Development of *Bacillus subtilis* spores and cells for surface display of proteins

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To

my parents

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Contents

Zusammenfassung

Die Möglichkeit der Verankerung von Proteinen auf der Oberfläche von Biopartikeln (Phagen, bakterielle und eukaryotische Zellen und bakterielle Sporen) hat Wissenschaftler veranlasst, eine Reihe verschiedener Verankerungssysteme zu entwickeln. Unter den verwendeten Systemen besitzen die Endosporen (Sporen) von *Bacillus subtilis* folgende Vorteile: einfache Produktion, Sicherheit, hohe Stabilität der Sporen bei ihrer Lagerung in getrockneter Form, viele Techniken der genetischen Manipulation verfügbar und weniger Probleme bei der Länge der verankerten Proteine im Vergleich zu Zell- und Phagen-basierten Systemen. Eine Strategie zur Nutzung von *B. subtilis*-Sporen um heterologe Proteine auf der Sporenoberfläche zu präsentieren besteht in der Nutzung von Proteinen der äußeren Sporenhülle (CotB, CotC, CotG) oder der inneren Sporenhülle (OxdD) unter Verwendung der Transkriptions- und Translations-Signale dieser Proteine als Carrier. Diese Strategie garantiert die Verankerung der Fusionsproteine auf der Sporenoberfläche, aber die Menge an produziertem Protein kann nicht kontrolliert werden.

Daher war das erste Ziel dieser Dissertation die Konstruktion eines effektiven Expressionssystems zur Verankerung von Proteinen auf der Oberfläche von Sporen. Es basiert auf der Substitution nativer Promotoren durch zwei verschiedene IPTG-induzierbare Promotoren. Das CotB-Protein wurde zur Verankerung benutzt, und die Expression des *cotB*-Gens erfolgte einmal über den eigenen Promotor, den P_{grac} und den P_{Sgrac} Promotor (beides IPTG-induzierbare Promotoren) als Teil von integrativen oder autonom replizierenden Plasmiden. Zwei verschiedene Reporter-Proteine, die α-Amylase Q von *B. amyloliquefaciens* (AmyQ) und GFP_{uv}, eine Variante von GFP aus der Qualle *Aequoria victoria* mit erhöhter Aktivität, wurden zunächst an das CotB-Protein, dann auch an CotC und CotG fusioniert. Bei nachfolgenden Messungen erwies sich der P_{grac}-Promotor als der Promotor der Wahl. Verglichen mit den nativen Promotoren führte er zu einer Verdoppelung der Menge verankertem Protein. Außerdem ist die Menge an verankertem Protein abhängig von der Gen-Dosis. Unter Verwendung des GFP-Tags konnten die drei Cot-Proteine zum ersten Mal direkt in der Sporenhülle lokalisiert werden.

Das zweite Ziel war die Entwicklung eines Sporen-basierten Expressions- und Reinigungssystems für rekombinante Proteine. Basierend auf diesem System sollten Proteine, die nach cytoplasmatischer Überexpression Aggregate bilden, auf der Sporenoberfläche verankert

werden. Dabei wurde zwischen dem Carrier-Protein (einem Cot-Protein) und dem rekombinanten Protein ein Mini-Intein eingefügt, welches in der Art und Weise verändert war, dass es nur noch die Spaltstelle zwischen dem Intein und dem rekombinanten Protein nach Aktivierung endonucleolytisch spaltet. Im vorliegenden Fall stammte das Mini-Intein von dem Ssp *dnaB*-Gen, wobei seine Protease-Aktivität durch Erniedrigung des pH-Wertes im LB-Medium induziert wurde. Im vorliegenden Fall wurde das Mini-Intein zwischen CotB and AmyQ eingebaut, und die Abspaltung von AmyQ an gereinigten Sporen durch Messung der α-Amylase Aktivität im Überstand gemessen. Die Ergebnisse zeigten, dass Sporen als Carrier für rekombinante Proteine benutzt werden können. Allerdings kommt es unter den getesteten Bedingungen auch zu einer pH-Wert unabhängigen Aktivierung der Protease-Aktivität des Mini-Inteins. Daher sind weitere Experimente notwenig, um das System zu optimieren.

Das dritte Ziel der Dissertation war die Entwicklung von Cellulose-Chips durch Verankerung von Cellulasen auf der Zelloberfläche und auf Sporen. Die Verankerung auf der Zelloberfläche sollte mittels eines Sortase-Systems erfolgen. Als Modell-Cellulase wurde CelA aus *Clostridium thermocellum* ausgewählt. Allerdings erwies sich die Verankerung von CelA auf der Zelloberfläche als ineffektiv. Dies könnte auf einer ineffizienten Interaktion zwischen der Cellulase und der Sortase hinweisen. Im Gegensatz dazu konnte CelA erfolgreich auf der Sporenoberfläche verankert werden, wenn CotB oder CotG als Carrier-Protein verwendet wurden. Um den Abbau von CMC nachzuweisen, mussten Zellen oder Sporen aus bis zu einem Liter präpariert werden. Dies deutet auf eine geringe Aktivität von CelA hin. Weitere Studien sollten mit anderen Cellulasen durchgeführt werden, um Cellulase-Chips zu optimieren.

Summary

Surface display has attracted the attention of researchers in developing efficient display systems expressing heterologous polypeptides on the surface of bioparticles such as phages, bacterial and eukaryotic cells and bacterial spores. Among these bioparticles, the endospore from *B. subtilis* has advantages, including feasibility of production, safety feature, the robustness of the bacterial spore allowing storage in the desiccated form, a technological platform supported by extensive tools for genetic manipulation and less size restrictions of the displayed proteins compared to cell- and phage-based systems. A strategy to engineer *B. subtilis* spores to display heterologous protein on their surface is to use outer spore coat proteins (CotB, CotC, CotG) or an inner-coat protein (OxdD) with the coat genes' transcriptional and translational signals as carriers (Isticato *et al.*, 2001; Mauriello *et al.*, 2004; Hinc *et al.*, 2010; Zhou *et al.*, 2008a; Potot *et al.*, 2010; Kim *et al.*, 2005a; Kwon *et al.*, 2007). This strategy guarantees the timing for fusion protein synthesis during coat formation, but the amount of produced fusion proteins cannot be controlled.

Therefore, the first aim of this doctoral thesis focused on construction of more effective expression systems for spore surface protein anchoring. A novel approach of substitution of native promoter by two different IPTG-inducible promoters to the increase the production of fusion protein is presented here. CotB was used and the expression of the *cotB* gene was regulated by either its own promoter, the P_{grac} and the P_{Sgrac} promoter in a series of plasmids which can be integrated into or replicated independently of the *B. subtilis* chromosomal DNA. Two reporter proteins, α-amylase Q from *B. amyloliquefaciens* (AmyQ) (Palva, 1982) and GFP_{uv} – an enhanced version from the GFP protein of the jellyfish *Aequorea victoria* (Crameri *et al.*, 1996), were fused downstream of the CotB protein. To assess the enhancement of GFPuv displayed on the spore surface, CotC and CotG were similarly examined. The results indicated that the P_{grac} promoter is a suitable, hence recommended as a promoter of choice. Substitution of the native promoter by P_{grac} promoter, the amount of proteins displayed per spore can be increased two-fold. Furthermore, the display of heterologous proteins on the spore surface when using different carriers is gene dosage dependent. And for the first time, the tendency of the three Cot proteins' localization on the spore coat compartment is reported using the GFPuv tag.

Second, a new *B. subtilis* spore-based system for protein expression and purification was developed. Using this system, proteins prone to form inclusion bodies can be anchored on the spore surface, separated by a mini-intein derived from the *SSp* DnaB, which was then used as self-cleaving tag for purification by shifting the pH and/or temperature conditions, with no addition of any proteases or thiol reagent (Mathys *et al.*, 1999). To construct the system, the mini-intein was fused downstream of the CotB protein, followed by the reporter protein AmyQ. By changing the pH of the buffer, the mini-intein self-cleaving process was induced followed by the release of α-amylase into the supernatants. This observation suggests the use of the *B. subtilis* spores as an effective and low cost tool for protein purification. However, concerns related to premature of the pH-inducible mini-intein and auto-release of coat protein raise the question about the stability of the fusion coat-heterologous protein on the spore surface using the system. Hence, further investigation is needed to achieve a usable spore-based purification system.

The last aim of the thesis was to apply the newly constructed *B. subtilis* spore display and the cell surface display systems (Nguyen and Schumann, 2006) to generate cellulose chips, in which enzymes were immobilized on the surface of microorganism cells or spores. The cellulase A (CelA) from *C. thermocellum* (Beguin *et al.*, 1985) was utilized as a model enzyme. Unfortunately, the results showed an ineffective anchoring of CelA on the cell wall. This indicates the unsuccessful creation of cell-based cellulase chip when using the SrtA transpeptidase. In contrast, CelA was verified to be successfully displayed on the spore surface using CotB and CotG, but not CotC, as carriers. In general, a large volume of culture (up to one liter) must be prepared containing both cells and spores displaying CelA on the surface to assure sufficient CMC degradation. This might indicate a low activity of CelA. Further works should be done in selection of cellulase and improvement of the systems to generate the more effective cellulase chips.

1 Introduction

1.1 Microbial surface display

Surface display is a molecular biological technique by which heterologous peptides and proteins are immobilized on the exterior of phages, cells or spores (Kim and Schumann, 2009). Since the first surface expression system based on the M13 phage was published by Smith in 1985 (Smith, 1985), many other phage and microbial display systems have been studied and developed (Kim and Schumann, 2009). Surface display technology has shown a wide range of biotechnological and industrial applications including: development of live vaccines (Lee *et al.*, 2000); screening displayed peptide libraries; antibody production; bioadsorbents for the removal of harmful chemicals and heavy metals; whole-cell biocatalysts by immobilizing enzymes; development of biosensors (Lee *et al.*, 2003;Benhar, 2001), etc. Thus, it has become more and more attractive to scientists.

1.1.1 Phage display

Bacteriophages (also simply called phages) are a diverse group of viruses that use prokaryotes as specific host-cells. Due to their genetic and structural simplicity and their ability to simply grow on bacterial hosts in the laboratory, phages have been extensively used in basic and applied life sciences since their discovery in the early twentieth century (Pennazio, 2006; Sulakvelidze *et al.*, 2001). With the advent of genetic engineering in the late 1970s, phage-based vectors were also among the first cloning vehicles (Maniatis *et al.*, 1978; Zacher, III *et al.*, 1980; Hines and Ray, 1980).

A technique for displaying foreign peptides or proteins on the surface of a phage particle is called phage display. In the first phage display system, developed in 1985, Smith cloned a fragment of the *Eco*RI restrictase gene in the middle section of the gene III to create fusions to the minor capsid protein p3 (product of gene III) of the non-lytic filamentous phage M13 and demonstrated that the fusion can be well tolerated by the phage. Moreover, Smith was able to enrich the 'fusion phage' by affinity capture with polyclonal antibodies against *Eco*RI, this technique was later called biopanning (Smith, 1985). Besides the M13 phages, display systems have also been developed for other phages like the fd filamentous phage (Smith and Petrenko, 1997; Kehoe and Kay, 2005), λ

(Sternberg and Hoess, 1995; Hoess, 2002), T4 (Efimov *et al.*, 1995; Ren and Black, 1998; Li *et al.*, 2006), and T7 (Houshmand *et al.*, 1999).

In most of the phage display systems, the proteins or peptides to be displayed are usually expressed as fusions with the coat protein pIII or pVIII of phages. Such fusion proteins are directed to the bacterial periplasm or inner-cell membrane by an appropriate signal sequence that is added to their N-terminus (Endemann and Model, 1995). During the phage assembly process, the fusion proteins are incorporated into the nascent phage particle. The genetic information encoding the displayed fusion protein is also packaged inside the same phage particle in the form of a single-stranded DNA (ssDNA) molecule. Hence, the genotype–phenotype coupling occurs before the phages are released into the extracellular environment, ensuring that phages produced from the same bacterial cell clone are identical. In this manner, huge phage display libraries can be created from batch-cloned gene libraries.

The first phage system was developed for affinity selection of protein fragment expressed from a cDNA library (Smith, 1985). Since, others have been constructed; the most well-known application for phage display turns out to be affinity selection of peptide/protein (McCafferty *et al.*, 1990; Marks *et al.*, 1991; Hoogenboom *et al.*, 1998; Makvandi-Nejad *et al.*, 2010). Antibody phage libraries are now the most widely used and most commercially successful application of phage display (Paschke, 2006). Many technical improvements have emerged in the field of antibody phage display. Phage display is also used for detection of biological threat agents (Petrenko and Vodyanoy, 2003; Turnbough, Jr., 2003) and finding new ligands to target proteins (Bratkovic *et al.*, 2005; Lunder *et al.*, 2005). Recently, it has been determined to be a useful tool to engineer protease inhibitors (Zani and Moreau, 2010). Phage display nowadays is the main method for the study of protein-protein, protein-peptide, and protein-DNA interactions.

1.1.2 Bacterial surface display

The bacterial display systems whose construction based on OmpA (Freudl *et al.*, 1986) and LamB (Charbit *et al.*, 1986) proteins of *E. coli* were firstly reported in 1986. Since then, many different bacterial display systems have been developed using both Gram-negative and Gram-positive bacteria as host strains (Benhar, 2001; Samuelson *et al.*, 2002).

1.1.2.1 Cell surface display in Gram-negative bacteria

A common character of Gram-negative bacteria is to have three principal layers in the envelope. They are the outer membrane (OM), the peptidoglycan cell wall, and the cytoplasmic or inner membrane (IM). The inner membrane has a typical phospholipid bilayer structure. The outer membrane consists of two different leaflets. The inner leaflet is composed of phospholipids and the outer leaflet is composed of glycolipids, principally lipopolysaccharide (Silhavy *et al.*, 2010). Therefore, target proteins need to be led to the envelope and must cross through the IM, periplasm and the OM before being able to be displayed on the surface of Gram-negative bacteria. In order to achieve surface exposure of heterologous proteins on Gram-negative bacteria, the primary choice is to use secretion-competent proteins as carriers for the protein of interest. This process is known to be critically dependent on properties of the heterologous peptide/protein to be displayed and the carrier (Sandkvist and Bagdasarian, 1996).

The OM proteins (OMPs) are promising carriers for this purpose. In *E. coli*, the OMPs such as the maltoporin LamB (Charbit *et al.*, 1986; Steidler *et al.*, 1993; Sousa *et al.*, 1998), the outer membrane protein OmpA (Freudl *et al.*, 1986; Yang *et al.*, 2008a; Verhoeven *et al.*, 2009) and the phosphate-inducible porin PhoE (Agterberg *et al.*, 1987; Agterberg *et al.*, 1990) are used as anchoring motifs. The Lpp-OmpA system, which is comprised of the signal sequence and first nine amino acids of the major lipoprotein, Lpp, fused to a region encompassing either three or five transmembrane helices of OmpA to form a chimera (Georgiou *et al.*, 1996; Francisco *et al.*, 1992; Francisco and Georgiou, 1994; Daugherty *et al.*, 1998), has been shown to give efficient translocation and surface anchoring of the fused gene products, resulting in a high number of chimeric surface proteins present in an accessible form on *E. coli* cells (Earhart, 2000; Yang *et al.*, 2008b; Yang *et al.*, 2008a). In other Gram-negative bacteria, OMPs have been widely employed as well as for developing surface display systems. For example, OmpS was used to localize the receptor-binding region of PapG protein on V*ibrio cholera* (Lang and Korhonen, 1997; Lang *et al.*, 2000). Part of the outer membrane protein OmpU, or Omp26La and the outer membrane lipoprotein Wza from *V. anguillarum* have been used to develop new surface display systems (Yang *et al.*, 2008c). Additionally to the OMPs, lipoproteins such as the TraT lipoprotein (Chang *et al.*, 1998; Taylor *et al.*, 1990), the peptidoglycan-associated lipoprotein PAL (Dhillon *et al.*, 1999) and the ice-nucleation protein INP (Jung *et al.*, 1998a; Xu *et al.*, 2008; Yang *et al.*, 2008b) are also used as anchoring motifs for exploiting the bacterial wall and membrane. Proteins from the filamentous

structures present on gram-negative bacteria, including fimbria protein FimA (Hedegaard and Klemm, 1989), pili protein F Pilin (Malmborg *et al.*, 1997), etc. have been employed for surface-expression purposes.

