Bacterial Immune Evasion Proteins as Laboratory Tools

R.A. Karssemeijer

Master thesis

Supervised by J.A.G. van Strijp

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Introduction

Every day we encounter millions of bacterial microbes. More than 2000 species colonize our respiratory and intestinal tract, billions are present on our skin and they form an essential part of our body(*12-13*). A small portion of these bacteria can become pathogenic and evolutionary mechanisms allowed us to evolve an elaborate immune system to clear those bacteria that are able to invade our body. As a first line of defense, our innate immune system is equipped with macrophages, neutrophils and NK cells that are able to phagocytose or kill infectious microbes. But they also activate our adaptive immune system which enables a longstanding line of defense characterized by antibody secreting B cells and cytotoxic and helper T lymphocytes.

Bacteria however are smart organisms that evolved alongside us and developed advanced strategies to thwart our host defense system. By creating a favorable environment for themselves they increase their odds for survival and their defense mechanisms challenge almost all arms of the immune system. *Staphylococcus aureus* (*S. aureus*) is one of the most characterized examples(*1*). It produces protein A that is capable of binding the Fc portion of immunoglobulins resulting in incorrectly opsonized bacteria that are not recognized by our immune system. Sortase A is an essential enzyme needed for anchorage of virulence factor to the bacterial cell wall. The bacteria secrete pore forming toxins that lyse target cells such as innate immune cells. CHIPS is a protein that inhibits neutrophil chemotaxis by blocking the formyl-peptide receptor and C5a. Furthermore, the bacteria express Efb on the cell surface, which binds C3 convertase and thus inhibits complement deposition on the surface. And SCIN, staphylococcal complement inhibitor, blocks both the classical and alternative complement pathway.

Interestingly, other bacteria have proteins with similar functions. *Streptococcus magnus* (*S. magnus*) and group C and G Streptococci produce protein L and G respectively(*14-15*). These proteins also bind immunoglobulin and thereby block complement deposition and phagocytosis similar to protein A, but there is no homology between the three proteins indicating that they are not evolutionary conserved.

The strategies that bacteria have created to escape our immune system have such an exceptional specificity and functionality that life scientists have found use for these proteins as laboratory tools. Protein A, G and L are widely used methods for the

purification of antibodies and pore forming toxins are used for selective permeabilisation and delivery of proteins into target cells. Different proteins and their function are described in table 1. The discovery of these proteins and their use as molecular tools is the focus of this thesis.

Effect	Name	Source	Activity	Laboratory use
Ig binding proteins	Protein A	*S. aureus*	Binds Fc part of IgG, IgM and IgA	*Antibody isolation, purification and detection*
	Protein G	*Group C and G Streptococcus*	Binds Fc part of IgG	*Antibody isolation, purification and detection*
	Protein L	*Peptostreptococcus magnus*	Binds Ig light chain	*Antibody isolation, purification and detection*
Labelling	Sortase A	*S. aureus*	Recognizes LPXTG motif	*Protein labeling*
Proteomics	IgA1 protease	*N. gonorroeae*	Cleaves IgA in the Fab region	*Protein purification*
	Adenylate Cyclase toxin	*B.pertussis*	Generates cAMP	*- Bacterial two hybrid system* *- Reporter protein*
G-protein signalling	Cholera toxin	*V. cholerae*	$G_s\alpha$ proteins	*Irriversible activation of G_s proteins*
	Pertussis toxin	*B. pertussis*	G_i proteins, G_o proteins	*Irreversibly inactivation of inhibitory G-proteins*
Pore formation	Streptolysin-O	*S. pyogenes*	Large pores	*Reversible membrane permeabilisation, siRNA transfection, protein delivery*
	Alpha-hemolysin	*S. aureus*	Small pores	*Erythrocyte lysis, biosensors in nanotechnology*
	Listeriolysin 0	*L. monocytogenes*	Large pores	*Antigen delivery*
Cell culture	Collagenase	*C. histolyticum*	Collagen destruction	*Cell separation, wound healing, Dupuyptren's disease*

Staphylococcus aureus

The bacteria

Staphylococcus aureus is a gram-positive coccus of the micococcaceae family discovered in the 1880s that is potentially pathogenic for humans. The name Aureus, gold in latin, is derived from the fact that *S. aureus* forms gold colonies when grown on blood agar plates. About 20% of the human population is a persistent asymptomatic carrier of the bacteria which can become part of the commensal flora of the nose and skin(*16*). However, infection can occur when the skin or mucosal barrier is breached and it can lead to a broad spectrum of symptoms from skin abcesses to severe pneumonia, meningitis or even sepsis. Over the last decades *S. aureus* has become increasingly resistant to antibiotics and infection with methicillin-resistant *Staphylococcus aureus* (MRSA) has become a major problem in health care settings.

The high pathogenicity of *S. aureus* is due to the expression of a large variety of virulence factors(*1, 16*). *S. aureus* expresses several MSCRAMMs that facilitate attachement to host tissues, some of which are covalently attached to the cell wall by the enzyme sortase. The organism also secretes proteases, lipases and nucleases to aid spreading of the infection, destructive toxins, superantigens and several immune evasion proteins.

The immune respons to *S. aureus* is largely dependent on the innate system, mediated by neutrophils and macrophages. Antibodies are formed upon infection but do not provide efficient protection(*1*). However, *S. aureus* has evolved many mechanisms to escape the innate immune system, such as Protein A, α-hemolysin and Sortase A.

Protein A

Discovery and immune evasion strategy of protein A

Protein A is an immune evasion protein of *S. aureus* capable of binding the Fc portion of antibodies. It was first discovered in 1959 as a cell wall component that formed a precipitate with sera from every individual, which was assumed to result from the fact that *S. aureus* is a commensal bacteria against which every individual produces antibodies. A few years later, Lofkvist et al. further identified this antigen and described a protein with a very simple aminoacid composition(*17-18*). However, Forsgren et al. showed in 1966 that the interaction between protein A and antibodies is non-specific(*19*). This was concluded from agar gel diffusion experiments in which

protein A forms a precipitation line with γ-globulins present in the agar. When protein A was incubated with γ-globulins prior to addition to agar, no precipitation was seen anymore because protein A was already saturated with antibody. However, incubation of protein A with only the heavy chain led to the same results while incubation with Fab fragments or light chains had no effect on precipitation. These results indicated that it is not the antigen binding site of the antibody but the Fc-region that is responsible for the interaction with protein A. This also explained why about 45% of the γ-globulins present in sera react with protein A, which is an unlikely amount for a specific antibody(19). Further studies confirmed the specific interaction between protein A and the Fc portion of IgG(20). Later studies also showed the ability to bind IgM and IgA(21).

The first reports of protein A as an immune evasion mechanism of *S. aureus* were published in 1967, when Gustafson et al speculated that the formation of protein A-antibody complexes might be beneficial for the bacteria as less antibody will be present in the initial stages of infection(22). This was further studied by Dossett et al. who showed that addition of protein A to IgG opsonised bacteria markedly reduced phagocytosis(23). This is accomplished by inhibition of both Cq1 deposition as well as direct antibody mediated phagocytos(24). This is caused by the incorrect conformation of the antibody for proper recognition by Fc receptors present on neutrophils and macrophages (figure 1).
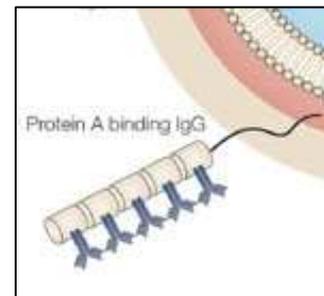
Figuur 1. Protein A
Protein A binds the Fc portion of immunglobulin. Fc receptors on innate immune cells are unable to recognize these antibodies. Picture edited from Foster(1)

Laboratory use of protein A

A little over 10 years since its discovery, studies were being published about using protein A as a laboratory tool. Hjelm et al were the first to realize that protein A could be used to isolate IgG from human sera, which until then could only be achieved by anti-IgG antibodies(25). By coupling protein A to sepharose they were able to recover 95% pure IgG from sera. This was quickly followed by a radioimmune assay to detect antibodies against specific antigens (26-27). In this assay, pure radio labelled antigen is mixed with human serum. Protein A containing *staphylococcus* is then added which

binds all IgG in the sera. If specific antibodies are present, radio labelled antigen will be detectable in the protein A-IgG fraction. This technique is also applied for the isolation and characterization of antigens using specific or unknown antibodies(*27-28*). Another interesting application is the use of labelled staphylococci expressing protein A for the detection of cell surface antigens(*29-30*). This technique was successfully used to determine the amount and subtypes of T and B cells in peripheral blood by using specific anti-sera, it was used for HLA typing and for cell separation based on different surface markers(*31-34*). Other applications of protein A include ELISA and western blotting(*35*).

