of manuscripts co-authored by the candidate and others, **the candidate is required to make an explicit statement in the thesis of who contributed to such work and to what extent;** supervisors must attest to the accuracy of such claims at the Ph.D. Oral Defense. Since the task of Examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of different authors of co-authored papers.

With regard to the above conditions, I have included as chapters of this thesis three reprints of original papers which have been published and one paper that has been submitted. Chapters 2, 3, 4, and 5 contain their own Summary, Introduction, Materials and Methods (Chapters 3, 4, and 5) or Experimental Procedures (Chapter 2), as well as Results, and Discussion sections. A preface which serves as the connecting text to bridge the manuscripts is found prior to Chapters 2, 3, 4, and 5. A chapter containing General Introduction and Literature Review (Chapter 1) and another containing General Conclusions and Opportunities for Research (Chapter 6) have

also been included. References are collated alphabetically (numbered or not) and found at the end of each chapter. Page numbers of the thesis are found on the bottom-center of each page and should be distinguished from page numbers of individual manuscripts.

The manuscripts, in order of their appearance in the thesis, are listed below. The contributions of co-authors with respect to experimental work are specified under each manuscript. I am responsible for the rest of the work presented in these manuscripts. Unless otherwise stated under each paper, the text that appears in the manuscripts were composed by me. The manuscripts were edited by Dr. J. W. Coulton. All of my research was conducted under the supervision of Dr. J. W. Coulton.

1. Srikumar, R., Chin, A. C., Vachon, V., Richardson, C. D., Ratcliffe, M. J. H., Saarinen, L., Käyhty, H., Mäkelä, P. H. & Coulton, J. W. (1992). Monoclonal antibodies specific to porin of *Haemophilus influenzae* type b: localization of their cognate epitopes and tests of their biological activities. *Molecular Microbiology* **6**, 665-676 (Chapter 2 of this thesis).

A. C. Chin cloned and expressed recombinant *Haemophilus influenzae* type b (Hib) porin in the baculovirus expression system. She also constructed a series of C-terminal deletions of Hib porin in the baculovirus expression system.

Dr. C. D. Richardson provided the baculovirus transfer vectors pJVETLZ and pJV.P10Z. He also assisted A. C. Chin in her project.

Dr. V. Vachon established an FPLC-based purification scheme for Hib porin.

Dr. M. J. H. Ratcliffe performed the flow cytometric analyses.

L. Saarinen, Drs. H. Käyhty, P. H. Mäkelä, and J. W. Coulton performed the microbiological tests of the monoclonal antibodies. They also provided text for the relevant sections.

2. Srikumar, R., Dahan, D., Gras, M. F., Ratcliffe, M. J. H., van Alphen, L. & Coulton, J. W. (1992). Antigenic sites on porin of *Haemophilus influenzae* type b: mapping with synthetic peptides and evaluation of structure predictions. *Journal of Bacteriology* **174**, 4007-4016 (Chapter 3 of this thesis).

D. Dahan sequenced the entire porin gene from Hib subtype 2L.

M. F. Gras provided some technical assistance.

Dr. M. J. H. Ratcliffe assisted with the flow cytometric analyses.

Dr. L. van Alphen provided the monoclonal antibody 23AA12.

3. Srikumar, R., Dahan, D., Gras, M. F., Saarinen, L., Käyhty, H., Sarvas, M., Vogel, L. & Coulton, J. W. (1993). Immunological properties of recombinant porin of *Haemophilus influenzae* type b expressed in *Bacillus subtilis*. *Infection*

and Immunity **61**, 3334-3341 (Chapter 4 of this thesis).

Dr. J. W. Coulton cloned and expressed recombinant Hib porin in *Bacillus subtilis*. Dr. M. Sarvas assisted.

D. Dahan assayed Hib porin in black lipid membranes. He also wrote the relevant sections.

M. F. Gras provided some technical assistance.

L. Saarinen and Dr. H. Käyhty performed bactericidal assays and tested the activity of antibodies in the infant rat model of bacteremia. They also provided text for the relevant sections.

L. Vogel performed the opsonophagocytosis assay and provided text for this section.

4. Srikumar, R., Arhin, F. F. & Coulton J. W. (1995). Site-directed mutagenesis of *Haemophilus influenzae* type b porin: alteration of pore properties by deletions in loop 3. (Submitted; Chapter 5 of this thesis).

Dr. F. F. Arhin performed several experiments that led to the construction of nontypeable *Haemophilus influenzae* and Hib porin deletion strains, and to the loop 3 and loop 4 deletions in Hib porin.

LIST OF FIGURES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

LIST OF TABLES

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 1

GENERAL INTRODUCTION AND LITERATURE REVIEW

1. Introduction

Haemophilus influenzae is a small, non-motile, rod-shaped Gram-negative bacterium. Some strains do not possess a detectable capsule and are referred to as nontypeable *Haemophilus influenzae*. These strains are generally a harmless component of the normal flora in the upper respiratory tract of humans. They may become pathogenic in older or immunologically-compromised individuals.

Other strains of *Haemophilus influenzae* are encapsulated and cause disease. They can be categorized into several serotypes based on the immunological properties of the capsular polysaccharide (type a through f). Of all the serotypes, *Haemophilus influenzae* type b (Hib) is the most important pathogen (Turk, 1984; van Alphen & Bijlmer, 1990). It is a causative agent of bacterial meningitis mainly in infants and young children. Until recently, it was the leading cause of bacterial meningitis in this population (Dajani *et al.*, 1979). Besides bacterial meningitis, Hib can also cause septicemia, epiglottitis, cellulitis, arthritis, osteomyelitis, pericarditis, and pneumonia (Wilfert, 1990).

The fatality rate for Hib meningitis is approximately 5%, and 40% of surviving children show significant neurologic

sequelae (Wilfert, 1990). In 1985, it was estimated that systemic disease caused by Hib occurred in 500 per 100,000 children in the United States before the age of five (Cochi *et al.*, 1985). Between 1985 and 1989, the incidence of disease in this population in the United States had diminished to 37 per 100,000 (Adams *et al.*, 1993). Anti-microbial therapy was partly responsible for reducing Hib disease. However, the emergence during the past decade of organisms resistant to several antibiotics including ampicillin, chloramphenicol, and the cephalosporins (Wilfert, 1990) posed a threat to the efficacy of treatment of these infections with these agents. This threat plus the strain imposed on the health care system (Hay & Daum, 1987) spurred researchers to focus studies on the prevention of Hib disease by immunization.

Immunity to Hib infection is derived by antibodies to the type b capsular polysaccharide, polyribosylribitol phosphate (PRP). Whereas a PRP vaccine was fairly effective in children over 24 months of age, the vaccine was clearly not effective in children under 18 months of age (Weinberg & Granoff, 1988). The deficiency of children under 18 months of age to mount an antibody response to a T cell-independent antigen such as PRP was the reason for its ineffectiveness. PRP was therefore conjugated to several proteins to create conjugate vaccines. By this conjugation it was expected that antibodies to PRP would be generated in a T cell-dependent fashion. PRP was conjugated to diphtheria toxoid (PRP-D), tetanus toxoid (PRP-

T), or to an outer membrane protein of *Neisseria meningitidis* (PRP-OMP) (Weinberg & Granoff, 1988). These conjugate vaccines have been in use since 1987 and have been useful in significantly reducing the incidence of Hib related disease in children including those under 18 months of age. Between 1987 and 1992, the incidence of Hib disease in the United States among children less than 5 years was reported to be 11 per 100,000 (Adams *et al.*, 1993).

Although these PRP-conjugate vaccines had a marked impact in the incidence of Hib disease, other candidates for the protein component were sought to create new conjugate vaccines. It seemed logical to identify a surface-exposed outer membrane protein of Hib for such an endeavour. The advantage of such a conjugate vaccine over the existing ones would be that antibodies to the outer membrane protein may contribute additionally to immune-protection against Hib disease.

Porins of Gram-negative bacteria are outer membrane proteins that are surface-exposed and are responsible for the molecular sieve properties of these cells (Nakae, 1976). They form water-filled channels which allow the diffusion of molecules such as sugars, amino acids, nucleosides, and hydrophilic antibiotics into the periplasmic space. Classical porins such as the outer membrane protein F (OmpF) of *Escherichia coli* reside in the outer membrane as trimers. In this thesis, the term porin is generally used to identify this

class of proteins. Monomeric outer membrane proteins that exhibit pore-forming activity have also been identified. The maximum size of a solute molecule that can permeate the pores defines a value termed the molecular mass exclusion limit. The variety of porins and their exclusion limits differ from one bacterial genus to another.

The outer membrane of Hib contains approximately 24 different proteins. Five proteins account for 80% of the outer membrane proteins (Loeb & Smith, 1982b) and are detected as major bands on sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). The nomenclature of the five major outer membrane proteins of Hib and their molecular masses by SDS-PAGE are the following: protein a or P1, 46 kilodaltons (kDa); protein b/c or P2, 38 kDa; protein e or P4, 28 kDa; protein d/f or P5, 34 kDa; and protein g or P6, 15 kDa (Granoff & Munson, 1986; Loeb & Smith, 1982a). The primary sequences of all five proteins from nontypeable *Haemophilus influenzae* (Hi) or Hib have been determined and some of their properties are known. Protein P1 is surface-exposed and is heat modifiable; on SDS-PAGE it migrates differently upon boiling in sample buffer. Although the function of P1 is not known, this protein exhibits 42% identity to the outer membrane protein, FadL, of *E. coli* (Black, 1991). FadL plays a role in the uptake of exogenous long-chain fatty acids in *E. coli*. P2 is a surface-exposed protein that functions as a porin and is the subject of this thesis. P4 is a lipoprotein

that is surface-exposed (Green *et al.*, 1991). P5 is heat modifiable and shows 50% identity to the outer membrane protein OmpA of *E. coli* (Munson *et al.*, 1993). Like OmpA, P5 is proposed to play a role in maintaining the integrity of the Hib outer membrane. P6 is a surface-exposed protein that was recently shown to bind to the *ompP6* gene thereby regulating its own expression (Sikkema *et al.*, 1992). Of the five major proteins, P6 is the most conserved among different strains. Due to their surface location and abundance, all these proteins have been studied extensively as potential vaccine components. Some of the minor proteins in the outer membrane of Hi or Hib that function in the acquisition of iron have been characterized. These proteins include haemin-binding protein (Hanson & Hansen, 1991), haemopexin-binding protein (Cope *et al.*, 1994), and transferrin-binding protein (Gray-Owen *et al.*, 1995).

The protein P2 (341 amino acids; M_r 37,782 daltons) is the most abundant in outer membrane of Hib. When the five major outer membrane proteins were tested for pore function, only the P2 protein exhibited channel activity (Vachon *et al.*, 1985). This prompted researchers to refer to the P2 protein simply as Hib porin.

Recently, the entire DNA sequence (1.83×10^6 base pairs) of nontypeable *Haemophilus influenzae* strain KW20 was determined (Fleischmann *et al.*, 1995). This is the first report of sequencing the complete genome of any bacterium.

Other than the *ompP2* sequence itself, none out of the 1,743 predicted coding regions in the Hi chromosome showed any homology to the porin gene. Since porin sequences from the same genus show similarities, this finding provided confirmation that the protein encoded by *ompP2* was the only porin in Haemophilus strains.

Haemophilus influenzae type b porin allows for the non-specific diffusion of solutes and has a slight preference for cations (Vachon *et al.*, 1986). It is expressed at high levels under all conditions tested (Loeb & Smith, 1982b). A genetically-altered Hib strain that did not express the porin protein was shown to be avirulent in an animal model of infection (Cope *et al.*, 1990), demonstrating the importance of porin in pathogenicity of Hib. Because of its surface location, abundance, constitutive expression, and role in disease, Hib porin may be a candidate for the construction of a new PRP-conjugate vaccine against Hib. Therefore, information concerning the immunogenicity of this protein combined with information on the protective ability of anti-Hib porin antibodies is essential for the evaluation of this protein's candidacy. An evaluation of the surface-exposed parts of Hib porin is also of interest since antibodies to these regions of the protein might be predicted to be protective.

The molecular architecture of Hib porin is currently unknown. Since this protein is the route of entry of

hydrophilic antibiotics, information on the structure of this protein may enable researchers to identify novel antibiotics that would be most effective for anti-microbial therapy against Hib.

Several other reasons warrant the determination of the topology and structure of Hib porin. The pore formed by Hib porin has a molecular mass exclusion limit of 1400 daltons (Vachon *et al.*, 1985), considerably larger than the value of 600 daltons for the pore formed by OmpF of *E. coli*. By liposome swelling assays (Vachon *et al.*, 1988), Hib porin appeared to have a greater pore diameter than that associated with the porins of *E. coli*. Unlike most classical porins, trimers of Hib porin are quite unstable and were detected by SDS-PAGE only when the proteins were previously cross-linked (Vachon *et al.*, 1988). What is the molecular organization of Hib porin that contributes to these unique features and how does that organization differ from other well characterized bacterial porins?

This thesis presents results of experiments designed to elucidate the structure of Hib porin and its native topological organization in the outer membrane. An understanding of the topology of this protein coupled with information pertaining to its immunological properties will be useful in the development of alternate strategies for combating Hib infections. The characterization of this protein will also allow for an examination of

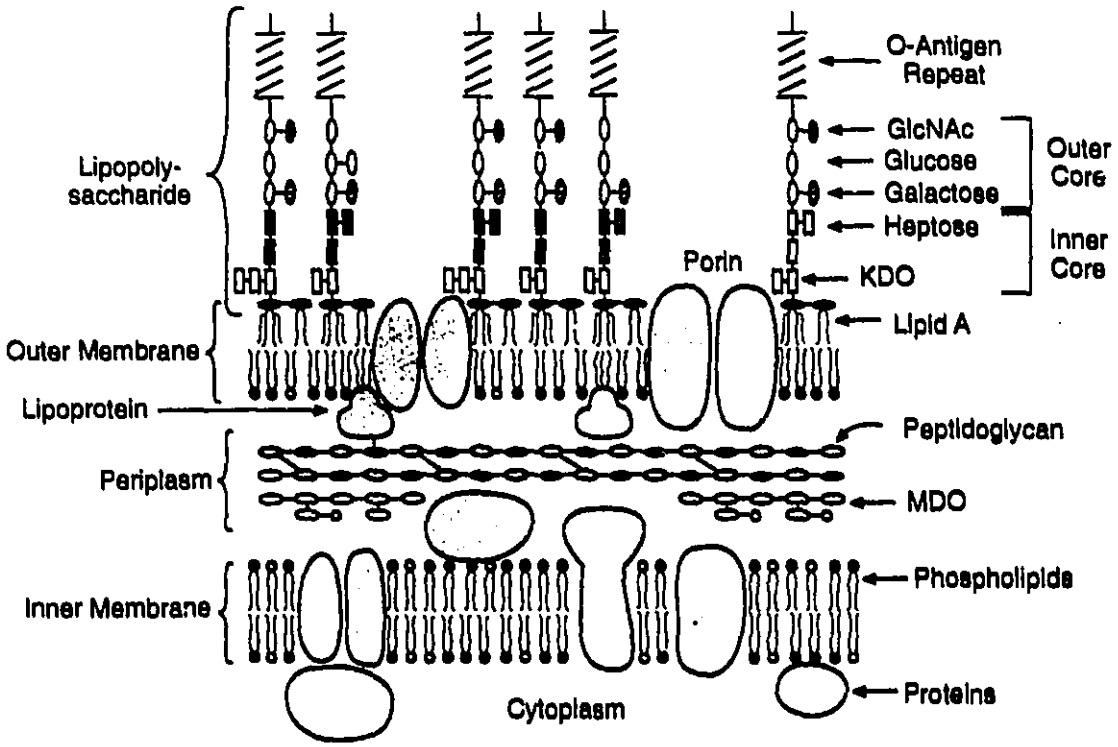
structure/function relationships of Hib porin.

2. The cell envelope of Gram-negative bacteria

The cell envelope of Gram-negative bacteria including *Escherichia coli*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, and *Haemophilus influenzae*, consists of three different layers: the outer membrane, the murein or peptidoglycan layer, and the cytoplasmic or inner membrane (Fig. 1). The inner membrane represents a real diffusion barrier and is composed of phospholipids and proteins. The inner membrane is a bilayer: it contains an outer leaflet and an inner leaflet of phospholipids. It is a symmetric bilayer with respect to its lipid content: both leaflets contain the same phospholipids with phosphatidyl ethanolamine being the major lipid (Meadow, 1975). The inner membrane contains several hundred proteins: these include proteins of the respiratory chain, those involved in transport across the inner membrane, components of the machinery for protein export and enzymes involved in the synthesis of the peptidoglycan layer.

The peptidoglycan layer is composed of a network of a hetero-polymer called murein consisting of amino sugars. The amino sugars (dimers of N-acetylglucosamine and N-acetylmuramic acid) form long linear strands that are covalently linked between muramyl residues by short tetrapeptides (Labischinski & Maihdof, 1994). This layer

Fig. 1. Molecular representation of the envelope of a Gram-negative bacterium. Ovals and rectangles represent sugar residues, whereas circles depict the polar head groups of glycerolphospholipids. MDO and KDO are membrane-derived oligosaccharides and 3-deoxy-D-manno-octulosonic acid, respectively. The core region depicted is that of *Escherichia coli* K-12. Reproduced with permission from Raetz, 1993.


O-Antigen
Repeat
GlcNAc
Glucose
Galactose
Heptose
KDO
Outer
Core
Inner
Core
Lipopoly-
saccharide
Porin
Lipid A
Outer Membrane
Lipoprotein
Peptidoglycan
Periplasm
MDO
Phospholipids
Inner Membrane
Proteins
Cytoplasm

provides rigidity to the cell envelope, contributes to the shape of the cell and prevents the bacteria from osmotic lysis. The space between the inner membrane and the peptidoglycan layer is referred to as the periplasmic space. The contents of the periplasmic space is the periplasm. The constituents of the periplasm include proteins that bind substrates in order to facilitate their transport across the inner membrane, several enzymes including those that hydrolyse β-lactam antibiotics, and membrane-derived oligosaccharides (MDO) that allows the organism to cope with changes in the osmolarity of the medium (Bayer & Bayer, 1994).

The outer membrane plays an important role in the physiology of the Gram-negative bacteria. It allows the passage of certain substances into the periplasmic space but impedes the passage of others: thus the outer membrane is said to be selectively permeable. The outer membrane is composed of lipids and proteins and its permeability properties can be attributed to these components. The outer membrane is a bilayer which is asymmetric with respect to its lipid content: the inner leaflet is composed of phospholipids much in the same manner as the inner membrane but the outer leaflet is composed exclusively of a special kind of lipid sugar called lipopolysaccharides (LPS) or lipooligosaccharides (LOS) in some bacteria (Benz, 1994). Some proteins in the outer membrane serve to maintain the structural integrity of this layer whereas others are involved in transport of substrates

into the periplasmic space.

Some Gram-negative bacteria also possess additional layers surrounding the outer membrane such as capsules (made up of polysaccharide) or S layers (made up of protein) (Hancock, 1991).

Most work on the cell envelope and its components have been carried out in *E. coli*. Therefore, most of information presented in this chapter is from this organism. Whenever possible, the relationships are extended to *Haemophilus influenzae* type b, the subject of this thesis, to point out similarities as well as differences. In special circumstances, information regarding other bacteria are also presented.

3. Structure and composition of the bacterial outer membrane

3.1. Lipopolysaccharides

Lipopolysaccharide is an amphiphilic molecule consisting of two or three components that are covalently associated and are proximal to distal in the following order. (i) A hydrophobic lipid A moiety (endotoxin), (ii) a core oligosaccharide region, and in some cases, (iii) an O-antigen chain (Hancock *et al.*, 1994). The basic structure of the lipid A moiety is a diglucosamine backbone to which between five and seven fatty acid acyl chains are linked through ester and amide bonds. The core oligosaccharide is comprised of 3-

deoxy-D-mannooctulosonate (KDO) and other heptose and hexose residues. The O-antigen which is most surface-exposed consists of a variable number of identical saccharide subunits. It is the O-antigen that shows the greatest amount of variability among Gram-negative bacterial species. In some species such as *H. influenzae*, the O-antigen is not extensive: such strains are referred to as rough and the polymer is referred to as lipooligosaccharides (Hancock *et al.*, 1994). The negatively-charged phosphate groups present in the sugars of the core oligosaccharide bind divalent cations (Yamada & Mizushima, 1980). Such cross connections of neighbouring LPS molecules form a rigid network that helps to stabilize the outer membrane (Hancock *et al.*, 1994).

3.2. Proteins

Approximately 50% of the outer membrane by weight is protein (Benz, 1994). A large portion of this belongs to a few species of major outer membrane proteins with molecular mass between 30 and 50 kilodaltons. These major outer membrane proteins (approximately 100,000 copies per cell) include those that primarily serve to stabilize the outer membrane such as OmpA of *E. coli* (Morona, R. *et al.*, 1984) and others that form channels in the outer membrane such as OmpF and OmpC of *E. coli* (Nikaido & Vaara, 1985) and *H. influenzae* type b porin (Vachon *et al.*, 1985). In some cases, both these roles may be accomplished by the same protein. Examples of

such proteins are OprF of *P. aeruginosa* and the major outer membrane protein (MOMP) of *Clamydia trachomatis* (Hancock *et al.*, 1994). The type and number of channel-forming proteins expressed in the outer membrane differ from one bacterial species to another. Another protein that is abundantly present in the outer membrane is lipoprotein. The lipid moiety allows these proteins to be inserted within the bilayer. About one-third of the lipoproteins are covalently attached to the peptidoglycan (Braun, 1975). Other outer membrane proteins such as OmpA, OmpF and OmpC of *E. coli* and OprF of *P. aeruginosa* have strong non-covalent associations with the peptidoglycan (Hancock *et al.*, 1994). These interactions produce a tight network between murein and the outer membrane that protects the bacterial cell from osmotic lysis (Hancock, 1991).

Besides the major proteins, the outer membrane also contains several minor proteins. Examples of these minor proteins are those that are responsible for the export of toxins out of the cell (TolC of *E. coli*; Benz, 1994) and the active transporters discussed below.

Due to their surface location, these outer membrane proteins are receptors for binding and entry of bacteriophages and anti-microbial agents such as colicins, toxins, and certain antibiotics. Phages that bind to porins, such as phage lambda to maltoporin or LamB of *E. coli*, have also been identified. A Hib porin-specific phage or anti-microbial

agent is yet to be identified.

4. Accessibility of solutes to the periplasmic space

4.1. Uptake across the outer membrane

Hydrophobic substances that manage to gain access into the periplasmic space do so by their ability to penetrate the lipid bilayer of the outer membrane. On the other hand, hydrophilic substances gain access into the periplasmic space via several proteins embedded within the outer membrane. These proteins can be categorised into three classes. (i) Non-specific channels, (ii) specific channels, and (iii) active transporters (Nikaido, 1992; Nikaido, 1994). The non-specific channels such as OmpF and OmpC of *E. coli* and Hib porin allow for the diffusion of small hydrophilic solutes up to a certain molecular mass into the periplasmic space. These non-specific channels exhibit a high rate of flux for the solutes. Although monomeric OmpA of *E. coli* has been recently shown to form channels (Sugawara & Nikaido, 1992), the rate of solute diffusion through OmpA is only about 1% compared to the OmpF and OmpC porins. It was concluded that the role of OmpA in *E. coli* is likely a structural one: it helps to maintain the integrity of the outer membrane. OprF from the outer membrane of *P. aeruginosa* also shows a solute flux rate similar to OmpA (Nikaido *et al.*, 1991). The role of OprF in outer membrane permeability to small, hydrophilic solutes may

be significant in *P. aeruginosa* since its outer membrane lacks other non-specific porins.

The specific channels facilitate the diffusion of one or more selected substrates. Examples of specific channels are LamB of *E. coli* for maltose and maltodextrins, Tsx of *E. coli* for nucleosides, and OprP of *P. aeruginosa* for phosphate (Nikaido, 1994a). These proteins usually have binding sites for the substrates within the channel. Therefore transport via the specific channels could proceed even when the substrate is not abundantly present in the medium. Active transporters are proteins that translocate larger substrates that cannot gain access to the periplasmic space via the channels. Examples are FhuA of *E. coli* for ferrichrome and BtuB of *E. coli* for vitamin B (Nikaido, 1994a). Active transporters bind specifically to one or more substrates but their translocation across the outer membrane into the periplasmic space requires the coupling of energy in the cytoplasm provided by the inner membrane protein TonB. Translocation via the active transporters can occur even when the concentration of the substrate is greater in the periplasmic space than in the external medium.

The expression of some of these proteins in the outer membrane are regulated in response to one or more environmental signals and consist of sophisticated control mechanisms (Nikaido, 1994a) whereas the expression of others are constitutive. Almost all specific channels are induced in

the presence of their substrates and many of the active transporters are induced under conditions that are limiting for essential nutrients such as iron. Non-specific porins from some bacterial species, such as OmpF, OmpC, and PhoE of *E. coli*, are subject to regulation whereas porins in others such as Hib porin are constitutively expressed. OmpF and OmpC are reciprocally regulated by the osmolarity of the medium. In medium containing low salt OmpF is preferentially expressed and in medium containing high salt OmpC is preferentially expressed (Nikaido & Vaara, 1985). Although PhoE is induced by low concentrations of phosphate (Overbeeke & Lugtenberg, 1980), the rate of flux of phosphate through PhoE was found to be similar to any anion of comparable size (Nikaido & Rosenberg, 1983). Hence PhoE is grouped with the non-specific porins.

4.2. Outer membrane permeability barrier

Many Gram-negative bacteria are resistant to a number of large, hydrophilic compounds and hydrophobic compounds. These include substances such as lysozyme, digestive enzymes, hydrophobic antibiotics (fusidic acids, erythromycin, and rifampicin) and detergents (bile salts). Due to the chemical properties of the LPS, the outer membrane has an unusually low permeability to the agents mentioned above. The fatty acid acyl chains of the lipid A can be packed much more densely than the glycerol phospholipids (Nikaido, 1994b). This

arrangement makes the outer membrane an effective barrier for hydrophilic agents. The permeability barrier for hydrophobic agents is due to LPS in the outer membrane. LPS is negatively charged and is crosslinked by divalent cations. The outer membrane can be made more permeable to many hydrophobic compounds by mutational alterations to the structure of LPS or by the disruption of the divalent cationic network using EDTA or competing polycationic molecules (Hancock *et al.* 1994). Since Hib produces LOS in the outer membrane, it is more sensitive to hydrophobic antibiotics than other Gram-negative bacteria that produce LPS (Hancock & Bell, 1988). The outer membrane of Hib was also shown to be more permeable to small hydrophilic solutes when compared with the outer membrane of *E. coli* and *P. aeruginosa* (Coulton *et al.*, 1983). This high degree of permeability was attributed to the presence of larger channels in the outer membrane of Hib.

Some large, hydrophilic substances such as siderophores (ferrichrome, ferric enterochelin) gain access into the periplasmic space by way of active transporters as discussed above. The entry of small, hydrophilic solutes (sugars, amino acids, inorganic ions, nucleosides, and antibiotics such as the β-lactams, chloramphenicol, tetracyclines, and quinolones) into the periplasmic space and the efflux of waste products to the exterior occurs by passive diffusion through porins, those that form both specific and non-specific channels. Porins form water-filled channels in the outer membrane. The sizes

of the channels formed by Gram-negative bacterial outer membrane proteins range from 6 to 23 angstroms (Hancock, 1987) and their exclusion limits range from 600 to 5000 daltons (Welte *et al.*, 1995). The water-filled nature of these channels make them poor routes for hydrophobic compounds whereas the channel size restricts the entry of large, hydrophilic substances. Mutations that alter the structure of these outer membrane components may also change the permeability properties of the outer membrane.

To classify solutes as being able or unable to permeate the porins based on their hydrophilicity and size is not always accurate. Such a simplistic and qualitative view can be quite misleading. Other physical and chemical properties of the solute molecules also influence the rates at which they permeate the channels. For example, the shape of the solute molecule as well as the charge that it imparts are factors that determine the rates of their diffusion through the porins.

5. Investigation of porin function

5.1. *In vivo* assays

Permeation of substances through the outer membrane in bacterial cells can be studied in several ways. (i) The uptake of radio-labelled substrates into the cell (Sonntag *et al.*, 1978); (ii) the growth of cells on defined substrates

which are usually the sole sources of any one nutrient group (Benson & Decloux, 1985); and (iii) susceptibilities of the bacteria to several anti-microbial agents such as antibiotics (Capobianco & Goldman, 1994). Since the type and number of porins expressed in bacterial species are different, the permeability properties of the outer membranes of different organisms cannot be compared by these methods. Even in the same species, changes to the conditions of growth can result in the differential expression of the amount, type and number of porins. Therefore, only under the same conditions, the permeability properties of a given porin and changes to the permeability after mutagenesis of that porin can be evaluated by these experiments. However, the interpretation of data using these assays may become difficult due to several complications. (i) The presence of more than one route of entry for the sclutes; (ii) in the case of mutated porins, whether or not the mutations change cellular physiology besides their alteration of channel properties; and (iii) the inactivation, efflux or transport across the inner membrane of the solutes.

5.2. Porin-deficient mutants

Porin-deficient, antibiotic-resistant mutants have been isolated from several Gram-negative bacteria. *E. coli* mutants deficient in OmpF and OmpC porins that showed resistance to cefazolin and moxalactam (Jaffé *et al.*, 1983), *S. typhimurium*

mutants deficient in the OmpC porin that showed resistance to cephalexin (Medeiros *et al.*, 1987), and *Serratia marcescens* mutants deficient in the 41,000-dalton porin that showed resistance to β-lactams (Goldstein *et al.*, 1983) are examples. Most of the porin-deficient mutants displayed increased resistance to hydrophilic antibiotics. The differences in sensitivities of these mutants over the wild-type strains as determined by their minimum inhibitory concentrations (MICs) were two-fold to more than 100-fold. Porin-deficient strains, that showed more than a 100-fold increase in the minimum inhibitory concentration of antibiotics over their wild-type counterparts, were proposed to contain inactivation systems for those antibiotics in both strains (Nikaido, 1989). When such a system was absent for a particular antibiotic, the minimum inhibitory concentrations for the wild-type and the porin-deficient strain were altered only slightly (Nikaido, 1989). Therefore the loss of porins in strains produces significant levels of resistance to antibiotics, especially in combination with the inactivation of those antibiotics.

Methods that complement the *in vivo* assays for porin function are provided by *in vitro* techniques, given the availability of purified porin.

5.3. *In vitro* assays

Classical porins of Gram-negative bacteria are major outer membrane proteins that form stable trimers. In fact,

most porins migrate as trimers when subjected to SDS-PAGE after incubation of the protein in SDS-containing sample buffer at room temperature. The samples have to be boiled in order to denature these porins into monomers. Moreover, most of them are tightly associated to the peptidoglycan layer. These properties provide a relatively simple method for the purification of these proteins. First bacteria are sonicated or passed through a French pressure cell. Subsequent centrifugation and collection of the pellet isolates the cell envelope. Detergents are then used to solubilize most components of the cell envelope. The insoluble material contains a few proteins either covalently attached to or non-covalently associated to the peptidoglycan. The porins can be released from this preparation either by digestion of the peptidoglycan by lysozyme or by extraction with salt. A final step of chromatography is usually undertaken in the presence of a suitable detergent in order to obtain porin in pure form.

