Unraveling the killing mechanism of complement's Membrane Attack Complex

Writing assignment about methods to study the Membrane Attack Complex in cell membranes of Gram-negative bacteria

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ABSTRACT

The host's innate immune system forms the first line of defense against invading microbes. Of major importance in innate immunity is the complement system which consists of plasma proteins to eliminate potential pathogens. Assembly of complement's Membrane Attack Complex (MAC) lyses bacteria through pore-formation in the bacterial membrane. Despite the fact that the MAC has been extensively studied, the exact killing mechanism of Gram-negative bacteria remains uninvestigated. This writing assignment focuses on approaches and methods to study killing mechanisms of other pore-forming complexes and to distinguish between outer and inner membrane disruption of Gram-negative bacteria. Several approaches are highlighted which may be useful to unravel the exact killing mechanisms of the MAC on Gram-negative bacteria.

INTRODUCTION

In everyday life, we are constantly exposed to (mostly harmless) microorganisms [1]. Infectious microbes are recognized by the host's immune system and only when the immune system fails to clear it, a microbe can become pathogenic. Immune cells and plasma proteins try to eliminate the invading pathogen before it can cause disease. Essential factors of the innate immune system are components of the complement system which immediately target invading microbes and hereby induce a cellular response by the recruitment and activation of phagocytes which can kill the bacteria (e.g. neutrophils, monocytes and macrophages) [2]. Furthermore, activation of the complement system results in direct killing of the microbe.

The complement system consists of roughly 30 plasma proteins that can either trigger phagocytosis of the pathogen or formation of the Membrane Attack Complex (MAC). The MAC is a pore-forming complex, inserted into the bacterial membrane with subsequent cell lysis [3]. It is formed after activation of the complement system which occurs via three major pathways. Pathogen associated molecular patterns (PAMPs) are recognized by recognition molecules of the classical, lectin or alternative pathway. In each case activation results in the formation of C3 convertases [4,5]. C3 convertases (C3bBb or C4b2a) cleave C3 to C3a and C3b with subsequent C5 convertase formation. C5 converstases (C3bBbC3b or C4b2aC3b) cleave C5 to C5a and C5b after which C5b is the trigger for formation of the MAC (C5b-C9) [3]. Activation products C3a and C5a are anaphylatoxins with proinflammatory and chemotactic functions [6–8]. A schematic overview of the MAC formation is depicted in figure 1 [9].

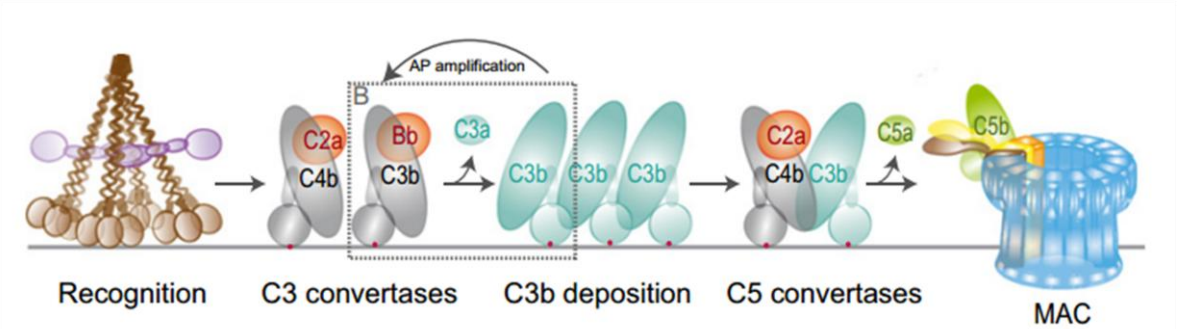

Figure 1. Schematic overview of the complement activation cascade. The bacterial surface is recognized by recognition molecules of the classical and lectin pathway, which results in formation of C3 convertases by cleavage of C4 and C2 to C4b2a. C3 convertases cleave C3 to C3a (anaphylatoxic) and C3b, which induces formation of another C3 convertase, C3bBb (alternative pathway). Activation of the AP results in amplification of the proteolytic cleavage cascade and eventually C3b molecules will bind C3 convertases to form C4b2aC3b or C3bBbC3b. These complexes now gain C5 convertase activity. C5 convertases cleave C5 to C5a and C5b, where C5b triggers formation of the Membrane Attack Complex (MAC) .

The MAC is of major importance in the first line of defense against invading bacteria. Bacterial cell wall characteristics distinguish Gram-positive and Gram-negative bacteria, of which Gram-negative bacteria are major targets of the MAC. The cell wall of Gram-negative

bacteria consists of an outer membrane (OM) with phospholipids and lipopolysaccharides (LPS), an inner membrane (IM) and a thin peptidoglycan layer (PG) in between [10]. Complement is activated by LPS on the bacterial surface, however also other surface characteristics can be complement stimulators [11,12].

Up to now, lots of papers have been published in which different aspects of the MAC were unraveled stepwise. Studies on phospholipid membranes, inner membrane vesicles and erythrocytes elucidated the following about MAC formation. When C5b is formed by C5 convertases, it binds C6 to form stable C5b-6 [13]. This bimolecular complex engages C7 which exposes a membrane binding site enabling the trimolecular complex to anchor into the membrane without macro pore-formation (Fig. 2) [14,15].

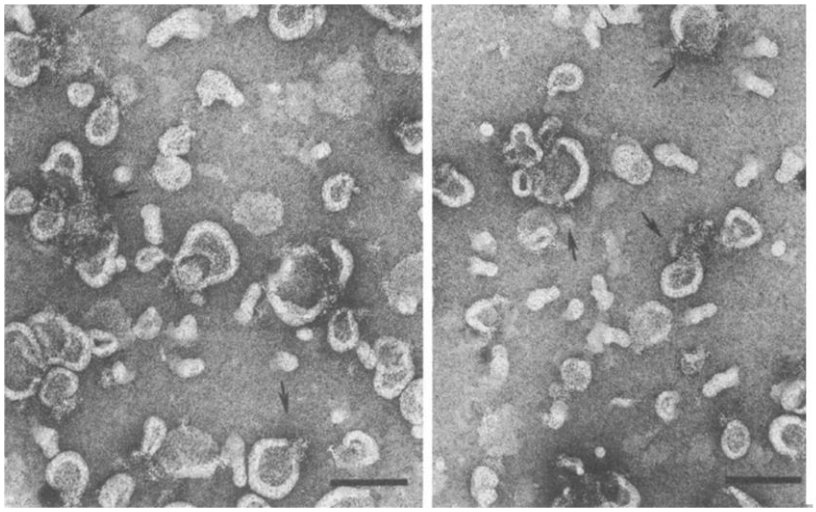

Figure 2. C7 binding to phospholipid vesicles. Preissner et al. published in 1985 that C7 is able to dimerize and polymerize and to subsequently insert into lipid bilayers. Neither C5b or C6 show these properties, which suggests that C7 is responsible for anchoring the trimolecular complex into the membrane . Arrows point at insertions of dimeric C7 into lipid bilayers resulting in disruption of the vesicles. Scale bars: 100 nm.

C8 is build up as a trimer (C8α, β and γ) where C8-β engages C5b-7. C5b is of major importance in this binding process [16]. C5b-8 has hemolytic activity, however is not yet bactericidal [17]. C9 binds to the C8-α/γ site to initiate autopolymerization of further C9 monomers [18]. Binding of one C9 molecule to C5b-8 ($(C5b\text{-}8)_1C9_1$) does not result in bactericidal activity of the complex. Bhakdi et al. (1987) state that multiple C9 molecules (at least 4) are required to make the complex ($(C5b\text{-}8)_1C9_4$) capable to kill bacteria [19]. It is suggested that in nature 12-18 C9 molecules are present in the MAC, which makes the complex about 100 Å wide and 160 Å high [20,21]. It was proposed that C5b-8 is important for penetration and destabilization of the OM, allowing C9 to form pores in the IM [22]. Wang et al. published that C9 is converted in the periplasm from protoxin to a cytotoxin, independent of the way it enters the periplasm. Electron microscopic images of poly-C9 complexes are depicted in figure 3 [23].

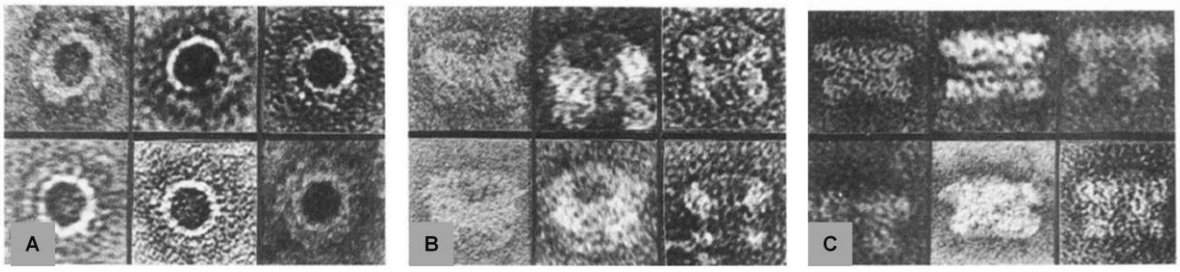

Figure 3. Electron microscopy of poly-C9 on a carbon film with magnifications of 585,000 times as published by DiScipio et al. A) Poly-C9 from top view looking down the channel (90 ±10 Å wide). B)Three quarters orientation of poly-C9 and C) Side view of poly-C9. The height of the complex is expected to be ± 150 Å .

More recently, it has been elucidated that specific domains of C6 to C9 may be responsible for binding interactions and pore-formation, referred to as membrane attack complex/perforin

(MACPF) domains. The MACPF domains of MAC subunits were shown to be similar to the ones of other pore-forming complexes e.g. perforin (a human cytolytic protein) and cholesterol-dependent cytolysins (secreted by Gram-positive bacteria). It is expected that these pore-forming complexes have common mechanisms of trans-membrane helix formation [22,24,25]. Hadders et al. (2007) published a hypothetical model of a complex consisting of multiple C8α monomers (Fig. 4). The hypothetical structure of this complex appears to be highly similar to the pore-forming structure of poly-C9, which may explain the hemolytic properties of C8 without C9 [22].