Nowadays, every pharmaceutical company offers numerous protein A products including columns, sepharose or agarose linked protein A, labelled protein A (biotin, HRP, B-galactosidas, gold, peroxidase), antibody purification kits and many more. Protein A coated beads are routinely used for isolation of immune complexes in classical Co-IP experiments and for isolation of antibodies on large scale.

And still new ingenious tools with protein A are being developed. For example, nanoparticles coated with the IgG binding domain of protein A, called ZZ-BNC can be used to enhance ELISA signals when the concentration of first antibody is low (figure 2) (*4*). Additionally this can be used for enhancing western blot detection but there are also clinical possibilities as ZZ-BNC nanoparticles can be used for antibody dependent delivery of therapeutic molecules to different tissues(*36*).

All the methods for using protein A described above prove that it is a versatile and indispensable tool for immunologists.
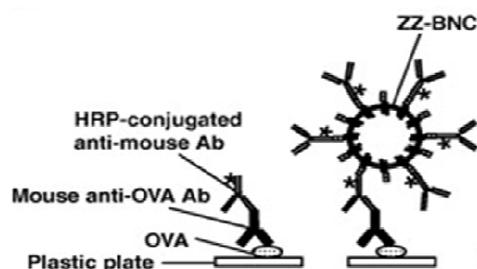
Figure 2. ZZ-BNC particles in ELISA
The use of ZZ-BNC particles coated with secondary antibody enhances the signal obtained from classical ELISA.
Picture edited from Iijima et al.(*4*)

Sortase A

Discovery and immune evasion strategy of sortase A

Many surface proteins of *S. aureus*, including protein A, are linked to the bacterial cell wall by sortase A, an enzyme that catalyzes surface anchoring through a transpeptidation reaction.

Sortase A recognizes and cleaves a special sorting signal, LPXTG, which is then linked to peptidoglycan in the cell wall. The significance of the sorting signal was first identified in protein A, which is no longer anchored to the cell wall upon deletion of this motif (*37*). Since the LPXTG motif is conserved in many cell surface proteins of gram positive bacteria, this suggested the presence of a general mechanism for protein sorting. By using different proteases, Edman degradation and mass spectrometry, Schneewind et al showed in 1995 that the LPXTG motif is cleaved between the threonine and the glycine (*38*). The free COOH-terminal of threonine is subsequently linked to the free NH2 group of a glycine residue in the peptidoglycan crossbridge of the cell wall of *S. aureus* (figure 3). The enzyme involved was still unknown though. Sortase A was identified a few years later using a sorting deficient strain of *S. aureus* which was transformed with a plasmid library of genomic DNA; transformation with
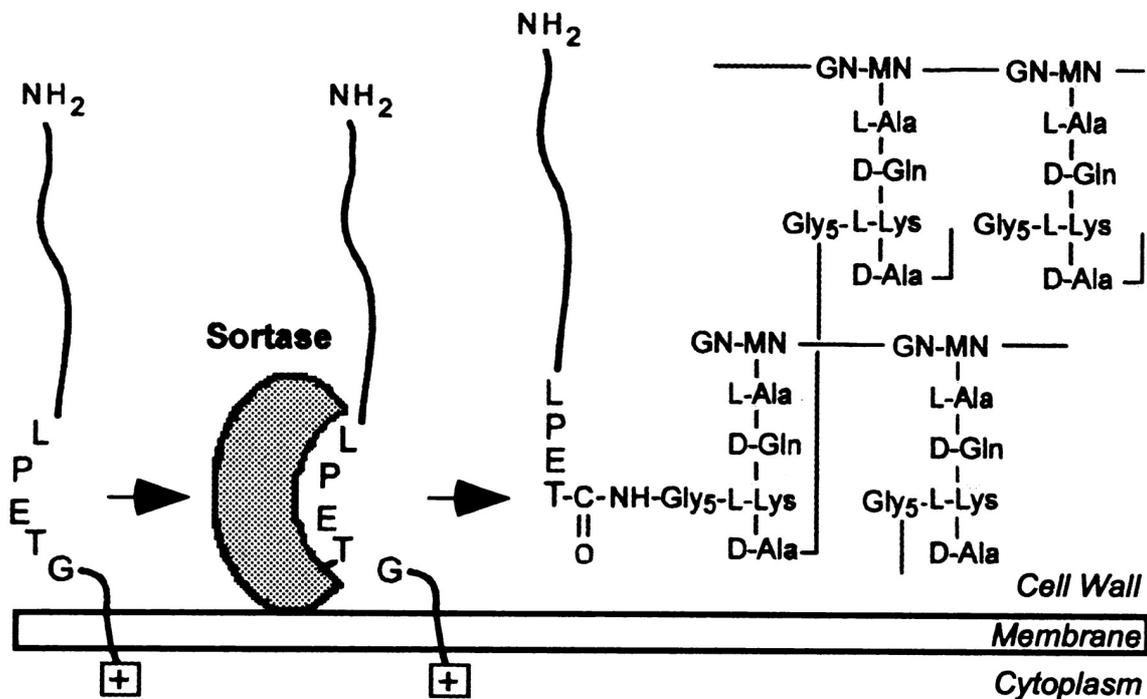
Figure 3. Sortase mediated reaction
Sortase A recognizes the LPETG signal on proteins as they are transported through the membrane. It cleaves the sequence between the threonine and glycine residue. It then attaches the free threonine residue to a free glycine residue in the peptidoglycan crossbridge of the cell wall. Picture copied from Ton-That et al. (*5*)

the *srtA* gene repaired the sorting defect (*39*). Further studies on this gene revealed many other gram-positive species containing *srtA* homologs and that especially the cysteine on position 184 is highly conserved (*39*). This residue is located in the active site and is needed for the formation of the acyl-enzyme intermediate between sortase and the COOH-teminal of threonine (*5*). Nowadays, the complete pathway of cell surface anchoring by sortase A is known. Proteins dedicated for this pathway contain an N-terminal signal sequence for translocation over the plasmamebrane by the Sec-pathway. The hydrophobic and charged domain around the C-terminal sorting signal is thought to retain the protein in the membrane during secretion after which sortase A, located in the plasmamebrane, is able to cleave the sorting signal and form an attachement to the peptidoglycan in the cell wall (figure 3) (*37, 40*).

Sortase A is essential for the pathogenicity of *S. aureus*. Strains missing a functional protein are severely hampered in producing lethal disease in a mouse model (*41*). This is partially explained by the fact that this strain no longer expresses protein A on the surface and is therefore unable to bind IgG. Other LPXTG bearing proteins of *S. aureus* include adhesins such as collagen-binding proteins and fibrinogen binding proteins. They are essential for attachment to other cells and the extracellular matrix and therefore contribute to the pathogenesis of *S. aureus*(*41*). Because of its role in anchoring these virulence factors to the cell wall, sortase A is an important immune evasion protein. Much research is focused on finding inhibitors to block sortase A as treatment for *S. aureus* infections (*42*). Since sortase A is not essential for growth of the bacteria but for the pathogenesis, inhibitors of sortase A are less likely to induce drug resistance then antiobiotics.

Laboratory use of Sortase A

Laboratory engineered protein modifications are nowadays an invaluable tool for molecular biologists. The specific transpeptidation reaction catalyzed by sortase A was therefore quickly redesigned for laboratory use (*43-44*).