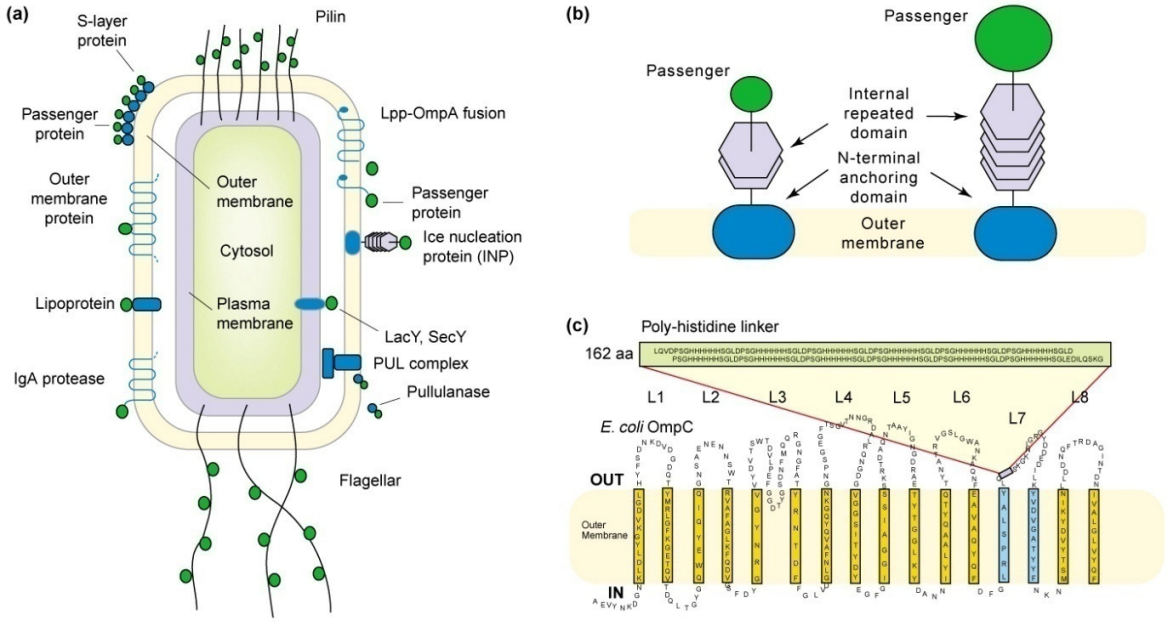

Fig 1.1 ***Cell surface display systems in Gram-negative bacteria.*** *Green circles represent heterologous passenger proteins. (a) Surface display systems developed in Gram-negative bacteria: S-layer protein, OmpC, PhoA, OmpF, OmpA, lipoprotein, IgA protease, pilin, Lpp–OmpA, INP and flagella. (b) Cell-surface display system using ice nucleation protein (INP), which is a representative example of the N-terminal fusion method. The INP is the most stable and useful carrier to express foreign proteins as large as 60 kDa. (c) Cell-surface display system using E. coli outer membrane protein OmpC, which is a representative example of the sandwich fusion method. In this system, poly-histidine (poly-His) peptides of up to 162 amino acids could be inserted into the seventh external loop (L7) of OmpC and could be efficiently exposed on the E. coli cell surface (Lee et al., 2003).*

There are many other different carriers such as autotransporters, secreted proteins and proteins from S-layers (Samuelson *et al.*, 2002; Lee *et al.*, 2003) have been employed for developing more efficient systems for displaying the proteins on Gram-negative bacteria (see ***Fig 1.1*** for more

detail). Each type of carrier has different characteristics and might thus be useful for specific applications. Therefore, this field can still be very attractive for researchers.

1.1.2.2 Cell surface display in Gram-positive bacteria

Unlike Gram-negative bacteria, Gram-positive bacteria only have a single membrane covered by a very thick cell wall in the envelope structure. This introduces a different situation for the surface display of target proteins since most of the anchoring motifs were developed based on surface proteins of Gram-positive bacteria. In more than 100 known cell-wall proteins of Gram-positive bacteria (Ton-That *et al.*, 1997), many share some conservative features needed for cell wall anchoring: an N-terminal signal peptide for translocation through the cellular membrane and a C-terminal cell wall sorting signal for anchoring to the cell wall of the bacteria (Schneewind *et al.*, 1992). The sorting signal has been extensively studied and it consists of: (i) a conserved pentapeptide motif, LPXTG (in single-letter amino acid code, where X denotes any amino acid); (ii) a hydrophobic stretch of 15–22 aa; and (iii) a short charged tail (6–7 aa) (Schneewind *et al.*, 1993). The LPXTG motif contains a cleavage site for sortase (SrtA), which is an enzyme of 206 amino acids that cleaves polypeptides between the threonine (T) and the glycine (G), then anchors the polypeptides covalently to the cell wall (Fischetti *et al.*, 1990; Marraffini *et al.*, 2006). The hydrophobic domain serves as a membrane-spanning region, the charged tail is a retention signal to prevent secretion of the polypeptide chain into the surrounding medium (Schneewind *et al.*, 1993). The sorting signal from Staphylococcal protein A (SpA) has been used to develope several surface display systems for *Staphylococcus xylosus* (Hansson *et al.*, 1992; Nguyen *et al.*, 1995; Liljeqvist *et al.*, 1997), *S. carnosus* (Liljeqvist *et al.*, 1997; Wernerus *et al.*, 2001), *Lactococcus lactis* (Steidler *et al.*, 1998) and *B. subtilis* (Nguyen and Schumann, 2006). The *S. gordinii* surface display system uses the sorting signal from the M6 protein of *Streptococcus pyogene1s* to achieve surface exposure of various chimeric surface proteins (Pozzi *et al.*, 1992; Medaglini *et al.*, 1995).

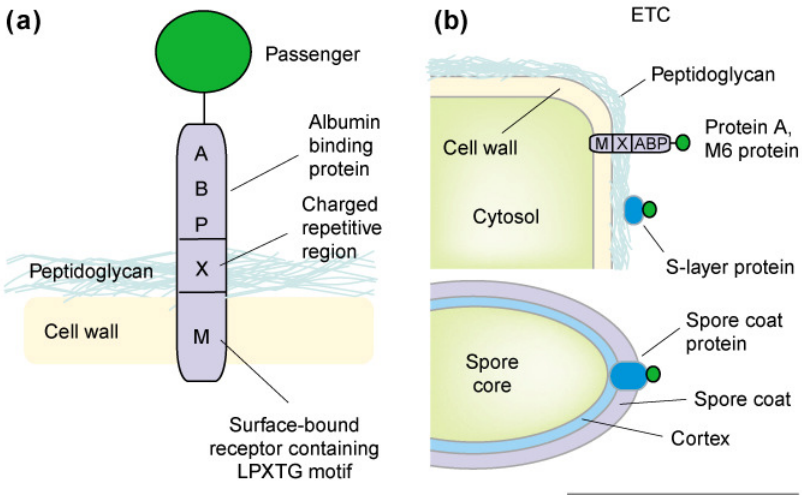

Fig 1.2 ***Cell-surface display systems in Gram-positive bacteria.*** *Green circles represent heterologous passenger proteins. (a) Cell-surface display system using staphylococcal protein A, which is a representative example of the N-terminal fusion method. (b) Schematic illustration of surface display systems constructed in Gram-positive bacteria (Lee et al., 2003).*

Besides the sorting signal from surface proteins, other types of carrier proteins have also been used. The S-layer homology (SLH) domain, which is present in singles or in multiples at the N-terminus of Gram-positive S-layer proteins and consist of residues of 70 amino acids, has been found to mediate association of SLH-domain-bearing proteins to the polymers of the secondary cell wall, which are linked covalently to the peptidoglycan layer. The SLH domain of the *B. anthracis* S-layer protein EA1 has been used to display levansucrase of *B. subtilis* and tetanus toxin fragment C in *B. anthracis* (Mesnage *et al.*, 1999a; Mesnage *et al.*, 1999b). The antigenic domain of the *Yersinia pseudotuberculosis* invasin has been displayed on *B. subtilis* by fusing to the cell membrane lipoprotein DppE or the cell wall bound autolysin modifier protein CwbA of this bacterium (Acheson *et al.*, 1997). The molecular chaperone PrsA of *B. subtilis,* whose location is predicted to be outside of cytoplasmic membrane, was also used for development of a membrane surface display system (Kim *et al.*, 2005b). Interestingly, the PgsA, a transmembrane protein derived from the poly-γ-glutamic acid synthetase complex (the Pgs-BCA system) of *B. subtilis* (Ashiuchi *et al.*, 1999) has been employed to develope several surface display systems for both Gram-negative (*E. coli*) (Narita *et al.*, 2006a; Narita *et al.*, 2006b) and Gram-positive (*Lactobacillus casei*) (Lee *et al.*, 2006; Poo *et al.*, 2006; Hou *et al.*, 2007) bacteria.

1.1.2.3 Bacterial spore surface display

The spore (endospore) is a tough and non-reproductive structure produced by certain genera of bacteria, which allows these bacteria to go dormant under unfavorable conditions. These are observed in *Bacillus* and related aerobic endospore-forming bacteria (a group of some 200 species, distributed over 25 genera) as well as by *Clostridium* (Fritze, 2004). The process of spore formation and spore coat assembly are quite similar in endospore-forming bacteria and were mentioned in section ***1.2*** in case of *B. subtilis*. Bacterial spore display systems may provide a durable supporting matrix similar to that of chemical polymer beads, which can easily and economically be produced in large quantities. Using endospores for surface display may also help to prevent the problem of size-limiting which seems the biggest disadvantages of other microbial surface display systems. Because the formation of endospores takes place within the sporulating cell, all components of the spore including anchored proteins are produced inside the cytoplasm and then directed on the spore surface. Thus, they do not have to cross the cell membrane and the potention of being stuck at cell membrane or breaking the cell are solved (Kim and Schumann, 2009). Unfortunately, no spore-surface proteins containing cell-wall-sorting signals have yet been isolated among the genera of *Bacillus*, which makes the strategy of using a versatile anchoring motif become incapable. Therefore, the spore-surface proteins are more promising for serving as carriers.

Compared to other *Bacillus* species, *B. subtilis* has more advantages in developing spore surface display systems due to the detailed knowledge of its spore structure (Driks, 1999; Henriques and Moran Jr, 2007), the availability and ease of advanced genetic tools (Cutting and Vander Horn, 1990) and genomic data (Kunst *et al.*, 1997) that facilitate the construction of recombinant spores. Therefore, most of spore surface display systems are developed with this species. As mentioned in section ***1.2.3***, the *B. subtilis* spores are surrounded by a coat, a proteinaceous structure organized into two layers, the inner and the outer coat (Errington, 1993; Errington, 2003; Driks, 2004). For construction of spore surface display systems, the outer-coat proteins were preferred (***Fig 1.3***). Several outer-coat proteins such as CotB (Isticato *et al.*, 2001; Duc *et al.*, 2007; Hinc *et al.*, 2010), CotG (Hinc *et al.*, 2010; Kim *et al.*, 2005a; Kim *et al.*, 2007; Kwon *et al.*, 2007) and CotC (Mauriello *et al.*, 2004; Hinc *et al.*, 2010; Zhou *et al.*, 2008a) were succesfully used as anchoring motifs for displaying different heterologous proteins on the *B. subtilis* spore surface. Recently, Potot *et al.* have proven the posibility of using an inner-coat protein, OxdD, as original anchoring motif to display proteins of biological interest at the *B. subtilis* spore surface (Potot *et al.*, 2010).

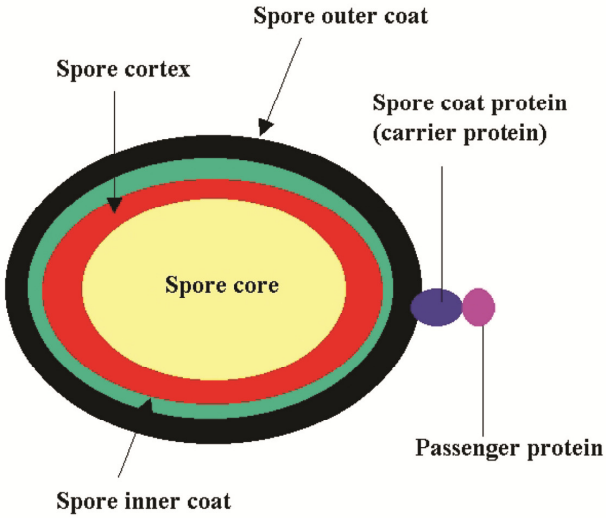

Fig 1.3 ***Spore-surface display using spore coat proteins.*** *The B. subtilis spore is composed of an internal core (yellow) surrounded by a peptidoglycan-like cortex (in red) and a proteinaceous coat sub-divided into an inner (green) and an outer (black) part. The fusion protein, composed of a carrier (blue) and a passenger (purple) part is exposed on the spore surface (Ricca and Cutting, 2003).*

In addition to *B. subtilis* systems, a surface display system using a 130 kDa protoxin, whose presence is abundant in the *B. thuringiensis* spore coat, as the carrier protein was constructed. This model has been succesfully used to display green fluorescent protein and a single-chain antibody (scFv) by substitution of the N-terminal portion of the protoxin with the heterologous proteins (Du *et al.*, 2005). Another system based on the InhA, an exosporium component of *B. thuringiensis* has been developed and shows the expression of green fluorescent protein and active ß-galactosidase on the spore surface (Park *et al.*, 2009).

1.1.3 Surface display in yeast

A significant feature of the yeast surface display system is its employment of a eukaryotic host possessing the secretory biosynthetic apparatus for promoting efficient oxidative protein folding and N-linked glycosylation. Most of the display systems in yeast up to now are developed in *Saccharomyces cerevisiae*. The cell wall of *S. cerevisiae* consists of two types of mannoproteins: SDS-extractable and glucanase-extractable mannoproteins. Many glucanase-extractable mannoproteins have been found to generally contain a putative glycosyl phosphatidylinositol (GPI)

attachment signal, which helps the proteins to be tranferred to the secretory pathway and anchored to the cell wall, at the C-termini (van, V *et al.*, 1997). Most of the cell-surface display systems developed for yeast are GPI anchor-dependent. *S. cerevisiae* α-agglutinin, whose component contains a putative GPI attachment signal (Roy *et al.*, 1991), has widely been used to display various peptides and proteins such as hepatitis B virus surface antigen, lipase, glucoamylase, green fluorescent protein (GFP) and blue fluorescent protein (BFP) (Schreuder *et al.*, 1996; Shibasaki *et al.*, 2001). Some newly identified yeast cell-wall proteins, such as Cwp1p, Cwp2p, Tip1p, Tir1p/Srp1p and Sed1p, have been proven capable of displaying α-galactosidase (van, V *et al.*, 1997) and GFP on the surface of *S. cerevisiae*.

1.1.4 Applications of microbial surface display

One of the most common applications of bacterial surface display has been the development of live vaccine-delivery systems, with cell-surface displayed heterologous antigens for the induction of antigen-specific antibody responses (Stover *et al.*, 1993; Haddad *et al.*, 1995; Nguyen *et al.*, 1995; Georgiou *et al.*, 1997; Titball *et al.*, 1997) for mucosal immunization. To generate live bacterial vaccines, two different types of bacteria have been used: (i) the normally pathogenic bacteria that have been subjected to attenuation, such as Gram-negative *Salmonella* spp (Dertzbaugh, 1998) and (ii) the Gram-positive *Mycobacterium bovis* strain BCG (Stover *et al.*, 1993; Langermann *et al.*, 1994), or non-pathogenic commensal or food-grade bacteria, such as *S. gordinii* and several staphylococcal and lactic acid bacteria, respectively (reviewed in (Fischetti *et al.*, 1996; Stahl and Uhlén, 1997; Wernerus and Stahl, 2004)). Non-pathogenic or food-grade bacteria for that purpose are more favorable due to the fact that they are generally regarded as safe (GRAS) for human use, inexpensive, easy to administer and capable of inducing both a local secretory IgA response at the site of pathogen entry and a systemic immune response (Pozzi and Wells J.M., 1997). To overcome the situation of less efficiency in generating strong antibody responses, several stratagies for improving the immune response have been described and involve the co-display of adhesins that will assist in targeting to the mucosal epithelium (Liljeqvist *et al.*, 1999; Cano *et al.*, 2000). The ability to withstand the harsh conditions (which can be expected during vaccine storage and transportation) can make spore-based vaccines be an attractive alternative. It has been shown that the heterologous antigens which were exposed on *B. subtilis* spores by being fused to outer-coat proteins, CotB and CotC, can stimulate the immune system of mice to produce antigen-specific antibodies (Isticato *et al.*, 2001; Duc *et al.*, 2007; Zhou *et al.*, 2008b).

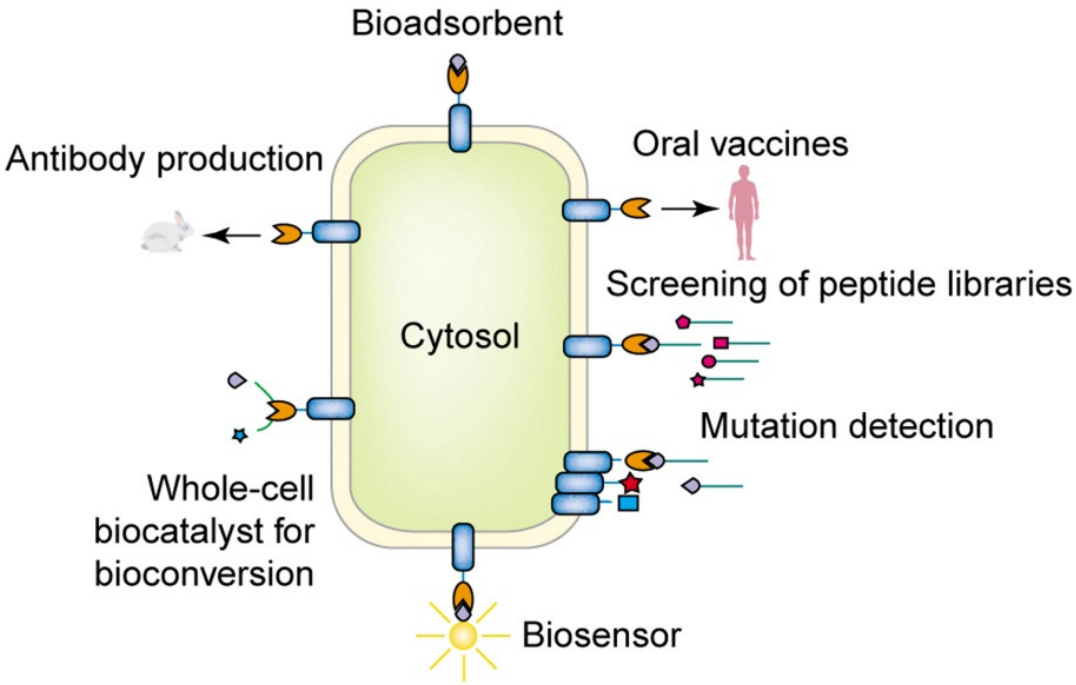

Fig 1.4 Applications of microbial cell surface display (Lee et al., 2003).