Several *in vitro* techniques are used to study pore function. The first method (Nakae, 1975) is based on the efflux of radio-labelled solutes from proteoliposomes: liposomes reconstituted with purified porins. By this assay it was concluded the molecular mass exclusion limit for *E. coli* OmpF, OmpC and PhoE porins was about 600 daltons (Jap & Walian, 1990) and that of Hib porin was about 1400 daltons (Vachon *et al.*, 1985).

The second method is termed liposome swelling assays

(Nikaido & Rosenberg, 1983). In this method, the rates of sugars of different sizes to permeate porin-reconstituted liposomes are determined. This method is considered more informative than the former method since it determines the relative rates of diffusion of a series of sugars, rather than the absolute rates. Initially the liposomes contain a large molecular weight sugar, such as dextran of 20 to 40 kilodaltons, that is unable to permeate porins. These liposomes scatter light and consequently exhibit high optical density. Liposomes are diluted into a solution containing dextran (isotonic with respect to the inside of liposomes) and the test sugar. If the sugar is able to diffuse into the liposomes, water will move along with it to correct for the change in osmotic pressure within the liposomes with respect to the exterior. The consequent swelling of liposomes can be detected as a decrease in the optical density. Usually, measurements are made for about 10 seconds after mixing. The initial rate of swelling is taken as a measure of rate of solute permeation. From the initial rate of swelling, the effective diameter of the porin channel can be calculated according to the theory of Renkin (Renkin, 1954). By this method, the pore formed by OmpF was estimated to be 11 angstroms (Nikaido & Rosenberg, 1983) and the pore formed by Hib porin was estimated to be 18 angstroms (Vachon *et al.*, 1988).

The third method is the assay of channel function in

black lipid membranes (Benz *et al.*, 1978). In this technique, the conductivity of ions through the porin channels is measured. The device consists of a chamber separated by a teflon sheet. A small hole in the teflon sheet connects the *cis* and *trans* sides of the chamber each containing an electrode. The chamber is filled with buffer and lipids are applied across the hole. When viewed through a microscope, the formation of a lipid bilayer is indicated by the field turning black to incident light. The purified porin sample is added to one side of the chamber after application of voltage across the two electrodes (10 to 100 mV). The protein spontaneously inserts into the bilayer and if channel formation occurs, ions flow through the pores to equilibrate the potential difference created. The conductance of ions is detected as current.

Depending on the dilution of the porin sample used in this assay, either single channel conductance measurements (more dilute protein) or macroscopic conductance measurements (more concentrated protein) can be obtained. Whereas in single channel conductance there are stepwise increments in current, in macroscopic conductance many porins insert simultaneously resulting in a concerted increase in the current. The macroscopic conductance method can also be used to study ion or substrate selectivity under zero applied potential.

Single channel conductances of OmpF porin, *Rhodobacter*

capsulatus porin, and Hib porin in 1M KCl were reported as 2 nanosiemens (nS) (Benz *et al.*, 1979), 3.3 nS (Benz, 1994), and 1.1 nS (Vachon *et al.*, 1986), respectively. It was proposed (Benz, 1994) that the difference in conductance between OmpF and *Rhodobacter capsulatus* porin may reflect the larger diameter of the latter channel (Cowan *et al.*, 1992; Weiss *et al.*, 1991). Although the solute size exclusion limit and channel size (liposome swelling assay) for Hib porin were greater than that for OmpF, the single channel conductance was lower. This anomaly has not yet been resolved, but it has been suggested that monomers of Hib porin may form functional channels (Dahan *et al.*, 1994). Due to the uncertainty of the oligomeric state of porin in these synthetic membranes, conductance measurements may not correlate with their channel dimensions estimated from liposome swelling assays, especially when comparing porins from different species.

6. Structure of porins

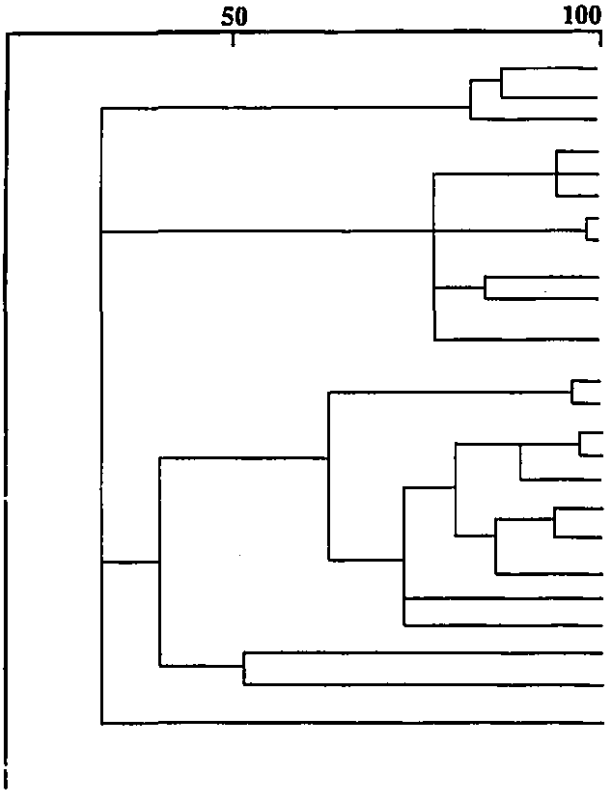
6.1. Primary structure

Several Gram-negative bacterial porin sequences have been determined. They include several species from the family Enterobacteriaceae, and from other genera including Pseudomonas, Chlamydia, Bordetella, Neisseria, Rhodobacter, Comomonas, and Haemophilus. Their primary structures show some common features. The molecular mass of the monomers of

these proteins range from 30 to 50 kilodaltons. The DNA sequences shows that they all contain a typical prokaryotic signal sequence present in proteins exported across the inner membrane. Unlike inner membrane proteins, the amino acid sequences of the proteins do not contain long stretches of hydrophobic amino acids that can span the membrane as α-helices. Hydrophilic (charged and uncharged) and hydrophobic amino acids occur evenly along the entire lengths of the porin proteins, a feature that is required for the formation of amphiphilic (alternating between hydrophobic and hydrophilic) β-strands. Usually there are no cysteines present in the sequences and the C-terminal amino acid is phenylalanine. This terminal phenylalanine was proposed to be important for proper localization of the proteins in the outer membrane (Struyvé *et al.*, 1991).

When porin sequences from Gram-negative bacteria were compared, significant global similarities were not present. Bacterial porins from the same species (*E. coli* OmpF, OmpC, and PhoE), from the same genera (*Neisseria meningitidis* class 3 porin and *Neisseria gonorrhoeae* PIA porin), and from related organisms (*E. coli* and *Salmonella typhimurium* OmpC) show sequence similarities (Jeanteur *et al.*, 1994). Based on the similarities of porin sequences, a phylogenetic tree (Fig. 2) was proposed (Jeanteur *et al.*, 1994). In general, these results are in agreement to the phylogenetic relationships derived by 16S ribosomal RNA analyses.

Fig. 2. Phylogenetic tree derived from similarity of porin sequences. Each branching point shows the level of maximum similarity between the groups that it connects. The protein name is the one most used in the literature describing the gene product. The species is that from which the gene was originally isolated. Reproduced with permission from Jeanteur *et al.*, 1994.



GENE	SPECIES	PROTEIN	ACC Nº
P2	H. influenzae	HIOOMP2B	M93270
P2	H. influenzae	HIOOMP2C	M93269
P2	H. influenzae	HIOOMP2A	M93268
PhoE	E. coli	ECOPHOE	V00316
PhoE	K. pneumonia	KLEBPHOE	M28295
PhoE	E. cloacae	CLOAPHOE	-------
Lc	PA-2	PA2LC	P07238
Nmpc	E. coli	ECONMPC	P21420
OmpC	E. coli	ECOOMPC	K00541
OmpC	S. typhi	STYOMPC	M31424
OmpF	E. coli	ECOOMPF	M74489
PorA	N. meningitidis	NMPORA	X52996
PorA1	N. meningitidis	NMPORA1	X52995
PIB1	N. gonorrhoeae	NGP1B	X52823
PIB2	N. gonorrhoeae	NGPROI	M21829
Por	N.lactamica	NLPORING	X65533
PorB	N. meningitidis	NMCLA3PRO	M68962
PorB	N. meningitidis	NMPOR3B	-------
PIA1	N. gonorrhoeae	NGOMPI	J03029
P2	N. meningitidis	NMPOR2	-------
Por	N.sicca	NSPOR	X65461
BPPor	B.pertussis	PBPORING	X58488
Omp32	C.acidovorans	COMVOR	-------
-------	R.capsulatus	RHODO	-------

6.2. Secondary structure

Secondary structures of porins have been determined by spectroscopy of purified porins. They include circular dichroism (CD) and fourier transform infrared spectroscopy (FTIR). The major secondary structures in these porins were β-sheets (50 to 60%). The rest were turns and undefined structures. A small amount of α-helices was also detected (Eisele & Rosenbusch, 1990; Nabedryc *et al.*, 1988).

The secondary structure of these porins have been predicted by using several algorithms (Jeanteur *et al.*, 1994). The folding pattern based on these predictive parameters showed several amphiphilic β-strands connected by hydrophilic loops that could form channel-producing β-barrels in the outer membrane. The predicted secondary structures have been tested experimentally by one of several ways: (i) mapping mutations that abolish substrate or phage binding to porin (Korteland *et al.*, 1985); (ii) mapping and localization of antibody binding sites of porin (Klebba *et al.*, 1990); (iii) limited proteolysis of intact cells and mapping cleavage sites in the outer membrane protein (Koebnik & Braun, 1993); and (iv) insertion of foreign epitopes in porin and their localization (Charbit *et al.*, 1991). These experiments have been most useful in the identification of regions in porins that are part of surface-exposed or buried loops thereby providing information on their topological organization.

Porins with β-sheet structure have also been identified

in the outer membranes of mitochondria and chloroplast (Welte *et al.*, 1995), as well as in the outer membrane of the bacterium *Thermotoga maritima* (Rachel *et al.*, 1990), which is considered ancestral to eubacterial species. It appears that the β-barrel motif that produces channels has been conserved through evolution.

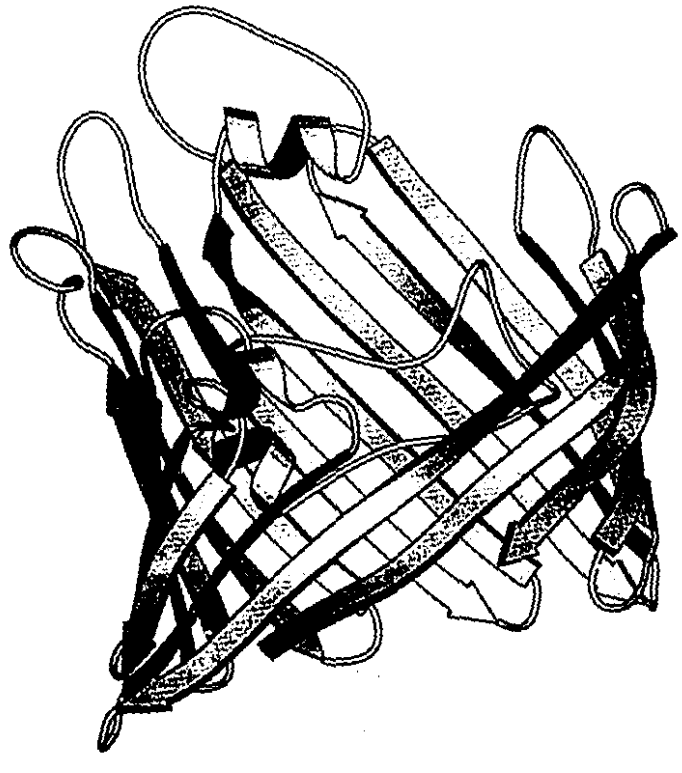
Outer membrane receptors of *E. coli*, FhuA and FepA, are involved in the uptake of siderophores ferrichrome and ferric enterochelin, respectively. Deletion of sequences in these receptors that are thought to be surface-exposed converted these receptors to non-specific channels (Killmann *et al.*, 1993; Rutz *et al.*, 1992). Therefore, it was suggested that the β-barrel motif may be universal to most if not all outer membrane proteins.

6.3. Three-dimensional structure

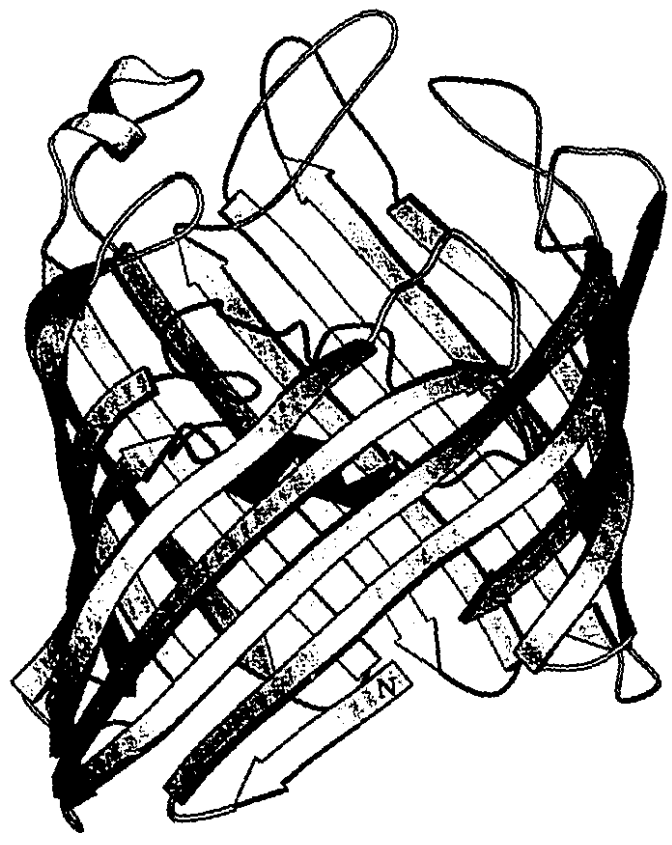
The structures of the non-specific channels *Rhodobacter capsulatus* porin (Weiss *et al.*, 1991), *Rhodopseudomonas blastica* porin (Kreusch *et al.*, 1994), and *E. coli* OmpF and PhoE (Cowan *et al.*, 1992) porins have been solved to atomic resolution by X-ray crystallography. The crystal structures confirmed the results of the topological analyses of these porins. Although the amino acid sequences of *R. capsulatus* porin and OmpF show very little homology, their folding pattern are remarkably similar (Fig. 3). Based on the crystal structures, the consensus folding pattern shows 16 anti-

Fig. 3. Ribbon diagrams of structures of porins. The monomers of the *Rhodobacter capsulatus* porin (A) and the *E. coli* OmpF porin (B) and the trimers of the *Rhodobacter capsulatus* porin (C) and the *E. coli* OmpF porin (D) are shown. The 16 β-strands are represented by arrows and the α-helices by coils. Loop 3 of OmpF is shaded in black for clarity. In the presentations of the monomers, the periplasmic space is below the molecules. For the trimers, the views are from the external side. In the *Rhodobacter capsulatus* porin trimer, the bound calcium atom in the molecular interface (black ball) and two calcium atoms bound to residues of loop 3 (grey balls) are also shown. The presentations of the *Rhodobacter capsulatus* porin and OmpF were reproduced with permission from Welte *et al.*, 1995, and Cowan & Schirmer, 1994, respectively.

A



B



C

D

parallel β-strands and loops that connect these strands on either side to form a β-barrel. The β-barrel structure forms a large channel. The β-strands are tilted 30 to 60 degrees in relation to the trimer axis. Each monomer produces a channel and therefore there are three channels in the trimer.

Within each β-strand, the side chains of amino acids on the side facing the membrane are hydrophobic and the amino acid side chains facing the channel are hydrophilic. However, close to the surface of the bilayer there is a preference for the aromatic amino acids tyrosine and phenylalanine. The side chains of these amino acids are thought to extend outward to the hydrophilic environment (tyrosine) or extend into the lipid (phenylalanine) thereby helping to anchor these proteins in the outer membrane.

The loops on the extracellular side are much longer and those on the side of the periplasmic space form tight turns. Most extracellular loops are surface-exposed. Although few in number, the α-helices found in the porin structures are restricted to the loops. Several acidic residues in the surface-exposed loops are thought to interact with the negatively charged LPS through divalent cation bridges (Cowan *et al.*, 1994). One important feature that is universal to all these porins relates to the role of loop 3.

As the distance between neighbouring β-strands is 4.5 angstroms, 16 β-strands should give rise to a backbone to backbone diameter of 23 angstroms. However, the pores are

narrowed at one point to a cross-section of 8 x 10 angstroms in *R. capsulatus* porin and 7 x 11 angstroms in *E. coli* OmpF (Nikaido, 1994a). The narrowing of the pores was attributed to loop 3 which in all cases folds back into the β-barrel and thereby produces a constriction. The solute-discriminating properties of these channels are expected to be influenced by the eyelet formed by this loop as opposed to the overall dimensions of the β-barrel. This organization produces a channel that has a wide entrance, a wide exit, and a constriction in the middle. Such an architecture provides for the efficient flux of small hydrophilic solutes but impedes the passage of larger ones.

A section through the channel parallel to the plane of the outer membrane shows several negative charges on one side of the eyelet and several positive charges on the other. Certain amino acids of the β-strands and amino acids of loop 3 contribute to this charge separation. Due to electrostatic forces of these charged residues, their side chains are extended maximally. This arrangement is thought to produce a predominantly hydrophilic channel that is well defined and has rigid shape.

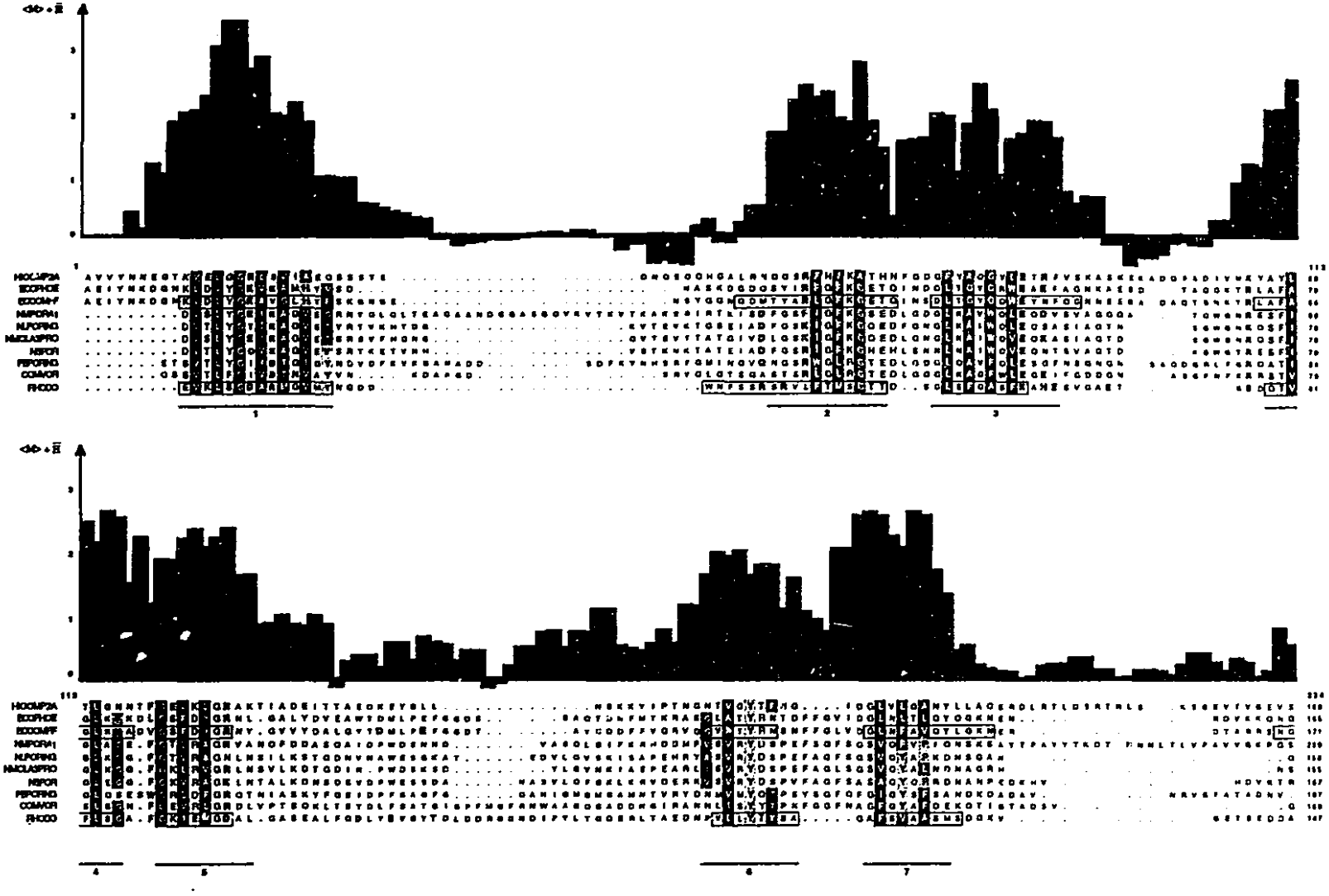
Hydrophobic interactions between monomers that bring about oligomerization was reported in both *R. capsulatus* porin and *E. coli* OmpF. Several other features, sometimes unique to one porin, also helps in trimer formation or in its stabilization. In OmpF, loop 2 from one subunit extends into

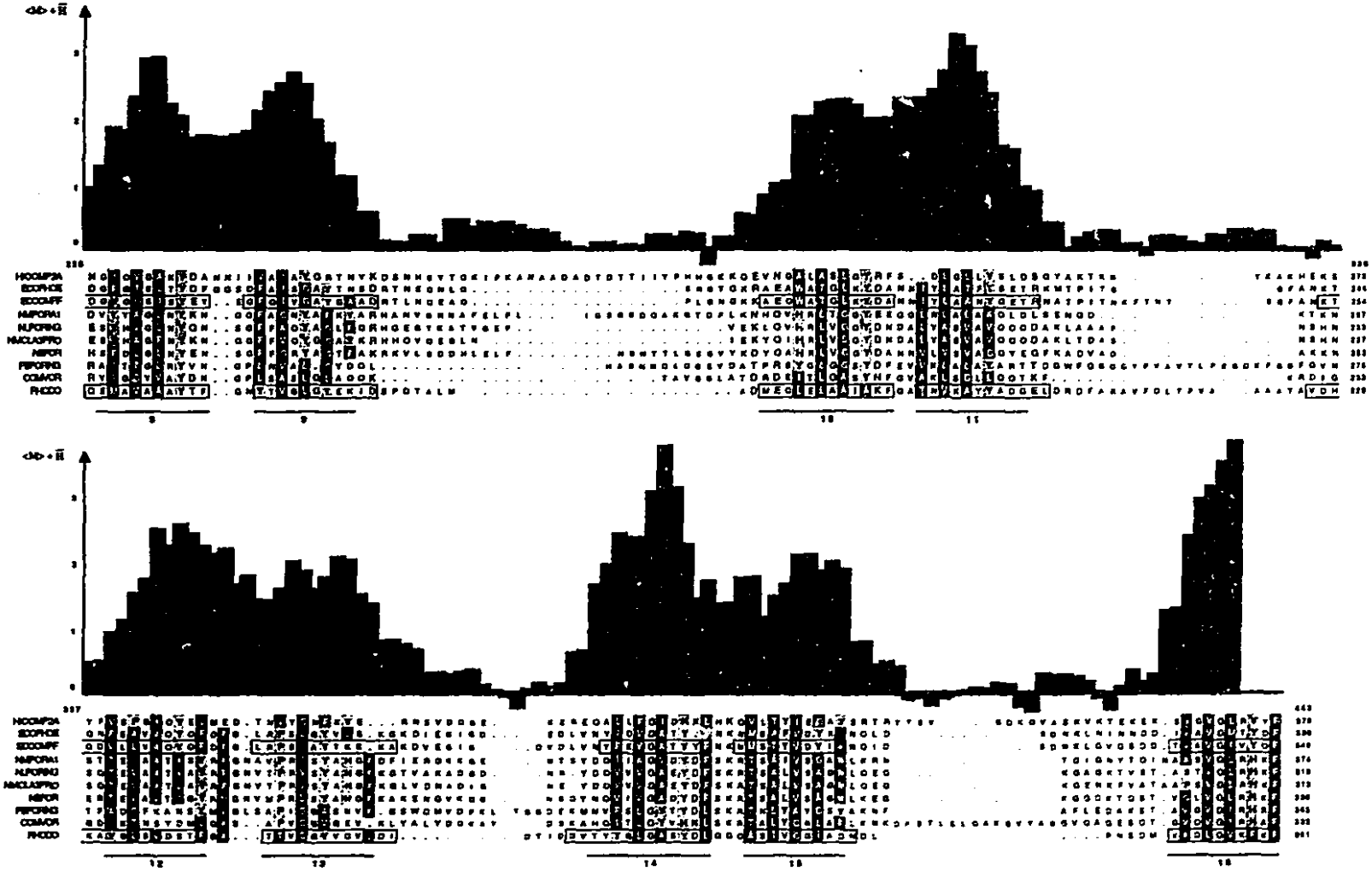
the other where it makes hydrophilic contacts. This feature is not seen in the *R. capsulatus* porin. In the *R. capsulatus* porin, the N-terminus of one monomer is bridged to the C-terminus of the other; the N-terminus to C-terminus contact in OmpF is within the same subunit. Unlike OmpF, the trimer of *R. capsulatus* porin is disrupted in the presence of EDTA (Nestel *et al.*, 1989). Since the structure of *R. capsulatus* porin shows several bound calcium ions (not found in OmpF), these calcium ions may help in the stabilization of the *R. capsulatus* porin trimer.

Of the specific channels, the high resolution structure of LamB of *E. coli* has been obtained (Schirmer *et al.*, 1995). Several of the properties of the non-specific porins are also seen in LamB. However, the structure shows 18 β-strands, and 3 loops (including loop 3) contribute to the constriction that is midway through the channel. Residues detected as part of the maltose and maltodextrin binding site are located within the channel; some of these amino acids are found in the internally-folded loop 3 (Klebba *et al.*, 1994).

The availability of crystal structures of porins and several porin sequences from other bacteria have prompted researchers to align the sequences and predict the structures of less well characterized porins. Three porins whose structures had been solved (*Rhodobacter capsulatus* porin and *E. coli* OmpF and PhoE porins) were part of such an alignment (Fig. 4). Also included was the porin protein from

Fig. 4. Alignment of porin sequences and prediction of membrane-spanning β-strands. The <M> + $\bar{H}$ (index of hydrophobicity and amphiphilicity) was plotted using the set of porin sequences shown in Fig. 2. The structures of three of these porins have been solved and the boxes indicate the true strands revealed by X-ray crystallography. In order to show the alteration of hydrophobic residues within β-strands, the hydrophobic residues are shaded black. Residues (Y, H, and P) that posses some polar properties are shaded grey. Charged residues involved in the eyelet are outlined. Each number (1 to 16) under the alignment denote a predicted β-strand. Reproduced with permission from Jeanteur *et al.*, 1994.





nontypeable *Haemophilus influenzae* which is approximately 80% identical to Hib porin. Although the homologies among the bacterial porins were superficially weak (Jeanteur *et al.*, 1991), a more careful examination revealed several regions of conservation (Fig. 4). The highest values for the hydrophobic and amphiphilic indexes (<M> + $\bar{H}$) were localized to these regions. In the crystal structures of porins, these regions corresponded to the 16 β-strands. The amino acids of the loops were variable; some conservation was present in residues of loop 3. During evolution, selection by antibodies and phages may have forced the amino acids of surface-exposed loops to evolve at a higher rate than others. Also, the external loops may simply have more freedom to change without altering pore function.

7. Structure-function relationship of porins

The three-dimensional structures of porins explain many of their functional properties. The two major porins in *E. coli*, OmpF and OmpC, exhibit high degree of homology. The channel formed by OmpF was determined to be slightly larger than that formed by OmpC (Nikaido, 1989). The alignment of the two porins showed that when compared to OmpC, there was a deletion in the sequences corresponding to loop 3 of OmpF.

Mutants of *E. coli* were selected for the expression of larger pores that allowed for the growth in maltose in strains

that lacked the maltose-specific porin LamB. In these mutants, several point mutations in OmpF (Benson *et al.*, 1988) and OmpC (Misra & Benson, 1988a; Misra & Benson, 1988b) that produced wider channels than their wild-type counterparts were identified. Remarkably, most of the point mutations alter one of the charged residues (many in loop 3) that form the electrostatically-rigidified eyelet mentioned above. This strategy also resulted in the selection of deletions in loop 3 of these porins that produced widening of the channel. The insertion of an antigenic determinant (7 amino acids) into loop 3 of *E. coli* PhoE porin resulted in a mutant PhoE whose channel was much narrower than wild-type PhoE (Struyvé *et al.*, 1993).

Whereas OmpF and OmpC porins show a preference for cations, PhoE porin shows a preference for anions. Site-directed mutagenesis of Lys residue at 125 (in loop 3) in PhoE to a Glu residue reversed the ion selectivity of this porin; it became cation selective similar to OmpF and OmpC (Bauer *et al.*, 1988).

The channels formed by several non-specific porins can be voltage gated. In most instances, voltage gating of porins occurred at potentials over 100 mV. The sequestration of cations by the negatively-charged membrane-derived oligosaccharides in the periplasm creates a potential across the outer membrane. This potential, referred to as Donnan potential, could not be greater than 100 mV (Nikaido, 1993).

Therefore, the physiological relevance of voltage gating is unclear at the present time. Nevertheless, it has been shown that mutations that change the voltage dependency of porins are those that change charged amino acids in the eyelet region including those of loop 3 or that result in small deletions in loop 3 (Delcour *et al.*, 1991; Rocque & McGroarty, 1990).

In Hib porin, a residue in the surface-exposed loop 4 (amino acid 166) has been shown to be critical for voltage gating (Dahan *et al.*, 1994). The molecular mass exclusion limit of Hib porin (1400 daltons) is larger than those of the four non-specific porins whose structures are known. Based on this, researchers (Welte *et al.*, 1995) suggested that loop 3 in Hib porin may not be involved in narrowing the channel, and that loop 4 may be part of the external channel entrance.

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PREFACE TO CHAPTER 2

We wished to identify the topological organization of *Haemophilus influenzae* type b (Hib) porin in the outer membrane. This chapter describes the generation of a panel of anti-Hib porin monoclonal antibodies (mAbs) as probes of Hib porin topology. The identification of regions in Hib porin recognized by these mAbs enabled the construction of a preliminary epitope-map of antibody reactivities. Some of these epitopes were localized to the bacterial cell surface. The immunological activities of these mAbs were also tested.

