THE DEVELOPMENT OF THE *PSEUDOMONAS AERUGINOSA* OUTER MEMBRANE PROTEIN OPRF AS A PRESENTATION VECTOR FOR FOREIGN ANTIGENIC DETERMINANTS

by

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ABSTRACT

A variety of systems have been developed to improve the presentation of foreign antigenic determinants ('epitopes') by inserting them in the context of carrier proteins. The goals of this study were to develop the *Pseudomonas aeruginosa* outer membrane protein OprF as a carrier for foreign epitopes and to study the effect of the mode of presentation on the antigenicity of the presented epitope. The model epitope used in this study was the 4-amino acid repeating epitope (NANP) of the circumsporozoite protein of the malaria parasite, *Plasmodium falciparum*. Linker-insertion mutagenesis was carried out to create 11 "permissive" sites which allowed the insertion of 4 extra amino acids. Two series of OprF::malarial epitope hybrid proteins, the positional hybrids and the multiple-repeat hybrids, were constructed by inserting oligonucleotides encoding the epitope into the linker-insertion sites of *oprF*. The effects of the insertion position and the length of the epitope on its antigenicity were studied by ELISA using outer membranes and by whole cell dot blot analysis. It was shown that the antigenicity of the epitope varied when inserted at different positions of OprF, while it increased with the length of the epitope at two of the three insertion positions studied. These data were employed to revise the membrane topology model of OprF and have improved our understanding of the epitopes recognized by the OprF-specific monoclonal antibodies. Generalizations about the influence of surrounding amino acids on the antigenicity of the inserted epitope are proposed. A targeted study of

immunogenicity showed that a 19-amino acid malarial epitope was significantly more immunogenic than a 7-amino acid epitope when inserted at an N-terminal insertion site of OprF. A parallel immunogenicity study of two versions of glutathione *S*-transferase (GST)::malarial epitope fusion proteins demonstrated that neither an 11- nor a 19- amino acid epitope fused to the C-terminus of GST was immunogenic. This study demonstrated for the first time that OprF can be used as a carrier to generate and detect anti-epitope antibodies in immunized animals and in immunoassays respectively.

TABLE OF CONTENTS

ABSTRACT ii

TABLE OF CONTENTS iv

LIST OF FIGURES x

LIST OF TABLES xiii

LIST OF ABBREVIATIONS xv

ACKNOWLEDGEMENTS xvii

INTRODUCTION 1

1. Epitope presentation systems 1

    1.1 Introduction 1

    1.2 *E. coli* outer membrane protein presentation systems 1

    1.3 Other presentation systems 5

2. Applications of epitope presentation systems 7

    2.1 Vaccines 7

    2.2 Immunopurification 8

    2.3 Detection and production of anti-peptide antibodies 9

    2.4 Construction of random libraries 9

3. The carrier protein: OprF 10

    3.1 Bacterial outer membrane 10

    3.2 General structures of porins 11

    3.3 OprF 12

4. The model epitope: the malarial epitope 17

4.1 Life cycle of the malaria causative agent: 17

Plasmodium falciparum 17

4.2 The tetrapeptide repeating epitope NANP 17

5. Factors affecting antigenicity and immunogenicity 20

5.1 Factors affecting antigenicity 20

5.2 Factors affecting immunogenicity 22

6. Aims of this study 24

METHODS AND MATERIALS 25

1. Bacterial strains, plasmids and media 25

2. General recombinant DNA techniques 25

3. General protein and immunological techniques 28

3.1 SDS-PAGE and immunoblottings 28

3.2 Antibodies 29

3.3 Indirect immunofluorescence labelling 29

3.4 Trypsin sensitivity assays 31

3.5 Protein assays 32

4. Construction of pRW3 32

5. Linker-insertion mutagenesis of *oprF* 32

5.1 Mutagenesis with kanamycin resistance cassette 32

5.2 Mutagenesis at the *SalI* site 36

5.3 Determination of linker-insertion sites 36

6. Construction of OprF::malarial epitope hybrid proteins 36

6.1 Positional hybrids 36

6.2 Multiple-repeat hybrids 39

7. DNA sequencing 40

8. Construction of glutathione *S*-transferase (GST)::malarial epitope fusion proteins 42

9. Isolation of outer membranes 42

9.1 Triton X-100 extraction 42

9.2 Sucrose gradient centrifugation 42

9.3 Removal of inclusion bodies 44

10. Expression of *oprF* and *oprF* derivatives in *E. coli* 45

10.1 Expression of *oprF* in different *E. coli* strains 45

10.2 Expression of an *oprF* derivative in different induction conditions 45

11. Protein purification 46

11.1 OprF::malarial epitope hybrid proteins 46

11.2 GST::malarial epitope fusion proteins 46

11.3 Extraction from SDS-polyacrylamide gel 47

12. Antigenicity studies 47

12.1 Outer membrane ELISA 47

12.2 Whole cell dot blot analysis 49

12.3 Statistical analyses 49

13. Immunization studies 50

13.1 Immunization with OprF::ME10aa215 and OprF 50

13.2 Immunization with OprF::MEaa26 multiple-repeat hybrids and GST::malarial epitope fusion proteins 50

13.3 Determination of antibody titers 50

13.4 Characterization of antisera by Western immunoblot analysis.. 51

RESULTS

Chapter one: Construction and characterization of OprF linker mutants.. 53

1.1 Introduction 53

1.2 Expression of *oprF* in *E. coli* 54

1.2.1 Construction of pRW3 54

1.2.2 Expression of *oprF* in different *E. coli* host strains.. 55

1.3 Semi-random linker mutagenesis with a kanamycin cassette... 58

1.4 Site-directed mutagenesis at the *Sal*I site 59

1.5 Determination of insertion sites 59

1.6 Expression and cellular localization of linker mutants 61

1.7 Monoclonal antibody reactivities of linker mutants 67

1.8 Membrane configuration of linker mutants in *E. coli* 69

1.8.1 Trypsin sensitivity assays 69

1.8.2 Immunofluorescence labelling 73

1.9 Summary 75

Chapter two: Construction, characterization and purification of OprF:: malarial epitope and GST::malarial epitope hybrid proteins. 77

2.1 Introduction..... 77

2.2 Construction of OprF::malarial epitope hybrid proteins..... 78

2.2.1 Positional hybrids..... 78

2.2.2 Multiple-repeat hybrids..... 80

2.3 Characterization of OprF::malarial epitope hybrid proteins..... 82

2.3.1 Expression of hybrid proteins..... 82

2.3.2 Cellular localization of hybrid proteins..... 84

2.3.3 Surface exposure of the epitope..... 88

2.3.4 Monoclonal antibody reactivity of hybrid proteins..... 91

2.4 Purification of OprF::malarial epitope hybrid proteins..... 93

2.4.1 Induction experiments..... 93

2.4.2 Detergent extractions..... 95

2.4.3 FPLC purification..... 98

2.4.4 Purification of inclusion body-contaminated outer membrane preparations..... 98

2.5 GST::malarial epitope fusion proteins..... 101

2.5.1 Construction and purification of fusion proteins..... 101

2.5.2 Binding of fusion proteins with epitope-specific monoclonal antibodies..... 103

2.6 Summary..... 103

Chapter three: Study of the effect of mode of presentation on antigenicity and immunogenicity 106

3.1 Introduction 106

3.2 Antigenicity study 107

3.2.1 Approaches 107

3.2.2 Position effect 108

3.2.3 Length effect 113

3.3 Immunogenicity study 119

3.3.1 Immunogenicity of OprF::ME10aa215 121

3.3.2 Immunogenicity of OprF::ME7aa26 and OprF::ME19aa26 124

3.3.3 Immunogenicity of GST::ME11 and GST::ME19 128

3.4 Summary 131

DISCUSSION 134

General 134

Linker-insertion mutagenesis 135

Effects of amino acid insertions in OprF 136

Membrane topology of OprF 140

Binding epitopes of OprF-specific monoclonal antibodies 144

Antigenicity and mode of presentation 147

Immunogenicity 151

REFERENCES 159

LIST OF FIGURES

Figure 1. Schematic diagram of the β-barrel structure of a porin..... 13

Figure 2. Proposed membrane topolopy model of OprF..... 15

Figure 3. The life cycle of *Plasmodium falciparum*..... 18

Figure 4. Stereo drawings of two of the predicted structures of the $(NANP)_6$ peptide..... 21

Figure 5. Construction of pRW3..... 33

Figure 6. Schematic representation of semi-random linker-mutagenesis with a kanamycin resistance cassette..... 34

Figure 7. Nucleotide and encoded amino acid sequences of the oligonucleotides used for the construction of OprF::malarial epitope hybrid protein..... 37

Figure 8. Construction of GST::malarial epitope fusion proteins..... 43

Figure 9. Expression of *oprF* in different *E. coli* host strains..... 56

Figure 10. Restriction mapping of linker-insertion sites..... 60

Figure 11. Cellular localization of OprF linker mutants..... 65

Figure 12. Expression of OprF linker mutants..... 66

Figure 13. Trypsin sensitivity of linker mutants in outer membranes..... 71

Figure 14. Expression of OprF::malarial epitope positional hybrids..... 83

Figure 15. Cellular localization of OprF::malarial epitope positional hybrids..... 85

Figure 16. Expression of OprF::malarial epitope multiple-repeat hybrids.. 87

Figure 17. Presence of inclusion bodies in outer membrane samples..... 89

Figure 18. Surface exposure of the malarial epitope..... 90

Figure 19. Western immunoblots of OprF::malarial epitope multiple-repeat hybrids..... 94

Figure 20. Expression of an *oprF* derivative in different induction conditions..... 96

Figure 21. Purification of OprF::malarial epitope hybrid proteins..... 97

Figure 22. FPLC profile of a MonoQ column separation of the octyl-POE/EDTA soluble OprF hybrid expressed by pRW307.1M..... 99

Figure 23. Removal of inclusion bodies from outer membrane preparations by octyl-POE extraction..... 100

Figure 24. Purification of GST::malarial epitope fusion proteins..... 102

Figure 25. Binding of GST::malarial epitope fusion proteins with epitope-specific monoclonal antibodies..... 104

Figure 26. Binding of an OprF-specific polyclonal serum and the malarial epitope-specific mAb pf2A.10 with OprF and OprF::malarial epitope hybrid..... 109

Figure 27. Effect of insertion position on the antigenicity of the malarial epitope..... 111

Figure 28. Effect of insertion of multiple copies of the malarial epitope on antigenicity at insertion sites aa^{26} and aa^{213} of OprF..... 114

Figure 29. Effect of the length of the epitope on its antigenicity at insertion site aa^{26} of OprF 116

Figure 30. Effect of the length of the epitope on its antigenicity at insertion site aa^{196} of OprF 117

Figure 31. Effect of the length of the epitope on its antigenicity at insertion site aa^{213} of OprF 118

Figure 32. ELISA titrations of anti-OprF and anti-malarial epitope responses induced in BALB/c mice immunized with OprF and OprF::ME10aa215 by ELISA 122

Figure 33. ELISA titrations of anti-OprF and anti-malarial epitope responses induced in C57BL/6J mice immunized with OprF::ME7aa26 and OprF::ME19aa26 by ELISA 125

Figure 34. Western immunoblot analysis of the sera from mice immunized with OprF::ME7aa26 and OprF::ME19aa26 127

Figure 35. ELISA titrations of anti-GST and anti-malarial epitope responses induced in C57BL/6J mice immunized with GST::ME11 and GST::ME19 129

Figure 36. Western immunoblot analysis of the sera from mice immunized with GST::ME11 and GST::ME19 130

Figure 37. Proposed membrane topology model of OprF 143

LIST OF TABLES

Table I.	Examples of epitope presentation systems.....	2
Table II.	Bacterial strains and plasmids.....	26
Table III.	OprF epitopes recognized by monoclonal antibodies.....	30
Table IV.	Summary of insertion sites of 11 linker-insertion mutants and one site-directed insertion mutant.....	62
Table V.	Summary of six of the deletion mutants isolated during linker-insertion mutagenesis.....	63
Table VI.	Summary of monoclonal antibody reactivity of linker mutants.	68
Table VII.	Summary of trypsin sensitivity assays of linker mutants in *E. coli* outer membranes, DH5α and C386 whole cells.....	72
Table VIII.	Results from indirect immunofluorescence labelling of *E. coli* C386 cells expressing OprF linker mutants.....	74
Table IX.	Summary of OprF::malarial epitope positional hybrids.....	79
Table X.	Summary of OprF::malarial epitope multiple-repeat hybrids..	81
Table XI.	Summary of monoclonal antibody reactivity of OprF::malarial epitope positional hybrids.....	92
Table XII.	Summary of antigenicity of the malarial epitope in OprF::malarial epitope positional hybrids.....	112
Table XIII.	Summary of antigenicity of the malarial epitope in OprF::malarial epitope multiple-repeat hybrids.....	120

Table XIV. Summary of antibody responses induced in mice immunized with wild type OprF or OprF::ME10aa215 123

Table XV. Summary of antibody responses induced in mice immunized with OprF::ME7aa26 and OprF::ME19aa26 126

Table XVI. Summary of antibody responses induced in mice immunized with GST::ME11 and GST::ME19 132

Table XVII. Predicted primary and secondary structures at the insertion sites 149

LIST OF ABBREVIATIONS

A	optical density
aa^n	amino acid position 'n'
amp	ampicillin
bp	base pair
BSA	bovine serum albumin
CSP	circumsporozoite protein
DEAE	diethylaminoethyl
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FCS	fetal calf serum
FMDV	foot-and-mouth disease virus
FPLC	fast protein liquid chromatography
GST	glutathione *S*-transferase
h	hour(s)
HIV-1	human immunodeficiency virus-1
IgG	immunoglobulin G
IPTG	isopropyl thio-β-D-galactopyranoside
kb	kilobase pair
kDa	kilodalton
Km	kanamycin

LPS	lipopolysaccharides
MSP	merozoite surface protein
mAb	monoclonal antibody
min	minute(s)
Octyl-POE	octyl-polyoxyethelene
OprF	*P. aeruginosa* major outer membrane protein F
oprF	gene encoding OprF
PBS	phosphate-buffered saline (0.14 M NaCl/2.7mM KCl/1.47 mM KH_2PO_4/20mM $NaHPO_4$ pH7.4)
PVDF	polyvinylidene difluoride
s	second(s)
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
TGEV	transmissible gastroenteritis coronavirus
wt	wild type

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INTRODUCTION

1. Epitope presentation systems

1.1 Introduction

Progress in molecular biology has allowed the engineering of heterologous proteins that carry components from two or more different host proteins. A variety of systems have been developed to improve the presentation of foreign antigenic determinants ('epitopes') by inserting them in the context of carrier proteins. Carrier proteins that have been utilized in these systems include bacterial outer membrane proteins, subunits of bacterial cellular appendages such as pili and flagella, bacterial secreted proteins, filamentous phage surface structural proteins, and viral surface coat proteins. Examples of these epitope presentation systems are listed in Table I. In general, the incorporation of passenger epitopes into appropriate sites in these carrier proteins does not seriously interrupt the structure and function of the carriers. As a result, the passenger epitopes are usually targeted to the same cellular compartment as the carrier proteins.

1.2 *E. coli* outer membrane protein presentation systems

It has been found that certain regions, identified as "permissive sites", of outer membrane proteins are flexible enough to accommodate extra amino acid sequences without affecting the biogenesis, folding and localization of these

Table I. Examples of epitope presentation systems

Carriers	Epitopes	References
***E. coli* outer membrane proteins**		
LamB	Hepatitis B virus pre S2 epitope	Charbit *et al.*, 1987
	Poliovirus C3 epitope	van der Werf *et al.*, 1990
	HIV-1 GP110 epitope	Charbit *et al.*, 1990
	HIV-1 V3 loop	Charbit *et al.*, 1993
	Chlamydia MOMP epitope	Hayes *et al.*, 1991
	VR1 and VR2 of class 1 OMP of *N. meningitidis*	McCarvil *et al.*, 1993
	Random peptides	Brown, 1992
PhoE	Foot-and-mouth disease virus (FMDV) VP1 epitope	Agterberg *et al.*,1990a
	Mycobacterial T-cell epitope	Janssen *et al.*, 1994a
OmpA	Malarial antigen fragments	Schorr *et al.*, 1991
	Antibody fragment	Francisco *et al.*, 1993
FhuA	Poliovirus C3 epitope	Moeck *et al.*, 1994
TraT	Poliovirus C3 epitope	Taylor *et al.*, 1990
Non-outer membrane proteins		
E. coli β-galactosidase	FMDV VP1 epitope	Broekhuijsen *et al.*, 1986
	Random peptides	Lenstra *et al.*, 1992
E. coli MalE	Poliovirus C3 epitope	Leclerc *et al.*, 1990
	HIV-1 V3 loop	Charbit *et al.*, 1993

To be continued.....

Table I. Examples of epitope presentation systems (continued)

Non-outer membrane proteins (continued)		
E. coli ClpG prepilin	TGEV spike protein S epitope	Der Vartanian *et al.*, 1994
E. coli Pap pili	IgG binding domain of protein A	Steidler *et al.*, 1993
E. coli P-fimbriae	FMDV VP1 epitope	Van Die *et al.*, 1990
E. coli Type 1 fimbriae	Hepatitis B surface antigen epitope	Hedegaard *et al.*, 1989
	FMDV VP1 epitope	Hedegaard *et al.*, 1989
	Poliovirus C3 epitope	Hedegaard *et al.*, 1989
Salmonella flagellin	Cholera toxin epitope	Newton *et al.*, 1989
	Hepatitis B surface antigen epitope	Wu *et al.*, 1989
	Influenza haemagglutinin epitope	McEwen *et al.*, 1992
Glutathione *S*-transferase	*P. aeruginosa* OprF and OprI fragments	von Specht *et al.*, 1995
	Malaria MSP fragments	Ling *et al.*, 1994
Filamentous phage coat proteins		
pIII	Malaria CSP repeating epitope	Cruz *et al.*, 1988
	Antibody variable domains	McCafferty *et al.*, 1990
	Antibody Fab fragments	Barbas *et al.*, 1991
	HIV-1 Gag p24	Tsunetsugu *et al.*, 1991
pVIII	HIV-1 p17 epitope	Minenkova *et al.*, 1993

To be continued.....

Table I. Examples of epitope presentation systems (continued)

Gram-positive system		
M6 protein (*S. pyogenes*)	E7 protein of human papillomavirus	Pozzi *et al.*, 1992
Protein A (*S. aureus*)	Malaria blood-stage antigen	Hansson *et al.*, 1992
	Streptococcal albumin binding receptor	Hansson *et al.*, 1992
Viral proteins		
Influenza A virus neuraminidase	Chloramphenicol acetyltransferase	Percy *et al.*, 1994
Hepatitis B surface antigen	HIV-1 antigenic determinant	Michel *et al.*, 1993
	Malaria CSP epitope	Rutgers *et al.*, 1988
Adenovirus hexon	Poliovirus VP1 capsid protein epitope	Crompton *et al.*, 1994
Cowpea mosaic virus	FMDV VP1 epitope	Usha *et al.*, 1993
Human rhinovirus 14	HIV-1 V3 loop	Smith *et al.*, 1994
Poliovirus VP1 protein	Human papillomavirus epitope	Jenkins *et al.*, 1990
	HIV-1 gp41 epitope	Evans *et al.*, 1989

proteins. Foreign epitopes that are inserted in the "permissive" surface-exposed loop regions of these outer membrane proteins have been shown to be detectable on the cell surface. The system using the *Escherichia coli* outer membrane protein LamB as the carrier is one of the most developed. LamB is the porin responsible for maltose uptake and the receptor for λ phage (Szmelcman and Hofnung, 1975). The poliovirus C3 epitope has been used as a passenger epitope to identify 11 permissive sites in LamB (Charbit *et al.*, 1991). Other foreign antigenic determinants such as the hepatitis B virus preS2, HIV-1 gp110 or V3 loop epitopes, or a *Chlamydia* Major Outer Membrane Protein (MOMP) epitope have also been inserted into selected permissive sites of this carrier protein (Charbit *et al.*, 1987; Charbit *et al.*, 1990; Hayes *et al.*, 1991). The phosphate-starvation-inducible porin PhoE of *E. coli*, has been used as a carrier to present the foot-and-mouth disease virus (FMDV) VP1 epitope and a *Mycobacterial* T cell epitope (Agterberg *et al.*,1991a; Janssen *et al.*, 1994a). Likewise, the outer membrane protein OmpA has been employed as a carrier to present malarial antigen fragments on the surface of a *Salmonella* vaccine strain (Schorr *et al.*, 1991). In most of these studies, the foreign epitopes in the context of the carrier proteins have been shown to be immunogenic in test animals.

1.3 Other presentation systems

Subunits of bacterial cellular appendages such as flagella and pili generally contain variable regions that allow the insertion of foreign amino acid

sequences. A number of viral epitopes have been inserted into the subunits of these appendages and are found to be incorporated into the corresponding structures. Epitopes have also been fused to the periplasmic proteins MalE and β-galactosidase, where the immunogenicity of the inserted epitopes has been reported (Leclerc *et al.*, 1990; Charbit *et al.*, 1993; Broekhuijsen *et al.*, 1986). In addition to these *E. coli* proteins, the *Salmonella* flagellin has been used to express epitopes from *Cholera* toxin, hepatitis B surface antigen and influenza haemagglutinin (Newton *et al.*, 1989; Wu *et al.*, 1989; McEwen *et al.*, 1992). The vaccine potential of an attenuated vaccine strain of *Salmonella* expressing a recombinant flagellin has been demonstrated.

A phage display system has also been developed to express foreign genetic information in the context of the bacteriophage structural surface proteins. Both the major coat protein pVIII and the minor coat protein pIII of the filamentous phage have been used to display various foreign epitopes (see Scott and Craig, 1994 for review). Furthermore, the potential of gram-positive bacterial surface proteins as carriers to present foreign epitopes on the surface of gram-positive bacteria has been investigated (Pozzi *et al.*, 1992; Hansson *et al.*, 1992). Another major category of these epitope presentation systems involves the use of viral coat proteins as carriers (Table I). A similar repertoire of foreign epitopes has been inserted into the antigenic regions of these coat proteins and the vaccine potentials of some of these systems have been studied.

The system using glutathione *S*-transferase (GST) as the carrier protein

deserves a special mention because it was also used in this study. The protein carrier was originally identified in *Schistosoma japonicum* (Smith *et al.*, 1986) and can be expressed as an active, soluble protein in *E. coli* (Smith *et al.*, 1988). This protein is a commonly used affinity tag for the purification of fusion proteins. The affinity of GST for reduced glutathione allows the purification of soluble GST fusion proteins by adsorption to glutathione beads and subsequent desorption using free reduced glutathione (Smith and Johnson, 1988). Due to the ease of purification, GST has also been used as a carrier to induce immune response against small peptides or antigenic fragments (*e.g.*, Ling *et al.*, 1994; von Specht *et al.*, 1995).

2. Applications of epitope presentation systems

2.1 Vaccines

Recombinant live bacterial vaccines consist of attenuated strains of enteric bacteria expressing heterologous peptides derived from pathogenic agents. It has been reported that when intact cells are used as immunogens, the inserted epitope must protrude sufficiently from the outer membrane to stimulate an antibody response (Leclerc *et al.*, 1991). Therefore, the insertion of the peptide within the surface-exposed loop of an outer membrane protein carrier is likely to facilitate the immunogenicity of the peptide. Moreover, the surface exposure of the inserted epitope may be advantageous because most of the strongly antigenic regions of outer membrane proteins reside in the surface-exposed loops; hence, the

location of the inserted epitope in these regions may enhance its interaction with B cells. Furthermore, the association of the peptide with surface moieties such as lipopolysaccharides (LPS) may provide an adjuvant effect to promote the immunogenicity of the peptide. The attenuated strains of *Salmonella* or *E. coli* can colonize the intestinal tract without causing infection to the host, and hence can provide a refuge for the recombinant protein so that it can persist to elicit a more lasting immune response. In these situations, the surface exposure of the inserted epitope may be beneficial for the targeting of the epitope to the gut-associated lymphoid tissues.

2.2 Immunopurification

The affinity purification of antigens or antibodies usually requires one of the ligands to be in an immobilized form. Traditionally, this involves the large scale purification of these molecules, followed by covalent linkage of the proteins to a solid-phase matrix. If the binding epitopes in these protein antigens have been identified, these epitopes can be inserted into an outer membrane protein carrier and expressed on the cell surface of bacteria. The resultant recombinant bacteria thus represent a source of readily available whole cell affinity adsorbent. The use of such a system can not only circumvent the necessity for large scale purification of the protein and the subsequent chemical linkage of the protein to a solid matrix, and hence provide a more time and cost efficient alternative for the preparation of reagents for affinity purification.

2.3 Detection and production of anti-peptide antibodies

Some diseases can be diagnosed by the presence of specific antibodies in the patient's serum. These antibodies are often directed against peptide antigens associated with the pathogens. If the peptide(s) that are reactive with these antisera are identified, then oligonucleotides encoding the peptide(s) can be genetically inserted into the DNA sequences of the carrier protein so as to express the peptide in the context of the carrier protein. Since chemically-synthesized peptides often do not bind to antibodies efficiently by themselves, this method is likely to improve the presentation of the peptide(s) for interaction with the respective antibodies. On the other hand, the peptide/carrier hybrid can also be used as an immunogen to raise anti-peptide antibodies. In this case the carrier protein is likely to provide a T cell epitope which is required for an effective antibody response, thus circumventing the need to synthesize the peptide chemically and then link it to a carrier protein. Combining these two applications, it has been demonstrated that the LamB and MalE epitope presentation systems can be used in a complementary fashion to induce and detect anti-peptide antibodies without the use of chemically synthesized peptides (Martineau *et al.*, 1991).

2.4 Construction of random libraries

Another application of epitope presentation systems is for the construction of random peptide libraries. These libraries can be used for the

identification of epitopes or 'mimotopes' (antigenic sequences that mimic epitopes) that bind to specific antibodies. Random libraries using phage pIII protein, β-galactosidase and rhinovirus as carriers have been constructed. These libraries, expressing 6- to 15- residue random peptides encoded by the inserted degenerate oligonucleotides, have been used to successfully identify linear and conformational epitopes recognized by specific antibodies (Stoute *et al.*, 1995; Lenstra *et al.*, 1992; Smith *et al.*, 1994). Likewise, random peptide libraries can also be used to identify substrate binding or adhesion motifs. For example, the screening of a LamB random peptide library has led to the successful identification of an iron-oxide adhesion domain (Brown, 1992). On the other hand, these vectors can also be used to express combinatorial antibody libraries which have shown potential for the identification of useful antigen binding domains (McCafferty *et al.*, 1990; Barbas *et al.*, 1991; Francisco *et al.*, 1993). The number of applications of the random library approach is likely to increase upon further study. Future advances in this area may lead to powerful applications in the area of drug design and to the development of diagnostic markers and vaccines.

3. The carrier protein: OprF

3.1 Bacterial outer membrane

The cell envelope of gram-negative bacteria is composed of an inner or cytoplasmic membrane and an outer membrane, separated by the peptidoglycan

layer and periplasm. Unlike the cytoplasmic membrane, the outer membrane is an asymmetric bilayer containing lipopolysaccharides on the outer leaflet and phospholipids or lipids in the inner monolayer. The outer membrane of gram-negative bacteria represents the primary barrier between the cell and its environment. Proteins associated with or embedded in the outer membrane perform a variety of cellular functions, including nutrient uptake, receptor activity and the maintenance of structural integrity. In addition, the outer membrane also functions as a permeability barrier to exclude the entry of harmful substances such as destructive enzymes and detergents, and it limits the passage of antibiotics. The high level of antibiotic resistance in *P. aeruginosa*, for instance, is attributed in part to the low permeability of the outer membrane (Nakae, 1995).

3.2 General structures of porins

Porins are outer membrane proteins that form transmembrane water-filled channels which allow the uptake of small hydrophilic molecules. According to their substrate specificity, porins can be divided into two functional classes. General or non-specific porins such as OmpF and OmpC in *E. coli* allow the general diffusion of water-soluble molecules smaller than the exclusion limit of the channels (Nikaido and Vaara, 1987). Specific porins such as the *P. aeruginosa* OprP and *E. coli* LamB proteins have specific binding sites for the uptake of phosphate ions and maltose respectively (Hancock and Benz, 1986; Szmelcman and Hofnung, 1975). Unlike other membrane proteins which have membrane-spanning

α-helical segments, porins have short amphipathic stretches of residues that traverse the outer membrane in β-sheet structure. To date, crystal structures of four porins have been resolved by X-ray diffraction. These porins include the *Rhodobacter capsulatus* porin, the *E. coli* general porin OmpF, the phosphate-starvation-inducible porin PhoE and the maltoporin LamB (Weiss *et al.*, 1991; Cowan *et al.*, 1992; Schirmer *et al.*, 1995). All of these established structures indicate that these proteins have β-barrel structures comprised of transmembrane anti-parallel β-sheet segments of 7-14 amino acids in length. The β-strands fold back and form a barrel structure which constitutes the framework of the channel. The structures of the *R. capsulatus* porin, OmpF and PhoE contain 16 β-strands while that of LamB contains 18 β-strands. The neighbouring strands are joined by long cell surface loops and small periplasmic turns (Cowan *et al.* 1992; Fig. 1). A common feature of these porins is that at least one of the surface loops folds back into the centre of the channel to form an eyelet region that constricts the size of the channel.

3.3 OprF

OprF is the major outer membrane protein of *P. aeruginosa* and is present in about 2×10^5 copies per cell (Angus *et al.*, 1982). The protein is 325 amino acids in length and has an apparent molecular mass of 35 kDa. Circular dichroism data revealed that OprF has 62% β-sheet structure, which is consistent with the predominance of β-sheet structure in the other outer membrane proteins (Siehnel

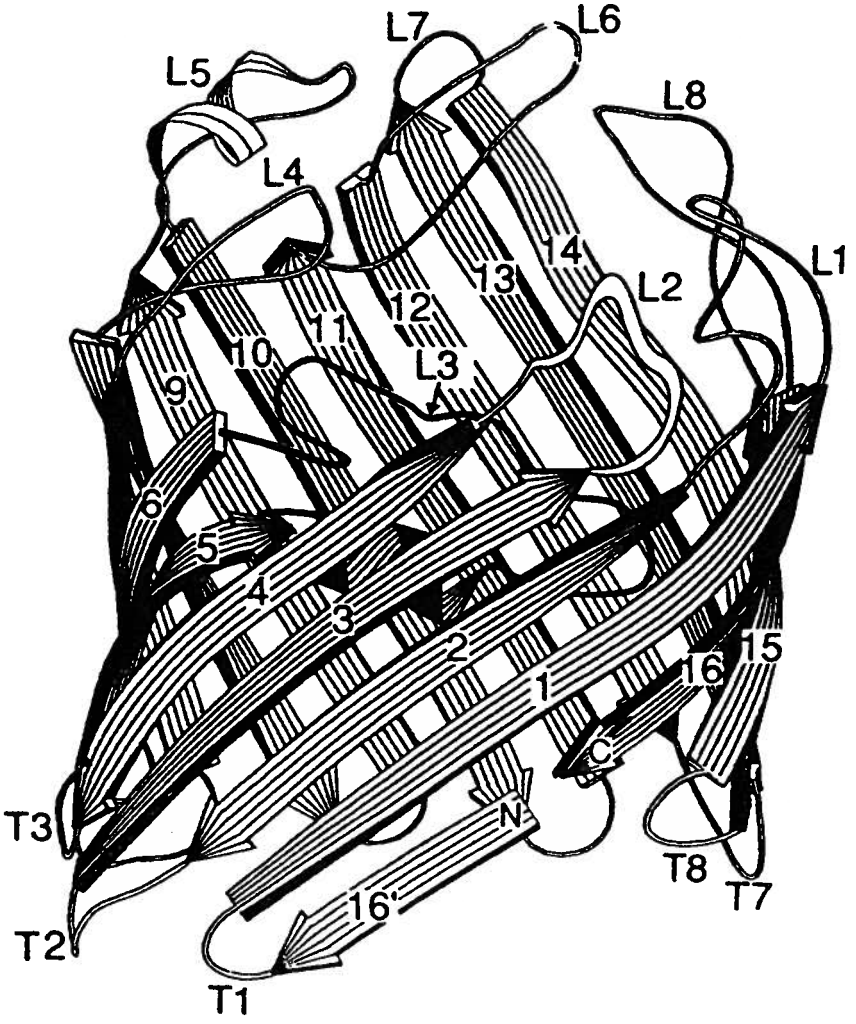


Figure 1. Schematic diagram of the β-barrel structure of a porin. Thick arrows represent transmembrane β-strands; L, surface loops; T, periplasmic turns; N, N-terminus; C, C-terminus. Reproduced with permission from Cowan *et al.*, 1992.

et al., 1990). Heat treatment of OprF increases the apparent molecular mass of the protein as monitored by SDS-PAGE, indicating the presence of a compact β-sheet structure (Siehnel *et al.*, 1990). The amino acid sequence of OprF contains four cysteine residues, and the mobility of the protein on SDS-PAGE is modifiable by treatment with 2-mercaptoethanol (Hancock and Carey, 1979). These findings suggested that the cysteine residues are involved in disulphide bond formation.

A number of approaches have been used to study and predict the membrane topology of OprF. These approaches include the prediction of secondary structures by computer programs, the comparison of amino acid sequences with other outer membrane proteins, Tn*PhoA* mutagenesis, *oprF* gene deletion analysis, and linker- and epitope-insertion mutagenesis (described in this study). The data generated from these studies have proven to be of use in confirming and refining the working model of OprF membrane topology. One of the most recent topology models of OprF is shown in Figure 2.

The primary amino acid sequence of OprF is distinct from the classical trimeric porins such as the *E. coli* OmpF, OmpC and PhoE porins (Duchene *et al.*, 1988). However, OprF shares strong C-terminal homology with the OmpA-related outer membrane proteins (Duchene *et al.*, 1988; Woodruff and Hancock 1989) as well as several proteins from unrelated species including the *B. subtilis* MotA (De Mot and Vanderleyden, 1994). The highly conserved C-terminal motif in these proteins has been proposed to share a common role in peptidoglycan association (De Mot and Vanderleyden, 1994).