Surface display has also been used for displaying antibody fragments and other binding proteins. Phage display is by far the major tool for the isolation and engineering of recombinant antibodies (Benhar, 2001). Initial attempts to display Fab fragments fused to pVIII, the phage major coat protein, were also successful (Gram *et al.*, 1992) although this site was then shown to be unsuitable for the efficient display of large polypeptides such as antibodies. Since then, many other systems have been optimized for antibody display and make it the leading tool in antibody engineering during the last decade (Benhar, 2001). The expression of functional antibodies on the surface of *E. coli* (Fuchs *et al.*, 1991; Little *et al.*, 1993; Francisco *et al.*, 1993; Maurer *et al.*, 1997) and Staphylococci (Gunneriusson *et al.*, 1996) has led to discussions of whether this strategy would be used to create inexpensive diagnostic tools or alternatives to the rapidly developing phage technology for the selection of peptides or recombinant antibody fragments from large libraries (Little *et al.*, 1993; Georgiou *et al.*, 1997). The Lpp-OmpA system was applied for the development of a quantitative immunoassay that utilizes *E. coli* bacteria expressing scFv antibody fragments attached to the cell surface (Chen *et al.*, 1996). This was also applied for antibody affinity

maturation by bacterial surface display of scFv libraries utilizing a useful technique: fluorescence-activated cell sorting (FACS) (Daugherty *et al.*, 1998). A possible advantage of the bacterial-display systems over the phage-display techniques is that bacterial selection can be accomplished through FACS technology using fluorescently labeled antigens. This avoids crucial steps in phage-display selection procedures such as immobilization of the antigen, elution of bound phage and reinfection of bacteria with eluted phages (Francisco and Georgiou, 1994). Phages, in general, are too small to be compatible with current FACS technology. Yeast cells have been also given the GRAS status, and are capable of displaying large proteins and expression of the antibody on the yeast cell surface can be monitored by FACS (Feldhaus and Siegel, 2004). Yeast display of proteins, specifically individual scFv clones, was originally presented by Boder and Wittrup (Boder and Wittrup, 1997). Yeast display was also used for the discovery and characterization of novel affinity reagents from a large (10^9) non-immune human scFv library (Feldhaus *et al.*, 2003).

One of the other less common applications is peptide display for mapping epitope. Phage display of random peptide libraries has been extensively utilized for epitope mapping (Dunn, 1996; Smith and Petrenko, 1997). In another example, a random peptide library in a conformationally constrained thioredoxin region was introduced into the flagellin gene of *E. coli* and thus exposed on the *E. coli* flagellum surface (Lu *et al.*, 1995). This system was capable of identifying the epitope sequences for the three antibodies tested.

Another application of microbial surface display is whole-cell biocatalysis. The targeting of biologically active proteins to microbial surfaces creates potential applications for biomedical and biotechnological use. A number of enzymes have been displayed on phage (Soumillion *et al.*, 1994; Maruyama *et al.*, 1994; Mikawa *et al.*, 1996; Pedersen *et al.*, 1998; Demartis *et al.*, 1999; Legendre *et al.*, 2000), bacteria (Francisco *et al.*, 1992; Strauss and Götz, 1996; Jung *et al.*, 1998b; Jung *et al.*, 1998a) and spores (Kwon *et al.*, 2007; Park *et al.*, 2009). A good practical example of what genetically enzyme-coated bacteria can be used for is in biosensor technology. Mulchandani *et al.* used recombinant *E. coli* cells with surface expressed organophosphorus hydrolase in a biosensor format for direct determination of organophosphate nerve agents: The bacteria were immobilized and connected to either a fiber-optic bundle (Mulchandani *et al.*, 1998a) or a potentiometric device (Mulchandani *et al.*, 1998b) for signal transduction. Both types of biosensors showed good sensitivity and selectivity, and exhibited very good stability when used repeatedly more than 75 times. More recently, bacterial surface display was applied for enzyme evolution. Large libraries of

engineered enzymes have been displayed on the surface of bacteria with the purpose of selecting enzyme variants with novel substrate specificities (Olsen *et al.*, 2000) or improved substrate catalysis (Kim *et al.*, 2000). Based on differences in growth rates, Kim *et al.* selectively screened for improved variants of carboxymethyl cellulose (CMCase) displayed on *E. coli*, when the bacteria were grown on CMCase plates (Kim *et al.*, 2000). The yeast *S. cerevisiae* has long been utilized in fermentation for the production of food, pharmaceuticals, bioactive compounds, alcohol, etc. Amylolytic enzymes, cellulose-lytic enzymes and lipases have been successfully displayed and their functional activities on yeast surfaces proven (Ueda and Tanaka, 2000; Shibasaki *et al.*, 2009). Hence, it helps enhancing the abilities and potentials of yeast as whole-cell biocatalysts.

A very interesting field of microbial surface display is in environmental application. The first reports about the use of various heavy-metal binding motifs displayed on the surface of *E. coli* were reported (Sousa *et al.*, 1996; Sousa *et al.*, 1998; Schembri and Klemm, 1998; Kotrba *et al.*, 1999). When these recombinant bacteria were tested for their ability to adsorb the heavy metal in a Cd^{2+}-millieu the results were quite optimistic with the increase of Cd^{2+} absorption in *E. coli* cells with surface-exposed heavy metal-binding peptides compared to the control cells. Gram-positive bacteria, such as *S. xylosus* (Samuelson *et al.*, 2000) and *S. carnosus* (Wernerus *et al.*, 2001) strains were also generated with surface-exposed chimeric proteins containing polyhistidyl peptides designed for binding to divalent metal ions. Such bacteria could perhaps be used for bioadsorption of heavy metal ions, potentially valuable for environmental (bioremediation) applications. A peptidoglycan-associated lipoprotein fused to an antibody fragment (scFv) specific to the herbicide and environmental pollutant atrazine, has been successfully targeted to the cell surface of *E. coli* (Dhillon *et al.*, 1999). A yeast metallothionein (YMT) and histidine oligopeptide (hexa-His) were displayed on the yeast cell surface using α-agglutinin (Kuroda *et al.*, 2001; Kuroda and Ueda, 2003). The hexa-His surface-displayed yeast adsorbed more copper ions and was more resistant to copper than the parent. Futhermore, it was possible to recover the copper ions adsorbed by whole cells with EDTA treatment without disintegrating the cells (Kuroda *et al.*, 2001). A comparison of different numbers of YMT tandem repeats indicated that a higher number of YMT expressed corresponded to a higher amount of Ca^{2+} bound on the yeast cells, as well as increased tolerance to cadmium toxicity (Kuroda and Ueda, 2003). The surface display of anti-pollutant antibodies may have a future role in the bioremediation of contaminated water or the development of pollutant-specific, whole-cell biosensors.

1.2 *Bacillus subtilis* spore

1.2.1 Sporulation in *B. subtilis*

Among Gram-positive bacteria, *B. subtilis* and a few of its relatives are among the best known experimental systems (Driks, 2002). One of the most studied features of *Bacilli* is the ability to form an endospore, which is a tough, non-productive and highly resistant structure. This cell type allows the organism to survive a wide range of extreme stresses and hard conditions such as starvation or terrestrial environments (Nicholson *et al.*, 2000). Sporulation, the process by which cells form spores, had been first observed more than 130 years ago (Koch, 1876). Since then, many studies have been carried out to understand the fundamental process of spore formation. The sporulation in *B. subtilis* proceeds through a well-defined series of morphological stages which can be easily followed by using light and electron microscopy. Within this process, hundreds of genes are involved, the transcription of which is temporally and spatially controlled by four DNA-binding proteins and five RNA polymerase sigma factors (Stragier and Losick, 1996). The formation of spores, as an outcome from sporulation, needs about 8 hours at 37 °C under laboratory conditions.

It is not easy for *B. subtilis* to start sporulation. At the initiation step, a key transcriptional regulatory protein, Spo0A, needs to be synthesized and activated by phosphorylation. However, this does not simply take place by one or two enzymes but through a complicated process, called phosphorelay, in which the phosphorylation of the first protein, Spo0F, is regulated by at least five different autokinases. Then, the $(PO_4)^{2-}$ group is transferred to Spo0B before being delivered to Spo0A. Spo0F~P and Spo0A~P are subjected to negative control by phosphatases (Errington, 2003). The phosphorelay itself must first be stimulated by so far unknown signals which are combined by *B. subtilis* from the internal and external environments such as cell density, nutrient starvation, cell cycle, etc. (Errington, 2003; Piggot and Hilbert, 2004). Phosphorylated Spo0A also plays a key role in sporulation during early mother-cell development (Fujita and Losick, 2003). Once the Spo0A is phosphorylated, the sporulation program is initiated. Spo0A~P can either directly activate or repress the transcription of more than 121 genes and indirectly control further 400 genes, all of which are sporulation-specific and/or stationary-phase genes (Molle *et al.*, 2003). In addition to Spo0A, the other key positive regulator of sporulation, sigma factor H (σ^H) is needed for the transition stage (the stage between exponential growth and stationary phase) and sporulation entry.

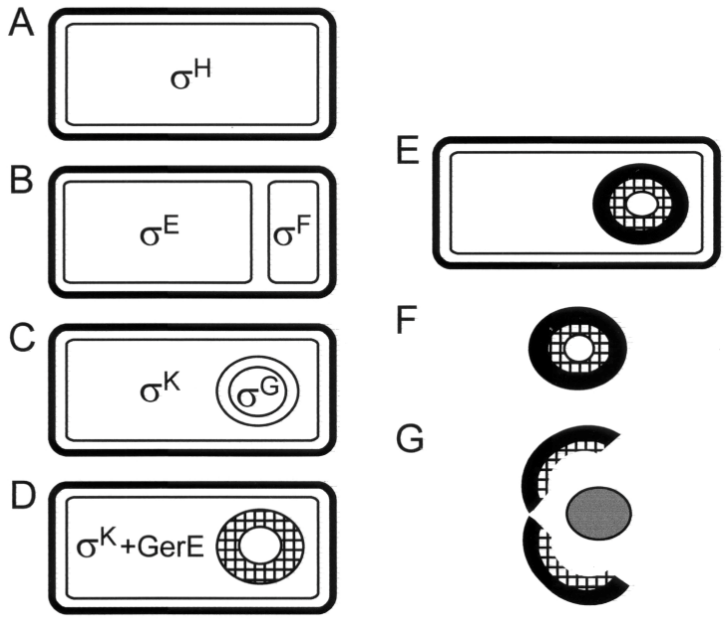

Fig 1.5 ***Stages of sporulation.*** *(A) Once the cell is committed to sporulation, the σ^H activity increases. (B) In the next stage, an asymmetrically positioned septum divides the cell into the forespore and mother-cell compartments. σ^F becomes active in the forespore, and σ^E in the mother-cell. (C) The forespore is engulfed into a membrane-bound protoplast. σ^G becomes active in the forespore, and σ^K directs gene expression in the mother cell. (D) The cortex (the hashed area) forms between the forespore membranes. GerE works in conjunction with σ^K to direct a final phase of gene expression. (E) The coat (the dark ring surrounding the hashed cortex) becomes visible by electron microscopy. (F) In the final stage of sporulation, the mother cell lyses and releases the mature spore into the environment. (G) When nutrients return to the medium, the spore can germinate and the cell can resume vegetative growth. This involves rehydration of the interior of the spore and cracking open of the coat (Driks, 1999).*

The remarked, crucial and morphological event for entering the sporulation decision of the cell is the asymetric division which is similar to cell division but under the regulation of several sporulation-specific factors. This step is started with the formation of axial filament chromatin, followed by segregation at one of the two copies of the chromosome into the forespore (also called prespore) compartment.

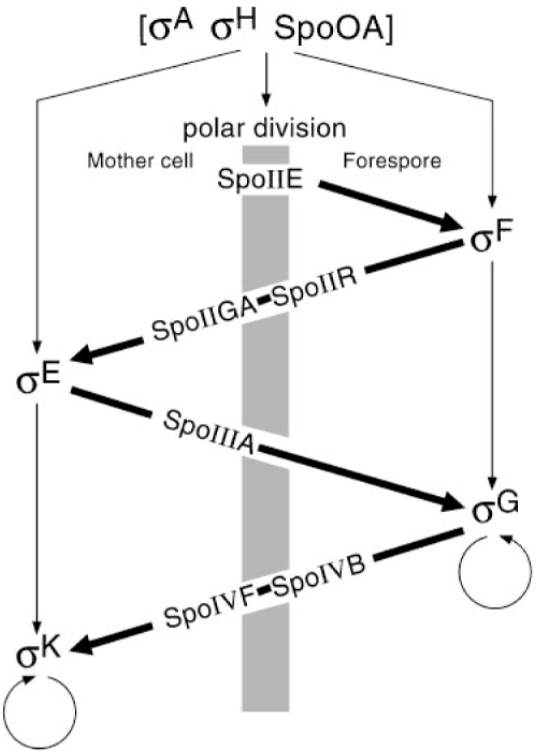

Fig 1.6 ***Crisscross regulation of cell-specific sigma factors.*** *The central shaded area represents the inter-compartmental boundary between the forespore and the mother cell, which is initially created through the combined action of the transcription factors σ^A, σ^H, and Spo0A. Sporulation is governed by two parallel pathways of intracellular gene control operating at the level of the transcription (thin arrows) of the genes for σ^G and σ^K and a crisscross pathway operating at the level of the activity (thick arrows) of all four factors. In the parallel pathways, σ^F turns on the gene for σ^G in the forespore, and σ^E turns on genes involved in the appearance and synthesis of σ^K in the mother cell. Both σ^G and σ^K stimulate their own synthesis by positive feedback loops. In the crisscross pathway, the activation of σ^F in the forespore leads to the appearance of σ^E in the mother cell. The σ^E factor in turn causes the activation of σ^G in the forespore. Finally, σ^G sets in motion a chain of events leading to the appearance of σ^K in the mother cell. All the proteins involved in the activation of the sigma factors are located at the boundary between the two cells (Stragier and Losick, 1996).*

The formation of the asymmetric septum will trigger expression of many sporulation-related genes under a criss-cross pattern of regulation that happens between the two compartment cells. The σ^F in the forespore is first activated; this event leads to the activation of σ^E in the mother-cell.

Together with the completion of engulfment of the forespore by mother-cell membrane, σ^E activates σ^G in the forespore; the σ^G in turn sets in motion a chain of events causing the appearance of σ^K in the mother-cell compartment. σ^K is required for a variety of events that occur late in sporulation including the synthesis of most coat proteins and also (together with σ^G) the production of the cortex (Sun *et al.*, 1989). This sigma factor and the DNA–binding protein GerE works together to regulate gene expression at the last known phase of the mother cell (Zheng and Losick, 1990). Additionally, the GerE protein also helps in controlling coat protein genes and may be involved in glycosylation of the coat (Roels and Losick, 1995). Throughout the intermediate and late stage of sporulation, it seems that the mother-cell and the forespore have communicated to each other by exchanging several sets of 'signals' so that the whole process could be well programmed. The coat assembly will take place at the late stage of sporulation. Finally, the mature spore is liberated by lysis of the mother- cell (Errington, 2003; Piggot and Hilbert, 2004). The mature spore can survive for years, but is also capable of resuming normal vegetative growth upon the return of favorable environmental conditions.

1.2.2 Spore morphology

The mature spore, after being released from mother cell, typically has a spherical or elliptical shape with a size of 1.2 x 0.8 µm on the average (Chada *et al.*, 2003). Under the electron microscope, the *B. subtilis* spore shows three main structures: the core, the cortex and the coat. The single bacterial chromosome, coated by low-molecular-weight proteins, is condensed within the core compartment. The core is surrounded by the the spore cortex, which consists of peptidoglycan whose structure is slightly different from that of the peptidoglycan in the vegetative cell wall. This compartment is synthesized between the two membranes and is one of the two most obvious morphological structures of the mature spore (Errington, 1993; Errington, 2003).

The spore coat is assembled around the cortex, playing the most important role in the spore's resistance to organic solvents and lysozyme. In thin sections, the coat appears as a series of concentric layers which are divided into two major layers: a thick, highly electron-dense outer coat and a thin, less electron-dense inner coat. Under the electron microscopy, the inner coat shows a fine lamellar appearance and stains lightly. It is composed of several layers (between two and five, usually about four) and is about 75 nm wide. The inner coat is surrounded by the outer coat, ranging from about 70 to 200 nm in width, stains more darkly than the inner coat and has a more coarsely

layered appearance (Driks, 1999). In addition to the two predominant coat layers, a third layer of density is often visible between the inner coat and the cortex on electron micrographs. This could be part of the coat and is designated as the undercoat (Aronson *et al.*, 1992). The coat component is mainly consistent of proteins which comprise about 10 % of the total dry weight of spores and 25 % of the total spore protein (Driks, 1999; Munoz *et al.*, 1978). Recently, another structure – a glycoprotein layer – has been observed surrounding the spore by using the ruthenium red staining technique (Waller *et al.*, 2004) (see ***Fig 1.7A***). This structure is intimately connected to the rest of the spore coat and may represent an initial exosporium at the surface of the spore (Chada *et al.*, 2003; Plomp *et al.*, 2005).

