Quorum Sensing inhibition to battle infectious diseases

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Introduction

Antibiotic crisis

In the year 1946, Alexander Fleming was laureated with a Nobel prize. He is celebrated for one of mankind´s most important discoveries he made already in 1928: Penicillin. A metabolite of a mold, set on a course to revolutionize the medical world.

Already in 1946, Alexander Fleming warned that:

> "The time may come when penicillin can be bought by anyone in the shops. Then there is the danger that the ignorant man may easily underdose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant."

These words were already more than a prophetic warning but based on the scientific understanding of these compounds and the understanding of evolution itself. In recent years it has become clearly evident that common antibiotics lose efficacy against a broad range of pathogens.

At the beginning of the last century Paul Ehrlich coined the word "magic bullet", for a compound which is able to kill bacteria in a directed way without affecting eukaryotic cells. With the discovery of antibiotics this dream became a reality, and the dream became a standard treatment, which is so effective that the people spend little thought on antibiotic treatments and the bacteria that cause diseases. Which in turn leads to an overuse of the once medical miracles and renders them ineffective. A major driving factor for this is the usage of antibiotics in farm animals. It is estimated that in the United States 80% of the annual antibiotics consumption is attributed to the use in livestock [1]. The overuse of antibiotics for farm animals contributes to the formation of multi resistant bacteria and the exposure of humans to these multi-drug resistant bacteria (MDR) are thought to contribute significantly to the raise of resistant clinical infections [2]. So, what are the alternatives for antibiotics? To answer this question, it is of utmost importance to understand the mechanisms of bacterial infections and finally how bacteria coordinate their "attacks" on hosts equipped with a highly efficient immune system? How do the simplest unicellular organisms know when to strike?

Bacterial communication

In the 1970´s sociobiology gained major recognition by publications of Edward O. Wilson. He proposed a definition of communication: "...the action of or cue given by one organism [the sender] is perceived by and thus alters the probability pattern of behavior in another organism [the receiver] in a fashion adaptive to either one of both participants"

(Wilson 1975). The common belief attributes communication to higher organisms of the animal kingdom, also including insecta. At that time nobody was considering bacteria being able to communicate or not to mention seeing bacteria as social beings.

However several years before, scientists described the fascinating bioluminescence of *Aliivibrio fischeri*, a Gram-negative bacterium living in almost every maritime environment on earth [3]. These bacteria live in a symbiotic relationship with the "Hawaiian Bobtail squid" *Euprymna scolopes*. The cephalopod takes a great effort to acquire, cultivate and maintain a pure culture of *A. fischeri* in a special light organ located in the squid's mantle. The interesting part about this finding was not only the emission of light per se, but more the timing of the bacteria when they "decided" to produce the necessary proteins for their luminescence after reaching a certain number. Today this "quorum sensing" (QS) is the paramount example of bacterial communication and the luminescence of *A. fischeri* became the textbook example. Accordingly, the QS key enzymes are named LuxI type synthases and LuxR type receptors referring to their involvement in bioluminescence [4]. Over the following decades scientists discovered more and more traits that are controlled by QS and hence controlled by the population density of microorganisms. The layout of QS systems is highly conserved and surprisingly simple, the basic QS circuit consists of signaling molecules the so-called auto inducers, proteins that synthesize the same – LuxI type synthases and proteins that recognize the autoinducers, the LuxR type response regulators. In most cases the LuxI type synthase is upregulated by the cognate response regulator LuxR in a positive feedback loop, which is also the explanation why the communication molecules are described as auto-inducers [5]. It is important to mention that next to the N-acyl-homoserine lactones there are also different forms of autoinducers used by different bacteria [6]. However, AHLs represents a bacterial language, which is spoken in different form by a lot of Gram-negative pathogens and thus makes them from a scientific perspective very interesting. QS systems in Gram-positive bacteria differ significantly from Gram-negative bacteria as they employ oligopeptides as signaling molecules and membrane bound two-component sensor kinases and cytoplasmic transcription factors [7].