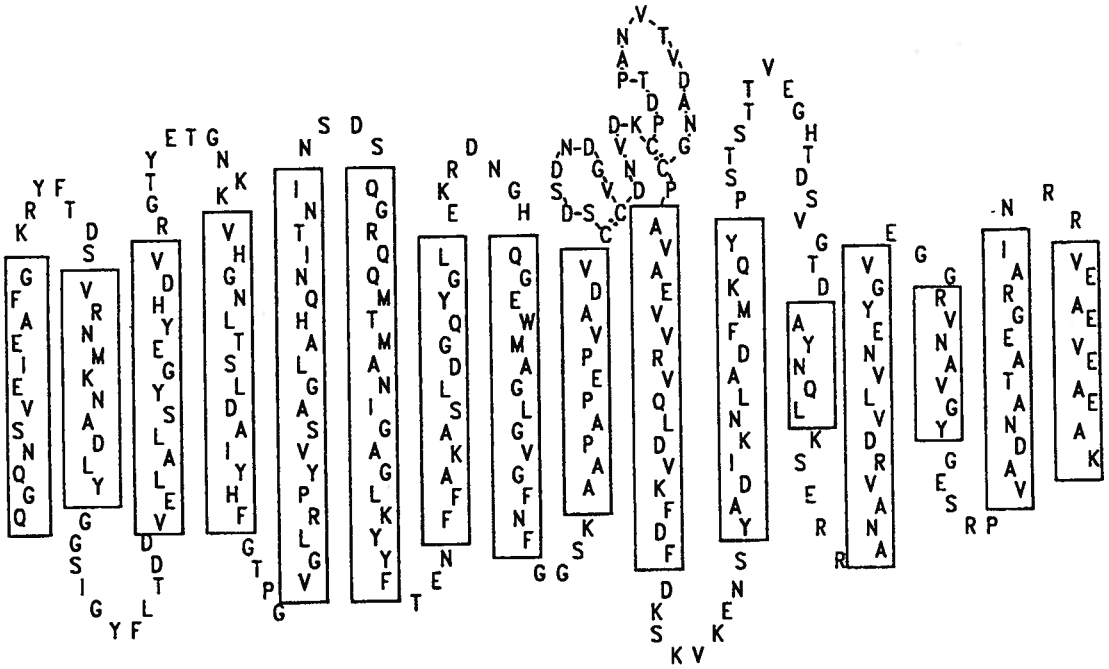


Figure 2. Proposed membrane topology model of OprF. The top of this model is proposed to face the exterior of the cell. The transmembrane β-strands are indicated by rectangular boxes. Reproduced with permission from Siehnel *et al.* (1990).

OprF serves a structural role in maintaining the cell shape and cell wall integrity (Gotoh *et al.*, 1989; Woodruff and Hancock, 1989). It has been demonstrated that OprF can complement the cell shape defect in an *E. coli* OmpA-deficient mutant (Woodruff and Hancock, 1989). It has also been reported that OprF is required for the growth of *P. aeruginosa* in low osmolarity medium (Nicas and Hancock, 1983). In addition to its structural role, OprF also functions as a porin which was proposed to be responsible for the molecular mass exclusion limit of 3000 to 9000 daltons through the outer membrane of *P. aeruginosa* (Hancock and Nikaido, 1978; Hancock *et al.*, 1979). However, other model membrane studies reported that OprF only forms small channels and that the exclusion limit of the *P. aeruginosa* outer membrane is too low for the penetration of disaccharides (342 daltons) (Caulcott *et al.*, 1984, Yoneyama and Nakae, 1986). More recent studies have shown quite conclusively that OprF forms channels that allow the diffusion of substrates of at least the size of tetrasaccharides in both model membrane (Nikaido *et al.*, 1991) and intact cell (Bellido *et al.*, 1992) experiments. One possible explanation to this paradox is that OprF forms channels of two different pore sizes (Woodruff *et al.*, 1986). *In vivo*, only a small portion of OprF form the large pores, and hence contributes to the low outer membrane permeability of *P. aeruginosa.*

Purified OprF has been shown to be a B cell mitogen (Chen *et al.*, 1980). Immunizing animals with purified or partially purified OprF preparations protects the animals from subsequent challenge with *P. aeruginosa* in various models. These findings suggested that OprF is immunogenic and a potential candidate for

a *P. aeruginosa* vaccine (Gilleland *et al.*, 1984; Matthews-Greer and Gilleland, 1987; Gilleland, *et al.*, 1988).

4. The model epitope: the malarial epitope

4.1 The life cycle of the malaria causative agent: *Plasmodium falciparum*

Malaria is a parasitic disease that afflicts hundreds of millions of people in a broad tropical band around the world. The disease is spread by *Anopheles* mosquitos infected with the protozoan parasite *Plasmodium falciparum*. The malaria parasite has a complex life cycle involving intracellular and extracellular stages in both the human host and the mosquito vector. The stage that infects man, the sporozoite, is present in the salivary gland of the mosquito and injected into the victim's bloodstream when the mosquito takes a blood meal. The sporozoite then finds its way to a liver cell, where it undergoes a series of transformations and is released into the blood stream of the victim as merozoite, which is the blood stage of the parasite. Each merozoite then invades a red blood cell and multiplies. Some merozoites become male and female gametocytes, which are then taken up by a mosquito. After further transformations, sporozoites appear in the mosquito's salivary gland and are ready for another infective cycle (Fig. 3).

4.2 The tetrapeptide repeating epitope NANP

The circumsporozoite protein (CSP) is the major surface antigen of the

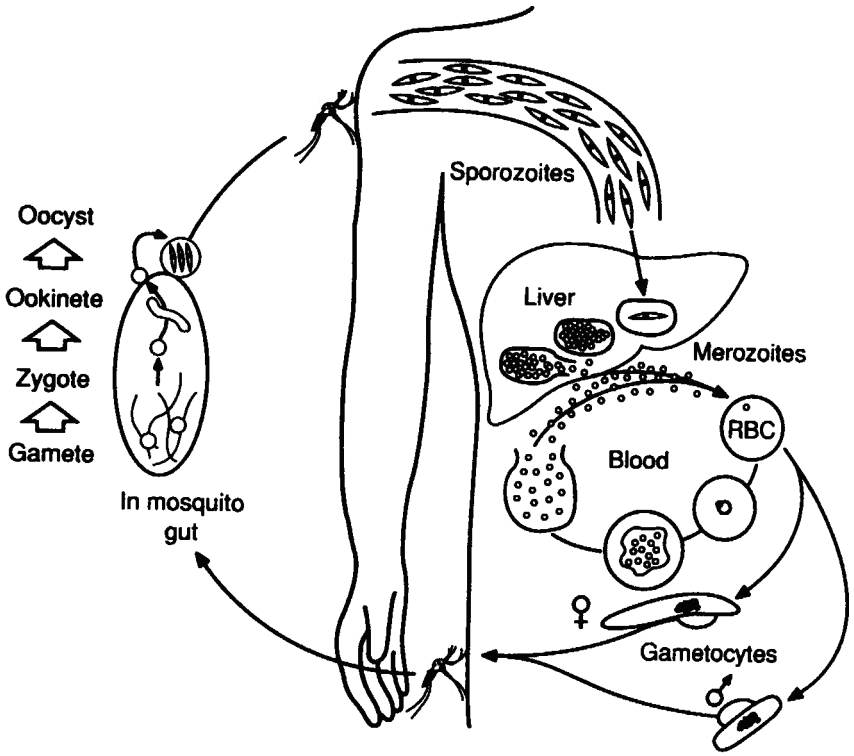


Figure 3. The life cycle of *Plasmodium falciparum*. Reproduced with permission from Fig. 83-3, p. 1037. Medical microbiology. S. Baron (ed.), Churchill Livingstone Publishing Co..

sporozoite stage of the parasite. The central portion of the protein contains 37 tandem repeats of the tetrapeptide Asn-Ala-Asn-Pro (NANP), with 4 interspersed Asn-Val-Asp-Pro (NVDP) variants (Dame *et al.*, 1984). It has been reported that the repetitive sequence encompasses the immunodominant region of the protein and antibodies raised against this region are potent inhibitors of invasion and of the development of sporozoites in cultured hepatocytes (Young *et al.*, 1985; Zavala *et al.*, 1985). Thus, the central tandem repeat region of CSP has engendered considerable interest as a potential candidate for a malaria vaccine. Both the whole CSP and the tandem repeat portion of the protein have been presented by carrier proteins such as the surface antigen of hepatitis B virus (Rutgers *et al.*, 1988) and the pIII protein of filamentous phage (Cruz *et al.*, 1988). It has been documented that in mice the repeat $(NANP)_{n>2}$ can only be recognized as a T cell epitope by animals with a H-2^b background (Good *et al.*, 1986). In view of this, identified T cell epitopes have been used in conjunction with the NANP repeating epitope in order to stimulate an anti-NANP response (Good *et al.*, 1987). The incorporation of the synthetic NANP peptides as multiple antigen peptide (MAP) has also been shown to be able to overcome the genetic restriction in non-responsive animals (Pessi *et al.*, 1991; Carvo-Calle *et al.*, 1993).

Due to the intense medical interest in the use of CSP as a component in a malaria vaccine, knowledge of the conformation of the repeating epitope comprising the immunodominant region of the protein will be useful in the design of an effective molecule. Various methods have been used to predict the three-

dimensional structure of the repeating tetrapeptide and different conclusions have been drawn. Theoretical investigation using energy minimization and molecular dynamics methods indicated that a right-handed helical conformation is likely to be adopted in aqueous solutions while a left-handed helical conformation should be favoured in non-polar environment (Gibson and Scheraga, 1986; Fig. 4). A similar study suggested that the most stable structure of the repeating tetrapeptide is a right-handed helix with 12 residues per turn (Brooks *et al.*, 1987). However, the Chou-Fasman predictive algorithm indicates a high β-turn content in the synthetic peptide $(NANP)_8$ (i.e. NANP repeated eight times). Circular dichroism measurements showed that the presence of prolines in these repeats induces an increase in the β-turn content (Fasman *et al.*, 1990). Proton nuclear magnetic resonance revealed that a repeating structural motif is formed by the NPNA (instead of NANP) cadence (Dyson *et al.*, 1990). To date, the X-ray crystallography structure of the CSP or the repeating region has not yet been resolved. The choice of the NANP repeat as the model epitope in this study was based on the simplicity of its repeating pattern, the well-documented immunodominance of the epitope, and the availability of epitope-specific antibodies from a collaborator.

5. Factors affecting antigenicity and immunogenicity

5.1 Factors affecting antigenicity

The antigenicity of a molecule refers to its ability to interact with

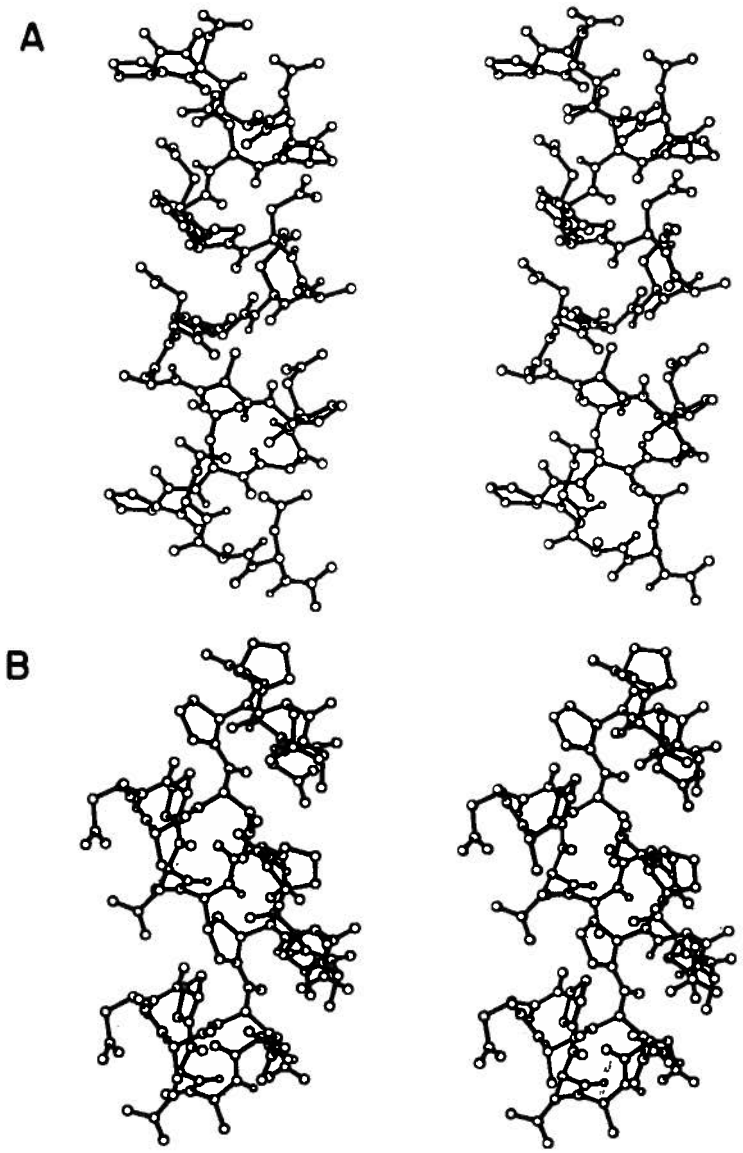


Figure 4. Stereo drawings of two of the predicted structures of the $(NANP)_6$ peptide. **A)** The left-handed helical conformation. **B)** The right-handed helical conformation. In both views, the carboxyl terminus is at the top. Reproduced with permission from Gibson and Scheraga, 1986.

antibodies. In principle, peptide antigenic determinants can be divided into two structural categories: the continuous epitope, which consists of a contiguous stretch of the amino acid sequence; and the conformational epitope, which is an assembled topographic site consisting of amino acid residues separated in the primary sequence but brought together during the folding of the protein. It is generally believed that the surface accessible regions of a protein usually contain antigenic determinants that can be recognized by the immune system, and thus stimulate the production of specific antibodies (Hopp and Woods, 1981). Since proteins in solution tend to fold in a way that exposes the hydrophilic amino acid residues to the surface, amino acid sequences with high local hydrophilicity are quite frequently predicted to have high antibody affinity (Berzofsky, 1985). The overall strength of an antibody-antigen interaction is governed by 3 major factors: the intrinsic affinity of the antibody for the epitope, the valency of the antibody and antigen, and the spatial configuration of the interacting compounds. With a monoclonal antibody of defined specificity, it seems logical that the presence of repeating epitopes may increase avidity and hence overall stability. However, the effects of multivalency may also involve spatial configurations that may impose steric constraints on the interactions.

5.2 Factors affecting immunogenicity

Immunogenicity, the ability to elicit an immune response, is determined by the intrinsic chemical structures of a molecule and by the ability of the host

animal to recognize the molecule. The mechanisms of an immune response can be divided into 2 categories: humoral, which mainly involves the production of circulatory antibodies, and cellular, which functions to target specific immune T cells against fungi, intracellular pathogens and cancer cells, *etc.*. In general, for an antigen to elicit a good antibody response, both a B cell epitope and a T cell epitope are required. The B cell epitope is recognized by B cell surface receptors to stimulate the production of antibodies of its own specificity. The T cell epitope, which results from antigen processing, is presented on the surface of an antigen presenting cell or B cell in conjunction with a MHC class II molecule for binding to specific T cell receptor. Clonal expansion of both B cells and T cells can then occur in parallel, leading to the production of specific antibodies by plasma cells. For a small antigen (hapten) which is not likely to encompass a T cell epitope, this epitope can be obtained by conjugating the hapten to a carrier protein. The whole hapten/carrier conjugate can then be used as an immunogen to elicit an anti-hapten antibody response. In addition to the intrinsic properties of a compound, the immunogenicity of the compound also depends on the extrinsic factors such as the processing pathways in antigen presenting cells, the set of MHC molecules available for antigen presentation, the presence of specific T suppressor cells, the delivery system and the route of adminstration, etc. (Gammon *et al.*, 1987; Gregoriadis, 1990; Monaco, 1992).

6. Aims of this study

A number of outer membrane proteins have been developed as carriers for foreign antigenic determinants in different epitope presentation systems. Limited studies have shown that the flanking amino acid residues and the length of the inserted epitope can affect the antigenicity and immunogenicity of the inserted epitope (Agterberg *et al.*, 1990a; Van der Werf *et al.*, 1990; Janssen *et al.*, 1994b). In light of these findings, it is conceivable that determining the optimal parameters for epitope presentation would increase the potential utility of these presentation systems.

The goals of this study were to develop the *P. aeruginosa* outer membrane protein OprF as a carrier for foreign antigenic determinants, to employ this protein to study the effect of the mode of presentation on the antigenicity of the presented epitope, and to perform a limited study correlating antigenicity with immunogenicity. The "permissiveness" of different regions of OprF to accommodate extra amino acid sequence was first examined by linker-insertion mutagenesis. The feasibility of OprF as a carrier for foreign antigenic determinants was then investigated by using the malarial tetrapeptide repeating epitope NANP as the model epitope. Also, the influence of the insertion position and the length of the inserted epitope on the antigenicity of the epitope was examined by using a series of OprF::malarial epitope hybrid proteins. Finally, this was correlated with the immunogenicity of the epitope presented in different ways in the context of OprF.

METHODS AND MATERIALS

1. Bacterial strains, plasmids and media

The bacterial strains and plasmids used in this study are listed in Table II. Bacterial strains were grown in Luria broth (LB) {1%(w/v) tryptone, 0.5%(w/v) yeast extract, 0.5%(w/v) NaCl} unless otherwise stated. Strains of C158 background were grown in Luria broth supplemented to a final concentration of 1.7% (w/v) NaCl and 0.1% (w/v) glucose to suppress the expression of OmpF and LamB respectively. When plasmids were present, media were supplemented with 75 µg/ml of ampicillin or with 50 µg/ml each of ampicillin and kanamycin. All media components were purchased from Difco Laboratories, Detroit, Michigan.

2. General recombinant DNA techniques

General DNA techniques were performed as described in Ausubel *et al.* (1987) and in Sambrook *et al.* (1989). Competent cells for transformation were prepared using the $CaCl_2$ method (Hanahan, 1983). DNA fragments were isolated either by elution from preparative agarose gel onto DEAE paper (Schleicher & Schuell) or by using the GENECLEAN kit (BIO 101 Inc. La Jolla, CA). Restriction enzymes and DNA modifying enzymes were purchased from Bethesda Research Laboratories (BRL, Burlington Canada) or Boehringer Mannheim (Mannheim,

Table II. Bacterial strains and plasmids

Strain or plasmid	Genotype, phenotype, or relevant properties	Source and/or reference
E. coli		
DH5αF'	*F'* Φ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17(r_K^-, m_K^-) *sup*E44 λ^- *gyr*A96 *thi*-1 *rel*A1	Hanahan, 1983
C158	*aroA ilv met his purE*41 *pro cyc*-1 *xyl*1 *lacY*29 *rpsL*97 *tsx*63 *ompA ompC*	Foulds & Chai, 1979
C386	*lpp ompA tsx*	Sonntag *et al.*, 1983
C466	*ara-14 leuB6 azi-6 lacY1 proC14 tsx-67* Δ*(ompT-fepC)266 entA403,* λ^- *trpE38 rfbD1 rpsL109 xyl-5 mtl-1 thi-1*	Elish *et al.*, 1988
C443 (DH5αF'IQ)	*F'* Φ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17(r_K^-, m_K^-) *sup*E44 λ^- *gyr*A96 *thi*-1 *rel*A1/F' *pro*AB$^+$ *lac*IqZΔM15zzf::Tn5[Km^r]	BRL
P. aeruginosa		
H103	PAO1 Cm^r prototroph	Hancock & Carey, 1979
H692	LPS A^- , B^-, same as rd 7513 in reference	Lightfoot & Lam, 1991

To be continued .....

Table II. Bacterial strains and plasmids (continued)

Strain or plasmid	Genotype, phenotype, or relevant properties	Source and/or reference
Plasmids		
pTZ18R/19R	cloning vector, Amp^r.	Pharmacia
pHJ13	pTZ18R with a 4.5 kb insert carrying 2 separate fragments of *oprF*.	Hancock lab
pRW1	pTZ19R with a 4.8 kb *Hin*dIII /*Eco*RI insert carrying the entire *oprF* in one continuous fragment.	This study
pRW3	pTZ19R with a 1.47 kb *Hin*dIII/*Kpn*I insert carrying the entire *oprF*	This study
pUC4KAPA	pUC type plasmid with a drug-resistance marker from Tn*903*, Km^r and Amp^r.	Pharmacia
pGEX-1N	glutathione *S*-transferase fusion protein expression vector	Pharmacia

Germany). Oligonucleotides were synthesized on an Applied Biosystems Incorporated (ABI, Foster City, CA) 392 DNA/RNA synthesizer according to manufacturer's instructions. Oligonucleotides were purified by passing through a Sep-Pak C_{18} cartridge (Waters, Division of Millipore) and eluting with 20% acetonitrile followed by ethanol precipitation. For oligonucleotides used in ligations, the sense and antisense strands of each set were annealed by heating an equal amount of each strand (100 μM) in 2 mM $MgCl_2$/50 mM NaCl/20 mM Tris-HCl pH7.5 at 90°C for 15 min, followed by gradual cooling to 23°C.

3. General protein and immunological techniques

3.1 SDS-PAGE and immunoblottings

SDS-PAGE procedures were performed as described in Hancock and Carey (1979). Colony and Western immunoblotting procedures were as described in Mutharia and Hancock (1985). The percentage of acrylamide used was 11% unless otherwise stated. The amount of proteins loaded per lane was normalized by protein assays (described in section 3.5). For some Western immunoblots, polyvinylidene difluoride (PVDF) membrane (Millipore Corporation, Bedford, MA) was used and immunodetection was done without blocking (Mansfield, 1994). Briefly, the PVDF membrane was air-dried completely after protein transfer, followed by incubation with primary antibody for 1 h at 37°C. After 2 x 10 s washing in PBS, secondary antibody was added and incubated for 30 min at 37°C,

followed by 2 x 10 s washing in PBS and colour development.

3.2 Antibodies

The OprF-specific mAbs and polyclonal serum used in this study were as described by Finnen *et al.* (1992). The epitopes recognized by the OprF-specific mAbs have been delineated by Rawling *et al.* (1995) and are summarized in Table III. The isolation of the malarial epitope-specific mAbs pf2A.10 and pf5A4.1 were described by Wirtz *et al.* (1987).

3.3 Indirect immunofluorescence labelling

Immunofluorescence labelling was performed as follows. Overnight cultures of strains containing the specified plasmids were harvested and washed twice in PBS. Slides were coated with poly-L-lysine (Sigma Chemical Co., St. Louis, Mo; average MW~25,000) by flooding with poly-L-lysine solution (1 mg/ml) in a moist chamber for 15-20 min and then rinsing thoroughly with distilled water. Samples of washed cells were smeared onto the poly-L-lysine coated slides and allowed to air dry briefly. Slides were then incubated with an OprF-specific mAb or a malarial epitope-specific mAb (both at 1/100 dilution) in PBS containing 1% fetal calf serum (FCS) for 30 min at 23°C. After washing with excess PBS, slides were incubated with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (BRL) at 1/20 dilution in PBS/1% FCS for 30 min at 23°C. Following PBS washing, one drop of mounting medium (Sigma) was added to the slides and the cells were

Table III. OprF epitopes recognized by monoclonal antibodies[a].

Monoclonal antibody	Amino acid positions of epitope	Type of epitope	Surface exposure of epitope[c]
MA7-1	55-62	linear	+
MA7-2	237-244	linear	+/–
MA7-3	188-230	conformational[b]	+
MA7-4	188-278	conformational	+
MA7-5	188-278	conformational	+
MA7-6	198-240	conformational	+
MA7-7	188-278	conformational	+
MA7-8	152-210	conformational	+
MA4-4	152-210	conformational	+
MA5-8	307-314	linear	+

[a] Data are summarized from Rawling *et al.*, 1995

[b] Defined by non-reactivity with overlapping 8-amino acid peptides synthesized on pins.

[c] +, surface-exposed; +/–, surface exposure only in an LPS altered rough strain.

examined under a Zeiss microscope fitted with a halogen lamp, a condenser and filters for fluorescence microscopy at 525 nm for emission of fluorescein isothiocyanate.

3.4 Trypsin sensitivity assays

E. coli C386-derived strains containing different pRW3-derived plasmids were grown in Luria broth supplemented with Amp (75 μg/ml) to an A_{600} of 0.8. Samples of 1.5 ml of the cultures were harvested and washed twice with 20 mM Tris-HCl pH 7.4 containing 5 mM $MgCl_2$, and then resuspended in 500 μl of the same buffer. Trypsin (TPCK treated, Sigma) was added to a final concentration of 0.1 mg/ml of cell resuspension, followed by incubation at 37°C for 60 min. Un-treated samples were incubated in the same conditions, except that trypsin was omitted. Proteolysis was stopped by heating at 88°C for 10 min in solubilization buffer (2% SDS, 10% glycerol, 62.5mM Tris-HCl pH6.8). OprF in outer membrane samples was digested at a trypsin concentration of 0.1 mg/ml. The reactions were carried out as described above. In both cases, the trypsinized samples were analyzed by SDS-PAGE and Western immunoblotting with the specified monoclonal antibodies. As controls, the cleavage of OprF in *P. aeruginosa* intact cells to a 28 kDa core fragment and the complete cleavage of bovine serum albumin by trypsin to low molecular weight peptides were demonstrated.

3.5 Protein assays

The modified Lowry assay was performed as described by Sandermann and Strominger (1972). The bicinchoninic acid (BCA) protein assay was performed in 96-well microtiter plates with a sample volume of 10 µl and the addition of 200 µl of BCA reagent (Sigma). The plates were then incubated at 37°C for 30 min and the A_{550} was determined with a BioRad model 3550 ELISA microplate reader.

4. Construction of pRW3

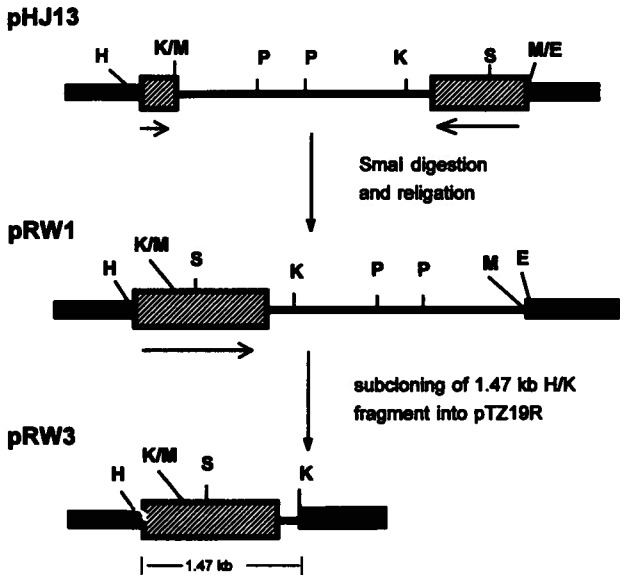
Figure 5 outlines the subcloning of *oprF*. The 4.5 kb *Sma*I fragment containing the 3' end of *oprF* was excised from pHJ13 and re-ligated in the opposite orientation into the vector to construct pRW1, so that the coding region of *oprF* was in one continuous fragment. The 1.47 kb *Hin*dIII/*Kpn*I partial fragment containing the entire *oprF* gene from pRW1 was then subcloned into the vector pTZ19R to obtain pRW3.

5. Linker-insertion mutagenesis of *oprF*

5.1 Mutagenesis with kanamycin resistance cassette

Figure 6 shows a schematic diagram of the procedures. The plasmid pRW3 was linearized separately by partial digestion with restriction enzymes: *Rsa*I, *Hae*III, *Tha*I or *Alu*I (Boehringer Mannheim), all of which leave blunt ends

A



B

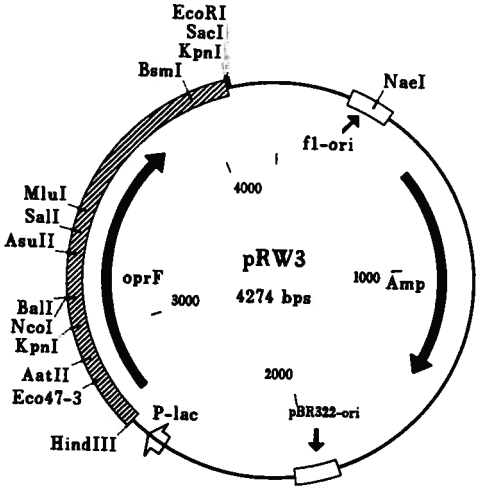


Figure 5. Construction of pRW3.

A. Schematic diagram of the subcloning of *oprF*. The thick solid line represents the cloning vector pTZ19R; the hatched box represents the OprF coding region. The arrows under each box indicate the direction of transcription of *oprF*. H, *Hin*dIII; K, *Kpn*I; S, *Sal*I; P, *Pst*I; M, *Sma*I; E, *Eco*RI. **B.** Restriction map of pRW3. The position and direction of transcription of *oprF* and the ampicillin resistance marker (Amp) are indicated. Abbreviations: f1-ori, f1 phage origin of replication; pBR322-ori, ColE1 origin of replication; P-lac, *lac* promoter; bps, base pairs.

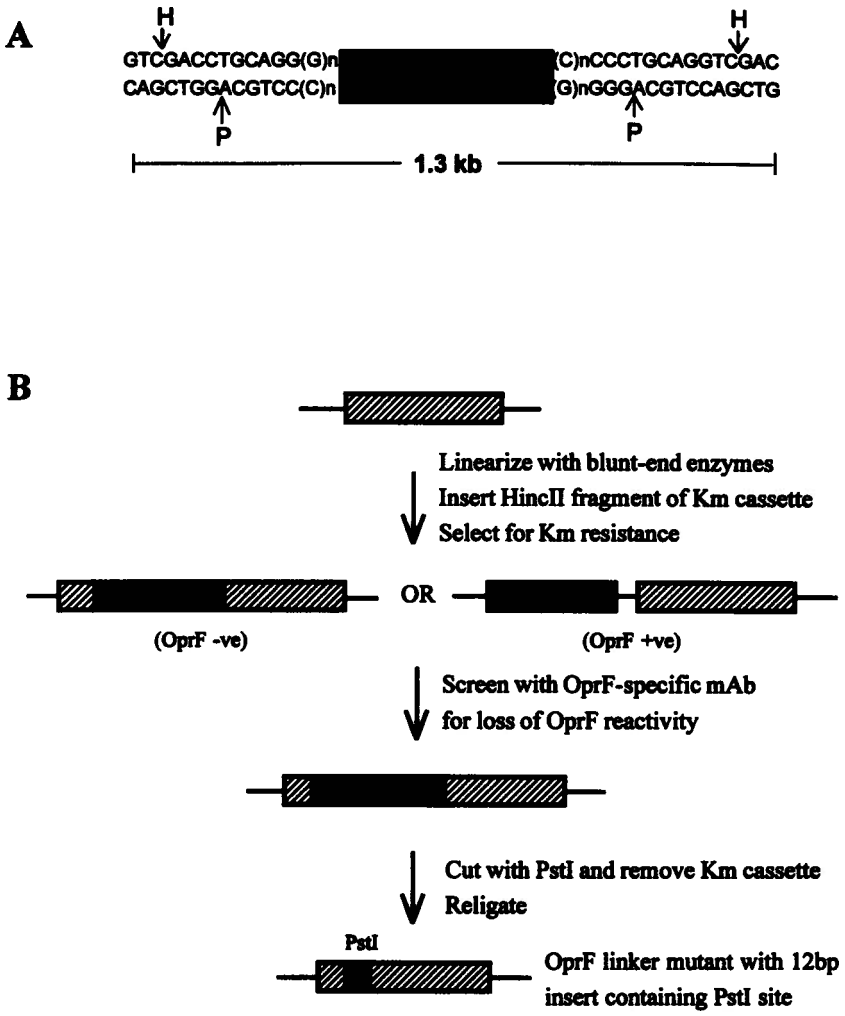


Figure 6. Schematic representation of semi-random linker-mutagenesis with a kanamycin resistance cassette.

A. The kanamycin resistance cassette used for the linker-insertion mutagenesis. The solid box represents the region that encodes an aminoglycoside 3'-phosphotransferase gene conferring kanamycin, neomycin, and G418 resistance. The nucleotide sequences in the flanking regions are specified to indicate the symmetric restriction enzyme sites. H, *Hinc*II; P, *Pst*I. **B.** Procedures for linker-insertion mutagenesis. The solid and hatched boxes represent the kanamycin resistance cassette and the *oprF* gene respectively; Km, kanamycin, OprF -ve, non-reactive with OprF-specific (C-terminal reactive) mAb MA5-8; OprF +ve, reactive with MA5-8.

after digestion. In the cases of *Alu*I and *Tha*I, partial digestions were performed in the presence of ethidium bromide (20 and 50 μg/ml respectively) to increase the recovery of DNA molecules cleaved at a single site. After partial digestions, the reaction mixtures were resolved by preparative agarose gels and the full sized linear form of the plasmid was isolated by elution onto DEAE paper (Schleicher & Schuell). The four pools of linearized pRW3, each corresponding to a separate restriction enzyme used, were ligated separately with a 1.3 kb *Hinc*II fragment containing the kanamycin resistance cassette from pUC4KAPA. Following ligation and transformation, cells were plated on Luria agar plates containing 50 μg/ml each of kanamycin and ampicillin. The doubly resistant colonies were further screened by colony immunoblotting for loss of expression of OprF using the OprF C-terminal-specific mAb MA5-8 (Rawling *et al*, 1995 and Table III). Plasmid DNA from the clones that did not express OprF were extracted and then digested with *Pst*I, which only recognized sites in the flanking sequences of the kanamycin resistance cassette, and hence cleaved the cassette from the plasmid. Following re-ligation of the *Pst*I digestion mixtures and transformation, recombinants were screened for kanamycin sensitivity and the recovery of immunoreactive OprF by colony immunoblotting using the same OprF-specific mAb. The OprF expressing ($OprF^+$), kanamycin sensitive (Km^s) clones presumably contained mutated forms of pRW3 with a 12 bp insertion at sites originally interrupted by the kanamycin resistance cassette.

5.2 Mutagenesis at the *Sal*I site

The plasmid pRW307 was constructed by inserting a self-hybridizing *Sal*I adaptor oligonucleotide (5'TCGACCTGCAGG3') which contained a *Pst*I site into the *Sal*I site corresponding to position 188 (aa^{188}) in the mature OprF sequence. As a result, the 4 amino acids DLQV were added after the valine residue at aa^{188}.

5.3 Determination of linker-insertion sites

The 12 bp insertion in the linker mutants carried a unique *Pst*I site. Plasmid DNA was prepared from 100 $OprF^{+}$, Km^{s} clones and the linker-insertion sites were mapped by restriction enzyme digest using double digestions with *Pst*I/*Hin*dIII and *Pst*I/*Sal*I, where *Hin*dIII and *Sal*I recognized unique sites at nucleotide positions -63 and +726 of the *oprF* gene coding sequence respectively (Duchene *et al.*, 1988). Clones with the same restriction pattern were grouped and the exact position of insertion was confirmed by DNA sequencing of at least one representative from each group.