Single labelling

Reporter proteins were already widely used for live cell imaging but a major disadvantage was the relative large size of the available probes, such as GFP and luciferase, which hindered the formation of fusion proteins. Mao et al showed for the first time that sortase A could be used for protein ligation (*43*). The target protein is

equipped with a C-terminal LPXTG motif which is incubated with a probe containing one or more N-terminal glycine residues. Addition of sortase A catalyzes the cleavage of the LPXTG motif and attachment of the probe at the glycine residue (figure 4a). The advantages of this technique are that only a small sequence needs to be added to the target protein, the ligation occurs after production of the protein, thus no interference with folding occurs, and the method is highly specific. Also, chemical probes containing glycine residues are easily produced(*45*). Popp at all extended this method for tagging proteins on the surface of living cells, a useful tool for studying protein interactions and trafficking (*44*),. However, sortase A mediated ligation can only attach the C-terminal LPXTG tag of the target protein to the N-terminal glycine residue of the probe. This approach is therefore limited to surface proteins with extracellular C-terminals, which is not very common. A way to overcome this problem would be the use of LPXTG-linked probes and surface proteins with a
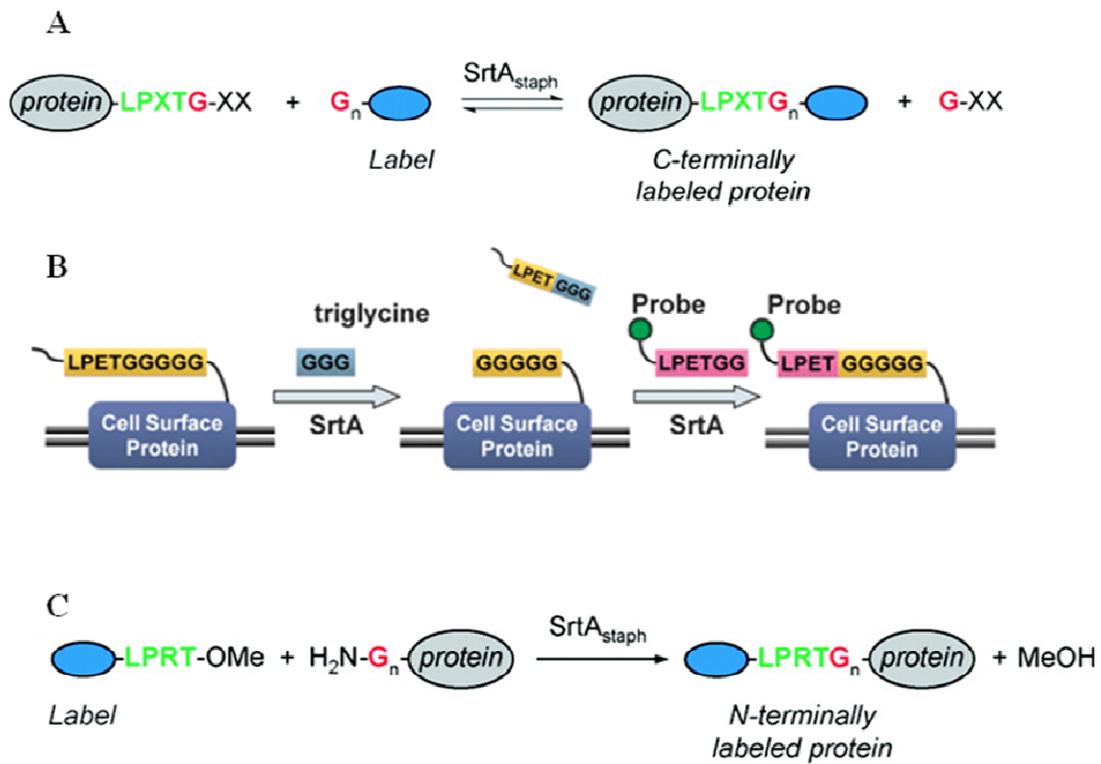
Figure 4. Sortagging on the cell surface
A. LPXTG-tagged surface proteins are linked to a molecular probe labeled with tryglycine resulting in C-terminal labeling. **B.** N-terminal labeling according to Yamamoto et al. requires two sortase steps. First, an LPXTG motif close to the N terminus of the protein is replaced by tryglycine using sortase. This is then linked to an LPXTG-tagged probe in the second reaction step resulting in N terminal labeling. **C.** N-terminal labeling according to Antos et al. is uses an LPRT labeled tag which is attached to a glycine residue on the C-terminal of a target protein
Pictures edited from Antos et al.(*8*) and Yamamoto et al.(*11*)

glycine sequence on the N-terminus. There are several obstacles to this approach however(*11*). First of all, sortase A requires an excess of the glycine linked substrate because the LPXTG motif is also slowly hydrolysed by sortase A in the absence of a glycine residues. An excess of glycine-linked probe is easily accomplished, but the amount glycine linked surface proteins cannot quickly be enhanced due to the limited capacity of cells. Secondly, constructing proteins with an N-terminal glycine tag is difficult as this is the site of many post-translational modifications which will remove or acetylate the N-terminus(*46*). Yamamoto et al. devised a very clever strategy to overcome these obstacles (*11*). They constructed a LPETGGGGG sequence close to the N-terminus of the cell surface protein. When cells expressing this protein were incubated with triglycine and sortase A, the LPET moiety was removed by sortase and replaced by triglycine, rendering a protein with an N-terminal glycine tag. In a second step, the N-terminal linked surface protein is ligated to an LPXTG-containing probe resulting in N-terminal labeling of the protein (figure 4b). With this technique, many cell surface receptors such as GPCR and EGFR can be studied in detail.

A few months later, another method was described for N-terminal labeling(*8*). Antos et al modified the B subunit of cholera toxin with an N-terminal signal sequence with a glycine linker in between. This results in an N-terminal glycine residue upon cleavage of the signal sequence in the periplasm, thus bypassing the obstacle of post-translational modifying changes removing the glycine residue. A labeled protein with an LPRT tag instead of the LPXTG tag was used to prevent the release of free glycine residues upon cleavage by sortase A which could compete with the less abundant glycine linked cholera toxin subunit (figure 4c).

Another interesting application of sortagging is the attachement of lipids to proteins (*47*). This enables the study of post translational lipid modifications but also membrane targeting of proteins not naturally present there.

Furthermore, given the recent advances in targeted therapies using liposomes, sortase A will enable quick and easy anchorage of proteins in these structures. Other applications of sortase A include the creation of circular proteins (*48*) and covalent attachement of proteins to solid surfaces (*49*).

Dual labelling

Besides *S. aureus*, many other gram-positive bacteria contain sortases(*50*). Since their mode of action is often distinct from that of sortase A$_{staph}$, this offers the opportunity

of dual labelling of proteins. By using sortase A$_{staph}$ for N-terminal labelling and sortase A$_{strep}$ for C-terminal labelling, two different chemical labels were attached to the same protein(*8*). This technique could be applied to study protein folding but it will also be possible to specifically label different proteins on the surface of a living cell to study their interaction by FRET analysis. For now, only Sortase A from staphylococcus and streptococcus has been efficiently exploited. However, the existence of many other sortases with different recognition sequences and the ability to change the recognition sequences using bioinformatics will have broad implications for the further use of sortases.

