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# ETUDE DE L'INTERACTION ENTRE GPJ, UNE PROTEINE DU BACTERIOPHAGE LAMBDA ET LAMB, UNE PROTEINE DE LA MEMBRANE EXTERNE DES BACTERIES GRAM-NEGATIVES

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A ma famille, à mes amis.

A la mémoire de mon oncle Ahmed.

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# Résumé

**<u>Titre :</u> Etude de l'interaction entre GpJ, une protéine du bactériophage Lambda, et LamB, une protéine de la membrane externe des bactéries gram-négatives.**

La fixation du bactériophage Lambda sur son récepteur cellulaire, LamB, est dûe à une protéine de sa queue appelée GpJ. Le but des travaux est d'étudier l'intéraction entre le bactériophage Lambda et LamB à travers l'étude du complexe entre LamB et GpJ exprimée en protéine de fusion. Pour ce faire, deux protéines de fusion sont utilisées : MBP-gpJ et His-gpJ.

MBP-gpJ est une protéine de fusion entre la Maltose Binding Protéine et l'extrêmité C-terminale de la protéine GpJ (résidu 684 à 1132), grâcieusement fournie par le Pr. Charbit (Paris, France). Grâce à la Technique du Film Noir (BLM), il a été permis d'observer que MBP-gpJ, après expression dans *E.coli* et purification, intéragit grâce au fragment de GpJ avec l'extrêmité extracellulaire de LamB. Cette intéraction se traduit par un blocage complet et réversible des canaux de LamB sauvage, mais également de mutants: LamB de *Shigella sonnei*, LamB Y118G et LamB Δ4+Δ6+Δ9v. Afin d'obtenir des informations sur la liaison de LamB avec uniquement le fragment de GpJ sans la partie MBP, une autre protéine de fusion a été réalisée: His-GpJ. His-gpJ représente l'extrêmité C-terminale de GpJ (684-1132) en fusion avec un 6×Histidine-tag. Cette protéine est exprimée sous forme de corps d'inclusion dans *E.coli*. Après purification et renaturation, une protéine de nouveau soluble peut être obtenue. Lors d'expériences de Film Noir, His-gpJ intéragit certes avec LamB, mais n'induit pas le blocage des canaux comme précedemment observé après ajout de MBP-gpJ. En parallèle, la formation d'un complexe entre His-gpJ et LamB sauvage, ainsi que de mutants a pu être confirmée au travers de travaux de SDS-PAGE et d'immunodétection par la présence de bandes de masse moléculaire élevée.

L'utilisation de mutants de LamB a par ailleurs permis d'essayer d'identifier la partie de LamB impliquée dans l'interaction avec le fragment C-terminal de GpJ, qui se révèle être différente de celle de GpJ dans la queue du bactériophage Lambda.

**<u>Mots clés:</u>** bactériophage Lambda, gpJ, LamB, technique du film noir (BLM), immunodétection.

# Summary

**Title : Study of the interaction between GpJ, a protein of the bacteriophage Lambda, and LamB, a protein of the outer membrane of gram-negative bacteria.**

The bacteriophage Lambda is a virus which infects bacteria carrying LamB protein in their outer membrane. GpJ, a protein of the tail of the phage, is involved in the binding to LamB. The study of the interaction between GpJ expressed as fusion protein and LamB was performed in order to investigate the interaction between the bacteriophage Lambda and LamB. The fusion proteins are called MBP-gpJ and His-gpJ.

MBP-gpJ is a chimeric protein representing Maltose Binding Protein connected to the C-terminal part of the GpJ protein (residue 684 until 1132), graciously given by Pr. Charbit (Paris, France). MBP-gpJ, expressed in *E.coli* and purified, bound to the exoplasmic side of LamB and LamB variants in planar lipid bilayer experiments and allowed a complete and reversible blockage of LamB channels. In order to obtain data about the binding of the GpJ fragment alone to LamB, an other fusion protein without MBP was created, called His-gpJ.

His-gpJ is the C-terminal part of GpJ (684-1132) in fusion with a 6×Histidine-tag, produced as insoluble form in *E.coli*. After renaturation, a soluble protein can be obtained. Without MBP, the GpJ fragment still bound to LamB in planar lipid bilayer experiments, but did not block significantly its channels, as previously observed after addition of MBP-gpJ. The interaction between His-gpJ and LamB or LamB mutants was also demonstrated on SDS-PAGE and immunodetection by the presence of high molecular mass bands.

Furthermore, the use of variants of lamB allowed to demonstrate that the C-terminal fragment of GpJ does not bind to the same area on the surface of LamB than GpJ involved in the tail of the Lambda phage.

**Keywords:** bacteriophage Lambda, GpJ, LamB, planar lipid bilayer (BLM), immunodetection.

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# CHAPTER I - Introduction

## Résumé (Summary)

La Maltoporine est une protéine de la membrane externe des bactéries Gram-négatives qui appartient à la famille des porines *(Benz et Bauer, 1988; Nikaido, 1992; 2003)*. Elle est impliquée dans le transport de maltooligosaccharides, des sucres, du milieu extracellulaire vers le périplasme (*Szmelcman et Hofnung, 1975; Palva, 1978; Boos et Schuman, 1998, Charbit, 2003*). Cette protéine est également le récepteur d'un virus à bactéries, le bactériophage Lambda (*Randall-Hazelbauer et Schwartz, 1973; Roa et Scandella, 1976)*. Le bactériophage Lambda appartient à la famille « Siphoviridae », qui se caractérise par une queue longue et non contractile. Il est utilisé couramment en biologie moléculaire depuis plusieurs dizaines d'années (*Hendrix et al., 1983; Sambrook et al., 1989*). Il l'est maintenant de plus en plus en biotechnologie, et ce principalement dans la lutte antibactérienne (*Merril et al., 1996; Duckworth et Gulig, 2002; Hoess, 2002; Projan, 2004*). Cette situation est paradoxale puisque il est utilisé pour sa capacité à véhiculer du matériel génétique dans une cellule hote alors les processus moléculaires impliqués dans son mécanisme d'infection demeurent assez mal connus. Mon objectif est justement d'étudier les mécanismes moléculaires impliqués dans l'infection du bactériophage Lambda, et plus spécifiquement sa première phase: l'interaction entre le virus et sa cellule-cible. Comprendre à terme comment une grande molécule hydrophile comme l'ADN viral peut passer à travers une double bicouche hydrophobe, présente un intérêt à venir non négligeable en biotechnologie, en permettant d'optimiser le transport de matériel à l'intérieur d'une cellule. On peut en effet imaginer dans le futur la création de nanocapsules chargées de substances toxiques qui seraient spécifiquement destinées à des bactéries, puisque celles-ci portent LamB à la surface de leur membrane. Il est également intéressant d'un point de vue fondamental de comprendre

un mécanisme d'infection, d'autant que les virus en possèdent plusieurs. Comme le phénomène d'éjection de l'ADN viral peut être reproduit *in vitro*, il a été établi que le phage n'a besoin que de LamB pour injecter son acide nucléique double brin hydrophile à travers la double bicouche que représente la membrane plasmique externe. Toutefois, la façon par laquelle l'ADN pénètre dans l'espace périplasmique demeure un mystère: après la fixation sur son récepteur cellulaire, le phage injecte-t-il son acide nucléique directement à travers la membrane, ou LamB est-elle utilisée comme canal ? Les travaux réalisés par l'équipe du Pr. Charbit (Paris, France) ont démontré que l'intéraction du bactériophage Lambda avec son récepteur cellulaire, LamB, est possible grâce au domaine C-terminal d'une protéine de sa queue, GpJ . Ce domaine C-terminal, exprimé en protéine de fusion avec la Maltose Binding Protéine se fixe en effet sur LamB à la surface de cellules (*Wang et al., 2000*). Mon travail de thèse s'est orienté vers deux axes :

- dans un premier temps, poursuivre l'étude de l'intéraction entre l'extrémité C-terminale de GpJ et LamB, principalement au moyen d'une technique électrophysiologique appelée BLM (« Black Lipid Mambrane », que l'on peut traduire par « Technique du Film Noir » en français) afin de savoir si la liaison du fragment de GpJ modifie les propriétés de transport (ions, maltooligosaccharides) de LamB. Ces travaux ont été réalisés avec deux protéines de fusion de l'extrêmité C-terminale de GpJ : d'abord avec MBP-gpJ puis avec His-gpJ. MBP-gpJ, gracieusement donnée par le Professeur Charbit (Paris, France), est une chimère où le fragment de GpJ est exprimé en fusion avec MBP (*Wang et al., 2000*). His-gpJ est une protéine de fusion entre le fragment de GpJ et un 6×His-tag afin de travailler sans MBP. Nous verrons que le fragment de GpJ se lie à LamB quelque soit la protéine de fusion utilisée, avec cependant des propriétés différentes.

- Identifier précisement la surface de LamB impliquée dans l'intéraction avec GpJ. Pour ce faire, plusieurs mutants ont été utilisés : LamB sauvage, LamB de la souche *Shigella sonnei* (*Roa et Scandella, 1976*), LamB Y118G (collection du laboratoire) et LamB Δ4+Δ6+Δ9v (*Andersen et al., 1999*), un mutant sur lequel les boucles extracellulaires L4, L6 and L9, indispensables à la liaison du phage Lambda, sont absentes. Nous verrons que le fragment de GpJ sur LamB ne se lie pas sur le même domaine de LamB que celui identifié pour GpJ intégrée dans le bactériophage Lambda entier.

# 1.1. Introduction to the Gram-negative bacteria

## 1.1.1. Plasma membrane of Gram-negative bacteria

The cell envelope of Gram-negative bacteria is composed of an inner and an outer membrane as well as the peptidoglycan existing in the periplasmic space in between (*Nikaido, 2003*; see Figure 1.1).

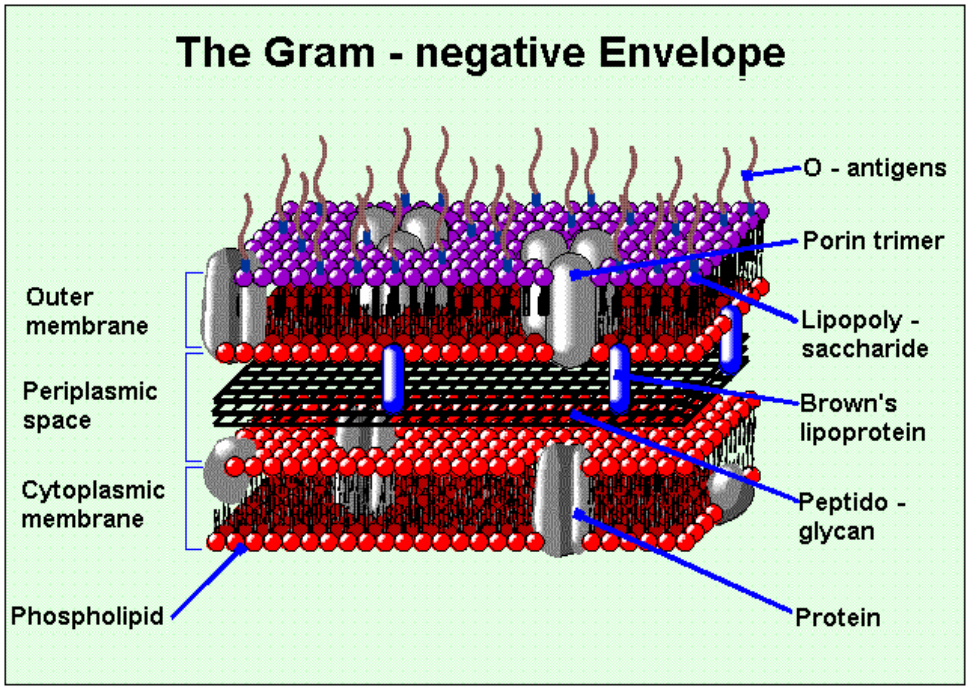


***Figure 1.1:*** *Schema of the plasma membrane of Gram-negative bacteria (laboratory collection)*

The inner membrane is composed of phospholipids and proteins. Proteins are mainly involved in the transport of compounds to the cytoplasm and to the respiratory chain export. The phospholipids, mainly phosphatidylethanolamine, phosphatidylglycerol and cardiolipin, are present in both layers of the 2 to 3 nm thick membrane (*Smit et al., 1975; Nikaido et Varaa, 1985*).

The outer membrane is composed of phospholipids, proteins, lipoproteins and lipopolysaccharides. Its thickness is around 7 nm. The distribution of the different compounds of the outer membrane is highly asymmetric (see Figure 1.1): the phospholipids and the

lipoproteins are located in the inner layer whereas the lipopolysaccharides (LPS) are found in the outer layer (*Kamio et Nikaido, 1976*). The lipopolysaccharides form a tight barrier to prevent the diffusion of detergents, proteases, lipases and hydrophobic antibiotics through the outer membrane (*Vaara et al*., 1990; *Plesiat et Nikaido, 1992*). The lipid A, also called endotoxin, is common to all LPS, while the hydrophilic core (O-antigen) varies within single species. The charge on the cell surface is negative mainly because of the presence of the phosphates of lipids and the acidic groups in the sugars of LPS. In the inner layer of the outer membrane, lipoproteins are present connecting the outer membrane to the peptidoglycan layer.

The peptidoglycan layer consists of a network of amino sugars (N-acetylglucosaminyl-N-acetylmuramyl dimers) and amino acids in the periplasmic space. This thin layer is responsible for the rigidity of the cell wall and prevents osmotic lysis. The periplasmic space represents an additional cellular compartment. It occupies around 20% of the total cell volume and plays an important physiological role: it prevents the osmotic lysis of the bacteria. Furthermore, binding proteins involved in the transport of solutes to the cytoplasm (e.g. maltose, phosphate) are located there. The periplasmic space is almost isoosmotic with the cytoplasm and an osmotic pressure is only present across the outer membrane.

### 1.1.2. Porins, channel forming proteins in the outer membrane of Gram-negative bacteria

Proteins represent about 50% of the total mass of the outer membrane of Gram-negative bacteria. They are involved in the maintenance of cell structure, binding of substances, adhesion to other cells and regulation of transport of nutrients and bactericidal agents (Koebnik *et al*., 2000; Benz, 2000; *Nikaido, 2003*). They can be classified into several categories: porins, lipoproteins, OmpA and minor proteins.

Porins are proteins involved in the uptake of hydrophilic nutritive compounds inside the periplasmic space *(Benz et Bauer, 1988; Nikaido, 1992; 2003)*. They can mediate also, like the OmpF *E. coli* protein, the transport of antibiotics (*Nestorovich et al., 2002*). Porins are divided in two classes: general diffusion and specific pores. General diffusion pores, for example OmpC and OmpF of *E. coli*, allow the entrance of molecules according to their molecule mass and a gradient of concentration. Specific pores, like LamB, ScrY, PhoE or Tsx, have a binding site for specific substrates inside the channel and facilitate the diffusion of these substrates through the outer membrane. The structure of several porins was solved by

X-ray crystallography. They present a barrel structure, with hydrophilic inner and outer loops. Most of them are organized as trimers (*Weiss et al., 1991; Cowan et al., 1992; Schirmer et al., 1995; Dutzler et al., 1996; Ferguson et al., 1998; Locher et al.,1998; Chimento et al., 2003*).

### 1.1.3. Maltoporin (LamB)

Maltoporin, also called LamB, is a specific channel protein involved in the transport of maltose and maltooligosaccharides (polymers of maltose) through the outer membrane. This protein is involved in the maltose uptake system (*Szmelcman et Hofnung, 1975; Palva, 1978; Boos et Schuman, 1998, Charbit, 2003*). Maltose binds to a soluble protein in the periplasmic space: the Maltose Binding Protein (MBP). The formed complex binds to the $MalFGK_2$ complex located in the inner membrane. The transport of the sugar across the inner membrane is controled by an ATP dependent process (see Figure 1.2).

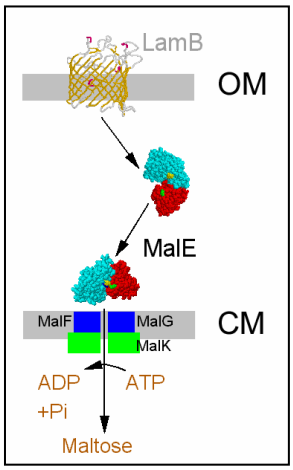


***Figure 1.2 :*** *Sugar translocation pathway. LamB mediated transport across the outer membrane (OM). Active uptake over the cytoplasmic membrane (CM) via the maltose-transport-complex (MTC). (taken and modified from:http://www.biologie.unihamburg.de/lehre/bza/kanal/transp/etransp.htm)*

#### 1.1.3.1. Structure

Maltoporin is a trimeric protein of the outer membrane of Gram-negative bacteria. Each monomer is composed of 421 residues. The structure was solved by X-ray crystallography at 3.1Å resolution (*Schirmer et al., 1995*). LamB is a water filled barrel with 18 antiparallel β-strands. 9 large and flexible loops are exposed to the extracellular side whereas small loops are orientated towards the inner one. The extracellular loops L1, L6 and especially L3 make a constriction inside the channel, which dimensions are about 5×7 Å (see Figure 1.3).

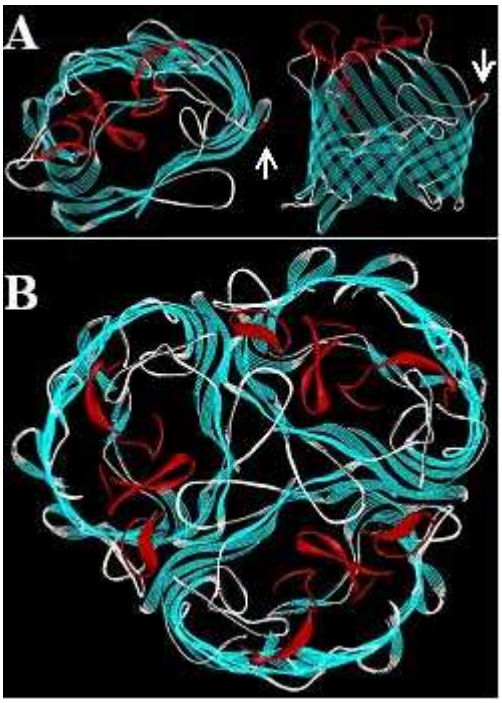


**Figure 1.3:** A: Top and side view of a monomeric LamB. B: Top view of LamB trimer. In red: outer loops L4, L6 and L9v. Arrows indicate the position of the residue 18 on the outer loop L1, involved in the stability of the trimer, as described by *Gehring et al., 1987*.

### 1.1.3.2. Maltose and maltooligosaccharide transport

Maltoporin is a specific diffusion porin due to a binding site inside the channel, called "greasy slide" (*Dutzler et al., 1996; Van Gelder et al., 2002*). It is composed of six aromatic amino acids (Y6, Y41, W74, F227, W358, W420) that line the channel lumen from the extracellular to the periplasmic opening (see Figure 1.4).

Point mutations affections of on those residues modify the diameter of the channel and the transport of maltose and maltooligosaccharides. In addition to the so called "greasy slide" there are several amino acid residues inside the channel that are involved in malto-oligosaccharide binding (*Dutzler et al. 1996*).

Mutations located in the loops L4 and L6 (*Charbit et al., 1984)* decrease significantly the maltose uptake suggesting that these loops are also involved in the transport of the sugar to the binding site inside the channel (see Table 1.1).

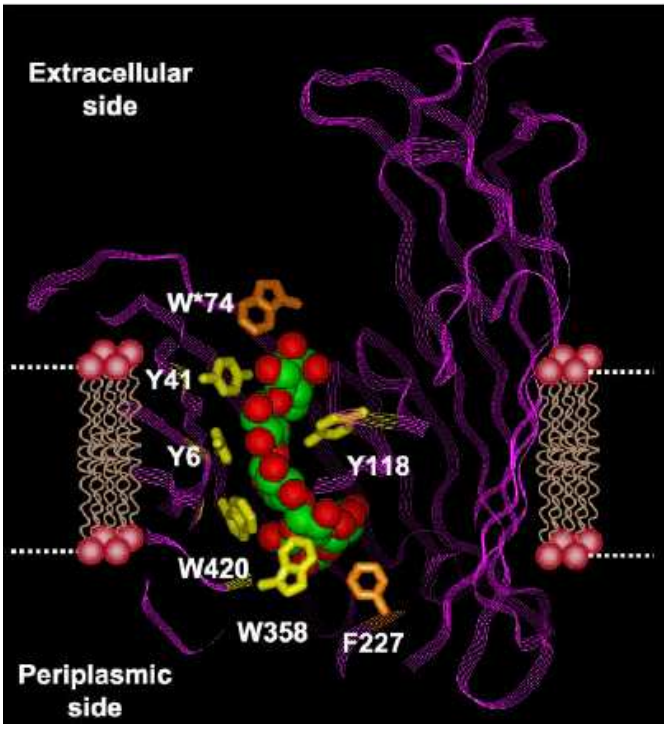


***Figure1.4:*** *Molecular modelisation of the "greasy slide" of the LamB protein (Danelon et al., 2003).*

*In vitro* studies of LamB showed that the transport of sugar is highly asymmetric. When LamB is reconstituted in an artificial membrane and maltose is added, maltose transport is five times higher when the sugar is added to the extracellular side of Maltoporin than to the periplasmic side (*Kullman et al., 2002; Danelon et al., 2003*).

| Loop | Residue | Substitution | Transport of maltose* |
|---|---|---|---|
| - | 18 | G 18V | 3 |
| Outer Loop 4 | 148 | E 148 K | 24 |
| Outer Loop 4 | 151 | G 151 D | 50 |
| Outer Loop 4 | 152 | S 152 F | 42 |
| Outer Loop 6 | 245 | G 245 R | 15 |
| Outer Loop 6 | 247 | S 247 L | 17 |
| Outer Loop 6 | 249 | G 249 D | 37 |
| Outer Loop 6 | 250 | S 250 F | 21 |

***Table1.1:*** *Description of residues on the outer loops of LamB important for the transport of maltose.*
** percentage of transport of maltose in comparison to LamB WT (Charbit et al., 1984).*

### 1.1.3.3. Phage receptor

Maltoporin is a specific diffusion porin involved in the transport of maltose and maltooligosaccharides. However Maltoporin is also called LamB because it is the receptor for a bacterial virus, called bacteriophage Lambda (LamB for **Lamb**da receptor: *Randall-Hazelbauer et Schwartz, 1973; Schwartz, 1975*). *In vitro* studies showed that the bacteriophage Lambda binds to LamB wild type (WT) and can even inject its DNA when ethanol or chloroform is added (*Roa et Scandella, 1976*). However, triggering of the ejection of the viral DNA without chloroform or ethanol is also possible using the LamB protein of the *E. coli pop154* strain. *Pop 154* is a derivative of *E. coli K-12* carrying the LamB region of *Shigella sonnei 3070.* The triggering of DNA occurs spontaneously when the temperature is raised up to 37°C (*Roa et Scandella, 1976*).

Mutations on the LamB gene can prevent the infection of the Lambda phage. Different mutant classes were described: Class I, II, and III (see Table 1.2).

| Phage Classes / LamB | Bacteriophage Lambda | | |
|---|---|---|---|
| | **λh$^+$ (WT)** | **λh** | **λhh$^*$** |
| **Wildtype (WT)** | + | + | + |
| **Class I** | - | + | + |
| **Class II** | - | - | + |
| **Class III** | - | - | - |

**Table 1.2:** Binding possibilities between different types of LamB and different types of bacteriophage Lambda. (+): interaction. (-): no interaction. (Charbit et al. 1984).