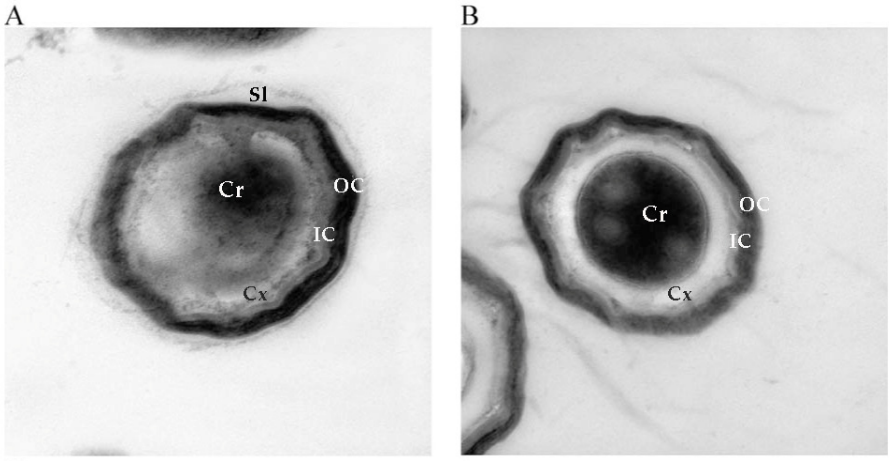

Fig 1.7 ***Electron microscopy of B. subtilis spores.*** *(A) Spore stained with ruthenium red. Glycoprotein surface layer (Sl), outer coat (OC), inner coat (IC), cortex (Cx) and core (Cr). (B) Spore without staining. Adaption from (Waller et al., 2004)*

While the spore core is metabolically inactive (Driks, 1999), the spore surface and the cortex structure together not only help the spore resistance to harsh environmental conditions but also prompt its response to molecules that trigger germination (Moir, 1981; Bourne *et al.*, 1991). These interactions between the spore and its environment seem to be modulated mainly by the spore-surface layers, which have biochemical roles as well as a structure basis. The structures of these outer spore layers are quite flexible, expanding or retracting in response to environmental parameters (Driks, 2003). In addition, the components of them are dominated mainly by proteins,

including several enzymes that enhance spore protection, modulate germination and are likely to affect the environment and behavior of neighboring organisms (Driks, 1999; Nicholson *et al.*, 2000).

1.2.3 The spore coat compartment

Compared to the other compartments, the spore coat's structure, composition, assembly, and function have been studied most extensively in *B. subtilis*. As previously mentioned, the spore coat has a lamellar structure with two main layers representing the majority of proteins (about 25 % of the total spore protein) with the number of proteins estimated to be more than 50 (Kim *et al.*, 2006). These coat proteins can be extracted from purified spores by either alkali treatment or treatment with reducing agents in the presence of detergents. However, as the resistance properties of the spore suggest, solubilization of the coat proteins is quite difficult, only about 70% of coat proteins can be solubilized (Takamatsu and Watabe, 2002). The coat extraction from *B. subtilis* spores can produce a collection of about 40 protein bands on one-dimensional denaturing polyacrylamide gels (SDS-PAGE) that range in size from ~6 kDa to larger than 70 kDa (Henriques and Moran, Jr., 2000; Takamatsu and Watabe, 2002). About 6 % of the soluble material is carbohydrate, and at least two proteins are glycosylated (Jenkinson *et al.*, 1981; Pandey and Aronson, 1979). The insoluble fraction of coat proteins contains highly cross-linked material including a cysteine-rich component encoded by the *cotVWXYZ* cluster, which is briefly described below (Zhang *et al.*, 1993). There are two types of irreversible covalent cross-links which have been detected in the coats: *o,o*-dityrosine bonds (Pandey and Aronson, 1979; Driks, 2003) and ε- (γ-glutamyl)-lysil isopeptide bonds (Kobayashi *et al.*, 1996). The formation of the ε- (γ-glutamyl)-lysil isopeptide cross-link is catalyzed by a transglutaminase (Lorand and Graham, 2003) and one coat-specific enzyme, Tgl, has been found to have this activity (Kobayashi *et al.*, 1998; Suzuki *et al.*, 2000). The *o,o*-dityrosine cross-link can be catalyzed by an enzyme with peroxidase activity. The CotA protein from *B. subtilis* has been demonstrated as a laccase (Hullo *et al.*, 2001), which belongs to a multicopper oxidase family of enzymes. However, the laccase activity of CotA has not yet been shown in conjunction with cross-links formation in the coat. Protein cross-links could be a critical feature in spore resistance. At least 20 coat proteins have demonstrated an enzymatic function or shown a similarity to known enzymes (Henriques and Moran Jr, 2007) and some are proven to have important roles in coat assembly by posttranslationally modifying proteins at the spore surface (e.g., Tgl and YabG protease) (Henriques *et al.*, 1998; Ragkousi and Setlow, 2004; Takamatsu *et al.*, 2000; Zilhao *et al.*, 2005) whereas others

affect spore protection (Henriques *et al.*, 1998; Martins *et al.*, 2002) or germination (Ragkousi *et al.*, 2003).

The first coat protein genes, which are *cotA*, *cotB*, *cotC* and *cotD*, were cloned by Losick *et al.* in 1987 (Donovan *et al.*, 1987). Since then, more than 20 other coat protein genes have been identified. Based on the known morphogenetic role of the proteins on the spore coat, they can be divided into three different groups: the major morphogenetic proteins, the intermediate morphogenetic proteins and the unknown morphogenetic role proteins (Driks, 1999).

Table 1.1 Proteins known to be located in the B. subtilis spore coat. *Estimates of molecular masses (MM) and isolelectric points (PI) are based on the amino acid sequences of the full-length proteins. Molecular masses (kDa) are based on migration in SDS-PAGE. Amino acids that comprise over 10% of the sequence and the presence of cell wall binding motifs (CWB) are indicated under 'sequence characteristics'. Sequence data comes from the B. subtilis ORF database (http://bacillus.tokyo-center.genome.ad.jp) (Takamatsu and Watabe, 2002).*

Name	*Length (aa)*	*MM*	*SDS-PAGE (kDa)*	*PI*	*Para-logues*	*Sequence characteristics*	*Function*	*Gene regulation*
CotA	513	58.5	66	6.3	–	–	unknown	σ^K, GerE
CotB	380	42.97	34, 59	10.3	–	S20%, K12%	unknown	σ^K, GerE
CotC	66	14.79	12	10.1	YnzH	K20%, Y18%, D10%	unknown	σ^K, GerE
CotD	75	8.84	11	7.7	–	H23%, P11%	Germi-nation	σ^K, GerE
CotE	181	20.98	24	4.2	–	E15%, V12%	protein assembly	σ^E,σ^K
CotF	160	18.73	5, 8, 19	7.9	YraD, YraF	L12%	unknown	σ^K
CotG	195	23.94	36	11.1	–	K28%, S20%, H11%, Y11%	assembly of CotB	σ^K, GerE
CotH	362	42.81	43	6.4	YisJ	–	protein assembly	σ^K

CotJA	82	9.74	9	9.8	–	P13%	assembly of CotJC	σ^E
CotJC	189	21.70	24	5.0	YdbD, YjqC	A11%, L10%	assembly of CotJA	σ^E
CotM	130	15.22	6	4.0	–	crystallin family	protein assembly	σ^K
CotS	351	41.05	41	7.1	YtaA	L11%, K11%	assembly of CotSA	σ^K, GerE
CotSA	377	42.91	43	8.1	YtcC	–	unknown	σ^K, GerE
CotT	82	10.13	8, 10	9.0	–	Y22%, P21%, G11%	Germi-nation	unknown
CotX	172	18.60	24	4.3	–	V11%, A10%, L10%, D10%	protein assembly	σ^K, GerE
CotY	162	17.84	26, 52, 78	4.9	CotZ	–	protein assembly	σ^K, GerE
CotZ	148	16.53	18	5.3	CotY	–	protein assembly	σ^K, GerE
SpoIVA	429	55.16	55	4.6	–	E11%	protein assembly	σ^E
SpoVID	575	64.96	66, 120	4.0	–	CWB motif, E21%, A10%	protein assembly	σ^E
YabG	290	33.29	33	9.6	–	–	protease	σ^K
YrbA	387	43.21	30, 31, 45	6.2	–	CWB motif, P15%	protein assembly	σ^E

The first group contains proteins whose absence would cause severe morphogenetic changes in the spore. CotE, SpoIVA and SpoIVD have been identified to belong to this category. CotE is a 24 kDa alkali-soluble coat protein and its location site is at the junction of the inner and outer coat layer which is shown under immunoelectron microscopy (Driks *et al.*, 1994). The *cotE* mutant

formes spores with no outer coat (the inner coat still remained), sensitive to lysozyme and somewhat impaired in germination (Zheng *et al.*, 1988). It was demonstrated that CotE is synthesized early in sporulation, well before the electron-dense coat structure appears, and immuno-electron microscopy studies showed that CotE takes up a discrete subcellular location at the juncture of the inner and outer coat layers just after the formation of the sporulation septum (Zheng and Losick, 1990). The assembly of CotS, an inner coat protein, was proved to be CotE-dependent (Takamatsu *et al.*, 1998). Recently, Isticato *et al.* also demonstrated the binding of CotE to CotC and CotU would mediate their interaction during the spore coat assembly process (Isticato *et al.*, 2010). SpoIVA is a 55-kDa protein, located on the mother-cell side of the forespore membrane which attaches the matrix to the forespore (Driks *et al.*, 1994; Piggot and Coote, 1976; Price and Losick, 1999). The *spoIVA* null mutant results in the coat's misassembly as it swirls within the mother-cell and abolishes cortex synthesis (Roels *et al.*, 1992; Stevens *et al.*, 1992). SpoIVD is a 64.8 kDa which is required for assembly of a normal spore coat (Beall *et al.*, 1993). The *spoIVD* mutation displayed the phenotype in which the coat was detached from the spore, this is quite similar to *spoIVA* mutation, but the cortex was intact (Driks *et al.*, 1994). Driks *et al.* suggested that SpoIVD is also required for maintenance of CotE during later stages, when most of the proteins are assembled into the coat (Beall *et al.*, 1993).

The morphogenetic proteins with intermediate roles are those whose deletion might cause a slight change in spore morphology. Several proteins that belong to this group have been identified. One of these is CotT. This protein is synthesized as a 10.1 kDa precursor, and then processed to be a 7.8 kDa coat protein. Its maturation could take place by a proteolytic activity similar to trypsin (Bourne *et al.*, 1991). An insertional mutation in *cotT* results in spores with morphological alteration as reducing thickness of the inner coat layers, suggesting that CotT is an inner-coat protein. This CotT mutation also causes slow germination in response to a mixture of fructose, glucose and asparagines but the spore is still normal in resistance properties (Zhang *et al.*, 1993). CotG is another member of this group. The *cotG* gene encodes a polypeptide of 24 kDa but the protein isolated for microsequencing by SDS-PAGE migrates as a 36 kDa protein (Henriques *et al.*, 1998). CotG has an unusual primary sequence which is organized into nine repeat of 13-amino acid residues whose consensus is H/Y-K-K-S-Y-R/C-S/T-H/Y-K-K-S-R-S (Sacco *et al.*, 1995). Both the lysine and tyrosine residues could participate in the formation of cross-links within CotG and with other coat proteins. The *cotG* null mutant fails to assemble not only CotG but also CotB, an outer coat protein,

suggesting that CotG may be a morphogenetic protein required for the incorporation of CotB into the coat (Henriques *et al.*, 1998). The CotM protein is related to the α-crystalline family of low molecular-weight heat-shock proteins, members of which can be substrates for transglutaminase-mediated protein cross-linking. *cotM* spores have lesser amounts of several coat proteins, including the outer coat protein CotC (but not the inner coat protein CotD). Under the electron microscope, the outer coat of a *cotM* mutant appears diffuse. Henriques *et al.* proposed that CotM is part of a cross-linked, insoluble skeleton that surrounds the spore and that CotM serves as a matrix for the assembly of additional outer coat material and also confers structural stability to the final structure (Henriques *et al.*, 1997). Another intermediate morphogenetic protein, CotH, plays a role in outer coat assembly, but in a different way from CotG and CotM (Naclerio *et al.*, 1996). A 42.8 kDa inner-coat protein, CotH, is expected to have a strong relationship to CotB and CotG due to the fact that the three genes are located close together on the chromosome (*cotH* lies between *cotB* and *cotG*) and are expressed under control of σ^K (Zilhao *et al.*, 1999). *cotH* spores have a small but detectable germination defect and normal resistance properties. The mutant spores are also pleiotropically deficient in the assembly of several coat proteins, including CotB, CotG and CotC. This result, together with the ultrastructural analysis of purified spores, suggested that CotH is needed for proper formation of both inner and outer layers of the coat (Zilhao *et al.*, 1999).

The coat proteins whose effects from their deletion have not been observed until now are classified in the unknown morphogenetic roles proteins group. The first elucidated coat proteins, CotA, CotB, CotC and CotD (whose molecular-weight are 65, 59, 12 and 11 kDa, respectively) belong to this group. The *cotA* gene is identical to a previously identified gene called *pig*, known to be responsible for sporulation-associated, pigment production (Donovan *et al.*, 1987), and also for a copper-dependent lacase (Hullo *et al.*, 2001). Insertional mutation in any of the four genes results in spores exhibiting the wild-type pattern of coat polypeptides, except for the absence of the product of the inactivated *cot* gene. Spores bearing null alleles of *cotA*, *cotB* or *cotC* have no obvious phenotype. However, *cotD* null mutant spores germinate more slowly than do wild-type spores (Donovan *et al.*, 1987). It has been shown that CotA, CotB, and CotC are probably outer-coat proteins and CotD is most probably an inner coat protein (Zheng *et al.*, 1988). Another coat protein, CotS, is also classified into this group (Driks, 1999). This 41 kDa coat protein is indicated to be an inner-coat protein by immunoelectron microscopy experiments (Takamatsu *et al.*, 1998). The absence of CotS causes no detectable effect on germination or resistence (Abe *et al.*, 1995). Several

other coat proteins have relatively subtle or intermediate effects on the coat when deleted. CotX, a protein with a predicted size of 18.6 kDa, falls into this class. *cotX* deleted spores are largely normal, except that they germinate slightly faster than wild-type spores (Zhang *et al.*, 1993). This protein is encoded by a *cotVWX* operon and has a significant sequence similarity with CotV. The component of CotX is rich in glutamine and lysine residues which might lead to the suggestion that CotX could be crosslinked via a transglutaminase-dependent formation of ε- (γ-glutamyl)-lysine crosslinks. Two other coat proteins, CotY and CotZ, are encoded immediately downstream of the *cotVWX* operon (Zhang *et al.*, 1994). They are cysteine rich, have significant identity and are transcribed from a single promoter. The predicted sizes of CotY and CotZ are 17.9 and 16.5 kDa, respectively. A *cotY* mutation results in a subtle germination defect that is similar to the *cotX* phenotype. Deletion of *cotXYZ* results in spores with a reduced outer-coat, altered surface properties, and increased accessibility to germinants (Zhang *et al.*, 1993). CotY is detected in the soluble fraction, as minor components with electrophoretic mobilities of 26 kDa. It also exists as 52 and 76 kDa dimeric and trimeric forms (with either itself or possibly CotZ). The multimeric forms of CotY probably result from disulfide crosslinks, since they can be completely reduced in the presence of 200 mM DTT (Zhang *et al.*, 1993).

1.2.4 Regulation of spore coat protein genes

Spore coat polypeptides are synthesized only in the mother-cell compartment beginning 3-4 h after onset of sporulation (t_{3-4}) and in a defined temporal order (Cutting *et al.*, 1991a; Jenkinson *et al.*, 1981; Sandman *et al.*, 1988; Zheng and Losick, 1990). The coat genes are controlled by a regulatory cascade (Zheng and Losick, 1990) and their transcription induced the successive appearance of the regulatory proteins σ^E, SpoIIID, σ^K, and GerE. While σ^K regulates the expression of most coat genes, the *cotE* gene alone has dual promoters for σ^E and σ^K-dependent RNA polymerase (Zheng and Losick, 1990).

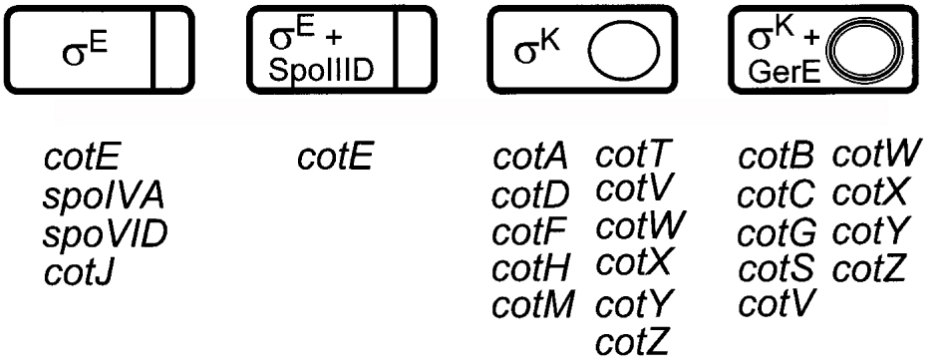

Fig 1.8 ***Program of mother cell gene expression.*** *The stages of sporulation with the related transcription factors that direct mother cell gene expression are shown within the mother cell compartment. Below each cell are the coat protein genes that are active at that time. The repressive functions of SpoIIID and GerE are not indicated (Driks, 1999).*

The coat protein genes are organized into four regulons of mother-cell-expressed genes (***Fig 1.8***). This guarantees the appearance in a particular sequence and in the correct compartment of the coat proteins during sporulation (Driks, 1999). The first regulon, which includes the *cotE* and *cotJ* operons, is activated immediately after the formation of the sporulation septum (Henriques *et al.*, 1995; Zheng and Losick, 1990). σ^E directs expression of these two coat protein genes. In addition, a significant amount of *cotE* expression is also under control of SpoIIID (Zheng and Losick, 1990). The second regulon contains a second promoter of *cotE* and the gene encoding σ^K (Zheng and Losick, 1990; Kroos *et al.*, 1989; Kunkel *et al.*, 1988). The third activated regulon contains a large set of coat proteins (Cutting *et al.*, 1991b; Henriques *et al.*, 1997; Naclerio *et al.*, 1996; Zhang *et al.*, 1994; Zheng and Losick, 1990). At the late stage of sporulation, the fourth and final regulon consists of *cotB*, *cotC*, *cotG*, and *cotS*, as well as genes that are also part of the third regulon, such as *cotV*, *cotW*, *cotX*, *cotY*, and *cotZ* are activated (Abe *et al.*, 1995; Sacco *et al.*, 1995; Takamatsu *et al.*, 1998; Zhang *et al.*, 1994; Zheng and Losick, 1990). Most coat gene expressions are regulated by either σ^K or both σ^K and GerE. GerE also down-regulates *cotA* and *cotM* (Sacco *et al.*, 1995; Zheng *et al.*, 1992). The SpoIIID can also repress both the cortex biosynthetic gene *spoVD* (Zhang *et al.*, 1997) and the coat protein gene *cotD* (Kroos *et al.*, 1989; Halberg *et al.*, 1995). Further intricate regulations in which late regulatory events modulate ones that were initiated earlier in the control of mother-cell gene expression have been observed. For example, σ^K down-regulates transcription of

the gene encoding σ^E, thereby helping to terminate the expression of σ^E-directed genes (Zhang and Kroos, 1997). These regulatory pathways guarantee the timing of production of sporulation proteins and may adjust to the level of coat protein gene expression as a function of the availability of energy or nutrients.

