c-di-GMP regulated cellulose synthesis in *Bacillus thuringiensis* 407

Cloning and expression of PilZ domains from the putative cellulose synthase BcsA

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Master thesis in Pharmacy

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Preface

This thesis was performed in the *Bacillus cereus* research group in the Department of Pharmaceutical Bioscience, The School of Pharmacy, in the period June 2016- May 2017. I want to give a big thanks to my supervisor Ole Andreas Løchen Økstad who guided me through this past year, and was always available when I had questions or needed help. I also want to thank chief engineer Ewa Jaroszewicz, Ph.D. student Sarah Finke, Ph.D. student Veronika Smith and post doc. Adam Heikal for always being helpful under laboratory work and for being available when I had questions.

This has been a hard but informative year, where I have learned a lot and gained a lot of experience while having a great year at the same time. It has been a pleasure to be a part of the *Bacillus cereus*- group who consist of professional researchers.

Big thanks to my closest that have always been there for me.

15. May 2017

Naya Ikram El Habbach

Summary

Species in the *Bacillus* group of organisms are known for their ability to make biofilm, producing an extracellular polymer substance matrix that provides a favourable environment where the cells can grow in multicellular communities on different types of surfaces. The matrix as well as other mechanisms results in the bacteria being more resistant against antimicrobial agents, making the cells almost impossible to eliminate by standard antibiotic treatment or disinfectant procedures. Biofilm formation in bacteria can cause complications in the food industry and in various clinical contexts.

Several members of the *Bacillus cereus* group are known to produce biofilm, including the human pathogen *B. cereus* and the insect pathogen *B. thuringiensis,* which is indistinguishable from *B. cereus* except for the presence of insect toxin genes. Therefore *B. thuringiensis* also has the potential to cause human disease. Bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) is a second messenger found in different Gram-positive and negative bacteria, including *Bacillus* species. c-di-GMP is involved in several signalling pathways and elevated levels of c-di-GMP have generally been associated with activation of biofilm formation. The *B. cereus* group has been shown to contain a wide range of genes encoding c-di-GMP metabolizing enzymes. c-di-GMP typically interacts with different downstream macromolecular targets, including the PilZ domain identified in the bacterial cellulose synthase subunit A (BcsA). *E. coli* carries bacterial cellulose synthesis (bcs) operons, and cellulose is produced as an extracellular matrix component of biofilm in the *E. coli* cells. Two motifs (RxxxR and N/DxxxG) in the PilZ domain, e.g. of YcgR from *E. coli,* are recognized to bind c-di-GMP. Studies in several Gram-negative species confirmed that PilZ domain proteins frequently play a role in the synthesis of exopolysaccharide during biofilm formation, however this has not been systemically examined in Gram-positive species, and is yet to be studied in the *B. cereus* group for which biofilm matrix composition has not been comprehensively investigated.

Two single PilZ domains were identified in the *B. thuringiensis* 407 putative cellulose synthase BcsA. DNA encoding the two domains was successfully cloned into an expression vector and expressed during growth of the bacterium. The expressed proteins were then isolated and we aimed to purify recombinant PilZ domains from BcsA to be used in a study

to identify potential regulatory mechanisms occurring through c-di-GMP binding. The PilZ domains (d1 and d2) of the BcsA protein (BcsA $PilZ_{d1d2}$) was successfully expressed as a His-tag polypeptide. The polypeptide could however unfortunately not be successfully purified within the scope of this work, possibly due to formation of inclusion bodies during expression, which is often the result of high level expression of protein. The use of high temperature and high inducer concentration during protein expression is often associated with the formation of inclusion bodies, and therefore inducing the BcsA $PilZ_{d1d2}$ protein at room temperature with lower concentrations of IPTG during expression were attempted. Unfortunately the BcsA $PilZ_{d1d2}$ polypeptide was still expressed in a pattern consistent with inclusion body formation. Additional approaches such as solubilization of inclusion bodies, refolding and purification of the proteins could be tried out in future strategies for obtaining purified BcsA $PilZ_{d1d2}$ polypeptide. Also the cloning, expression and purification of the full BcsA protein could be a possible approach for studying the activity of the PilZ domains and their potential connection with c-di-GMP binding and function in biofilm formation or other cellular functions in *Bacillus cereus* group bacteria.

Abbreviations

bp: base pair

C: Celsius

DNA: Deoxyribonucleic acid

GMP: Guanosine monophosphate

g: Gram

GTP: Guanosine triphosphate

kb: Kilo bases (1000 base pair)

kDa: Kilo Dalton

kHz: Kilo Hertz

L: Liter

mg: Milligram

ml: Milliliter

MQ: MilliQ (ultrapure water)

mRNA: Messenger ribonucleic acid

OD: Optical density

rpm: Revolutions per minute

rRNA: Ribosomal ribonucleic acid

V: Volt

µg: Microgram

µl: Microliter

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1 Introduction

1.1 Genus *Bacillus*

Ferdinand Cohn was the first to describe the B*acillus* genus and cited *Bacillus subtilis* as the first species. Cohn described the organism as thin thread-like with motile activity, and also mentioned its spore forming ability (Soule, 1932). Member of the genus *Bacillus* are part of the *Bacillaceae* family, and are comprised of many heterogeneous organisms grouped together without any defining characters that are commonly shared. This is a problem, which has resulted in repeated redefinition of the genus. 16s RNA gene sequencing of the B*acillus* genus showed that the *B. subtilis* group and the *Bacillus cereus* group are the two largest clades and both contain more than three species (Bhandari, Ahmod et al., 2013).

Bacillus species are aerobic or facultatively anaerobic Gram-positive or Gram-variable bacteria which grow at optimal temperatures between 25-37°C. The range in size can vary from 0.5 to 1.2µm in diameter and 2.5 to 10µm in length. The G+C content in the DNA has been seen to vary from 32-69% in different species (Drobniewski, 1993). The bacteria are found in many environmental reservoirs like decaying organic matter, fresh and marine waters, vegetables, the intestinal tract of invertebrates and more (Bottone, 2010). Some of the species can grow in extremes of acidity and alkalinity, ranging from pH 2 to 10 (Drobniewski, 1993). Their ability to produce spores gives the advantage of being able to live in many different habitats, and makes the bacteria refractory to extreme environmental conditions so as to being able to withstand heat, freezing, drying and radiation (Bottone, 2010). The *Bacillus* species have a wide range of useful physiological characteristics like production of many types of enzymes and metabolites, and hence are used in medical, pharmaceutical, agricultural and industrial processes. The production of antimicrobial peptides in the species has for example led to the production of two well-known antibiotics; bacitracin and polymyxin (Turnbull, 1996).

1.2 The *Bacillus cereus* group

The *Bacillus cereus* group of organisms consists of at least seven species *Bacillus cereus (B. cereus) sensu stricto, Bacillus anthracis (B. anthracis), Bacillus thuringiensis (B. thuringiensis), Bacillus weihenstephanensis, Bacillus mycoides (B. mycoides)*, *Bacillus pseudomycoides (B. pseudomycoides)* (Jensen, Hansen et al., 2003) and *B. cytotoxicus* (Guinebretière, Auger et al., 2013). The *Bacillus cereus* group organisms are widely distributed in the environment and seem to adapt to very different habitats (Guinebretière, Thompson et al., 2008), ranging from soil to water and from plants and animals to food (Liu, Lai et al., 2017). Sequence analysis of members of the *Bacillus cereus* group shows *that B. cereus, B. anthracis* and *B. thuringiensis* could be considered genetically as one species (Helgason, Økstad et al., 2000) however pathogenicity pattern towards different host have kept the species distinction. Differences in pathogenicity in the different species are often encoded by genes carried on plasmids (David A. Rasko, Michael R. Altherr et al., 2005). Often only a single feature separate species and this can be difficult when trying to separate between the members. For example when *B. thuringiensis* loses its delta-endotoxin (plasmid) it can not be differentiated from *B. cereus* (Drobniewski, 1993). Not all members of the group are highly virulent (Jensen, Hansen et al., 2003). The major differences that are seen between the members of the *Bacillus cereus* group are not dependent on the level of sequence divergence or gene content, but are dependent on small variation in gene expression (David A. Rasko, Michael R. Altherr et al., 2005), and *B. cereus* and *B. thuringiensis* strains are phylogenetically intermixed (figure 1.1).

Many pathological characteristics such as spore formation, enzyme production of for example proteases and beta-lactamases (Bottone, 2010), colonization ability and persistence in a host (Kamar, Gohar et al., 2013) have been identified in this group of organisms (David A. Rasko, Michael R. Altherr et al., 2005). The transcriptional regulator Phospholipase C Regulator (PlcR) controls many of the virulence factors found in these species, like for example the expression of enterotoxins, haemolysins, phospholipases and proteases (Gohar, Faegri et al., 2008). plcR transcription is autoinduced, and PlcR needs PapR and OppABCDF (oligopeptide permease system) to function. PapR is a propeptide that is under transcriptional control of PlcR, and is transported out of the cell to form the active peptide (Gohar, Faegri et al., 2008) by photolytic cleavage during transport (Pomerantsev, Pomerantseva et al., 2009). When the peptide is active OppABCDF captures it back into the cell where it binds to PlcR

forming the active transcriptional regulator. These three factors function as a quorum-sensing system for the bacterium, and are activated shortly before the bacterium reaches its stationary phase (Gohar, Faegri et al., 2008). plcR is present in all members of the *B. cereus* group, and has been shown to regulate genes dispersed in the chromosome (Agaisse, Gominet et al., 1999). However mutations identified in the plcR gene in *B. anthracis* has resulted in plcR regulated genes not being expressed in this species, which is a characteristically difference separating it from *B. cereus* and *B. thuringiensis* (Agaisse, Gominet et al., 1999).

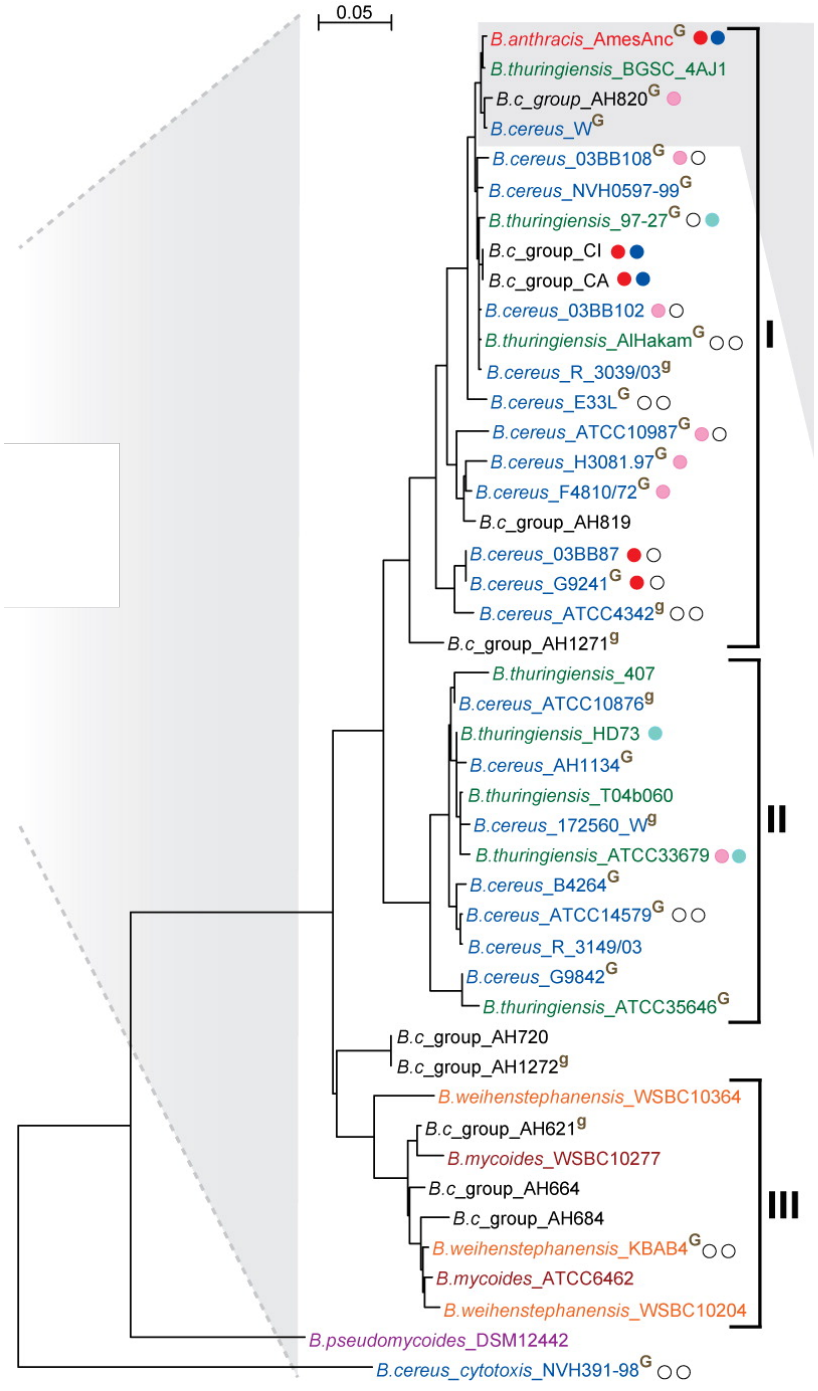


Figure 1.1 Phylogenetic relationship among 45 isolates of the *B. cereus* group bacteria extracted from the multilocus sequence typing (MLST) supertree of 1105 isolates. The tree is based on a sequence data from all five published MLST schemes for the *B. cereus group.* The three main phylogenetic clusters of the *B. cereus* are marked by roman numerals I-III (Kolstø, Tourasse et al., 2009).

1.2.1 B. anthracis

The *B. anthracis* species is mostly found in the environment as metabolically dormant spores that do not replicate, and therefore the genetic evolution of *B. anthracis* is limited to the short periods of time from infection of a host where they are in a vegetative state until host death. Due to these circumstances *B. anthracis* evolves very slowly (Pilo & Frey, 2011), and displays a low genetic variability and highly clonal evolution (Thierry, Tourterel et al., 2014). *B. anthracis* is the cause of the severe disease anthrax in a variety of mammalian hosts, including humans (Carlson, Bourgis et al., 2015). Different routes like inhalation or subcutaneous and gastrointestinal entry of the spores can cause the infection (Carlson, Bourgis et al., 2015). The most common route of infection is cutaneous entry trough open wounds, cuts or abrasions. Ingestion of vegetative *B. anthracis* in poorly cooked meat is often the cause of gastrointestinal infection (Mazumdar, 2009). It is believed that when the spores enter the host they are taken up by alveolar macrophages where they germinate, but has yet to been shown in human macrophages (Dixon, Fadl et al., 2000). However murine alveolar phagocytes have shown to ingest *B. anthracis* endospores that then germinate within the macrophage and grow into vegetative bacilli. The vegetative bacilli can then escape from the phagocytic vesicles of the macrophage and replicate freely in the host cell cytoplasm before they release themselves from the macrophage (Weiner & Hanna, 2003). Very little is known about the bacterial factors that regulate germination and escape of *B. anthracis* spores within macrophages (Barua, McKevitt et al., 2009), but if the vegetative cell enters the bloodstream it can grow rapidly and lead to death due to septicaemia or pulmonary complications (Carlson, Bourgis et al., 2015).

B. anthracis contains two virulence plasmids called pXO1 and pXO2. pXO1 encodes three toxic protein factors: the protective antigen (PA), the lethal factor (LF) and oedema factor (EF) (Jensen, Hansen et al., 2003) encoded by the genes *PagA, lef* and *cya* (Okinaka, Cloud et al., 1999). These toxins can lead to cell death by interfering with signal transduction pathways and inhibiting normal immune system functioning (Mazumdar, 2009). pXO2 encodes a poly-D-glutamic acid capsule which enables the bacteria to withstand phagocytosis (Jensen, Hansen et al., 2003). Three genes *capB, capC* and *capA,* are required for the synthesis of the capsule (Okinaka, Cloud et al., 1999). Both pXO1 and the pXO2 plasmids are necessary for full virulence, and without one of them the bacteria are attenuated. Growth outside a host often leads to the loss of the pXO2 plasmid (Jensen, Hansen et al., 2003).

1.2.2 B. cereus

B. cereus is found to be present in many environments such as different type of soil, sediments, dust, plants (Stenfors Arnesen, Fagerlund et al., 2008) and food (Chaabouni, Barkallah et al., 2014), and is therefore described as being of ubiquitous presence in nature (Stenfors Arnesen, Fagerlund et al., 2008). *B. cereus* ability to grow and multiply on several different habitats displays a wide diversity of virulence factors, growth behaviour and survival characteristics (Chaabouni, Barkallah et al., 2014). The natural niche for *B. cereus* is probably the gut microflora of invertebrates, but it has also been found in mosquito larvae and various soil-dwelling pests (Jensen, Hansen et al., 2003). If the vegetative cell form is released from a host into the environment e.g. such as soil, it can sporulate and survive until another host takes it up (Bottone, 2010), hence *B. cereus* species develop adaptive strategies depending on the habitat it encounters (Chaabouni, Barkallah et al., 2014).

B. cereus is a well-known cause of food poisoning (Bottone, 2010; Jensen, Hansen et al., 2003), often due to its ability to contaminate dairy products, rice or pasta (Helgason, Økstad et al., 2000), and hence its ingestion by humans *B. cereus* could be seen as a part of the transitory human intestinal flora. (Bottone, 2010). *B. cereus* can cause two types of food poisoning, either inducing diarrhoea and abdominal pain (diarrheal syndrome) or nausea and vomiting (emetic syndrome) (Jensen, Hansen et al., 2003). If viable cells or spores are ingested the vegetative form of the bacterium can produce and secrete one or several protein enterotoxins and induce the diarrheal syndrome (Bottone, 2010). *B. cereus* produces two different three-component enterotoxins; haemolysin (HBL) and the so-called non-haemolytic enterotoxin (NHE) which has later been shown to be haemolytic (Senesi & Ghelardi, 2010). Vegetative cells can also produce emetic toxin, a plasmid-encoded cyclic peptide (cereulide) that can be secreted directly into food products. Emetic toxin in ingested food can also cause food poisoning (Bottone, 2010). Another toxin that has been reported in some strains (*B. cytotoxicus*), is the cytotoxin CytK that can cause necrotic enteritis, which could be lethal to humans (van Schaik, Tempelaars et al., 2004). In addition to food poisoning *B. cereus* also causes a number of systemic and local infections in humans such as meningitis, pneumoniae, cutaneous infections and endocarditis (Bottone, 2010).

1.2.3 B. thuringiensis

B. thuringiensis is known for its ability to produce insecticidal crystal proteins (ICPs) (H. Li, Hu et al., 2016). A number of chemical compounds contains the insecticidal toxins of this species and are used commercially to control insects important to agriculture and public health (Ibrahim, Griko et al., 2010). Formulations containing *B. thuringiensis* are applied in high amounts on crops in different parts of the world, and in Brazil *B. thuringiensis* based products are used in approximately 150 000 acres (Schünemann, Knaak et al., 2014).

During sporulation *B. thuringiensis* produces large crystal protein inclusions inside the cell, also referred to as δ- endotoxins. The locations of the genes encoding δ- endotoxins are on transferable plasmids and are the only principle feature separating *B. thuringiensis* from *B. cereus* (Jensen, Hansen et al., 2003). δ- endotoxins form two multigenic groups called *cry* and *cyt* (Pardo-López, Soberón et al., 2013). Cry and Cyt endotoxins do not share significant sequence homology, but both endotoxins seem to cause cell lysis in the insect midgut due to pore formation (Guerchicoff, Delécluse et al., 2001). The Cry proteins have been classified into 70 subgroups, and are parasporal inclusion proteins which hereby exhibit a toxic effect to the target organism (Pardo-López, Soberón et al., 2013). A number of other insecticidal factors have also been studied in *B. thuringiensis*, and these consist of additional insecticidal proteins, proteases, chitinases, exotoxins and lipases (Guerchicoff, Delécluse et al., 2001). Even though *B. thuringiensis* has been rendered safe for use as a bioinsecticidal formulation there has been reported some outbreaks of foodborne disease (Scarano, Virdis et al., 2009) Probably because a large number of *B. thuringiensis* strain can produce the same enterotoxins and other virulence factors of *B. cereus,* and therefore it has been suggested to exclude enterotoxigenic strains of *B. thuringiensis* from bioinsecticidal formulations (Scarano, Virdis et al., 2009). Example of other reported cases where *B. thuringiensis* has caused infection in humans are the isolation of *B. thuringiensis* from two patients with deep burns, a needle-stick hand injury and corneal ulcers due to *B. thuringiensis*-based pesticides splashed into the eye. The two latter cases occurred during the handling of fluids containing highly concentrated *B. thuringiensis* spores and δ- endotoxins (Damgaard, Granum et al., 1997).

1.3 Biofilm formation in bacteria

Bacteria can grow on surfaces and produce multicellular communities held together by a self-produced extracellular matrix, a process known as biofilm formation (figure 1.2) (López, Vlamakis et al., 2010). The microorganisms produce an extracellular polymeric substance (EPS) matrix, which provides an optimal environment for the cells, where the cells exhibit reduced growth rate and can exchange genetic material, and also communicate through quorum sensing (Donlan, 2002). The matrix can account for over 90% of the dry mass in a biofilm, while the microorganisms account for less than 10% (Flemming & Wingender, 2010). The matrix protects the cells against desiccation, oxidizing or charged biocides, some antibiotics and metallic cations, ultraviolet radiation, many protozoan grazers and host immune defences (Flemming & Wingender, 2010). EPS can vary between different biofilms depending on which microorganisms that are present and environmental factors. The matrix typically contains extracellular polysaccharides, proteins, nucleic acids, lipids and other biopolymers such as humic substances (Flemming & Wingender, 2010). Open water channels separate the microbial cells in a biofilm. These channels act as a circulatory system for delivery of nutrition and oxygen, and the removal of metabolic waste products. Because of this regulation, a chemical concentration gradient of nutrients, waste products and secreted signalling compounds is formed that leads to many unique microenvironments within the microbial biofilm (Fengjun, Feng et al., 2013)

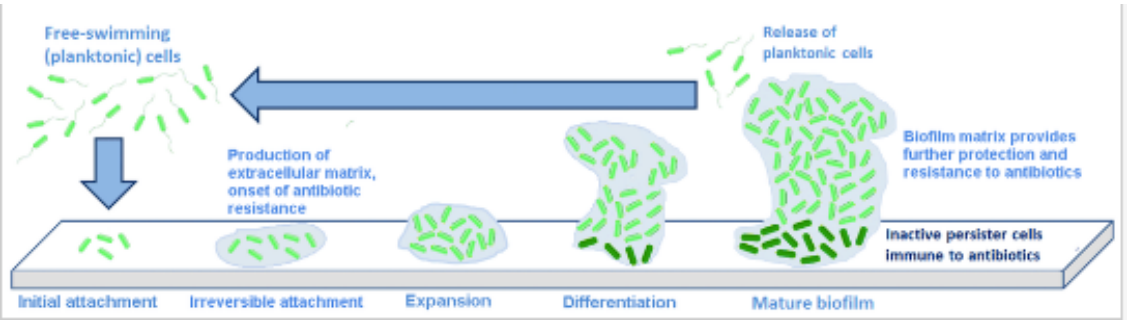


Figure 1.2: The life cycle of bacteria in biofilms. Stages in the biofilm formation of bacteria are illustrated in the figure. Free-flowing cells can settle on surfaces where they eventually initiate production of an extracellular matrix of carbohydrate, protein and/or DNA, increase antimicrobial resistance and form a microcolony of cells in a multicellular community (Harper, Parracho et al., 2014).

When a biofilm forms the bacterial cells may become almost impossible to eliminate (Thieh-Fah, 2012). Microbial biofilms are highly resistant to antibiotics (figure 1.2) and host immune defences, and it has been estimated to account for up to 80% of all microbial infections in the human body (Fengjun, Feng et al., 2013). Therefore, biofilm have become an increasing environmental healthcare issue and are causing many types of problems, ranging from dental plaque, urinary tract infections and contamination of medical instruments such as catheters, to biofouling of oceangoing vessels (Newman, Rodrigues et al., 2013). There has been reported up to 100 to 1000- fold increase in antimicrobial tolerance in biofilms compared to planktonic cells. This tolerance includes failure of antibiotics to penetrate biofilms, slow growth rate, altered metabolism, persister cells, oxygen gradient, and EPS. Many antibiotics exhibit a mechanism of action directed toward interrupting replication, transcription, translation and cell wall synthesis of growing bacteria. Reduced growth rates leading to dormant cells increases the antimicrobial tolerance (Olsen, 2015). The antibiotic tolerance in a biofilm is a complex phenomenon that is most likely the result of multiple mechanisms of resistance working together (Thieh-Fah, 2012).

1.3.1 Quorum sensing

Quorum sensing is an important factor during biofilm development, and is a form of cell-cell communication mechanism that synchronizes gene expression in response to population cell density (Solano, Echeverz et al., 2014). The cells communicate by releasing, sensing and responding to small signal molecules called autoinducers. These signal molecules are diffusible and interact with a sensor transcriptional activator to initiate gene expression (Y.-H. Li & Tian, 2012). Many different functions are under the control of quorum sensing, and these include motility, conjugation, competence, sporulation, virulence and biofilm formation (Hammer & Bassler, 2003). During quorum sensing, bacterial cells cooperate to obtain group-specific benefits (Y.-H. Li & Tian, 2012). Quorum sensing can contribute to increased pathogenesis by activation of virulence genes such as toxins and proteases. The microorganisms in a biofilm can by working together use quorum sensing to build a defence to protect themselves from for example antibiotics (LaSarre & Federle, 2013).

PlcR is a master quorum sensing regulator in *B. cereus* (Grenha, Slamti et al., 2012). PlcR constitutes the quorum sensor and PapR the signalling molecule. Together they form a PlcR-PapR complex and this coordinate gene expression to cell density (Slamti, Perchat et al.,

2014a). When the cells enter sporulation PlcR-regulated genes are repressed (Dubois, Faegri et al., 2012) and NprR is sequentially activated. The NprR regulon includes genes encoding degrading enzymes and lipopeptide involved in biofilm formation (Slamti, Perchat et al., 2014a). The NprR regulon has also shown to activate genes involved in necrotrophism in Bacilli, and is essential for the survival of Bacilli in the cadaver of infected insects (Slamti, Perchat et al., 2014b). NprR is active later in the infection process and after the death of the host and encodes degradative enzymes (Dubois, Faegri et al., 2012) and has been seen to be activated in *B. thuringiensis* after host death allowing a necrotrophic lifestyle of the bacteria by allowing the vegetative cell to use the insect cadaver as a bioincubator and to survive (Verplaetsea, Slamtia et al., 2015). NprR binds a signalling peptide called NprX, which leads to its activated form that controls the expression of genes essential for biofilm formation, survival and sporulation (Dubois, Perchat et al., 2013).

Signal molecules called autoinducers are produced and released by quorum sensing bacteria and can alter gene expression in response to cell density (Miller & Bassler, 2001). Genes encoding Pfs- and LuxS-like enzymes, which can lead to the formation of Autoinducer-2, are seen to be present in *B. cereus, B. thuringiensis* and *B. anthracis* (Auger, Krin et al., 2006). A study performed on the effect of AI-2 in biofilm formation was tested for *B. cereus* and showed that AI-2 had an inhibitory effect on the formation of biofilm in a concentration-dependent matter (Auger, Krin et al., 2006).

1.3.2 Biofilm formation in *Bacillus subtilis*

Biofilm formation in *B. subtilis* is more comprehensively studied than in the *B. cereus* group of organisms (Newman, Rodrigues et al., 2013). Flagellated, motile single cells of *B. subtilis* switches state during biofilm formation to growth of long chains of nonmotile cells that form parallel bundles (Lemon, Earl et al., 2008). There has been reported multiple mechanisms and genes that are involved in the formation of the biofilm, but the master regulator system governing biofilm formation in *B. subtilis* is the *sin*R repressor (Newman, Rodrigues et al., 2013). SinR is a DNA-binding protein that is activated during late growth of *B. subtilis*. It is needed for development of competence and motility, and for the production of autolysin (Bai, Mandic-Mulec et al., 1993). SinR plays a role in repressing transcription of the *eps* operon, which is necessary for exopolysaccharide production, and hence inhibits the formation of biofilm. SinI acts the opposite, and activates the *eps* operon (Kearns, Chu et al., 2005). High

levels of SinI promote the expression of the *slrR* gene that is under the transcriptional repression by SinR. *tapA* and *eps* promoters are stimulated by SlrR induction, and SlrR is therefore an activator of matrix production and biofilm formation. The *tapA-sipW-tasA* operon encodes the main protein components TapA and TasA of the biofilm matrix. SlrR binds to SinR and forms a complex that inhibits the binding of SinR to the *eps* and *tapA* promoter regions, and also represses the transcription of genes required for motility and autolysins (Cairns, Hobley et al., 2014).

Activation of the transcription factor *spo0A* is central to biofilm initiation (Cairns, Hobley et al., 2014) Increased levels of SinR has been seen to repress the expression of *spo0A,* which is needed for sporulation (Mandic-Milec, Doukhan et al., 1995). It has also been seen that increased levels of sinR inhibits the production of extracellular proteases (Bai, Mandic-Mulec et al., 1993). Sporulation regulatory proteins *spo0A* positively control *sinI* (Kearns, Chu et al., 2005), and during sporulation increased expression of *sinI* down-regulates SinR (Bai, Mandic-Mulec et al., 1993). SinI disrupts the SinR dimer by interacting with the C-terminal end of SinR and thereby prevents SinR from binding to DNA (Cervin, Lewis et al., 1998). SinR is expressed throughout growth, while SinI is expressed during stationary phase (Newman, Rodrigues et al., 2013).

The CodY regulator represses many genes that are expressed early in stationary phase in *B. subtilis*. CodY has also been reported to regulate *spo0A*. It is believed that CodY is activated when there is a decrease in growth rate due to nutrient-limiting conditions (Ratnayake-Lecamwasam, Serror et al., 2001b). It regulates over 200 genes that are repressed during presence of excess nutrients, many of which encode components of metabolic pathways (Barbieria, Voigtb et al., 2015). When nutrient levels drop GTP is degraded to ppGpp and ppGpp. CodY allows the cell to adapt to nutrient limitation by sensing intracellular GTP concentrations and regulates early-stationary-phase and sporulation genes (Ratnayake-Lecamwasam, Serror et al., 2001a). Another key regulator found in *B. subtilis* is AbrB. AbrB regulates expression of different genes with different biological function including biofilm formation, antibiotics production, motility, development of competence for DNA uptake, synthesis of extracellular enzymes and sporulation. AbrB is expressed at high levels during lag to exponential phase, and decreases when the cell enters stationary phase. Spo0A represses *abrB* transcription (Chumsakul, Takahashi et al., 2010).

1.3.3 Biofilm formation in *Bacillus cereus*

B. cereus has the ability to produce a variety of biofilms depending on the environment it is in, and can grow as an immersed or floating pellicle biofilm or as a ring strongly sticking to the recipient wall at air-liquid interface (Majed, Faille et al., 2016; Wijman, de Leeuw et al., 2007). In most isolates of the *B. cereus* group of organisms, biofilms are found as floating pellicles, but are also capable to stick on immerged abiotic surfaces or even be present on living tissue. Biofilms are suspected to play a key role in the ubiquitous distribution of *B. cereus* because of its ability to colonize different types of environments (Majed, Faille et al., 2016). *Bacillus* biofilms are found on every food contact surfaces of open or closed equipment such as pasteurizers, filling machines, storage thanks and more. *B. cereus* and *B. thuringiensis* are also been found in association with other microorganisms in biofilms, like for example *B. cereus* in dental plaque of multispecies biofilm (Majed, Faille et al., 2016).

The majority of cells in *B. cereus* biofilm are in a vegetative form, but during the course of biofilm formation sporulation can also occur (Ryu & Beuchat, 2005). This suggests a potential role of biofilm-derived spores, and has been observed in contamination of food (Majed, Faille et al., 2016). These spores have greater resistance to environmental stress and antimicrobial compounds. Spores can also attach to inert surfaces and lead to germination, growth and then biofilm formation (Ryu & Beuchat, 2005). Depending on which *B. cereus* strain is in the biofilm it secretes metabolites, surfactants, bacteriocins, enzymes and toxins. These secreted products can act on the biofilm itself or on the environment (Majed, Faille et al., 2016). Different features are seen to affect biofilm formation in different strains of *B. cereus*, and these include differences in genes for polysaccharide capsule production, genes involved in flagella production, environmental conditions and temperature (Wijman, de Leeuw et al., 2007). Motile *B. cereus* cell can increase nutrient exchange in biofilm by producing channels in the matrix. Motile *B. cereus* cells can also contribute to colony spreading, or even occur in depth of pre-existing biofilms because of their ability to create channels in the matrix. Suppression of motility in a *B. cereus* strain which forms biofilms at the air-liquid interface resulted in the formation of submerged biofilm, which indicates that motility play an essential role in biofilm formation (Majed, Faille et al., 2016).

The *B. cereus* species share a large number of transcriptional factors, including the sporulation regulator Spo0A, the stress response sigma factor σ^B, and the phase-transition

regulators SinI, SinR, CodY and AbrB which are also found in *B. subtilis* (Fagerlund, Dubois et al., 2014). SinR was reported to supress biofilm production and was necessary for swimming motility in *B. thuringiensis*, while SinI had the opposite effect. This SinI/SinR antirepressor/repressor mechanism shows similarities with *B. subtilis* (Fagerlund, Dubois et al., 2014). Although some of the molecular mechanisms involved in biofilm formation in *B. cereus* are shared with *B. subtilis,* there have been seen differences between the two species regarding biofilm structure, the effectors of matrix formation and the regulation pathways controlling them (Majed, Faille et al., 2016). *B. cereus* exhibit the pellicle formation biofilm mode which is similar to many of the genes known to be required for biofilm formation in *B. subtilis,* however *B. cereus is* also able to form submerges, surface-associated biofilms, that are not dependent on the genes required for pellicle formation (T. Gao, Foulston et al., 2015).