Class I and II are point mutations, where residues on the outer loops L4, L6 and L9 are substituted (*Hofnung et al., 1976; Katsura, 1976; Clément et al., 1983; Charbit et al., 1984; Charbit et al., 1994*). Class I LamB mutants prevent the binding of the bacteriophage Lambda "wildtype", also called λh$^+$, and subsequently its infection. In order to infect again the bacteria, Lambda phage can adapt to mutation of the Class I LamB (*Hofnung et al., 1976*). These mutants able to bind to LamB Class I (and also to LamB wildtype) are called λh. The growth of λh phages can be prevented by a second point mutation on the LamB gene: these mutants are called Class II LamB mutants. However, Lambda phage can adapt after mutation to the Class II LamB, and is able to infect again the bacteria (*Clément et al., 1983; Charbit et*

*al., 1994; Werts et al., 1994*) carrying Class II LamB. Those mutants are called λhh*, and are also able bind to LamB WT and to Class I mutants. Class III mutations are nonsens mutations on lamB gene. Class III mutants block the growth of every type of Lambda phage, because a large part of the cell receptor is deleted (*Hofnung et al., 1976*).

## 1.2. Bacteriophages

### 1.2.1. Bacteriophage families

Bacteriophages, discovered by F.W. Twort (1915) and F. d'Herelle (1917) are pathogen viruses that attack bacteria (*Brussow et Hendrix, 2002; Hendrix, 2003; Weinbauer, 2004*). The attack of the cell by a bacteriophage is called infection. They are the largest known virus group. For more than fifty years, the bacteriophages were strongly studied and were classified into 19 families (see Table 1.3). The name "phagos" in greek means "to eat". Their aim is to replicate and to destroy (lyse) the cell: they are a real "bacteria contagious disease" infinitely contagious from a microbial lysed to a sensitive cell.

### 1.2.2. Bacteriophage receptors

Bacteriophages adsorb first non specifically to the surface of the host cell by reversible binding (*Letellier et Santamaria, 2004*). Then they recognize a specific receptor and bind irreversibly to it. Many surface-localized phage receptors have been identified: including flagella, pili, capsules, lipopolysaccharides (for C21, ϕ5, ϕW, K19, P1, T2, T3, T4, and U3 phage: *Hancock et al., 1976*) and proteins (Lambda, K10, T5, TuIa, TuIb).

Most of the protein receptors are porins (*Wandersman et Schwartz, 1978; Roa, 1979*). It is known that the same porin can be the receptor of different phages (LamB for the Lambda and K10 phages, Fhu A for the T1, T5 and Φ80 phages, OmpC for the TuIb, Me1, PA2, 434 and T4 phages). In the same way, a phage can adapt by mutation to bind to an other porin than its "natural" cell receptor (*Moreno et Wandersman, 1980*). This observation can be explained by the fact that porins have an ancestral common structure and that there are homologue sequences in proteins of the tail of different phages (protein Gp37 of TuIa, TuIb, SV14 phages and stf of Lambda phage; *Tétart et al., 1996*).

| Family | Characteristics |
|---|---|
| **Corticoviridae** | Icosaedral capsid with layer, circular supercoiled dsDNA |
| **Cysoviridae** | Enveloped, icosaedric capsid, lipids three molecules of linear dsDNA |
| **Fuselloviridae** | pleomorphic, envelope, lipids, no capsid, circular supercoiled dsDNA |
| **Inoviridae genus Inovirus** | long filaments with helical symmetry, circular ssDNA |
| **Inoviridae genus Plectrovirus** | short rods with helical symmetry, circular ssRNA |
| **Leviviridae** | quasi-icosaedral capsid, one molecule of linear ssRNA |
| **Lipothrixviridae** | Enveloped filaments, lipids, linear dsDNA |
| **Myoviridae, A1** | tail contractile, head isometric |
| **Myoviridae, A2** | tail contractile, head elongated (length/width ratio : 1.3-1.8) |
| **Myoviridae, A3** | tail contractile, head elongated (length/width ratio : 2 or more) |
| **Plasmaviridae** | pleomorphic, envelope, lipids, no capsid, circular supercoiled dsDNA |
| **Podoviridae, C1** | tail short and non-contractile, head isometric |
| **Podoviridae, C2** | tail short and non-contractile, head elongated (length/width ratio : 1.4) |
| **Podoviridae, C3** | tail short and non-contractile, head elongated (length/width ratio : 2.5 or more) |
| **Rudiviridae** | helical rods, linear dsDNA |
| **Siphoviridae, B1** | tail long and non-contractile, head isometric |
| **Siphoviridae, B2** | tail long and non-contractile, head elongated (length/width ratio : 1.2-2) |
| **Siphoviridae, B3** | tail long and non-contractile, head elongated (length/width ratio : 2.5 or more) |
| **Tectiviridae** | icosahedral capsid with inner lipoprotein vesicle, linear dsDNA, « tail » produced for DNA injection |

**Table 1.3: Different bacteriophage families.**

Bacteriophage Ecology Group (http://www.mansfield.ohio-state.edu/~sabedon/names.htm).

# 1.3. The Lambda phage

Bacteriophage Lambda is a tailed virus of the “Siphoviridae, B3” family. Known for many decades, it is one of the most frequently used phages or vectors in biology (*Hendrix et al., 1983*; *Sambrook et al., 1989*). The Lambda family of bacteriophages continues to provide significant insights into the understanding of basic biological processes, as well as useful technological innovations (*Friedman et Court, 2001; Jepson et March, 2004*). In this context it is interesting to note that phages have emerged recently as an alternative vehicle for the

surface display of peptides and proteins (*Hoess, 2002*) and a potential treatment for bacterial infections (*Merril et al., 1996; Duckworth et Gulig, 2002; Projan, 2004*).

### 1.3.1. Description

A low resolution structure of the bacteriophage Lambda has been solved since the middle of the seventies by electronic microscopy (see Figure 1.5). The phage is composed of two parts: the head and the tail.

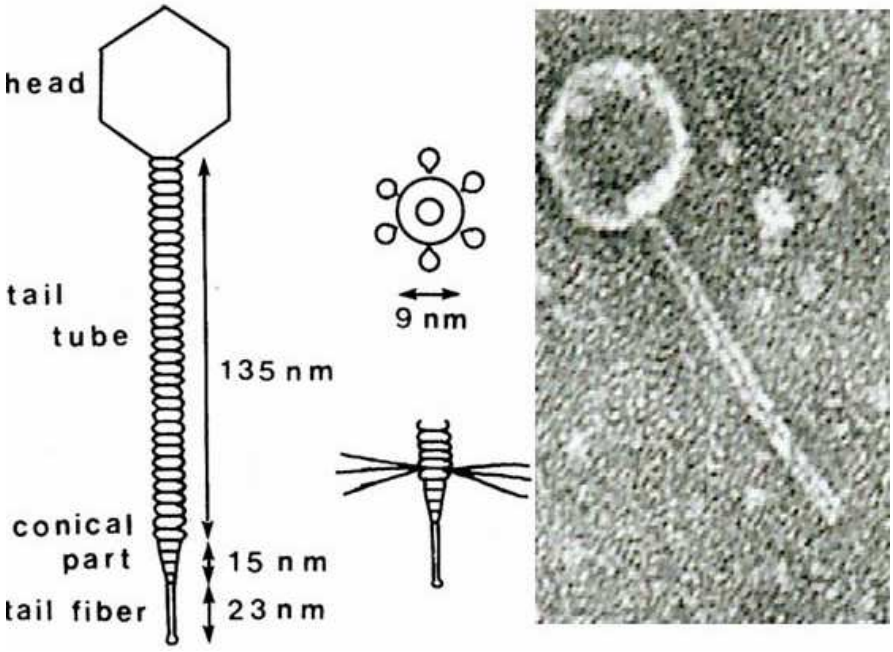


***Figure 1.5:*** *(left) Schema of the bacteriophage Lambda. Source:* Lambda II. (1983) Cold Spring Harbor Laboratory. *(right) Electronic microscopy photo of a Lambda phage (laboratory collection, negative coloration, x 91000 fold)*

The viral tail is divided in three parts: a long, flexible and non-contractile tube (135nm), a conic part (around 15nm) and a terminal fiber (23nm). The main tube is constituted by GpV. The GpV protein is the main component of the tail tube: approximately 200 copies per phage. It possesses a diameter of 9 nm and is empty inside (3 nm diameter). 6 knobs are present around the core. Side fibers are attached to the tail tip (see Figure 1.5). The ending part of the tail is constituted of the GpJ, GpI, GpL, GpK, GpH as pH*, GpG and GpM proteins (see Figure 1.6). A maturation step occurs to allow the binding of GpH to the other proteins of the tail. The maturated GpH form is called $pH^*$. Many proteins of the tail ($pH^*$, GpG, GpV) are involved in the mechanism of injection of DNA (*Katsura et Kühl, 1976*). The role of many

proteins of the tail has been investigated (*Tsui et al., 1983, Hendrix et al., 1983*). GpJ is the only one shown to be involved in the binding of the Lambda phage to LamB (*Wang et al., 1998*).

The eicosaedric (20 sides) head protects a double stranded, linear DNA of 48501pb (*Sanger et al., 1982*). This DNA seems to be organised as DNA B (right helix ; 10,5 bp/turn, diameter of around 20Å). The structure of GpW, a protein involved in the architecture of the head of the virus and of an intermediate of the viral capside has been solved by crystallography (*Murialdo et al., 2003, Wikoff et al., 2003*).

The binding between the head and the tail is mediated by two proteins: GpZ and GpU. Mutations on the Z gene prevent the ejection of DNA. If a protein of the phage is missing, the assembling stops. Some mutants of the Lambda phage have been performed to obtain just the tail: λ phage $U^-$ or $Z^-$ ( *Thomas et al., 1978).*

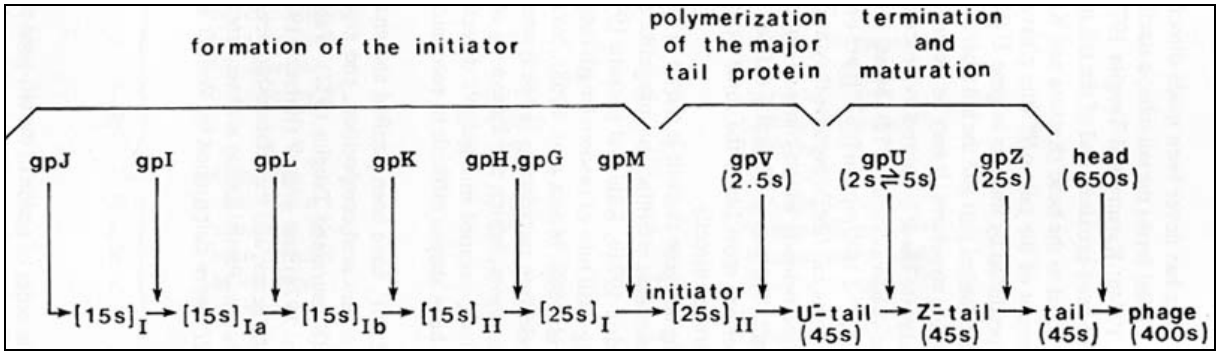


***Figure 1.6:*** *Assembly of proteins of the Lambda phage. (Katsura, 1983)*

## 1.3.2. General mechanism of the infection

The general infection processes are well known whereas the mechanisms at the molecular level remain less understood (*Hendrix et al., 1983*). Bacteriophage Lambda is a temperate virus. A temperate virus, in response to special stimuli, has two possible behaviours: to be in a lysogenic phase or to be in a lytic phase.

- lysogenic phase (late replication)

The phage decreases its capacities of development and integrates into the chromosome of the cell host. It is then called prophage. Thus, the prophage is passively replicated during

generations of *E. coli* and then take back its lytic activity. The signal which allows to awake the phage *in vivo* remains unknown. I*n vitro*, this signal can be ultraviolet radiation.

- lytic phase (early replication)

The phage multiplies inside the bacteria and releases proteins called transglycosylases which lead to lysis of the host cell and allow the liberation of a lot of new synthesized phages.

A lytic cycle is represented in the Figure 1.7. A Lambda phage particle attaches to the host cell and injects its linear, duplex DNA molecule through the plasma membrane. The molecule circularises by base-pairing of complementary single-stranded ends. The resulting nicked circle is closed by DNA ligase. To start the replication step, the DNA must be supercoiled. Supercoils are introduced by DNA gyrase. The replication is bi-directional. The DNA produced by rolling-circle replication can be cut and packed into phage heads. It is compressed by an active ATP dependent processus, using basic molecules (*Gosule et Schellman, 1976; Hud et Downing, 2001*). Those molecules, mainly polyamines, bind to the phosphate backbone and allow the compression of the nucleic acid inside the viral head. The addition of tails completes the maturation of phage particles capable of initiating a new cycle of infection. A single cycle of productive growth generates approximately 100 copies of the viral genome.

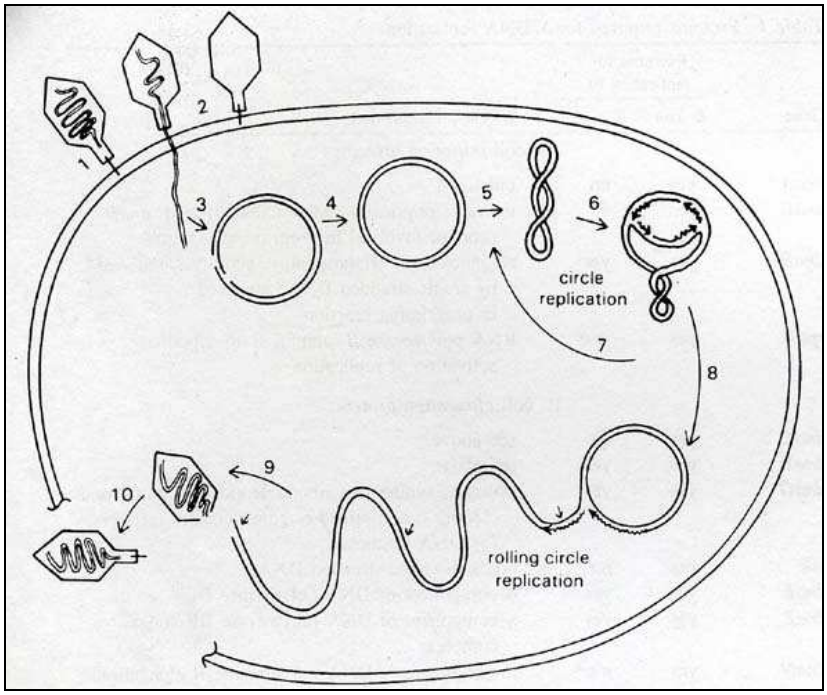


***Figure 1.7:*** *Main steps of the lytic phase of the bacteriophage Lambda (Hendrix et al., 1983).*

## 1.3.3. Different steps of infection

The first steps of infection are the binding of the phage to the outer membrane of the host cell, the triggering of the ejection and the passage of the viral DNA through the outer membrane, the periplasmic space and the inner membrane. These first steps were described by *Roa et Scandella, 1976*:

**phage + LamB ↔ [phage-LamB] (1)**

**[phage-LamB] → [phage-LamB]$^{*}$ (2)**

**[phage-LamB]$^{*}$ → phage DNA + [ghost receptor] (3)**

The phage binds to LamB, and forms a reversible complex: reaction (1). Reaction (2) corresponds to the so called "inactivation" of the phage: the complex between LamB and the Lambda phage becomes irreversible, and the processes that will lead to the triggering of the DNA start to occur. This step can be reproduced *in vitro* upon addition of chloroform or ethanol or when LamB from *Shigella sonnei* is used and the temperature is raised up to 60°C (*Parkinson et Huskey, 1971*). This step is followed by triggering the ejection of DNA: reaction (3).

## 1.3.4. Binding to the host cell and passage through the outer membrane

### 1.3.4.1. Binding

Binding is the first step of the infection mechanism (see Figure 1.8). Divalent ions in general and $Mg^{2+}$ ions in particular are known to increase *in vivo* the affinity of the phage to its cell receptor (*Schwartz, 1976*). $K_d$ of the complex between the phage and LamB on entire cells is estimated by titration to be $5.10^{-12}$ M in the presence of 2 mM $Mg^{2+}$ (*Schwartz, 1975*). The diffusion coefficient of the virus in the medium is to $5.10^{-8} cm^2 s^{-1}$. The phage is about 50 times larger than the size of LamB. When it binds to LamB, it covers such a big area on the surface of the cell that 1/3 of the total receptors remain free when the cell is completely covered of phages.

Bacteriophage Lambda binds to the LamB protein using GpJ (**g**ene **p**roduct **J**), a protein of the terminal part of the tail (*Wang et al., 1998*). Its oligomerization step is not known, but it seems to be dimeric or trimeric (*Roa et Scandella, 1976*). The 20% of theC-terminus end of

gpJ are involved in the binding step *(Wang et al., 2000)*. Some point mutations on this part of the J gene and especially on residues 1040, 1077, and 1127 are known to prevent the phage from binding *(Werts et al., 1994)*.

Electronic microscopy revealed the formation of the complex between the bacteriophage Lambda via the GpJ protein and LamB (*Roa et Scandella, 1976; Roessner et Ihler, 1984*).

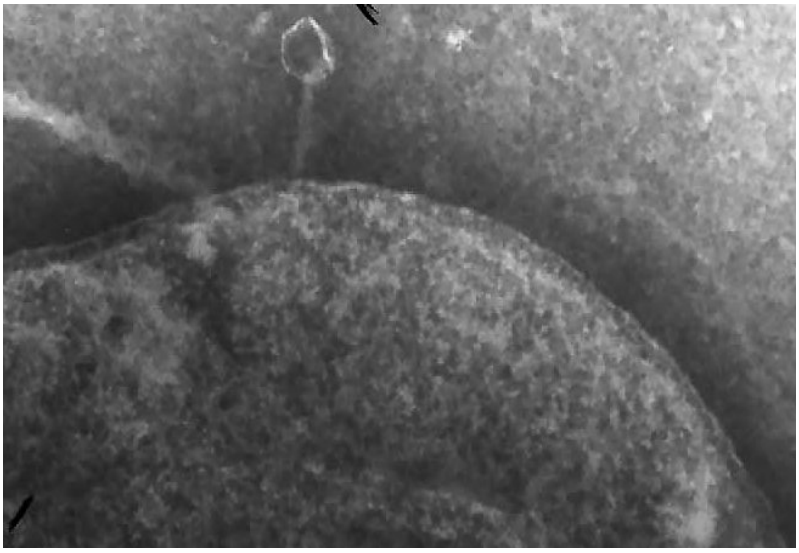

***Figure 1.8: Lambda phage bound on the surface of an E. coli cell. (laboratory collection).***

### 1.3.4.3. Triggering of the injection of DNA

After the binding step, a "delay time" always occurs bevore the triggering of the injection of the DNA. This delay time can be reduced by temperature increase with a maximum at 37°C. When the temperature is + 4°C, the time is unlimited and the ejection of the DNA is never triggered.

The triggering of the DNA injection remains an open question. It is possible to prevent it by the addition of diamines (cadaverin or putrescein: *Harrison et Bode, 1975*), or by point mutation on the lamB or J gene. A mutation of only one residue of LamB can be sufficient to prevent infection (*Hofnung et al., 1976; Katsura, 1976; Clément et al., 1983; Charbit et al., 1984; Charbit et al., 1994*).

Studies of the triggering signal leading to DNA injection started efficiently when LamB of *Shigella sonnei* was discovered (*Scandella et Roa, 1976),* because the triggering of DNA can be controlled: it occurs when the temperature is raised to 37°C. This effect is due to seven amino acids on the loop L9 different to LamB of *E. coli* (*Roessner et Ihler, 1987*). The DNA

injection can be induced *in vitro* using only LamB reconstituted in liposomes (*Roessner et al., 1983; Roessner et Ihler, 1986*). The lag time leading to *in vitro* injection into liposomes is about one minute *in vitro* (*Novick et Baldeschwieler, 1988*) in contrast to ten minutes *in vivo* (*Mackay et al., 1976*).

The triggering of DNA induces modifications in the tail of the bacteriophage Lambda. This difference of morphology was clearly observed: the same phage proteins have a different protease sensibility if they are analysed before or after DNA injection. GpJ is the protein of the extremity of the fiber and pH* is a protein in the extremity of the conic part of the tail. Before the triggering of the injection of DNA, GpJ is protease sensitive and pH* is protease resistant, whereas GpJ is protease resistant and pH* is protease sensitive after injection of the DNA (see table 1.4). Differences in the morphology of the phage are confirmed by electronic microscopy: after the injection of DNA, the tail of the phage appears shorter, suggesting that the end part of the tail gets injected inside the outer membrane of the host cell (*Roessner et Ihler, 1984*).

| | **DNA injection** | **Distance between phage head and plasma membrane** | **Proteinase K treatment (sensible)** | |
|---|---|---|---|---|
| | | | **pH*** | **gpJ** |
| **Type I** | no | almost 170 nm | sensitive | resistant |
| **Type II** | yes | almost 150 nm | resistant | sensitive |

***Table 1.4****: Evidence of two pattern of the Lambda phage before and after ejection of the DNA.*
*Type I: bacteriophage Lambda before injection of the DNA.*
Type II: bacteriophage Lambda after injection of the DNA. (*Roessner et Ihler, 1984*).

## 1.3.5. DNA passage across the inner membrane

The *E. coli* ptsM gene codes for inner membrane proteins involved in mannose uptake and phosphorylation (*Williams et al., 1986; Esquinas-Rychen et Erni, 2001*). They are also involved in the DNA injection of the bacteriophage Lambda (*Elliott et Arber, 1978*). The mutations in this gene that do not allow the infection of Lambda phage are called pel$^-$ (**pe**netration of **l**ambda). The study of the energetics of the injection process indicates that the entrance of the DNA into the cytoplasm seems to occur by simple diffusion (*Filali-Maltouf et Labedan, 1985*). Otherwise, the ptsM gene is not indispensable to the passage of the inner

membrane, because the phage can use another protein of the inner membrane when the gene is suppressed. Lambda phage mutants that can recover their infection ability are called λhp. They possess mutations on the genes V or H coding for proteins of the tail. This suggests, in agreement with the data of *Roessner et Ihler, 1984*, that the tail of the Lambda phage can interact with the inner membrane and especially pH*, the maturated form of the gpH protein (*Roa et Clément, 1980*).

### 1.3.6. Other investigated infection mechanisms

The early step of all the infection mechanisms seems to be smilar: after the binding, an unknown stimulus induces a conformational change in part of the tail proteins thus allowing DNA injection. The difference in osmotic pressure in the compressed nucleic acid within the phage head and the outer medium seems to be the main driving force involved in injection (*Evilevitch et al., 2003*). For example, the potential force generated by the compression of the DNA inside the phage head has been estimated to be 60 atmospheres for the ϕ29 phage, ten times the pressure inside a champagne bottle (*Smith et al., 2001*).

T4 phage binds specifically to lipopolysaccharides of the outer membrane of the host cell and injects its DNA using its contractile tail in less than one minute (transfert rate: 4000bp/s, *Letellier et al., 1999*). Gp5, a three part protein organized as a trimer, plays an essential role in the cell-puncturing device involved in the mechanism of injection of the DNA (*Arizaka et al., 2003*). Its structure has been recently solved by crystallography (*Kanamaru et al., 2002*). The initiation of the infection activates a lysozyme activity of a domain of the protein to digest the peptidoglycan allowing the access of the tail to the inner membrane of the host cell.

T5 phage has a non-contractile tail. It binds to FhuA, a protein involved in the transport of ferrichrome and antibiotics (*Bonhivers et al., 1998; Ferguson et al., 1998; Locher et al., 1998*). The protein of the tail involved in the interaction with FhuA is pb5. This protein was recently cloned and purified, and the complex was studied *in vitro* (*Plancon et al., 2000*). The injection of DNA occurs in two steps during around ten minutes. The phage injects first about 20% of its DNA inside the host cell. Afterwards, two proteins are synthetised, that allow the entrance of the rest of the DNA (*Letellier et al., 2004*).

T7 phage has also a non-contractile tail. At present, it is the only known case where the transcription of the totality of the genome (40 kb) occurs during the process of injection,

suggesting that proteins inside the cytoplasm could help in some cases the entrance of the nucleic acid inside the host cell (*Letellier et al. 1999*).