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Monoclonal antibodies specific to porin of *Haemophilus influenzae* type b: localization of their cognate epitopes and tests of their biological activities

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Summary

The major outer membrane protein of *Haemophilus influenzae* type b (Hib) is porin (M_r 38000, 341 amino acids). To identify antigenic determinants on Hib porin that might be exposed at the bacterial cell surface, seven mouse monoclonal anti-Hib porin antibodies were generated. The monoclonal antibodies were tested for their binding to intact cells by flow cytometry; all but one bound to the cell surface. Digestions of Hib porin with cyanogen bromide, hydroxylamine or trypsin generated fragments, the identities of which were confirmed by microsequencing of the amino termini. Following electrophoresis and immunoblotting of the fragments, the specificities of the monoclonal antibodies for their cognate sequences were determined. The porin gene *ompP2* was expressed in the baculovirus expression vector system; the recombinant porin was recognized by all of the monoclonal antibodies. Deletions were created by omega mutagenesis of *ompP2*, generating proteins truncated after amino acids 139, 174, 182, and 264. These deletion proteins were tested for reactivities with the monoclonal antibodies, thereby establishing the boundaries of three antigenic determinants that were recognized by the monoclonals: domain (i), amino acids 104–130; domain (ii) amino acids 162–174; and domain (iii), amino acids 267–341. The biological activities of monoclonal antibodies that were representative of these three classes were tested for their bactericidal activity in complement-mediated lysis of whole cells. The monoclonal antibodies were also tested for their immunoprotective properties in the infant rat model of bacteraemia. Although the monoclonal antibodies were surface-binding, they were neither bactericidal nor protective.

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Introduction

Haemophilus influenzae type b (Hib) is the most common cause of bacterial meningitis in infants under 18 months. To elicit protective antibodies against this disease, much attention has been directed to the capsular polysaccharide polyribosyl ribitol phosphate (PRP) of this bacterium and its use as a vaccine (Granoff *et al.*, 1986). Because PRP alone was poorly antigenic in young infants most susceptible to disease, a new generation of vaccines was developed based on conjugation of PRP to carrier proteins. These polysaccharide–protein conjugates are of recognised value in preventing disease (Eskola *et al.*, 1990; Mäkelä *et al.*, 1990). Other surface components of Hib have also been considered as vaccine candidates (Granoff and Munson, 1986). Studies performed with an experimental infant rat model demonstrated that antibodies specific for non-capsular surface components might play a role in humoral defence mechanisms. A key observation was that antibodies directed against non-capsular antigens were able to protect against the experimental infection (Munson *et al.*, 1983). One of the non-capsular antigens is porin (M_r 38000), the most abundant protein in the outer membrane of this bacterium.

We have studied the Hib porin extensively and have shown that it has a molecular mass exclusion limit to solutes greater than 1400Da (Vachon *et al.* 1985), a cross-sectional diameter of 0.9nm (as determined by reconstitution into black lipid membranes; Vachon *et al.*, 1986), and a trimeric organization in the outer membrane (Vachon *et al.*, 1988). Furthermore, Hib porin is surface-exposed and conserved; monoclonal antibody Hb-2 recognized native porin at the surface of intact cells (Hamel *et al.*, 1987b) and reacted with 453/455 type b strains collected from a wide variety of sources (Hamel *et al.*, 1987a). Information on the structural and antigenic conservation of Hib porin (Hansen *et al.*, 1989b) has been extended to the cloning and sequencing of the porin gene

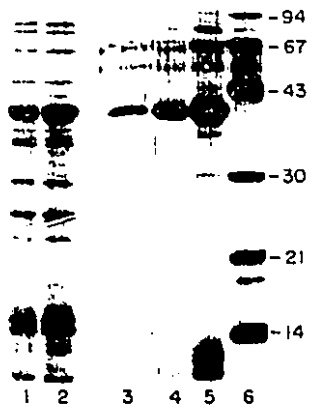

Fig. 1. Purification of porin from Hib strain ATCC9795. Samples were run on 13% SDS–PAGE and silver stained. Lanes 1 and 2: 1500 and 3000ng of porin prepared by the procedures of Vachon *et al.* (1985) and previously appearing as a single band after staining with Coomassie Brilliant Blue staining. Lanes 3, 4 and 5: 250, 500, 1000ng of FPLC-purified porin prepared as described in the *Experimental procedures*. Lane 6: molecular mass markers in kDa.

(Hansen *et al.*, 1989a; Munson and Tolan, 1989). Additional DNA sequence data have been recently generated for Hib porin genes from prototype strains of three major clone families of different outer membrane protein (OMP) subtypes: 2L, 3L, and 6U. These gene sequences showed variations of 1, 0, and 13 nucleotides respectively, over the entire sequence (Munson *et al.*, 1989) relative to the first reported porin sequence. The variations correspond to 1, 0, and 10 amino acid changes in primary sequence relative to porin from OMP subtype 1H.

To extend the information on porin of Hib beyond its biophysical behaviour and its gene structure, we wished to identify regions of porin that might be surface-exposed and accessible to antibodies. Our approach was to generate a panel of anti-Hib porin monoclonal antibodies (mAbs), to map the epitopes on porin that were recognized by them, and to identify those epitopes that are at the bacterial cell surface. Because polyclonal antibodies against Hib porin have been reported to be bactericidal and protective, the mAbs were also tested for their biological activities in complement-mediated bactericidal tests and immunoprotection in the infant rat model of infection.

Results

Monoclonal antibodies against Hib porin

The purification scheme for porin of Hib strain ATCC9795 as described by Vachon *et al.* (1985) yielded a single species of protein when stained with Coomassie Brilliant Blue. Upon silver staining of the preparations, several contaminating bands appeared (Fig. 1). The use of fast-protein liquid chromatography (FPLC) improved the purification to produce one major species by silver staining, provided high yields (10mg per run), and showed some contamination with lipopolysaccharide (LPS) of less than 100ng per μg of FPLC-purified protein. LPS could not be removed even by repeated passage of the sample over the MonoQ column.

BALB/c mice were immunized with FPLC-purified Hib porin suspended in Freund's incomplete adjuvant. Polyclonal sera were collected from the mice and analysed for the presence of Hib porin-specific antibodies by immunoblotting. These sera reacted strongly with the 38kDa porin and weakly with other OMPs. To generate stable hybridomas from the cells, the porin-challenged splenocytes were fused with the SP2/0 myeloma cell line. Tissue-culture supernatants from the hybridomas were screened for antibodies by means of the enzyme-linked immunosorbent assay (ELISA) using outer membrane preparations from Hib and FPLC-purified Hib porin as coating antigens, and the positive ones were further analysed for their capacity to recognize denatured porin by immunoblotting. OMPs were solubilized in electrophoresis sample buffer by boiling, electrophoresed on a 12% acrylamide gel, and transferred to nitrocellulose. Immobilized proteins were reacted first with the culture supernatants and then with a secondary anti-mouse immunoglobulin conjugated to peroxidase. If a band of 38kDa was observed in this second screen, then the hybridoma was cloned by limiting dilution and retested in the above two assays. Using these strategies, seven anti-Hib porin monoclonal antibodies, mAb POR.1 to mAb POR.7, were generated, each specifically reacting with a band in the preparation of the OMPs that co-migrated with purified Hib porin. The mAbs differed according to the reciprocal titres of their reactivities against FPLC-purified Hib porin and were of different IgG isotypes (Table 1).

Surface binding of monoclonal antibodies

While the ab[illegible] results demonstrated that mAbs POR.1 to POR.7 reacted with Hib porin in outer membrane vesicles (ELISA) and with denatured porin (immunoblotting), it was important to assess whether the seven monoclonals bound to native Hi[illegible] porin at the bacterial cell surface. Flow cytometry is a sensitive technique for assessing the surface location of cellular macromolecules by their reaction with specific antibodies. Intact bacteria were mixed with a mAb, washed free of unbound mAb, and the surface-bound mAb was detected with anti-mouse kappa light-chain mAb 187.1 (Yelton *et al.*, 1981) conjugated to fluorescein or anti-mouse IgG heavy chain conjugated to fluorescein. Surface-bound fluorescence on 20 000 cells

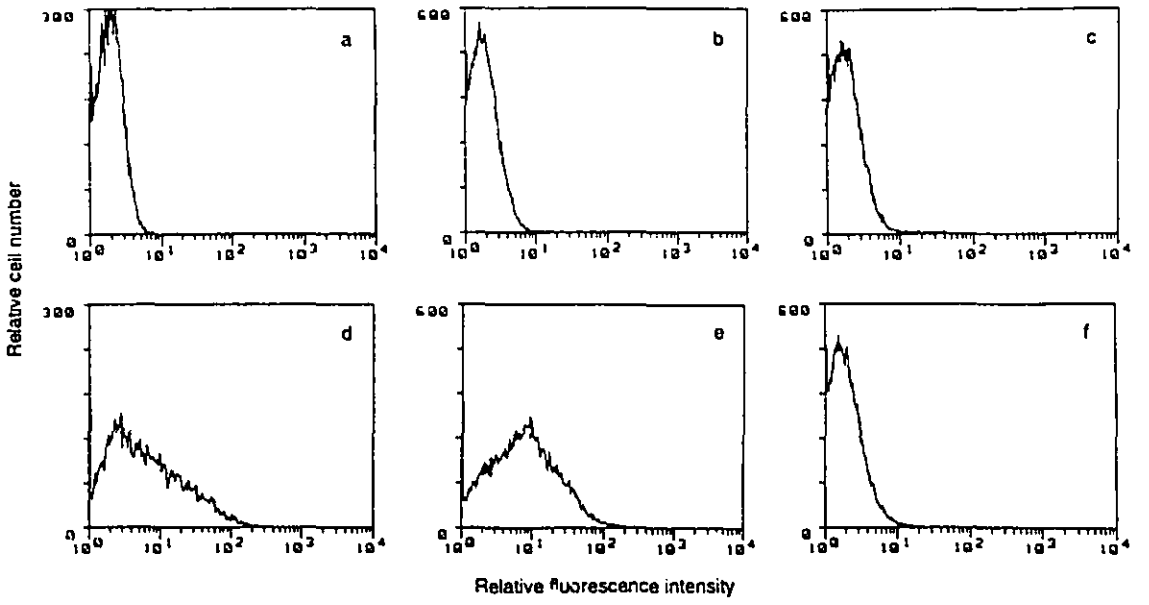

Fig. 2. Flow cytometry of *Haemophilus influenzae* type b with anti-Hib porin monoclonal antibodies. Bacteria were prepared and stained as described in the *Experimental procedures*. Panel a, Hib strain ATCC9795 stained only with secondary antibody conjugated to FITC; panels b and c, Hib strain DL42/2F4 stained with mAb POR.4 and mAb POR.1, respectively; panels d, e and f: Hib strain ATCC9795 stained with mAb Hb-2 and mAb POR.4 and mAb POR.1, respectively.

per sample was assessed by flow cytometry. As a control for non-specific binding of antibody, we used Hib strain DL42/2F4$^-$, a porin-deletion mutant. No staining of strain DL42/2F4$^-$ was observed with any of mAbs POR.1 to POR.7 or with mAb Hb-2 (Fig. 2). When Hib strains ATCC9795 or RH3527 were assayed, there was no detectable staining with mAb POR.1. However, all other POR.2 to POR.7 mAbs and mAb Hb-2 stained these cells, as shown by the increase in relative fluorescence intensity. *H. influenzae* strain RH3528 is a non-capsulated variant derived from RH3527 and was stained by mAbs POR.2 to POR.7 and mAb Hb-2 but not by mAb POR.1.

Identification of immunoreactive amino acid sequences of Hib porin

To identify the primary amino acid sequences with which the seven mAbs reacted, several chemical and enzymatic digestions were conducted using FPLC-purified protein. The digestion products were separated by sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS–PAGE), blotted onto nitrocellulose, and probed with each mAb. Cyanogen bromide digested the Hib porin at a unique Met-266 to two fragments of 30kDa and 8kDa. Different patterns of mAb reactivity were distinguished: (i) mAbs POR.1, POR.2, POR.3, POR.4, and POR.5 reacted with the amino-terminal 30kDa fragment; (ii) mAbs POR.6 and POR.7 reacted with the carboxy-terminal 8kDa fragment.

Cleavage with hydroxylamine occurs primarily between Asn–Gly bonds. In Hib porin there are three potential cleavage sites for this reagent: between amino acids 74/75, 181/182, and 218/219. Digestion conditions were selected to favour partial cleavages and so a family of five fragments was observed upon staining of the electropherograms. These fragments were 30, 24, 20, 18, and 14kDa

Table 1. Monoclonal antibodies against porin of *H. influenzae* type b.

mAb designation	Reciprocal Titre of Antibody Reactivity: bacterial porin[a]	recombinant porin BEVS[b]	Immunoglobulin subclass
POR.1	10 000	25 000	IgG1, κ
POR.2	5000	5000	IgG1, κ
POR.3	5000	5000	IgG1, κ
POR.4	1000	5000	IgG1, κ
POR.5	1000	1000	IgG3, κ
POR.6	1000	500	IgG2a, κ
POR.7	100	500	IgG2a, κ
Hb-2	500	500	IgG2a, κ

a. FPLC-purified bacterial porin (10 μg ml^{-1}) from strain ATCC9795 in carbonate buffer was adsorbed to the ELISA plate, overnight, at room temperature; dilutions of mAbs were reacted with antigen, followed by a secondary antibody conjugated to alkaline phosphatase. The reciprocal titre is expressed as the dilution of mAb which gave an absorbance reading of at least 0.2 over background for the colorimetric assay of enzymatic activity.

b. Lysate from Sf9 insect cells infected with AcPOR virus (1.5 μg ml^{-1}) was adsorbed to the ELISA plate. Conditions for primary and secondary reactions with antibodies and with substrate were the same as for the reaction with FPLC-purified porin.

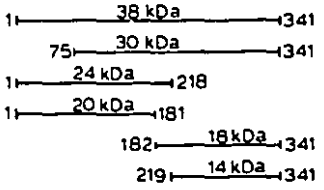

Fig. 3. Cleavage of Hib porin by hydroxylamine and identification of the cleavage products by microsequencing. Five polypeptides were generated by partial digestion with hydroxylamine: 30, 24, 20, 18, and 14 kDa. Each polypeptide was sequenced from its amino terminus and was oriented relative to the others by sequence and by molecular size. The numerical values at the ends of each line represent the confirmed amino-terminal amino acids and the assigned carboxy-terminal amino acids, based upon the location of Asn–Gly linkages in the primary sequence of porin.

(Fig. 3). When these five porin-derived peptides were immunoblotted and probed with mAbs, the following reactivities were detected: mAbs POR.1, POR.2, POR.3, POR.4, POR.5, POR.6 and POR.7 reacted with the 30 kDa fragment; mAbs POR.1, POR.2, POR.3, POR.4, and POR.5 reacted with the 24 and the 20 kDa fragments; mAbs POR.6 and POR.7 reacted with the 18 and 14 kDa fragments. To confirm the sites of cleavage, microsequencing of the first five amino acids of each fragment was conducted. These sequences were then matched with the primary amino acid sequence of Hib porin as follows: Gly-75–Ser–Asp–Asn–Phe, 30 kDa fragment; Ala-1–Val–Val–Tyr–Asn, 24 and 20 kDa fragments; Gly-182–Ile–Gln–Val–Gly, 18 kDa fragment; Gly-219–Val–Leu–Ala–Thr, 14 kDa fragment. These data indicate that the boundaries of epitopes recognized by mAbs POR.1, POR.2, POR.3, POR.4, and POR.5 are between amino acids 75 and 181 and that the boundaries of epitopes recognized by mAbs POR.6 and POR.7 are between amino acids 219 and 341.

Although porins from other bacteria are generally resistant to enzymatic cleavage (Rosenbusch, 1990), porin of Hib was cleaved by trypsin when the digestion was performed overnight in detergent. Trypsin was predicted to cleave this protein more than 40 times at the Arg and Lys residues. After overnight incubation and with a high amount of trypsin, seven cleavage products (and sometimes eight) were resolved by SDS–PAGE on a 20% gel followed by silver staining. Although these fragments were not always of the same intensity, their relative mobilities and therefore molecular sizes could be accurately estimated. The trypsin cleavage products were 19.5, 19.1, 18.9, 18.4, 17.1, (15.5), 15.0, and 14.0 kDa (Fig. 4A). Monoclonal antibodies POR.6 and POR.7 reacted with both the 19.5 and 19.1 kDa fragments (Fig. 4B). Monoclonal antibodies POR.2, POR.3, POR.4, and POR.5 reacted only with the 18.9 and 15.5 kDa fragments and mAb POR.1 reacted with all six trypsin fragments between 18.9 and 14.0 kDa. Microsequencing of the peptides showed that the amino termini were Ala-171–Gly–Glu–Val–Arg, 19.5 kDa fragment; Ile-176–Gly–Glu–Ile–Asn, 19.1 kDa fragment; Ala-1–Val–Val–Tyr–Asn, 18.9, 18.4, and 17.1 kDa fragments; Gln-33–Gln–His–Gly–Ala, 15.5, 15.0, 14.0 kDa fragments. Considering the locations and the relative susceptibilities of trypsin-sensitive cleavage sites between amino acids 156 and 175 and using the sizes of trypsin cleavage products calculated from the primary sequence, tentative assignments were made for the carboxy-termini of the fragments. The proposed C-terminal residues are: Phe-341 for the 19.5 and 19.1 kDa fragments; Arg-175 for the 18.9 and 15.5 kDa fragments; Lys-170 for the 18.4 and 15.0 kDa fragments; Lys-161 for the 17.1 and 14.0 kDa fragments. A summary of these assignments is found in Fig. 4C. From these data it is proposed that mAb POR.1 reacts with some amino acid sequences up to Lys-161, that mAbs POR.2, POR.3, POR.4, and POR.5 react with sequences between Gly-162 and Arg-175, and that mAbs POR.6 and POR.7 recognize some amino acids in the carboxy-terminal half of Hib porin. Instead of digesting Hib porin in solution, experiments were conducted to digest Hib porin by trypsin that was immobilized in a polyacrylamide gel. One of the many

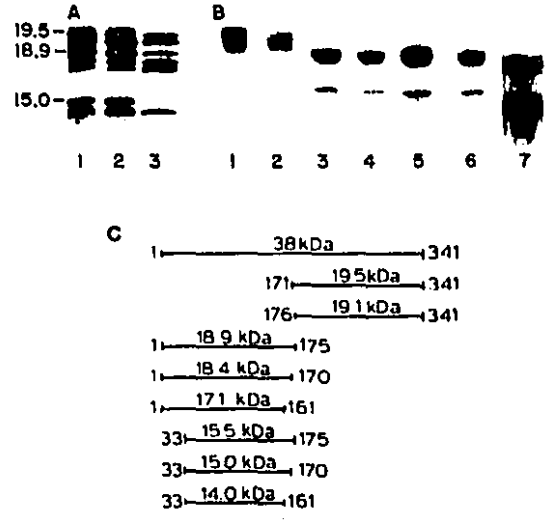

Fig. 4. Cleavage of Hib porin by trypsin and identification of monoclonal antibody reactivities against the cleavage products.
Panel A. Hib porin in solution was digested with trypsin, aliquots were removed at 1, 2, and 16 h of incubation (lanes 1, 2 and 3), electrophoresed on a 20% SDS–PAGE, and silver stained to identify the fragments. The molecular masses (kDa) of three of the fragments are indicated for reference in the left margin.
Panel B. Reaction of anti-Hib porin mAbs against trypsin digestion fragments. Lanes 1 to 7: mAbs POR.6, POR.7, POR.2, POR.3, POR.4, POR.5, and POR.1, respectively.
Panel C. Orientation of fragments from trypsin cleavage of Hib porin. Fragments were sequenced from their amino-termini; the first amino acid from the sequence data is the numerical value on the left of each line. Carboxy-termini were assigned, based upon sizes of the fragments; the numerical value on the right of each line indicates the carboxy-terminus of the cleavage fragments.

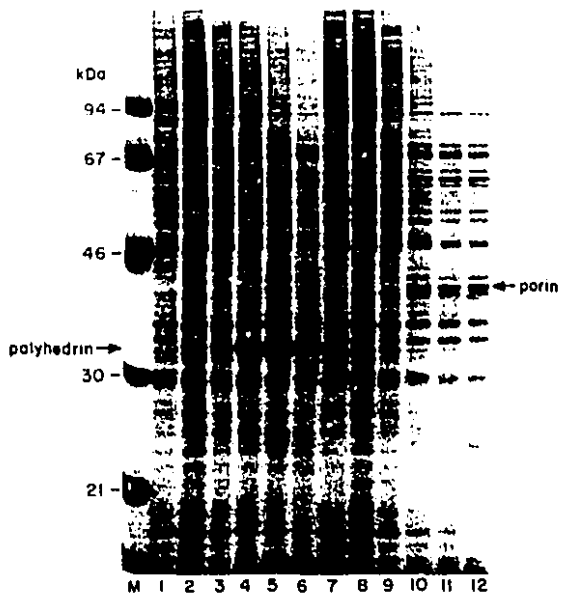

Fig. 5. Identification of proteins in extracts of Sf9 insect cells infected with wild-type baculovirus AcNPV (lanes 1–6) or with recombinant virus AcPOR (lanes 7–12). Replicate Sf9 cultures were infected with virus at a multiplicity of infection of 10:1 and samples were taken at the following hours, post-infection: 0 (lanes 1 and 7); 12 (lanes 2 and 8), 24 (lanes 3 and 9); 48 (lanes 4 and 10); 72 (lanes 5 and 11); and 96 (lanes 6 and 12). Samples were lysed in electrophoresis sample buffer, sheared by passage through a fine-guage needle, and electrophoresed on a 10% acrylamide slab gel. Proteins were stained with Coomassie Brilliant Blue. Molecular mass standards (in kDa) are indicated in the column labelled 'M'. The arrow in the left-hand margin indicates the position of polyhedrin; the arrow in the right-hand margin indicates the position of recombinant porin.

fragments of this partial proteolysis had a M_r of 27000 and an amino terminus of Ala-104–Lys–Thr–Ile–Ala; such a fragment would extend to Phe-341. This 27000 porin fragment reacted with mAb POR.1 as well as with mAbs POR.2, POR.3, POR.4, POR.5, POR.6 and POR.7. Such a result served to narrow further the domain of reactivity for mAb POR.1 to between Ala-104 and Lys-161.

Recombinant porin from baculovirus expression vector system

An independent verification that the mAbs were reacting against the Hib porin protein and not against contaminating materials such as LPS was obtained by expressing the porin gene *ompP2* in a non-bacterial expression vector system. The baculovirus expression vector system (BEVS) is an attractive system because no LPS is synthesized by Sf9 insect cells. The *ompP2* gene was cloned into pJV.P10Z, one of a new generation of baculovirus transfer vectors which possess the following features: the polyhedrin promoter drives the expression of the foreign gene; the gene for β-galactosidase is divergently transcribed from the P10 promoter and in the presence of a chromogenic indicator serves to identify recombinant plaques on a lawn of Sf9 cells. Blue plaques were purified by three cycles of plaque purification and a single isolated plaque was amplified to give a high-titre lysate designated AcPOR. Because of the cloning strategy that was used, the nucleotide sequence of recombinant porin was extended to create four amino acids at the amino terminus Met–Gly–Thr–Pro followed by Val-2-Val-3-Tyr-4–Asn-5– – –Phe-341 of the mature porin protein.

The time course of synthesis of recombinant porin from BEVS was monitored by infecting Sf9 cells with a stock of recombinant virus AcPOR. For comparison, wild-type baculovirus AcNPV was used to infect a parallel culture of Sf9 cells. Samples were extracted at various times post-infection and the total proteins of the infection mixture were identified by SDS–PAGE (Fig. 5). Up to 24 hours post-infection (hpi), the spectrum of proteins in cells infected with AcNPV or with AcPOR was indistinguishable. At 48 hpi, a major protein corresponding to polyhedrin (33 kDa) appeared in the lysate of cells infected with AcNPV. The relative amounts of this protein were equivalent in the samples taken up to 96 hpi. For Sf9 cells infected with AcPOR, a novel species of protein appeared at 48 hpi and was of the M_r anticipated for recombinant porin (38000). No polyhedrin protein was detectable in these same samples, thereby confirming the purity of the AcPOR virus stocks. Again the amounts of this recombinant protein were comparable in all samples up to 96 hpi.

Two assays were used to identify the presence of recombinant porin and its reactivities with mAbs. First, samples of the above extracts were blotted onto nitrocellulose, probed with each of the mAbs POR.1 to POR.7 and detected with a secondary antibody conjugated to alkaline phosphatase. Extracts from mock-infected cells and from cells infected with AcNPV served as controls. For the latter two extracts, no bands were detected by immunoblotting. All mAbs reacted with recombinant porin by Western blotting. The kinetics of synthesis of recombinant porin (Fig. 6) matched the appearance of the novel protein species of M_r 38000 that was identified by staining with Coomassie Brilliant Blue staining. Recombinant porin first appeared at 48 hpi and was also observed in samples taken at 72 and 96 hpi. Recombinant porin co-migrated with FPLC-purified Hib porin from the bacterial outer membrane. The absence of lower molecular-weight species on Western blotting suggested that there was little or no degradation of recombinant porin.

A second assay showed that recombinant porin reacted in a solid-phase system in a manner similar to porin isolated from Hib. Extracts of Sf9 insect cells infected with AcPOR were applied to ELISA plates and then tested with increasing dilutions of mAbs POR.1 to POR.7. The

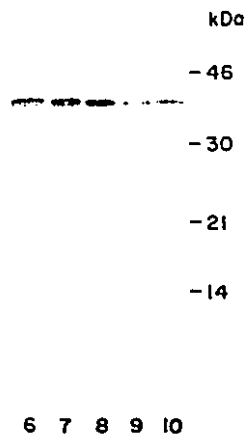

Fig. 6. Kinetics of synthesis of recombinant porin in BEVS. At different times post-infection of Sf9 insect cells by AcPOR, the samples were collected and lysed. After separation by SDS–PAGE, proteins were transferred to nitrocellulose, probed with mAb POR.1, and detected with a secondary anti-mouse mAb conjugated to alkaline phosphatase. Mock-infected cells (lane 1) and cells infected with AcNPV (lane 2) were used as negative controls. Porin isolated from Hib was the positive control for immunoreactivity: 100ng (lane 9) and 250ng (lane 10). Recombinant porin was not detected in samples at 0 (lane 3), 12 (lane 4), or 24 (lane 5) hpi. It appeared first in the sample taken at 48hpi (lane 6) and was also detected in samples at 72 and 96hpi (lanes 7 and 8 respectively). Molecular mass (in kDa) markers are indicated in the right margin.

reciprocal titres of mAb reactivities are shown in Table 1 and these values are comparable with those obtained for the reactivities of mAbs against Hib porin.

Truncated porin proteins from BEVS

Because we were able to synthesize stable, full-length recombinant porin without apparent endogenous proteolysis by Sf9 insect cells, it was of interest to generate stable carboxy-terminal deletion proteins in the BEVS and to use these deletion proteins to confirm the domains of reactivities of the panel of seven mAbs. By omega mutagenesis, the transcription and translation termination signals from gene *32* of T4 phage were inserted as a spectinomycin/streptomycin-resistance cassette at four hexanucleotide recognition sites *Dra*I, *Sna*BI, *Eco*RI, and *Ase*I within the porin gene. The insertions of the omega cassette at these positions within *ompP2* were expected to generate proteins that terminated after amino acids 139, 174, 182 and 264 of the primary sequence of mature porin. These four deletion proteins were designated POR139, POR174, POR182, and POR264 and they were used to clarify the domains of reactivities of the mAbs with particular reference to the carboxy-terminal boundaries.

Mouse polyclonal anti-porin IgG and mAb POR.1 recognized all four deletion proteins (POR139, POR174, POR182, POR264) as well as full-length recombinant porin from BEVS and bacterial porin (Fig. 7). Monoclonal antibodies POR.2, POR.3, POR.4 and POR.5 did not recognize the truncated protein POR139 but they showed a positive reaction on immunoblotting against proteins POR174, POR182, and POR264. Monoclonal antibodies POR.6 and POR.7 did not react with the truncated protein POR264 but were positive against full-length recombinant porin. These data served to establish the following carboxy-terminal boundaries of reactivity: mAb POR.1, amino acid 139; mAbs POR.2, POR.3, POR.4, and POR.5, amino acid 174; mAbs POR.6 and POR.7, amino acid 341.

Biological activities of mAbs

Because six mAbs were demonstrated to bind to the surface of Hib, we tested the mAbs for their biological activities in two different experimental systems. Bactericidal assays provided an indication of the ability of the mAb to bind complement and to activate complement-mediated killing of intact cells. The immunoprotection of the mAbs was assessed in the infant rat model of bacteraemia; passive transfer of protective antibodies, prior to intraperitoneal challenge with a dose of Hib, results in clearance of the infecting bacteria. Antibodies that are not protective are unable to abrogate bacteraemia, the index of infection.

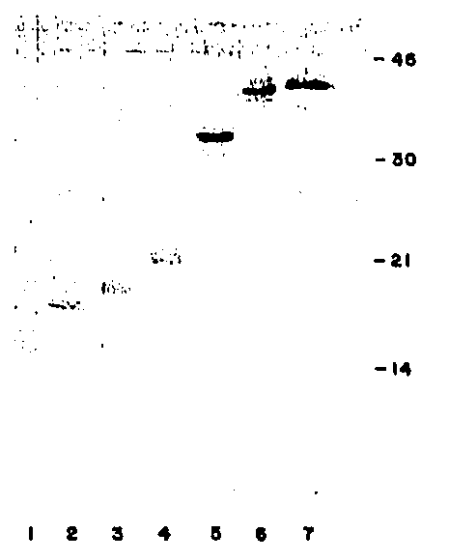

Fig. 7. Identification of recombinant carboxy-terminal deletion proteins and their immunoreactivities with monoclonal antibodies. Four truncated genes were created by omega mutagenesis of *ompP2* and the deletion proteins were expressed in BEVS. The following samples were subjected to the SDS–PAGE: lane 1: mock-infected Sf9 cells; lanes 2, 3, 4 and 5: Sf9 cells infected with recombinant virus that expressed POR139, POR174, POR182, and POR264, respectively; lane 6: Sf9 cells infected with AcPOR; lane 7, FPLC-purified porin from Hib. Proteins were electrophoresed, transferred to nitrocellulose, probed with mAb POR.1, and detected with an enzyme-linked secondary antibody. Molecular mass (in kDa) markers are indicated in the right margin.

Table 2. Passive protection of infant rats by antibodies against PRP or against porin.

Antibody	Bacteraemia[a]	High-level bacteraemia[b]	Geometric mean titre (c.f.u. × 10^3 ml^{-1} blood)
None	11/11 (100%)	11/11 (100%)	60.2
Anti-PRP	0/11 (0%)	0/11 (0%)	0.05
POR.1	8/8 (100%)	8/8 (100%)	38.7
POR.2	9/9 (100%)	8/9 (89%)	28.2
POR.5	9/9 (100%)	9/9 (100%)	60.0
POR.6	9/9 (100%)	9/9 (100%)	47.0
Hb-2	9/9 (100%)	9/9 (100%)	33.6

a, b. Values listed are numbers of rats showing viable c.f.u. of Hib in blood samples at 18 hours post-challenge; high-level bacteraemia is defined as greater than 2.5 × 10^3 c.f.u. ml^{-1} of blood.