1.3.4 Bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP)

c-di-GMP is a second messenger that regulates several processes in bacteria including virulence, motility, biofilm formation, cell-cell signalling, and host colonization (figure 1.3) (Chena, Chaia et al., 2012; Römling, Galperin et al., 2013; Schirmer, 2016). c-di-GMP has shown to modulate lifestyle changes of many bacteria, as seen in the establishment of multicellular biofilm community where it modulates transition of a motile cell to the sessile state. It can also modulate the transition of a cell from a virulent state in acute infections, to a less virulent but more resilient state characteristic of chronic infectious disease (Römling, Galperin et al., 2013). It is best characterized in Gram-negative bacteria where elevated levels of intracellular c-di-GMP has been associated with reduction in motility and activation of biofilm formation, and has been demonstrated in for example *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella enterica* serovar typhi (Valentini & Filloux, 2016).

c-di-GMP is synthetized from two molecules of GTP by enzymes called diguanylate cyclases (DGCs) and is degraded into 5'-phosphoguanylyl-(3'-5')-guanosine (pGpG) and/or GMP by phosphodiesterases (PDEs) (figure 1.3) (Römling, Gomelsky et al., 2005). DGCs carry a GGDEF active site motif and PDEs carry either EAL or HD-GYP domain (Valentini & Filloux, 2016). HD-GYP domain-containing proteins belong to the HD superfamily of metal-dependent phosphohydrolases. This enzyme hydrolyzes c-di-GMP in a two-step reaction, producing a final product of two GMP (Valentini & Filloux, 2016). A GGDEF domain and an EAL domain may often be found in the same protein, where the GGDEF domain is

located N-terminally to the EAL domain. These two domains are also often located C-terminally to multiple sensory and signal transduction domains (Römling, Gomelsky et al., 2005) N-terminal sensor domains control the activity of a majority of GGDEF/EAL/HD-GYP domain proteins, and are often integrated in the cytoplasmic membrane. The N-terminal sensor domains may contain periplasmic loops that bind small ligands or other periplasmic protein (Hengge, Gründling et al., 2015). Environmental factors such as for example oxygen, amino acids, electrons and photons are believed to stimulate and regulate the activity of DGC or PDE proteins. c-di-GMP seems to be the common second-messenger for these external signals (Camilli & Bassler, 2006). Production of GGDEF domain proteins results in elevated c-di-GMP concentrations, which leads to the production of adhesive matrix components and elevated multicellular behaviour. Overproduction of EAL domain proteins conversely leads to the opposite, and suppresses biofilm formation and autoaggregation, but some motility is still left activated (Römling, Gomelsky et al., 2005). This could be due to flagella that has also been shown to play a distinct role during biofilm formation (Hengge, Gründling et al., 2015).

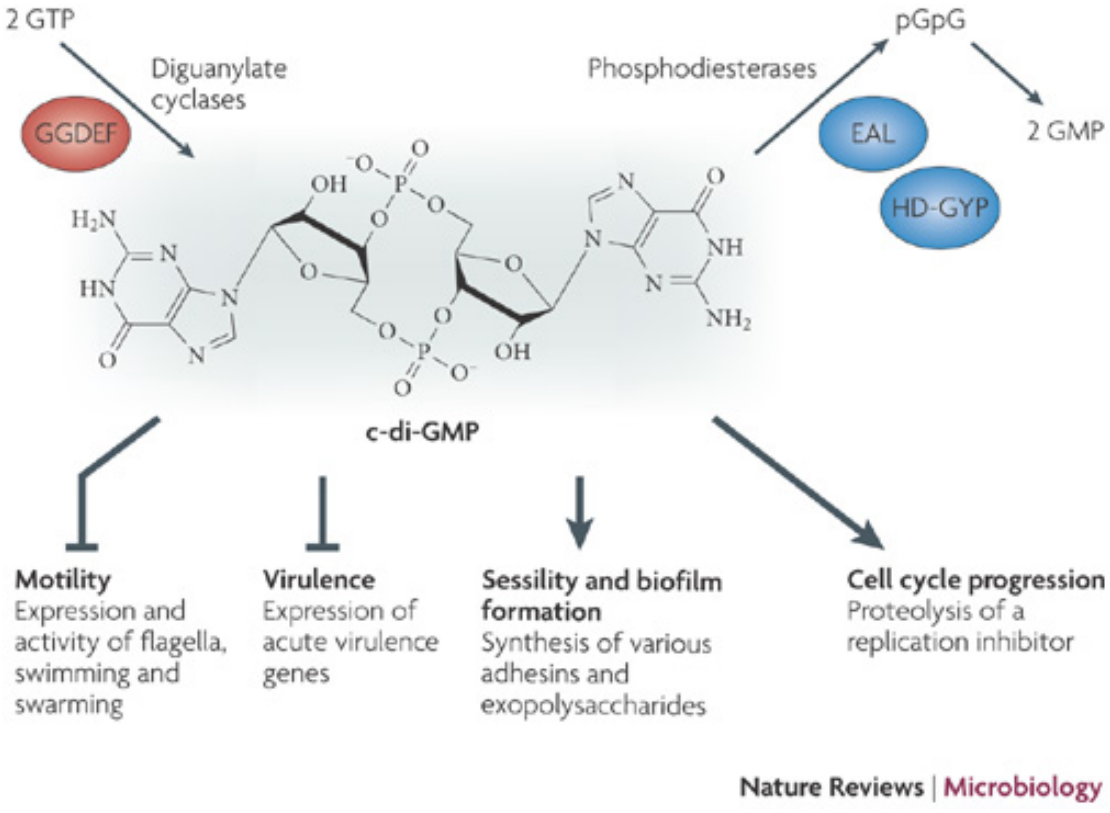


Figure 1.3 Synthesis and breakdown of c-di-GMP, and c-di-GMPs role in different pathways. c-di-GMP regulates several processes in the bacteria and functions as a second-messenger (Hengge, 2009).

c-di-GMP interacts with downstream macromolecular targets. c-di-GMP-binding proteins identified include members of the PilZ domain containing family (figure 1.4) and transcription factors. The mechanism of action of many other c-di-GMP binding proteins is still unknown. Riboswitches that bind to c-di-GMP have also been identified as part of the signalling pathway. These are noncoding RNA elements that bind to specific small molecule ligands (Shanahan, Gaffney et al., 2011), including molecules such as metabolites, coenzymes, amino acids and nucleobases (Furukawa, Gu et al., 2012). Binding of ligands induces structural rearrangements within the RNA which causes changes in the expression levels of the downstream genes (Shanahan, Gaffney et al., 2011). Increasing or decreasing c-di-GMP concentrations results in physiological changes in these genes (Furukawa, Gu et al., 2012).

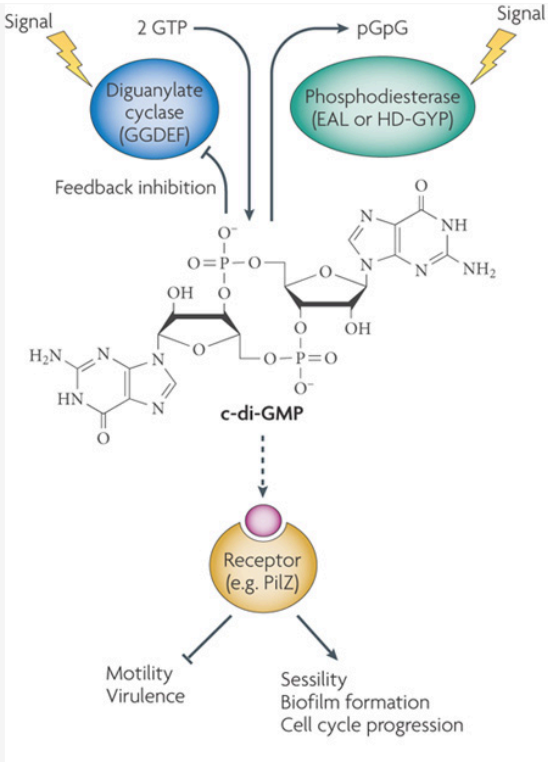


Figure 1.4 Binding of c-di-GMP to the PilZ receptor. Production and breakdown of c-di-GMP and its binding to the PilZ receptor (Schirmer & Jenal, 2009).

B. cereus group bacteria carry a set of genes named *cdg*; cyclic diguanylate, encoding enzymes putatively involved in c-di-GMP synthesis and breakdown, and some of which are essential for effective biofilm formation in an oxygenated environment (Fagerlund, Dubois et al., 2014). Most of the Cdg proteins carry genes encoding GGDEF and/or EAL domains. CdgF and CdgB overexpression resulted in increase in c-di-GMP concentration, and hence indicates DGC activity (Fagerlund, Smith et al., 2016) Not all *B. cereus* group strains are

motile or produce biofilm, but the motile strain *B. thuringiensis* 407 which is often used as a model strain in biofilm studies, harboured all identified c-di-GMP signalling genes except for *cdg*M and *cdg*K (Fagerlund, Dubois et al., 2014). Results showed that *B. cereus* group bacteria carry a wider range of genes encoding c-di-GMP metabolizing enzymes compared to *B. subtilis*, however further studies are necessary to provide more links between Cdg proteins and c-di-GMP signalling and phenotypes important for the *B. cereus* group in their different habitats, including sporulation, motility, toxicity and biofilm formation (Fagerlund, Smith et al., 2016).

1.3.5 Cellulose synthesis and regulation by c-di-GMP

Bacterial cellulose is a linear extracellular polysaccharide produced as long nanofibers, and has been seen in biofilm of several bacteria (Omadjela, Narahari et al., 2013; Zhijiang & Guang, 2011). Bacterial cellulose is synthetized from cytosolic UDP-activated glucose (UDP-Glc) (McNamara, Morgan et al., 2015), by membrane-embedded glycosyltransferases (Omadjela, Narahari et al., 2013) and has a basic structure of fibril that consists of β-1→4 glucan chain held together by inter- and intra- hydrogen bonding (Esa, Tasirin et al., 2014). Bacterial cellulose display several properties like high water uptake capacity, high crystallinity, and an ultrafine nanofibril network structure (Zhijiang & Guang, 2011).

Cellulose is a known component of EPS in many bacteria such as *Escherichia coli, Salmonella spp., Acetobacter spp., Rhizobium spp.*, and *Vibrio spp* (Ziemba, Shabtai et al., 2016). Expression of bacterial cellulose synthesis genes is generally stimulated during biofilm formation, where cellulose mediates cell-cell interactions, cell adherence, and biofilm formation on biotic and abiotic surfaces (Römling & Galperin, 2015b). In *Vibrio fischeri* biofilms cellulose showed to have a significant impact on the mechanical properties and morphological characteristics of the biofilm, and was shown to increase the total volume of the biofilm (Ziemba, Shabtai et al., 2016). In rhizobia the bacterial cellulose was shown to be involved in attachment of the bacteria to plant roots and biofilm maturation (Augimeri, Varley et al., 2015). Another study done on bacterial cellulose showed that statically cultured bacterial cellulose formed a pellicle, composed of small amounts of nanofibril which held around 99% water (Zhijiang & Guang, 2011). The pellicle formation of *Gluconacetobacter xylinus* grown at air-liquid interface has been studied closer, and it was seen that newly synthetized cellulose was predominantly located at the top surface of the pellicle which

shows that cellulose production is primarily dependent on oxygen supply. The cellulose containing pellicle of *G. xylinus* had a porous network at the bottom surface (liquid-media interface) and densely packed macrofibrils at the top surface (air-media interface) after 7 day of incubation (Lee, Gu et al., 2015). Hence, bacterial cellulose in *G. xylinus* leads to formation of a pellicle which has a dens surface on one side and a gelatinous layer on the other side (Jozala, Pértile et al., 2015). Bacterial cellulose has shown to aid in attachment, adherence and colonization of a substrate. The bacterial cellulose produced when *G. xylinus* colonized fruit protected the bacteria from desiccation and from damaging effect of UV radiation. The bacterial cellulose in *G. xylinus* also resulted in outcompeting fungi and other bacteria (Augimeri, Varley et al., 2015). Cellulose production in bacteria has been suggested to aid the bacteria in position close to surfaces of culture solutions, as well as protecting the bacteria form ultraviolet radiation and entry of enemies and heavy metal-ions (Vazquez, Foresti et al., 2013).

The cellulose synthase (CeS) complex contains bacterial cellulose synthase subunit A (BcsA), B (BcsB), C (BcsC) and D (BcsD), and these are identified as the activators of bacterial cellulose production through binding to c-di-GMP. Subunits are all encoded by genes within a single operon (Ji, Wang et al., 2016). The four-gene *bcs*ABCD operon was first identified in *Komagataeibacter (Acetobacter) xylinus*. BcsA and BcsB are essential for the bacterial cellulose synthesis, while BcsC and BcsD are involved in exporting the glucan molecules and packing them at the cell surface (Römling & Galperin, 2015b). BcsC and BcsD form a channel in the cell membrane to translocate the molecules (McNamara, Morgan et al., 2015). BcsA is predicted to include nine transmembrane helices, a glycosyltransferase domain, and a PilZ domain (Pultz, Christen et al., 2012). The PilZ domain in the BcsA subunit has been identified to bind to c-di-GMP (Römling & Galperin, 2015b), and is known as the catalytic subunit of the cellulose synthase complex (Choua & Galperinb, 2016). When this binding occurs the active site (glycosyltransferase domain) becomes accessible for the UDP glucose substrate (Römling & Galperin, 2015b). In a study it was observed that binding of c-di-GMP to BcsA occurred with high affinity and activated cellulose synthesis, and functioned as a potent inducer of biofilm formation. When c-di-GMP concentrations was increased it did no alter BcsAs affinity for UDP-Glc, but increased the apparent catalytic rate of BcsA in vitro with at least 10-fold (Omadjela, Narahari et al., 2013).

Studies in *Salmonella* have shown that the BcsA subunit of cellulose synthase is encoded in one of the bacterial cellulose synthesis (bcs) operons. Cellulose, a β-1,4-D- glucose polymer, is a main component of the extracellular matrix of the *Salmonella* biofilm, and high levels of c-di-GMP has been associated with its production (Zorraquino, García et al., 2012). The PilZ domain in the BcsA subunit in *Salmonella* is thought to bind c-di-GMP and regulate the enzymatic activity of a periplasmic cellulose synthesis domain (Zorraquino, García et al., 2012). *E. coli* has also been found to encode the bacterial cellulose synthesis (bcs) operons (Römling & Galperin, 2015a), which affects biofilm formation capability of the organism by expressing cellulose as an extracellular matrix component (Bokranz, Wang et al., 2005). The YcgR protein carrying PilZ domains in *E. coli* was also found to bind c-di-GMP, and individual PilZ domains were observed to bind c-di-GMP, only with less affinity. This indicates that PilZ domain alone is sufficient for binding to c-di-GMP (D.A. Ryjenkov, 2006). Studies in several species of bacteria confirmed that PilZ domain proteins play a role in the synthesis of exopolysaccharide during biofilm formation. Binding of the PilZ domain to c-di-GMP leads to structural changes in the protein, which initiates a downstream signal transduction cascade. Another function observed for BcsA was inhibition of motility, which is believed to occur directly by inactivation of the flagellar motor or indirectly by synthesis of cellulose (Zorraquino, García et al., 2012).

1.4 Background for the study

c-di-GMP was first identified as an allosteric activator of cellulose synthase (BcsA) in the bacterium *Gluconacetobacter xylinus* (Römling, Gomelsky et al., 2005). A PilZ domain was identified in the cellulose synthase BcsA of this bacterium. (Cotter & Stibitz, 2007). PilZ domains are widely distributed in bacterial genomes, and bacterial genomes generally encode one or multiple PilZ domain proteins (Yang, Tian et al., 2015). The PilZ domain was also identified in the YcgR protein in *E. coli* (Zorraquino, García et al., 2012). This domain showed similarities with a single-domain PilZ protein from *Pseudomonas aeruginosa*. In *Pseudomonas aeruginosa* the PilZ protein is involved in pilus formation and twitching motility (Dmitri A. Ryjenkov, Simm et al., 2006).

GGDEF and EAL domains are often found in species with PilZ domain proteins, and this shows a form of correlation between c-di-GMP synthesis and PilZ (Zorraquino, García et al.,

2012). Analysis of the *B. subtilis* genome showed that the genome has the potential to encode four DGCs, two PDEs, a single bifunctional protein with GGDEF and EAL domains, and one PilZ domain c-di-GMP receptor protein, YpfA (X. Gao, Mukherjee et al., 2013). Two motifs RxxxR and N/DxxxG in the PilZ domain of YcgR from *E. coli* are recognized to bind c-di-GMP, and the R residue in the RxxxR motif has been identified as the crucial site for the PilZ domain to bind c-di-GMP (Yang, Tian et al., 2015). YpfA is the only predicted protein in *B. subtilis* that contains a PilZ domain, and is a homolog of the YcgR receptor from *E. coli* (X. Gao, Mukherjee et al., 2013). YpfA is involved in regulating swarming motility and has a small and possibly indirect effect only on biofilm formation through binding of c-di-GMP (Yun Chena, Chai et al., 2012)

1.5 Aims for the study

In this study we hypothesized that the individual PilZ domains found in *B. thuringiensis* BcsA could bind c-di-GMP, thereby potentially promoting cellulose production (figure 1.5.1). Two single PilZ domains (one potentially partial) are found in the *B. thuringiensis* 407 BcsA protein (fig. 1.5). *B. thuringiensis* 407 which is a strain cured of the insert toxin plasmid and which is effective in biofilm formation, was used as the model strain in this study. We aimed to clone different combinations of the PilZ domains from *bcsA* into an expression vector and express and purify overexpressed protein following growth of the bacterium and investigate c-di-GMP binding by thermophoresis. Results in this thesis were going to be used in a larger study to identify different mechanisms of biofilm formation through c-di-GMP in the pathogenic species of the *Bacillus* group of organisms (figure 1.6).

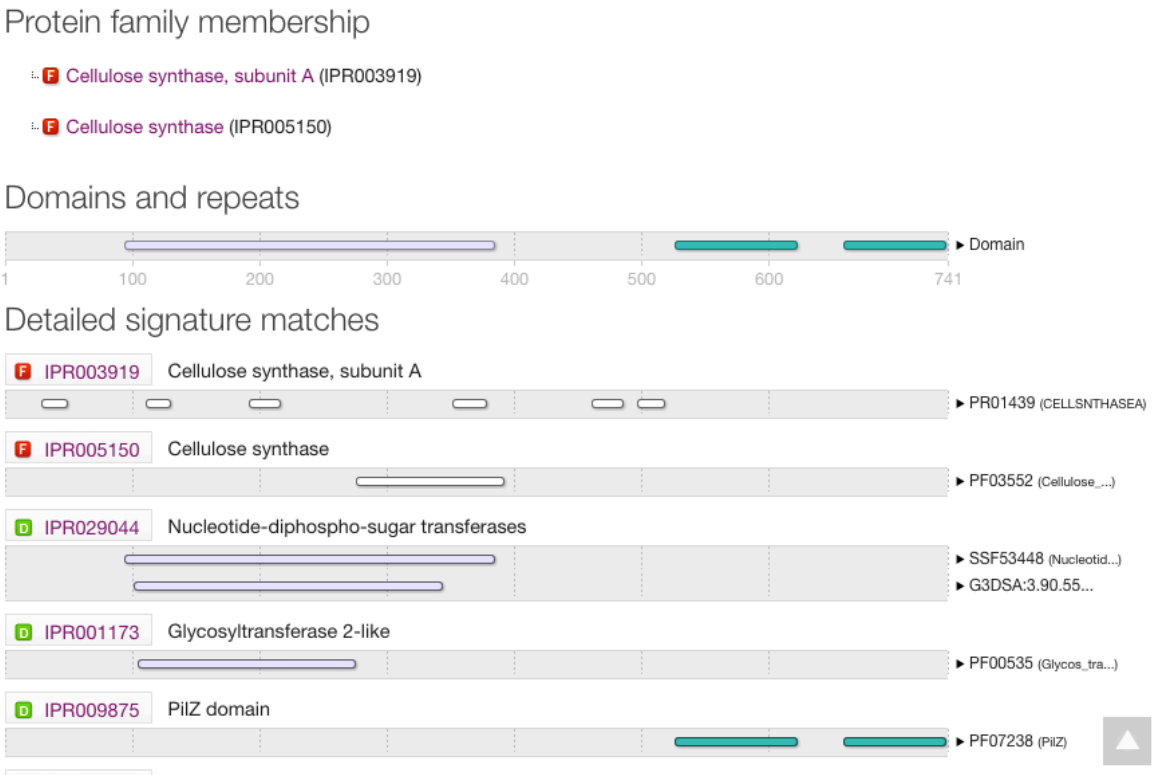


Figure 1.5 Illustration of protein domains in the BcsA protein from *B. thuringiensis* 407. The figure shows PilZ domains (green) and a cellulose synthase domain (grey), as analysed in InterPro (InterPro, 2017a).

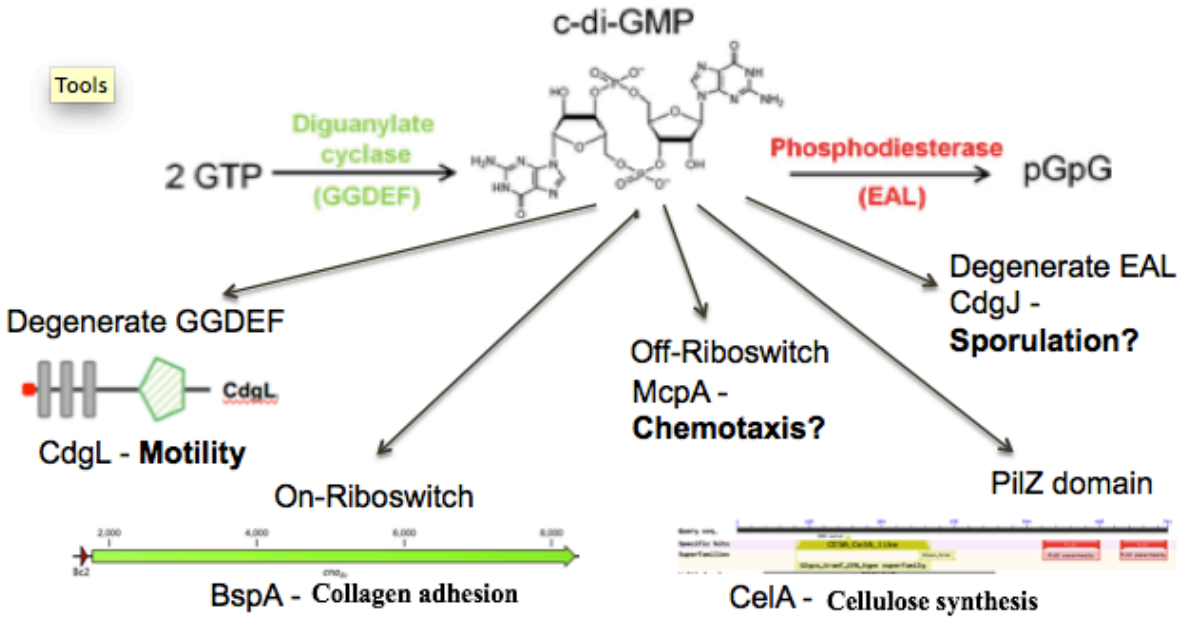


Figure 1.6 Illustration of the different putative c-di-GMP effectors and the corresponding phenotypes potentially affected in *Bacillus* group bacteria, based on preliminary experiments and interpretation of bioinformatics data (Ole Andreas Økstad, personal communication) (Fagerlund, Smith et al., 2016). Drawing made by Ole Andres Økstad.

2 Materials

2.1 Bacterial strains

Table 2.1 Bacterial strains used in this work.

Bacterial strain	**AH-code from internal strain collection**	**Growth medium used**
***B. thuringiensis* 407 (wildtype)**	1423	Luria-Bertani broth (LB)
B. thuringiensis* 407 Δ*bcsA	1871	LB
One shot® TOP10 Chemically Competent *Escherichia coli*	-	LB
***Escherichia coli BL21* (DE3)**	1498	LB

2.2 Primers

Table 2.2 Primers used for cloning.

Primers	**Sequence (5' to 3')**
BcsA_PilZ_start1_F	GTGCCTCCATGGAAAGACCGCGTTTTCGAA
BcsA_PilZ_stop1_R	GTGCCTCTCGAGTTAGTGATGGTGATGGTGATGCG CTGTGAATTTTAATCCAGCA
BcsA_PilZ_stop2_R	GTGCCTCTCGAGTTAGTGATGGTGATGGTGATGAACC GGTTTACTAAATAGATGAAGAATGAG
BcsA_PilZ_start2_F	GTGCCTCCATGGCACCAAGACAATTTCGCAG
BcsA_full_F	GTGCCTCATATGAGCCTTTCTATATACGAAGGTT

Table 2.3 Gene fragments size and primers used.

Domains/ Name	**Primers**	**Product length**
Both PilZ domains ($PilZ_{d1d2}$)	BcsA_PilZ_start1_F BcsA_PilZ_stop1_R	704 bp
1st PilZ domain ($PilZ_{d1}$)	BcsA_PilZ_start1_F BcsA_PilZ_stop2_R	365bp
2nd PilZ domain ($PilZ_{d2}$)	BcsA_PilZ_start2_F BcsA_PilZ_stop1_R	305bp
Full *bcsA* gene	BcsA_full_F BcsA_PilZ_stop1_R	2226bp

Table 2.4 Primers used in DNA-sequencing:

Primers	**Sequence (5' to 3')**
T7_promoter	TAATACGACTCACTATAGGG
T7_terminator	GCTAGTTATTGCTCAGCGG

2.3 Restriction map for vectors: pET-28a(+), pET-30b(+) and pET-26b(+).

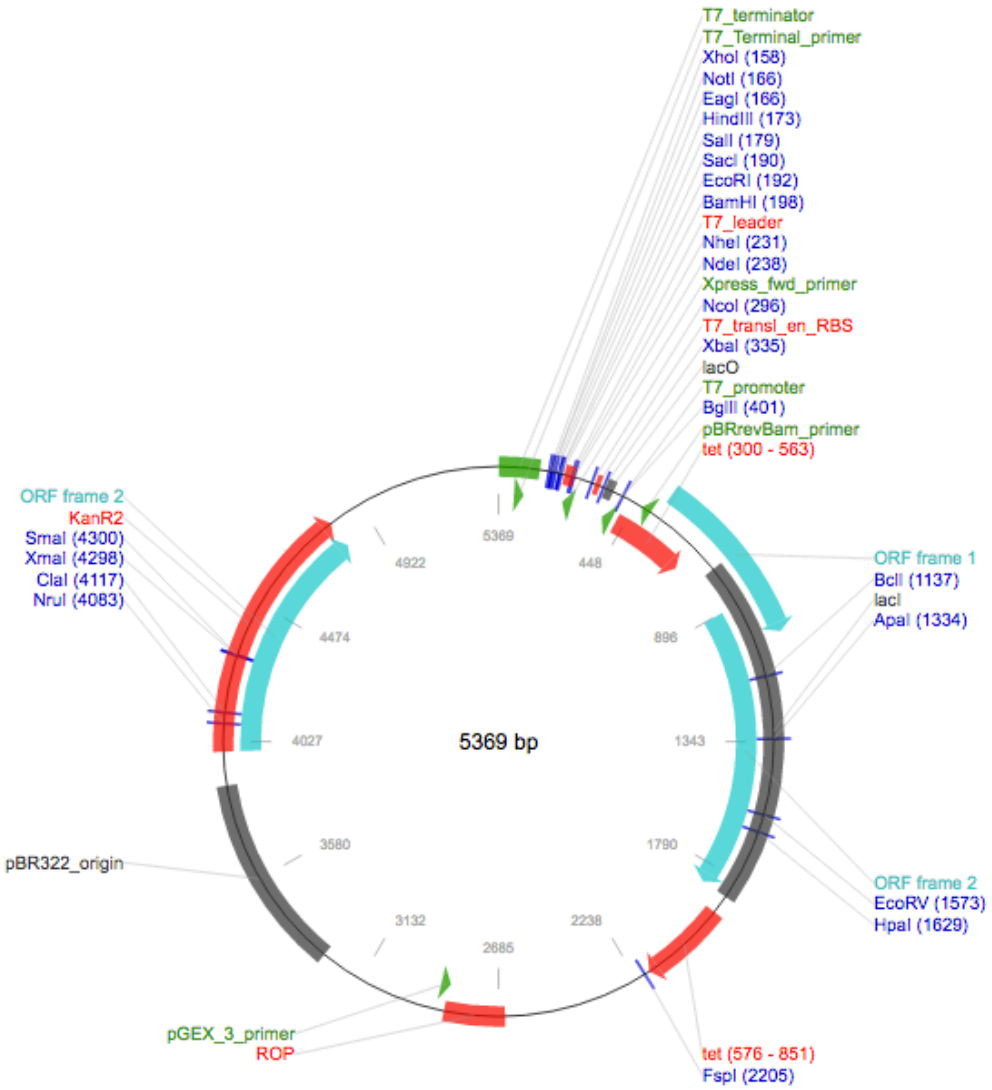


Figure 2.1 Restriction map and vector features for pET-28a(+) (Addgene, 2017b).

pET-28a(+) sequence landmarks:

T7 promoter: 370-386

T7 transcription start: 369

His•Tag coding sequence: 270-287

T7 terminator: 26-72
Information taken from: (Novagen, 2017b).

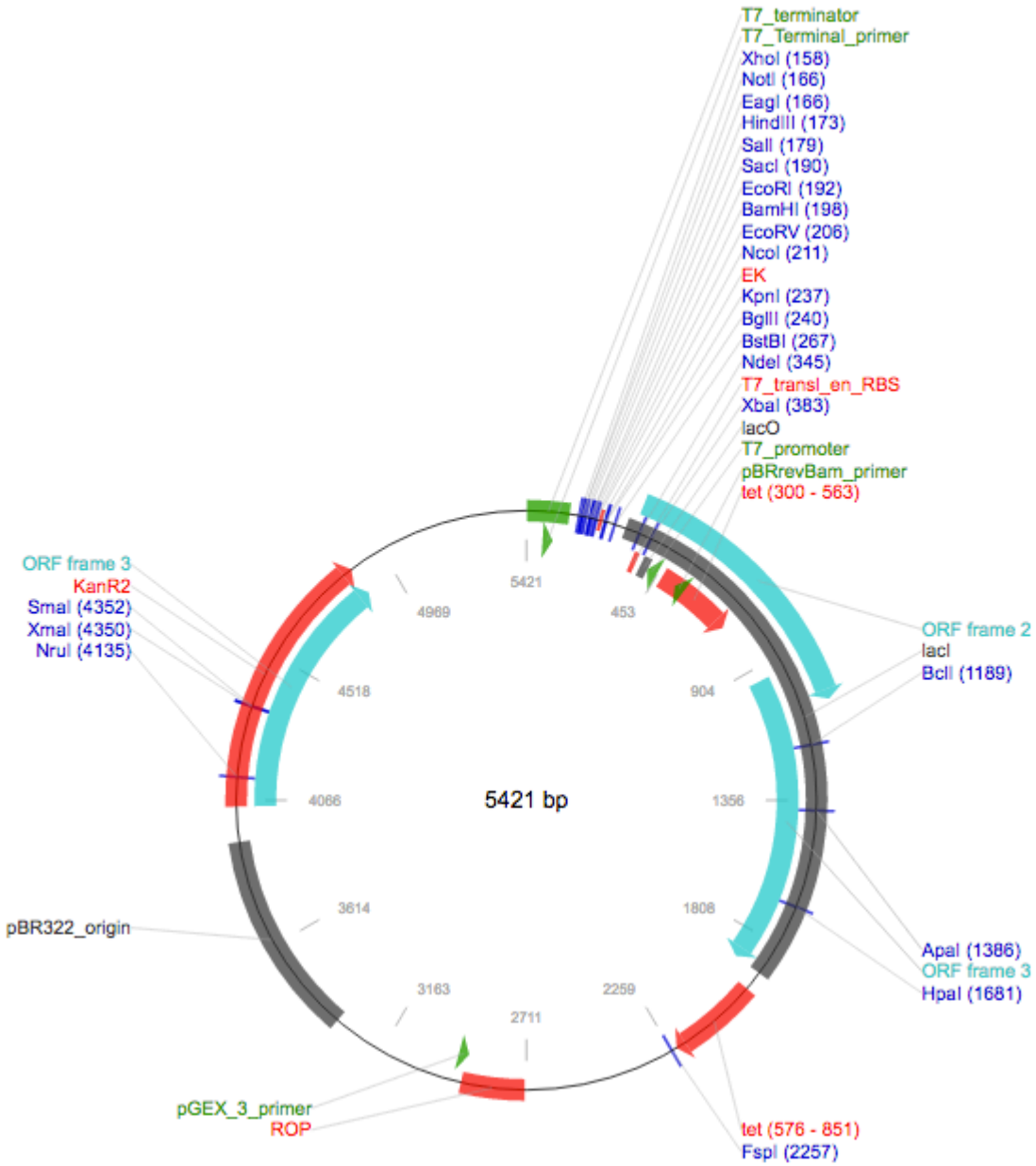


Figure 2.2 Restriction map and vector features for pET-30b(+) (Addgene, 2017c)

pET-30b(+) sequence landmarks:

T7 promoter: 419-435

T7 transcription start: 418

His•Tag coding sequence: 327-344

T7 terminator: 26-72

Information taken from: (Novagen, 2017c).