Acinetobacter baumannii

In 2017 the WHO released the "Priority pathogens List". It includes Gram-negative and Gram-positive bacteria classified as pathogenic or facultative pathogenic. The hierarchy of the bacteria within the list is based on the urgency to develop antimicrobials against the different species from medium to critical. *Acinetobacter baumannii* is the

number one pathogen on this list and is classified under "critical". *A. baumannii* is a Gram-negative gammaproteobacterium with a coccoid shape, it is non fermentative and it is not able to form spores [8]. *A. baumannii* is considered an emerging nosocomial pathogen found in IC units around the world. It is classified as a highly virulent pathogenic bacterium, which forms together with other notorious pathogens the ESKAPE-group, which is an acronym formed by the members of this group (Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter spp*.) [9]. Infections caused by this bacterium include hospital acquired respiratory tract infections, urinary tract infections, nosocomial meningitis and soft tissue infections [10]. In this background it is fascinating that reports about community acquired infections are extremely scarce even though it can be isolated from environmental samples from soil and freshwater all over the world [8]. Many strains can be classified MDR since isolates showed resistance towards several classes of antibiotics, such as aminoglycosides, cephalosporins, penicillins and meropenem [11,12]. Infections with carbapenem resistant *A. baumannii* strains show a mortality rate of 33.7%, even though the patients received an appropriate antibiotic treatment [13]. This highlights the need for finding additional treatment options for this pathogen. However, in contrast to other pathogens the precise reason why and how *A. baumannii* is such an extremely dangerous pathogen is unsolved. There is no mayor virulence factor such as a toxin identified, which could serve as an explanation why it is so dangerous in nosocomial environments [10]. Researchers suggested the clinical perseverance can be explained by its ability to withstand and survive very harsh conditions. It was described as a persist and resist strategy, which is underscored by its remarkable ability to withstand desiccation on abiotic surfaces and its resistance to disinfectants [14]. Another way of shielding itself from environmental challenges is its ability to form a biofilm. It is able to attach to abiotic surfaces in an very efficient way using pili [15]. As a matter of fact, it could be shown, that *A. baumannii´*s ability to attach to abiotic surfaces and the formation of biofilms is for a major part controlled by its quorum sensing system [16]. The main QS system in *A. baumannii* follows the "classic" QS circuit blueprint. It is consisting of AbaI, a LuxI type AHL synthase, and the corresponding response regulator AbaR. A lux-box DNA consensus sequence in front of the AbaI gene hints that this system is auto induced by its own autoinducer 3-OH-C12-homoserine lactone.

Pseudomonas aeruginosa

P. aeruginosa is an opportunistic pathogen and predominantly leads to infections in immune compromised patients and patients with an

already existing primary disease. The cause of infections can range from milder skin irritations over chronic lung infections towards acute life-threatening infections[17,18]. It is for example causing an inflammation of body hair follicles, the medical term for this is "hot tub folliculitis", since the patients infect themselves in hot tubs or water slides, which are perfect incubators for freshwater bacteria [17]. This skin rash does not require any treatment and is normally not dangerous to the hosts. Unfortunately, this mild disease is rather the exception than the rule. On the other hand *P. aeruginosa* is accountable for severe hospital acquired infections such as "hospital acquired pneumonia", urinary tract infections as a result of catheterization as well as blood stream infections [19]. A comprehensive study from the United States shows that 7.5% of all healthcare associated infections are caused by *P. aeruginosa* [20]. As a result of its clinical importance, *P. aeruginosa* is considered to be a model organism for many virulence related traits.

P. aeruginosa is known to man since the mid-19th century, when physicians described a greenish discoloration in wound bandages. In the year 1882, Carle Gessard was the first person to connect the characteristic color to a bacterium and reported in a publication entitled "On the Blue and Green Coloration of Bandages" his findings [21]. Still today *P. aeruginosa* is a pathogen of interest in the medical field.

P. aeruginosa and *A. baumannii* are the top ranks of the abovementioned WHO priority pathogens list and their common ground is their multi-drug resistance and the very interesting fact, that both organisms are described as opportunistic pathogens who are widespread in all kinds of ecological niches.

