

# MogR regulates flagellar synthesis, cell motility and biofilm formation in the *Bacillus cereus* group

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Malin Josefsen



# ABSTRACT

Many bacterial species move by means of rotating flagella. The flagellum is a complex organelle, built up by dozens of different proteins, and its synthesis is usually tightly regulated by the cell. Motility plays a role mostly in the initial phase of an infection. The combination of motility and chemotaxis gives bacteria the opportunity to detect and search for nutrients and also provide the opportunity to colonize, when the environment is favorable. When the bacteria have found their target, adherence occurs and the initial phase of infection has started. In *Listeria monocytogenes*, a highly flagellated Gram-positive food-borne pathogen which causes life-threatening infections in humans, flagellar gene expression is down-regulated when the bacteria encounter temperatures above 37°C. This has never been observed before in any bacteria, and at 37°C, rendering the bacteria non-motile. A regulator protein, MogR, has been identified that directly binds to the *flaA* promoter region and thus functions as a repressor of motility gene expression. It is suggested that MogR, located immediately upstream of the motility gene cluster in *L. monocytogenes*, is essential for temperature-dependent transcription of the motility genes. The *Bacillus cereus* group is the only other set of organisms known to encode MogR homologs except for *L. monocytogenes*. Therefore, it has been speculated that organisms in the *Bacillus cereus* group and *Listeria* share a novel regulatory mechanism for flagella production, where MogR functions as a master regulator to control expression of flagellar motility genes.

*Bacillus anthracis*, *Bacillus cereus* and *Bacillus thuringiensis* are members of the *Bacillus cereus* (sensu lato) group within the gender *Bacillus*. The bacteria are Gram-positive, rod shaped and spore-forming. While *B. anthracis* is well known for causing the lethal disease anthrax and has been used as a biological warfare weapon, *B. cereus* is an important source of food poisoning and a growing problem for the dairy industry, while *B. thuringiensis* has been used worldwide as a biopesticide.

Results from the current thesis show that a MogR overexpression strain becomes non-motile at 37°C after observing motility during growth curves, and a larger number of genes, 109 in total, were down-regulated in the MogR overexpression strain by microarray analysis. The results from the microarray analysis showed that a total of 26 genes, including *mogR* itself, were up-regulated in the MogR overexpression strain as compared with a vector control strain. MogR was shown to positively affect the transcription of a gene encoding for a predicted collagen adhesion protein, an ortholog to *B. cereus* ATCC 14579 BC1060 which is

located upstream of a riboswitch, that gets positively regulated upon binding of c-di-GMP, a well-known second messenger molecule. Also, an ortholog to *B. cereus* ATCC14579 BC0422, annotated as a methyl-accepting chemotaxis protein (located upstream of a c-di-GMP "off" riboswitch) was down-regulated in the MogR overexpression strain. Results from biofilm assays indicate that the MogR overexpression strain also produce significantly more biofilm than the empty vector control strain, particularly at 37 °C.



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# 1 INTRODUCTION

## 1.1 The *Bacillus* genus

The *Bacillus* genus belongs to the family *Bacillaceae*, and was first described and classified by Ferdinand Cohn in 1872 [1]. Bacteria belonging to this group are ubiquitous in the environment, are saprophytes [2] and are common in soil. They are Gram-positive bacteria, rod-shaped and range from 0,5 by 1,2 to 2,5 by 10µm in diameter[3]. The *Bacilli* are aerobic or facultative anaerobic and the G+C content can vary from 32-78%. In a human host, species such as *B. cereus* can remain in a vegetative form, but when in nature, both vegetative cells and spores are found [4]. If the spores come in contact with organic matter, or inside an animal or insect host, they may germinate [4]. Spores can survive in extreme environment, as they are resistant to heat, dehydration and other physical stresses [5].

## 1.2 The *Bacillus cereus* group

The *Bacillus cereus* group (*Bacillus cereus sensu lato*) contains *Bacillus anthracis*, *Bacillus thuringiensis*, *Bacillus cereus (sensu stricto)*, *Bacillus mycoides*, *Bacillus pseudomycoides*, *Bacillus weihenstephanensis* and the newest member of this group, *Bacillus cytotoxicus*. Two strains of *B. cytotoxicus*, NVH 391/98 and INRA AF2 [6], are pathogens. *B. anthracis* and *B. thuringiensis* are pathogens of mammals and insects respectively, and will therefore create infection, when infecting their respective hosts. *B. cereus* is an opportunistic pathogen, which often gives foodborne disease or infects immunocompromised patients. *B. thuringiensis*, *B. anthracis* and *B. cereus* are closely related, and are the most frequently studied species in the *B. cereus* group[1].

*B. mycoides*, *B. pseudomycoides* and *B. weihenstephanensis* are considered largely non-pathogenic [7]. *B. cereus* strains are well know for having the potential of holding several plasmids in the cell at the same time, but the different *B. cereus* species are largely defined by differences in their plasmids, while the chromosome in all regarding species has been shown to be similar – both in gene content and gene order[8].

### 1.2.1 *Bacillus anthracis*

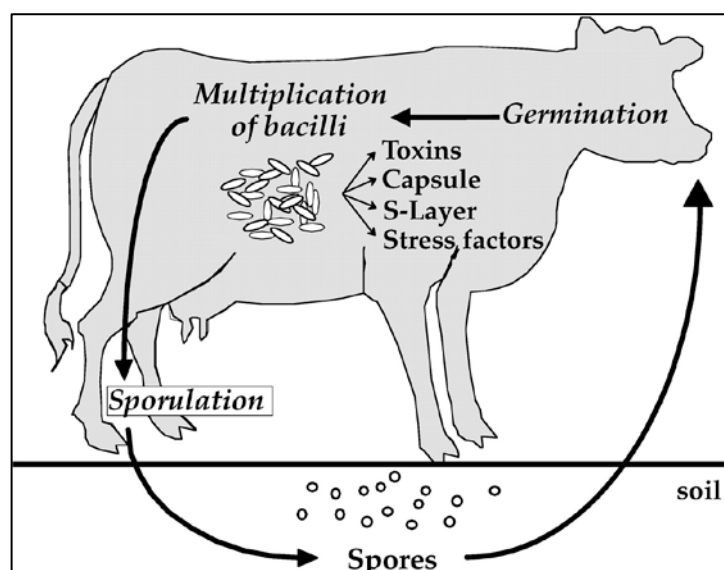
In the environment, *Bacillus anthracis* remains as a dormant spore in the soil [1]. *B. anthracis* are the cause of the disease anthrax and has become known as a biological warfare weapon [9]. At the end of the nineteenth century, Robert Koch and Louis Pasteur proved *B. anthracis* to be the etiological agent of anthrax [10].

Anthrax is primarily a disease seen in herbivores, but *B. anthracis* may cause infections in all warm-blooded animals, included humans [10]. Today, anthrax is hyperendemic or endemic in Africa, Asia and parts of South America. Scandinavia is largely free from the disease ([http://www.vetmed.lsu.edu/whocc/mp\\_world.htm](http://www.vetmed.lsu.edu/whocc/mp_world.htm)).

For *B. anthracis* to get its virulence, there are two large plasmids that are essential for toxicity, pXO1 and pXO2. pXO1 encodes at least three toxic factors; the protective antigen (PA), the lethal factor (LF) and oedema factor (EF) [11]. pXO2 encodes for a poly D glutamic acid capsule enabling the bacterium to withstand phagocytosis [11].

*B. anthracis* spores are able to survive for a long time in soil. Spores ingested by herbivores, probably by contaminated grass, germinate inside the host and produces vegetative cells (Figure 1) [10]. When inside the host, the spores germinate and form vegetative cells rapidly in the regional lymphoid glands [12]. In the final living hours of the animal, the vegetative cells are released in large numbers in the blood, expressing virulence factors (Figure 1) [10]. When the animal dies and shed the bacilli, the bacilli will sporulate when in contact with air [10].

Humans can be infected in three ways; by inhaling endospores, inoculation of endospores through a break in the skin, or by ingestion. The cutaneous form is the most common way [10] of being infected with anthrax, giving black painless eschars, accompanied by surrounding edema [12].



**Figure 1. Illustration of the life cycle of *B. anthracis*.** Herbivores ingest the spores; the spores germinate inside the host to produce vegetative cells. Shed bacilli resporulate. Taken from Mock et al. [10]

### **1.2.2 *Bacillus thuringiensis***

*Bacillus thuringiensis* is an insect pathogen, and used worldwide as a biopesticide. The bacteria produce two different families of toxins, called Cry and Cyt, which show a selective spectrum of virulence activity [13]. This discovery led to development of biopesticides, specific for the orders Lepidoptera, Diptera and Coleoptera [14] and contributes to killing the larvae. Most of the toxin genes in *B. thuringiensis* are located on large plasmids, but the plasmids can be lost, making *B. thuringiensis* indistinguishable from *B. cereus* [7].

### **1.2.3 *Bacillus weihenstephanensis*, *Bacillus mycoides* and *Bacillus pseudomycoides***

*Bacillus weihenstephanensis* was proposed in 1998, as a psychrotolerant *B. cereus* strain [15], and possesses a 16S rRNA signature for psychrotolerance and the cold shock protein gene *cspA* [16]. *Bacillus weihenstephanensis* has the ability to grow at low temperatures – at 6°C in BHI (brain heart-infusion), but not at 43°C. This means that *B. weihenstephanensis* has the ability to survive and grow at refrigerated temperature 6°C [16, 17], and the bacteria are shown to grow in whole liquid eggs at low temperature [18]. Only two strains of *B. weihenstephanensis*, MC67 and MC118, have been identified to produce enterotoxins [16, 19].

*Bacillus mycoides* is a non-motile rhizoid-growing bacterium. Rhizoid-forming bacteria are defined on agar-plates having filaments or root-like structures that may extend several centimeters from the inoculation site [11].

*Bacillus pseudomycoides* was first discovered in 1998, after researching *B. mycoides* as described by Nakamura [20]. They found that *B. mycoides* diverged into two groups, after revealing *B. pseudomycoides* can be distinguished from *B. mycoides* by differences in 12:0 iso and 13:0 anteiso fatty acid [20].

### **1.2.4 *Bacillus cytotoxicus***

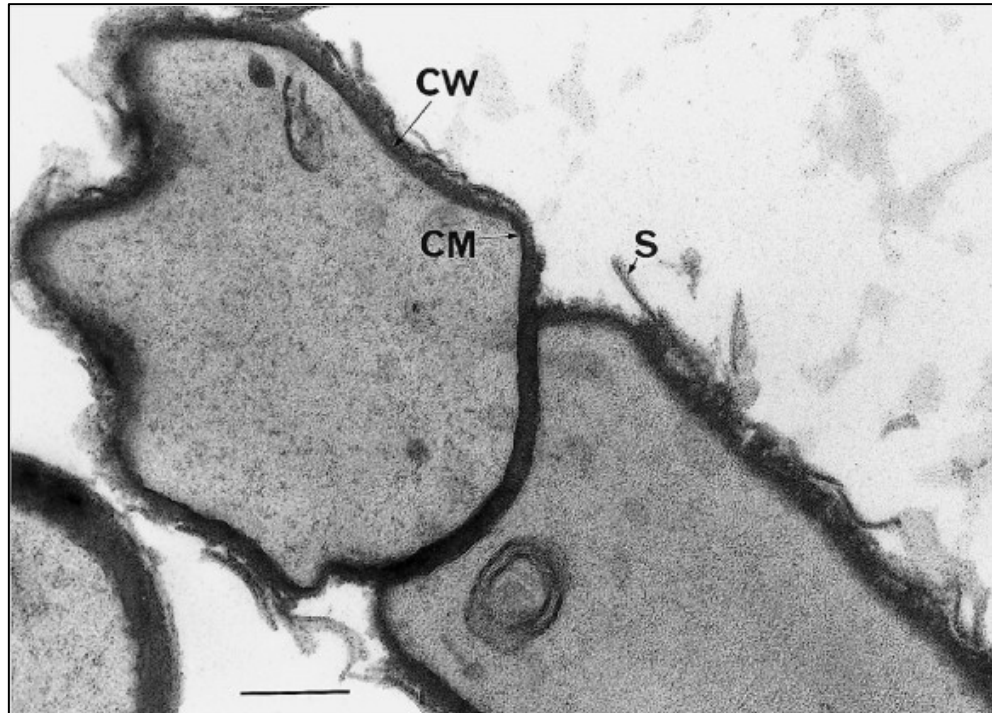
In 1998, an endospore-forming bacterium was isolated during a food-poisoning outbreak in France. Based on 16S rRNA-analysis it was shown to belong to *B. cereus sensu lato*, with over 97% similarity to the other members of the group. Since 1998, four other strains have been isolated. With genome similarity, DNA-DNA hybridization and physiological and biochemical tests, it was possible to genotypically and phenotypically differentiate *Bacillus*

*cytotoxicus* from the six other *Bacillus cereus*-members. The enterotoxin cytotoxin K was also discovered, which is a diarrheic toxin. Only two strains, NVH 391/98 and INRA F2, are pathogens [6], and are highly cytotoxic. *B. cytotoxicus* has a growth interval from 20°C to 50°C, and is therefore the first thermotolerant bacterium in the *Bacillus cereus* group.

### 1.2.5 *Bacillus cereus*

*B. cereus* (Figure 2) is an opportunistic pathogen, and is well known to cause food-borne human infections [21]. Since *B. cereus* is ubiquitous in the environment, lives in the soil, and forms spores, the bacterium is a big contamination problem in the dairy industry. *B. cereus* contaminate the milk by contaminating udders of cows, via soil and dung during grassing [5], with fast germinating spores [22]. Additionally, *B. cereus* has been isolated from a wide range of foods and food ingredients, including dried foods, vegetables and rice [5]. *B. cereus* can also be a problem for food industry equipment, such as dairy pipe lines; the spores can form biofilms, and such protect vegetative cells and spores from sanitizers [5].

Non-gastrointestinal infection can be divided into five groups; (I) local infections – burns and operation wounds [4], (II) ocular infections – endophtalmitis, which often causes permanent vision loss [23], (III) central nervous system infections – meningitis and encephalitis [3, 4], (IV) bacteremia and sepsis – often transient, but in immunosuppressive patients an infection can be fatal [24, 25] and (V) respiratory tract infections – not common, but there are case reports with *B. anthracis* [4]. When *B. cereus* is the cause of nosocomial infections, it is most likely a contaminant, and has contaminated, for instance, hospital linen and catheters [26, 27]. *B. cereus* produces  $\beta$ -lactamases, and is therefore resistant to  $\beta$ -lactamase sensitive antibiotics, including cephalosporin. It is susceptible to aminoglycosides, clindamycin, vancomycin, chloramphenicol and erythromycin.



**Figure 2. Ultrastructure of a *B. cereus* sporangium.** CW= cell wall, CM = cell membrane S= S-layer  
Taken from Kotiranta et al. [28]

*B. cereus* has a virulence regulon consisting of 45 genes [29], which mostly code for extracellular toxins and degradative enzymes [30]. PlcR is the major virulence regulator [5] which controls the transcription of the genes encoding for the potential virulence factors, and is a pleiotropic regulator [31]. PlcR is part of a quorum sensing system, which allows *B. cereus* to regulate virulence genes on the basis of self-cell density [5, 32]. The bacteria synthesize autoinducers, signal molecules that is specific for each bacterium, which diffuses over the cell envelope. When the autoinducer has reached high enough concentration, the signal molecule binds to specific activators inside the cell, and triggers specific gene transcription [32]. The peptide PapR is an autoinducer for *B. cereus*. When the concentration of bacterial cell density is high enough, the PapR concentration inside the cell increases, the PapR/PlcR complex binds the PlcR box and activate transcription [5]. The PlcR-box (conserved palindromic sequence) is found upstream of the transcriptional start site of all PlcR regulated genes, and it is suggested that the PlcR box regulates the transcription for at least 15 virulence genes in *B. cereus* [31]. *plcR* is autoregulated, but is also under the control of the sporulation factor Spo0A.



Emetic and diarrheal syndromes are associated with *B. cereus*. The diarrheal illness is generally mild, and is thought to be an infection caused by vegetative cells [5]. The patient may have ingested contaminated foods containing spores or vegetative cells. When inside the host, *B. cereus* produces enterotoxins in the small intestine [5]. The diarrheal syndrome is normally over after 12-24 hours after infection. In 1955, Hauge proved the toxicity and disease of *B. cereus* by inoculating vanilla sauce with *B. cereus*. After 24 hours incubation time, Hauge consumed 200 ml of the sauce. 13 hours later, Hauge got severe abdominal pains, diarrhea and rectal tenesmus, and this lasted more or less continuously for 8 hours [33]. The diarrheal syndrome can be caused by three cytotoxins; hemolysin BL (Hbl), non-haemolytic enterotoxin (Nhe) and cytotoxin K (cytK). Hbl and Nhe are three-component toxins, while CytK belongs to the  $\beta$ -barrel pore-forming toxins. The enterotoxins and hemolysin are activated by *plcR* [29].

The emetic syndrome arises when the patient gets intoxicated with the emetic toxin, cereulide. The emetic disease has a rapid onset of 30 minutes to 5 hours [5], and gives nausea and vomiting, occasionally diarrhea, and lasts less than 24 hours [28]. Most cases caused by emetic intoxication occur from food products in which boiled rice is the main ingredient [34]. High emetic toxin production is also reported in starchy foods, such as noodles [34]

The emetic toxin is named cereulide, is a cyclic dodecadepsipeptide with molecular mass 1,2 kDa, and is the only virulence factor that is not encoded on the bacterial chromosome [31]. Cereulide is produced by a non-ribosomal peptide synthetase, encoded by the *ces* gene cluster, and is located on a megaplasmid [5] of the same family as pXO1 in *B. anthracis*. Cereulide is resistant to gastric acid and proteolytic enzymes in the intestinal tract [5].

In *B. cereus* several potential virulence factors has been characterized. Among the virulence factors we find degradative enzymes, like proteases and phospholipases, and non-specific toxins, such as enterotoxins and hemolysin [35, 36]. Plasmids with high similarity to pXO1 and, in some cases, pXO2, have been identified in *B. cereus* isolates from pulmonary anthrax-like disease [37]. *B. cereus* G9241 was isolated from a welder, and contains the plasmids pBCXO1, pBC218 and pBClin29. The plasmid pBCXO1 is >99% similar to pXO1. The genes that encode PA (*pag*), EF (*aya*) and LF (*lef*) are all present. pBC218 is not similar in sequence with the capsule-encoding pXO2, but encodes for a putative polysaccharide capsule operon. pBClin29 contains genes that encode putative phage proteins [37].

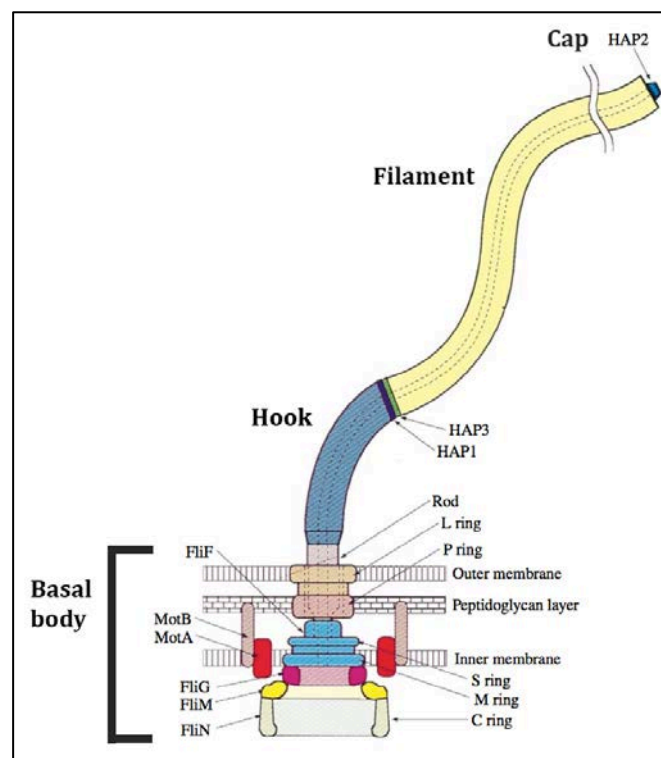
## 1.3 Motility in bacteria

Assisted movement for bacteria is mediated primarily by flagella, a whiplike structure which rotates and makes the cell motile. Many flagellated bacteria have more than one mode of cell locomotion [38]. In most cases, the cells move independently through liquid (called swimming) or on surfaces (called swarming). In this way, the bacteria can move towards favorable environments in a process called chemotaxis [39]. When the motor of the flagella spins counterclockwise (CCW), the flagella make a bundle and push the bacteria forward. This is termed run. When the flagellated bacteria alters course, the motor spins clockwise (CW) and is termed tumble [38]. Only five proteins are thought to be involved in rotation – FliG, FliM, FliN and MotA and MotB (Figure 3). FliG, FliM and FliN forms a large complex called “the switch complex”, and function to regulate CW and CCW reversals, but is also essential for flagellar assembly and rotation [40]. MotA and MotB forms the stator in the motor of the flagellum [41]. The direction the flagellum is rotating is determined by environmental stimuli, such as pH, temperature and chemicals (e.g. sugars, amino acids). Swimming cells can respond to changes in chemical concentrations by moving up and down a chemical gradient [38, 42]. This is called chemotaxis. A two-component phosphorelay signalling cascade, consisting of an associated histidine kinase, CheA, and a response regulator, CheY, is modified by chemicals binding to receptors at the cell membrane. CheY-P binds to FliM, and modulates the direction of the motor rotation [42]. The energy for the flagellar motor is generated by a proton ( $H^+$ ) or sodium ( $Na^+$ ) gradient created by the static motor force generators that surround the base components of the flagellum [40, 43].

## 1.4 Flagellar assembly

In *Salmonella enterica*, which is the most extensively studied flagellated bacterium [40], a total of about 50 genes is required for flagellar assembly and function [44]. In other bacterial species, including Gram positive bacteria, the total of genes and component proteins required is about the same [44]. The flagella assemble outside of the cell, since it is an extracellular structure. Assembly proceeds by addition of one subunit protein at a time at the distal end of the structure, and subunits are secreted through a type III secretion system, from the cytosol and to the outside of the cell [44]. The type III secretion system is a protein complex, formed by gene products in the very first step of flagellar assembly. Among the 14 genes which are required for this first step, more than half are necessary for forming this protein complex [44].

The flagellum is divided into three parts: i) the basal body ii) the hook and iii) the filament. The basal body consists of three parts: the rod, the motor and the switch complex (Figure 3). The basal body is the foundation of the structure, and is located in the bacterial membrane, and is also the part which gives the necessary torque of the filament [43]. The first subunit to be made is the MS-ring complex (consisting of the M- and S-ring) in the inner membrane. The MS-ring acts as a foundation for the rest of the basal body at the thick cell-wall of Gram-positive bacteria [45], and is the smallest flagellar structure [44]. The C-ring and C-rod attach to the cytoplasmic face of the M-ring, and makes up a complex that starts secreting other flagellar proteins to continue flagellar construction [44].