Alpha-hemolysin

Discovery and immune evasion strategy of alpha-hemolysin

S. aureus produces many toxins including α-hemolysin, a 33.2kDa protein also known as α-toxin. This cytolytic toxin was identified in the early 20th century as the major exotoxin produced *S. aureus* and was later identified as the first bacterial pore-forming toxin(*51*). The name hemolysin is derived from the lytic effect on red blood cells. It was discovered however that α-hemolysin is also toxic for lymphocytes, macrophages, fibroblasts, muscle cells and mast cells, thereby compromising the host immune system(*52-53*). Nowadays the structure of the pores created by α-hemolysin has been elucidated (figure 5). The secreted toxin is monomeric but upon binding the
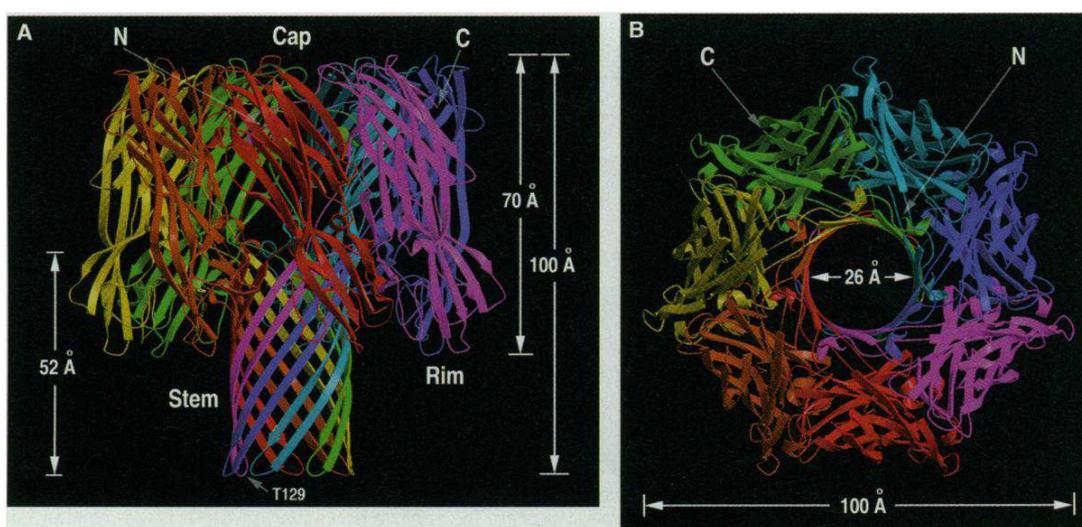
Figure 5. Structure of α-hemolysin pore
A. Ribbon presentation with each subunit of the heptamer in a different color. **B.** View from the top of the pore. The size of the β-barrel pore ranges from 14-46Å.
Picture copied from Song et al.(*9*)

plasmembrane, it quickly oligomerizes to form a water filled heptameric channel. This consists out of a membrane compassing β-barrel, an external cap composed out of a β-sandwich and a rim that is probably connected to the lipid-bilayer (*9, 53*). The interior of the β-barrel is hydrophilic and ranges from 14-46Å in diameter. Water, ions and small organic molecules can diffuse through these pores accounting for the observed lytic effect.

Due to the pore forming capacity, this toxin is an important virulence factor of *S. aureus* but it also aids immune escape. As mentioned above, α-hemolysin has the ability to lyse immune effector cells and it induces apoptosis in lymphocytes (*52, 54*). Recent studies also describe a role for α-hemolysin in phagosomal escape (*55-56*). Kubica et al. show that α-hemolysin is essential for the intracellular survival of the pathogen in macrophages. They propose that the toxin is needed to escape the phagosome and also for efficient lysis of the host cell after replication of the bacteria. This view is supported by a recent study in which α-hemolysin is necessary for escape of *S. aureus* into the cytosol in cystic fibrosis epithelial cells (*57*). However, α-hemolysin is not the only mediator in this process as the presence of the toxin alone is not sufficient to mediate escape (*56, 58*).

Laboratory use of alpha-hemolysin

Although initially recognized as a tool for controlled cell permeabilization, α-hemolysin has not yet been used extensively for this purpose although there have been studies using this toxin to selectively lyse erythrocytes. A more interesting use of α-hemolysin is in the field of nanotechnology. This started when Russo et al. engineered a 1000nm nanopore from α-hemolysin which is reversibly opened by the addition of Zn^{2+}. As the pore can be modified in its conductibility, it can be used to regulate the intracellular environment(*59*).

Pores can also function as highly sensitive stochastic sensing devises. By engineering adaptors in the lumen of the pore, binding of ions(*60*), DNA(*61*) and proteins(*62*) can be measured through a voltage change across the membrane due to constriction of the pore (figure 6) (*7*). These so called biosensors can be used for many purposes including detection of toxins and drug residues in patients or for sensing signaling molecules, ions or other chemical substrates. This is still a field of study and researchers are especially interested in creating lab-on chip technology with these chemically modified α-hemolysin nanopore as biosensors. This would even enable

researchers to measure intracellular levels of substrates in living cells by attaching this chip to a microscopic needle.

Because the size of these nanopores is only 2.6nm, DNA and RNA can be transported through the pore in a single nucleotide fashion which is a promising tool for DNA sequencing(*63*). Indeed, it is possible to discriminate between different nucleotides using α-hemolysin biosensors although the sensitivity remains to be optimized, especially for measuring homopolymer sequences(*64*). Other problems that are still under investigation are the optical read-out of the system, the fabrication and stability of the pores and reducing the speed of translocation to increase the sensitivity (*65*). However, no fundamental drawbacks to using this approach have been found yet and the potential advantages are vast; the DNA sequence length of a single run is very high (up to 50.000 nucleotides), the costs are low (<$1000/genome) and the process requires very little preparation of the samples (*65*). Therefore, DNA sequencing through α-hemolysin nanopores is a very attractive prospect that will acquire much attention over the next coming years.
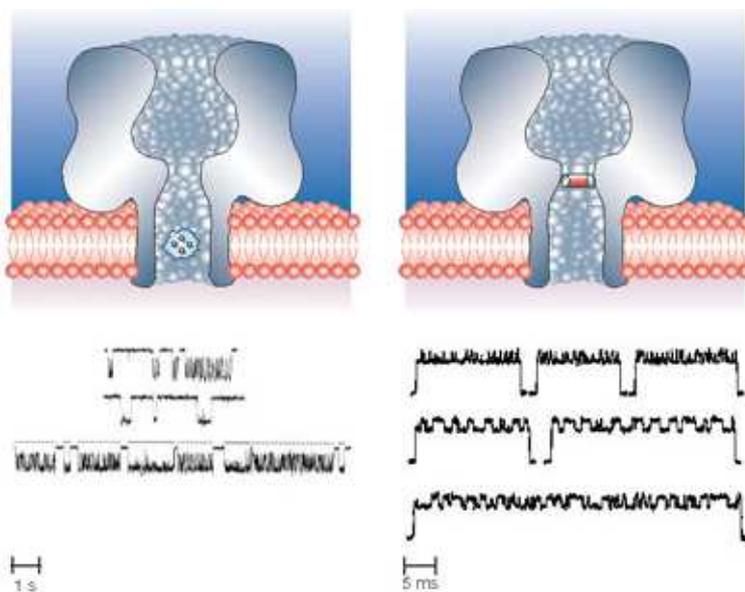
Figure 6. Use of α-hemolysin as biosensor
A. A genetically engineered binding site for metal ions (left) or organic molecules (right) will bind different substrates each producing obstruction of the channel to a different extend. The voltage change can be measured and gives a specific profile for each substrate. Picture copied from Bayley et al.(*7*)

Streptococci

The bacteria

Streptococci are gram positive bacteria which form chain like structures upon division. They can be divided into groups based on their hemolytic capacity: alpha-hemolytic, beta-hemolytic and non-hemolytic streptococci(*66*). Alpha-hemolytic streptococci include *S. pneumonia*, the causative agent of pneumonia, meningitis and otitis media. Beta-hemolytic streptococci are further classified into group A, B C and G by Lancefield serotyping, based on antigenic cell wall carbohydrates(*66*). Especially the group A streptococci are highly pathogenic in humans.

Streptolysin O

Discovery and immune evasion strategy of streptolysin-O

S. pyogenes, the major pathogen of Group A streptococcus, produces the toxin streptolysin-O (SLO). This toxin was identified in the early 20th century as a major hemolysin although the mechanism of the membrane damage was not yet known (*67*). Similar toxins were identified in other gram positive bacteria, including listeriolysin O and tetanolysin and this family of proteins all share some common characteristics such as the ability to bind membrane cholesterol and the appearance of small holes in the membrane visualized by electron microscopy (*68*). Researchers believed that these holes resulted from the extraction of cholesterol from the membrane by these toxins (*69*). However, Bhakdi et al, who also identified α-hemolysin and complement pores, described for the first time that SLO forms a 30nm wide transmembrane channel(*68*).