The Hib strain ATCC9795 was avirulent for the infant rat since intraperitoneal challenge of 40 or 400 or 4000 colony-forming units (c.f.u.) did not result in bacteraemia. Upon injection of the same numbers of c.f.u. of Hib strain RH3527, bacteraemia was routinely established. This strain was therefore chosen for both biological assays. In addition, a spontaneously derived non-capsulated derivative (RH3528) was tested as a second target strain for bactericidal activities. Some mAbs were selected for assay according to the primary porin sequences to which the antibodies bound: POR.1, POR.2 and POR.5, and POR.6. As controls, mouse anti-Hib porin mAb Hb-2 (Hamel *et al.*, 1987b), a human pool of anti-PRP antibodies, and mouse anti-LPS mAb 3DA11 were used.

Hib strain RH3527 was resistant to 25% human complement and was not lysed by any of the monoclonal or polyclonal antibodies alone. In the presence of complement plus anti-PRP antibodies up to a dilution of 1/128, 50% killing of the input number of Hib strain RH3527 was observed. There was no bactericidal effect if complement had been heat-inactivated. In the presence of complement plus each of the mAbs POR.1, POR.2, POR.5, POR.6, or Hb-2, no reduction in c.f.u. was detected, even with the lowest dilution (1/100) which corresponded to 0.5 μg of affinity-purified mAb. This lack of bactericidal activity by the mAbs was not a strain-dependent phenomenon since the same results were obtained with Hib strain Eagan. Nor was the phenomenon related to the inhibition of complement action by the presence of capsule. *H. influen-* strain RH3528, resistant to only 5% complement, was not lysed by any of the antibodies alone, but was efficiently killed by 5% complement plus anti-LPS mAb 3DA11 (IgG3) up to 1/3000 dilution. Again, 5% complement plus either mAb POR.1, POR.2, POR.5, POR.6 or Hb-2 was tested and shown not to be bactericidal against strain RH3528.

Infant rats in seven groups (with a minimum of eight rats per group) were injected with saline or anti-PRP antibodies (400 ng per animal) or mAbs POR.1, POR.2, POR.5, POR.6 or Hb-2 (all affinity-purified, up to 8 μg per rat). Upon administering a challenge dose of 4000 c.f.u. of Hib strain RH3527 followed by an interval of 18 hours, the geometric mean titre for the control group indicated high-level bacteraemia; no rats were bacteraemic when anti-PRP antibodies were passively transferred before challenge. The geometric mean titres for rats receiving anti-porin mAbs indicated that none of these antibodies conferred protection against bacteraemia (Table 2).

Discussion

The major pore-forming protein in the outer membrane of *H. influenzae* type b plays an essential role in permitting the transmembrane passage of water-soluble solutes into the periplasm. Our initial characterization of the biophysical behaviour of porin (Vachon *et al.*, 1986) has recently been extended by the cloning and sequencing of the *ompP2* gene (Hansen *et al.*, 1989a; Munson and Tolan, 1989). Although the primary sequences for the porin gene from four OMP subytpes of Hib are now available (Munson *et al.*, 1990), there is little information on the relationship between these variations of sequence and the immunobiological response that they elicit. There is also a paucity of information about the native topological organization of porin of Hib as it is oriented in the outer membrane. An obvious goal in the characterization of porin of Hib is to confirm the role of this protein in pathogenesis and immunity to infection. The studies in this paper were prompted by the often-cited report that polyclonal antibodies against porin of Hib have protective activity in the infant rat model of bacteraemia (Munson *et al.*, 1983). We wish to provide some answers to the following questions: what antigenic determinants on porin of Hib elicit an immune response; can the epitopes recognized by antibodies be assigned to the outer or inner surface of the outer membrane; what amino acid sequences on Hib porin are recognized by antibodies; do the antibodies against bacterial porin also recognize recombinant porin; and what is the role of such antibodies in conferring immunoprotection against bacteraemia?

Generating a panel of seven monoclonal antibodies against porin of Hib (also designated OmpP2) has provided experimental tools with which to answer some of these questions. Our mAbs reacted with both native and denatured OmpP2, and all but one was shown by flow cytometry to react to porin of Hib and to porin on a non-capsulated strain of *H. influenzae*. Taken together, these results implied that linear amino acid sequences at the cell surface were recognized by the mAbs and that strategies of protein fragmentation could be used to identify the cognate sequences. Such an analysis would have been more difficult if conformational epitopes had

been recognized by the mAbs (Hansen *et al.*, 1989b). A combination of chemical and enzymatic digestions of OmpP2 gave information about the primary sequences to which the mAbs bound. The mAbs were tentatively assigned to three groups based upon their patterns of reactivities. While protein fragmentation gave clearly different patterns of recognition by mAbs, some caution was appropriate because of the report of the isolation of anti-Hib porin mAbs (Gulig *et al.*, 1983) which were later confirmed to be directed against LPS (Gulig and Hansen, 1985). The cloning and expression of *ompP2* in the BEVS, an LPS-free system, provided unequivocal evidence that the mAbs were reactive against Hib porin and not against any other biopolymer of *Haemophilus*. In the BEVS, four stable carboxy-terminal deletion proteins were synthesized: POR139, POR174, POR182 and POR264. These four proteins reacted variously with the mAbs and permitted accurate identification of the carboxy-terminal boundaries for mAb reactivities. Our data serve to identify the following domains of mAb reactivities: (i) mAb POR.1 reacts with some amino acids between Ala-104 and Phe-139; (ii) mAbs POR.2, POR.3, POR.4, and POR.5 react with some amino acids between Gly-162 and Val-174; and (iii) mAbs POR.6 and POR.7 react with some amino acids between Glu-267 and Phe-341. From our results with flow cytometry, we propose that domain (i) is either buried in the membrane or is on the periplasmic face of the outer membrane, that domain (ii) is a surface-exposed loop, and that domain (iii) or, more likely, part of this sequence is also oriented as a surface-exposed loop. Because domains (i) and (iii) are long stretches of 36 and 75 amino acids, respectively, further fine-structure mapping of these regions is warranted. Domain (ii) of 13 amino acids is close to the minimum length of sequences required for antibody recognition.

Coinciding with our success in generating and characterizing surface-reactive mAbs, there appeared another report in which mAbs against OmpP2 were isolated and used for essentially epidemiological purposes (Martin *et al.*, 1990). Most of the mAbs that were isolated in their study also appeared to be surface-reactive. No studies were reported that related to the biological effects of the mAbs, i.e. bactericidal tests or animal-protection studies. It was of interest that their mAbs reacted with encapsulated bacterial clones previously assigned to phylogenetic division I (Musser *et al.*, 1990) whereas none reacted with clones in primary division II.

Some recent mapping of B-cell epitopes on OmpP2 identified four distinct immunogenic and antigenic regions (Martin *et al.*, 1991). We have used computer-assisted methods (PC-Gene) to predict antigenic determinants on OmpP2. The three highest points of hydrophilicity are Lys-326–Lys-331 (average hydrophilicity, Ah = 2.43), Lys-161–Arg-166 (Ah = 2.03), and Lys-165–Lys-170 (Ah = 2.03). Six of our seven mAbs map to these regions and some of their mAbs map to these regions.

A surprising result of our studies is that even though six of the mAbs were surface-reactive, none was able to mediate complement-dependent lysis of Hib. This lack of bactericidal effects was not attributable to some steric hindrance of the capsule as was previously shown for anti-P1 antibodies (van Alphen *et al.*, 1986), since flow cytometric analysis demonstrated binding of mAbs both to capsulated and non-capsulated *H. influenzae*; nor were the mAbs immunoprotective in an infant rat model of bacteraemia. It is possible that antibodies against other surface-located antigenic determinants on porin of Hib might elicit different biological effects compared with those we observed. On the other hand, some revision of the conclusions of Munson *et al.* (1983) — that anti-porin antibodies are bactericidal and protective —may be necessary. Recombinant porin will be of value in extending our observations.

Experimental procedures

Bacterial strains and culture conditions

H. influenzae type b (Hib) strains ATCC9795 (Vachon *et al.*, 1985), Eagan (Moxon *et al.*, 1974) and RH3527 (also designated 760705; van Alphen *et al.*, 1983) were used in our studies. They are of OMP subtypes 1H, 1L, and 3L, respectively, and therefore have the same primary sequences of porin (Hansen *et al.*, 1989a; Munson *et al.*, 1989), a result confirmed by our nucleotide sequencing. A spontaneously isolated variant of RH3527 is designated RH3528 and is devoid of capsular polysaccharide. Hib strain DL42/2F4 was constructed by insertion mutagenesis of the porin gene *ompP2* and was characterized as porin-deficient (Cope *et al.*, 1990). The identity of all strains of *H. influenzae* was confirmed by their growth requirements for haemin and NAD^+. The capsular serotype was determined by the latex agglutination kit, commercially available from Wellcome Laboratories. Strains were grown either on chocolate agar supplemented with 1% Isovitalex (BBL) or in brain–heart infusion medium supplemented with Fildes, Enrichment Agar (Becton Dickinson).

Preparation of antigens

Vesicles containing OMPs from Hib were prepared using the lithium chloride extraction procedure (Johnston *et al.*, 1976). Alternatively, OMPs were extracted from whole cells of Hib with 2% cetyl trimethyl ammonium bromide (CTB). For purification of the 38 kDa porin, the CTB extract was subjected to chromatography on DEAE-Sepharose (Vachon *et al.*, 1985). Porin was further purified by FPLC using a MonoQ HR 5/5 column (Pharmacia LKB Biotechnology Inc.). The protein solution from the DEAE-Sepharose column containing 50 mM Tris-HCl pH 8.0, 10 mM ethylenediaminetetraacetic acid (EDTA), and 0.05% Zwittergent Z-3,14 was washed with 80% ethanol and suspended in 50 mM Tris-HCl pH 8.0, 0.05% Zwittergent Z-3,14. A sample of 30 mg in 10 ml was loaded on to the column. The salt concentration of the elution buffer was raised to 0.35 M using a 2 ml NaCl gradient. A number

of protein-containing peaks were eluted at this salt concentration. When no other proteins could be eluted at 0.35 M NaCl, the salt concentration was raised in a gradient fashion to 1.0M. A major peak which contained highly purified porin was eluted at 0.65M NaCl.

Anti-porin monoclonal antibodies (mAbs)

BALB/c mice were injected intraperitoneally with 10 μg of FPLC-purified porin in Freund's incomplete adjuvant (Gibco Laboratories). At two weeks and four weeks after primary immunization, animals received injections of 10 μg of porin, also in incomplete adjuvant. Four days before fusion, a booster dose of 30 μg of OMPs in 0.01 M phosphate buffer, pH 7.5, 0.85% NaCl (PBS) was given intravenously. Spleen cells from immunized mice were fused in a ratio of 10:1 with non-secreting hypoxanthine-guanine phosphoribosyl transferase-deficient SP2/0 myeloma cell line in a solution containing 50% (w/v) polyethylene glycol (PEG1450; Eastman Kodak) in Dulbecco's modified Eagle's medium (Flow Laboratories). Fused cells at a concentration of 10^5 cells ml^{-1} were distributed into 96-well tissue-culture plates which already contained a feeder layer of 4×10^3 murine macrophages. On day 10–12, hybridoma supernatants were tested for reactivity against vesicles containing OMPs. Antibody-secreting cells were subcloned by limiting dilution. The class and subclass of mAbs were determined with reagents prepared by Southern Biotechnology Associates.

Purification of mAbs using affinity chromatography

Mouse anti-Hib porin mAbs were purified by using a rat anti-mouse kappa mAbs 187.1 (Yelton *et al.*, 1981) column. Ascites fluid was diluted five times with PBS. The sample was passed through a filter of 0.45 μm pore size (Nucleopore), loaded onto the column, and washed with PBS. Bound anti-porin mAbs were eluted with 0.1M sodium acetate pH2.8, 0.15M NaCl. The absorbance (at 280nm) of the collected fractions was measured and peak fractions were pooled. After addition of 1M Tris-HCl pH8.2 to neutralize the pH, the pooled fractions were dialysed against PBS. The total protein was measured using the dye binding assay. The mAbs were then brought to a concentration of 0.5 mg ml^{-1}. Dilutions ranging from 1/100 to 1/100 000 were used in ELISA and in Western immunoblotting analysis.

Flow cytometry

Bacteria from fresh overnight cultures were washed in Tris-buffered saline and suspended in the same buffer to 10^9 cells ml^{-1}. To 2×10^6 bacteria was added affinity-purified mAb at 1/100 dilution, corresponding to 5 μg ml^{-1} of antibody (Bentley and Klebba, 1988). After incubation for 60min at 37°C, cells were pelleted by brief centrifugation in a microcentrifuge, washed, and further incubated with fluorescein-labelled anti-mouse Ig and analysed using a FACScan (Becton Dickinson) with Consort 30 software. Acquisition gates were set on forward scatter and side scatter to exclude lysed cells and cell aggregates. To minimize background noise, all solutions including the sheath fluid were filtered through a 0.22 μm filter. Twenty thousand cells were analysed for each sample.

Cleavage of Hib porin by cyanogen bromide

Two-microgram amounts of FPLC-purified Hib porin were solubilized in 25 μl of 70% acetic acid. Cyanogen bromide (250 μg) (Pierce) was added to each reaction vial and incubated at room temperature in the dark for 18h (Gross, 1967). Upon completion of the reaction acetic acid and cyanogen bromide were removed from the reaction medium by vacuum concentration. The protein pellets were resuspended in 25 μl of sample buffer, boiled for 5min, and analysed by SDS–PAGE.

Hydroxylaminolysis of Hib porin

Aliquots of 5 μg of FPLC-purified Hib porin were subjected to SDS–PAGE and the gel was stained with Coomassie Brilliant Blue. Gel pieces were excised, transferred to 1.5ml vials and washed four times with 5% methanol at 4°C to remove SDS. The gel pieces were brought almost to dryness in a vacuum concentrator. To the vials containing the gel pieces was added 300 μl of cleavage solution; the samples were incubated at 45°C for 3h. Cleavage solution was 2 M hydroxylamine hydrochloride (Baker), 6 M guanidine hydrochloride, and 15 mM Tris in 4.5 M LiOH pH 9.0 (Bornstein and Balian, 1977). The reaction was stopped by adding 150 μl of 70% acetic acid to each vial. The gel pieces were washed four times with 5% methanol at 4°C to remove the cleavage solution. The gels were brought to near dryness; 100 μl of sample buffer was added to each vial and boiled for 5min. Peptide fragments were released from the gels by analysis on a second SDS–PAGE. For amino-terminal sequencing and for staining of fragments with Coomassie Brilliant Blue, the above protocol was repeated with 50 μg of porin as starting material.

Limited trypsin digestion of Hib porin in solution or in gels

Two-microgram amounts of FPLC-purified Hib porin in 5 μl of 50mM Tris-HCl pH8.0, 0.05% Zwittergent Z-3,14 were placed in reaction vials. TPCK-treated trypsin (200ng) (Sigma) in 10 μl of 50mM Tris-HCl pH8.0 was added to each vial and the vials were incubated at 37°C for up to 16h (Smyth, 1967). Sample buffer (25 μl) was added; boiling for 5min terminated the reaction. Peptide fragments resulting from the digestion were analysed by SDS–PAGE. For amino-terminal sequencing, 20 μg of porin was digested with 2 μg of TPCK-treated trypsin.

Alternatively, FPLC-purified Hib porin was immobilized in acrylamide gels and subjected to trypsin digestion. Gel pieces containing 5 μg amounts of porin were incubated in 300 μl of 50mM Tris-HCl pH8.0 containing 500ng of TPCK-treated trypsin. The reaction was at 37°C for 1h. For sequencing purposes, 50 μg of Hib porin was treated with 5 μg of TPCK-treated trypsin. Gel pieces were prepared and the digestion products analysed by the same protocols as in hydroxylaminolysis.

Amino-terminal sequencing of peptide fragments

Cleavage products were subjected to SDS–PAGE and fragments transferred electrophoretically onto Immobilon-P (Millipore) membranes. The membranes were washed several times with distilled water to remove traces of glycine from the transfer buffer. Bands were cut out and subjected to gas-phase sequencing

(Hewick *et al.*, 1981) in a PI 2090E Integrated Micro-Sequencing System (Porton Instruments Inc.). Edman degradations were conducted according to procedures recommended by the manufacturer. PTH-amino acid analysis employed gradient elution (Solvent A: triethylamine/acetic acid/tetrahydrofuran pH4.0: solvent B: acetonitrile) from a reversed-phase Hewlett Packard Amino Quant (200 × 2.1mm) column with a flow rate of 0.2ml min^{-1} at 42°C. Five sequential cycles of the amino terminus of each fragment were sequenced.

Molecular cloning of ompP2

Chromosomal DNA of Hib strain ATCC9795 was digested with *Pvu*II and size-fractionated by electroelution from an agarose gel. A library of chromosomal fragments was constructed in M13 mp18 that had been digested with *Sma*I. The library was screened using an oligonucleotide (5'-GCTGTTGTTTATAA-CAACGAAGGG-3') complementary to amino acids at the amino terminus of Hib porin. A *Pvu*II fragment containing the entire porin sequence but lacking codons for the signal sequence was isolated as a 2.0kbp insert. The Hib porin gene was excised from the RF form of M13 by digestion with *Kpn*I and *Ssp*I as a fragment of 1130bp and subcloned into pBluescript (Stratagene) that had been restricted with *Kpn*I and *Sma*I. The resulting plasmid was designated pACC01. The restriction map of the insert matched the information reported by Hansen *et al.* (1989a) and by Munson and Tolan (1989).

Improved baculovirus expression vectors were recently developed to expedite screening of recombinants (Vialard *et al.*, 1990) and a recent version of this class of vectors, pJV.P10Z, was used in our experiments. This vector contained promoters derived from the P10 and the polyhedrin genes of *Autographica californica* nuclear polyhedrosis virus. P10 promoter directed synthesis of a β-galactosidase and the polyhedrin promoter controlled the synthesis of the *ompP2* gene. The unique *Nhe*I cloning site of this plasmid necessitated modification of the 5'- and 3' sequences of the porin gene. To generate the *Nhe*I ends on *ompP2*, a synthetic adaptor was added to the 5' end and consisted two oligonucleotides: 5'-CTAGCTATAAATATGGGT-AC-3' and 5'-CCATATTTATAG-3'. When these two oligonucleotides were annealed, they generated *Nhe*I and *Kpn*I sites at their 5' and 3' ends: internal sequences introduced an AT-rich region just upstream of the ATG start site. The AT-rich sequence may serve as a polymerase-binding site. This sequence also corresponds to those necessary for maximal levels of expression of another foreign gene (Matsura *et al.*, 1987).

pACC01 was digested with *Kpn*I and *Bam*HI to excise the entire Hib porin gene. The DNA fragment containing *ompP2* was gel-purified and ligated with annealed adaptor at a molar ratio of 10:1 (adaptor:porin). The adaptor-modified fragment with *Nhe*I–*Bam*HI ends was force-cloned into pBR322 cut with *Nhe*I and *Bam*HI, thereby creating pACC02 as an intermediate plasmid containing *ompP2*. pACC02 was digested with *Bam*HI, backfilled with the Klenow fragment of DNA polymerase I, and ligated to an 8bp *Nhe*I linker that was added in 100-fold molar excess. The linker-ligated porin gene was then subjected to excess *Nhe*I digestion to remove multiple linkers from the 3' end of the *ompP2* gene. This linker-ligated fragment was subcloned into pBR322 cut with *Nhe*I and was designated pACC03 (5523bp). The *Nhe*I insert of pACC03 (1161bp) was ultimately transferred into the 13kbp baculovirus transfer plasmid, pJV.P10Z, creating pACC33. Two primers that were complementary to the vector's 5'-promoter sequences (5'-CGTAACAGTTTTGTAATAAA-3') and 3'-end sequences (5'-GTGAGTTTTTGGTTCTTGCC-3') were used for DNA sequencing to confirm the orientation of the porin gene with respect to the polyhedrin promoter and to confirm the fidelity of the subcloning through intermediate plasmids.

To generate a series of deletion mutants of *ompP2*, pACC03 was digested with *Dra*I or *Sna*BI or *Eco*RI or *Ase*I. Restriction with the first two enzymes yielded blunt ends; restriction with the latter two enzymes generated cohesive ends that were back-filled with the Klenow fragment of DNA polymerase. Each of the linearized, blunt-ended plasmids was ligated with the omega cassette derived from pHP45 (Prentki and Krisch, 1984). This cassette of 2kbp contains the spectinomycin/streptomycin-resistance genes and in either orientation provides a large stable stem-loop structure, making it an efficient transcription terminator. The blunt-end ligation of the cassette resulted in the recircularization of the plasmid, now increased in size to 7.5kbp. The cassette-modified *ompP2* genes were purified as *Nhe*I fragments and ligated into pJV.P10Z cut with *Nhe*I.

DNA transfections, plaque assays, and isolation of recombinant virus

pACC33 and the four transfer vectors containing omega-modified *ompP2* genes were purified by CsCl gradient ultracentrifugation and were separately transfected into Sf9 cells together with wild-type viral DNA (Summers and Smith, 1987) by using the calcium phosphate precipitation technique. Plaque assays were performed on culture plates, such that the infected cells were overlaid with 1% Sea Plaque agarose in Grace's medium and containing 150μg of Bluo-Gal per ml of overlay. Blue plaques, easily recognized after incubation for 5d at 27°C, were picked and diffused overnight in Grace's medium. Plaque assays were repeated on 10-fold dilutions of diffused virus, again using Bluo-Gal in the overlay. Three rounds of plaque assays were sufficient to generate recombinant virus free of wild-type virus. Isolated recombinant virus was finally amplified to a titre 10^7 p.f.u. ml^{-1}.

Microbiological tests

Bactericidal activity was tested as described earlier (Käyhty *et al.*, 1988). Bacteria were incubated for 60min, 37°C in microwells with dilutions of serum or mAbs and 5 or 25% human serum as a complement source. The complement source did not kill the bacteria without added antibody. After incubation, aliquots of the bacterial suspensions were plated in duplicate on a chocolate agar plate and incubated overnight at 37°C in a CO_2 incubator. The last dilution that gave 50% killing was taken as the bactericidal antibody titre.

To determine whether passively given mAbs were protective *in vivo*, infant rat experiments were performed as described earlier (Smith *et al.*, 1973). The antisera or mAbs were given intraperitoneally (i.p.) 2h before the challenge Hib which was also given i.p. Bacteraemia was detected 24h later by plating 20μl of neat or 100μl of 1:10 and 1:100-diluted blood.

Analytical procedures

Sodium dodecyl sulphate/ polyacrylamide gel electrophoresis (SDS–PAGE) was used to assess the purity of Hib porin following FPLC, to resolve fragments derived from chemical or enzymatic digestions of Hib porin, and to resolve proteins in samples of outer membrane vesicles or insect cell lysates. The gel compositions were adopted from protocols of Lugtenberg *et al.* (1975). Gels were stained for proteins with Coomassie Brilliant Blue or by the more sensitive method of Morrissey (1981) for silver staining. The molecular mass standards were from Pharmacia LKB Biotechnology Inc. Alternatively, gels were silver stained to identify lipopolysaccharide (LPS) by the procedure of Tsai and Frasch (1982). The amounts of LPS that remained bound to Hib porin samples were estimated by comparing the staining pattern to standards of 50, 100, 200 and 300 ng on the same gels.

Following SDS–PAGE, proteins were transferred electrophoretically (Towbin *et al.*, 1979) to nitrocellulose paper (Schleicher & Schuell, Inc.). Rainbow markers (Amersham Canada Ltd) were also electrophoretically transferred and used on immunoblots to estimate the sizes of reactive fragments. The nitrocellulose paper was blocked with 1% (w/v) skimmed milk or with 1% bovine serum albumin in Tris-saline, and the immobilized proteins were incubated with (i) hyperimmune serum, or (ii) concentrated culture supernatants or (iii) affinity-purified monoclonal antibodies. The developing antibodies were alkaline phosphatase-labelled anti-mouse immunoglobulins (Cappel Laboratories). The enzymatic reactivity of alkaline phosphatase was detected by addition of substrates NBT and BCIP (BioRad Laboratories).

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PREFACE TO CHAPTER 3

As probes of the topological organization of *Haemophilus influenzae* type b (Hib) porin, we previously generated monoclonal antibodies (mAbs) against the isolated protein and screened them for their reactivities with intact bacteria. Surface-reactive mAbs bound to two regions of Hib porin: amino acids 162 to 174, and amino acids 267 to 341. Another region between amino acids 104 and 139 was strongly antigenic but not surface-exposed. This chapter describes work on defining precisely the amino acid sequences that are recognized by each of these mAbs. These data were used in conjunction with predictions of porin secondary structure to generate a working model for the topological organization of Hib porin at the bacterial cell surface.

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Antigenic Sites on Porin of *Haemophilus influenzae* Type b: Mapping with Synthetic Peptides and Evaluation of Structure Predictions

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The major surface-located protein in the outer membrane of *Haemophilus influenzae* type b (Hib) is porin, molecular mass, 38 kDa, 341 amino acids. To define precisely the molecular reactivities of nine mouse monoclonal antibodies (MAbs) against Hib porin, overlapping hexapeptides corresponding to the entire sequence of porin were synthesized. The epitopes recognized by the MAbs were mapped by enzyme-linked immunosorbent assay to stretches of 6 to 11 amino acids. Antigenic sites between amino acids 112 and 126, 148 and 153, 162 and 172, and 318 and 325 were identified. The antigenic sites between amino acids 162 and 172 and between amino acids 318 and 325 were determined by flow cytometry to be on the bacterial cell surface. Four MAbs, POR.2, POR.3, POR.4, and POR.5, that react with amino acids 162 to 172 were able to discriminate among porins from the three major outer membrane protein subtypes of Hib, i.e., 1H, 2L, and 6U. A model for the topological organization of Hib porin was created by calculating the hydrophobicity, amphiphilicity, and turn propensity in its amino acid sequence. Determination of the molecular reactivities of the anti-Hib porin MAbs provided substantive evidence for the orientation of selected regions of porin in the outer membrane of Hib.

Porins of gram-negative bacteria form transmembrane water-filled channels that permit diffusion of hydrophilic solutes across the outer membrane (2, 20). By acting as molecular sieves, they display unique features of selective permeability such that the maximum size of a solute molecule that can permeate the pores defines a value termed the molecular weight exclusion limit. Solutes lower in molecular weight than this value diffuse readily through porins, whereas solutes whose molecular weights exceed the value of the exclusion limit are impeded in their passage. The variety of porins and their exclusion limits differ from one bacterial genus to another.

Most porins are organized in the outer membrane as trimers which are unperturbed by detergents such as sodium dodecyl sulfate (SDS) (2, 22). Porins are also an unusual class of membrane proteins because the primary amino acid sequences of their monomers contain a high density of charged residues and there are no clearly hydrophobic stretches which would be predicted to span a membrane as an alpha helix (13, 22). It has therefore been proposed that the secondary structure of porin is organized as transmembrane beta strands (2, 22), a proposal for which there is now experimental support (12, 30). Interest in the structural organization of porins has been heightened by the identification of sequence alignments, from which some predictions can be made as to membrane-spanning strands. For example, the alignment of eight sequences from the enteric porin family generated information about alternating conserved (beta strand) and variable (loop) regions (14). By comparison, the porins from neisseriae are not as strongly conserved, but six sequence alignments still had few ambiguities. When these sequence similarities were then considered along with plots of hydrophobicity and hydrophobic moment and with turn predictions, it was possible to propose transmembrane beta strands and loops as the hallmarks of porin structure (14). One of the requirements of such detailed structural analysis is that several porin sequences from a given bacterial genus are known. Amino acid sequence information is available for three porin variants of *Haemophilus influenzae* type b (Hib) (18, 19). However, these data are insufficient to construct a *Haemophilus* porin family.

The pore formed by Hib porin has a molecular size exclusion limit of 1,400 Da (26), considerably higher than the value of 600 Da for the pore formed by OmpF of *Escherichia coli*. In addition, by liposome swelling assays, Hib porin appears to have a greater pore diameter than that associated with the porins of *E. coli* (25). Unlike most porins, trimers of Hib porin are quite unstable and were visualized by SDS-polyacrylamide gel electrophoresis (PAGE) only when the proteins were previously cross-linked (25). Recently, however, by image reconstruction of two-dimensional crystals (4), the trimeric organization of Hib porin has been clearly demonstrated.

As probes of the topological organization of Hib porin, we previously generated monoclonal antibodies (MAbs) against the isolated protein and screened them for their reactivities with intact bacteria. Surface-reactive MAbs bound to two regions of Hib porin: amino acids 162 to 174 and 267 to 341. Another region, between amino acids 104 and 139, was strongly antigenic but not surface exposed (23). We have extended these studies on Hib porin by defining precisely the amino acid sequences that are recognized by each of our seven MAbs. To expand our study, we obtained from other laboratories two different MAbs against Hib porin and identified the amino acids that they recognize. These data

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were used in conjunction with predictions of porin secondary structure to generate a working model for the topological organization of Hib porin at the bacterial cell surface.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Three Hib strains were used in this study, and they differ by outer membrane protein (OMP) subtype: Hib ATCC 9795 is OMP subtype 1H (23, 26); Hib MCH 5539, a clinical isolate provided by K. Knowles, Montreal Children's Hospital, is OMP subtype 2L; and Hib 1481 is OMP subtype 6U (27). All strains were grown on chocolate plates or in liquid medium consisting of brain heart infusion plus hemin (1.0 μg/ml) and NAD^+ (1.0 μg/ml) at 37°C.

Hexapeptide synthesis. Overlapping hexapeptides were synthesized on solid-phase supports as described by Geysen et al. (6, 7), by using a commercially available epitope-mapping kit (Cambridge Research Biochemicals, Gadbrook Park, Northwich, Cheshire, United Kingdom). Peptides were synthesized on polyethylene pins which were prepared by the supplier of the kit to contain distally fluorenylmethyl oxycarbonyl (Fmoc)-β-alanine coupled to the polyethylene matrix via hexamethylene diamine and acrylic acid. All of the amino acids used were provided by the manufacturer and were in the form of Fmoc-protected pentafluorophenyl active esters, except serine and threonine, which were Fmoc-protected oxobenzotriazine active esters. All amino acids had their side chains protected with *t*-butyl derivatives, except arginine, which had a methoxy-trimethylphenylsulfonyl side-chain-protecting group. The catalyst for the coupling reaction, hydroxybenzotriazole, was also provided by the supplier. Dimethylformamide (DMF; Accusolv; Anachemia) was the solvent used in the synthesis, and it was freed from contaminating amines by standing over type 4A molecular sieves (Anachemia) for 6 weeks in the dark before commencement of synthesis. Amine levels in this DMF were monitored by measuring the UV absorbance of the sample treated with 1-fluoro-2,4-dinitrobenzene (28). The final concentration of free amines in the DMF used for the synthesis was less than 2 ppm. All other solvents were of the highest purity levels and were used without any further treatment. On the basis of the amino acid sequence of Hib porin, a schedule for the synthesis of overlapping hexapeptides over the entire sequence of this protein was created by using the software provided.