6. Construction of OprF::malarial epitope hybrid proteins

6.1 Positional hybrids

Since the linker insertions occurred in different reading frames, three sets of synthetic oligonucleotides were required to accommodate the three possible reading frames at the *Pst*I sites (Fig. 7A). In the course of cloning, it was realized that

A. Phase 1

```
     P   N   A   N   P   N   A   N   P   N   A   G   H   A
    CCG AAC GCC AAC CCG AAC GCC AAC CCG AAC GCC GGG CAT GCA
ACGTGGC TTG CGG TTG GGC TTG CGG TTG GGC TTG CGG CCC GT
```

Phase 2

```
    N   P   N   A   N   P   N   A   N   P   N   A   C
    AC CCG AAC GCC AAC CCG AAC GCC AAC CCG AAC GCA TGC A
ACGTTG GGC TTG CGG TTG GGC TTG CGG TTG GGC TTG CGT
```

Phase 3

```
      N   A   N   P   N   A   N   P   N   A   L   D   V   Q
    G AAC GCC AAC CCA AAC GCG AAT CCG AAT GCT CTA GAC TTG CA
ACGTC TTG CGG TTG GGT TTG CGC TTA GGC TTA CGA GAT CTG A
```

B. 7-amino acid insert

```
       N   P   N   A   N   P   N
TCGAAAC CCG AAC GCT AAT CCA AAT
    TTG GGC TTG CGA TTA GGT TTAGATC
```

11-amino acid insert

```
       N   P   N   A   N   P   N   A   N   P   N
TCGAAAC CCG AAC GCT AAT CCA AAC GCC AAC CCT AAT
    TTG GGC TTG CGA TTA GGT TTG CGG TTG GGA TTAGATC
```

15-amino acid insert

```
       N   P   N   A   N   P   N   A   N   P
TCGAAAC CCG AAC GCT AAT CCA AAC GCC AAC CCC
    TTG GGC TTG CGA TTA GGT TTG CGG TTG GGG

 N   A   N   P   N
AAT GCA AAT CCG AAT
TTA CGT TTA GGC TTAGATC
```

19-amino acid insert

```
       N   P   N   A   N   P   N   A   N   P   N
TCGAAAT CCA AAC GCC AAC CCG AAC GCA AAC CCC AAT
    TTA GGT TTG CGG TTG GGC TTG CGT TTG GGG TTA

 A   N   P   N   A   N   P   N
GCA AAT CCT AAC GCG AAC CCA AAT
CGT TTA GGA TTG CGC TTG GGT TTAGATC
```

Figure 7. Nucleotide and encoded amino acid sequences of the oligonucleotides used for the construction of OprF::malarial epitope hybrid proteins.

A. The three sets of oligonucleotides used for the construction of the positional hybrids. Each set encodes the malarial epitope sequence in one of the three possible reading frames at the *Pst*I cleavage sites. The *Pst*I compatible ends for the ligation into the *Pst*I sites generated by the linker-insertion mutagenesis procedures are in italics. The unique restriction enzyme sites engineered in the oligonucleotides are underlined (*Sph*I in frames 1 and 2, *Xba*I in frame 3). **B.** The four sets of oligonucleotides encoding different lengths of the repeating epitope. All four sets carried *Xho*I and *Xba*I sites (in italics) on each end respectively for the ligation into the corresponding sites generated by the *Pst*I adaptors for directional cloning. The amino acid sequences are indicated in one letter code.

maintaining the same codon usage in each NANP repeat caused the problem of hairpin loop formation when more than one copy of the insert was ligated in opposite orientation. Therefore, a different codon usage for the NANP repeats was chosen for the Phase 3 oligonucleotides. Each set of the annealed synthetic oligonucleotides encoding the malarial epitope was ligated into the *Pst*I sites of the various *oprF* linker mutant plasmids. After transformation, the recombinants were screened by colony immunoblotting with the OprF-specific, N-terminus reactive mAb MA7-1 (Rawling *et al.*, 1995, and Table III) and the malarial epitope-specific mAb pf2A.10 (Wirtz *et al.*, 1987) separately. Plasmid DNA from transformants that reacted positively with both monoclonal antibodies were extracted and analyzed by restriction analysis and DNA sequencing.

6.2 Multiple-repeat hybrids

Three sites of OprF (aa^{26}, aa^{196} and aa^{213}) were chosen for further study of the length effect of the epitope on its antigenicity and immunogenicity. Hybrid OprF::malarial epitope plasmid constructs carrying different lengths of the epitope were generated as follows:

i) Insertion of *Pst*I adaptors: The three selected sites were of two different reading frames. To simplify the cloning procedures and the number of oligonucleotides required, two sets of adaptors were synthesized and inserted into the *Pst*I sites of the corresponding linker mutants. The insertion of the adaptors created two unique sites for directional cloning (*Xba*I and *Xho*I) and also adjusted the reading frames at the

three chosen sites so that only one set of malarial epitope oligonucleotides would be required for all three sites.

ii) Insertion of malarial epitope encoding oligonucleotides: Four sets of oligonucleotides, representing 7 amino acids (NPNANPN), 11 amino acids $\{(NPNA)_2NPN\}$, 15 amino acids $\{(NPNA)_3NPN\}$ and 19 amino acids $\{(NPNA)_4NPN\}$ of the malarial epitope were synthesized (Fig. 7B). The oligonucleotides contained *Xho*I and *Xba*I compatible ends and were designed so that the *Xho*I site would be destroyed after the ligation. Transformants were screened by colony immunoblotting with the malarial epitope-specific mAb pf2A.10. Plasmid DNA from positive clones was extracted and the incorporation of the oligonucleotides was confirmed by restriction digest analysis.

The resultant hybrid proteins were designated as OprF::ME(X)aa(Y), where ME refers to malarial epitope, X refers to the number of amino acids inserted, and Y refers to the amino acid position of the insertion.

7. DNA sequencing

Automated DNA sequencing was carried out with the Applied Biosystems Incorporated (ABI, Foster City, CA.) model 373A DNA sequencing system using the polymerase chain reaction and dye-terminator chemistry as described by the manufacturer's protocols. Sequence analyses were performed using the ABI 675 DNA sequence editor program. Template DNA was prepared using Qiagen columns

(Qiagen Inc., Chatsworth, CA 91311) according to the manufacturer's protocols. To determine the exact position of the insertion sites of the representative *oprF* linker-insertion mutants, plasmid DNA from the corresponding kanamycin resistant clones was used as template. The sequencing primers used were 21 mer oligonucleotides (5'ATGTAACATCAGAGATTTTGA3' and 5'TATGAGTCAGCAACACCTTCT3') that hybridized to opposite strands of the kanamycin resistance cassette, approximately 50 bp from the ends of the cassette (Oka *et al.*, 1981). The directions of extension from these primers were outward from the cassette so that the *oprF* sequences flanking the insertion sites could be identified. DNA sequencing to determine the number and orientation of the malarial epitope insert in the *oprF*::malarial epitope hybrid plasmids was carried out by using primers that hybridized to *oprF* gene sequences at appropriate distances upstream of the insertion sites. The sequences of the primers(5' → 3') were: FP1, ^{19}TTAGGCGTTGTCATCGGCTCG^{39}; FP2, ^{402}AACATGGCCAACATCGGCGCT^{422}; FP3, ^{577}CCGGAACCGGTTGCCGACGTT^{597}; FP4, ^{879}GAGCGTCGTGCCAACGCCGTT^{899}; FP5, ^{702}GTCGTACGCGTACAGCTGGACGTG^{725} (numbers in superscripts indicate the positions of the first and last nucleotides in the *oprF* gene sequence as described by Duchene *et al.*, 1988).

8. Construction of glutathione *S*-transferase (GST)::malarial epitope fusion proteins

Two sets of annealed synthetic oligonucleotides, each encoding 11 and 19 amino acids corresponding to the malarial epitope, were ligated into the *Bam*HI and *Eco*RI sites of the pGEX cloning vector (Fig. 8). Transformants were screened by colony immunoblotting with the malarial epitope-specific mAb pf2A.10. The resultant fusion proteins, GST::ME11 and GST::ME19, expressed 11 amino acids {$P(NANP)_2NA$} and 19 amino acids {$P(NANP)_4NA$} respectively at the C-terminus of glutathione *S*-transferase (GST).

9. Isolation of outer membranes

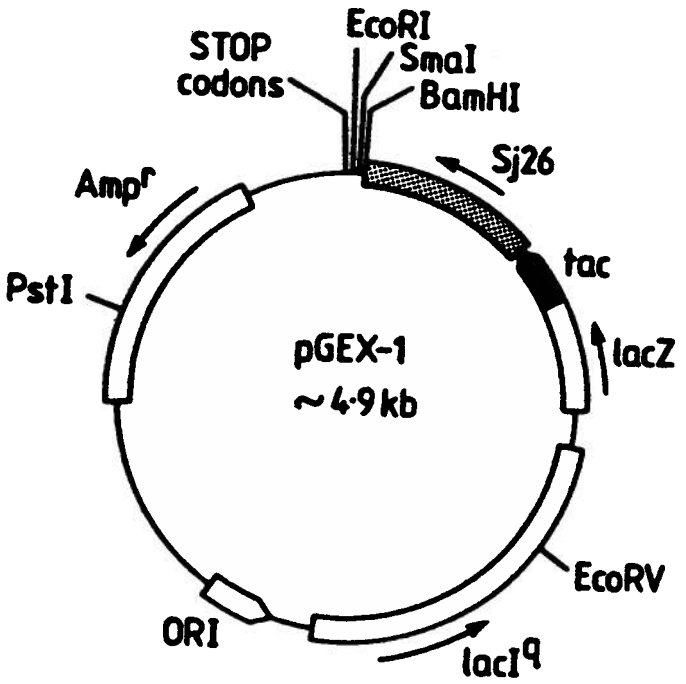
9.1 Triton X-100 extraction

The outer membranes of OprF linker mutants were isolated by selective Triton X-100 solubilization of cell envelopes as described by Schnaitman (1971).

9.2 Sucrose gradient centrifugation

Overnight cultures (1 L) of C158-derived strains were harvested and resuspended in 10 ml of 20% sucrose, 10 mM Tris-HCl pH 8.0. Deoxyribonuclease I (50 μg/ml) was then added to each cell resuspension, followed by incubation at 23°C for 20 min. Cell lysis was achieved by two passages through a French

A



B

11 amino acids

```
 D   P   N   A   N   P   N   A   N   P   N   A   Q   L
GAT CCG AAC GCC AAT CCG AAT GCG AAC CCA AAC GCA CAG CTG
     GC TTG CGG TTA GGC TTA CGC TTG GGT TTG CGT GTC GAC TTAA
```

19 amino acids

```
 D   P   N   A   N   P   N   A   N   P   N   A   N   P   N
GAT CCG AAC GCC AAT CCG AAT GCG AAC CCA AAT GCT AAC CCC AAC
     GC TTG CGG TTA GGC TTA CGC TTG GGT TTA CGA TTG GGG TTG

 A   N   P   N   A   Q   L
GCA AAT CCT AAC GCA CAG CTG
CGT TTA GGA TTG CGT GTC GAC TTAA
```

Figure 8. Construction of GST::malarial epitope fusion proteins.

A. Schematic representation of the pGEX-1 cloning vector. Abbreviations: Sj26, the gene encoding a 26 kDa glutathione *S*-transferase (GST); tac, tac promotor; Amp^r, β-lactamase gene; ORI, origin of replication. **B.** The nucleotide and amino acid sequences of the oligonucleotides encoding the malarial epitope. The oligonucleotides carried *Bam*HI and *Eco*RI compatible ends (in italics) for directional cloning into the multiple cloning sites of pGEX-1. Fig. 8a is reproduced from Smith and Johnson, 1986, with permission.

pressure cell at 15,000 psi. The lysed cells were centrifuged at 1,700 × g for 10 min to remove cell debris. The supernatants were then applied onto a 2-step sucrose gradient {50%/70%(w/v)} and centrifuged at 100,000 × g in a SW28 rotor (Beckman) for at least 6 h at 4°C. The lower band that formed at the interface of the 50% and 70% sucrose layers was collected and the sucrose was diluted with at least two volumes of distilled water, followed by centrifugation at 200,000 × g in a 60Ti rotor (Beckman) for 1 h. The final pellets were resuspended in 1 ml of distilled water. The protein concentration in each sample was determined by a modified Lowry protein assay.

9.3 Removal of inclusion bodies

Outer membrane samples containing the series of OprF::malarial epitope multiple-repeat hybrid proteins at aa[26] were contaminated with inclusion bodies. The membrane bound form of these hybrid proteins was obtained by octyl-polyoxyethelene (octyl-POE) extraction. Briefly, the loosely-bound proteins in the preparations were removed by resuspending the outer membrane pellets in 0.5% octyl-POE, followed by incubation at 37°C for 30 min and centrifugation at 4°C for 15 min at 13,000 × g. The OprF::malarial epitope hybrids were released by resuspending the insoluble fractions in 3% octyl-POE, 10mM EDTA, followed by incubation at 37°C for 30 min and centrifugation in a microfuge at 4°C for 15 min at 13,000 × g. Extraction with 3% octyl-POE, 10mM EDTA was repeated. The membrane bound form of the proteins was found to be contained in the

supernatant.

10. Expression of *oprF* and *oprF* derivatives in *E. coli*

10.1 Expression of *oprF* in different *E. coli* strains

The plasmid pRW3 was transformed into different *E. coli* host strains by the $CaCl_2$ method (Hanahan, 1983). IPTG was added at 1 mM final concentration to mid-log phase cultures and growth was continued at 37°C for another 4 h. Un-induced cells were harvested at the same time as the induced cultures. Cell envelopes were prepared by centrifugation of whole cell lysates at 200,000 × g for 1 h. The expression level of *oprF* was examined by Western immunoblotting of cell envelope samples with the OprF C-terminal-specific mAb MA5-8.

10.2 Expression of an *oprF* derivative in different induction conditions

Fresh LB broth (50 ml in 250 ml flask) was inoculated at 1/100 dilution with an overnight stationary phase culture of C158 containing pRW307.1M. IPTG, at 0.2 mM or 1 mM final concentration, was added to cultures either at the time of inoculation or when the cells were at mid-log phase (A_{600} ~0.5-0.6). IPTG induction was carried out at 30°C or 37°C for 3 h or 16 h as indicated. Outer membranes were prepared using differential Triton X-100 extraction as described in Section 9.1. Expression of OprF::hybrid protein was examined by Western immunoblotting with an OprF-specific mAb.

11. Protein purification

11.1 OprF::malarial epitope hybrid proteins

OprF and OprF::malarial epitope hybrid proteins were purified from plasmid-containing derivatives of *E. coli* strain C158. Outer membrane samples containing OprF or OprF::malarial epitope hybrid proteins were prepared by a 2-step sucrose gradient centrifugation as described in Section 9.2. The samples were then extracted sequentially with 0.5% octyl-POE, 3% octyl-POE /50 mM NaCl, and 3% octyl-POE/10 mM EDTA. Extractions were performed by resuspending the insoluble fractions in the detergent solutions, followed by incubation at 37°C for 1 h and centrifugation at 200,000 × g for 1 h. Supernatants from the 3% octyl-POE/10 mM EDTA extractions contained predominantly OprF or OprF::malarial epitope hybrid proteins. The detergent-extracted proteins were further purified by FPLC using an anion exchange column, MonoQ (Pharmacia), and elution with an NaCl gradient. Column buffer contained 0.5% octyl-POE, 10 mM EDTA, 10 mM Tris-HCl pH8.0. Purified OprF or OprF::malarial epitope hybrid proteins were eluted in the flow through fractions while the contaminants bound to the column.

11.2 GST::malarial epitope fusion proteins

The GST::malarial epitope fusion proteins were purified by affinity chromatography using glutathione agarose beads as described in Smith and Johnson, 1988. Briefly, the procedures involved breaking of cells using a French-

pressure cell, centrifugation to remove cell debris, incubation of the cell supernatant with glutathione agarose beads to allow binding of the GST::malarial epitope fusion protein to the matrix, washing away of non-binding proteins, and elution of the fusion protein with 5-10 mM reduced glutathione. The anti-GST polyclonal serum was kindly provided by Dr. Michael Gold (Department of Microbiology and Immunology, U. of British Columbia).

11.3 Extraction from SDS-polyacrylamide gel

Protein samples were separated by preparative SDS-PAGE (11%). Bands of interest were excised and the proteins were eluted into 0.1% SDS, 10mM EDTA, 10mM Tris-HCl pH8.0 by incubation at 4°C for 16 h. The eluted proteins were quantitated by SDS-PAGE, followed by Coomassie blue staining and measurement of the intensity of the bands by scanning densitometry using a protein + dna Imageware apparatus (protein + dna Imageware Systems, PDI, NY, U.S.A.). The concentration of the samples was extrapolated from a standard curve obtained from protein samples with known concentrations.

12. Antigenicity studies

12.1 Outer membrane ELISA

Outer membrane samples containing OprF::malarial epitope hybrid proteins were diluted to various concentrations (from 0.5 to 20 μg/ml) in carbonate

buffer (15mM Na_2CO_3/35mM $NaHCO_3$/3mM NaN_3 pH 9.6). Dilutions of the outer membrane samples (100 µl) were used to coat the bottom of 96 well plates by incubation at 4°C for 16 h. The wells were then washed twice with PBS containing 5 mM $MgCl_2$ and blocked by incubation with 3% BSA/PBS at 37°C for 1 h. After washing, 100 µl of primary antibody (1/2000 of rabbit-anti-OprF antiserum or 1/2000 of pf2A.10) was added. After incubation (37°C, 1 h) and washing, 100 µl of horseradish peroxidase-conjugated secondary antibody was added to each well (37°C, 1 h). 3,3'5,5' Tetramethylbenzidine (TMB) (Pierce Chemical Co., USA) was used as a chromogenic substrate and the reactions were stopped after 5-10 min by the addition of 1 M H_3PO_4. The A_{450} readings of the wells were obtained using a BioRad ELISA microplate reader (model 3550) with a 450 nm filter. To normalize the expression levels of the hybrid proteins, each index was the ratio of the A_{450} readings when pf2A.10 was used as the primary antibody to the A_{450} readings when the OprF-specific polyclonal antibody was used as the primary antibody. For each experiment, a plot of A_{450} readings versus the concentrations of coating antigen was drawn for each antibody, only values that corresponded to the linear portion of the binding curve were used for the calculation of antigenicity indices.

Due to the presence of inclusion bodies in the outer membrane samples containing the multiple-repeat hybrids carrying an insertion at aa^{26}, the membrane bound protein solubilized in 3% octyl-POE was used in ELISA. The samples were diluted at least 40 fold in carbonate buffer and the concentration of the detergent in the other samples was adjusted so as to standardize the effect of the detergent

on the antigen-antibody interactions in all of the samples.

12.2 Whole cell dot blot analysis

Mid-logarithmic growth phase cells of strain C158 expressing the hybrid plasmids were harvested, washed twice with PBS and diluted in PBS to $1x10^8$, $2x10^7$, $4x10^6$, and $8x10^5$ cells/µl. One µl of each cell resuspension was spotted onto nitrocellulose filters, and the blotting procedures were performed as described in Mutharia and Hancock (1983). The intensities of the dots were quantitated by densitometry with the protein + dna Imageware (PDI) systems using the Quantity One software. Each antigenicity index was the mean of the ratios of anti-malarial epitope reactivity to anti-OprF reactivity obtained from four sets of dots representing different numbers of cells ($8x10^5$ to $1x10^8$ cells).

12.3 Statistical analyses

The antigenicity indices of the inserted epitope in the positional hybrids were compared by using F-tests. The differences discussed in the text as significant had p values <0.05. The relationship between the antigenicity and the length of the epitope was analyzed by linear regression. The value of correlation coefficient (r) lies between -1 to +1, where $r=0$ indicates no linear relationship, $r>0$ indicates a positive linear relationship (the closer to 1, the stronger the correlation), and $r<0$ indicates a negative linear relationship.

13. Immunization studies

13.1 Immunization with OprF::ME10aa215 and OprF

Two groups of 6-8 week old female BALB/c mice (H-2^d background) were immunized subcutaneously with 10 μg of FPLC-purified OprF or OprF::ME10aa215 with Titermax™ (CytRx Corp., Norcross, Georgia) on days 0 and 14. On day 28, the animals were injected with $2x10^8$ cells of heat-killed *E. coli* expressing the corresponding OprF or OprF::malarial epitope hybrid. Serum samples were obtained by tail-bleeding on days 7, 21 and 35. The control group was injected with 100 μl of PBS for all three injections.

13.2 Immunization with OprF::MEaa26 multiple-repeat hybrids and GST::malarial epitope fusion proteins

Groups of 6-8 week old female C57BL/6J mice (H-2^b background) were immunized subcutaneously with 20 μg of immunogens on days 0 and 21 and with 10 μg of immunogens on day 35 each suspended with 200 μg of Adjuvax (Alpha-Beta Technology, Worcester, MA) as an adjuvant in 200 μl total volume. Serum samples were obtained by tail-bleeding on days 0 and 28 and by whole body bleed on day 45.

13.3 Determination of antibody titers

The anti-OprF titer in serum samples was determined by ELISA using

FPLC-purified OprF from *P. aeruginosa* as the coating antigen (500 ng/ml). The anti-malarial epitope titer was determined by using affinity-purified GST::ME19 (2 μg/ml) or gel-purified OprF::ME19aa26 (1 μg/ml) as the coating antigens. GST::ME19 and OprF::ME19aa26 were chosen because these proteins demonstrated highest binding to the malarial epitope-specific mAbs in ELISA as compared to the corresponding proteins carrying the shorter versions of the epitope. In addition, the anti-malarial epitope peptide titer was determined by ELISA using the chemically synthesized peptide NANPNANPNANP $(NANP)_3$ (API, Edmonton, Alberta) as the coating antigen. The peptide (10μg/ml) in PBS was covalently linked to the wells of Reacti-Bind™ maleic anhydride activated polystyrene plates (Pierce) by incubation at 4°C for 16 h. The secondary antibody used in the assays was horse radish peroxidase-conjugated goat anti-mouse IgG (heavy and light chains) (BioRad).

13.4 Characterization of antisera by Western immunoblot analysis

To detect the presence of anti-OprF antibodies in the antisera, FPLC-purified OprF (20 μg per gel) was resolved by SDS-PAGE and was transferred onto PVDF membrane, the filter was then cut into slices and incubated with serum samples from the immunized animals at 1/1000 dilution or with a 1/3000 dilution of MA7-2 as a positive control. The presence of anti-GST antibodies in the antisera was detected in a similar manner using affinity-purified GST (20 μg per gel). The presence of anti-malarial epitope antibodies in the groups immunized with

GST::malarial epitope fusion proteins or OprF::malarial epitope hybrid proteins was detected by using outer membrane preparation containing OprF::ME10aa196 (50 μg per gel) or affinity-purified GST::ME19 fusion protein (20 μg per gel) respectively. Antisera from immunized animals were diluted 1/100. The malarial epitope-specific mAb pf2A.10 (1/3000 dilution) was used as a positive control. Subsequent incubations with secondary antibody and enzymatic staining were carried out as described by Mutharia and Hancock (1983).

RESULTS

Chapter one: Construction and characterization of OprF linker mutants

1. Introduction

Linker-insertion mutagenesis, either random or site-directed, has been employed to study the topology of several *E. coli* outer membrane proteins, including the maltoporin LamB (Boulain *et al.*, 1986), the phosphate-starvation-inducible porin PhoE (Bosch and Tommassen, 1987) and the major peptidoglycan-associated protein OmpA (Freudl *et al.*, 1986). The mutagenesis introduces extra amino acid residues at specific or non-specific sites of these membrane proteins, while leaving the rest of the proteins intact. As a result, it represents a more subtle modification of the protein in comparison to the other genetic approaches employed to study membrane topology such as alkaline phosphatase and β-galactosidase gene fusions (Manoil, 1991). In general, the extended surface loop regions are more likely to accommodate extra amino acids without gross perturbation of the protein structure. Indeed, the 3-dimensional structures of PhoE and LamB confirmed that all of the known insertion sites occurred within these loops (Cowan *et al.*, 1992; Schirmer *et al.*, 1995).

This chapter describes the expression of *oprF* in *E. coli*, a semi-random linker-insertion mutagenesis of *oprF* and the results of the characterization of the

linker mutants. The data obtained have raised the possibility that certain regions of OprF can be used to express longer foreign amino acid sequences.

1.2 Expression of *oprF* in *E. coli*

When attempts to produce recombinant OprF were initiated, plasmid vectors that allowed stable expression of cloned genes in *P. aeruginosa* were not available; hence, *E. coli* was chosen as the background strain for the expression of *oprF*. Earlier attempts to subclone *oprF* into high copy number plasmids were unsuccessful, probably due to the efficient expression of *oprF* from its own promoter in *E. coli* leading to over-expression lethality (Woodruff *et al.*, 1986). Plasmids that contained *P. aeruginosa* DNA containing *oprF* sequence were already available in this laboratory. However, the linker mutagenesis procedure required an OprF-encoding plasmid that did not contain a *Pst*I site. Therefore, the subcloning of *oprF* was necessary to generate a plasmid that would allow the expression and the linker mutagenesis of *oprF* in *E. coli*.

1.2.1 Construction of pRW3

The plasmid pHJ13, made by Helen Jost in this laboratory, was used for the initial subcloning of *oprF*. The plasmid contained two fragments of *P. aeruginosa* chromosomal DNA containing respectively the 5' and 3' portions of the OprF coding sequence inserted in the cloning vector pRK404 (Methods and

materials section 4, Fig. 5A). The putative -10 site of *oprF* in pHJ13 was mutated by adding a G:C nucleotide pair between nucleotides -9 and -10 to create a *Hin*dIII site. This procedure weakened the *oprF* promoter, avoiding the over-expression lethality in *E. coli*. To put the *oprF* coding region in one continuous reading frame, the *Sma*I fragment in pHJ13 had to be inserted in a reverse orientation (Fig. 5A). Therefore, the plasmid pHJ13 was digested with *Sma*I and the fragments were re-ligated. The final plasmid, pRW3, contained a 1.47 kb *Hin*dIII/*Kpn*I fragment carrying the entire *oprF* gene with a mutated promoter in the cloning vector pTZ19R. The transcription of the gene was in the same direction as the *lac* promoter (Fig.5B). In addition to the elimination of the *Pst*I sites, the subcloning also removed most of the chromosomal DNA flanking *oprF*, thus reduced the size of the plasmid by approximately 3 kb and could potentially improve the efficiency of further genetic manipulation.

1.2.2 Expression of *oprF* in different *E. coli* host strains

Since a certain genetic background in the host strain might be advantageous for the optimal expression of *oprF*, the plasmid pRW3 was transformed into different *E. coli* host strains to examine expression levels. The cell envelope samples of strains carrying pRW3 were analyzed by SDS-PAGE (Fig. 9). In most of the strains examined, the band corresponding to OprF was not readily observed in Coomassie blue stained gel (Fig. 9), indicating that the expression level of *oprF* in these *E. coli* strains was modest at best. Western immunoblotting with

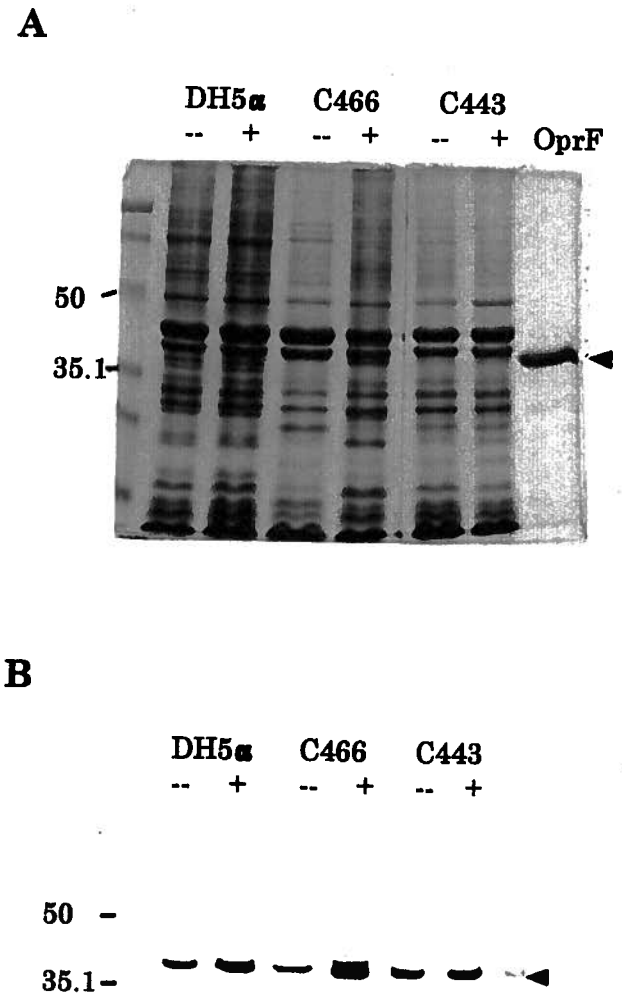


Figure 9. Expression of pRW3 in different *E. coli* host strains.

A. SDS-PAGE of cell envelope proteins from strains expressing pRW3. Protein samples (20μg/lane) were heated at 100°C for 10 min in solubilization buffer (2% SDS, 10% glycerol, 62.5mM Tris-HCl pH6.8) before loading. **B.** Western immunoblot of the same samples with an OprF-specific mAb MA7-1. Symbols: --, un-induced; +, induced with 1mM IPTG at 37°C for 4 h. Each lane contained 20μg of proteins. Numbers on the left indicated the positions of the relevant molecular mass standards (kDa). The position of OprF is indicated by an arrow head.

an OprF-specific monoclonal antibody showed that the expression level of *oprF* varied in different host strains. For instance, the un-induced levels of OprF in DH5α and C443(DH5αIQ) were comparatively higher than that in the membrane protease OmpT-deficient strain (C466). Similar levels of expression in the DH5α and DH5αIQ strains were unanticipated since the presence of the *lac* repressor was expected to suppress the expression from the *lac* promoter under un-induced condition. Although the basal expression level of *oprF* was lower in C466 ($OmpT^-$), it increased more significantly than that in the two DH5α strains upon IPTG induction. Since C466 is OmpT-deficient, the lack of this membrane protease might permit tolerance of a higher amount of OprF in the outer membrane of *E. coli*.

In the course of the subcloning, the 1.47 kb *Hin*dIII/*Kpn*I fragment containing *oprF* was also incorporated into the cloning vector pTZ18R, resulting in the transcription of the gene in the opposite orientation to the *lac* promoter. Expression of *oprF* from this plasmid was not observed, implying that the mutated *oprF* promoter was not functional. Furthermore, when *oprF* was in the same orientation as the *lac* promoter, the level of OprF production was increased upon IPTG induction, suggesting that the transcription of the gene was under the control of the *lac* promoter. Since DH5α is a widely used strain for genetic manipulation and since the basal level of *oprF* expression appeared to be sufficient for our purposes, it was used as the background strain for most of the characterization in the later stages of this study.

1.3 Semi-random linker mutagenesis with a kanamycin resistance cassette

The kanamycin resistance cassette used for the mutagenesis contained the gene encoding an aminoglycoside 3'-phosphotransferase, which confers kanamycin resistance. The gene was flanked by symmetric restriction enzyme sites. The restriction enzyme sites included *Pst*I, which were flanked by *Hin*cII, a blunt-end cutting enzyme (Methods and materials section 5, Fig. 6A). The plasmid pRW3 was linearized separately by partial digestion with 1 of 4 blunt-end cutting restriction enzymes as described in Methods and materials section 5.1. There were a total of 74 cleavage sites in pRW3 that were recognized by the four enzymes utilized, and 37 of these were within *oprF*. Low enzyme concentrations and/or ethidium bromide were used to favour the production of singly cut plasmids. After ligation of the restriction enzyme-linearized plasmid pRW3 with the 1.3 kb *Hin*cII fragment of the kanamycin resistance cassette, plasmid DNA from 100 clones that appeared to have the insertions within *oprF* were digested with *Pst*I. The *Pst*I digestion removed the kanamycin resistance cassette but left behind a residue of 12 nucleotide pairs in length, which was between the *Hin*cII sites and *Pst*I sites flanking both sides of the cassette (Fig. 6A). After re-ligation, 44 of the 100 kanamycin sensitive clones had regained the ability to produce OprF, as determined by colony immunoblotting with the OprF C-terminal-specific mAb MA5-8. Presumably, these clones represented insertion sites in OprF that could

accommodate the insertion of 4 extra amino acids without affecting the production of the protein. The rest of the kanamycin sensitive clones were unable to produce immunoreactive OprF or produced OprF that reacted only weakly with MA5-8 on colony immunoblots. Restriction enzyme analysis was used to map the insertion sites in each of the 100 plasmids. Sites in the 44 OprF-expressing plasmids could be placed into 10 unique groups (*e.g.* Fig. 10). The remaining 56 plasmids included those which demonstrated gene rearrangements or deletions, probably due to multiple cleavages of pRW3 by the restriction enzymes prior to ligation with the kanamycin resistance cassette.

1.4 Site-directed mutagenesis at the *Sal*I site

The unique *Sal*I site corresponding to amino acid position 188 (aa^{188}) of the mature OprF was a potentially interesting site to study because of its location in the cysteine-containing region. To obtain the same 4-amino acid insertion at the *Sal*I site, a 12 bp adaptor containing a *Pst*I site was inserted. The characterization of this mutant was performed simultaneously with the rest of the linker-insertion mutants.