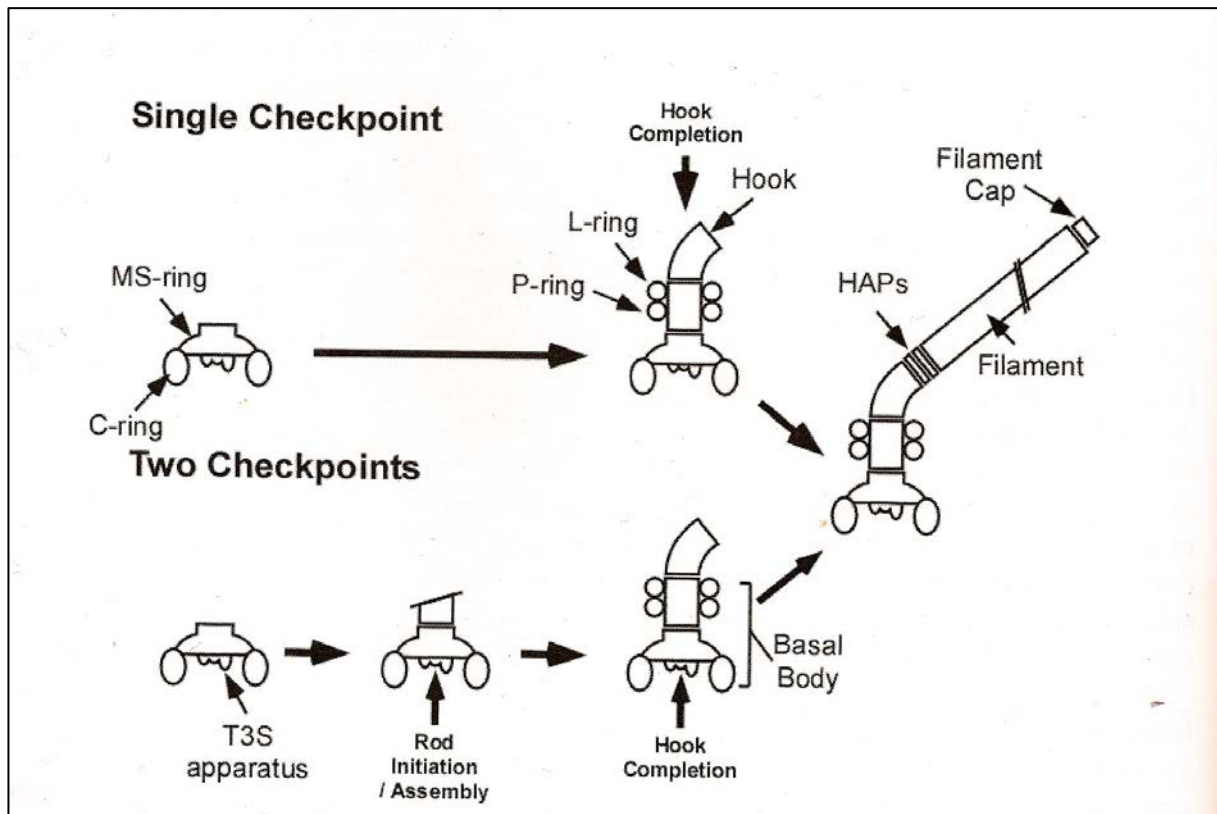


**Figure 3. Schematic diagram of the flagellum.** The different colours represent different protein components. The central sections are shown for FliG, FliM and FliN. Dotted lines represent hidden structures including part of the rod and the central channel that extends all the way from FliF to the distal end of the filament. Taken from Namba, K. and F. Vonderviszt. [46]

The C-ring (Figure 3) is necessary for torque generation, and works through interactions with the chemotaxis protein CheY and motor force generation [45]. The next structure assembled, onto the MS-ring complex, is the rod (Figure 3), which connects the hook to the motor [41]. The rod is made up of five proteins: FliE, FlgB, FlgC, FlgF and FlgG. The rod grows through the peptidoglycan layer, with aid from the capping protein FlgJ [44], but is soon stopped by the outer membrane, which functions as a physical barrier. When the outer ring complex

makes a hole in the membrane and removes this barrier, the hook (Figure 3) starts to assemble [44]. Initiation of the rod is used as a checkpoint that coordinates flagellar gene expression with assembly [47] (Figure 4). The motor is made of a stator, MotA and MotB, and the rotor, FliG, and the MS-ring, FliF (Figure 3) [41]. The switch complex consists of FliM and FliN.

The hook is a short and curved segment, as seen in figure 3, composed of the FlgE protein [48]. When the basal body is complete, the hook assembles onto the rod. The hook is the first component of the flagellum that is visible on the cell surface. Hook-basal body completion is the second assembly checkpoint utilized to coordinate flagellar assembly (Figure 4) [47]. This checkpoint coincides with the activation of a complete type III secretion apparatus required for the assembly of the rest of the flagellum [47]. After the hook is completed, the hook-associated proteins (HAPs) assemble to form the hook-filament junction (Figure 3). When FliD (filament cap protein or HAP2), which is located at the distal end of the filament, caps the hook-filament junction, the filament assembly begins [47]. The filament is made by tens of thousands of polymerized copies of flagellin (FliC), and is the most visible part of the bacterium [41]. The filament subunit flagellin is incorporated at the growing filament only in the presence of FliD [47]; without this cap, exported flagellin molecules are lost to the medium [44]. When the flagellin incorporates to form the filament, they form a hollow tubular structure, which may resemble a shell, which goes all the way down to the MS-ring [45]. The filament cap protein *FliD*, guides the flagellin, the filament subunits, into the growing filament.



**Figure 4. The assembly checkpoints utilized by flagellar systems** to coordinate the assembly pathway and flagellar gene expression. Assembly starts with the assembly of the MS-ring, C-ring and type III secretion apparatus. Flagellar systems utilize either a single checkpoint, associated with hook completion, or utilize a second checkpoint intermediate basal body checkpoint. This second checkpoint coincides with the initiation of rod assembly. After hook-basal body-completion, filament assembly will complete the flagellar structure.

Taken from Brown et al. [47]

## 1.5 Motility as a virulence factor

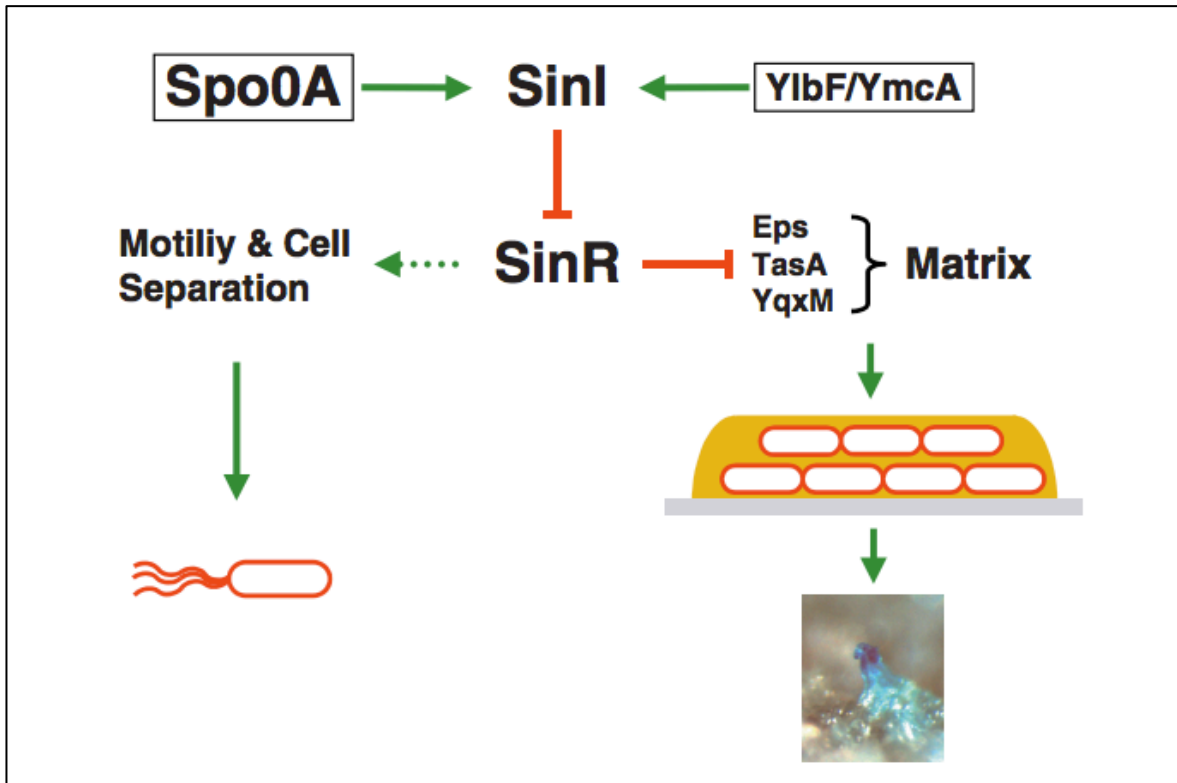
Motility and virulence are linked together by complex regulatory systems in the bacterial cell [49]. Motility plays a role mostly in the initial phase of an infection. The combination of motility and chemotaxis (bacteria sense different chemical gradients) gives bacteria the opportunity to detect and search for nutrients. This also gives the bacterial cell the opportunity to colonize, when the environment is favorable. When bacteria have found their target, adherence occurs and the initial phase has started. Motile, flagellated bacteria will be tethered, and it is suspected that tethered flagella serves as a mechanical signal to the bacteria so the regulation program for host contact can be started [49]. When the infection has been established, the flagellated bacteria down-regulate their motility, and switch from flagellar motility gene expression to producing an exopolysaccharide matrix [30, 50].

Type III secretion system, (T3SS) and the flagellar system are closely related structurally and functionally, and these systems coexist in many pathogens [49]. When both T3SS and flagellar system are present, they are not expressed at the same time [49]. This means that when the bacteria have found the target and adhered to the surface, the bacterial cell switches off their motility and gets full virulence. Both features play a major role in establishing and maintaining the infection. Flagellin-genes are mostly on the bacterial chromosome, while virulence-associated TTSS genes are mostly localized on pathogenicity islands or on plasmids [49].

Flagellin genes can be considered as a major “danger signal” for the innate immune system against Gram-negative bacteria in the host. Bacteria with flagella are recognized by the innate immune system through Toll-like receptor (TLR) 5, and are a target for host recognition. Toll-like receptors are a class of receptors that are part of the innate immune system, and are present in many types of leukocytes, and each type of TLR is specific for different types of common pathogen components [51]. It has been showed that TLR5, located on the intestinal epithelium [51], recognizes flagellin as their PAMP (pathogen-associated molecular pattern) [52]. When TLR5 recognizes flagellin and becomes activated, NF $\kappa$ B (nuclear factor kappa B) and TNF- $\alpha$  (tumor necrosis factor  $\alpha$ ) are mobilized. When NF $\kappa$ B is released, it activates genes encoding inflammatory cytokines, which induces the innate immune response. TNF- $\alpha$  is a cytokine that has several functions in the immune system, but when activated as a result of TLR-stimulation, it can have both harmful and beneficial consequences. Within a local area of infection, TNF- $\alpha$  gives increased levels of plasma proteins into the tissue, which give phagocyte and lymphocyte migration into the tissue and increased platelet adhesion to the blood vessel wall. Phagocytes come to the infected tissue, and phagocytosis of bacteria happens. The local vessels will occlude, and the infection is contained [51]. But, the activation of TNF- $\alpha$  can also give sepsis, if TNF- $\alpha$  is spread widely throughout the body. This happens if the infection develops in the blood. The systematic release of TNF- $\alpha$  and the effect it has on the vessels, with increased blood flow, vascular permeability and endothelial adhesiveness for white blood cells and platelets, is released simultaneously and may induce septic shock, a state of shock that can lead to organ failure and death [51]. Some species, like *Salmonella* spp., regulate their flagellin biosynthesis by changes in the composition of the flagella by phase variation mechanisms [53].

## 1.6 Involvement of motility and flagella in biofilm formation

Most bacteria live as part of biofilms in the natural environments [30]. Biofilms can be defined as “communities of microorganisms that are attached to a surface” [54], where the biofilm can comprise a single or multiple microbial species [54]. Bacteria can be up to a 1000-fold more resistant to antibiotics when growing in a biofilm [55]. Bacterial biofilms on prosthetic valves are the leading cause of endocarditis in patients who have undergone heart valve replacement [55], and biofilms can contaminate food of animal origin [56]. Flagella are involved in the first step of biofilm formation, which is when the bacteria make contact with a surface. When the bacteria have settled, biofilm formation can begin. In *Listeria monocytogenes* flagella has been shown to function as an adhesive tool, and in addition promotes attachment, as motility might repulse forces between the cell and surface [30]. *B. cereus* shares many regulatory mechanisms with *Bacillus subtilis*. In *B. subtilis*, flagella are not required as an adhesive tool to surfaces, but motility was shown to be important in pellicle formation [57]. *B. cereus* behaves the same way, as the studies done by Houry et al. [30] show. They found that in non-motile mutants there was a delay in pellicle formation. This delay was a result of negative feedback regulatory mechanisms mediated by components of the flagellar apparatus, and not a defect in motility. Both DegU/DegR and SinI/SinR regulators are acting at the transcriptional levels to control biofilm formation in *B. subtilis*. This results in a switch between motility and a production of exopolysaccharide matrix. SinR is a master regulator that controls the transition between motile cells and biofilm formation (Figure 5) [58], with SinI acting as an antagonist for SinR. In motile cells, SinR represses the transcription of genes responsible for matrix production. When the conditions become favorable for biofilm formation, SinR activity becomes antagonized by SinI (Figure 5) [59]. This results in biofilm matrix production and loss of motility [59]. DegU activates transcription of flagellar genes and pellicle formation genes at different times [60]. SinI/SinR is also found in the *B. cereus* group, and preliminary studies imply that c-di-GMP is important in Gram-negative bacteria and biofilm regulation in *B. cereus* (Fagerlund et al., unpublished).



**Figure 5. Regulation of biofilm formation in *B. subtilis*.** SinR is considered the master regulator of the switch between the planktonic state and biofilm state. In motile cells, SinR represses the transcription of genes responsible for matrix production, and thereby promotes cell separation and motility. Spo0A is a transcriptional regulator inducing expression of SinI, when nutrient limitation is sensed. SinI, YlbF and YmcA functions to directly or indirectly antagonize SinR-activity, leading to expression of matrix genes. Taken from Lemon et al. [59]

In *B. cereus* ATCC 14579 there are four *fla*-genes. The first three are in an operon, whilst the fourth comes after an intrinsic terminator sequence downstream [30]. In *B. cereus* 407 there are two *fla*-genes, separated by a terminator [30]. When Houry et al. [30] examined the role of flagellin genes in biofilm formation they found that only the first genes in the *fla* locus in *B. cereus* ATCC 14579 and *B. thuringiensis* 407 were expressed in the early stationary phase of biofilm formation. In planktonic cultures, in *B. thuringiensis* 407, *flaA* expression increased throughout the exponential phase, and peaked during the transition between the exponential and stationary phase. After this, the expression decreased sharply. They also investigated *flaA* expression in air-liquid interface in glass tubes, where *flaA* expression started out high in 24 hour old biofilms, but after the biofilm aged, the *flaA* expression decreased [30]. This result is likely to reflect either a decrease in motile bacteria in the biofilm, or a decrease in flagellin synthesis, but motility is not completely abolished among bacteria in the biofilm. Therefore, in young biofilms, flagellin genes are expressed. Motile bacteria are located at the



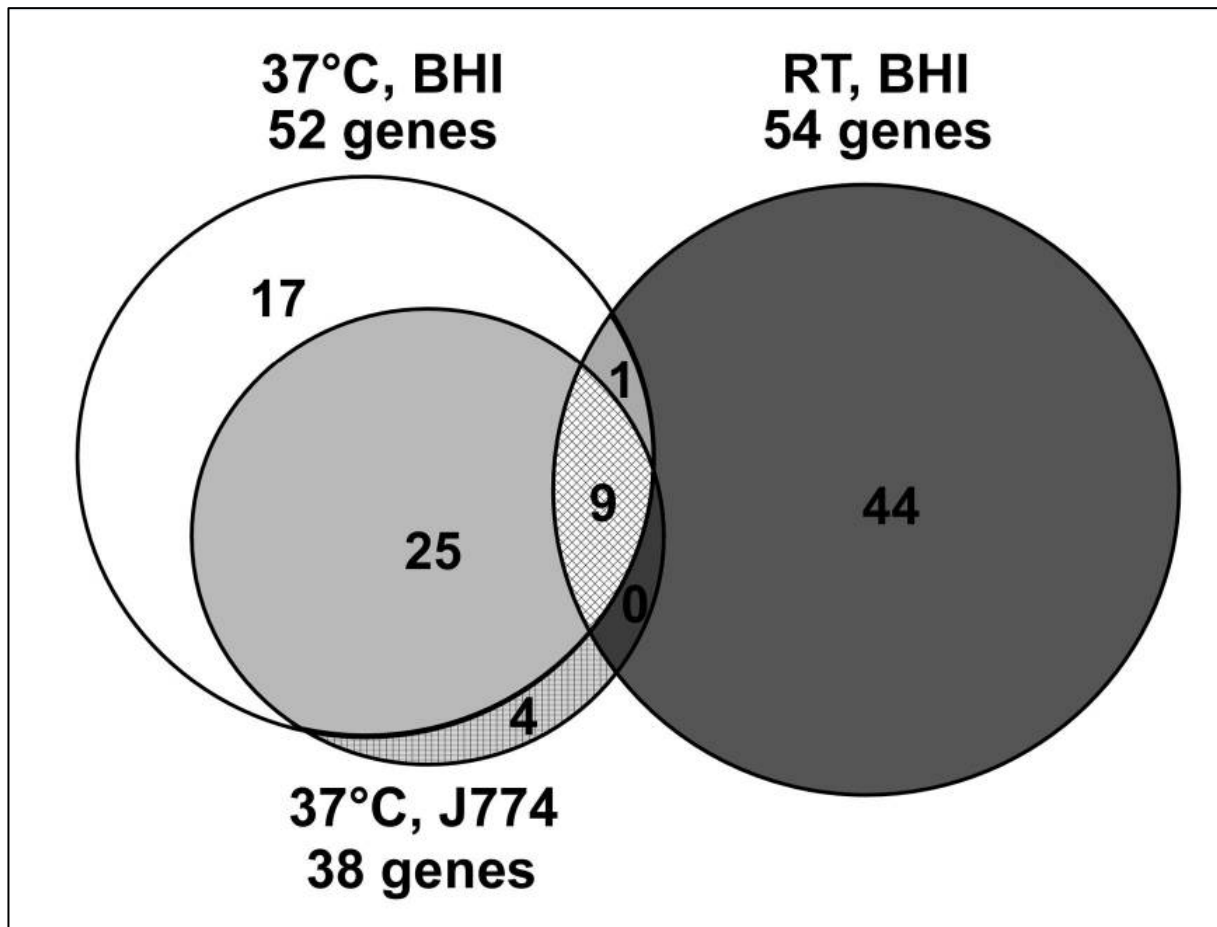
edge of the biofilm, and can extend the biofilm limits and promote biofilm formation. This gives greater surface coverage. Also, motility dramatically increase the importance of recruitment of the growing biofilm, and rapid recruit met of planktonic cells in biofilm [30].

## 1.7 MogR

*Listeria monocytogenes* is a Gram-positive food-borne pathogen which may cause life-threatening infections in fetuses, newborns and immunocompromised patients [50]. This bacterium is highly flagellated, and scientists have seen that *L. monocytogenes* flagellar gene expression is down-regulated when it encounter temperatures above 37°C [61-63]. This has never been observed before in any bacteria [63]. At 37°C, *L. monocytogenes* becomes non-motile, and it is therefore proposed that this type of down-regulation serves as an adaptive mechanism to avoid host recognition and mobilization of the innate immune response [61]. Gründling et al. [61] identified a regulator protein that directly binds to the *flaA* promoter region which functions as a repressor of motility gene expression, and is needed for full virulence. This protein, Lmo0674, is located immediately upstream of the motility gene cluster in *L. monocytogenes* [61]. Deletion of Lmo0674 abolished temperature regulation of the *flaA* promoter, suggesting that Lmo0674 is essential for temperature-dependent transcription of motility genes [61]. Since results indicate that Lmo0674 is a repressor of motility genes, Lmo0674 was given the name MogR.

Gründling et al [61] measured LD<sub>50</sub> for *L. monocytogenes* strain EGD<sub>e</sub> and strain EGD<sub>e</sub>Δ674, which is containing an in-frame deletion in the lmo0674 gene [61]. LD<sub>50</sub> in EGD<sub>e</sub> was 3-5x10<sup>3</sup>, while LD<sub>50</sub> in EGD<sub>e</sub>Δ674 was ≈1x10<sup>6</sup>, which is a 250-fold decrease in virulence upon *mogR* deletion. These results indicated that MogR is required for full virulence in *L. monocytogenes*.

To determine if MogR represses flagellar motility gene expression in a temperature-dependent manner, three experiments were done. To identify MogR-regulated genes, they compared the transcriptional profile in the ΔmogR strain against wild type during growth in brain-heart infusion (BHI) broth at 37°C and room temperature (RT), as well as in J774 macrophages at 37°C [61].



**Figure 6. Venn diagram representation of MogR-repressed genes.** MogR regulons defined during growth in BHI broth during infection of J774 macrophages at 37 °C were largely identical. This implies that the main function of MogR is to repress flagellar motility genes at 37 °C regardless if the bacteria are growing extracellular or within host cells. The transcriptional gene profile obtained during growth at room temperature (RT) differed substantially from growth at 37 °C. At RT, more MogR activated genes were identified than repressed genes when cultured at RT. Only nine genes were repressed during all growth temperatures. Taken from Shen et al. [63]

Fifty-two genes were identified as being MogR regulated at least 3,5-fold in MogR-negative bacteria relative to wild type during growth in BHI at 37°C (Figure 6). Of these putative 52 MogR-repressed genes, 39 genes were flagellar motility genes or located in operons encoding for flagellar motility. In J774 macrophages they identified 39 genes as being regulated at least 3,5-fold or greater from microarray analysis. Thirty-eight genes were repressed by MogR, and was either located within the major flagellar motility gene cluster, or in an operon with additional flagellar motility genes [63].

These findings were largely overlapping, implying that MogR represses flagellar motility genes at 37°C. The obtained results from growth at RT, discovered that more genes were

activated MogR than repressed (Figure 6). Fifty-four genes were repressed at an average of 4,9-fold. In contrast, the average fold-change for genes identified as MogR-repressed during growth at 37°C was 21-fold in BHI-broth and 27-fold in J774 macrophages. This means that MogR-repression activity was reduced during growth at RT. Only nine genes (Figure 6) were repressed at all three conditions. These nine genes were either related to flagellar motility or located within an operon with additional flagellar motility genes, but they were less repressed during growth at RT than at 37 °C.