The first step in the synthesis involved removal of the protecting group from the Fmoc-β-alanine of each pin to facilitate subsequent amino acid coupling. This was achieved by mild base treatment of the pins with 20% piperidine in DMF. Following deprotection and washing, the pins were immersed in 96-well polyethylene trays, each well containing 100-μl aliquots of solution. The solution consisted of the appropriate Fmoc amino acid ester (30 mM) and hydroxybenzotriazole dissolved in DMF. Amino acid coupling was carried out overnight at room temperature. The deprotection and coupling were repeated in this manner until a peptide of the required length was generated on each pin. The direction of synthesis resulted in a peptide whose C terminus was proximal to the common β-alanine and whose N terminus was distal. The N terminus of each peptide was deprotected and immediately acetylated by reaction with a mixture of DMF-acetic anhydride-triethylamine (5:2:1, vol/vol/vol) to remove the unnatural charge. Finally, the side chains of amino acids in the peptide were deprotected and neutralized by reaction with trifluoroacetic acid-phenol-ethanedithiol (38:1:1, vol/vol/vol), followed by washing in a bath containing 5% di-isopropylethylamine in dichloromethane. After the derivatized pins were washed in a methanol bath for 18 h, they were dried in vacuo. This procedure allowed for synthesis of a set of 336 consecutive and overlapping hexapeptides corresponding to the complete sequence of 341 amino acids of Hib porin, OMP subtype 1H.

Also synthesized in parallel were two control tetrapeptides, Pro Leu Ala Gln (PLAQ) and Gly Leu Ala Gln (GLAQ). The success of synthesis was confirmed by demonstration of the reactivity of a previously characterized test antibody (Cambridge Research Biochemicals) to the positive control, PLAQ, and lack of reactivity to the negative control, GLAQ.

Immunological assays. The immunoreactivity of the solid-phase peptides was assayed by enzyme-linked immunosorbent assay (ELISA) as recommended by the manufacturer. Pins with the immobilized peptides were placed in microtiter plates (Maxisorp F96; Nunc) containing 200 μl of blocking solution (2% bovine serum albumin, 0.1% Tween 20 in phosphate-buffered saline, 0.05% sodium azide) for 1 h at room temperature. Following blocking, the pins were incubated overnight at 4°C with 150 μl of antiporin MAbs appropriately diluted in blocking solution. After being washed four times with 0.05% Tween 20 in phosphate-buffered saline, the pins were incubated with 150 μl of a secondary antibody (rabbit anti-mouse immunoglobulin G [heavy and light chains] conjugated to horseradish peroxidase; ICN) appropriately diluted in blocking solution (minus sodium azide) for 1 h at room temperature. The washes were repeated, and the pins were incubated with 0.5 mg of the substrate, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma), per ml in disodium hydrogen P_i-citric acid buffer, pH 4.0, for 30 min in the dark. The microtiter plates were read on a Multiscan MC (Titertek; Flow Laboratories) ELISA plate reader at 405 nm. After immunoassay, the immobilized peptides were freed from bound antibody by ultrasonication in hot SDS and β-mercaptoethanol and finally washed in methanol. The peptides were then reused for subsequent immunoassays (7).

Reactivity of MAbs to isolated porin on microtiter plates was assayed by ELISA as described for immunoreactivity of immobilized peptides, with the following modifications. Blocking and incubation steps were performed in 0.5% milk, the secondary antibody was a MAb (the rat anti-mouse κ light chain conjugated to alkaline phosphatase), and the substrate was 2 mg of *p*-nitrophenylphosphate (GIBCO, BRL) per ml in diethanolamine buffer, pH 9.5.

Flow cytometry. Details of our conditions for flow cytometry were published recently (23). Bacteria from fresh overnight cultures were washed in Tris-buffered saline and suspended in this buffer to 10^9 cells per ml. An affinity-purified MAb (concentration, 0.5 mg/ml) at a 1/100 dilution was mixed with 2×10^8 cells and incubated at 37°C for 1 h. Bacteria were pelleted in an Eppendorf microcentrifuge, washed, and incubated with anti-mouse immunoglobulin conjugated to fluorescein. Analysis of labelled cells used a FACScan (Becton Dickinson) with Consort 30 software. For each sample, 2×10^4 cells were analyzed.

Modelling of Hib porin. Hydrophobicity and amphiphilicity plots of Hib porin were generated by using a computer program called AMPHI (11). The hydrophobicity scale (h) for every amino acid used in this program is the consensus scale created and first reported by Kyte and Doolittle (16). $H_T(i)$ and $H_D(i)$ values were calculated by using the following

equations: $H_\gamma(i) = [h(i \pm 3) + h(i \pm 2) + h(i \pm 1) + h(i)]/7$ and $H_\beta(i) = [h(i \pm 4) + h(i \pm 2) + h(i)]/5$.

Two types of transmembrane beta strands with distinct values of average hydrophobicity $[H_\gamma(i)]$ or average amphiphilicity $[H_\beta(i)]$ were anticipated for Hib porin: residues which interact with the hydrophobic membrane bilayer on either side of the beta strand $[H_\gamma(i) \geq 1.6]$; and residues which interact with the hydrophobic bilayer on one side and with the hydrophilic surface of the pore's interior on the other side of the beta strand $[H_\beta(i) \geq 1.6$ and $H_\beta(i + 1) \leq 0.4]$. After beta strands in Hib porin within such defined limits were identified, other potential beta strands were predicted because they displayed relatively high values for $H_\gamma(i)$ and $H_\beta(i)$. Once identified, candidates for beta strands and loops were evaluated by turn prediction.

Turns in Hib porin were identified by using the method of Paul and Rosenbusch, which was adapted for proteins displaying beta structure (21), with a slight modification for the minimum number of residues required to satisfy the conditions for turn prediction. Constituent amino acids were divided into three groups. Turn promoters include Asn (N), Asp (D), Glu (E), Gly (G), Pro (P), and Ser (S); turn blockers comprise Ala (A), Gln (Q), Ile (I), Leu (L), Met (M), Phe (F), Trp (W), Tyr (Y), and Val (V); turn-indifferent residues are Arg (R), Cys (C), His (H), Lys (K), and Thr (T). Segments of four or more residues in which at least one was turn promoting and none was turn blocking were identified as turns.

RESULTS

Epitope scanning of Hib porin, 1H. We elected to synthesize overlapping hexapeptides for the entire sequence of 341 amino acids of Hib porin (9, 19) and to use these hexapeptides to define the molecular reactivities of our seven MAbs (23). A primary requirement for such analysis is that the MAbs react with linear epitopes as opposed to conformational determinants. This criterion was satisfied by our seven MAbs because they reacted with SDS-denatured porin on Western blotting (immunoblotting) and a subset of the MAbs reacted with truncated porin proteins that were expressed in the baculovirus expression vector system (23). All of the MAbs were purified on a monoclonal anti-mouse κ light chain immunoaffinity column (32), and some range finding was then necessary to determine conditions for optimum dilutions of the MAbs in their reactivities with the hexapeptides. Insufficient dilution of a MAb contributed to high background values of ELISA data; appropriate dilutions showed clearly a single peak of reactivity over a small number of hexapeptides (one to six) and a uniform, low background over all other regions of the sequence.

Affinity-purified MAb POR.1 (concentration, 0.5 mg/ml) at a dilution of 1/5,000 reacted with six contiguous hexapeptides of the 336 hexapeptides tested (Fig. 1). The reactivities of MAb POR.1 spanned the region Asp-116 to Asp-126 (Fig. 2). No other hexapeptide bound MAb POR.1, and nonspecific binding to all other regions of the sequence was appropriately low. MAb POR.4 recognized five hexapeptides, those between Gly-162 and Ala-171 (Fig. 2). The pattern of reactivity of MAbs POR.2 and POR.3 to the hexapeptides was identical to that of POR.4. By comparison with these results, a subtly different reactivity was determined for MAb POR.5: it bound to three hexapeptides that included the amino acid sequence from Lys-165 to Gly-172 (Fig. 2). MAb POR.6 was tested and found to react with hexapeptides between Thr-318 and Val-325 (Fig. 2). MAb POR.7 showed

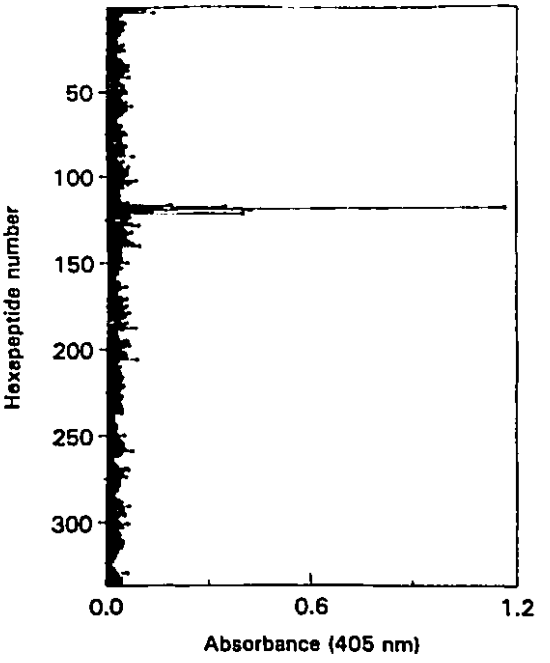

FIG. 1. Epitope scanning with hexapeptides corresponding to the sequence of Hib porin, OMP subtype 1H. The sequence of 341 amino acids was synthesized as 336 sequential overlapping hexapeptides on solid-phase supports. Hexapeptides were incubated with POR.1, washed free of unbound MAb, and reacted with a secondary antibody as described in Materials and Methods. ELISA values are expressed as A_{405} units measured after 30 min of incubation.

a pattern of reactivity identical to that of POR.6. All of these reactivities are therefore consistent with our previous information, which assigned the sequences that were recognized by the seven MAbs to three distinct regions of Hib porin.

Also tested was mouse MAb Hb-2, a surface-reactive anti-porin MAb that has been shown to react with 451 of 453 type b strains from a worldwide collection of *H. influenzae* (8). MAb Hb-2 did not react with fast protein liquid chromatography (FPLC)-purified porin on Western blotting nor did it react with any of the hexapeptides. This result is consistent with the suggestion that Hb-2 recognizes some conformational determinant(s) of Hib porin.

To amplify the analysis of Hib porin by epitope scanning, we tested mouse MAbs from different laboratories which reacted positively with the porin of Hib ATCC 9795. Two MAbs, 9F5 (10) and 23AA12 (27), were previously characterized as recognizing buried or otherwise inaccessible regions of Hib porin, the locations of which were unknown. Both MAbs 9F5 and 23AA12 were first tested for recognition of full-length recombinant porin and four C-terminal deletions of porin generated in the baculovirus expression vector system. These deletion proteins were POR139 (containing amino acids 1 to 139 of the mature Hib porin), POR174, POR182, and POR264. All of these recombinant proteins were previously used to designate regions of antibody recognition (23). MAb 9F5 reacted on Western blotting with recombinant porin and all four truncated porin proteins. MAb 23AA12 did not react with POR139 but gave a positive signal with POR174, POR182, POR264, and full-length recombinant porin (data not shown). Such data enabled

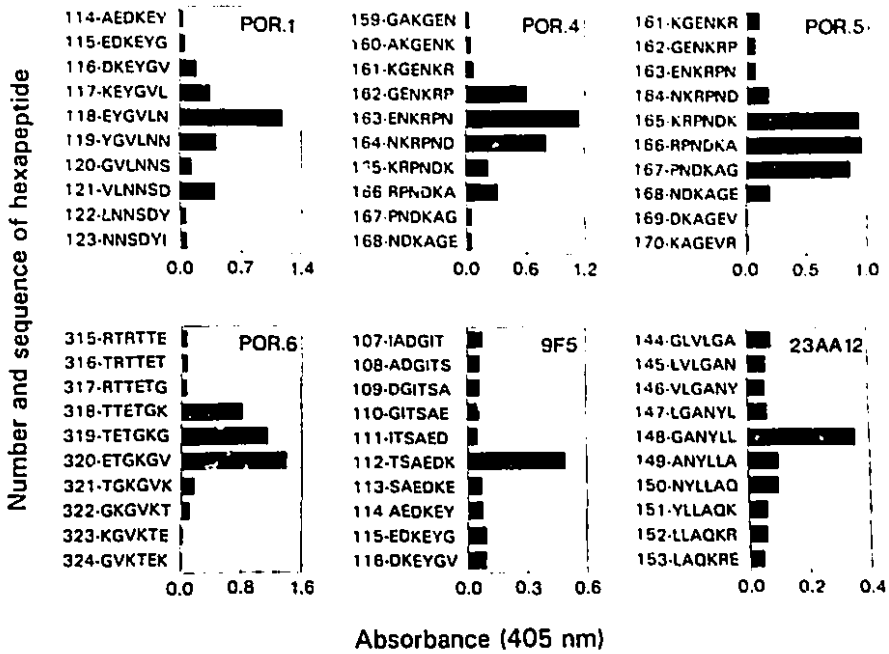

FIG. 2. ELISA reactivities of anti-Hib porin MAbs. The entire set of hexapeptides was subjected to epitope scanning with six anti-Hib porin MAbs. Only the region of positive ELISA reactivity (A_{405}) is shown in each panel. The numerical value that identifies each hexapeptide sequence corresponds to the amino acid in the sequence of Hib porin (OMP subtype 1H) at the amino end of a given peptide.

preliminary localization of epitopes recognized by these MAbs: 9F5 between Ala-1 and Phe-139 and 23AA12 between Lys-140 and Val-174. Epitope scanning identified the primary sequences to which these MAbs bound. MAb 9F5 recognized Thr-112 to Lys-117, and MAb 23AA12 recognized Gly-148 to Leu-153 (Fig. 2).

Altered immunoreactivities of MAbs against porins. Variations in the mobility patterns of OMPs on SDS-PAGE have been employed as a means of classifying Hib isolates (1). This diversity of patterns extends to the differing mobilities of porin, and these differences on SDS-PAGE have now been correlated with amino acid sequence changes of porin (18). We wished to determine whether any of the panel of MAbs would discriminate between variants of porin. Three bacterial strains were used. They differ in porin protein amino acid sequence. Hib ATCC 9795 has 1H porin having the prototype sequence (9, 19); Hib MCH 5539 has 2L porin, a change of Arg-166 to Gln compared with 1H porin (18); Hib 1481 has 6U porin, a total of 10 amino acid sequence changes compared with 1H porin: Phe-96 to Leu, Gly-162 to Met, Glu-163 to Ala, Arg-166 to Leu, Asp-169 to Asn, Asp-193 to Asn, Ser-210 to Ala, Lys-214 to Thr, Ile-249 to Val, and Glu-320 to Gly (18). Our DNA sequence data confirmed these amino acid changes. Porin protein was extracted from each of these three strains and purified by FPLC on a MonoQ column to apparent homogeneity (23). A single band appeared on silver staining of 500 ng of purified protein. Each of these three purified proteins was then used in a solid-phase ELISA to assess the immunoreactivities of the MAbs against different porins. The reciprocal titers determined by ELISA provided comparisons of the reactivities of MAbs against the sequence variants.

All of the MAbs from POR.1 to POR.7 reacted with FPLC-purified Hib porin 1H (Table 1). Identical reactivity patterns were displayed by MAbs POR.1, POR.5, POR.6, and POR.7 against 1H porin and 2L porin. MAbs POR.2, POR.3, and POR.4 showed a ninefold decrease in reactivity to 2L porin compared with 1H porin. In marked contrast to these data, we were unable to detect by ELISA any reactivity of MAbs POR.2, POR.3, POR.4, and POR.5 with 6U porin. The reactivities of MAbs POR.1, POR.6, and POR.7 with isolated 1H porin, 2L porin, and 6U porin were identical, as indicated by the reciprocal titers of antibody reactivities on ELISA.

Recognition of different Hib porins on intact cells. While the above information on the binding of MAbs with isolated porin identified marked differences in reactivity, it did not

TABLE 1. Reactivities of MAbs against porin variants of Hib

MAb	Reciprocal titer of antibody reactivity[a] with OMP subtype:		
	1H	2L	6U
POR.1	8,100	8,100	8,100
POR.2	8,100	900	<100
POR.3	8,100	900	<100
POR.4	2,700	300	<100
POR.5	900	900	<100
POR.6	2,700	2,700	2,700
POR.7	300	300	300

[a] FPLC-purified porin (5 μg/ml) from the three different Hib strains in carbonate buffer was adsorbed to an ELISA plate overnight at room temperature. Threefold dilutions (starting from 1/100) of affinity-purified MAbs (concentration, 0.5 mg/ml) were reacted with antigen, followed by a secondary antibody conjugated to alkaline phosphatase. The reciprocal titer is expressed as the dilution of a MAb that gave an absorbance of at least 0.2 over the background for the colorimetric assay of enzymatic activity.

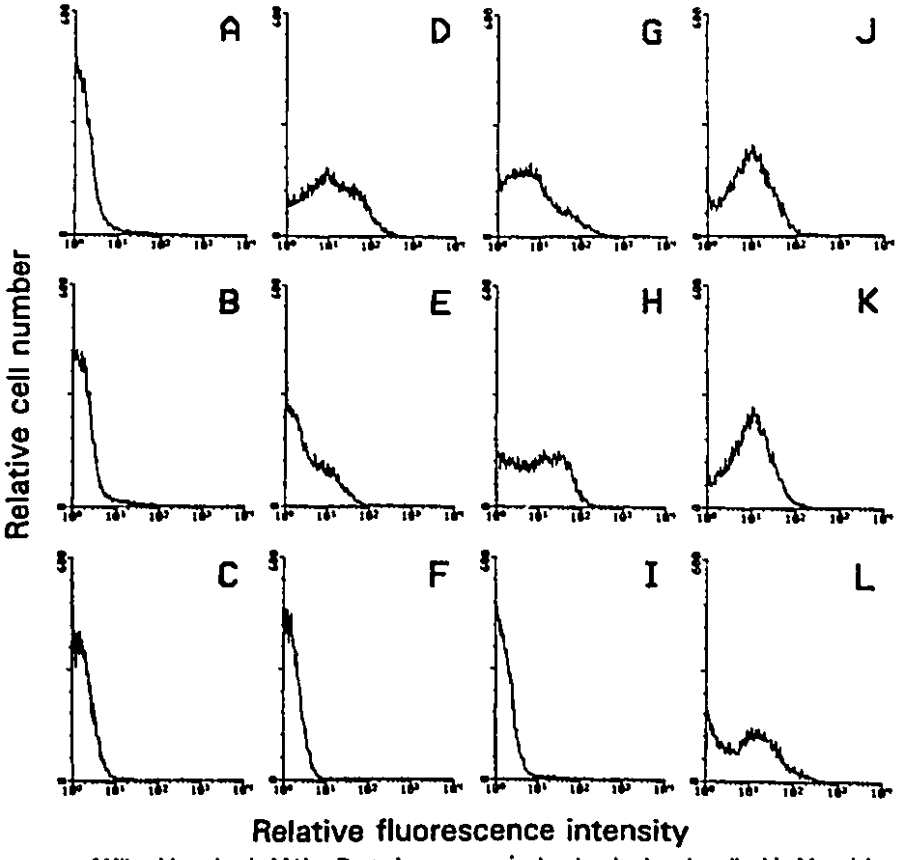

FIG. 3. Flow cytometry of Hib with antiporin MAbs. Bacteric were prepared and stained as described in Materials and Methods. Three strains of Hib were analyzed: ATCC 9795 (OMP subtype 1H) in panels A, D, G, and J; MCH 5539 (OMP subtype 2L) in panels B, E, H, and K; and 1481 (OMP subtype 6U) in panels C, F, I, and L. The MAbs tested were POR.1 in panels A, B, and C; POR.4 in panels D, E, and F; POR.5 in panels G, H, and I; and POR.6 in panels J, K, and L.

provide information as to whether the cognate epitopes were exposed at the bacterial cell surface or were buried in the outer membrane and therefore inaccessible to antibodies. To probe the topological orientation of the amino acid sequences that were recognized by our panel of MAbs, we employed flow cytometry of intact Hib. After reaction of freshly grown bacterial cells with a selected MAb, the cells were exhaustively washed and reacted with a fluorescent secondary antibody. Fluorescence-activated cell sorting (FACS) identified whether a population of cells was labelled or not labelled by the primary MAb. All experiments included two controls: Hib ATCC 9795 reacted positively with MAb Hb-2; Hib DL42/2F4$^-$ is a porin deletion strain (3) and was negative by flow cytometry for all of the anti-porin MAbs tested. Data from FACS scans with four MAbs are presented (Fig. 3) because they represent the clearest distinctions between MAb reactivities with sequence variants of Hib porin. MAb POR.1 was uniformly negative by FACS analysis of Hib which contains 1H, 2L, or 6U porin (Fig. 3A, B, and C). Even though this MAb reacts strongly with the isolated porins, the epitope to which MAb POR.1 binds is therefore not surface exposed. MAb POR.4 reacted positively with cells with 1H porin, to an intermediate extent with intact Hib that contains 2L porin, and negatively with intact cells with 6U porin (Fig. 3D, E, and F). Reactivity of MAbs POR.2 and POR.3 with intact bacteria was similar to that of POR.4. MAb POR.5 was positive with cells displaying either 1H or 2L porin and negative with Hib displaying 6U porin (Fig. 3G, H, and I). MAb POR.6 reacted positively by FACS analysis with all three Hib strains (Fig. 3J, K, and L), and so did MAb POR.7.

Epitope scanning of Hib porin 2L. The reactivities of MAbs POR.2, POR.3, and POR.4 with FPLC-purified 2L porin on ELISA (Table 1) and with intact Hib of OMP subtype 2L by FACS analysis (Fig. 3) were clearly lower than in parallel experiments that analyzed 1H porin. To determine whether this differential reactivity was attributable to a change in the secondary structure or to a difference in direct recognition of primary sequences, epitope scanning of the Hib 2L porin was undertaken. Since the 2L porin differs by a single amino acid change of Arg-166 to Gln (18), all possible overlapping hexapeptides that incorporated Gln-166 were synthesized. The six new hexapeptides for the sequence of Hib 2L porin were those from Lys-161 to Gln-166 and Gln-166 to Ala-171. We then asked whether MAbs POR.2, POR.3, POR.4, and POR.5 would recognize the hexapeptides of the Hib 2L porin sequence. Whereas MAb POR.4 reacted with the sequence Gly-162 Glu Asn Lys Arg Pro Asn Asp-169 from Hib 1H porin (Fig. 2), it did not react at all to the sequence in which Arg-166 had been changed to Gln (Fig. 4). The identical

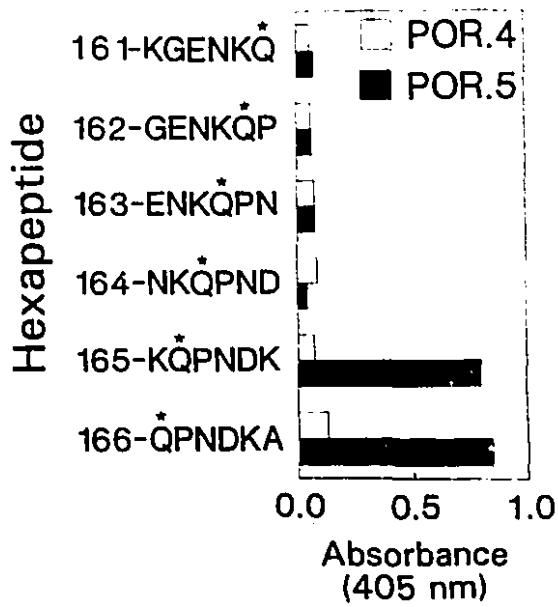

FIG. 4. Epitope mapping with hexapeptides corresponding to the sequence of Hib porin, OMP subtype 2L. The asterisk indicates the unique change (Arg-166 to Gln) in the primary sequence of 2L porin compared with the primary sequence of 1H porin. The ELISA values were determined after reaction with anti-Hib porin MAbs POR.4 and POR.5.

result was seen for MAbs POR.2 and POR.3. MAb POR.5 bound to the sequence Lys-165 Arg Pro Asn Asp Lys Ala Gly-172 from Hib 1H porin (Fig. 2); the change of Arg-166 to Gln had no effect on the ability of MAb POR.5 to recognize the hexapeptides which incorporated this sequence change (Fig. 4).

Model for topological organization of Hib porin. Bacterial porins are generally considered to be composed of beta strands that traverse the membrane and loops that connect the beta strands on either side of the membrane. Individual beta strands can either be buried in the hydrophobic milieu of the membrane or be arranged such that one side faces the lipid membrane and the other side faces the interior of the channel. In the latter case, the side contacting the membrane must be predominantly hydrophobic and the other side must be predominantly hydrophilic so as to provide a hydrophilic lining to the pore. This orientation is referred to as amphiphilic (11). Hydrogen bonding between neighboring strands then leads to formation of a closed structure, a beta barrel, which can function as a pore across the membrane. Loops connecting beta strands, on the other hand, are exposed to the aqueous environment on either side of the membrane and must therefore be predominantly hydrophilic. To connect neighboring beta strands, the loops encounter turns along their sequence (21). Owing to this and other constraints in secondary structure, the loops on any one side of the membrane are expected to protrude to various extents and therefore are not equally accessible at the surface. The ultimate refinement for the structure of porins has come from analysis of crystals of *Rhodobacter capsulatus* porin (29, 30).

We predicted beta strands for Hib porin of OMP subtype 1H, the prototype sequence used in our studies, by identifying hydrophobic and amphiphilic stretches in its primary

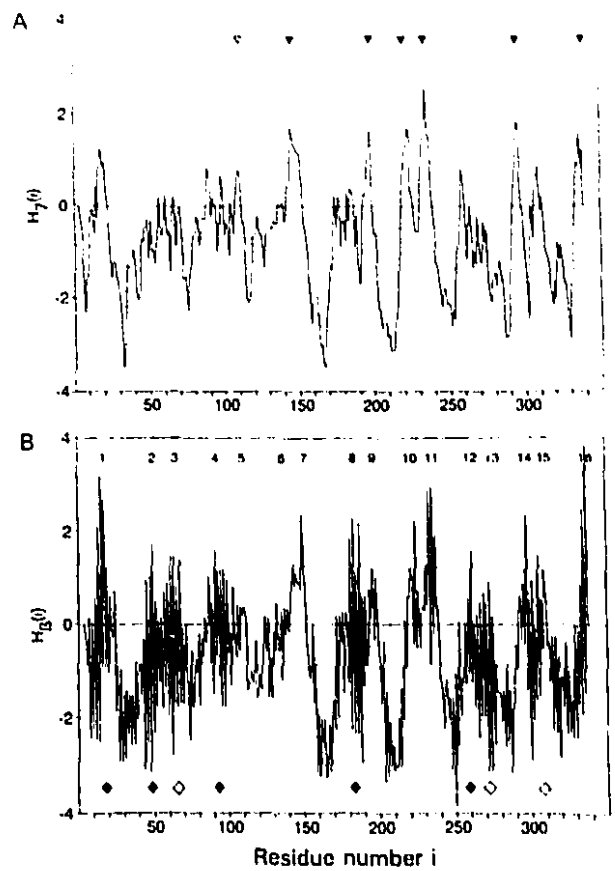

FIG. 5. Plots of structure predictions for Hib porin. By using a window of seven neighbors, the hydrophobicity $[H_\gamma(i)]$ (A) and amphiphilicity $[H_\beta(i)]$ for beta strands (B) were plotted. Peak values that predict hydrophobic (▼) and amphiphilic (◆) beta strands are within the stringency limits specified by Jähnig (see Results). Other hydrophobic (▽) and amphiphilic (◇) beta strands are proposed because the regions display relatively high values for $H_\gamma(i)$ and $H_\beta(i)$, respectively. The potential beta strands are numbered 1 to 16.

sequence as described by Jähnig (11). To predict hydrophobic stretches in Hib porin, the average hydrophobicity of each neighboring seven residues $[H_\gamma(i)]$ sliding over the entire sequence was plotted (Fig. 5A). A positive value of $H_\gamma(i)$ for a given sequence denotes hydrophobicity, while a negative value denotes hydrophilicity. Six membrane-spanning hydrophobic beta strands (Fig. 5A, closed triangles) were predicted over regions which displayed peak values of $H_\gamma(i) \geq 1.6$. Amphiphilic regions in Hib porin were identified by plotting the average amphiphilicity of each of the five alternate amino acids within nine-residue segments $[H_\beta(i)]$ sliding over the entire sequence (Fig. 5B). Alternate amino acids in a sequence would be distributed to one side of a hypothetical membrane-spanning beta strand. Five amphiphilic beta strands (Fig. 5B, closed diamonds) were identified over regions which displayed values of $H_\beta(i) \geq 1.6$ and $H_\beta(i + 1) \leq 0.4$ as specified by Jähnig (11). These limits for predicting hydrophobicity and amphiphilicity were based on the analyses of a large number of membrane proteins. Such pattern searches within these defined limits were useful in predicting 11 beta strands. Since analyses of other porins

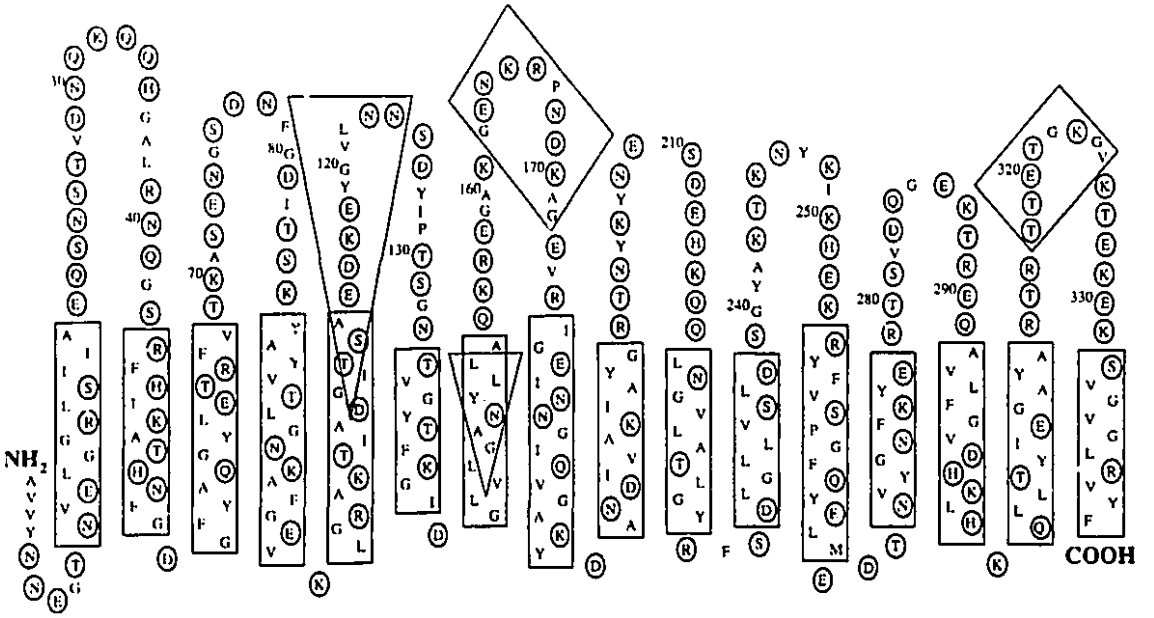

FIG. 6. Topology of Hib porin. The structure of Hib porin, OMP subtype 1H, was predicted from analyses of its hydrophobicity, amphiphilicity, and reactivity with MAbs. Sixteen beta strands corresponding to sequences in Hib porin defined by the numbers in Fig. 5 are shown as rectangles. For each beta strand, the right side shows residues that are proposed to face the lining of the pore; the left side shows residues that are proposed to interact with the outer membrane bilayer. Some reference numbers are shown for amino acids positioned in loops. Significantly hydrophilic residues D, E, H, K, N, Q, R, S, and T are circled. Surface-located epitopes are enclosed in parallelograms. Epitopes buried or inaccessible to antibodies are enclosed in triangles.

of gram-negative bacteria revealed greater than 11 beta strands (14, 30), we attempted to identify other potential beta strands in Hib porin in a manner analogous to that proposed by Jähnig (11). To do this, we predicted four beta strands which corresponded to relatively hydrophobic (Fig. 5A, open triangles) and/or relatively amphiphilic (Fig. 5B, open diamonds) regions, even though the peak values of $H_7(i)$ and $H_{11}(i)$ in these regions were not within the limits specified above. Fifteen beta strands (Fig. 5B, no. 1, 2, 3, 4, 5, 7, 8, 9, 10, 11, 12, 13, 14, 15, and 16) were located by using the plots generated by the program AMPHI. By inspection of the amino acid sequence of Hib porin for amphiphilic stretches, the last of the beta strands (Fig. 5B, no. 6) is proposed because it was close to beta strand 7 and conformed to the emerging pattern (14) which found pairs of closely linked antiparallel beta strands, each pair being separated by long stretches of hydrophilic residues (see Discussion). Beta strand 6 for either OmpF-OmpC of *E. coli* or the porin of *Neisseria meningitidis* was also not readily predicted in the study of bacterial porins (14). The determination of the three-dimensional structure of OmpF will provide detailed clarification (22a). Thus, 16 beta strands of 9 to 14 amino acids were predicted for Hib porin.