## 1.4. Presentation and aim of the work

Even if the bacteriophage Lambda is commonly used in molecular biology (*Hendrix et al., 1983; Sambrook et al., 1989*), and now in biotechnology (*Merril et al., 1996; Duckworth et Gulig, 2002; Hoess, 2002; Projan, 2004*), the molecular processes of its infection mechanism remain unknown. The understanding how a large hydrophilic molecule like the viral DNA can pass across the plasma membrane (a double and hydrophobic bilayer) of the Gram-negative bacteria is not understood, and can be useful in future, especially for the optimisation of the transport of material across membranes carrying reconstituted LamB. Furthermore, it is also interesting to understand a viral infection in general: viruses have a huge number of mechanism, and Lambda phage is only one…

We want to understand the mechanism at the molecular level, and focus on the first step of the infection: the binding step. The phage binds to LamB using a protein of its tail called GpJ (*Wang et al., 1998*). The C-terminal extremity of this protein is involved in binding with LamB: 40% of the C-terminus were expressed in fusion with the Maltose Binding Protein (MBP); this fusion protein was called MBP-gpJ and bound to the surface of entire *E. coli* cells (*Wang et al., 2000*). This work was the first step towards an *in vi*tro study of the interaction between LamB and GpJ.

My work is divided in two parts:

- further investigation of the interaction between the C-terminal part of GpJ and LamB, mainly by the use of an electrophysiological technique called BLM (Black Lipid Membrane) or planar lipid bilayer (*Van Gelder et al., 2000*). I wanted to investigate if the binding of the GpJ fragment modifies the channel properties of the LamB protein. We used fusion proteins called MBP-gpJ and His-gpJ. MBP-gpJ is a chimera protein of the GpJ fragment in fusion with Maltose Binding Protein, graciously given of Pr. Charbit (Necker, Paris, France). His-gpJ is a fusion protein of GpJ fused His-tag, made in our laboratory, in order to work only with the GpJ fragment, without the MBP part of the fusion protein. The characterisation of the interaction between LamB and

His-gpJ was also demonstrated, confirming the binding of the GpJ fragment in fusion with MBP.

- investigate accurately the area on the surface of LamB involved in the binding step with the GpJ fragment, some mutants were used : LamB WT, LamB from *Shigella sonnei* (*Roa et Scandella, 1976*), LamB Y118G (laboratory collection), and LamB Δ4+Δ6+Δ9v (*Andersen et al., 1999*), a mutant with deletions on the outer loops L4, L6 and L9, necessary for the interaction with the Lambda phage.

# CHAPTER II – Material and methods

## Résumé (Summary)

**- Production des protéines: LamB sauvage, LamB de *Shigella sonnei*, LamB Y118G, LamB Δ4+Δ6+Δ9v, MBP-gpJ et His-gpJ.**

LamB sauvage ainsi que ses mutants sont produites dans les conditions décrites par Klebba et collaborateurs (*Klebba et al., 1994*). La protéine MBP-gpJ est obtenue dans les conditions décrites par Wang et collaborateurs (*Wang et al., 2000*). His-gpJ est obtenue par sous-clonage du fragment de gpJ de MBP-gpJ dans le vecteur d'expression pBAD/$His_B$ (*Invitrogen$^{TM}$ life technologies*). Des bactéries chimiocompétentes TOP 10F' (Invitrogen) sont transformées par ce nouveau plasmide contenant le fragment de GpJ. Comme la protéine de fusion His-gpJ est porduite sous forme de corps d'inclusion, elle est ensuite purifiée en conditions dénaturantes.

**- Technique du Film Noir (BLM).**

La technique du film noir est une technique d'électrophysiologie qui utilise le courant électrique comme sonde de perméabilisation de la membrane (*Benz et al., 1978*). Elle est particulièrement utilisée pour l'étude de protéines canal reconstituées dans une membrane modèle (*Van Gelder et al., 2000*). Dans notre étude, elle va servir principalement à savoir si le fragment de GpJ exprimé en protéine de fusion bloque les canaux de LamB, et empêche ainsi le transport des maltooligosaccharides. Cette étude est réalisée à l'échelle de la molécule unique ainsi qu'avec plusieurs dizaines de molécules de LamB insérées dans la membrane. Dans le cas de la formation d'un complexe réversible, les constantes d'association et de dissociation du complexe peuvent de plus être obtenues comme l'ont décrit Nekolla et collaborateurs (*Nekolla et al., 1994*).

**- Dichroisme Circulaire.**

Le Dichroïsme Circulaire est une technique qui donne des informations sur les élements de structure secondaire de protéines ou d'acides nucléiques (*Greenfield, 2004*). Il est utilisé ici afin d'obtenir des informations structurales sur les protéines de fusion MBP-gpJ et His-gpJ.

**- Immunodétection.**

Le protocole est semblable à celui décrit par Sambrook et collaborateurs (*Sambrook et al., 1989*)

**- Microscopie électronique à transmission.**

*E.coli* exprimant LamB sauvage ou un mutant de LamB et le bactériophage Lambda (rapport environ 10 :1) sont incubés 1 heure à température ambiante. L'échantillon ensuite est observé par contraste négatif par coloration à l'acétate d'uranyle afin d'observer la fixation de phages à la surface de bactéries exprimant différentes sortes de LamB.

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# 2.1. Protein production

## 2.1.1 LamB production

### 2.1.1.1. Expression of LamB WT, from *Shigella sonnei*, and LamB Δ4+Δ6+Δ9v

LamB WT is extracted from TOP 10F' (Invitrogen©) cells, LamB from *Shigella sonnei* from the pop154 strain (*Roa et Scandella, 1976*) and LamB Δ4+Δ6+Δ9v from JM501 cells transformed by pAC-1 derivated plasmide IPTG (AppliChem) inducible (*Charbit et al., 1998; Andersen et al., 1999*). Top 10F' (Invitrogen ©) and pop154 cells are grown in 200 ml Luria-Bertani (LB) medium (Difco), 1% maltose (AppliChem) until the end of the exponential phase ($OD_{600nm}$ ≈1,2).

Transformed JM501 cells are grown in 200 ml LB, 100 µg/ml ampicillin (Applichem), 1% maltose. They are induced by IPTG 0.3mM at an $OD_{600nm}$ of 0.4 and grown until the end of the exponential phase.

#### 2.1.1.2. Purification of LamB from different sources

Media and chemicals were described previously (Klebba *et al.*, 1994). Cells are washed with 10mM Tris pH 7.5, 100mM NaCl, disrupted with the French Pressure cell and centrifuged (15min, 6000g, 4°C). The supernatant is ultracentrifuged (1h, 48000 rpm, rotor Ti-70, 4°C): the membrane containing LamB is found in the pellet. The pellet is twice resuspended in Tris buffer 10mM pH 7.5, 100mM NaCl, 0.4% LDAO (Fluka) and twice ultracentrifuged (30min, 48000 rpm, rotor Ti-70, 20°C). The obtained supernatants are passed over an amlyose-sepharose (*New England Biolabs*) column, equilibrated with the same buffer. Elution is performed with a buffer Tris HCl 20mM pH 7.4, 200mM NaCl and 10% maltose.

### 2.1.2. Production of the MBP-gpJ protein

#### 2.1.2.1. Expression of the MBP-gpJ protein

The MBP-gpJ protein was graciously given by Pr. A. Charbit (Necker, Paris, France). Expression and purification of « MBP-gpJ (684-1132) » were performed as described by *Wang et al., 2000*. *E. coli* JM 501 cells are transformed by a pMal$^{TM}$-c2X plasmid (New England Biolabs) expressing the end part (residues 684-1132) of the J gene of the Lambda phage.

Cells are grown in 200 ml of LB medium, 1% glucose, 100µg/ml ampicillin grow until the end of the exponential phase. When $OD_{600nm}$ is 0.5, induction with IPTG (0.3mM) is performed.

#### 2.1.2.2. Purification of MBP-gpJ

Cell culture is washed in Tris buffer pH 7.4, NaCl 200mM and resuspended in Tris buffer pH 7.4, NaCl 200 mM + antiproteases (protease inhibitor cocktail P 8465, Sigma). Cells are disrupted (three times, 1000 bars) with the French Pressure cell. After centrifugation (9000g, 30min, 4°C), the supernatant is passed through an amlyose-sepharose (*New England Biolabs© Inc.*) column, equilibrated with the same buffer. Elution is performed with a Tris-buffer (Tris HCl 20mM pH 7.4, 200mM NaCl) complemented with 10mM maltose.

## 2.1.3. Production of the His-gpJ protein

### 2.1.3.1. Construct coding for His-gpJ_(684-1132)

The J gene (684-1132), coding for 40% of the C-terminus end of the J protein of the bacteriophage Lambda, called GpJ_(684-1132)**,** was cut out from the pMal-GpJ_(684-1132), described by *Wang et al., 2000*, using a EcoRI-HindIII digestion in Tango buffer (Fermentas). The isolated fragment was inserted in a pBAD/His B multiple Cloning Site (*Invitrogen$^{TM}$ life technologies*) digested with the same restriction enzymes (see Figure 2.1). Chemo-competent bacterial TOP 10F' cells (Invitrogen ©) were transformed by the construction coding for His-gpJ_(684-1132) and positive clones were selected on ampicillin 100µg/ml LB-agar medium. Analysis of the sequence of the new obtained gene confirmed the sequence of the end-terminal extremity of the J gene (from the residue 684 until the residue 1132) and reveals furthermore the addition at its N-terminal extremity of a small sequence of amino acids: MGGS**HHHHHH**GMASMTGGQQMGRDLYDDDDKDPSSRSAAGTIWEF…

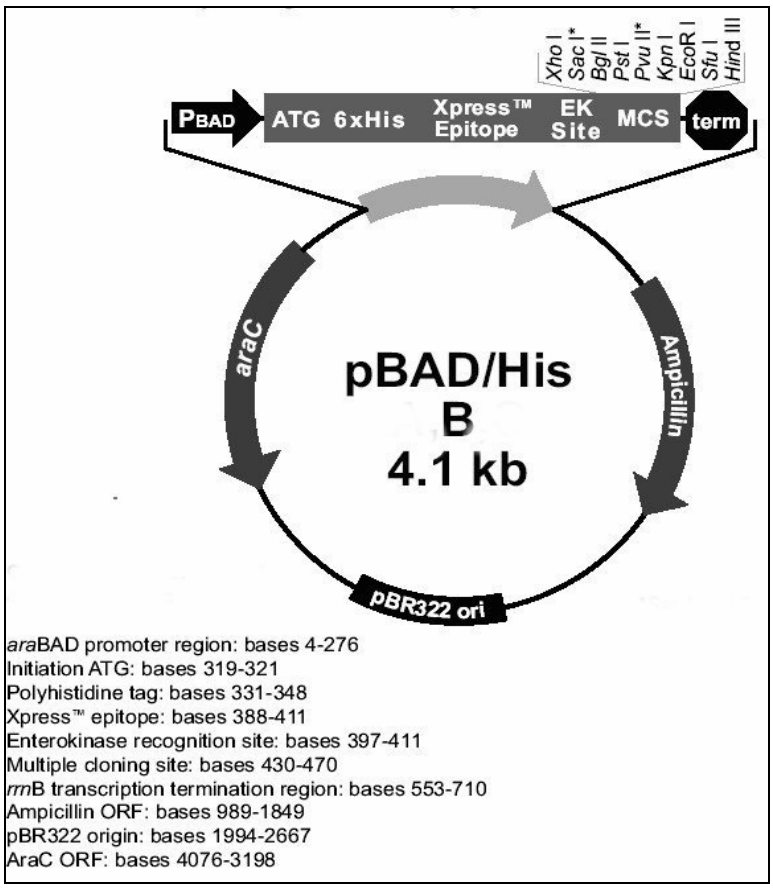



***Figure 2.1:*** *Plasmid used for the His-gpJ construction. The GpJ (684-1132) fragment is inserted between the EcoRI and Hind III digestion sites. (Invitrogen$^{TM}$ life technologies, pBAD/His instruction manual version F).*

#### 2.1.3.2. Expression of the His-gpJ protein

Production of the new fusion protein was performed as described in the *Invitrogen$^{TM}$ life technologie* pBAD/His B instruction manual version F.

Cells were grown in 500ml LB media, 100µg/ml ampicillin at 37°C. Induction with 0.2% arabinose (Roth) was performed at an $OD_{600nm} \approx 0.4$. Three hours after induction, cells are washed in Tris 20 mM pH 7.5, 100mM NaCl, and pelleted after centrifugation (6000g, 15min, 4°C).

#### 2.1.3.3. Purification of the His-gpJ protein

Cells are resuspended in a Tris-buffer: Tris 20 mM pH 8, 100 mM NaCl + antiproteases (protease inhibitor cocktail P 8465, Sigma) and disrupted (three times, 1000 bars) by the French pressure cell. The fusion protein is produced in an insoluble form (inclusion bodies). Following *Wang et al., 1998,* the GpJ protein was found in inclusion bodies and active protein after renaturation.

The pellet was solubilized in a Tris buffer (Tris 20 mM, pH 8, 200 mM NaCl, 8 M urea) and centrifugated (15min, 6000g, 4°C). The obtained supernatant was incubated with Ni-NTA Agarose (*Qiagen*) equilibrated with the same buffer overnight at 4°C. The resin was washed 10 times in a Tris-buffer (Tris 20 mM pH 8, 200 mM NaCl, 20 mM imidazole), and the elution was performed in a Tris-buffer (Tris 20 mM pH 8, 200 mM NaCl, 300 mM imidazole). The elution fractions were dialyzed against Tris HCl 20mM pH 8, 200mM NaCl to remove imidazole which disturbs the $OD_{280nm}$ measurements investigating the protein concentration.

## 2.2. Lipid Bilayer Experiments

### 2.2.1. Technique

Black lipid bilayer membranes were formed from 1% solution of diphytanoyl phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) in n-decane as described previously (*Benz et al., 1978*). This instrumentation consists of a Teflon chamber with two aqueous compartments filled with unbuffered electrolyte solution - usually 1M KCl - and pH 6. The

temperature was kept at 20°C. The compartments are connected via a small circular hole with a diameter of about 0.1 - 1mm across which the membranes are formed (see Figure 2.2).

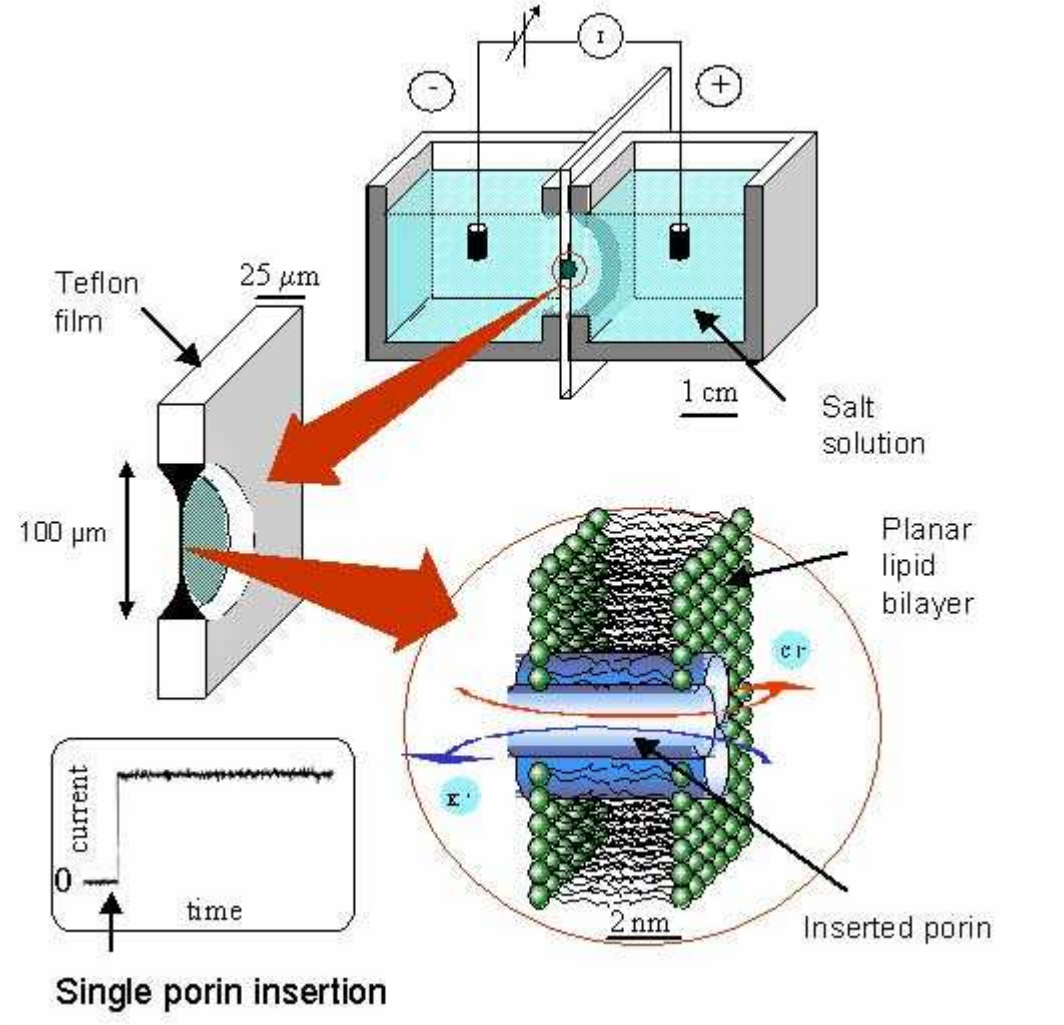



***Figure 2.2:*** *Schema of a bilayer set-up. A teflon chamber is separated by a wall containing a hole of about 0.5 mm diamter in two compartments, filled with an electrolyte buffer. A potential is applied. At the level of the hole, an artificial membrane is spread and prevents the passage of ions. After the insertion of a LamB trimer inside the membrane, ions can pass across the reconstituted channel and a current specific for the passage of ions through LamB can be detected.*

The current was measured with a pair of Ag/AgCl electrodes with salt bridges connected in series with a voltage source and a current amplifier (Keithley 427 with a four pole filter or a home made current to voltage converter containing a Burr Brown operational amplifier with a three-pole filter). The feedback resistors of the current amplifier were between 0.01 and 10

gigaΩ. The amplified signal was monitored with a strip chart recorder in order to measure the membrane current.

Maltoporin was added from concentrated stock solution to one side of the membrane (cis-side). The reconstitution of channels in the black lipid membrane was monitored on a strip chart recorder resulting in a stepwise increase of the membrane current. The membrane potential was held at +20mV throughout the experiments.

## 2.2.2. Titration of LamB by MBP-gpJ

Shortly after the membrane turned black, the channel forming protein was added to the aqueous compartment (final concentration $10^{-12}$M). The membrane conductance increased stepwise due to the reconstitution of channels. After a few minutes, the conductance increase slowed down. When the conductance was nearly constant the titration experiment started and MBP-gpJ was added in defined concentrations to one or both sides of the membrane. Subsequently the membrane conductance decreased in a dose-dependent manner as a result of the channel block for ions because of binding.

In recent publications the properties of substrate-gated or ligand-gated channels has been studied in detail (Benz *et al.*, 1987; *Nekolla et al.*, 1994; Jordy *et al.*, 1996; Bachmeyer *et al.*, 2001). It has been demonstrated that the translocation of the carbohydrates through channels can be described by a simple one-site, two barrier channel. Examples are LamB (*Läuger, 1973; Benz et al., 1987; Benz et Hancock, 1987*) or CymA (Pajatsch *et al.*, 1999). We used this model to investigate the binding between LamB and MBP-gpJ or LamB and His-gpJ. This model assumes a binding-site for the substrate in the center of the channel with symmetrical barriers for the on-rate constants of substrate binding. The rate constant $k_1$ describes the jump of the substrate from the aqueous phase (concentration $c$) to the binding-site, whereas the inverse movement is described by the rate constant $k_{-1}$.

The stability constant of the binding between a substrate and the binding-site inside the channel is $K = k_1/k_{-1}$. Furthermore, we assume that only one substrate molecule can bind to the binding-site at a given time (Benz *et al*., 1987). This means that a substrate molecule can bind to the channel only when the binding-site is free. The substrate-gated channel (given by $P$) is open when no substrate $L$ is bound and closed when it is occupied to form the non- or low-conducting substrate-channel complex $PL$:

$$P + L \underset{k_{-1}}{\overset{k_1 c}{\rightleftharpoons}} PL \qquad (1)$$

The probability, $p$, that the binding-site is occupied by a substrate molecule and the channel does not conduct ions or is in the low-conductance state is given by:

$$p = \frac{K \cdot c}{1 + K \cdot c} \qquad (2)$$

and that it is free and the channel is in its high conductance-state is given by:

$$1 - p = \frac{1}{1 + K \cdot c} \qquad (3)$$

The conductance, $G(c) = I_m / V_m$, of a LamB containing membrane in the presence of a substrate with the stability constant, $K$, and a ligand concentration, $c$, is given by the probability that the binding-site is free:

$$G(c) = G_{\max} \frac{1}{K \cdot c + 1} \qquad (4)$$

where $G_{\max}$ is the membrane conductance before the start of the addition of the ligand to the aqueous phase. Eqn. (4) may also be written as:

$$\frac{G_{\max} - G(c)}{G_{\max}} = \frac{K \cdot c}{K \cdot c + 1} \qquad (5)$$

which means that the titration curves can be analyzed using Lineweaver-Burke plots as has been shown in previous publications (Benz *et al*., 1987; Andersen *et al.*, 1995). The half saturation constant, $K_S$ is given by the inverse stability constant.

### 2.2.3. Noise analysis measurements

The ion current noise of the channel was analysed as previously described (*Nekolla et al., 1994*). The amplified time resolved current signal was monitored by a strip chart recorder and

simultaneously fed through a low pass filter (4 Pole Butterworth Low-Pass Filter) into an AD-converting card of an IBM-compatible PC. The digitized data were analyzed with a home-made fast Fourier transformation program, which yielded identical results as compared to a commercial digital signal analyzer (Ono Sokki CF 210). The spectra were composed of 400 points and they were averaged either 128 or 256 times. The spectra were analyzed using commercial graphic programs. The measurements of current noise presented here are based on small perturbations of the number of closed channels due to microscopic variations involved in the chemical reaction between the phage protein and its binding site, which can be monitored by current fluctuations. For the derivation of the rate constants of phage protein binding they were fitted to a Lorentzian function (*Verveen et De Felice, 1974; De Felice 1981*). Its reaction rate $1/\tau$ is given by:

$$\frac{1}{\tau} = 2\pi \cdot f_c = k_1 \cdot c + k_{-1} \quad (6)$$

$f_c$ is the corner frequency of the power density spectrum, $S(f)$, given by a "Lorentzian" function. Lorentzian spectra correspond to the noise expected for a random switch with different on and off probabilities, which are coupled by a chemical reaction (Verveen et DeFelice, 1974; Conti et Wanke, 1975; DeFelice, 1981):

$$S(f) = S_0 \Big/ \left(1 + \left(f / f_c\right)^2\right) \quad (7)$$

$S_0$ is the plateau value of the power density spectrum at small frequencies. It is given by (*Verveen et De Felice, 1974*):

$$S_0 = 4 \cdot N \cdot i^2 \cdot p \cdot (1 - p) \cdot \tau \quad (8)$$

$N$ is the total number of channels (blocked and unblocked) within the membrane $i$ is the current through one single open channel and $p$ is the probability that the channel is occupied by MBP-gpJ (i.e. closed).