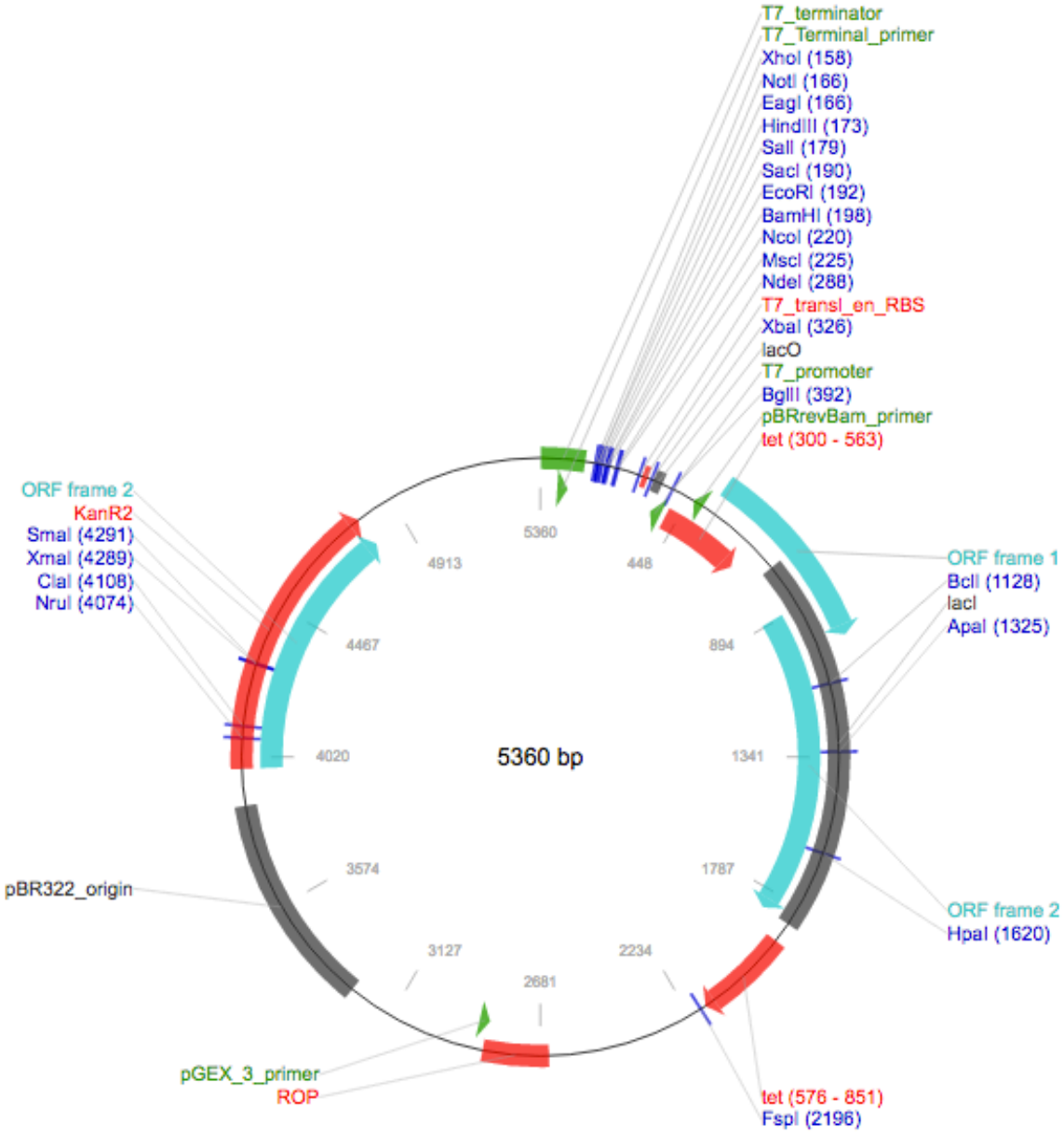


Figure 2.3 Restriction map and vector features for pET-26b(+) (Addgene, 2017a).

pET-26b(+) sequence landmarks:

T7 promoter: 361-377

T7 transcription start: 360

His•Tag coding sequence: 140-157

T7 terminator: 26-72

Information taken from:(Novagen, 2017a)

2.4 DNA Marker

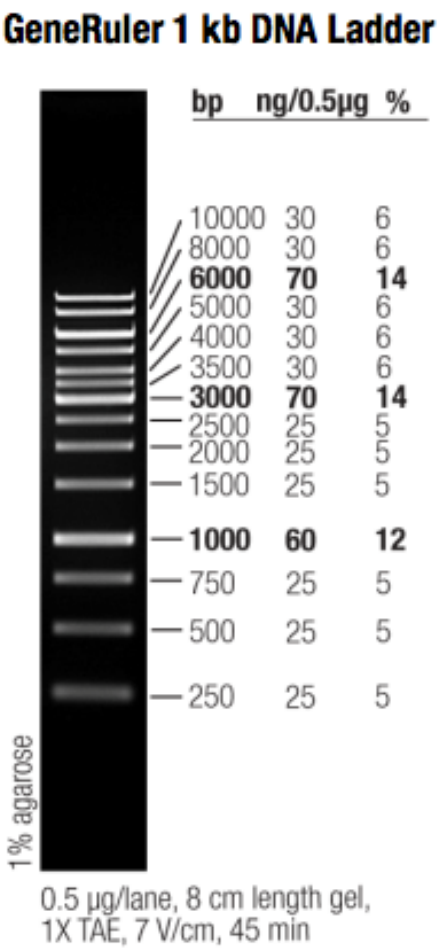


Figure 2.4: Thermo Scientific GeneRuler 1 kb DNA Ladder. Can be used for sizing and approximate quantification of DNA concentration by agarose gel electrophoresis. DNA-fragments of sizes ranging from 250-10000bp in a 0.5µg sample can be measured with this type of ladder (ThermoFisherScientific, 2016).

2.5 Protein markers

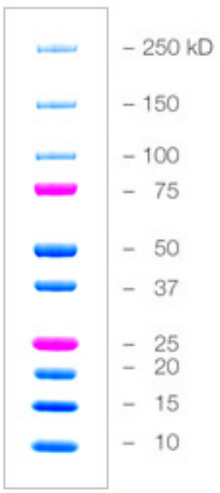


Figure 2.5: Precision Plus Protein™ Dual Color Standard. Proteins sizing from 10-250 kDa can be measured with this type of marker (Bio-Rad, 2017a)

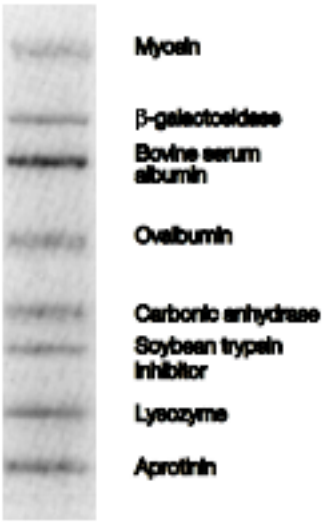


Figure 2.6: Prestained SDS-PAGE Standard, broad range, contains a mixture of 8 natural proteins ranging from approximately 7 to 200 kDa (Bio-Rad, 2017b).

2.6 Antibodies

Primary antibody:

α-5X His antibody produced in mouse 0.2 mg/ml (Sigma-Aldrich).

Secondary antibody:

Goat Anti-Mouse IgG (H+L) HRP-conjugate 0.8 mg/ml (Bio-Rad).

2.7 Enzymes

Antarctic phosphatase enzyme (Thermo Fischer Scientific)

DNase I (Sigma-Aldrich)

*Eco*RI (New England Biolabs)

*Hinc*II (New England Biolabs)

Lysozyme (Sigma-Aldrich)

Mutanolysin (Sigma-Aldrich)

*Nco*I (New England Biolabs)

*Nde*I (New England Biolabs)

Phusion DNA Polymerase (Thermo Fischer Scientific)

RNase A (Sigma-Aldrich)

T4 DNA ligase (New England Biolabs)

*Xho*I (New England Biolabs)

2.8 Kits used

E.Z.N.A.® Gel Extraction Kit (Omega Bio-Tek):

Binding Buffer (XP2)

HiBind® Mini Column

2 ml Collection tubes

SPW Wash Buffer (20ml Absolute alcohol Prima added)

Elution Buffer

E.Z.N.A Plasmid Mini Kit I (Omega Bio-Tek):

HiBind® DNA Mini Columns

2 ml Collection tubes

Solution I (100 µl RNase A added before use)

Solution II

Solution III

HBC Buffer (10 ml isopropanol added before use)

DNA Wash Buffer (60ml Absolute alcohol Prima added before use)

Elution Buffer

3M NaOH (From section 2.9)

Genomic-Tip 100G Midi Kit (QIAGEN):

Buffer B1 (Bacterial lysis buffer): 50mM Tris-Cl pH 8.0, 50mM EDTA pH 8.0, 0.5% Tween®-20, 0.5% Triton X-100.

Buffer B2 (Bacterial lysis buffer): 3M guanidine HCl, 20% Tween-20.
Buffer QBT (Equilibration buffer): 750mM NaCl, 50mM MOPS pH 7.0, 15% isopropanol, 0.15% Trion X-100.
Buffer QC (Wash buffer): 1.0 M NaCl, 50mM MOPS pH 7, 15% isopropanol.
Buffer QF (Elution buffer): 1.25M NaCl, 50mM Tris-Cl pH 8.5, 15% isopropanol.
QIAGEN Genomic-tip 100/G

Pierce BCA Protein Assay Kit (Thermo Scientific)

BCA Reagent A: 500 ml containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1M sodium hydroxide.
BCA Reagent B: 25mL containing 4% cupric sulfate.
Albumin Standard Ampule, 2mg/ml: 10x1 ml ampules containing bovine serum albumin (BSA) at 2mg/ml concentration in 0.9% saline and 0.05% sodium azide.

Trans-Blot® Turbo™ RTA Mini Nitrocellulose Transfer Kit

40 mini-sized nitrocellulose membranes (7x 8.5 cm)
80 transfer stacks
1 L 5x transfer buffer
2 gel trays for wetting and equilibrating membranes and transfer stacks

2.9 Reagents and solutions

Absolute alcohol Prima (Kemetol)

Acrylamide/Bis solution 30% (Bio-Rad)

Agar Bacteriological (agar no.1) (Oxoid)

Agarose (Sigma–Aldrich)

Ammoniumpersulphate (Chemi–Teknik)

Antarctic phosphatase 10x buffer (Thermo Fischer Scientific)

Bio–Safe ^{TM}Coomassie G 250 Stain (Bio–Rad)

Bromophenolblue (Merck)

Calcium chloride dihydrate ($CaCl_2$ X $2H_2O$) (VWR)

Coomassie Brilliant Blue G-250 Stain (Bio-safe^{TM}-Bio-Rad)

Cut Smart buffer® (New England Biolabs)

6x DNA Loading dye (Thermo Fischer Scientific)

dNTP mix, 10mM (Qiagen)

dNTP set (100mM each) (Invitrogen)

Dry milk (Sigma-Aldrich)

Ethidiumbromid (EtBr) (Sigma-Aldrich)

Ethylenediaminetetraacetic acid (EDTA) (M&B laboratory chemicals)

Glycerol (VWR International Ltd)

Glycine (Sigma–Aldrich)

Hydrogenchloride (HCl) (VWR)

IPTG (Fermentas)

Imidazole (Fluka)

Iron(II) sulphate heptahydrate ($FeSO_4$ X $7H_2O$) (Sigma-Aldrich)

Kanamycin (Sigma-Aldrich)

Magnesium sulphate ($MgSO_4$ x $7H_2O$) (VWR)

β-mercaptoethanol (Sigma)

Methanol (MeOH) (VWR)

Mutanolysin (Sigma-Aldrich)

Phenylmethylsulfonyl fluoride (PMSF) (Sigma–Aldrich)

Potassium chloride (KCl) (Emsure)

Phusion HF 5x Buffer (New England BioLabs)

Sodium chloride (NaCl) (Merck)

Sodium dodecyl sulphate (SDS) (Sigma–Aldrich)

Sodium hydroxide (NaOH) (Merck)

Sodium phosphate dibasic dihydrate (Na_2HPO_4 X $2H_2O$) (Emsure)

10x T4 DNA ligase buffer (New England Biolabs)

Tetramethylethylenediamine (TEMED) (Sigma–Aldrich)

Tris/Glycine Buffer (electrophoresis buffer) (10 X TG) (Bio-Rad)

Tris/Glycine SDS buffer (transfer buffer) (10 X TGS) (Bio-Rad)

Trizma® – Base (Sigma–Aldrich)

Trizma® hydrochloride (Sigma–Aldrich)

Tryptone (Oxoid)

Tween-20® Detergent (Calbiochem)

Yeast extract (Oxoid)

2.10 Prepared Solutions

Ammoniumpersulphate (APS) (10 %)

- 0.1g ammoniumpersulphate dissolved in 1ml MQ water in an eppendorf tube and immediately used, or stored at -20°C.

1% Dry milk in 1x TBST

- 0.1 g dry milk
- 10 ml 1x TBST

  prepared right before use.

Elution buffer pH 8.0 (For protein isolation):

- 0.6 ml Imidazole (1M)
- 1 ml Na_2HPO_4 x $2H_2O$ (0.5M)
- 1.2 ml NaCl (5M)

  4ml MQ water was added, and then pH was adjusted to 8.0. MQ water was then added to 10ml total volume.

70 % Ethanol

- 7 ml Absolute alcohol Prima
- 3 ml MQ water.

  Mixed and stored at 4 °C.

Ethidiumbromid (5mg/ml)

- 0.5g ethidiumbromid dissolved in 100ml MQ water and packed in aluminum foil. Container with mix was put under stirring with magnet in a couple of hours to ensure total dissolving of EtBr. Solution was stored at 4°C.

Ethylenediaminetetraacetic acid (EDTA) (0.5M) pH 8.0

- 181.1g EDTA added to 800ml MQ water, and then added 21g NaOH. MQ water was then filled till 1000ml. Solution was autoclaved and stored at room temperature.

50 % Glycerol, sterile

- 100.5ml glycerol (99.5 %) added to 99.5ml MQ water. Solution was autoclaved and stored at room temperature.

Imidazole (1M)

- 0.6808g Imidazole dissolved in 10ml MQ water, sterile filtered and then stored at room temperature.

IPTG (100mM)

- 0.238g IPTG dissolved in 10ml MQ water, sterile filtered and distributed in 1ml aliquots. Stored at -20°C.

Kanamycin (25mg/ml)

- 2.5g kanamycin dissolved in 100ml MQ water, sterile filtered and distributed in 1ml aliquots. Aliquots were stored at -20°C.

4x Laemmli-buffer

- 25 ml 20% SDS
- 14.7 ml glycerol 87%
- 9.4 ml 2 M Tris-HCl (pH 6.8)
- 0.01 ml bromophenol blue

- 0.9 ml MQ water

  Mixed and stored at room temperature.

2x Laemmli-buffer

- 2 ml 4x Laemmli buffer
- 2 ml MQ water

  Mixed and used immediately before use.

Lysis buffer pH 8.0 (For protein isolation)

- 0.4 ml Imidazole (1M)
- 4 ml Na_2HPO_4 x $2H_2O$ (0.5M)
- 2.4ml NaCl (5M)

  40ml MQ water was added, and then pH was adjusted to 8.0. MQ water was then added to 40ml total volume.

Lysozyme 100mg/ml

- 1g lysozyme dissolved in 10ml MQ water and used immediately.

MQ H_2O

- Autoclaved MilliQ filtered water.

NaCl (5M)

2.92g NaCl dissolved in 10ml MQ water**,** sterile filtered and then stored at room temperature.

Na_2HPO_4 x $2H_2O$ (0.5M)

- 0.690g Na_2HPO_4 x $2H_2O$ dissolved in 10ml MQ water, sterile filtered and then stored at room temperature.

Phenylmethylsulfonyl Fluoride (PMSF) (100mM)

- 0.174g PMSF was dissolved in 1ml isopropanol and stored at -20 °C.

Proteinase K (20 mg/ml)

- 0.02g lyophilized Proteinase K powder was dissolved in 50mM Tris-HCl (pH 8.0), and potassium acetate to 1.5mM. Solution was stored at -20 °C.

2 x SDS loading buffer

- 50µl β-mercaptoethanol
- 950µl 2x Laemmli-buffer

Mixed in eppendorftube. This buffer was made right before use.

10 % Sodium dodecyl sulfate (SDS)

- 10 ml 20 % SDS
- 10 ml MQ water

Mixed well and stored in 50ml NUNC-tube at room temperature.

20 % Sodium dodecyl sulfate (SDS)

- 200g SDS
- 1000ml MQ water

SDS dissolved in MQ water under low heat and stirring. Solution was made in sterile glass flask and stored at room temperature.

Sodium hydroxide (NaOH) (3M)

- 1.8g NaOH was dissolved in 15ml MQ water in a sterile glass flask and stored at room temperature.

10 X TBST (Tris-buffered Saline with Tween)

- 24.23 g Trizma (200mM)
- 87.66 g NaCl (1.5M)
  10 ml Tween-20 (1%)
  pH 7.5
  MQ water added to 1L total volume. Stored at room temperature.

1 X TBST

- 1 part of 10X TBST with 9 parts of MQ water.
  Stored at room temperature.

TEN-buffer (Tris-EDTA-NaCl buffer)

- 10ml Tris pH 8.0 (1M)
- 2ml 0.5M EDTA
- 5.846g NaCl
  Components were dissolved in 900ml MQ water, pH adjusted to 8.0. MQ water was then added to 1000ml total volume.

1x TG: Tris/glycine Buffer (ELFO-buffer, for protein electrophoresis).

- 100 ml 10x TG (Bio-Rad)
- 900 ml distilled water

1M Tris (pH 6.8)

- 30.29 g Trizma base
  Dissolved in 200 ml MQ water and pH adjusted to 6.8. MQ water was then added to 250ml total volume. The solution was autoclaved and stored in a glass flask at room temperature. pH was controlled and adjusted again before solution was used in the experiment.

1.5M Tris (pH 8,8)

- 45,43g Trizma base
  Components was dissolved in 200ml MQ water and pH adjusted to 6,8. After adjusted pH MQ water was added until 250ml reached. The solution was autoclaved and stored in glass flask at room temperature. pH was controlled and adjusted again before solution was used in the experiment.

TSS buffer:

- 1.0g PEG 8000
- 0.5ml DMSO
- 40mM Mg^{2+}*
  *9.9g $MgSO_4$ X $7H_2O$ dissolved in 100ml MQ water. 1ml of this was used.

Dissolved in 10ml LB medium, sterile filtered, and stored at -20 °C.

Wash buffer pH 8.0 (For protein Isolation):

- 0.4 ml Imidazole (1M)
- 2 ml Na_2HPO_4 x $2H_2O$ (0.5M)
- 1.2 ml NaCl (5M)

14ml MQ water was added, and then pH was adjusted to 8.0. MQ water was then added to 20ml total volume.

2.11 Growth media

2.11.1 LB agar

- 10g Tryptone
- 5g yeast extract
- 10g NaCl
- 15,0g Nr 1 Bacterial agar

Dissolved in 950 ml MQ H_2O, pH adjusted to 7,0 with 5,8M HCl, and then volume was adjusted to 1000ml with MQ H_2O.

Autoclaved and stored at 4 °C.

LB-agar plates with added antibiotic: antibiotic in desired concentration was added to melted LB agar cooled down to 50 °C before the agar plate was made.

2.11.2 LB medium

- 10g Tryptone
- 5g yeast extract
- 10g NaCl

Dissolved in 950 ml MQ H_2O, pH adjusted to pH 7,0 with 5,8M HCl, and then volume was adjusted to 1000ml with MQ H_2O.

Autoclaved and stored at 4 °C.
Antibiotic in LB medium: antibiotic in desired concentration was added to LB medium before use.

2.11.3 SOC medium

- 20g Tryptone
- 5g yeast extract
- 0.5g NaCl

Dissolved in 950ml MQ H_2O, and 250mM KCl was added. pH adjusted to 7,0 with 5,8 M HCl, and then volume was adjusted to 1000ml with MQ H_2O. Solution was then autoclaved, and after cooling of solution 5ml sterile $MgCl_2$ (2M) and 20 ml sterile glucose (1M) was added. Stored at -20°C.

2.12 Gels for protein and DNA electrophoreses

2.12.1 Agarose gel (1%)

For small gel:

- 0.4g agarose
- 40 ml 1x TAE

For bigger gel:

- 2g agarose
- 200 ml 1xTAE

The mix is first heated up so the agarose powder dissolves in a microwave. After heating it is cooled down to approximately 50°C, and EtBr is added to the solution. For small gel 4µl EtBr (5mg/ml) is added, and for big gel 20µl EtBr (5mg/ml) is added. This is then immediately pored over to gel-chamber and left to harden. After this it could be used immediately or stored in buffer 1xTAE for a couple of days.

2.12.2 Sodium dodecyl sulphate Polyacrylamide gel (SDS-PAGE)

Resolving gel 12% acrylamide (2gels):

- 6.6 ml MQ H_2O
- 8.0 ml 30% acrylamide mix

- 5.0 ml 1.5 M Tris (pH 8.8)
- 0.2ml 10% SDS
- 0.008 ml TEMED
- 0.2 ml 10% ammoniumpersulphate

Components are first mixed in this order, and then poured over to form the gel in the instrument/chamber. MQ water is poured over the gel-mix to smooth shape. The gel was left for 30minutes to harden. Then the water was removed, and water drops dried with fiber paper.

5% stacking gel (2 gels):

- 3.4 ml MQ H_2O
- 1.0 ml 30% acrylamide mix
- 1.5 ml 0,5 M Tris-HCl (pH 6.8)
- 0.06 ml 10% SDS
- 0.006 ml TEMED
- 0.06 ml ammoniumpersulphate

Components was mixed in this order, and applied over the 12% acrylamide gel. The instrument to make wells in the gel was put on after the mixture was pored over. The gel was left for 30min to harden, and was always used the same day.

2.13 Equipment and apparatus

- Applied Biosystems 2700 Thermal cycler, PCR system, (Thermo Fisher Scientific)

- Bio-Rad tubes, 250ml or 500ml (Bio-Rad).

- Centrifuge apparatus:
  - For eppendorftubes: Biofuge Heraeus Pico (Kendro).
  - For eppendorftubes: Mikro 200/200R centrifuge (Hettich)
  - For NUNC-tubes: Rotina 420R (Hettich)
  - For Bio-Rad tube: Beckman Centrifuge, Rotor: Avanti^{TM} Ja-25,5/JS-21 (Beckman).

- CLARIOstar Microplate reader (BMG Labtech)

- Cuvettes for OD_{600} measuring (VWR)

- Electrophoresis power suppliers:
  -Model 200 / 2.0 Electrophoresis Power Supply (Bio-Rad)
  - Power Pac 300 Electrophoresis Power Supply (Bio-Rad)

- Electroporation systems:
  - Gene Pulser® II, Pulse Controller Plus, Capacitance extender plus. Electroporation systems (Bio-Rad).

- Eppendorftubes, 1.5ml.

- Heat blocks:
  - Digital dry block heaters (Boekel Scientific)

- Incubators:
  - Innova 4330 Refrigerated Incubator Shaker (New Brunswick Scientific)
  - GFL 3031 Shaking Incubator (GFL)
  - Innova 4200 Incubator shaker (New Brunswick Scientific).

- Mini-Protean® TGX™ Precast gels (Bio-Rad).

- NUNC-tube: 15ml or 50ml (Corning Incorporation)

- Photos of DNA and protein gels taken with: Gel Doc XR^+ (Bio-Rad) and Image Lab software used (Bio-Rad).

- Sartorius 1265 MP / A 7073-03 Digital Electronic Scale, .001g to 400g.

- Semi-micro disposable cuvettes, polystyrene, capacity 1.5 ml. (Sigma-Aldrich)

- Sub-Cell® GT Cell (Bio-Rad)
- Ultrasonicator: Vibra cell TM (Sonics & Materials Inc.)
- UV-spectrophotometer: BioPhotometer (Eppendorf).
- Vortex ms3 basic (IKA)
- Water Bath (GFL)

3 Methods

3.1 Phylogenetic three

A phylogenetic tree representing 123 selected isolates of the *B. cereus* group was constructed to see if they contain the *bcsA* gene by using Tourasse-Helgason MLST scheme. The *bcsA* sequence of *B. thuringiensis* Bt407 was used to find similar sequences among the 123 selected isolates by using the BLAST sequences in the MLST scheme (N.J. Tourasse & Helgason, 2015). Distance computations, tree building and visualization was done using SEAVIEW4 (Gouy, Guindon et al., 2010) The seven phylogenetic clusters within the 123 *B. cereus* isolates were referred to as group I to VII (Guinebretière, Thompson et al., 2008; Nicolas J. Tourasse, Helgason et al., 2011). This was performed in cooperation with Ph.D. Sarah Finke.

3.2 Making of starter cultures

Starter cultures are made for storing desired bacteria at a specific cell density, so that it is not lost and can always be plated/incubated when needed. The desired bacterial strain was plated on LB-agar plates, and in some cases with selection. This was then left to incubate (at 30°C for *B. thuringiensis* and at *37*°C for *E. coli*) over night. From the over night plate a single colony was picked and left to incubate in 1ml LB medium for around 4 hours (225rpm 30°C), with use of selection in some cases. A spectrophotometer was used to measure OD_{600}. When early exponential-phase (OD_{600} ~ 0.8) was reached, a glycerol stock was made, by adding glycerol to 20%. This was done by mixing 600µl bacteria-culture with 400µl 50% glycerol and mixed together by vortexing for around 20 seconds in an eppendorf tube and then stored at -80°C.

Aseptic work was always employed during the making of starter cultures and glycerol stocks.

3.3 Isolation of genomic DNA

The QIAGEN Genomic DNA kit was used for the isolation of genomic DNA from *B. thuringiensis* 407 (section 2.8). The QIAGEN kit is optimized for use with bacterial cultures that are grown in LB medium. This kit contains QIAGEN Genomic tip, which allows efficient purification of genomic DNA from a large variety of biological samples. The column ensures that only DNA binds. Degraded RNA, cellular proteins and metabolites are not retained in the column. The QIAGEN Genomic-tip is washed with a medium-salt buffer (Buffer QC) that removes remaining contaminants (proteins, RNA), and also disrupts nonspecific interactions and allows removal of nucleic acid-biding proteins. The DNA is then eluted from the QIAGEN Genomic-tip with a high-salt buffer (Buffer QF) (Qiagen, 2001).

The QIAGEN Genomic-tips contain an anion-exchange resin. Positively charged DEAE groups on the surface of the resin binds to DNA that has a negatively charged phosphatase backbone. The QIAGEN Genomic-tip 100/G was used, and has a binding capacity of 100µg of DNA (Qiagen, 2001).

Reagents:

- B1 – 3.5 ml
- B2 – 1.2 ml
- QBT – 4 ml
- QC – 15 ml
- QF – 5 ml
- Isopropanol – 3.5 ml
- 70% ethanol – 2 ml
- RNase A stock solution * – 7 µl
- Lysozyme stock solution (100mg/ml) * – 80 µl
- Proteinase K stock solution – 100 µl
  (section 2.8)

B. thuringiensis 407 was streaked to single colonies from glycerol stocks and incubated on LB agar plates over night at 30°C. The next day a single colony was inoculated from the plate into 5ml LB-medium in a Nunc-tube at 225rpm shaking and 30°C over night.

The over-night culture was inoculated at 1:100 concentration into new LB-medium (for example 140µl over-night culture in 14ml LB-medium) and grown to OD_{600} ~ 0.3 at 30°C and 225rpm. The cells were harvested and the protocol from the QIAGEN kit was followed to isolate genomic DNA (protocol 1, p. 40-43 -, protocol 2, p. 44-46).

Modifications used relative to the QIAGEN Genomic DNA protocol 1 :

Three times the amount of lysozyme stock solution (240µl ,100mg/ml) was used, and this was then incubated for 30 minutes at 37°C. Then 200U Mutanolysin (40µl of a 5U/µl stock) was added and incubated for 30 minutes at 37°C. Proteinase K (100µl, 20 mg/ml) was added, and the QIAGEN protocol was followed. These changes were introduced in the protocol because *B. thuringiensis* 407 has a thick cell wall and hence requires stronger lysis conditions. Mutanolysin is an N-acetylmuramidase that cleaves β-N-acetylmuramyl-(1→4)-N-acetylglucosamine binding in the peptidoglycan polysaccharides in the cell wall, and is used for lysis of Gram-positive bacteria (SIGMA-ALDRICH, 2016).

Modifications used relative the QIAGEN Genomic DNA protocol 2:

After eluting the genomic DNA with buffer QF, the eluate was distributed in 14 eppendorf tubes. Instead of isopropanol, 14mL 100% ethanol was used for RNA precipitation (distributed between the 14 eppendorf tubes). After addition of ethanol the samples were centrifuged at 16000 xg for 10 minutes at 4°C. Ethanol was then removed carefully and 500µl cold 70% ethanol was added to each eppendorf tube, and centrifuged at 16000 xg for 10 minutes at 4°C. All traces of ethanol were removed before adding elution buffer. The use of ethanol to precipitate DNA has shown to increase the stability of the DNA. Tris-HCl (pH 8.5)(elution buffer from the «E.Z.N.A Plasmid Mini Kit I») was used to dissolve the genomic DNA and was left over night at room temperature, and then stored at -20°C.

3.4 Plasmid isolation

For plasmid isolation the «E.Z.N.A Plasmid Mini Kit I» (Omega Bio-Tek) was used (section 2.8). In the kit protocol, Solution II disrupts the hydrogen bonds in the DNA and leads to single stranded chromosomal DNA and plasmid, which upon neutralization within 5 minutes allows that the small plasmid can be renatured in the right conformation. If this is not done the plasmid will form into an insoluble mass, which is precipitated together with the proteins. Solution III neutralizes solution II (Birnboim & Doly, 1979).

For plasmid isolation, an over night culture of *E. coli* was made by inoculating one single colony (bacterial strain with plasmid to be isolated) in 5ml LB medium with selection at 37°C and 225rpm. The next day the cells were harvested at 4500 xg for 10 minutes at 4°C and the protocol for plasmid isolation step 3-23 (OMEGA-bio-tek, 2013) was then followed.

The DNA was eluted in 2x 25µl elution buffer (Tris-HCl, pH 8.5) in an eppendorf tube and stored at -20°C.

3.5 Digesting plasmid-DNA with restriction enzymes

Restriction enzymes are DNA-cutting enzymes also known as endonucleases. Each restriction enzyme cuts in a specific site in the DNA sequence (Bloch & Grossmann, 2001). This can for example be used to insert DNA fragments into plasmids.

Each enzyme digestion consists of 4 components; DNA, restriction enzyme(s), a buffer and water to adjust volume. Total volume used in these experiments was 50µl. 1µl of each restriction enzyme (10 units) was used and 5µl of the buffer (10X concentrated). Incubation time was 2.5-3 hours at 37°C,

Restriction enzymes used were from New England Biolabs: *Eco*RI, *Hinc*II, *Nco*I, *Nde*I, *Xho*I
Buffers used were from New England Biolabs: 10X NEBuffer 3.1, 10X NEBuffer for *Eco*RI

Optional steps used sometimes after digestion:

- Enzyme inactivation:
  Incubation at 80°C for 20 minute to heat inactivate restriction enzymes.

- Dephosphorylation:

  To each tube with digested DNA add 2µl Antarctic phosphatase buffer (10x concentration) and 1µl Antarctic phosphate enzyme (5 units per 20µl reaction). Then incubate for 30 minutes at 37°C.

  - Dephosphorylation removes the 5´ phosphate that is generated after digestion by restriction enzyme and reduces the occurrence of vectors being re-closed by intramolecular ligation, ideally leading to vector ligation to inserted gene only. It is important that the PCR insert contains a 5´phosphate when ligation is being done, because 5´phosphate is necessary for the ligation to the 3´hydroxyl group of the vector (BioLabs, 2017a).

3.6 Agarose gel electrophoresis

Gel electrophoresis is used to separate nucleic acids based on their molecular size. The gel is made of agarose, which forms pores that the molecules can diffuse through. DNA and RNA molecules have a negative charge because of the phosphate backbone, and an electrical field is used to pull the molecules through the gel toward a positive pole. Small molecules will move faster through the pores in the gel. (Koontz & Laura, 2013). After gel electrophoresis, bands can be detected to decide the size of the molecules using different types of markers.

1% agarose gel was used with EtBr to visualize DNA (Section 2.12.1) and 1x TAE buffer. Generuler 1kb DNA ladder was used as marker, and 1-2 size markers were used for each gel electrophoresis run. Thermo Fisher Scientific 6x DNA loading dye (bromophenol blue and xylene cyanol FF) was added to samples to be separated to give a visual tracking of the migration. The loading dye also contains glycerol, which makes the samples heavier and allows them to sink to the bottom of the well after gelization. Another component of the loading dye is EDTA, which binds divalent metal ions and inhibits metal-dependent nucleases (ThermoFisherScientific, 2017a). 3µl dye was added per 10µl sample used.
The electrophoresis apparatus was set at 70 volt, and was left to run 1-2 hours before results were viewed. UV-detection was used to detect bands in the gel, which were recorded using a Gel Doc XR$^+$ (Bio-Rad) and Image Lab Software (Bio-Rad).

3.7 Cloning of gene fragment in expression vectors

3.7.1 PCR-amplification of PilZ domain fragments.

Three gene fragments, which code for $PilZ_{d1}$, $PilZ_{d2}$ and $PilZ_{d1d2}$ sub-domains in *bcsA* BTB_RS08505, were PCR amplified with a C-terminal 6x His-Tag. Primer sequences can be found in Table 2.2.

Expected product length of the PCR products:

- $PilZ_{d1}$ fragment: 365bp
- $PilZ_{d2}$ fragment: 305bp
- $PilZ_{d1d2}$ fragment: 704bp

PCR reaction:

Component	50 µl reaction volume	Final conc.
H_2O	add to 50 µl total volume	
5x Phusion HF Buffer*	10 µl	1x
10 mM dNTPs	1 µl	200 µM each
Primer A**	2.5 µl	0.5 µM
Primer B**	2.5 µl	0.5 µM
Template DNA B. thuringiensis 407	2 µl	
Phusion DNA Polymerase	0.5 µl	0.02 U/µl

The primer stock concentration were 10µM, hence:

0.5µM/10µM x 50 =2.5 µl volume of each primer to get a final concentration of 0.5µM.

As a negative control, MQ water replaced template DNA.

Table 3.1 Settings for PCR amplification.

Cycle step	3-step protocol		Cycles
	Temp.	Time	
Initial denaturation	98°C	1 minute	1
Denaturation	98°C	10 s	30x Denaturation, primer annealing and extension.
Annealing	65°C	30 s	
Extension	72°C	15 s/kb	
Final extension	72°C	7 minutes	1

Reactions were held at 4°C.