1.5 Determination of insertion sites

The exact linker-insertion sites in at least one representative from each

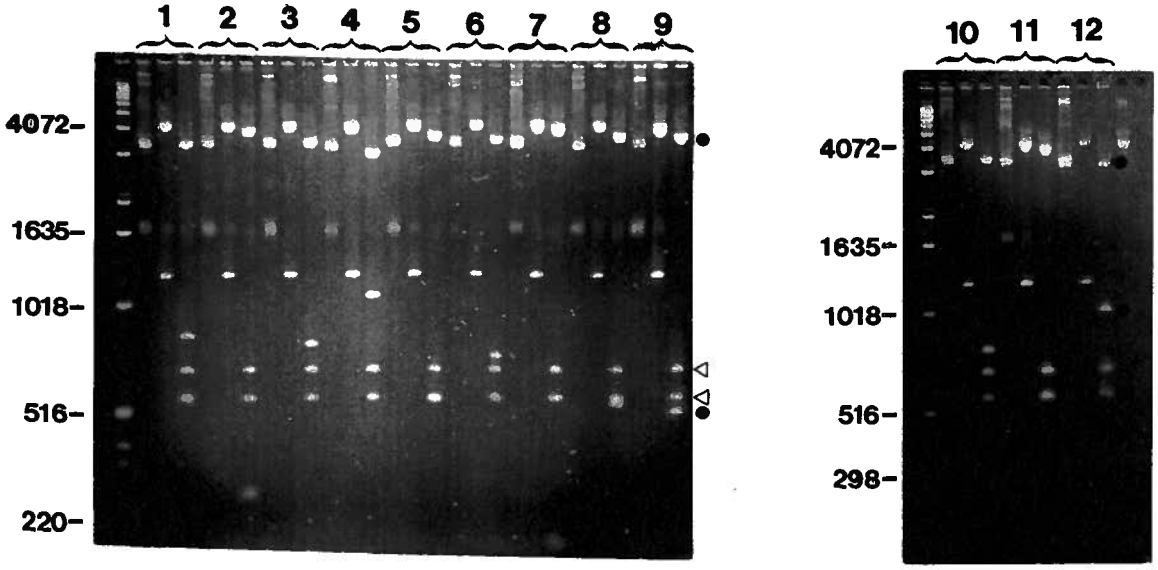


Figure 10. Restriction mapping of linker-insertion sites.

Restriction digest patterns of plasmid DNA from 12 of the kanamycin resistant clones. Each three lanes correspond to plasmid DNA from one clone treated in the following ways (from left to right): i. uncut, ii. *Pst*I digest, iii. *Pst*I/*Hin*dIII double digest. Each plasmid had 2 *Pst*I sites, one on each side of the cassette, and 2 *Hin*dIII sites, one within the cassette and one at the promotor region of *oprF*. In the *Pst*I digest lanes, the 1.3 kb and the 4.3 kb fragments represent the kanamycin cassette and the pRW3 plasmid respectively. In the *Pst*I/*Hin*dIII double digest lanes, the 600 bp and 700 bp fragments (marked by open triangles) correspond to the kanamycin cassette while the ~4 kb and the fourth fragments (marked by solid circles) correspond to pRW3. The sizes of the fourth fragments indicated the distance of the insertion sites from the *Hin*dIII site. Numbers on the left indicate the positions of molecular size markers (bp).

of the 10 groups of mutant plasmids encoding immunoreactive OprF were determined by DNA sequencing. The nucleotide positions of the linker insertions and the identities of the 4 inserted amino acids for 11 linker-insertion mutants and 1 site-directed insertion mutant (pRW307) are summarized in Table IV. Seven of the mutant plasmids that did not express immunoreactive OprF were also analyzed by sequencing (Table V). The results revealed that two of the mutant plasmids had incorporated the 12 bp insert at nucleotide positions +433 and +795 of *oprF* respectively, but the reading frames at these insertion sites both led to the translation of stop codons from the 12 bp insert. The other four mutant plasmids analyzed represented deletions of part of the *oprF* sequence, and the study of these mutants was not further pursued in this work. Only one (pRW303) of the seven mutant plasmids analyzed showed the incorporation of a 12 bp insert in the OprF coding region without any other genetic alteration or change of reading frame. The inability of this clone to demonstrate an OprF positive phenotype on colony or Western immunoblots suggested the "non-permissiveness" of this insertion site.

1.6 Expression and cellular localization of linker mutants

In addition to the signal peptide, the primary sequence of mature membrane proteins is believed to carry the targeting signal for the export of these proteins across the bacterial membrane (MacIntyre and Henning, 1990). Therefore, it is possible that insertion of extra amino acids in the primary sequence of OprF

Table IV. Summary of insertion sites of 11 linker-insertion mutants and one site-directed insertion mutant and the identities of the inserted amino acids.

Plasmids	Insertion sites (nucleotides)[a]	Insertion sites (amino acid)[a]	Amino acids inserted	Apparent mol. mass[e] (kDa)
pRW301	77	Gly-2	TCRS	41
pRW302	148	Ala-26[c]	PAGP	36
pRW303	198	Glu-42	DLQV	NE
pRW305	463	Ala-131[c]	PAGP	40
pRW306	476	Gly-135	TCRS	35
pRW307[b]	636	Val-188	PAGP	35
pRW308	658	Ala-196[c]	PAGP	35
pRW309	710	Arg-213	TCRS	35
pRW310	717	Gln-215	DLQV	35
pRW311	764	Ser-231[d]	TCRS	36
pRW312	939	Arg-290	TCRS	35
pRW314	1001	Gly-310	TCRS	28

[a] Position 1 is the translational start site (Duchene *et al.*, 1988). The amino acid numbers correspond to the mature native OprF.

[b] pRW307 was generated by inserting a *Sal*I adaptor that contained a *Pst*I site into the *Sal*I site corresponding to aa[188].

[c] The alanine residue at these insertion sites was replaced by a glycine.

[d] The serine residue at the insertion site was replaced by an arginine.

[e] The apparent molecular mass of wild type OprF expressed by pRW3 is 35kDa. NE, no expression.

Table V. Summary of six of the deletion mutants isolated during linker-insertion mutagenesis.

Plasmids	Insertion sites (nucleotides)[a]	Insertion sites (amino acid)	Amino acids inserted or mutations
pRW304	433	Tyr-121	stop codon[b]
pRW313	1001	Gly-310	deletion[c]
pRW315	795	Tyr-245	stop codon[b]
pRW316	898	Val-279	TCRS + 24 aa [d]
pRW317	399	Ala-114	PAGP + 83 aa[d]
pRW318	296	Gly-78	DLQV + 97 aa[d]

[a] Position 1 is the translational start site (Duchene *et al.*, 1988).

[b] The first codon encoded by the linker is a stop codon.

[c] The linearized pRW3 was cleaved at multiple sites so that the rest of the coding region was deleted.

[d] The linearized pRW3 was cleaved at multiple sites. The extra amino acids encoded were due to a frame shift and represented the translated sequence before the first stop codon was encountered.

might affect its transport to the outer membrane. To examine the cellular localization of the OprF linker mutants, the outer membrane of *E. coli* containing the linker mutant plasmids was isolated by using Triton X-100 extraction procedure (Schnaitman, 1971). SDS-PAGE analysis of the outer membrane fractions demonstrated the presence of OprF and OprF linker mutants, suggesting the association of these proteins in the outer membrane of *E. coli* (Fig.11). The electrophoretic mobility of all of the linker mutants was modified by pre-treatment with 2-mercaptoethanol, indicating that the inserted amino acid residues did not perturb the formation of the OprF cysteine disulphide bonds (Fig. 11, lanes 6 & 10).

The apparent molecular mass of the OprF linker mutants carrying an insertion at aa^{2} and aa^{131} (encoded by plasmids pRW301 and pRW305 respectively) was noticeably greater than that of the wild type (Table IV). The protein expressed by pRW305 migrated with a mobility similar to that of the heat-modified (unfolded) form of OprF (Fig. 12, lane 3), suggesting that the incorporation of the 4-amino acid linker may have increased the susceptibility of the protein to denaturation by heating in SDS. Plasmids pRW309 (aa^{213}) (Fig. 11, lane 9) and pRW311 (aa^{231}) (data not shown) each directed the expression of an intense band with an apparent molecular mass of 70 kDa, likely corresponding to the trimeric form of OprF (Mutharia and Hancock, 1985). After 2-mercaptoethanol treatment, a much more intense monomer band with an apparent molecular mass of 35 kDa was observed in the same samples (e.g. Fig. 11, lane 5), suggesting that insertion of the linker at aa^{213} and aa^{231} may enhance the association of SDS-stable oligomers.

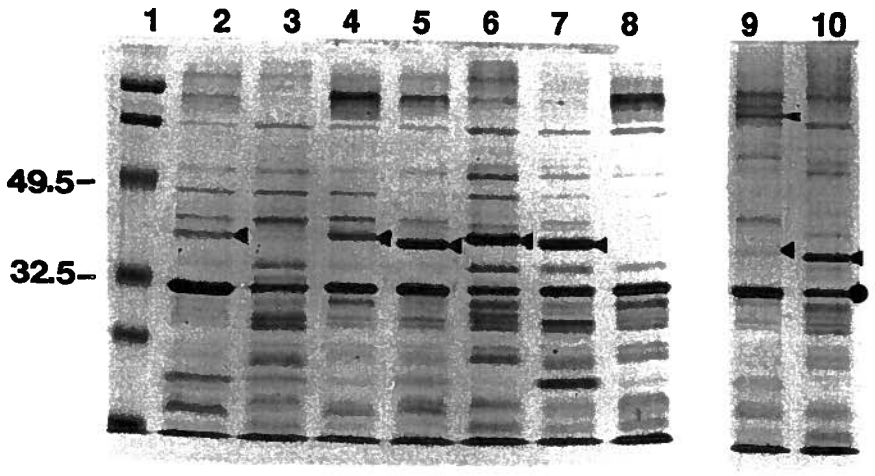


Figure 11. Cellular localization of OprF linker mutants.

SDS-PAGE of outer membrane samples of *E. coli* DH5αF' strains carrying the pRW3-derived plasmids. Samples were prepared by using Triton X-100 extraction procedures (Schnaitman, 1971) and were incubated at 37°C for 10 min in solubilization buffer with (lanes 2-8) or without (lanes 9 and 10) 4% 2-mercaptoethanol before loading. The gel was stained with Coomassie blue after electrophoresis. Each lane contained ~16 μg protein from each sample. Plasmids present in the lanes were: 2, pRW302 (aa^{26}); 3, pRW305 (aa^{131}); 4, pRW306 (aa^{135}); 5, pRW309 (aa^{213}); 6, pRW310 (aa^{215}); 7, pRW3; 8, pTZ19R; 9, pRW309; 10, pRW310. The amino acid positions of the insertions are indicated in the brackets. OprF monomer bands are indicated by triangles; the OprF SDS-stable trimer is indicated by an arrow head; the position of OmpA is indicated by a solid circle. Positions of relevant molecular mass markers (kDa) are indicated on the left. Due to the low level of expression, protein expressed by pRW305 (lane 3) was localized by Western immunoblotting.

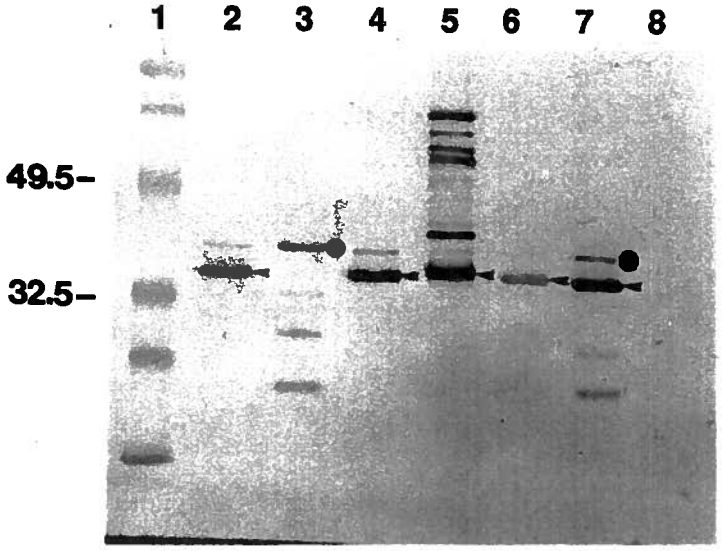


Figure 12. Expression of OprF linker mutants.

Western immunoblot analysis of outer membrane samples of *E. coli* DH5αF' strains expressing the pRW3 derived plasmids. The OprF-specific mAb MA7-5, which recognizes an epitope that is not interrupted by the insertions, was used. Samples were heated at 100°C for 10 min in solubilization buffer before loading. Each lane contained about 8 μg protein. Plasmids present were, lanes: 2, pRW302; 3, pRW305; 4, pRW306; 5, pRW309; 6, pRW310; 7, pRW3; 8, pTZ19R. Numbers on the left indicate the positions of the relevant molecular mass standards (kDa). OprF monomer bands are indicated by arrow heads. The position of the heat-modified form of OprF is indicated by a circle. Bands corresponding to oligomeric and LPS-associated forms of OprF are visible in some lanes.

The level of production of two of the linker mutants, encoded by pRW302 and pRW306, was noticeably lower than that of the others, as determined by their abundance relative to the other *E. coli* proteins (Fig. 11, lanes 2 & 4), indicating that insertions at these sites may lead to reduced protein production or unstable products. Mutants with insertion sites at the C-terminal end of the proteins (e.g. those encoded by pRW312 and pRW314) produced OprF variants that were substantially but not completely degraded to smaller fragments, including a predominant 28 kDa fragment (Table IV). This confirmed the results of Finnen *et al.* (1992), that the C-terminal regions of OprF were required for the resistance of the protein to cellular proteases.

1.7 Monoclonal antibody reactivity of linker mutants

Most of the OprF-specific mAbs available in the laboratory recognized conformational epitopes and thus can be used as probes to examine the general conformation of the OprF linker mutants. Outer membrane samples containing the linker mutants were analyzed by Western and colony immunoblotting using the series of OprF-specific monoclonal antibodies (Table VI). The results demonstrated that the OprF derivatives expressed by 5 of the plasmids (pRW301, 302, 306, 309 and 310) were reactive with all 10 monoclonal antibodies, indicating the retention of native OprF structure. In 6 other mutants, specific OprF epitopes were disrupted by the insertion of the 4-amino acid linker. However, the reactivity of

Table VI. Summary of monoclonal antibody reactivity of OprF linker mutants.

Plasmid	Insertion site (aa position)	Monoclonal antibody reactivity[a]									
		7-1	7-2	7-3	7-4	7-5	7-6	7-7	7-8	4-4	5-8
pRW301	Gly-2	w	+	+	+	+	+	+	+	+	+
pRW302	Ala-26	+	+	+	+	+	+	+	+	+	+
pRW303	Glu-42	-	-	-	-	-	-	-	-	-	-
pRW305	Ala-131	-	+	+	+	+	+	+	+	+	+
pRW306	Gly-135	+	+	+	+	+	+	+	+	+	+
pRW307	Val-188	+	+	+	+	+	+	+	-	+	+
pRW308	Gly-196	+	+	+	+	+	+	+	-	-	+
pRW309	Arg-213	+	+	+	+	+	+	+	+	+	+
pRW310	Gln-215	+	+	+	+	+	+	+	+	+	+
pRW311	Ser-231	+	+	-	-	-	+	-	+	+	+
pRW312	Arg-290	+	+	-	-	-	+	-	+	+	+
pRW314	Gly-310	+	w	-	-	-	w	-	+	+	-

[a] Measured by colony immunoblot and Western immunoblot analyses of outer membrane samples. Symbols: +, reactivity equivalent to wild type OprF expressed by pRW3; -, no reactivity; w, weak reactivity.

these mutated proteins with the majority of the monoclonal antibodies suggested the retention of substantial native OprF structure in these mutants. OprF expressed by pRW303 was an exception. Despite the fact that DNA sequencing demonstrated that only 12 bp were inserted and no premature stop codon or change in reading frame occurred, it did not produce any OprF product that could be detected by the OprF-specific monoclonal antibodies on immunoblots or visualized in Coomassie blue stained SDS-PAGE gel of outer membrane samples and whole cell lysates. Thus it was assumed that this site was "non-permissive" for the insertion of 4 amino acids.

1.8 Membrane configuration of linker mutants in *E. coli*

To permit conclusions regarding the structure of OprF to be drawn based on linker-insertion mutagenesis in *E. coli,* it was necessary to examine whether the structure of OprF and its linker mutants in *E. coli* reflected that of OprF in *P. aeruginosa.* To further examine the configurations of the OprF derivatives in the outer membrane of *E. coli*, trypsin accessibility assays and indirect immunofluorescence labelling experiments were conducted.

1.8.1 Trypsin sensitivity assays

Outer membrane porins tend to be protease resistant (Paul and Rosenbusch, 1985) by virtue of their extensive β-sheet structure with linking

surface loops that are tightly packed or folded in towards the porin channel (Weiss *et al.*, 1991; Cowan *et al.*, 1992; Schirmer *et al.*, 1995). It was previously demonstrated that purified OprF or OprF in outer membrane preparations are partly cleaved by trypsin to a core 28 kDa fragment, and that increasing concentrations of trypsin or increasing length of treatment time fails to cause further proteolysis (Mutharia and Hancock, 1985).

Trypsin treatment of outer membranes from *E. coli* DH5αF' expressing the parental plasmid pRW3 resulted in substantial retention of full-sized OprF and partial proteolysis to a 28 kDa fragment that could be detected by the OprF-specific mAb 7-1 (Fig. 13, lanes 8, 9, and 10). Similar results were obtained after trypsin treatment of outer membranes from cells containing plasmids pRW302 and pRW306 (data not shown), which encoded OprF linker mutants with N-terminal insertions in OprF (Fig. 13, lane 1, Table VII). Mutant proteins with C-terminal insertions in OprF (i.e. those encoded by pRW309, pRW310, pRW311 and pRW312) were completely cleaved to the 28 kDa fragment after trypsin treatment (Fig. 13, lanes 4-7; Table VII). Proteins encoded by plasmids pRW307 and pRW308, which carried insertions in the central cysteine disulphide region (aa^{188} and aa^{196} respectively), were cleaved by trypsin to a 24 kDa fragment, instead of (pRW307; Fig. 13, lane 2) or in addition to (pRW308; Fig. 13, lane 3) the 28 kDa fragment. Based on previous studies (Finnen *et al.*, 1992), such a 24 kDa fragment might be expected if cleavage occurred near aa^{190} within the cysteine-containing region, suggesting localized modification of OprF by these insertions rendered this region

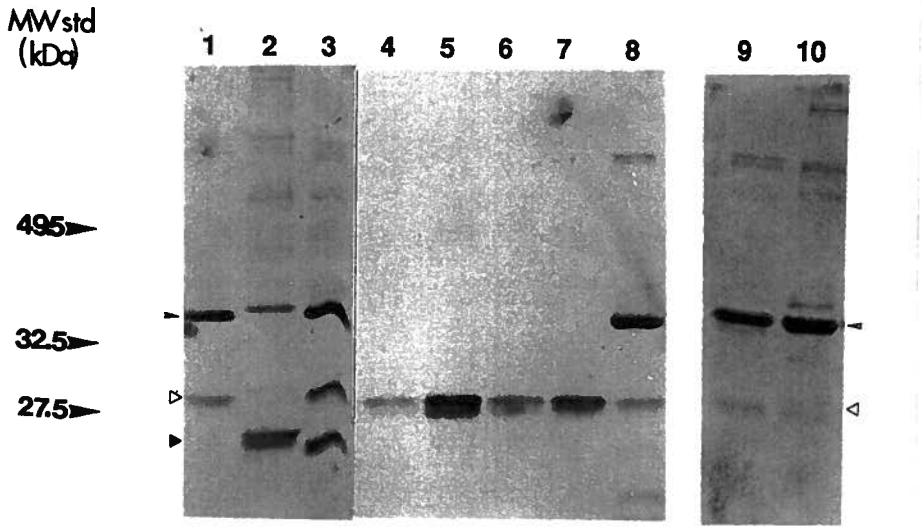


Figure 13. Trypsin sensitivity of linker mutants in outer membranes.

Western immunoblot analysis of trypsinized outer membrane samples containing OprF linker mutants. Samples were treated with trypsin (0.1 mg/ml) at 37°C for 60 min, and then heated at 88°C for 10 min in solubilization buffer. The plasmids corresponding to the OprF linker mutants contained in each lane are: 1, pRW302; 2, pRW307; 3, pRW308; 4, pRW309; 5, pRW310; 6, pRW311; 7, pRW312; 8, pRW3; 9, pRW3; 10, pRW3 (untreated). OprF monomer band is indicated by an arrow head. The 28 kDa and 24 kDa trypsin-resistant core fragments are indicated by open and solid triangles, respectively. The OprF N-terminal specific mAb MA7-1 was used for immunodetection. The positions of relevant molecular mass standards (kDa) are indicated on the left.

Table VII. Summary of trypsin sensitivity assays of OprF linker mutants in *E. coli* outer membrane, DH5α and C386 whole cells.

Plasmid	Apparent mol. mass[a] (kDa)	Apparent mol. mass after trypsin treatment[a] (kDa)		
		Outer membrane	DH5α whole cell	C386 whole cell
pRW3	35	35, 28	35	28
pRW301	41	ND	ND	ND
pRW302	36	36, 28	36	36, 28
pRW305	40[b]	24, 20, 18[b]	40[b]	ND
pRW306	35	35, 28	35	ND
pRW307	35	24, 35	35	24, 28
pRW308	35	35, 24, 28	35	24, 28
pRW309	35	28	35	28
pRW310	35	28	35, 28	ND
pRW311	36	28	ND	ND
pRW312	35	28	28	28
pRW314	28[c]	ND	28	ND

[a] As estimated on Western immunoblot with MA7-1. Where more than one band appeared, they are listed in order of abundance. ND, not determined.

[b] Tested with MA4-4 since this mutant OprF derivative was non-reactive with MA7-1.

[c] 35 kDa was observed as a minor band.

susceptible to trypsin. Plasmid pRW305 (aa^{131}) expressed an OprF linker mutant that showed unexpected trypsin-cleavage pattern with a predominant 24 kDa product and minor 20 kDa and 18 kDa products (Table VII). This implied a substantial localized disruption of OprF structure, consistent with the observed susceptibility to heat denaturation.

Trypsin treatment of whole cells of *E. coli* DH5αF' expressing the wild type or mutant *oprF* plasmids did not result in proteolysis of OprF or its linker mutant derivatives (Table VII), with the exceptions of those C-terminal insertion mutants encoded by plasmids pRW310, pRW312 and pRW314. On the other hand, the proteolysis patterns of OprF and the linker mutants in whole cells of C386 (*ompA, lpp*) resembled that of these proteins in isolated outer membranes. Since the C-terminal of OmpA is highly homologous to that of OprF, the differences in the results from these two host strains might have been due to the interaction of this region of OmpA and OprF, affecting the accessibility of the trypsin cleavage site in OprF and some of its mutants.

1.8.2 Immunofluorescence labelling

To examine whether the OprF linker mutants had surface-exposed regions, immunofluorescence labelling was carried out using *E. coli* strains expressing selected linker mutant plasmids and monoclonal antibodies which bind to surface epitopes in the N-terminus (MA7-1), central region (MA7-8), and C-terminus (MA5-8) of OprF (Table VIII). To avoid the presence of OmpA, which

Table VIII. Results from indirect immunofluorescence labelling of intact *E. coli* C386 cells containing different plasmids.

Plasmid	Immunofluorescence with monoclonal antibodies[a]		
	MA7-1	MA7-8	MA5-8
no plasmid	-	-	-
pRW3	++	++	+
pRW302	++	++	+
pRW307	++	+	+
pRW308	+	-	+
pRW312	++	++	+

[a] ++,positive labelling ; +, weak labelling; -,negative labelling.

might affect the accessibility of OprF epitopes, an OmpA-deficient strain (C386) was used as the background strain for the expression of the plasmids. In each case, regardless of the trypsin susceptibility of the respective OprF mutants in intact cells, immunoreactivity followed precisely the pattern observed in both colony immunoblots and Western immunoblots (Table VI). Taken together, the OprF linker mutants were probably inserted in the outer membrane in the native conformation, as reflected by their trypsin resistance and surface exposure.

1.9 Summary

The results presented here demonstrated the identification of 11 unique "permissive" sites in OprF, which were sites that allowed the insertion of 4 extra amino acids without grossly affecting the production, folding and stability of the protein. The characterization of OprF linker mutants provided compelling evidence that the general conformation and membrane configuration of these proteins in *E. coli* were very similar to that of OprF in *P. aeruginosa*. The evidence included: 1). The presence of the mutant proteins in purified *E. coli* outer membranes, 2). The apparently correct formation of disulphide bonds as judged by the 2-mercaptoethanol modifiability of the OprF mutants, 3). The reactivity of the proteins with at least three of the mAbs MA7-3 through MA7-8 and MA4-4, which apparently recognize conformational epitopes (Finnen *et al.*, 1992; Rawling *et al.*, 1995), 4). The demonstration of a trypsin-resistant core structure in most mutants

contained in outer membranes and the general resistance of OprF to trypsin cleavage in intact cells, and 5). The correct surface localization of some of the OprF epitopes as examined by immunofluorescence labelling of intact cells with OprF-specific monoclonal antibodies.

The information obtained from this study established the "permissiveness" of the characterized linker-insertion sites in OprF, thus opening up the possibility that OprF could be used as a carrier for the presentation of foreign amino acid sequences. The 12 bp insertion in *oprF* resulted from the linker mutagenesis procedure provided a unique *Pst*I site, which was useful for the cloning of foreign DNA sequences.

Chapter two: Construction, characterization and purification of OprF::malarial epitope and GST::malarial epitope hybrid proteins

2.1 Introduction

Epitope-insertion studies have been used to demonstrate the potential of outer membrane proteins as carriers for the expression of foreign antigenic determinants (Charbit *et al.*, 1991; Agterberg *et al.*, 1990b). The examination of the surface exposure of the inserted epitope can also provide information about the membrane topology of the carrier protein. To further investigate the flexibility and limitations of OprF as a carrier for epitope presentation, an epitope-insertion study was carried out using the four-amino acid repeating epitope (NANP) of the circumsporozoite protein of the human malarial parasite, *Plasmodium falciparum*, as a model epitope. In this chapter, the construction and characterization of two series of OprF::malarial epitope hybrid proteins, the positional and multiple-repeat hybrids, will be described. In addition, the construction and characterization of two versions of GST::malarial epitope fusion proteins, which were used to monitor the anti-malarial epitope response in serum samples from immunized animals, will also be described.

2.2 Construction of OprF::malarial epitope hybrid proteins

Previous linker-insertion mutagenesis had identified "permissive" sites in OprF that can accommodate 4 extra amino acid residues. To further explore the "permissiveness" of the sites, a 10-amino acid malarial epitope was genetically inserted into these sites to generate a series of OprF::malarial epitope positional hybrids. In addition, 4 different lengths of the malarial epitope were inserted into 3 of the "permissive" sites to generate a series of multiple-repeat hybrids. These two series of OprF::malarial epitope hybrids not only helped to explore the "permissiveness" of the OprF insertion sites, they also provided a tool for the study of the effects of insertion position and length of the epitope on epitope presentation in the OprF system.

2.2.1 Positional hybrids

The previous linker-insertion mutagenesis study generated a series of *oprF* linker mutants that carried a unique *Pst*I site at different positions of the gene. To construct the series of OprF::malarial epitope hybrid proteins expressing the epitope at different positions of OprF, oligonucleotides encoding the malarial epitope sequence (NANPNANPNA) were inserted into the *Pst*I sites of the *oprF* linker mutants. Three sets of oligonucleotides were required to accommodate the three possible reading frames at the *Pst*I sites. The positions and reading frames of the insertions were confirmed by DNA sequencing. Table IX summarizes the

Table IX. Summary of OprF::malarial epitope positional hybrids.

Plasmid	Insertion site	Amino acids inserted[a]	Surface exposure of the epitope[d]
pRW302.1M	Ala-26	PAP(**ME**)[a]GHAGP	+
pRW302.2M	Ala-26	PA{P(**ME**)GHA}$_2$GP	
pRW306.2M	Ala-135[b]	TC{NP(**ME**)C}$_2$RS	+
pRW307.1M	Val-188	DLQ(**ME**)LDVQV	+
pRW308.1M	Ala-196	PAP(**ME**)GHAGP	+
pRW309.1M	Arg-213	TCNP(**ME**)CRS	+
pRW309.3M	Arg-213	TC{NP(**ME**)C}$_3$RS	
pRW310.1M	Gln-215	DLQ(**ME**)LDVQV	+
pRW311.1M	Ser-231	TCNP(**ME**)CRS	+
pRW311.5M	Ser-231	TC{NP(**ME**)C}$_5$RS[c]	
pRW312.1M	Arg-290	TCNP(**ME**)CRS	+
pRW312.4M	Arg-290	TC{NP(**ME**)C}$_4$RS[c]	
pRW314.1M	Gly-310	TCNP(**ME**)CRS	+

[a] **ME**=NANPNANPNA.

[b] The site at aa^{135} was also found to be permissive for the expression of two copies of the epitope insert, but a hybrid that carried a single copy of the epitope was not obtained and therefore this site was not included in this study.

[c] The numbers of insert in these cases were estimated by 2% agarose gel.

[d] +, the malarial epitope was detectable on the cell surface by indirect immunofluorescence studies using a malarial epitope-specific monoclonal antibody.

series of OprF::malarial epitope positional hybrids and the identities of the inserted amino acid residues. The amino acid residues immediately flanking the malarial epitope varied according to the reading frame at the linker-insertion sites. In the course of cloning, hybrids that had incorporated multiple copies of the insert were also isolated. However, due to the volume of work involved and the clarity of presentation, only two of these hybrids were chosen for further characterization.

2.2.2 Multiple-repeat hybrids

To study the length effect on epitope presentation in the OprF system, OprF::malarial epitope hybrids with different lengths of the epitope insert were required. Three "permissive" sites in OprF (aa^{26}, aa^{196} and aa^{213}) were selected for the construction of such multiple-repeat hybrids. The choice of these sites was based on their positions in the protein (the N-terminus, the middle region and the C-terminus) and the stability of the corresponding positional hybrids. Table X summarizes the multiple-repeat hybrids constructed in this study. These hybrids carried 7, 11, 15 and 19 amino acids corresponding to the malarial epitope, each with an increment of one tetramer repeat. In addition, nine flanking amino acid residues, which were the result of the previous linker-insertion mutagenesis procedures and genetic cloning, were also added.

Table X. Summary of OprF::malarial epitope multiple-repeat hybrids.

Insertion site	Plasmid	Amino acids inserted[a]
Ala-26	pRW302.7	PAARNPNANPNLDAGP
	pRW302.11	PAAR(NPNA)$_2$NPNLDAGP
	pRW302.15	PAAR(NPNA)$_3$NPNLDAGP
	pRW302.19	PAAR(NPNA)$_4$NPNLDAGP
Ala-196	pRW308.7	PAARNPNANPNLDAGP
	pRW308.11	PAAR(NPNA)$_2$NPNLDAGP
	pRW308.15	PAAR(NPNA)$_3$NPNLDAGP
	pRW308.19	PAAR(NPNA)$_4$NPNLDAGP
Arg-213	pRW309.7	TCTRNPNANPNLDCRS
	pRW309.11	TCTR(NPNA)$_2$NPNLDCRS
	pRW309.15	TCTR(NPNA)$_3$NPNLDCRS
	pRW309.19	TCTR(NPNA)$_4$NPNLDCRS

[a] The amino acid residues corresponding to the malarial epitope are underlined. The flanking amino acids PA__GP and TC__RS were the results of the previous linker-insertion mutagenesis procedures.

2.3 Characterization of OprF::malarial epitope hybrid proteins

To examine if the "permissive" sites previously identified by linker-insertion mutagenesis were "permissive" for the expression of the longer epitope sequence, the OprF::malarial epitope hybrid proteins were characterized in terms of their expression, cellular localization and reactivity with the series of OprF-specific monoclonal antibodies.

2.3.1 Expression of hybrid proteins

The expression of the hybrid proteins was examined by Western immunoblotting of whole cell lysates of strains carrying the hybrid plasmids. The hybrid plasmids containing the epitope-encoding oligonucleotides at eight different sites expressed proteins that were reactive with both OprF-specific and malarial epitope-specific mAbs on Western immunoblots (Fig. 14). The apparent molecular mass of these proteins was slightly higher than that of native OprF, which was consistent with the presence of additional malarial epitope sequences in the hybrid proteins. Plasmids pRW302.2M (aa^{26}) and pRW309.3M (aa^{196}) encoded proteins of higher apparent molecular mass than plasmids carrying a single copy of the insert at the same sites (Fig.14, compare lanes 1 & 2, and lanes 5 & 6). The lanes corresponding to plasmids pRW307.1M (aa^{188}) and pRW308.1M (aa^{196}) (lanes 3 and 4) showed a more prominent upper band which apparently corresponded to the heat-modified form of the protein. This implied that the presence of extra amino

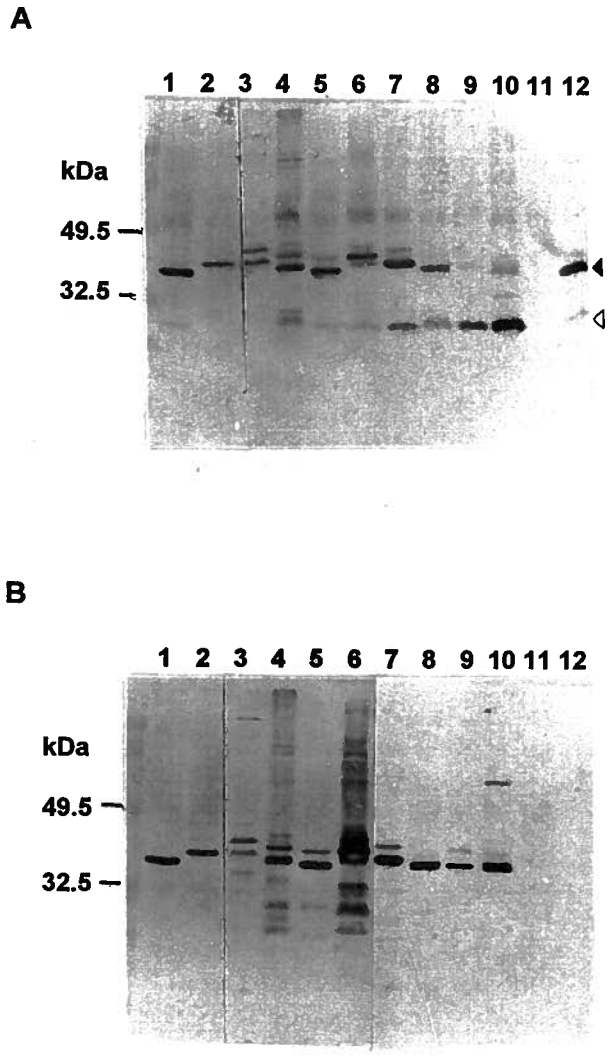


Figure 14. Expression of OprF::malarial epitope positional hybrids.