### **1.7.1 Temperature-dependent regulation of the *flaA* promoter region**

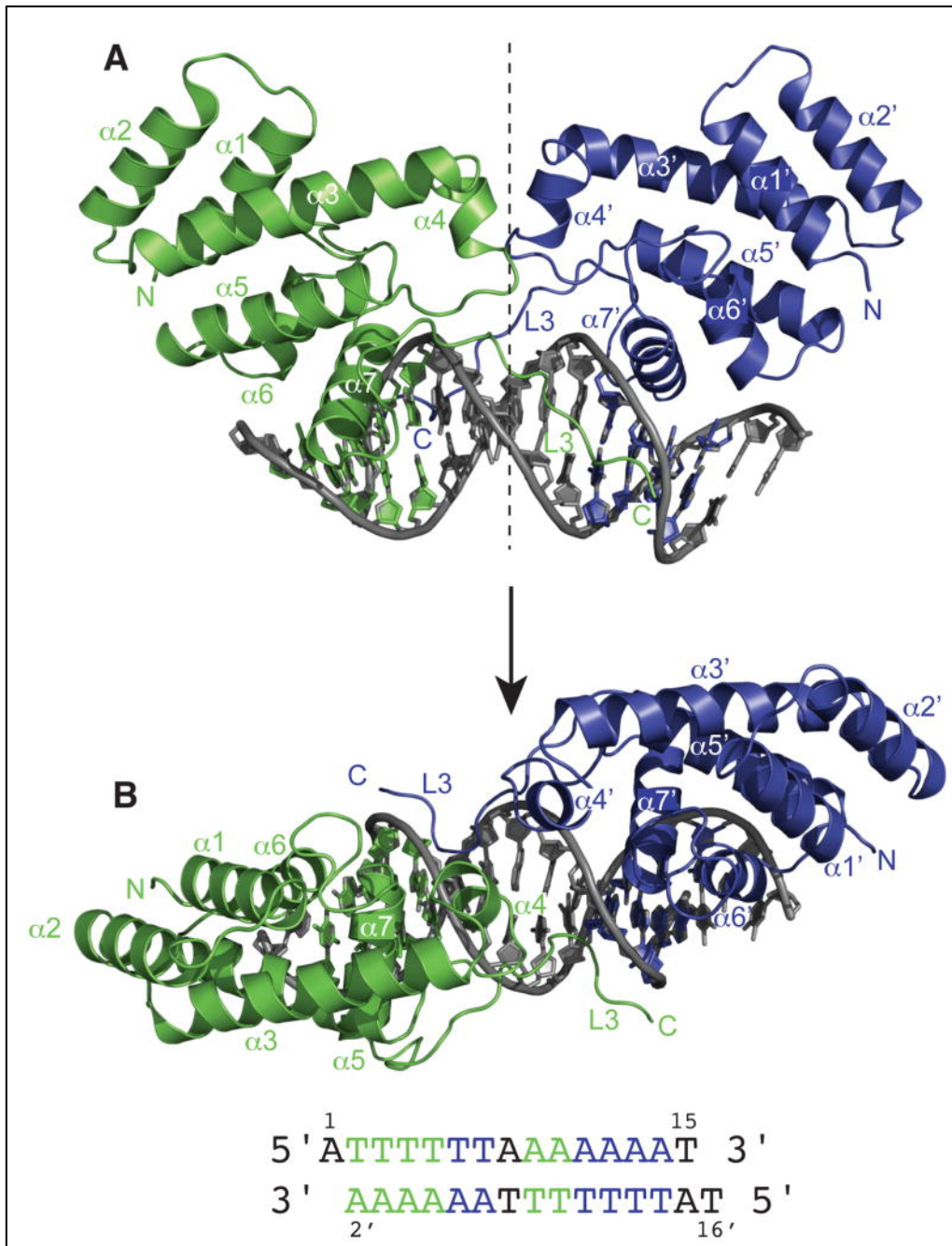
During growth at low temperatures in *L. monocytogenes*, a response regulator, DegU, mediates modulation of MogR repression activity [62], and functions as an indirect antagonist of MogR. At 25 °C the *degU* gene product is suggested to be a transcriptional activator of *flaA* [64]. DegU is also required for flagellar gene transcription at low temperatures [62], and autorepresses its own synthesis by binding directly to its promoter region [65]. DegU modulates the ability of MogR to repress flagellar motility genes at low temperatures [62], and DegU in general functions as a response regulator which mediates modulation of MogR repression activity at low temperatures [62].

In addition, Lmo0688, a putative flagellin glycosyltransferase, also functions as an antagonist of MogR-mediated repression at low temperatures. Because of its biofunctional nature, Lmo0688 got the name GmaR, to indicate its role as both glycosyltransferase and motility anti-repressor [62]. GmaR permits flagellar gene expression at low temperatures by directly binding to MogR and inhibiting its ability to bind target sequences in flagellar promoter regions. By forming a stable protein-protein complex with MogR, this GmaR:MogR-complex will inhibit the binding activity of MogR. The GmaR:MogR anti-repressor complex resembles the SinI:SinR-complex, where SinI anti-repressor functions by preventing SinR to bind to its target [62]. The GmaR:MogR complex only forms at low temperatures – when *L. monocytogenes* is grown at 37°C, MogR will bind directly to its targets at the *flaA* promoter region, and hinder flagellar gene expression, and thereby counter motility.

### 1.7.2 MogR binding sites

MogR binds to the *flaA* promoter region, and in addition directly binds the genes *lmo0675*, *cheY*, *lmo0703*, *lmo0723* and *lmo1699* in *Listeria monocytogenes* to repress transcription [63]. MogR binds to three regions within the *flaA* promoter region DNA. At low MogR concentrations, MogR protects the DNA comprising the -35 promoter element, whilst at higher concentrations, MogR also protects the regions proximal and distal to the -35 promoter element [63].

When MogR down-regulates flagellar motility gene expression during infection, it binds to a specific recognition sequence of thymine and adenins. The promoter regions contain multiple MogR binding sites – TTTTNNNNNAAAA – separated by 1, 2 or 3 helical turns [63, 66]. Earlier results have shown that a minimum of two MogR binding sequences are required for *flaA* repression in *L. monocytogenes* [63]. The best characterized MogR binding site at the *flaA* promoter region contains the sequence 5′ TTTTTTAAAAAAA 3′ (Figure 7) [66]. When MogR is absent in *L. monocytogenes*, all flagellar motility genes are constitutively transcribed, and no transcriptional activator has been identified [63]. The *B. cereus* group is the only other set of organisms known to encode MogR orthologs. Therefore, it has been speculated that the *B. cereus* group and *Listeria* share a novel regulatory mechanism for flagella production, where MogR functions as a master regulator to control expression of flagellar motility genes [63].



**Figure 7. Structure of the MogR-DNA complex.** Domain A is shown in green and domain B in blue. Two views of the complex, related by 90°, showing the overlapping DNA contacts of both domains. (A) Side view of the complex. (B) Top view of the complex. The core-binding site for each domain is shown with the corresponding color and the nucleotide numbering scheme is indicated.

Taken from Shen et al. [66]

## 2 BACKGROUND AND AIM OF THE THESIS

Flagella are cell surface structures that are critical for motility and virulence in many bacteria. In *Listeria monocytogenes*, MogR has been identified as a regulator protein that directly binds to the *flaA* promoter region and thereby functions as a repressor of motility gene expression at 37 °C. The MogR protein is needed for full virulence in *L. monocytogenes*, and *mogR* is located immediately upstream of the motility gene cluster [61].

*Bacillus thuringiensis* 407 (Cry<sup>-</sup>) (Bt407) is commonly used as a model strain for studying genetics of *B. thuringiensis*, and the genome has been sequenced (NCBI: ACMZ01000000). The original wild type strain, carrying Cry toxin genes, was cured for the Cry-encoding plasmid by Dr. Olivia Arantes, thus producing a strain that no longer forms insecticidal crystals [67], resulting in Bt407.

By analysis of the Bt407 genome sequence, the strain was shown to carry a gene encoding for a putative MogR protein. Preliminary experiments performed to create a knock out of MogR have not been successful, which could mean that the *mogR* gene is essential. The aim of this study was to perform an analysis of changes in global gene expression upon MogR overexpression, and to understand what functions MogR serve in *B. cereus* at different growth temperatures. In this respect it was also of interest to make an overexpression construct of a MogR variant where putative key amino acids were modified by site-directed mutagenesis, to be used in further functional studies.

# 3 MATERIALS

## 3.1 Bacterial strains

Strain	Description
Bt407 wild type	<i>Bacillus thuringiensis</i> 407 (Cry $\delta$ ), wild type
<i>Escherichia coli</i> pHT304-pXyl-mogR	Plasmid pHT304-pXyl-mogR clone 2 in <i>E. coli</i> XL1-blue MRF' cells
Bt407 pHT304-pXyl-empty vector	Bt407 containing empty pHT304-pXyl vector. Control for over-expression strains.
Bt407 pHT304-pXyl-mogR	Bt407 wild type containing pHT304-pXyl over-expressing MogR
<i>Escherichia coli</i> XL1-Blue + pHT304-pXyl-mogRmut	<i>Escherichia coli</i> XL1-Blue containing plasmid carrying mutated <i>mogR</i> gene
<i>Escherichia coli</i> XL1-Blue MRF' 1	<i>Escherichia coli</i> XL1-Blue used as host cells in transformation

## 3.2 Vectors

pHT304 is a low-copy number *E. coli/Bacillus* shuttle vector with a plasmid copy-number of  $4 \pm 1$  in *B. cereus*, in which *xylR* and the *xylA* promoter from *B. subtilis* has been inserted into the pHT304 cloning site [68], allowing xylose-inducible expression of downstream cloned genes. It contains genes giving resistance to erythromycin and ampicillin.

Erythromycin was used as a resistance marker in *B. thuringiensis*, while erythromycin or ampicillin were used in *E. coli*.

## 3.3 Primers

Name	Sequence (5' to 3')
mogRmut-F1	tccaaaaacagaaagtcaattggcagctacgtattataaattgaaaaacgtg
mogRmut-R1	cacgtttttcaattataatcgtagctgccaattgactttctgttttgg
MogR-F	gggatgcgagcatatgaaaa

M13-R	caggaacagctatgac
pHT304pXyl-F2	ggttgatcagcgatatccac
Oligonucleotide control primer #1	ccatgattacgccaagcgcgcaattaaccctcac
Oligonucleotide control primer #2	gtgagggttaattgcgcgcttggcgtaatcatgg

### 3.4 Enzymes

*DpnI* (Stratagene)

*PfuUltra* High Fidelity polymerase (Stratagene)

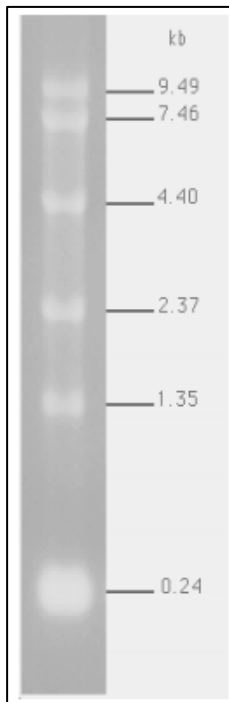
RNase A (Sigma Aldrich)

Superscript III Reverse Transcriptase (Invitrogen)

Turbo DNase (Ambion)



### 3.5 Molecular weight standards



**Figure 8: 0.24-9.5 Kb RNA Ladder**  
(Invitrogen product description)

### 3.6 Reagents and solutions provided

Acetic acid (Merck)

Acetone (VWR)

Agar bacteriological (No. 1) (Oxoid)

Agarose (Sigma-Aldrich)

Ampicillin (Sigma-Aldrich)

$\beta$ -mercaptoethanol (Sigma-Aldrich)

Bactopeptone (Becton, Dickinson and Company)

Boric acid ( $H_3BO_3$ ) (Sigma-Aldrich)

Bovine Albumin fraction V Solution (BSA) 7,5% (Gibco)

Crystal violet (Sigma-Aldrich)

Cy3 dye (GE Healthcare)

Cy5 dye (GE Healthcare)

dATP (Quiagen)

dCTP (Quiagen)

dGTP (Quiagen)

dTTP (Quiagen)

DEPC-treated water/RNase free water (Ambion)

Di-sodium hydrogen phosphate 2-hydrate ( $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ ) (Merck)

6 x Loading Dye (Fermentas)

Erythromycin (Sigma-Aldrich)

Ethanol (Arcus kjemi)

Ethidium bromide (EtBr) (Sigma-Aldrich)

Ethylenediaminetetraacetic acid (EDTA)

Ficoll 400 (Sigma-Aldrich)

Formamide (Sigma-Aldrich)

Glucose (Merck)

Hydrochloric acid (HCl) (VWR)

Isopropanol (Kemetyl)

Magnesium chloride ( $\text{MgCl}_2$ ) (Sigma)

Methanol (Merck)

Monopotassium phosphate ( $\text{KH}_2\text{PO}_4$ ) (Merck)

Sodium hydroxide (NaOH) (VWR)

Orange G (Merck)

PEG 8000

Potassium chloride (KCl) (Merck)

pWhitescript 4.5-kb control plasmid (Stratagene)  
QuickSolution reagent (Stratagene)  
Random hexamers (Applied Biosystems)  
10 × Reaction Buffer (Finnzymes)  
10 × Reaction buffer (Stratagene)  
20 × Saline-sodium citrate (SSC) buffer (Gibco)  
Salmon sperm DNA (1 mg/ml) (Sigma-Aldrich)  
Sodium acetate, water free (NaOAc) (Merck)  
Sodium acetate (NaOAc) (3 M, pH 5.5) (Ambion)  
Sodium chloride (NaCl) (Merck)  
Sodium dodecyl sulfate (SDS) (Sigma-Aldrich)  
Tris-HCl (10 mM, pH 8.5) (Elution Buffer, Qiagen)  
Tryptone (Oxoid)  
10 × Turbo DNase-buffer (Ambion)  
XL10-Gold β-mercaptoethanol mix (Stratagene)  
Xylose (Sigma-Aldrich)  
Yeast extract (Oxoid)

### **3.7 Solutions prepared in the lab**

Ampicillin (100 mg/ml)

2 g ampicillin

Milli Q water (MQ-dH<sub>2</sub>O) added to 20 ml

The solution was sterile-filtered, aliquoted in sterile eppendorf tubes and stored at -20°C.

Crystal violet solution (0.3% w/v)

0.3 g crystal violet (Methyl violet)  
dH<sub>2</sub>O added to 100 ml

Dissolved on a magnetic stirrer over night, sterile-filtered and stored at room-temperature.

dNTP-mix (final concentration 10 mM each)

10 µl dATP (100 mM)

10 µl dCTP (100 mM)

10 µl dGTP (100 mM)

10 µl dTTP (100 mM)

60 µl MQ-dH<sub>2</sub>O

The components were mixed and stored at -20 °C.

Erythromycin (100 mg/ml)

2 g erythromycin

Ethanol (96%) added to 20 ml

The solution was aliquoted in sterile eppendorf tubes and stores at -20 °C.

Ethidium bromide (5 mg/ml)

0.5 g ethidium bromid (EtBr) was added to 100 ml MQ-dH<sub>2</sub>O. The container was wrapped in aluminium foil and the mixture was stirred magnetically for several hours to ensure dissolution of the dye. The solution was stored at 4 °C.

Sodium hydroxide (1M)

40 g Sodium hydroxide (NaOH)

MQ-dH<sub>2</sub>O added to 1 L

The solution was stored at room temperature.

### Orange mix

10 g Ficoll 400

0.125 g Orange G

2 ml EDTA (0.5 M, pH 8.0)

The components were dissolved in 50 ml MQ-dH<sub>2</sub>O and sterile filtrated. The solution was aliquoted in sterile eppendorf tubes and stored at -20°C.

### Phosphate buffered saline (PBS)

8 g NaCl

0.2 g KCl

0.27 g KH<sub>2</sub>OPO<sub>4</sub>

1.78 g Na<sub>2</sub>HPO<sub>4</sub> X 2H<sub>2</sub>O

987.5 ml dH<sub>2</sub>O

pH was adjusted to 7.4 with HCl and the solution was autoclaved and stored at 4 °C.

### 20% Sodium Dodecyl Sulphate (SDS)

200 g SDS

MQ-dH<sub>2</sub>O added to 1 L.

The components were carefully heated to help the dissolution process, and the solution was stored at room temperature.

### Sodium acetate (NaOAc) (3 M, pH 5.2)

246.09 g NaOAc (3 M)

dH<sub>2</sub>O added to 800 ml

~100 ml Acetic acid

dH<sub>2</sub>O added to 950 ml

pH was adjusted to 5.2 and the volume was adjusted to 1 L with dH<sub>2</sub>O. The solution was autoclaved.

50 x TAE (Tris/acetat/EDTA buffer)

242.0 g Tris-Base

57.1 ml acetic acid (17.5 M)

100 ml EDTA (0.5 M pH 8.0)

MQ-dH<sub>2</sub>O added to 1 L.

The solution was stored at room temperature.

TSS

1.0 g PEG 8000

0.5 ml DMSO

20–50 mM Mg<sup>2+</sup>

Solved in 10 ml LB, sterile filtrated. Stored at 4 °C.

Xylose (1 M)

7.5 g xylose was dissolved in 50 ml MQ-dH<sub>2</sub>O and the solution was sterile-filtered, aliquoted in sterile eppendorf tubes and stored at -20 °C.

## **3.8 Growth media**

Bactopeptone medium

10 g bactopeptone

5 g yeast extract

10 g NaCl

The components were dissolved in dH<sub>2</sub>O and the volume was adjusted to 1 L. The solution was autoclaved and stored at 4 °C.

### Luria-Bertani (LB)-agar

10 g Tryptone

5 g Yeast extract

10 g NaCl

pH was adjusted to 7.0 with 5.8 M HCl. No. 1 bacteriological agar (12.5 g) was added and the volume was adjusted to 1 L with MQ-dH<sub>2</sub>O. The solution was autoclaved and then cooled to approximately 45-50 °C before being plated out. The plates were stored at 4 °C.

### Luria-Bertani (LB)-medium

10 g Tryptone

5 g yeast extract

10 g NaCl

MQ-dH<sub>2</sub>O added to 1 L

pH was adjusted to 7.0 with 5.8 M HCl. The solution was autoclaved and stored at 4 °C.

### Super Optimal broth with Catabolite repression (SOC)-medium

20 g Tryptone

5 g Yeast extract

0.5 g NaCl

The components were added to 950 ml MQ-dH<sub>2</sub>O and the mixture was shaken until dissolution. KCl (250 mM, 10 ml) was then added and pH was adjusted to 7.0 with HCl (5.8 M). The volume was adjusted to 1 L with MQ-dH<sub>2</sub>O and the solution was autoclaved. After allowing the solution to cool down, 5 ml sterile MgCl<sub>2</sub> (2 M) and 20 ml sterile glucose (1 M) was added. The solution was stored at -20 °C.

## 3.9 Commercial kits

### E.Z.N.A Plasmid Miniprep Kit 1 (Omega Bio-Tek)

Alkaline-SDS was used to lyse the bacterial cells. DNA reversibly binds to the HiBind matrix allowing contaminants to be removed (E.Z.N.A plasmid miniprep kit-I Handbook).

Contents:     HiBind Miniprep columns  
                  Solution I  
                  Solution II  
                  Solution III  
                  Buffer HB  
                  DNA Wash buffer, concentrate (diluted with ethanol before use)  
                  Elution buffer

### E.Z.N.A Gel Extraction Kit (Omega Bio-Tek) – Protokol for Cleanup DNA from Enzyme Reactions (PCR, enzyme reaction)

The following protocol was used for recovery of DNA from of enzyme reactions, among these PCR.

Contents:     HiBind DNA Mini Column  
                  2 ml Collection tube  
                  Binding Buffer  
                  SPW Wash Buffer, concentrate (diluted with ethanol before use)  
                  Elution Buffer

### RNeasy Mini Kit (Qiagen)

Up to 100 µg of RNA can be isolated. RNA binds to a silica-based membrane and a high-salt buffer system is used. A highly denaturing guanidin-thiocyanate-containing buffer is present to inactive RNases (RNeasy Mini Handbook, RNAprotect Bacteria Reagent Handbook).



Contents: RNeasy mini spin columns  
Buffer RLT  
Buffer RW1  
Buffer RPE (4 volumes of 96 % ethanol was added before use)

### FairPlay III Microarray Labeling Kit

cDNA is synthesized from RNA, purified, labeled with fluorescent dyes and then repurified.  
(Fairplay III Labeling Kit Instruction Manual)

Contents: 10 x AffinityScript RT buffer  
20 x dNTP mix with amino allyl dUTP  
DTT (0.1 M)  
RNase block (40 U/ $\mu$ l)  
AffinityScript HC Reverse Transcriptase  
Glycogen (20 mg/ml)  
DMSO (high purity)  
2 x Coupling Buffer  
DNA-binding solution  
Microspin cups  
2 ml receptacle tubes

### QuikChange® Site-Directed Mutagenesis Kit (Stratagene)

The QuikChange site-directed mutagenesis kit is used to make point mutations, switch amino acids, and delete or insert single or multiple amino acids.

Contents: PfuTurbo® DNA polymerase (2.5 U/  $\mu$ l)  
10 $\times$  reaction buffer  
*Dpn*I restriction enzyme (10 U/ $\mu$ l)  
Oligonucleotide control primer #1 [34-mer (100 ng/ $\mu$ l)]  
Oligonucleotide control primer #2 [34-mer (100 ng/ $\mu$ l)]

pWhitescript™ 4.5-kb control plasmid (5 ng/ μl)  
dNTP mix  
XL1-Blue supercompetent cells  
pUC18 control plasmid (0.1 ng/μl in TE buffer)

#### QuikChange® II XL Site-Directed Mutagenesis Kit (Stratagene)

The basic procedure utilizes supercoiled double-stranded DNA (dsDNA) vector with an insert of interest and two synthetic oligonucleotide primers, both containing the desired mutation. The oligonucleotide primers are extended during PCR (Chapter 4.7). Extension of the oligonucleotide primers generates a mutated plasmid containing staggered nicks. Following PCR, the product is treated with *DpnI*, and afterwards transformed into competent cells (provided from kit). (Taken from QuickChange II XL Site-Directed Mutagenesis Kit Manual)

Contents: *Pfu Ultra* High Fidelity (HF) polymerase  
10× reaction buffer  
*DpnI* restriction enzyme  
Oligonucleotide control primer #1  
Oligonucleotide control primer #2  
pWhitescript™ 4.5-kb control plasmid  
QuikSolution™ reagent  
dNTP mix  
XL10-Gold® ultracompetent cells  
XL10-Gold® β-mercaptoethanol mix (β-ME)  
pUC18 control plasmid

#### ElectroTen-Blue Electroporation Competent Cells (Agilent Technologies)

The ElectroTen-Blue Electroporation Competent Cells are a derivative of the XL1-Blue MRF' strain that can withstand much higher levels of electrical current. These electroporationready cells are thawed, mixed with DNA, and electroporated. StrataClean

Resin is used to prepare DNA ligation reactions for electroporation. (Taken from the ElectroTen-Blue Electroporation Competent Cells manual)

Contents:     ElectroTen-Blue Electroporation Competent Cells  
              pUC18 control plasmid (0.1 ng/μl in TE buffer)  
              StrataClean Resin

### **3.10 Various equipment**

Centrifuge, rotor radius = 85 mm (Hettich)

Centrifuge, rotor radius = 184 mm (Nunc-tubes) (Hettich)

Cuvettes for electroporation (Genepulser Cuvettes 0,2 cm electrode, Bio-Rad)

Cuvettes for measuring OD<sub>600</sub> (VWR)

Cuvettes for measuring RNA/DNA concentration: UVette 220-1600 nm (Original Eppendorf)

Electroporator Gene Pulser II (Bio-Rad)

Filtered pipette tips (ART)

Genepix 4000b Microarray Scanner (Axon)

Genepix 7.0 Microarray analysis program (Axon)

Hybridization chambers

Lifter slips

Microarray slides (Information in chapter 4.5)

Micron filter device: Amicon 0,5 ml 30 K Ultracel 30 K Membrane Millipore

Nunc-tubes (15 ml, 50 ml) (Corning Incorporated)

PCR Machine (Applied Biosystems GeneAmp PCR System 2700 + DEN GRØNNE)

PCR tubes (0,5 ml, thin wall) (Sarstedt)

Photo box for gels: Gel Doc 1000 (Bio-Rad)

Plate scanner used in biofilm assay: HTS 7000 Plus Bio Assay Reader (Perkin Elmer)

Plates used in biofilm assay: HTS 7000 Plus Bio Assay Reader (Perkin Elmer)

Precellys machine (Bertin technologies)

Precellys tubes VK01 (Bertin technologies)

PXE 0,2 Thermal Cyclers (Thermo Electron Corporation)

RNase Away (Sigma)

UV-spectrophotometer, Biophotometer (Eppendorf)

# 4 METHODS

## 4.1 Growth of bacteria

### 4.1.1 *Bacillus thuringiensis* Bt407

Bacteria from frozen stocks stored at -80 °C were streaked on LB agar-plates, with the appropriate antibiotics when needed and incubated at 37 °C over night. The plates were stored in a refrigerator (4°C) in plastic bags when not in use. One single colony was inoculated in 10 ml fresh LB and incubated at 37 °C with rotation at 225 rpm over night. Antibiotics and xylose were added when appropriate. Over night culture (100 µl) was transferred to 9.9 ml LB medium and proper antibiotics and xylose were added. OD<sub>600</sub> was measured in a UV-spectrophotometer. Samples were taken every hour and/or every half hour during the exponential growth phase. At each time point 100 µl of the culture was sampled and diluted with 900 µl LB-medium in a 1 ml plastic cuvette. Motility was studied in a microscope by observing the bacteria at each sampling point. Growth experiments were either performed in Erlenmeyer flasks with aluminium foil caps, or in 50 ml Nunc tubes.