After every PCR the products were purified using the E.Z.N.A Gel extraction kit using the protocol for cleanup DNA from enzyme reactions (page 13-14) (OMEGA-bio-tek, 2012) and was then analyzed on 1% agarose gel to confirm correct DNA fragment.

3.7.2 Ligation of PCR-products (PilZ domain fragments) in the expression vector pET-28a(+).

PCR-products (section 3.7.1) and pET-28a(+) vector from a plasmid miniprep (section 3.4) were restriction enzyme digested and ligated.

Cutting PCR products:
25µl purified PCR product
18µl MQ H_2O
5µl 10x NEBuffer 3.1
1µl *Nco*I
1µl *Xho*I
Total volume: 50µl

Cutting pET-28a(+) vector:

35µl purified pET-28a(+) plasmid miniprep

8µl MQ H_2O

5µl 10x NEBuffer 3.1

1µl *Nco*I

1µl *Xho*I

Total volume: 50µl

The reactions were left to incubate for 2.5 hours at 37°C. After cutting an optional step with dephosphorylation (section 3.5) was used for vector DNA. The digested DNA was then purified using the E.Z.N.A Gel extraction kit: Protocol for cleanup DNA from enzyme Reactions (page 13-14) (OMEGA-bio-tek, 2012).

After cleanup the samples were checked using 1% agarose gel electrophoresis to estimate concentrations and integrity following enzyme treatment.

Ligation:

Approximately 3 times the molar amount of digested gene fragment was used relative to the amount of digested plasmid. These were added together with 1µl 10x DNA ligase buffer, 1µl T4 DNA ligase and MQ water was added till total 10µl volume reaction. Incubated at 16°C over night.

3.7.3 Transformation of ligated constructs into *E. coli* host cells

One shot® TOP10 electrocompetent *E. coli* cells were used for transformation, and 1µl from each ligation reaction was added directly into different tubes with 50µl One Shot® TOP10 *E. coli* cells, and mixed carefully together. All tubes were always left on ice and the mix was then transferred to 0.2 cm cuvette for electroporation.

Settings used: 400**Ω** resistance –2.5 kV voltage - 25µF capacitance.

After each electroporation 2x 500µl SOC medium (preheated to 37°C) was added into the cuvette, and mixed carefully by pipetting up and down. Everything was then transferred to 50 ml NUNC tubes and left to incubate at 37°C and 225rpm for 3 hours.

After 3 hours the cultures were plated on LB agar medium plates at different volumes (50µl, 100µl and around 850µl) with selection (25µg/ml Kanamycin) and incubated over night at 37°C.

3.8 Bioinformatics: Sequencing

3.8.1 Sanger-Sequencing of clones

The clones were sequenced by Sanger-Sequencing to confirm that the right constructs were cloned in the expression vector pET-28a(+). T7 promoter and T7 terminator primers (table 2.4) were used to sequence the clones from both directions, and compared to *bcsA* consensus sequence to confirm the right construct of gene fragments.

3.8.2 Prediction of disulphide bonds by DISULFIND software

DISULFIND software was used to predict cysteines disulphide bonding and connectivity in desired protein by analysing the amino sequence (DISULFIND, 2017)

3.9 Growth curve

B. thuringiensis 407 (wild type) (AH1423) and *B. thuringiensis* 407 Δ*bcsA* (AH1871) were plated out on agar plates from glycerol stock (-80°C) and grown over night at 30°C. The next day one colony was transferred to a NUNC-tube with 5 ml LB medium. Duplicates of each cultures were made, and these were incubated over night at 30°C and 225rpm. The over night cultures were diluted to an OD_{600} ~ 0.05 the next day, and inoculated in 100 ml LB medium in volumetric baffled Erlenmeyer flasks and left to incubate at 30°C and 225rpm. OD_{600} was measured at different time points, with the first measurement done after 1 hour, then every 30minutes.

Around 1 ml culture was transferred to a cuvette for each measurement, and OD_{600} was measured in a spectrophotometer. Some of the samples at later time points needed to be diluted with LB medium to get OD_{600} volumes between 0.1 and 0.8, where the spectrophotometer is in the linear range. Blank test with 1 ml LB medium was used to calibrate the spectrophotometer. Before each measurement the samples were mixed in the cuvette by using parafilm to seal the cuvette and turning the cuvette upside down 2-3 times.

3.10 Making of chemically competent *E. coli* BL21 cells and transformation for expression of recombinant protein

E. coli BL21 (DE3) (AH1498) cells were plated out on LB agar plate over night at 37°C from freezer stock. Over night culture was made, by transferring one single colony from the plate in to 5 ml LB medium, and incubated at 37°C and 225rpm over night. 10 ml LB medium was inoculated with 100µl over night culture and left to grow to OD_{600} ~ 0.4, at 37°C and 225rpm. The cells were then harvested at 5000 xg for 5 minute at 4°C. The supernatant was removed and the cell pellet was resuspended in 1/10 volume cold TSS (section 2.10). The resuspended cells were then aliquoted to 100µl aliquots in eppendorf tubes, and frozen directly on dry ice/ethanol before being stored at -80°C. Cells should last about 6 weeks at -80°C.

Competent cells could be used immediately after preparation or taken out from -80°C freezer stock and put on ice. 1µl of plasmid prep was added to 100µl chemically competent *E. coli* BL21 (DE3) (AH1498) cells, and then left to incubate 30 minutes on ice. After incubation the cells were placed in a heat block at 42°C for 1 minute, and then placed straight on ice. The whole sample was transferred to a 15 ml NUNC-tube with 0.9 ml preheated SOC medium (37°C), and left to incubate at 37°C and 225rpm for 1 hour. After incubation the sample was plated on LB-agar with 25µg/ml Kanamycin in three different volumes to get single colonies (50µl, 100µl and the rest of the sample). Plates were left over night at 37°C.

3.11 Protein expression, isolation and purification

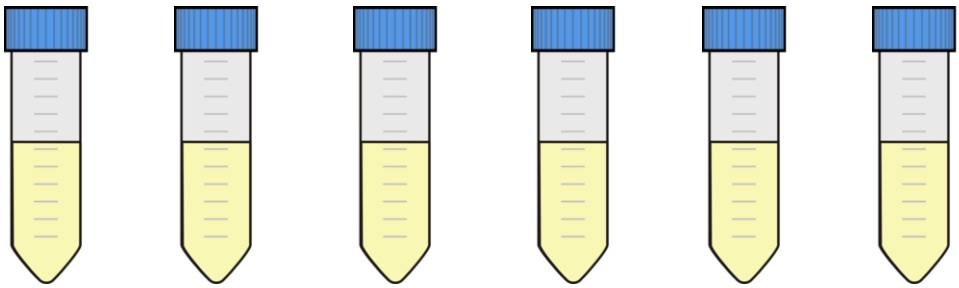
pET-28a(+) contains a T7/*lac* promoter, which is inducible by Isopropyl- β-D-thiogalactoside (IPTG). IPTG binds to the *lac* repressor and alter its conformation so that the repression on the *lac* operator is removed. (Bell & Mitchell, 2000). When repression is removed, the *lac* promoter will be able to activate transcription of the T7 polymerase gene in the *E. coli* BL21 (DE3) chromosome (BioLabs, 2017b), and hence transcription of cloned genes is started.

3.11.1 Induction using variable amounts of IPTG

Over night culture of transformed *E. coli* BL21 (DE3) (AH1498 cells) (section 3.10) was

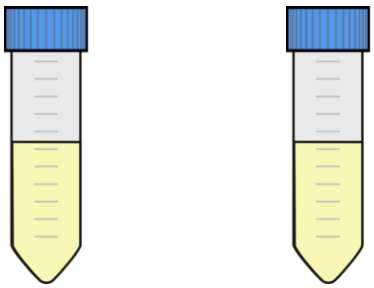
made, by inoculating one single colony in 10 ml LB medium with 25µg/ml Kanamycin in a NUNC-tube. This was incubated over night at 37°C and 225rpm.

The over night culture was diluted to OD_{600} = 0.1 in 5 ml LB medium with 25µg/ml Kanamycin in six separate cultures (figure 3.11.1.1), in NUNC tubes.



0.00 mM 0.125 mM 0.250 mM 0.500 mM 0.750 mM 1.00 mM

Figure 3.1 A illustrate of the six separate cultures induced with different concentrations of IPTG. Cultures were left to grow until OD_{600} ~ 0.5 was reached, at 37°C and 225rpm. Figure collected from (clker, 2017).



0.00mM 1.00 mM

Figure 3.2 The setup was repeated in the same way as over, using 1 mM IPTG and incubation at 28°C and 225rpm. Figure collected from (clker, 2017).

When OD_{600}~ 0.5 was reached (figure 3.1 and 3.2) cells were lysed at different time points. An aliquot of cells (100µl if $OD_{600} \geq 1$, 200µl if $OD_{600} \leq 1$) were taken out at different time points and transferred to eppendorf tubes. This was done at 1h, 1.5h, 2h, 2.5h, 3h, 4h and 5h (for samples at 28°C, only up till 3h was done). The aliquot was centrifuged at 13000 xg for 5 minutes to harvest cells. The supernatant was removed and the pellet was re-suspended in 50µl 2x SDS-PAGE buffer. This was left to incubate at 95°C for 10 minutes, and then spun

down at 13000 xg for 5 minutes. Supernatant (25µl) was taken out for SDS PAGE (can be stored at -20°C).

3.11.2 Protein expression

Over night culture of transformed *E. coli* BL21 (DE3) (AH1498 cells) (section 3.10), were made by inoculating one single colony in 10 ml LB medium with 25µg/ml Kanamycin in a NUNC-tube. This was incubated over night at 37°C and 225rpm. Cells were harvested by centrifugation 6 minutes at 45000 xg at 4°C. The supernatant was then removed and the pellet was resuspended in 10 ml preheated (37°C) LB medium with 25µg/ml kanamycin to remove toxic substances that the cell has made during incubation. 1:50 (2 ml) of this over night culture was then inoculated in 100 ml LB medium with 25µg/ml kanamycin in a 500 ml baffled flask, and left to grow at 37°C and 225rpm until OD_{600} ~ 0.5.

3 baffled flasks were used:
1) For OD_{600} measurements (working flask).
2) Sample flask to be induced
3) Control flask (Negative control)

After reaching OD_{600} ~ 0.5 mM IPTG was added to the sample flask, and induction was left to run for 2.5 hours at 37°C and 225rpm. After induction the cultures were transferred to separate 100 ml Bio-Rad flasks and the cells were harvested by centrifugation at 4000 rpm (Beckman, JA 25, 50 rotor) at 4°C for 20minutes. The supernatant was removed, and the pellet was then washed in 30 ml TEN buffer, transferred to thick Bio-Rad tubes and centrifuged at 10 000 rpm (Beckman, JA 25, 50 rotor) for 10minutes. Here the experiment could be stopped by removing supernatant and the tubes with pellet could be stored at -80°C.

3.11.3 Protein isolation

The cell pellets from protein expression (3.11.2) were defrosted on ice and water for 15 minutes (negative control sample and induced sample). PMSF was added to 1 mM end concentration in lysis buffer, and 4 ml of this was then added to each sample. The cell pellets were resuspended by careful pipetting. Fresh stock of lysozyme-solution 1 mg/ml (400 µl) was then added to each sample, and this was left to incubate on ice for 30 minutes. The

samples were then sonicated with frequency 50 kHz 6 times for 10 seconds, with 10 seconds in between each sonication (Vibra cell TM; Sonics & Materials Inc.). Samples were always left on ice to minimize protein degradation. After sonication 20µg/ml RNase (0.8µl of 100 mg/ml stock) and 10µg/ml DNase (4µl from 10 mg/ml stock) was added to each sample to reduce the viscosity. This was left to incubate on ice for 10 minutes. After incubation the samples were centrifuged 20 minutes at 10 000 rpm (Beckman, JA 25, 50 rotor), at 4°C. The supernatant was transferred to a new tube and sterile filtered through a 0.45µm filter, and for testing on SDS-PAGE 30µl of supernatant was transferred to an eppendorf tube and stored at -80°C. The rest of the supernatant was also stored at -80°C to be continued working on (section 3.11.4). Cell debris (rests of the cell in the bottom of the tube) was transferred to eppendorf tubes and stored at -80°C. This was also going to be tested on SDS-PAGE.

3.11.4 Protein purification on Ni-resin column

The supernatants from section 3.11.3 were defrosted on ice and water. 1 ml HisPur Ni-NTA Resin (from Thermo Fisher Scientific; stored at 4°C) was added to each of the two supernatant samples. These were left to incubate on shaking-platform on moderate "shaking" for 1 hour at 4°C. Tubes were laid flat on the plate (not left standing). Supernatant with HisPur Ni-NTA Resin was transferred to a column (Bio-Rad Poly-prep, chromatography columns, cat. 731-1550) and was left to flow through. The flow through (lysis buffer eluate) was collected, and stored, so it could be tested on SDS-PAGE. The columns were then each washed 2 times with 4 ml washing buffer containing PMSF (for 100 ml washing buffer, 1 ml of 100 mM PMSF stock solution was added). This flow through was also collected in two separate tubes to be tested on SDS-PAGE, and these were labelled "Wash1" and "Wash2". 50µl PMSF-solution of 100 mM stock was added to 5 ml elutionbuffer. The proteins were eluted from the column in 10 separate eppendorf tubes, and for each sample 250µl elution buffer with added PMSF was used.

Concentrations of the eluted proteins were measured in a UV- visualizing spectrophotometer at 280nm. Normally it was a high concentration of protein in eluate nr.2.

Tubes with eluted protein were stored at -80°C (so it could later be checked on SDS-PAGE).

3.12 Detection of protein on SDS PAGE

Denatured proteins are separated by their molecular weight in SDS-PAGE (Sodium dodecyl sulphate-polyacrylamide gel electrophoresis). The polyacrylamide gel consists of a stacking gel and a resolving gel. The stacking gel has a higher porosity and lower pH (6.8) than the resolving gel (pH 8.8). The higher porosity is used in the stacking gel so that the proteins are up concentrated here, and not separated based on molecular weight until they reach the resolving gel. In the resolving gel the proteins migrate based on molecular weight through the smaller pores of the gel in response to an electric field (Bio-Rad, 2016).

SDS is used as an anionic detergent to denature proteins, and also bind noncovalently to the denaturized proteins and hence give a negative charge. β -mercaptoethanol is added to the SDS-buffer to disrupt disulfide bonds in the protein (figure 3.3). This leads to the unfolding of protein so that they can be separated based by their molecular size (Bio-Rad, 2016).

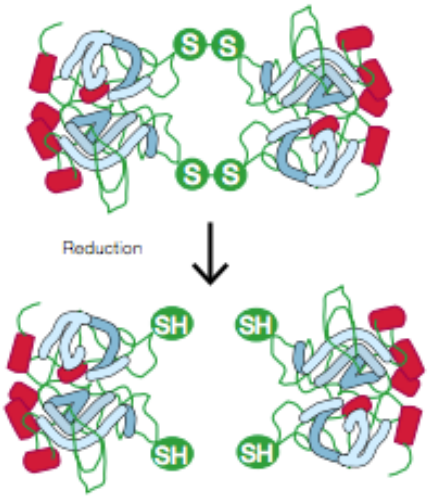


Figure 3.3 Disruption of disulfide bonds in protein by 2-mercaptoethanol. The addition of 2-mercaptoethanol leads to the reduction of cysteines in the protein.
Figure from: Bio-Rad – A guide to Polyacrylamide Gel electrophoresis and detection (Bio-Rad, 2016)

3.12.1. SDS-PAGE

All the samples stored from section 3.11 were first defrosted on ice and water, and then mixed with 2x SDS loading buffer in a 1:1 ratio. The samples were then heated at 95°C in a heat block for 5 minutes. The heat will promote denaturation of proteins, so that the SDS detergent can bind through out the denaturised proteins with its hydrocarbon-tail. β-mercaptoethanol will break down the disulfide bonds in the protein (SIGMA-ALDRICH, 2017). After heating the samples were spun down so that condensation in the lid would go down in the tube, and put on ice before SDS-PAGE. The samples were then applied on the

gel with a protein standard (section 2.5) and separated for 15 minutes at 90V, and then 60 minutes at 150V. 1 X ELFO-buffer (section 2.10) was used for the electrophoresis.

3.12.2. Staining of SDS-PAGE gel with Coomassie Brilliant Blue

After SDS-PAGE, the polyacrylamide gel was washed 3 times with MQ water for 5 minutes each on a shaking platform. MQ water was then removed and 50 ml Coomassie Brilliant Blue (Bio-Rad) was added to stain the gel for 1 hour on shaking platform. The stain was removed and the gel washed for 30 minutes with MQ water. Protein was detected under UV-light using a Gel Doc XR^{+} (Bio-Rad).

3.13 Western Blot

For this method the Trans-Blot® Turbo^{TM} RTA Mini Nitrocellulose Transfer Kit from Bio-Rad was used. Western Blot is based on the transfer of protein samples from a gel to a membrane and the detection of these (Towbin, Staehelin et al., 1979). The proteins were first separated by polyacrylamide gel electrophoresis and then transferred/blotted to a nitrocellulose membrane (VWR). The Trans-Blot® Turbo^{TM} RTA Mini Nitrocellulose Transfer Kit contains membranes, 3MM paper and transfer buffer. The protocol in the kit was followed. First the membrane and the transfer stacks were soaked in Trans-Blot Turbo transfer buffer, and then assembled as shown in figure 3.4 (Bio-Rad, 2017).

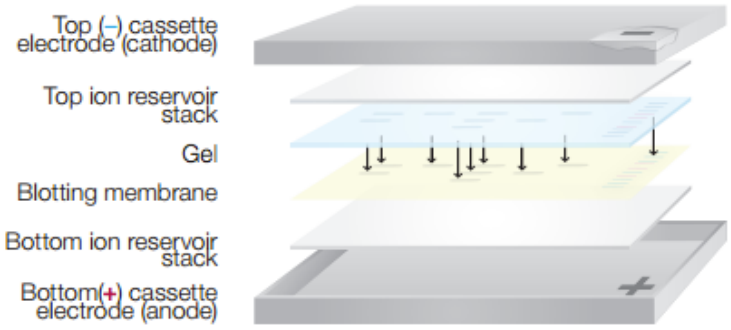


Figure 3.4 Assembly of the assembled transfer pack. This assembly was used for the blotting of proteins to the membrane. Figure from Trans-Blot® Blotting system Instruction manual (Catalog number 170-4155).

The transfer of the proteins from the gel to the membrane was run for 10 minutes using the Trans-Blot Turbo system. The preprogramed protocol for 1.5mm gel was used (Bio-Rad, 2017)

To check if the proteins were transferred to the membrane Ponceau staining solution was used in sufficient amount to cover the membrane. The membrane was put in a small tub where it was stained for 5 minutes. If proteins were transferred they will appear as pink on the membrane. After control of the transfer, the membrane was washed with MQ-water for 1 minute (several times if necessary) to remove the Ponceau stain.

The membrane with transferred proteins was put in a NUNC tube and washed with 25 ml 1x TBST buffer for 5 minutes on a shaking platform at room temperature. After washing the membrane was blocked in 5 ml 1x TBST with 1% dry milk for 1 hour on shaking platform at room temperature. Blocking is a necessary step in Western blotting because this prevents the antibodies from binding non-specifically to the membrane (Mahmood & Yang, 2012). After blocking the membrane was washed 3 times with 15 ml 1x TBST 5 minutes on a shaking platform at room temperature to remove excess blocking solution. The membrane was then incubated with primary antibody (α-5X His antibody produced in mouse (Sigma-Aldrich) 0.2 mg/ml) diluted 1:1000 in 10 ml 1x TBST with 1% dry milk, at 4°C on a shaking platform over night.

The next day the incubation solution was removed and the membrane was washed 3 times with 15 ml 1x TBST for 5 minutes on shaking platform at room temperature. The membrane was then incubated for 2 hours with a secondary antibody (Goat Anti-Mouse IgG (H+L) HRP-conjugate Bio-Rad) diluted 1:10 000 times in 10 ml 1xTBST with 1% dry milk on shaking platform at room temperature. After incubation the solution was removed and the membrane was again washed 3 times with 15 ml TBST for 5 minutes on the shaking platform at room temperature.

Around 1-2 ml Luminata Classico Western HRP Substrate was then added to cover the membrane and left on for 5 minutes before detection. The Chemi Genius 2 Bio Imaging System (Syngene) was used for the detection.

3.14 Quantification of protein bands by BCA (Bicinchoninic Acid) protein assay

The Pierce BCA Protein Assay Kit from Thermo Scientific™ was used for quantity protein bands. This kit is based on bicinchoninic acid (BCA), which is a detergent-compatible formulation. Protein reduces Cu^{2+} to Cu^{1+} in alkaline medium (the biuret reaction). Amino acid residues such as cysteine, tyrosine and tryptophan present in proteins cause this reduction. By using a reagent that contains BCA, a colometric (purple-colored reaction) detection of Cu^{1+} can be obtained. The detection works by chelation of two molecules of BCA with one cuprous ion (Cu^{1+}), which absorbs light at 562nm. By using Bovine serum albumin (BSA) at known concentrations to generate a standard curve the concentration of unknown protein can be determined (ThermoFisherScientific, 2017c). CLARIOstar Microplate reader (BMG Labtech) was used for the detection of protein.

4 Results

4.1 Distribution of the *bcsA* gene in strains of the *B. cereus* group

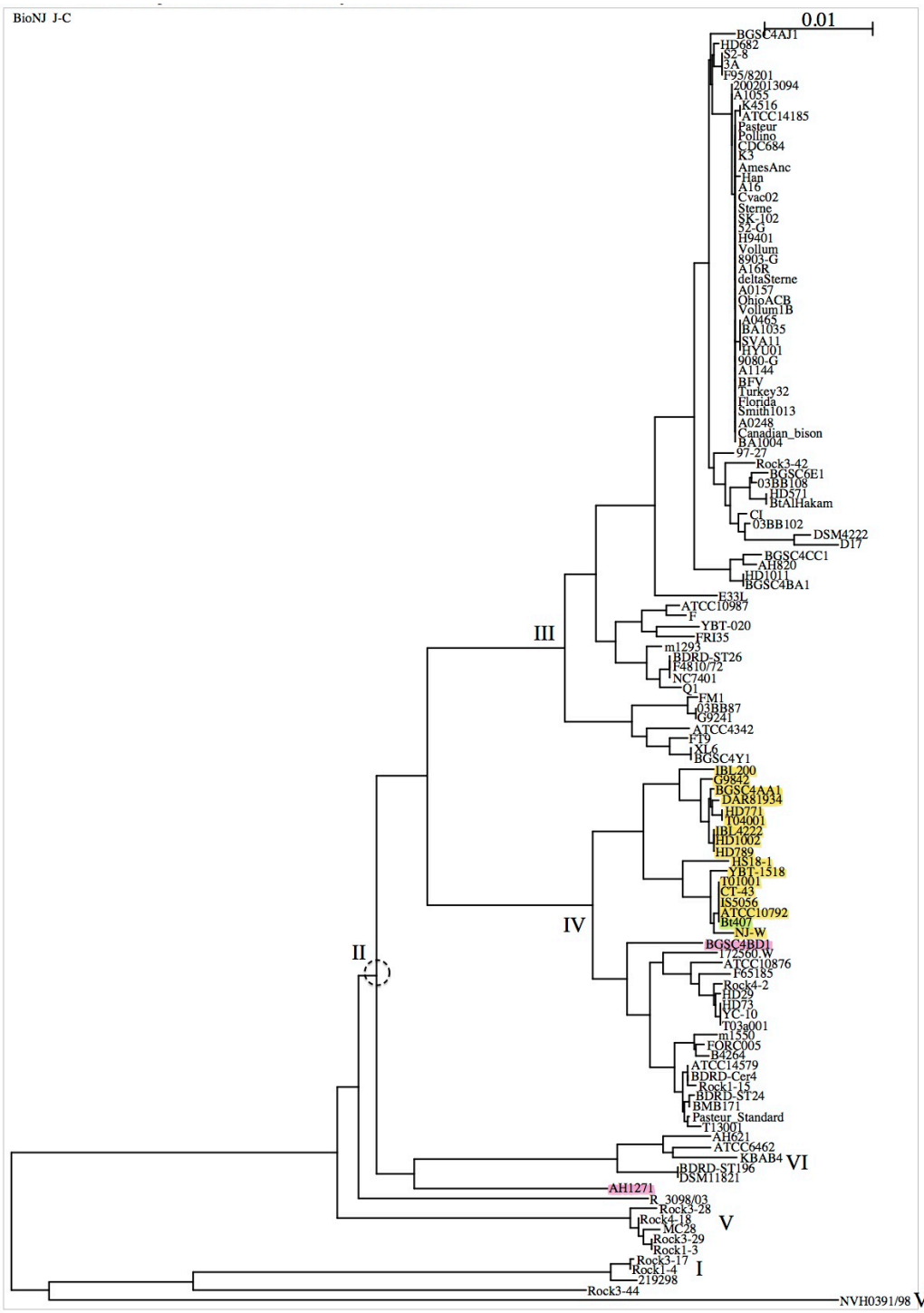


Figure 4.1 A phylogenetic tree of 123 sequenced strains representing the *B. cereus* group. Distribution of the *bcsA* gene in the *B. cereus* group is highlighted, and the phylogenetic clusters within the *B. cereus* group are referred to as I-VII.

Phylogenetic tree of 123 sequenced strains representing the *B. cereus* group (section 3.1), referred to as group I-VII (figure 4.1). The *bcsA* sequence of *B. thuringiensis* Bt407 was used to find similar sequences in the 123 strains. *B. thuringiensis* Bt407 is marked as green, strains containing similar sequence of *bcsA* and was confirmed to be *bcsA* by GenBank (NCBI, 2017) are marked yellow, while strains containing similar sequence but was not confirmed to be *bcsA* is marked with pink (figure 4.1). All the strains marked had almost 100% similar sequence of the *bcsA* sequence of *B. thuringiensis* Bt407, except for *B. cereus* AH1271, which had 89.99% similarity (Appendix C). The matched sequence of *B. cereus* AH1271 was identified as a framshifted pseudo gene of the *bcsA*. *B. thuringiensis serovar huazhongensis* BGSC 4BD1 matched the *bcsA* sequence of *B. thuringiensis* Bt407 with 97.89%, but was annotated as a hypothetical protein (NCBI, 2017).

4.2 Growth curves of *B. thuringiensis* 407 wildtype and *B. thuringiensis* 407 Δ*bcsA*

Growth curves were performed to get an indication of potential growth differences between *B. thuringiensis* 407 wildtype and a *B. thuringiensis* 407 Δ*bcsA* gene deletion mutant (section 3.9). This was done three times on three separate days to obtain independent experiments. The results were combined and averages of the measurements from the three days were calculated (table 4.1).

Table 4.1: Measured OD_{600} values of *B. thuringiensis* 407 wild type and *B. thuringiensis* 407 Δ*bcsA* during growth until stationary phase was reached. Combined result (average) of three independent experiments giving an indication of growth patterns.

Time (hours)	*B. thuringiensis* 407 wildtype OD_{600}	*B. thuringiensis* 407 Δ*bcsA* OD_{600}	Dilutions factor of measured culture (in LB medium)
0	0,045	0,047	-
1	0.126	0.127	-
1,5	0,237	0,213	-
2	0,477	0,445	-
2,5	0,936	0,875	1:2
3	1,789	1,617	1:10
3,5	3,028	2,751	1:10
4	4,648	4,116	1:20
4,5	6,335	6,079	1:20
5	9,150	8,825	1:20
5,5	11,356	10,871	1:20
6	14,254	13,399	1:20
6,5	15,889	15,476	1:40
7	17,2845	17,313	1:40
7,5	18,551	18,133	1:40
8	19,760	20,160	1:40
8,5	20,124	19,793	1:40
9	20,700	20,478	1:40
9,5	20,547	20,240	1:40
10	19,850	18,470	1:40

A plot of the measurements was made for *B. thuringiensis* 407 wild type and *B. thuringiensis* 407 Δ*bcsA* gene deletion mutant (figure 4.2 and 4.3), showing that deletion of *bcsA* does not significantly affect growth of the bacteria at these growth conditions.

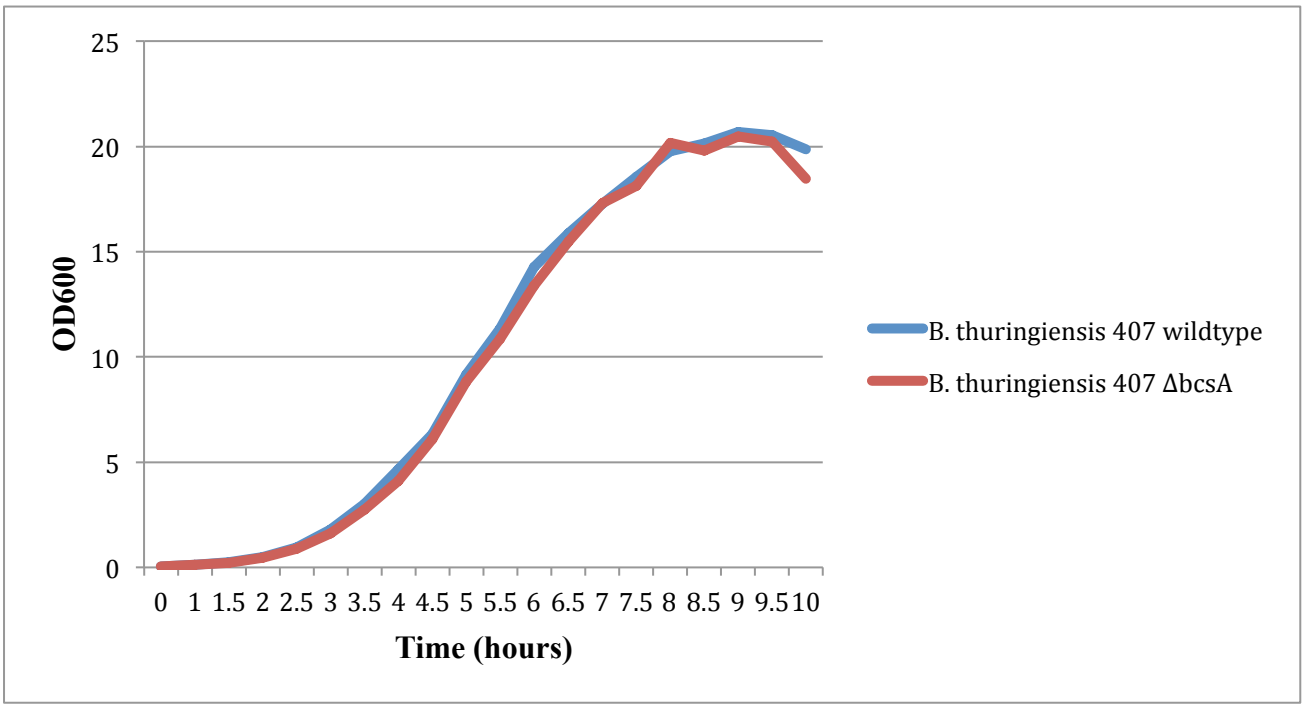


Figure 4.2 Plot of *B. thuringiensis* 407 wild type and *B. thuringiensis* 407 Δ*bcsA* growth. The plot shows growth curves for *B. thuringiensis* 407 wild type and *B. thuringiensis* 407 Δ*bcsA* gene deletion mutant until stationary phase was reached.

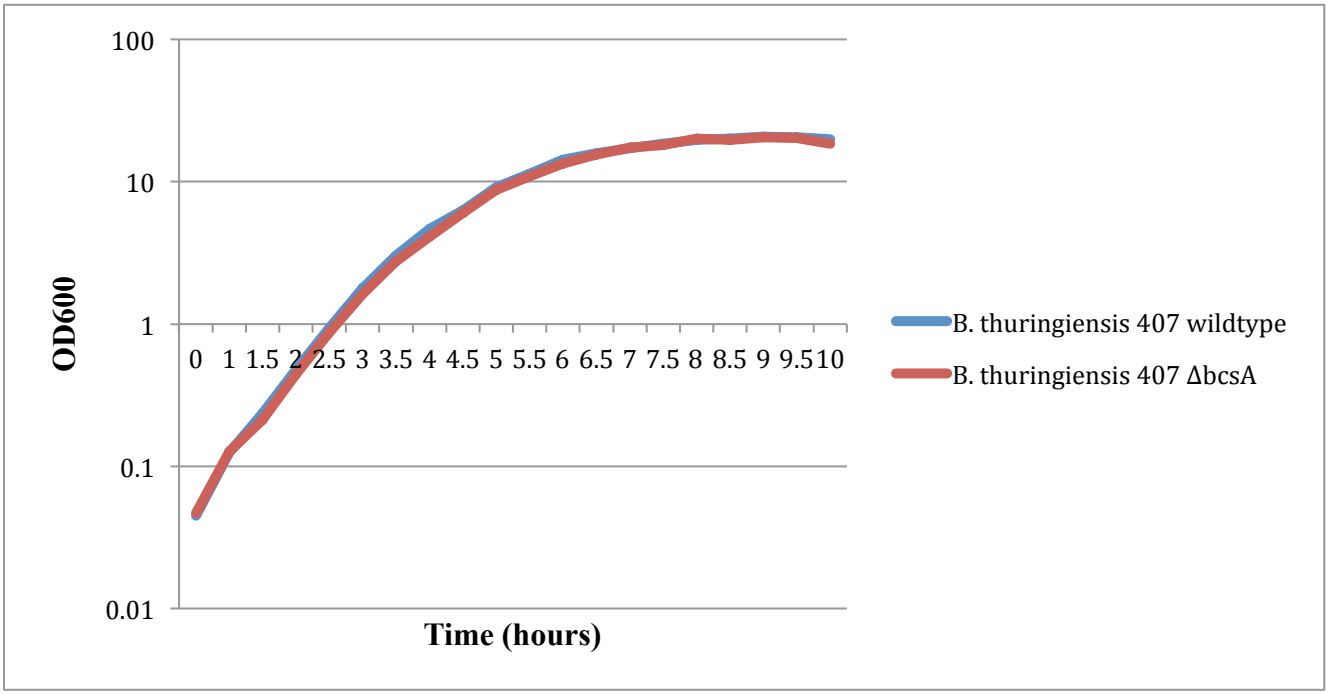


Figure 4.3 Logarithmic plot of *B. thuringiensis* 407 wild type and *B. thuringiensis* 407 Δ*bcsA* growth curves. Logarithmic plot showing growth of *B. thuringiensis* 407 wild type and the *B. thuringiensis* 407 Δ*bcsA* gene deletion mutant until stationary phase was reached.