Loops in the Hib porin sequence are proposed by locating stretches of hydrophilic amino acids and by identifying residues that promoted turns. Regions exhibiting hydrophilic character (Fig. 5A) served to identify loops in the Hib porin sequence. In addition, the degree of amphiphilicity in these regions (Fig. 5B) was significantly lower. Finally, we subjected the Hib porin sequence to turn predictions as described by Paul and Rosenbusch (21), by locating stretches of four or more amino acids in which at least one residue was turn promoting and none was turn blocking. Stretches of amino acids that are predicted to promote turns occurred exclusively in regions that had previously been assigned to loops on the basis of hydrophilicity. In contrast, stretches of amino acid sequences that are predicted to block turns occurred primarily in stretches that had been previously predicted to be beta strands.

On the basis of these considerations, a model for the topological organization of Hib porin in the outer membrane was generated (Fig. 6). The model proposes 16 beta strands. Fourteen of these strands are sufficiently amphiphilic to interact with the bilayer of the outer membrane on one side and to face the interior of the hydrophilic channel on the other. Two strands (Fig. 6, beta strands 7 and 10) are more hydrophobic than amphiphilic and are predicted to span the outer membrane without being directly exposed to the interior of the pore. The model also shows eight large loops on one side of the predicted beta barrel and eight short loops on the other side. These loops connect neighboring beta strands and are predominantly hydrophilic. Two of the large loops are located at the outer leaflet of the outer membrane on the basis of our determination of the reactivities of anti-porin MAbs against intact cells of Hib.

DISCUSSION

Experiments reported here were designed to extend our observations on the molecular reactivities of seven mouse MAbs against Hib porin and to generate information on the folding of the protein in the outer membrane. Initial characterization of our MAbs was based upon their recognition of fragments of porin that were obtained by chemical and enzymatic digestions. In addition, omega mutagenesis of porin gene *ompP2* (23) created 3′ deletions and the truncated

porin derivatives were expressed in the baculovirus expression vector system. Our initial resolution of the antigenic determinants which were recognized by the MAbs varied considerably: 36 residues for region i between amino acids 104 and 139, 13 residues for region ii between amino acids 162 and 174, and 75 residues for region iii between amino acids 267 and 341 (23). To refine this analysis, 336 overlapping synthetic hexapeptides were generated for the entire Hib porin sequence and tested for recognition by Hib porin MAbs. This strategy of epitope scanning generated data that were entirely consistent with our previous assignments. MAb POR.1 reacted with six hexapeptides, thereby narrowing region i to the sequence between amino acids 116 and 126. MAbs POR.2, POR.3, POR.4, and POR.5 reacted with hexapeptides between amino acids 162 and 172 and was therefore close to the earlier boundaries proposed for region ii. MAbs POR.6 and POR.7 recognized hexapeptides which included the eight amino acids between 318 and 325 and served to define the limits of region iii. Two other MAbs were analyzed by epitope scanning and found to react with amino acids 112 to 117 (MAb 9F5) and 148 to 153 (MAb 23AA12). All of these assignments were possible because linear epitopes and not conformational epitopes are recognized by the nine MAbs. This contrasts with the lack of reactivity with hexapeptides shown by Hb-2, a MAb that recognizes conformational determinants.

Region ii is of particular interest because of the amino acid sequence variations it displays. If the porin of OMP subtype 1H is taken as the prototype sequence, then the sequence of the porin of OMP subtype 2L differs by a single amino acid change, Arg-166 to Gln. This unique change can be distinguished by MAbs POR.2, POR.3, and POR.4; there was a ninefold decrease in the reactivity of these MAbs with FPLC-purified 2L porin in the solid-phase assay (ELISA) by comparison with 1H porin. Such a decrease in reactivity was also reflected in the results from flow cytometry, in which MAbs POR.2, POR.3, and POR.4 showed diminished surface binding to intact cells of Hib MCH 5539 (2L) compared with Hib ATCC 9795 (1H). By epitope scanning, these three MAbs were unreactive with hexapeptides when they were changed in sequence to reflect th: Arg-166-to-Gln substitution. Different results for MAb recognition therefore reflect different stringencies that are inherent to the assays. Synthetic hexapeptides present a limited number of amino acids for antibody recognition and provide a context of restricted structural flexibility (28). Isolated protein and protein in its native conformation at the bacterial surface present the same amino acids but in different contexts. Our data indicate that the primary sequence to which these three MAbs bind is between Gly-162 and Ala-171 in the 1H porin sequence and that these MAbs are unable to bind to hexapeptides in this region containing the Arg-166-to-Gln change. However, when these MAbs encounter the native sequence of the Hib 2L porin (isolated 2L porin or porin on intact cells of Hib with OMP subtype 2L), the presence of other residues may be able to compensate to a certain extent the Arg-166-to-Gln substitution and thereby allow for some binding. These additional residues may directly participate in the structure of the epitope, or in their presence, the epitope may better assume a conformation required for antibody binding. Porin of OMP subtype 6U has four changes in sequence within region ii: Gly-162 to Met, Glu-163 to Ala, Arg-166 to Leu, and Asp-169 to Asn. These multiple changes are sufficient to abolish the reactivities of MAbs POR.2, POR.3, and POR.4 against the isolated Hib 6U porin and against intact Hib 1481, which displays this protein at the cell surface.

MAb POR.5 also recognizes amino acids within region ii but has a specificity different from that of MAbs POR.2, POR.3, and POR.4. The hexapeptides with which it reacted (amino acids 165 to 172) were slightly shifted from those recognized by MAbs POR.2, POR.3, and POR.4 (amino acids 162 to 171). MAb POR.5 binding to hexapeptides was refractory to the Arg-166-to-Gln change, and by flow cytometry MAb POR.5 reacted positively with Hib ATCC 9795 and MCH 5539. Nevertheless, POR.5 did not react with Hib 1481, which contains two changes in its porin sequence between amino acids 165 and 172. Taken together, these data indicate the utility of our anti-Hib porin MAbs that are directed against region ii of porin: they differentiate minor and specific differences among strains of the major Hib OMP subtypes 1H, 2L, and 6U.

The porin of *R. capsulatus*, another gram-negative bacterium, has been crystallized, and the structure has been solved to a resolution of 1.8 Å (30). The monomer subunit of this porin consists of 16 beta strands which form a completely antiparallel beta barrel in which all strands are connected to their neighbors by loops. The connecting loops are generally longer at the top of the barrel, corresponding to the extracellular surface, and shorter at the bottom, corresponding to the periplasmic surface (29). A recent report that analyzed the structure of a family of bacterial porins by using sequence alignments and structure prediction also arrived at the same consensus for the arrangement of bacterial porins (14). Those researchers predicted 16 beta strands which are arranged in pairs, with the largest connecting loops on the extracellular side and very short turns on the periplasmic side.

Porin of *H. influenzae* was not included in the above study (14), since the 18% homology of Hib porin with OmpF of *E. coli* was considered to be at the lower limit of significance for meaningful comparison. Could algorithms similar to those used by Jeanteur et al. (14) be applied to predict the topological organization of Hib porin? Such predictions might then be substantiated by our mapping and localization of MAb binding sites on Hib porin. To search for amphiphilic regions in porin of Hib, we used a program (AMPHI) developed by Jähnig (11), a program that is similar to that first described by Eisenberg et al. (5) and was used to predict the structure of OmpF (15). We used the Jähnig method because of its demonstrated validity for predicting secondary structures of OMPs (11). Moreover, when we subjected the amino acid sequence of *R. capsulatus* porin (31) to secondary-structure prediction by using this program, the highest values for hydrophobicity [$H_7(i)$] and amphiphilicity [$H_\beta(i)$] corresponded to regions of beta strands as determined by analysis of the crystal structure of this protein. Once we had identified potential beta strands for Hib porin on the basis of hydrophobicity and amphiphilicity data (Fig. 5A, B), we constructed a model for the organization of Hib porin in the outer membrane (Fig. 6). Beta strands of 9 to 14 amino acids were predicted. From analyses of secondary structures of other porins (12, 30), this range appears to be a conservative estimate for the length of beta strands that traverse the membrane. We then subjected the model of Hib porin to verification by identifying turns by using the method of Paul and Rosenbusch (21). The choice of three amino acids as the minimum requirement for constituting a turn, as proposed by Paul and Rosenbusch (21), would falsely identify turns that may otherwise be within beta strands. Because Gln is the only hydrophilic amino acid that acts as a turn blocker in beta-pleated sheets, a sequence of three hydrophilic amino acids which can be within a beta strand

(two of the amino acid side chains facing the interior of the pore and the other facing the lipid membrane, where it could contribute to hydrogen bonding) would most often be identified as a turn. For example, a portion of the sequence of *R. capsulatus* porin, Val-227 Asp His Lys Ala Tyr Gly Leu Ser Val Asp Ser Thr Phe-240, has been shown to be a beta strand (31). This sequence contains two stretches of amino acids, Asp His Lys and Asp Ser Thr, both of which would be predicted to be turns according to the criterion proposed by Paul and Rosenbusch. To circumvent this bias, we used four amino acids as the minimum number required to constitute a turn. When applied to Hib porin, the choice of this criterion resulted in identification of turns and turn clusters exclusively in regions that had been previously assigned to loops. It is noteworthy that all but one (residue position 193) of the amino acid changes among porins of the three OMP subtypes of Hib (1H, 2L, and 6U) fall within surface-exposed loops predicted for Hib porin. This finding is consistent with the notion that sequence variations occur primarily in surface-exposed loops (14).

Direct evidence for exposure of two of the largest loops to the exterior stemmed from the fact that some of our anti-porin MAbs which bound to amino acids 162 to 172 and 318 to 325 also reacted with intact cells of Hib. Such data supported the proposed orientation of the side consisting of the large loops to the outside and the side corresponding to the tight connections of beta strands to the periplasm. The epitope recognized by MAb 23AA12 (amino acids 148 to 153) has been located within a hydrophobic beta strand which spans the membrane (Fig. 6). The region of Hib porin corresponding to amino acids 112 to 126 was recognized by two anti-porin MAbs and predicted to be part of an external loop. However, this region is not surface exposed, as determined by the nonreactivity of these MAbs with porin in intact cells. Nor did these MAbs react with outer membrane vesicles (data not shown), indicating that their cognate epitopes are not exposed on the side of the periplasm. The region encompassing these sequences may be part of an unusually hydrophilic beta strand. Alternatively, this loop could be folded into the pore and therefore the sequences would be unavailable for antibody binding. A region designated c in PhoE of *E. coli* is thought to form a loop. Evidence for this proposal was based on structure predictions and on sequence variations when this region was compared with the corresponding regions in OmpF and OmpC. However, no surface exposure could be detected for this region (24). A loop of 44 amino acids in a corresponding region in the porin of *R. capsulatus* was located within the pore (30). We speculate that the loop in Hib porin encompassing amino acids 112 to 126 is located similarly.

Coinciding with our success in generating and characterizing MAbs against Hib porin (23), there appeared another report of MAbs against this protein (17). Although the epitopes of MAb recognition defined by those studies are less well refined than ours, there is some correspondence of regions recognized by the MAbs derived from both studies. Of particular interest are their group C MAbs which map to a region between amino acids 148 to 174. Like some of our MAbs (POR.2, POR.3, and POR.4), group C MAbs were able to discriminate between Hib strains of OMP subtypes 1H and 2L. They reacted with OMP subtype 1H and not with OMP subtype 2L. Another of their MAbs, P2-17, mapped to a region between amino acids 28 and 55, which is distinct from any region of MAb recognition defined by our study. Further refinement of the recognition sites for their MAbs would be useful in evaluating our model of the topological organization of Hib porin.

Even though the amino acid sequence of Hib porin shows little homology to any of the known porins from gram-negative bacteria, our model of Hib porin is in agreement with the emerging consensus for the channel-forming motif of porins. The predictive value of the several algorithms used in this study is clearly substantiated by our MAb mapping data. By reconstitution of Hib porin with dimyristoyl phosphatidylcholine, we recently succeeded in obtaining two-dimensional crystalline sheets. Our present crystals are sufficiently well ordered to allow reconstruction of images at a resolution of about 30 Å (4). These combined approaches will ultimately guide us towards a high-resolution structure of Hib porin.

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PREFACE TO CHAPTER 4

We wished to extend our investigations on the immunological properties of *Haemophilus influenzae* type b (Hib) porin described in Chapter 2. In this chapter, we describe studies on the properties of lipooligosaccharide-free recombinant Hib porin expressed in *Bacillus subtilis*. We assayed the immune response against recombinant Hib porin and the immunological value of anti-recombinant porin antibodies in immunoprotection. The biophysical activity of this recombinant Hib porin was also tested and compared to that of native Hib porin.

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

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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

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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.

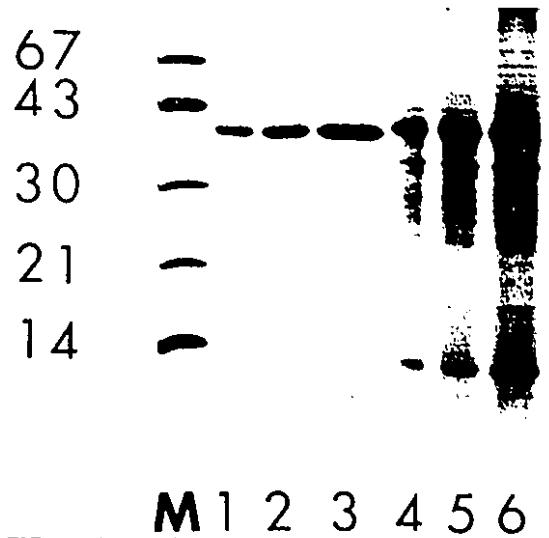

FIG. 1. Identification of recombinant Bac porin. Protein samples were loaded onto SDS-15% PAGE gels and revealed on the gel by silver staining. Lane M, size marker proteins (in kilodaltons); lanes 1 to 3, porin purified from the outer membrane of Hib (250, 500, and 1,000 ng, respectively); lanes 4 to 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

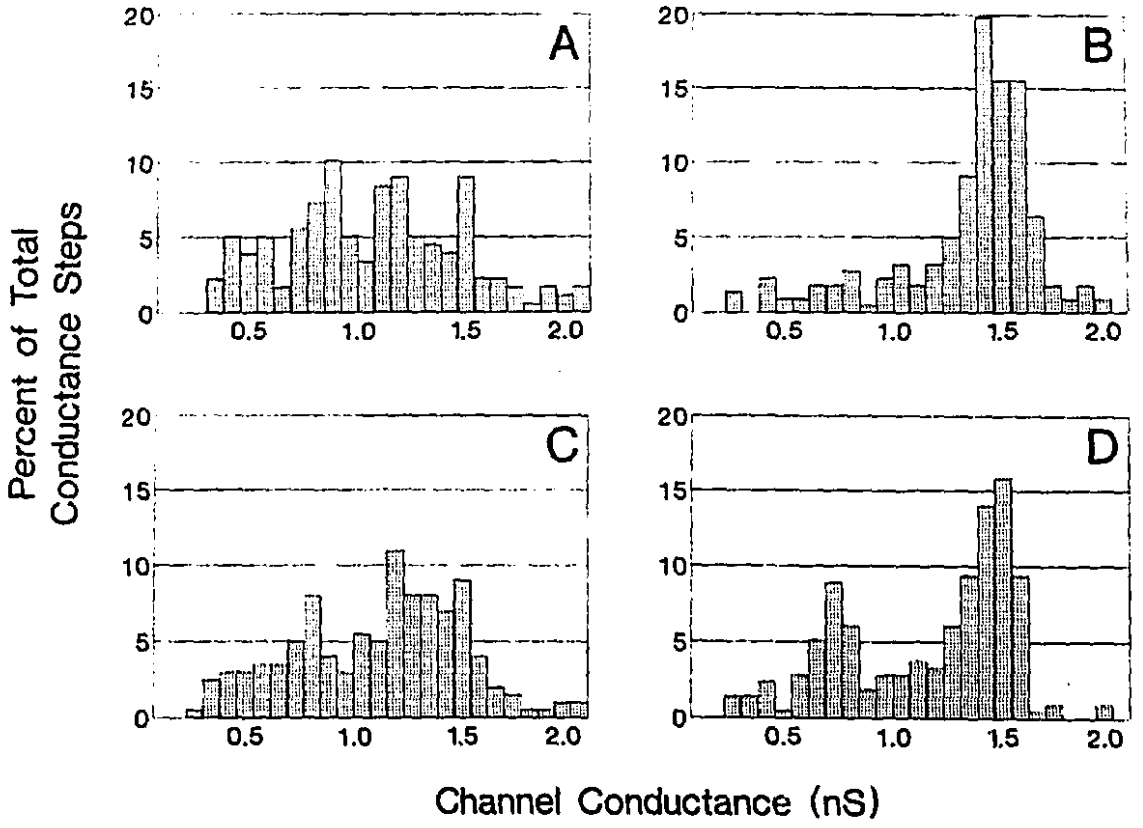

FIG. 2. *Comparison of channel conductances of Hib porin and Bac porin in planar bilayers. Conductance steps were recorded at a transmembrane potential of 10 mV in 1 M KCl. (A) FPLC-purified Hib porin at 2 μg/μl in 50 mM Tris-HCl (pH 8.0)–0.1% Zwittergent Z-3,14 was diluted 500-fold into 50 mM Tris-HCl (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

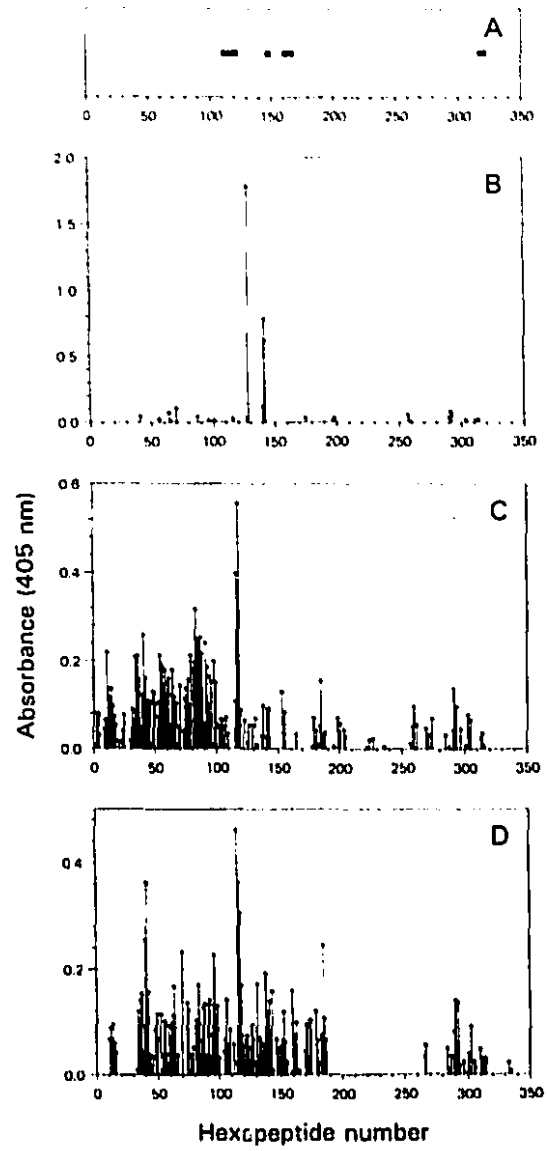

FIG. 3. Epitope scanning with 336 overlapping hexapeptides corresponding to the sequence of Hib porin, OMP subtype 1H, with purified antibodies raised against Hib porin or Bac porin. (A) Regions of the hexapeptides recognized by four different groups of mouse anti-Hib porin MAbs as described previously (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 (10^5 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.

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

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PREFACE TO CHAPTER 5

From our previous studies (Chapter 3), a region in *Haemophilus influenzae* type b (Hib) porin, between residues 112 and 126, was not surface-exposed. This region was predicted to be part of the third loop in our topological model for Hib porin. This chapter describes work that was designed to test the hypothesis that, analogous to other well characterized porins, the third loop in Hib porin folds into the lumen of the pore and produces a narrowing of the channel. Genetic deletions and insertions were constructed in the Hib porin sequences corresponding to the third loop. These mutant porins were used to transform a nontypeable *Haemophilus influenzae* strain that was deleted for porin. The sensitivities of these strains to various anti-microbial agents were compared.

Site-directed mutagenesis of *Haemophilus influenzae* type b porin: alteration of pore properties by deletions in loop 3

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1. Summary

A protein called porin (341 amino acids; M_r 37 782 Da) located in the outer membrane of *Haemophilus influenzae* type b (Hib) allows for the diffusion of small solutes up to a molecular mass of 1400 Da into the periplasmic space. In order to examine the role of loop 3 in the structure of Hib porin, site-directed mutagenesis of the cloned Hib porin gene was undertaken. Hib porin containing 6 or 12 amino acid deletions in loop 3 was expressed in a nontypeable *Haemophilus influenzae* strain deleted for porin. Strains expressing such mutant porins showed significant increases in sensitivities to several anti-microbial agents as compared to wild-type Hib porin. Deletion of 12 amino acids showed more pronounced phenotypes than deletion of 6 amino acids. Therefore deletions in loop 3 caused alterations in pore properties. By comparison, strains expressing mutant porins with 6 or 12 amino acid deletions in the surface-located loop 4 showed no changes in sensitivities to the anti-microbial agents. The C3 epitope of the polioviral VP1 capsid protein was inserted into loop 3 and loop 4 of Hib porin. Whereas the C3 epitope in strains expressing C3 insertion in loop 4 was surface-exposed, the C3 epitope in strains expressing C3 insertion in loop 3 was inaccessible at the surface. Such mutagenesis experiments provided support to the notion that loop 3 in Hib porin folds back into the pore and produces a constriction of the channel.

2. Introduction

The outer membrane of Gram-negative bacteria forms a selective permeability barrier to substances present in their environment. Solutes such as sugars, amino acids, nucleosides, and small antibiotics diffuse easily across the outer membrane whereas substances such as proteins, detergents, and large antibiotics do not gain easy access into the periplasmic space. Porins are trimeric proteins located in the outer membrane and are largely responsible for the molecular sieve properties of this bilayer. They form water-filled channels which allow the diffusion of hydrophilic molecules into the periplasmic space (Benz *et al.*, 1978; Nakae, 1976; Nikaido & Vaara, 1985). The maximum size of a solute molecule that can permeate the pores defines a value termed the molecular mass exclusion limit. Solutes lower in molecular mass than this value diffuse readily through porins into the periplasmic space, whereas solutes whose molecular mass exceed the value of the exclusion limit are impeded in their passage. The variety of porins and their exclusion limits differ from one bacterial genus to another (Jeanteur *et al.*, 1994).

Haemophilus influenzae type b (Hib) is an encapsulated Gram-negative bacterium that until recently was the most frequent cause of meningitis in infants under 18 months. The most abundant outer membrane protein in Hib is porin and is

encoded by the *ompP2* gene. Whereas the outer membrane of *E. coli* contains at least three proteins (OmpF, OmpC, and PhoE) that are general diffusion channels (Nikaido, 1992), only one of the six major outer membrane proteins from Hib has been shown to form pores. The pore formed by Hib porin has a molecular mass exclusion limit of 1400 Da (Vachon *et al.*, 1985), considerably larger than the value of 600 Da (Nikaido, 1992) for the pore formed by OmpF of *E. coli*. By liposome swelling assays, Hib porin has a greater pore diameter (Vachon *et al.*, 1988) than the porins of *E. coli*.

Based on the crystal structures of *Rhodobacter capsulatus* porin (Weiss *et al.*, 1991), *Rhodopseudomonas blastica* porin (Kreusch *et al.*, 1994), and *E. coli* OmpF (Cowan *et al.*, 1992), PhoE (Cowan *et al.*, 1992), and LamB (Schirmer *et al.*, 1995) porins, the folding pattern of the consensus bacterial porin can be predicted to be comprised of (i) 16 or 18 anti-parallel β strands that traverse the outer membrane forming a β barrel and (ii) loops that connect the β strands on either side of the membrane. The connecting loops on the extracellular surface are generally longer than the loops on the periplasmic surface.

Based on parameters of hydrophilicity and amphiphilicity, we previously generated a computer-derived model for the secondary structure of Hib porin (Srikumar *et al.*, 1992b). Even though the amino acid sequence of Hib porin showed little homology to any of the known porins of Gram-negative bacteria

(Jeanteur *et al.*, 1994), our model of Hib porin was in agreement with the emerging consensus for the channel-forming motif of porins. Our model for Hib porin predicted (i) 16 anti-parallel β strands that traverse the outer membrane and (ii) eight long loops that connect the β strands on one side of the membrane. A panel of seven mAbs against Hib porin (Hansen *et al.*, 1989b; Srikumar *et al.*, 1992a; van Alphen *et al.*, 1991) provided data which (i) supported the computer-assisted predictions of Hib porin's secondary structure and (ii) allowed for the orientation of the Hib porin model such that the eight long, connecting loops were assigned to the extracellular surface. These studies established two surface-exposed regions in Hib porin, amino acids 162-172 and 318-325. In the topological model for Hib porin, these two regions were part of the fourth loop (loop 4) and the eighth loop (loop 8), respectively. Two regions between residues 112-126 and residues 148-153 were buried or inaccessible at the surface of the outer membrane. The region between residues 112-126 was predicted to be part of the third loop in the topological model for Hib porin (Srikumar *et al.*, 1992b).

In the consensus model for the folding of bacterial porins, there is an indication that the third loop (loop 3) contributes to the narrowing of the channel. In the *Rhodobacter capsulatus* porin, *Rhodopseudomonas blastica* porin, and *E. coli* OmpF, PhoE, and LamB porins, loop 3 folds back into the channel and produces a constriction of the channel.

This feature is predicted to control pore sizes, thereby establishing their molecular mass exclusion limits. Mutants of *E. coli* were selected genetically for the expression of pores larger than those that have a molecular mass exclusion limit of 600 Da (Benson & Decloux, 1985). In these mutants, several deletion and point mutations in loop 3 of the OmpF (Benson *et al.*, 1988) and OmpC (Misra & Benson, 1988a; Misra & Benson, 1988b) porins were identified. The region encompassing amino acids 112-126 in Hib porin and predicted to be part of loop 3 was not surface-exposed (Srikumar *et al.*, 1992b). Our hypothesis is that loop 3 forms the constriction within the barrel of Hib porin, analogous to other characterized bacterial porins.

To test the hypothesis that loop 3 of Hib porin folds into the lumen of the pore and produces a narrowing of the channel, genetic deletions and insertions were constructed in the Hib porin sequences corresponding to loop 3 and loop 4. Plasmids carrying the cloned Hib porin gene or carrying mutations in the Hib porin gene were used to transform a nontypeable *Haemophilus influenzae* (Hi) strain that was deleted for porin. The sensitivities of these strains to various anti-microbial agents were compared.

3. Materials and Methods

Media, enzymes and reagents. Unless otherwise stated,

Haemophilus strains were grown on chocolate agar plates {36 g l^{-1} GC base (Difco), 10 g l^{-1} haemoglobin, and 20 ml l^{-1} Vitox supplements (Oxoid)} containing 150 μg ml^{-1} of bacitracin. Liquid cultures of Haemophilus strains were grown in brain heart infusion (BHI; Oxoid) broth supplemented with haemin (10 μg ml^{-1}) and NAD (10 μg ml^{-1}); this medium is designated supplemented BHI (sBHI). Media used to grow *E. coli* strains have been described (Sambrook *et al.*, 1989). Unless otherwise stated, antibiotic concentrations used for selection of chromosomal or plasmid markers in Haemophilus and *E. coli* were 20 μg ml^{-1} of kanamycin and 10 μg ml^{-1} of tetracycline. The concentration of ampicillin used for selection of plasmid-encoded resistance in *E. coli* was 100 μg ml^{-1}. Oligonucleotides were synthesized at the Sheldon Biotechnology Centre, McGill University.

Bacterial strains and plasmids. The strains and plasmids used in this study are listed in Table 1. Nontypeable *Haemophilus influenzae* strain DB117 is a recombination-deficient derivative from parent KW20.