Biofilm

Bacterial biofilms are omnipresent in nature, they can be found on all kinds of surfaces in almost every ecological niche on our planet [22]. Especially in nosocomial environments biofilms pose a major threat to patients worldwide. It is estimated that biofilms are responsible for 60% of all infections treated by physicians [23]. Bacteria also colonize surfaces of indwelling medical devices like catheters or infusion catheters and in this way the pathogens have an entryway into a patient host. Since catheters are often applied to immune compromised patients the impact the bacteria can have in the human body is even worse [24]. In material science researchers explore for many years materials and surface structures to eliminate or reduce bacterial attachment and colonialization on medically relevant surfaces [25].

Furthermore, the bacterial biofilm offers some level of protection against antibiotic treatment as well the host immune system. Bacteria organized in biofilms show fundamental differences with planktonic bacteria in tolerance towards antibiotics and the clearance through

immune cells [26]. This protective effect is for one caused by an extracellular matrix and for the other by different metabolic states of the bacteria persisting within the biofilm [27]. The extracellular matrix consists of external DNA, which is believed to be a structural component and different polysaccharides as well as proteins and lipids [28]. Biofilms should not be seen as an impenetrable shield which is completely cutting off the bacteria from there surrounding environment, but rather as a complex matrix with several micro-environments, which are highly dynamic [29]. *P. aeruginosa* secretes ß-lactamases into the outer matrix layer, which are actively degrading ß-lactam antibiotics [30]. Especially in patients with chronic lung diseases like cystic fibrosis (CF) the therapeutic management of pathogens and biofilms significantly increases the survival of patients. Commonly, lungs of CF patients are colonized first by Staphylococcus aureus and *Haemophilus influenza*. Another common pathogen that was identified in the mucus of CF patients is the *Burkholderia cepacia* complex (Bcc). In the later stages of the disease in 50% of the patients the dominating bacterial species is *P. aeruginosa* [31]. For *P. aeruginosa* it could be shown that while colonizing CF patient´s lungs the bacteria are organized in biofilms and can be detected in the intraluminal mucus [32]. This highlights that in order to control infectious disease it is crucial to understand biofilms and ultimately control biofilms.

QQ Treatment

The inhibition of the QS system in pathogenic bacteria offers an alternative approach aimed at attenuating the virulence. [33]. Facing the emergence of many MDR bacterial strains throughout IC units worldwide, quorum sensing inhibition can offer a valuable treatment strategy [34]. Research within the last decades showed in many cases that virulence factors of pathogenic bacteria could be significantly reduced by inhibiting the QS system, like in the case of *P. aeruginosa*. Recent publications highlight that the QQ provoked attenuation of virulence factors mediates a prolonged survival in rodent models. Hraiech et al, reported in their publication from 2014 a beneficial effect of the lactonase Sso-Pox-I on the course of a *P. aeruginosa* pulmonary infection in a rat model [35]. Also, in a later study from 2018 it could be shown by Utari et al, that the employment of the Ntn-hydrolase PvdQ shows a significant better survival rate of mice, previously challenged with a *P. aeruginosa* pulmonary infection. It could be shown that the enzyme does not have a toxic effect on epithelial cell lines and also does not cause any abnormalities in the physiology of the tested mice [36].

The term quorum quenching is applied to all forms of interference with the QS system of bacteria. More precisely, there are several ways

to disrupt this system. One option is the inhibition of the synthesis of the auto inducer molecules [37]. Another way is to target the messaging molecules directly outside of the cell [38]. Lastly the interference with the signal recognition also leads to the disconnection of the QS dependent virulence factors. Even though these mechanisms show significant differences in the way how QQ is achieved the result basically remains the same. All QQ treatments have in common the aim to attenuate virulence factors. This means that in contrast to antimicrobial treatments the compounds or enzymes do not target vital bacterial physiological processes, but render cells avirulent and alive. The actual clearance of the infection is achieved by the host´s immune system. The formation of microbial resistance towards this treatment is hypothesized to be low, since QQ targets non-essential pathways that are normally active to coordinate group behaviors in bacterial population while still attenuation virulence factors. Conversely QQ does not exert a strong selective pressure on the organisms and this means in turn that a formation of resistance against QQ treatment is less likely to appear. This is beneficial for example in the case of managing chronic infections such as respiratory infections in immune compromised patients and those suffering from cystic fibrosis. In this work the emphasis is on the extracellular degradation of auto inducer by enzymes.