4.3 Plasmid preparation of vectors pET30b(+), pET28a(+) and pET-26b(+).

Plasmid preparation of the vectors pET-30b(+), pET28a(+) and pET-26b(+) were made so that they could be used for cloning of the PilZ domain encoding fragments of *bcsA*. The pET-28a(+) vector was used during later cloning experiments. The cloned products were going to be used further to express and purify the PilZ domains for studies of c-di-GMP binding .The plasmid vectors were first isolated from *E. coli* host cells by using the E.Z.N.A Plasmid Mini Kit I (section 3.4).

To make sure that the correct plasmid vectors were isolated, a restriction enzyme digestion reaction was set up (section 3.5). 1µg DNA from the vector plasmidprep was digested with the restriction enzymes *Eco*RI (0.5µl) and *Hinc*II (1µl) (table 4.2) for 1 hour at 37°C. 5-10 units of restriction enzyme per 1µg plasmid-DNA was used for cutting.

Table 4.2: Locations of restriction sites for *Eco*RI and *Hinc*II in the pET-vectors.

Plasmid	Enzyme	Digestion site
pET-30b	*Eco*RI	192
pET-30b	*Hinc*II	181, 1682
pET-28a	*Eco*RI	192
pET-28a	*Hinc*II	181, 1629
pET-26b	*Eco*RI	192
pET-26b	*Hinc*II	181, 1620

After digestion the DNA fragments were analyzed by 1% agarose gel electrophoresis (section 3.6). As expected digestion with *Eco*RI produced one fragment of ~5000bp for pET-30b(+) and one fragment of ~5000bp for pET-28a(+) (figure 4.4). A fragment of ~5000bp was produced after digestion of pET-26b(+), but a high molecular fragment was also observed (figure 4.4). This larger band in the gel could be binding of enzyme to the substrate or plasmid that has not been digested. Digesting with *Hinc*II lead to the formation of two bands as expected for the three vectors at around ~3500-4000bp and ~1500bp (figure 4.4). Several bands seen in the non-digested vectors could be due to different migration rate between different conformational shapes of the plasmid. Supercoiled plasmids migrate faster through the gel than the relaxed form of the plasmid. Bands could also be breakdown of the plasmid, and some signs of single stranded DNA are seen in the lanes ~5000bp (figure 4.4).

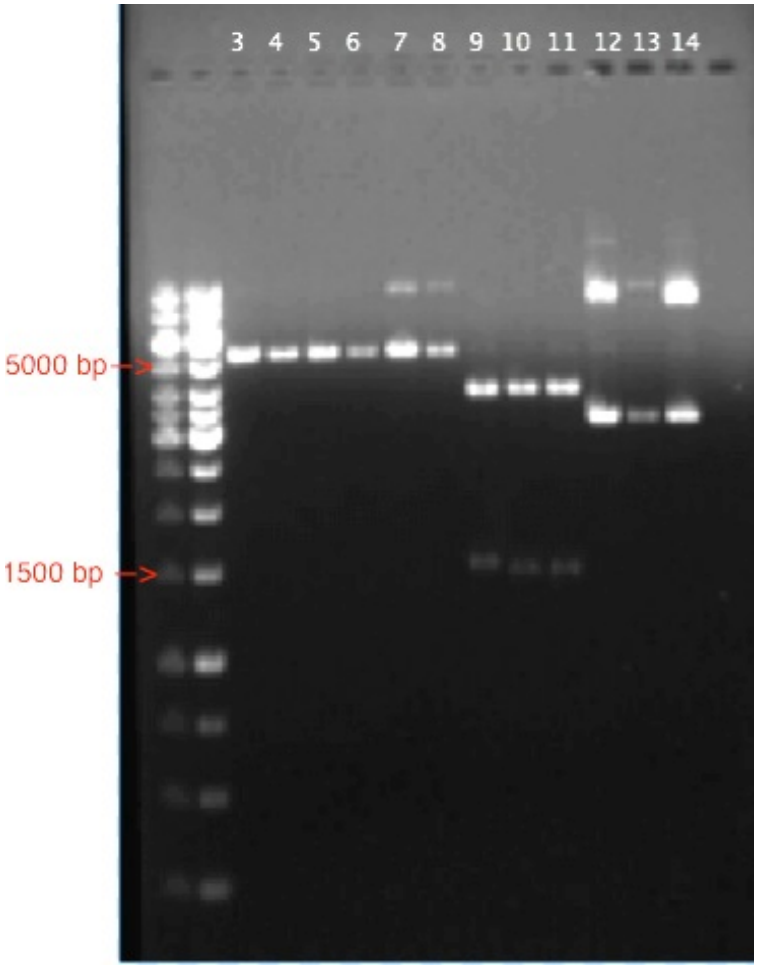


Figure 4.4: Gel electrophoresis of plasmid vector DNA isolated from *E. coli* host cells in a 1% agarose gel. Lane 1 and 2: Generuler 1 kb DNA ladder (3µl and 5µl respectively). Lane 3: 7µl pET-30b(+) digested with *Eco*RI. Lane 4: 3.5µl pET-30b(+) digested with *Eco*RI. Lane 5: 7µl pET-28a(+) digested with *Eco*RI. Lane 6: 3.5µl pET-28a(+) digested with *Eco*RI. Lane 7: 7µl pET-26b(+) digested with *Eco*RI. Lane 8: 3.5µl pET-26b(+) digested with *Eco*RI. Lane 9: 10µl pET-30b(+) digested with *Hinc*II. Lane 10: 10µl pET-28a(+) digested with *Hinc*II. Lane 11: 10µl pET-26b(+) digested with *Hinc*II. Lane 12: 1µl of pET-30b(+). Lane 13: 1µl of pET-28a(+). Lane 14: 1µl of pET-26b(+).

4.4 Isolating genomic DNA from *B. thuringiensis* 407.

To perform PCR amplification of the PilZ domains of the *bcsA* gene, genomic DNA from *B. thuringiensis* 407 was needed as a DNA template. Genomic DNA was isolated using the QIAGEN Genomic tip 100/G kit (section 2.8.) from a 10ml over night culture of *B. thuringiensis* 407 inoculated from a single colony (section 3.3).

4.5 Cloning of PilZ domains from *bcsA* into pET-28a(+) vector.

Three DNA fragments $PilZ_{d1}$, $PilZ_{d2}$ and $PilZ_{d1d2}$ (consisting of both $PilZ_{d1}$ and $PilZ_{d2}$) (table 2.3) were PCR amplified ahead of cloning into vector (section 3.7.1). In each case the reverse primer used in the PCR amplification of these fragments contained a 6X His-Tag.

4.5.1. PCR amplification

Three PCR-products were amplified, with negative controls (section 3.7.1).

PCR 1: $PilZ_{d1d2}$
PCR 2: $PilZ_{d1d2}$ negative control (without template DNA).
PCR 3: $PilZ_{d1}$
PCR 4: $PilZ_{d1}$ negative control (without template DNA)
PCR 5: $PilZ_{d2}$
PCR 6: $PilZ_{d2}$ negative control (without template DNA).

Experiment 1:

All three fragments were PCR amplified (table 4.3).

Table 4.3: PCR settings used for PCR amplification experiment 1.

Cycle step	3-step protocol		Cycles
	Temp.	Time	
Initial denaturation	98°C	1 min	1
Denaturation	98°C	10 s	30x Denaturation, primer annealing and extension.
Annealing (see 5.3)	64°C	30 s	
Extension (see 5.4)	72°C	15 s	
Final extension	72°C	7 min	1

The PCR products were analyzed on 1% agarose gel (section 3.6) to control that the right PCR-products were obtained. A band at ~700bp for $PilZ_{d1d2}$ was the only band observed in the gel. The PCR amplification of the other two PilZ domains ($PilZ_{d1}$ and $PilZ_{d2}$) was not successful, and no bands were observed (figure 4.5), because of this another experiment was performed for the $PilZ_{d1}$ and $PilZ_{d2}$ domains.

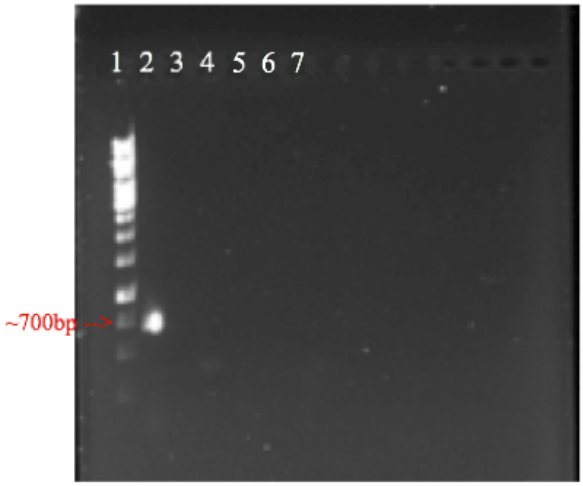


Figure 4.5: PCR amplified products on 1% agarose gel, experiment 1. Lane 1: 3µl size marker; Generuler 1 kb DNA ladder. Lane 2: 10µl $PilZ_{d1d2,}$ band observed at ~700bp. Lane 3: 10µl $PilZ_{d1d2}$ negative control, no band observed as expected. Lane 4: 10µl $PilZ_{d1,}$ no band observed. Lane 5: 10µl $PilZ_{d1,}$ negative control, no band observed as expected. Lane 6: 10µl $PilZ_{d2,}$ no band observed. Lane 7: 10µl $PilZ_{d2,}$ negative control, no band observed as expected.

Experiment 2:

The second experiment was only performed for the $PilZ_{d1}$ DNA fragment and the $PilZ_{d2}$ DNA fragment because $PilZ_{d1d2}$ DNA fragment was successfully PCR amplified in experiment 1. Annealing temperatures were changed and an increase in the number of cycles from 30 to 35 was used. Annealing temperature was changed because 64°C did not result in any product, and could therefore be the wrong annealing temperature for these DNA fragments. Increase of cycles can increase the yield of DNA fragment obtained and was therefore also changed in this experiment. Two separate PCR reactions were set up and were named experiment 2.1 and experiment 2.2 (table 4.4 and 4.5 respectively).

Table 4.4: PCR settings used for PCR amplification experiment 2.1.

Cycle step	3-step protocol		Cycles
	Temp.	Time	
Initial denaturation	98°C	1 min	1
Denaturation	98°C	10 s	35 x Denaturation, primer annealing and extension.
Annealing (see 5.3)	63°C	30 s	
Extension (see 5.4)	72°C	15 s	
Final extension	72°C	7min	1

Table 4.5: PCR settings used for PCR amplification experiment 2.2.

Cycle step	3-step protocol		Cycles
	Temp.	Time	
Initial denaturation	98°C	1 min	1
Denaturation	98°C	10 s	35 x Denaturation, primer annealing and extension.
Annealing (see 5.3)	65°C	30 s	
Extension (see 5.4)	72°C	15 s	
Final extension	72°C	7 min	1

The PCR products from experiment 2.1 and 2.2 were analyzed on 1% agarose gel (section 3.6) to control that the correct PCR-products were amplified (figure 4.6). The $PilZ_{d1}$ was successfully PCR amplified when using annealing temperature at 65°C with 35 cycles and bands were observed in the gel at ~350bp (lane 4 and 5), but it was not obtained when using annealing temperature at 63°C (lane 2 and 3). For $PilZ_{d2}$ both annealing temperatures (63°C

and 65°C) were sufficient for PCR amplification and bands were observed at ~300bp in the gel (lane 8-11). No DNA was observed in any of the negative controls as expected (lane 12 and 13) (figure 4.6).

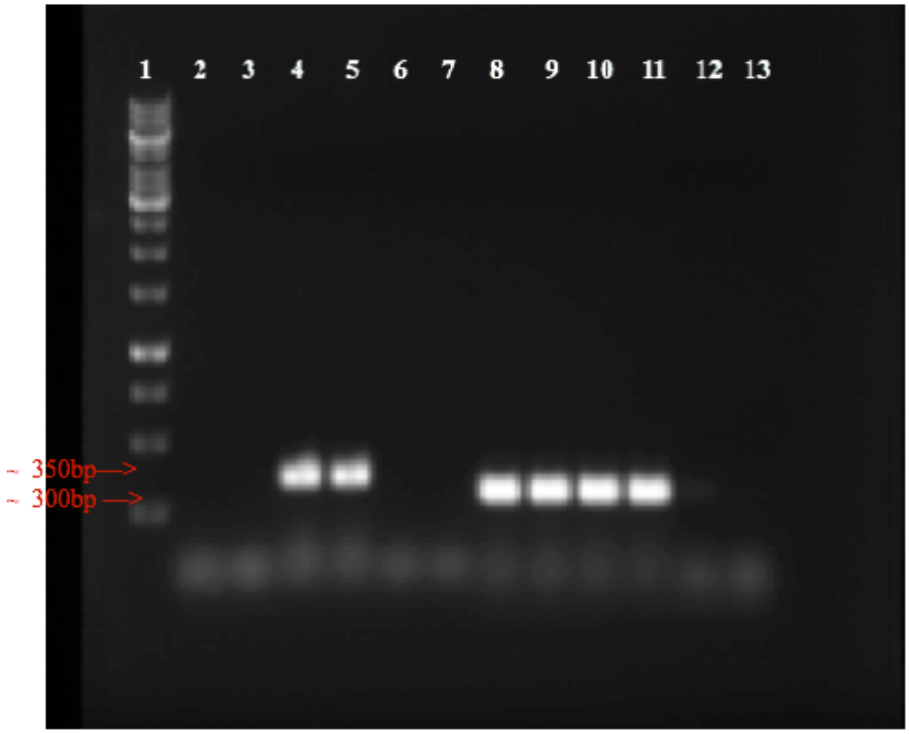


Figure 4.6: PCR amplified products on 1% agarose gel, experiment 2. Lane 1: 3µl size marker; Generuler 1 kb DNA ladder. Lane 2: 2µl $PilZ_{d1}$ from experiment 2.1. Lane 3: 5µl $PilZ_{d1}$ PCR product from experiment 2.1. Lane 4: 2µl $PilZ_{d1}$ PCR product from experiment 2.2. Lane 5: 5µl $PilZ_{d1}$ PCR product from experiment 2.2. Lane 6: 5µl $PilZ_{d1}$ negative control PCR product from experiment 2.1. Lane 7: 5µl $PilZ_{d1}$ negative control PCR product from experiment 2.2. Lane 8: 2µl $PilZ_{d2}$ PCR product from experiment 2.1. Lane 9: 5µl $PilZ_{d2}$ PCR product from experiment 2.1. Lane 10: 2µl $PilZ_{d2}$ PCR product from experiment 2.2. Lane 11: 5µl $PilZ_{d2}$ PCR product from experiment 2.2. Lane 12: 5µl $PilZ_{d2}$ negative control PCR product from experiment 2.1. Lane 13: 5µl $PilZ_{d2}$ negative control PCR product from experiment 2.2.

All the PCR products from experiment 1 and experiment 2 were then purified using the E.Z.N.A Gel Extraction Kit-protocol for Purification of DNA from Enzymatic reactions (section 2.8), and then analysed on 1% agarose gel (section 3.6) to see if the PCR products were successfully purified (figure 4.7).

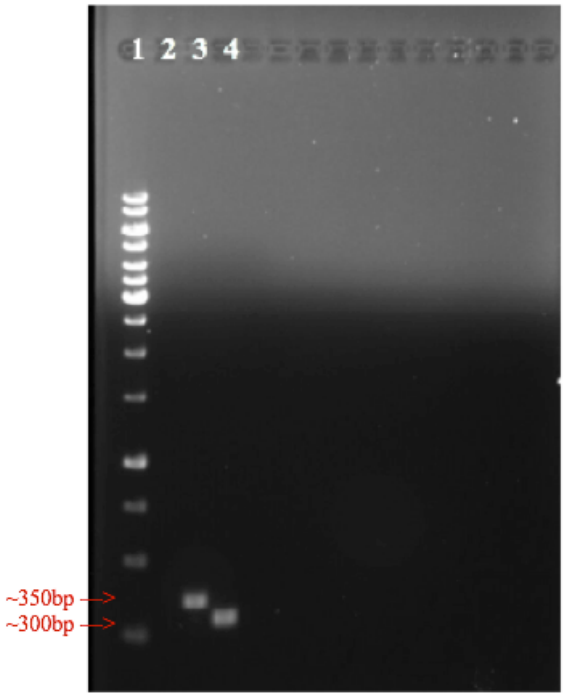


Figure 4.7: PCR purified products on 1% agarose gel, experiment 1 and experiment 2. Lane 1: 3µl size marker; Generuler 1 kb DNA ladder. Lane 2: 2µl purified $PilZ_{d1d2}$ PCR product. Lane 3: 2µl purified $PilZ_{d1}$ PCR product. Lane 4: 2µl purified $PilZ_{d2}$ PCR product. Lane 5: 10µl negative control from purification.

Bands from the purified $PilZ_{d1}$ PCR product at ~350bp (lane 3) and the purified $PilZ_{d2}$ PCR product at ~300bp (lane 4) were observed, and hence $PilZ_{d1}$- and $PilZ_{d2}$ DNA fragments were successfully purified from the PCR reactions. No bands from the purified $PilZ_{d1d2}$ PCR product (lane 2) were observed for unknown reasons (figure 4.7). Most likely no DNA was purified from the experiment 1 due to mistakes done during the purification protocol, for example use of incorrect amount of binding buffer or too low amount of DNA from the PCR product were obtained in PCR experiment 1 etc. No DNA was observed in the negative control as expected (lane 5) (figure 4.7). Another experiment was performed for amplifying and purifying $PilZ_{d1d2}$ DNA fragment since it was not obtained like the two other DNA fragments of $PilZ_{d1}$ and $PilZ_{d2}$ as seen in figure 4.7.

Experiment 3:

Another PCR amplification for the $PilZ_{d1d2}$ DNA fragment (table 4.6) with some changes from experiment 1 (table 4.3) was performed since the purification from experiment 1 did not result in any product (figure 4.7). Annealing temperature was increased to 65°C and cycles were increased from 30 to 35 for denaturation, primer annealing and extension (table 4.6). These changes were made because they gave successful results for the PCR amplification of $PilZ_{d1}$ and $PilZ_{d2}$ DNA fragments, and were therefore believed to give better results for the PCR amplification of $PilZ_{d1d2}$ DNA fragment. Four reactions were performed for the PCR

amplification of $PilZ_{d1d2}$ DNA fragment to obtain higher amounts of DNA for the purification.

Table 4.6: PCR settings used for PCR amplification experiment 3.

Cycle step	3-step protocol		Cycles
	Temp.	Time	
Initial denaturation	98°C	1 min	1
Denaturation	98°C	10 s	35 x Denaturation, primer annealing and extension.
Annealing (see 5.3)	65°C	30 s	
Extension (see 5.4)	72°C	15 s	
Final extension	72°C	7 min	1

The PCR products from experiment 3 were analyzed on 1% agarose gel (section 3.6) to control that the correct PCR-products were amplified. PCR amplification of the $PilZ_{d1d2}$ DNA fragment was successful because clear bands at ~700bp were seen at lane 2-5, and with no sign of DNA in the negative control in lane 6 like expected (Figure 4.8).

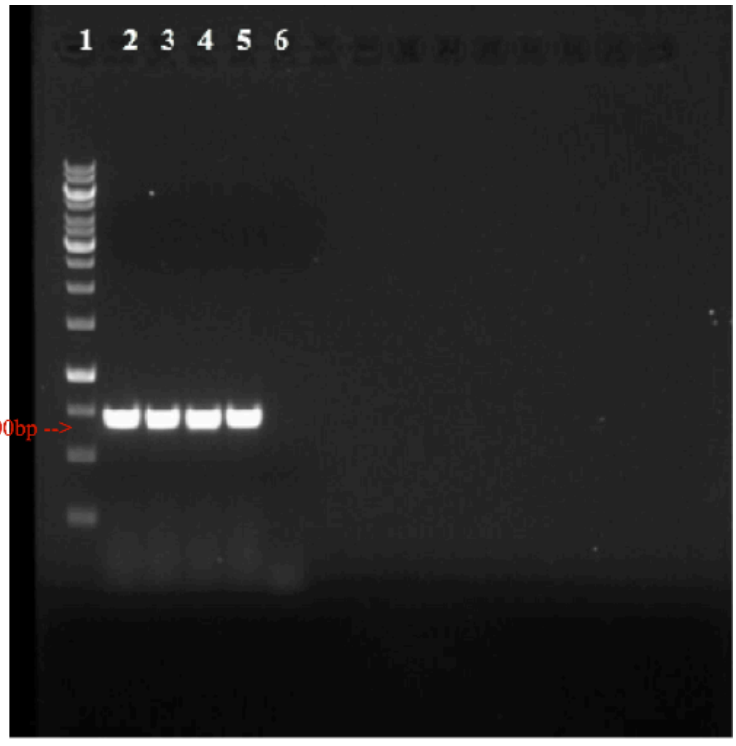


Figure 4.8 PCR amplified products on 1% agarose gel, experiment 3. Lane 1: 3µl size marker; Generuler 1 kb DNA ladder l. Lane 2: 5µl $PilZ_{d1d2}$ PCR product from experiment 3. Lane 3: 5µl $PilZ_{d1d2}$ PCR product from experiment 3. Lane 4: 5µl $PilZ_{d1d2}$ PCR product from experiment 3. Lane 5: 5µl $PilZ_{d1d2}$ PCR product from experiment 3. Lane 6: Negative control from PCR reaction experiment 3.

The PCR products from the four reactions (figure 4.8) were pooled into one sample and purified using the E.Z.N.A Gel Extraction Kit-protocol for Purification of DNA from Enzymatic reactions (section 2.8), and then analysed on 1% agarose gel (section 3.6) to see if the PCR products were successfully purified. A clear band was observed at ~700bp for the pooled and purified $PilZ_{d1d2}$ PCR products and hence the $PilZ_{d1d2}$ DNA fragment was successfully purified from the PCR reactions (figure 4.9).

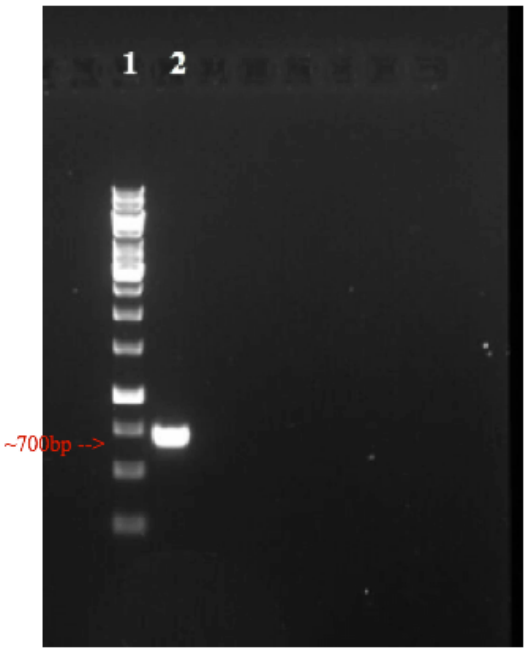


Figure 4.9: PCR purified products on 1% agarose gel, experiment 3. Lane 1: 3µl size marker; Generuler 1 kb DNA ladder. Lane 2: 2µl of pooled and purified $PilZ_{d1d2}$ PCR products from experiment 3.

Restriction enzyme digestion and ligation of PCR-products into pET-28a(+)-vector:

pET-28a(+) vector was used for the cloning of the DNA fragments $PilZ_{d1}$, $PilZ_{d2}$ and $PilZ_{d1d2}$, and the purified PCR products were ligated into the vector. The successfully purified PCR products (25µl) ($PilZ_{d1}$ and $PilZ_{d2}$ figure 4.7 and $PilZ_{d1d2}$ figure 4.9) and pET-28a(+) (35µl) were double digested with the restriction enzymes *Nco*I and *Xho*I for 2.5 hours at 37°C (section 3.7.2). The amounts were chosen to ensure that there was enough DNA and vector for ligation. After digestion a dephosphorylation was performed to reduce vector religation (vectors that can self-ligate) (section 3.5).

Digested DNA fragments were then purified using the E.Z.N.A Gel Extraction Kit- Protocol for Purification of DNA from Enzymatic Reactions (section 2.8) followed by analysis on 1% agarose gel electrophoresis (section 3.6) to calculate the concentration of DNA. A clear band was seen for the digested pET-28a(+) vector (lane 2), and only a faint band was visible for

the digested $PilZ_{d1d2}$ (lane 3). No bands were observed for the $PilZ_{d1}$ and the $PilZ_{d2}$ (lane 4 and 5 respectively) (figure 4.10).

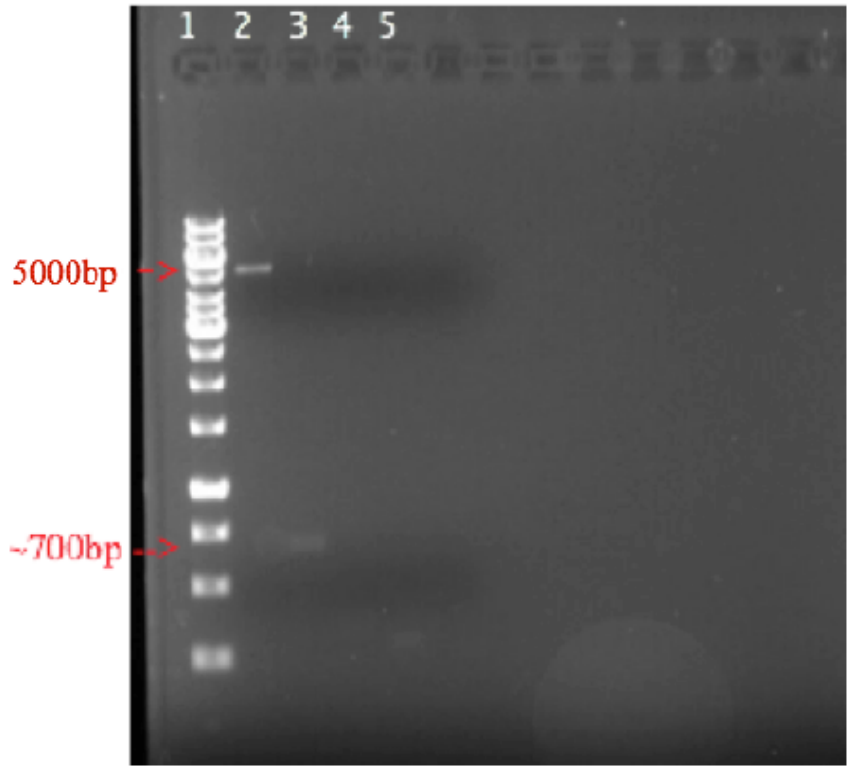


Figure 4.10: 1% agarose gel of digested and purified PCR products and pET-28a(+) vector. Lane 1: 3µl size marker; Generuler 1 kb DNA ladder. Lane 2: 1µl *Nco*I and *Xho*I digested and purified pET-28a(+) fragment. Lane 3: 1µl *Nco*I and *Xho*I digested and purified $PilZ_{d1d2}$ fragment. Lane 4: 1µl *Nco*I and *Xho*I digested and purified $PilZ_{d1}$ fragment. Lane 5: 1µl *Nco*I and *Xho*I digested and purified $PilZ_{d2}$ fragment.

Ligation of $PilZ_{d1d2}$ into pET-28a(+) vector:

The concentration of the digested and purified pET-28a vector and $PilZ_{d1d2}$ fragment were calculated visually based on the fluorescence intensity of the respective bands in the gel (figure 4.10), and was used to calculate the amount of each product needed for ligation. 3µl of the size marker Generuler 1 kb DNA ladder was used, this correlates to 0.3µg DNA. Based on the intensity of the bands seen for pET-28a(+) vector and $PilZ_{d1d2}$ fragment (figure 4.10) they were estimated to have the following concentrations:

1) pET-28a(+) ~ 20ng/µl.
2) $PilZ_{d1d2}$ ~ 10ng/µl.

3) Calculations:

pET-28a(+) → 5369bp

$PilZ_{d1d2}$ → 704 bp

5369bp/704bp = 7.63. → The pET-28a(+) has an approximately 7.63 times higher molar mass relative to $PilZ_{d1d2.}$

2µl of pET-28a(+) was used in the ligation = approximately 40ng DNA
6µl of $PilZ_{d1d2}$ was used in the ligation = approximately 60ng DNA

This was equivalent to (60/40)*7.63 =11.45 times molar excess of insert ($PilZ_{d1d2)}$ to vector (pET-28a(+) DNA)

The following ligation reactions were set up in separate eppendorftubes:
Ligation reaction I: 6µl $PilZ_{d1d2}$, 2µl pET-28a(+), 1µl T4 DNA ligase buffer (10x), 1µl T4 DNA ligase.
Ligation reaction II: 6µl MQ H_2O, 2µl pET-28a(+), 1µl T4 DNA ligase buffer (10x), 1µl T4 DNA ligase. (Negative empty vector religation control).

The two samples were left to incubate at 16°C over night (section 3.7.2).

Transformation of ligated products into *E. coli* host cells:

The ligated products were then transformed into One Shot® Top10 *E. coli* host cells (section 3.7.3). The transformed cells were plated out in different volumes (50µl, 100µl and around 850µl) on LB agar plates with 25µg/ml Kanamycin and incubated over night at 37°C.

The next day colony growth was observed on the LB agar plates and the number of colonies from transformed cells with ligation reaction I ($PilZ_{d1d2}$_pET-28a(+)) was compared to the plates with transformed cells from the empty vector religation control (ligation reaction II). On the plate with 50µl $PilZ_{d1d2}$_pET-28a(+) transformants 641 colonies were counted, while on the same plate with empty vector transformants 403 colonies were counted. A somewhat higher number of colonies were observed on the plates from the ligation where insert DNA was included; hence the transformation was believed to be potentially successful. To

investigate this, plasmid DNA was prepared followed by restriction digestion. Eight single colonies from the plate with $PilZ_{d1d2}$_pET-28a(+) transformants were used to make over night cultures. Plasmid DNA was then isolated (section 3.4) and digested with the same restriction enzymes as before (section 3.5) to check if the vector plasmid contained the $PilZ_{d1d2}$ fragment. The digested samples were then analysed by 1% agarose gel electrophoresis (section 3.6). Four colonies contained the correct construct (lane 3, 4, 5 and 8), because bands were observed at ~5000bp for pET-28a(+) and ~700bp for $PilZ_{d1d2}$ (figure 4.11)

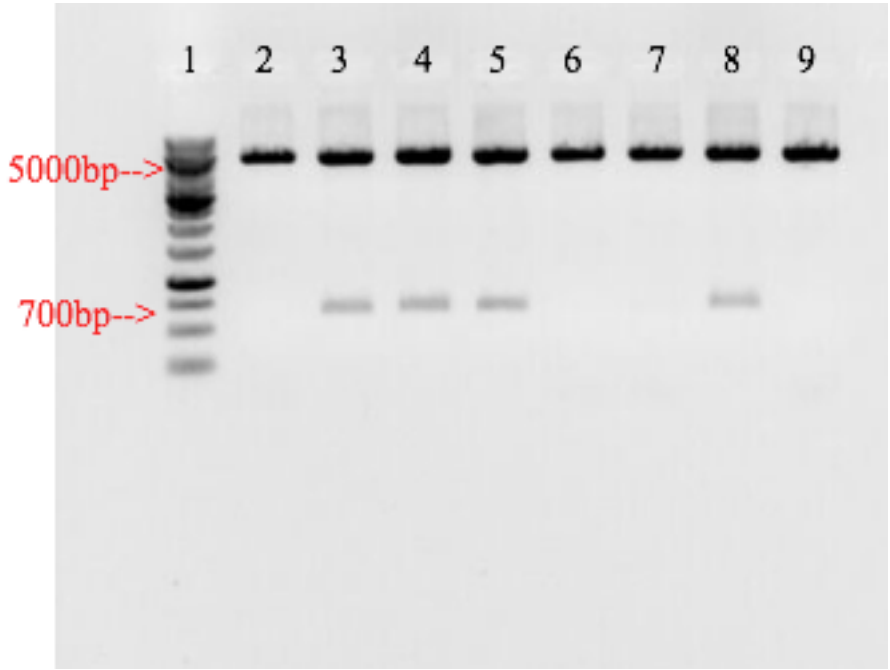


Figure 4.11: 1% agarose gel electrophoresis of plasmid DNA from eight colonies transformed with DNA following ligation of $PilZ_{d1d2}$ fragment into pET-28a(+).
Lane 1: Size marker: 3µl Generuler 1 kb DNA ladder. Lane 2-9: 20µl digested samples from the plasmids isolated from the eight colonies (plate with $PilZ_{d1d2}$_pET-28a(+) transformants). Lane 3, 4, 5, and 8 contained plasmid DNA with an insert of the expected size.

Remains of overnight cultures from clones potentially carrying the correct $PilZ_{d1d2}$ construct (figure 4.11) were used to make freezer stock cultures (section 3.2). The clones were named $PilZ_{d1d2}$_pET-28a(+)_2, $PilZ_{d1d2}$_pET-28a(+)_3, $PilZ_{d1d2}$_pET-28a(+)_4 and $PilZ_{d1d2}$_pET-28a(+)_7.