## 2.3. Immunodetection

After migration on SDS PAGE, proteins are transferred on a nitrocellulose membrane (Schleicher & Schuell, Protran®) in Towbin buffer (25 mM Tris, 152mM glycin, 20% methanol) 45minutes, 100V, 350mA constant. The membrane was blocked with 5% non fat dried milk powder in TBS-T buffer (20mM Tris pH 7.6, 140mM NaCl, 1% Tween 20). Two types of immunodetection were performed: a detection anti-6xHis-tag and a detection anti-MBP. The membrane was first incubated with the primary antibody in TBS-T (dilution ratio:1/3000) and afterwards with the secondary antibody in TBS-T (dilution ratio:1/3000) for 1 hour at room temperature. For the anti-6xHis-tag detection, the primary antibody was a mouse Ig (Amersham Bioscience®) and the secondary antibody is an antimouse immunoglobulin, horseradish peroxidase linked to whole antibody (Amersham Bioscience ®). For the anti-MBP detection, the primary antibody was an anti-MBP serum from rabbit (New England Biolabs ®) and the secondary antibody is an anti rabbit immunoglobulin, horseradish peroxidase linked to whole antibody (Amersham Bioscience ®). Immunodetection was performed using the ECL™ Western Blotting Detection Reagents and Hyperfilm™ (Amersham Biosciences ®).

## 2.4. Electron transmission microscopy

Cells (Top10F' expressing LamB WT, pop154 expressing LamB from *Shigella sonnei* or JM501 transformed by pAC-1/LamB Δ4+Δ6+Δ9v for LamB Δ4+Δ6+Δ9v) and bacteriophage Lambda were incubated in SM buffer (Tris 20mM pH 7.5, 100mM NaCl, 10mM $MgSO_4$, 1% gelatine (Sigma)) for 1 hour at room temperature.

## 2.5. Circular dichroism measurements

Circular Dichroism is a method providing data about the secondary structure of proteins (*Greenfield, 2004*). The CD spectrum was recorded between 190 and 320 nm in a mark VI (Jobin-Yvon) spectrometer and using a 1mm wide cuvette. The temperature was set at 20 °C. The spectrum of MBP-gpJ (4µM) was performed in a Phosphate buffer (Phosphate 20 mM pH 7.6, 200 mM NaCl, 1mM EDTA) and the spectrum of His-gpJ (18.5 µM in the beginning of the experiment) was performed in a Tris buffer (Tris 20 mM pH 7.6, 200 mM NaCl).

# CHAPTER III - Results

## Résumé (Summary)

**- Production et caractérisation des protéines: LamB sauvage, LamB de *Shigella sonnei*, LamB Y118G, LamB Δ4+Δ6+Δ9v, MBP-gpJ et His-gpJ.**

Les différents types de LamB, dont les protocoles de production sont établis, sont facilement obtenus, et avec une très grande pureté. Par contre, MBP-gpJ se révèle être une protéine instable qui semble se dégrader dans le temps. La protéine His-gpJ se trouve être exprimée en corps d'inclusion. Après purification en conditions dénaturantes et renaturation, une protéine soluble et qui pourra par la suite intéragir avec LamB sera obtenue.

**- Etude de l'intéraction entre LamB et le fragment C-terminal de GpJ par la Technique du Film Noir (BLM).**

Etude de l'intéraction entre LamB et MBP-gpJ.

MBP-gpJ induit un blocage complet et réversible des canaux de LamB qui permet de démontrer une intéraction entre ces deux protéines. L'effet de MBP-gpJ sur LamB est qualifié d'« asymétrique », puisque observé principalement lorsque MBP-gpJ est ajouté d'un seul côté de la membrane (le côté cis, celui où LamB est ajouté). Ce type de résultat est également observable avec les mutants de LamB. La constante d'association entre LamB sauvage et MBP-gpJ a pu être évaluée à $1.10^8$ 1/M dans 1M KCl. Enfin, la force ionique (concentration de KCl et de $Mg^{2+}$) n'a aucun effet sur le complexe.

Etude de l'intéraction entre LamB et His-gpJ.

Pas de blocage significatif de LamB après ajout de His-gpJ comme ce fut le cas après ajout de MBP-gpJ: le passage des ions et des maltooligosaccharides à travers LamB n'est pas perturbé. Toutefois, le fait que l'intéraction entre MBP-gpJ et LamB n'est plus possible en présence de His-gpJ montre que His-gpJ se lie à LamB bien que ses canaux ne soient pas bloqués par la présence de cette protéine à sa surface.

**- Vérification de la formation du complexe entre LamB et His-gpJ.**

L'intéraction entre LamB (ou un de ses mutants) et His-gpJ est confirmée par immunodétection. Ces résultats montrent également que les boucles externes L4, L6 et L9v de LamB ne sont pas indispensables à la liaison du fragment de GpJ alors qu'elles le sont à celle du phage Lambda. De plus, la structure en trimère de LamB est indispensable pour la liaison au fragment de GpJ.

---

# 3.1. Expression and characterisation of the required proteins

The proteins used to investigate the interaction between LamB and the C-terminal fragment of GpJ were expressed as fusion protein and produced by ourselves. Here we described how they were expressed and characterised.

## 3.1.1. LamB from different sources and mutants

LamB Wildtype (WT), LamB from *Shigella sonnei*, and LamB Δ4+Δ6+Δ9v were purified as described in Materials and Methods. Pure LamB Y118G was generous gift from Dr. F. Orlik. Purity of the samples was investigated by SDS-PAGE (see Figure 3.1). The trimer (unboiled sample) of LamB WT, from *Shigella sonnei* and Y118G had an apparent molecular weight a bit upper to 66kDa, and the monomer (boiled sample), an apparent molecular weight of 45 kDa. The trimer (unboiled sample) of LamB Δ4+Δ6+Δ9v had an apparent molecular weight of 66 kDa, and the monomer (boiled sample), an apparent molecular weight between 30 and 45 kDa, due to the deletion on the loops. Subsequently, the proteins were reconstituted in planar lipid bilayer to confirm their channel forming activity.

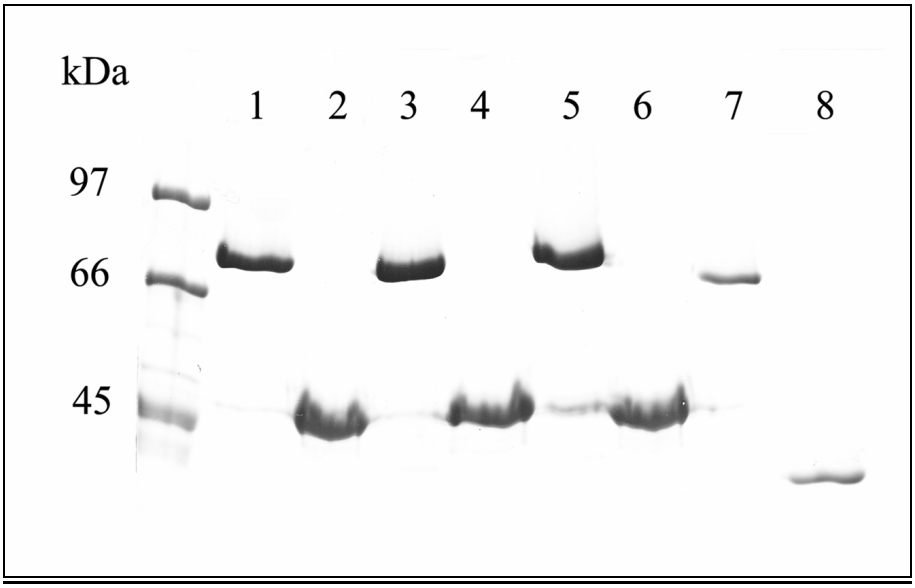



**Figure 3.1: 10% SDS-PAGE of the purified LamB and mutant LamB proteins. The gel was stained with Comassie brilliant blue.**

Lane 1: 5 µg purified LamB wildtype of *E. coli* solubilised at 30°C for 5 minutes in 5 µl sample buffer. Lane 2: 5 µg purified LamB wildtype of *E. coli* solubilised at 100°C for 5 minutes in 5 µl sample buffer. Lane 3: 5 µg purified LamB wildtype of *S. sonnei* solubilised at 30°C for 5 minutes in 5 µl sample buffer. Lane 4: 5 µg purified LamB wildtype of *S. sonnei* solubilised at 100°C for 5 minutes in 5 µl sample buffer. Lane 5: 5 µg purified LamB mutant Y118G solubilised at 30°C for 5 minutes in 5 µl sample buffer. Lane 6: 5 µg purified LamB mutant Y118G solubilised at 100°C for 5 minutes in 5 µl sample buffer. Lane 7: 3 µg purified LamB mutant Δ4+Δ6+Δ9v solubilised at 30°C for 5 minutes in 5 µl sample buffer. Lane 8: 3 µg purified LamB mutant Δ4+Δ6+Δ9v solubilised at 100°C for 5 minutes in 5 µl sample buffer.

## 3.1.2. The MBP-gpJ protein

### 3.1.2.1. Production and purification

The induction of the $p_{tac}$ promotor of the plasmid allowed the overexpression of the MBP-gpJ protein with a molecular weight close to 97 kDa on SDS-PAGE (see Figure 3.2A).

At concentrations above 44µM, the protein precipitated. The variation of the ionic strength (between 50 and 400 mM) had no effect on the stability of MBP-gpJ. At pH close to 8 less precipitation was observed than at pH 7. The theoretical pI of the protein is about 6.7 (see Table 3.1 for characteristics). Furthermore, the protein remained active around 2 weeks when it was stored at + 4°C or longer if it was conserved at – 20 °C. As described by *Wang et al., 2000*, the fractions were not completely pure after the maltose elution step. In order to obtain a pure protein sample, a further purification step was performed by gel elution. However, after

investigation of the purity by immnunodetection, the protein appeared not to be completely pure, suggesting its instability or a possible degradation (see Figure 3.2B).

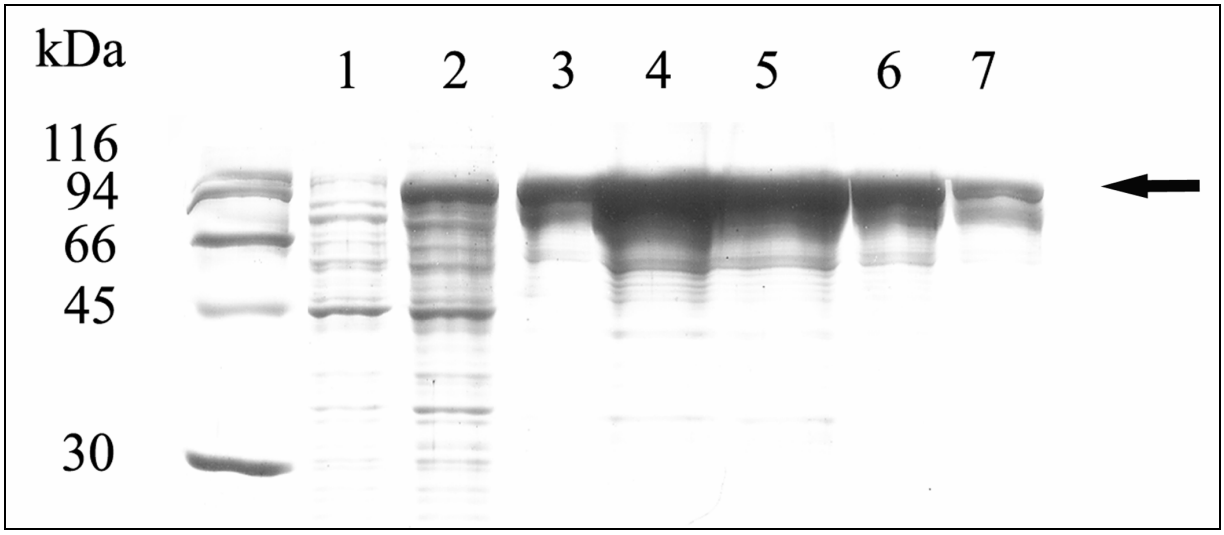



**Figure 3.2A: 10% SDS-PAGE of the purification steps of MBP-gpJ.**

Lane 1: 5 µl of total cell extract of induced *E. coli* cells was solubilised at 100°C for 5 minutes in sample buffer. Lane 2: 5 µl of total cell extract of non-induced *E. coli* cells was solubilised at 100°C for 5 minutes in sample buffer. The arrow shows the position of MBP-gpJ. Lanes 3-7: Protein content of different fractions of the amylose-sepharose column after elution with buffer supplemented with maltose. 15 µl of the fractions were solubilised at 100°C for 5 minutes in sample buffer. The gel was stained with Comassie brilliant blue.

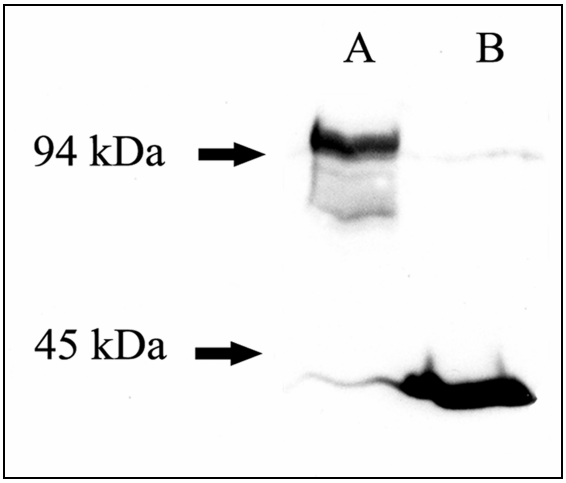



**Figure 3.2B: Western blot of MBP-gpJ and Maltose Binding Protein** (lanes A and B respectively).

The proteins were run on a 10% SDS-PAGE and blotted onto a nitrocellulose membrane as described in "Material and Methods".

```
MDVIKKKHWW QSDALKWSVL GLLGLLVGYL VVLMYAQGEY LFAITTLILS
SAGLYIFANR KAYAWRYVYP GMAGMGLFVL FPLVCTIAIA FTNYSSTNQL
TFERAQEVLL DRSWQAGKTY NFGLYPAGDE WQLALSDGET GKNYLSDAFK
FGGEQKLQLK ETTAQPEGER ANLRVITQNR QALSDITAIL PDGNKVMMSS
LRQFSGTQPL YTLDGDGTLT NNQSGVKYRP NNQIGFYQSI TADGNWGDEK
LSPGYTVTTG WKNFTRVFTD EGIQKPFLAI FVWTVVFSLI TVFLTVAVGM
VLACLVQWEA LRGKAVYRVL LILPYAVPSF ISILIFKGLF NQSFGEINMM
LSALFGVKPA WFSDPTTART MLIIVNTWLG YPYMMILCMG LLKAIPDDLY
EASAMDGAGP FQNFFKITLP LLIKPLTPLM IASFAFNFNN FVLIQLLTNG
GPDRLGTTTP AGYTDLLVNY TYRIAFEGGG GQDFGLAAAI ATLIFLLVGA
LAIVNLKATR MKFDIEGRIS EFGNYRLTVR AVNAWGQQGD PASVSFRIAA
PAAPSRIELT PGYFQITATP HLAVYDPTVQ FEFWFSEKQI ADIRQVETST
RYLGTALYWI AASINIKPGH DYYFIRSVNT VGKSAFVEAV GRASDDAEGY
LDFFKGKITE SHLGKELLEK VELTEDNASR LEEFSKEWKD ASDKWNAMWA
VKIEQTKDGK HYVAGIGLSM EDTEEGKLSQ FLVAANRIAF IDPANGNGNE
TPMFVAQGNQ IFMNDVFLKR LTAPTITSGG NPPAFSLTPD GKLTAKNADI
SGSVNANSGT LSNVTIAENC TINGTLRAEK IVGDIVKAAS AAFPRQRESS
VDWPSGTRTV TVTDDHPFDR QIVVLPLTFR GSKRTVSGRT TYSMCYLKVL
MNGAVIYDGA ANEAVQVFSR IVDMPAGRGN VILTFTLTST RHSADIPPYT
FASDVQVMVI KKQALGISVV
```

**Number of amino acids:** 970

**Molecular weight:** 106849.7

**Theoretical pI:** 6.71

**Total number of negatively charged residues (Asp + Glu):** 86
**Total number of positively charged residues (Arg + Lys):** 85

**Extinction coefficients:**

Conditions: 6.0 M guanidium hydrochloride
0.02 M phosphate buffer
pH 6.5

The first table lists values computed assuming ALL Cys residues appear as half cystines, whereas the second table assumes that NONE do.

| Wavelength | 276 nm | 278 nm | 279 nm | 280 nm | 282 nm |
|---|---|---|---|---|---|
| Ext. Coefficient ($M^{-1}$ $cm^{-1}$) | 155090 | 157054 | 156200 | 154430 | 149840 |
| Abs. 0.1% (= 1g/l) | 1.451 | 1.470 | 1.462 | 1.445 | 1.402 |

| Wavelength | 276 nm | 278 nm | 279 nm | 280 nm | 282 nm |
|---|---|---|---|---|---|
| Ext. Coefficient ($M^{-1}$ $cm^{-1}$) | 154800 | 156800 | 155960 | 154190 | 149600 |
| Abs. 0.1% (= 1g/l) | 1.449 | 1.467 | 1.460 | 1.443 | 1.400 |

**Table 3.1: Characteristics of the MBP-gpJ protein.**
Characteristics obtained by the program Expasy ProtParam Tool. (http://au.expasy.org/tools/protparam.html)

### 3.1.2.2. Secondary structure data of MBP-gpJ : CD-spectra and structure prediction

The CD spectra of MBP-gpJ (4µM) were investigated in order to obtain some structural data. Figure 3.3 shows a spectrum with two peaks at 205 and 220nm indicating a typical α-helical pattern. A structure prediction performed with the GOR IV program confirmed the presence in majority of α-helix inside the MBP-gpJ protein (cf. Figure 3.4: 38.5% of α-helices, 17.41% extended strand, 44.07% random coiled), and indicated that the secondary structure of the GpJ fragment (residue 522 until residue 970) should be also mainly composed of α-helices (see CD spectra of the His-gpJ protein Figure 3.7). However, the GpJ fragment is connected to the Maltose Binding Protein, which structure has been solved. The latter is mainly composed of α-helices, and dominates a part of the spectrum of the MBP-gpJ.

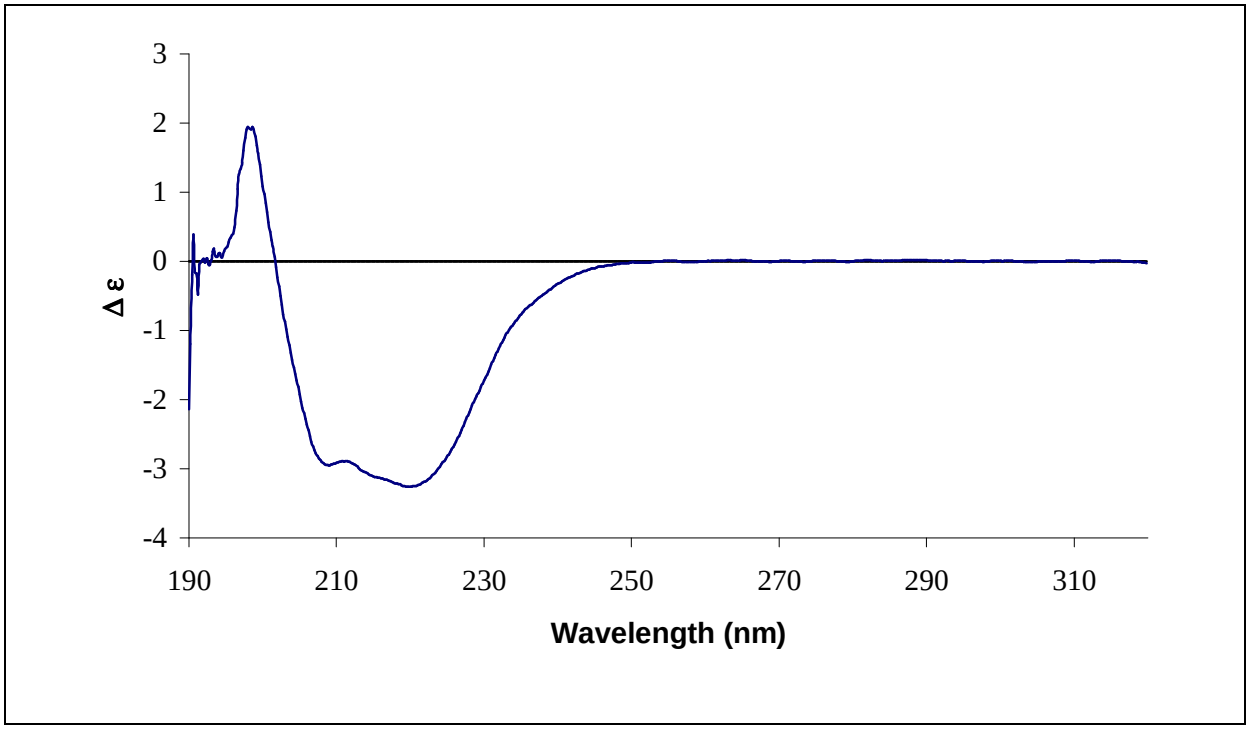
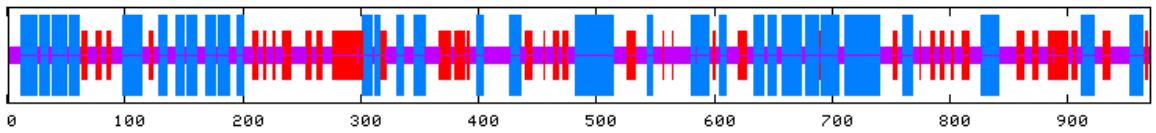



**Figure 3.3: CD spectrum of MBP-gpJ,** (4µM) performed in a Phosphate buffer (Phosphate 20 mM, pH 7.6, 200mM NaCl, 1mM EDTA.

### 3.1.2.3. Cleavage of the MBP part of the MBP-gpJ protein

A protease (factor Xa) sensitive site in the link on the pMal-2cX plasmid is located between the MBP and the GpJ part. In order to work only with the fragment of the GpJ protein alone, the MBP-gpJ protein was digested with the factor Xa. Surprisingly, the analysis of the bands by SDS-PAGE revealed the presence of the MBP and the factor Xa, but not of the GpJ part (data not shown). It was concluded that the fragment was degraded or not stable, as previously

observed with MBP-gpJ. Under those conditions, we decided to make a new fusion protein, with a different tag than the MBP. We chose a 6×His-tag, because it is a smaller tag, and is not involved in the maltose operon, like the Maltose Binding Protein.




```
         10         20         30         40         50         60         70
          |          |          |          |          |          |          |
MDVIKKKHWWQSDALKWSVLGLLGLLVGYLVVLMYAQGEYLFAITTLILSSAGLYIFANRKAYAWRYVYP
ccccccccccchhhhhhhhhhhhhhhccchhhhhhhccchhhhhhhhhhhhhccchhhhhhhhhceeeeeec
GMAGMGLFVLFPLVCTIAIAFTNYSSTNQLTFERAQEVLLDRSWQAGKTYNFGLYPAGDEWQLALSDGET
cccccceeeeccccceeeecccccccccchhhhhhhhhhhhhhhhhcccccceeecccccchhhhhhhcccc
GKNYLSDAFKFGGEQKLQLKETTAQPEGERANLRVITQNRQALSDITAILPDGNKVMMSSLRQFSGTQPL
ccchhhhhhhhchhhhhhhhhhcccccchhhhhhhhhhchhhhhhhhhhcccccchhhhhhccccccccce
YTLDGDGTLTNNQSGVKYRPNNQIGFYQSITADGNWGDEKLSPGYTVTTGWKNFTRVFTDEGIQKPFLAI
eeecccceeecccccceecccccceeeeeeecccccccccccccceeeeecccceeeeecccccccceeee
FVWTVVFSLITVFLTVAVGMVLACLVQWEALRGKAVYRVLLILPYAVPSFISILIFKGLFNQSFGEINMM
eeeeeeeeeeeeeeeeeceeehhhhhhhhhchhhhheeeeecccccccchhhhhhhccccccccchhhhh
LSALFGVKPAWFSDPTTARTMLIIVNTWLGYPYMMILCMGLLKAIPDDLYEASAMDGAGPFQNFFKITLP
hhhhhccccccccccceeeeeeeeeeccceeeeeeeeceeecccccchhhhhhhccccccccccccccc
LLIKPLTPLMIASFAFNFNNFVLIQLLTNGGPDRLGTTTPAGYTDLLVNYTYRIAFEGGGGQDFGLAAAI
ccccccchhhhhhhhhccccceeeeeecccccccccceecccccceeeeecccceeeeecccccchhhhhhhh
ATLIFLLVGALAIVNLKATRMKFDIEGRISEFGNYRLTVRAVNAWGQQGDPASVSFRIAAPAAPSRIELT
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ccchhhhhhhhhcccchhhhhhhcccchhhhhhhhhhhhhhhhhhcchhhhhhhhhhhhcehhhhhhhhh
VKIEQTKDGKHYVAGIGLSMEDTEEGKLSQFLVAANRIAFIDPANGNGNETPMFVAQGNQIFMNDVFLKR
hhhhhhccccchhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhcccccccccccceeeecccchhhhhhhhhc
LTAPTITSGGNPPAFSLTPDGKLTAKNADISGSVNANSGTLSNVTIAENCTINGTLRAEKIVGDIVKAAS
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AAFPRQRESSVDWPSGTRTVTVTDDHPFDRQIVVLPLTFRGSKRTVSGRTTYSMCYLKVLMNGAVIYDGA
hhccccccccccccccceeeeeecccccccceeeeecccccccceeeeeceeeeeeeeeeecccceeeecc
ANEAVQVFSRIVDMPAGRGNVILTFTLTSTRHSADIPPYTFASDVQVMVIKKQALGISVV
cchhhhhhhhhhhccccccceeeeeeecccccccccccccccchhhhhhhhhhhhceeec
```

Sequence length : 970

Alpha helix (h) : 357 is 36.80%

Beta bridge (b) : 0 is 0.00%

Extended strand (e) : 197 is 20.31%

Random coil (c) : 416 is 42.89%

**Figure 3.4: Secondary structure prediction of the MBP-gpJ protein.**

Structure prediction performed with GOR IV.