Molecular biological techniques. Restriction endonuclease digestions, ligations, and DNA manipulations were performed as described in Sambrook *et al.*, 1989. *E. coli* strain DH5α or Hi strain DB117 were used as hosts for large scale isolation of plasmid DNA using the plasmid maxi kit (Qiagen). DNA was extracted from agarose gels using the Geneclean kit (Bio 101). For transformation of *E. coli*, cells

TABLE 1. Bacterial strains, phages, and plasmids used in this study

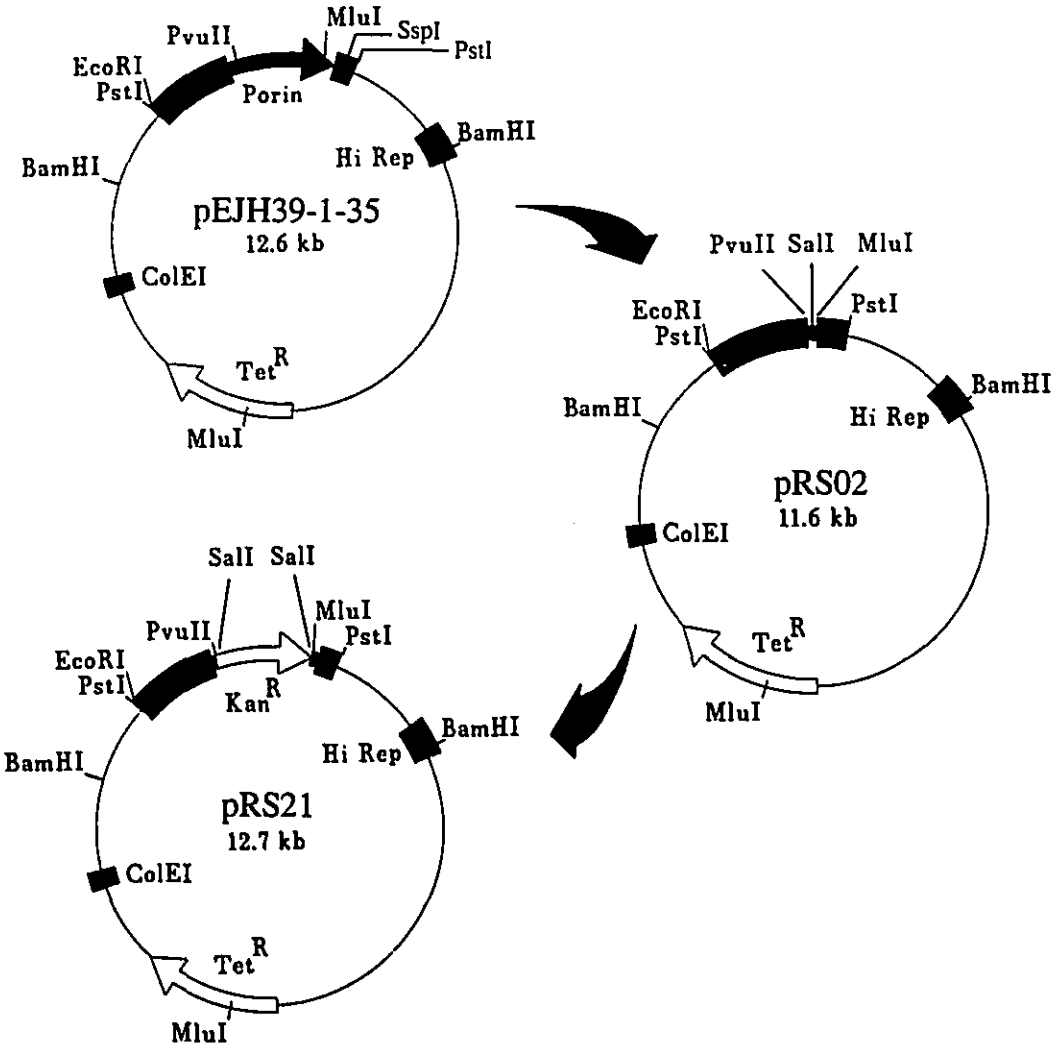
Strain, phage, or plasmid	Relevant characteristics	Source or reference
***E. coli* strains**		
DH5α	*supE44 ΔlacU169 (ϕ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Bethesda Research Laboratories
CJ236	*dut1 ung1 thi-1 relA1*/pCJ105(*cam*^{r} F$'$)	Bio-Rad
MV1190	*Δ(lac-proAB) thi supE Δ(srl-recA)306::Tn10*(tet^r) F$'$ [*traD36 proAB lacI*^{q} *lacZΔM15*]	Bio-Rad
Haemophilus strains		
ATCC9795	Wild-type Hib subtype 1H *ompP2*$^+$	Vachon *et al.*, 1985
KW20	Wild-type Hi Rd *ompP2*$^+$ *rec-1*$^+$	Alexander & Leidy, 1951
DB117	KW20 *rec-1*	Setlow *et al.*, 1972
DL42	Wild-type Hib subtype 1H *ompP2*$^+$ *rec-1*$^+$	Hansen *et al.*, 1989a
DL42/2F4$^-$	DL42 *ompP2*	Cope *et al.*, 1990
RSFA21	KW20 *ΔompP2 kan*^{r}	This study
RSFAb21	DL42 *ΔompP2 kan*^{r}	This study
RS01	RSFA21 containing pEJH39-1-35	This study
RS03 to RS08	RSFA21 containing pRS03 to pRS08	This study
Phage		
M13K07	M13 carrying a mutation in gene II	Bio-Rad
Plasmids		
pBluescript SK(-)	Phagemid *bla*$^+$	Stratagene
pUC-CI	pACYC184 Ω(*Eco*RI::*Eco*RI pUC4K 1.1 kb *kan*^{r})	Vieira & Messing, 1982
pEJH39-1-35	pGB103 Ω(*Pst*I::*Eco*RI-*Pst*I DL42 2.5 kb *ompP2*$^+$) ColE1 Hi Rep	Cope *et al.*, 1990
pFFA02	pBluescript SK(-) Ω(*Pvu*II::*Pvu*II-*Ssp*I pEJH39-1-35 1 kb sequences coding for mature Hib porin)	This study
pFFA03 to pFFA08	pFFA02 carrying mutations in sequences coding for mature Hib porin	This study
pRS02	pEJH39-1-35 Ω(*Pvu*II-*Mlu*I::*Pvu*II-*Sal*I-*Mlu*I adaptor)	This study
pRS03 to pRS08	pEJH39-1-35 *ompP2*	This study
pRS21	pRS02 Ω(*Sal*I::*Sal*I pUC-CI 1.1 kb *kan*^{r})	This study

were made competent with calcium chloride (Sambrook *et al.*, 1989). Haemophilus strains were made competent for DNA uptake using calcium chloride by a similar method (Barcak *et al.*, 1991). To induce natural competence in Haemophilus, strains were grown under conditions described previously (Barcak *et al.*, 1991).

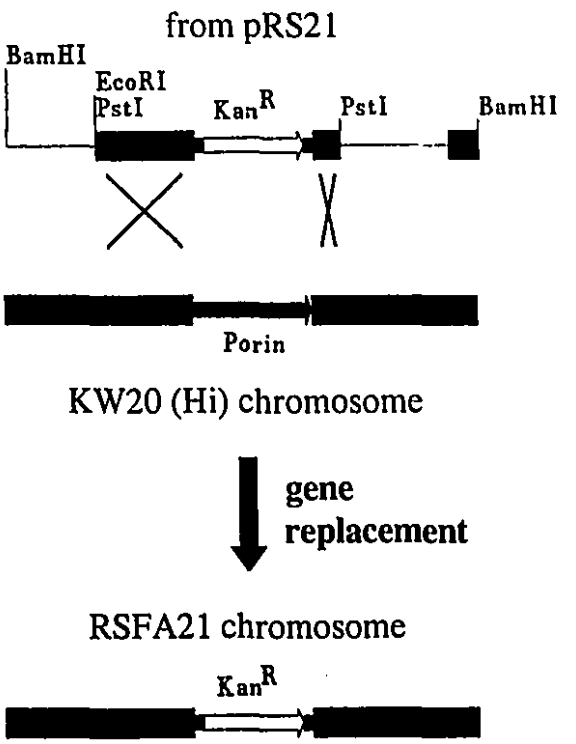
Construction of a nontypeable *Haemophilus influenzae* strain deleted for its porin gene. The entire *Haemophilus influenzae* type b porin gene (*ompP2*) together with upstream sequences (1.1 kb at the 5′-end of the gene) and downstream sequences (0.37 kb at the 3′-end of the gene) are contained in the shuttle vector pEJH39-1-35 (Fig. 1a) as an *Eco*RI-*Pst*I fragment. This plasmid was linearised with *Pvu*II and then subjected to partial digestion with *Mlu*I. The digestion products were electrophoresed on an agarose gel; the 11.6 kb *Pvu*II-*Mlu*I DNA fragment was isolated. A double-stranded DNA adaptor with *Pvu*II and *Mlu*I ends containing an internal *Sal*I site was constructed by annealing two single-stranded oligonucleotides: 5′-CTG GTC GAC A-3′ and 5′-CG CGT GTC GAC CAG-3′. Ligation of the adaptor to the 11.6 kb DNA fragment created a novel *Sal*I site. The ligation mixture was used to transform Hi strain DB117; selection was for tetracycline resistance. Plasmid DNA was isolated from transformants and was designated pRS02 (Fig. 1a). Plasmid pRS02 had 98 % of the coding sequences for Hib porin removed, but retained sequences flanking the excised porin gene. In order to overcome *Sal*I

Fig. 1. Genetic construction of Hi deleted for porin. The strategy used is described in Methods. A: Construction of pRS21 starting from pEJH39-1-35. ColEI and Hi Rep are sequences required for replication in *E. coli* and Haemophilus, respectively. The *Ssp*I restriction site identified in pEJH39-1-35 is not unique to the plasmid. B: Mechanism of homologous recombination that resulted in gene replacement and the generation of porin-deleted, kanamycin-resistant Hi strains (Hi Δ Porin, Kan^R).

A


MluI
SspI
PvuII
PstI
EcoRI
PstI
Porin
BamHI
Hi Rep
BamHI
pEJH39-1-35
12.6 kb
ColEI
Tet R
MluI
PvuII SalI MluI
EcoRI
PstI
PstI
BamHI
BamHI
Hi Rep
pRS02
11.6 kb
ColEI
Tet R
MluI
SalI SalI
MluI
PvuII
PstI
EcoRI
PstI
Kan R
BamHI
Hi Rep
BamHI
pRS21
12.7 kb
ColEI
Tet R
MluI

B


from pRS21
BamHI
EcoRI
PstI
Kan^R
PstI
BamHI
Porin
KW20 (Hi) chromosome
gene
replacement
RSFA21 chromosome
Kan^R
Hi ΔPorin, Kan^R

site modification in Hi strain DB117, pRS02 was used to transform *E. coli* strain DH5α and plasmid DNA was re-isolated. *E. coli*-derived pRS02 was digested with *Sal*I and ligated to a 1.1 kb *Sal*I-restricted DNA fragment containing a kanamycin resistance gene derived from transposon *Tn*903. The ligation mixture was used to transform *E. coli* strain DH5α with selection for tetracycline and kanamycin resistance. The plasmid in which the kanamycin resistance cassette with its own promoter had been cloned in the same orientation as the Hib *ompP2* promoter was designated pRS21 (Fig. 1a).

Plasmid pRS21 was digested to completion with *Bam*HI and the digestion mixture was used to transform naturally-competent Hi strain KW20; selection was for kanamycin resistance. Kanamycin-resistant transformants were analyzed for their expression of porin in the outer membrane. From Hi strains that were deficient for porin expression, chromosomal DNA was subjected to Southern blotting using the Hib porin gene and the kanamycin resistance gene as probes.

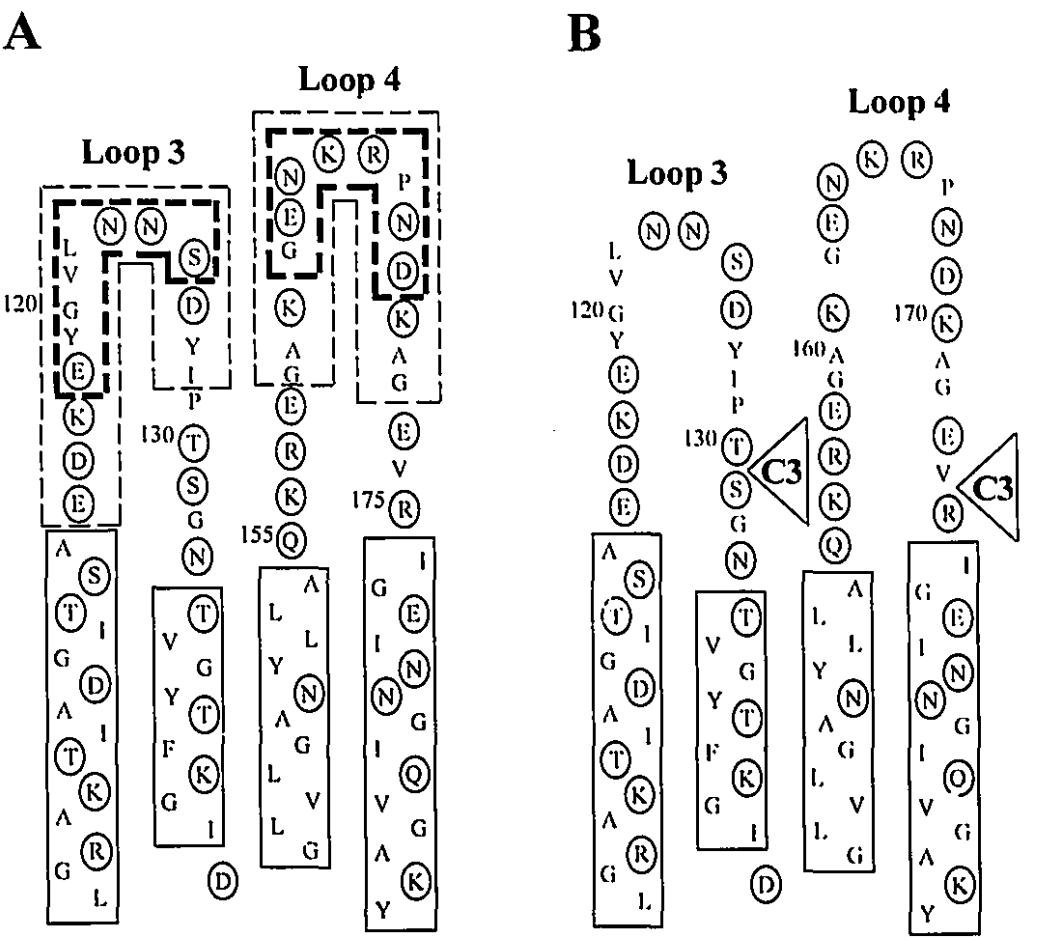
Southern hybridization. Genomic DNA was isolated from Haemophilus strains by a microscale procedure (Barcak *et al.*, 1991). DNA restriction fragments were separated electrophoretically in 0.7 % agarose gels containing TAE (0.01 M Tris-acetate plus 0.01 M EDTA) buffer and transferred to Nytran hybridization membranes (Schleicher & Schuell). Enzymes and the digoxigenin (DIG) DNA labelling kit (The Genius system) for Southern hybridization were obtained from

Boehringer Mannheim. Southern hybridizations were carried out with DIG-labelled probes by following the manufacturer's instructions. Hybridizations were at 68 °C and a final wash with 0.1X SSC containing 0.1 % SDS at 68 °C for 15 min was included to reduce the background. The concentration of the probes during the hybridization steps was 25 ng ml^{-1} of hybridization solution.

Mutagenesis of cloned Hib porin. Plasmid pEJH39-1-35 was digested with *Pvu*II and *Ssp*I. The 1.1 kb *Pvu*II-*Ssp*I DNA fragment was isolated (Fig. 1a); it contained only the coding sequences for the mature form of Hib porin. This DNA fragment was ligated to the 2.5 kb *PvuII* fragment from pBluescript SK(-) and used to transform *E. coli* strain DH5α; selection was for ampicillin resistance. A recombinant plasmid in which the *Pvu*II-*Ssp*I DNA fragment from pEJH39-1-35 was cloned in the same orientation as the *lacZ* gene in pBluescript SK(-) was designated pFFA02. It was used for mutagenesis experiments.

(i) Deletion mutagenesis. Site-directed deletions in Hib porin were constructed with the Muta-Gene Phagemid *In Vitro* Mutagenesis kit, Version 2 (Bio-Rad), using single-stranded mutagenic oligonucleotides. Two regions in Hib porin were selected for mutagenesis (Fig. 2); they correspond to loop 3 and loop 4 in the proposed topological model for Hib porin (Srikumar *et al.*, 1992b). Plasmid pFFA02 was used to transform *dut ung E. coli* strain CJ236. After infection of a transformant with helper phage M13K07, uracil-containing

Fig. 2. Mutations in Hib porin. A portion of our published model (Srikumar *et al.*, 1992b) for the secondary structure of Hib porin is shown. It encompasses β strand 5 to β strand 8. A: Deletions in loop 3 and loop 4 of Hib porin. The eight amino acids (---) and the fourteen amino acids (---) of Hib porin deleted in each loop are enclosed. B: C3 epitope insertions in loop 3 and loop 4 of Hib porin. The point of insertion of the C3 epitope in each loop is indicated.


A
Loop 4
Loop 3
120
130
155
175
B
Loop 4
Loop 3
120
130
160
170
C3
C3

single-stranded phagemid DNA was isolated and used for the mutagenesis. Two mutagenic oligonucleotides created deletions of 6 amino acids and 12 amino acids in loop 3: 5′-ACA AGT GCA GAA GAT AAA GAG CTC GAC TAT ATT CCT ACT AGT-3′ and 5′-GAT GGC ATA ACA AGT GCA GAG CTC CCT ACT AGT GGT AAT ACC-3′, respectively. Two mutagenic oligonucleotides created deletions of 6 amino acids and 12 amino acids in loop 4: 5′-AAG CGT GAG GGT GCA AAA GAG CTC AAG GCT GGT GAA GTA CGT-3′ and 5′-TTA GCA CAA AAG CGT GAG GAG CTC GAA GTA CGT ATA GGT GAA-3′, respectively. Each mutagenic oligonucleotide also incorporated a unique *Sac*I site (underlined, Fig. 3a). After *in vitro* mutagenesis, the reactions were used to transform *E. coli* strain MV1190. Plasmids were isolated from transformants of the four deletion mutagenesis experiments, mapped using restriction enzyme digestions, and candidates corresponding to each mutation were confirmed by DNA sequencing. Plasmids (pFFA05 to pFFA08, Table 1) were digested to completion with *Pvu*II and *Mlu*I and the 1.0 kb *Pvu*II-*Mlu*I DNA fragments containing the mutations were isolated. These fragments were ligated to the 11.6 kb *Pvu*II-*Mlu*I DNA fragment from pEJH39-1-35 and used to transform calcium chloride-competent Hi strain deleted for porin (RSFA21, Table 1), with selection for kanamycin and tetracycline resistance.

(ii) C3 insertion mutagenesis. Plasmid pFFA02 contains unique sites for the restriction enzymes *Spe*I and *Sna*BI within the coding sequences for the mature form of Hib porin.

Fig. 3. Sequences of mutant Hib porins. The regions of the mutations in Hib porin are shown. A: Plasmids carrying deletions in Hib porin; pRS05: 6 amino acid deletion in loop 3, pRS06: 12 amino acid deletion in loop 3, pRS07: 6 amino acid deletion in loop 4, and pRS08: 12 amino acid deletion in loop 4. B: Plasmids carrying C3 insertions in Hib porin; pRS03: C3 epitope insertion in loop 3 and pRS04: C3 epitope insertion in loop 4. The first nucleotide of the local sequence is preceded by a number indicating its position in the published sequence of the Hib porin gene (Munson & Tolan, 1989); the position of the last nucleotide is also indicated. The wild-type Hib porin nucleotide sequences are shown in lower case letters; upper case letters correspond to the additional sequences of the mutations. The corresponding amino acid sequences are indicated above the nucleotide sequence; the numbers above the amino acids denote their position in mature Hib porin. The amino acid sequence of the C3 epitope is italicised.

A

```
                116 117          126 127                    113 114          129 130
                asp lys GLU LEU asp tyr                     ser ala GLU LEU pro thr
pRS05    639-gat aaa GAG CTC gac tat-674      pRS06    630-agt gca GAG CTC cct act-683
                    SacI                                        SacI

                160 161          170 171                    157 158          173 174
                ala lys GLU LEU lys ala                     arg glu GLU LEU glu val
pRS07    771-gca aaa GAG CTC aag gct-806      pRS08    762-cgt gag GAG CTC gaa gta-815
                    SacI                                        SacI
```

B

```
                129 130                                                        131 132
                pro thr SER ASP ASN PRO ALA SER THR THR ASN LYS ASP LYS THR ser gly
pRS03    678-cct aCT AGT GAT AAC CCG GCG TCG ACC ACT AAC AAG GAT AAG Act agt ggt-689
                                        SalI

                173 174                                                    175 176
                glu val ARG ASP ASN PRO ALA SER THR THR ASN LYS ASP LYS arg ile
pRS04    810-gaa gta cGT GAT AAC CCG GCG TCG ACC ACT AAC AAG GAT AAG Cgt ata-821
                                        SalI
```

Restriction sites for *Spe*I and *Sna*BI are found within sequences encoding amino acids of loop 3 and loop 4 of Hib porin, respectively. These sites were used to construct in-frame insertions of the C3 epitope of the VP1 protein of poliovirus (Charbit *et al.*, 1991; Moeck *et al.*, 1994) in Hib porin. Plasmid pFFA02 was digested with either *Spe*I or *Sna*BI and ligated to double-stranded oligonucleotide linkers SpeI-C3 or SnaBI-C3. Linker SpeI-C3 was constructed by annealing two single-stranded oligonucleotides: 5′-CT AGT GAT AAC CCG GCG TCG ACC ACT AAC AAG GAT AAG A-3′ and 5′-CT AGT CTT ATC CTT GTT AGT GGT CGA CGC CGG GTT ATC A-3′; linker SnaBI-C3 was constructed by annealing two single-stranded oligonucleotides: 5′-GT GAT AAC CCG GCG TCG ACC ACT AAC AAG GAT AAG C-3′ and 5′-G CTT ATC CTT GTT AGT GGT CGA CGC CGG GTT ATC AC-3′. Linkers SpeI-C3 and SnaBI-C3 created the codons for the C3 epitope in-frame to the codons for Hib porin (Fig. 3b). The ligation mixtures were used to transform *E. coli* strain DH5α with selection for ampicillin resistance. Plasmids from transformants were isolated, mapped, and sequenced. Plasmids (pFFA03 and pFFA04, Table 1) were digested to completion with *Pvu*II and *Mlu*I, ligated to the 11.6 kb *Pvu*II-*Mlu*I DNA fragment from pEJH39-1-35 and treated as described for deletion mutagenesis.

DNA sequence determination. For dideoxy sequencing (Sambrook *et al.*, 1989), the T7 Sequencing kit (Pharmacia) was used. One oligonucleotide was adequate for all DNA sequencing

across regions encoding amino acids corresponding to loop 3 and loop 4. The primer was 5′-T(584) GAA GTA AAA CTT GGT CGT (603)G-3′ where the numbers are according to the published sequence of the Hib porin gene (Munson & Tolan, 1989).

Preparation of outer membrane vesicles, SDS-PAGE, and immunoblotting. Outer membrane vesicles were obtained by treatment of Haemophilus cells with Tris-lysozyme-EDTA (Hantke, 1981). Samples of vesicles containing outer membrane proteins were suspended in electrophoresis sample buffer (with 2 % SDS), heated for 5 min at 100 °C, and run on 10 % polyacrylamide gels. For immunoblotting, outer membrane proteins were subjected to SDS-PAGE as described above, transferred to nitrocellulose paper (Schleicher & Schuell) and treated as described previously (Srikumar *et al.*, 1992b). The dilutions of primary and secondary antibodies used for immunoblotting were 1/2000.

Flow cytometry. Bacteria from mid-log phase cultures were washed in PBS and suspended in PBS to 2 x 10^9 cells per ml. Affinity-purified anti-Hib porin mouse mAbs (Srikumar *et al.*, 1992a) or anti-peptide (C3 epitope of poliovirus) rabbit hyper-immune serum (Moeck *et al.*, 1994) at 1/100 dilution was mixed with 2 x 10^8 cells and incubated at room temperature for 1 h. Bacteria were pelleted, washed, and incubated at room temperature for 1 h with anti-mouse or anti-rabbit immunoglobulins conjugated to fluorescein. Bacteria were diluted ten-fold in PBS and analyzed for green fluorescence

intensity using a FACScan flow cytometer (Becton Dickinson) with LysisII software. For each sample, 10^4 cells were analyzed.

Assays of sensitivities to arti-microbial agents. Bacteria were grown for 6 h, 37 °C in sBHI containing 10 mM magnesium sulfate. For the selection of antibiotic resistance markers, sBHI contained kanamycin (10 $\mu g\ ml^{-1}$) and tetracycline (5 $\mu g\ ml^{-1}$). Bacteria were centrifuged, suspended in 50 mM magnesium sulfate, and stored over-night on ice. Bacteria (100 μl) were mixed with 3 ml molten sBHI top agar (0.7 % bacto-agar) and spread over sBHI agar (1.5 % bacto-agar) plates. After solidifying, absorbent discs (6.5 mm diameter, gel blot paper GB002, Schleicher & Schuell) were placed on the agar and appropriate volumes (2-10 μl) of anti-microbial agents dissolved in distilled water were pipeted onto the discs. Following over-night incubation at 37 °C, the diameters of the zones of growth inhibition were measured. For strains RSFA21 and RS06 (Table 1), the amount of cells added to the molten sBHI top agar was four-fold greater than that used for the other strains.

4. Results

A nontypeable *Haemophilus influenzae* strain deleted for its porin gene. Plasmid pRS21 was constructed by replacing the sequences coding for the mature form of Hib porin in

pEJH39-1-35 with a kanamycin resistance cassette (Fig. 1a). The kanamycin resistance cassette in this construct was flanked by sequences upstream and downstream of the Hib *ompP2* gene. A *BamHI* fragment derived from pRS21 and containing the kanamycin resistance cassette was used to transform naturally-competent nontypeable Hi. This transformation yielded kanamycin-resistant, porin deletion mutants. Such mutants were predicted to arise by homologous recombination which results in gene replacement of the wild-type chromosomal copy of the porin gene with the kanamycin resistance cassette (Fig. 1b).

Initial characterization of the Hi strains deleted for porin was based on growth properties of recombinants in comparison to strain DL42/2F4$^-$. DL42/2F4$^-$ is a porin-minus Hib strain previously constructed by linker mutagenesis (Cope *et al.*, 1990). On chocolate plates, DL42/2F4$^-$ grew poorly when compared to wild-type strains DL42 and KW20. After transformation of the naturally-competent Hi strain KW20, most transformants (≈ 98 %) were kanamycin-resistant and exhibited retarded growth characteristics compared to the wild-type strain. SDS-PAGE analysis and Western blotting of outer membrane preparations from these transformants showed that gain of kanamycin resistance coincided with loss of porin expression (Fig. 4, lane 4 and Fig. 5a, lane 4). Southern blot analyses (Fig. 6) confirmed the replacement of the chromosomal copy of the Hi porin gene with the kanamycin

Fig. 4. Outer membrane proteins of strains expressing mutant Hib porins. Protein samples were resolved by SDS-PAGE (10 % gel) and stained with Coomassie blue. Lane M: 7500 ng of molecular weight markers; lane 1: 2500 ng of FPLC-purified Hib porin (Srikumar *et al.*, 1992a); lanes 2 and 3: 7500 ng of outer membrane proteins (OMPs) from strains DL42 and KW20, respectively; lane 4: 3750 ng of OMPs from Hi porin deletion (Hi Δ porin) strain RSFA21; lanes 5 to 11: 7500 ng of OMPs from strains RS01, RS05, RS06, RS07, RS08, RS03, and RS04, respectively. Captions on the top indicate sample characteristics. For example, six amino acid deletion in loop 3 of Hib porin is referred to as Loop 3 Δ6aa and the C3 epitope insertion in loop 3 of Hib porin is referred to as Loop 3 C3.

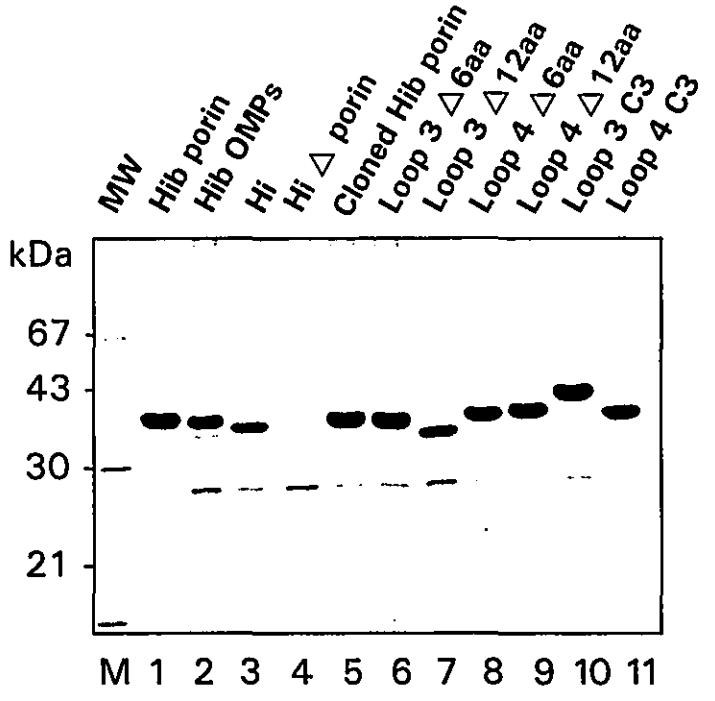

MW
Hib porin
Hib OMPs
Hi
Hi △ porin
Cloned Hib porin
Loop 3 △6aa
Loop 3 △12aa
Loop 4 △6aa
Loop 4 △12aa
Loop 3 C3
Loop 4 C3
kDa
67
43
30
21
M 1 2 3 4 5 6 7 8 9 10 11

Fig. 5. Western blots of outer membrane proteins. Samples that were twenty-fold less and in the same order as shown in Fig. 4 were resolved by SDS-PAGE. The proteins were transferred onto nitrocellulose and probed with antibodies and detected with secondary antibodies conjugated to alkaline phosphatase. Anti-Hib porin mAbs POR.1, POR.4, and POR.6 were used in panels a, b, and c, respectively. Anti-C3 polyclonal antibodies were used in panel d. Epitope scanning performed previously (Srikumar *et al.*, 1992b) showed that POR.1, POR.4, and POR.6 recognized Hib porin sequences between amino acids Asp-116 and Asp-126 (Loop 3), Gly-162 and Ala-171 (Loop 4), and Thr-318 and Val-325 (Loop 8), respectively.