4.6 Sequence analysis of plasmids with correct construct.

Four plasmid preparations (section 3.4) were sent to GATC (Germany) for sequencing, and these were:

1) $PilZ_{d1d2}$_pET-28a(+)_2
2) $PilZ_{d1d2}$_pET-28a(+)_3
3) $PilZ_{d1d2}$_pET-28a(+)_4
5) $PilZ_{d1d2}$_pET-28a(+)_7

Each sample was sequenced in two directions with T7-promoter primer (table 2.4) and T7-terminator primer (table 2.4). The constructs $PilZ_{d1d2}$_pET-28a(+)_2 and $PilZ_{d1d2}$_pET-28a(+)_7 were confirmed to be 100% identical to the *B. thuringiensis* 407 *bcsA* DNA sequence in GenBank, with no mutations in the DNA-segment between the start codon ATG to the 6X His-tag, (sequences were controlled and confirmed by Ph.D. student Sarah Finke). Confirmed clones were used in later expression experiments.

4.7 Transformation of $PilZ_{d1d2}$ into *E. coli* BL21 (DE3) cells

The $PilZ_{d1d2}$_pET-28a(+)_7 clone was transformed into chemically competent *E. coli* BL21 (DE3) cells: 1µl of plasmid $PilZ_{d1d2}$_pET-28a(+)_7 preparation was added to 100µl chemically competent *E. coli* BL21 (DE3) cells (section 3.10). For each transformation three different volumes (50µl, 100µl and the rest of the sample) were plated on LB-agar plates with 25µg/ml Kanamycin. Plates were incubated over night at 37°C, and colonies were observed the following day.

4.8 Expression and isolation of BcsA $PilZ_{d1d2}$

E. coli BL21 (DE3) cells transformed with the $PilZ_{d1d2}$_pET-28a(+)_7 construct were used to express the $PilZ_{d1d2}$ domain gene fragment by IPTG induction (section 3.11.2), followed by isolation of total protein from the cell (section 3.11.13) and protein purification (section 3.11.14).

One single colony from the transformed *E. coli* BL21 (DE3) cells with $PilZ_{d1d2}$_pET-28a(+)_7 construct was used following protocol for protein expression section 3.11.2. The

samples were induced with 1 mM IPTG for 2.5 hours 37°C and 225rpm. After induction the cells were harvested and stored at -80°C (section 3.11.2). The next day total protein was isolated and the His-tagged $PilZ_{d1d2}$ peptide was purified on a Ni-resin column (section 3.11.3-3.11.4) and analysed by SDS-PAGE (section 3.12). 10µl of the Precision Plus Protein™ Dual Color Standards or the Prestained SDS-PAGE standards Broad Range Bio-Rad was used as size marker (section 2.5). Cell debris obtained from the protein isolation was first mixed with 100µl MQ water to dissolve, and then 5µl was loaded to the wells (figure 4.12), this was performed because the cell debris material could not be directly pipetted, and hence this step was necessary to dissolve the cell debris for application to wells in the SDS-PAGE gel. Lysis buffer flow through and wash buffers from the $PilZ_{d1d2}$ peptide purification on Ni-resin column was also analysed on the SDS-PAGE to see if any sign of peptide was visible in these fractions (figure 4.12). The gel was then stained with Coomassie Brilliant Blue (Bio-Rad) to detect protein (Section 3.12.2). The expected molecular weight for the BcsA_$PilZ_{d1d2}$ peptide was predicted to be around 26 kDa.

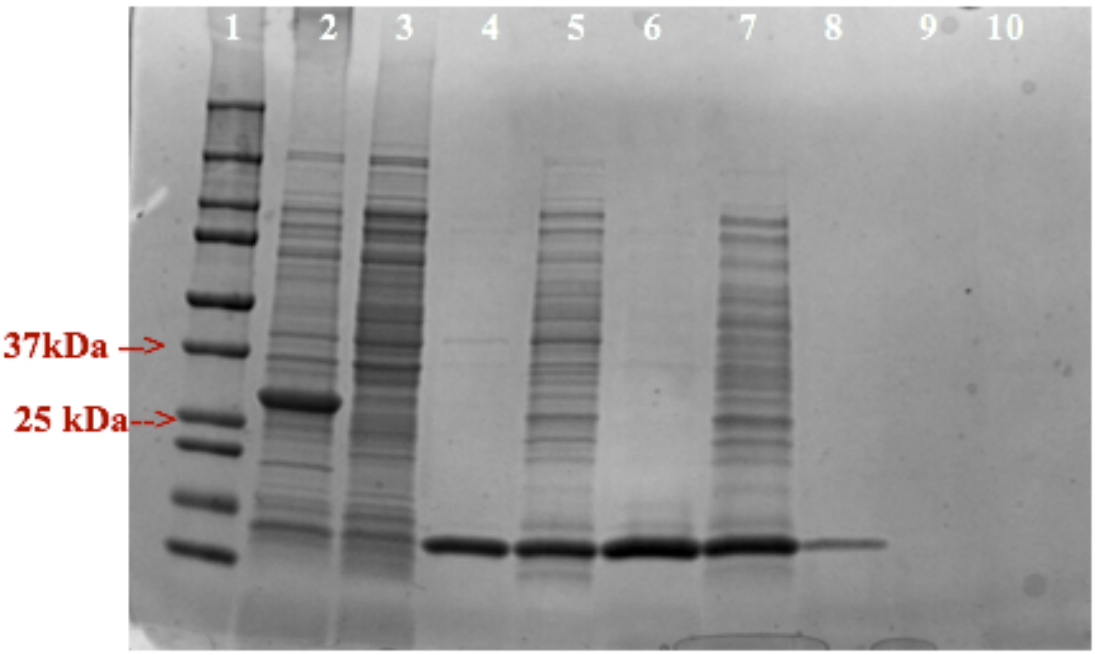


Figure 4.12: SDS-PAGE of samples from isolation and purification of the His-tagged BcsA $PilZ_{d1d2}$ peptide. Lane 1: 10µl Precision Plus Protein™ Dual Color Standards, Bio-Rad (section 2.5). Lane 2: 5µl of the cell debris fraction from protein isolation (IPTG-induced sample). Lane 3: 5µl of the cell debris fraction from protein isolation from non-induced sample. Lane 4: 5µl of the supernatant fraction from protein isolation (IPTG-induced sample). Lane 5: 5µl of the supernatant from protein isolation (non-induced sample). Lane 6: 10µl lysis buffer flow through from protein purification (IPTG-induced sample). Lane 7: 10µl lysis buffer flow through from protein purification (non-induced sample). Lane 8: 10µl of

first wash buffer flow through from protein purification (IPTG-induced sample). Lane 9: 10µl of second wash buffer flow through from protein purification (induced sample). Lane 10: 10µl eluted protein (IPTG-induced sample). All the samples were mixed with 2X SDS loading buffer at a 1:1 concentration.

Results obtained by the gel showed that the induced cells are harder to lyse due to less protein observed in the supernatant fraction compared to the non-induced cells (figure 4.12). Also the correct peptide size of the BcsA $PilZ_{d1d2}$ was only observed in the cell debris of the induced sample at ~26 kDa, and was not seen in the non-induced sample which indicate that the $PilZ_{d1d2}$ peptide is only expressed in the induced sample (figure 4.12). Some unknown peptide was observed in the wash buffer and flow through of the IPTG-induced and non-induced samples, which was believed to be peptide produced by the *E. coli* BL21 (DE3) cells. This peptide was not seen when purified with Ni-resin column, which indicates that it is not breakdown of the $PilZ_{d1d2}$ protein as no his-tagged protein was seen in the IPTG-induced eluted protein sample (lane 10) (figure 4.12). The formation of inclusion bodies or cells not lysing properly was believed to be the reason for the BcsA $PilZ_{d1d2}$ peptide not seen in the soluble fraction due to only being present in the cell debris of induced sample, and because of these results it was decided to induce the $PilZ_{d1d2}$ protein using variable amounts of IPTG for expression to see if this could have any affect on the expression of the $PilZ_{d1d2}$ protein (section 4.9), and also an optimization of cell lysis was performed (section 4.10).

4.9 Optimization of IPTG concentration and length of recombinant protein expression

Several IPTG concentrations and time points were used to decide how to continue with protein expression and isolation (section 3.11.1) of the $PilZ_{d1d2}$ protein. An over night culture was made of the *E. coli* BL21 (DE3) cells containing the $PilZ_{d1d2}$_pET-28a(+)_7 construct. The protocol in section 3.11.1 was followed for inducing protein expression and cell lysis, and the protocol in section 3.12 was used for protein detection by SDS-PAGE (figure 4.13 and 4.14). All samples were induced at 37°C (figure 4.13 and 4.14) except for samples in lane 9 and 10 which were induced at 28°C (figure 4.14).

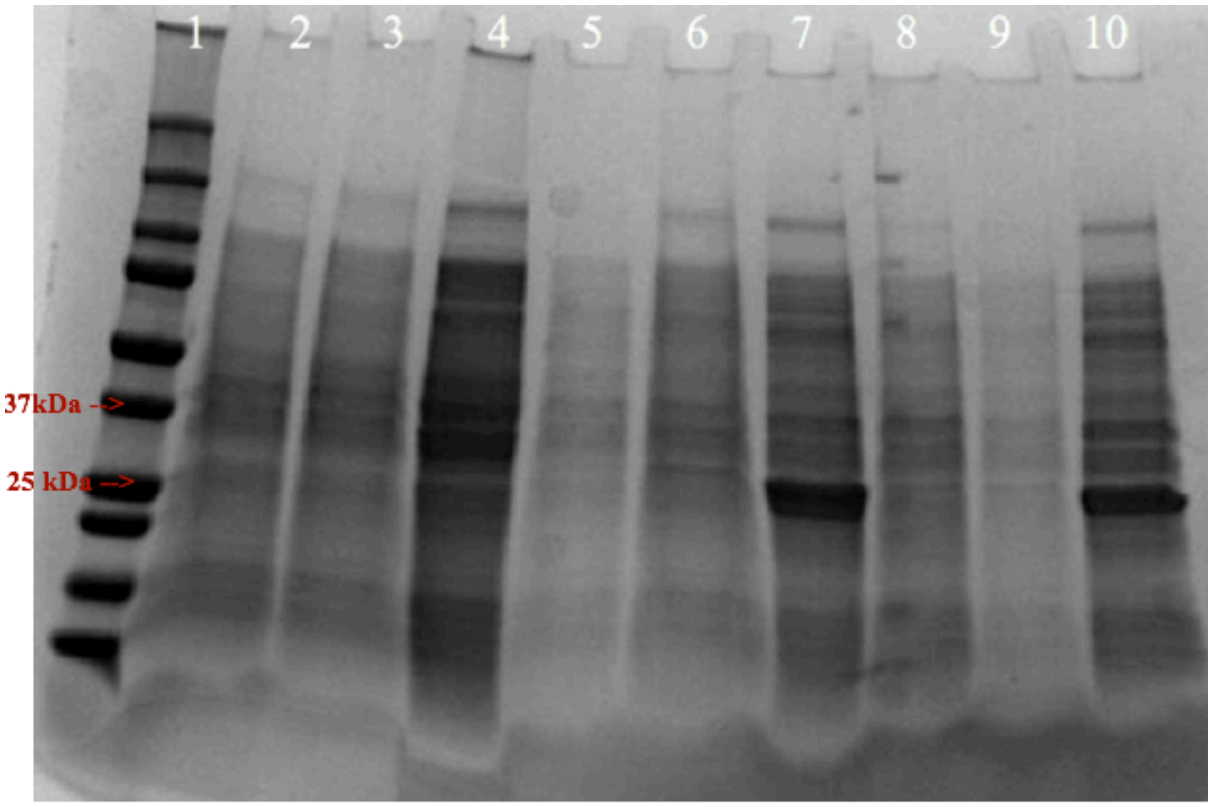


Figure 4.13. SDS-PAGE of protein isolated from *E. coli* BL21 (DE3) carrying the $PilZ_{d1d2}$_pET28a(+)_7 construct. Lane 1: 10µl Precision Plus Protein™ Dual Color Standards, Bio-Rad (section 2.5). Lane 2: 20µl of non-induced sample from culture at 1.5h after incubation of other samples with IPTG. Lane 3: 20µl of non-induced sample from culture 2.5 after incubation of other samples with IPTG. Lane 4: 20µl of non-induced sample from culture 2.5h after incubation of other samples with IPTG. Lane 5: 20µl of sample from culture induced with 0.125 mM IPTG 1.5h after incubation with IPTG. Lane 6: 20µl of sample from culture induced with 0.125 mM IPTG 2h after incubation with IPTG. Lane 7: 20µl of sample from culture induced with 0.125 mM IPTG 2.5h after incubation with IPTG. Lane 8: 20µl of sample from culture induced with 0.25 mM IPTG 1.5h after incubation with IPTG. Lane 9: 20µl of sample from culture induced with 0.25 mM IPTG 2h after incubation with IPTG. Lane 10: 20µl of sample from culture induced with 0.25 mM IPTG 2.5h after incubation with IPTG.

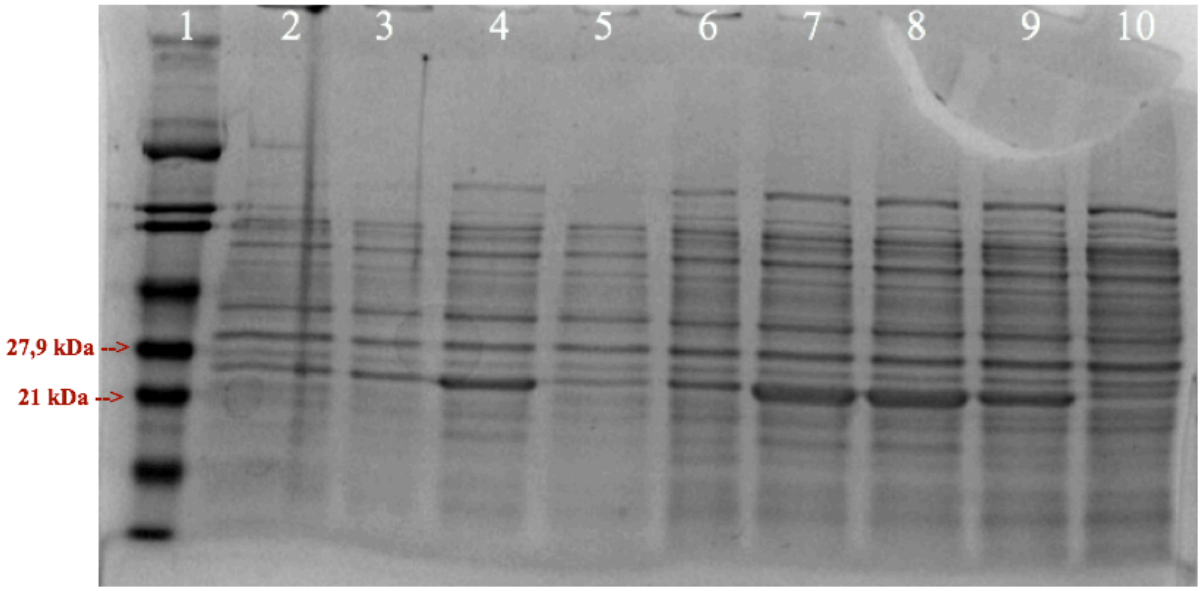


Figure 4.14. SDS-PAGE of protein isolated from *E. coli* BL21 (DE3) carrying the $PilZ_{d1d2}$_pET28a(+)_7 construct. Lane 1: 10µl Prestained SDS-PAGE standards Broad Range Bio-Rad (section 2.5). Lane 2: 20µl of sample from culture induced with 0.5 mM IPTG 1.5h after incubation with IPTG. Lane 3: 20µl of sample from culture induced with 0.5 mM IPTG 2h after incubation with IPTG. Lane 4: 20µl of sample from culture induced with 0.5 mM IPTG 2.5h after incubation with IPTG. Lane 5: 20µl of sample from culture induced with 0.75 mM IPTG 1.5h after incubation with IPTG. Lane 6: 20µl of sample from culture induced with 0.75 mM IPTG 2h after incubation with IPTG. Lane 7: 20µl of sample from culture induced with 0.75 mM IPTG 2.5h after incubation with IPTG. Lane 8: 20µl of sample from culture induced with 1 mM IPTG 2.5h after incubation with IPTG. Lane 9: 20µl of sample from culture induced with 1 mM IPTG at 28°C 2h after incubation with IPTG. Lane 10: 20µl of sample from culture induced with 1 mM IPTG at 28°C 2.5h after incubation with IPTG.

It was concluded that 0.5 mM IPTG and 2.5h were going to be used for expression of the $PilZ_{d1d2}$ protein (~26 kDa) in *E. coli* BL21 (DE3) cells, because the protein was expressed in high enough amounts under these conditions. The use of 1 mM IPTG resulted in overexpression of the $PilZ_{d1d2}$ protein that could lead to inclusion body formation (figure 4.14). A new protein expression, isolation and purification of the $PilZ_{d1d2}$ protein was therefore performed the same way as before (section 4.8) but with the change of IPTG concentration from 1 mM to 0.5 mM, and a new SDS-PAGE was performed for detection of protein (section 3.12) (figure 4.15).

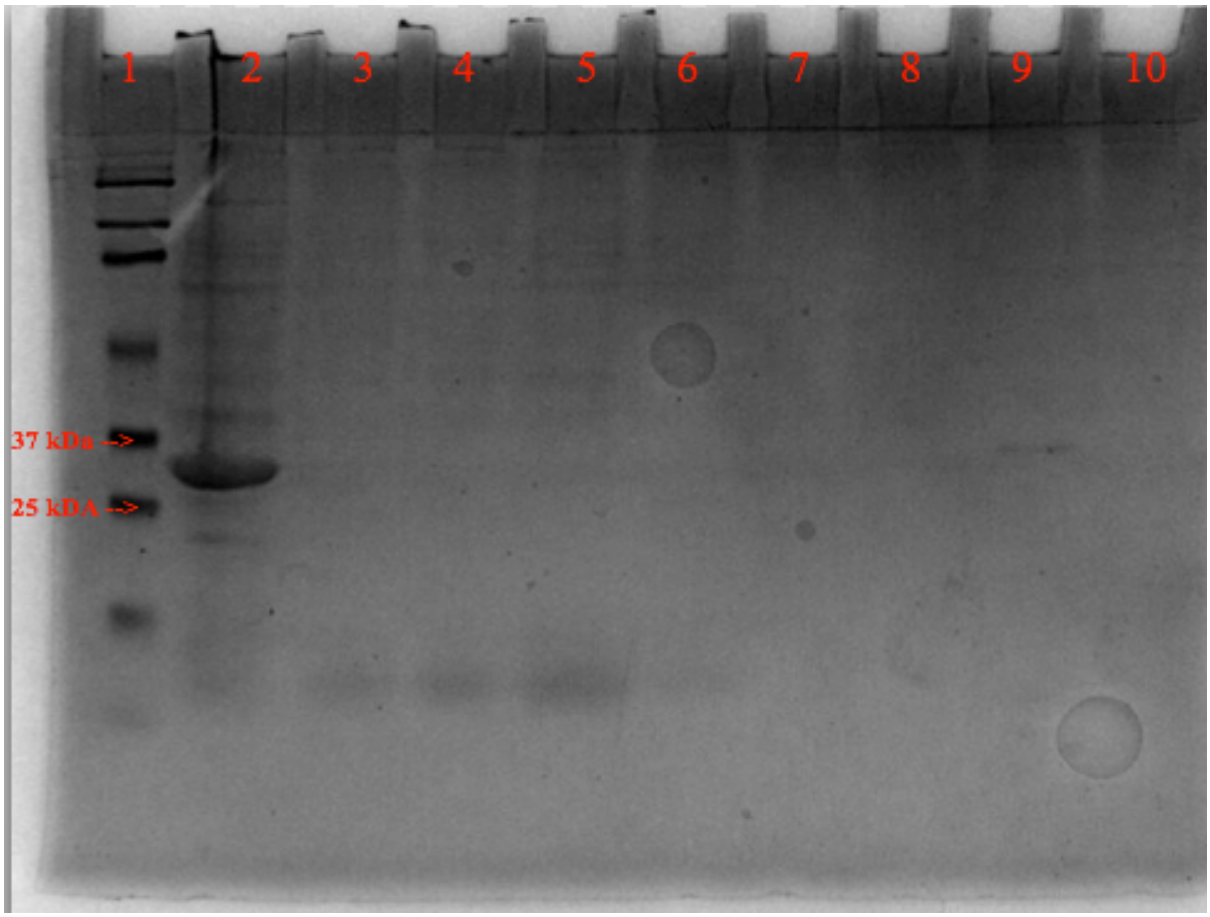


Figure 4.15 SDS-PAGE of samples from PilZ$_{d1d2}$ protein induction, isolation and purification using 0.5 mM IPTG. Lane 1: 5µl Precision Plus Protein^{TM} Dual Color Standards, Bio-Rad (section 2.5). Lane 2: 5µl cell debris from protein isolation (IPTG-induced sample). Lane 3: 5µl supernatant from protein isolation (IPTG-induced sample). Lane 4: 5µl supernatant from protein isolation (non-induced sample). Lane 5: 10µl lysis buffer flow through from protein purification (IPTG-induced sample). Lane 6: 10µl of first wash buffer flow through from protein purification (IPTG-induced sample). Lane 7: 10µl of second wash buffer flow through from protein purification (IPTG-induced sample). Lane 8: Eluted protein (1) from sample induced with IPTG. Lane 9: Eluted protein (4) from sample induced with IPTG. Lane 10: Eluted protein (4) from NON-induced sample.

Cells were not lysing properly with the initial experiment set up since only the cell debris was observed on the SDS-PAGE gel (figure 4.15), and it was therefore needed to solve this problem before continuing with protein expression and isolation of the PilZ$_{d1d2}$ protein (section 4.10.1).

4.10 Optimization of induced *E. coli* BL21 (DE3) cell lysis, BCA protein assay, SDS-PAGE and Western Blot on BcsA_PilZ$_{d1d2}$ peptide.

4.10. 1 Optimization of induced *E. coli* BL21 (DE3) cell lysis

Since the BcsA PilZ$_{d1d2}$ peptide could not be isolated and purified by the initial experiment set up (section 4.8 and 4.9) changes in the protocol were made. Samples were induced the same way as before (section 3.11.2) but with the use of 0.5 mM IPTG instead (section 4.9). Since it was thought that the cells were not being lysed properly (figure 4.12 and 4.15) 16ml lysisbuffer (section 2.10) was used instead of 4 ml with a corresponding adjustment in volume of lysozyme solution added (section 2.10) (1.6 ml instead of 400µl) in the protein isolation method (section 3.11.3.). Cells were divided into four fractions, and different cell lysis methods were tried:

Method 1) Sonicated with frequency 50 kHz, in 10 seconds intervals with 10 seconds rest in between each sonication, repeated 6 times.
Method 2) Sonicated with frequency 50 kHz, in 15 seconds intervals with 15 seconds rest in between each sonication, repeated 6 times.
Method 3) Sonicated with frequency 60 kHz, in 10 seconds intervals with 10 seconds rest in between each sonication, repeated 6 times.
Method 4) Precellys bead beater (5400 rpm -2x30 sec –pause 20 sec). After lysis the tube was centrifuged at 500 xg for 1 minute. Supernatant and celldebris were removed carefully into a new eppendorftube. The eppendorftube with the mix was centrifuged at 10 000 xg for 5 minutes to separate the celldebris from the supernatant. Supernatant was transferred to new eppendorftube.

An increase of viscosity of the suspension occurs after sonication of cells due to cell lysis and release of cell content, and hence an observation of viscosity was performed after cell lysis, which had not been performed in earlier tries of BcsA PilZ $_{d1d2}$ protein isolation (section 4.8 and 4.9). All methods (1-4) resulted in increased viscosity.

The celldebris and supernatant obtained from the four fractions were used for BCA protein assay (section 4.10.2) and Western blot protein detection (section 4.10.3).

4.10.2 Quantification of protein content in samples ahead of SDS-PAGE by using BCA (Bicinchoninic Acid) protein assay.

BCA protein assay was done on the fraction samples (section 4.10.1) using The Pierce BCA Protein Assay Kit from Thermo Scientific™ (section 3.14) to quantify total protein in each sample ahead of SDS-PAGE analysis. The fraction samples from method 1-4 (12µl; for methods see 4.10.1) were diluted 5 times in lysis buffer (section 2.10). Lysis buffer was also used for dilution of the protein standard. Two parallels of each standard and each sample (25µl volume) were analyzed following the protocol in the kit.

Bovine serum albumin (BSA) standard (section 2.8):

1. 0.0 µg/ml (Blank).
2. 2000 µg/ml
3. 1000 µg/ml
4. 500 µg/ml
5. 250 µg/ml
6. 125 µg/ml
7. 62.5 µg/ml
8. 25 µg/ml

Diluted samples containing $PilZ_{d1d2}$:

1. Cell debris fraction, method 1
2. Supernatant fraction, method 1
3. Cell debris fraction, method 2
4. Supernatant fraction, method 2
5. Cell debris fraction, method 3
6. Supernatant fraction, method 3
7. Cell debris fraction, method 4
8. Supernatant fraction, method 4

Table 4.7 BCA protein Assay results from samples from lysis method 1-4. Columns 1 and 2: parallels (technical replicates) of protein samples from the following methods: A: Cell debris fraction, method 1. B: Supernatant fraction, method 1. C: Cell debris fraction, method 2. D: Supernatant fraction, method 2. E: Cell debris fraction, method 3. F. Supernatant fraction, method 3. G: Cell debris fraction, method 4. H: Supernatant fraction, method 4.

	1	2	Average (5 X diluted sample)	Average (undiluted sample)	Concentration (µg/µl)	Sample volume equivalent to 10µg protein/
A	1697.438	1923.994	1810.716	9053.580	9.053580	1.105 µl
B	922.884	1002.707	962.796	4813.980	4.812980	2.077 µl
C	39.356	30.161	34.759	173.795	0.173795	57.540 µl
D	932.275	996.446	964.361	4821.805	4.821805	2.074 µl
E	458.034	428.296	443.165	2215.825	2.215825	4.513 µl
F	785.542	794.346	789.944	3949.720	3.949720	2.532 µl
G	320.496	244.391	282.445	1412.225	1.412225	7.081 µl
H	710.806	673.634	692.220	3461.100	3.461100	2.889 µl

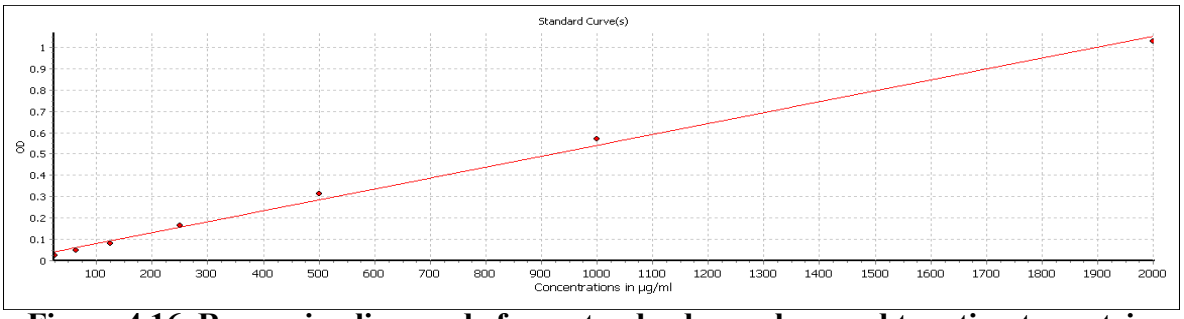


Figure 4.16. Regression line made from standard samples, used to estimate protein concentrations in cell debris and supernatant fractions from method 1-4 (BCA protein Assay).

Based on the results from the BCA protein Assay the amount equal to 10µg protein of each sample was calculated (table 4.7). The results also showed that there were no big differences between the methods in the amount of protein that came into the supernatant fraction. The samples were then run on SDS-PAGE (section 3.12) applying volumes equivalent to 10µg protein in each well (figure 4.17).

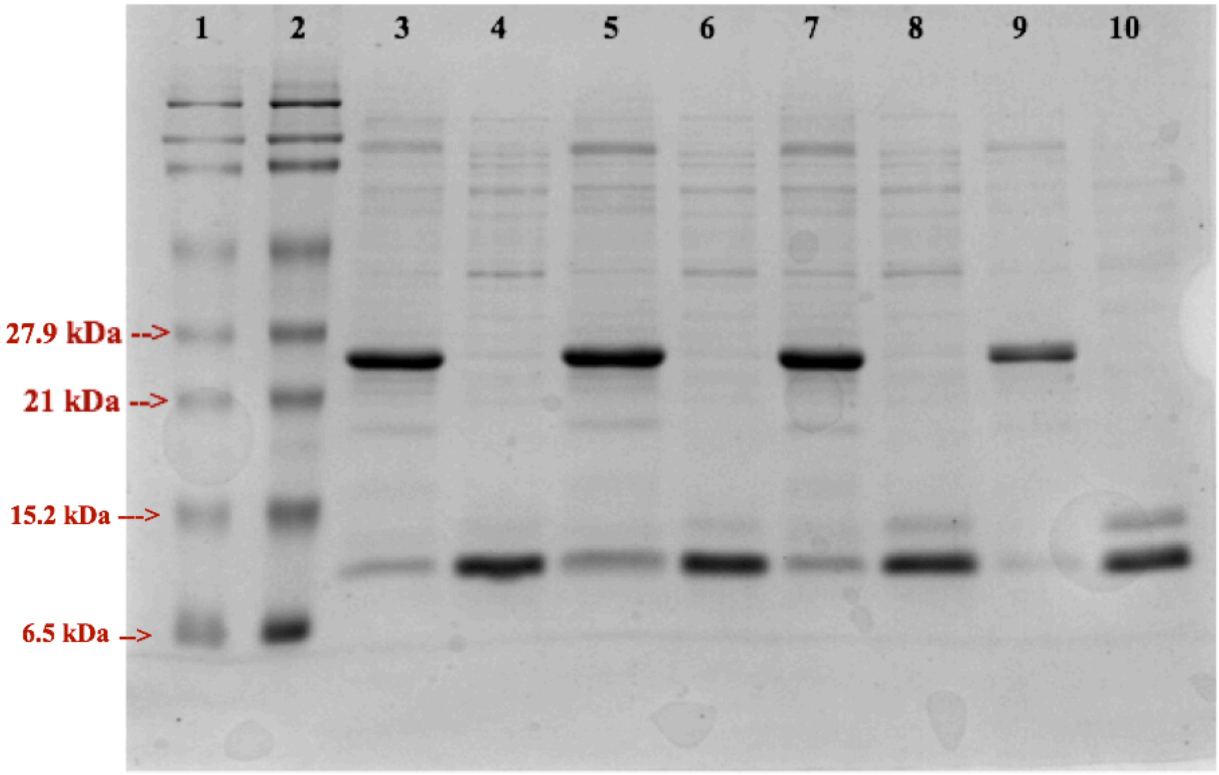


Figure 4.17. SDS-PAGE of protein isolated from *E. coli* BL21 (DE3) carrying the $PilZ_{d1d2}$_pET28a(+)_7 construct from fractions, method 1-4. Lane 1: 5µl Prestained SDS-PAGE standards Broad Range Bio-Rad (section 2.5). Lane 2: 10µl Prestained SDS-PAGE standards Broad Range Bio-Rad (section 2.5). Lane 3: Cell debris fraction from lysing method 1. Lane 4: Supernatant fraction from protein isolation using method 1. Lane 5: Cell debris fraction from lysing method 2. Lane 6: Supernatant fraction from protein isolation using method 2. Lane 7: Cell debris fraction from lysing method 3. Lane 8: Supernatant fraction from protein isolation using method 3. Lane 9: Cell debris fraction from lysing method 4. Lane 10: Supernatant fraction from protein isolation using method 4. – 10µg protein was added to each well (table 4.7), MQ water was used to adjust volume to 10µl. All the samples were mixed with 2X SDS loading buffer at a 1:1 concentration (total volume 20µl).

From this it was concluded that the cells were now indeed lysing since peptides were being isolated in the soluble fractions, but the $PilZ_{d1d2}$ protein was most likely forming inclusion bodies within the cell (figure 4.17). Optimization of cell lysis showed that method 1 and method 2 gave the marginally best results for cell lysis of induced cells due to more protein released from the cells as observed in the soluble fraction (supernatant) (figure 4.17). Also using same amount of protein and not volume when applying to SDS-PAGE gel gave a better indication of cell lysis, and also makes the possibility of comparing cell lysis methods more clear.

4.10.3 Western blot of $PilZ_{d1d2}$

A western blot was performed on the samples using antibodies directed towards the His-tag to investigate whether there was any sign of the His-tagged $PilZ_{d1d2}$ domains of the BcsA protein in the supernatant fractions of method 1-4 (section 3.13).

Regrettably, there were some complications with the Western blot analysis due to the antibodies apparently not giving specific binding to the His-tag (high number of bands). There was however observed a His-tagged protein of the expected peptide size for the BcsA $PilZ_{d1d2}$ protein (~26 kDa) in the supernatant fraction, and matching in size the most intense band in the cell debris fraction, which may indicate that soluble protein of BcsA $PilZ_{d1d2}$ was to some extent separated from the cell during cell lysis but in very low amounts. The highly expressed ~ 11 kDa peptide observed by SDS-PAGE in the supernatant fraction (figure 4.17) did not give a signal with the His-tag antibodies in the Western blot, and indicates that this was not a His-tagged protein, strengthening the theory of it being a peptide produced by the *E. coli* BL21 (DE3) cells (section 4.8). Attempts were made to determine if a corresponding protein of this size (~ 11 kDa), was found in other studies of *E. coli* BL21 (DE3) recombination gene expression in the literature but none matched the size.