### 4.1.2 *E. coli* XL1-Blue

Bacteria from frozen stocks stored at -80 °C were streaked onto LB agar-plates, with the appropriate antibiotics when needed and inoculated over night at 37 °C. One single colony was inoculated in 5 ml LB-medium rotating at 225 rpm at 37 °C. Nunc tubes of 50 ml were used.

## 4.2 Preparation of RNA

In general, when working with RNA, special precautions have to be taken. Ribonucleases (RNases) are very stable and active enzymes that are difficult to inactivate, and even small amounts of RNases are sufficient to destroy RNA. Therefore, it is advised to use RNase-free plasticware, change gloves often and keep isolated RNA on ice at all times. RNeasy Mini Kit

(Qiagen) was used for isolation and purification of RNA. The maximum capacity of the RNeasy mini spin column is 100 µg of RNA.

Samples were harvested from bacterial cultures grown to desired optimal densities. The appropriate volume of harvested culture is dependent on the optical density of the culture. A volume of 1 ml with OD<sub>600</sub> of 3 has shown to be suitable by previous experiments in the laboratory. The samples (2 ml each) were added to tubes containing equal volumes of ice cold methanol, this to stop growth immediately and to fix transcripts. The tubes were centrifuged at 2800 x g for 20 minutes. Supernatants were discarded and pellets stored at -80 °C until use. The pellets were thawed on ice and any rests of supernatants were removed. The pellets were resuspended in 1 ml Buffer RLT containing 10 µg/ml β-mercaptoethanol and transferred to Precellys tubes. The cells were lysed with the Precellys machine. The Precellys machine was programmed as followed; 5800 rpm, 30 seconds of length, 20 seconds pause with two runs. The suspensions were centrifuged at 16 000 x g at 4 °C for 1 minute, and beads and debris discarded. The supernatants were transferred to eppendorf tubes containing 700µl 70% ethanol. 700 µl was transferred to the RNeasy mini kit column, and centrifuged at 16 000 x g at room temperature (RT) for 20 seconds. Flow-through was discarded, the columns were reloaded with the remaining sample and centrifuged as before. RW1 Wash buffer (350 µl) was added to each column, and centrifuged. A DNase I mix was prepared, consisting of RDD (70 µl) and DNase I (10 µl) and added to the columns and incubated for 15 minutes at RT. RW1 Wash buffer (350 µl) was added to the columns after the incubation, and centrifuged as before. Old collection tubes were changed to fresh collection tubes. Buffer RPE (500 µl) was added to the columns and centrifuged. Flow-through was discarded. This step was repeated once more with Buffer RPE (500 µl), but this time centrifuged for 2 minutes. The empty columns were centrifuged at 16 000 x g for 1 minute to be sure that all liquid is discarded from the columns. The columns were transferred to RNase-free 1,5 ml eppendorf tubes, RNase-free water (50 µl) were added, and centrifuged for 16 000 x g for 20 seconds. Another 50 µl of RNase-free water was added to the columns, incubated at RT for 1 minute and centrifuged at 16 000 x g for 1 minute. The RNA samples were immediately placed on ice.

The concentration and purity of the RNA samples were measured with spectrophotometry (Chapter 4.3) and the integrity of the RNA molecules was examined with agarose gel electrophoresis (Chapter 4.4).

### **4.3 Spectrophotometry**

To assess concentration of preparations of RNA and DNA, absorbance was measured at  $A_{260}$ . For single stranded RNA, one  $A_{260}$  unit corresponds to 40  $\mu\text{g}$  of RNA per ml (1  $A_{260}$   $\Rightarrow$  40  $\mu\text{g}/\text{ml}$ ). For double stranded DNA, one  $A_{260}$  unit corresponds to 50  $\mu\text{g}$  of DNA per ml (1  $A_{260}$   $\Rightarrow$  50  $\mu\text{g}/\text{ml}$ ). In addition,  $A_{280}$  was measured to control for proteins, content which has a peak at this wavelength, since protein can be a contaminant in the preparation. Ratios of 1.8 to 1.9 indicate highly purified preparations of DNA, whilst ratios of 1.9 to 2.0 indicate highly purified preparations of RNA [69]. When assessing the density of bacterial cultures, 600 nm wavelength was used.

### **4.4 Agarose gel electrophoresis**

Agarose gel electrophoresis is a method for separating DNA or RNA molecules by casting agarose gels with ethidium bromide (EtBr) and exposing the molecules to an electric field. The negatively charged molecules will migrate towards the anode during the electrophoresis, and larger molecules will migrate more slowly than the smaller molecules, because of greater friction. EtBr binds to the nucleic acids and is used to detect the molecules as it fluoresces in UV-light when bound. A gel-loading buffer is added to the samples before applying them to the gel, providing color and density, and electrophoresis buffer is added to the electrophoresis tank to provide ions and buffer capacity. The appropriate concentration of agarose to be used depends on the size of the DNA or RNA molecules to be analyzed. In table 3, appropriate concentrations of agarose for separating of DNA molecules according to Voytas [70], are shown.

**Table 3. Appropriate Agarose Concentrations for Separating DNA Fragments of Various Sizes [70]**

Agarose (%)	Effective range of resolution of linear DNA Fragments (kb)
0.5	1 to 30
0.7	0.8 to 12
1.0	0.5 to 10
1.2	0.4 to 7
1.5	0.2 to 3

#### **4.4.1 RNA electrophoresis**

The integrity and size distribution of total RNA purified is determined by agarose gel electrophoresis and EtBr staining. The respective ribosomal bands should appear as sharp bands on the stained gel, where 23S ribosomal RNA bands should be present with twice the intensity compared to the 16S RNA band (RNeasy midi kit protocol). If the ribosomal bands appear as a smear of smaller sized RNAs, it is likely that the RNA sample has suffered major degradation during preparation. Agarose gels were made by heating the appropriate amount of agarose in electrophoresis buffer until complete dispersion. The electrophoresis buffer used in RNA gels was 1 x TAE (Chapter 3.7). EtBr was added to a final concentration of 10µg/ml. The heated gel solution was poured into a closed gel-casting platform, containing a comb forming the slots for sample application. When the gel had hardened, the platform was transferred to the electrophoresis tank, and electrophoresis buffer was added so that the gel was just covered. For RNA preparations, orange mix was added to the samples. Before the RNA samples were applied to the gel, the samples were incubated at 65 °C for 5 minutes, and placed on ice. The samples with total volumes of 6 µl (5 µl RNA-sample and 1 µl orange mix) were pipetted into the slots of the gel. A RNA Ladder was added to one of the slots for estimation of molecule size. The samples were exposed to voltage (80 V) for approximately an hour depending on the gel volume, and the gel was transferred to a photo box, exposed to UV light and photographed.

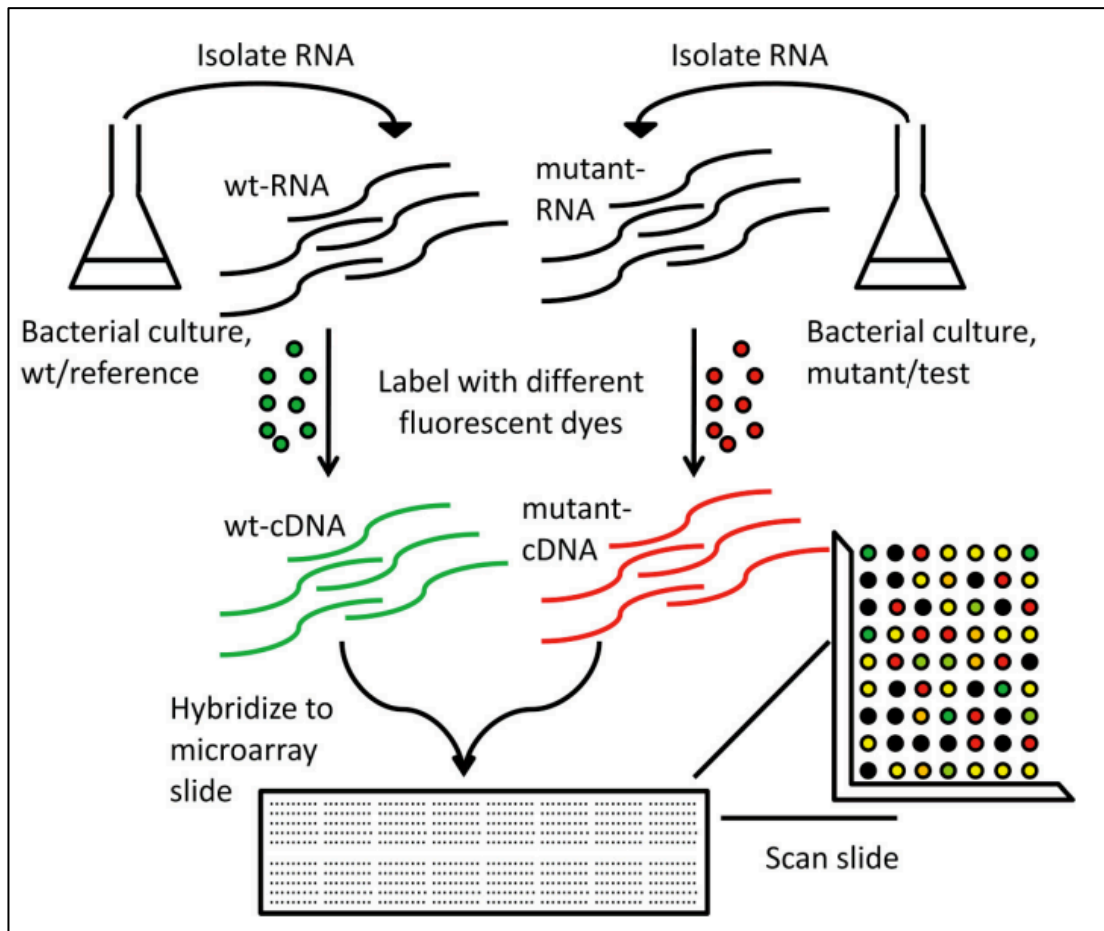


## 4.5 Microarray analysis

The principle behind microarray analysis is hybridization. Two strands of DNA hybridize if they are complementing each other, where adenine binds to thymine and guanine binds to cytosine. Figure 9 shows a general overview of the microarray experiment. RNA is isolated from the bacterial strains to be compared, for example from a control strain and an overexpression strain. RNA is converted to cDNA. Depending on the array, one or two samples are fluorescently labeled (Figure 9), denatured and applied onto the slide, where the samples will bind to the matching probes by hybridization [71]. For two-color arrays, which are used in this experiment, a competitive hybridization will occur between the two labeled samples [71]. After several washing steps, where unhybridized material is washed away [72], the slide is scanned (Figure 9). The intensity of the fluorescent signal from the probes is measured, which indicates the amount of the matching nucleotide [71]. For two-color arrays, the scanning is done at two different wavelengths (635 nm and 532 nm), giving two values for each probe. This gives a ratio between the red and green signal that shows the signal intensities of the two samples to be compared.

The technique has its origin from Northern and Southern blotting [72], and DNA arrays are massive parallel version of these techniques.

The FairPlay III Microarray Labeling Kit (Stratagene) was used for synthesizing, labeling and purifying cDNA. The microarray slides used in this experiment are spotted arrays, custom printed on aminosilyl-covered glass slides [71]. Different probes can be attached within  $\mu\text{m}$  of each other, so it is possible to place many probes on the same surface [72]. The probes are 70-mer oligos designed for *B. anthracis* Ames, with supplementary probes for *B. anthracis* A2012 and *B. cereus* ATCC14579. Negative and positive controls (housekeeping genes) are included for each strain [71].



**Figure 9. A general overview of the microarray experiment.** Bacterial cultures from two different bacterial strains, e.g. reference strain and overexpression strain are harvested and RNA is isolated. RNA is converted to cDNA through reverse transcription and labeled with two different fluorescent dyes. The reference and overexpression strains are hybridized to the microarray slide, containing matching probes that represent the bacteria's entire sets of genes. The slides are scanned at two different wavelengths and ensure detection from the two different fluorescent dyes. The expression of the two samples can thus be compared. Drawing taken from Karoline Fægri [71].

#### 4.5.1 Precipitation of RNA

RNA (20 µg) obtained with the RNeasy mini kit (Chapter 3.9) was volume adjusted to 100 µl with RNase-free water. Sodium acetate (3M, pH 5.5, 11 µl) was added to each sample and mixed by vortexing. Ethanol (96%, 280 µl) was added and mixed again. The sample was precipitated at -80 °C over night.

#### 4.5.2 cDNA preparation

The precipitated RNA was centrifuged at 16 000 x g at 4 °C for 20 minutes. The supernatants were pipetted off and discarded. The samples were washed once with ice-cold ethanol (70 %, 500 µl) and centrifuged as before for 10 minutes. The supernatants were completely pipetted off and the pellets allowed to air dry for 5 minutes on a heating block adjusted to 37 °C, followed by air-drying at room temperature (RT) for 5 minutes. Each RNA sample was resuspended in RNase-free water (8 µl) and put on ice for the rest of the procedure. Random hexamer (5 µl) was added to each sample, and mixed by vortexing and incubated on a heating block (70 °C) for 5-10 minutes, and put directly back on ice to cool down. A master mix was prepared for synthesis of cDNA for each sample, consisting of the following reagents (FairPlay III Microarray Labeling Kit (Stratagen)):

2 µl 10×AffinityScript reaction buffer

1 µl 20×dNTP mix with amino allyl dUTP

1.5µl 0.1 M DTT

0.5µl RNase Block (40 U/µl)

2.5µl 200U/µl Affinity Script HC Reverse Transcriptase

Master mix (7.5µl) was added to the sample and incubated on a heating block (42 °C) for 60 minutes. Sodium hydroxide (1M, 10 µl) was added to the sample, and incubated on a heating block (70 °C) for 10 minutes and cooled down slowly to RT. The sample was briefly spun down. Hydrochloric acid (1M, 10 µl) was added to the sample. Sodium acetate (3M, pH 5.2, 4 µl) and glycogen (20 mg/ml, 1 µl) were added to the sample, and mixed by vortexing. Ice-cold ethanol (96 %, 100 µl) was added, and the sample was precipitated at -20 °C over night. The reactions were centrifuged at 16 000 x g at 4 °C for 20 minutes. The supernatant was pipetted off and discarded. Ice-cold ethanol (70 %, 500 µl) was added to wash the sample and centrifuged as before for 10 minutes. The supernatant was completely pipetted off, and the pellet was allowed to air-dry for 5 minutes on a heating block (37 °C).

### **4.5.3 Resuspension of Cy3 and Cy5 dyes**

DMSO (45  $\mu$ l) was added to each of the tubes containing Cy3 and Cy5 dyes. The tubes were gently vortexed and briefly spun down. All lights were turned off in the lab during the procedure when handling the fluorescent dyes.

### **4.5.4 NHS-Ester Containing Dye Coupling Reaction**

The cDNA pellet was resuspended in 2xcoupling buffer (5  $\mu$ l) and heated on a heating block (37 °C) for 15 minutes. Occasionally the tube was mixed by vortexing and briefly spun down a couple of times during the incubation. The samples were dye-swapped, meaning that MogR overexpression strain cDNA was colored with Cy3 and empty vector control strain cDNA colored with Cy5 in two of the biological replicates, and MogR overexpression strain with Cy5 and empty vector control strain cDNA with Cy3 in the two remaining biological replicates. The tubes were incubated for 30 minutes at RT in the dark.

### **4.5.5 Dye-Coupled cDNA Purification**

1xTE Buffer (pH 7.6, 90  $\mu$ l) and 200  $\mu$ l of DNA binding solution: 70% ethanol (1:1) was added to each sample of labeled cDNA, and thoroughly mixed by vortexing. Each mixture was transferred to a microspin cup (FairPlay kit) placed in a 2 ml receptacle tube and centrifuged at 16 000 x g for 30 seconds. The flow-through was discarded. DNA binding solution/70 % ethanol-solution (200  $\mu$ l) was added once and ethanol (75%, 750  $\mu$ l) twice, for washing, to the mixture and centrifuged as before. After the last wash, the flow-through was discarded and the tubes centrifuged as before for 30 seconds to remove traces of ethanol. The microspin cups were transferred to fresh eppendorf tubes and Tris-HCl (10mM, pH 5.8, 50  $\mu$ l) was added to elute the labeled cDNA. The mixture was incubated for 5 minutes and centrifuged for 30 seconds at maximum speed. This elution step was repeated twice, for a total of three times, by using the elute from the previous elution again. The sample pairs were combined to be applied to the slide, and stored in the dark at 4 °C until the filter devices were ready to use.

#### **4.5.6 Concentration of labeled cDNA**

For each combined sample/each slide to be prepared, one micron filter device (Chapter 3.10) was prepared by adding MQ-water (500 µl) and centrifuged for 12 minutes at 13 700 x g, and flow-through was discarded. The sample of combined labeled cDNA was added to the column, and centrifuged at 13 700 x g for approximately 10 minutes. The elutes were collected by placing the columns upside down in fresh tubes and centrifuged at 1000 x g for 2 minutes. The final volume has to be exactly 27 µl. If the volume was higher than 27 µl, the elute was transferred back to the filter device and concentrated further. If final volume was lower than 27 µl, MQ-water was added to the eluate, up to 27 µl. The elutes were stored in the dark at 4 °C until the slide was ready to use.

#### **4.5.7 Prehybridization**

For each slide to be prepared, 50 ml prehybridization solution was made and filled in Nunc tubes (50 ml). The buffer consisted of the following reagents:

12.5ml 20×SSC (Chapter 3.6)

250µl 20% SDS

666µl BSA (7,5%)

36.6ml MQ dH<sub>2</sub>O

The prehybridization solution was pre-warmed at 42 °C for 15 minutes. One slide was placed in each tube and incubated in the same water bath for 30 minutes. The slide was then transferred to one Nunc tube containing MQ-water (50 ml) and washed by gentle inversion for 30 seconds. This step was repeated twice more. The slide was transferred to one Nunc tube containing isopropanol (50 ml) and washed for exactly 30 seconds. The slide was directly transferred to a Nunc tube containing two sheets of folded lens papers in the bottom and immediately centrifuged for 4 minutes at 200 x g at 22 °C for 4 minutes. Immediately after centrifugation, the slides were transferred to empty Nunc tubes with the lid slightly open so the isopropanol could evaporate. The slides could be used immediately or stored for a maximum of 1.5 hours.

#### 4.5.8 Hybridization

Hybridization buffer (53  $\mu$ l) was made to each eluated sample of labeled and combined cDNA/each slide, containing the following reagents:

24  $\mu$ l formamide (100%)

20  $\mu$ l 20xSSC

1  $\mu$ l SDS (8%)

8  $\mu$ l salmon sperm DNA (1 mg/ml)

27  $\mu$ l labeled and combined cDNA

Hybridization buffer (53  $\mu$ l) was added to each sample of labeled and combined cDNA (27  $\mu$ l) to a total volume of 80  $\mu$ l. The mixture was incubated on a heating block (95 °C) for 2 minutes, briefly spun down and incubated on a heating block (42°C) for 20 minutes. One lifter slip per slide to be analyzed was washed in ethanol (70%) and dried using lens paper. The prehybridized slide was placed on a 42 °C heating block with the array side pointing upwards. The prewashed lifter slip was placed on top of the array with the protuberant borders facing downwards so that a small space between the lifter slip and the slide was created. The lifter slip had to be placed correctly, to cover the area that was containing the printed microarray oligonucleotides. Half of the volume (40  $\mu$ l) was pipetted slowly at the edge of the lifter slip, so the sample was distributed evenly onto the array. Each of two small cavities of the hybridization chamber were filled with 5 x SSC (30  $\mu$ l, chapter 3.6) and the slide was placed in the middle of the chamber. The lid was attached to the hybridization chamber by six screws and tightened carefully. Holding the hybridization chamber horizontally at all times, the hybridization chamber was submerged in a water bath (42 °C) and incubated for 17-18 hours.

Nunc tubes were filled with wash buffer 1 and wash buffer 2 were preheated to 42 °C for approximately 15 minutes. The wash buffers consisted of;

##### Wash buffer 1:

12.5ml 20xSSC

250 $\mu$ l SDS (20%)

MQ-water added to 500ml  
and sterile filtered.

#### Wash buffer 2:

1.5ml 20×SSC

MQ dH<sub>2</sub>O added to 500 ml  
and sterile filtered.

The hybridization chamber was removed from the water bath (42 °C), placed on paper towels, gently dried off and disassembled. The slide with lifter slip was transferred gently to a Nunc tube containing water buffer 1 (50 ml). The lifter slip was carefully removed without disturbing the slide and washed by gentle inversion for 5 minutes. This step was repeated twice. The slide was transferred to a Nunc tube containing wash buffer 2 (50 ml) and washed in the same manner twice. The slide was transferred to a Nunc tube containing isopropanol (50 ml) and washed for exactly 30 seconds. The slide was then directly transferred to a Nunc tube containing two sheets of folded lens paper in the bottom and centrifuged at 200 x g for 4 minutes at 22 °C. Immediately afterwards, the slide was transferred to an empty Nunc tube and allowed to air-dry for a couple of minutes to let the isopropanol evaporate. All Nunc tubes were covered in aluminum foil the whole time.

The slides were scanned using a GenePix 4000b scanner (Axon) with the software GenePix Pro. The intensities of red and green beams (635 nanometers and 532 nanometers respectively) were adjusted so the ratio between the red and green signals intensities was as close to 1 as possible.

#### **4.5.9 Analysis**

The raw image was first analyzed by gridding. This is performed by positioning a grid consisting of circles corresponding to each probe. Spots of poor quality and “flames” were marked (flagged “bad”) to make it possible to ignore them during the analysis. The boundaries of each spot were determined, and intensities from the spots and the background were calculated for each wavelength. Further analysis was performed using the statistical computing platform R, version 2.13.0 (<http://www.r-project.org/>) and the package LIMMA (Linear models for microarray data) [71].

After the raw image analysis, relevant information was exported from GenePix Pro to R. Appropriate cut-off values for red and green signal intensities were decided based on histograms showing intensities for red and green spot signals and background, respectively.

These cut-off values ensured removal of the background signals in further analysis, and a small offset (usually in the size range 10-50) was added to all signal intensities to stabilize the spots [71]. Spots of poor quality and very small spots were excluded from the analysis. The genes wished to be identified were those differently expressed in the reference strain and the overexpression strain corresponding to the spots with different signal intensities when exposed to different wavelengths. Control spots were removed so they did not influence the analysis, and normal spots were given a quality weight based on the spread in signal intensity within the spot. A normalization step removed the systemic bias from the data, i.e. bias arising from variation in the technology. Dye bias was corrected through dye-swap. Technical replicates were averaged and given a quality weight based on the original spots, and these averages were compared across multiple slides. These values were used to estimate  $\log_2$  expression ratios and significance [71]. Probes for twelve typical housekeeping genes were used as positive controls for the slide, which were separately analyzed to obtain expression ratios. From the final list of genes obtained from the microarray analysis, only genes with false discovery rate (FDR) corrected p-values < 0.05 were considered differentially expressed.

## **4.6 Atomic force microscope (AFM)**

### **4.6.1 Preparation of cells**

The cell culture of the empty-vector control strain and MogR overexpression strain were grown over night in LB-medium with erythromycin (10  $\mu\text{g/ml}$ ) at 37°C and shaking (220 rpm). The day after, 100  $\mu\text{l}$  over night culture was inoculated in 10 ml LB-medium at 37 °C with erythromycin (10  $\mu\text{g/ml}$ ) and xylose (10 mM) to an OD<sub>600</sub> of 3. One ml of each of the over night cell cultures were centrifuged at 4000 rpm for 3 minutes, the supernatant removed, and pellet resuspended in 1 ml physiological NaCl. The cells were centrifuged once more, and the pellet was resuspended in 1 ml physiological NaCl. From this a sample solution was made: 15  $\mu\text{l}$  washed cell suspension, 5  $\mu\text{l}$  100mM MgCl<sub>2</sub> and 30  $\mu\text{l}$  MQ water.