As mentioned, the majority of studies that research the MAC were performed on erythrocytes or artificial membranes. The ones that were performed on living bacteria generally measure cell viability. Though, Bhakdi et al. (1987) also measured OM integrity of *E. coli* cells by measuring β-lactamase activity [19].

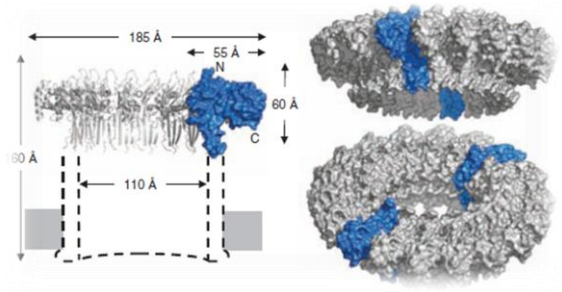

Figure 4. Hypothetical model of a pore-forming complex formed by poly-C8α MACPF domains. Left: cross section of a ring of 18 C8α monomers, in which the MACPF domains form the ring structure. Right: two orientations of the ring structure formed by the MACPF domains. This hypothetical model shows much similarity to the main characteristics of a poly-C9 pore (depicted in figure 2).

Despite the fact that much is known about the MAC, the exact mechanism of membrane disruption in Gram-negative bacteria remains unexplained. The questions that still need to be investigated are 1. Which MAC subunits are present in the bacterial OM and/or the IM? and 2. Which membrane is disrupted by MAC subunits or combinations of subunits? Studies propose that C5b-8 lyses the OM (given the fact that it is capable to lyse erythrocytes) which enables C9 to perforate the IM. However, it is not clear how C9 molecules would be translocated through the periplasm to the IM. Moreover, it may be possible that also the C5b-8 complex is translocated to the IM. Eventually, two questions remain: 3. How many C9 molecules are present in the MAC inside the bacterial membrane and 4. Where and when is the MAC formed? To answer these questions we need new approaches to study the MAC, in which it is of major importance to use living bacteria instead of artificial membranes or erythrocytes.

Studies that have focused on other complexes capable to disrupt cell membranes of Gram-negative bacteria e.g. antimicrobials, bacterial secretion systems or phages show presence of structures and measure membrane disruption via numerous methods. Furthermore, several methods have been investigated to distinguish between the IM and OM of Gram-negative bacteria. Comparable methods may be useful to study the MAC.

This writing assignment provides a clear overview of how to study complexes inside the membrane of Gram-negative bacteria and may be useful to design a project to unravel the exact killing mechanisms of complement's MAC.

1. Which MAC subunits are present in the bacterial OM and/or the IM?

To investigate whether the bacterial OM or the IM is targeted and/or disrupted by specific combinations of MAC subunits, three major groups of methods are distinguished. Methods can a) monitor membrane binding of a molecule of interest, b) measure integrity of the membrane or c) correlate peptide binding with membrane integrity [26]. These three approaches to measure interaction mechanisms of a complex with the bacterial membrane will be discussed extensively in this writing assignment.

Numerous methods to visualize protein complexes or label membranes of living bacteria use fluorescence. Fluorescence microscopy is based on molecular absorption of light after which the molecule reaches an excited state. Emission of fluorescent light occurs when (some) energy is released again [27]. Re-radiated of light occurs at a lower energy level. Each fluorescent probe has its own absorption and emission spectrum and can be measured by flowcytometry or different types of microscopes which can provide real-time observations of living cells. The labeling of protein complexes allows us to effectively localize them in living bacteria. In addition, membrane labeling may be useful for co-localization studies with labeled complexes. Unfortunately, membrane disruption may not always be detected as the probes typically only give an overall measure of the membrane [28]. Several methods to label membranes of Gram-negative bacteria or subunits of the MAC are discussed below. Moreover, assays to measure membrane disruption are discussed.

(a) Monitor membrane binding of a molecule of interest

Labeling the bacterial membrane

As explained earlier, the membrane of Gram-negative bacteria consists of an OM with LPS, an inner membrane and a periplasmic space each of which may be targets for labeling. Small molecules can be labeled to LPS, surface peptidoglycans, surface proteins or peptidoglycan in the periplasmic space. The structural aspects of Gram-negative membranes are depicted in figure 5 together with a few labeling methods [10,29,30].

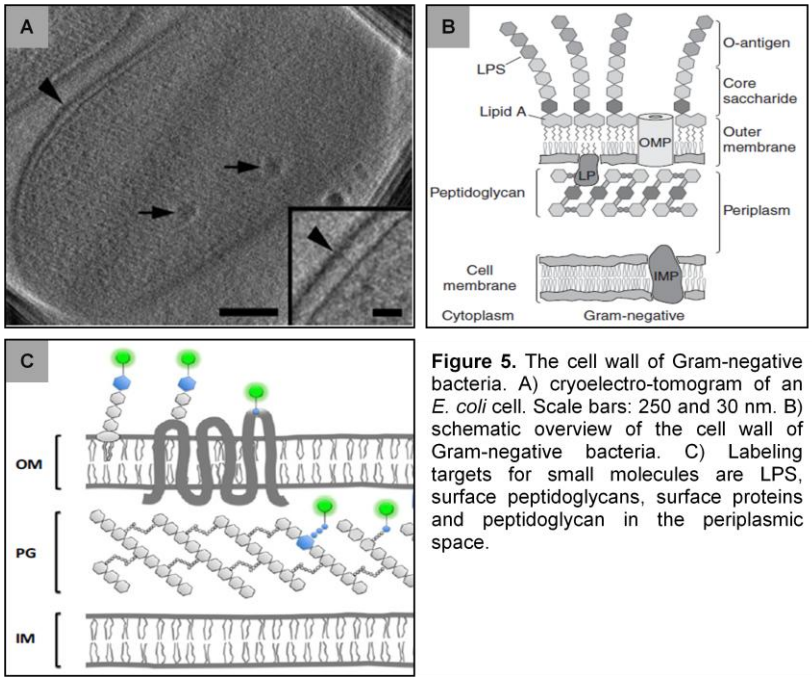

Figure 5. The cell wall of Gram-negative bacteria. A) cryoelectro-tomogram of an *E. coli* cell. Scale bars: 250 and 30 nm. B) schematic overview of the cell wall of Gram-negative bacteria. C) Labeling targets for small molecules are LPS, surface peptidoglycans, surface proteins and peptidoglycan in the periplasmic space.

A promising method to label membranes of Gram-negative bacteria is Metabolic Glycan Labeling (MGL). MGL labels LPS on the bacterial OM and enables tagging with a probe of interest. To realize this, a monosaccharide with a 'bio-orthogonal chemical reporter' (a target for tagging and visualization) is incorporated into glycans of LPS. There is no need for genetically modified bacteria, however, the modified LPS needs to be metabolically assimilated by cellular biosynthetic machinery of the bacterium itself. Subsequently, the orthogonal chemical reporter can be used for tagging and visualization by chemical ligation with a label. DuMont et al. (2012) have identified an essential component of LPS to label the OM of Gram-negative bacteria; 3-deoxy-D-

mannose-octulosonic acid (KDO). KDO is present in the inner core of LPS and is a major target for labeling (Fig. 6A). Cell penetration of modified KDO is efficient to (partly) replace natural KDO of LPS on the OM, given that free KDO is present during an intermediate step of LPS formation. Another advantage of this method is that metabolically active bacteria are marked very easily since modified LPS will only be assimilated by viable bacteria. So after all, fluorescence will be concentrated on the surface of living bacteria (Fig. 6B). Previous to these findings, L-Fucose of the O-antigen used to be labeled (Fig. 6A), however limitations were that this antigen is not conserved among Gram-negative bacteria [31].

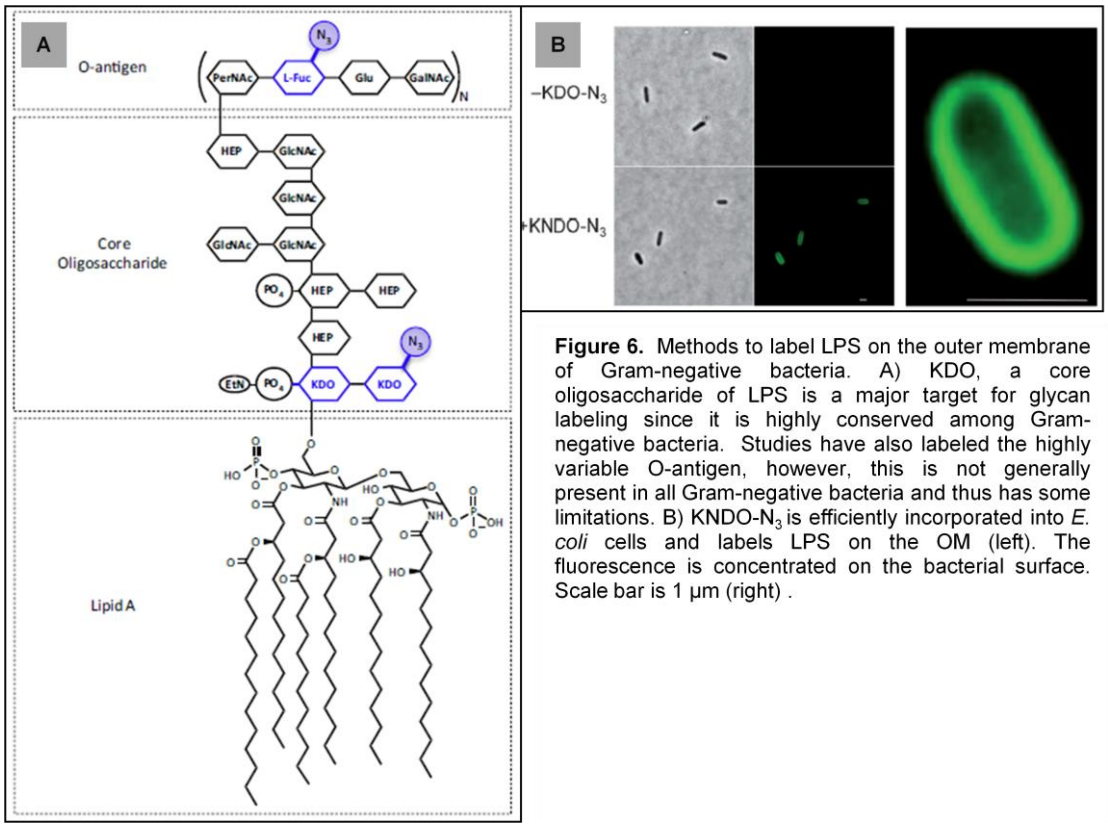

Figure 6. Methods to label LPS on the outer membrane of Gram-negative bacteria. A) KDO, a core oligosaccharide of LPS is a major target for glycan labeling since it is highly conserved among Gram-negative bacteria. Studies have also labeled the highly variable O-antigen, however, this is not generally present in all Gram-negative bacteria and thus has some limitations. B) KNDO-N_3 is efficiently incorporated into *E. coli* cells and labels LPS on the OM (left). The fluorescence is concentrated on the bacterial surface. Scale bar is 1 µm (right) .