## 3.1.3. The His-gpJ protein

### 3.1.3.1. Production and purification

The gene GpJ_(684-1132) of the MBP-gpJ protein was subcloned into a new vector. The expressed protein presented 40 % C-terminus end of GpJ, which carried a 6×His-tag attached to its N-terminal end. The protein was followed during the different steps of the production and the purification using SDS-PAGE analysis. After production, immunodetection by anti-6×His-tag antibodies indicated that the His-gpJ protein was expressed as insoluble inclusion bodies. The purification of the His-gpJ protein was performed under denaturating conditions (see Figure 3.5).

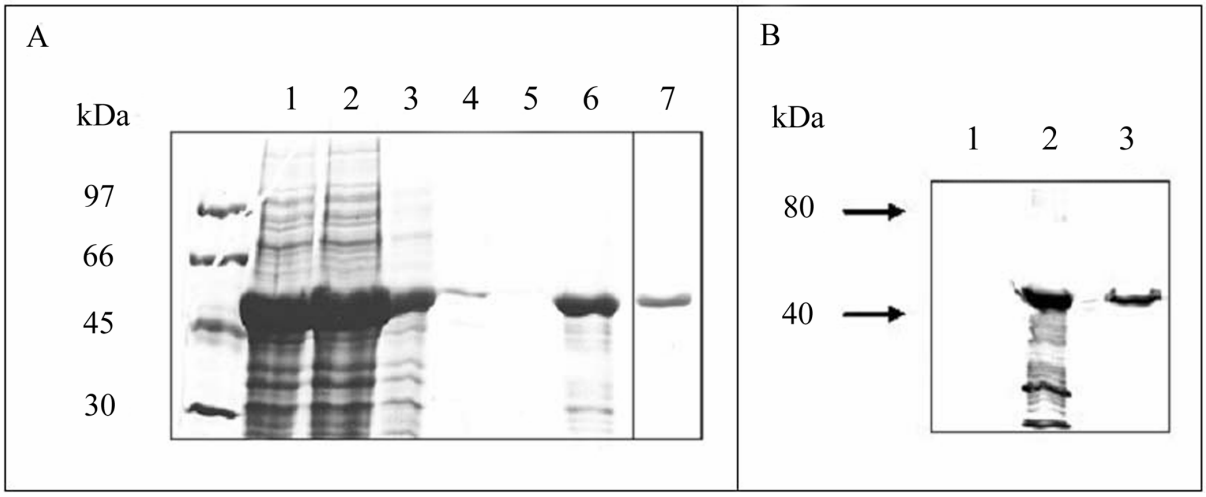



**Figure 3.5: Purification of His-gpJ.**

**A: 10% SDS-PAGE of the purification steps of His-gpJ.**

Lane 1: 20µl solubilised inclusion bodies (in 8 M urea, 20 mM Tris-HCl, pH 8) were dissolved at 100°C in 10 µl sample buffer. Lanes 2 to 5: Supernatants of subsequent washing steps with 100 mM NaCl, 20mM Tris-HCl, pH 8, 20µl of the solutions were dissolved at 100°C in 10 µl sample buffer. Lane 6: Supernatant of the steps with 100 mM NaCl, 20mM Tris-HCl, pH 8, supplemented with 300 mM imidazole, 20µl of the solutions were dissolved at 100°C in 10 µl sample buffer. Lane 7: 3 µg pure His-gpJ obtained by preparative SDS-PAGE was solubilised in 10 µl sample buffer. The gel was stained with Comassie brilliant blue.

**B: Western blot of His-gpJ**. Cells expressing His-gpJ were disrupted and centrifuged. Lane 1: Analysis of the supernatant. Lane 2: Analysis of the pellet. Lane 3: Analysis of the His-gpJ purified by gel elution. The proteins were run on a 10% SDS-PAGE and blotted onto a nitrocellulose membrane as described in the "Material and Methods" section.

After elution, a pure and soluble protein with a molecular weight of ca 50 kDa was obtained. This result was in accordance with the expected value of 54 kDa calculated from the sequence (see Table 3.2 for the His-gpJ characteristics), the immunodetection analysis, and the fact that this band was not found when the purification was performed with cells that did not express the plasmid carrying the GpJ_(684-1132) gene (data not shown). All those controls convinced us that the protein obtained after purification was the His-gpJ protein. Furthermore, the protein remained active around 2 weeks after stored at + 4°C or longer if it was conserved at -20 °C, as it was the case for the MBP-gpJ protein.

MGGSHHHHHH GMASMTGGQQ MGRDLYDDDD KDPSSRSAAG TIWEFALGNY RLTVRAVNAW
GQQGDPASVS FRIAAPAAPS RIELTPGYFQ ITATPHLAVY DPTVQFEFWF SEKQIADIRQ
VETSTRYLGT ALYWIAASIN IKPGHDYYFI RSVNTVGKSA FVEAVGRASD DAEGYLDFFK
GKITESHLGK ELLEKVELTE DNASRLEEFS KEWKDASDKW NAMWAVKIEQ TKDGKHYVAG
IGLSMEDTEE GKLSQFLVAA NRIAFIDPAN GNGNETPMFV AQGNQIFMND VFLKRLTAPT
ITSGGNPPAF SLTPDGKLTA KNADISGSVN ANSGTLSNVT IAENCTINGT LRAEKIVGDI
VKAASAAFPR QRESSVDWPS GTRTVTVTDD HPFDRQIVVL PLTFRGSKRT VSGRTTYSMC
YLKVLMNGAV IYDGAANEAV QVFSRIVDMP AGRGNVILTF TLTSTRHSAD IPPYTFASDV
QVMVIKKQAL GISVV

**Number of amino acids:** 495

**Molecular weight:** 54040.8

**Theoretical pI:** 5.98

**Total number of negatively charged residues (Asp + Glu):** 55
**Total number of positively charged residues (Arg + Lys):** 48

**Extinction coefficients:**
Conditions: 6.0 M guanidium hydrochloride
0.02 M phosphate buffer
pH 6.5

The first table lists values computed assuming ALL Cys residues appear as half cystines, whereas the second table assumes that NONE do.

| Wavelength | 276 nm | 278 nm | 279 nm | 280 nm | 282 nm |
|---|---|---|---|---|---|
| Ext. Coefficient ($M^{-1}$ $cm^{-1}$) | 63645 | 64527 | 64230 | 63560 | 61720 |
| Abs. 0.1% (= 1g/l) | 1.178 | 1.194 | 1.189 | 1.176 | 1.142 |

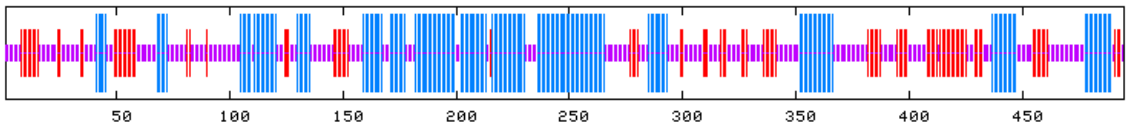
| Wavelength | 276 nm | 278 nm | 279 nm | 280 nm | 282 nm |
|---|---|---|---|---|---|
| Ext. Coefficient ($M^{-1}$ $cm^{-1}$) | 63500 | 64400 | 64110 | 63440 | 61600 |
| Abs. 0.1% (= 1g/l) | 1.175 | 1.192 | 1.186 | 1.174 | 1.140 |

**Table 3.2: Characteristics of the His-gpJ protein.**

Characteristics obtained by the program Expasy ProtParam Tool. (http://au.expasy.org/tools/protparam.html)

### 3.1.3.2. Structural characteristics of His-gpJ

The investigation of the structural characteristics of His-gpJ allowed us to obtain data from 40% of the C-terminal extremity of the GpJ protein alone. Secondary structure predictions using the GOR4 automated program suggested a structure presenting mainly α–helices for the GpJ fragment (34% α–helix, 20% extended strand and 46% random coil, see Figure 3.6) as for the MBP-gpJ protein.




```
         10         20         30         40         50         60         70
          |          |          |          |          |          |          |
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eeeeeeccceeeecccchhhhhhhhhhhccccccceeeeeeecccccccccccccccchhhhhhhhhhhh
GISVV
ceeec
```

Sequence length : 495

Alpha helix (h) : 169 is 34.14%

Extended strand (e) : 99 is 20.00%

Random coil (c) : 227 is 45.86%

**Figure 3.6: Prediction of the secondary structure of the His-gpJ protein.**

Structure prediction performed with the logiciel GOR IV.

This prediction was not completely confirmed by CD measurements of the His-gpJ protein by far UV light. Figure 3.7 showed different patterns of His-gpJ depending on its concentration.

At a concentration of 18.5µM, the spectrum shows a peak at 225nm which was difficult to be interpreted as it was not characteristic for typical secondary structure elements (Figure 3.7, trace 1). However, after diluting the sample five times (3.8µM), the spectrum of His-gpJ changed: the peak at 225nm was shifted to lower wavelengths (Figure 3.7, trace 2) indicating a change of secondary structure of the protein in function of its concentration.

An other spectrum of the five times diluted sample (3.8µM) was performed 30 minutes after the one showed at figure 3.7, trace 2 in order to investigate if there is an evolution of the secondary structure in time. The spectrum was still more shifted to lower wavelengths, and two peaks at 205 and 220nm seemed to appear (Figure 3.7, trace 3) suggesting the presence of α-helices in this range of concentration.

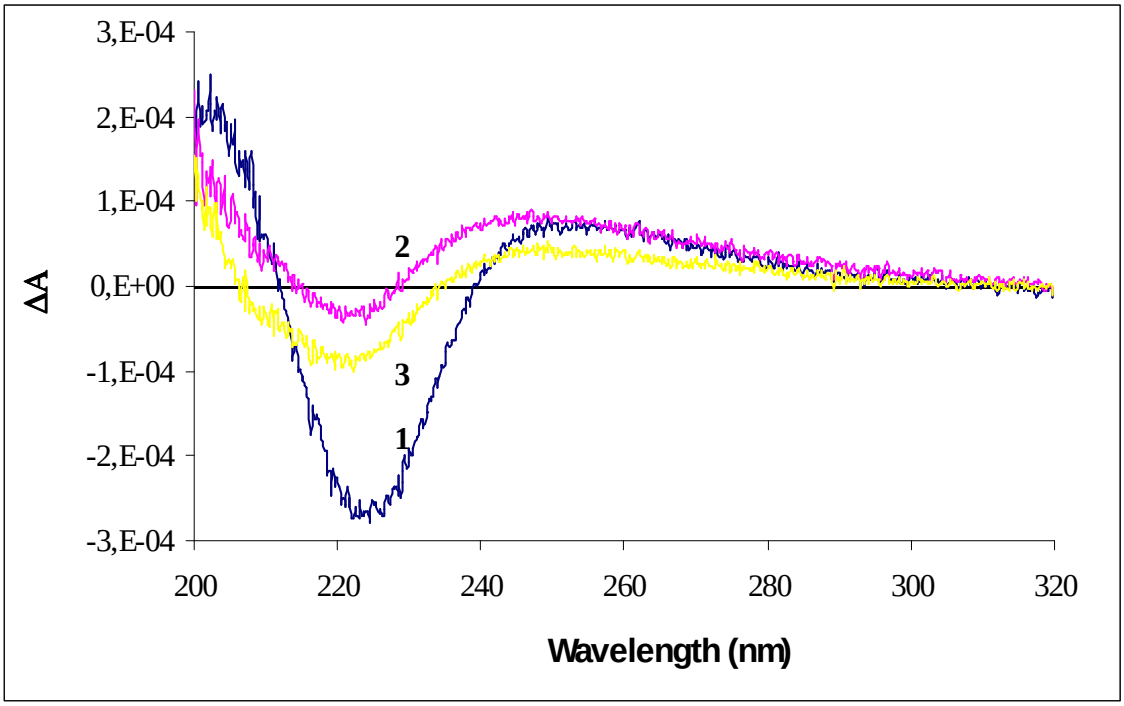


**Figure 3.7: CD-spectra of His-gpJ** in a Tris-buffer (Tris 20mM pH 7.6, 200mM NaCl). Trace 1: the concentration of His-gpJ is 18,5 µM. Trace 2: the concentration of His-gpJ is 3.8µM (sample of the trace 1 diluted 5 times): an α-helix pattern is appearing. It is more evident when the spectrum is done again after 30 minutes (trace 3), indicating that the secondary structure of the protein changes with its concentration.

The CD-spectra of His-gpJ at "low" concentration (about 4µM) showed a totally different pattern as the one observed at "higher" concentration (about 20µM). Because the His-gpJ protein was pure, we concluded that the differences of secondary structure of His-gpJ were

due to the difference of concentration, suggesting a possible oligomerisation step at about 20µM (see paragraph 4.5, Discussion).

## 3.2. Bilayer experiments

### 3.2.1. Effect of MBP-gpJ on the LamB protein at single molecule level

In order to elucidate the interaction of MBP-gpJ with LamB we reconstituted LamB in planar lipid bilayer. LamB ($10^{-14}$M) was added to the cis compartment of the cell. Figure 3.8A and 3.8B showed the typical behaviour of MBP-gpJ on LamB. In the Figure 3.8A, the insertion of a single channel in the membrane is seen by a sudden increase in current. The addition of MBP-gpJ to the cis compartment of the cell prevents the passage of ions through LamB WT trimer. A complete and reversible blockage of the channels was observed.

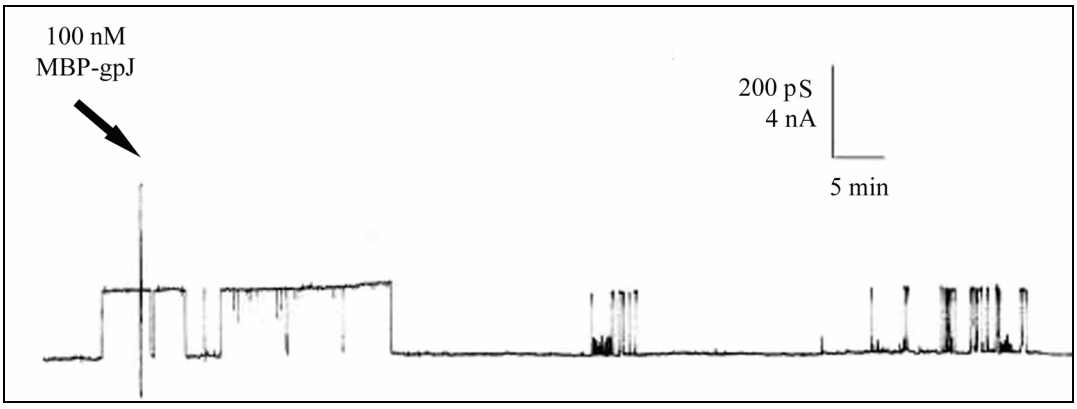


**Figure 3.8A: Effect of MBP-gpJ on LamB wildtype measured on the single-channel level.**

MBP-gpJ was added in a concentration of 100 nM to the cis-side of a diphytanoyl phosphatidylcholine/n-decane membrane (arrow) after the reconstitution of one wildtype LamB channel. Note that the channel opened only for a short time. The membrane was formed from diphytanoyl-phosphatidylcholine/n-decane. The aqueous phase contained 1M KCl and less than 1 pM LamB or LamB Y118G mutant. The applied voltage was 20mV at the cis-side; T = 20°C.

In the Figure 3.8B, a mutant of LamB, Y118G, was used. The passage of ions through this mutant, also called conductance value, is about 900 nS and is about six times higher than the one of LamB WT (156 pS). This mutant would allow to achieve a better signal/noise ratio.

Furthermore the mutation on the residue 118 does not affect the binding of the bacteriophage Lambda. In this experiment, five trimers of LamB Y118G were inserted inside the membrane. The addition of MBP-gpJ to the cis-side of the cell allowed a complete and reversible blockage of the LamB channels: even if they were switching in time between a fully open and fully closed state, they remained principally closed.

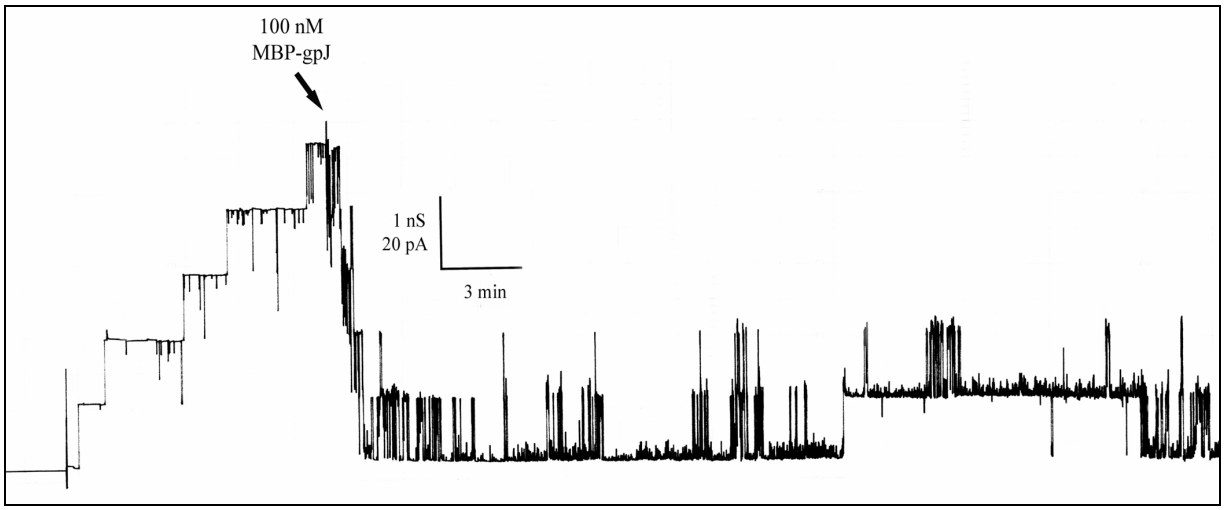


**Figure 3.8B: Effect of MBP-gpJ on LamB mutant Y118G measured on the single-channel level.** MBP-gpJ was added in a concentration of 100nM to the cis-side of a membrane (arrow) after the reconstitution of five Y118G mutant LamB channels. The channels closed and showed rapid flickering. The membrane was formed from diphytanoyl-phosphatidylcholine/n-decane. The aqueous phase contained 1M KCl and less than 1 pM LamB or LamB Y118G mutant. The applied voltage was 20mV at the cis-side; T = 20°C.

In order to investigate if the interaction with MBP-gpJ disturbed the reconstitution of LamB inside the membrane, LamB and MBP-gpJ were pre-mixed at approximatively equal concentration and added to the cis-side of the cell. At this concentration of LamB, one would usually expect the insertion of about 100 to 1000 channels. We observed that the reconstitution of LamB inside the membrane required more time, and that only a few channels were inserted inside the membrane. Furthermore the conductance of the reconstituted LamB proteins was also affected: it switched strongly between a fully open and a fully closed state due to the presence of the MBP-gpJ protein as it was previously observed after addition of MBP-gpJ to LamB proteins inserted in a membrane (see Figure 3.8C).

In conclusion, MBP-gpJ blocks completely and reversibly the LamB trimer channels. The interaction is possible when LamB was in the bulk or was inserted inside the membrane.

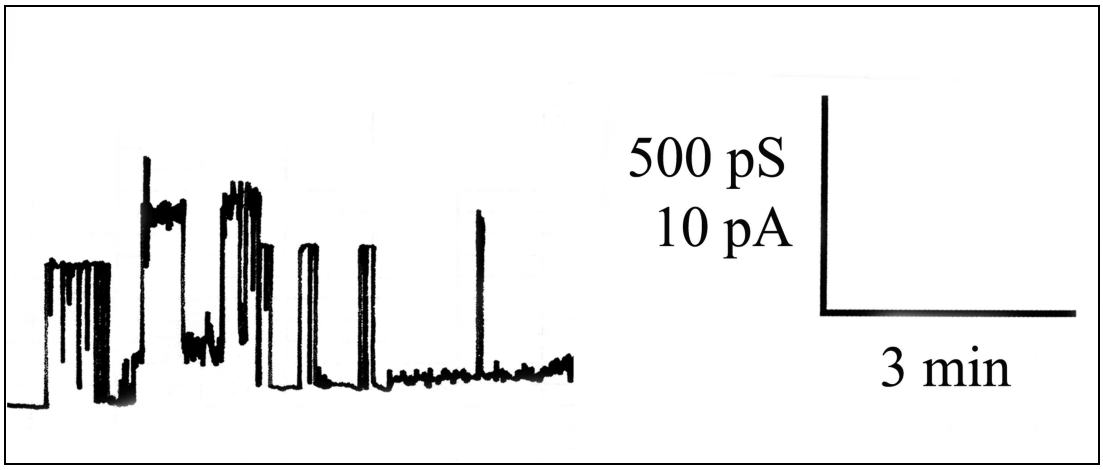


**Figure 3.8C: Effect of MBP-gpJ on LamB wildtype measured on the single-channel level when LamB and MBP-gpJ are previously mixed.**

LamB and MBP-gpJ were mixed at equal concentration. The mixture was added at a concentration of about 100 nM to the cis-side of a black membrane about 15 minutes before the recording started. Note that under these conditions only a small number of rapid switching channels was observed. The membrane was formed from diphytanoyl-phosphatidylcholine/n-decane. The aqueous phase contained 1M KCl and less than 1 pM LamB or LamB Y118G mutant. The applied voltage was 20mV at the cis-side; T = 20°C.

## 3.2.2. Titration of LamB by the MBP-gpJ protein

### 3.2.2.1. Effect on LamB WT and LamB from *Shigella sonnei*

We observed that the addition of MBP-gpJ allowed a complete and reversible blockage of the Maltoporin channels. In order to investigate the stability constant K of the interaction between LamB and MBP-gpJ titration experiments of the closure of LamB after addition of MBP-gpJ were performed. LamB WT (final concentration about $10^{-12}$M) was added to the cis-side of the cell. About 150 LamB channels got inserted in a diphytanoyl phosphatidylcholine/n-decane membrane. After reaching a stable number of inserted proteins, MBP-gpJ was added first to the trans, and afterwards to the cis compartment of the cell. The binding of MBP-gpJ resulted in a decrease of the conductance of LamB WT after a short time (see Figure 3.9). The same effect was also observed with LamB from *Shigella sonnei* (data not shown).