Ten  $\mu\text{l}$  sample for AFM was applied to a microscopy slide to use an optical microscope to control that the concentration of cells was sufficient.



#### 4.6.2 Sample for AFM

Ten  $\mu\text{l}$  of sample solution was applied on a piece of freshly cleaved mica, incubated at room temperature for 10 min, followed by a wash with 10x 100  $\mu\text{l}$  sterile filtered MQ water. The samples were then dried by applying a soft jet stream of nitrogen.

AFM imaging was performed in intermittent contact mode in air, using the NanoWizard instrument from JPK (Berlin, Germany). The scanning probes used were NSC35/AIBS purchased from Micromash (Estonia).

### 4.7 Polymerase chain reaction (PCR) for site-directed mutagenesis

The polymerase chain reaction (PCR) technique was developed by Kary Mullis in 1983, and has since been an important tool in biotechnology. With PCR, scientists could make large quantities of DNA fragments without cloning it [73]. PCR consists of three steps, which are 1) denaturation, 2) priming and 3) extension. During denaturation the target DNA is separated into two single strands by breaking the hydrogen bonds [74] carried out at a high temperature. During priming, a mixture consisting of DNA primers (which are oligonucleotides usually about 20 nucleotides long), DNA polymerase and dNTP-mix is added to the target DNA. The target DNA will anneal to the primers rather than to each other, since the primers are in excess. The temperature is lowered, so the target DNA can bind to primers by forming hydrogen bonds. The last step is extension, where the DNA polymerase replicates the strands and produces more DNA fragments. These steps, as described above, are repeated 30-40 times, so that large quantities of target DNA are produced.

For each PCR reaction, the following components were mixed (Table 4, table 5):

Sample	Negative control
1 $\mu\text{l}$ template	1 $\mu\text{l}$ template
2,5 $\mu\text{l}$ forward primer (10 $\mu\text{M}$ )	2,5 $\mu\text{l}$ forward primer (10 $\mu\text{M}$ )
2,5 $\mu\text{l}$ reverse primer (10 $\mu\text{M}$ )	2,5 $\mu\text{l}$ reverse primer (10 $\mu\text{M}$ )
1 $\mu\text{l}$ dNTP mix (10 $\mu\text{M}$ each, see chapter 3.7)	1 $\mu\text{l}$ dNTP mix (10 $\mu\text{M}$ each, see chapter 3.7)
5 $\mu\text{l}$ 10 $\times$ Reaction Buffer (Agilent)	5 $\mu\text{l}$ 10 $\times$ Reaction Buffer (Agilent)
37 $\mu\text{l}$ MQ-dH <sub>2</sub> O	37 $\mu\text{l}$ MQ-dH <sub>2</sub> O
1 $\mu\text{l}$ Pfu Ultra HF (Agilent)	1 $\mu\text{l}$ MQ-dH <sub>2</sub> O

**Table 5. Overview of reagents added in each PCR solution for first mutagenesis with the QuikChange II XL Site-Directed Mutagenesis Kit.**

Sample	Control plasmid
1 µl template	2 µl pWhitescript4.5-kb control plasmid
2,5 µl forward primer (10 µM)	1,25 µl oligonucleotide control primer #1
2,5 µl reverse primer (10 µM)	1,25 µl oligonucleotide control primer #2
1 µl dNTP mix (10 µM each, see chapter 3.7)	1 µl dNTP mix (10 µM each, see chapter 3.7)
5 µl 10 × Reaction Buffer (Agilent)	5 µl 10 × Reaction Buffer (Agilent)
3 µl QuikSolution	3 µl QuikSolution
34 µl MQ-dH <sub>2</sub> O	36,5 µl MQ-dH <sub>2</sub> O
1 µl Pfu Ultra HF (Agilent)	1 µl MQ-dH <sub>2</sub> O

The following cycling conditions were used when performing PCR to mutate selected amino acids:

**PCR cycling with the QuikChange II Site-directed mutagenesis kit:**

95 °C for 30 seconds

18 cycles of: 95 °C for 30 seconds, 55 °C for 1 minute, 68 °C for 9 minutes  
(1 minute/kb of plasmid length)

68 °C for 5 minutes

4 °C →

**PCR cycling with the QuikChange II XL Site-directed mutagenesis kit:**

- i) 95 °C for 1 minute  
18 cycles of 95 °C for 1 minute, 60 °C for 50 seconds, 68 °C for 9 minutes  
(1 minute/kb of plasmid length)  
68 °C for 7 minutes  
4 °C →

or:

- ii) 95 °C for 1 minute  
18 cycles of 95 °C for 1 minute, 55 °C for 50 seconds, 68 °C for 9 minutes  
(1 minute/kb of plasmid length  
68 °C for 7 minutes  
4 °C →

#### **4.7.1 PCR purification**

Purification of DNA from liquid solutions (PCR reactions) was done with a commercial kit from Omega Bio-Tek (E.Z.N.A). The protocol issued from the manufacturer was followed:

The sample volume was adjusted to 200 µl with water, 200 µl of Binding Buffer was added, and thoroughly mixed by vortexing. The sample was applied to the membrane matrix for DNA absorption, and centrifuged at 10 000 x g for 30 seconds. The column was washed with 700 µl SPW Washing buffer and centrifuged at 10 000 x g for 30 seconds. This step was repeated twice, so any salt sensitive downstream applications were washed away entirely. After centrifuging the empty column for 2 minutes at 13 000 x g, the DNA was eluted with 30 µl Elution Buffer after being applied directly onto the membrane matrix and incubated at room temperature for 1-2 minutes.

## **4.8 Treatment with *DpnI***

*DpnI* specifically cleaves fully methylated G<sup>Me6</sup> ATC sequences [75]. After mutagenesis-PCR (Chapter 4.7), *DpnI* restriction enzyme can be added to the product, and will digest the bacterial DNA used as template for amplification, but not the newly synthesized DNA-fragments. *DpnI* resistant molecules are rich in the desired mutant, and are then transformed into *E.coli* by transformation.

**Following mutagenesis with the QuikChange II Site-directed mutagenesis kit (Chapter 4.7):**

*DpnI* restriction enzyme (2 µl) was added to the eluated sample and negative control

respectively. 10 x NEBuffer 4 (3.6  $\mu$ l) was then added to the mixture and incubated on a heating block (37 °C) for approximately 2 hours.

**Following mutagenesis with the QuikChange II XL Site-directed mutagenesis kit (Chapter 4.7):**

*DpnI* restriction enzyme (1  $\mu$ l) was added directly to each sample. The solution was gently and thoroughly mixed by pipetting up and down several times, and spun down in a microcentrifuge for 1 minute. The reactions were immediately incubated on a heating block (37 °C) for 1 hour.

## **4.9 Transformation**

### **4.9.1 Chemical transformation**

First, the cells were prepared for transformation. *E. coli* XL1-Blue from frozen stocks were streaked out on LB-plates containing 12  $\mu$ g/ml tetracycline and incubated at 37 °C over night. One single colony was inoculated in 5 ml LB-medium with 12  $\mu$ g/ml tetracycline and incubated over night at 37 °C with shaking at 225 rpm. Over night culture of chemically competent cells (100  $\mu$ l) was reinoculated in 9,9 ml LB with the appropriate antibiotic at 37 °C with shaking at 225 rpm. The cells were grown to an OD<sub>600</sub> of 0,4. The cells were centrifugated at 5000 rpm for 5 minutes at 4 °C. The cell pellet was resuspended in 1 ml cold TSS (Chapter 3.7) and 100  $\mu$ l cells were aliquoted and frozen directly on a dry ice/ethanol bath. The cells could be used directly after preparation or stored at -80 °C. If stored at -80 °C, the cells were thawed on ice for 10-15 minutes before use. The sample DNA was added directly to the cells (2  $\mu$ l) and incubated on ice for 30 minutes. The cells were then placed in a 42 °C water bath, then directly back on ice. Prewarmed SOC-medium (900  $\mu$ l) was added and the cells were spun down in a centrifuge for 1 minute at maximum speed (16 000 x g). The supernatant was discarded until 100  $\mu$ l was left in the tube. The cells were then incubated at 37 °C with rotation at 225 rpm for 1 hour, and plated out at LB agar plates with 100 mg/ml ampicillin and incubated at 37 °C over night.

### 4.9.2 Electroporation

Electroporation was carried out with ElectroTen-Blue Electroporation Competent Cells from Agilent Technologies. The DNA samples were first treated with StrataClean resin, which removes modifying enzymes. The StrataClean was vortexed to be completely resuspended, and 5  $\mu\text{l}$  was transferred to the DNA samples to be transformed. The mixture was vortexed for 15 seconds, centrifuged at 2500 x g for 1 minute, and the supernatant carefully removed. This procedure was repeated once more, and the collective supernatant was used in the following electroporation reaction. The electrocompetent cells were gently thawed on ice and 30  $\mu\text{l}$  cells were aliquoted into prechilled Eppendorf tubes. Experimental DNA (2  $\mu\text{l}$ ), negative control (2  $\mu\text{l}$ ) and 1  $\mu\text{l}$  pUC18 control plasmid (diluted 1:10 with sterile, distilled water) was transferred to each of the three tubes. Each DNA-cell mixture was transferred to a chilled electroporation cuvette with 0,2 cm gap, tapping the top of the cuvette until the mixture settled evenly to the bottom. The cells were electroporated at 2,25 kV, 22,5  $\mu\text{F}$ , 400  $\Omega$ . After electroporation SOC-medium (960  $\mu\text{l}$ , preheated to 37  $^{\circ}\text{C}$ ) was immediately added, and the cells were incubated for 90 minutes at 37  $^{\circ}\text{C}$  while shaking at 225 rpm. Different amounts of cell suspension (10  $\mu\text{l}$  and 100  $\mu\text{l}$ , 900  $\mu\text{l}$  following spin-down of cells) were streaked out on LB plates containing 100 mg/ml ampicillin. The plates were incubated at 37  $^{\circ}\text{C}$  over night.

### 4.9.3 Chemical transformation for QuikChange II XL Site-Directed Mutagenesis Kit

XL10-Gold Ultracompetent Cells, provided by the manufacturer in the QuikChange XL-kit (Chapter 3.9) was used for the transformation. After storage at -80  $^{\circ}\text{C}$ , 45  $\mu\text{l}$  of the cells were aliquoted to prechilled Eppendorf tubes. 2  $\mu\text{l}$   $\beta$ -mercaptoethanol mix were added to the cells, and incubated on ice for 10 minutes. *DpnI*-treated mogR plasmid DNA (1  $\mu\text{l}$  and 1  $\mu\text{l}$  of 1:10 dilution MogR plasmid DNA) and the control reaction (pWhitescript 4.5 kb control mutagenesis reaction, 2  $\mu\text{l}$ ) were transferred to the cells and incubated on ice for 30 minutes. The tubes were heat-pulsed for 30 seconds in a 42  $^{\circ}\text{C}$  water bath and immediately put back on ice for 2 minutes, and 500  $\mu\text{l}$  SOC-medium was added to each tube. The tubes were incubated at 37  $^{\circ}\text{C}$  for 1 hour with shaking at 225 rpm, and 10  $\mu\text{l}$  and 100  $\mu\text{l}$  were streaked out on an LB plate with 100 mg/ml ampicillin, and after centrifuging the rest of the volume in the tube, the supernatant was pipetted off, the cell pellet (~50  $\mu\text{l}$ ) was resuspended, and streaked out on a

LB plate containing 100 mg/ml ampicillin. For the control reaction, 100  $\mu$ l transformed cells were spread out on LB-ampicillin agar containing 80  $\mu$ g/ml X-gal and 20 mM IPTG. All transformation plates were incubated at 37 °C over night.

## 4.10 Isolation of plasmids

The “E.Z.N.A Plasmid Miniprep Kit 1” (Omega Bio-Tek) was used for isolation of plasmids from *E. coli* strains.

One single colony was inoculated in 5 ml LB-medium with the appropriate antibiotic at 37 °C and shaking at 225 rpm over night in a 50 ml Nunc tube, and then centrifuged at 2800  $\times$  g for 10 minutes. The supernatant was discarded. The pellet was resuspended in 500  $\mu$ l Solution I and vortexed until no clumps of cells were visible. Solution II (250  $\mu$ l) was added and the tube was carefully inverted until a clear lysate was obtained. Solution III (350  $\mu$ l) was then added followed by immediate inversion several times until a white flocculate formed. The tube were centrifuged at 11 500  $\times$  g for 10 minutes. Columns were placed in a collection tube and prepared by adding 100  $\mu$ l 45 Equilibration Buffer and centrifuging at 11 500  $\times$  g for 1 minute. The supernatant was carefully transferred to the column and centrifuged at 11 500  $\times$  g for 1 minute. Flow-through was discarded, and 500  $\mu$ l Buffer HB was added before the column was centrifuged as above. Flow-through was discarded, and 700  $\mu$ l DNA Wash Buffer was added. The tube was centrifuged, the flow through discarded, and this wash step was repeated once. The empty column was then centrifuged at 11 500  $\times$  g for 2 minutes and transferred to fresh eppendorf tubes. Elution Buffer (30  $\mu$ l) was added to the column and centrifuged at 11 500  $\times$  g for 1 minute. The elute was pooled and the concentration and purity of DNA was measured with UV-spectrophotometry (Chapter 4.3).

## 4.11 Sequencing of plasmid DNA

To prepare plasmid DNA for sequencing, a miniprep was done as described in chapter 4.10, and sequencing was done at the ABI-lab at the Department of Biology, University of Oslo.

An 8-strip PCR tube was used. The purpose of sequencing was to confirm that the right sequence was amplified during PCR. Separate reactions with forward and reverse primers respectively, were made.

For each PCR-tube, the following components were mixed:

1  $\mu$ l miniprep (200 ng)

7  $\mu$ l MQ-water

2  $\mu$ l primer (5 $\mu$ M)

## 4.12 Biofilm screening assay

Bt407 forms biofilm at the air-liquid interface, and the biofilm adhere to plastic. Flexible, 96 well U-bottomed PVC microtiter plates were used. The microtiter plate assay has been used successfully in biofilm screening of various *B. cereus* strains [76, 77].

On the first day of the assay, bacteria were streaked out on LB agar plates and incubated over night at 30°C or 37 °C. On the second day, one single colony was inoculated in 5 ml LB medium and incubated over night at 30°C and 37 °C with shaking at 225 rpm. On the third day, 50  $\mu$ l over night culture was inoculated in 5 ml LB with the appropriate antibiotic and 1 mM xylose for 3 hours. The pre-culture was then diluted 1:200 in fresh bactopectone medium (Chapter 3.8), and 125  $\mu$ l was added to each well of a PVC microtiter plate. Each strain of bacteria was added to sixteen wells, i.e. to two entire columns of the plate. The first column functioned as a blank by adding bactopectone medium only. The plates were closed with corresponding lids and placed in a plastic chamber containing moist filter paper. After positioning the lid on the chamber, the lid was sealed off with plastic wrap, making the chamber air-tight. The chambers were placed at 30°C or 37 °C for the proper number of hours.

When harvesting the plates, the wells were first observed, by seeing if the blank column was clear, and if pellicles and similar growth could be observed.

The wells were first washed once with 150  $\mu$ l PBS (Chapter 3.7). Crystal violet solution (0,3%, 130  $\mu$ l, Chapter 3.7) was then added to each of the wells, and the plates were incubated for 20 minutes at room temperature. The wells were then washed three times with 180  $\mu$ l PBS. Acetone:ethanol (1:4, 150  $\mu$ l) was then added to each of the wells. From each well, 75  $\mu$ l was transferred to the wells of a 96 polystyrene plate with flat bottoms to which 75  $\mu$ l

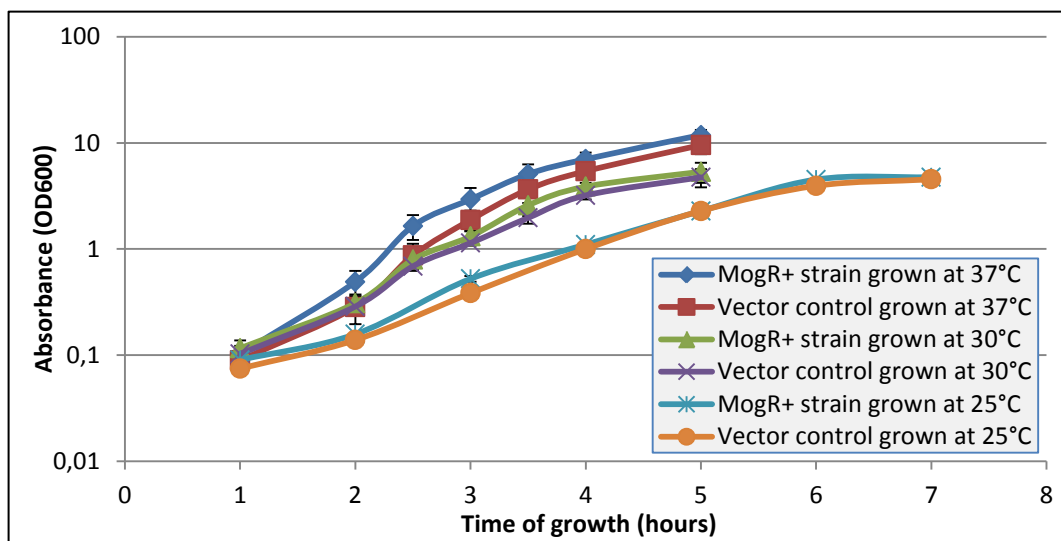
acetone:ethanol (1:4) was already added, thereby diluting the samples 1:2. Absorbance was measured at 492 nm with an HTS 7000 Bio Assay Reader.



# 5 RESULTS

## 5.1 MogR overexpression completely inhibits motility in Bt407

In order to study the role of the MogR protein in *B. cereus*, *mogR* had previously been cloned into the low-copy shuttle vector pHT304-pXyl and introduced into *B. thuringiensis* Bt407, allowing xylose-inducible expression of MogR (A. Fagerlund, unpublished; Chapter 3.1). To examine whether overexpression of MogR affected cell growth, growth of the MogR overexpression strain in liquid culture was compared with that of the vector control strain carrying the pHT304-pXyl empty vector (i.e. without the *mogR* gene inserted), as described in chapter 4.1. To induce expression of MogR, xylose at 10mM was added to the cultures. Growth was examined at four different temperatures: 25°C, 30°C, 37°C and 42°C. At 42°C, no growth was observed even after incubation for >16 hours. The results presented in figure 10 show that overexpression of MogR did not affect growth at either of the temperatures examined.



**Figure 10: Growth curves of a *B. thuringiensis* (Bt407) MogR overexpression strain (MogR+) and an empty vector control strain.** Cells were grown in LB medium with erythromycin (10 µg/ml) and xylose (10 mM), at 25 °C, 30°C or 37 °C with rotation at 225 rpm. The means and standard errors of three independent experiments are shown.

The effect of MogR overexpression on motility was studied by visual examination in a light microscope of samples of bacteria collected during growth in culture. The degree of motility at each examined time-point was scored on a scale from 0 to 5, where 0 indicates no observed motility and 5 indicates the maximum observed degree of movement (Chapter 4.1).

Interestingly, Bt407 cells overexpressing MogR were completely immotile throughout the growth curve at the three tested temperatures (25°C, 30°C, and 37°C) (Table 6). The motility of the vector control strain appeared to be maximal at around late exponential phase/early stationary phase at all three tested temperatures, with the highest degree of motility at 25°C observed after 6-7 hours of growth, and at 30°C and 37°C after 3 hours of growth (Table 6).

**Table 6. Motility of *B. thuringiensis* (Bt407) MogR overexpression (MogR+) and vector control strains observed at the indicated times during growth in liquid culture at 25°C, 30°C and 37°C.** The results are ranked on a scale between 0 and 5, where 5 is maximum motility and 0 is no observed motility, and are based on three biological replicates.

		1h	2h	2.5h	3h	3.5h	4h	5h	6h	7h
25°C	MogR+ strain	0	0		0		0	0	0	0
	Vector control	1	1		1		2	3	4	4
30°C	MogR+ strain	0	0	0	0	0	0	0		
	Vector control	0	1	2	4	3	2	1		
37°C	MogR+ strain	0	0	0	0	0	0	0		
	Vector control	0	3	4	5	3	2	1		

## 5.2 Analysis of changes in global gene expression as a result of MogR overexpression

A genome-wide transcriptional microarray analysis comparing the Bt407 MogR overexpression strain with the vector control strain was performed to investigate the effect of *mogR* expression on global gene expression. The samples to be compared were collected after 3 hours of growth at 37°C, as the highest degree of motility in the vector control strain was observed at this time-point and temperature (Table 6). Cells were grown as described in chapter 4.1, and as before, 10 mM xylose was added to the cultures to induce expression of MogR. Four biological replicates of both the empty vector control strain and MogR overexpression strain were collected as described in chapter 4.1. OD<sub>600</sub> was measured upon harvesting (Table 7) and motility was observed in a light microscope as a control, and the

MogR overexpression strain was nonmotile, while the empty vector control was confirmed to be highly motile at the point of harvesting cells.

**Table 7. OD<sub>600</sub> values of cultures at the time of harvest for microarray analysis.** Four biological replicates (1-4) of each strain were analyzed.

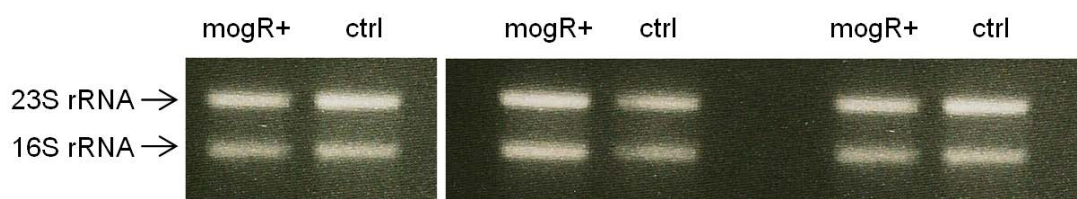
Set	<i>B. thuringiensis</i> (Bt407) empty vector control	<i>B. thuringiensis</i> (Bt407) MogR overexpression strain
1	3,03	3,10
2	3,17	3,70
3	2,76	3,50
4	3,73	2,74

### 5.2.1 Isolation of RNA from the empty vector control and MogR overexpression strains

RNA was isolated by using the RNeasy mini kit (Qiagen, chapter 3.9), and eluated in 100 µl RNase free DEPC-water (Chapter 4.2). Concentration and purity was measured (Chapter 4.3) using UV-spectrophotometry (Table 8). The purity of RNA should have an A<sub>260</sub>/A<sub>280</sub> ratio of 1,9 to 2,1, according to the Qiagen protocol. The A<sub>260</sub>/A<sub>280</sub> ratios given in table 8 are within this range, indicating that the purity of the RNA was good. RNA integrity was analyzed by agarose gel electrophoresis (Chapter 4.4). The ribosomal bands were sharp and no degradation was observed (Figure 11). The prepared RNA was thus of sufficiently good quality for use in microarray analysis.

**Table 8. Concentration measurements of RNA isolated from four biological replicates (1-4) each of the Bt407 empty vector control strain and the MogR overexpression strain (MogR+).** The ratio of the absorbance at A<sub>260</sub> and A<sub>280</sub> was calculated to estimate purity.