Enzymatic quorum quenching – PvdQ

Quorum sensing inhibition with the help of enzymes degrades auto-inducers outside of the cell or modifies the signal in a way that it is not recognized by the cognate response regulator. In both scenarios the external degradation or modification of the AIs leads to a lower titer of auto-inducers inside the bacterial cell which consequently will alter the expression profile of the bacteria [39]. In case of AHLs from Gram-negative bacteria the degradation can be achieved in two ways. Firstly, AHL lactonases can be used to cleave the homoserine lactone ring. Interestingly lactonases belong to the same superfamily as ß-lactamases, as they share a characteristic Zn^{2+} - HXHXDH binding motif [40]. Since lactonases recognize the homoserine lactone ring and cleave the same, the length of the acyl chain plays a minor role in the recognition of the AHLs. Lactonases can degrade a broader spectrum of AHLs with different acyl chain lengths [38]. However it is important to take into account that the lactone ring of the AHLs can recirculate at acidic pH, this means that under certain conditions the degradation of the autoinducers is reversible [41].

Secondly, it is also possible to modify AHLs in a way, that they are not recognized by the response regulator anymore. Oxidoreductases can oxidize ω-1, ω-2 and ω-3 carbons and hence are able to modify

in the case of AHLs the carboxyl group at the third carbon atom of the acyl side chain of AHLs [42].

It is also possible to interfere with QS signals of Gram-positive bacteria namely the AI-2 short peptides. The signal transduction of AI-2 is explained in chapter 2 and will not be discussed here in detail. It is speculated that if a specialized kinase is added to the bacterial culture externally, they will recognize AI-2 and phosphorylate the peptide. This in turn is thought to have the consequence that the signaling molecule will not be taken up by the cell and is not able to trigger the QS response in the cytosol.

PvdQ is an Ntn-hydrolase which is a part of the pyoverdine biosynthetic cluster. The maturation of pyoverdine takes place in the periplasm where PvdQ cleaves an acyl chain from the acetylated precursor PVDIq [43]. Pyoverdine is a fluorescent siderophore, which gives *P. aeruginosa* cultures the characteristic fluorescent green color. PvdQ belongs to the superfamily of N terminal nucleophile hydrolases, which is a diverse group of enzymes comprising amongst others penicillin acylases, aspartylglucosaminidases and the 20s proteasome. As different as the enzymes might look, they share common traits such as the ability of cleaving amide bonds and the presence of an N-terminal catalytic nucleophile (threonine, serine, or cysteine) [44]. Further these enzymes exist as an inactive pro-enzyme and undergo an autocatalytic maturation process [45]. Overall the sequence similarity amongst the hydrolases is low, however the N terminal nucleophile is nested in an characteristic αββα fold, which is common throughout the whole enzyme superfamily [44,46]. PvdQ is able to cleave AHLs at the amid bond between the homoserine lactone ring and the acyl chain. The crystal structure shows next to the characteristic abba fold and the N-terminal Serine and extended hydrophobic pocket, which can harbor the acyl chain of long chained AHLs and thus determines the specificity of the enzyme towards AHLs. Protein engineering studies show that upon modification of the pocket also short chained AHLs are accepted as substrate [47]. PvdQ is a potent QQ enzyme and it could be shown that it is able to attenuate virulence factors of *P. aeruginosa*.

A subclass of CF patients is shown to also develop a chronic infection with *B. ce*pacia complex. The Bcc complex can be isolated in the sputum of CF patients and leads to an significant decrease of long term survival of the patient [32]. *B. cepacia* predominantly produces the AHLs N-octanoylhomoserine lactone (C8-HSL) and N-hexanoylhomoserine lactone (C6-HSL) [48]. Both of these auto-inducers are not recognized by the QQ acylase PvdQ. Previous work by Koch at al, demonstrated that it is possible to alter the substrate binding pocket of PvdQ to also accept shorter chain AHLs such as C8-HSL. Further evaluation of the mutated enzyme in the context of QQ confirmed that C8-HSL is accepted as a substrate and it was

demonstrated that it could rescue *G. mellonella* larvae upon infection with *B. cepacia* [47].