Western immunoblots of whole cell lysates of *E. coli* DH5αF' strains expressing various OprF::malarial epitope hybrid proteins after reaction with **A)** an OprF specific monoclonal antibody MA7-1 and **B)** a malarial epitope-specific mAb pf2A.10 . Samples were resuspended in solubilization buffer and heated at 100°C for 10 min before loading. Plasmids expressed in the samples in lanes were: 1, pRW302.1M; 2,pRW302.2M; 3, pRW307.1M; 4, pRW308.1M; 5, pRW309.1M; 6, pRW309.3M; 7, pRW310.1M; 8, pRW311.1M; 9, pRW312.1M; 10, pRW314.1M; 11, pTZ19R; 12, pRW3. The bands corresponding to wild type OprF and the N-terminal degradation product are indicated by a solid and an open triangle, respectively. In some lanes, the bands corresponding to OprF dimers, oligomers and protease degradation products are visible. The positions of relevant molecular mass standards (kDa) are indicated on the left.

acids in the cysteine-containing region of OprF might have affected the local conformation, and thus rendered the protein more susceptible to heat denaturation (Hancock and Carey, 1979). Lanes 7 to 10 demonstrated an increase in abundance of the 28 kDa degradation product, which failed to react with the malarial epitope-specific mAb, suggesting that it represented the N-terminal part of these proteins lacking the malarial epitope sequences. This result was consistent with previous findings that C-terminal perturbations rendered these OprF derivatives more susceptible to cellular proteases (Finnen *et al.*, 1992).

Oligonucleotides encoding the malarial epitope were also inserted into sites corresponding to aa^{2} and aa^{131} of OprF, but no hybrid proteins were detected on Western immunoblots. These two sites appeared to be either "non-permissive" for the insertion of more than 4 amino acids (encoded by the linker insertion) or "non-permissive" for the expression of the malarial epitope sequence.

2.3.2 Cellular localization of hybrid proteins

The insertion of extra amino acid residues into the mature sequence of a membrane protein affects its primary and possibly secondary structure and therefore might interfere with its transport to the native subcellular compartment. SDS-PAGE analysis of outer membrane preparations from strains carrying the hybrid plasmids was performed to examine the outer membrane localization of the OprF::malarial epitope hybrid proteins. It was shown that the hybrid proteins were associated with the outer membranes (Fig. 15). Due to the genetic background of

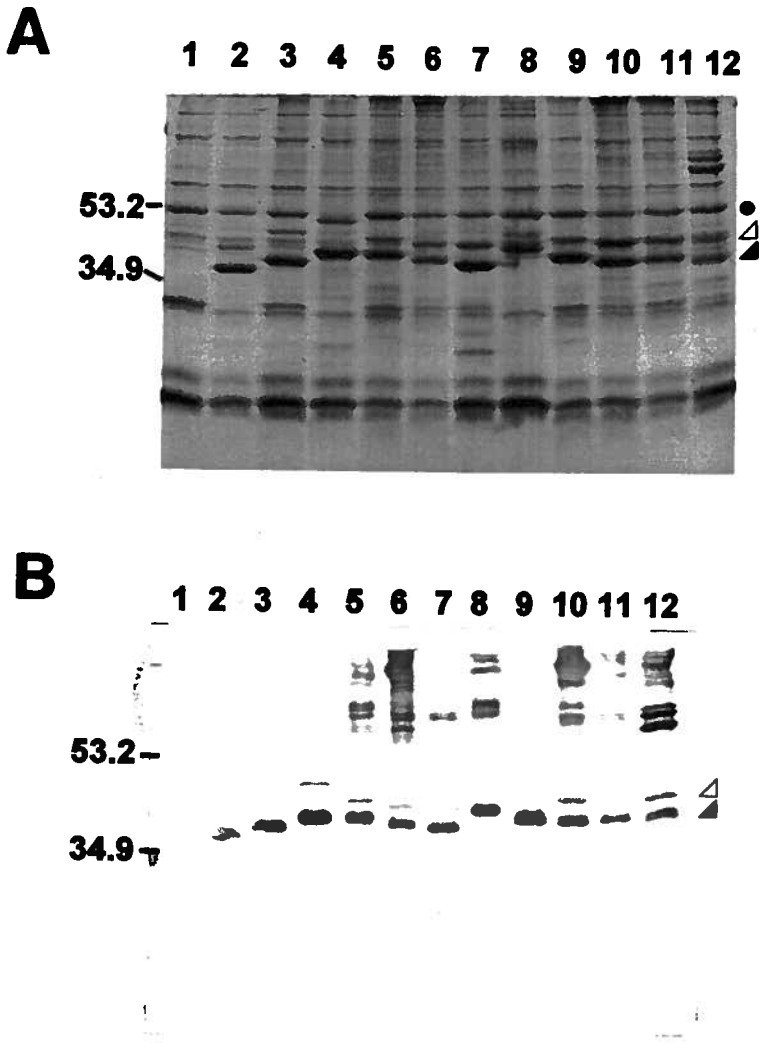


Figure 15. Cellular localization of OprF::malarial epitope positional hybrids.

A. Sucrose gradient outer membrane preparations of *E. coli* C158 (*ompA, ompC, phoE*) strains expressing OprF::malarial epitope positional hybrid proteins. Samples were heated at 100°C for 10 min in solubilization buffer before loading. The gel was stained with Coomassie blue after electrophoresis. **B.** Western immunoblot analysis of the same samples with MA7-1. Plasmids expressed in the strains were: 1, pTZ19R; 2, pRW3; 3, pRW302.1M(aa^{26}) ; 4, pRW302.2M(aa^{26}); 5, pRW307.1M(aa^{188}); 6, pRW308.1M(aa^{196}); 7, pRW309.1M(aa^{213}); 8, pRW309.3M(aa^{213}); 9, pRW310.1M(aa^{215}); 10, pRW311.1M(aa^{231}); 11, pRW312.1M(aa^{290}); 12, pRW314.1M(aa^{310}). The amino acid positions of the insertion sites are in brackets. The positions of relevant molecular mass standards (kDa) are indicated on the left. The positions of the native and heat-modified forms of the proteins are indicated by solid and open triangles respectively. The band corresponding to an *E. coli* outer membrane protein is indicated by a solid circle. Bands corresponding to OprF oligomers are visible in some lanes.

the host strain (*ompA, ompC, phoE*) and the growth conditions of the bacterial cultures used for the outer membrane preparations (high osmolarity in the presence of 0.1% glucose to suppress the production of OmpF and LamB respectively), the hybrid proteins appeared to be one of the major species present in the preparations (Fig. 15). The slight differences in apparent molecular mass of the hybrids might have been due to the interactions between the inserted amino acid residues and the local OprF amino acid sequence which affected the electrophoretic mobilities of these proteins. Western immunoblot analysis of the same outer membrane preparations revealed minor and comparable levels of protease degradation products in all of the samples, implying that the 28 kDa product observed in the whole cell lysates was not associated with the outer membrane (Fig. 15B).

Similar to the positional hybrids, the OprF::malarial epitope multiple-repeat hybrids were also expressed in the outer membrane of *E. coli* (Fig. 16). The length increment of the inserted epitope in each set of the hybrids was reflected by the stepwise increase in the apparent molecular mass of the hybrid proteins. The series of multiple-repeat hybrid proteins carrying insertions at aa^{26} formed inclusion bodies which fractionated with the outer membrane. The bands corresponding to the inclusion body form of the hybrid proteins migrated at higher apparent molecular mass than the membrane bound form. In addition, while the membrane bound form of the proteins was 2-mercaptoethanol modifiable due to the presence of disulphide bonds, the gel mobility of the inclusion body form of the proteins was not affected by 2-mercaptoethanol, indicating the absence of

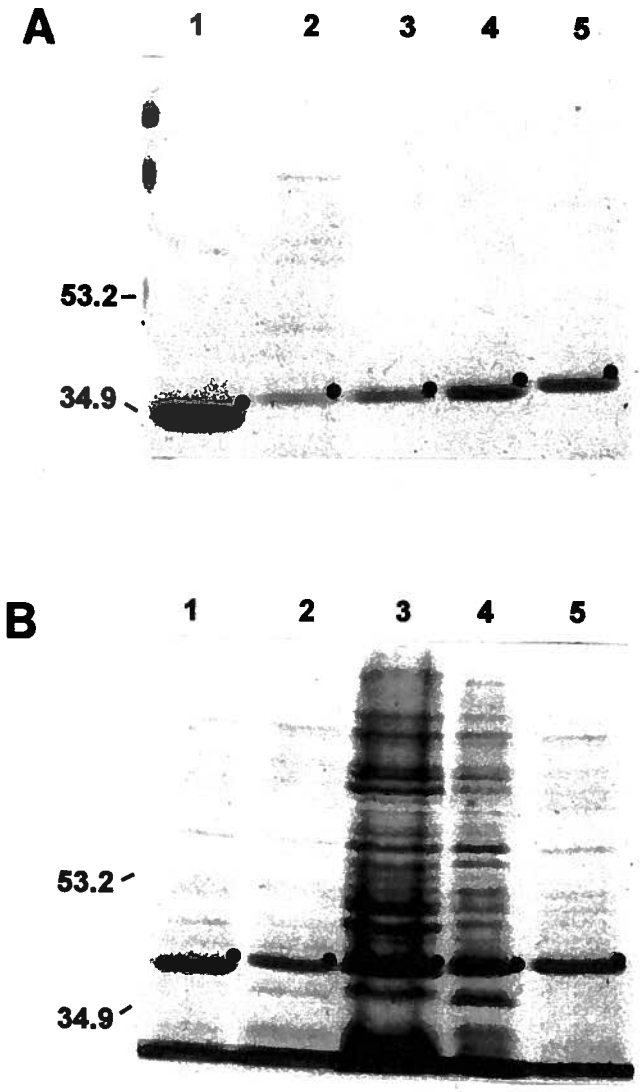


Figure 16. Expression of OprF::malarial epitope multiple-repeat hybrids.

SDS-7.5%PAGE outer membrane preparations carrying insertions at **A).** Ala-26 and **B).** Arg-213 of OprF. Samples were heated at 37°C for 10 min in solubilization buffer before loading. The gels were stained with Coomassie blue after electrophoresis. Lane 1, OprF with no insert; lanes 2 to 5 represent hybrids carrying 7, 11, 15 and 19 amino acids of the epitope respectively. Samples corresponding to the aa^{26} hybrids were obtained by octyl-POE extraction of outer membrane samples. The positions of relevant molecular mass standards (kDa) are indicated on the left. Bands corresponding to OprF or OprF hybrid proteins are marked with solid circles.

disulphide bonds in these proteins (Fig. 17).

2.3.3 Surface exposure of the epitope

According to the membrane topology model of OprF, most of the malarial epitope insertion sites were proposed to be in the surface-exposed loop regions of OprF. In other words, the inserted epitope should have been detectable on the *E. coli* cell surface. Previous studies have shown that the presence of OmpA, an *E. coli* outer membrane protein that shares C-terminal homology with OprF (Duchene *et al.*, 1988; Woodruff and Hancock, 1989), appeared to mask the binding of mAbs to OprF in intact *E. coli* cells (Martin *et al.*, 1993). Therefore, an OmpA-deficient strain C386 was chosen for the expression of the hybrid plasmids. The surface exposure of the malarial epitope was examined by indirect immunofluorescence labelling of whole cells containing the hybrid proteins with the malarial epitope-specific monoclonal antibody, followed by a secondary antibody that was conjugated to a fluorescent dye (Fig. 18). Due to the limitation of the instruments used in fluorescence microscopy, only the cells that were at the same depth of field appeared fluoresced in Figure 18. However, examination of the slides by varying the depths of field showed that the majority of cells (>90%) were labelled. The malarial epitope expressed at all eight "permissive" sites was detectable on the cell surface (Table IX), which suggested the placement of the insertion sites in the surface-exposed loop regions in the topology model of OprF. These results were consistent with the general assumption that surface loop regions are more likely to

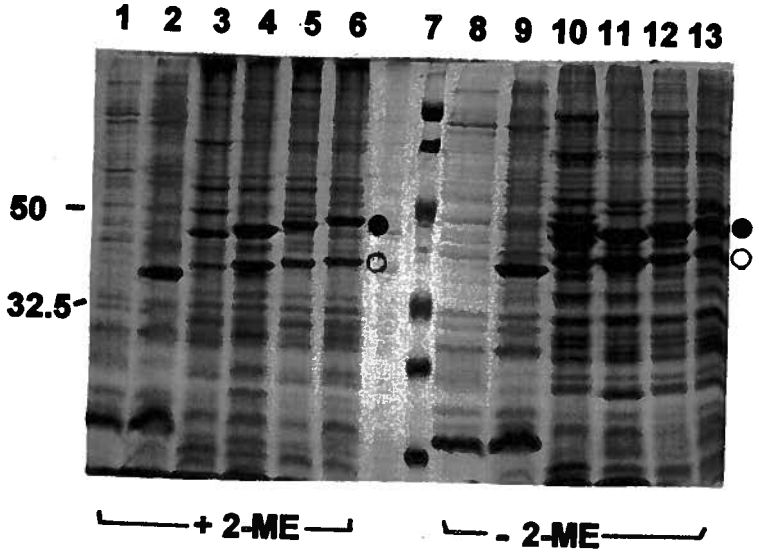
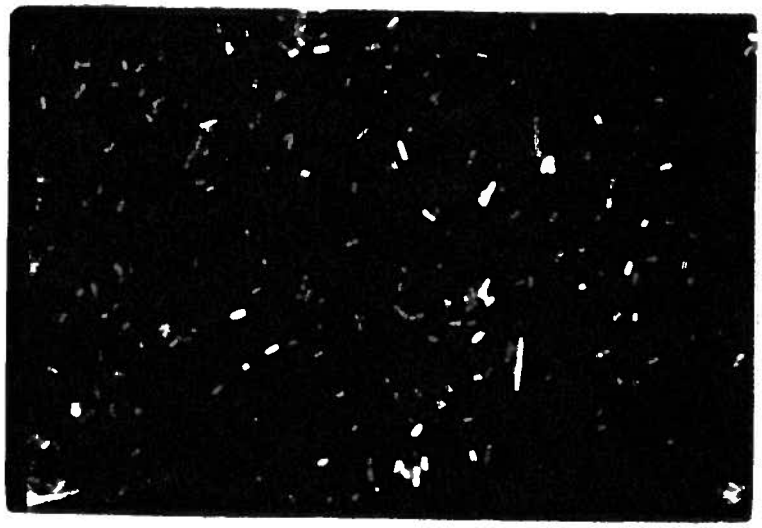


Figure 17. Presence of inclusion bodies in outer membrane samples.

SDS-PAGE of sucrose gradient outer membrane preparations containing both the membrane bound and inclusion body forms of the OprF::malarial epitope multiple-repeat hybrid proteins carrying the inserted epitope at aa^{26}. Samples were heated at 100°C for 10 min in solubilization buffer without (lanes 1 to 6) or with 4% 2-mercaptoethanol (2-ME) (lanes 8-13) before loading. The gel was stained with Coomassie blue after electrophoresis. Samples contained in the lanes: 1 & 8, outer membrane samples from strain expressing pTZ19R; 2 & 9, OprF; 3 & 10, OprF::ME7aa26; 4 & 11, OprF::ME11aa26; 5 & 12, OprF::ME15aa26; 6 & 13, OprF::ME19aa26; 7, molecular mass standards. Solid and open circles indicate the positions of the inclusion body and membrane bound forms of the proteins respectively. The positions of relevant molecular mass standards (kDa) are marked on the left.

A



B

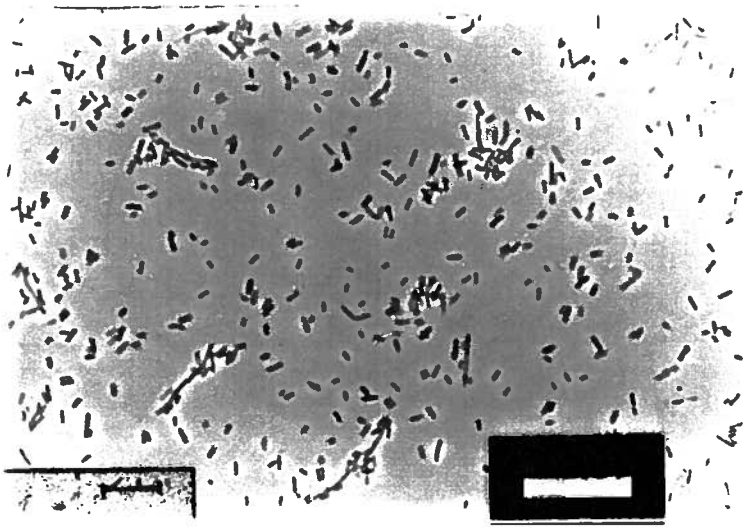


Figure 18. Surface exposure of the malarial epitope.

Indirect immunofluorescence labelling of C386 (pRW302.1M) with the malarial epitope-specific monoclonal antibody pf2A.10. **A)** Labelled cells observed under fluorescence microscopy. **B)** the same field observed under phase contrast . The scale bar at the bottom left corner indicates 20μm. The dark box at the bottom right corner was an artefact from the scale bar slider.

be flexible enough to accommodate foreign peptide sequences (Hofnung, 1991).

2.3.4 Monoclonal antibody reactivity of hybrid proteins

The insertion of foreign amino acid residues might affect the local conformation of the protein and hence disrupt the antibody binding sites of OprF. To examine the effect of epitope insertion in OprF on antibody binding, the outer membrane samples containing the hybrid proteins were analyzed by Western immunoblotting with ten OprF-specific mAbs and two malarial epitope-specific mAbs (Table XI). In general, the pattern of mAb reactivities of the OprF::malarial epitope hybrid proteins was similar to that of the OprF linker mutants. However, while the linker insertion at aa^{188} disrupted only the MA7-8 epitope, the epitope insertion at the same site disrupted the MA4-4 epitope in addition to the MA7-8 epitope. This difference suggested that the insertion of a longer amino acid sequence caused more extensive disruption of the local secondary structure, thus destroying both the MA4-4 and MA7-8 epitopes. Moreover, while the linker insertion at aa^{310} (encoded by pRW314) disrupted the binding of MA7-3 and MA7-5, epitope insertion at the same sites restored weak reactivities with these two mAbs, suggesting that the insertion of the malarial epitope might have restored the antibody binding site(s) to a conformation that resembled to that of the native OprF environment. This speculation was consistent with previous conclusions that these two monoclonal antibodies recognize conformational epitopes (Rawling *et al.*, 1995). Malarial epitope inserted at all eight sites was recognized by the two malarial

Table XI. Summary of monoclonal antibody reactivity of OprF::malarial epitope hybrid proteins contained in outer membrane samples

Plasmids	Insertion sites (aa position)	Monoclonal antibody reactivity[a]											
		MA7-1	MA7-2	MA7-3	MA7-4	MA7-5	MA7-6	MA7-7	MA7-8	MA4-4	MA5-8	pf2A.10	pf5A4.1
pRW3	-	+	+	+	+	+	+	+	+	+	+	-	-
pRW302.1M	Ala-26	+	+	+	+	+	+	+	+	+	+	+	+
pRW302.2M	Ala-26	+	+	+	+	+	+	+	+	+	+	+	+
pRW307.1M	Val-188	+	+	+	+	+	+	+	-	-	+	+	+
pRW308.1M	Gly-196	+	+	+	+	+	+	+	-	-	+	+	+
pRW309.1M	Arg-213	+	+	+	+	+	+	+	+	+	+	+	+
pRW309.3M	Arg-213	+	+	+	+	+	+	+	+	+	+	+	+
pRW310.1M	Gln-215	+	+	+	+	+	+	+	+	+	+	+	+
pRW311.1M	Ser-231	+	+	w	w	w	+	w	+	+	+	+	+
pRW312.1M	Arg-290	+	+	w	-	-	+	-	+	+	+	+	+
pRW314.1M	Gly-310	+	+	w	-	w	+	-	+	+	-	+	+

[a] Determined by Western immunoblot analyses of outer membrane samples. Symbols: +, reactivity equivalent to wild type OprF expressed by pRW3; -, no reactivity; w, weak reactivity.

epitope-specific mAbs.

Western immunoblot analysis of the outer membrane samples containing the multiple-repeat hybrids with an OprF-specific polyclonal antiserum demonstrated that the increase in length of the insert did not increase the amount of degradation products in the outer membrane (Fig. 19A). The hybrid proteins remained reactive with the OprF-specific mAbs MA7-1, MA7-2, MA7-6, MA7-8 (except for the series carrying insertions at aa^{196}) and MA5-8, of which MA7-6 and MA7-8 recognized conformational epitopes, indicating that the overall secondary structure of OprF was still conserved in these hybrid proteins (Fig. 19B).

2.4 Purification of OprF::malarial epitope hybrid proteins

Purified forms of the hybrid proteins could be useful as antigens for the study of antibody binding and as immunogens for immunogenicity studies. Therefore, efforts were made to establish a protocol for the purification of OprF or OprF::malarial epitope hybrids from *E. coli*. To simplify the purification procedures, the *E. coli* strain C158 (*ompA, ompC, phoE*) was used as the background strain for the purification.

2.4.1 Induction experiments

Since the expression of *oprF* was under the control of the *lac* promoter from the cloning vector, different IPTG induction conditions were investigated to

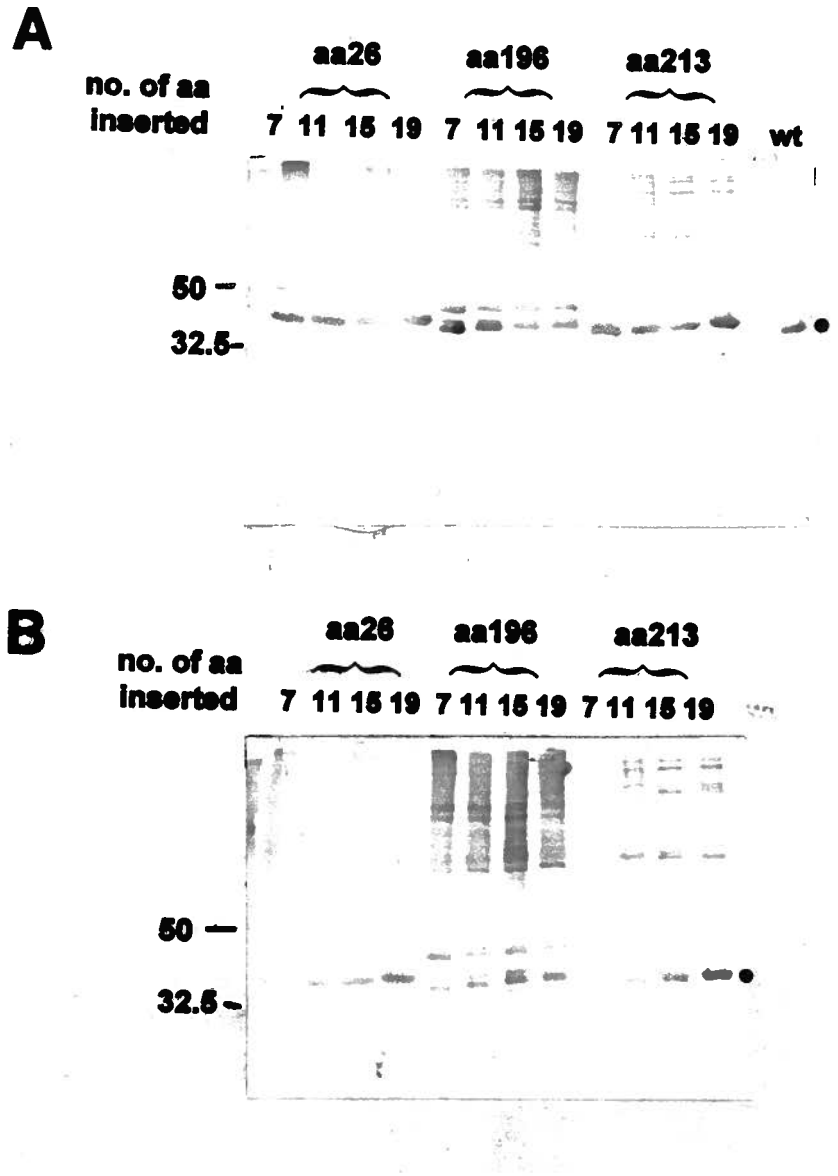


Figure 19. Western immunoblots of OprF::malarial epitope multiple-repeat hybrids with A) an OprF-specific polyclonal antibody and B) an OprF-specific mAb MA7-6. Each set of four lanes represented hybrids carrying an insertion at the site indicated; wt, OprF with no insert. Samples carrying the aa^{26} hybrids were supernatants from 3% octyl-POE extraction of outer membrane samples, while samples carrying the aa^{196} and aa^{213} hybrids were sucrose gradient outer membrane preparations. The percentages of acrylamide used in the SDS-PAGE prior to Western transfer in A and B are 11% and 9% respectively. Samples were heated at 100°C for 10 min before loading. Bands corresponding to oligomeric and LPS-associated forms of OprF are visible in some lanes. The position of OprF is indicated by a ●. The positions of relevant molecular mass standards (kDa) are indicated on the left.

maximize the level of OprF production. Figure 20 shows the amount of OprF hybrid proteins in the outer membrane preparations of cultures grown under different induction conditions. Induction at 30°C for 3 h with 0.2 mM or 1 mM of IPTG did not increase the expression level significantly (lanes 3 and 4). On the other hand, induction with 1 mM IPTG at 37°C or with 0.2 mM IPTG for 16 h at 30°C increased the expression level considerably (lanes 5 to 7). Prolonged induction appeared to lead to higher levels of protein production and degradation (lane 7).

2.4.2 Detergent extractions

The purification protocol for OprF was as described in Methods and materials section 11.1. Outer membrane samples prepared by sucrose gradient method were extracted with detergent in the presence of NaCl and EDTA sequentially. The three detergents tested, Triton X-100, Zwittergent 3-16 and octyl-POE, gave similar results. Octyl-POE was chosen because of its mild nature which was hypothesized to preserve the structure of OprF after extraction. Figure 21 shows a Coomassie blue-stained gel of samples from the sequential detergent extraction procedures. The addition of EDTA released the tightly bound outer membrane proteins including OprF or the OprF hybrid proteins (lanes 7&8). The amount of the OprF hybrid protein in the residual insoluble fraction was significantly reduced (lane 9) as compared to that in the initial outer membrane preparations (lane 3).

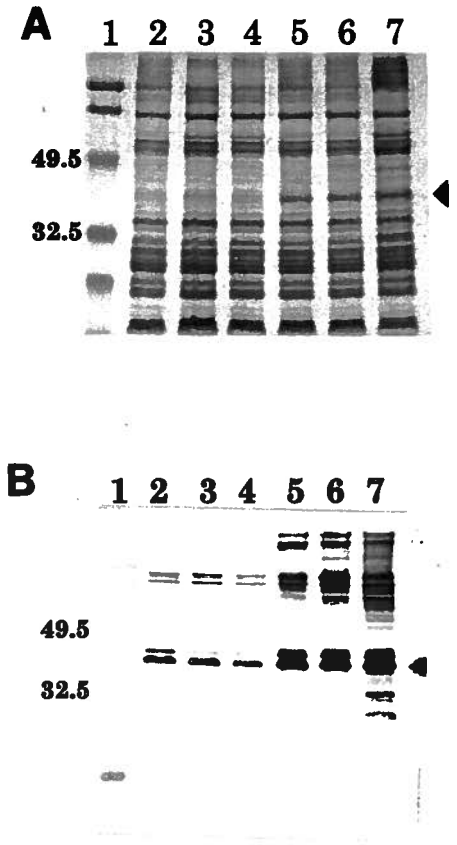


Figure 20. Expression of an *oprF* derivative in different induction conditions.

A. SDS-PAGE of outer membrane samples containing the hybrid protein OprF::ME10aa188. Samples were heated at 37°C for 10 min before loading. The gel was stained with Coomassie blue after electrophoresis. **B.** Western immunoblot of the same samples with the OprF-specific mAb MA5-8. Samples were heated at 100°C for 10 min before loading. Lanes: 1, molecular mass standards; 2, un-induced; 3, 0.2 mM IPTG at 30°C for 3 h; 4, 1mM IPTG at 30°C for 3 h; 5, 1mM IPTG at 37°C for 3 h; 6, 1mM IPTG at 37°C for 6 h (i. e. added at the time of inoculation); 7, 0.2mM IPTG at 30°C for 16 h. IPTG was added to the cultures during the logarithmic growth phase unless otherwise stated. The position of the hybrid protein is indicated by an arrow head. The positions of relevant molecular mass standards (kDa) are indicated on the left.

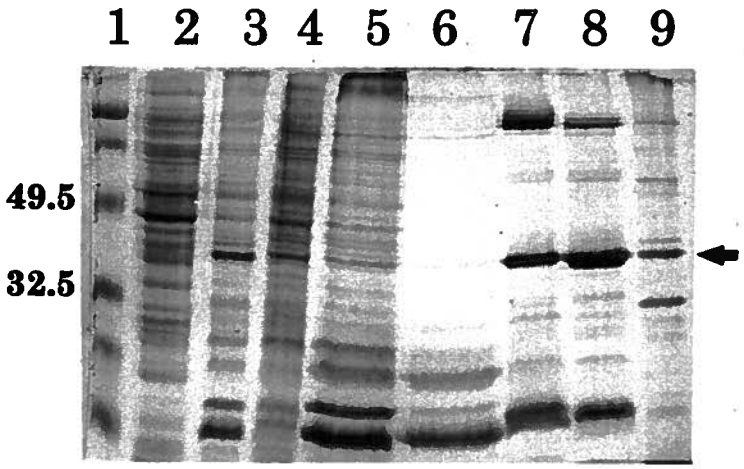


Figure 21. Purification of OprF::malarial epitope hybrid proteins.

SDS-PAGE analysis of samples from different steps of the purification of the OprF hybrid protein from C158 expressing pRW307.1M. The gel was stained with Coomassie blue after electrophoresis. Lanes: 1, molecular mass standards; 2, whole cell lysates; 3, sucrose gradient outer membrane fraction; 4, supernatant from 0.5% octyl-POE extraction; 5 & 6, supernatants from two sequential 3% octyl-POE/1M NaCl extractions; 7 & 8, supernatants from two sequential 3% octyl-POE/10mM EDTA extractions; 9, insoluble fraction of 3% octyl-POE/10mM EDTA extraction. The positions of relevant molecular mass standards (kDa) are indicated on the left. The position of the OprF hybrid protein is indicated by an arrow.

2.4.3 FPLC purification

Based on our laboratory's experience of OprF purification from *P. aeruginosa*, a single chromatography step with an anion exchange column by FPLC was introduced and found to be sufficient to yield purified OprF. Therefore, the OprF hybrids contained in the solubilized fraction after 3% octyl-POE/10mM EDTA extraction were further purified by FPLC. In these experiments, OprF did not bind to the anion exchange column while the major contaminants in the samples bound and were eluted at a NaCl concentration of 0.3 M and 1 M respectively. Figure 22 shows the FPLC profile of the samples eluted from MonoQ column, indicating that OprF was the predominant species in the flow through fractions.

2.4.4 Purification of inclusion body-contaminated outer membrane preparations

Attempts to prevent the formation of inclusion bodies in strains expressing the multiple-repeat hybrids at aa^{26} by growing the cultures at 30°C and harvesting at early-log growth phase were unsuccessful. Detergent extraction was therefore used to isolate the membrane bound protein from the inclusion bodies (Piers *et al.*, 1993). It was found that extraction with 3% octyl-POE and 10mM EDTA selectively released the membrane bound protein into the supernatant while leaving the inclusion body form in the insoluble fraction (Fig. 23).

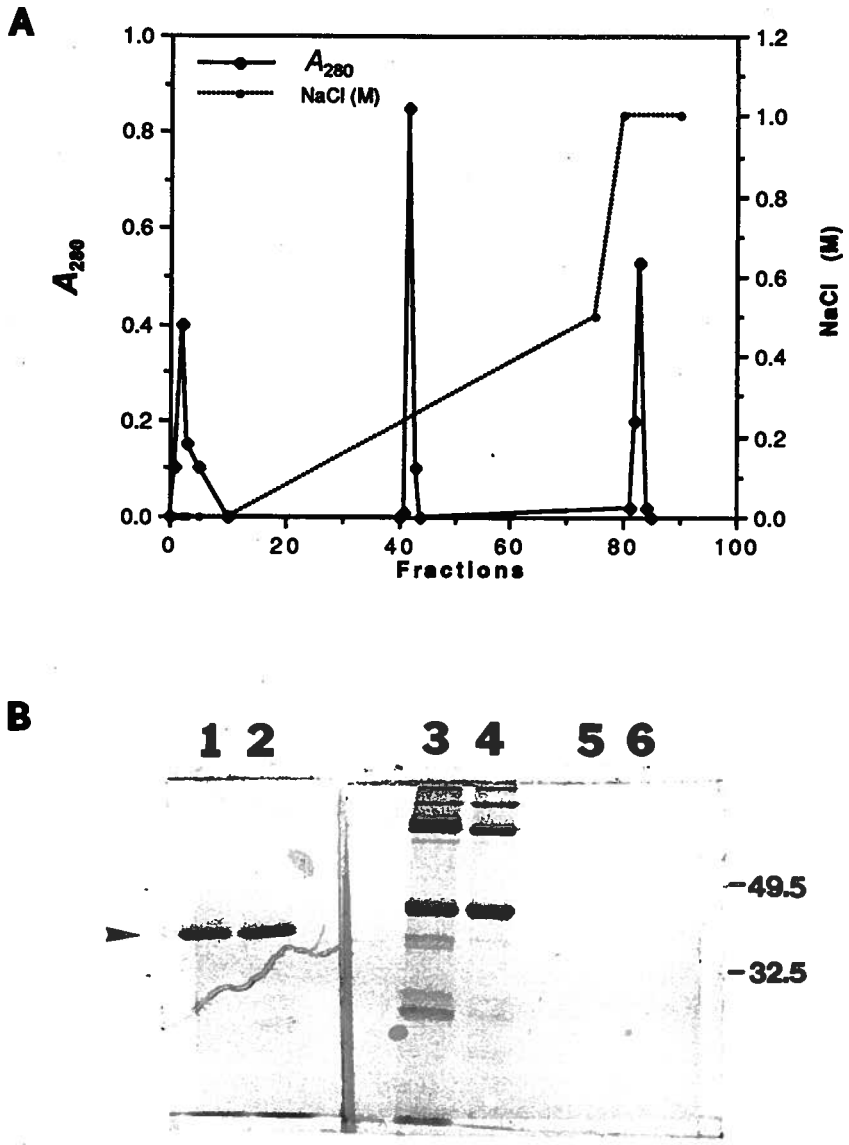


Figure 22. FPLC profile of a MonoQ column separation of the octyl-POE/EDTA soluble OprF hybrid expressed by pRW307.1M.