SLO is an important virulence factor and immune evasion protein of *S. pyogenes*. Besides its direct lytic effect on cells, SLO was also shown to specifically enhance macrophage apoptosis(*70*). Timmer et al. show that expression of SLO is both necessary and sufficient for the observed increase in macrophage apoptosis. *S. pyogenes* must first be internalized, after which apoptosis is induced by SLO pores formed in mitochondrial membranes after escape from endocytic vesicles. Both in vitro as well as in vivo studies were done to verify the effect of SLO on macrophages and the authors also indicate SLO to be involved in neutrophil apoptosis(*70*). This would enable the bacteria to evade two important lines of first defense of the human immune system. Another study indicates the contribution of SLO in mediating escape from lysosomal killing(*71*). They show that SLO expressing *S. pyogenes* do not co-

localize with lysosomal markers in constrast to SLO mutant strains. SLO negative strains are taken up by lysosomes and killed by acidification. In WT strains, there is excessive fusion between lysosomes and the cell membrane, which might be a repair mechanism for the SLO induced membrane damage. The author argue that this might be the mechanism through which SLO strains escape lysosomal uptake, although further studies need to be done(71). A disadvantage of this study is that only epithelial cells were studied and no professional antigen presenting cells.

Laboratory use of streptolysin-O

Due to the exceptionally large size of the pores created by SLO, it has been a useful tool for membrane permeabilization and transportation of proteins up to 150kDa(72). A large pitfall however is the lethality of the large pores for the cells under investigation. Therefore, a reversible permeabilization strategy was devised using Ca^{2+} to repair the lesions(73). This enables researchers to administer large proteins into live cells and to study their behaviour. The technique is not only applicable for protein transport, also DNA and RNA can easily be transported into cells. SLO permeabilization was effectively used for siRNA transfection and even hard-to transfect cells were permissible(74). siRNA mediated techniques are nowadays very useful and SLO provides an easy to use delivery mechanism, which is very promising for future applications.

Combining protein delivery via SLO with other sophisticated techniques, Ogino et al. were able to obtain structural information of proteins inside living cells by NMR analysis(75). This requires injection of the target proteins as overexpression of isotopically labelled proteins in eukaryotic cells is impossible. This technique will be very useful to obtain structural information from inside the cell that might result from protein-protein interactions or post-translational modifications. There are many other applications for this natural permeabilization agent and the laboratory use of SLO is already widespread.

Protein G

Discovery and immune evasion strategy of protein G

Protein G is an immunoglobulin binding protein from group C and G streptococci, similar to protein A from *S. aureus*. It was discovered in 1984 by Bjork et al. who purified the protein and discovered that it binds to all classes of human IgG as well as

IgG from other species (*14*). The protein has three repeated Fc binding regions, similar to protein A, which contains 5 homologous regions(*76*). Interestingly, there is no sequence homology between protein A and protein G indicating that their functional similarities developed by convergent evolution due to the advantage they offer to the bacteria.

Laboratory use of protein G

Protein G is different from protein A in its immunoglobulin binding capacity. Whereas protein A only binds human IgG1, IgG2 and IgG4, protein G is able to bind all classes of humen IgG with a higher affinity. Furthermore, protein G is especially more capable of binding rat and goat IgG, making it a more useful laboratory tool as these are widely used antibodies. However, an advantage of protein A is the capability to also bind IgA, IgM and IgE. Therefore, a hybrid molecule was created, composed out of both protein A and protein G, which has the combined binding capacities of these proteins separately(*77*). Nowadays protein A, protein G and protein A/G are all commercially available and used depending on the type and selectivity of the assay performed.

Protein L

Over the years, many other Ig binding proteins have been identified in gram-positive bacteria. The only one used frequently for research purposes is protein L, a cell wall molecule of *Peptostreptococcus magnus*. This anaerobic gram-positive bacterium is a normal commensal of the gastrointestinal tract but it can become pathogenic and cause a variety of symptoms. Protein L was discovery only a few years after protein G and has the advantage of interacting with the kappa light chain instead of the heavy chain which is bound by protein A and G (*78*). Due to this specific property, protein L can bind all classes of immunoglobulins, only restricted to those containing kappa light chains. This makes it an extremely useful tool for isolation of FaB and scFv fragments, which do not contain Fc regions(*79*). ScFvs are small antibody fragments composed of only the variable domains fused together by a linker. They have received much attention due to their therapeutic use, their small size enabling efficient tissue penetration and economic production. Their small size also allows them to target antigenic sites inaccessible for whole antibodies(*80*). Research now focuses on their use as intrabodies, intracellular antibody fragments capable of suppressing

intracellular proteins (*80-81*). This could be very useful against tumour cells by inhibition of crucial oncogenes. Efficient purification of scFvs is therefore of great importance and protein L is the main tool to do so.

Clostridium histolyticum

The bacteria

Clostridium histolyticum is a gram-positive anaerobic species and the causative agent of gas gangrene in humans.

Collagenase

Discovery and immune evasion strategy of collagenase

Collagenase is a zinc metalloproteinase capable of cleaving collagen in its native form. About 30% of all mammalian proteins are collagen, making it the most abundant protein and its main function is providing tissue structure. It is the main component of the extracellular matrix but it is also found inside cells. Collagen molecules together form long fibres and each collagen molecule has a typical aminoacid sequence with a glycine residue at approximately every third position, which is also the cleavage site for collagenase (roche)(*82-83*).

C. histolyticum uses collagenase to bypass one of the basic innate immune defences that our body has developed: the skin. Therefore, the bacteria is able to quickly invade tissues and escape circulating immune cells(*83*). Destruction of the extracellular matrix could also help in the spreading of the toxins produced. Collagenase production is therefore one of the immune evasion strategies of the bacteria that is essential for colonization and spreading in host tissues.

Laboratory use of collagenase

Collagenase from *C. histolyticum* has been a widely used enzyme for tissue separation since it became commercially available in 1960s (*84*). A number of cell types has been isolated using collagenase including myocytes from the heart, pancreat islets, mammary epithelium and osteoblasts, which can then be used for the generation of primary cell cultures(*85*).

Clinical use of collagenase

Collagenase for *C. histolyticum* has acquired special attention due to its therapeutic opportunities. It is used for isolation of pancreatic islet cells which are subsequently used for transplantation as treatment of diabetes type 1 (*84*), although this is still an experimental treatment. Collagenase was also shown to be effective for wound

healing, especially for debridement and enhanced closure of the wound (*86-87*). Recently, collagenase was shown to be an effective therapy for patients with Dupuytren's disease, a progressive genetic disease of excessive collagen production with an estimated prevalence between 3-6% among whites(*88*). Patients suffer from collagen nodules in the hand ultimately leading to limited hand function and treatment options are limited to surgery. Injection of Xiaflex, the drug name for collagenase from *C. histolyticum*, significantly reduced symptoms in a phase 3 clinical trial.

These promising studies all show the usefulness of collagenase in laboratory and medical settings.

Vibrio cholerae

The bacteria

Vibrio cholerae is a major pathogen that has killed more than a million people since its first outbreak in 1816. There have been many outbreaks since then and still many people become infected each year, especially in third world countries.

V. cholerae is a gram negative bacterium that causes watery diarrhea quickly leading to dehydratation and death(*89*). The main virulence factor involved is the cholera toxin.

Cholera toxin

Discovery and immune evasion strategy of cholera toxin

Cholera toxin (CT) belongs to the family of AB toxins. The pentameric B-subunit binds the sphingolipide GM1 on intestinal epithelial cells and the heterodimeric A subunit forms the active part(*89*). Upon binding GM1, CT is taken up in endosomes and winds up in the ER through retrograde transport. Here the toxin disassembles into the A_1 and the A_2-B subunits and the A_1 translocates to the cytosol via the SEC pathway. The A_1 subunit causes ADP-ribosylation of $G_s\alpha$, which blocks the intrinsic GTPase activity of this G-protein and thus leads to constitutive activation (figure 8) (*89-91*). The target of $G_s\alpha$ is adenylate cyclase and overactivation of this protein causes overproduction of cAMP, which is responsible for the clinical symptoms of cholera. It causes the excessive secretion of ions followed by water through activation of PKA amongst others.