A few other strategies have been developed to label membranes of Gram-negative bacteria among which the labeling of surface proteins with Fuc1A. Fuc1A can be ligated to a chemical probe through click-chemistry which allows identification (Fig. 7) [32]. Furthermore, surface proteins can be labeled through incorporation of noncanonical amino acids which can be ligated after expression on the surface. Finally, Gram-negative bacteria have the ability to take up a modified version of L-alanyl-g-D-glutamyl-L-lysine which is naturally present in the turnover of peptidoglycan. Hereby the peptidoglycan layer can be labeled [29].

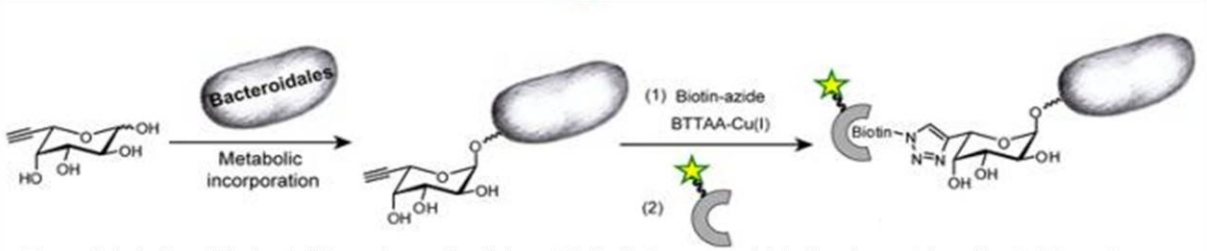

Figure 7. Labeling of the bacterial membrane. Fuc1A is metabolically incorporated into the glycoproteins of bacterial membranes and subsequently the tagged sugar is ligated to an azide labeled probe which allows identification. (published by Besanceney-Webler et al., 2011).

Labeling the MAC subunits

To localize MAC subunits inside the bacterial membrane, individual subunits can be labeled. Among pore-forming complexes, antimicrobial peptides (AMPs) are extensively studied. These approaches to visualize complexes inside the bacterial membrane may give new insights to study the MAC. Both binding of AMPs to the membrane and their antimicrobial effects have been studied, but unfortunately also in this research area exact killing mechanisms often remain unclear. Despite the fact that lots of studies have been performed on artificial membranes, there are some methods to study living bacteria. A few methods to study peptides on/in the bacterial membrane are summed up below.

Subunits of the MAC may bind to the OM of the bacterial cell wall without disrupting it. A very easy method to study the correlation of peptide binding and cell viability is to use fluorescently labeled peptides and cell-impermeable dyes like trypan blue (TB). Peptide binding and cell viability can be measured by flowcytometry or confocal microscopy. Figure 8A depicts an example of an *E. coli* cell, incubated with a fluorescently labeled AMP [33]. TB could be used to simultaneously study cell viability, since it stains dead cells blue.

As an alternative, (fluorescently) labeled antibodies can be used to localize MAC subunits. Immunoelectron microscopy is a promising method to visualize structures on/in the bacterial membrane. In a study of Podda et al. (2006), *E. coli* cells were incubated with an AMP with cytoplasmic targets (so no membrane permeabilization effects) and fixed afterwards. Sections were incubated with an antibody specific against the AMP and eventually with a gold-IgG complex. Analysis of these sections with transmission electron microscopy (TEM, explained later) shows very clear spots at the location of the AMPs (Fig. 8B) [34]. Moreover, Leptihn et al. (2009) visualized an AMP by combining high-resolution imaging, single molecule observations *in vivo* and functional assays. Bacteria were treated with nanogold-labeled S1 and fixed afterwards. Again, TEM was used to observe treated bacteria and to localize the nanogold labeled S1. This assay clearly shows presence of the AMP of interest in the bacterial membrane and can simultaneously distinguish the IM and OM by the high resolution of TEM (Fig. 8C) [35].

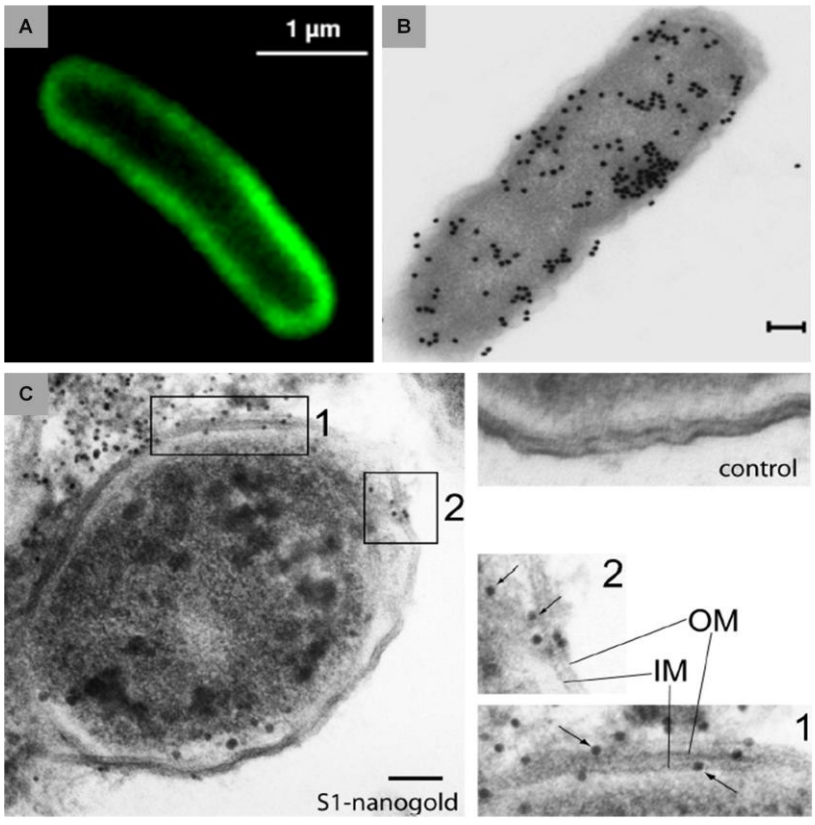

Figure 8. *E. coli* cells incubated with AMPs. A) Incubation of *E. coli* cell with an AMP labeled to BODIPY (fluorescent), imaged by confocal microscopy. Addition of trypan blue (TB) would give indication of cell viability. Scale bar: 1 µm . B) *E. coli* cell incubated with an AMP, a specific antibody and eventually with a gold-IgG complex, imaged by TEM. Scale bar: 0.1 µm. C) *E. coli* cell incubated with nanogold labeled AMP, fixed and visualized by TEM. Scale bar: 100 nm, right panel shows 2x magnification .

The assays described above can be useful to determine if C5b-8 targets the OM and stays there, or if this complex is translocated to the IM together with C9 in the process of pore-formation. Furthermore, it can be elucidated if C9 is present in the IM only or also in the OM. If these labeling methods could be combined with bacterial membrane labeling or it would be possible to label each individual subunit separately, we might be able to perform co-localization studies.

2. Which membrane is disrupted by MAC subunits or combinations of subunits?

(b) Measure integrity of the bacterial membranes

Quite an old method (1984) to study membrane disruption is to use the probe 1-N-phenylnaphthylamine (NPN) [36]. Given the shift in fluorescence only after membrane permeabilization, NPN is a useful tool to study disruption upon addition of pore-forming complexes. The shift in fluorescence is due to a changed environment of NPN from hydrophilic to hydrophobic. One drawback is that fluorescence may also increase upon environmental changes, but this can be corrected for with Triton X-100. Addition of Triton X-100 to the cells increases fluorescence of free NPN only, which indirectly provides information about cell-bound NPN [37]. NPN can partition into the outer, and probably also the inner membrane so it will not be useful to distinguish between OM and IM disruption. It is uncharged, lipophilic and is, upon disruption, inserted deeply into the lipid bilayers of Gram-negative bacteria. Fluorescence of NPN can be measured with an LS-50 fluorimeter [38,39].

A very promising method to study membrane disruption and distinguish between the bacterial OM and IM is a tetraphenylphosphonium (TTP) assay in combination with potassium (K+) measurements. According to Yasuda et al. (2002), an increased uptake of the TPP ion can be monitored from cells with a disrupted OM using a TPP sensitive electrode. LPS on the OM normally prevents TPP from entering the cell and only upon OM disruption TPP is able to cross the OM where it can easily pass the (intact) IM and accumulate inside the cell. A huge advantage is that IM disruption measurements can be performed in the same assay. IM disruption can be measured with a potassium electrode since K+ is only able to leave the cell upon IM disruption. So by combining these assays, OM disruption is measured by TPP accumulation inside the cells and only if the IM is also disrupted, K+ will be released from the cells. Eventually also TPP will be released again. The principle of these methods is depicted in figure 9 [40]. The TPP/K+ assay is a highly promising approach to study the MAC, given the ability to distinguish between OM and IM disruption.