Galleria mellonella infection model

Galleria mellonella is the caterpillar of the greater wax moth. It got its name from the fact that the caterpillar eats honeycombs in its natural habitat. The life cycle of the greater wax moth spans from a couple of weeks to several months, it is a holometabolous insect and develops through four distinct life stages: egg, larva, pupa, and adult moth. Mature moths lay their eggs in beehives, were the larvae hatch and can cause major damage to beehives. This makes them a parasite in the eyes of beekeepers all over the planet. It is estimated that *G. mellonella* is responsible for financial losses of several million dollars in the U.S alone and is even connected to the decline of feral and cultivated bee populations worldwide [49]. However, at the same time it is also appreciated by many people as a bait for fishing. From a researcher's perspective *G. mellonella* is extremely interesting since it is an established animal model to study host pathogen interactions. Over the last years infection models were developed for many important pathogens like *P. aeruginosa*, *A. baumannii*, *Burkholderia cepacia, Burkholderia mallei, Proteus mirabilis, Francisella tularensis, Bacillus cereus*, as well as pathogenic fungi like *Aspergillus fumigatus, Candida albicans* [50]. *G. mellonella* is a very promising non-vertebrate animal model which is an alternative to mammalian animal models and it has less ethical implications. Infection model experiments can be performed faster and more cost effective, which is a big benefit in early stages of for example drug development. The larvae have several biological and technical advantages over other non-mammalian animal models such as nematodes. Biologically advantages are that as an insect *G. mellonella* has a more advanced immune system and thus the results are more transferable to higher organisms. It´s complex innate immune system resembles the principles of the mammalian immune response, it has specialized cells in the hemolymph which are able of phagocytosing bacteria. It also shows a humoral immune response producing lysozyme and antimicrobial peptides [51]. Part of the immune response is the formation of melanin, which has the benefit that it is possible for the researcher to assess the reaction of the immune system by looking at the color change of the individuals.

Technical advantages of *G. mellonella* towards other non-vertebrate animal models are for example its size. For experiments larvae are chosen, which have a body weight of 250 – 350mg and a body length of around 2cm making the handling relatively easy and offering the advantage that the bacterial load to induce infections can be injected with high precision to each larva individually. In contrast to the

Caenorhabditis elegans model, where only oral infection is possible, which makes it hard to assess what the actual bacterial load per individuum is [50,51]. Finally, it is possible to perform *G. mellonella* experiments at a temperature range from 15°C to 37°C which offers the benefit that the bacteria can be grown under temperature conditions which would resemble the body temperature of mammals. In conclusion *G. mellonella* is a well characterized cost effective and quick to perform animal model, which can be a great help as a pilot system before moving on to more specialized and advanced animal models.

Scope of the thesis

In this work the focus lays on the QS mediated expression of virulence factors and more specifically the concept of attenuating the virulence of pathogenic bacteria by enzymatic quorum quenching. The approach of utilizing QQ as a treatment option will be explored in the following chapters. **Chapter 2** gives an overview about enzymatic quorum quenching with emphasis on the bacterial biofilm and its implications in infectious diseases. Following this, **Chapter 3** puts the focus on Ntn-hydrolases and asks the question about their physiological functions. **Chapter 4** shows a concept of how the QQ acylase PvdQ can be immobilized on medical relevant surfaces to offer a preventive measure against *P. aeruginosa* biofilm formation. **Chapter 5** shifts the focus from *P. aeruginosa* towards the pathogen *A. baumannii* and demonstrates that PvdQ is able to interfere with its biofilm formation. Further we could show that the impairment of the biofilm formation shows a significant prolonged survival of individuals in a *G. mellonella* infection model. In **Chapter 6** we explore the attenuation of the virulence factor pyoverdine of *P. aeruginosa* with small molecule inhibitors. We could identify a potent inhibitor of PvdQ, which is an important enzyme for the maturation of a pyoverdine precursor.