Although coat protein gene regulation appears as a hierarchical cascade, which might lead to a possiblitity that coat assembly is a consequence of the order of coat protein gene expression, the formation of the coat is not regulated by just transcriptional events. By analyzing the locations of the inner and outer coat proteins and of the timing of the appearance of the coat proteins, it has been shown that the inner coat protein genes are not necessarily expressed before the outer coat protein genes. Additionally, the genes encoding any one layer of the coat are not necessarily members of a single sporulation gene regulon. For example, *cotE* is among the first known coat protein genes to be expressed, but the gene product appears in the outermost layer of the coat as opposed to an inner layer, as might have been anticipated from the early expression of *cotE* (Little and Driks, 2001; Isticato *et al.*, 2010). Furthermore, *cotD*, which codes for an inner coat protein, and *cotA*, which encodes an outer coat protein, are both expressed in the middle phase of the mother cell gene expression (Donovan *et al.*, 1987; Zheng *et al.*, 1988). Most strikingly, the lacking GerE spores, which are therefore unable to activate the final phase of mother-cell gene expression, still have some outer coat but no inner coat (Moir, 1981; Jenkinson *et al.*, 1981). It is obvious that gene-regulatory controls play an important role in coat assembly; however, there must be other additional mechanisms that take part in regulation of coat assembly. The effects of altering the timing of coat protein gene regulation still remain to be determined (Driks, 1999).

1.2.5 Model for coat assembly

With several parts of spore coat protein regulation and expression remain unknown, the coat assembly process has not been completely understood. However, the model for coat assembly has been proposed by Dirks and then complemented by Takamatsu and Watabe. This model has four majors steps, starting with the binding of SpoIVA to the forespore surface followed by the formation of the precoat, a σ^K -dependent phase of inner and outer coat layer assembly and a σ^K -plus-GerE-dependent phase of inner and outer coat layer assembly that includes postassembly modification of the coat. SpoIVA, SpoVID, CotE and YrbA (SafA) have played crucial roles in this model (Driks, 1999; Takamatsu and Watabe, 2002).

The model for spore coat assembly which was given by Henriques and Moran is somewhat similar yet more detailed (***Fig 1.9***) (Henriques and Moran Jr, 2007). The first step of this model is attachment of the coat to the forespore outer-membrane, in which SpoIVA is synthesized in mother-cell early in sporulation and localized at the mother-cell surface of the division septum. At latter stages, following engulfment of the forespore by the mother-cell, the protein forms a shell that surrounds the forespore (Driks *et al.*, 1994; Pogliano *et al.*, 1995; Price and Losick, 1999). The production of SpoIVA is necessary for the assembly of both the spore cortex and the coat layer (Roels *et al.*, 1992; Stevens *et al.*, 1992). The localization of SpoIVA requires a 26-amino-acid-long σ^E-controlled sporulation protein, SpoVM (Price and Losick, 1999). The appearance of SpoIVA also helps in fixing another key coat morphogenetic protein, SpoVID, into the mother-cell side of the forespore membrane (Ozin *et al.*, 2001; Driks *et al.*, 1994; Beall *et al.*, 1993). Another protein, which is also required for the localization of SpoVID, is the proline-rich SafA protein. SafA has an N-terminal LysM domain, a peptidoglycan binding domain, and localizes at the cortex/coat interface in mature spores (Ozin *et al.*, 2000). The C-terminal region sequence of this protein is similar to inner coat proteins such as CotT, CotD, and CotJA (Bourne *et al.*, 1991; Donovan *et al.*, 1987; Henriques *et al.*, 1995; Seyler, Jr. *et al.*, 1997). The structure of SafA suggests its function in bridging the cortex and coat, with its LysM domain binding to the former, and its C-terminal half capable of binding to other coat proteins (Ozin *et al.*, 2000; Ozin *et al.*, 2001).

The second step of the model is the formation of the precoat (matrix) layer. The precoat consists of CotE, whose localization requires SpoIVA, to form a layer with size ~75 nm parallel to the plane of the septum and on its mother cell side. The region delimited by the positions of SpoIVA and CotE is called the matrix (Driks *et al.*, 1994; Webb *et al.*, 1995). The components of the matrix are not completely known but it most likely contains proteins that are synthesized early and recruited under SpoIVA control (CotE-independent) and those proteins also require SpoVID or SafA. CotJA and CotJC are possible candidates since both their expressions are σ^E-independent and both are present in spores of *cotE gerE* mutant strains (Henriques *et al.*, 1995; Seyler, Jr. *et al.*, 1997). When engulfment is completed, all these proteins form a shell encircling the forespore.

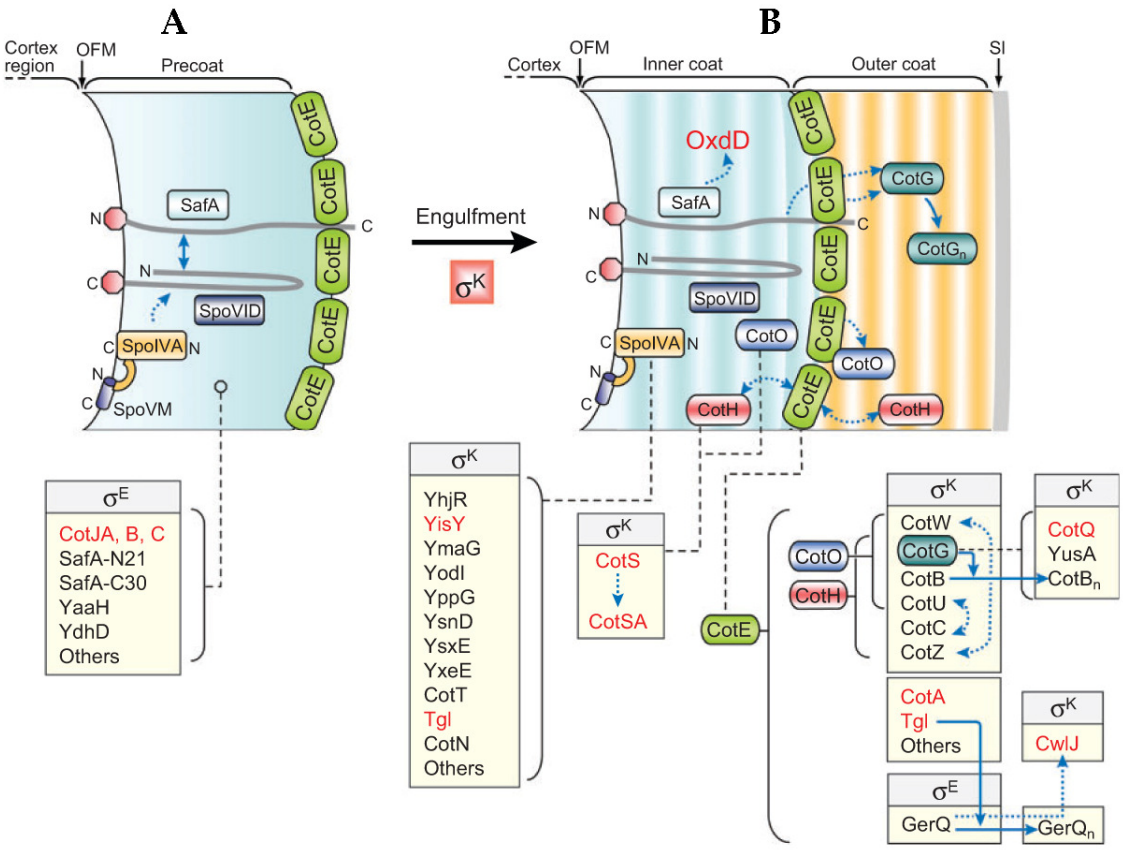

Fig 1.9 Proposed model for assembly of the B. subtilis spore coat by Henriques and Moran. *(A) The early stages in coat assembly, during which the proteins involved in attachment of the coat to the surface of the developing spore are localized to or close to the outer forespore membrane (OFM) and CotE is localized to the edge of the precoat region. Proteins produced under the control of σ^E are thought to be part of the precoat. (B) The assembly of the inner and outer layers of the spore, which take place following engulfment completion and the activation of σ^K. Morphogenetic proteins are shown within boxes where their main function is exerted. Broken arrows represent dependencies for assembly or postulated interactions, whereas solid arrows indicate direct interactions. The subscript n indicates multimerization of the indicated protein. Note that certain proteins (e.g., CotH, CotO, or Tgl) may be present in different layers of the coat. Enzymes assembled within the coat layers are shown in red. Sl, glycosylated surface layer (Henriques and Moran Jr, 2007).*

The formation of the precoat has set up the next step of coat assembly. The precoat material will be converted into the characteristic inner layer, and, in parallel, the outer coat layer is also

assembled. All the processes are dependent on the activation of σ^K (Driks, 1999; Henriques and Moran, Jr., 2000). The inner-coat proteins such as CotD, CotT, CotH, CotS and OxdD (oxalate decarboxylase) are presumably assembled into the matrix. Outer-coat proteins such as CotA and CotF are assembled around the shell of CotE in a CotE-dependent manner (Costa *et al.*, 2004; Zheng and Losick, 1990). CotT may interact with CotE and participate in defining the precise width of the final inner coat (Chada *et al.*, 2003). At least 24 proteins or about 40% of the total number of coat components known to date are assembled in a CotE-dependent manner and may be associated primarily with the outer coat layers (Kim *et al.*, 2006). The assembly process also needs additional morphogenetic proteins, CotH and CotO, to work in cooperation with CotE. Possibly, CotH locates alongside CotE in the shell that surrounds the forespore and is able to direct assembly of a subset of the outer coat and still remain attached to the inner coat in a CotE-dependent manner (Naclerio *et al.*, 1996; Zilhao *et al.*, 1999). CotH may function in part in the mother cell cytoplasm, perhaps as a protease inhibitor for the stabilization of CotG and CotC (Isticato *et al.*, 2004; Zilhao *et al.*, 2004).

The expression of several coat proteins is later under the control of GerE and σ^K. Some of the most abundant coat proteins, including CotB, CotG, and CotC, are belonging to this group of proteins. All three are found to be mainly associated with the outer coat (Donovan *et al.*, 1987; Isticato *et al.*, 2004; Kim *et al.*, 2005a; Sacco *et al.*, 1995), and the details of their assembly are quite interesting. CotC can form multimeric species with itself and the nearly identical CotU protein at the spore surface. CotC and CotU have been determined already to be interactive, with the mediation of CotE, during the coat assembly (Isticato *et al.*, 2008; Isticato *et al.*, 2010). CotG is present in the coat as a major form of about 36 kDa, but undergoes extensive multimerization following spore release from the mother-cell (Zilhao *et al.*, 2005; Zilhao *et al.*, 2004). CotB also forms multimers and one of which, an abundant form of about 64 kDa (this formation was previously reported as 59 kDa protein by Donovan *et al.* (Donovan *et al.*, 1987)), is presumed to result from its direct interaction with CotG (Zilhao *et al.*, 2004). It has been shown that the repetitive nature of the CotG protein, and perhaps also of CotB, is crucial in guiding the ordered assembly and patterning of the outer coat layer (Henriques *et al.*, 1998; Zilhao *et al.*, 2004). The appearance of the inner-coat is shown to be GerE-dependent, which means there is at least one unknown inner-coat morphogen becoming active during this time. There is also evidence that there is a brief σ^K-dependent period of morphogenesis that builds a small part of the outer coat, which is rapidly followed by the assembly of the full inner and outer coats (Driks, 1999).

After the formation of inner and outer coat layers, the coat is set to the maturation phase. In this final stage of coat assembly, the correctly positioned coat proteins are further cross-linked and modified. This step of maturation helps the spore in resistance to lytic enzymes and noxious chemicals and ensures normal germination. GerQ, a 20 kDa protein which is necessary for the proper localization of CwlJ (an enzyme important in the hydrolysis of the peptidoglycan cortex during spore germination) was found to cross-link into high-molecular-mass complexes in the spore coat late in sporulation. The assembly of GerQ is dependent on the coat morphogenetic proteins CotE and SpoIVA (Ragkousi *et al.*, 2003). This protein contains three N-terminal lysine residues, which can serve as lysine donor to form ε-(γ-glutamyl)-lysine isopeptide bonds with a glutamine acceptor from another protein (Monroe and Setlow, 2006). The coat-associated transglutaminase, Tgl, is responsible in mediating the cross-linking of GerQ and another unknown substrate protein which provides a glutamine acceptor (Ragkousi and Setlow, 2004). Tgl is assembled onto the spore surface soon after its synthesis in the mother-cell under σ^K-control but that the complete insolubilization of at least two of the Tgl-controlled polypeptides occurs several hours later (Zilhao *et al.*, 2005). There are evidences for cooperation between the YabG protease, Tgl and other cross-linking enzymes at the spore surface. The YabG protease generates substrates from precursor proteins or otherwise facilitates their access to Tgl and the other cross-linking enzymes (Kuwana *et al.*, 2006). The activity of the cross-linking enzymes might be prevented until all the components of the coat are in place. Mother-cell lysis, which is catalyzed by autolysins, might then function as a checkpoint for the final stages in maturation of the spore surface (Monroe and Setlow, 2006). Because cross-linking activity on the spore coat can be influenced by several environmental conditions, the maturation of spores can follow different paths, and it is still unknown whether disulfide bonds can be formed during sporulation or be enzymatically facilitated.

1.3 Intein and protein splicing

1.3.1 Configuration of the intein

The first two inteins were described by two independent groups in 1988 (Bowman *et al.*, 1988; Shih *et al.*, 1988). Since then, many other inteins have been discovered and studied. By the midth of 2010, more than 600 of inteins have been reported (data from the intein registry InBase at http://www.neb.com/neb/inteins.html (Perler, 2002)). At the early time, inteins were called as protein intron, intervening protein sequence, spacer sequence, or protozyme (Anraku and Hirata,

1994; Cooper and Stevens, 1995; Davis *et al.*, 1992; Abanes-De Mello *et al.*, 2002; Gu *et al.*, 1993; Hirata and Anraku, 1992; Hodges *et al.*, 1992; Kane *et al.*, 1990). The term "intein" (internal protein) was adapted in 1994 to define a protein sequence embedded in-frame within a precursor protein sequence which is then excised precisely and its flanking sequences are joined with a peptide bond to produce the mature spliced protein (***Fig 1.10***). The flanking sequences are termed N- and C-exteins (external proteins). The spliced or excised intein is referred to the free intein to distinguish it from the fused intein present in the precursor (Perler *et al.*, 1994; Liu, 2000).

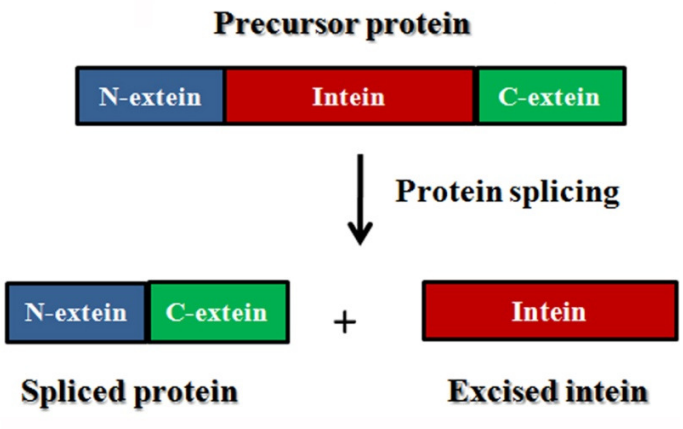

Fig 1.10 ***Schematic illustration of protein splicing with the role of inteins****. Inteins active in protein splicing to cut themselves out from the precursor protein and create the peptide bond between the two flanking exteins.*

Inteins are found in proteins from members of all three kingdoms: Eukaryotes, Bacteria, Archaea and also in viral proteins (Perler, 2002). The host proteins of inteins have diverse functions, including metabolic enzymes, DNA and RNA polymerases, proteases, the vacuolar-type ATPase, etc., but enzymes involved in DNA replication and repair appear to dominate (Liu, 2000). Inteins are found between 134 and 608 amino acids long (Perler, 2002). Within the host protein, inteins appear to prefer conserved regions, for example, nucleotide-binding domains (Pietrokovski, 2001). Based on their structure and function, inteins are classified in three groups (Liu, 2000; Gogarten *et al.*, 2002):

- ***Large inteins*** are bifunctional proteins with a protein splicing domain and a central endonuclease domain. The elements necessary for splicing are present at the N- and C-terminal regions of the inteins. The 50 kDa intein of the 69 kDa vacuolar membrane

ATPase subunit from *S. cerevisiae* (*Sce* VMA intein) is a typical example for large intein (Hirata *et al.*, 1990; Kane *et al.*, 1990).