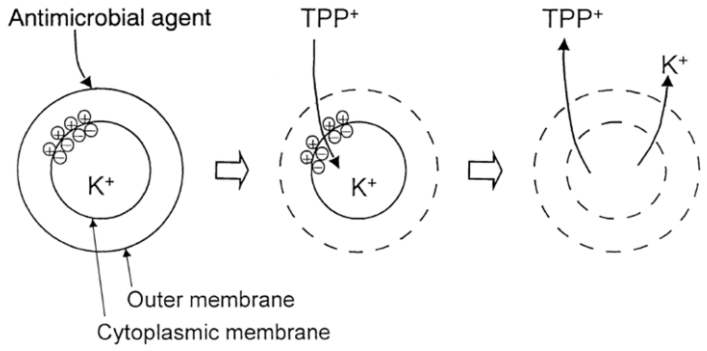

Figure 9. Principle of membrane permeabilization studies based on membrane permeability for TPP and K+ as published by Yasudu et al. TPP is able to enter the cell when the outer membrane is disrupted. Only upon inner membrane disruption, also K+ is released from the cell.

One more method that may be promising to visualize membrane disruption in Gram-negative bacteria upon addition of MAC subunits is Fluorescence Resonance Energy Transfer (FRET). FRET is used to elucidate interaction between two proteins of interest by labeling one protein with a donor and another protein with an acceptor fluorophore. Interaction between the labeled subunits results in transfer of energy between the donor and acceptor

fluorophore, with subsequent emission of fluorescent light. Obviously, FRET could be helpful to study interaction between MAC subunits, however, it may also be useful to study membrane disruption.

In recent publications, Tat (a secretion system that is present on the IM of Gram-negative bacteria) was labeled with Alexa 532 (a donor fluorophore). Labeling of protein (complexes) specific for the IM *in vivo* would allow us to study OM disruption since a protein labeled with an acceptor fluorophore (e.g. mCherry) that will only be able to reach the IM upon OM disruption, will become fluorescent only if the OM is disrupted by MAC subunits. Unfortunately, the mentioned Tat labeling with Alexa 532 was performed on inner membrane vesicles (IMV) and not on living bacteria [41]. Though, promising is that Tat of living *E. coli* cells has been labeled *in vivo* with other fluorescent tags like green/cyan/yellow fluorescent fusion proteins [42].

A major problem of *in vivo* FRET assays is to specifically label the protein of interest. There are several methods to label proteins in living bacteria. One of the possibilities is cysteine or lysine labeling. Moreover, the C-terminus or N-terminus can be labeled with a fusion protein which can subsequently be labeled with a variety of fluorescent proteins [43]. As mentioned, labeling with fluorescent probes can be very unspecific. This is why the expression of 'green fluorescent protein (GFP) fusions' is commonly used in living bacteria. CFP (cyan fluorescent protein) and YFP (yellow fluorescent protein) are among the most used acceptor/donor pair. The fusion protein should obviously not interfere with the function of the target protein [44].

To study OM disruption by MAC subunits, a donor GFP fusion protein should be expressed on the outside of the IM or in the periplasmic space. However, proper expression of the fusion protein has some limitations. The two main export systems from the bacterial cytoplasm to the periplasmic space are the general secretory pathway (Sec) and the twin-arganine translocation pathway (Tat, discussed earlier). If the fusion protein is secreted by the Sec pathway (whereby GFP is folded in the periplasmic space) GFP was shown to be present in its non-fluorescent state [45]. In contrary, Tat can export folded proteins from the cytoplasm to the periplasmic space which yields presence of active GFP proteins between the IM and the OM. The Tat secretion system would thus be more favorable to transport a fusion protein with a donor GFP towards the periplasmic space. An alternative could be the super folder variant of GFP (sfGFP), which has been shown to be active also upon Sec mediated secretion [46]. In our case, the fusion protein should contain an active donor fluorophore that is not yet fluorescent but becomes fluorescent in contact with an acceptor fluorophore. Different types of microscopy (described later) can be used to localize the fluorescent proteins.

Taken together, in the FRET assays mentioned above fluorescence will be detected only when subunits of the MAC are able to disrupt the OM. OM disruption could allow a protein labeled with an acceptor to enter the periplasmic space where the fusion protein with a donor fluorophore is present (Fig. 10). Combination of FRET assays with an intracellular staining for IM disruption would clarify if only the OM, or both OM and IM are disrupted [47]. FRET assays can provide a time-dependent estimation of what happens to the inner and outer membrane of Gram-negative bacteria upon addition of different combinations of MAC subunits e.g. C5b-7, C5b-8, C5b-9 or individual subunits.

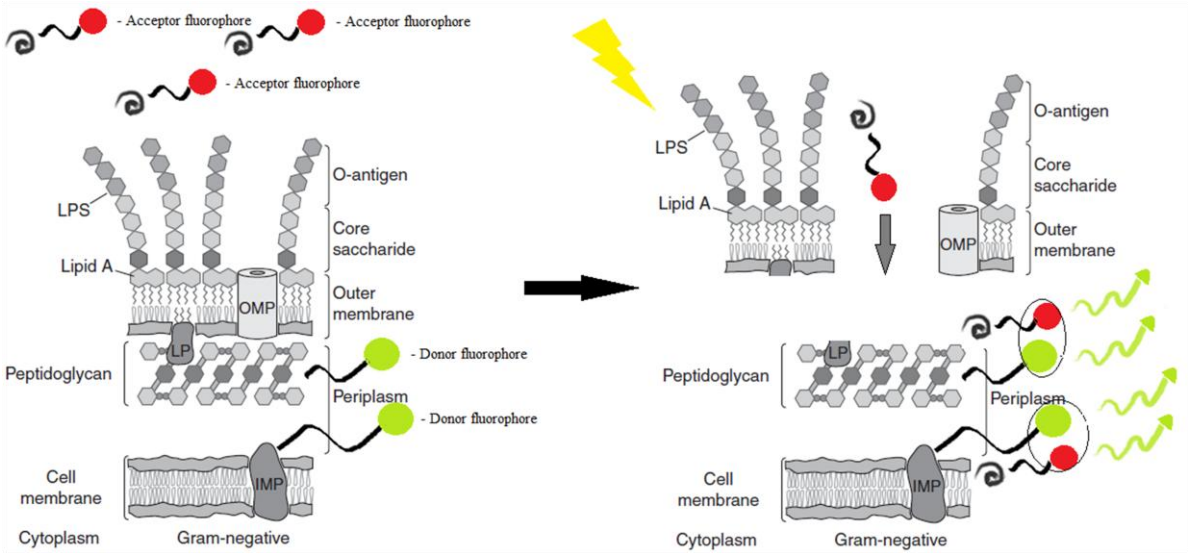

Figure 10. Principle of Fluorescence Resonance Energy Transfer (FRET). Either an Inner Membrane Protein (IMP) or a periplasmic protein is labeled with a donor fluorophore. The acceptor fluorophore is labeled to a protein which is not able to pass the OM. Only upon membrane disruption, the acceptor and donor fluorophore come close enough together to transfer energy which results in the emission of light.

Quite an easy, but maybe less specific approach to study OM and IM disruption has been published by Berney et al., 2007. The LIVE/DEAD BacLight Kit was developed to stain either live or dead bacterial cells but it turned out that this kit is also functional to distinguish between OM and IM disruption of Gram-negative bacteria. The bacterial OM (partly) forms a barrier for the green fluorescent protein SYTO9, which is able to cross the IM. Upon OM disruption, SYTO9 enters the cell more easily, resulting in a more homogeneous cluster of SYTO9 positive cells. When this staining is combined with a propidium iodide (PI) staining which is only able to cross the IM when it is disrupted, we are able to investigate OM an IM disruption separately [48]. SYTO9 and PI intensity can be measured with flowcytometry (Fig. 11).

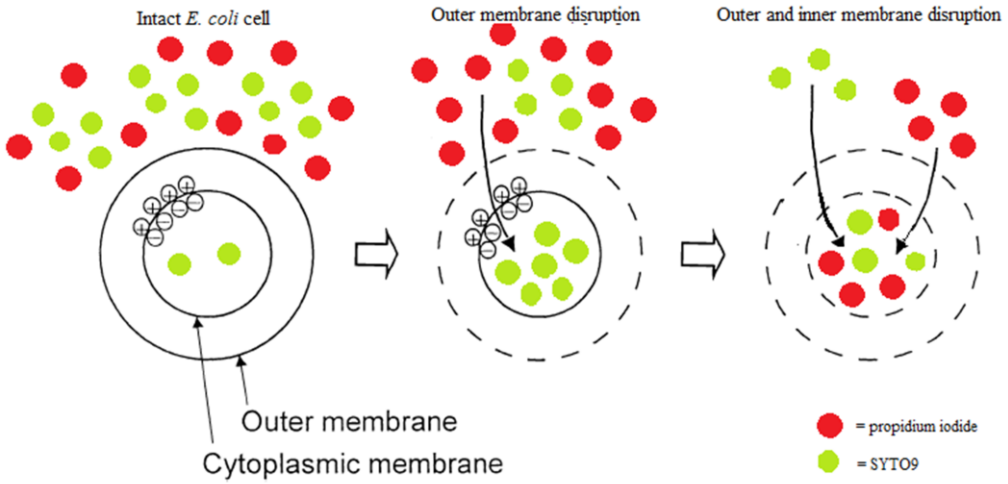

Figure 11. Schematic overview of the LIVE/DEAD Baclight Kit. An intact outer membrane of *E. coli* cells partly forms a barrier for SYTO9. Upon disruption, more SYTO9 is able to enter the cell which can be detected. Only upon IM disruption, propidium iodide (PI) can enter the cell, which gives an indication for IM disruption.