4.11 Induction and isolation of $PilZ_{d1d2}$ using different conditions

It was decided to make a last attempt at isolating the protein by inducing expression of BcsA $PilZ_{d1d2}$ during growth at room temperature over night with 0.5 mM IPTG and 0.1 mM IPTG, respectively; to see if this allowed protein expression without formation of inclusion bodies: An over night culture was inoculated 1:50 into 25 ml LB medium with 25µg/ml Kanamycin and was left to grow until OD_{600} ~0.2 in 100 ml baffled flasks at room temperature and 225rpm, before induction. Parallel to this cells were grown at 37°C and induced with 0.1 mM IPTG: An overnight culture was inoculated 1:50 into 25 ml LB medium with 25µg/ml Kanamycin and was left to grow until OD_{600} ~0.5 in 100 ml baffled flasks at room temperature and 225rpm, before induction with 0.1 mM IPTG for 2.5 hours. The next day protein isolation was performed for all the samples by using the same method as before with

the use of 4 ml lysisbuffer (section 2.10) and 400µl of lysozyme solution (section 2.10) per 25ml bacterial culture, and samples were sonicated (50 kHz) for six times 10 seconds, with 10 seconds rest in between. The samples were analyzed on SDS-PAGE (section 3.12)

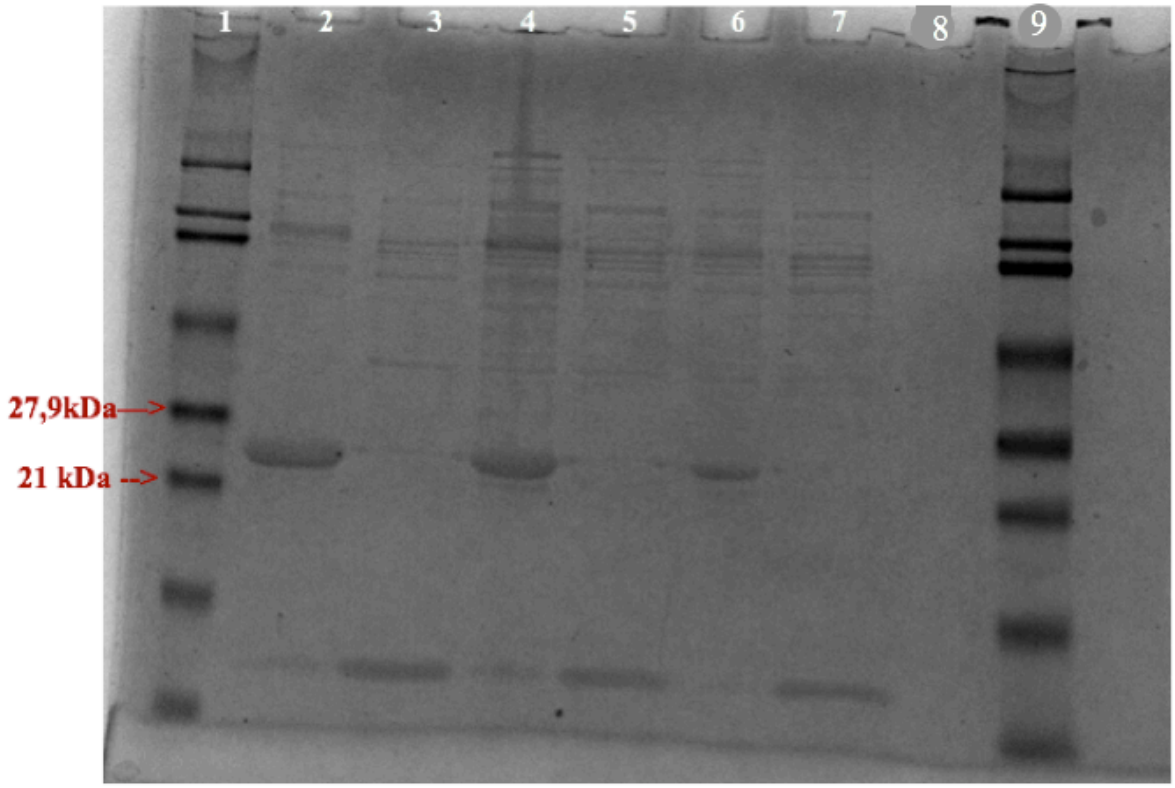


Figure 4.18. **SDS-PAGE of protein isolated from *E. coli* BL21 (DE3) carrying the $PilZ_{d1d2}$_pET28a(+)_7 construct.** Lane 1: 5µl Prestained SDS-PAGE standards Broad Range Bio-Rad (section 2.5). Lane 2: 10µl cell debris fraction from protein isolation (0.1 mM IPTG induced sample at 37°C). Lane 3: 10µl supernatant fraction from protein isolation (0.1 mM IPTG induced sample at 37°C). Lane 4: 10µl cell debris from protein purification (0.1 mM IPTG induced sample at room temperature). Lane 5: 10µl supernatant from protein isolation (0.1 mM IPTG-induced sample at room temperature). Lane 6: 10µl cell debris from protein isolation (0.5 mM IPTG-induced sample at room temperature) Lane 7: 10µl supernatant from protein isolation (0.5 mM IPTG-induced sample at room temperature). Lane 9: 10µl Prestained SDS-PAGE standards Broad Range Bio-Rad (section 2.5). - All the samples were mixed with 2X SDS loading buffer at a 1:1 concentration.

Independent of cultures and expression of the BcsA $PilZ_{d1d2}$ peptide was still only found in presumed intact form in the cell debris fraction, and it was believed that this was due to the formation of inclusion bodies (figure 4.18).

4.12 MALDI-TOF Mass spectrometry analysis of main protein bands from protein isolated samples

Main protein bands from the cell debris and supernatant fractions were cut out of the gel (bands from lanes labelled with 7 and 8; figure 4.17) and sent for analysis by MALDI-TOF MS at the Proteomics service, Department of Bioscience (Professor Berndt Thiede) to identify the nature of each peptide.

Results returned from the Proteomics Service Facility informed that the tryptic peptides from the main band from the cell debris fraction (band from lane 7; figure 4.17) sequencing in the cell debris gave a match to the *B. thuringiensis* 407 BcsA protein (Appendix D), confirming that the main band in the cell debris fraction after IPTG induction was indeed BcsA. However, there was no match for the small peptide cut out from the supernatant fraction (band from lane 8; figure 4.17) to any protein in *B. thuringiensis* 407. Unfortunately we did not receive a NCBI blast of the unknown peptide by the MALDI-TOF MS analysis, and it is therefore still unclear what main protein is present in the fraction.

4.13 Disulphide-bond prediction in $PilZ_{d1d2}$

The amino acid sequence of $PilZ_{d1d2}$ protein was used to predict protein disulphide bonds formation by cysteine residues *in silico* using DISULFIND software (section 3.8.2). The results showed the appearance of four cysteine residues and two predicted disulphide bond connections in the amino acid sequence (figure 4.19). The formations of disulphide bonds are necessary for the refolding of protein inside the cell. If the proteins do not refold in the correct conformation this could lead to inclusion body formation, and it was therefore interesting to take a closer look at the possible formation of disulphide bonds in the $PilZ_{d1d2}$ protein.

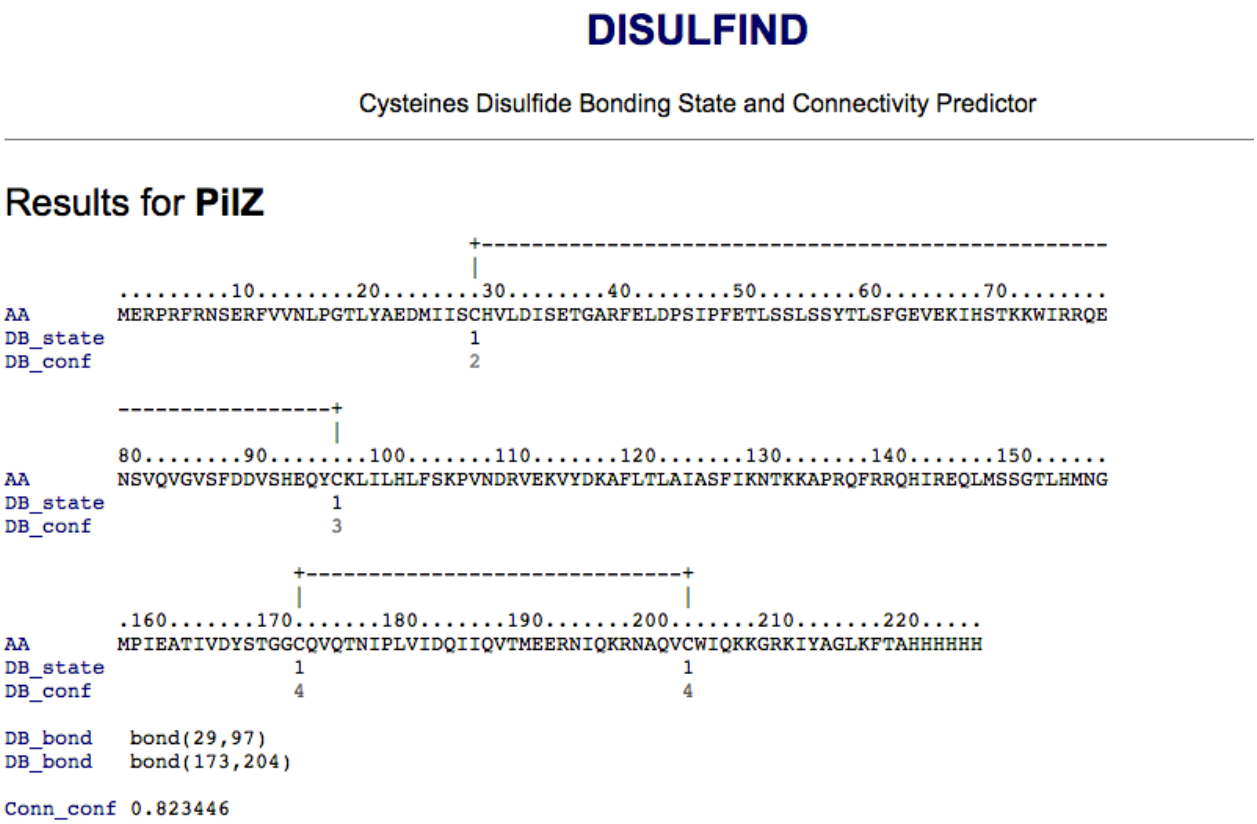


Figure 4.19 Predicted disulphide-bonds in PilZ$_{d1d2}$ protein sequence using DISULFIND software. Settings used were: Predicted bonding state + connectivity pattern (DISULFIND, 2017).

4.14 Cloning of the *bcsA* gene fragment into pET-26b(+).

Since there were several problems expressing and isolating the PilZ$_{d1d2}$ protein probably due to inclusion body formation in the cell, it was decided to try to clone the full *bcsA* gene carrying both PilZ domains and the cellulose synthase domain. Some proteins require all the subunits to be produced in order to form their proper conformation, and when individual subunits are cloned independently of the protein it can either rapidly degrade or form inclusion bodies.

4.14.1. Cloning of *bcsA* into pET-26b(+).

The full *bcsA* gene fragment (table 2.3) were PCR amplified (section 3.7.1) ahead of cloning into vector. The reverse primer used in the PCR amplification of the fragment contained a 6X His-Tag.

The PCR-products were amplified, with negative controls (section 3.7.1; Table 4.8):

PCR 1: *bcsA* gene fragment
PCR 2: *bcsA* gene fragment
PCR 3: *bcsA* gene fragment
PCR 4: *bcsA* gene fragment
PCR 5: *bcsA* gene fragment
PCR 6: *bcsA* gene fragment
PCR 7: *bcsA* gene fragment
PCR 8: *bcsA* gene fragment
PCR 9: *bcsA* negative control (without template DNA)

Table 4.8 PCR settings used for PCR amplification of *bcsA*.

Cycle step	3-step protocol		Cycles
	Temp.	Time	
Initial denaturation	98°C	1 minute	1
Denaturation	98°C	10 s	
Annealing (see 5.3)	64,4°C	30 s	30
Extension (see 5.4)	72°C	45 s	
Final extension	4°C	hold	1

The BcsA gene fragment PCR products were analyzed on 1% agarose gel (section 3.6) to control that the right PCR-products were obtained.

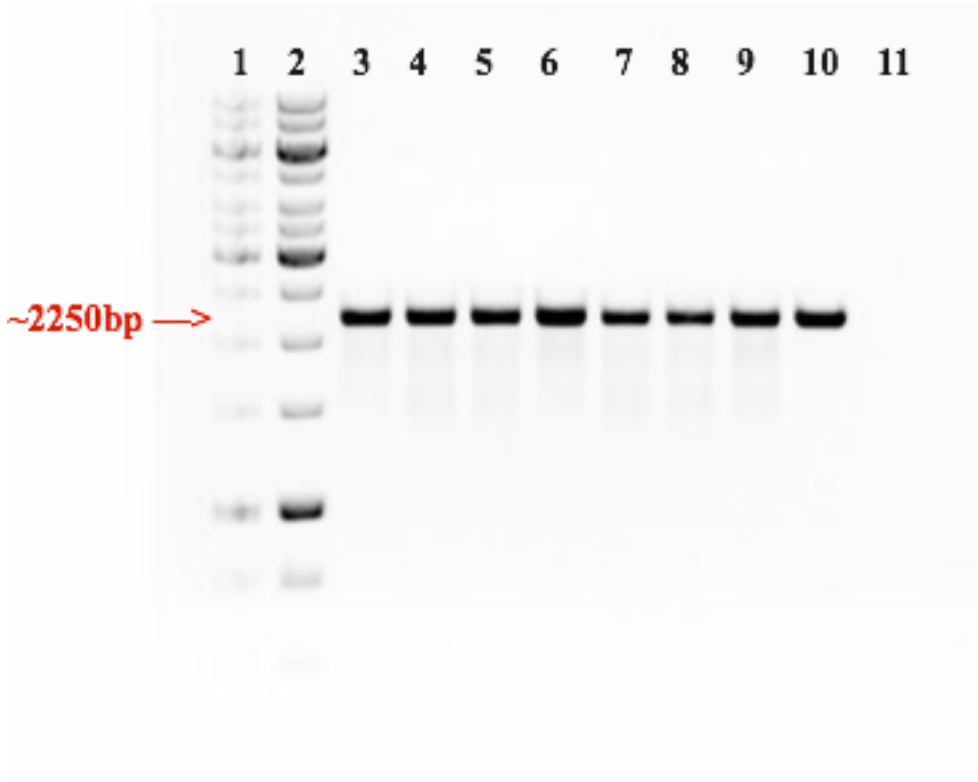


Figure 4.20 PCR amplified products of *bcsA* gene fragment on 1% agarose gel. Lane 1: 3µl size marker: Generuler 1 kb DNA ladder. Lane 2-10: 2µl of *bcsA* PCR reactions 1-8 respectively. Lane 11: 2µl of *bcsA* negative control.

The *bcsA* gene fragments from PCR reaction 1-8 were successfully PCR amplified and was seen at ~2250bp (figure 4.20). The PCR products from PCR reaction 1 and 2 were pooled together and were named BcsA_1 and the same was done for PCR product from PCR reaction 7 and 8, and were named BcsA_2. The pooled reactions BcsA_1 and BcsA_2 were then purified using the E.Z.N.A Gel Extraction Kit-protocol for Purification of DNA from Enzymatic reactions (section 2.8). The other PCR products were stored at -20°C for later use.

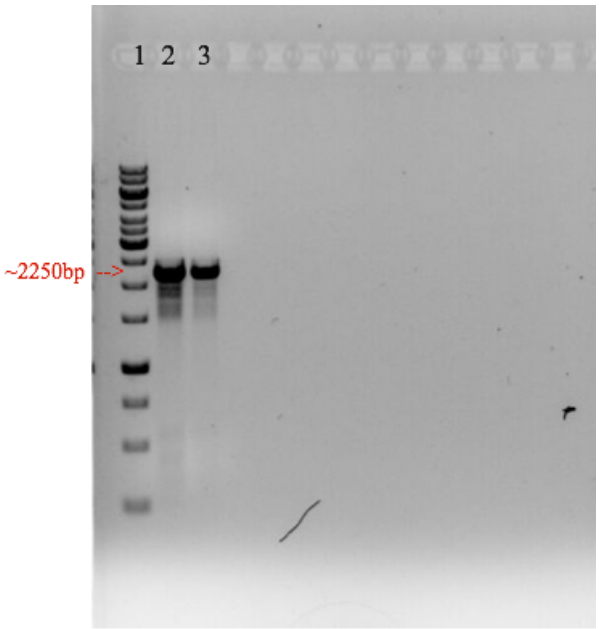


Figure 4.21. PCR purified products of *bcsA* on 1% agarose gel. Lane 1: 3µl size marker; Generuler 1 kb DNA ladder. Lane 2: 2µl purified BcsA_1 PCR product. Lane3: 2µl purified BcsA_2 PCR product.

The purification was not 100% successful due to other bands observed in the gel (figure 4.21) but even though the samples were not successfully purified, it was decided to try continuing working with BcsA_1 and BcsA_2 since the samples were going to be purified again after digestion with restriction enzymes.

Restriction digestion and ligation of *bcsA* PCR-products into pET-26b(+)-vector:

pET-26b(+) vector was used for the cloning of the *bcsA* DNA fragment, and the purified PCR products were ligated into the vector. The purified PCR product (25µl) (BcsA_1 and BcsA2; figure 4.21) and pET-26b(+) (35µl) were double digested with the restriction enzymes *Nde*I and *Xho*I for 2.5 hours at 37°C (section 3.5). The amounts were chosen to ensure that there was enough DNA and vector for ligation. After digestion the restriction enzymes were deactivated at 80°C for 20 minutes (section 3.5). The digested samples were then purified using the E.Z.N.A Gel Extraction Kit-protocol for Purification of DNA from Enzymatic reactions (section 2.8) followed by analysis on 1% agarose gel electrophoresis (section 3.12) to calculate the concentration of DNA.

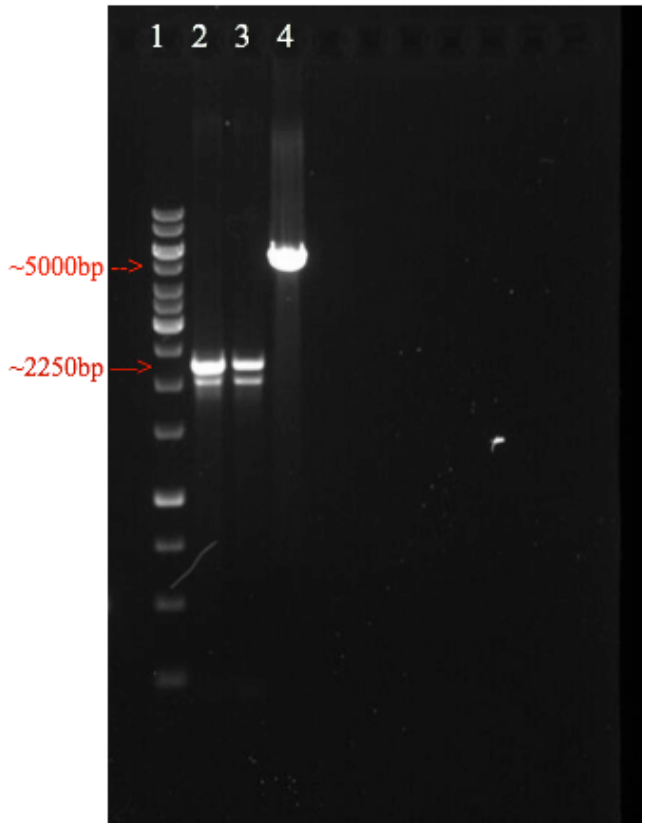


Figure 4.22. 1% agarose gel of digested and purified PCR products and pET-26b(+) vector. Lane 1: 3µl size marker; Generuler 1 kb DNA ladder. Lane 2: 3µl *Nde*I and *Xho*I digested and purified BcsA_1 fragment. Lane 3: 3µl *Nde*I and *Xho*I digested and purified BcsA_2 fragment. Lane 4: 3µl *Nde*I and *Xho*I digested and purified pET.26b(+).

A clear band was seen for the digested pET-26b(+) vector (lane 4), BcsA_1 (lane 2) and Bcsa_2 (lane 3). Some unknown bands were observed in lane 2 and 3 in the gel, and could be contamination of DNA (figure 4.22). It was decided to continue to the ligation step of insert to vector because most likely only the BcsA insert would ligate to pET-26b(+)

Ligation of *bcsA* gene fragment into pET-26b(+).

The concentration of the digested and purified pET-26b(+) vector and *bcsA* fragments were calculated visually based on the fluorescence intensity of the respective bands in the gel (figure 4.22), and was used to calculate amount of each product needed for ligation. 3µl of the size marker Generuler 1 kb DNA ladder was used, this correlates to 0.3µg DNA. Based on the intensity of the bands seen for pET-26b(+) vector and the *bcsA* gene fragments (figure 4.22) they were estimated to have the following concentrations:

1) pET-26b(+) ~ 200ng/µl.
2) BcsA_1 ~ 375ng/µl.
3) BcsA_2 ~ 225ng/µl

Calculations:

pET-26b(+) → 5360bp

bcsA → 2226 bp

5360bp/2226bp = 2.41. → The pET-26b(+) has an approximately 2.41 times higher molar mass relative to *bcsA*.

2µl of pET-26b(+) was used in the ligation = approximately 400ng DNA
5µl of BcsA_1 was used in the ligation = approximately 1875ng DNA
6µl of BcsA 2 was used in the ligation = approximately 1350ng DNA

This was equivalent to (1875/400)*2.41 = 11.30 times molar excess of insert (BcsA_1) to vector pET-26b(+) DNA.

This was equivalent to (1350/400)*2.41 = 8.13 times molar excess of insert (BcsA_2) to vector pET-26b(+) DNA.

The following ligation reactions were set up in separate eppendorftubes:

Ligation reaction I: 5µl BcsA_1, 2µl pET-26b(+), 1µl T4 DNA ligase buffer (10x), 1µl T4 DNA ligase.
Ligation reaction II: 6µl BcsA_2, 2µl pET-26b(+), 1µl T4 DNA ligase buffer (10x), 1µl T4 DNA ligase.
Ligation reaction III: 6µl MQ H_2O, 2µl pET-26b(+), 1µl T4 DNA ligase buffer (10x), 1µl T4 DNA ligase. (Negative empty vector religation control).

The samples were left to incubate at 16°C over night (section 3.7.2).

Transformation of ligated products into *E. coli* host cells:

The ligated products were then transformed into One Shot® Top10 *E. coli* host cells (section 3.7.3). The transformed cells were plated out in different volumes (50µl, 100µl and around 850µl) on LB agar plates with 25µg/ml Kanamycin and incubated over night at 37°C. Colony growth was observed the following day on the LB agar plates and the number of colonies from transformed cells with ligation I and II (BcsA_1_pET-26b(+) transformants and BcsA_2_pET-26b(+) transformants, respectively) were compared to the plates with empty vector religation control (ligation reaction III). None of the plates showed any significant difference, and a low amount of colonies were observed on all the plates. The transformation did not work for these reactions.

Observed colony growth on LB agar plates (ligation reaction I-III):

- BcsA_1_pET-26b(+) transformants (ligation reaction I):
  50µl plate → 8 colonies - 100µl plate → 38 colonies - 850µl plate→ 204colonies
- BcsA_2_pET-26b(+) transformants (ligation reaction II):
  50µl plate → 10 colonies - 100µl plate → 38 colonies - 850µl plate → 229colonies
- Negative empty vector religation control (ligation reaction III):
  50µl plate → 10 colonies - 100µl plate → 32 colonies - 850µl plate→ 215colonies

It was decided to not continue working on these samples and due to time limitations a second transformation was not performed.

5. Discussion

5.1 Cloning and expression of PilZ domains

In this work it was aimed to clone the regions of *bcsA* encoding the $PilZ_{d1}$, $PilZ_{d2}$ and $PilZ_{d1d2}$ domains (Appendix B), respectively, into a pET series expression vector for protein isolation and purification as a His-tagged recombinant protein. The $PilZ_{d1d2}$ region was successfully cloned (section 4.6; Appendix A), however several attempts at cloning the other two domains individually were unfortunately not successful (section 4.5). As part of optimizing the cloning process, heat inactivation and phosphatase removal before ligation was tried as well as increasing the total DNA concentration, but no positive clones were achieved possibly due to inefficient ligation. Another possible solution for optimization which was not tried out in the scope of this work, was to vary the molar mass ratio of vector to insert systematically and stepwise from 1:1 to 1:10 (BioLabs, 2017d). Due to time limitations this was however not done.

The cloned $PilZ_{d1d2}$_pET-28a(+) construct was successfully transformed into *E. coli* BL21 (DE3) expression cells (section 4.7). Expression was induced with IPTG, and isolated protein was then tested on SDS-PAGE (section 4.8). A peptide of the expected size for $PilZ_{d1d2}$ (~26 kDa) was visible in the cell debris fraction but not in the supernatant fraction from the protein isolation from the first steps of protein isolation procedure (figure 4.12). The peptide was confirmed by MALDI-TOF MS to be $PilZ_{d1d2}$ showing that the d1d2 domain was successfully produced in the *E. coli* cells (section 4.12; Appendix D).

Although expression of $PilZ_{d1d2}$ was clearly detected, the purification was however not successful during the work in this thesis. At one point it was believed that the cells were not lysing properly and that this could have been the reason that no the BcsA $PilZ_{d1d2}$ protein was found in the soluble fraction (supernatant) (figure 4.12 and 4.15). Figure 4.12 shows that the induced cells did not release soluble proteins as seen with the non-induced cells after cell lysis in the soluble fraction (supernatant), suggesting that induced cells requires a stronger cell lysis method. Therefore an optimization of cell lysis was performed (section 4.10.1). To isolate proteins from a cell it is important that the cells are completely lysed, so that the protein can be separated from the cell debris. The disruption of the bacterial cells causes the

release of intracellular compounds and the solubilisation of particulate matter (Foladori, Laura et al., 2007), which then can be isolated and purified. One possible explanation for low-efficiency lysis could be that the sonication step was not working during the first rounds of protein isolation (section 4.9; figure 4.15), potentially due to too low ultrasonic frequency for cell lysing being used. An increase of viscosity in the suspension occurs after sonication due to cell lysis and release of cell content such as nucleic acids (Santos, Belo et al., 1996). Ultrasound irradiation (sonication) causes cell lysis by liquid shear and cavitation (EMBL, 2017). The collapse of cavitation bubbles within or near the bacteria causes cell wall damage. Within the bacterial cell shear forces are induced by microstreaming which weakens or disrupts the bacterial cell, and also chemical attack on the cell wall structure due to formation of free radicals during cavitation occurs during sonication which increases the disruption of the bacterial cells (Joyce, Al-Hashimi et al., 2011). Several attempt at optimizing cell lysis were performed (section 4.10.1), by using different settings for the sonication, however after observing soluble proteins in the supernatant fraction of induced *E. coli* BL21 (DE3) cells as seen in figure 4.17 it was concluded that the cells were lysing properly, due to protein having separated from the cells. Also after sonication it was observed visually that the viscosity of the suspension was increased (section 4.10), which indicated cell lysis. This had not been performed earlier (section 4.8 and 4.9).

A high expressed peptide was seen in the supernatant fraction on the SDS-PAGE gel but was not the expected size (~ 11 kDa) (figure 4.12 and 4.17) for the BcsA_PilZ$_{d1d2}$ protein (~26k kDa) and could be protein contaminants from the *E. coli* BL21 (DE3) cells. *E. coli* is most commonly used for high-yield expression of recombinant protein, however when *E. coli* cell disruption occurs this yields a suspension of membrane fragments/vesicles that contains membrane proteins, soluble proteins, remaining intact cells, various cell debris and other material as contaminants, which can be removed during purification (GE-Healthcare, 2007). The main protein found in the supernatant fractions as observed on the SDS-PAGE gel (figure 4.12 and 4.17) was most likely a soluble protein expressed at high levels in the *E. coli* BL21 (DE3) cells. This is supported by the fact that purification on resin column removed the protein contaminant (figure 4.12; lane 10).

It was initially contemplated that the protein found in the supernatant fraction (figure 4.12 and 4.17) could represent breakdown of the BcsA $PilZ_{d1d2}$ protein, but a closer look at the experiments showed that it most likely was not. The use of PMSF inhibits protease activity and protein breakdown, and was always used during the experiments. Also the experiments were always performed on ice, so there would not be any enzyme activity or breakdown of proteins. Furthermore the MALDI-TOF MS analysis of the supernatant fraction showed conclusively, that the protein found was not the *B. thuringiensis* 407 BcsA $PilZ_{d1d2}$ protein, and that it did not have any match to other *B. thuringiensis* 407 proteins in the database (section 4.12). Western blot did not give any signal of the protein found in the supernatant fraction with the His-tag antibodies used. Hence it was concluded that it most likely was not breakdown of BcsA $PilZ_{d1d2}$ protein, but most likely a high expressed *E. coli* protein as stated above (section 4.10.3).

Since the protein found in the main band of the cell debris was the BcsA_$PilZ_{d1d2}$ protein as confirmed by the MALDI-TOF MS analysis (section 4.12; Appendix D), and the protein found in the supernatant fraction did not have any match with the BcsA_$PilZ_{d1d2}$ protein it was believed that the protein only found in the cell debris could be formation of inclusion bodies in the cell.

Overexpression of genes in bacteria often leads to insoluble aggregates whose product fails to attain a soluble bioactive conformation. These insoluble aggregates are termed inclusion bodies (Zhu, Gong et al., 2013), and are seen as molecules densely packed in the form of particles (S. M. Singh & Panda, 2005). This is often a limitation of recombinant protein production in *E. coli* (Zhu, Gong et al., 2013), and are often seen as the result of high level expression of protein (A. Singh, Upadhyay et al., 2015). The increase of local concentration of naive polypeptides chains emerging from the ribosomes during overexpression of foreign proteins (Upadhyay, Murmu et al., 2012) in *E. coli* is believed to overwhelm the bacteria with a high metabolic burden. This can lead to insufficient folding of the polypeptides due to low supply of chaperones (Basu, Li et al., 2011). Also hydrophobic surfaces exposed on the protein can lead to protein-protein interaction and inclusion body aggregates (Upadhyay, Murmu et al., 2012). The PilZ protein contains hydrophobic surfaces that are believed to be the reason for the interaction and binding to c-di-GMP, which has been studied for the PilZ domain of *Pseudomonas aeruginosa* (Shin, Ryu et al., 2011). This implicates that the PilZ

domain contains hydrophobic surfaces, which when in an unfolded state could potentially form inclusion bodies (Fink, 1998).

Single aggregated protein species are often highly enriched when found in bacterial inclusion bodies (Rajan, Illing et al., 2001). 80-95 % of the inclusion body material usually accounts for the target protein. The rest of the inclusion body material could be contamination of adsorbed material from the cell lysis like for example outer membrane proteins, ribosomal components and a small amount of phospholipids and nucleic acids (Baneyx & Mujacic, 2004). Biological activity is seen to be devoid in proteins that have resulted in inclusion body formation, and they need to be solubilized, refolded and purified to recover (S. M. Singh & Panda, 2005). Inclusion bodies are insoluble and very difficult to recover without harsh denaturants and protein-refolding procedures (ThermoFisherScientific, 2017b). The use of high concentration of denaturants such as urea or guanidine hydrochloride along with a reducing agent such as β-mercaptoethanol is generally used for solubilisation of inclusion bodies. Refolding of the proteins is then done by slow removal of denaturants in the presence of an oxidising agent (S. M. Singh & Panda, 2005). This process is time consuming and was not possible to perform within the time frame of this thesis, but it could be a possible way to isolate the protein and could be tried in future work. Also an important note is that the process of isolating inclusion bodies from the cell most of the time lead to poor recovery of functional protein (A. Singh, Upadhyay et al., 2015). Because of this other methods were tried, such as the attempts to clone each PilZ domain separately (section 4.5).

Cysteine residues in proteins can form disulphide bonds, which play a role in stability, folding and function of proteins. Disulphide formation which plays a role in the stabilization of the protein is one of the most important post-translational modification events (Thangudu, Manoharan et al., 2008). The formation of correct disulphide bonds is important for many recombinant proteins. If the formation of disulphide bonds is not performed it can lead to protein misfolding and aggregating into inclusion bodies. Cysteine oxidation and formation of disulphide bonds takes place in the periplasm of *E. coli* while formation of disulphide bonds in the cytoplasm is rare (Rosano & Ceccarelli, 2014). This is due to disulphide bond formation that can not be promoted in the reducing environment of the cytoplasm (Basu, Li et al., 2011). By using the DISULFIND bioinformatics software it was predicted that the BcsA $PilZ_{d1d2}$ protein does contain four cysteine residues and hence possibly two disulphide bonds

(section 4.13). The *E. coli* BL21 (DE3) cells used in these experiments are however mutated to enable disulphide bond formation in the cytoplasm (BioLabs, 2017c), and because of this, lacking disulphide bond formation should in theory not be a problem.

The use of high temperature during protein expression and high inducer concentration can often exhaust bacterial protein quality control system (A. Singh, Upadhyay et al., 2015) which do not allow the bacteria to support all post-translational modification needed for protein folding, and hence often results in protein aggregation (Baneyx & Mujacic, 2004). Partially folded and misfolded protein molecules can aggregate and form inclusion bodies (A. Singh, Upadhyay et al., 2015). Lowering the temperature during protein expression generally decreases hydrophobic interactions between protein chains (Fink, 1998), and decreases protein translation rate in concert with the metabolic rate of the bacteria. A lower temperature during protein expression has been reported to be successful for protein folding within the bacterial cytoplasm in several cases (Basu, Li et al., 2011). It has been seen that some recombinant proteins precipitate when expressed at 37°C, but are soluble when expressed at 15-25°C. This is most likely because slower rates of protein expression allow newly transcribed recombinant protein time to fold properly (A. Singh, Upadhyay et al., 2015). Because of this it was tried to induce the cells at room temperature and using lower inducing concentrations of IPTG for the BcsA_PilZ$_{d1d2}$ protein expression (section 4.11), but unfortunately the BcsA_PilZ$_{d1d2}$ protein was always exclusively found in the cell debris, probably due to formation of inclusion bodies. Inducing at 15°C could also be tried for future experiments, but due to the high degree of the formation of inclusion bodies the best approach could perhaps be to induce the cells to form high yields of inclusion bodies that could subsequently be solubilised and refolded, or alternatively clone the complete *bcsA* gene for expression.