CT is not only responsible for the clinical symptoms of the disease; it also modulates the host immune system. It does so by inhibiting the production of interleukin 12 from monocytes and dendritic cells through inhibition of IRF8(*92-93*). IL-12 is an important stimulator of Th1 driven immune response and it stimulates the production of IFN-γ, both of which are inhibited by addition of CT in vivo(*92*). The inhibition of a Th1 driven immune response contributes to the escape of *V. cholerae*. The bacteria itself remains in the bowel where it is not recognized by cells of the immune system. CT however resides intracellular and the inhibition of the Th1 response prevents the activation of NK cells and cytotoxic T cells ensuring that toxin infected cells are not destroyed. This allows the toxin to cause the watery diarrhea which aids in the secretion of the multiplied bacteria from the bowel for spreading to new individuals.

Immune evasion by inhibition of IL-12 is not restricted to *V. cholerae*; an end product of bacterial metabolism, sodium butyrate, was shown to have the same effect as does adenylate cyclase toxin from *Bordella pertussis*(*94-95*).

Laboratory use of cholera toxin

The characteristics of both the A and the B subunit of cholera toxin have led to the generation of excellent laboratory tools.

Activation of G-protein signalling

Many signal transduction pathways are initiated by G-protein coupled receptors and the A subunit of cholera toxin has been an invaluable tool to study their action(*96*). The toxin was discovered to have an activating effect on adenylate cyclase though the presence of G-proteins was not known at that time. Therefore, the toxin has been very useful in identifying the structure and function of the heterotrimeric G-proteins(*97*). As the toxin specifically activates some G-proteins but not all, this has been used to identify the specific pathways involved upon receptor activation. Nowadays, many different G-proteins are known but the toxin is still used to study new pathways and the effect of overactivation. It is commercially available at many pharmaceutical companies and it is extensively used.

Membrane labelling

The B subunit of cholera toxin is used for molecular studies of cell membranes. Its receptor, ganglioside GM1, is part of cell membranes where it is specifically enriched in lipid rafts. Lipid rafts are membrane microdomains that are thought to organize the assembly of molecules necessary for signal transduction cascades but their precise function is still under debate and study. The fluorescently labelled B subunit is frequently used for detection of lipid rafts and associated molecules can be analysed by fluorescent microscopy or FRET(*98*). Recently, cholera toxin was used to study localisation and endocytosis of MHCII molecules in lipid rafts(*99*). However, the use of cholera toxin as a lipid raft marker is not the only possibility as the B subunit can be used as a neuronal tracer (figure 7). Neurons contain many GM1 molecules and, when injected close to these cells, cholera toxin is taken up and transported to the cell body by retrograde transport. Using labelled toxin or a secondary antibody, the cellular organisation and connectivity of neurons can be

studied(*100*). Cholera toxin was also fused to saporin to selectively kill the cells that bind the toxin, thus enabling functional studies of the neurons (*101*).
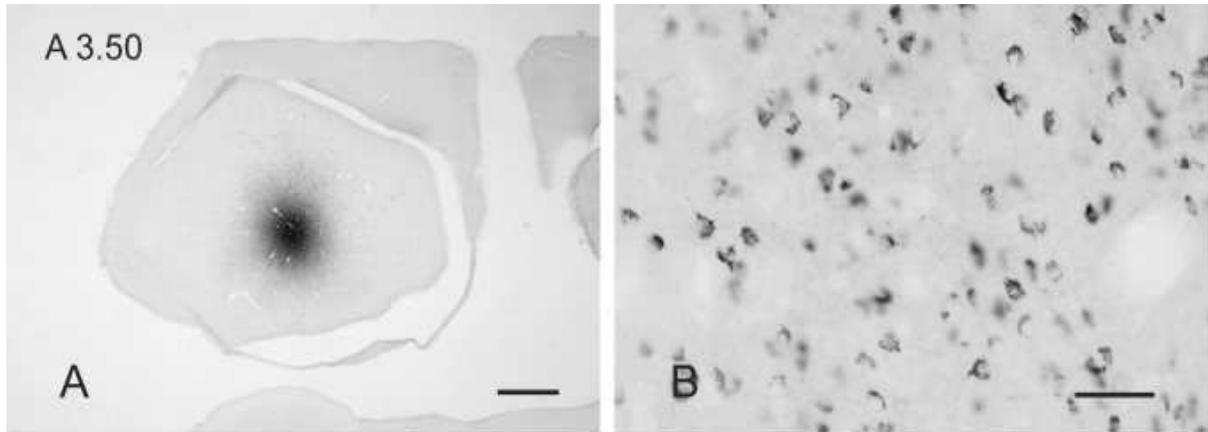
Figure 7. Cholera toxin as a neuronal tracer.
A. The B-subunit of cholera toxin was injected caudal in the nidopallium caudalis centralis (NCC) of a pigeon. **B.** Nerves to the front of the injection site are labeled by the injection, illustrating the direction and location of the nerves in this region. Picture was edited from Atoji et al. (*6*)

Bordella pertussis

The bacteria

Bordella pertussis is a gram negative bacterium that causes whooping cough in humans. It infects the respiratory epithelial cells where it produces toxins to destroy the cells. Many people used to die from the disease but this was greatly reduced by the introduction of an effective vaccine. However, according to the WHO 39 million people still get infected annually worldwide. The bacteria produce many toxins, but especially pertussis toxin and adenylate cyclase toxin have found their way into biomedical research laboratories.

Pertussis toxin

Discover and immune evasion strategy of pertussis toxin

Pertussis toxin (PT) is an AB toxin that shares many similarities with CT. It is composed out of a single A subunit and 5 B subunits(*102*). The B subunit binds carbohydrate receptors on the cell surface and the toxin is subsequently internalized via endocytosis. It is transported from the Golgi network to the ER, where the toxin disassembles and the A subunit translocates into the cytosol (*103*). Here it catalyzes the transfer of ADP to a cysteine residue of the alpha subunit of heterotrimeric G-proteins which blocks the release of GDP and thus leaves the protein inactive.

However, PT inactivates G_i and G_0, which are inhibitory G-proteins. Therefore, PT also indirectly activates adenylate cyclase, like CT (figure 8).

PT was shown to be essential for immune evasion of *B. pertussis* by inhibiting chemokine production from alveolar macrophages in mice, which inhibits chemotaxis of immune effector cells like neutrophils (*104*). PT may also block neutrophil chemotaxis directly
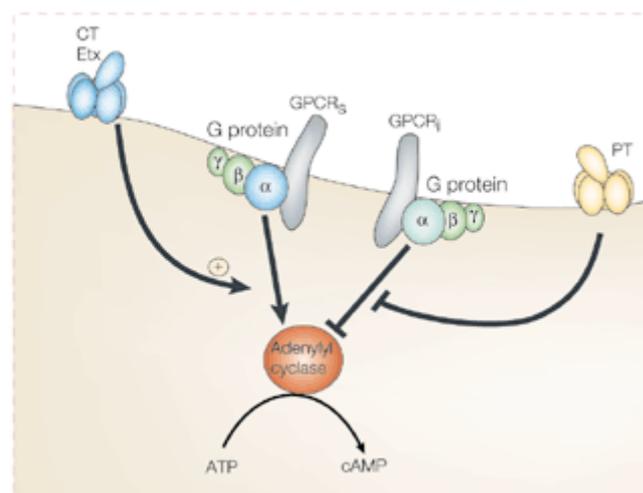
Figure 8. Toxins affecting G-protein signalling Cholera toxin (CT) causes overactivation of activating G-proteins while pertussis toxin (PT) inhibits inhibitory G-proteins, both resulting in overactivation of adenylate cyclase. Picture edited from Schiavo et al. (*2*)

since most chemokine receptors signal via G_i proteins, but this is still under investigation(*105*).

Recently it was shown that the B subunit also has effector functions independent of the A subunit. It is capable of reducing CXCR4 receptor levels on circulating T lymphocytes, therefore blocking their migration(*106*). Thus, PT is an essential virulence factor to escape host immunity by blocking migration of immune cells to the site of infection.

Laboratory use of pertussis toxin

PT is used to study G-proteins in a similar way as CT. It has been essential in the discovery of G_i and G_o proteins and there distribution. Smooth muscle cells for example were discovered to be dependent on G_i proteins for contraction by studies using PT(*107*). The toxin can also be used for direct visualization of the presence of G-proteins by using ^{32}P labelled ADP. Incorporation of this molecule in G-proteins can then be measured radiographically(*107*).