Finally, destabilization of the OM after treatment with MAC subunits can be detected by the release of OM specific components. One component we could think of is LPS. For example, SDS-PAGE gels can be used to reveal released LPS of treated cells. These results can be compared with intracellular staining of the DNA which shows if the IM is also disrupted or not (as mentioned earlier) [36].

Each assay mentioned above may be useful to study the effects of MAC subunits on the bacterial OM and IM separately. C5b-7, C5b-8 or C5b-9 complexes or individual components can bind the OM, disrupt the OM (and bind the IM) or disrupt both the OM and the IM. Though, what remains unclear in the approaches described until now is how many subunits are present in the MAC. Methods to unravel protein stoichiometry of the MAC will be discussed below.

3. How many C9 molecules are present in the Membrane Attack Complex?

Studies have tried to unravel how many C9 molecules are present in the MAC, which is considered to be 12-18. However, these studies were performed on erythrocytes or artificial membranes and not on living bacteria [21]. Protein stoichiometry (the amount of subunits in a bigger complex) of the MAC as it is formed inside the bacterial membrane could be unraveled by several techniques which will now be discussed.

Combination of high resolution X-ray crystal structures and lower resolution cryo-electron microscopy could be useful to visualize complexes at a resolution of 20-24 Å. Studies that have unraveled the structural data of the bacteriophage capsid (PRD1) used atomic models to improve cryo-EM images [49]. However, combining these techniques is extremely challenging and the crystal structure of the MAC should be known before we would be able to use this assay.

A more suitable technique to determine stoichiometry of a protein complex is to perform a membrane strep–protein interaction experiment (membrane-SPINE). Membrane-SPINE is a technique that combines fixation of bacterial cells (by formaldehyde) and purification of Strep-tagged target proteins. Formaldehyde can easily penetrate membranes and make a 'snap shot' of the membrane protein interactions. Protein complexes can be purified by their strep-tag and subsequently mass spectrometric assays can determine the molecular weight of the complex [50]. Furthermore, cross-linking by formaldehyde is reversible so also immunoblot analysis can provide information about the MAC stoichiometry [51]. MAC subunits could be labeled with a strep-tag to perform this assay. Principle of membrane-SPINE is depicted in figure 12.

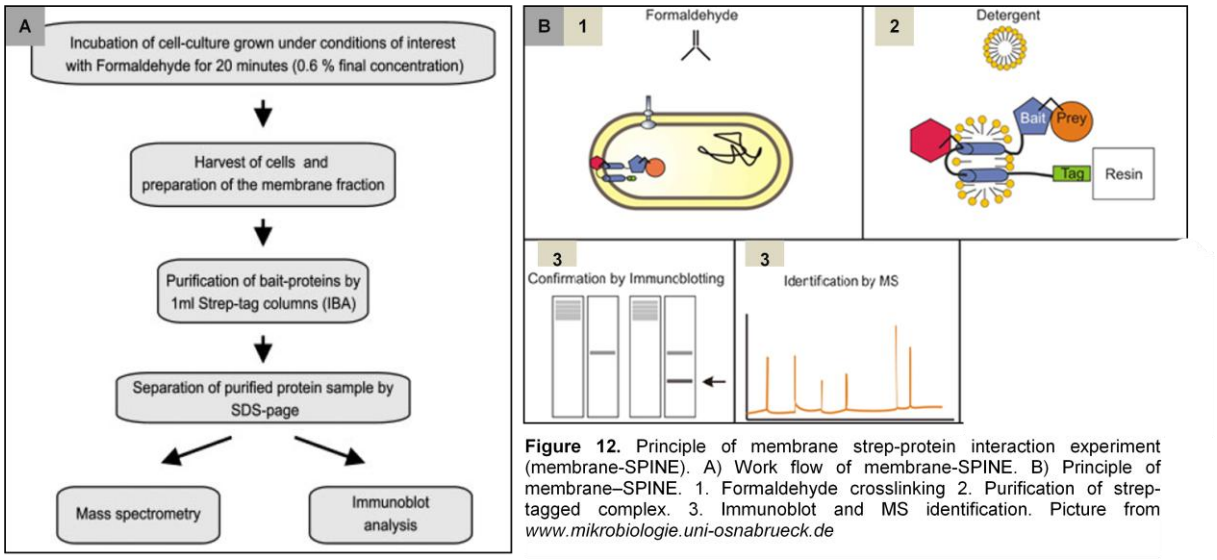

Figure 12. Principle of membrane strep-protein interaction experiment (membrane-SPINE). A) Work flow of membrane-SPINE. B) Principle of membrane–SPINE. 1. Formaldehyde crosslinking 2. Purification of strep-tagged complex. 3. Immunoblot and MS identification. Picture from *www.mikrobiologie.uni-osnabrueck.de*

Herzberg et al. demonstrated that indeed membrane-SPINE is highly suitable to isolate protein complexes with high purity and no background in an assay where they isolated a transcription regulator together with its interaction partner from a Gram-positive bacterium.

They state that this assay can also be performed with *E. coli* cells. Note that the experiments were performed with bacterial proteins and not with added pore-forming complexes [52].

Other methods to provide information on the stoichiometry of protein complexes are analytical ultracentrifugation and blue native electrophoresis. Blue Native PAGE (BN-PAGE) is mostly used with isolated membrane complexes to determine mass by binding of Coomassie brilliant blue (CBB) (which contributes to the observed mass but can be corrected for). Membranes are suspended and solubilized first without dissociation of multi-protein complexes. These complexes can eventually be analyzed by BN-PAGE. Analytical ultracentrifugation relies on mass properties of complexes by sedimentation with which hydrodynamic and thermodynamic characterization can be performed. Heuberger et al. (2002) state that both analytical ultracentrifugation and BN-PAGE studies have been performed with Gram-negative bacteria [53].

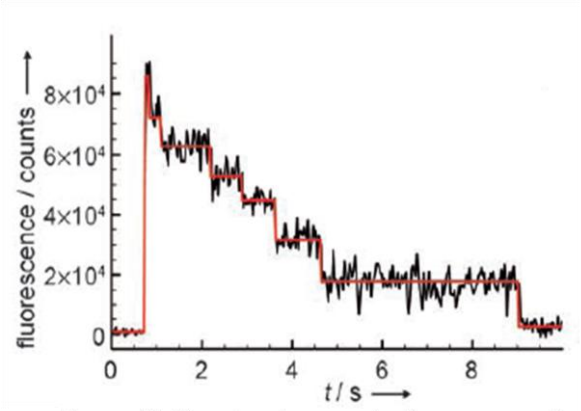

Figure 13 Stepwise decrease in fluorescence after photobleaching. Each step of decrease in fluorescence counts for one molecules present in a complex. Less steps than molecules can be measured, however, there will never be more steps than molecules in the complex.

A relatively new and promising technique to unravel the stoichiometry of a protein complex is Single-Molecule Fluorescence (SMF) [54]. With SMF, protein oligomerization can be measured *in vivo.* One SMF method is to 'simply count the number of photobleaching steps within a single complex'. Photobleaching is the loss of fluorescence in a molecule by changes in the structure which is followed by a light-induced chemical reaction. If a complex is photobleached, each molecule will lose its fluorescence stepwise. These steps can be counted. The principle of this stepwise loss in fluorescence is depicted in figure 13 [54]. Here we address if SMF could be used to study complexes inside the bacterial membrane.

Leake et al. (2006) performed photobleaching experiments with living *E. coli* cells. A gene of interest was replaced by the same gene fused to GFP. This resulted in expression of GFP labeled to a membrane protein of interest. Cytoplasmic GFP and autofluorescence was measured and corrected for. The illumination intensity to photobleach the entire complex and to bleach an individual GFP in the complex was determined. The intensity to bleach the entire protein complex, divided by the intensity to bleach one GFP estimates the amount of molecules in the complex. As mentioned, fluorophores can also simply be counted after bleaching of complete complexes [55]. Fluorescence recovery after photo-bleaching (FRAP) and fluorescence loss in photobleaching (FLIP) can be used to determine movement/mobility of fluorescently labeled proteins (Fig. 14) [42,56]. The measurements described above can all be performed under physiological conditions [42,55,56].

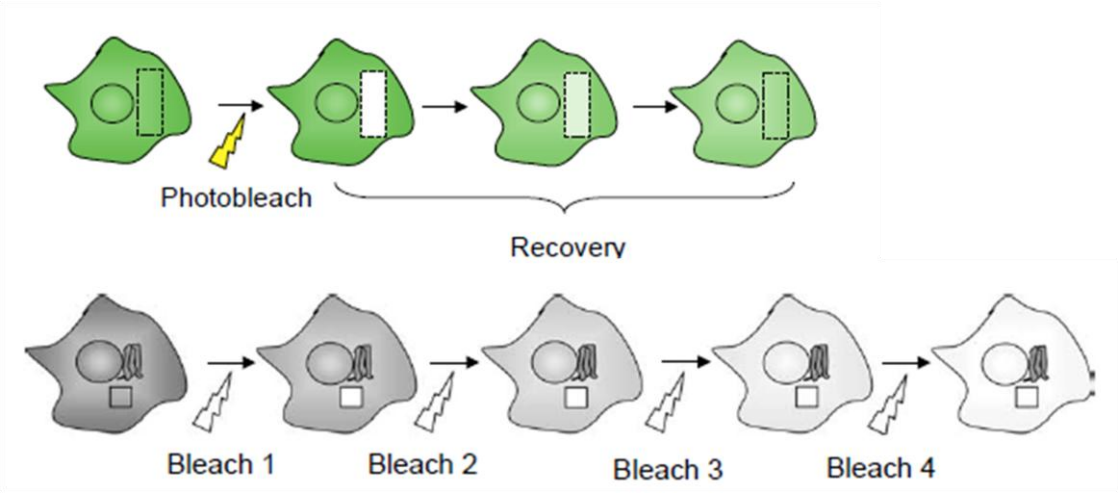

Figure 14. Principle of fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleacing (FLIP) . A) FRAP: A region of interest is photobleached. Afterwards the diffuse recovery of fluorescent proteins is determined. B) FLIP: A specific region is bleached several times which will be recovered by fluorescent proteins elsewhere in the cell. Loss in fluorescence of other parts of the cell indicates the movement towards the bleached area .