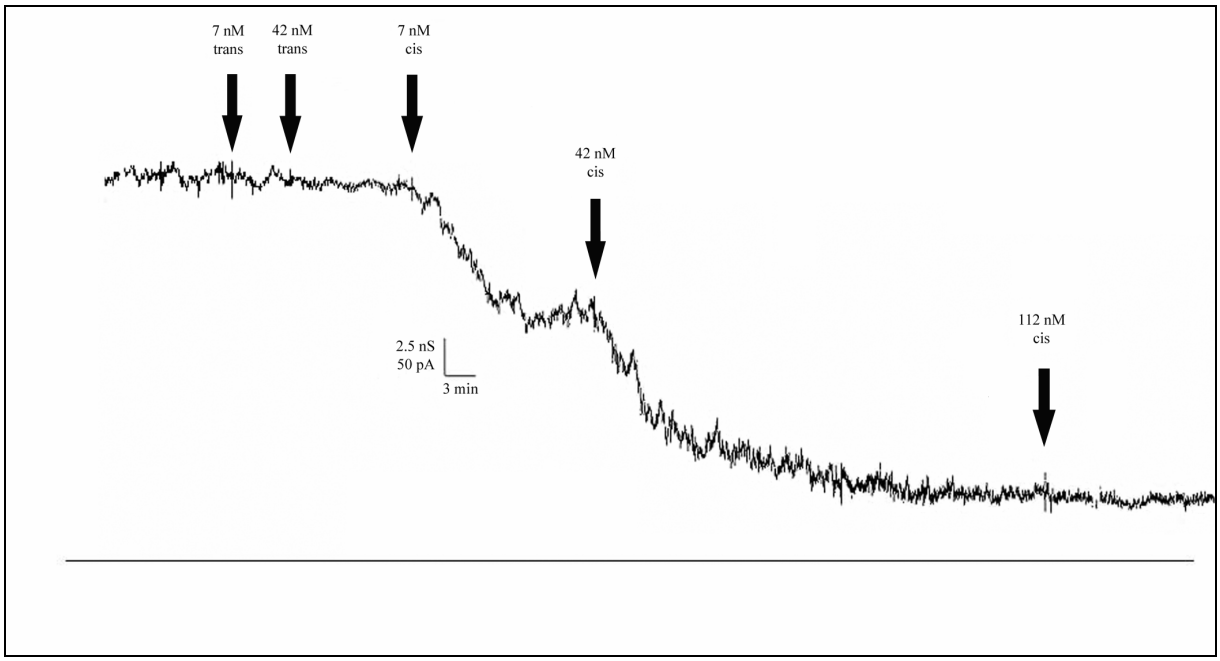


**Figure 3.9: Titration of LamB-mediated conductance with MBP-gpJ.**

LamB was added to the cis-side of a black-diphytanoyl-phosphatidylcholine/n-decane membrane at a concentration of 100 pM. The experiment started when about 150 channels were reconstituted in the membrane. Increasing concentrations of MBP-gpJ were first added to the trans-side (arrows), and then to the cis side of the cell (arrows). The aqueous phase contained 1M KCl and about 10pM LamB. The applied voltage was +20mV at the cis-side; T = 20°C.

We observed that the effect of MBP-gpJ on LamB was asymmetric. If MBP-gpJ protein was added to the trans-side, the decrease of the conductance of LamB was not very significant: between 0 and 15 %. In contrast, MBP-gpJ added to the cis-side caused a strong decrease: the affinity of the fusion protein to LamB is very high as shown at Figure 3.10. In Figure 3.10, a Lineweaver-Burke plot (see equation (5), Material and Methods) of the titration experiment of Figure 3.9 and of similar measurements is shown. The stability constant for MBP-gpJ binding to LamB was about $8 \cdot 10^7$ 1/M. The half saturation constant, $K_s$, is given by the inverse stability constant 1/K. The half saturation constant for MBP-gpJ binding to the LamB channel was about 13 nM. Unfortunately, the fit of the titration data of Figure 3.9 and similar experiments using equation (5) was not satisfactory as the solid line in Figure 3.10 clearly indicated. This could be explained either by the assumption that the LamB channels did not close completely when MBP-gpJ was bound, which is rather unlikely when the results of Figure 3.8A were considered, or by the assumption that some channel flickering was still possible at very high MBP-gpJ concentrations (see Figure 3.8A).

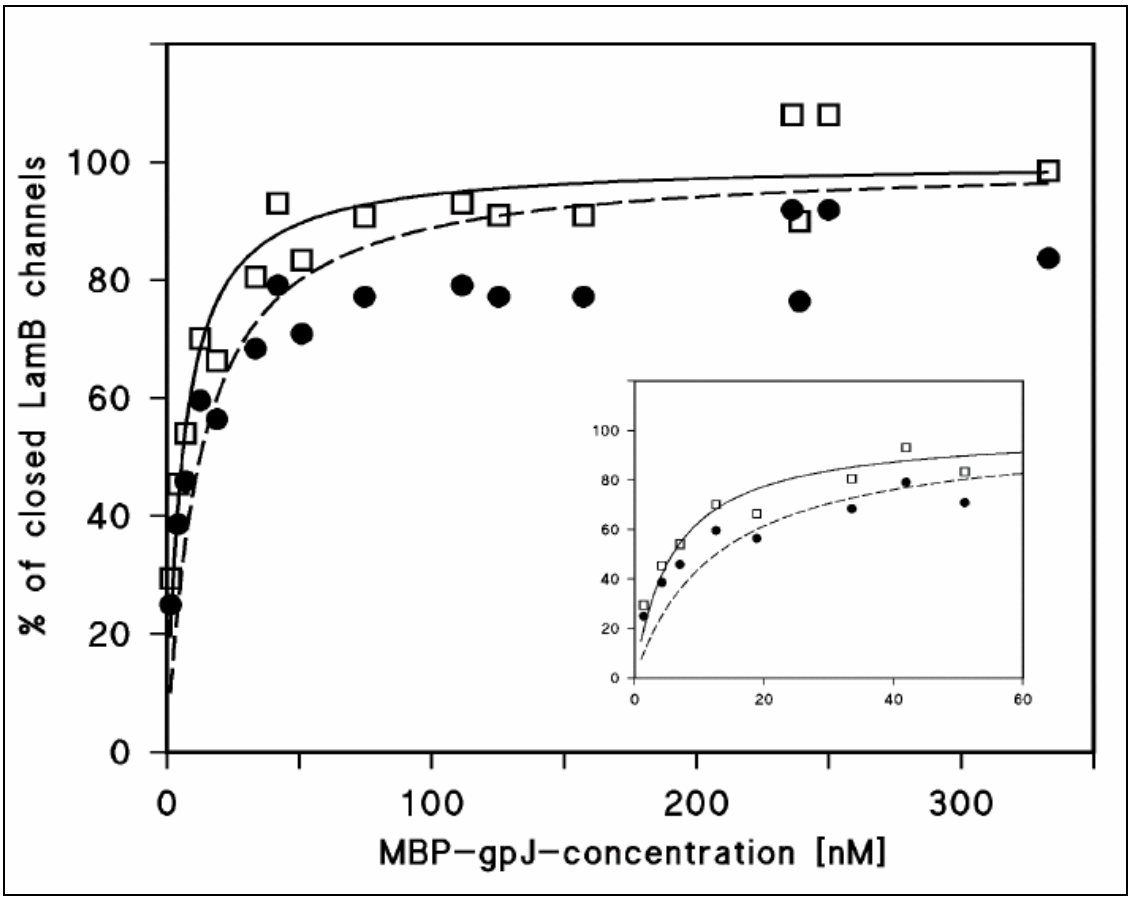


**Figure 3.10: Fit of the results of the titration experiment shown in Figure 3.9 and similar experiments with the equation (5), (full circles; broken line) or with the equation (6) (open squares, solid line).**

The fit of the data using the equation (5, Material and Methods section) yielded the following parameters: Ks = (13±2.5) nM (K = $7.95 \cdot 10^7$ 1/M); that using the equation (6) the following ones: Ks = (5.8±0.07) nM (K = $1.7 \cdot 10^8$ 1/M); $G_\infty$ = 85±3% of $G_{max}$. The inset shows the fits of the data for MBP-gpJ concentration up to 60nM. For further explanations, see text. This figure represents the results of 3 experiments performed in 1M KCl. The applied voltage was 20mV at the cis-side; T = 20°C.

According Figure 3.10, binding of GpJ allows a small but finite conductance. Taking the finite conductance into account gives a correction to equation (5), as described by the equation (6):

$$\frac{G_{\max} - G(c)}{G_{\max} - G_\infty} = \frac{K \cdot c}{K \cdot c + 1} \qquad (6)$$

Where $G_\infty$ is the conductance at very high MBP-gpJ concentration, i.e. the fraction of the conductance that did not seem to be blocked by MBP-gpJ. Accounting for the finite

conductance yielded a stability constant, *K*, of about $1.7 \cdot 10^8$ 1/M ($K_S$ = 5.8 nM) for 3 titration experiments of the same type. On average 85 ± 3% of the total conductance was blocked by MBP-gpJ.

A negative control experiment by adding MBP to both compartments of the cell did not show a significant decrease of the conductance of LamB (see Figure 3.11). We concluded that the decrease of the conductance of LamB was due to the GpJ fragment of the MBP-gpJ fusion protein. In addition we performed a control measurement in 0.1 M KCl, closer to the physiological conditions. The stability constant was in these experiments approximately the same as in 1 M KCl indicating that the ionic strength had a minor influence on the interaction between MBP-gpJ and LamB (data not shown).

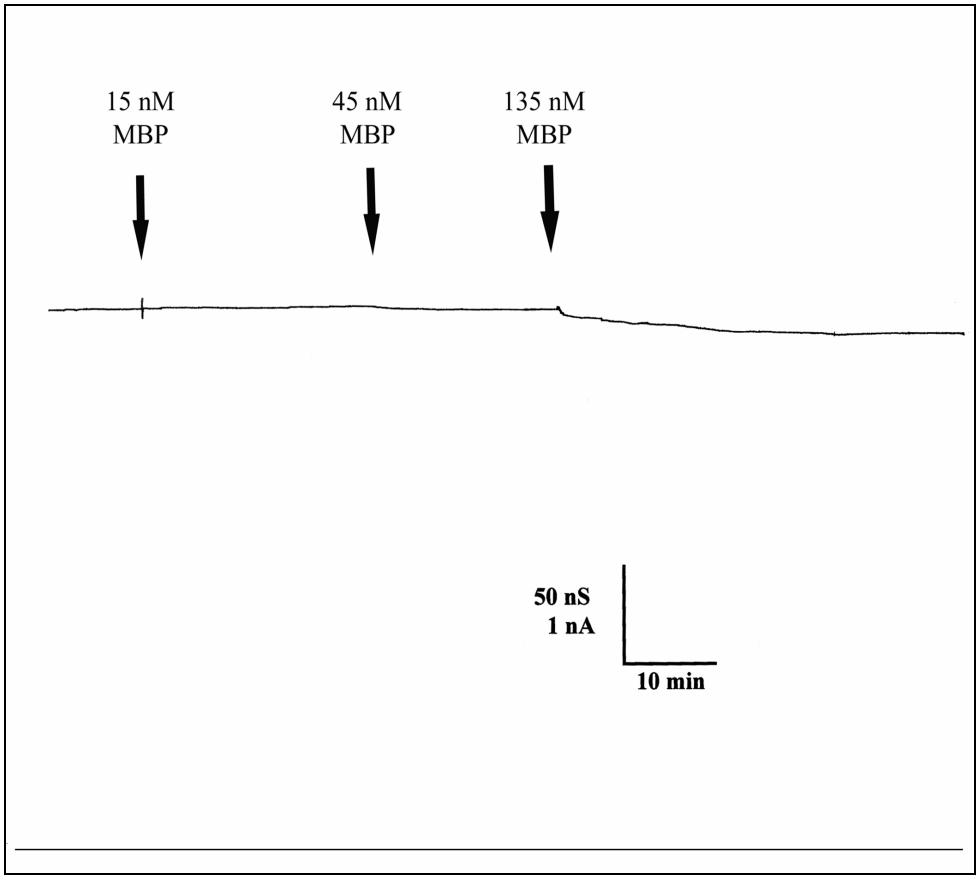


**Figure 3.11: Effect of MBP on LamB-mediated membrane conductance.** LamB was added to the cis-side of a black diphytanoyl-phosphatidylcholine/n-decane membrane in a concentration of $10^{-12}$M. The experiment started when about 2000 channels were reconstituted in the membrane. Increasing concentrations of MBP were added to the cis-side of the membrane (arrows). The aqueous phase contained 1M KCl. The applied voltage was +20mV at the cis-side; T = 20°C. Note that the addition of MBP led to an only insignificant decay of membrane conductance.

In conclusion, the effect of MBP-gpJ on LamB is asymmetric. Because GpJ binds to the outer loops of LamB, MBP-gpJ can be used as a probe of the orientation of LamB inside the membrane (see paragraph 4.1, Discussion). The stability constant K of the interaction between LamB and MBP-gpJ is about $1 \cdot 10^8$ 1/M ($K_S$ about 10 nM), and on average 85 ± 3% of the LamB channels were blocked after addition of MBP-gpJ. Furthermore, the ionic strength has a minor influence on the binding of MBP-gpJ to LamB.

### 3.2.2.2. Effect on LamB with loop deletion Δ4+Δ6+Δ9v

Recent experiments have shown that some residues of LamB mainly located on the outer loops L4, L6 and L9 are involved in the infection mechanism of the bacteriophage Lambda: the deletion of one or more loops prevent the binding of the phage (*Andersen et al., 1999*). In order to investigate if those loops are involved in the binding step of the GpJ protein, measurements were done with a mutant of LamB carrying deletions of the outer loops L4, L6 and some residues of L9 and MBP-gpJ. Unfortunately, channels formed by this mutant were rather unstable, which means that we observed a slow decrease (current drift) following the initial rapid reconstitution of channels. This means that precise information about the stability constant for MBP-gpJ binding to this mutant was not possible. However, we could demonstrate that ion transport through LamB Δ4+Δ6+Δ9v could be blocked by the addition of MBP-gpJ. Figure 3.12 shows the reconstitution of around 100 LamB channels in a membrane. An excess of MBP-gpJ added to the cis-side allowed after a short time a significant decrease of the conductance of around 100 LamB channels inserted in a membrane, as previously shown for LamB WT. This result indicated clearly that the interaction of the MBP-gpJ protein with LamB does not require the presence of the outer loops L4, L6, and a part of L9.

In conclusion, the binding site of GpJ is not located on the outer loops L4, L6 and a part of L9, what are necessary for the binding of the Lambda phage (see paragraph 4.6 of the Discussion).

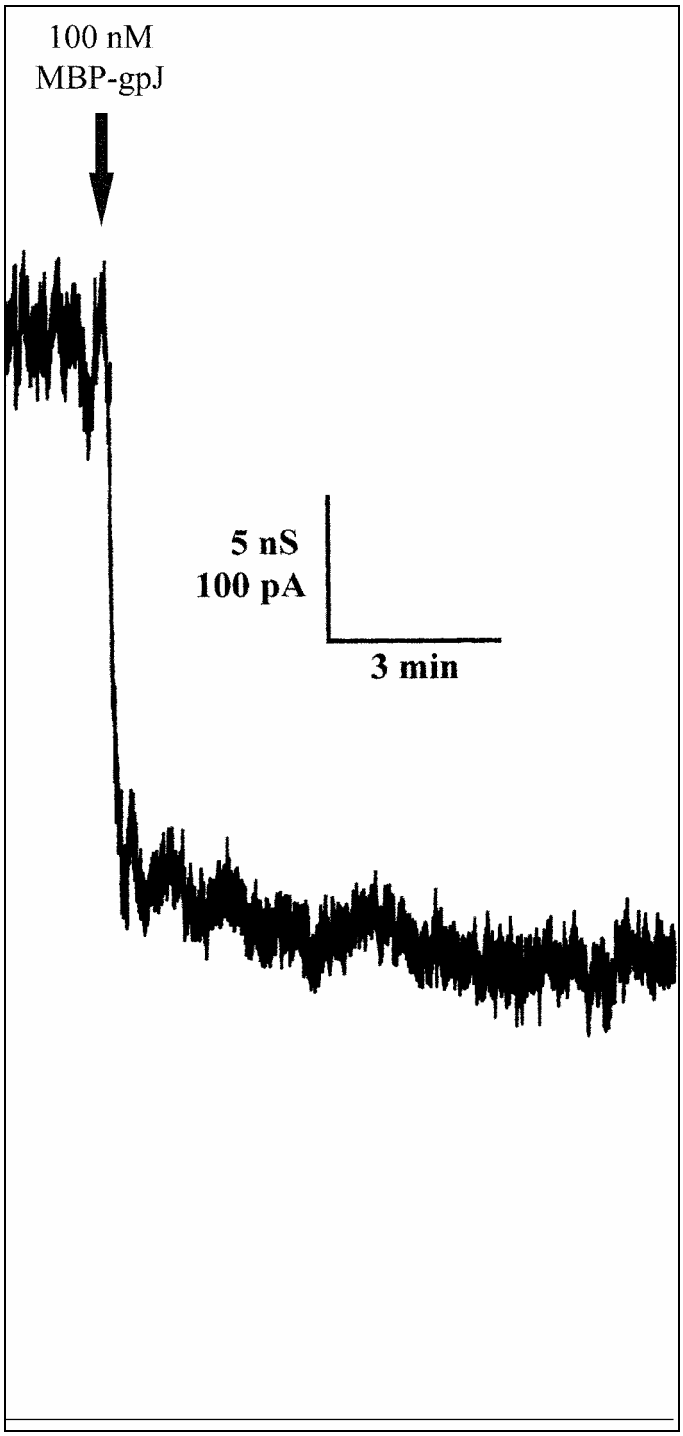


**Figure 3.12.**

**Effect of MBP-gpJ on the conductance mediated by the LamB-mutant Δ4+Δ6+Δ9v.**

The mutant was added to the cis-side of a black diphytanoyl-phosphatidylcholine/n-decane membrane in a concentration of about 10pM. When about 260 channels were reconstituted in the membrane, MBP-gpJ was added to the cis-side of the membrane in a concentration of 100nM (arrow). The aqueous phase contained 1M KCl and about 10pM Δ4+Δ6+Δ9v mutant. The applied voltage was +20mV at the cis-side; T = 20°C.

### 3.2.2.3. Effect of $Mg^{2+}$ ions and EDTA on the complex formation between LamB and MBP-gpJ

Divalent ions, such as $Mg^{2+}$ have a strong influence on the binding step of the infection: the presence of $Mg^{2+}$ is required for the interaction of the bacteriophage Lambda with its cell receptor, LamB (*Schwartz, 1975; 1976*). In order to investigate whether divalent ions are involved in the interaction between LamB and the C-terminal part of the GpJ, we tested the effect of $Mg^{2+}$ ions on the complex between LamB and MBP-gpJ. The experiment was performed in a buffer of 1M KCl supplemented with $Mg^{2+}$ (from 5 to 20mM final concentration) or EDTA (from 5 to 20mM final concentration). EDTA is a divalent ions chelator, known to prevent the formation of the complex between the bacteriophage Lambda and LamB. After reconstitution of a few channels inside the membrane, the reversible blockage of LamB was still observed after addition of MBP-gpJ. In addition, titration experiments of LamB with MBP-gpJ in presence of $Mg^{2+}$ or EDTA were also performed. The stability constant was almost the same as in 1 M KCl (data not shown), indicating that the presence of $Mg^{2+}$ ions is not required for the formation of the complex between LamB and the MBP-gpJ protein. Furthermore, the minor influence of the $Mg^{2+}$ ions is in agreement with the minor influence of the ionic strength on complex formation between LamB and the MBP-gpJ protein (see paragraph 3.2.2.1).

In conclusion, $Mg^{2+}$ ions are not clearly involved in the interaction between LamB and MBP-gpJ *in vitro*, whereas they are necessary for the interaction between LamB and the bacteriophage Lambda *in vivo*.

### 3.2.2.4. Current noise analysis measurement of the complex formation between LamB and MBP-gpJ

Titration experiments previously described (see paragraph 3.2.2.1) allowed to obtain the stability constant K of the interaction between LamB and MBP-gpJ. In order to investigate the kinetic constants of the on and off reaction rates of the interaction between LamB and MBP-gpJ, we performed noise analysis measurements of the complex between those two proteins. The ion current noise was analysed as described previously (*Nekolla et al., 1994*). The background spectrum was recorded before the first addition of MBP-gpJ. Around 150 channels were inserted in the membrane. Prior to addition of MBP-gpJ the reference spectrum was recorded. The difference spectra could be fitted to single Lorentzian functions (equation 7). Figure 3.13 demonstrates that the corner frequencies of the Lorentzians were small, in the range of 1Hz and less. Unfortunately this value were not precise enough to

calculate the kinetic constants via the on and off reaction rates. However, the increase of noise after addition of the MBP-gpJ protein indicated that the complex between LamB and MBP-gpJ is reversible.

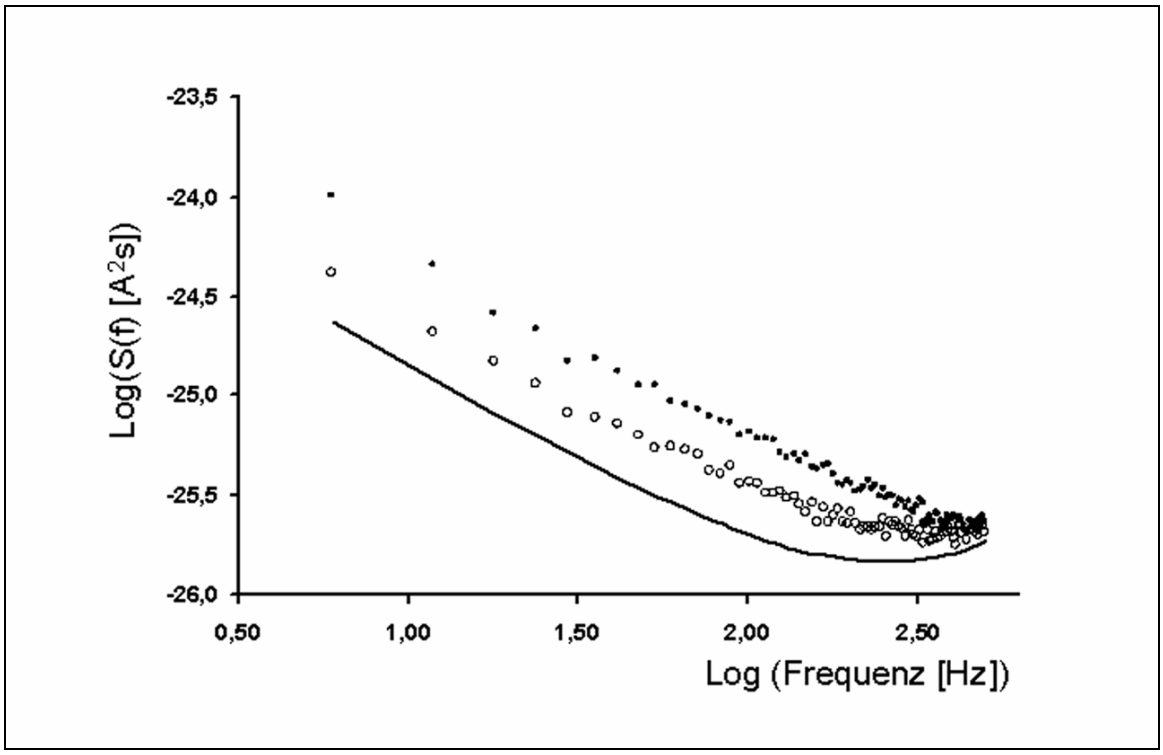


**Figure 3.13. Power density spectra of MBP-gpJ induced current noise of around 150 LamB channels.**
Full line and empty circles show the control (1M KCl). Full circles: the aqueous phase contained 7nM MBP-gpJ.