Set	RNA concentration, vector control	RNA Concentration, MogR+	A <sub>260</sub> /A <sub>280</sub> vector control	A <sub>260</sub> /A <sub>280</sub> MogR+	A <sub>260</sub> vector control	A <sub>260</sub> MogR+
1	0,250µg/µl	0,238µg/µl	1,96	1,93	0,11	0,13
2	0,648µg/µl	0,584µg/µl	2,04	2,10	0,29	0,33
3	0,886µg/µl	0,492µg/µl	1,92	1,89	0,40	0,22
4	0,716µg/µl	1,297µg/µl	1,87	1,95	0,33	0,61



**Figure 11.** Representative examples of results from agarose gel electrophoresis analysis performed to inspect the integrity of isolated RNA samples from the Bt407 MogR overexpression strain (lanes labeled mogR+) and the Bt407 empty vector control strain (lanes labeled ctrl). The ribosomal 23S rRNA and 16S rRNA bands are indicated by arrows.

### 5.2.2 Analysis of differential gene expression by microarray experiments

Isolated RNA samples from four biological replicates were used for the microarray experiments and converted to cDNA, as described in chapter 4.5.1 and 4.5.2 respectively. Each set of cDNA was differentially labeled with the fluorescent dyes Cy3 and Cy5, and competitively hybridized to microarray slides. The cDNA from the MogR overexpression strain was labeled with Cy3 and the empty vector cDNA with Cy5 in sets 1 and 3, and MogR overexpression cDNA was labeled with Cy5 and empty vector cDNA with Cy3 in sets 2 and 4. Following hybridization and washing of the slides, they were scanned in a microarray scanner. During scanning, the intensities of the scanning beams from each wavelength, 635 nm and 532 nm, were adjusted to achieve a ratio as close to 1 as possible between the total sum of the green (Cy3) signal and the total sum of the red (Cy5) signal (the count ratio) from the raw image. Table 9 shows count ratios and intensities for each slide used in the analysis.

**Table 9. The count ratio for scanned slides 1-4 used in microarray experiments comparing effect of MogR overexpression strain on global gene expression.** PTM gain for the red (635) and green (532) channel is also shown.

Slide	PTM Gain 635	PTM Gain 532	Count ratio
1 (nr. 37)	720	610	0,98
2 (nr. 41)	800	640	1,01
3 (nr. 45)	720	540	1,01
4 (nr. 46)	660	540	1,03

Further analysis of data was done using the statistical computing platform "R" (<http://www.r-project.org>) and the statistical software package LIMMA (Linear models for microarray data) [78]. Histograms showing the distribution of intensities from the red and green background, as well as red and green spots, are shown in Appendix A. Minimum intensities to be included in the downstream analysis for the red and green spots were set based on the mentioned histograms, to ensure that background signals were not interfering with the results. Cut-off values of 200 were chosen both for red and green spots. From the final list of genes obtained from the microarray analysis, only genes with false discovery rate (FDR) corrected p-values < 0.05 were considered differentially expressed.

The results from the microarray analysis showed that a total of 25 genes, including *mogR* itself, were up-regulated in the strain overexpressing MogR as compared with the vector control strain. A larger number of genes, 91 in total, were down-regulated in the MogR overexpression strain, and are thus putatively repressed, either directly or indirectly, by MogR. The complete list of differentially expressed genes (p-values < 0.05) from the microarray experiment is shown in Appendix B.

As expected, the *mogR* gene was found to be significantly up-regulated (two-fold) in the MogR overexpression strain compared to the empty-vector control strain (Table 10). More interesting was the observation that all other differentially regulated genes within the Bt407 motility gene loci (bthur0002\_15350 to bthur0002\_15790, and bthur0002\_43710 to bthur0002\_43720), encoding different flagellar and motility proteins, were down-regulated upon MogR overexpression (Table 10). This strongly indicates that MogR acts as a motility gene repressor in Bt407, like it does in *L. monocytogenes* [63].

Other interesting data obtained from the microarray experiment was the observation that several known virulence factors, including the genes encoding phospholipase C and HBL components B and L1, were down-regulated in the MogR overexpression strain (Table 11). Like the virulence related genes, stress genes were also observed to be down-regulated in the MogR overexpression strain (Table 12). In contrast, three genes putatively involved in promoting biofilm formation, were up-regulated in the MogR overexpression strain (Table 13). Of these, the gene encoding SinI was two-fold up-regulated in the MogR overexpression strain. SinI is an antagonist of SinR, which is known to repress expression of biofilm matrix genes in *B. subtilis* and *B. cereus* ([30], Gohar et al., unpublished data). Furthermore, two genes known to carry upstream c-di-GMP responsive riboswitches in their 5' untranslated

regions (BC1060, BC0422; Table 13), were oppositely regulated upon MogR expression (Table 13).

<b>Table 10. Genes within the Bt407 motility gene loci (bthur0002_15350 to bthur0002_15790, and bthur0002_43710 to bthur0002_43720) differentially regulated in a microarray experiment comparing gene expression in a MogR overexpression strain with that in an empty vector control strain. All listed genes have FDR-corrected p-values&lt;0.05.</b>				
<b>Locus tag in Bt407</b>	<b>Locus tag in ATCC14579</b>	<b>Predicted function</b>	<b>log<sub>2</sub> (fold change)*</b>	<b>Average expression<sup>#</sup></b>
bthur0002_15380	BC1628	Chemotaxis protein, histidine kinase CheA	-1,80	8,79
bthur0002_15400	BC1630	hypothetical protein	-0,92	7,99
bthur0002_15460	BC1636	Flagellar hook-associated protein 1, FlgK	-1,67	8,12
bthur0002_15490	BC1639	Flagellar (assembly) protein FliS	-0,68	8,37
bthur0002_15520	BC1642	Flagellar basal-body rod protein FlgC	-2,87	8,89
bthur0002_15540	BC1644	Flagellar MS-ring protein FliF	-2,31	8,63
bthur0002_15550	BC1645	Flagellar motor switch protein FliG	-1,09	7,94
bthur0002_15590	BC1649	Flagellar hook-length control protein FliK	-1,47	8,29
bthur0002_15630	BC1653	hypothetical protein	-1,37	8,52
bthur0002_15640	BC1654	Chemotaxis signal transduction protein CheV	-1,80	8,43
bthur0002_15650	BC1655	mogR	0,75	8,56
bthur0002_15660	BC1656	flagellin FlaB	-1,04	7,87
bthur0002_15670	BC1657	flagellin FlaA	-3,45	9,11
bthur0002_15680	BC1660	Putative lytic murein transglycosylase	-0,75	8,12
bthur0002_15690	BC1661	Flagellar motor switch protein (fliN-homolog)	-0,99	8,14
bthur0002_15730	BC1665	Flagellar biosynthesis protein FliP	-1,86	8,11
bthur0002_15740	BC1666	Flagellar biosynthesis protein FliQ	-1,23	7,99
bthur0002_15750	BC1667	Fflagellar biosynthesis protein FliR	-1,02	7,73
bthur0002_15760	BC1668	Flagellar biosynthesis protein FlhB	-1,60	8,10
bthur0002_15770	BC1669	Flagellar biosynthesis protein FlhA	-1,43	8,64

bthur0002_43710	BC4512	Flagellar motor protein MotB (H <sup>+</sup> -coupled stator)	-3,01	9,02
bthur0002_43720	BC4513	Flagellar motor protein MotA (H <sup>+</sup> -coupled stator)	-1,74	8,20

\*Fold change is the ratio of expression in the MogR overexpression strain divided by the expression in the empty-vector control strain  
#Average expression is a measurement of how much mRNA is present for the specific gene in the array. The higher the value, the more mRNA is present.

**Table 11. Virulence genes differentially regulated in microarray experiment** comparing gene expression in a MogR overexpression strain with that in an empty vector control strain. All listed genes have FDR-corrected p-values<0.05.

Locus tag in Bt407	Locus tag in ATCC14579	Predicted function	log <sub>2</sub> (fold change)*	Average expression #
bthur0002_5080	BC0598	Helix-turn-helix domain protein	-0,89	8,29
bthur0002_5820	BC0666	Immune inhibitor A	-1,03	8,42
bthur0002_5870	BC0670	Phospholipase C (PC-PLC)	-2,32	8,38
bthur0002_5880	BC0671	Sphingomyelinase C (Smase)	-1,47	7,82
bthur0002_28440	BC3102	Hbl component B	-1,13	8,81
bthur0002_28450	BC3103	Hbl component L1	-1,59	8,79

\*Fold change is the ratio of expression in the MogR overexpression strain divided by the expression in the empty-vector control strain  
#Average expression is a measurement of how much mRNA is present for the specific gene in the array. The higher the value, the more mRNA is present.

**Table 12. Stress genes differentially regulated in microarray experiment** comparing gene expression in a MogR overexpression strain with that in an empty vector control strain. All listed genes have FDR-corrected p-values<0.05.

Locus tag in Bt407	Locus tag in ATCC14579	Predicted function	log <sub>2</sub> (fold change)*	Average expression #
bthur0002_41690	BC4313	hypothetical protein; similar to Molecular chaperone GrpE (heat shock protein)	-0,75	11,59
bthur0002_41700	BC4314	Heat-inducible transcription repressor hrcA	-0,67	9,59
bthur0002_2270	BC0294	10 kDa chaperonin; similar to co-chaperonin GroES	-1,06	11,98
bthur0002_2280	BC0295	60 kDa chaperonin 2; similar to chaperonin GroEL	-0,58	9,32
bthur0002_9210	BC1004	RNA polymerase sigma-B factor	-1,05	9,43

\*Fold change is the ratio of expression in the MogR overexpression strain divided by the expression in the empty-vector control strain

# Average expression is a measurement of how much mRNA is present for the specific gene in the array. The higher the value, the more mRNA is present.

**Table 13. Genes associated with biofilm formation and predicted c-di-GMP regulated genes** differentially regulated in microarray experiment comparing gene expression in a MogR overexpression strain with that in an empty vector control strain. All listed genes have FDR-corrected p-values<0.05.

Locus tag in Bt407	Locus tag in ATCC14579	Predicted function	log <sub>2</sub> (fold change)*	Average expression#
bthur0002_3430	BC0422	Methyl-accepting chemotaxis protein (with upstream "off" c-di-GMP riboswitch)	-1,31	8,44
Not annotated	BC1060	collagen adhesion protein with upstream c-di-GMP "on" riboswitch	3,02	9,01
bthur0002_11930	BC1283	SinI	1,08	8,29
bthur0002_51490	BC5278	Capsular polysaccharide biosynthesis	0,71	8,96

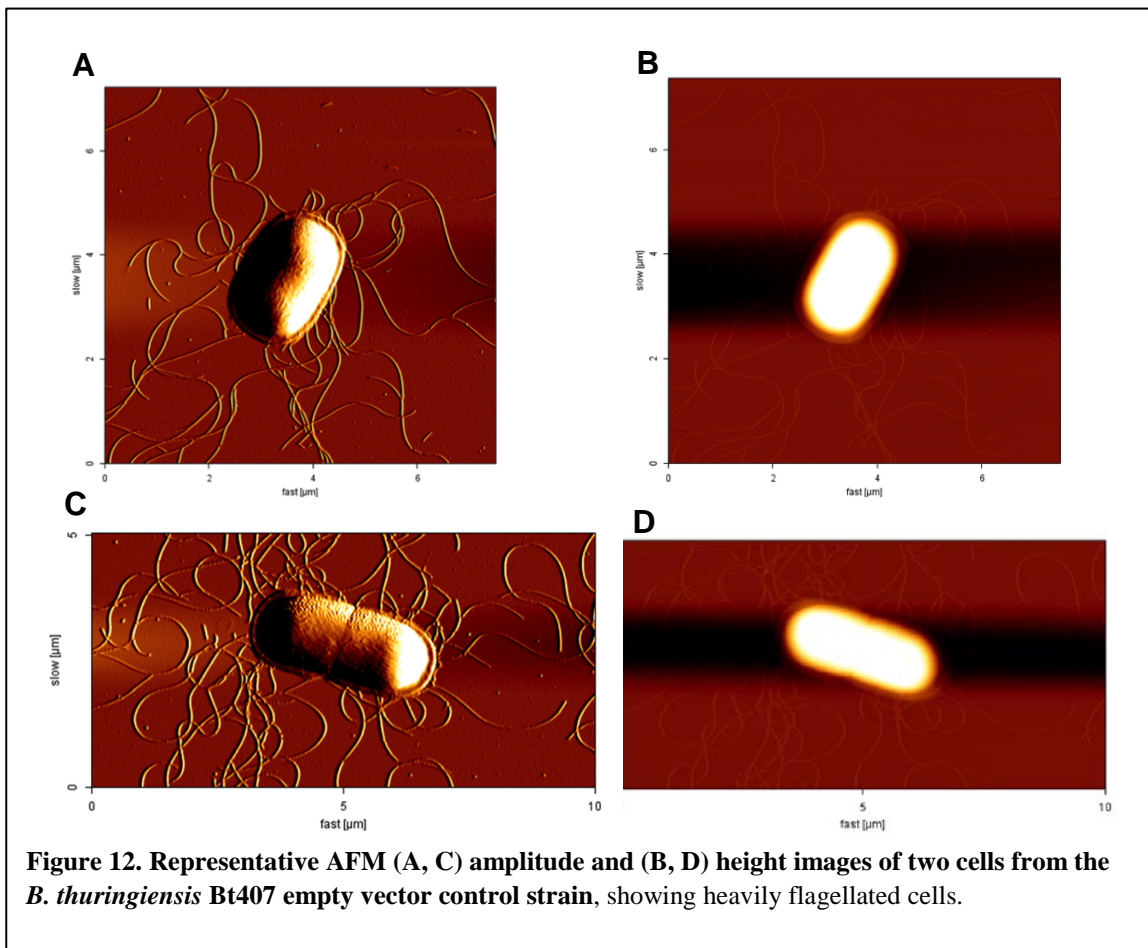
\*Fold change is the ratio of expression in the MogR overexpression strain divided by the expression in the empty-vector control strain.

# Average expression is a measurement of how much mRNA is present for the specific gene in the array. The higher the value, the more mRNA is present.

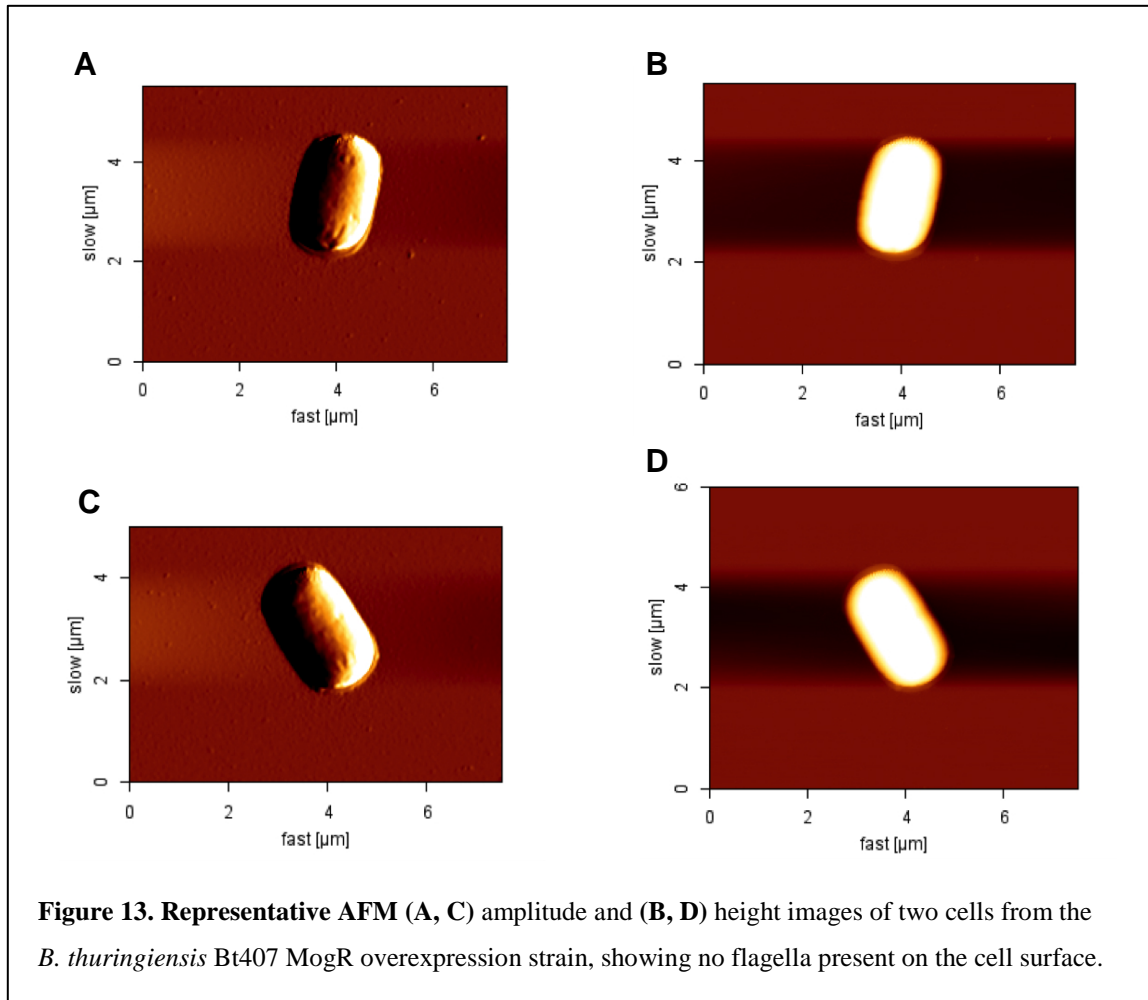


### 5.3 Atomic force microscopy (AFM) imaging of Bt407 MogR overexpression strain and Bt407 empty vector control strain

As cells overexpressing MogR were non-motile (Table 6), and since a large proportion of the genes down-regulated in the MogR overexpression strain were associated with motility (Table 10), cells were examined using Atomic Force Microscopy (AFM) to investigate whether cells overexpressing MogR lacked flagella. The procedure was done as described in chapter 4.6, and Ida K. Hegna performed the AFM-microscopy.



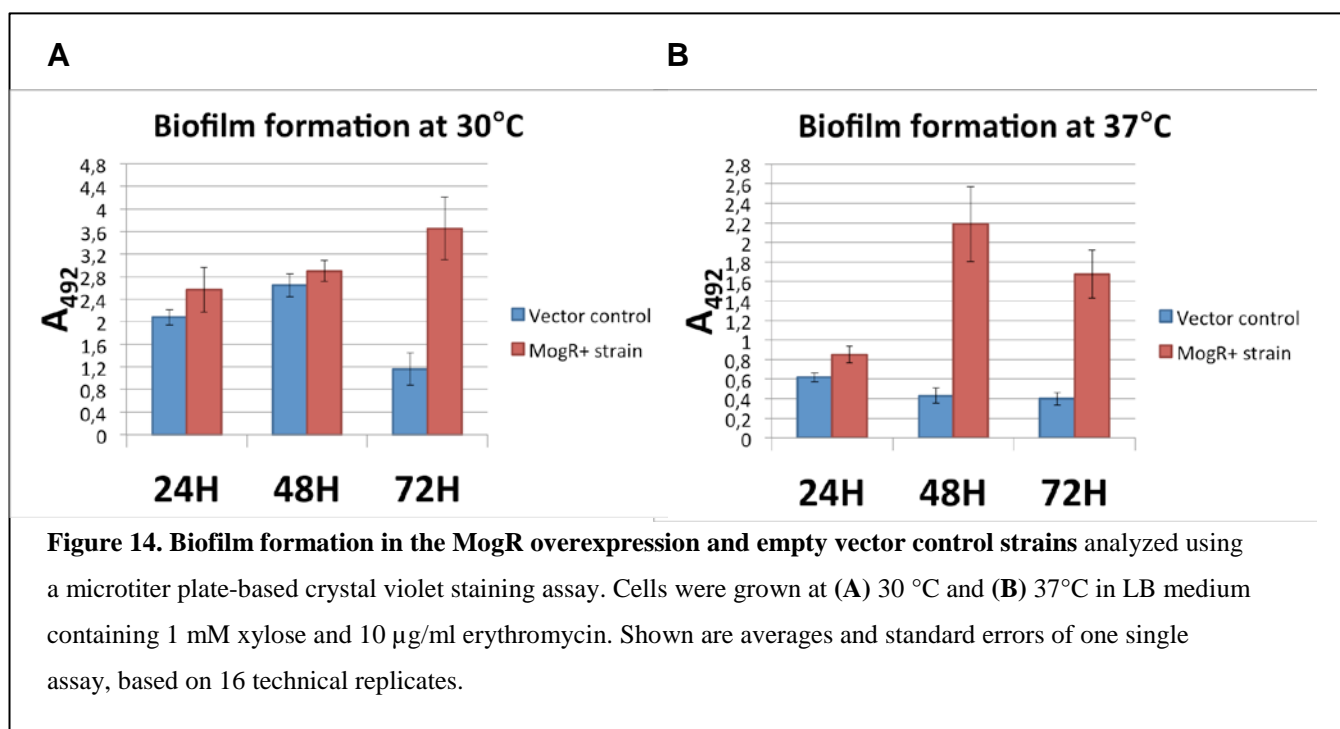
Our results show that the empty vector control strain was heavily flagellated when grown at 37 °C. In contrast, no flagella were observed on cells of the Bt407 MogR overexpression strain at 37 °C (Figure 12). Results shown in figure 13 confirm that in the Bt407-pXyl-mogR, flagella were not assembled on the cell surface. This is in accordance with the microarray



results, which showed down-regulation of flagellum genes in the MogR overexpression strain (Table 10). During the course of the AFM experiment more than 100 cells were investigated, and only one occurrence of partially flagellated bacteria was shown in the MogR overexpression strain sample.

## 5.4 Effect of MogR overexpression on biofilm formation

Microarray results had indicated that MogR might possibly play a role in the regulation of biofilm formation, given the effect of MogR overexpression on transcription of several biofilm-related genes (Table 13). Therefore, a biofilm formation screening assay was performed, using the MogR overexpression and vector control strains, to see whether overexpression of MogR affected the ability of Bt407 to form biofilms. Biofilm formation was analyzed after 24, 48 and 72 hours of growth at both 30 °C and 37 °C, in a microtiter plate assay as described in chapter 4.12. Xylose at 1 mM was added to the strains to induce expression of MogR. Results are shown in figure 14, based on one biological replicate only, with 16 technical replicates for each strain. At 37 °C, the MogR overexpression strain produced significantly more biofilm than the empty vector control, at all three tested time-points in the one experiment performed (Figure 14B). At 30 °C however, the MogR overexpression strain and the empty vector control strain produced a similar amount of biofilm at 24 and 48 hours, but at 72 hours, the MogR overexpression strain produced significantly more biofilm compared with the empty vector control strain (Figure 14A). Thus, based on an initial screening experiment, it appears that MogR overexpression results in increased biofilm formation in Bt407, and that this effect was most pronounced at 37°C.