Since the His-tag was placed C-terminally in the BcsA_PilZ$_{d1d2}$ construct during this master thesis work, a closer look if this could affect protein expression was warranted. Previously experiments have been done to investigate if the presence and position of a His-tag had an impact on the formation of inclusion body formation for three model proteins expressed in *E. coli*. The model proteins were uridine phosporylase (UP) from *Aeropyrum pernix* K1, and (+)-γ- lactamase and (−)-γ-lactamase from *Bradyrhizobium japonicum* USDA 6 (Zhu, Gong et al., 2013). The expression vectors pET-28a(+) and pET-30a(+), and *E. coli* BL21 (DE3)

competent cells were also used in these experiments, similar to the work in this thesis . The cells were cultivated in 50ml LB medium containing 20μg/ml kanamycin and were grown in shaking incubator at 37 °C to OD_{600} of 0.5-0.8, and induced with IPTG for an additional 4h. The cell suspensions were then sonicated and centrifugated to separate the soluble and insoluble fractions, and then analysed on SDS-PAGE (Zhu, Gong et al., 2013). It was observed for the UP protein, only the version without a His-tag was successfully expressed in a soluble form, while the UP protein with C- and N-terminal His-tags were expressed as inclusion bodies in the cell. The model protein (−)-γ-lactamase gave a similar pattern as the model protein UP. For the (+)-γ-lactamase protein both the versions with an N-terminal His-tag and without a His-tag were expressed in a soluble form, while the version with a C-terminal His-tag was expressed as an inclusion body in the cell, which indicated that the His-tag at the C-terminus had a negative effect on protein folding (Zhu, Gong et al., 2013). The results in that study provides substantial evidence that the presence of a histidine tail can affect the expression, folding and/or stability of the recombinant proteins, since all the native proteins were expressed in a soluble form (Zhu, Gong et al., 2013). This suggests that the His-tag sequence could potentially interfere with the folding of the BcsA_$PilZ_{d1d2}$ protein in the cytoplasm, and may be a possible reason for the inclusion body formation observed when expressing the protein. Expression from new clones containing an N-terminal His-tag, expressing a his-tagged version of bcsA and/or expressing the native form of BcsA_$PilZ_{d1d2}$ could be tried instead of cloning and expression of the BcsA_$PilZ_{d1d2}$ with a C–terminal his-tagged protein, as was done here.

Cloning of the whole BcsA fragment, instead of just the single domains could be a possible way to go to isolate the whole protein without the formation of inclusion bodies. The $PilZ_{d1d2}$ protein may potentially form inclusion bodies due to not refolding correctly after expression induction. The individual PilZ domain from the *E. coli* YcgR protein was proved to be insoluble during protein purification, and was therefore purified from the inclusion bodies, and resulted in a ≥90% purity of the PilZ domain proteins (Dmitri A. Ryjenkov, Simm et al., 2006), this could be tried in this case also, or alternatively express the full BcsA protein instead, as a possible way for the protein to refold in a correct conformation that does not lead to aggregation of the protein in the cell. Some proteins require all the subunits to be produced in order to form their proper conformation, and when individual subunits are cloned independently the protein can either rapidly degrade or form inclusion bodies.

This was for example observed for penicillin acyclase and the host integration factor proteins in *E. coli* (Kane & Hartley, 1988). Because of this the *bcsA* gene was PCR amplified and purified, and was going to be cloned into pET-26b(+) to be continued working on (section 4.14).

Even though there are several options to go forward for isolating the BcsA protein, none of them are guaranteed to actually work. Even after careful selection of plasmid and host it is not possible to fully predict if a protein will be obtained in high amounts and in a soluble active form (Structural Genomics, Architecture et Fonction des Macromolécules et al., 2008). Some proteins are isolated quickly from the cell, while others need several steps and require more complicated processes. This probably warrants a multiscale approach for further work to express and isolate BcsA protein for future studies of c-di-GMP binding – trying out the various methods mentioned above in parallel experiments.

5.2 Distribution of *bcsA* in the *B. cereus* group

Nineteen out of the 123 sequenced strains representing the *B. cereus* group (figure 4.1) contained a *bcsA* gene sequence, based on more than 90% sequence similarity and a full length BLAST hit to the *B. thuringiensis* Bt407 *bcsA* sequence (Attachment C). Strikingly, all the strains carrying *bcsA* were within the same phylogenetic cluster (Cluster IV) as shown in figure 4.1, except for *B. cereus* AH1271 which maps to cluster II. It is not very common to see this type of phylogenetic clustering within the *B. cereus* group when analysing gene sequence similarities. The strains within cluster IV represent several *B. thuringiensis* strains that are often found in soil with a saprophytic lifestyle, or as an opportunistic pathogen when ingested by insects (NCBI). We can not rule out that the *bcsA* gene in these strains is involved in niche adaption where cellulose synthesis might be necessary. *B. thuringiensis serovar huazhongensis* BGSC 4BD1 matched the *bcsA* sequence of *B. thuringiensis* Bt407 with 97.89%, but was annotated as a hypothetical protein in GenBank.

Several bacterial species harbour the BcsA protein (InterPro, 2017b). The strong phylogenetic clustering observed for *bcsA a*mong *B. cereus* group strain could imply that the *bcsA* gene has been acquired during evolution by the *B. cereus* strains by horizontal gene transfer. Acquisition of DNA by horizontal gene transfer can provide the bacteria a selective advantage like for example local adaption to environmental conditions (Bedhomme, Perez

Pantoja et al., 2017). Horizontal gene transfer of plasmids has been observed among the *B. cereus* group of organisms, and also extensive recombination between species and a low degree of clonality are seen in *B. cereus* and *B. thuringiensis* strains (Böhm, Huptas et al., 2015).

B. cereus AH1271, which showed 89.99% identity with the *bcsA* gene sequence of *B. thuringiensis* 407 was identified as a framshifted pseudogene. Pseudogenes share a close similarity to paralogous functional genes, but are in general not expressed in the cell (Ji Chang, Huang et al., 2013). Pseudogenes can be formed in bacteria because of inactivation and degradation of genes that no longer are needed in the host environment (Lerat & Ochman, 2005), and are often seen in bacterial species undergoing processes such as niche selection or host specialization (Williams, Slayden et al., 2009). This could be the case of the *B. cereus* AH1271 strain, which also is observed in another phylogenetic cluster (II) than the other strains that matched the *bcsA* gene sequence.

Growth curves were performed to get an indication of potential growth differences between *B. thuringiensis* 407 wild type and a *B. thuringiensis* 407 Δ*bcsA* gene deletion mutant (section 4.2), and showed that *bcsA* did not affect growth of the bacteria in the rich growth medium (figure 4.2 and 4.3). If the time frame of this thesis had allowed it, the investigation of whether *bcsA* affects growth in nutrient-poor media could have been performed. Also in previous surveys, preliminary data showed that *bcsA* deletion does not affect biofilm mass in our models (ring and pellicle models) (Sarah Finke, preliminary data). Further work in this respect could include a study of whether biofilm structure is affected by *bcsA* deletion, for example by confocal microscopy.

5.3 Conclusion and future work

In conclusion BcsA is a non-essential protein under rich conditions, and has a very restricted distribution in the *B. cereus* group, being phylogenetically highly clustered. The $PilZ_{d1d2}$ expressed well in *E. coli* BL21 (DE3) host cells, but even under low growth temperatures and low induction conditions seemed to form inclusion bodies in the cell. Future studies to obtain purified protein for thermophoresis could include cloning and expressing the full BcsA protein, varying the position of the His-tag, or trying to reconstitute protein from inclusion bodies formed from the $PilZ_{d1d2}$ recombinant construct.

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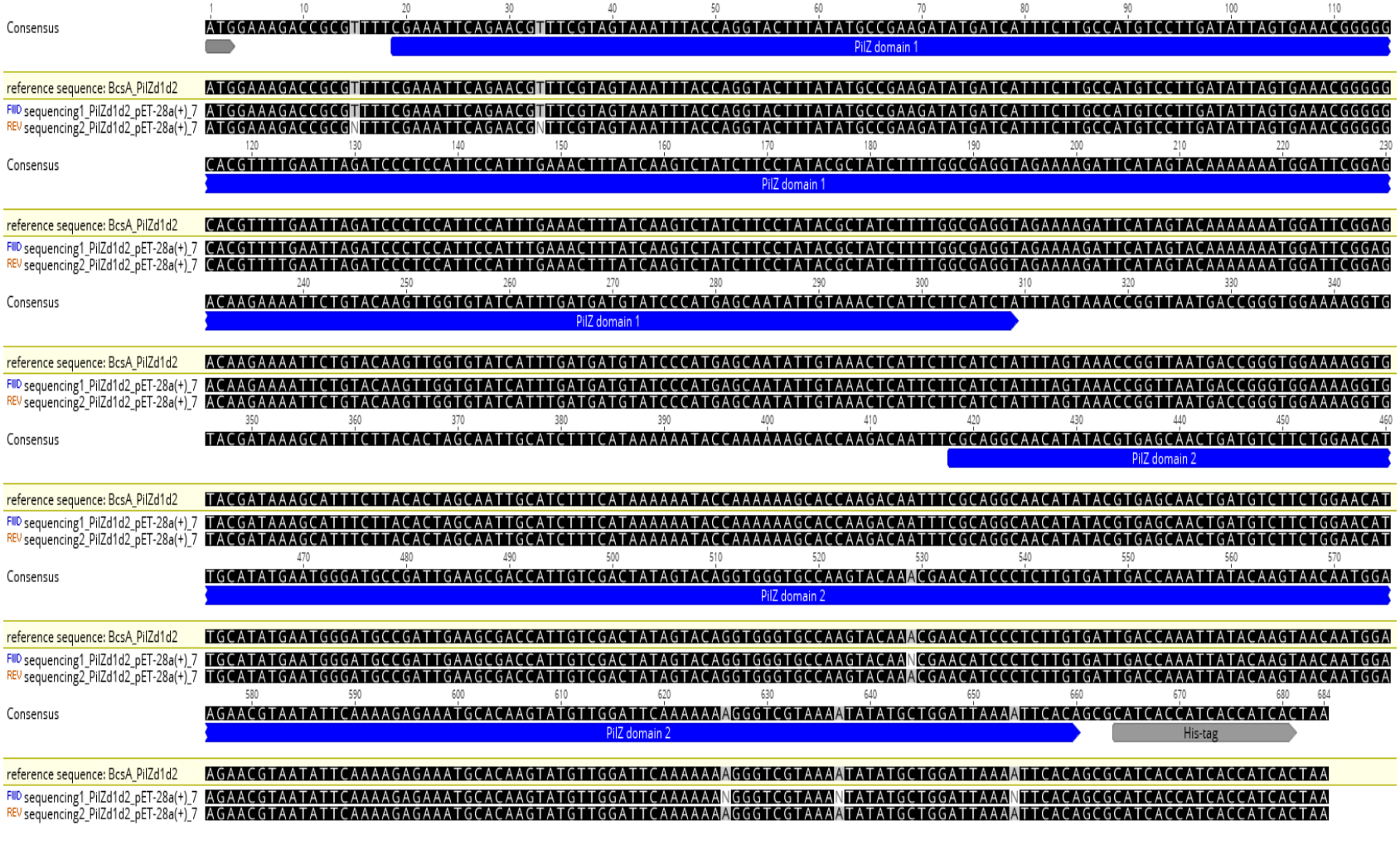
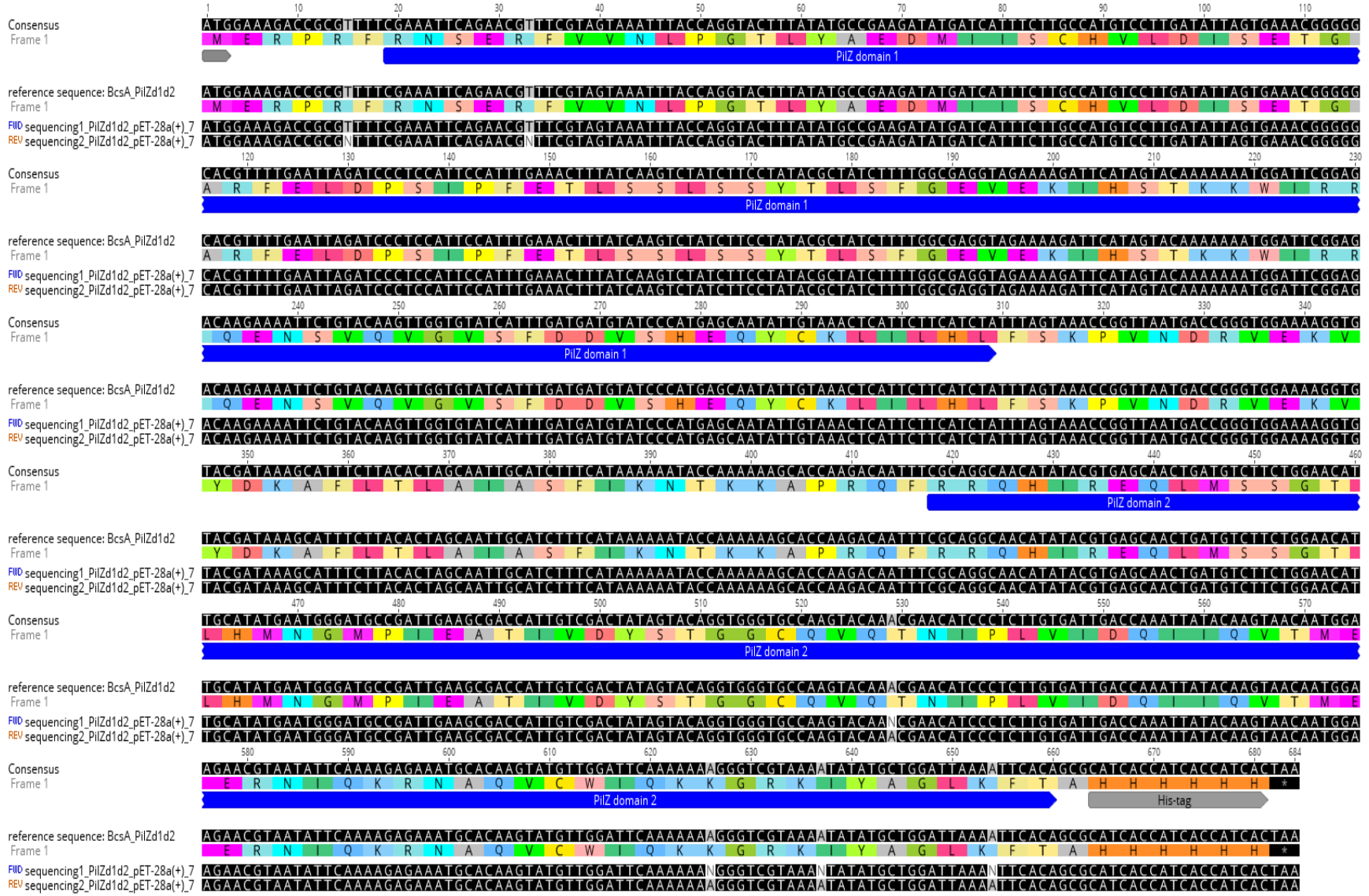
Appendices

Appendix A

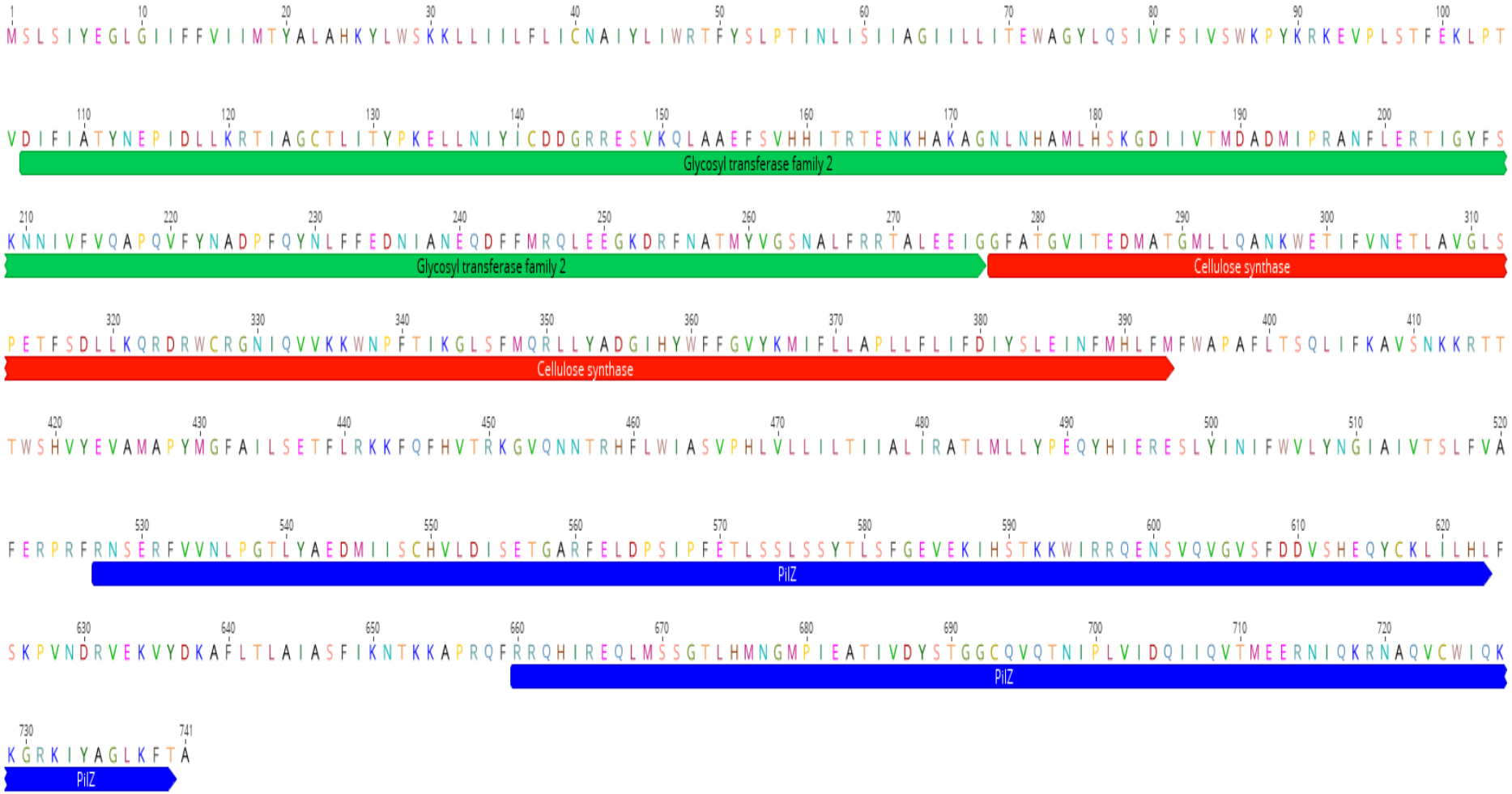
Assembly of DNA sequences obtained for the BcsA_PilZ$_{d1d2}$_pET28a(+) cloned construct used for expression studies (made by Ph.D. student Sarah Finke)



Assembly of DNA sequences obtained for the BcsA_PilZ$_{d1d2}$_pET28a(+) cloned construct used for expression studies with protein translation (made by Ph.D. student Sarah Finke)



BcsA protein with domain annotations according to Pfam (http://pfam.xfam.org/) showing cellulose synthase domain (red) and PilZd1 and PilZd2 domains (blue) (made by Ph.D. student Sarah Finke).



Appendix B

>bcsA:BTB_RS08505_409187965:c1658112-1655687 DNA sequence from *Bacillus thuringiensis* Bt407, complete genome.

ATTTTGTGAT AATGTTTGAC TTGGAAATAA CTAAATCTTT ATAGATGAAT AATATAAAGT TTTTTCCTAT
TTCCCACTCT ATTCATAAAG GAGGAATTGT

1 ATGAGCCTTT CTATATACGA AGGTTTAGGT ATTATTTTTT TCGTAATCAT TATGACTTAC GCCCTTGCAC

71 ACAAATATTT ATGGTCAAAA AAATTACTTA TTATTCTATT TCTCATTTGT AATGCTATTT ATCTTATTTG

141 GCGAACTTTT TATTCGTTAC CAACTATTAA TCTCATAAGT ATTATCGCTG GTATTATATT GTTAATTACA

211 GAATGGGCTG GTTACTTACA ATCTATCGTT TTCAGCATTG TTTCATGGAA GCCATATAAA CGGAAAGAAG

281 TACCATTATC CACTTTCGAA AAACTACCTA CTGTCGACAT ATTTATTGCA ACTTACAATG AACCGATCGA

351 TTTGTTAAAA CGTACTATAG CTGGTTGTAC GTTGATTACT TATCCGAAAG AGTTACTAAA CATTTATATT

421 TGTGACGACG GGCGCCGTGA AAGTGTAAAG CAACTTGCGG CAGAGTTTTC TGTTCATCAC ATAACTCGCA

491 CCGAAAATAA GCATGCAAAA GCTGGAAATT TAAATCATGC GATGCTTCAT TCAAAAGGTG ATATTATCGT

561 AACAATGGAT GCTGATATGA TACCACGAGC TAACTTTTTA GAGCGAACGA TTGGTTATTT TTCTAAAAAT

631 AATATTGTAT TCGTTCAAGC ACCACAAGTT TTTTACAATG CTGATCCTTT CCAATATAAT TTGTTTTTTG

701 AAGATAACAT CGCCAATGAA CAAGATTTCT TTATGCGCCA GTTAGAAGAA GGAAAAGATC GCTTTAATGC

771 TACGATGTAT GTTGGTAGTA ACGCTCTATT TCGCCGCACC GCTCTTGAAG AAATTGGAGG ATTTGCGACT

841 GGAGTTATTA CTGAGGATAT GGCAACTGGT ATGCTACTAC AAGCTAACAA ATGGGAAACT ATATTTGTCA

911 ATGAAACACT TGCTGTTGGA TTATCCCCAG AAACATTTAG TGATTTATTA AAACAACGGG ACCGCTGGTG

981 TAGAGGGAAT ATTCAAGTCG TAAAAAAGTG GAATCCTTTC ACTATAAAAG GATTATCTTT TATGCAGCGT

1051 CTTTTATATG CAGATGGAAT TCATTATTGG TTTTTCGGTG TTTATAAAAT GATATTTTTA CTAGCACCGC

1121 TATTGTTTTT AATATTTGAT ATTTATAGTT TAGAAATTAA TTTTATGCAC TTATTTATGT TCTGGGCACC

1191 TGCTTTTTTA ACATCACAGC TTATTTTCAA AGCTGTTTCT AATAAAAAAC GAACAACTAC GTGGAGTCAT

1261 GTGTACGAAG TTGCTATGGC TCCTTATATG GGATTTGCTA TTTTATCAGA AACTTTCTTA CGTAAAAAAT

1331 TTCAATTTCA TGTGACAAGA AAGGGAGTTC AAAATAATAC AAGGCATTTT TTATGGATTG CAAGCGTCCC

1401 TCACCTCGTA TTACTCATTT TAACAATTAT TGCACTGATT CGGGCAACAC TTATGCTCTT ATATCCTGAG

1471 CAATATCATA TTGAAAGAGA GAGCTTATAT ATTAATATTT TCTGGGTACT ATACAACGGG ATAGCTATTG

1541 TCACTTCACT ATTTGTAGCA TTTGAAAGAC CGCGTTTTCG AAATTCAGAA CGTTTCGTAG TAAATTTACC

1611 AGGTACTTTA TATGCCGAAG ATATGATCAT TTCTTGCCAT GTCCTTGATA TTAGTGAAAC GGGGGCACGT

1681 TTTGAATTAG ATCCCTCCAT TCCATTTGAA ACTTTATCAA GTCTATCTTC CTATACGCTA TCTTTTGGCG

1751 AGGTAGAAAA GATTCATAGT ACAAAAAAAT GGATTCGGAG ACAAGAAAAT TCTGTACAAG TTGGTGTATC

1821 ATTTGATGAT GTATCCCATG AGCAATATTG TAAACTCATT CTTCATCTAT TTAGTAAACC GGTTAATGAC

1891 CGGGTGGAAA AGGTGTACGA TAAAGCATTT CTTACACTAG CAATTGCATC TTTCATAAAA AATACCAAAA

1961 AAGCACCAAG ACAATTTCGC AGGCAACATA TACGTGAGCA ACTGATGTCT TCTGGAACAT TGCATATGAA

2031 TGGGATGCCG ATTGAAGCGA CCATTGTCGA CTATAGTACA GGTGGGTGCC AAGTACAAAC GAACATCCCT

2101 CTTGTGATTG ACCAAATTAT ACAAGTAACA ATGGAAGAAC GTAATATTCA AAAGAGAAAT GCACAAGTAT

2171 GTTGGATTCA AAAAAAGGGT CGTAAAATAT ATGCTGGATT AAAATTCACA GCGTAA

AAAAAGAAAA GAAGCGTACT GCTTCTTTTC TTTTTTACAT GCTAAAATAC

TTATTAAAGG GGGAATTTAT ATGGAAAATG CTTCACAAAA GGCGAATCAT

Marked in blue: predicted PilZ domains
Marked in Yellow: upstream/downstream sequence

>sequence cloniert $PilZ_{d1d2}$

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ATCCCTCCATTCCATTTGAAACTTTATCAAGTCTATCTTCCTATACGCTATCTTTTGGCGAGGTA
GAAAAGATTCATAGTACAAAAAAATGGATTCGGAGACAAGAAAATTCTGTACAAGTTGGTGTATC
ATTTGATGATGTATCCCATGAGCAATATTGTAAACTCATTCTTCATCTATTTAGTAAACCGGTTA
ATGACCGGGTGGAAAAGGTGTACGATAAAGCATTTCTTACACTAGCAATTGCATCTTTCATAAAA
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BcsA protein sequence NCBI Fasta

>gi|445981167|ref|WP_000059022.1| cellulose synthase [*Bacillus thuringiensis*]
MSLSIYEGLGIIFFVIIMTYALAHKYLWSKKLLIILFLICNAIYLIWRTFYSLPTINLISIIA
GIILLITEWAGYLQSIVFSIVSWKPYKRKEVPLSTFEKLPTVDIFIATYNEPIDLLKRTIA
GCTLITYPKELLNIYICDDGRRESVKQLAAEFSVHHITRTENKHAKAGNLNHAMLHS
KGDIIVTMDADMIPRANFLERTIGYFSKNNIVFVQAPQVFYNADPFQYNLFFEDNIAN
EQDFFMRQLEEGKDRFNATMYVGSNALFRRTALEEIGGFATGVITEDMATGMLLQA
NKWETIFVNETLAVGLSPETFSDLLKQRDRWCRGNIQVVKKWNPFTIKGLSFMQRLL
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KKRTTTWSHVYEVAMAPYMGFAILSETFLRKKFQFHVTRKGVQNNTRHFLWIASVP
HLVLLILTIIALIRATLMLLYPEQYHIERESLYINIFWVLYNGIAIVTSLFVAFERPRFRN
SERFVVNLPGTLYAEDMIISCHVLDISETGARFELDPSIPFETLSSLSSYTLSFGEVEKIH
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>BcsA PilZ$_{d1d2}$_design_proteinsequence (from ATG start codon to 6x His-tag)
MERPRFRNSERFVVNLPGTLYAEDMIISCHVLDISETGARFELDPSIPFETLSSLSSYTL
SFGEVEKIHSTKKWIRRQENSVQVGVSFDDVSHEQYCKLILHLFSKPVNDRVEKVYD
KAFLTLAIASFIKNTKKAPRQFRRQHIREQLMSSGTLHMNGMPIEATIVDYSTGGCQV
QTNIPLVIDQIIQVTMEERNIQKRNAQVCWIQKKGRKIYAGLKFTAHHHHHH*

> BcsA PilZ$_{d1}$_design_proteinsequence (from ATG start codon to 6x His-tag)
MERPRFRNSERFVVNLPGTLYAEDMIISCHVLDISETGARFELDPSIPFETLSSLSSYTL
SFGEVEKIHSTKKWIRRQENSVQVGVSFDDVSHEQYCKLILHLFSKPVHHHHHH*

> BcsA PilZ$_{d2}$_design_proteinsequence (from ATG start codon to 6x His-tag)
MAPRQFRRQHIREQLMSSGTLHMNGMPIEATIVDYSTGGCQVQTNIPLVIDQIIQVTM
EERNIQKRNAQVCWIQKKGRKIYAGLKFTAHHHHHH*

Appendix C

BLAST sequence search result using the *Bacillus thuringiensis* Bt407 *bcsA* gene sequence as query of complete genome sequences from the *B. cereus* group, for phylogenetic tree construction (made by Ph.D. student Sarah Finke)

Query sequence id	Subject sequence id	Strain	Percentage of identical matches	Alignment length	Number of mismatches
gi|409187965:c1658012-1655787	NC_018877.1	*Bacillus thuringiensis* Bt407	100.00	2226	0
gi|409187965:c1658012-1655787	NZ_CM000748.1	*Bacillus thuringiensis serovar thuringiensis* str. T01001	100.00	2226	0
gi|409187965:c1658012-1655787	NC_017208.1	*Bacillus thuringiensis serovar chinensis* CT-43	100.00	2226	0
gi|409187965:c1658012-1655787	NZ_CM000753.1	*Bacillus thuringiensis serovar berliner* ATCC 10792	100.00	2226	0
gi|409187965:c1658012-1655787	NC_020376.1	*Bacillus thuringiensis serovar thuringiensis* IS5056	100.00	2226	0
gi|409187965:c1658012	NZ_CM000739.1	*Bacillus cereus* AH1271	89.99	2227	222
gi|409187965:c1658012-1655787	NZ_CM000749.1	*Bacillus thuringiensis serovar sotto* T04001	97.75	2227	48
gi|409187965:c1658012-1655787	NZ_CM000758.1	*Bacillus thuringiensis* IBL 200	97.71	2226	51
gi|409187965:c1658012-1655787	NZ_CM000759.1	*Bacillus thuringiensis* IBL 4222	97.75	2226	50
gi|409187965:c1658012-1655787	NC_018508.1	*Bacillus thuringiensis* HD-789	97.75	2226	50

gi|409187965:c1658012-1655787	NZ_CP009351.1	*Bacillus thuringiensis* HD1002	97.75	2226	50
gi|409187965:c1658012-1655787	NZ_CP010577.1	*Bacillus thuringiensis serovar morrisoni* BGSC 4AA1	97.75	2226	50
gi|409187965:c1658012-1655787	NC_011772.1	*Bacillus cereus* G9842	97.80	2226	49
gi|409187965:c1658012-1655787	NZ_CM001804.1	*Bacillus thuringiensis* DAR 81934	97.80	2226	49
gi|409187965:c1658012-1655787	NC_018500.1	*Bacillus thuringiensis* HD-771	97.84	2226	48
gi|409187965:c1658012-1655787	NZ_CM000756.1	*Bacillus thuringiensis serovar huazhongensis* BGSC 4BD1	97.89	2226	47
gi|409187965:c1658012-1655787	NZ_CP012099.1	*Bacillus thuringiensis* HS18-1	97.98	2226	45
gi|409187965:c1658012-1655787	NZ_CP012483.1	*Bacillus cereus* NJ-W	99.42	2226	13
gi|409187965:c1658012-1655787	NC_022873.1	*Bacillus thuringiensis* YBT-1518	99.91	2226	1
gi|409187965:c1658012-1655787	NZ_CM000725.1	*Bacillus cereus* BDRD-ST196	86.84	76	10
gi|409187965:c1658012-1655787	NZ_CM000725.1	*Bacillus cereus* BDRD-ST196	76.39	72	16
gi|409187965:c1658012-1655787	NZ_CM000719.1	*Bacillus cereus* AH621	78.57	70	15
gi|409187965:c1658012-1655787	NC_018491.1		85.02	574	75

gi|409187965:c1658012-1655787	NZ_CP009746.1		86.84	76	10
gi|409187965:c1658012-1655787	NZ_CP009746.1		76.39	72	16
gi|409187965:c1658012-1655787	NC_018491.1		91.89	37	2
gi|409187965:c1658012-1655787	NC_018491.1		90.91	33	3

Appendix D

Results obtained from analysis of the main protein band from the cell debris fraction, by MALDI-TOF MS analysis at the Proteomics service, Department of Bioscience (Professor Berndt Thiede).

mass	position	#MC	peptide sequence
6167,044	12-67	1	FVVNLPGTLYAEDMIISCHV LDISETGARFELDPSIPFET LSSLSSYTLSFGEVEK
5924,911	142-194	1	QHIREQLMSSGTLHMNGMPI EATIVDYSTGGCQVQTNIPL VIDQIIQVTMEER
5873,889	146-198	1	EQLMSSGTLHMNGMPIEATI VDYSTGGCQVQTNIPLVIDQ IIQVTMEERNIQK
5390,609	146-194	0	EQLMSSGTLHMNGMPIEATI VDYSTGGCQVQTNIPLVIDQ IIQVTMEER
3930,944	78-111	1	QENSVQVGVSFDDVSHEQYC KLILHLFSKPVNDR
3649,799	14824	1	NSERFVVNLPGTLYAEDMII SCHVLDISETGAR
3588,8	41-72	1	FELDPSIPFETLSSLSSYTL SFGEVEKIHSTK
3163,58	12-40	0	FVVNLPGTLYAEDMIISCHV LDISETGAR
3022,482	41-67	0	FELDPSIPFETLSSLSSYTL SFGEVEK
2554,163	77-98	1	RQENSVQVGVSFDDVSHEQY CK
2398,062	78-98	0	QENSVQVGVSFDDVSHEQYC K
1908,107	99-114	1	LILHLFSKPVNDRVEK
1805,91	213-227	1	IYAGLKFTAHHHHHH
1800,031	115-130	1	VYDKAFLTLAIASFIK
1637,963	119-133	1	AFLTLAIASFIKNTK
1551,901	99-111	0	LILHLFSKPVNDR
1294,777	119-130	0	AFLTLAIASFIK
1245,652	199-208	1	RNAQVCWIQK
1217,646	200-209	1	NAQVCWIQKK
1160,525	219-227	0	FTAHHHHHH
1089,551	200-208	0	NAQVCWIQK
991,5254	1-7	1	MERPRFR
880,4774	112-118	1	VEKVYDK
808,406	43045	1	FRNSER