## 3.2.3. Effect of His-gpJ on the conductance of LamB

The work performed with LamB and MBP-gpJ demonstrated that the GpJ (684-1132) fragment in fusion with MBP allows the reversible and complete blockage of LamB channels reconstituted in planar lipid bilayer. In order to be sure that the blockage of LamB is only due to the GpJ fragment, and that the MBP part is not involved in this process, planar lipid bilayer experiments were performed with LamB and the His-gpJ protein. LamB (final concentration $10^{-14}$M) was added to the cis-side of the cell. Subsequently some channels were inserted inside the membrane. Surprisingly the addition of His-gpJ did not lead to reversible blockage of LamB as observed with MBP-gpJ. The addition of His-gpJ prevented after a short time the insertion of additional LamB channels inside the membrane, and some channel flickering was observed, suggesting an interaction between these two proteins (Figure 3.14). The interaction

of LamB with MBP-gpJ was clear because of a direct proof: MBP-gpJ prevented the passage of ions across LamB. In order to demonstrate that His-gpJ binds to LamB in planar lipid bilayer, we used an indirect proof. At a first we investigated the effect of MBP-gpJ on the complex between LamB and GpJ. In the following we investigated the effect of His-gpJ on the transport of sugar (maltopentaose) through LamB.

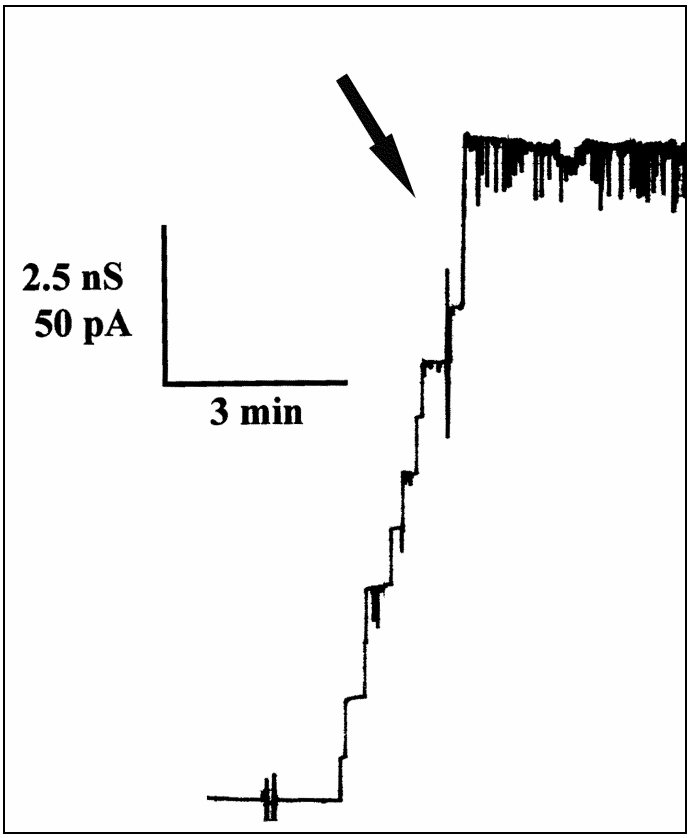


**Figure 3.14. Effect of His-gpJ on the LamB mutant Y118G measured on a single-channel level.**

His-gpJ was added in a concentration of 50µM to the cis-side of a diphytanoyl-phosphatitylcholine/n-decane membrane (arrow) after the reconstitution of nine mutant LamB channels. The aqueous phase contained 1M KCl and about 10pM Y118G mutant. The applied voltage was +20mV at the cis-side; T = 20°C. Note that the reconstitution of further channels stopped shortly after the addition of His-gpJ and that the open channels show rapid flickering.

### 3.2.3.1. Effect of MBP-gpJ on the complex between LamB and His-gpJ

We demonstrated previously that MBP-gpJ protein blocks the LamB channels. In order to demonstrate that His-gpJ binds to LamB, we investigated the binding constant K of the interaction between LamB and MBP-gpJ in presence of His-gpJ. We expected that the binding of His-gpJ to LamB would disturb the interaction between LamB and MBP-gpJ, and

subsequently modify the affinity constant of the complex between these two proteins. This experiment performed in 1M KCl is described in Figure 3.15. LamB (final concentration $10^{-14}$M) was added to the cis-side of the cell, and some channels got inserted inside the membrane. After reaching a stable number of inserted proteins (about 80), His-gpJ was first added to the cis-side: the conductance of the LamB channels is not affected as previously observed. After 40 minutes, increasing amounts of MBP-gpJ were added to the cis-side to allow a titration of LamB as performed paragraph 3.2.2.1. Surprisingly, the addition of MBP-gpJ had only a minor influence on the blockage of the LamB channels, even if the final concentration of MBP-gpJ was efficient to close about 85% of LamB conductance as previously observed in our titration experiments without His-gpJ. We concluded that the binding of the MBP-gpJ protein to LamB was prevented because His-gpJ was already bound to LamB. The increase of the concentration of MBP-gpJ allowed a progressive blockage of the LamB channels, but the blockage effect turned out to be clearly not significant. This result was an indirect proof, confirming the binding of His-gpJ to LamB.

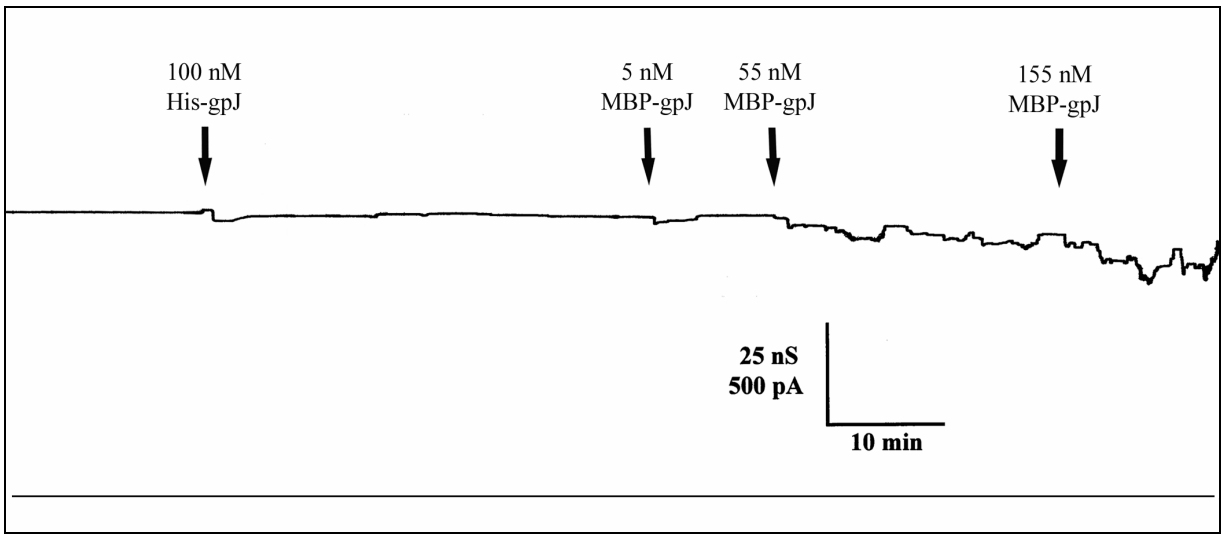


**Figure 3.15: Effect of His-gpJ on the titration of LamB-mediated conductance with MBP-gpJ.**

LamB was added to the cis-side of a black diphytanoyl-phosphatidylcholine/n-decane membrane in a concentration of $10^{-12}$M. The experiment started when about 250 LamB channels were reconstituted in the membrane. First His-gpJ was added to the cis-side of the membrane in a concentration of 100nM while stirring to allow equilibration (left side arrow). About 30 minutes later, MBP-gpJ was added to the cis-side in increasing concentrations from 5nM to 155nM (arrows). The aqueous phase contained 1M KCl. The applied voltage was 20mV at the cis-side; T = 20°C. Note that the addition of His-gpJ prevented the block of the LamB channels by MBP-gpJ.

### 3.2.3.2. Effect of maltopentaose on the complex between LamB and His-gpJ

His-gpJ binds to LamB, and prevents the binding of MBP-gpJ. The binding of the bacteriophage Lambda to LamB disturbs the transport of sugars (*Van Gelder et al., 2000; Berrier et al., 2000*). We investigated the effect of sugar (maltopentaose) on the complex between LamB and His-gpJ in order to investigate whether the binding of His-gpJ disturb the transport of sugar across the LamB channels. LamB (final concentration $10^{-14}$M) was added to the cis-side of the cell, and channels got inserted into the membrane. In this experiment, we performed a titration of LamB by maltopentaose in presence of His-gpJ. After reaching a stable number of inserted proteins, His-gpJ was added to the cis-side. After 30 minutes, increasing amounts of maltopentaose were added only to the cis-side. In an additional experiment, we performed a titration of LamB with maltopentaose without His-gpJ as reference. The stability constant of the complex between LamB and the maltopentaose without His-gpJ was about 770 1/M (Ks = 1.3 mM) whereas the stability constant of the complex between LamB and the maltopentaose in presence of His-gpJ was about 630 1/M (Ks = 1.6 mM, see Table 3.3). The binding constant was not significantly modified. We concluded that the binding of His-gpJ does not affect the transport of sugar across LamB channels.

| Titration of LamB Y118G with maltopentaose | Reference (without His-gpJ) | + 100 nM His-gpJ |
|---|---|---|
| K (1/M) | 770 | 630 |
| $K_s$ (mM) | 1.3 | 1.6 |

**Table 3.3: Investigation of the transport of maltopentaose by LamB Y118G in presence or in absence of the His-gpJ protein.** The experiment is inspirited from the one described by *Benz et al., 1986*. However, addition of substrates was only performed to the cis-side of the membrane because the outer loops of LamB are mainly orientated to this compartment. For the reference, increasing amounts of maltopentaose were added to the cis-side of the membrane after the end of the insertion of channels in the membrane to allow the titration of LamB. For the experiment in presence of His-gpJ, 100nM of His-gpJ were first added to the cis compartment of the cell after the end of the insertion of channels in the membrane, and then the titration with maltopentaose is performed. This table represents the results of 3 independent experiments performed in 1M KCl. The applied voltage was +20mV at the cis-side; T = 20°C.

We concluded that His-gpJ binds to LamB because the affinity of MBP-gpJ to LamB decreased strongly in presence of His-gpJ. Furthermore, His-gpJ has a different behaviour

than MBP-gpJ because His-gpJ does not block the channels of LamB: ions and small compounds like maltopentaose can pass through even if His-gpJ is bound to the outer face of LamB.

## 3.3. Evidence for the complex between LamB and His-gpJ by SDS-PAGE and immunodetection

In addition to the planar lipid bilayer experiments, the interaction between LamB and His-gpJ was investigated on SDS-PAGE and by immunodetection. Similar experiments (dot blot) were already performed with LamB and MBP-gpJ (*Wang et al., 2000*) showing the formation of a complex. When LamB wildtype and His-gpJ were mixed together and loaded on SDS-PAGE, three bands were observed: two corresponding to these two proteins, and a new band with an apparent molecular weight of around 100 kDa (see Figure 3.16A).

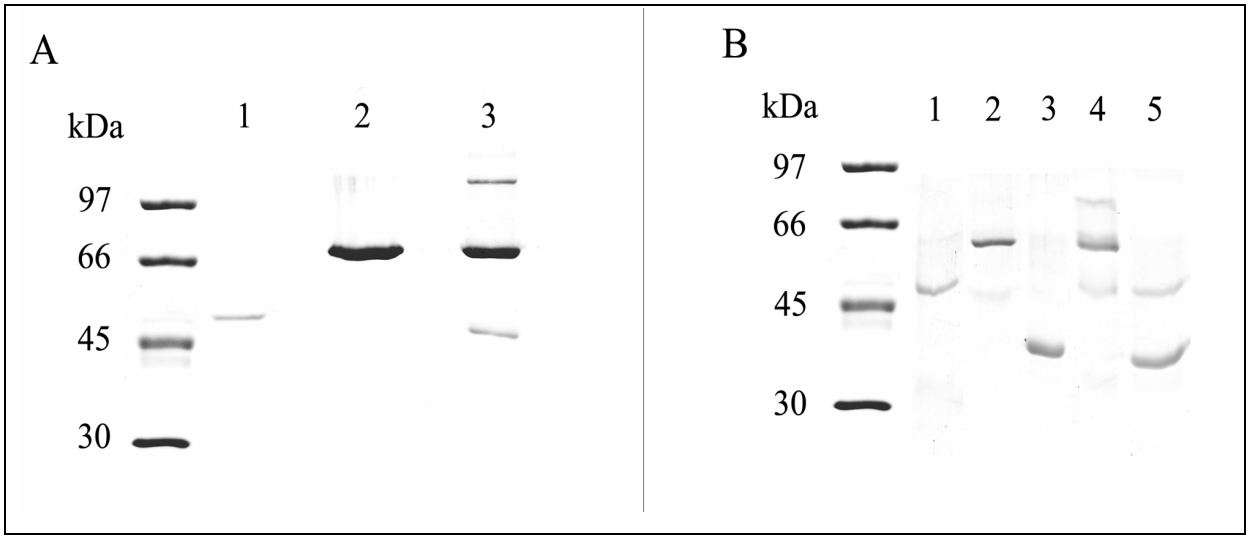


**Figure 3.16. SDS-PAGE 10% acrylamide of complexes between LamB wildtype or mutant Δ4+Δ6+Δ9v and His-gpJ. The gel was stained with Comassie brilliant blue.**

A: Lane 1: 5 µg purified His-gpJ solubilised at 30°C for 5 minutes in 5 µl sample buffer. Lane 2: 5 µg purified LamB wildtype solubilised at 30°C for 5 minutes in 5 µl sample buffer. Lane 3: 5µg purified LamB wildtype + 5 µg purified His-gpJ solubilised at 30°C for 5 minutes in 5 µl sample buffer.

B: Lane 1: 5 µg purified His-gpJ solubilised at 30°C for 5 minutes in 5 µl sample buffer. Lane 2: 3 µg purified LamB Δ4+Δ6+Δ9v solubilised at 30°C for 5 minutes in 5 µl sample buffer. Lane 3: 3 µg purified LamB Δ4+Δ6+Δ9v solubilised at 30°C for 5 minutes in 5 µl sample buffer. Lane 4: 3µg purified LamB Δ4+Δ6+Δ9v + 5 µg purified His-gpJ solubilised at 30°C for 5 minutes in 5 µl sample buffer. Lane 5: 3µg purified LamB Δ4+Δ6+Δ9v + 5 µg purified His-gpJ solubilised at 100°C for 5 minutes in 5 µl sample buffer.

This new band with a higher molecular weight than LamB was also observed by immunodetection against 6×His-tag and disappeared completely when LamB WT and His-gpJ were boiled (see Figure 3.17).

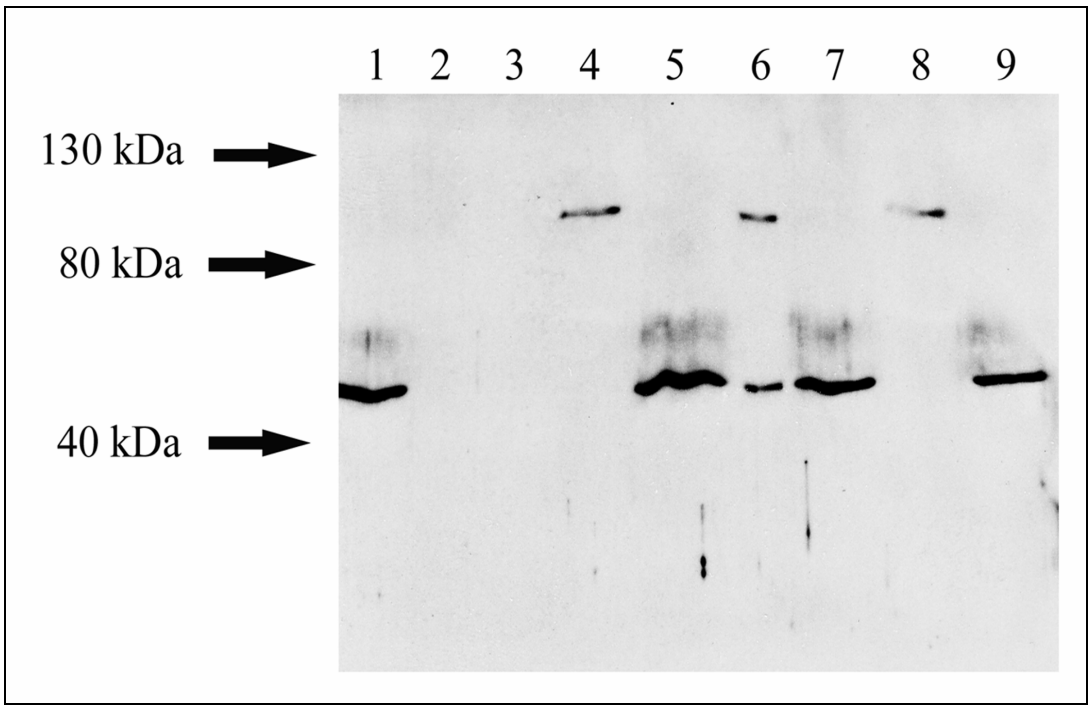


**Figure 3.17. Western blot analysis: Detection complexes between LamB and the LamB mutant Δ4+Δ6+Δ9v with His-gpJ.** The proteins were run on a 10% SDS-PAGE and blotted onto a nitrocellulose. Mouse antibodies against the hexa-histidyl-tag were used in a dilution of 1:3000.
Lane 1: 5 µg purified His-gpJ solubilised at 30°C for 5 minutes in 5 µl sample buffer. Lane 2: 5 µg purified LamB wildtype solubilised at 30°C for 5 minutes in 5 µl sample buffer. Lane 3: 3 µg purified LamB Δ4+Δ6+Δ9v solubilised at 30°C for 5 minutes in 5 µl sample buffer. Lane 4: 5 µg purified LamB wildtype + 5 µg purified His-gpJ solubilised at 30°C for 5 minutes in 5 µl sample buffer. Lane 5: 5 µg purified LamB wildtype + 5 µg purified His-gpJ solubilised at 100°C for 5 minutes in 5 µl sample buffer. Lane 6: 3 µg purified LamB Δ4+Δ6+Δ9v + 5 µg purified His-gpJ solubilised at 30°C for 5 minutes in 5 µl sample buffer. Lane 7: 3 µg purified LamB Δ4+Δ6+Δ9v + 5 µg purified His-gpJ solubilised at 100°C for 5 minutes in 5 µl sample buffer. Lane 8: 5µg purified His-gpJ solubilised at 100°C for 5 minutes in 2.5 µl sample buffer + 5µg purified LamB wildtype solubilised at 30°C for 5 minutes in 2.5 µl sample buffer. Lane 9: 3 µg purified LamB Δ4+Δ6+Δ9v mixed with 5 µg purified His-gpJ solubilised at 100°C for 5 minutes in 5 µl sample buffer.

Furthermore, this band was also found if just the His-gpJ protein was boiled, but not when LamB was boiled, indicating that the trimer structure of LamB is necessary for the formation of the complex (see paragraph 4.6, Discussion). The same experiments were performed with

LamB and LamB Δ4+Δ6+Δ9v mixed with the His-gpJ protein (see Figures 3.16B and 3.17), and LamB from *Shigella sonnei* mixed with His-gpJ (data not shown). In the both cases, a new high molecular band was also observed when these LamB mutants were used.

We concluded that LamB interacts clearly with the GpJ fragment without the MBP part and that the outer loops L4, L6 and a part of L9 are not necessary for the formation of the complex between LamB and the 40% of the C-terminal extremity of the GpJ protein as observed in planar lipid bilayer experiments.

## 3.4. Electron microscopy

The planar lipid bilayer and the immunodetection experiments demonstrated that GpJ interacts with LamB, even if the loop deleted form was used. In contrast, the bacteriophage Lambda can not infect bacteria carrying LamB with loop deletion Δ4+Δ6+Δ9v (*Andersen et al., 1999*). Is the binding of the phage to LamB with loop deletion Δ4+Δ6+Δ9v really impossible, or does it bind and not infect the bacteria? In order to answer this question, cells expressing LamB with loop deletion Δ4+Δ6+Δ9v and bacteriophage Lambda were mixed, and observed by transmission electronic microscopy. No phage could be observed on the surface of the cells, indicating that the binding to the outer membrane cells carrying loop deleted mutants of LamB should not be possible. Control experiments were performed with complexes between Lambda phage and Top10F' or Pop154 cells, carrying LamB WT and LamB from *Shigella sonnei*, respectively. In both cases, phages could be seen on the surface of the outer membrane of the cells. We concluded that the outer loops L4, L6 and L9v of LamB are necessary for the binding of the bacteriophage Lambda whereas the C-terminal fragment of GpJ does not require them to bind to LamB, as demonstrated by our planar lipid bilayer and immunodetection experiments.

# CHAPTER IV - Discussion

## Résumé (Summary)

**- Reconnaissance spécifique de LamB par GpJ: orientation de LamB en BLM.**

MBP-gpJ et His-gpJ intéragissent avec LamB lorsque ces protéines sont ajoutées du côté cis de la membrane lors d'expériences de BLM. Quelle face de LamB est exposée du côté cis de la membrane ? Andersen et collaborateurs ont démontré que dans nos conditions expérimentales, les boucles extracellulaires de LamB sont orientées principalement du côté cis de la membrane (*Andersen et al., 2002*). Par conséquent, les expériences de Film Noir démontrent que MBP-gpJ et His-gpJ se lient à l'extrêmité extracellulaire de LamB.

**- La MBP peut-elle intéragir avec LamB?**

MBP-gpJ intéragit-elle avec LamB par son extrêmité MBP? Les travaux réalisés avec His-gpJ démontrent que le fragment GpJ se lie à LamB. De plus, un contrôle négatif indique que la MBP a une action minime sur les canaux de LamB. Par conséquent, l'effet de MBP-gpJ observé sur LamB est dû à son extrêmité GpJ et non MBP.

**- Intéraction entre LamB et le fragment de GpJ.**

L'intéraction entre LamB et le fragment de GpJ a été étudiée par immunodétection ainsi que par la technique du Film Noir. D'après les expériences de Film Noir, le complexe entre LamB et MBP-gpJ est réversible. Le fait qu'il puisse être observé par immunodétection démontre une forte association entre LamB et MBP-gpJ, suggèrant que la constante de dissociation $k_{off}$ du complexe soit très faible.

**- Possible effet de la MBP sur le fragment de GpJ.**

MBP modifie les propriétés de stabilité du fragment de GpJ : His-gpJ est produite sous forme de corps d'inclusion, tout comme la protéine GpJ entière (*Wang et al., 1998*), alors que MBP-gpJ est produite sous forme soluble et fonctionnelle (*Wang et al., 2000*). De plus, contrairement à MBP-gpJ, le bactériophage Lambda ne bloque pas les canaux de LamB (*Berrier et al., 2000; Van Gelder et al., 2000*). Par conséquent, His-gpJ est un modèle d'étude plus proche du phage Lambda que MBP-gpJ. Le fait que MBP-gpJ puisse bloquer les canaux de LamB en BLM et pas His-gpJ est probablement imputable à la MBP.

**- Les protéines de fusion de GpJ sont-elles oligomérisées?**

Il est établi que GpJ est présente à l'état d'oligomère dans la queue du phage. Toutes les tentatives pour obtenir des informations sur l'état d'oligomérisation de MBP-gpJ et His-gpJ n'ont pas donné de résultat clair et définitif. Les travaux d'immunodétection entre le trimère de LamB et His-gpJ semblent cependant suggérer que la stoechiométrie de la réaction est 1:1.