## 5.5 Functional analysis of MogR - Mutating predicted active site residues in the MogR overexpression clone (first mutagenesis)

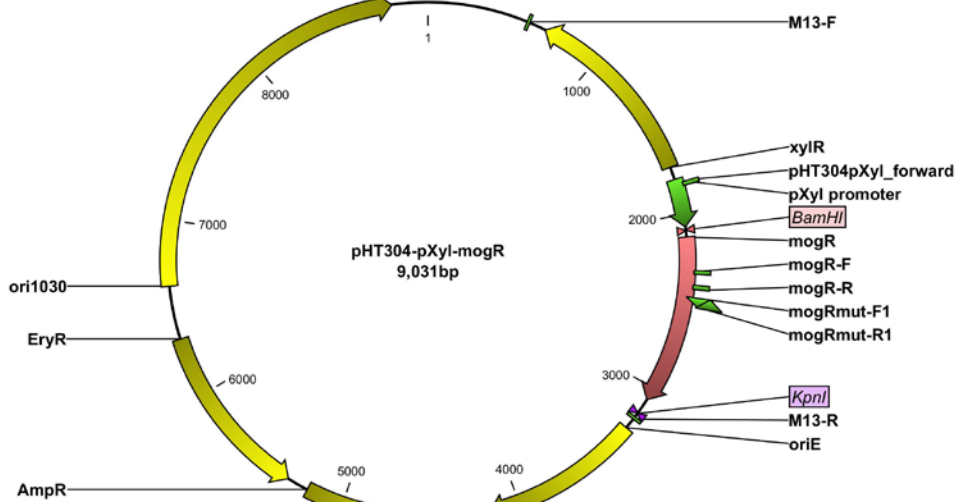
In this experiment we aimed to mutate the *mogR* gene to potentially inactivate the putative MogR active site. Based on an alignment between the *L. monocytogenes* and Bt407 MogR proteins, the codons in the Bt407 gene corresponding to the six amino acid residues that were shown in *L. monocytogenes* MogR to make base-specific contacts with the MogR recognition site [66] were identified (Figure 15). Two of these six residues were selected for mutation, marked in green in figure 15. If mutation of these amino acids was successful, the outcome was expected to be an inactivated protein. The codons “caa” and “aat”, encoding glutamine and asparagine respectively, were to be changed to “gca” and “gct” codons, both encoding alanine.

```
1 atgtatcacc acacagcaat taatgtatta ggtcttttgc aaaacatgtc
51 aaataataaa atgaacgata tgcaactaga ggtggaatta aaaaaaatag
101 aaaaagaatt ccaagtgaag tatgaagaat tagttgattt acataataga
151 atggtattat ttcaaataga tatagaaaaa aatggcgagg tgcgagcata
201 tgaaaaatca gcaattacat ggctgaagtc tgaactagag ctactgtatg
251 aagtgtatca attttgtcaa cgtcacgggt taaacatttt aaatatttca
301 aaatacgtaa gtaaaaatga actaaatctt tttccaaaaa cagaaagtca
351 attgcaaaa acgtattata aattgaaaaa acgtgaaata ccgtttgaaa
401 atattgaaaa acagaaacca ggcgaaagc gaaaatatat accagtaaaa
451 gagacaatcg ttacaagaaa acaagaaaac gagtacgaat taaaagaaga
501 aatccagcat aaagaggatg aaaaaaatct tgtaacagtt atatctggta
551 ttgttgataa ctttgaaaca attaataaat gtagtgaaag gaaagaagac
601 gaactacatc agtttatgga aggtatttat aagctttcta gcatggcagc
651 agggcgttct agagaagcaa agaatacacg tagcttagaa ggtgaattgc
701 agtcgttaca actagaaaat gaaaggctaa aacgagagaa agaagagctt
751 gtacaagata ttaaaggatg gacgcagctt ttaattcatt tcattacaag
801 ttctgatatc gatcaaatc gtaaattacc aactttcgta caagattgta
851 aaatggaatt acataaacta gggctatata atgcacaaga cggtaaaatg
901 aaaattatgg ttgatcgtag cggacaagtt atgaccgtaa cacagtaa
```

**Figure 15. DNA sequence encoding MogR.** The codons marked in yellow are codons encoding amino acids that were predicted to be in direct contact with bases in the DNA. This prediction was based on comparison of the Bt407 MogR sequence with that of *L. monocytogenes* MogR, for which the residues making base-specific contacts with DNA has been determined [66]. The codons highlighted in green are those that were chosen for alanine mutation (from glutamine and asparagine, respectively).

### 5.5.1 PCR

The mutations were to be introduced by site-directed mutagenesis, as described in chapter 4.7, which involves performing PCR with primers containing the desired mutations, and are otherwise complimentary to the site in *mogR* where mutations are to be introduced (Figure 16). The primers (*mogRmut-F* and *mogRmut-R*, see chapter 3.3) were designed specially for this experiment by Annette Fagerlund. The original plasmid, containing the original *mogR* gene, is referred to as pHT304-pXyl-*mogR*, while the intended construct, with the successfully incorporated mutations, will be referred to as pHT304-pXyl-*mogRmut*. The PCR reaction was run as described in chapter 4.7, and a negative control, without added DNA polymerase, was included.



**Figure 16. Location of primer annealing sites on the pHT304-pXyl-*mogR* plasmid.** The complementary overlapping *mogRmutF1* and *mogRmutR1* primers anneal to opposite strands of DNA at the location where the mutations are intended to be introduced into the pHT304-pXyl-*mogR* plasmid to create pHT304-pXyl-*mogRmut*. Picture made by Annette Fagerlund.

### 5.5.2 Purification of PCR product and treatment with *DpnI*

Following PCR, the DNA was purified with the E.Z.N.A PCR Purification kit (Chapter 4.7.1) and treated with *DpnI* restriction enzyme (Chapter 4.8). The *DpnI* restriction enzyme cleaves fully methylated G<sup>Me6</sup> ATC sequences, and will digest the bacterial DNA used as template for amplification, but not the newly synthesized PCR-generated DNA-fragments.

*DpnI* restriction enzyme (2 µl) was added to the eluted sample and negative control, respectively. The negative control, without added DNA polymerase in the PCR reaction, was used to confirm that the polymerase replicates the strains and produces more DNA fragments in the sample reaction, but not in the negative control.

## 5.6 Transformation of first mutagenesis

### 5.6.1 Chemical transformation

Competent *Escherichia coli*, *E. coli* XL-1 Blue (Chapter 3.1), were prepared and transformed as described in chapter 4.9.1, with 2 µl sample plasmid DNA from each reaction.

No colonies were detected after 24 hours of growth at 37°C from either reaction.

### 5.6.2 Electrotransformation

As the chemical transformation did not give any colonies, an electroporation method was carried out. In order to perform electrotransformation of plasmid DNA in the mutagenesis procedure, ElectroTen-Blue Electroporation Competent cells (Agilent) were used.

The electroporation was performed as described in chapter 4.9.2, and cells (30 µl) were transformed with 2 µl plasmid DNA, 2 µl negative control or 1 µl pUC18 control plasmid (0.1 ng/µl), respectively. Table 14 shows the time constant from the electroporations. A high time constant should be >8, and indicated that a sufficient low ion content is present in the cell suspension transformed.

<b>Table 14. Time constant of electroporation.</b>		
pHT304-pXyl:: <i>mogR</i> mut mutagenesis reaction	Negative control	pUC18 control plasmid
9,04	9,06	10,2

Colonies could only be observed from the pUC18 control plasmid transformations after 24 hours of growth at 37°C. The cells were counted and the transformation efficiency (colony forming units (CFU)/μg transformed DNA) was estimated to be  $1,31 \times 10^{10}$  CFU/μg for the pUC18 reaction. This number corresponds well with the number according to the manufacturer, which says that a transformation efficiency  $\geq 3 \times 10^{10}$  is estimated, so this was considered an expected number. This indicated that there was nothing wrong with the competent cells.

A second identically repeated mutagenesis experiment did not produce any colonies from the pHT304-pXyl::*mogR*mut plasmid, nor from the negative control plasmid.

## **5.7 Mutating predicted active seats in *mogR* - Second mutagenesis experiment**

As the above described attempt to create the intended mutations were unsuccessful using the QuikChange II Site-directed Mutagenesis kit (Stratagene), it was decided to try to perform the mutagenesis reaction using the QuikChange II XL site-directed Mutagenesis Kit (Stratagene) instead. This kit is, according to the manufacturer, optimized for large and difficult templates.

### **5.7.1 PCR and treatment with *DpnI***

Mutation reactions were again carried out by PCR, as described in chapter 4.7. Three mutagenesis reactions containing pHT304-pXyl-*mogR* DNA template (1 μl) were set up (Chapter 4.7). The two mutagenesis primers, designed for the experiment (*mogR*mut-F and *mogR*mut-R, see chapter 3.3) by Annette Fagerlund were added to each sample. A control mutagenesis reaction, using the pWhitescript 4.5-kb plasmid and mutagenesis primers included in the QuikChange II XL site-directed Mutagenesis Kit, was also set up. The four mutagenesis reactions were set up and cycled in PCR reactions as described in chapter 4.7. Of

the three reactions with pHT304-pXyl-mogR DNA template, one was cycled using the PCR program with 55°C annealing temperature, and the two others were cycled using the PCR program with 60°C annealing temperature (see chapter 4.7). The control reaction was cycled using the PCR program with 60 °C annealing temperature.

Following PCR, the mutagenesis reactions were treated with 1 µl *DpnI* restriction enzyme (Chapter 4.8) as provided by the manufacturer (Stratagene), by incubation on a heating block at 37 °C for 1 hour.

## 5.8 Transformation of second mutagenesis

Chemical transformation, using XL10-Gold Ultracompetent Cells (Stratagene) was performed as described in chapter 4.9.3.

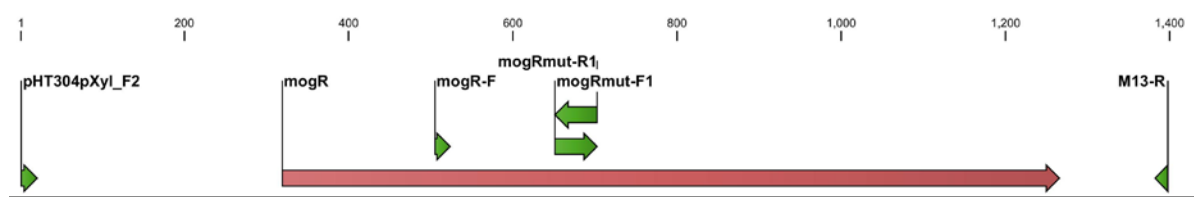
Plates from transformants of the pHT304-pXyl::*mogR*mut plasmid transformants and from the control plasmid transformations were observed after 24 hours of growth at 37°C. For the control reaction, in which the pWhitescript 4.5-kb plasmid was mutated, the agar plates were confluent with growth, thus the number of obtained colonies could not be counted. For the transformations of pHT304-pXyl::*mogR*mut plasmid, 12 CFU (annealing temperature 55 °C, 1 µl plasmid DNA), 16 CFU (annealing temperature 60 °C, 1 µl plasmid DNA) and 66 CFU (annealing temperature 60°C, 1 µl plasmid DNA) were observed.

## 5.9 Sequence analysis of pHT304-pXyl::*mogR*mut

To confirm that the intended mutations in *mogR* had been achieved, plasmid DNA was prepared from 6 clones, and DNA samples were sent for DNA sequence analysis at the ABI-lab (Department of Biology and Molecular Biosciences, University of Oslo). Two single colonies from each of the pHT304-pXyl::*mogR*mut plasmid transformation plates were picked and inoculated in 5 ml LB with ampicillin (100 µg/ml), six in total. The next day, minipreps were prepared from the cultures as described in chapter 4.10, and eluted in 30 µl Elution buffer. Template DNA (200 ng) was mixed with 2 µl primer (5 µM) for each sample (Chapter 4.12), where the primers named MogR-F, M13-R, and pHT304pXyl\_F2 were used. pHT304pXyl\_F2 and MogR-F anneals upstream of the intended mutagenesis site in *mogR*,



while M13-R anneals downstream of this site, as shown in Figure 17. The insert in each pHT304-pXyl::*mogRmut* clone was sequenced with an ABI 3730 high-throughput capillary electrophoresis sequencer, and the sequence files were read in CLC Main Workbench 6. The analysis confirmed that the expected base mutations had been achieved in the mutagenesis reaction (data not shown). Therefore, in the MogRmut protein encoded on the new pHT304-pXyl-mogRmut plasmid, the amino acids glutamine and asparagine have been successfully mutated to alanine.



**Figure 17.** The position of the *mogR* gene and the sequencing primers (*mogR*-F, pHT304pXyl\_F2, M13-R) and the PCR mutagenesis primers (*mogRmut*-F1, *mogRmut*-R1). Figure made by Annette Fagerlund.

# 6 DISCUSSION

## 6.1 Effect of MogR overexpression on growth and motility

We showed that MogR controls motility in *B. cereus* by making an overexpression strain in *Bacillus thuringiensis* Bt407 (done by Annette Fagerlund) and observing motility in a light microscope at 37 °C. Our results showed that upon overexpression of MogR, motility was completely inhibited in Bt407. This strongly indicates that MogR acts as a motility gene repressor in Bt407, like it does in *L. monocytogenes*[63], but from the results in this thesis MogR may also regulate motility at both 25°C and 30 °C. This was not the case in *L. monocytogenes*, where GmaR represses MogR at lower temperatures [79]. Interestingly, in *B. thuringiensis* Bt407, which does not harbor a GmaR homologue, MogR expression is indeed regulated in a growth phase dependent manner (Fagerlund et al, unpublished). Accordingly, the AFM analysis confirmed that the MogR overexpression strain did not produce flagella at 37 °C.

## 6.2 Microarray analysis indicates MogR as a co-regulator for motility and virulence

A microarray experiment comparing a MogR overexpression strain to an empty vector control strain was carried out with harvested samples from the early exponential phase, when empty vector was most motile. In total, 136 genes were found to be significantly up- or down regulated with FDR-corrected P-values <0,05 (Appendix A). In this section, some of the most interesting genes will be discussed. For the whole array list or the array list with genes with FDR-corrected P-values <0,05, see Appendix B.

### 6.2.1 MogR may affect the expression of motility genes

All proteins for flagellar assembly and other motility related genes (with FDR-corrected P-values<0.05) were significantly down-regulated in the microarray analysis in the MogR

overexpression strain. MogR may thus function as a repressor for motility genes in *Bacillus thuringiensis* Bt407 at 37 °C, as described for *Listeria monocytogenes* [61]. The expression of the gene *flaA*, encoding flagellin, was found to be 10,92 fold lower expressed in the MogR overexpression strain compared to in the empty vector control strain. Also, the flagellar motor protein MotB was found to be 8,05 fold lower expressed in the MogR overexpression strain compared to in the empty vector control strain. These two genes are necessary for flagellar assembly and function, and if they are not present, the flagella most likely cannot be assembled and be active [41].

### **6.2.2 MogR may affect the expression of virulence genes**

All virulence genes shown to be differentially regulated in the microarray analysis were down-regulated. This may indicate that the MogR overexpression strain is less virulent than the control strain. It seems that MogR regulates virulence genes, including toxin genes, and this result supports earlier studies suggesting that virulence and motility is co-regulated [80]. Therefore it has been hypothesized that coordinated regulation of motility and virulence genes [81, 82] occurs in various bacterial pathogens. MogR repression may thus be the reason that motility and virulence genes are down-regulated at the same time in the transcriptional analysis.

### **6.2.3 MogR may affect the expression of stress related genes**

The microarray experiment showed, as expected, that *mogR* was up regulated in the MogR overexpression strain, but in addition there were several stress genes down regulated in response to MogR overexpression, among these homologs to the molecular chaperone systems DnaK and GroE, belonging to class I heat shock genes [83]. Proteins in the heat shock response generally play a role in protein folding, assembly and repair and prevention of aggregation both under stress and non-stress conditions [83].

The DnaK chaperone system has two important roles in stressed cells where proteins are becoming unfolded: i) they act as “holders”, probably by binding to exposed hydrophobic patches on the proteins to prevent aggregation from occurring and ii) by interacting with the protein ClpB they function as “refolders”, to refold aggregated proteins back to their active form [84]. The GroE chaperones (encoded by the genes *groES* and *groEL*) are particularly important in enabling cells to cope with stresses which makes proteins unfold [84].

The HrcA protein is a transcriptional repressor of class I heat shock genes [85]. In *B. subtilis*, HrcA is known to regulate the expression of the complex *dnaK* operon, consisting of *hrcA-grpE-dnaK-dnaJ* [86] and the *groES-groEL* operon [85].

The alternative sigma-B ( $\sigma^B$ ) factor, which controls expression of the class II heat shock genes [87], was also down-regulated upon overexpression of MogR.

In our analysis, the *clpB* class III heat shock gene did not come up in the array list, but is down-regulated in *Listeria*. [88]

It thus seems that MogR represses cellular stress responses. Why this happens, one can only speculate, but it has been suggested that in *L. monocytogenes*, MogR directly regulates stress responses in a similar manner to how it regulates motility [89]. Another hypothesis is that the down-regulation of stress genes in the MogR overexpression strain may be an indirect effect of the cells no longer having to produce flagella, which is a fairly energy-costly for the cells. The flagella production could cause stress in the cells, and the chaperone systems mentioned above may therefore be needed for correct protein folding and activation. Another hypothesis may be that, when the cells produce flagella there are many proteins involved, and therefore many chaperones need to be present. Since there are no flagella in the MogR overexpression strain, the cell may down-regulate the genes necessary for chaperone expression.

#### **6.2.4 MogR may affect the expression of biofilm related genes**

Three genes possibly related to biofilm formation were up regulated in response to MogR overexpression. These genes were *sinI* (bthur0002\_11930), a chain length regulator gene located within a potential exopolysaccharide (EPS) synthesis locus (bthur0002\_51490), and a putative cell wall-expressed MSCRAMM adhesion protein (an ortholog to BC1060 from *B. cereus* ATCC 14579, present but not annotated in Bt407).

In *B. subtilis*, SinI has been shown to be an antagonist of the master regulator of biofilm matrix genes, SinR. In *B. subtilis*, activation of the 15 gene EPS-operon *epsA-O* requires the action of SinI (and YlbF and YmcA), which are collectively responsible for reversing, or otherwise counteracting, SinR-mediated repression [58]. In *B. subtilis*, SinR also represses the expression of the an operon encoding the major protein component of the biofilm matrix, TasA [90]. In *B. cereus* and *B. anthracis*, SinR similarly represses expression of CalY, a homologue of TasA shown to be required for biofilm formation in Bt407 (Gohar *et al.*,

unpublished; [91]. The observation that *sinI* was up-regulated as a response to MogR overexpression in Bt407 indicates that MogR may be involved in controlling regulatory pathways leading to the formation of biofilms in *B. cereus*.

There are no published results showing that there are complete or partial candidate loci that are able to synthesize EPS matrix components contributing to biofilm formation in *B. cereus* and *B. thuringiensis*. However, Bt407 contains a locus (genes bthur0002\_51500 to bthur0002\_51320), referred to as the EX locus, which based on predicted gene functions may possibly be involved in production of an extracellular polysaccharide. This locus is similar to the *epsA-O* operon of *B. subtilis*, which has been shown to be required for biofilm formation in that species [92], in that both loci contain the so-called EAR element. The EAR element is a co-transcriptional genetic control required for expression of *eps* genes, which is located between the second and third gene in the *eps* operon, is always located within biofilm or capsular polysaccharide operons and is required for *eps* expression in *B. subtilis* [93]. The observation that the EX locus contains an EAR element may indicate that this locus could play a similar role in *B. cereus* as it does in *B. subtilis*. Thus, the observation that a capsular polysaccharide synthesis protein in the EX-locus (bthur0002\_51490) was up-regulated in the MogR overexpression strain may suggest that MogR promotes production of increased amounts of biofilm.

BC1060 is annotated as an adhesion protein in several *Bacillus cereus* group genomes, and the gene encoding this protein contains a cyclic-di-GMP (c-di-GMP) responsive “on” riboswitch, named Bc2 in *Bacillus cereus* ATCC 14579 [94], in the 5’ untranslated region of the mRNA. C-di-GMP is a second messenger shown to play a central role in controlling the transition from a motile, planktonic lifestyle, to a sessile biofilm-associated lifestyle in various bacteria, and usually, increased levels of intracellular c-di-GMP promote biofilm formation [95]. The domain structure of the BC1060 adhesion protein, referred to as BspA herein, is typical for the MSCRAMM (microbial surface components recognizing adhesive matrix molecules) protein family, which are anchored at the surface of bacteria [96]. A *bspA* deletion mutant in Bt407 has previously been shown to produce reduced amounts of biofilm (Fagerlund et al., unpublished results). Up-regulation of *bspA* in the MogR overexpression strain thus further indicates that MogR may promote production of increased amounts of biofilm, which also was confirmed in the biofilm screening performed in this thesis (see discussion below).

The Bt407 orthologue to the ATCC 14579 gene BC0422, encoding a methyl accepting chemotaxis protein (MCP), was down-regulated as a response to MogR overexpression. Similarly, MCP genes have also been shown to be regulated by MogR in *L. monocytogenes* [63]. MCPs are receptors that sense temporal changes in chemical gradients. If an MCP is activated by binding to an attractant in *B. subtilis*, the histidine kinase CheA becomes activated, and CheA activates the response regulator CheY. If the cell moves towards lower concentrations of attractants, there is lack of CheY activity, which results in tumbling (Chapter 1.3). This could suggest that MogR is involved in regulating the chemotactic response, at least towards certain classes of attractants. Interestingly, BC0422 also contains an upstream c-di-GMP responsive “off”-riboswitch as response to c-di-GMP [94]. The observation that MogR overexpression results in increased expression of a gene with an upstream c-di-GMP responsive “on” riboswitch (*bspA*), while at the same time represses expression of a gene with an upstream c-di-GMP responsive “off” riboswitch (BC0422), could potentially suggest that MogR influences c-di-GMP levels in the cell, and which again could influence the expression of BC1060 and BC0422 through the upstream c-di-GMP responsive riboswitches.

### **6.3 MogR overexpression causes increased biofilm formation**

The up-regulation of three biofilm-related genes in the MogR overexpression strain, and the potential positive effect of MogR expression on the cellular c-di-GMP levels, suggested that MogR may influence the ability to form biofilms. This was confirmed using a crystal violet microtiter-plate assay, in which the MogR overexpression strain showed increased ability to form biofilm compared with the vector control strain.

After discovering the results from the microarray analysis, where SinI, the antagonist of the master regulator of biofilm formation SinR, was up-regulated, a biofilm screening assay was done to see how MogR overexpression strain behaved.

It was also decided to do the assay based on the fact that motility was necessary for biofilm formation as stated by Houry et al [30].

The results showed that after 24, 48 and 72 hours the MogR overexpression strain produced significantly more biofilm than the empty vector control strain at 37 °C. This was perhaps a surprising result, in light of stationary biofilm assays, which has previously shown that motility was important for biofilm formation [30]. The effect of MogR on biofilm formation may seem to be temperature dependent, as significantly more biofilm was produced in the MogR overexpression strain at 37 °C than at 30 °C.

## 6.4 Conclusions

Results from the current thesis indicate that MogR is a contributor to down regulation of motility-, virulence and stress genes. Strikingly, biofilm genes known to be involved in biofilm formation were up-regulated in the MogR overexpression strain. Since BC1060 is up-regulated and BC0422 is down-regulated, indicates that MogR may be influence c-di-GMP levels in the cells. The mechanism behind this up- and down-regulation remains unclear.

The biofilm formation assay showed a significant increased formation of biofilm in the MogR overexpression strain at 37 °C than at 30 °C. In light of the observation that BC1060 is up-regulated in the MogR overexpression strain, and that this gene is known to contribute biofilm formation, it may seem possible that MogR also promotes to biofilm formation in the *B. cereus* group.

## 6.5 Future perspectives

It would be of great interest to run biofilm formation assays and motility assays with the strain expressing the MogR variant in which two of the putative MogR active site residues have been mutated (MogRmut), and for which an overexpression construct was constructed in this thesis. Also, it would be of interest to perform a bioinformatics analysis to identify putative conserved MogR binding sequences upstream of the genes differentially expressed in the microarray analysis. Furthermore, it would be interesting to perform an electrophoresis mobility shift assay (EMSA) to see if MogR actually binds to the predicted binding sequence upstream of these genes.