A. Elution profile with a NaCl gradient. B. SDS-PAGE of samples corresponding to the peak fractions. Lanes: 1 & 2; 3 & 4; 5 & 6; correspond to samples from the first, second and third peaks (from left to right) respectively. The position of the OprF hybrid protein is indicated by an arrow. The positions of relevant molecular mass standards (kDa) are indicated on the right.

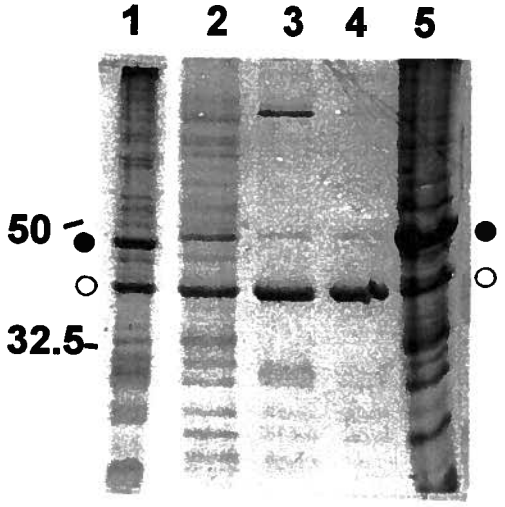


Figure 23. Removal of inclusion bodies from outer membrane preparations by octyl-POE extraction.

SDS-PAGE of OprF::ME19aa26-containing samples from the extraction. The gel was stained with Coomassie blue after electrophoresis. Lanes: 1, outer membrane sample from a sucrose gradient separation; 2, supernatant from 0.5% octyl-POE extraction; 3 & 4, supernatants from the two sequential 3% octyl-POE/10mM EDTA extractions; 5, insoluble fraction after octyl-POE extractions. Solid and open circles indicate the positions of the inclusion body and membrane bound forms of the protein respectively. The positions of relevant molecular mass standards (kDa) are marked on the left.

2.5 GST::malarial epitope fusion proteins

Not only are synthetic peptides costly, they quite often do not interact efficiently with antibodies. Therefore, the genetic construction of 2 versions of GST::malarial epitope fusion proteins was undertaken to provide a source of the epitope for the detection of anti-malarial epitope antibodies in the sera of immunized animals in the later stage of this study.

2.5.1 Construction and purification of fusion proteins

The fusion proteins were constructed by cloning hybridized oligonucleotides encoding the malarial epitope into the 3' end of the GST coding region in the cloning vector pGEX-1N (Fig. 8). The resulting fusion proteins, GST::ME11 and GST::ME19, expressed 11 {$P(NANP)_2NA$} and 19 {$P(NANP)_4NA$} amino acids respectively corresponding to the epitope at the C-terminus of the carrier protein. The fusion proteins were expressed in *E. coli* DH5α and purified by affinity chromatography using glutathione agarose beads as described by Smith and Johnson (1988). Figure 24 shows that both fusion proteins had higher apparent molecular mass than GST, in agreement with the presence of the extra malarial epitope amino acid residues. Both fusion proteins were recognized by an anti-GST polyclonal antiserum and the malarial epitope-specific mAb pf2A.10 on Western immunoblots (Fig. 24).

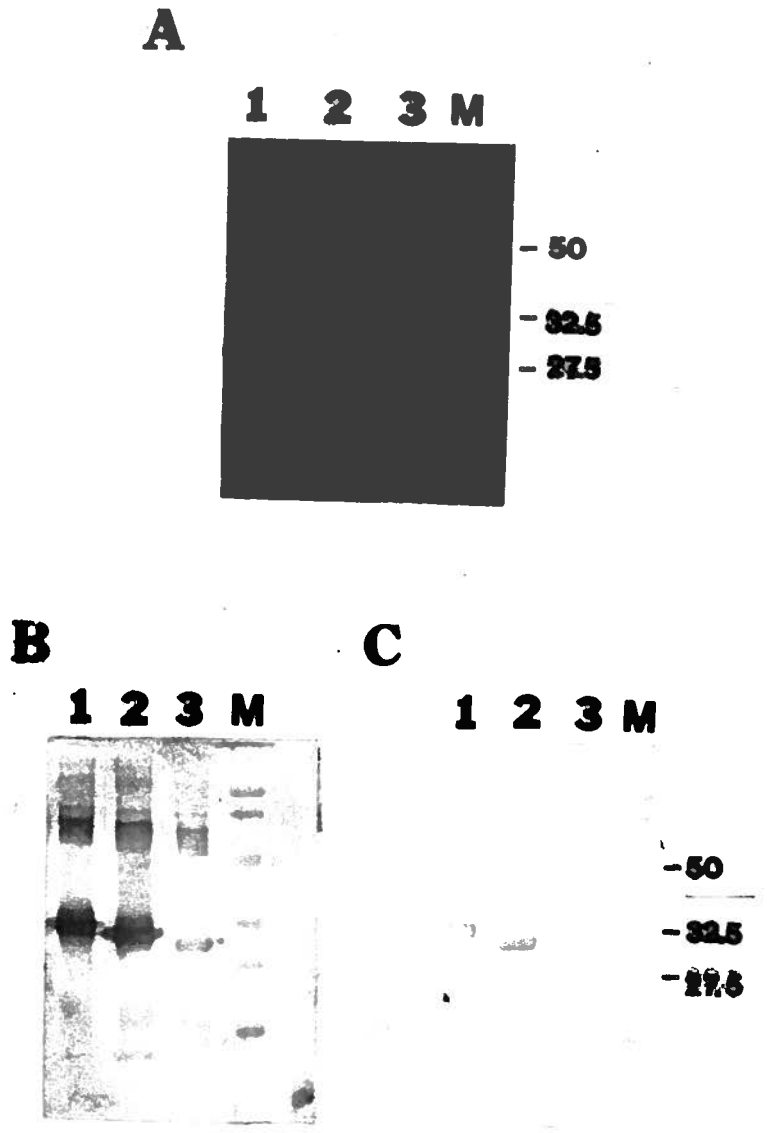


Figure 24. Purification of GST::malarial epitope fusion proteins

A. SDS-PAGE of GST::malarial epitope fusion proteins purified by affinity chromatography. The gel was stained with Coomassie blue after electrophoresis. B. Western immunoblot of the affinity-purified protein with an anti-GST polyclonal serum. C. the same blot reacted with the malarial epitope-specific mAb pf2A.10. Lanes: 1, GST::ME19; 2, GST::ME11; 3, GST; M, molecular mass standards. Samples were heated at 100°C for 10 min in solubilization buffer before loading. The positions of the relevant molecular mass standards (kDa) are indicated on the right.

2.5.2 Binding of fusion proteins with epitope-specific monoclonal antibodies

Since the GST::malarial epitope fusion proteins were constructed for use as coating antigens in ELISA to determine the anti-malarial epitope titers in the sera of immunized animals, the level of binding of these proteins with the epitope-specific mAbs in ELISA was also examined. As extrapolated from the binding curves, GST::ME11 had K_m values of 1.25 μg/ml and 0.5 μg/ml for the epitope-specific mAbs pf2A.10 and pf5A4.1 respectively, whereas GST::ME19 had K_m values of 0.83 μg/ml and 0.35 μg/ml for pf2A.10 and pf5A4.1 respectively (Fig. 25). This suggested that GST::ME19 had a slightly higher affinity for the malarial epitope-specific monoclonal antibodies, which might have been due to the presence of the longer epitope sequence permitting more appropriate folding.

2.6 Summary

In this chapter, it was demonstrated that OprF could be used as a carrier for the expression and surface exposure of a malarial epitope. Two series of OprF::malarial epitope hybrids were constructed genetically. The first series, the positional hybrids, consisted of OprF hybrid proteins that expressed the malarial epitope at different permissive sites within OprF. The second series, the multiple-repeat hybrids, consisted of hybrids that expressed four different lengths of the repeating epitope at one of the three selected sites in OprF. Eight "permissive" sites were identified which could accommodate and express the model malarial

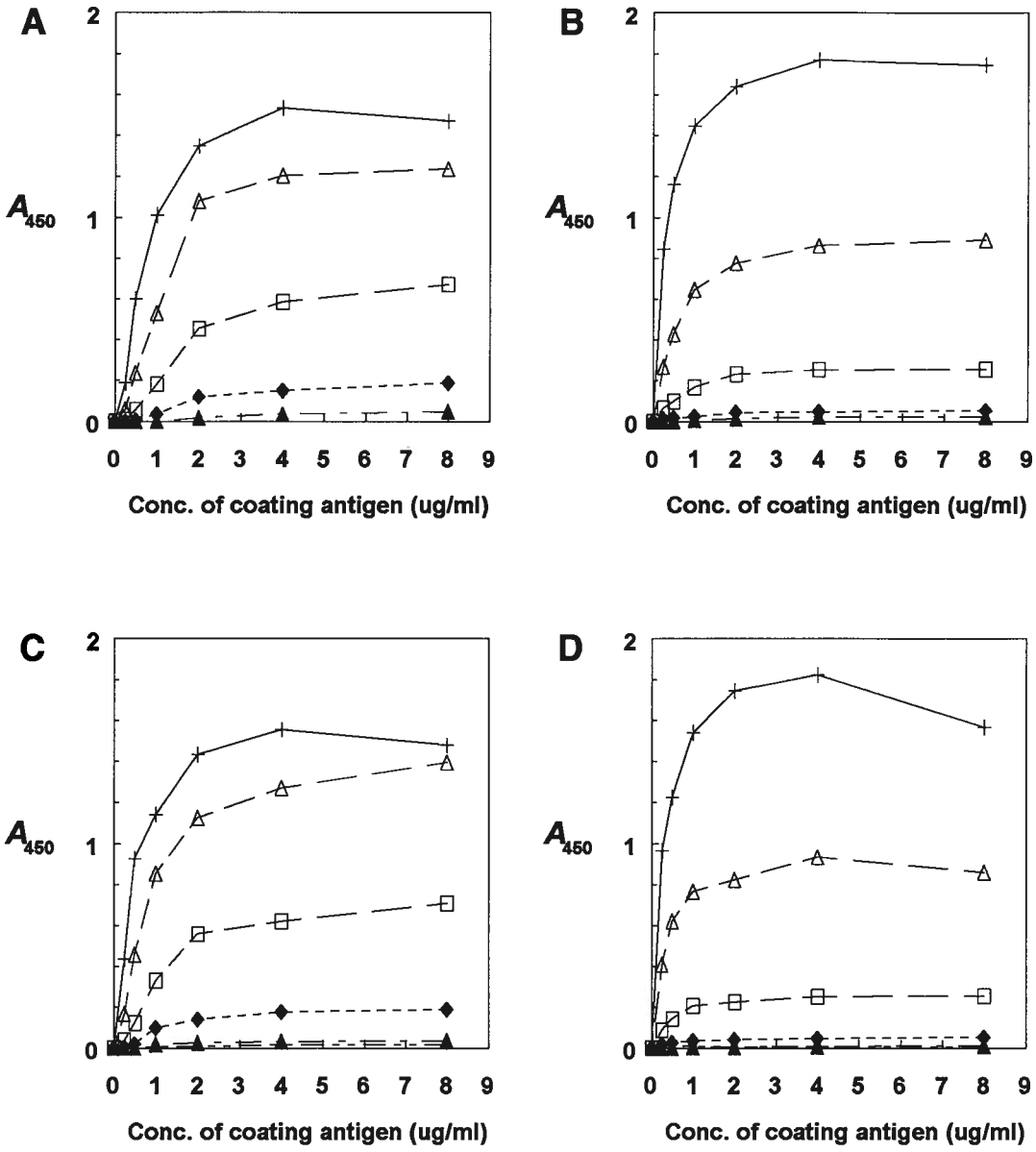


Figure 25. Binding of GST::malarial epitope fusion proteins with epitope-specific monoclonal antibodies.

ELISA of GST::ME11 with pf2A.10 **(A)** and pf5A4.1 **(B)**; and ELISA of GST::ME19 with pf2A.10 **(C)** and pf5A4.1 **(D)**. Curves represent different dilutions of the antibodies: +, 1:1000; ▵, 1:5000; □, 1:25000; ♦, 1:125000; ▲, 1:625000.

epitope sequence. Insertion of the epitope sequence in the cysteine-containing region of OprF increased the heat sensitivity of the hybrid proteins, while insertions in the C-terminus of the protein rendered the hybrid proteins more susceptible to degradation by cellular proteases. All of the hybrids were expressed in the outer membrane and the inserted epitope at each of the "permissive" sites was detectable on the cell surface. Western immunoblot analysis of the hybrid proteins with the series of OprF-specific mAbs indicated that the proteins retained substantial wild type conformation. Furthermore, a protocol for the purification of OprF or OprF hybrid proteins from *E. coli* was also established. In addition to the OprF::malarial epitope hybrids, two versions of GST::malarial epitope fusion proteins were also constructed. Both fusion proteins were reactive with the two epitope-specific mAbs tested.

The availability of the two series of OprF::malarial epitope hybrid proteins provided a set of tools for the study of the effects of insertion position and length of the epitope on epitope presentation in the OprF carrier system, while the GST::malarial epitope fusion proteins represented a source of easily-purified epitope for the analysis of anti-epitope response in serum samples from immunized animals.

Chapter three: Effects of mode of presentation on antigenicity and immunogenicity.

3.1 Introduction

Limited studies of two of the outer membrane protein epitope presentation systems have shown that the nature of the flanking amino acid sequences and the length of the inserted epitopes could influence the antigenicity (i.e., the ability to interact with antibody) and the immunogenicity (i.e. the ability to stimulate an immune response) of the epitopes (Agterberg *et al.*, 1990b; Van der Werf *et al.*, 1990). These findings suggested that more extensive investigations of the position and length effects of epitope insertion in carrier proteins will help us to exploit the effectiveness of such presentation systems.

The potential of OprF as a carrier protein for the presentation of a foreign malarial epitope was clear from the results described in the previous chapter. To further our understanding of the flexibility and limitations of the OprF system, the two series of OprF::malarial epitope hybrid proteins were used to examine the effects of the insertion position and the length of the epitope on epitope presentation in the OprF system. In this chapter, a broad survey of antigenicity of the inserted epitope is described and this led to a targeted study of immunogenicity of the epitope.

3.2 Antigenicity study

The antigenicity of a molecule refers to its ability to interact with antibodies. In the OprF epitope presentation systems, the accessibility of the insertion sites, the nature of flanking amino acid residues and the length of the inserted epitope might affect the interaction of the epitope with its specific antibodies. Therefore, a study was undertaken to compare the antigenicity of the epitope presented in different lengths and at different positions of OprF.

3.2.1 Approaches

In the process of establishing an assay to evaluate the antigenicity of the epitope, a whole cell ELISA and an antigen competition assay were employed. In the whole cell ELISA, *E. coli* cells expressing the hybrid proteins were used as the coating antigens to capture a malarial epitope-specific monoclonal antibody. The levels of malarial epitope-specific antibody binding to the various strains of *E. coli* cells were quantified and used as measurements for the antigenicity of the presented epitope. In the antigen competition assay, *E. coli* cells expressing the hybrids were used to adsorb the malarial epitope-specific antibody. The residual titers of the epitope-specific antibody were then measured by ELISA using the GST::malarial epitope fusion protein, GST::ME19, as the coating antigen. The amount of malarial epitope-specific antibody adsorbed by the various strains of *E. coli* cells was used as an indication of the antigenicity of the epitope in the

corresponding OprF::malarial epitope hybrid proteins. However, neither assay gave consistent results, presumably due to the high backgrounds in control bacteria lacking the malarial epitope. This background could be caused by the non-specific binding of the epitope-specific monoclonal antibodies, pf2A.10 and pf5A4.1, to surface components on *E. coli* cells in these assays. It was found that pf5A4.1 was more highly reactive with *E. coli* outer membrane preparations in ELISA than pf2A.10. Therefore, only pf2A.10 was used in the antigenicity assays. The relative antigenicity of the inserted epitope was measured by whole cell dot blot analysis and outer membrane ELISA using the epitope-specific mAb pf2A.10 and an OprF-specific polyclonal antiserum. These assays were quite reproducible and the background readings were low under the conditions utilized. Binding titration curves of the two antibodies to OprF and an OprF::malarial epitope hybrid protein are shown in Figure 26.

3.2.2 Position effect

The antigenicity of single copies of a 10-amino acid malarial epitope (NANPNANPNA) expressed at different positions of OprF was compared by whole cell dot blot analysis and ELISA using outer membranes. To take into account the various expression levels of the hybrid proteins in the outer membrane, the antigenicity index of each inserted epitope was calculated as the ratio of the anti-epitope reactivity to the anti-OprF reactivity of the corresponding hybrid protein. Both assays indicated that the epitope had different relative affinities for the

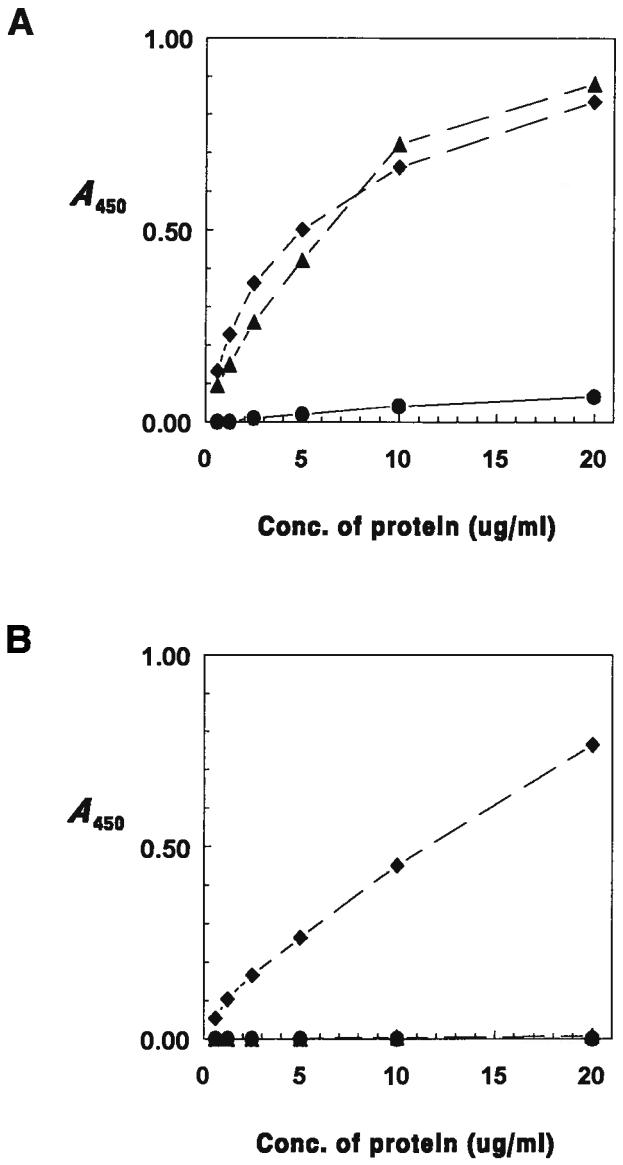


Figure 26. Binding of an OprF-specific polyclonal serum **(A)** and the malarial epitope-specific mAb pf2A.10 **(B)** with outer membranes from *E. coli* expressing the following OprF variants. Symbols: ●, vector control; ▲, OprF; ♦, OprF::ME7aa196.

malarial epitope-specific mAb pf2A.10 when expressed at different positions in OprF (Fig. 27). However, generally similar antigenicity patterns were observed for given mutants in the context of both whole cells and isolated outer membranes. For example, the malarial epitope inserted at aa^{215} or aa^{310} consistently demonstrated low relative antigenicity, whereas insertions at aa^{188} or aa^{196} were significantly more antigenic in both assays. In contrast, the epitope inserted at aa^{26} was more antigenic in whole cell dot blot analysis, while the epitope inserted at aa^{213} and aa^{290} was significantly more antigenic when assayed by outer membrane ELISA. The dissimilarity could conceivably be due to the difference in the presentation of the epitope in whole cells as compared to an outer membrane environment. Although the hybrid proteins were likely to be in their native configuration in the outer membrane preparations, the isolation procedures might have removed part of the surface moieties such as lipopolysaccharides (LPS). The presence of cell surface LPS could promote the presentation of the inserted epitope for antibody binding at aa^{26} while reducing the accessibility for antibody binding at aa^{213} and aa^{290}. As a result, significant dissimilarities in antigenicity indices were observed when assayed in whole cell and outer membrane environments. The antigenicity indices of the epitope in the positional hybrids are summarized in Table XII.

Due to the cloning procedures, the flanking sequences of the epitopes inserted at aa^{213}, aa^{231}, aa^{290} and aa^{310} contained cysteine residues, indicating that they might participate in disulphide bond formation. However, results from preliminary survey showed that the antigenicity of the epitope inserted at aa^{213} was

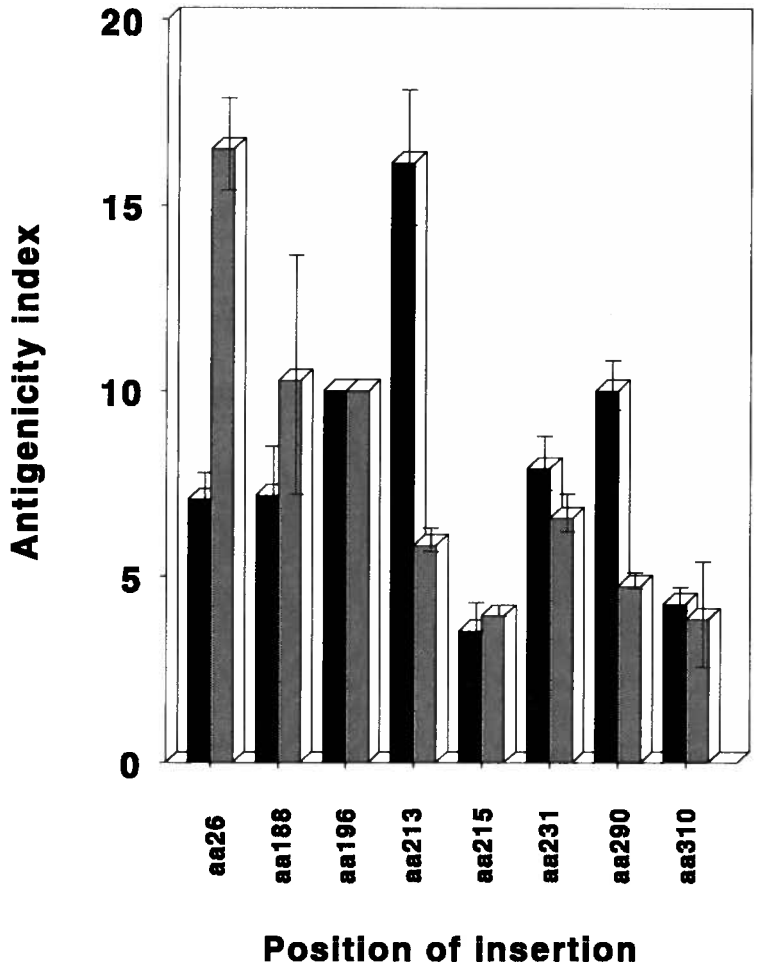


Figure 27. Effect of insertion position on the antigenicity of the malarial epitope.

Solid and hatched bars represent results from outer membrane ELISA and whole cell dot blot analyses respectively. To allow comparisons between the two methods, the antigenicity index of the epitope inserted at aa^{196} was used as an internal standard and arbitrarily set to 10 and the rest of the values were adjusted accordingly. Values were the means and standard deviations from six independent experiments for outer membrane ELISA and three independent experiments for dot blot analyses. The indices that were discussed in the Results section as being significantly different were confirmed by F-tests ($P<0.05$).

Table XII. Summary of antigenicity of malarial epitope in the OprF::malarial epitope positional hybrids.

Plasmid	Insertion site[a]	Amino acids inserted[b]	Antigenicity index[c]	
			Outer membrane	Whole cell
pRW3	-	wt	0	0
pRW302.1M	Ala^{26}	PAP(***ME***)GHAGP	7.06±0.58	16.49±1.24
pRW302.2M	Ala^{26}	PA{P(***ME***)GHA}$_2$GP	15.58±1.19	25.60±3.05
pRW307.1M	Val^{188}	DLQ(***ME***)LDVQV	7.18±1.03	10.28±3.22
pRW308.1M	Ala^{196}	PAP(***ME***)GHAGP	10	10
pRW309.1M	Arg^{213}	TCNP(***ME***)CRS	16.14±1.82	5.84±0.32
pRW309.3M	Arg^{213}	TC{NP(***ME***)C}$_3$RS	20.23±1.91	12.82±2.44
pRW310.1M	Gln^{215}	DLQ(***ME***)LDVQV	3.52±0.63	3.93±0.14
pRW311.1M	Ser^{231}	TCNP(***ME***)CRS	7.91±0.74	6.57±0.51
pRW312.1M	Arg^{290}	TCNP(***ME***)CRS	10.01±0.67	4.74±0.21
pRW314.1M	Gly^{310}	TCNP(***ME***)CRS	4.25±0.31	3.83±1.42

[a] Position of the amino acid preceding the insertion. At insertion sites Ala^{196} and Ser^{231}, the preceding amino acids were replaced by a glycine and arginine respectively.

[b] ***ME*** = NANPNANPNA, the outer two amino acids on both sides of the flanking sequences (i.e. PA_GP, DL_QV or TC_RS) were the results of the previous linker-insertion mutagenesis procedures.

[c] Results are presented as means±standard deviations as described in Figure 27 legend. Antigenicity indices were calculated as described in Methods and materials section 12.

not affected by the presence of 2-mercaptoethanol, suggesting that the flanking cysteine residues were likely not be involved in disulphide bonding, and/or that disulphide bonding did not affect the presentation of the epitope for antibody binding.

3.2.3 Length effect

The antigenicity of insertions containing 2 and 3 copies of the 10-amino acid epitope at aa^{26} and aa^{213} was also measured. Insertion of multiple copies of the epitope at both sites resulted in a significant increase in antigenicity (Table XII, rows 2&3; 6&7). The hybrid carrying 2 copies of the epitope insert at aa^{26} (encoded by pRW302.2M) consistently demonstrated an antigenicity index which was approximately two-fold higher than that of the hybrid expressing one copy of the insert at the same site (encoded by pRW302.1M), indicating that the presence of an additional copy of the epitope insert enhanced the ability of the inserted epitope to bind antibodies (Fig. 28). Similarly, the hybrid expressing three copies of the insert at aa^{213} (encoded by pRW309.3M) also demonstrated higher antigenicity than the hybrid expressing one copy of the insert at the same site (encoded by pRW309.1M) (Fig. 28). The presence of an additional copy of the epitope might have improved the exposure of the epitope and thus its accessibility for antibody binding, or it might have increased the valency of antibody binding. The lesser influence of multiple insertions at aa^{213} on the antigenicity might imply that this site is already relatively well-exposed in the OprF protein, or that the effects of multivalency in this case

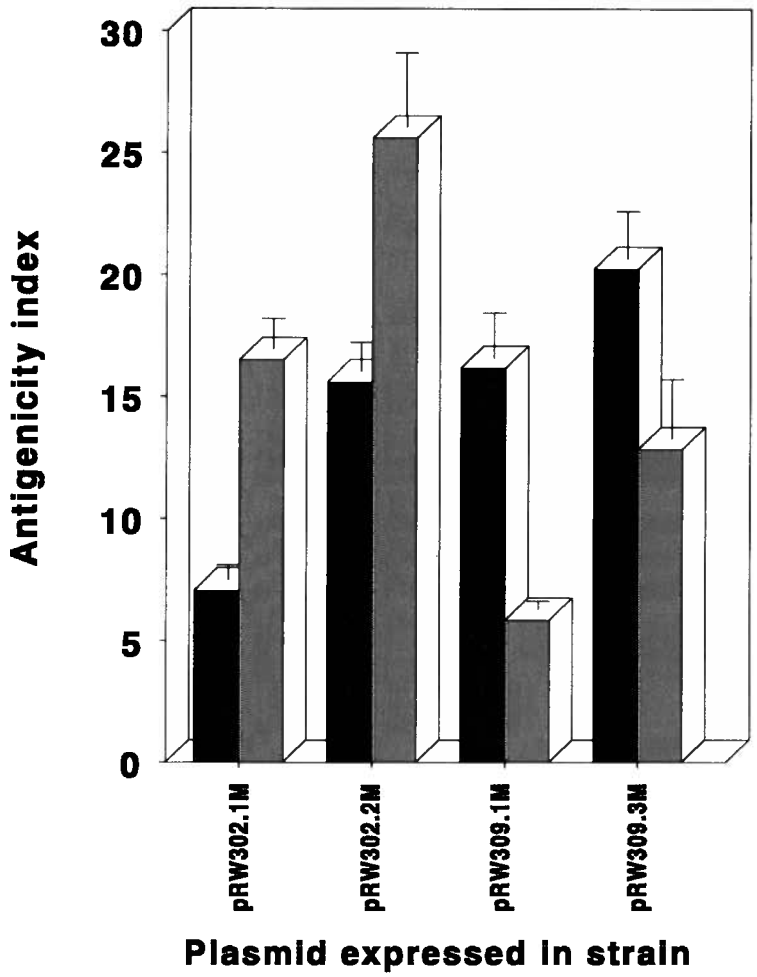


Figure 28. Effect of insertion of multiple copies of the malarial epitope on antigenicity at insertion sites aa^{26} and aa^{213} of OprF.

Plasmids pRW302.1M and pRW302.2M encoded hybrid proteins carrying one and two copies of the epitope at aa^{26} respectively; plasmids pRW309.1M and pRW309.3M encoded hybrid proteins carrying one and three copies of the epitope at aa^{213} respectively. Solid and hatched bars represent results from outer membrane ELISA and whole cell dot blot analyses respectively. Values presented are as described in Table XII foot notes (c).

might have induced steric hindrance which limited the accessibility of the epitope to antibody. In light of this finding, hybrids with increasing number of repeats of the tetramer unit at three different sites of OprF were constructed to investigate the effect of the length of the epitope on its antigenicity.

The three sets of multiple-repeat hybrids were constructed to express 7, 11, 15 or 19 amino acids of the malarial epitope sequence at either aa^{26}, aa^{196} or aa^{213} of OprF. At insertion sites aa^{26} and aa^{196}, the antigenicity of the epitope increased as the length of the epitope increased (Figs. 29 & 30). The assays for each set were repeated three times and the r (correlation coefficient) values by linear regression in each independent experiment were between 0.9314 and 0.9877, and between 0.9453 and 0.9875 for insertion sites aa^{26} and aa^{196} respectively. In linear regression, r lies between -1 and +1, and when r is close to one this indicates a positive linear relationship (Ott, 1988). Therefore, these results demonstrated a significant, positive relationship between the length of the epitope and its antigenicity in these two cases. On the other hand, the antigenicity of the four lengths of the epitope inserted at aa^{213} was not significantly different and did not seem to vary with the length of the insert (Fig. 31). According to the results obtained from the antigenicity study of the positional hybrids, the epitope inserted at aa^{213} was comparatively more antigenic than that inserted at aa^{26} and aa^{196}, probably due to the better exposure of the epitope at this site. Considering this result, the shortest version of the epitope inserted at aa^{213} might already be adequately accessible for antibody binding; hence the increase in length did not

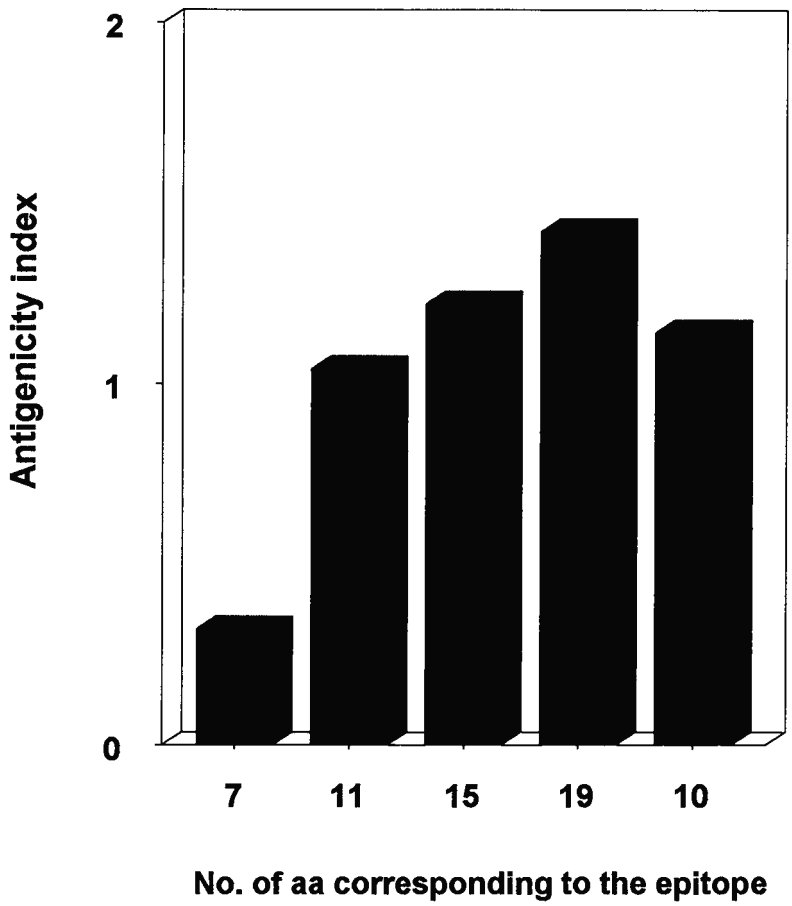


Figure 29. Effect of the length of the epitope on its antigenicity at insertion site aa[26] of OprF.

The data from one representative experiment is shown. The r value (correlation coefficient) from linear regression analysis in this experiment was 0.9314.