Thus far, CT and PT have been essential tools for studying the molecular details of G-protein signalling.

Adenylate cyclase toxin

Discovery and immune evasion strategy of adenylate cyclase toxin

Besides PT, *B. pertussis* secretes another toxin with important immune evasion properties. Adenylate cyclase toxin (CyaA) is an exotoxin that catalyzes the production of cAMP, thereby activating many intracellular signalling cascades. The C-terminal of CyaA binds the CD11b/CD18 integrin on the surface of innate immune cells. The N-terminal of CyaA, containing the adenylate cyclase activity, is then translocated into the cytosol (*108*). Here it is activated via a calmodulin dependent mechanism and causes over production of cAMP.

The excessive cAMP levels potently reduce the capacity of dendritic cells (DCs) to produce IL12 and therefore inhibit the Th1 response and the production of many pro-inflammatory cytokines such as interferon. But CyaA also increases IL 10 production in DCs via enhancement of ERK and p38MAPK phosphorylation (*95, 109*). This specific cytokine profile stimulates the activation of T_{reg} cells that suppress the immune system and induce a tolerogenic response, aiding the escape of the bacteria.

CyaA also targets phagocytes, in which it inhibits phagocytosis, chemotaxis and eventually leads to apoptosis (*110-112*).

Interestingly, CT uses the same mechanism of immune escape although CyaA toxin has a more profound effect. This might be due to the receptor expression levels, which are restricted to immune cells for CyaA while receptors for CT are widely distributed thus limiting the amount of toxin available to infect phagocytic cells.

Furthermore, CyaA has the same effect as PT by increasing the intracellular cAMP levels. However, PT is dependent on other cellular events as it only removes the inhibitory signal, while CyaA directly increases cAMP levels. CyaA is therefore more potent, but the two toxins do act in concert.

Other parts then the known enzymatic part of the toxins may also aid in immune escape as was shown for the B subunit of PT, thus establishing a difference between the two toxins.

Laboratory use of adenylate cyclase toxin

Bacterial two hybrid system

CyaA has been used to create a bacterial-two hybrid system for detecting protein-protein interaction(*113*). This is accomplished by fusing the T18 and T25 catalytic domains of CyaA to two proteins of interest and expression in *E. coli*. These T18 and T25 are capable of catalyzing cAMP production but only in the presence of calmodulin, which is absent in *E. coli*. When the two proteins of interest interact with each other, the T18 and T25 catalytical domain is restored, imitating the calmodolin effect, and cAMP production will occur which is linked to the activation of a reporter gene. There are advantages of using this system over the classical yeast-two hybrid system like the high transformation efficiency in *E. coli* enabling screening large and complex libraries. Also, yeast-two hybrid screens are based on restoration of a transcription factor upon interaction of the two proteins, which must therefore be able to translocate into the nucleus. This is no longer necessary with the bacterial system, where the cytosolic cAMP is produced as an intermediate.

Reporter protein

CyaA is used as a reporter protein to detect efficient insertion of bacterial proteins in eukaryotic cells. This is based on the fact that CyaA function requires calmodulin which is not present in bacteria. By fusing a recombinant CyaA without the

translocation domain, to a protein of interest, entering into eukaryotic cells can be detected by the presence of calmodulin there. This will activate the CyaA part and cAMP will be produced and can be measured. The system was created for studying the Yersinia type III secretion system. By using recombinant CyaA fused to Yersinia secretion proteins missing certain elements, the essential secretion signals and other functional domains involved were discovered(*114-116*).

This method has been further exploited for screening bacteria for secreted and membrane associated proteins. Tu et al. inserted the CyaA gene in a mini-TN5 transposon which can insert the gene anywhere on the bacterial DNA(*117*). When functional fusion proteins are created of CyaA and bacterial membrane associated or secreted proteins, this can be detected when calmodulin and ATP are present in the medium surrounding the bacteria. This is a very useful tool to detect novel virulence factors of bacteria.

Neisseria gonorrhoeae

The bacteria

Neisseria gonorrhoeae is a gram negative bacterium causing the sexually transmitted disease Gonorrhea. It is related to the *N. meningitidis*, the causative agent of meningitis. Both pathogens colonize human mucosal surfaces.

IgA1 protease

Discovery and immune evasion strategy of IgA1 protease

Several Neisseria strains as well as hemophilus influenza secrete IgA1 proteases that specifically cleave human IgA1 at the hinge region and therefore contribute to the escape of the pathogen. The enzyme is a serine protease excreted into the surroundings where it cleaves the proline rich hinge region in the Fab part by recognition of pro-pro-X-pro sequences.

The protein is thought to play a role in immune evasion by inhibiting agglutination and recognition by effector cells mediated by IgA. Also, the Fab region of Iga that remains bound to the bacterial surface after cleavage by IgA1 protease, blocks antigenic sites for intact antibodies. The IgA1 protease is very immunogenic, although antibodies from previous infections are not effective against new strains because of the heterogeneity of the protease(*118*). Additional studies to indentify the precise immunological implications are needed but are hindered by the limited use of animal models due to the selectivity of the protease for human IgA1.

Laboratory use of IgA1 protease

As the IgA protease from *N. gonorrheoe* is very site specific, it can be useful as an endopeptidase in laboratory settings. The farmaceutical company MoBitec has developed a strategy for the expression and purification of proteins using this protein. First, a fusion protein is made between the protein of interest and beta-lactamase with the signal sequence PRO-PRO-Y-PRO in between them. This construct is transfected in bacterial cells were production occurs. Beta-lactamase then enables efficient purification from the lysed cells using phenylboronate columns, from which the protein of interest can be removed by proteolytic cleavage by IgA1 protease.

Listeria monocytogenes

The bacteria

Listeria monocytogenes is a gram positive foodborne bacterium that is capable of intercellular survival in eukaryotic cells. Outbreaks occur on a regularly basis and cause gastroenteritis in healthy individuals. Different surface proteins of the bacteria allow it to enter cells via the host endocytic pathway, but the pathogen quickly escapes from the endosomes into the cytosol using the pore forming toxin listeriolysin-O (LLO) (*13*). The bacteria can also infect macrophages which ingest the pathogen via phagocytosis. Nutrients in the cytosol allow the bacteria to replicate and it infects new cells by an exceptionally inventive strategy. It produces the protein ActA that induces actin polymerization which propels the bacteria through the cell membrane into a neighboring cell. Here the action of LLO allows the escape from the vacuole into the cytosol and the whole process is repeated (figure 9).

A.

B.
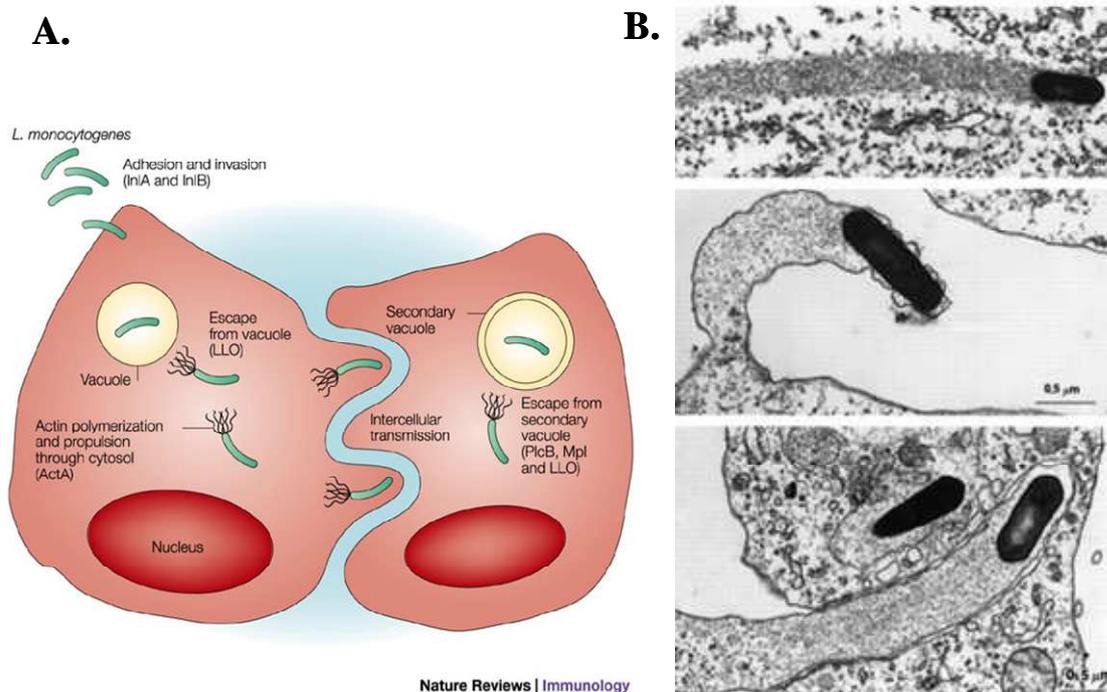
Figure 9. Listeria Monocytogenes infection
A. L. Monocytogenes enters cells via endocytic or phagocytic pathway and escapes from vacuole using LLO. In cytosol, actA expression causes actin polymerisation and infection to neighbouring cell. Here, LLO and other proteins are needed to escape secondary vacuole. Picture copied from Pamer 2004(*3*) **B.** Electron microscopy images of actin polymerisation (top), cell spreading (middle and below). Picture copied and edited from Cossart et al. (*10*)