- ***Mini-inteins*** consist of only the self-splicing domain and lack of an endonuclease domain. Many mini-inteins have been engineered by deletion of the endonuclease domain from a large inteins (e.g. the 20-30 kDa versions of *Sce* VMA intein (Chong and Xu, 1997) and the intein from the *Synechocystis* sp. *dnaB* gene (*Ssp* DnaB intein) (Wu *et al.*, 1998b)). However, there are also mini-inteins found to be naturally occurring inteins (e.g. the mini-intein from *M. xenopi* gyrase A – *Mxe* Gyr A intein (Telenti *et al.*, 1997)). This group of inteins has been widely used in molecular biology and biotechnology, especially in development of protein purification systems.
- ***Split inteins*** have the N- and C-terminal self-splicing elements encoded by two separately transcribed and translated genes. The splicing elements, after produced, are capable of self-association and catalyze the protein-splicing activity (*trans*-splicing). It is possible for a split intein to be a mini-intein, too. For example, the mini-intein *Ssp* DnaE is a natural split intein whose N-terminal and C-terminal fragments were found in two separate coding regions (Wu *et al.*, 1998a).

Details of conserved motifs, structures and functions of inteins have been reviewed (Gogarten *et al.*, 2002; Liu, 2000; Elleuche and Poggeler, 2010). Comparative analysis of intein sequences reveals similarities in some regions, with conserved residues only at the N- and C-termini. Most inteins begin with Ser or Cys and end in His-Asn, or in His-Gln. The first amino acid of the C-extein is an invariant Ser, Thr or Cys, but the residue preceding the intein at the N-extein is not conserved (Perler, 2002). However, residues proximal to the intein-splicing junction at both the N- and C-terminal exteins were recently found to accelerate or attenuate protein splicing (Amitai *et al.*, 2009).

1.3.2 Mechanism of protein splicing

Protein splicing, firstly reported in 1990 (Kane *et al.*, 1990; Hirata *et al.*, 1990), is a form of post-translational processing that consists of the excision of an intein from a precursor protein, accompanied by joining of the flanking polypeptide sequences, the exteins, by a peptide bond. This has been shown as an intramolecular process which is catalyzed entirely by amino acid residues contained in the intein and requires no coenzymes or sources of metabolic energy (Paulus, 2000). Details about the chemical reactions involved in protein splicing are reviewed in (Paulus, 2000;

Gogarten *et al.*, 2002; Liu, 2000; Elleuche and Poggeler, 2010; Saleh and Perler, 2006). Briefly, the protein splicing mechanism involves the following four steps (***Fig 1.11***):

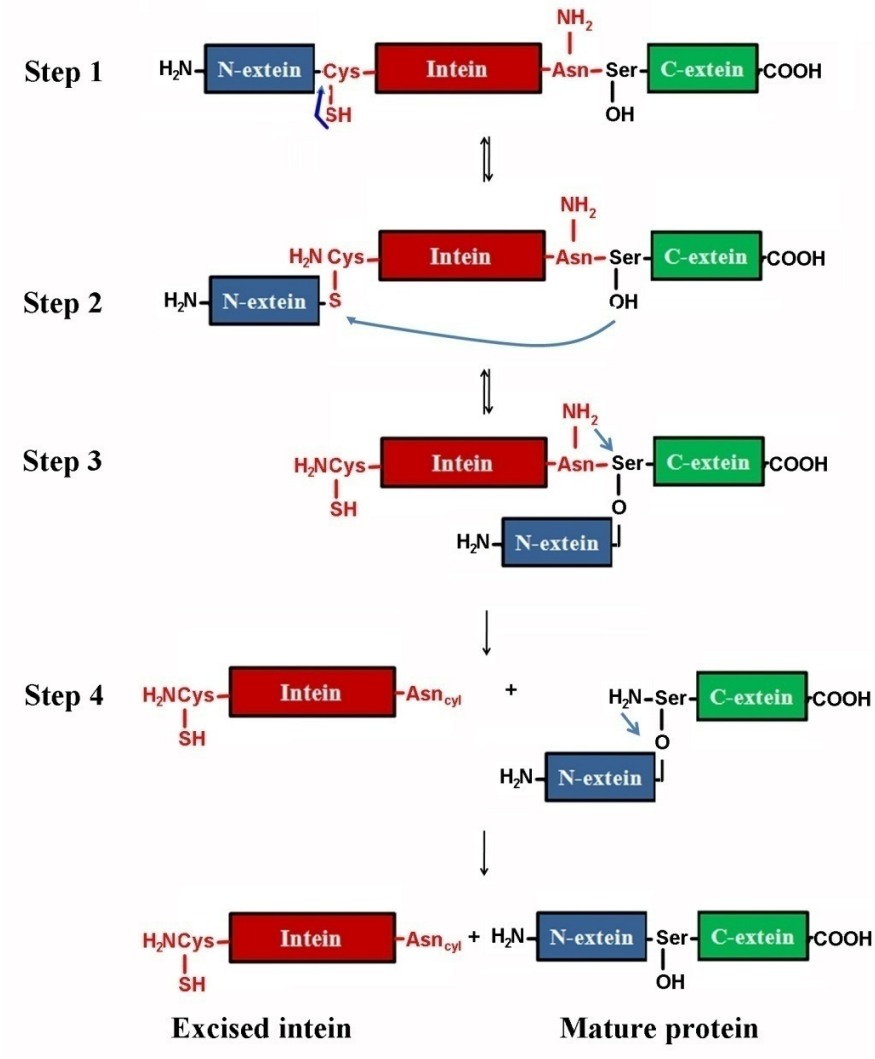

Fig 1.11 ***Protein splicing mechanism.*** *Four reaction steps occur in the protein splicing pathway: N-S acyl rearrangement of the peptide bond at the intein N-terminus (step 1), transesterification between the nucleophilic residue at the C-extein and the thioester (step 2), Asn-cyclization and peptide bond cleavage (step 3), N-O acyl shift rearrangement of the ester to a peptide bond (step 4). Adaption from (Elleuche and Poggeler, 2010).*

- Step 1: The amino-terminal splice junction of the intein is activated by an N-O or N-S acyl shift that leads to an ester or (thio)ester intermediate. As a result of this rearrangement, the

N-extein binds to the oxygen of a Ser or to the sulfur of a Cys residue at the amino-terminal splice junction.

- Step 2: Cleavage of the ester at the amino-terminal splice junction occurs through attack of a nucleophilic residue located at the carboxy-terminal splice junction. This transesterification results in a branched protein intermediate.
- Step 3: The cyclization of the conserved Asn residue at the C-terminus of the intein releases the intein and links the exteins by a (thio)ester bond.
- Step 4: The rearrangement of the (thio)ester bond by a spontaneous S–N or O–N acyl shift results in formation of a peptide bond between the two exteins.

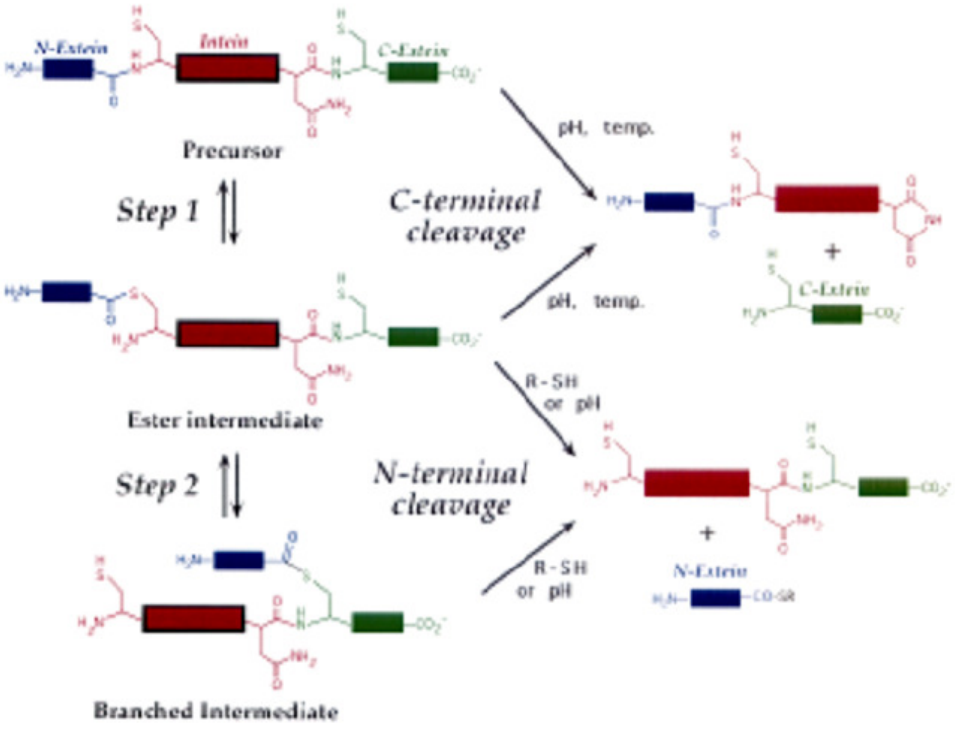

Fig 1.12 ***Side reactions of the protein splicing pathway*** *that lead to pH, temperature, or thiol-induced cleavage at the N- or C-terminal splice junctions. Only the first two steps of protein splicing are shown (Paulus, 2000).*

Side reactions of protein splicing can occur that lead to cleavage of inteins at their N- or C-terminal under certain circumstances (***Fig 1.12***). Cyclization of Asn can proceed independently when the normal route of the splicing reaction is inhibited and N-terminal splice junction cleavage can happen by hydrolysis or nucleophilic attack of the thioester or ester linkage (Chong *et al.*, 1996;

Chong *et al.*, 1997; Mathys *et al.*, 1999; Paulus, 2000). By appropriate amino acid substitution(s), an intein can be modified to catalyze only cleavage at either or both of its termini when being induced by addition of thiol reagent, e.g. dithiothreitol (DTT), or changing pH and/or temperature (Xu *et al.*, 2000; Evans, Jr. *et al.*, 1999; Mathys *et al.*, 1999). These induced cleavage reactions are powerful protein engineering tools because they are self-catalyzed and thus do not require the use of accessory enzymes.

The mechanism described above is a pathway for many standard inteins (classified as class 1 inteins). Recently, new classes of inteins lacking of the N-terminal Ser or Cys residue has been described as class 2 and class 3 inteins which cannot perform the acyl shift that starts the splicing reaction in class 1 inteins. Hence, they perform a splicing process where the initial steps are altered until forming the standard branched intermediate (Southworth *et al.*, 2000; Johnson *et al.*, 2007; Tori *et al.*, 2010).

1.3.3 Engineered inteins and mini-inteins used in protein purification

Understanding the protein splicing mechanism of inteins provided very useful tools for molecular biology and biotechnology. Since its discovery, inteins have been used for many applications. Among them are the ligation of peptides and proteins using the natural splicing activity of inteins, known as intein-mediated protein ligation, or expressed protein ligation (Evans, Jr. *et al.*, 1999; Muir *et al.*, 1998; Severinov and Muir, 1998). Furthermore, inteins have been used for segmental labeling of proteins for NMR analysis, cyclization of proteins, controlled expression of toxic proteins, conjugation of quantum dots to proteins, and incorporation of non-canonical amino acids (Arnold, 2009; Charalambous *et al.*, 2009; Oeemig *et al.*, 2009; Seyedsayamdost *et al.*, 2007; Zuger and Iwai, 2005).

Production of large amounts of highly purified recombinant proteins is an important task in molecular biology and biotechnology. Many systems and methods for overexpression and purification have been developed (Marino, 1989; Baneyx, 2004; Schumann, 2007; Schumann and Ferreira, 2004; Terpe, 2006). Since a wide range of affinity tags have been developed, the protein purification process is greatly simplified. Originally, the systems are developed to isolate proteins using affinity columns or beads. The tags, at the DNA level, are fused to the target gene. The fusion of different protein and peptide tags can also improve the solubility and folding of the target protein (Terpe, 2003; Waugh, 2005). Inteins are also utilized as a self-cleaving affinity tag for protein purification and this becomes one of their most important applications.

The first commercial intein-mediated purification system with an affinity chitin-binding tag has been developed by New England Biolabs (NEB), known as IMPACT. A modified *Sce* VMA1 intein was fused at its C-terminus to the chitin-binding domain (CBD), and at its N-terminus to the protein of interest (Chong *et al.*, 1997). This intein is mutated to block the splicing reaction after the N−S acyl shift, and prevents C-terminal cleavage. In a first step, addition of thiols will initiate the N-terminal cleavage, which leads to release of the target protein with an activated thioester at the C-terminus, while the intein-CBD remains bound to the column. In the second step, thiols and the small peptide are subsequently removed by dialysis.

The *Ssp* DnaB intein, available as part of the pTWIN1 and pTWIN2 vectors (NEB), is a pH-induced cleaving intein. This intein has been used with various affinity and non-chromatographic purification tags, including the CBD (Esipov *et al.*, 2008; Zhao *et al.*, 2008; Sharma *et al.*, 2006), phasing (Wang *et al.*, 2008), and regenerated amorphous cellulose binding tag (Hong *et al.*, 2008) to effectively purify proteins. Another pH-inducible intein is the ΔI-CM mini-intein, which is derived from the *Mtu* RecA intein (Wood *et al.*, 1999). Several proteins have been successfully purified using this intein in conjunction with different non-chromatographic tags (Banki *et al.*, 2005a; Banki *et al.*, 2005b). Compared to others, pH-induced inteins are the most economical because they only require a shift in the buffer pH to induce cleavage. Various self-cleaving purification tags and their potential industrial applications have been recently reviewed (Fong *et al.*, 2010).

Removing the affinity tag after isolation of the fusion proteins is one of the important steps in proteins purification. Conventionally, a site-specific endoprotease is often used. For industrial applications, the removal of the affinity tag by endoproteases is the most costly step in protein production, and can interfere with the biological activity of the purified component (Wood *et al.*, 2005). Therefore, intein-mediated bioseparation has become an excellent vehicle for affinity-tag-based protein purification techniques, and is an alternative to conventional cleavage by site-specific endoproteases.

1.4 Cellulases and their applications

1.4.1 Cellulose and cellulose degradation enzymes

Life on Earth depends on photosynthesis, which results in production of plant biomass. Cellulose, the most abundant component of plant biomass, is found in nature almost exclusively in plant cell walls, although it can be produced by some animals (e.g., tunicates) and a few bacteria. In

a few cases (notably cotton bolls), cellulose is present in a nearly pure state. In most cases, the cellulose fibers are embedded in a matrix of other structural biopolymers, primarily hemicelluloses and lignin, which comprise 20 to 35 and 5 to 30% of the plant dry weight (Lynd *et al.*, 1999; Sjostrom, 1993). Chemically, cellulose is a linear condensation polymer consisting of D-anhydro-glucopyranose joined together by β-1,4-glycosidic bonds with a degree of polymerization (DP) from 100 to 20,000 (Krassig, 1993; Tomme *et al.*, 1995; Zhang and Lynd, 2004a). Anhydrocellobiose is the repeating unit of cellulose. Coupling of adjacent cellulose chains and sheets of cellulose by hydrogen bonds and van der Waal's forces results in a parallel alignment and a crystalline structure with straight, stable supra-molecular fibers of great tensile strength and low accessibility (Demain *et al.*, 2005; Krassig, 1993; Nishiyama *et al.*, 2003; Notley *et al.*, 2004; Zhang and Lynd, 2004b; Zhbankov, 1992). Therefore, although abundant in nature, cellulose is a particularly difficult polymer to degrade (Mansfield *et al.*, 1999). Degradation of hemicellulose, pectin and lignin is generally easier.

Cellulose molecules can be hydrolysed by a class of enzymes called cellulases, which are produced primarily by fungi, bacteria, protozoa, and some plants and animals (Norkrans, 1963). In micro-organisms, there are two types of enzyme systems for cellulose degradation which have been observed. In the case of aerobic fungi and bacteria, several individual endoglucanases, exoglucanases and ancillary enzymes, are secreted which, together, can work synergistically to hydrolyze cellulose, therefore called non-complexed systems. In anaerobic microorganisms, a different type of system has evolved that involves the formation of a large, extracellular enzyme complex called the cellulosome (or complexed system). The cellulosome consists of scaffolding proteins and many bound cellulosomal enzymes (Bayer *et al.*, 1985). The scaffolding proteins are large nonenzymatic proteins that usually contain a number of cohesin domains (Coh) and cellulose binding domains. However, hydrophilic domains, dockerin II domains, the enzyme coding domain, and a number of unidentified domains whose functions remain unknown have also been observed in some of the scaffolding proteins (Doi *et al.*, 2003). The structure and function of cellulosomes have been reviewed (Doi *et al.*, 2003; Doi and Kosugi, 2004; Bayer *et al.*, 1985; Bayer *et al.*, 2004).