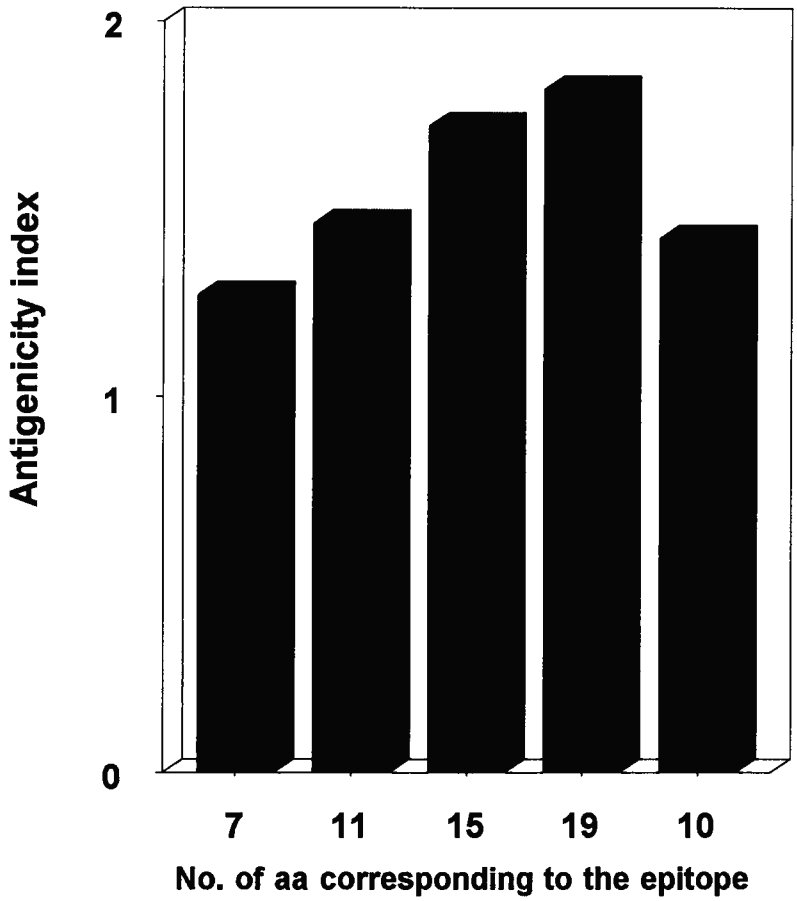


Figure 30. Effect of the length of the epitope on its antigenicity at insertion site aa^{196} of OprF.

The data from one representative experiment is shown. The r value (correlation coefficient) from linear regression analysis in this experiment was 0.9875.

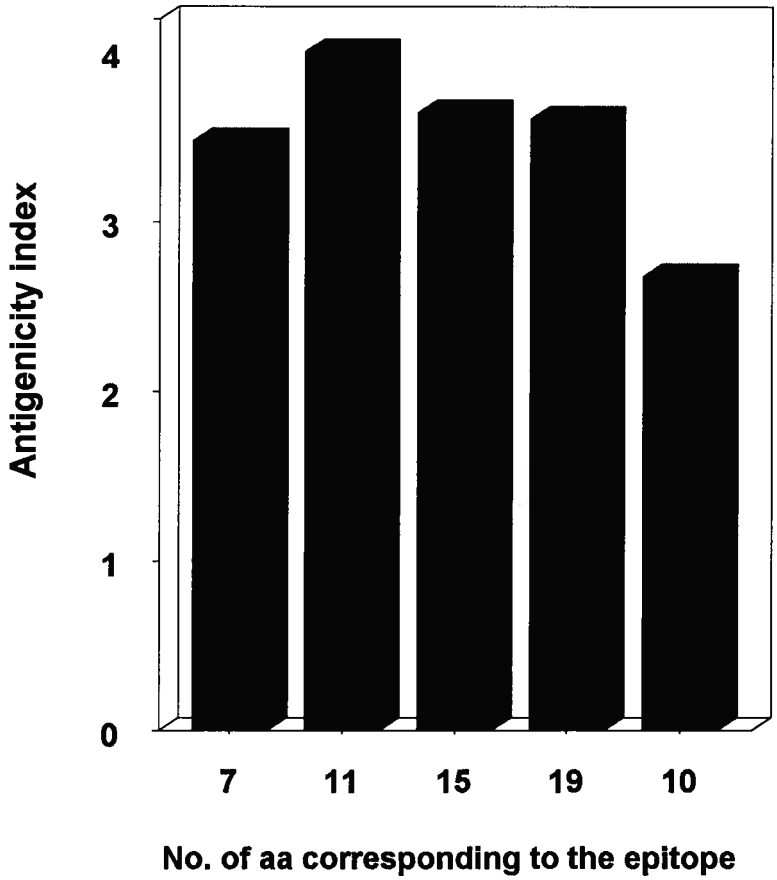


Figure 31. Effect of the length of the epitope on its antigenicity at insertion site aa^{213} of OprF.

The data from one representative experiment is shown. The r value (correlation coefficient) from linear regression analysis in this experiment was 0.0171.

lead to a significant improvement in antigenicity. On the other hand, in the case of insertion sites aa^{26} and aa^{196}, the longer inserted sequence might increase the antigenicity by improving the exposure of the epitope. This seemed to be particularly obvious at aa^{26}, where a more than two-fold increase in antigenicity was observed as the length of the inserted epitope was increased from 7 to 11 amino acids (Fig. 29). The antigenicity of the inserted malarial epitope in the multiple-repeat hybrids is summarized in Table XIII.

3.3 Immunogenicity study

The immunogenicity of a molecule refers to its ability to elicit an immune response. This study concentrated on the antibody responses against the immunogens. In general, to elicit a good antibody response, the immunogen is required to have a B cell epitope which binds to receptors on B cell surface, and a T cell epitope that can be recognized by MHC class II molecules and presented to T helper cells (Guillet *et al.*, 1986; Brown *et al.*, 1988). In an epitope presentation system, the flanking amino acid sequences and length of an epitope might affect its interaction with B cell receptors as well as its processing and presentation by MHC molecules. This section describes the immunogenicity of a 10-amino acid epitope inserted at aa^{215} and two different lengths of the epitope inserted at aa^{26} of OprF. In addition, the immunogenicity of the two lengths of the malarial epitope fused to the C-terminus of GST was also investigated.

Table XIII. Summary of the antigenicity of the malarial epitope in OprF::malarial epitope multiple-repeat hybrids.

Insertion site	Plasmid	Amino acids inserted[a]	Antigenicity indices from independent experiments (*r values*)[b]		
			1st	2nd	3rd
Ala-26	pRW302.7	PAARNPNANPNLDAGP	0.25	0.16	0.26
	pRW302.11	PAAR(NPNA)$_2$NPNLDAGP	0.8	0.6	0.48
	pRW302.15	PAAR(NPNA)$_3$NPNLDAGP	0.94	0.84	1.01
	pRW302.19	PAAR(NPNA)$_4$NPNLDAGP	1.09	0.94	1.3
			(*0.931*)	(*0.959*)	(*0.988*)
Ala-196	pRW308.7	PAARNPNANPNLDAGP	0.53	1.27	0.81
	pRW308.11	PAAR(NPNA)$_2$NPNLDAGP	0.73	1.46	0.91
	pRW308.15	PAAR(NPNA)$_3$NPNLDAGP	0.8	1.72	1.52
	pRW308.19	PAAR(NPNA)$_4$NPNLDAGP	1.26	1.82	1.71
			(*0.945*)	(*0.988*)	(*0.961*)
Arg-213	pRW309.7	TCTRNPNANPNLDCRS	1.17	1.55	3.48
	pRW309.11	TCTR(NPNA)$_2$NPNLDCRS	1.42	2	4.01
	pRW309.15	TCTR(NPNA)$_3$NPNLDCRS	1.21	1.86	3.65
	pRW309.19	TCTR(NPNA)$_4$NPNLDCRS	1.37	1.54	3.61
			(*0.415*)	(*-0.096*)	(*0.017*)

[a] The amino acid residues corresponding to the malarial epitope are underlined. The flanking amino acids PA__GP and TC__RS were the results of the previous linker-insertion mutagenesis procedures.
[b] r is the correlation coefficient value by linear regression, where r >1 indicates a positive linear relationship.

3.3.1 Immunogenicity of OprF::ME10aa215

The immunogenicity of the epitope inserted at aa^{215} was examined by immunizing BALB/c mice with OprF::ME10aa215. The two control groups included animals immunized with wild type OprF or PBS. The animals were immunized on days 0 and 14 with FPLC-purified proteins and on day 28 with $2x10^8$ heat-killed *E. coli* cells expressing the corresponding proteins. The anti-OprF and anti-malarial epitope antibody responses were determined by ELISA using purified OprF and GST::ME19 and $(NANP)_3$ peptide as coating antigens respectively (Methods and materials 13.3). No significant anti-OprF and anti-malarial epitope titers were detected in antisera after two injections. Significant anti-OprF response was observed in all animals after the third injection, whereas an anti-epitope response was only observed in one of the five animals immunized with OprF::MEaa215 (Fig. 32). However, characterization of the antiserum from the responsive animal by Western immunoblotting failed to demonstrate the presence of anti-malarial epitope antibodies in this serum. Therefore, the anti-epitope response detected could have been due to non-specific binding of the antiserum to the coating antigen. No significant anti-OprF or anti-epitope titers was detected in the pre-immune sera or the sera from the PBS control group. These results indicated that despite the fact that the malarial epitope inserted at aa^{215} of OprF was antigenic; it was not immunogenic when administered to BALB/c mice. The titers of the antisera are summarized in Table XIV.

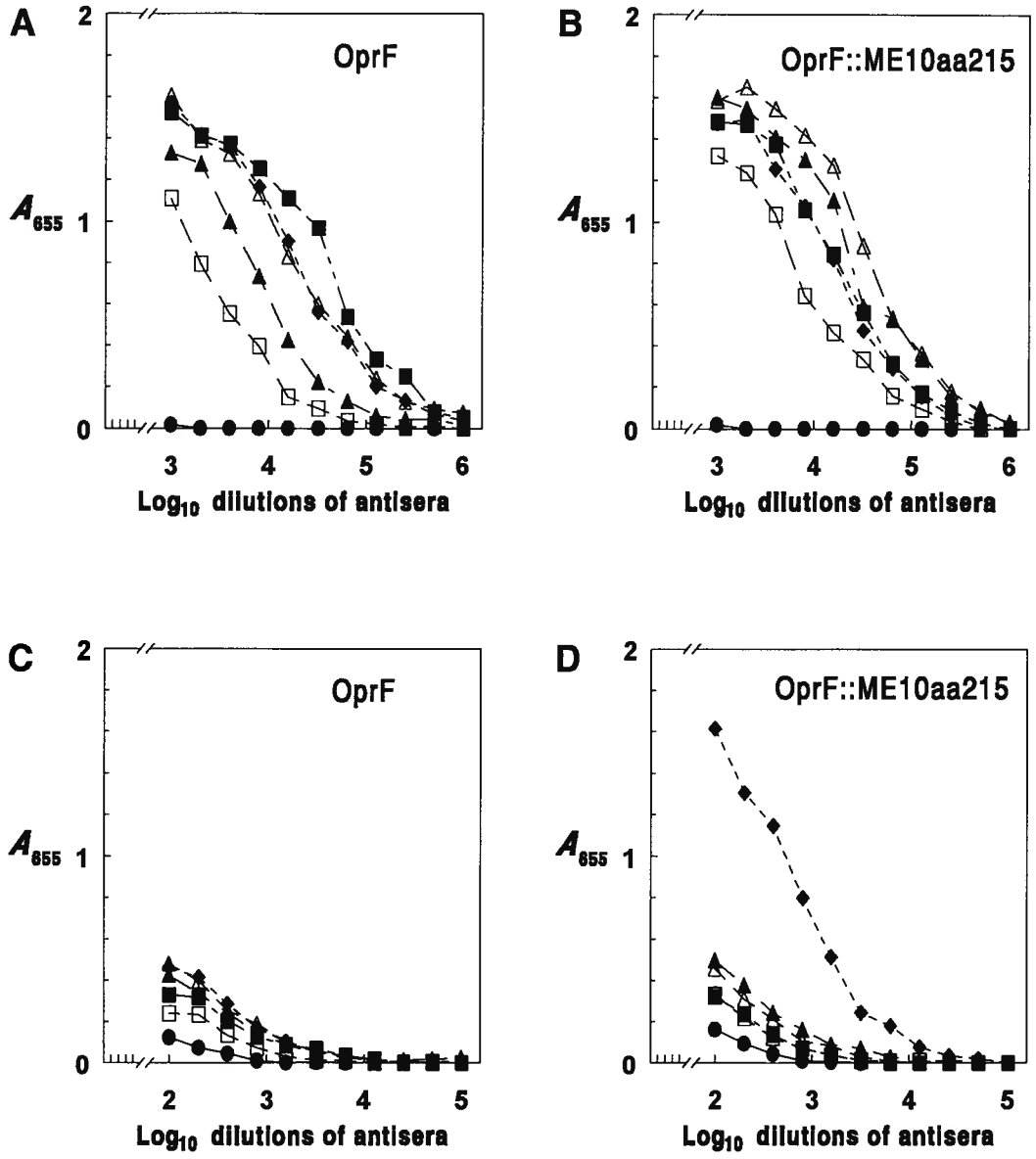


Figure 32. ELISA titrations of anti-OprF and anti-malarial epitope responses induced in BALB/c mice immunized with OprF and OprF::ME10aa215 by ELISA.

A and B, anti-OprF response; C and D, anti-malarial epitope response. The anti-OprF response and anti-malarial epitope responses were measured using purified OprF and GST::ME19 as coating antigens respectively. The immunogens used in each groups are indicated on the graphs. Symbols: ●, pooled pre-immune sera; △, □, ♦, ▲, ■; serum samples from five different animals.

Table XIV. Summary of antibody responses induced in mice immunized with wild type OprF or OprF::ME10aa215.

Immunogens	Animals	ELISA titers[a]		
		Anti-OprF	Anti-GST::ME19	Anti-$(NANP)_3$
OprF::ME10-aa215	a	5.00 ± 0.61	3.4 ± 0.96	<2
	b	4.70 ± 0.37	2.4 ± 0.35	<2
	c	4.70 ± 0.37	2.8 ± 0.35	<2
	d	5.30 ± 0.37	2.8 ± 0.46	<2
	e	5.20 ± 0.12	2.4 ± 0.35	<2
OprF	a	5.37 ± 0.57	<2	ND[b]
	b	4.37 ± 0.41	<2	ND
	c	5.27 ± 0.64	<2	ND
	d	4.97 ± 0.71	<2	ND
	e	5.57 ± 0.41	<2	ND

[a] Titers are reported as the log of dilutions of antisera that gave twice of the A_{655} readings of the pre-immune serum at 100-fold dilutions. Anti-OprF and anti-GST::ME19 responses were determined using purified OprF and GST::ME19 as coating antigens respectively. Anti-$(NANP)_3$ was determined using a synthetic peptide NANPNANPNANP as coating antigen. Antisera were taken after three injections. The reported values are mean values ± standard deviations from three independent assays.

[b] ND, not determined

3.3.2 Immunogenicity of OprF::ME7aa26 and OprF::ME19aa26

To examine the effect of the length of the epitope on its immunogenicity, two OprF::malarial epitope hybrids, OprF::ME7aa26 and OprF::ME19aa26, carrying a 7- and a 19- amino acid malarial epitope respectively at aa^{26}, were used as immunogens in an immunization study. C57BL/6J mice were immunized with 20 μg of the gel-purified immunogens on days 0 and 21 and with 10 μg of the immunogens on day 35. Control groups included animals injected with wild type OprF or PBS. The anti-OprF and anti-malarial epitope antibody responses were determined by ELISA using purified OprF, GST::ME19 and $(NANP)_3$ peptide as coating antigens (Methods and materials 13.3). The antisera taken after the second injection showed significant anti-OprF titers ($>10^4$), but no anti-epitope titers were detected in the same sera. After 3 injections, the anti-OprF titers increased to $>10^5$ in all three groups, while a significant anti-malarial epitope response was only detected in animals immunized with OprF::ME19aa26 and a weak anti-malarial epitope response was detected in one of the five animals (animal a) immunized with OprF::ME7aa26 (Fig. 33, Table XV). As controls, the anti-OprF titers were similar in all three groups. Characterization of the antisera by Western immunoblotting demonstrated the presence of anti-OprF and anti-malarial epitope antibodies in the antisera that showed the corresponding antibody response in ELISA (Fig. 34). Neither the pre-immune sera nor antisera taken from the PBS control group showed any significant anti-OprF and anti-malarial epitope response. The results suggested that the 19-amino acid epitope inserted at aa^{26} was significantly more

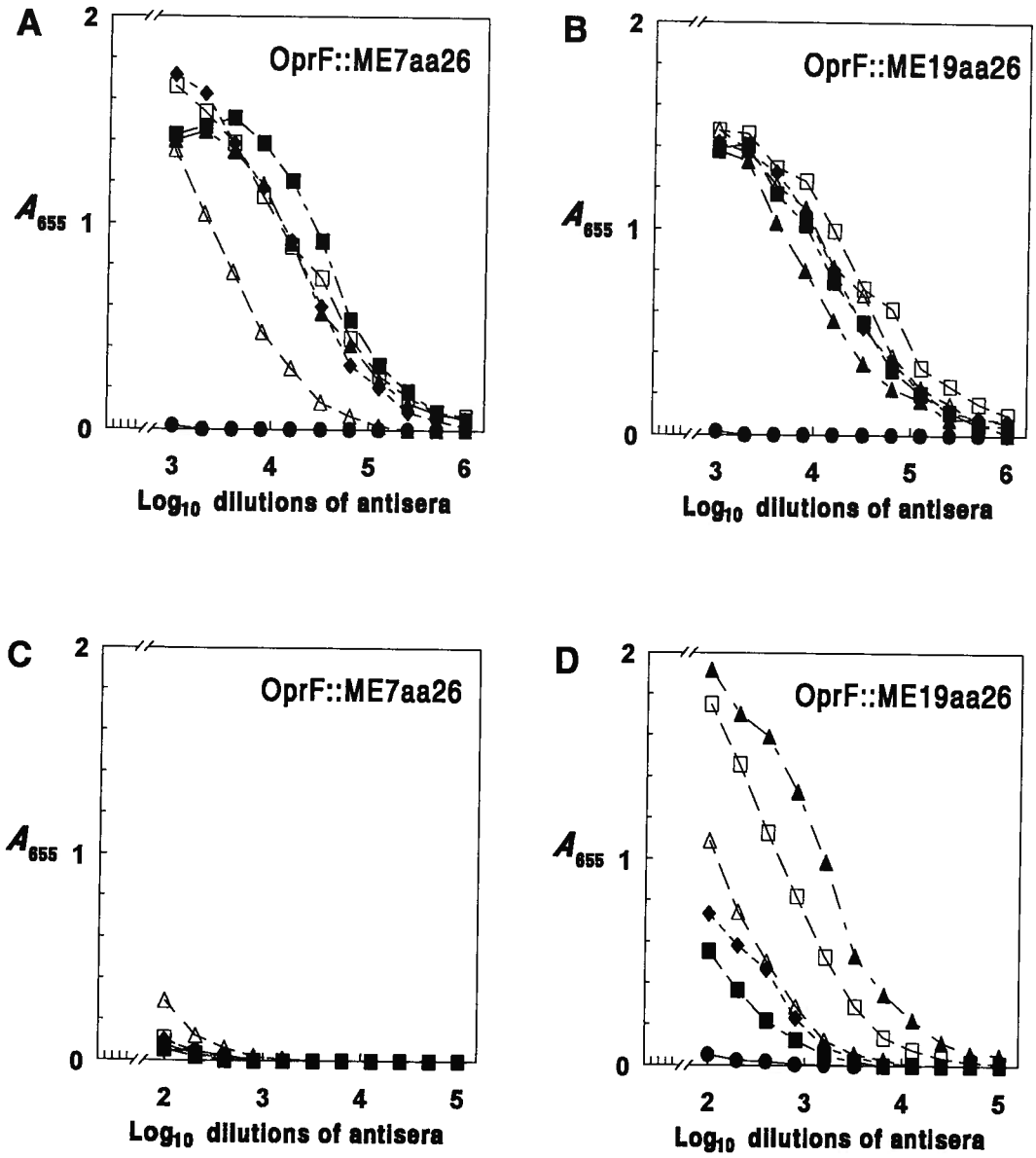


Figure 33. ELISA titrations of anti-OprF and anti-malarial epitope responses induced in C57BL/6J mice immunized with OprF::ME7aa26 and OprF::ME19aa26 by ELISA.

A and B, anti-OprF response; C and D, anti-malarial epitope response. The anti-OprF and anti-malarial epitope responses were measured using purified OprF and GST::ME19 as coating antigens respectively. The immunogens used in each groups are indicated on the graphs. Symbols: ●, pooled pre-immune sera; △, □, ◆, ▲, ■; serum samples from five different animals.

Table XV. Summary of antibody responses induced in mice immunized with OprF::ME7aa26 and OprF::ME19aa26.

Immunogens	Animals	ELISA titers[a]		
		Anti-OprF	Anti-GST::ME19	Anti-$(NANP)_3$
OprF	a	5.91 ± 0.17	<2	<2
	b	5.61 ± 0.17	<2	<2
	c	5.71 ± 0.30	<2	<2
	d	5.11 ± 0.60	<2	<2
	e	5.11 ± 0.52	<2	<2
OprF::ME7-aa26	a	4.61 ± 0.35	2.30 ± 0.00	<2
	b	5.31 ± 0.62	<2	<2
	c	5.31 ± 0.35	<2	<2
	d	5.31 ± 0.62	<2	2.00 ± 0.00
	e	5.71 ± 0.30	<2	<2
OprF::ME19-aa26	a	5.71 ± 0.30	3.40 ± 0.35	<2
	b	5.71 ± 0.30	3.71 ± 0.46	2.20 ± 0.17
	c	5.51 ± 0.46	3.20 ± 0.31	<2
	d	5.31 ± 0.62	4.21 ± 0.35	2.50 ± 0.17
	e	5.71 ± 0.30	3.05 ± 0.26	2.15 ± 0.21

[a] Titers are reported as the log of dilutions of antisera that gave twice of the A_{655} readings of the pre-immune serum at 100-fold dilutions. Antisera were taken after three injections. The antigens used were as described in Table XIV. The reported values are mean values ± standard deviations from three independent assays.

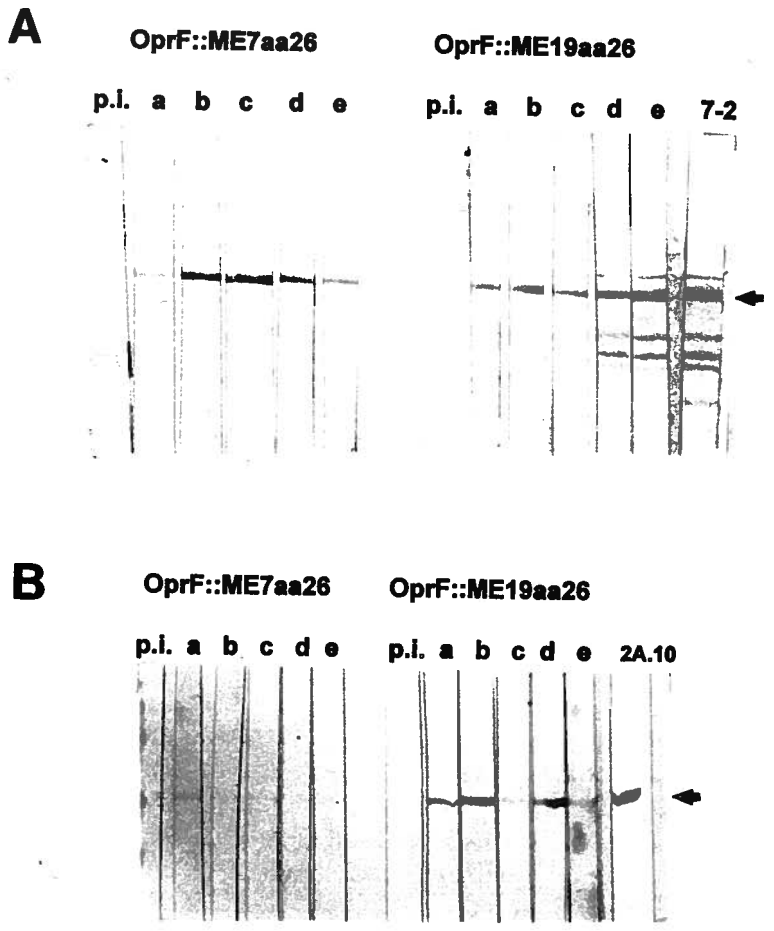


Figure 34. Western immunoblot analysis of the sera from mice immunized with OprF::ME7aa26 and OprF::ME19aa26.

A). Western immunoblot using purified *E. coli* OprF as the antigen with antisera from individual immunized mice at 1/1000 dilution. **B).** Western immunoblot using GST::ME19 as the antigen with antisera from individual immunized mice at 1/100 dilution. Lanes: p.i., pooled pre-immune sera; a,b,c,d,e; sera from five mice immunized with the indicated proteins. Arrows represent native OprF (A) and GST::ME19 (B) respectively. Positive controls used were MA7-2 and pf2A.10 (both at 1/3000 dilution) for (A) and (B) respectively.

immunogenic than the 7-amino acid epitope inserted at the same site. The titers of the antisera are summarized in Table XV.

3.3.3 Immunogenicity of GST::ME11 and GST::ME19

Due to the ease of genetic cloning and the simplicity of the subsequent purification of the fusion proteins, glutathione *S*-transferase is quite frequently used as a carrier to enhance the immunogenicity of small peptide immunogens (Ling *et al.*, 1994; von Specht *et al.*, 1995). Since two versions of GST::malarial epitope fusion proteins were already available, the immunogenicity of the epitope in these proteins was also studied so as to allow comparison between the GST and OprF carrier systems. The control groups in this experiment were immunized with GST or PBS. The anti-GST and anti-malarial epitope titers were determined by ELISA using gel-purified GST and OprF::ME19aa26 as coating antigens respectively. Animals immunized with GST or the GST fusion proteins developed a significant anti-GST response after 2 injections. The anti-GST and anti-malarial epitope responses in antisera taken after 3 injections are shown in Figure 35. All three groups immunized with the protein immunogens generated significant anti-GST titers (~10^5); however, no anti-malarial epitope response was observed in the groups immunized with the fusion proteins. Figure 36 shows the presence of anti-GST antibodies in the antisera by Western immunoblotting. The same antisera did not react with OprF::ME19aa26 on Western immunoblotting, indicating the absence of malarial epitope-specific antibodies. The inability of the GST fusion

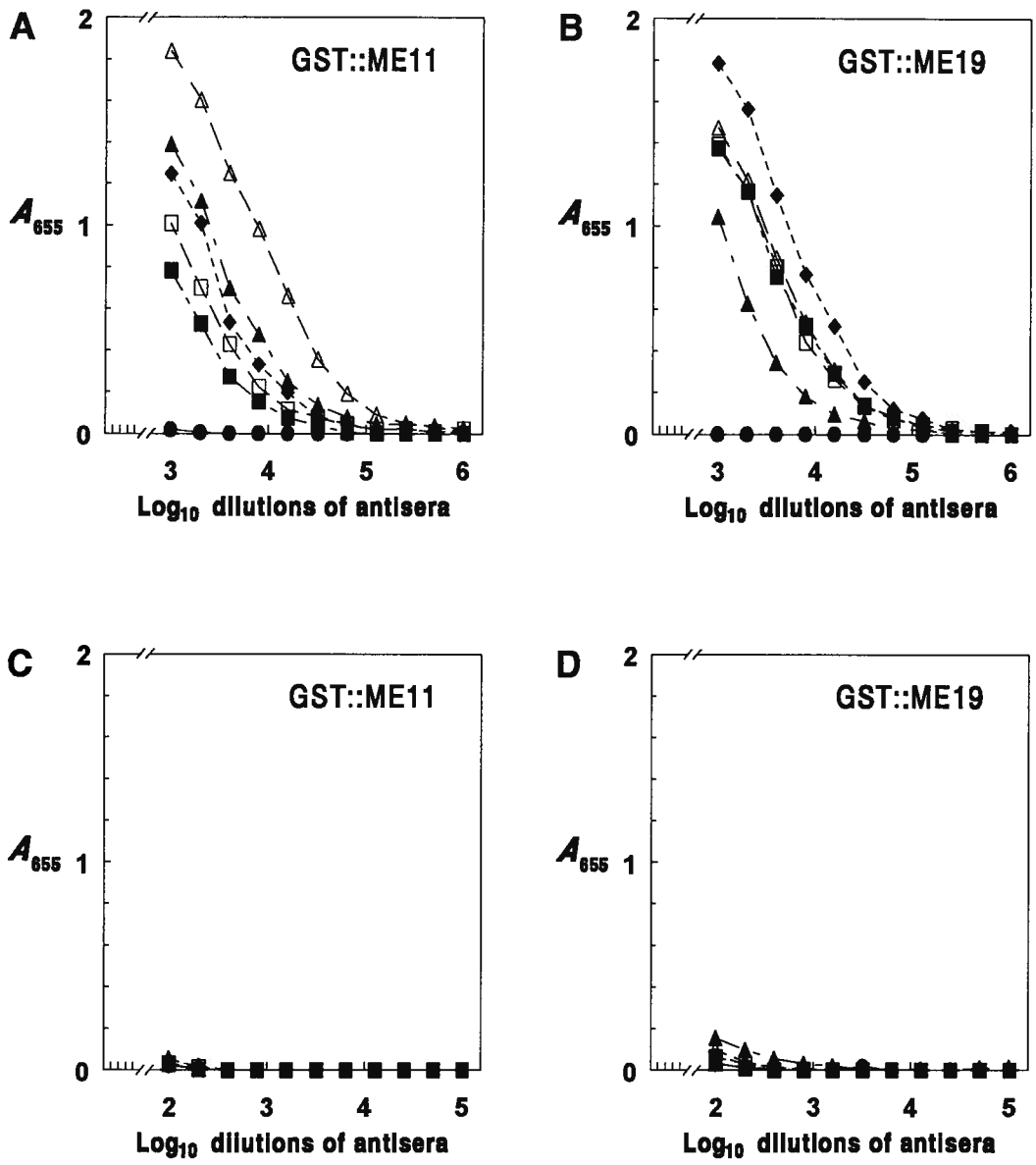


Figure 35. ELISA titrations of anti-GST and anti-malarial epitope responses induced in C57BL/6J mice immunized with GST::ME11 and GST::ME19.

A and B, anti-GST response; C and D, anti-malarial epitope response. The immunogens used in each group are indicated on the graphs. The anti-GST and anti-malarial epitope responses were measured using purified GST and OprF::ME19aa26 as coating antigens respectively. Symbols: ●, pooled pre-immune sera; △, □, ♦, ▲, ■, serum samples from five different animals.

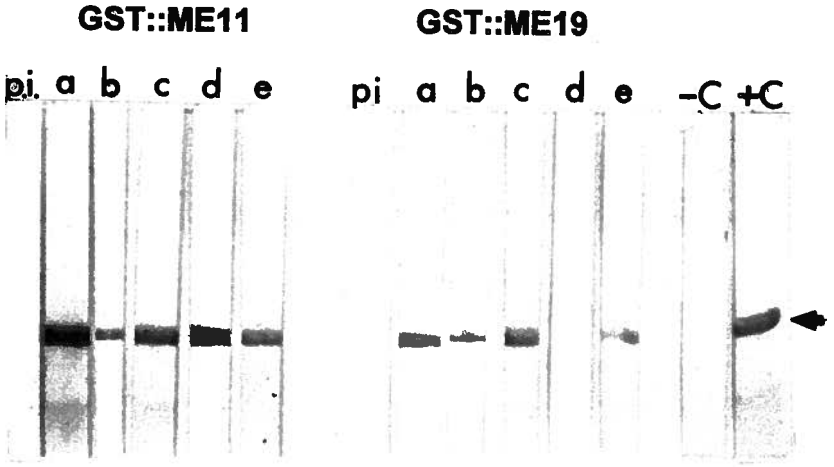


Figure 36. Western immunoblot analysis of the sera from mice immunized with GST::ME11 and GST::ME19.

Affinity-purified GST was used as the antigen. Antisera examined were from individual immunized mice at 1/1000 dilution. Lanes: p.i., pooled pre-immune sera; a,b,c,d,e, sera from five mice immunized with the indicated proteins; -C, negative control using pf2A.10; +C, positive control using an anti-GST antiserum. The arrow indicates the position of GST.

proteins to elicit an anti-malarial epitope response was unexpected. A possible explanation could be that the folding of the epitope in the GST fusion proteins did not allow the epitope to be readily recognized by the components of the immune system. The titers of the antisera are summarized in Table XVI.

3.4 Summary

In this chapter, the antigenicity of the malarial epitope presented at different positions and in different lengths in the OprF epitope presentation system was studied. The malarial epitope inserted at different positions of OprF displayed different binding affinities for an epitope-specific mAb. For example, the malarial epitope inserted at aa^{188} or aa^{196} was consistently more antigenic than that inserted at aa^{215} or aa^{310}, while insertions at aa^{26} or aa^{213} were significantly more antigenic in the whole cell and outer membrane environments respectively. Insertion of multiple copies of the epitope at aa^{26} and aa^{213} resulted in higher levels of antibody binding than with single copy; possibly due to the increase in valency and/or better presentation of the binding epitope. Among the three sets of multiple-repeat hybrids, insertions at aa^{26} and aa^{196}, but not at aa^{213} demonstrated an increase in antigenicity with the increase in length of the epitope. This suggested that the correlation between length and antigenicity of the epitope was site-dependent in the OprF system.

The immunogenicity of the epitope genetically inserted in OprF or fused

Table XVI. Summary of antibody responses induced in mice immunized with GST::ME11 amd GST::ME19.

Immunogens	Animals	ELISA titers[a]		
		Anti-GST	Anti-OprF::ME19-aa26	Anti-$(NANP)_3$
GST	a	4.81 ± 0.53	<2	<2
	b	4.10 ± 0.46	<2	<2
	c	4.81 ± 0.61	2.15 ± 0.21	<2
	d	4.41 ± 0.46	<2	<2
GST::ME11[b]	a	4.90 ± 0.76	2.10 ± 0.17	<2
	b	4.00 ± 0.76	<2	<2
	c	4.20 ± 0.61	<2	<2
	d	4.40 ± 0.63	<2	<2
	e	3.80 ± 0.63	<2	<2
GST::ME19[b]	a	4.30 ± 0.76	2.35 ± 0.31	<2
	b	4.20 ± 0.91	2.00 ± 0.00	<2
	c	4.41 ± 0.76	2.00 ± 0.00	<2
	d	3.80 ± 0.76	2.20 ± 0.17	<2
	e	4.30 ± 0.76	<2	<2

[a] Titers are reported as described in Table XV footnotes. Antisera were taken after three injections. The anti-GST and anti-OprF::ME19aa26 responses were determined by using purified GST and OprF::ME19aa26 as coating antigens. The anti-$(NANP)_3$ response was determined by using the synthetic peptide NANPNANPNANP as coating antigen. The reported values are mean values ± standard deviations from three independent assays.