If we want to perform photobleaching assays to determine the amount of C9 molecules in the MAC, each C9 molecule should be labeled before the MAC is formed. Labeling of the subunits can be confirmed with SDS-PAGE, Coomassie blue staining and autoradiography. Subsequent to formation of the MAC with these labeled molecules, the amount of photobleaching steps can be measured. There will be variable amounts of photobleaching steps per complex but there will never be more steps than the amount of subunits present in one complex. We should make sure that only one complex is imaged each time.

4. Where and when is the Membrane Attack Complex formed?

Microscopy methods

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) are frequently used to visualize bacterial membrane disruption. We can distinguish a surface technique to visualize whole bacteria (SEM) and a technique to visualize the OM and IM in sectioned samples (TEM) (Fig. 15A-D) [57]. Light microscopes have resolutions of about 200 nm, where SEM and TEM reach maximum resolution of 1-5 nm [58]. SEM and TEM require fixed materials by rapid freezing or chemical cross-linking. TEM can provide 3D information by imaging series of sections of one cell. One disadvantage of electron microscopy is the use of a high dose of high-energy electrons which may disrupt the sample. This is why focused ion beam (FIB) technology may be favorable over conventional methods due to the absence of mechanical deformation (Fig. 15E). The main difference with SEM/TEM is that FIB uses ions instead of electrons, which reduce the sample damage [30].

Among electron microscopy methods is cryo-electron tomography. Tomography is extensively used to visualize Gram-negative bacteria at high resolution. Thin specimens can be visualized in frozen-hydrated state. Tomograms provide 3D information of unstained and unfixed cells with a resolution of 6-8 nm. It is mainly used to visualize protein complexes in their natural state/environment (Fig. 15F) [59]. It would be extremely useful if the structure of the MAC would have been solved already as a template to search for it in the tomogram [60]. Unfortunately, this structure has not yet been unraveled which is why we should find other methods to identify the MAC inside the bacterial membrane in tomograms.

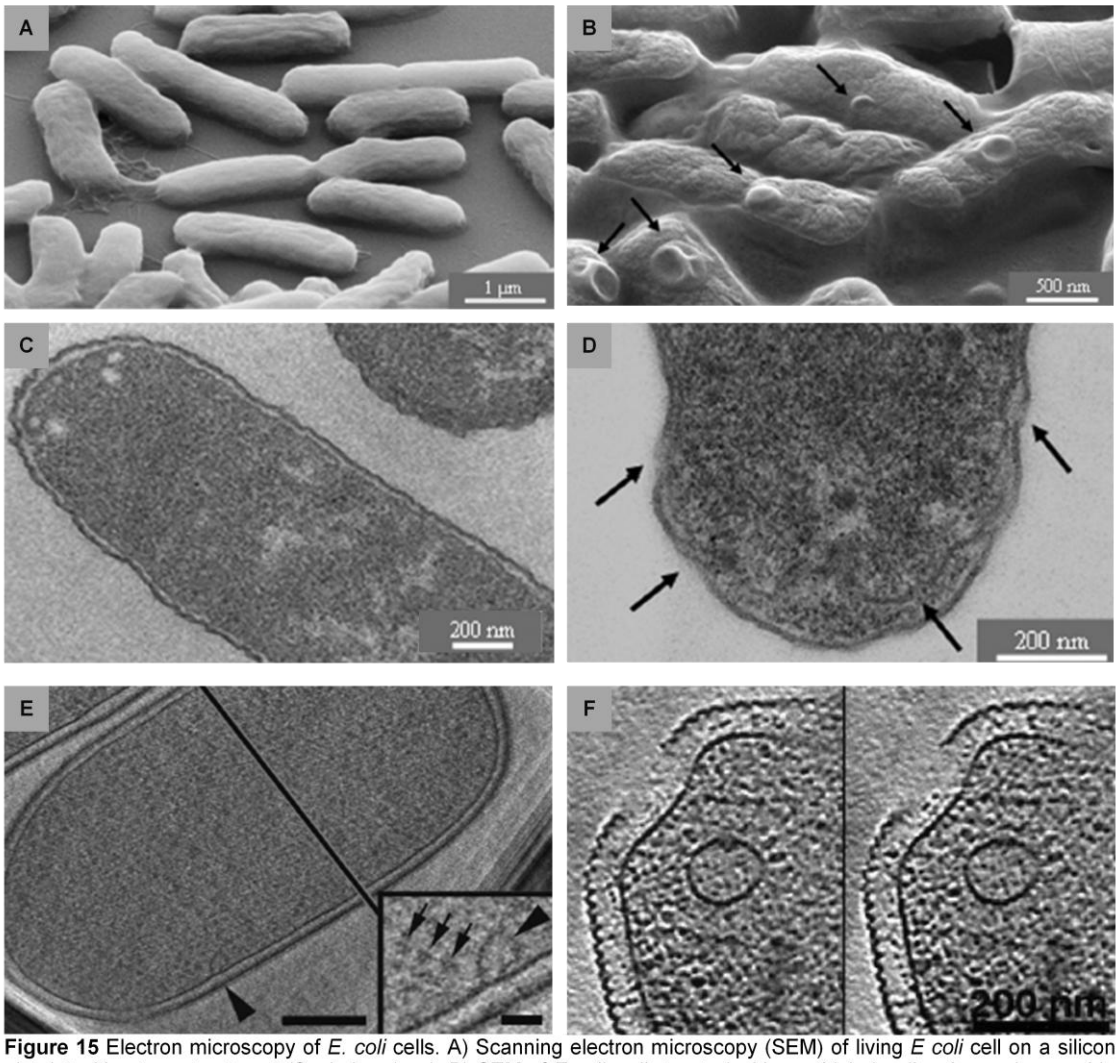

Figure 15 Electron microscopy of *E. coli* cells. A) Scanning electron microscopy (SEM) of living *E coli* cell on a silicon platelet without any treatment (Scale bar 1µm). B) SEM of *E coli* cells treated with peptidyl-glycylleucine-carboxyamide (PGLa). Blisters are observed on the cell surface after treatment (Scale bar 500 nm). C) Transmission electron microscopy (TEM) of untreated *E. coli* cell (Scale bar 200 nm). D) TEM of *E. coli* cell treated with PGLa. Arrows point at disrupted membranes. Furthermore, electrodense material has accumulated in the periplasm (Scale bar 200 nm). E) Focused ion beam of *E. coli* cell. Arrows point at ribosomes and a vesicle attached to the inner membrane. (Scale bar = 250 nm and 30 nm). F) Tomograph of a *Pyrodictium abyssi* cell (Scale bar 200 nm).

(c) Correlate peptide binding with membrane integrity

Several types of fluorescence microscopy super resolution techniques that can achieve nanoscale resolution have been developed in the last two decades among which stimulated emission depletion (STED) microscopy, structured illumination microscopy (SIM), stochastic optical reconstruction microscopy (STORM) and photoactivated localization microscopy (PALM) [61,62]. These methods can provide information about cellular dynamics in living cells with low diffraction barriers (which lead to unfocused imaging) and increased resolution when compared to the resolution of conventional light microscopy (±250nm). The diffraction barrier, which is nothing more than light outside the focus point, excites all molecules in a circle of 250nm, after which this area becomes fluorescent. A reduced diffraction barrier can be obtained by modulation of the excitation light which will also result in an increased resolution of the technique. In SIM, the sample is illuminated with a striped light pattern to reduce the diffraction barrier. Only half of the focus point is now excited (and thus fluorescent) which increases the resolution to 100 nm. In case of STED, the environment of the focus point is saturated with another laser to 'switch the fluorophores off' and only the focus point is excited to become fluorescent. The best resolution that has been achieved with STED is 20-30 nm. PALM/STORM is used to visualize single molecules to nanometer precision. Single

fluorophores are excited to become fluorescent randomly and are located. This process is repeated with several emission cycles after which patterns of fluorescence can be unraveled. One big disadvantage of PALM/STORM is that moving structures will become blurry due to the changed location of fluorophores. PALM mostly uses photoactivatable probes that are genetically expressed, where STORM uses pairs of cyanine dyes (but also other dyes can be used) that can be labeled to for example antibodies. We might also be able to label these dyes to MAC subunits. Figure 16 depicts the principle of STORM. The achieved resolution is ±20nm. Still, the electron microscopy methods (SEM/TEM) reach the highest resolution. An overview of these fluorescence microscopy super resolution techniques is depicted in figure 17 [63].

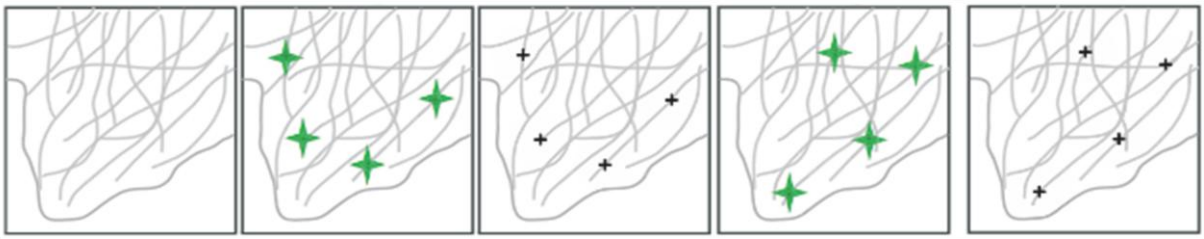

Figure 16 Principle of STORM. Cellular components are labeled with photoswitchable probes. Single molecules are switched on and located. Afterwards, new molecules are switched on. This is repeated several times after which a pattern of fluorescent probes (and thus the structure of interest) can be unraveled. Picture published on *http://nrs.harvard.edu/urn-3:HUL.InstRepos:9453705*

Combination of upper mentioned fluorescence and electron microscopy methods provides the advantages of both techniques in one assay. Therefore, to determine where the MAC is formed in real time, correlative fluorescence electron microscopy can be extremely useful. Some correlative light/fluorescence and electron microscopy (CLEM/CFEM) methods will be discussed below. Given that fluorescent nanoparticles can be used to study single molecules in living cells, this may allow us to localize and visualize the MAC in Gram-negative bacteria.