**- Partie de LamB impliquée dans l'intéraction avec le fragment de GpJ.**

Le fragment de GpJ peut intéragir avec LamB Δ4+Δ6+Δ9v. Il est cependant établi que les principaux résidus de LamB impliqués dans l'intéraction avec le bactériophage Lambda sont localisés sur les boucles extracellulaires L4, L6 et L9. Par conséquent, le fragment de GpJ ne se lie pas à la surface de ces boucles. Si GpJ ne se lie pas sur les boucles extracellulaires, et devrait le faire avec la partie en tonneau de la protéine. En effet, l'intéraction entre LamB et His-gpJ n'est plus possible lorsque la structure trimérique de LamB est altérée. Toutefois, la zone précise d'intéraction reste encore à être déterminée.

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## 4.1. Specific recognition of LamB by GpJ: orientation

MBP-gpJ blocks LamB completely and reversibly in planar lipid bilayer experiments. The effect of MBP-gpJ on LamB is asymmetric and closing of channels was only observed when MBP-gpJ is added to the cis-side of the cell. An interesting point is to know the orientation

after reconstitution inside an artificial membrane in order to understand to which side of LamB MBP-gpJ binds to. Recent investigations of the orientation in planar lipid bilayer experiments were based on the main characteristics of LamB: it is known for many years to be the cell receptor for the bacteriophage Lambda (*Randall-Hazelbauer et Schwartz, 1973*) and to be involved in the transport of maltooligosaccharides across the outer membrane of Gram-negative bacteria (*Benz et al., 1988; Charbit, 2003*). The orientation of LamB could not be demonstrated clearly using this phage: Van Gelder and coworkers described that the LamB outer loops are oriented towards the trans-side (*Van Gelder et al., 2000*) whereas Berrier and coworkers found them to be orientated towards the cis-side of the membrane (*Berrier et al., 2000*).

Previous studies on reconstituted LamB channels revealed in planar lipid bilayer experiments a significant asymmetry in conductance: the single channel conductance of LamB varies of 20 % (*Kullmann et al., 2000*). More interesting was the observation on the asymmetric transport of oligomaltosides through LamB. On a single molecular level, the $k_{on}$ rates were different according to the side where the sugar was added *(Kullman et al., 2002)*. The crystallisation of complexes between LamB and maltooligosaccharides revealed a specific translocation pathway (*Dutzler et al., 1996; Van Gelder et al., 2002*). However, the evidence of the asymmetry of the channel does not allow any conclusion concerning the orientation of the protein itself.

We were particularly attentive to the works performed by Andersen (*Andersen et al., 2002)*. After addition of LamB only to the cis-side and the end of the incorporation of proteins inside the membrane, the pH was lowered on either side of the membrane, the cis- or the trans-side, the response to pH was asymmetric, suggesting preferential orientation of channels and pH-dependent closure. In experiments performed with LamB mutants in which major external loops L4, L6, and a part of L9 were deleted, the closure of the protein was not observed, suggesting that those loops are involved in the closure mechanism. Furthermore, the insertion of LamB in the membrane was asymmetric: between 70 and 80% of the proteins reconstituted in the membrane had their outer loops oriented to the cis-side. Here in our investigation we used the same setup and material than Andersen and coworker (*Andersen et al., 2002*), and our observation of a partial orientation was in agreement with their results: the orientation of LamB in artificial membranes was confirmed by titration with MBP-gpJ yielding blockage after addition of MBP-gpJ to the cis-side. Furthermore the orientation of the outer loops on the cis-side was also confirmed by single molecule studies of LamB with modified

maltohexaose: when the reducing extremity of maltohexaose was modified, the transport of sugar is prevented when it is added to the cis-side *(Danelon et al., 2003)*.

In agreement with the works of Andersen, Berrier and Danelon (*Andersen et al., 2002, Berrier et al., 2000; Danelon et al., 2003*), we concluded that LamB is mainly inserted with its outer loops oriented towards the cis-side (side of protein addition) after reconstitution in planar lipid bilayer experiments. In consequence, the blockage effect observed after addition of MBP-gpJ to LamB reconstituted in membrane is due to an interaction with its extracellular side.

## 4.2. Does the MBP protein interact with LamB?

The interaction between MBP-gpJ and the extracellular side of LamB was clearly demonstrated. An important and necessary control for this conclusion was to show that the effect of the MBP-gpJ fusion protein on LamB was due to the gpJ fragment, and not to the MBP part. The Maltose Binding Protein was used as a tag for the expression and the purification of the C-terminal end of the GpJ protein, and should not be involved in the interaction with LamB. *In vivo*, MBP is a protein which belongs to a large complex involved in the transport of maltose and oligomaltosides from the outer medium to the cytoplasm of the bacteria (*Boos et Schuman, 1998*). Sugar (maltose and maltooligosaccharides) passed through the outer membrane using Maltoporin, and bound to the MBP protein in the periplasmic space. The binding of the MBP protein to LamB was investigated *in vivo* (*Brass et al., 1985*), and by electronic microscopy on the surface of *E. coli* cells using an anti-MBP antibody (*Wang et al., 2000*). In both cases, no interaction was observed, denying an evidence of a binding of the MBP protein to LamB. In planar lipid bilayer experiments, some authors observed that addition of MBP in the trans-side allowed a significant decrease of the conductance of LamB (*Neuhaus et al., 1984*) whereas some others did not see any effect (*Benz et al., 1986*). In order to elucidate this conflicting observation, we tested the effect of the Maltose Binding Protein to LamB in planar lipid bilayer experiments: as in earlier measurement performed by Benz (*Benz et al., 1986*), we observed no significant decrease of the conductance of LamB in presence of MBP. In addition to this control experiment, our results with the His-gpJ protein demonstrated clearly an interaction of the GpJ fragment with LamB without the MBP part. Consequently we concluded that the effect observed after addition of MBP-gpJ protein is not due to the binding of the MBP protein to LamB but really due to the GpJ part.

## 4.3. Interaction of LamB with GpJ

The planar lipid bilayer results obtained with MBP-gpJ and His-gpJ confirmed that the C-terminal domain of GpJ binds to LamB. What type of interaction is there between LamB and the GpJ fragment? MBP-gpJ completely and reversibly blocks the LamB channels, indicating the formation of a reversible complex. However, the kinetic constants of association and dissociation of this complex, respectively called $k_{on}$ and $k_{off}$ could not be obtained.

The immunodetection experiments revealed that the complex between His-gpJ to LamB remained stable after around two hours migration time under an electric field and in presence of 0,1% SDS. The complex between MBP-gpJ and LamB is so stable that it can be observed in immunodetection experiments, as previously described by Wang and coworkers (*Wang et al., 2000*). In order to be in agreement with all the experimental results, we can conclude that the interaction between LamB and the C-terminal fragment of GpJ is reversible. However, the observation of the complex in immunodetection experiments is possible because the association constant $k_{on}$ is credibly very high whereas the dissociation constant $k_{off}$ is very low.

Another interesting observation was that $Mg^{2+}$ ions had a low effect on the complex between the fusion proteins carrying GpJ and LamB although they are necessary for the binding of the Lambda phage (*Schwartz, 1975; Schwartz, 1976*). This indicates that $Mg^{2+}$ ions are lowly involved in the interaction between the C-terminal fragment of GpJ and LamB in *in vitro* experiments.

## 4.4. Possible effect of the MBP fused to the GpJ fragment

Some differences were observed between MBP-gpJ and His-gpJ. In the planar lipid bilayer experiments, the presence of His-gpJ did not show any significant effect on the conductance of LamB whereas the addition of MBP-gpJ allowed its reversible and complete closure. The binding of the entire Lambda phage, also investigated in planar lipid bilayer experiments, did not modify the conductance of LamB (*Berrier et al., 2000; Van Gelder et al., 2000*). This suggests that the behaviour of the His-gpJ protein is closer to the entire Lambda phage than MBP-gpJ. Furthermore, MBP-gpJ was expressed as soluble protein (*Wang et al., 2000*) whereas His-gpJ was overexpressed in inclusion bodies. The GpJ protein was recently cloned and purified, and is produced as insoluble form in inclusion bodies (*Wang et al., 1998*). The gpJ protein seemed to have a behaviour closer to His-gpJ than MBP-gpJ. The differences of

both GpJ fusion proteins can only be explained by the presence of the Maltose Binding Protein. MBP (about 45kDa) is almost as larg as the gpJ fragment (about 45kDa), and has an effect on the gpJ fragment concerning stability and blockage of LamB.

We concluded that MP-gpJ and His-gpJ both bind to LamB. However, MBP-gpJ is a chimeric protein which properties are different compared to gpJ alone. His-gpJ seemed to be a better study model because its behaviour is in agreement to the behaviour of the bacteriophage Lambda and gpJ described in the litterature.

## 4.5. Possible oligomerisation of the fusion proteins carrying the GpJ fragment?

MBP-gpJ blocked completely a trimer of LamB in planar lipid bilayer experiments. Interestingly the binding occured also for the loop deletion mutant LamB Δ4+Δ6+Δ9v. This suggested that the closing of Maltoporin was not due to a collapse of the outer loops, but presumably to the binding effect of MBP-gpJ. Was the blockage of the trimer due to a monomer or a multimer of MBP-gpJ? On one hand, nature suggests a possible oligomerisation of the GpJ protein: in the extremity of the Lambda phage. Although its exact state is not very well known, it seems to be a dimer or a trimer (*Roa et Scandella, 1976*). On the other hand, MBP is not oligomerised (*Boos et al., 1998*). Our fusion proteins carry only 40% of the C-terminal extremity of the GpJ protein. We tried to investigate the oligomerisation state of the MBP-gpJ protein with different techniques, like techniques such as Small Angle Neutron Scattering, Analytical Centrifugation and Native Gel Electrophoresis. Unfortunately, in all cases, it was not possible to obtain any data for the complex formation between LamB and MBP-gpJ or the oligomerisation step of the MBP-gpJ protein since the fusion proteins tended to precipitate.

Secondary structure data could be obtained with His-gpJ: CD spectra measurements showed different pattern depending on the His-gpJ concentration. Does this result suggest a dissociation of the complex by dilution? This is however not a significant proof for an oligomerisation. Furthermore the immunodetection experiments showed a complex between LamB and His-gpJ with an apparent molecular weight close to 100kDa. SDS-PAGE experiments showed that the apparent molecular weight of LamB alone is close to 66kDa whereas the His-gpJ's one is close to 45kDa, suggesting that monomeric His-gpJ interacts with LamB.

Although the oligomerisation state of GpJ is not well known, some phage proteins that do bind to the surface of the host cell have been studied: pb5 and Gp5. Pb5, a protein of the tail of bacteriophage T5, binds to FhuA, its cell receptor: in this case, a monomer of pb5 interacts with PhuA, which is also a monomer (*Plancon et al., 2002*). Gp5 is a protein of the extremity of the tail of the bacteriophage T4, involved in the first steps of its infection mechanism, its structure has been solved by crystallography (*Kanamaru et al., 2002*) and indicates that this protein is organized as a trimer.

We concluded that the oligomerisation state of the fusion proteins carrying the GpJ fragment could not be clearly analysed. However, the immunodetection experiments suggested that the stoichiometry of the interaction between the LamB trimer and His-gpJ could be 1:1.

## 4.6. Recognition site of LamB involved in the binding with GpJ

The part of the LamB protein involved in the binding of the bacteriophage Lambda was investigated for many years by mutagenesis (*Hofnung et al., 1976; Charbit et al., 1984*). Mutants with variable infection efficiency have been isolated (see Table 1.2). Point mutations on the LamB gene that prevent the bacteriophage Lambda adsorption are located on the outer loops L1, L4, L6 and L9 (see Table 4.1).

| Outer loop of LamB | Residue(s) involved in the binding of λh$^+$ | Residue(s) involved in the binding of λh |
|---|---|---|
| L1 | (18) | |
| L4 | 146, **148**, **151**, **152**, 154, 155, **163**, 164 | **151** |
| L6 | 245, 247, 249, 250, 259 | **245**, **247** |
| L9 | 382, 386, 387, 389 | |

***Table 4.2:*** *Position of different residues of the LamB protein involved in the binding to the Lambda phage. In red: residue which mutation is sufficient to prevent completely the binding of the phage. The residue 18 is in parentheses because it affects the stability of the LamB trimer and may thus have long-range effects. Hoffnung et al. 1976 ; Clément et al., 1983; Charbit et al., 1984; Gehring et al., 1987 ; Charbit et al., 1988; Dargent et al., 1988; Charbit et al., 1994; Werts et al., 1994.*

Two classes of mutants have been described: the Class I and the Class II mutants. Class I mutants block the growth of Lambda wild-type host range (λh$^+$). The Lambda phage (λh phages) can adapt after mutation to the Class I LamB proteins and infect again the cells. Mutagenesis allowed the production of Class II LamB proteins, that block the infection of the

λh phage. Lambda phage can once again adapt after mutation to the Class II LamB, and recover its infection activity. This type of mutants is called λhh$^{*}$. The mutations on LamB allowing the Class II phenotype are located on the outer loops L4 and L6 (see Table 4.1).

The work performed with Class I and Class II mutants allowed the identification of binding areas of different importance on the surface of LamB. If mutations on L4, L5 and L6 prevent the adsorption of all the types of phage, mutations on L9 prevent only the adsorption of the phage λh$^{+}$ (*Andersen et al., 1999*). The mutation of only one amino acid between the residues 148, 151, 152 and 163 for the Class I and between the residues 148, 151, 245 and 247 for Class II is sufficient to prevent the binding of the Lambda phage. Furthermore mutations of residues 148, 245 and 247 the phage can bind again to LamB after mutations on residues of the C-terminal extremity of the GpJ protein (*Werts et al., 1994*). Those residues located on the outer loops L4 and L6 are important and directly involved in the binding step of the entire Lambda phage.

For many years the binding site has been investigated by structure predictions of LamB (*Gehring et al., 1987*). Since the structure of LamB has been solved at 3.1Å resolution by X-ray crystallography (*Schirmer et al., 1995)*, it was possible to identify more accurately an area involved in the binding site by molecular modelisation using the mutagenesis data. This area covers a large part of the outer side of the LamB protein, and those large and flexible loops L4, L6 and L9 are easily accessible to an interaction with the Lambda phage. Our works performed with His-gpJ demonstrated that the C-terminal part of GpJ bound strongly to LamB, even if the outer loops L4, L6 and a part of L9, described to be important for the binding of the entire phage (*Andersen et al., 1999*), are deleted. Why has the C-terminal end of GpJ the ability to bind to LamB Δ4+Δ6+Δ9v whereas the entire Lambda phage has not? Presumably because GpJ is involved in a large, complex and voluminous structure in the extremity of the tail of the phage (*Hendrix et al., 1983*) whereas our work was only performed with a fusion protein carrying a fragment of around 50 kDa of GpJ. This means that the amino acids located on the loops L4, L6 and L9v are not necessary for the binding of the C-terminal part of the GpJ protein alone.

Our results demonstrate that the binding area on the surface of LamB remains barely known and has to be more clearly identified. In order to identify a possible new area involved in the binding of the C-terminal extremity of the GpJ protein, our immunodetection results demonstrated the formation of the complex between LamB and GpJ could be prevented when LamB was previously boiled. A monomer of LamB represents a water-filled barrel with

eighteen antiparallel β-strands and nine large and flexible outer loops. The trimeric structure of LamB is necessary for the formation of the complex between LamB and GpJ and suggests that the investigated area involved in the binding is not only located on the outer and flexible loops of LamB, but also surely somewhere on the surface of its barrel structure. The binding site of LamB is then surely not exclusively located in the known area covering the outer loops L4, L6 and a part of L9.

# CHAPTER V - Conclusion

## Conclusion et perspectives

Le but des travaux est l'étude de l'intéraction entre le bactériophage Lambda et LamB, son récepteur cellulaire. Etant donné que le phage Lambda se lie à LamB en utilisant le domaine C-terminal de GpJ, une protéine de sa queue, l'intéraction entre LamB et le domaine C-terminal (résidue 684 à 1132) de GpJ exprimé sous forme de protéine de fusion a constitué notre modèle d'étude. Les deux protéines de fusion utilisées s'appellent respectivement MBP-gpJ et His-gpJ. MBP-gpJ contient le domaine C-terminal de GpJ en fusion avec la Maltose Binding Protéine. His-gpJ est l'extrêmité C-terminale de GpJ en fusion avec un 6×Histidine-tag. Ce fragment intéragit avec LamB puisque MBP-gpJ et His-gpJ se lient toutes les deux à la partie exoplasmique de LamB sauvage ou de ses mutants (LamB de *Shigella sonnei*, LamB Y118G et LamB Δ4+Δ6+Δ9v) comme démontré lors des expériences de Film Noir ou d'immunodétection.

La liaison entre GpJ et LamB est de type réversible. La force ionique a peu d'effet sur ce complexe. Les constantes cinétiques d'association et de dissociation $k_{on}$ et $k_{off}$ n'ont pas pu être déterminées, mais la valeur du $k_{on}$ doit être élevée et celle du $k_{off}$ doit être très faible, puisque le complexe est observable par immunodétection.

Une différence majeure a été observée entre MBP-gpJ et His-gpJ. MBP-gpJ peut bloquer les canaux de LamB tandis que His-gpJ ne le peut pas:

- La liaison entre LamB et MBP-gpJ se traduit par un blocage complet et réversible des canaux de LamB sauvage lors d'expérience de BLM. K, la constante d'association de ce complexe entre MBP-gpJ et LamB sauvage, est estimée à environ $1\times10^8$ 1/M ($K_s$ environ 10nM) dans 1M KCl. En moyenne 85% des canaux de LamB peuvent être fermés par MBP-gpJ.

- His-gpJ n'induit pas de blocage visible des canaux de LamB: le transport d'ions et d'maltooligosaccharides n'est quasiment pas perturbé en présence de His-gpJ. Toutefois, l'intéraction entre His-gpJ et la face exoplasmique de LamB de LamB est forte et peut être démontrée de manière indirecte en empêchant la liaison de MBP-gpJ.

Etant donné que le bactériophage Lambda ne bloque pas les canaux de LamB (*Van Gelder et al., 2000 ; Berrier et al., 2000*), His-gpJ semble avoir un comportement plus proche de celui du phage Lambda que MBP-gpJ. En effet, le fragment de GpJ sans MBP ne bloque pas les canaux de LamB. La différence de comportement entre MBP-gpJ et His-gpJ est donc vraisemblablement imputable à la MBP. Afin de vérifier cette hypothèse, l'étude de l'intéraction entre la protéine GpJ entière et LamB par la Technique du Film Noir sera indispensable dans le futur. GpJ a en effet été clonée et partiellement purifiée (*Wang et al., 1998*).

Une preuve directe de la formation du complexe entre LamB et GpJ peut être observée par SDS-PAGE et par immunodétection: l'intéraction est très forte, et n'a pu être rompue que lorsque la structure 3D du trimère de LamB a été altérée.La surface de LamB impliquée dans l'intéraction avec GpJ demeure peu connue: les boucles L4, L6 et L9v de LamB ne sont pas impliquées dans la liaison avec GpJ alors qu'elles sont indispensables à l'intéraction avec le bactériophage Lambda. De plus, la liaison de GpJ avec LamB dépend de la structure en trimère du récepteur cellulaire. Afin de mieux appréhender cette région de LamB impliquée dans l'intéraction avec GpJ, des travaux à l'aide de mutants de LamB doivent être réalisés, les mutations se situant préférentiellement sur des résidus de la face extracellulaire proches de la structure en tonneau de la protéine.

# Conclusion and outlook

The aim of the work was the study of the binding of the bacteriophage Lambda to LamB, its cell receptor. Because the Lambda phage binds to LamB using GpJ, a protein of its tail, the study model was the interaction between LamB and the C-terminal extremity (residue 684 to 1132) of GpJ co-expressed as a fusion protein. Here we used two fusion constructs called MBP-gpJ and His-gpJ respectively. MBP-gpJ contains the C-terminal end of GpJ in fusion with the Maltose Binding Protein. His-gpJ is the C-terminal end of GpJ in fusion with a 6×Histidine-tag. This fragment interacts with LamB because MBP-gpJ and His-gpJ both bind both to the exoplasmic side of LamB wildtype or variants (LamB from *Shigella sonnei*, LamB Y118G and LamB Δ4+Δ6+Δ9v) as described in the planar lipid bilayer and the immunodetection experiments.

The interaction between GpJ and LamB is reversible. The effect of the ionic strength on this complex is very low. The kinetic association and dissociation constants, called $k_{on}$ and $k_{off}$ respectively, could not be obtained, but the $k_{on}$ value should be high and the $k_{off}$ value should be low, because the complex is observable by immunodetection.

A major difference was observed between MBP-gpJ and His-gpJ. MBP-gpJ can block the LamB channels whereas His-gpJ can not:

- MBP-gpJ allowed clearly a complete and reversible blockage of the channels of LamB wildtype. The binding constant K of the complex between LamB wildtype and MBP-gpJ was about $1\times10^8$ 1/M ($K_s$ about 10nM) in 1M KCl. About 85% of the channels could be closed and the ionic strength had no influence on the interaction.
- In contrast to MBP-gpJ, His-gpJ did not show a visible blockage of LamB in bilayer experiments. Furthermore it did not disturb the oligomaltosides transport through LamB channels. However, the binding of His-gpJ to the exoplasmic side of LamB could be demonstrated by an indirect proof: if His-gpJ is first added to LamB, the addition of MBP-gpJ did not result in a significant decrease of conductance.

The GpJ fragment expressed without MBP does not block the LamB channels anymore. Furthermore the addition of bacteriophage Lambda did not show any blockage of LamB channels in planar lipid bilayer experiments (*Van Gelder et al., 2000 ; Berrier et al., 2000*). The difference between MBP-gpJ and His-gpJ on the blockage of the LamB channels is obviously the MBP, which modifies a little the properties of GpJ. In order to validate this

hypothesis, the study of the interaction between the entire GpJ protein and LamB in planar lipid bilayer experiments has to be performed. Indeed, GpJ was cloned and partially purified (*Wang et al., 1998*).

A direct proof for the complex between His-gpJ and LamB wildtype was observed on SDS-PAGE and immunodetection: the binding was very strong, and the interaction was only prevented if the 3D structure of the LamB trimer was altered. The area of LamB involved in the interaction with the gpJ protein is not well known: the outer loops L4, L6 and L9v of LamB are not involved in the interaction with the GpJ fragment although they are important for the binding of the Lambda phage. Furthermore, the binding of the gpJ fragment to LamB can be prevented after denaturation of LamB, indicating the importance of its 3D structure. In order to investigate this “binding area”, the interaction between MBP-gpJ or His-gpJ and LamB mutants has to be performed. In agreement with the immunodetection results, the mutations have to be done on residues located on the surface of the barrel structure of LamB.

# CHAPTER VI - Appendix

## 6.1. Referencies

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## 6.2. Regular publications

**Berkane, E.**, F. Orlik, A. Charbit, C. Danelon, D. Fournier, R. Benz, and M. Winterhalter. Nanopores: Maltoporin Channel as a Sensor for Maltodextrin and Lambda-Phage.
Article submitted to Journal of Nanobiotechnology, in process.

**Berkane, E.**, F. Orlik, J.F. Stegmeier, A. Charbit, M. Winterhalter and R. Benz.
*In vitro* Study of the Interaction between gpJ, a Protein of the Tail of the Bacteriophage Lambda, and Maltoporin (LamB), its Cell Surface Receptor.
Article submitted

Neves, P., **E. Berkane**, P. Gameiro, M. Winterhalter and B. de Castro.
Interaction between Quinolinones Antibiotics and Bacterial Outer Membrane Porin OmpF.
*Biophys. Chem.* (2005) **113(2)**: 123-8.