[b] The malarial epitope sequence fused to the C-terminus of GST were $DP(NANP)_2NAQL$ and $DP(NANP)_4NAQL$ for GST::ME11 and GST::ME19 respectively.

to GST was also investigated. Despite its ability to interact with the malarial epitope-specific mAb, a 10-amino acid epitope inserted at the C-terminal insertion site aa^{215} of OprF was not immunogenic. A 19-amino acid epitope inserted at the N-terminal insertion site aa^{26} was able to elicit a significant anti-malarial epitope antibody response, whereas a 7-amino acid epitope inserted at the same site was only weakly immunogenic. For the GST-malarial epitope fusion proteins, it was found that neither an 11- nor a 19-amino acid epitope fused to the C-terminus of GST could stimulate an anti-malarial epitope response in immunized animals.

DISCUSSION

General

This study demonstrated the potential of OprF, the major outer membrane protein of *P. aeruginosa*, as a carrier for the presentation of foreign antigenic determinants. Semi-random linker-insertion mutagenesis was conducted to investigate the "permissiveness" of different regions of OprF to accommodate extra amino acid residues. The repeating epitope (NANP) of the circumsporozoite protein of the malarial parasite, *P. falciparum*, was used as a model epitope to further explore the usefulness of the insertion sites to express foreign antigenic determinants. The antigenicity of the malarial epitope inserted at different positions and in different lengths in OprF was compared. A targeted study was also undertaken to examine the immunogenicity of the inserted epitope in selected OprF::malarial epitope hybrid proteins.

The results of the linker-insertion and epitope-insertion studies have generated useful information about the membrane topology of OprF. In addition, the analysis of the reactivities of the OprF linker mutants and the OprF::malarial epitope hybrid proteins with the series of OprF-specific monoclonal antibodies have improved our understanding of the binding epitopes of these antibodies. This study represents the first attempt to compare the antigenicity of the presented epitope in **eight** different insertion sites of the carrier protein in an epitope presentation

system. Moreover, it is also the first study to systematically investigate the effects of insertion position and length of the epitope on its antigenicity in an epitope presentation system. Furthermore, this study revealed, for the first time, that OprF can be used as a carrier for a foreign epitope to generate and detect anti-epitope antibodies in immunized animals and in immunoassays respectively.

Linker-insertion mutagenesis

According to restriction enzyme site analysis, there were 37 sites within *oprF* that were potential targets for the linker-insertion mutagenesis. However, only 13 unique sites (including 2 in which the inserted 12 nucleotide pairs were translated to a stop codon) were identified after the screening of 100 clones by restriction enzyme digest analysis. Although attempts were made to identify sites where the insertion of the 4-amino acid linker resulted in no detectable OprF product (i.e. "non-permissive" sites), only one such site was identified. Six of the seven putative "non-permissive" clones analyzed by DNA sequencing showed that the lack of detectable OprF product was the result of deletions or the incorporation of stop codons translated from the 12 bp linker due to the reading frame at the insertion sites. It was possible that insertion sites that were close to each other have been overlooked by the restriction digest analysis. In addition, it was also likely that more unique sites could have been identified by more exhaustive screening.

Effects of amino acid insertions in OprF

Although the results of the characterization of the OprF linker mutants and the OprF::malarial epitope hybrid proteins suggested that these OprF variants shared many similarities to the wild type, insertion of extra amino acids at specific sites did alter the wild type properties to a limited extent. For example, the insertion of 4 amino acids at aa^2 of the mature protein directed the expression of a 41 kDa product which was of a higher apparent molecular mass than the wild type OprF (35 kDa) (Table IV). It is noted that one of the inserted amino acids was arginine. A number of studies have reported that the incorporation of positive charges at the N-terminal end of the mature protein impedes the export of membrane proteins (MacIntyre and Henning, 1990; Geller *et al.*, 1993; Struyvé *et al.*, 1993b). These studies suggested that the positively-charged residues might affect the export of proteins by disrupting membrane potential and interacting with polar head groups of acid phospholipids, thus arresting the transport across the cytoplasmic membrane. On the other hand, it has also been documented that the presence of positive charges at the extreme N-terminus of the mature protein can interfere with signal peptide processing (Li *et al.*, 1988). Therefore, one can speculate that this 41 kDa band corresponding to the OprF variant carrying an insertion at aa^2 represented OprF with the signal peptide still attached. The presence of this OprF variant in the outer membrane preparation might have been caused by contamination of the preparation with inclusion bodies, which were

formed as a consequence of defects in folding and the export pathway (Marston, 1986). The insertion of a 10-amino acid malarial epitope at aa^{2} did not lead to any detectable product. If the insertion of 4 amino acids already interfered with the export pathway, it is conceivable that the insertion of an additional 10 amino acids might lead to a more severe effect, resulting in the degradation of the cytoplasmic intermediate.

The series of multiple-repeat hybrids carrying an insertion at site aa^{26} also induced incomplete formation of inclusion bodies as indicated by the migration of some of the protein at the expected position on SDS-PAGE (Fig. 17). The inclusion body form of these proteins differed from the membrane associated form in two aspects. First, the apparent molecular mass of the inclusion bodies was higher than that of the membrane associated form of the proteins. As mentioned above, this could be due to the presence of signal peptide as a result of defective signal peptide cleavage. Second, the inclusion body form was not modified by 2-mercaptoethanol, indicating the absence of disulphide bond. Since the proteins required for disulphide bond formation in compartments external to the inner membrane, DsbA and DsbB, are located in the periplasm and cytoplasmic membrane respectively (Bardwell, 1994), this observation is consistent with the cytoplasmic localization of the inclusion bodies. The partial defect in export of this series of OprF::malarial epitope multiple-repeat hybrids was apparently not due to the length of the inserted sequence because the corresponding positional hybrid with a 10-amino acid insertion at the same site was completely inserted into the

outer membrane. Comparison of the flanking amino acid sequences of the positional hybrid and the multiple-repeat hybrids revealed that an arginine residue was introduced at the N-terminal side of the malarial epitope in the multiple-repeat hybrids as a result of the cloning procedures. It has been documented that positive charges introduced at positions up to +20 of the mature protein are able to inhibit export (Kuhn *et al.*, 1994). Therefore, it is possible that the introduction of the arginine residue at aa^{26} of the mature OprF might lead to the same effect. It should be noted that the insertion of arginine residues at other sites (e.g. aa^{213}, aa^{231}, and multiple-repeat hybrids at aa^{196}) did not affect the localization of the protein, indicating that this effect was site-specific.

Linker insertion at aa^{131} directed the production of an OprF variant of molecular mass identical to that of the heat-modified, unfolded form of OprF and with a distinct trypsin-cleavage pattern (Figs. 12 and 13). Thus it can be assumed that this insertion influenced the SDS stability of the protein, probably by inducing a slight change in membrane configuration. This decrease in stability may have explained the result that the insertion of a 10-amino acid epitope at this site did not lead to any detectable product. Taking together the results of the linker-insertion and epitope-insertion studies, this might reflect the inflexibility of this region of OprF to accommodate extra amino acid sequences.

Linker insertions at aa^{188} and aa^{196} led to OprF variants with a different trypsin-cleavage pattern in outer membranes (Fig. 13), indicating exposure of a trypsin-accessible cleavage site in or adjacent to the cysteine disulphide loop. This

suggested that the insertion of 4 amino acids caused alterations in the local conformation, a notion supported by the loss of reactivities of these OprF variants with the OprF disulphide bond-sensitive antibodies MA4-4 and MA5-8, and yet the retention of reactivities with the rest of the OprF-specific antibodies (Table VI). Likewise, the OprF::malarial epitope hybrid proteins with epitope insertions at aa^{188} or aa^{196} retained reactivity with most of the OprF-specific mAbs except for MA4-4 and MA7-8 (Table XI). Therefore, by the same token, one can assume that the decrease in heat stability in these OprF variants was due to localized instability, instead of an impact on the general folding of the β-sheet structure (Fig. 14).

It has been shown in this study that linker insertions at the C-terminal region of OprF demonstrated enhanced susceptibility to trypsin cleavage in the outer membrane while epitope insertions at the same region increased susceptibility of the hybrid proteins to cellular proteases. The increase in susceptibility could be due to the introduction of a new protease cleavage site or the increase in accessibility of existing cleavage site(s). Since the degradation product observed in all the cases had the same apparent molecular mass of 28 kDa and a cleavage product of the same apparent molecular mass was observed in wild type OprF, it seems more likely that the insertion of extra amino acids increased the accessibility of an existing cleavage site. This is reminiscent of the previous finding by Finnen *et al.* (1992) that C-terminal perturbations render the resulting OprF mutants more prone to protease action.

In cases where cysteine residues were present in the linkers translated as TCRS, interactions of these exogenous cysteines with the cysteines in OprF might disrupt the protein structure by forming alternate disulphide bonds. However, the observations that the OprF variants with 4-amino acid insertion at aa^{135} and aa^{213} migrated with similar mobility on SDS-PAGE, showed similar 2-mercaptoethanol modifiability and reacted with MA7-8 and MA4-4, which recognize epitopes sensitive to reduction of the OprF disulphide bonds, suggested that the cysteine residue present in the linkers of these variants did not participate in disulphide bonding with the endogenous cysteine residues. A recent study has reported on cysteine substitution mutagenesis of LamB and the subsequent examination of the reactivity of the mutated proteins with different monoclonal antibodies before and after thiol treatment (Notley *et al.*, 1994). The data obtained provided new information to extend previous maps of the monoclonal antibody binding sites. Therefore, using the same approach, the cysteine residues introduced in the linkers might provide similar information to refine the mapping of the OprF epitopes recognized by specific antibodies.

Membrane topology of OprF

Siehnel *et al.* (1990) previously presented a model for OprF based on the apparent existence of two disulphide bridges between the four cysteines of OprF (Hancock and Carey, 1979), the β-turn prediction rules of Paul and Rosenbusch

(1985), and circular dichroism data suggesting that 62% of the secondary structure of OprF is in the form of β-sheet, a value typical for outer membrane proteins (Cowan *et al.*, 1992; Schirmer *et al.*, 1995). This model has been tested in part by Tn*phoA* mutagenesis and deletion analysis of the *oprF* gene (Finnen *et al.*, 1992).

Sequence comparison studies have revealed that the sequences in the surface loop regions of outer membrane proteins are hypervariable. These regions are possibly least spatially constrained because of their surface location. Both of these properties lead to the assumption that these regions are more likely to tolerate extra amino acid insertions (Charbit *et al.*, 1991). Based on this assumption, linker- and epitope-insertion studies of other outer membrane proteins have suggested that the "permissive" insertion sites are most likely to be within the loop regions of the proteins. Interestingly, the 3-dimensional structures of PhoE and LamB, which were resolved after the insertion studies, proved this assumption to be correct (Cowan *et al.*, 1992; Schirmer *et al.*, 1995). Therefore, the insertion sites characterized in this study should have similar value in defining OprF topology.

With the data presented here, a new topological model of OprF was constructed partly based on the assumption that the "permissive" sites for linker insertion should be in the loop regions of the protein. It was considered that the site at aa^{2} was probably periplasmic since all outer membrane proteins studied to date have N-termini that are in the periplasm. The insertion site at aa^{42} was considered "non-permissive" since it resulted in no detectable product. Thus this

site was placed within the membrane. The remaining 9 sites were placed in surface loops. Linker insertion in five of these sites (aa^{188}, aa^{196}, aa^{231}, aa^{290} and aa^{310}) interrupted the binding of specific monoclonal antibodies that have been shown to bind to surface-exposed epitopes (Martin *et al.*, 1993), and were thus placed in the surface-exposed loop regions. The placement of aa^{310} was supported by its location within a flexible protein segment adjacent to a variable sequence (Jeanteur *et al.*, 1991). The location of this site was further confirmed by a subsequent study which showed that the insertion point is within the linear, surface-exposed epitope for MA5-8 (Rawling *et al.*, 1995). Insertions in the other 4 sites did not interrupt the binding of specific monoclonal antibodies, but these sites were assigned on the surface in keeping with the precedent in LamB and PhoE that "permissive" insertion sites are usually found in the surface loop regions. Moreover, the results obtained from the malarial epitope-insertion study showed that the epitope inserted at these sites was detectable on the cell surface (Table IX), which supported the surface localization of these sites. A revised topological model of OprF, which is constructed in part based on the data from this study, is shown in Figure 37.

The OprF topology model indicates that the insertion site at aa^{215} is at the end of a transmembrane segment. This segment has high homology to the transmembrane segment 8 of OprF and to the consensus 16th transmembrane β-strand of the porin superfamily (Jeanteur *et al.*, 1991), and thus appears almost certain to be in the membrane. However, this site was found to be "permissive" for the incorporation of both the 4-amino acid linker and the 10-amino acid epitope.

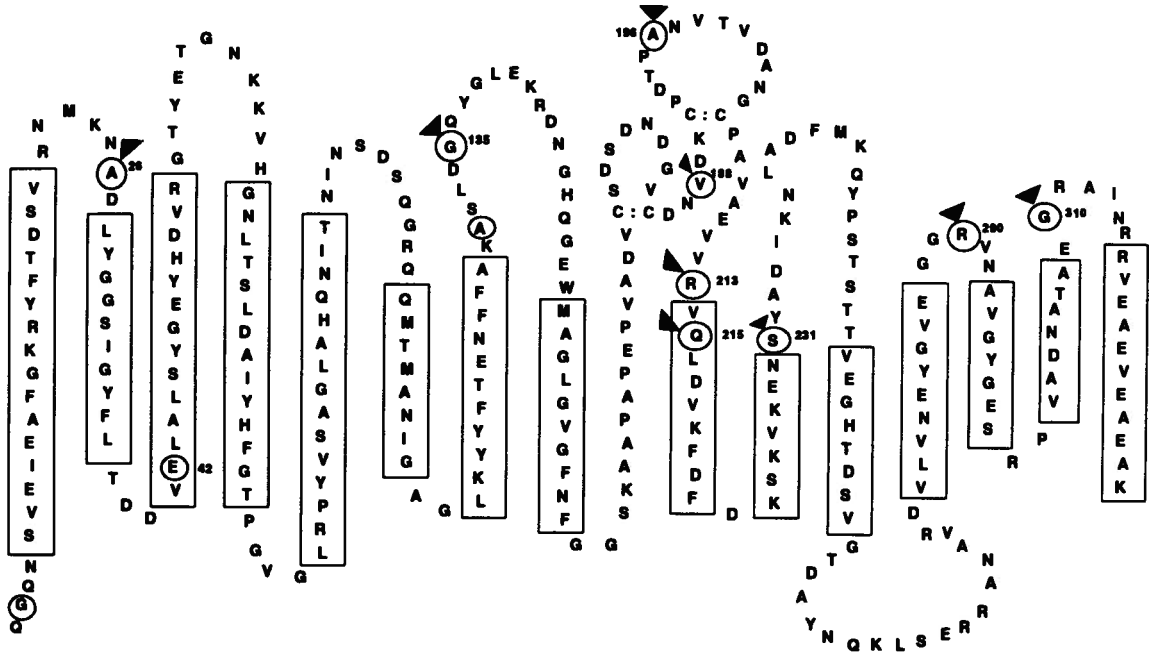


Figure 37. Proposed membrane topology of OprF.

The linker-insertion sites are circled and the permissive malarial epitope-insertion sites are indicated by solid triangles. The top of this model is proposed to face the exterior of the cell. The transmembrane β-strands are indicated by rectangular boxes.

Examination of the inserted sequences suggested the possibility that sufficient β-strand character was maintained in these sequences, which might have enabled the extension of this strand and hence the exposure of the epitope on the cell surface.

Insertions at aa^{213} and aa^{231} appeared to promote OprF trimer stability in the absence of 2-mercaptoethanol (Fig. 11). In the 3-dimensional structures of PhoE and LamB, it was revealed that the surface loop L2 is responsible for trimer association by extending from one monomer to an adjacent monomer. This leads to the speculation that the two surface loops of OprF where the insertions occurred might also be involved in OprF trimer/oligomer association. Extending these loops by 4 amino acids might enable them to reach farther to the adjacent monomer, thus enhancing trimer stability. On the other hand, the reduction of the disulphide bond by 2-mercaptoethanol might have loosened the secondary structure in a manner that resulted in the dissociation of the trimers.

Binding epitopes of OprF-specific monoclonal antibodies

The examination of the reactivities of the linker-insertion and epitope-insertion derivatives of OprF with the series of OprF-specific monoclonal antibodies also provided information about the binding epitopes of these antibodies. Recently, Rawling *et al.* (1995) had delineated the OprF epitopes recognized by a series of OprF-specific monoclonal antibodies available in our laboratory. The results of this study were based on data generated by monoclonal antibody reactivities with

overlapping synthetic peptides on pins and cyanogen bromide and papain cleavage fragments of OprF. Since the insertions of the 4-amino acid linker and the malarial epitope only disrupted a localized region of the protein while apparently leaving the rest of the protein intact, these approaches represented a more subtle way to define the boundary and nature of the binding epitopes.

This study provided new information about the conformational epitopes recognized by the OprF-specific mAbs MA4-4 and MA7-8. Rawling *et al.* suggested that epitope(s) recognized by these antibodies are located at the same region from aa^{152}-aa^{210}. According to the linker-insertion study, insertion of 4 amino acids at aa^{196} disrupted the epitopes for both MA4-4 and MA7-8, while insertion at aa^{188} disrupted only the MA7-8 epitope (Table VI), implying that these antibodies bind to overlapping but distinct epitope(s). Moreover, based on the observation that the presence of 2-mercaptoethanol abolished the binding of these monoclonal antibodies to OprF, the binding of MA4-4 and MA7-8 are believed to require the presence of disulphide bond(s) (Mutharia and Hancock, 1983). In the present study, most of the OprF variants carrying linker and epitope insertions in the cysteine-containing region (aa^{188} and aa^{196}) resulted in the loss of MA4-4 and MA7-8 reactivities. The insertion of extra amino acid residues in this region could potentially have interfered with the formation of disulphide bond and/or affected the conformation of the disulphide bond-containing region. The observation that these variants still retained similar 2-mercaptoethanol modifiability as wild type OprF, and that the 4-amino acid insertion at aa^{188} still retained MA4-4 reactivity suggested that the

disulphide bonds were correctly formed. Hence, it is likely that the loss of MA4-4 and MA7-8 reactivity was due to a change in the secondary structure (conformation) of the amino acids in the disulphide bond-containing region rather than the disruption of disulphide bond formation. Therefore, it appears likely that the binding of MA4-4 and MA7-8 requires both the correct formation of the disulphide bonds and the correct secondary structure in this region.

This study also shed new light on the conformational epitopes recognized by the OprF-specific mAbs MA7-3, MA7-4, MA7-5 and MA7-7. The insertion of 4 amino acids at the C-terminus of OprF (aa^{231}, aa^{290} and aa^{310}) abolished the binding of all of these monoclonal antibodies (Table VI). Interestingly, based on the reactivity of protease digested peptides carrying 1-275 amino acids, Rawling *et al.* (1995) have delineated the MA7-3 epitope to aa^{188}-aa^{230} and the MA7-4, MA7-5 and MA7-7 epitopes to aa^{188}-aa^{278}. The results presented here revealed that although sites aa^{290} and aa^{310} do not comprise the epitope(s), changes at these sites could still affect the conformation of the epitope(s). Alternatively, the presence of extra amino acids at these sites might have reduced the accessibility of the epitope(s) for antibody binding. A number of studies have reported that changes at sites distant from the epitopes can interfere with the binding of antibodies to the epitopes (McCutcheon *et al.*, 1993; Collawn *et al.*, 1988). Therefore, it is possible that the insertion of the 4-amino acid linker at aa^{290} and aa^{310} induced conformational changes in the epitopes recognized by MA7-3, MA7-4, MA7-5 and MA7-7, and consequently interrupted the binding of these antibodies. The insertion of a 10-

amino acid epitope at aa^{290} and aa^{310} resulted in weak binding of MA7-3, while disrupted binding of MA7-4, MA7-5 and MA7-7 (Table XI). This is consistent with the results from Rawling *et al.* (1995), which suggested that MA7-3 recognizes a different epitope than MA7-4, MA7-5 and MA7-7.

Based on monoclonal antibody reactivities of overlapping synthetic peptides on pins, Rawling *et al.* concluded that MA7-1, MA7-2 and MA5-8 recognize linear epitopes of OprF. In general, the disruption of the linear binding epitopes by insertions in this study was consistent with the epitope boundaries as defined by this previous study (Rawling *et al.*, 1995 and Table III). The only exception was that the OprF linker mutant carrying an insertion at aa^{131} was not reactive with MA7-1 (Table VI), whose epitope has been mapped to aa^{55} to aa^{62} of OprF. Since insertion at aa^{131} appeared to cause minimal changes in OprF membrane configuration in that the binding of the majority of mAbs was unaffected, the loss of MA7-1 reactivity of this mutant might have been due to the masking of the MA7-1 epitope as a result of the extension of an adjacent loop.

Antigenicity and mode of presentation

The larger number of sites examined in this study permitted us to attempt to correlate the measured antigenicity of the epitope at the various insertion sites with the primary and secondary structures at these sites. The possible structures of each insertion site were analyzed using various structure

prediction methods (Table XVII). The structures at each insertion site were analyzed in the context of the entire protein, as well as in a segment of the sequence including the six OprF amino acid residues flanking either side of the insertion. When only the flanking residues were taken into consideration, the Gascuel and Golmard Basic Statistical Methods (GGBSM) analysis (Gascuel and Golmard, 1988) predicted that three or more amino acids were in extended conformation on at least one of the flanking sequences of insertion sites aa^{196}, aa^{290} and aa^{213}, where epitope insertion showed medium to high antigenicity (Fig. 27). The antigenic determinant program of Hopp and Woods (1981) predicted that these sites have comparatively low to medium hydrophilicity on both sides of the flanking regions. When analyzed in the context of the entire amino acid sequence, the insertion sites that exhibited high relative antigenicity were found in regions that were generally predicted to be more flexible in their local secondary structure and to have higher coil propensity (Karplus and Schulz, 1985). Although the correlations were not universal, the general trend of extended conformation and high flexibility of the local sequences at insertion sites which resulted in high relative antigenicity of the inserted epitope seemed to suggest that these features might improve the accessibility of the epitope.

It is noteworthy that epitope inserted at aa^{213} and aa^{215} demonstrated significantly different antigenicities despite the fact that these insertion sites are only 2 amino acids apart (Fig. 27). The proposed location of aa^{215} is at the cell surface end of a transmembrane segment of OprF. Based on the proposed model

Table XVII. Predicted primary and secondary structures at the insertion sites.

Insertion sites	No. residues in extended conformation[a]		Average hydrophilicity[c]		Probability of coil conformation[a,e] (%)	Flexibility B[norm][d,e]
	Right[b]	Left[b]	Right	Left		
Ala-26	0	0	0.45	0.33	55	1.09
Val-188	0	0	0.42	1.03	35	0.98
Ala-196	0	3	0.43	-0.4	72	1.08
Arg-213	3	4	0.34	0.53	25	1.01
Gln-215	0	1	0.28	0.53	27	0.95
Ser-231	0	0	1.78	0.42	30	1.08
Arg-290	0	6	0.77	0.75	47	1.07
Gly-310	0	0	0.3	1.15	35	1.05

[a] as predicted by the GGBSM program (Gascuel and Golmard, 1988).
[b] right and left of the flanking sequences respectively.
[c] as predicted by the Antigenic Determinant program (Hopp and Woods, 1981). The highest and lowest hydrophilicity values of the various regions of the entire protein are 2 and -1.5, respectively.
[d] as predicted by the Flexpro program (Karplus and Schulz, 1985), the numbers cited are the B[norm] values. The B[norm] values of the whole protein range from 0.820 to 1.129.
[e] analyzed in the context of the entire protein.

of OprF topology, it can therefore be hypothesized that part of the inserted epitope might be at the membrane interface and/or that the exposure of the epitope was shielded by the protruding surface loops, thus resulting in low antigenicity.

The results of antigenicity studies suggested that LPS plays a role in the presentation of epitope for antibody binding at aa^{26}, aa^{213} and aa^{290}. This indicated that these regions of OprF are involved in LPS association. Therefore, depending on the type of antigen preparation chosen (i.e. whole cells or outer membranes), the choice of insertion sites for optimal antigenicity may vary. The relatively high antigenicity of the inserted epitope at aa^{213} and aa^{290}, and the fact that most OprF-specific monoclonal antibodies recognize epitope(s) that are located in this region, suggested the immunodominance of this region of OprF. This notion is supported by a recent report which identified seven B cell epitopes, two of which are surface-exposed, in the C-terminal region (aa^{190}-aa^{350}) of OprF, confirming that this part of the protein is rich in B cell epitopes (von Specht *et al.*, 1995).

The antigenicity of epitope insertions at aa^{26} and aa^{196} increased with the length of the epitope, while that at aa^{213} did not (Table XIII). This may be explained by the degree of exposure of the insertion sites. Since the 10-amino acid epitope inserted at aa^{213} was comparatively more antigenic, it appears that this site is already well-exposed. On the other hand, the 10-amino acid epitope inserted at aa^{26} and aa^{196} only displayed relatively low to medium antigenicity, suggesting mediocre exposure at these sites. Therefore, it appears that increasing the length of the epitope at aa^{26} and aa^{196} might have improved its exposure, thus facilitating

its presentation for antibody binding. This is reminiscent of the insertion of the FMDV epitope into the third loop of PhoE, which is a loop that is hidden inside the pore region of the protein. Researchers found that the insertion of one copy of the epitope does not result in the surface exposure of the epitope. However, insertion of multiple copies of the epitope forces this loop out of the pore, leading to exposure of the epitope (Struyvé *et al.*, 1993a).

Since the epitope-specific monoclonal antibody pf2A.10 was used in the antigenicity assays, one should keep in mind that the antigenicity of the epitope discussed in this study should only refer to its binding to this monoclonal antibody. Survey experiments using two other epitope-specific monoclonal antibodies to evaluate the correlation between antigenicity and the length of the epitope at aa^{196} and aa^{213} revealed the same findings as those observed using pf2A.10 (i.e. positive correlation at aa^{196} and no correlation at aa^{213}). These results implied that the trend of antigenicity observed using pf2A.10 may apply to the binding of other antibodies in general.

Immunogenicity

In general, the basic mechanisms of an immune response involve the production of antibodies and the development of cellular immunity. This study concentrated on the examination of antibody (more specifically, IgG) response against the immunogens. Therefore, the scope of immunogenicity discussed in the

context of this study is limited to the induction of antibody response. The immunogenicity of the inserted malarial epitope in five selected proteins, representing two different carriers (OprF and GST), was investigated in this study. The inserted epitope in only one of the proteins demonstrated significant immunogenicity. A number of factors might have caused the lack of immunogenicity of the inserted epitope. In this section I will attempt to address some of the possibilities.

A 19-amino acid epitope inserted at aa^{26} of OprF (OprF::ME19aa26) was significantly more immunogenic than a 7-amino acid epitope inserted at the same site (OprF::ME7aa26) (Table XV). Since no anti-malarial epitope response was detected in the immunized animals after the first injection, it indicated that the response was T cell-dependent. To generate a T cell-dependent response, the immunogen is required to have a B cell epitope which binds to the antigen receptor on B cells, as well as a T cell epitope that can be recognized by MHC class II molecules and presented to T helper cells (Guillet *et al.*, 1986; Brown *et al.*, 1988). The difference in immunogenicity of the 2 versions of malarial epitope inserted at the same position of OprF could be due to a number of factors. For instance, the increase in length of the epitope might have increased the accessibility of the epitope for binding to the B cell antigen receptor, as was indicated by the results of the antigenicity study which demonstrated a positive correlation between length and antigenicity at aa^{26}. Alternatively, since B cell activation requires the crosslinking of B cell antigen receptors, the poor immunogenicity of the shorter

version of the epitope might reflect its poor ability to elicit B cell receptor signalling. Nevertheless, it is quite possible that all of the factors mentioned above played a role in determining the immunogenicity of the epitope in this experiment. The examination of the immunogenicity of the epitope in OprF::ME11aa26 and OprF::ME15aa26 will further define the minimal length requirement for the immunogenicity of the epitope and/or the relationship between antigenicity and immunogenicity.

Using the same logic, the low antigenicity of the epitope inserted at aa^{215} could have been indicative of the lack of immunogenicity of this epitope in the context of OprF::ME10aa215. However, it is not likely that the intrinsic factors such as the antigenicity of the epitope were the sole factors affecting its immunogenicity in this case. Using the filamentous phage pIII protein as carrier, Cruz *et al.*, (1988) reported that although the inserted malarial epitope is antigenic *in vitro*, it is not necessarily immunogenic when administered to mice. An earlier study had also revealed that the malarial epitope is recognized as a T cell epitope only in mouse strains with $H\text{-}2^b$ and $H\text{-}2^k$ backgrounds (Good *et al.*, 1986). Since significant anti-OprF response was elicited in the BALB/c mice ($H\text{-}2^d$) used in this study, it indicates that OprF could recruit the required T cell response for the epitope. Therefore, the non-responsiveness of the animals immunized with OprF::ME10aa215 was likely due to inadequate B cell activation. Of course, the use of a lower dosage (10 μg instead of 25 μg as used in the other study) and the use of adjuvant might have also contributed to the non-responsiveness of the

immunized animals. However, the impact of these factors could not be easily evaluated by the simple design of the experiment in this study.

Successful antigen processing and presentation of the T cell epitope are two essential steps involved in an efficient antibody response. It has been documented that sequences outside a minimal epitope can affect the products of processing and thus may determine whether a peptide is selected for presentation (Van der Werf *et al.*, 1990; Del Val *et al.*, 1991; Janssen *et al.*, 1994b). Now that an effective immunization protocol has been established, similar studies using the other 2 series of multiple-repeat hybrids carrying the epitope at aa^{196} and aa^{213} might provide information about the length requirement at these two sites. A comparison of such results should elucidate the position effect (if one exists) on the immunogenicity of the epitope in the OprF presentation system. Moreover, these results might also increase our general understanding of the effect of flanking amino acid residues on immunogenicity. However, such studies were beyond the scope of the current thesis.

The inability of the GST::malarial epitope fusion proteins to generate an anti-malarial epitope response was unexpected (Table XVI). Since the protocol utilized for the immunogenicity study of the GST::malarial epitope fusion proteins was the same as that for the OprF::MEaa26 hybrid proteins, it is not likely that extrinsic factors such as the genetic background of the animals, the use of adjuvant, and the route of delivery were involved in the non-responsiveness of the immunized animals. The length of the epitope should not be a limiting factor either because

the length of the epitope in GST::ME19 is comparable to that in OprF::ME19aa26. One possible explanation could be that the conformation of the epitope in the GST::malarial epitope fusion proteins prevents its interaction with the components of the immune system. Based on the previous experience in this laboratory, attempts made to release a fusion peptide from the GST protein by protease cleavage at an engineered recognition site were unsuccessful, presumably due to the folding of the epitope in the fusion protein which limits the accessibility of the cleavage site (Piers, 1993). Similarly, antigen processing also requires cleavage of the original proteins by proteases. Therefore, one is tempted to speculate that the folding of the epitope *in vivo* might have rendered the protease cleavage site inaccessible for antigen processing, resulting in its failure to elicit an efficient antibody response.

In spite of its inability to stimulate an anti-malarial epitope antibody response, the GST::malarial epitope fusion proteins were useful for the detection of anti-epitope titers in antisera from animals immunized with the OprF::malarial epitope hybrid proteins. ELISA using the fusion protein as coating antigen for the determination of anti-malarial epitope titers was more sensitive than that using the synthetic peptide $(NANP)_3$ as coating antigen (Table XV). The same difference in sensitivity was also observed in ELISA using OprF::malarial epitope hybrid protein as coating antigen as compared to that using the same synthetic peptide (Table XVI). On the other hand, the synthetic peptide could be recognized by both of the malarial epitope-specific mAbs pf2A.10 and pf5A4.1, suggesting that it is antigenic.

These findings are consistent with the general knowledge that synthetic peptides alone do not interact effectively with antibodies, probably due to inadequate presentation.

Despite the apparent versatility of the OprF epitope presentation system, there were difficulties encountered in the course of this study that can be potential pitfalls of the system. For example, when *E. coli* cells expressing the OprF hybrid proteins were used as antigens in ELISA, the non-specificity binding of the epitope-specific monoclonal antibodies to whole cells was too high to permit any meaningful interpretation of the data. This problem was more prominent with the mAb MA5A4.1, which appeared to have lower affinity for the inserted epitope. Titration study of this monoclonal antibody with *E. coli* outer membrane containing OprF or one of the OprF::malarial epitope hybrid proteins showed that the dilution of the antibody that allowed reasonable sensitivity also resulted in significant background reactivity. Therefore, if whole cells containing the OprF hybrid proteins are to be used as antigens for the detection of anti-epitope antibody, the non-specificity of antibody binding should be carefully monitored.

The instability of the plasmids encoding OprF::malarial epitope multiple-repeat hybrids in C158 became a significant concern at the later stage of this study. Recombinant C158 strains had the tendency to lose the plasmids upon subculturing and subsequent growth in liquid medium. The ability of the host strain alone to grow in the presence of ampicillin remains puzzling. Since hydrophilic β-lactam antibiotics such as ampicillin are believed to be taken up by the bacterial cells via

the porin pathway, the lack of porins in the strain C158 may increase the ampicillin resistance of the bacteria. An attempt to enhance plasmid stability by raising the ampicillin concentration in the medium to 200 μg/ml was unsuccessful. Therefore, efforts to improve the stability of the recombinant plasmids in the host strain would be necessary to ensure the efficiency of the OprF epitope presentation system.

Using the malarial epitope as a model epitope, this study has shown that the OprF epitope presentation system can be used to raise and detect malarial epitope-specific antibodies. In addition, it has also been demonstrated that the OprF and GST systems can be used in a complementary fashion as a set of simple and flexible tools to induce and monitor an anti-peptide response without the use of synthetic peptides. The ability of OprF to promote immunogenicity of a foreign epitope and its potential as a vaccine against *P. aeruginosa* infections suggested that it has the possibility for the development of a multivalent vaccine.

In the course of this study, and using one of the OprF linker mutant plasmid vectors described here, a *Pseudomonas* pilin epitope was inserted in the context of OprF and shown to be recognized by its specific antibody in ELISA (B. Finlay, personal communication). Currently, OprF is being used to express random fragments of the filamentous haemagluttinin (FHA) gene of *Bordetella pertussis* and has shown some promising results for the mapping of antibody binding epitope(s) and functional domains of the protein (A. Siebers and B. Finlay, unpublished results). Attempts are also being made to utilize OprF as a carrier for

a neutralizing epitope of TSST-1, the Toxic Shock Syndrome Toxin-1 of *Staphylococcus aureus* (E. Rubinchik and A. Chow, personal communication). The increased use of the OprF epitope presentation system should expand the repertoire of foreign epitopes that can be presented and further explore the potential applications of this system.

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