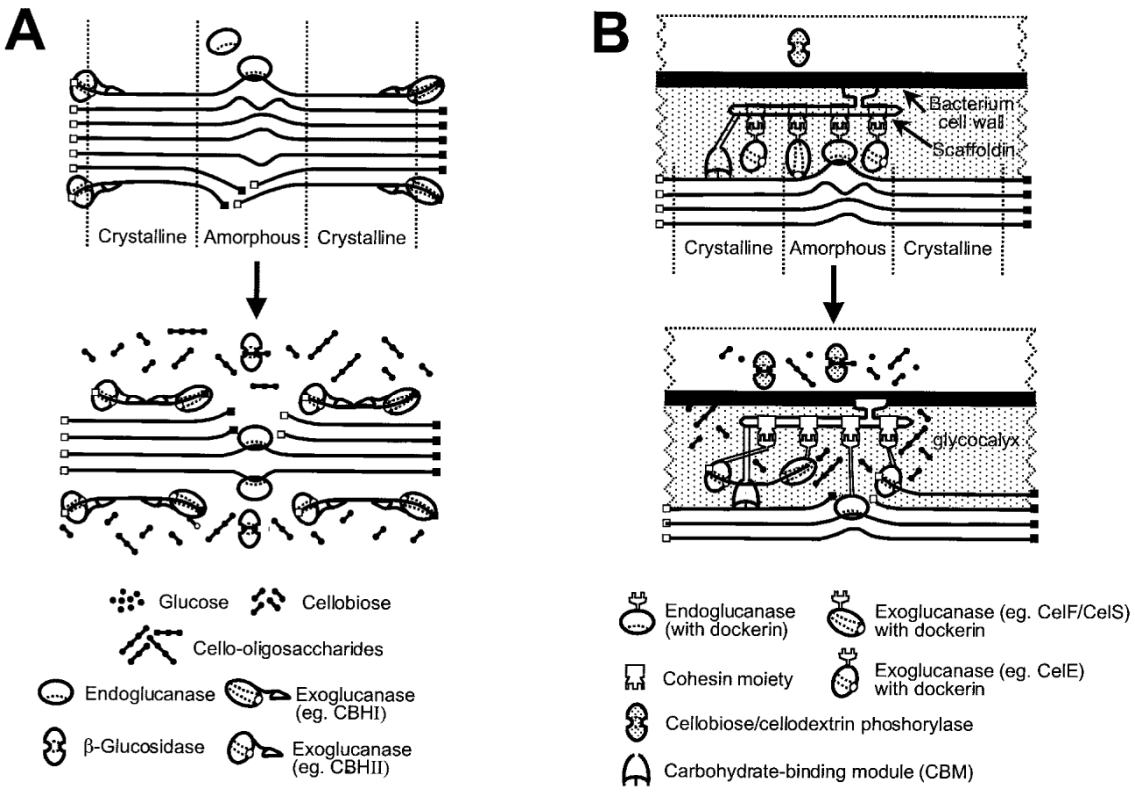

Fig 1.13 The hydrolysis of amorphous and microcrystalline cellulose by noncomplexed (A) and complexed (B) cellulase systems. *The solid squares represent reducing ends, and the open squares represent nonreducing ends. Amorphous and crystalline regions are indicated. Cellulose, enzymes, and hydrolytic products are not shown to scale (Lynd et al., 2002).*

Components of cellulase systems were first classified based on their mode of catalytic action and have been more recently classified based on structural properties (Henrissat *et al.*, 1998). Three major types of enzymatic activities are found: (i) endoglucanases or 1,4-ß-D-glucan-4-glucanohydrolases (EC 3.2.1.4), (ii) exoglucanases, including 1,4-ß-D-glucan glucanohydrolases (also known as cellodextrinases) (EC 3.2.1.74) and 1,4-ß-D-glucan cellobiohydrolases (cellobiohydrolases) (EC 3.2.1.91), and (iii) ß-glucosidases or ß-glucoside glucohydrolases (EC 3.2.1.21). Endoglucanases catalyze randomly at internal amorphous sites in the cellulose polysaccharide chain, generating oligosaccharides of various lengths and consequently new chain ends. Exoglucanases act in a processive manner on the reducing or nonreducing ends of cellulose polysaccharide chains, liberating either glucose (glucanohydrolases) or cellobiose (cellobiohydrolase) as major products. Exoglucanases can also act on microcrystalline cellulose,

presumably peeling cellulose chains from the microcrystalline structure (Teeri, 1997). ß-Glucosidases hydrolyze soluble cellodextrins and cellobiose to glucose (see ***Fig 1.13***).

1.4.2 Applications of cellulases

Cellulosic materials are particularly attractive because of their relatively low cost and plentiful supply. Since the early 1980s, biotechnology of cellulases has developed, first in animal feed, later followed by food applications (Voragen, 1992). Later on, cellulases have proven their utilities in the textile industry for cotton softening and denim finishing (Godfrey, 1996), in the detergent market for color care, cleaning, and anti-deposition, in the food industry for mashing, and in the pulp and paper industries for de-inking, improvement, and fiber modification (Godfrey and West, 1996; Bhat, 2000). The cellulase market is expected to expand dramatically when cellulases are used to hydrolyze pretreated cellulosic materials into sugars, which can be fermented to produce commodities such as ethanol and other bio-products on a large scale (Hahn-Hagerdal *et al.*, 2006). Some of the most promising applications of cellulases are listed in this part.

In food industry, cellulases are used in extraction and clarification of fruit and vegetable juices, production of fruit nectars and purées, and in the extraction of olive oil (Bhat, 2000). They are also used in carotenoid extraction for producing food coloring agents (Cinar, 2005).

The ß-glucanases, especially from *Trichoderma*, appear to be suitable for the production of high quality beer from poor quality barley, thus show a great benefit in the brewing industry (Galante *et al.*, 1998b). In the early 1980s, it was suggested that *Trichoderma* ß-glucanase could be successfully used for wine making from grapes infected with *Botrytis cinerea* (Dubordieu *et al.*, 1981; Villetaz *et al.*, 1984). This fungus generally attacks nearly ripe grapes under conditions of certain temperatures and humidity, and produces a high molecular mass soluble ß-(1,3) glucan with short side chains linked through ß-(1,6) glycosidic bonds, which alleviates several problems during wine filtration. A ß-glucanase from *Trichoderma harzianum* was also found to be useful for hydrolysis of glucans from yeast, which have caused adverse effects during filtration and clarification of wine (Galante *et al.*, 1998b).

Cellulases have achieved their worldwide success in textile and laundry because of their ability to modify cellulosic fibres in a controlled and desired manner, so as to improve the quality of fabrics. Cellulases have now become the third largest group of enzymes used in textile and laundry industry (Galante *et al.*, 1998a; Galante *et al.*, 1998a). Bio-stoning and bio-polishing are the best-

known current textile applications of cellulases (Belghith *et al.*, 2001). Cellulases are also increasingly used in household washing powders, since they enhance the detergent performance and allow the removal of small, fuzzy fibrils from fabric surfaces and improve the appearance and colour brightness (Uhlig, 1998).

Cellulases have been used for different purposes in the pulp and paper industry. Cellulase and hemicellulase mixtures have been used for the modification of fibre properties for improving drainage, beatability and runnability of the paper mills (Noe *et al.*, 1986; Pommier *et al.*, 1990). The addition of cellulase and hemicellulase after beating is to improve the drainage properties of pulps, which determine the speed of paper mills. A commercial cellulase/hemicellulase preparation, named Pergalase-A40, from *Trichoderma* has been used by many paper mills around the world for the production of release papers and wood-containing printing papers (Pommier *et al.*, 1990). For de-inking purpose, most of the published literatures are dealt with cellulases and hemicellulases (Bajpai, 1999).

The most important application currently being investigated is the utilization of lignocellulosic wastes for the production of biofuel. Currently, the US and Brazil are leaders in the production of starch/sugar-based fuels from corn and sugarcane crops, respectively. However, starch raw materials will not be sufficient enough to meet increasing demand and are a controversial resource for bioconversion (Greene *et al.*, 2004; Maki *et al.*, 2009). With the increasing demands for energy and the shrinking energy resources, the utilization of plant biomass for the production of biofuel offers a renewable alternative. A potential application of cellulase is the convertion of cellulosic materials to glucose and other fermentable sugars, which in turn can be used as microbiol substrates for the production of single cell proteins or a variety of fermentation products like ethanol. Theoretically, this is all quite possible; however, technologically, it is not an easy task because of various technological gaps. Cellulosic bioconversion is a multi-step process requiring a multi-enzyme complex for efficient bioconversion into fermentable sugars. However, there is no known organism capable of producing all the necessary enzymes in sufficient quantities. In addition, there is a lack of biocatalysts that can work efficiently and inexpensively at high temperatures and/or low pH conditions used in the bioconversion of lignocellulosic material to bioethanol. Moreover, there is a great need for cost-effective fermentation of derived sugars from cellulose and also from hemicellulose (Wyman *et al.*, 2005). Therefore, there is a lot of work ahead for researchers to efficiency use promising resources – plant biomass.

1.5 Aims of the thesis

Since first developed by G.P. Smith in 1985 (Smith, 1985), bacterial surface engineering has become more attractive to scientists because of its usefulness in various fields of biotechnological application. As mentioned before, the *B. subtilis* endospore has some advantages compared to phages and whole cells in recombinant proteins surface display. For this reason, it has recently gained a lot of attentions from scientists in developing new systems. The conventional method for anchoring a protein on the *B. subtilis* spore surface is to fuse the coding region of target protein with a gene which encodes any of the outer spore coat protein. For expression of the fusion, the native promoter of the carrier gene can be used (Kim *et al.*, 2007; Ricca and Cutting, 2003). As a result, the target protein would be produced in the cytoplasm and anchored on the spore surface together with the carrier coat protein when cells start to sporulate. When the mother cells lyse, spores will be released with the target protein displayed on surface. This method guarantees that the fusion protein will be synthesized at the right time for spore coat formation; however, the amount of produced fusion proteins cannot be controlled. Therefore, the major aim of this doctoral thesis was to construct more effective expression systems for spore surface protein anchoring. First, the native promoter of carrier coat gene was substituted by IPTG-inducible promoters. Second, the copy number of fusion genes was also increased. The efficiency of the new systems was evaluated through activities of reporter proteins. A new *B. subtilis* spore-based system for protein expression and purification was then developed, taking advantage of N-terminus DnaB mini-intein of *Synechocystis* sp. (Mathys *et al.*, 1999). The last aim of the thesis was to apply the newly constructed *B. subtilis* spore display and the cell surface display systems (Nguyen and Schumann, 2006) to generate cellulose chips which can degrade solubilized CMC substrates.

2 Materials and methods

2.1 Materials

2.1.1 Bacterial strains

The bacteria strains used in the course of this work are listed in the Table 2.1

Table 2.1 Bacterial strains used in this work

Name	Description	Reference
DH10B (*E. coli*)	F^-, *mcrA*, Δ(*mrr*, *hsdRMS*, *mcrBC*), ϕ80d (*lacZΔM15*, Δ*lacX74*), *deoR*, *recA1*, *araD139*, Δ(*ara*, *leu*)$_{7697}$, *galK*, λ^-, *rpsL*, *endA1*, *nupG*	Bethesda Research laboratories
XL1 Blue (*E. coli*)	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' *proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^R)]	Stratagene
1012	*leuA8 metB5 trpC2 hsdRM1*	(Saito *et al.*, 1979)
WW02	1012 *amyE*::*neo* (Neo^R)	(Wehrl *et al.*, 2000)
WB800N	*nprE aprE epr bpr mpr* :: *ble nprB* :: *bsr* Δ*vpr wprA* :: *hyg cm* :: *neo*; Neo^R (WB800 pB-*cat*5-*neo*-*cat3*)	(Nguyen, 2006)
WB800Spec	WB800N *amyE*::*spec* ($Spec^R$)	This work
QAS03	WB800N *amyE*::P_{cotB}-*cotB-linker* (Cm^R)	This work
QAS06	WB800N *amyE*::P_{cotB}-*cotB-linker-amyQ* (Cm^R)	This work
QAS52	WB800N *amyE*::P_{cotB}-*cotB-gfp*$_{uv}$ (Cm^R)	This work
QAS43	WB800N *amyE*::P_{grac}-*cotB-gfp*$_{uv}$ ($Spec^R$)	This work
QAS48	WB800N *amyE*::P_{grac}-*cotC-gfp*$_{uv}$ ($Spec^R$)	This work
QAS49	WB800N *amyE*::P_{grac}-*cotG-gfp*$_{uv}$ ($Spec^R$)	This work
QAM121	WB800N *amyE*::P_{cotB}-*cotB-miniInt* (Cm^R)	This work
QAS40	WB800N *amyE*::P_{cotB}-*cotB-miniInt-amyQ* (Cm^R)	This work

QAS15	WB800N *amyE*::P_{cotB}-*cotB-celA* (Cm^R)	This work
QAS16	WB800N *amyE*::P_{cotG}-*cotG-celA* (Cm^R)	This work
QAC07	WB800N *thrC::PdnaK-srtA* ($Spec^R$)	This work

2.1.2 Plasmids

The plasmids used during this work are listed in the Table 2.2

Table 2.2 Plasmids used during this work

Name	Description	Ref.
pDG1730	Integration vector for *B. subtilis* at *amyE*, $Spec^R$	(Guérout-Fleury *et al.*, 1996)
pDG1731	Integration vector for *B. subtilis* at *thrC*, $Spec^R$	(Guérout-Fleury *et al.*, 1996)
pDG364	Integration vector for *B. subtilis* at *amyE*, Cm^R	(Cutting and Vander Horn, 1990)
pHT01	Plasmid- based expression vector for *B. subtilis*, containing IPTG – inducible P_{grac} promoter, Cm^R	(Nguyen *et al.*, 2007)
pBG01	Plasmid-based expression vector for *B. subtilis*, containing strong, IPTG–inducible P_{Sgrac} promoter, Cm^R	BayGenetics
pSDJH-cotG-GFP_{uv}	Template for gfp_{uv} gene	(Kim *et al.*, 2007)
pKTH10	pUB110 with *amyQ,* template for *amyQ* gene	(Palva, 1982)
pCT105	pBR322 + *celA*, template for *celA*	(Cornet *et al.*, 1983)
pSW01	pDG364 with P_{cotB} promoter and *cotBΔ* gene, Cm^R	(Wendel, 2007)
pHCMC01	pMTLBs72 with *trpA* transcriptional terminator	(Nguyen *et al.*, 2005)

pQAS03	pSW01 with a flexible linker (G-G-G-G-S) coding region translationally fused to *cotBΔ* gene, Cm^R	This work
pQAS05	pDG364 with P_{cotG} promoter, *cotG* gene and a flexible linker (G-G-G-G-S) coding region translationally fused to *cotG* gene, Cm^R	This work
pQAS07	pHCMC01 with P_{cotB} promoter and *cotBΔ* gene, Cm^R	This work
pQAS52	gfp_{uv} gene translationally fused to c*otBΔ* gene in pQAS03, Cm^R	This work
pQAS17	pHT01 with *cotBΔ* gene under the control of P_{grac} promoter, Cm^R	This work
pQAS18	pHT01 with *cotC* gene under the control of P_{grac} promoter, Cm^R	This work
pQAS19	pBG01 with *cotBΔ* gene under the control of P_{Sgrac} promoter, Cm^R	This work
pQAS20	pHT01 with *cotG* gene under the control of P_{grac} promoter, Cm^R	This work
pSK01	gfp_{uv} gene translationally fused to *cotBΔ* gene in pQAS17, Cm^R	(Krauß, 2009)
pQAS43	Fusion *cotBΔ*-gfp_{uv} gene under control of P_{grac} promoter in pDG1730, $Spec^R$	This work
pQAS48	Fusion *cotC*-gfp_{uv} gene under control of P_{grac} promoter in pDG1730, $Spec^R$	This work
pQAS49	Fusion *cotG*-gfp_{uv} gene under control of P_{grac} promoter in pDG1730, $Spec^R$	This work
pQAS06	*amyQ* gene translationally fused to *cotBΔ* gene in pQAS03, Cm^R	This work
pQAS32	*amyQ* gene translationally fused to *cotBΔ* gene in	This work

	pQAS17, Cm^R	
pQAS34	*amyQ* gene translationally fused to *cotBΔ* gene in pQAS19, Cm^R	This work
pQAS53	*amyQ* gene translationally fused to *cotBΔ* gene in pQAS07 , Cm^R	This work
pMTB121	*miniInt* gene (mini-intein) translationally fused to *cotBΔ* gene in pSW01, Cm^R	M. T. Batista
pQAS40	*amyQ* gene translationally fused to *miniInt* gene in pMTB121, Cm^R	This work
pQAS41	pBG01 with fusion *cotBΔ-miniInt-amyQ* gene, Cm^R	This work
pQAS54	pHCMC01 with the fusion *cotBΔ-miniInt-amyQ* gene under control of P_{cotB} promoter, Cm^R	This work
pNDH11	Plasmid-based expression vector for anchoring protein on the *B. subtilis* cell wall, containing P_{xylA} promoter, S_{amyQ} signal sequence and *fnbB94* sorting sequence, Cm^R	(Nguyen, 2006)
pQAC01	pDG1731 with P_{dnaK} promoter, $Spec^R$	This work
pQAC02	pDG1731 with *srtA* gene under control of P_{dnaK} promoter , $Spec^R$	This work
pQAC07	pNDH11 with *celA* gene translationally fused to and flanked by S_{amyQ} signal sequence and *fnbB94* sorting sequence, Cm^R	This work
pQAS15	*celA* gene translationally fused to *cotBΔ* gene in pQAS03	This work
pQAS16	*celA* gene translationally fused to *cotG* gene in pQAS05, Cm^R	This work
pQAS24	*celA* gene translationally fused to *cotBΔ* gene in pQAS17, Cm^R	This work

pQAS25	*celA* gene translationally fused to *cotG* gene in pQAS20, Cm^R	This work
pQAS27	*celA* gene translationally fused to *cotC* gene in pQAS18, Cm^R	This work