Note that the type of labeling is dependent on the research question and should always be chosen carefully. First, the required spatial and temporal resolution must be predicted. (Spatial is the ability to distinguish between two small objects and temporal is the precision with respect to time). In our case, a complex of about 100 Å wide and 160 Å high must be visualized. The MAC is expected to be present in the bacterial membrane so we should especially visualize this area. Next, the most suitable tag should be chosen and the methods to label the MAC or the membrane must be determined. These choices will also define the most appropriate type of microscopy to visualize the MAC inside the bacterial membrane.

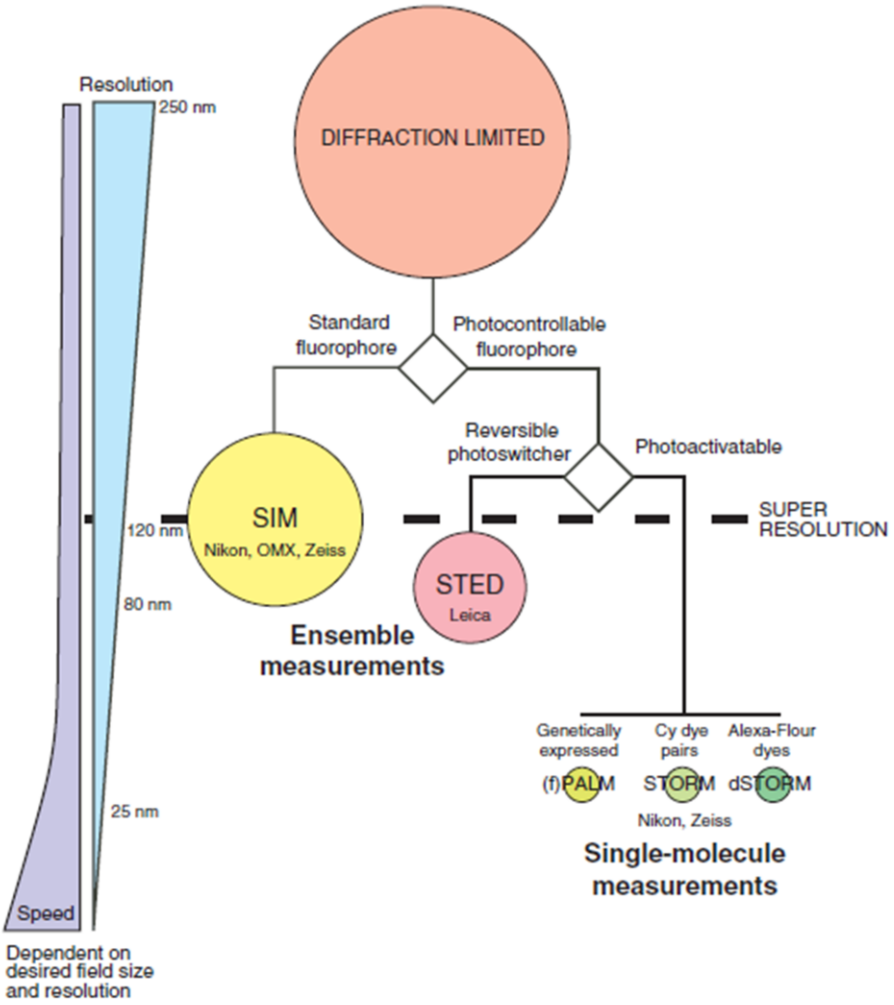

Figure 17. Super resolution microscopy methods using fluorescence. Either ensembles of molecules can be measured which results in resolutions between 80 and 120 nm or single molecules can be measured with a resolution of about 20 nm. In each technique, resolution is increased by a reduced diffraction barrier compared to conventional light microscopy (published by Galbraith et. al., 2011).

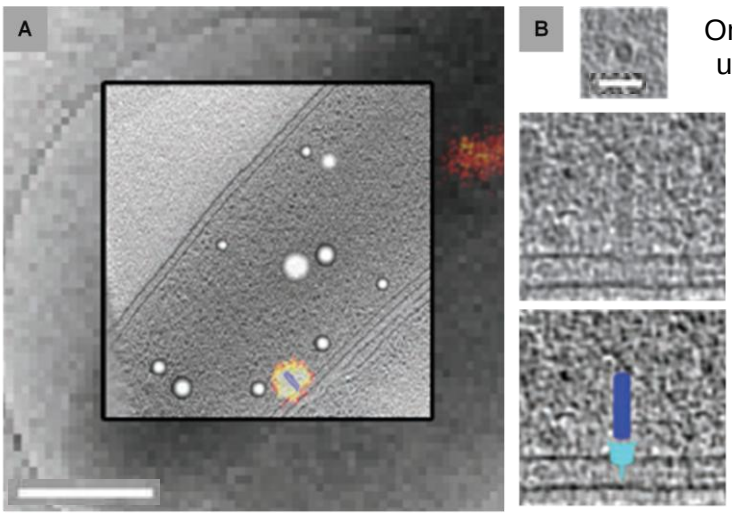

Figure 18 Correlated cryo-PALM-CET of an *M. Xanthus.* A) Combination of low-resolution Electron microscopy, a cryo-PALM signal and cryo-tomographic slice. B) Slices of T6SS as observed in A in which T6SS is highlighted with purple and blue. Scale bars A: 400 nm, B: 5 nm.

One example of CFEM that has been used to visualize structures in the bacterial membrane is Cryo-PALM-CET (Fig. 18). It is used to localize structures within a cryo-tomogram with photoactivated localization microscopy. PALM localizes genetically expressed probes in the cell, so might not be that useful to study the MAC. On the other hand, STORM is used to visualize labeled structures. It has the same resolution and properties as PALM and thus may be very promising to visualize the MAC. STORM can be combined with cryo-electron tomography in which living cells can be used without chemical fixations or

permeabilizations. As mentioned earlier, one drawback is that electron microscopy can induce damage to non-fixed cells. For visualization after fixation, the use of thin slices (<500nm) reduces background and denaturation of the sample is fully overcome by adding 10 Ficol PM 70 and 10% ethylene glycol [64].

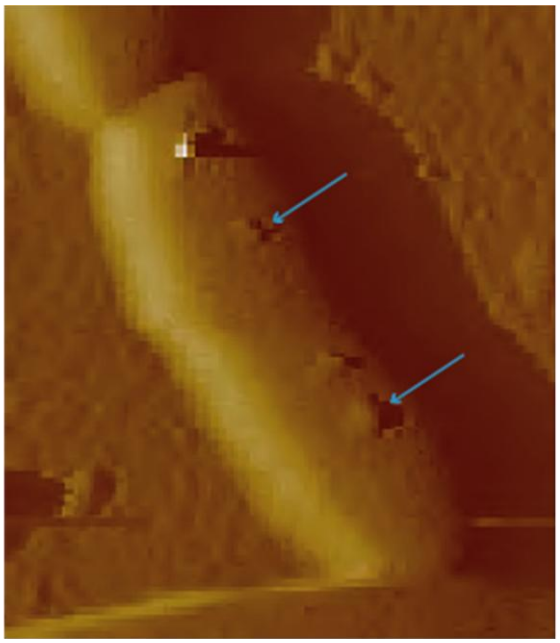

Figure 19 Atomic Force Microscopy (AFM) of an MK8A44 cell. Blue arrows point at outer membrane vesicles that are present on the bacterial surface. Image is of an area of 4 µm (published by Lonergan et. al., 2013.

Finally, one more method to characterize Gram-negative bacteria at very high resolution is Atomic Force Microscopy (AMF). With AFM we are able to characterize the integrity of the bacterial cell wall (Fig. 19). AFM can elucidate pore-like structures of approximately 65 nm and has high resolution compared to other techniques. It can be performed on living bacteria to determine membrane disruption. The reason why AMF is not used so commonly is that there are difficulties with immobilizing living bacterial cells [65].

Taken together, fluorophores can be labeled to subunits of the MAC and visualized by fluorescence light microscopy in combination with electron microscopy to determine the location of each subunit. Using different fluorophores labeled to each subunit of the complex may also allow us to perform co-localization studies. Combination of fluorescence and cryo-electron tomography is useful to image in real-time where each subunit is present in the bacterial membrane. These methods have been used to visualize for example bacterial secretion systems such as T6SS [64,66]. An overview of the microscopy methods discussed above is depicted in table 1.

Table 1: Resolutions, advantages and disadvantages microscopy techniques.

Technique	Resolution	Pro	Con
Light microscopy	200-250 nm	- Commonly used/less expensive than electron microscopy	- Low resolution due to high diffraction barrier
Scanning electron microscopy (SEM)	1-5 nm	- High resolution	- Only surface disruption images so not able to distinguish between OM and IM
Transmission electron microscopy (TEM)	1-5 nm	- High resolution - Able to visualize labeled proteins in the bacterial membrane. - Able to distinguish between OM and IM.	- Requires fixed samples that are sectioned, so not able to visualize living bacteria.
Focused ion beam (FIB)	1-5 nm	- Highly similar to SEM/TEM (high resolution) - Absence of mechanical deformation of the sample.	