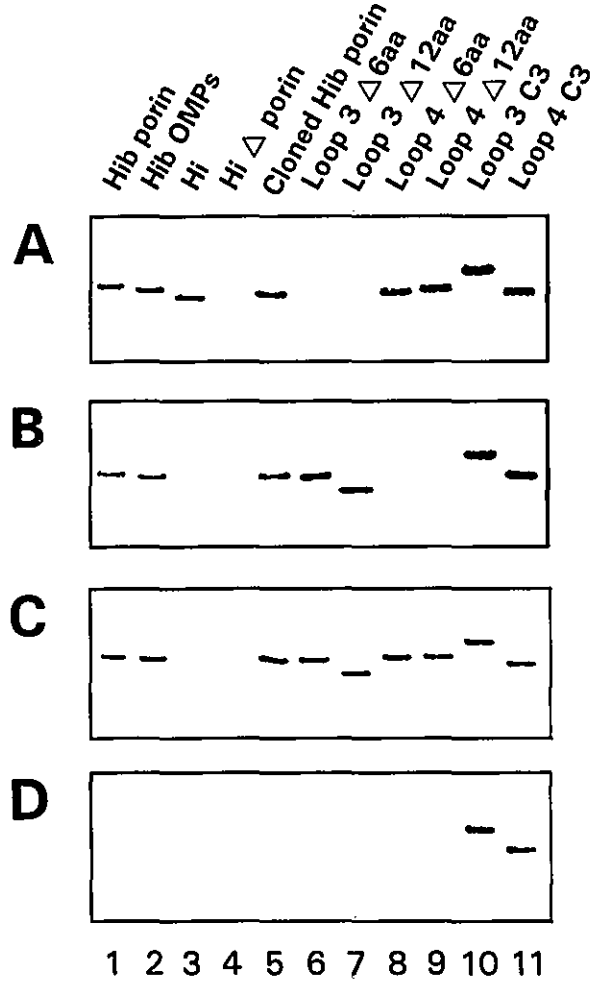
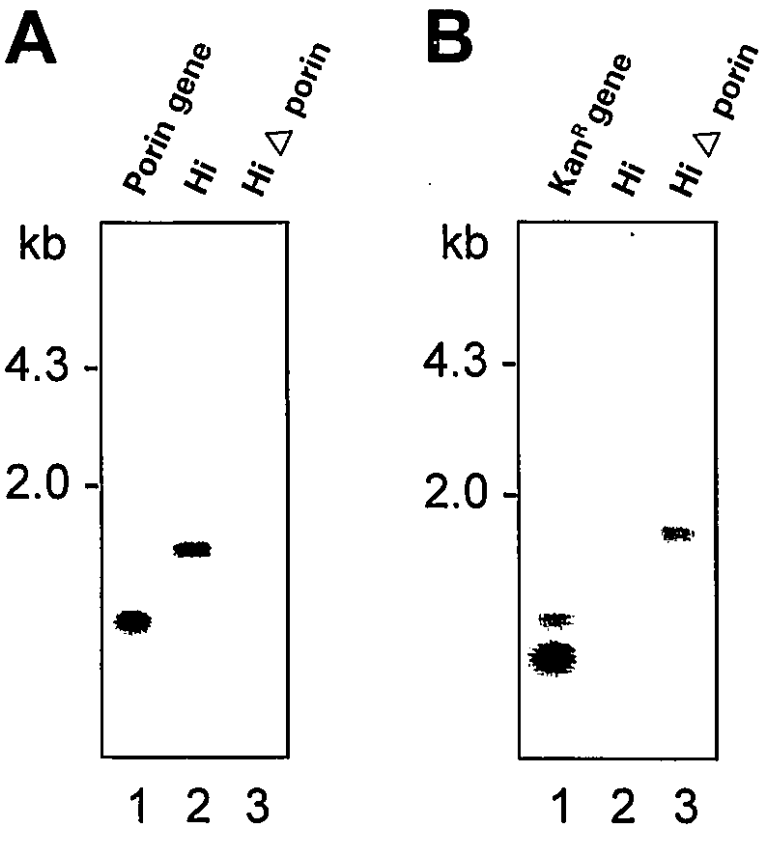

Hib porin
Hib OMPs
Hi
Hi △ porin
Cloned Hib porin
Loop 3 △ 6aa
Loop 3 △ 12aa
Loop 4 △ 6aa
Loop 4 △ 12aa
Loop 3 C3
Loop 4 C3
A
B
C
D
1 2 3 4 5 6 7 8 9 10 11

Fig. 6. Southern blots of restriction enzyme-digested genomic DNA. DNA samples were subjected to agarose gel electrophoresis using 0.7 % gels. Lane 1a: 3 ng of 1.0 kb *Pvu*II-*Mlu*I porin fragment from pEJH39-1-35; Lane 1b: 3 ng 1.1 kb *Sal*I kanamycin resistance (Kan^R) cassette from pUC-CI; Lanes 2 and 3: 5000 ng of *Pvu*II-digested genomic DNA from KW20 and RSFA21, respectively. After separation by electrophoresis, the DNA samples were transferred onto nylon membranes, hybridized with digoxigenin (DIG)-labelled probes and detected with anti-DIG antibodies conjugated to alkaline phosphatase. Probes used were: 1.0 kb porin fragment (panel a) or 1.1. kb Kan^R cassette (panel b).


A
Porin gene
Hi
Hi △ porin
kb
4.3
2.0
1 2 3
B
Kan^R gene
Hi
Hi △ porin
kb
4.3
2.0
1 2 3

resistance cassette. The Hib *ompP2* probe hybridized to a chromosomal DNA fragment from wild-type Hi strain KW20 (Fig. 6a, lane 2) but not to any chromosomal DNA fragment from the porin deletion strain RSFA21 (Fig 6a, lane 3). Conversely, the kanamycin resistance gene probe hybridized to a chromosomal DNA fragment from the porin deletion strain RSFA21 (Fig. 6b, lane 3) but not to any chromosomal DNA fragment from wild-type Hi strain KW20 (Fig. 6b, lane 2).

Mutations in Hib porin. By site-directed mutagenesis, we introduced deletions and insertions in the proposed loop 3 and loop 4 of Hib porin (Fig. 2). The sequences of the mutations and the resulting mutant Hib porins are shown in Fig. 3. The specific amino acids deleted were those that corresponded to epitopes recognized by the anti-Hib porin mAbs POR.1 (loop 3) and POR.4 (loop 4) (Srikumar *et al.*, 1992b). Since all four oligonucleotides used for construction of the deletions contained an extra six nucleotides corresponding to a *Sac*I site, each deletion protein gained two additional amino acids Glu and Leu. For example, in the loop 3, 6 amino acid deletion, eight amino acids of Hib porin were removed and replaced by the amino acids Glu and Leu; hence there was a net deletion of six amino acids. The ends of the linkers SpeI-C3 and SnaBI-C3 were compatible for insertion at *Spe*I and *Sna*BI restriction sites, respectively. Thus, in addition to the eleven amino acids of the C3 epitope, two extra amino acids in the loop 3 C3 mutant protein and an extra amino acid in the

loop 4 C3 mutant protein were introduced; hence there were net insertions of 13 amino acids and 12 amino acids in the loop 3 C3 and loop 4 C3 mutant proteins, respectively.

Plasmids containing the cloned Hib porin gene (pEJH39-1-35, Table 1) or containing deletions and insertions in Hib *ompP2* (pRS03 to pRS08, Table 1) were used to transform the porin deletion strain RSFA21. Proteins expressed in the transformants (RS01 and RS03 to RS08, Table 1) were detected by SDS-PAGE of total cell lysates (data not shown). In order to determine the cellular location of the mutant proteins, outer membrane vesicles were prepared, run on polyacrylamide gels and stained with Coomassie blue. The levels of porin expression in strain RS01 (multiple copies of Hib *ompP2*; Fig. 4, lane 5) were two-fold greater compared to wild-type strains DL42 and KW20 (single copy of *ompP2*; Fig. 4, lanes 2 and 3). The levels of expression of mutant porins in strains RS05, RS06, RS07, RS08, RS03, and RS04 (Fig. 4, lanes 6 to 11) were the same as porin expression in strain RS01.

By SDS-PAGE, porin from Hib strain DL42 migrated more slowly than porin from Hi strain KW20 (Fig. 4, lanes 2 and 3). Deletions in loop 3 (Fig. 4, lanes 6 and 7) or C3 epitope insertion in loop 3 of Hib porin (Fig. 4, lane 10) altered the mobility of the resulting mutant porins when compared with the migration of wild-type Hib porin. Deletions in loop 4 (Fig. 4, lanes 8 and 9) or C3 insertion in loop 4 (Fig. 4, lane 11) did not alter the mobility of the resulting mutant porins when

compared with the wild-type Hib porin.

Immunoblotting of mutant porins. Three anti-Hib porin mAbs (POR.1, POR.4, and POR.6; Srikumar *et al.*, 1992a) and a polyclonal antibody against the C3 epitope of poliovirus (928; Moeck *et al.*, 1994) were used to analyze mutant proteins. Whereas all three anti-Hib porin mAbs reacted with porin from DL42, only POR.1 reacted with porin from KW20 (Fig. 5a, lane 3). In the porins from strains DL42 and KW20, the amino acid sequences in the region corresponding to the epitope (Srikumar *et al.*, 1992b) recognized by POR.1 are conserved. MAb POR.1 did not react with mutant Hib porins from strains RS05 and RS06 (Fig. 5a, lanes 6 and 7) and mAb POR.4 did not react with mutant Hib porins from strains RS07 and RS08 (Fig. 5b, lanes 8 and 9). Genetic deletions of sequences coding for the epitopes recognized by mAbs POR.1 and POR.4 therefore abolished the reactivities of these mAbs to the corresponding mutant porins. Because sequences coding for the epitope recognized by mAb POR.6 were present in the genetic constructs pRS05, pRS06, pRS07, pRS08, pRS03, and pRS04, mAb POR.6 reacted with all the mutant Hib porins (Fig. 5c, lanes 6 to 11). Anti-C3 polyclonal antibodies reacted only with mutant Hib porins from strains RS03 and RS04 (Fig. 5d, lanes 10 and 11). Only the proteins from the mutant strains expressing Hib porins with C3 epitope insertions showed reactivity to the anti-C3 antibodies.

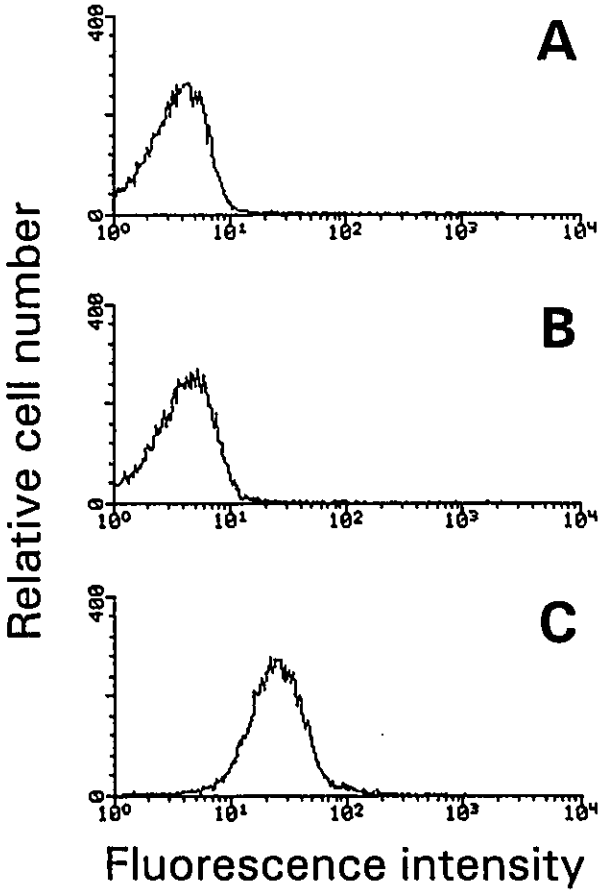
Flow cytometric analyses. Anti-Hib porin mAb POR.6,

previously shown to bind to a surface-exposed epitope in Hib porin (Srikumar *et al.*, 1992a), was used to stain intact bacterial cells of strains expressing the mutant porins. MAb POR.6 recognized all the mutant strains (RS03 to RS08) at the cell surface (data not shown) confirming the outer membrane localization of all mutant proteins. When anti-C3 polyclonal antibodies were used, the C3 epitope in the mutant Hib porin expressed in strain RS04 was detected by flow cytometry (Fig. 7c) but not the C3 epitope in the mutant Hib porin expressed in strain RS03 (Fig. 7b).

Growth properties of strains expressing mutant Hib porins. Haemophilus strains were grown on chocolate plates for 16 h, 37 °C. Single colonies of strains DL42, KW20, RS01, RS03, RS04, RS07, and RS08 grew well and to the same colony sizes. Growth of strain RS05 was slightly retarded when compared to the other strains; its colony size was smaller. Growth of RS06 on chocolate plates was severely affected: after 16 h at 37 °C, this strain grew as pinpoint colonies. This pattern of growth exhibited by RS06 was identical to the growth pattern exhibited by RSFA21 and porin-minus Hib strain DL42/2F4$^-$ (data not shown).

When Haemophilus strains were grown in sBHI liquid medium, strains DL42, KW20, RS01, RS03, RS04, RS07, and RS08 exhibited similar growth characteristics; they all grew to saturation ($A_{650} \approx 1$) after 6 h at 37 °C. Strain RS05 grew slightly slower than the above-mentioned strains but, upon

Fig. 7. Flow cytometry of Hib. Bacteria were reacted with rabbit anti-C3 polyclonal antibodies followed by anti-rabbit IgG (heavy and light chains) conjugated to fluorescein. Three strains were analyzed. Panels a, b, and c: RS01, RS03, and RS04, respectively.


A
B
C
400
0
10^0
10^1
10^2
10^3
10^4
Relative cell number
Fluorescence intensity

further incubation, it reached the same level of saturation. Strain RS06 grew more slowly and never reached the same level of saturation as the other strains. The growth characteristics of RS06 in sBHI liquid medium were again similar to that of RSFA21 and the porin-minus Hib strain DL42/2F4$^-$ (data not shown). Compared to the other mutations in Hib porin, deletion of the 6 amino acids in loop 3 of Hib porin caused only a slight change in the growth property of strain RS05; deletion of 12 amino acids in loop 3 of Hib porin resulted in a drastic change in the growth phenotype of strain RS06. This defect in growth exhibited by strains RSFA21, DL42/2F4$^-$, and RS06 was partly compensated by growing these strains in medium containing 10 mM magnesium sulfate.

Sensitivities to anti-microbial agents. Wild-type and mutant Haemophilus strains were tested for their sensitivities to ten different anti-microbial agents (Table 2). Except for rifampicin, there were no significant differences in sensitivities to the anti-microbial agents when the wild-type Hib strain DL42 was compared to the wild-type Hi strain KW20; these assays did not discriminate between wild-type porins from Hi or Hib. When RSFA21 was compared to the wild-type strains, there were no pronounced differences in sensitivities to the anti-microbial agents. The presence or absence of porin in the outer membrane of this bacterium did not change the sensitivities to these anti-microbial agents. There were no significant differences in the sensitivities to anti-

TABLE 2. Sensitivities to anti-microbial agents

Strain	Porin characteristic†	Diameter* of zone of growth inhibition (mm)									
		Anti-microbial agent‡									
		C	P	N	S	B	E	R	SDS	SDC	CTB
DL42	W.t. Hib porin	10	13	10	11	12	11	8	11	10	11
KW20	W.t. Hi porin	11	13	10	10	12	11	10	12	10	11
RSFA21	Hi Δ porin	11	11	10	11	14	12	11	13	10	11
RS01	Cloned Hib porin	11	12	9	10	12	12	10	12	10	11
RS05	Loop 3 Δ6aa	11	13	10	12	16	16	14	16	12	11
RS06	Loop 3 Δ12aa	13	15	11	15	22	19	17	17	15	12
RS07	Loop 4 Δ6aa	11	12	9	10	12	12	10	12	10	11
RS08	Loop 4 Δ12aa	11	12	9	10	12	12	10	12	10	11
RS03	Loop 3 C3	11	12	9	10	13	14	12	12	10	11
RS04	Loop 4 C3	10	12	9	10	12	12	11	12	10	11

*Assays were performed as described in Materials and Methods. Each value for the diameter of zone of growth inhibition is an average of values from three separate experiments. The standard deviation in these instances were between 0 and ±2.

†As described in the legend for Fig. 4.

‡The anti-microbial agents, their molecular masses, and the amounts used per experiment are the following: C: chloramphenicol (M_r 323, 0.6 μg); P: penicillin (M_r 372, 0.2 μg); N: neomycin (M_r 614, 10 μg); S: streptomycin (M_r 582, 2 μg); B: bacitracin (M_r 1411, 1500 μg); E: erythromycin (M_r 734, 3 μg); R: rifampicin (M_r 823, 1 μg); SDS: sodium dodecylsulfate (M_r 288, 45 μg); SDC: sodium desoxycholate (M_r 415, 750 μg); and CTB: hexadecyl trimethyl ammoniumbromide (M_r 365, 500 μg).

microbial agents between strain DL42 (single copy of Hib *ompP2*) and strain RS01 (multiple copies of Hib *ompP2*). The two-fold increase in Hib porin levels observed in strain RS01 compared to wild-type strain DL42 did not contribute to any changes in sensitivities to these anti-microbial agents. The sensitivities of the strains, RS03, RS04, RS07, and RS08, to the anti-microbial agents were similar to those of strains DL42, KW20, and RS01. The sensitivities of strain RS05 to several of the anti-microbial agents (bacitracin, erythromycin, rifampicin, and SDS) were significantly greater than the sensitivities of strains DL42, KW20, and RS01 to these agents. The sensitivities of the strain RS06 to bacitracin, erythromycin, rifampicin, SDS, and sodium desoxycholate were even greater than the sensitivities of RS05 to these agents. Also, the sensitivities of RS06 to the other anti-microbial agents were greater than the sensitivities of strains DL42, KW20, and RS01 to these agents. Taken together, these data indicate that deletions in loop 3 of Hib porin results in increased sensitivities to selected anti-microbial agents when compared to wild-type Hib porin. Deletions in loop 4 of Hib porin showed no difference in sensitivities to the anti-microbial agents when compared to wild-type Hib porin.

5. Discussion

Our studies were designed to create genetically engineered Hib porins that display altered pore properties. One pre-requisite for such studies was a Haemophilus strain which no longer expressed wild-type porin. DL42/2F4⁻ is a porin-minus Hib strain that was previously characterized by Cope *et al*. Its chromosomal sequences coding for porin were inactivated by insertion of an out-of-frame *Eco*RI linker. However, this mutation in DL42/2F4⁻ allowed revertants to be isolated by their rapid growth on chocolate plates. By SDS-PAGE and staining with Coomassie blue, the expression of a major outer membrane protein in these revertants was demonstrated. Immunoblotting showed that this major outer membrane protein was related to but not identical to wild-type Hib porin (data not shown). Deletions across the *Eco*RI site of *ompP2* in DL42/2F4⁻ restored the reading frame of the coding sequences and resulted in re-expression of porin. To create a stable mutation in *ompP2* that permanently abolished porin expression, we removed 98 % of the coding sequences for porin from the chromosome of Hi strain KW20. Such a strain was ideal for the expression and characterization of mutant Hib porins.

The *ompP2* sequences of several Hi strains (Duim *et al*., 1994; Sikkema & Murphy, 1992) were compared to the *ompP2* sequence of Hib (Hansen *et al*., 1989a; Munson & Tolan, 1989).

Approximately 75-85 % homology at the DNA level was observed. Since the kanamycin resistance cassette in pRS21 was flanked by sequences upstream and downstream of Hib *ompP2*, the genetic relatedness between Hi and Hib was sufficient to give rise to porin deletion strains in Hi by homologous recombination.

Studies that compared the *ompP2* sequences of several Hi strains (Duim *et al.*, 1994; Sikkema & Murphy, 1992) and Hib strains (Munson *et al.*, 1989) showed that sequences coding for the amino acids in loop 4 and loop 8 were among the most variable. However, sequences coding for amino acids in loop 3 were conserved. This accounts for the differences in reactivities of the anti-Hib porin mAbs POR.1, POR.4, and POR.6 to the porins from Hi and Hib.

Using the same protocol as described for the construction of RSFA21, 98 % of the coding sequences for porin were deleted in the chromosome of Hib strain DL42 (data not shown). Such a porin deletion strain in Hib was designated RSFAb21. The strain RSFA21 was chosen over the strain RSFAb21 for expression and characterization of mutant Hib porins because Hi strains were more amenable to genetic manipulation than Hib strains and strain RSFA21 grew better than strains DL42/2F4⁻ and RSFAb21.

All the mutant proteins were localized to the outer membrane. Deletions or C3 epitope insertions in the selected regions of Hib porin did not affect the processing and outer membrane assembly of the resulting mutant proteins. The

accommodation of an additional 13 amino acids (C3 epitope plus Ser and Thr) in the region between residues 115-133 in Hib porin supports our proposal that this region in Hib porin forms a loop. By SDS-PAGE, deletions and the C3 insertion in loop 3 produced some changes in mobilities of these mutant proteins as compared to wild-type Hib porin. Similar mutations in loop 4 did not result in any structural alterations when compared to wild-type Hib porin. We are not adequately able to explain these differences.

By flow cytometry, the C3 epitope inserted in loop 4 of Hib porin was surface-exposed, a result that matched our earlier work (Srikumar *et al.*, 1992a; Srikumar *et al.*, 1992b). However, the C3 epitope inserted in the proposed loop 3 of Hib porin was not surface-exposed. Such a result further strengthens our claim that loop 3 folds back into the β barrel formed by Hib porin (Srikumar *et al.*, 1992b; Srikumar *et al.*, 1993), and is therefore inaccessible to antibodies.

The role of major outer membrane proteins, including porins, in outer membrane stability and bacterial growth has been demonstrated by others (Hancock *et al.*, 1994). The outer membrane was stabilized by the interaction of proteins with the underlying peptidoglycan (Hancock *et al.*, 1994). Compared to the other strains, RSFA21 and RS06 displayed retarded growth characteristics. In both strains growth was enhanced by the addition of 10 mM magnesium sulfate. The integrity of the outer membrane of RSFA21 may be compromised by the absence

of the major protein, porin. These results suggest a role for Hib porin in the maintenance of outer membrane integrity, perhaps by interaction of porin directly or indirectly with the peptidoglycan. Because RS06 showed similar growth phenotypes as RSFA21, one or more of the amino acid residues 115-128 of Hib porin may be important for the stability of the outer membrane.

To correct for differences in growth of strains for the anti-microbial sensitivity assays, it was necessary to adjust the amounts of cells initially added to the sBHI plates. After over-night incubation of plates at 37 °C, it was desirable to obtain even levels of confluence for all strains. Compared to the other strains, the use of four-fold excess of cells for strains RSFA21 and RS06 was required to achieve comparable levels of cell density the following day. Compared to others, strains expressing the mutant Hib porins, loop 3, 6 amino acid deletion and loop 3, 12 amino acid deletion, showed increased sensitivities to several anti-microbial agents. We propose that the deletions in loop 3 made the channel larger, resulting in an increased influx of antibiotics into the periplasmic space. This interpretation is supported by other studies that characterized pore function of outer membrane proteins (Benson *et al.*, 1988; Capobianco & Goldman, 1994; Klebba *et al.*, 1994; Misra & Benson, 1988a; Rutz *et al.*, 1992). Deletion of 12 amino acids in loop 3 of Hib porin showed more pronounced sensitivities than deletion

of 6 amino acids in loop 3 of Hib porin. Deletion of 12 amino acids may have further increased the pore size as compared to deletion of 6 amino acids. Alternatively, the more pronounced phenotypes exhibited by RS06 may be the outcome of a combination of characteristics namely a larger pore size and an unstable outer membrane. Compared to deletions in loop 3, deletions in loop 4 did not show any difference in phenotypes when compared to wild-type Hib porin. Deletion of amino acid sequences in the surface-exposed loop 4 did not alter the pore sizes of these mutant Hib porins. Unexpectedly, the porin deletion strain RSFA21 displayed similar levels of sensitivities to the anti-microbial agents as compared to the wild-type strains. Such a result suggests that porin is not the unique route of entry for these anti-microbial agents. As these assays are not useful in detecting mutant porins that give rise to smaller pores, it was not surprising that strains expressing the C3 epitope insertion in loop 3 of Hib porin showed the same phenotypes as wild-type strains. Whether the C3 epitope inserted into loop 3 of Hib porin produces a further constriction of the pore remains to be examined.

By the disc assays, the smaller, hydrophilic antibiotics did not show significant differences in sensitivities among strains. The use of more-sensitive techniques may allow for their discrimination. Since the growth properties of these strains are different, comparisons of the minimum inhibitory concentrations (MICs) for the anti-microbial agents is not

possible. Bacitracin is a large, hydrophilic antibiotic. Erythromycin and rifampicin are larger and more hydrophobic than the other antibiotics (except bacitracin) used in this study. Bacitracin, erythromycin, and rifampicin showed significant changes in their growth inhibition among strains. Anionic detergents SDS and sodium desoxycholate also showed significant differences in their levels of growth inhibition. Hib porin forms a channel that is relatively large compared to *E. coli* OmpF and OmpC, and it is the most abundant outer membrane protein. Differences in levels of growth inhibition in strains expressing larger pores than that formed by wild-type Hib porin can be expected with larger antibiotics as opposed to smaller ones. The 12 amino acid deletion in loop 3 that removed several charged residues (three negative charges and one positive charge) may have made the channel less hydrophilic. Such a channel would enhance the uptake of hydrophobic antibiotics and detergents. However, the cationic detergent hexadecyl trimethyl ammoniumbromide (CTB) did not show any change in its level of growth inhibition. Perhaps, the uptake of the positively-charged CTB is not enhanced through these mutant channels.

Because our model for the topology of Hib porin is consistent with the emerging structural consensus for porins as derived from high resolution X-ray analysis, a sequence alignment was made for Hib porin *versus* *E. coli* OmpF (unpublished results, Coulton, J., Diederichs, K., Srikumar,

R. & Welte, W.). Identities were found for 59 amino acids and they were evenly distributed over the entire lengths of the two proteins. Superposition of the two sequences identified significant differences in the lengths of loop 3; loop 3 of Hib porin was much smaller than that of OmpF. This observation is in correspondence with the deduced molecular mass exclusion limits for Hib porin (1400 Da) and OmpF (600 Da). Our model predicts that deletions of 6 amino acids and 12 amino acids in loop 3 should increase the molecular mass exclusion limit of Hib porin to some value greater than 1400 Da. Further analyses of purified mutant Hib porins will provide detailed information about the structural context of loop 3 in defining the molecular mass exclusion limit of Hib porin.

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CHAPTER 6

GENERAL CONCLUSIONS AND OPPORTUNITIES FOR RESEARCH

We proposed (Chapter 3) a model for the folding of *Haemophilus influenzae* type b (Hib) porin in the outer membrane. Even though the amino acid sequence of Hib porin showed little homology to any of the known porins of Gram-negative bacteria, our model of Hib porin was in agreement with the emerging consensus for the channel-forming motif of porins. We generated a panel of monoclonal antibodies (mAbs) as probes of Hib porin topology (Chapter 2). These anti-Hib mAbs provided substantive data which confirmed the computer-assisted predictions of Hib porin's secondary structure. Loop 4 and loop 8 in the Hib porin model were shown directly to be surface-exposed (Chapter 3).

The prediction for the folding of Hib porin (Jeanteur *et al.*, 1994), which was based on the alignment of several porin sequences (Chapter 1), was in agreement with our model for 15 of the 16 β-strands. Only β-strand 5 was not in concurrence in both studies. In the original report of the modelling of Hib porin (Chapter 3), β-strand 5 was predicted with the least confidence and its assignment was the most difficult. Perhaps β-strand 5 is not properly located in our model and is more N-terminal as is suggested by the sequence alignment data. This would imply that loop 3 is longer than what was originally our

prediction. Construction of new deletion mutations in Hib porin that remove amino acid sequences more N-terminal than the loop 3 deletions (Chapter 5) may aid in the proper assignment of boundaries for β-strand 5 and loop 3 in Hib porin.

Other studies on nontypeable *Haemophilus influenzae* (Hi) porin, which shares approximately 80% identity with Hib porin, have confirmed our model for the folding of Hib porin. Based on the Hib porin model, the topological organization of Hi porin was predicted (Sikkema & Murphy, 1992). Using mAbs, the surface exposure of loop 5 and loop 8 in Hi porin was directly demonstrated (Haase *et al.*, 1994). The comparison of several heterogenic porin sequences from Hi strains (Duim *et al.*, 1994; Sikkema & Murphy, 1992) and Hib strains (Munson *et al.*, 1989) showed that regions corresponding to amino acid-hypervariability occurred primarily in the proposed loop 1, loop 2, loop 4, loop 5, and loop 8 of the consensus model for the folding of Haemophilus porins.

Upon injection of purified Hib porin or recombinant Hib porin, the antibody response was predominantly against non-native epitopes of Hib porin (Chapter 4). Such a result may be due to the unstable nature of this outer membrane protein when isolated from its membrane environment. The inability of these anti-Hib porin antibodies to protect against infection by this bacterium was therefore not surprising.

Antibodies against Hi porin and Hib porin that recognized

surface-exposed epitopes have been reported by others. Some of these antibodies were bactericidal *in vitro* and others were protective in animals. However, in all cases the antibodies were immunologically active only against the homologous strain and not against heterologous strains of the same serotype (Haase *et al.*, 1994; Munson *et al.*, 1983; van Alphen *et al.*, 1991). The hypervariability of the surface-exposed loops of Hi and Hib porins generates strain specific antibodies. Taken together, these observations do not lend support for the candidacy of Hib porin in vaccine development.

To ascertain if loop 3 in Hib porin played the same role as in other well characterized bacterial porins, we undertook site-directed mutagenesis of cloned Hib porin gene (Chapter 5). Strains expressing mutant Hib porins with deletions in loop 3 were more sensitive to anti-microbial agents than strains expressing wild-type Hib porin. Such experiments provided support to our hypothesis that loop 3 in Hib porin is involved in narrowing of the channel, analogous to other bacterial porins. The anti-microbial sensitivities of the Hib porin deletion mutant was similar to the wild-type strain. Other studies that compared the sensitivities of wild-type strains and porin deletion strains to anti-microbial agents have reported similar results: significant differences in sensitivities to a particular antibiotic was evident only when an inactivation system for that antibiotic was present in these strains (Nikaido, 1989). Nevertheless, to prove

unambiguously that the deletions in loop 3 of Hib porin produce larger channels, these mutant porins will be purified and tested for channel formation by *in vitro* techniques such as solute efflux from proteoliposomes, liposome swelling assay, and black lipid membranes (Chapter 1).

It is also important to demonstrate that, compared to wild-type Hib porin, the secondary structures of these mutant porins have not been altered. Our hypothesis regarding the role of loop 3 in Hib porin predicts that deletions in loop 3 should not modify the β-barrel motif of these mutant porins. Spectroscopic studies (circular dichroism and fourier transform infrared spectroscopy) of purified mutant Hib porins will be undertaken and the spectra will be compared to those of purified Hib porin.

We have constructed a system in which to express cloned Hib porin or mutant forms of it in a homologous but porin-free background (Chapter 5). Such a system could be used in the future for the expression and characterization of novel mutants of Hib porin. Because the growth of Haemophilus strains are drastically affected in the absence of their characterized porins (Chapter 5), it would be interesting to identify other proteins that could restore the growth phenotype of these strains. Candidates from the outer membrane of the homologous organism as well as from the outer membrane of heterologous organisms could be tested. These experiments are predicted to provide new insights into the

role of Hib porin in the outer membrane of this bacterium.

Several questions with respect to the structure and function of Hib porin remain unanswered. These questions relate to the differences of Hib porin when compared with the other well characterized porins. Why are the trimers of Hib porin not as stable as *E. coli* OmpF or the *Rhodobacter capsulatus* porin? Why is the exclusion limit and channel size of Hib porin greater than that of OmpF and *Rhodobacter capsulatus* porin? Is loop 3 shorter in Hib porin compared to the others? Can the length of loop 3 alone determine the channel size of Hib porin or are specific amino acid interactions important? As seen in the specific channel LamB of *E. coli*, are other loops involved in the formation of the eyelet of Hib porin? Some of these questions can be resolved by site-directed mutagenesis of Hib porin as described above, but others have to await the structural determination to high resolution of this protein.

The purification scheme described for Hib porin (Chapter 2) has been successfully amplified to generate large amounts of purified protein. The availability of large quantities of purified Hib porin has led naturally to our collaboration on the structural biology of membrane proteins. One of our objectives is the determination of the molecular architecture of Hib porin using X-ray crystallography. In collaboration with another research group, optimal conditions for crystal growth of Hib porin were recently established. Ultimately,

the high resolution structure of this protein will allow for some critical evaluation of our predictions of Hib porin structure and function, and will direct us to test structural determinants which relate to the organization of membrane proteins.

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