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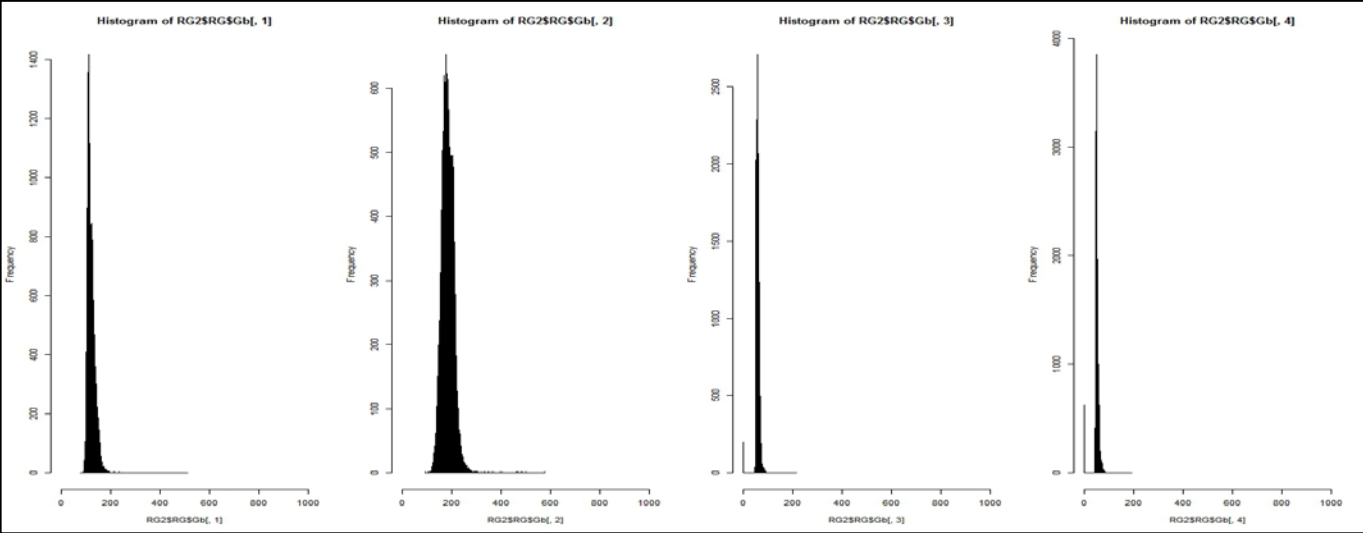
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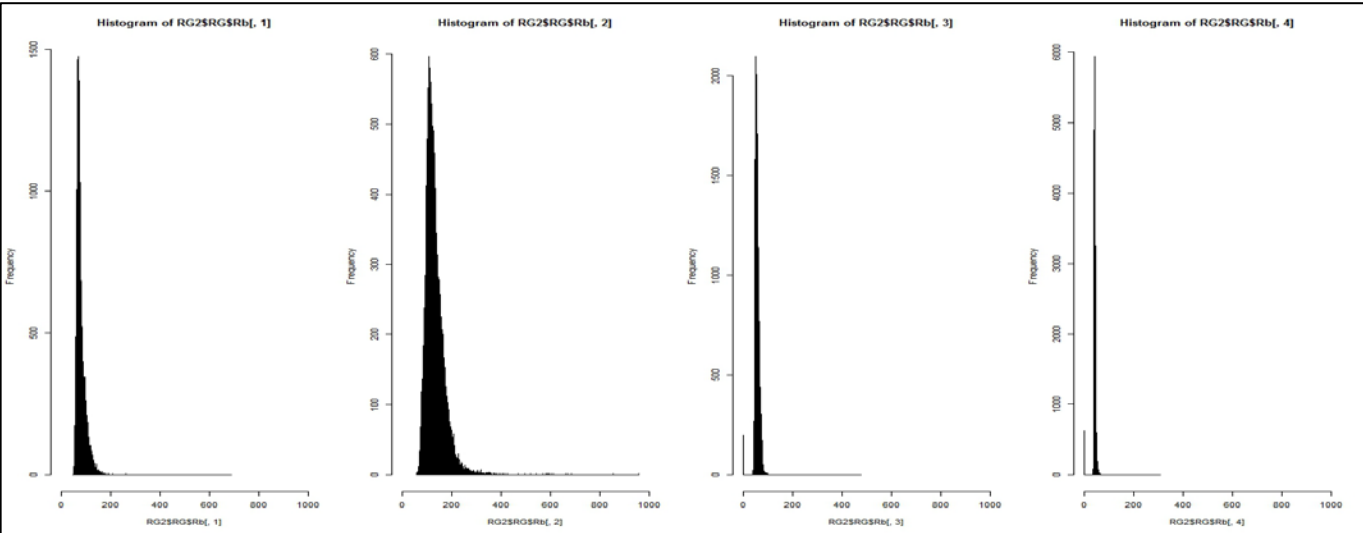
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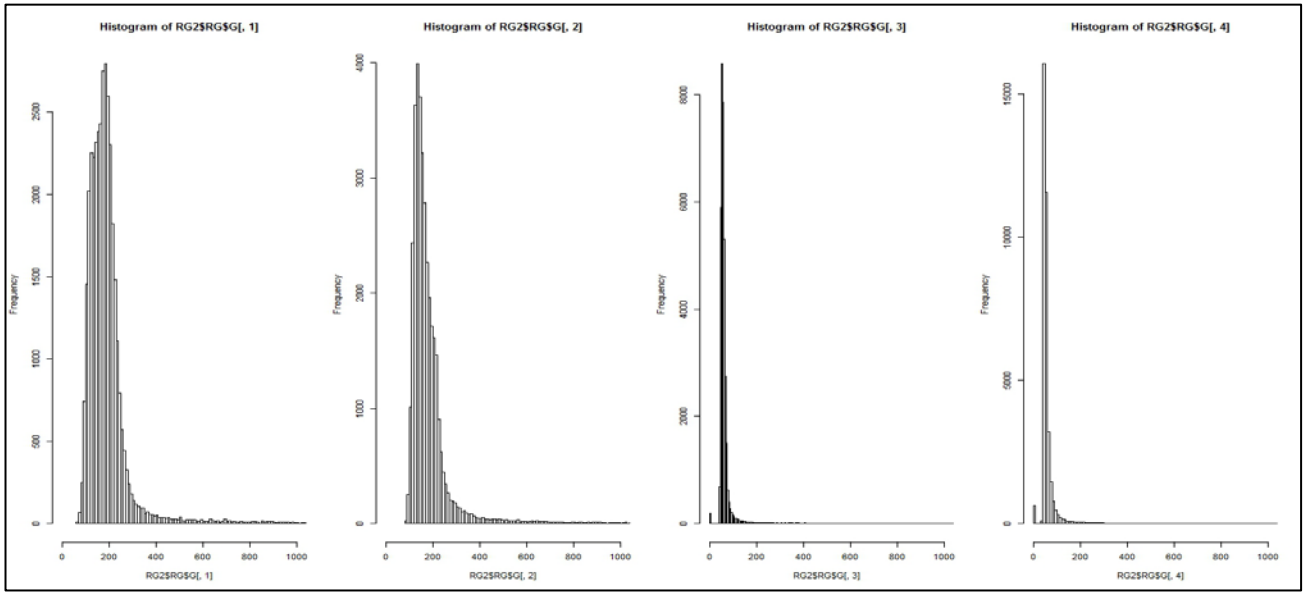
# Appendix A



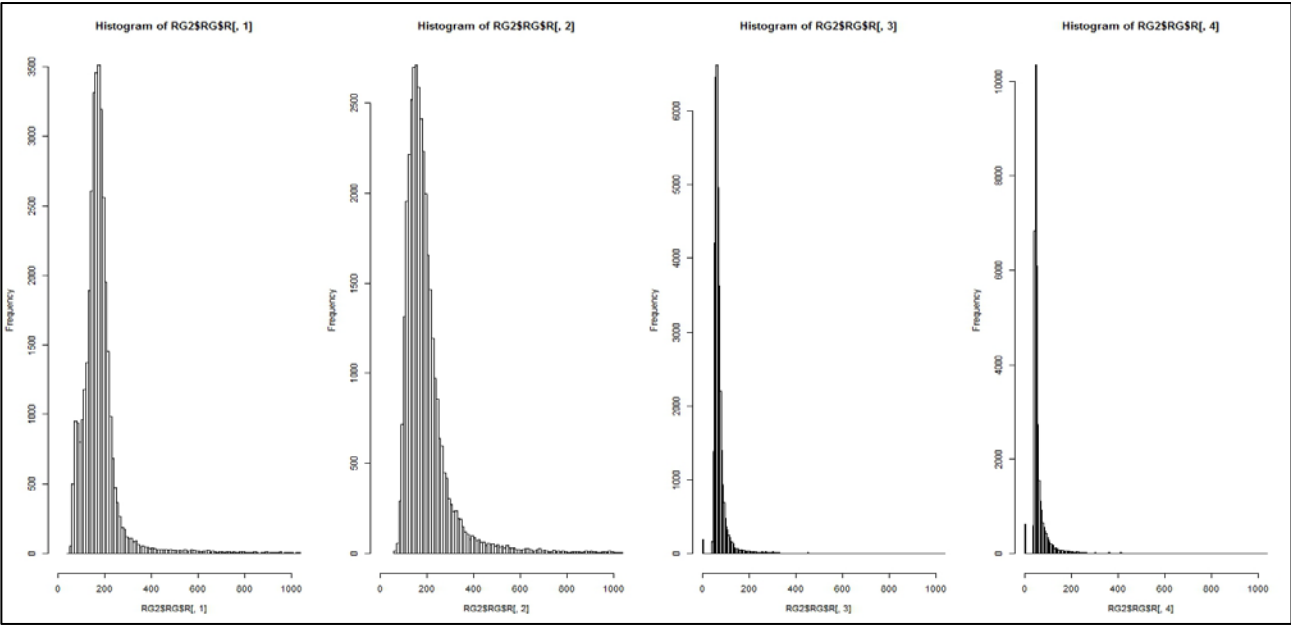
**Figure 18. Histograms from analysis of microarray experiment** comparing the Bt407 MogR overexpression strain with the empty vector control strain showing distribution of green background signal intensities. Histogram 1 to 4 represent slide 1 to 4 respectively.



**Figure 19. Histograms from analysis of microarray experiment** comparing the Bt407 MogR overexpression strain with the empty vector control strain showing distribution of red background signal intensities. Histogram 1 to 4 represent slide 1 to 4 respectively.



**Figure 20. Histograms from analysis of microarray experiment** comparing the Bt407 MogR overexpression strain with the empty vector control strain showing distribution of green spot signal intensities. Histogram 1 to 4 represent slide 1 to 4 respectively.



**Figure 21. Histograms from analysis of microarray experiment** comparing the Bt407 MogR overexpression strain with the empty vector control strain showing distribution of red spot signal intensities. Histogram 1 to 4 represent slide 1 to 4 respectively.



## Appendix B

Genes differentially regulated in a microarray experiment comparing gene expression in the Bt407 MogR overexpression strain with that in the empty vector control strain. All listed genes have FDR-corrected p-values<0.05.

<b>Genes up-regulated in microarray experiment comparing gene expression in the MogR overexpression strain with that in the empty vector control strain. All listed genes have FDR-corrected p-values&lt;0.05.</b>				
Locus tag in Bt407	Locus tag in ATCC14579	Predicted function	log <sub>2</sub> (fold change) *	Average expression #
bthur0002_4190	BC0492	hypothetical protein; similar to pyruvate formate lyase-activating enzyme 1	0,92	7,86
bthur0002_7510	BC0813	3D domain protein	0,96	9,57
Not annotated	BC1060	collagen adhesion protein with upstream c-di-GMP "on" riboswitch	3,02	9,01
bthur0002_10910	BC1174	3-oxoacyl-[acyl-carrier-protein] synthase	0,96	10,54
bthur0002_11250	BC1208	Glycosyl transferase, group 2	1,23	10,60
bthur0002_11930	BC1283	SinI	1,08	8,29
bthur0002_14550	BC1553	Penicillin-binding protein, 1A; similar to Membrane carboxypeptidase (penicillin-binding protein)	0,62	10,32
bthur0002_15650	BC1655	mogR	0,75	8,56
bthur0002_16890	BC1771	fibronectin-binding protein	0,82	8,35
bthur0002_17120	BC1794	Oligopeptide transporter, periplasmic-binding protein; similar to ABC-type oligopeptide transport system	2,01	7,23
bthur0002_37850	BC1853	hypothetical protein	2,00	7,28
bthur0002_20180	BC2164	Isoleucyl-tRNA synthetase 2	1,00	10,38
bthur0002_21110	BC2238	hypothetical protein	1,95	8,32
bthur0002_23190	BC2483	Butyryl-CoA dehydrogenase	1,81	9,63
bthur0002_38230	BC3972	Pyruvate dehydrogenase complex E1 component, beta subunit	0,71	11,05
bthur0002_39240	BC4083	Xanthine/uracil permease; similar to Xanthine/uracil/vitamin C permease	0,73	8,86
bthur0002_39260	BC4085	Pyrimidine-nucleoside phosphorylase	0,80	8,59
bthur0002_39270	BC4086	Purine nucleoside phosphorylase	0,87	8,54

bthur0002_47410	BC4870	L-lactate dehydrogenase 2	0,66	8,46
bthur0002_t1090	BC4880	tRNA-Met	0,61	8,98
bthur0002_49980	BC5138	Phosphoglycerate kinase, Pkk	0,83	9,80
bthur0002_50000	BC5141	Central glycolytic genes regulator, CggR	0,94	8,11
bthur0002_50180	BC5158	Integral membrane protein	1,58	8,50
bthur0002_51040	BC5238	Glycine betaine transporter; similar to Choline-glycine betaine transporter	1,10	7,60
bthur0002_51490	BC5278	Capsular polysaccharide biosynthesis	0,71	8,96

\*Fold change is the ratio of expression in the MogR overexpression strain divided by the expression in the empty-vector control strain.

# Average expression is a measurement of how much mRNA is present for the specific gene in the array. The higher the value, the more mRNA is present.

**Genes down-regulated in microarray experiment comparing gene expression in the MogR overexpression strain with that in the empty vector control strain. All listed genes have FDR-corrected p-values<0.05.**

Locus tag in Bt407	Locus tag in ATCC14579	Predicted function	log <sub>2</sub> (fold change)*	Average expression #
bthur0002_10300	BC1113	Sigma-M negative effector	-1,61	8,69
bthur0002_11020	BC1185	Extracellular solute-binding protein family 5; similar to ABC-type oligopeptide transport system, periplasmic component	-0,93	9,40
bthur0002_11090	BC1193	Oligoendopeptidase F	-2,39	8,34
bthur0002_12250	BC1316	hypothetical protein; similar to chromosome segregation protein PRK03918; similar to PhaR of Bacillus UniRef RepID=Q52JK1_BACTU	-0,66	8,54
bthur0002_13320	BC1424	Ferredoxin--nitrite reductase	-1,62	8,98
bthur0002_14730	BC1570	hypothetical protein; similar to Xanthine/uracil permeases	-1,21	8,33
bthur0002_15380	BC1628	Chemotaxis protein, histidine kinase CheA	-1,80	8,79
bthur0002_15380	BC1628	Chemotaxis protein, histidine kinase CheA	-1,08	11,24
bthur0002_15400	BC1630	hypothetical protein	-0,92	7,99
bthur0002_15460	BC1636	Flagellar hook-associated protein 1, FlgK	-1,67	8,12
bthur0002_15490	BC1639	Flagellar (assembly) protein FliS	-0,68	8,37
bthur0002_15520	BC1642	Flagellar basal-body rod protein FlgC	-2,87	8,89
bthur0002_15540	BC1644	Flagellar MS-ring protein FliF	-2,31	8,63

bthur0002_15540	BC1644	Flagellar MS-ring protein FliF	-1,05	8,18
bthur0002_15550	BC1645	Flagellar motor switch protein FliG	-1,09	7,94
bthur0002_15590	BC1649	Flagellar hook-length control protein FliK	-1,47	8,29
bthur0002_15630	BC1653	hypothetical protein	-1,37	8,52
bthur0002_15640	BC1654	Chemotaxis signal transduction protein CheV	-1,80	8,43
bthur0002_15660	BC1656	flagellin FlaB	-1,04	7,87
bthur0002_15670	BC1657	flagellin FlaA	-3,45	9,11
bthur0002_15680	BC1660	Putative lytic murein transglycosylase	-0,75	8,12
bthur0002_15690	BC1661	Flagellar motor switch protein (fliN-homolog)	-0,99	8,14
bthur0002_15730	BC1665	Flagellar biosynthesis protein FliP	-1,86	8,11
bthur0002_15740	BC1666	Flagellar biosynthesis protein FliQ	-1,23	7,99
bthur0002_15750	BC1667	Fflagellar biosynthesis protein FliR	-1,02	7,73
bthur0002_15760	BC1668	Flagellar biosynthesis protein FlhB	-1,60	8,10
bthur0002_15770	BC1669	Flagellar biosynthesis protein FlhA	-1,43	8,64
bthur0002_17050	BC1789	Methyl-accepting/DNA response regulator; similar to putative two-component response-regulatory protein YehT PRK11697; similar to Methyl-accepting/DNA response regulator, putative of Bacillaceae UniRef RepID=Q81S18_BACAN	-1,27	11,11
bthur0002_18510	BC1991	S-layer protein / Peptidoglycan endo-beta-N-acetylglucosaminidase; similar to Uncharacterized protein involved in cytokinesis, contains TGc (transglutaminase/protease-like) domain COG5279; similar to S-layer protein / Peptidoglycan endo-beta-N-acetylglucosaminidase of Bacillus cereus group UniRef RepID=Q3EWN8_BACTI	-0,80	8,50
bthur0002_19200	BC2056	hypothetical protein; similar to Membrane protein implicated in regulation of membrane protease activity COG1585; similar to Putative uncharacterized protein of Bacillus cereus group UniRef RepID=A0RD59_BACAH	-0,74	8,75
bthur0002_19420	BC2076	GCN5-related N-acetyltransferase; similar to hypothetical protein PRK13688; similar to GCN5-related N-acetyltransferase of Bacillus cereus group UniRef RepID=A9VST6_BACWK	-1,47	8,83

bthur0002_22430	BC2379	hypothetical protein; similar to DNA-binding transcriptional repressor PuuR PRK09943; similar to DNA-binding protein of Firmicutes UniRef RepID=A0RE37_BACAH	-1,54	8,39
bthur0002_2270	BC0294	10 kDa chaperonin; similar to co-chaperonin GroES	-1,06	11,98
bthur0002_2280	BC0295	60 kDa chaperonin 2; similar to chaperonin GroEL	-0,58	9,32
bthur0002_23140	BC2478	ABC transporter, ATP-binding protein	-3,19	7,74
bthur0002_23600	BC2529	Oxidoreductase, zinc-binding dehydrogenase; similar to Threonine dehydrogenase and related Zn-dependent dehydrogenases	-1,97	8,58
bthur0002_24210	BC2659	Glutamate-rich protein grpB	-1,72	7,52
bthur0002_26050	BC2832	Aldehyde dehydrogenase; similar to bifunctional acetaldehyde-CoA/alcohol dehydrogenase	-2,10	10,91
bthur0002_26150	BC2842	hypothetical protein	-0,73	8,40
bthur0002_26500	BC2881	hypothetical protein; similar to Integral membrane protein CcmA involved in cell shape determination	-2,10	10,82
bthur0002_27060	BC2925	Nucleotidyltransferase	-2,27	10,60
bthur0002_27440	BC2959	malate:quinone oxidoreductase	-0,67	8,35
bthur0002_27500	BC2964	Transcriptional regulator	-2,12	10,57
bthur0002_28440	BC3102	Hbl component B	-1,13	8,81
bthur0002_28450	BC3103	Hbl component L1	-1,59	8,79
bthur0002_31820	BC3461	hypothetical protein	-1,33	8,34
bthur0002_33180	BC3586	Extracellular solute-binding protein family 5; similar to ABC-type oligopeptide transport system, periplasmic component	-1,15	7,73
bthur0002_3430	BC0422	Methyl-accepting chemotaxis protein (with upstream "off" c-di-GMP riboswitch)	-1,31	8,44
bthur0002_34850	BC3762	Fibronectin type III domain protein	-0,84	8,22
bthur0002_36120	BC3835	Ribonuclease; similar to ribonuclease HII	-1,24	8,84
bthur0002_36440	BC3867	hypothetical protein; similar to bifunctional phosphopantothenoylecysteine decarboxylase/phosphopantothenate synthase	-1,12	9,74
bthur0002_38170	BC3965	hypothetical protein	-0,89	9,19
bthur0002_38180	BC3966	hypothetical protein	-2,57	9,14

bthur0002_40080	BC4154	Hypothetical membrane associated protein	-1,47	9,46
bthur0002_40470	BC4198	Uncharacterized peptidase yqhT; similar to aminopeptidase	-0,51	9,89
bthur0002_41140	BC4260	Glucokinase	-1,85	10,79
bthur0002_41480	BC4293	hypothetical protein	-0,71	9,33
bthur0002_41630	BC4307	30S ribosomal protein S21	-0,81	11,87
bthur0002_41690	BC4313	hypothetical protein; similar to Molecular chaperone GrpE (heat shock protein)	-0,75	11,59
bthur0002_41700	BC4314	Heat-inducible transcription repressor hrca	-0,67	9,59
bthur0002_42070	BC4861	hypothetical protein	-1,60	8,40
bthur0002_42470	BC4389	Helicase, RecD/TraA; similar to ATP-dependent exoDNase (exonuclease V), alpha subunit - helicase superfamily I member	-1,53	8,39
bthur0002_43710	BC4512	Flagellar motor protein MotB (H <sup>+</sup> -coupled stator)	-3,01	9,02
bthur0002_43720	BC4513	Flagellar motor protein MotA (H <sup>+</sup> -coupled stator)	-1,74	8,20
bthur0002_44530	BC4594	Citrate synthase 2	-0,63	10,31
bthur0002_45480	BC4683	Ribosomal-protein-serine acetyltransferase	-1,06	10,16
bthur0002_46260	BC4756	Glycosyl transferase, group 1	-1,58	7,36
bthur0002_46310	BC4762	Phosphoenolpyruvate carboxykinase	-1,26	10,97
bthur0002_49500	BC5048	Ferritin and Dps-like protein	-1,66	8,73
bthur0002_4980	BC0587	Acetyltransferase, GNAT	-1,21	8,38
bthur0002_5010	BC0590	hypothetical protein	-0,73	9,20
bthur0002_50710	BC5211	PTS system, lactose/cellobiose family IIC subunit	-1,23	10,79
bthur0002_5080	BC0598	Helix-turn-helix domain protein	-0,89	8,29
bthur0002_50910	BC5228	Glycolate permease glcA; similar to L-lactate permease	-0,84	9,87
bthur0002_51090	BC5243	hypothetical protein	-0,87	8,21
bthur0002_51510	BC5280	(3R)-hydroxymyristoyl-[acyl-carrier-protein] dehydratase	-0,68	9,80
bthur0002_52490	BC5380	Iron compound ABC transporter	-0,84	8,57
bthur0002_5520	BC0643	Amino-acid permease rocC	-0,63	8,60
bthur0002_5820	BC0666	Immune inhibitor A	-1,03	8,42
bthur0002_5830	NA	hypothetical protein	-1,22	7,47
bthur0002_5870	BC0670	Phospholipase C (PC-PLC)	-2,32	8,38
bthur0002_5880	BC0671	Sphingomyelinase C (Smase)	-1,47	7,82
bthur0002_62790	BC4020	hypothetical protein; similar to Lipoprotein	-0,98	8,52
bthur0002_7310	BC0791	Pyridine nucleotide-disulphide oxidoreductase; similar to	-1,54	7,27

		coenzyme A disulfide reductase		
bthur0002_7720	BC0834	hypothetical protein	-1,95	7,54
bthur0002_8270	BC0888	N-acetylmuramoyl-L-alanine amidase family 2; similar to N-acetylmuramoyl-L-alanine amidase	-1,50	8,34
bthur0002_9170	BC1000	Integral membrane protein	-1,01	8,49
bthur0002_9200	BC1003	Serine-protein kinase rsbW	-0,91	9,38
bthur0002_9210	BC1004	RNA polymerase sigma-B factor	-1,05	9,43
bthur0002_9520	BC1030	hypothetical protein; similar to N-acetylmuramoyl-L-alanine amidase	-0,51	9,57
bthur0002_9940	BC1061	hypothetical protein	-0,82	8,35
*Fold change is the ratio of expression in the MogR overexpression strain divided by the expression in the empty-vector control strain.				
# Average expression is a measurement of how much mRNA is present for the specific gene in the array. The higher the value, the more mRNA is present.				