Cryo-electron tomography	6-8 nm	- Visualizes protein complexes in their natural state/environment - Prevents damage by the frozen state of the sample	- Difficult to identify the MAC without knowing the exact structure.
Structured illumination microscopy (SIM)	100 nm	- Higher resolution than light microscopy	- Relatively low resolution compared to other methods - Not able to visualize single molecules
Stimulated emission depletion microscopy (STED)	20-30 nm	- Higher resolution than light microscopy	- Not able to visualize single molecules
Photoactivated localization microscopy (PALM)	20 nm	- Visualizes single molecules	- Visualizes structures that are genetically expressed by the bacteria - Moving structures become blurry which disables proper visualization - Lower resolution than electron microscopy
Stochastic optical reconstruction microscopy (STORM)	20 nm	- Visualizes single molecules - Used to visualize labeled subunits so could be useful to study MAC subunits	- Moving structures become blurry which disables proper visualization - Lower resolution than electron microscopy
Correlative fluorescence electron microscopy	1-10 nm	- High resolution of TEM/CET - Fluorescent localization of specific subunits by PALM/STORM - CET: Frozen samples prevent images to become blurry (no movement)	
Atomic force microscopy (AFM)	(up to) 0.1 nm	- Very high resolution - Visualize membrane damage of living Gram-negative bacteria (on the surface)	- Not commonly used due to difficulties with immobilizing bacteria - Only surface disruption images so not able to distinguish between OM and IM - No combination with fluorescence

CONCLUSION

Taken together, quite a few approaches and methods are available to study pore-forming complexes in Gram-negative bacteria and may be promising to study different aspects of MAC induced pore-formation. Of major importance is that the question 'how the MAC kills Gram-negative bacteria' should be divided into sub-questions, given that each method described in this thesis only elucidates one or two aspects of the total mechanism.

Until now it is unclear which MAC subunits target the OM and/or the IM. To localize the MAC subunits inside the bacterial membrane, both the bacterial membrane and MAC subunits can be labeled. LPS, peptidoglycans and surface proteins are targets to visualize the OM. Co-localization studies with fluorescently labeled subunits of the MAC may allow us to study if these subunits co-localize with the OM or pass the OM towards the IM. Unfortunately, these methods only give a rough indication on where the fluorescent subunits are present and may not be detailed enough to answer our question. Though, if we could label the periplasm, we might be able to see if the subunits are localized outside or inside the fluorescent layer.

A method whereby labeling of the bacterial membrane is not required is the use of nanogold labeled subunits or antibodies which can be visualized by transmission electron microscopy. Studies with nanogold labeled antimicrobial peptides or labeled antibodies revealed at very high resolution if the peptides were present in the OM, the periplasmic space or the IM. This approach is expected to be highly suitable to localize MAC subunits, but the question is if we should use antibodies or direct labeling. With labeled antibodies we might have higher chance on nonspecific data, which is absent if subunits are labeled directly. Furthermore, an antibody against each individual subunit is required, which still binds the subunit inside the bacterial membrane. The antibody binding site should not be hidden when the subunit is assembled in the MAC. Direct labeling sounds favorable over antibody mediated labeling but it should obviously not interfere with the function of each specific subunit or with the assembly of the MAC. If we are able to label each individual subunit of the MAC without disturbing its function, visualization inside the bacterial membrane may be possible. Transmission electron microscopy can image nanogold labeled subunits in sectioned samples at high resolution and clearly distinguish between the bacterial OM and IM.

To learn if MAC subunits are present in the OM, the OM and the IM or the IM only, the MAC must be formed with one labeled subunit each time to localize C5b, C6, C7, C8 and C9 separately. This would allow us to elucidate if C5b-8 targets the OM and stays there or if it is translocated to the IM. Furthermore we will be able to address if C9 targets the OM and the IM or only the IM. Note that these assays provide information about binding and not directly about membrane disruption.

Another remaining question is which membrane is disrupted by subunit combinations*. As* mentioned earlier, it is proposed that C5b-8 lyses the OM after which C9 lyses the IM. To prove this, we need to distinguish between OM and IM disruption for which several assays are available. Two approaches that may be quite easy to perform are the TPP/K+ and the BacLight Kit assay. Each of these assays can distinguish between OM and IM disruption by membrane permeability for specific substances. In the TPP/K+ assay, intracellular TPP accumulation can be measured upon OM disruption. Only if the IM is also disrupted, K+ is released from the cell. In BacLight Kit assays, a more heterogeneous population of SYTO9 positive cells can be observed upon OM disruption (by flowcytometry) and only upon IM disruption, cells will become PI positive. These measurements can answer if there is OM or both OM and IM disruption upon addition of subunit combinations e.g. C5b-6, C5b-7, C5b-8 or C5b-9 or individual components such as C8 and C9. Given that the BacLight Kit assay measures an increase in the heterogeneity of the SYTO9 population these results may be

less specific since there will always be a variable amount of positive cells. The TPP/K+ assay may be more suitable since the obtained data is expected to be more black or white.

Upper mentioned experiments provide indirect information about membrane disruption. In contrary, FRET can be used to visualize membrane disruption in real time. It can study OM disruption by labeling the IM or the periplasmic space with a donor fluorophore which only becomes fluorescent upon OM disruption. Fluorescence intensity can be measured or cells can be visualized by fluorescence microscopy, which would also allow us to combine the assay with a straining for IM disruption. Fusion proteins can be expressed on the IM or in the periplasmic space, but unfortunately there are some limitations. Proper expression of a fusion protein may be limited by the way it is transported to the membrane/periplasmic space. Furthermore, the donor fluorophore should be presented in such a way that the acceptor fluorophore can reach it upon OM disruption. The acceptor fluorophore should be labeled to a protein that is only able to pass the OM when it is disrupted.

If we are able to properly express the donor fluorophore, label the acceptor fluorophore to the right protein and combine this assay with a staining for IM disruption, this assay would be perfect to visualize membrane disruption in real time. Addition of MAC subunit combinations would either result in no fluorescence, fluorescence of the membrane or both fluorescence of the membrane and intracellular staining. FRET assays may be more challenging to perform but will also provide more innovative data than the TPP/K+ assay.

Studies propose that in nature 12-18 C9 subunits are present per MAC. However, this needs to be proven inside the bacterial membrane. To find out how many subunits are present in the MAC (formed in the bacterial membrane) two assays are promising. For membrane-SPINE, MAC subunits must be labeled with a Strep-tag by which the complex can be purified from the bacterial membrane after fixation with formaldehyde. Purified complexes can be analyzes by mass-spectrometry. Besides, photobleaching assays are promising to determine stoichiometry of a complex. C9 molecules must be labeled with photobleachable fluorophores after which the number of photobleaching steps can be counted. Photobleaching is expected to be more suitable than membrane-SPINE, given that only the C9 molecules are labeled and thus counted. With membrane-SPINE, the purity of the isolated complex should be determined to make sure the measurements are reliable.

Moreover, BN-PAGE and ultracentrifugation are mentioned in the writing assignment. These techniques may be useful to determine the mass of the complex but will only provide indirect evidence about the number of molecules. In addition, mobility of the subunits inside the bacterial membrane can be visualized by use of photobleaching. If, for example, C8 or C9 would be present in both the OM and the IM and we are able to photobleach the IM only, we could measure how long it takes before the area is recovered by fluorescent C8 or C9 molecules from the OM.

Finally, to measure where and when the MAC is formed, several high resolution microscopy techniques are available each of which has advantages and disadvantages for specific research question. TEM and CET can visualize the OM and IM separately, where TEM visualizes fixed samples and CET is able to visualize samples in their frozen state to reduce sample damage. SEM and AFM can visualize bacterial surface damage. FIB uses ions instead of electrons, which prevents mechanical deformation of the sample.

Though, finding MAC subunits in these images can be extremely challenging. A solution is the combination of electron and fluorescence microscopy. Several fluorescence methods are discussed in this writing assignment, of which STORM turns out to be the most suitable to visualize individual MAC subunits. Dyes can be labeled to subunit specific antibodies or directly to MAC subunits to localize them individually. Cryo-STORM-CET assays provide information about where and when the MAC is assembled e.g. if specific areas of the

membrane are preferred over others/if dividing cells may be favorable or not. CET is considered to be the most suitable microscopy technique since it can visualize samples at high resolution and distinguish the IM and OM. Furthermore, it prevents the sample from being disrupted and from moving (which results in blurry images). Measuring different time-points may also provide a time-schedule of MAC assembly. The assay that combines fluorescence- and high-resolution microscopy could also confirm the data of the nanogold localization of MAC subunits if we will be able to highlight in which membrane the fluorescence is located.

The methods discussed above could all be very helpful to stepwise unravel the exact killing mechanisms of the MAC. If we are able to properly perform these assays, we should be able to answer the questions which membrane is targeted and disrupted by each subunit or subunit combinations, how many subunits are present in the MAC and where and when the MAC is formed in living bacteria.

These answers will not only offer new insights in how complement kills bacteria, but also in other research areas. The way the host has evolved to target Gram-negative bacteria may offer new insights in the development of antibiotics. Despite the fact that much is known about antibiotics and AMPs, often exact killing mechanisms remain unclear. If we are able to setup a project to completely solve the killing mechanisms of pore-forming complexes, this might be very useful to answer the same questions in other studies. Given that common mechanisms have been elucidated already among pore-forming toxins, it would not be surprising if the killing mechanisms of different pore-forming complexes are highly similar. In addition, increased human susceptibility to Gram-negative bacterial infections might be a result of impaired MAC induced killing. Knowing the exact killing mechanisms of the MAC could help to solve these problems. The experimental setup may also be of big assistance to study immune evasion strategies or antibiotic resistance of Gram-negative bacteria since the discussed experiments can help to specifically address at which point the pore-formation process is impaired.

Taken together, with a proper experimental setup to unravel the killing mechanisms of the MAC we will not only obtain knowledge on how complement kills Gram-negative bacteria, but we may also facilitate the design of experiments in other research areas and provide new insights in how pore-forming complexes can lyse Gram-negative bacteria. Furthermore, it could be useful to study immune evasion strategies and antibiotic resistance of Gram-negative bacteria.

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