Clearing of the bacteria is dependent on macrophages and especially memory CD8$^+$ T-cells which kill infected cells. Innate immunity is usually not sufficient as the bacteria quickly escape the phagosomes before maturation into functional lysosomes. The adaptive response mediated by CD8$^+$ T cells is needed to effectively kill infected cells([119]).

Listeriolysin-O

Discovery and immune evasion of listeriolysin-O

The gene for LLO is structurally and genetically homologous to streptolysin-O which was critical for the discovery of the gene([120]). LLO contributes to host evasion as it allows the phagosomal escape of the bacteria. LLO is thought to form a β-barrel pore in the vacuole membrane and the optimal pH is below 6. This pH optimum restricts the active enzyme to the phagosome and ensures that the protein is inactive in the cytosol to prevent lysis of the cell membrane, which would cause cell death([121]). The pH dependency is regulated by three amino acids, which cause denaturation of the protein in a neutral environment.

Laboratory use of listeriolysin-O

LLO can be encapsulates in liposomes and used for the cytoplasmic delivery of macromolecules([122]). When liposomes are endocytosed by target cells, LLO can mediate the escape into the cytosol. This can be used for efficient delivery of drugs, but it was also identified as a vaccination strategy against tumours and intracellular pathogens, as delivery of antigen to the cytosol causes antigen presentation via MHC class I([122-123]). Using listeriolysin as antigen delivery strategy has been extensively studied and most studies use a recombinant *L. monocytogenes*, in which essential virulence factors like ActA have been deleted. This bacterium is non pathogenic though it is still capable of infection and escape to the cytosol through LLO. Antigen sequences incorporated into the bacterial genome will be expressed and presented in MHC class I inducing a CD8$^+$ T cell response though antigen presentation though MHC class II will also occur from the phagosomal compartment([124]). The efficacy of this strategy has been studied in many mouse models and the first clinical trial was recently published ([123, 125-126]).

LLO is also used for the delivery of shRNA for therapeutic gene silencing. In a strategy called transkingdom RNAi, non-pathogenic *E. coli* are equipped with a TRIP

plasmid. This plasmid encodes the shRNA, LLO and Invasin. Invasin is needed for target cell binding via integrins causing endocytosis. LLO mediates escape from the endocytic vesicles enabling the release of shRNA into the cytosol where it binds target mRNA(*127-129*). This technique is very promising for treatment of cancer through silencing of key oncogens. But it may also serve as a strategy to study in vivo gene function in mouse models.

Discussion

It is remarkable how many bacterial immune evasion proteins are nowadays used as laboratory tools. From protein labelling to membrane permeabilisation to nanotechnology, these proteins appear to have found a role in many disciplines. Currently, bioinformatical approaches allows us to structurally modify these evasion proteins to enable their use for different purposes. Cholera toxin was shown to have useful adjuvant properties for vaccines due to its strong activation of Th2 responses, but administration has several toxic side effects. Researchers are trying to create chemically engineered proteins that contain the adjuvant activities of cholera toxin without the side effects (*130-131*).

This is not the only bacterial evasion protein that has possible clinical applications. Clinical studies using protein A immunoadsorption have been used for treatment of chronic lymphocytic leukemia, rheumatoid arthritis, Wegener's disease and many others, especially when patients are resistant to conventional therapies(*132*). And currently researchers are investigating the use of adenylate cyclase toxin for antigen delivery, using the N-terminal translocation domain(*133*). Also, adenoviral injection of the streptolysin-O gene in tumor cells induces necrotic cell death thus providing a potential cancer therapy(*134*). Furthermore, as mentioned above, lysteriolysin O and collagenase also have therapeutic relevance. Thus, bacterial immune evasion proteins provide us with excellent tools to combat disease.

However, these bacterial proteins are also used for less obvious purposes. For example, protein A is used in the meat industry to measure meat tenderness(*135*). Protein A covalently linked to gold electrodes is used to immobilize calpastatin antibodies, a marker for protein proteolysis and hence tenderness. This enables measurements of capastatin levels in meat samples which is useful to determine the quality and the price of meat.

The α-hemolysin nanopore has found a non-biological utility in the detection of explosives. These stochastic biosensors have been redesigned for the detection of TNT contamination, a highly explosive substance, and for revealing components of liquid explosives (*136-137*).

The IgA1 protease from *N. gonorrhoeae* can be used to remove heavy metal contamination from soil and water (*138-139*). The transportation domain of the IgA

protease is fused to a metallothioneine, a cysteine rich protein that can capture heavy metals. Introducing this fusion protein in bacteria will results in expression of the metallothioneine on the cell surface because of the transportase domain. When these bacteria are added to contaminated soil, they immobilize the heavy metals which will reduce the toxicity. These are merely a few examples of the non-biological uses of these proteins and we are only just beginning to discover these proteins.

The above mentioned examples show the use of bacteria immune evasion proteins in many different settings. Future research will undoubtedly find much more interesting purposes for them. An interesting idea is the ability to combine these different proteins. Sortagging has the disadvantage that proteins need to reside on the cell surface as sortase A is not present in the cytosol. Perhaps it might be possible to combine sortagging with reversible permeabilisation using streptolysin-O to extend this method to intracellular labelling. Streptolysin pores can accommodate proteins up to 150kDa and sortase A is very small, only around 25kDa. However, the labelled probe must also be transported into the cytosol and excess must be washed away, which might pose a problem. But a solution to these problems might be found in the near future.

Nowadays, rapid sequencing techniques combined with computational tools allow us to more easily identify new bacterial immune evasion proteins. As illustrated by protein G, L and M, many species have evolved proteins with similar functions although there is no sequence homology between them. Searches based on structural homology may therefore be able to find functionally related proteins in other species. Furthermore, many bacterial virulence factors are located on pathogenicity islands, specified genomic islands which can be transferred to closely related strains(*140*). Modern techniques can recognize these islands and this will inevitably lead to the identification of new immune evasion proteins. Given the extraordinary use of these proteins for life scientists, medicine and other disciplines, the discovery of new evasion proteins and the details of their molecular function should have a high priority. Who knows how many useful new proteins will emerge; tens or hundreds or thousands? It is not unimaginable that the latter will turn out to be true. New bacteria are still being discovered and we have not even been able to identify the molecular mechanisms of bacteria that we've known for a hundred years.

Furthermore, the arms race between bacteria and humans will probably never end. They will constantly attempt to find new ways to counter our defence system while we try to eliminate their presence altogether. We have formed a parasitic relationship with these bacteria; they need us for their survival but we rely on their sophisticated strategies for our laboratory tools, environmental purposes and therapeutic applications. It is remarkable how we can change immune evasion proteins nowadays to our own benefit and discoveries in the near future will undoubtedly expand this emerging field further.

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