2.1.3 Oligonucleotides

The oligonucleotides used during this work are listed in the Table 2.3

Table 2.3 Oligonucleotides used in the course of this work

Name	Sequence (5' to 3')	Description
ON01	AGCTTGGCGGAGGCGGATCAATCGATG	Forward strand of the linker
ON02	AATTCATCGATTGATCCGCCTCCGCCA	Reverse strand of the linker
ON03	GGCCATGGATCCAGTGTCCCTAGCTCCGAGA	5' end of the promoter region of *cotG*
ON04	GGCCATGAATTCTGAACCCCCACCTCCTTTGTATTTCTTTTTGACTACCC	3' end of the *cotG*
ON05	GGCCATGAGCTCACGGATTAGGCCGTTTGTCC	5' end of the promoter region of *cotB*
ON06	GGCCATGGATCCGGATGATTGATCATCTGAAGATTT	3' end of the *cotB*
ON07	GGCCATAGATCTATGAGCAAGAGGAGAATGA	5' end of the *cotB*
ON08	GGCCATAGATCTATGGGTTATTACAAAAAATACAAAGAAG	5' end of the *cotC*
ON09	GGCCATGGATCCGTAGTGTTTTTTATGCTT	3' end of the *cotC*
ON10	GGCCACTATAGATCTATGTCCCATTCTG	5' end of the *cotG*
ON11	GGCCATGGATCCTTTGACTACCCAGCAATT	3' end of the *cotG*

ON12	GGTATAAACTTTTCAGTTGCAGACAAAGAT	Reverse primer for pHCMC01-derived plasmid
ON13	CCGGTAGAATTCATGAGTAAAGGAGAAGAACT	5’ end of the gfp_{uv}
ON14	CCGGTAGAATTCATGAGTAAAGGAGAAGAACT	3’ end of the gfp_{uv}
ON15	CCGGTAGACGTCATGAGTAAAGGAGAAGAACT	5’ end of the gfp_{uv}
ON16	CCCATAGACGTCTCATTATTTGTAGAGCTCATC	3’ end of the gfp_{uv}
ON17	GGCCTAATCGATGTAAATGGCACGCTGATGCAGTA	5’ end of the *amyQ*
ON18	GGCCTAATCGATGACCTTGTCATACGGCTGAAAAAA	3’ end of the *amyQ*
ON19	GGCCTAGGATCCGTAAATGGCACGCTGATGCAGTA	5’ end of the *amyQ*
ON20	GGCCTAGACGTCTTTATTTCTGAACATAAATGGAGACGGA	3’ end of the *amyQ*
ON21	GCCATGCGGCCGCCGTAAATGGCACGCTGAT	5’ end of the *amyQ*
ON22	GGCCATGCGGCCGCGTTTTTATTACCTTATTTCTG	3’ end of the *amyQ*
ON23	GGCCATTCTAGAATGAGCAAGAGGAGAATG	5’ end of the *cotB*
ON24	GGCCATGAATTCGCAGGTGTGCCTTTTAACAC	5’ end of the *celA*
ON25	GGCCATATCGATCTAATAAGGTAGGTGGGGTATG	3’ end of the *celA*
ON26	GCATGACGTCGCAGGTGTGCCTTTTAACAC	5’ end of the *celA*
ON27	GGCCATGACGTCTAATAAGGTAGGTGGGGTATG	3’ end of the *celA*
ON28	AGTAGTGGATCCTTATTAGGCAATGAAGTT	5’ end of the promoter region of *dnaK* (P_{dnaK})
ON29	GTGTAAGCTTCATCATCACCTCTGTTAGC	5’ end of the promoter

		region of *dnaK* (P_{dnaK})
ON30	GGCCATAAGCTTATGTTAAAGAAAACAATTGCA	5' end of the *srtA*
ON31	GGCCATGAATTCTTATTTACTAGGGAAATATTTATT	3' end of the *srtA*

2.1.4 Antibiotics

Information is given in Table 2.4 concerning the antibiotics solutions, which were used in the course of this work.

Table 2.4 Antibiotic solutions used in this work

Antibiotic	Concentration of stock solution (mg/ml)	Dissolved in	Final concentration (µg/ml)
Ampicillin	50 - 100	70% ethanol	100
Chloramphenicol	20	ethanol	10
Erythromycin	1 or 100	ethanol	1 or 100
Neomycine	10	water	10
Spectinomycin	100	water	100 water 100

2.1.5 Enzymes

- Roche: Alkaline phosphatase, Lysozyme, Proteinase K, RNase A
- New England Biolabs: Taq DNA polymerase, T4 DNA-Ligase
- Fermentas: Restriction enzymes

2.1.6 Antibodies

Table 2.5 Antibodies used in this work.

Name	Dilution for immunofluorescence	Dilution for Immunoblotting	Reference
α-AmyQ	1:1000	1 : 50000	V. Kontinen
α-CelA	1:300	1 : 2000	(Cornet *et al.*, 1983)
α-GFP	1:500	1 : 5000	Clontech
α-SrtA		1:10000	O. Schneewind
Anti-Rabbit IgG		1: 10000	Amersham™
Fluor®488 donkey anti-rabbit IgG	1: 600		Molecular probes
Cy3 donkey anti-rabbit IgG	1: 600		Molecular probes

2.1.7 Media

- LB medium: 1 % ($^{w}/_{v}$) tryptone, 0.5 % ($^{w}/_{v}$) yeast extract, 1 % ($^{w}/_{v}$) NaCl
- Sporulation medium DSM: 0.8 % ($^{w}/_{v}$) Difco Nutrient Broth, 0.1 % ($^{w}/_{v}$) KCl and 0.025 % $MgSO_4.7H_2O$. Adjust the pH to 7 with KOH. After autoclaving, the medium was supplied with 1 mM $Ca(NO_3)_2$, 0.01 mM $MnCl_2$ and 0.01 mM $FeSO_4$
- Sporulation medium 2x SG: 1.6 % ($^{w}/_{v}$) Difco Nutrient Broth, 0.2 % ($^{w}/_{v}$) KCl, and 0.05 % $MgSO_4.7H_2O$. Adjust the pH to 7 with KOH. After autoclaving, the medium was supplied with 0.1 % glucose, 1 mM $Ca(NO_3)_2$, 0.1 mM $MnCl_2$ and 0.01 mM $FeSO_4$
- Antibiotics (Table 2.4), 0.5 % ($^{w}/_{v}$) insoluble starch or 0.5 % CMC ($^{w}/_{v}$) were added when necessary
- Agar was added to 1.5 % ($^{w}/_{v}$) to prepare plates

2.1.8 Chemicals and biochemicals

- Amersham: Amonium persulphate, hyperfilm ECL

- Fermentas: DNA ladder and protein ladder
- Roche: Blocking reagent, protease inhibitor cocktail, Xgal
- Roth: Acetic acid, agar, agarose, aquatic phenol, chloroform, ethidium bromide, isopropanol, MOPS, potasium acetate, potasium phosphate, polyacrylamide, sodium phosphate, sodium chloride, starch, sodium dodecyl sulphate (SDS), sucrose, Tris, xylose, yeast extract
- Merck: D-glucose, soluble starch
- Sigma: Carboxyl methylcellulose (low viscosity), Renografin (Sodium diatrizoate dihydrate)

2.1.9 Kits

- Qiagen: PCR purification kit, gel-extraction kit, midi purification kit
- Peq Lab: Peq GOLD Cycle-Pure Kit
- Pierce: Super Signal® West Pico Chemiluminescent Substrate

2.2 Methods

2.2.1 General methods

2.2.1.1 PCR

The purpose of a PCR (Polymerase Chain Reaction) is to make a huge number of copies of a DNA sequence, e.g., a gene, to provide enough DNA for cloning. The first DNA polymerase used for PCR was from *E. coli* (Saiki *et al.*, 1985). Then, PCR became effective by the isolation of a thermostable DNA polymerase from *Thermus aquaticus* (Saiki *et al.*, 1988). During the PCR, DNA is denatured at high temperature and specific oligonucleotide primers are annealed and elongated at lower temperature in a cyclic manner.

2.2.1.2 Cloning

All the steps necessary for cloning were carried out as described by using standard methods (Sambrook and Russell, 2001). Preparation of competent *E. coli* cells and transformation were carried out as standard heat shock transformation (Inoue *et al.*, 1990) and electroporation

(Dower *et al.*, 1992); PCR for screening of plasmids and preparation of plasmid DNA by the alkaline lysis method with SDS have been described (Birnboim and Doly, 1979; Ish-Horowicz and Burke, 1981). The correct DNA sequence of all inserts into plasmids was verified by sequencing and carried out by SeqLab, and only plasmids with the correct DNA sequence were used in further experiments.

2.2.1.3 Growth and collection of samples

During this work, *B. subtilis* strains were grown in LB or 2x SG medium with the appropriate antibiotic(s) when necessary in a water-bath shaker (~200 rpm) at 37 °C. Overnight cultures in 3 ml LB medium in glass tubes were transferred partially to Erlenmeyer flasks containing medium to an OD_{578} of 0.05-0.08.

In case of cell samples collection, when an OD_{578} of 0.8 was reached (set as t = 0), the inducer (0.5% of xylose) was added. Aliquots were removed and centrifuged, and either the pellet and/or the culture supernatant were collected. Further samples were taken at different time points after induction as indicated in the experiments. Normally, a certain amount of cells was collected corresponding to 1.2 or 2.5 of OD_{578}.

For spore samples collection, after 6 h of inoculation at 37 °C, 0.2X of a protease inhibitors cocktail (Roche Diagnostics) solution was added (1X is equal to 1 tablet of protease inhibitor cocktail in 50 ml volume). The culture was then divided into subcultures where one was further grown in the absence and the others in the presence of the inducer (IPTG concentrations were indicated in each experiment). After 24 h of inoculation at 37 °C, whole cells, sporulating cells and spores were harvested by centrifugation.

2.2.1.4 Spore purification method

Single colonies of appropriate *B. subtilis* strains were grown overnight in LB at 37 °C, well shaken. Sporulation of *B. subtilis* strains was induced by the exhaustion method (using 2x SG medium) (Nicholson and Setlow, 1990). The spores were purified by the Renografin (sodium diatrizoate, S-4506, Sigma) gradient method (Nicholson and Setlow, 1990) with some modifications. After 24 h of inoculation at 37 °C, vegetative cells, sporulating cells and spores were harvested and washed several times with double distilled water (ddH_2O). The pellets were resuspended in 0.2 ml of 10% Renografin, and then gently layered over 1 ml of 50% renografin in a new 1.5 ml Eppendorf tube. This small gradient column was centrifuged for 15 min at 11000

rpm. After centrifugation, three bands appear in the Eppendorf tube with purified spores present at the bottom, vegetative cells reside in the top layer and just beneath the top layer, sporulating cells form a band. After careful removal of all the debris containing vegetative cells, sporulating cells and broken cells, the purified spores remained at the bottom of the tube. The spore pellets were then washed several times with ddH_2O to get rid of the residual Renografin. Purified spores were directly used for subsequent experiments such as FACS analysis or spore coat protein extraction.

2.2.2 Protein methods

2.2.2.1 Extraction of denatured total cellular lysate from *B. subtilis*

For the extraction of denatured cell lysate from *B. subtilis* cells (2.5 of OD_{578}) prepared as described in 2.2.1.3 were resuspended in 100 µl of TM buffer (50 mM Tris-HCl, pH 8.0, 16 mM $MgCl_2$) containing 0.5 mg/ml lysozyme and incubated at 37°C for 5 min. Then, 50 µl of 3x sample loading buffer (0.135 M Tris/HCl, 30% glycerol, 3% SDS, 0.03% bromophenol blue, 0.15 M DTT) was added to the suspension and frozen until use. Before the samples were used, they had been heated for 5 min at 95°C, and 15 µl of each sample were used for SDS-PAGE.

2.2.2.2 Preparation of proteins from the *B. subtilis* cell wall fraction

The cell wall fraction from *B. subtilis* was prepared with a cell fractionation method which has been described previously (Merchante *et al.*, 1995; Helfrich *et al.*, 2007). *B. subtilis* cultures corresponding to an OD_{578} of 10 were taken and the cells were collected by centrifugation. The pellets were washed twice with TM buffer (50 mM Tris-HCl, pH 8.0, 16 mM $MgCl_2$). To release the protein from the cell wall, whole cells were resuspended in 0.2 ml of TMS buffer (50 mM Tris-HCl, pH 8.0, 16 mM $MgCl_2$, 33 % ($^{w}/_{v}$) sucrose) containing 0.2 mg/ml lysozyme and incubated at 37°C for 45 min. The protoplast fraction was discarded by centrifugation (12000 rpm, 15 min, room temperature). The supernatant was then used for SDS-PAGE by addition of loading buffer or for determination of cellulase activity.

2.2.2.3 Extraction of *B. subtilis* spore-coat proteins

Purified spores corresponding to an OD_{600} of 5 were decoated by treatment for 60 min at 70 °C with 100 µl of ST solution (1% sodium dodecyl sulfate (SDS), 50 mM dithiothreitol (DTT)). Then, the spores were sedimented by centrifugation (13000 rpm, 5 min, 4 °C). The coat

protein extract was then used directly for SDS-PAGE by addition of 20 µl of 6x loading buffer and heating at 95 °C for 5 min.

2.2.2.4 Measurement of protein concentrations

The method of Bradford was used for the measurement of the protein concentrations from cell extracts (Bradford, 1976).

2.2.2.5 Protein electrophoresis using discontinuous SDS-PAGE

The electrophoretic separation of proteins according to their molecular mass was performed as first described by Laemmli (Laemmli, 1970).

2.2.2.6 Immunoblotting analysis

For the immunochemical detection of proteins using antibodies, the proteins were transferred, after their electrophoretic separation, onto a nitrocellulose membrane using electroblotting (Towbin *et al.*, 1979). The electrophoretic transfer of proteins to nitrocellulose membranes was achieved by "Semi-Dry-Blotting" between graphite plate electrodes in a "Fast-Blot" apparatus (Biorad). The procedure for detection of labelled proteins followed the instruction of ECL Western blot (Amersham Biosciences). The signals were detected by the LAS4000 machine (*Fujifilm*) and the collected pictures were analyzed by Multi Gauge Ver3.1 Software (*Fujifilm*).

To compare the expression level of proteins on the cell or spore surface, equal amounts of sample should be used for each lane. For cell surface samples, the cell wall fraction from cell of OD_{578} of 2.5 were applied. In case of spore surface samples, the spore coat extraction from purified spores with an OD_{600} of 1 were applied in each lane.

2.2.2.7 Activation of the mini-intein for the release of target protein

The spores from examined strains were purified, washed three times with intein washing buffer (50 mM Tris-HCl, 0.5 M NaCl and 1 mM EDTA, pH 8.5). For activation of the mini-intein, the amount of spores at OD_{600} of 5 per ml were incubated in intein cleavage buffer (50 mM Tris-HCl, 0.5 M NaCl and 1 mM EDTA, pH 8.5), well shaken at 25°C. Samples were collected at different times, supernatants and spores were separated by centrifugation.

2.2.3 Visualization of protein expression via plate assays

2.2.3.1 α-Amylase (AmyQ)

Single colonies of the *B. subtilis* strains carrying the AmyQ coding gene on a plasmid or integrated into chromosomal DNA were grown for 24 h either on LB plates containing 0.5 mM IPTG (when P_{grac} or P_{Sgrac} promoters were used) or DSM plates (when native promoters were used) with 0.5 % insoluble starch, then stained with I_2/KI solution (Nicholson and Chambliss, 1985). Pictures were taken by a digital camera.

2.2.3.2 GFPuv

Single colonies of the *B. subtilis* strains carrying the GFP_{uv} coding gene present on a plasmid or integrated into chromosomal DNA were grown for 24 h either on LB plates containing 0.5 mM IPTG (when P_{grac} or P_{Sgrac} promoters were used) or DSM plates (when native promoters were used). These plates were then observed under an MZFLIII microscope (Leica) using the GFP2 filter. The pictures were recorded directly through the object lens of the microscope by a digital camera.

2.2.3.3 Cellulase A (CelA)

Single colonies of the *B. subtilis* strains carrying plasmid pQAS07 or pNDH11 were grown for 24 h on LB plates containing 0.5 % xylose and 0.5 % CMC and stained with 1% Congo red solution (Cornet *et al.*, 1983). Pictures were taken by a digital camera.

2.2.4 Quantitative and qualitative analysis of protein expression

2.2.4.1 Visualization of surface immobilized proteins by Confocal microscope

Alive cells and purified spores were washed twice in AP buffer (100 mM Tris-HCl pH 7.4, 100 mM NaCl, 0.25 mM $MgCl_2$). For immunofluorescence staining, first the cells/spores were blocked in AP buffer containing 3 % BSA (APB buffer) then resuspended in APB buffer containing the primary antibody raised in rabbits against *B. amyloliquefaciens* α-amylase (at a dilution of 1:1000) or *C. thermocellum* cellulase A (at a dilution of 1:300) for 2 h on ice. After being washed three times with cold AP buffer, the pellets were incubated with anti-rabbit IgG Alexa conjugate (Alexa Fluor®488 donkey anti-rabbit IgG; Molecular Probes) in APB buffer at a dilution of 1:600 for 2 h on ice. The cells/spores were then washed again three times with cold

AP buffer. Generally, no staining process was needed for the samples with GFPuv expression; the spores/cells were used directly for visualization.

For visualization of cells/spores under the confocal microscope, a 5 µl aliquot of the stained cells or spores suspension was mixed with 10 µl of 1% agarose and spread onto a glass slide. The fluorescence images were acquired by a Leica SP2 or SP5 confocal microscope (Leica Microsystems, Germany) and processed by using Adobe Photoshop (Adobe Systems Inc., San Jose, CA, USA).

2.2.4.2 FACS analysis

The spores displaying GFPuv from different cultures were purified, then washed three times with ddH_2O. Dilutions of 10^5 spores per ml in ddH_2O were directly examined under a Beckman-Coulter FC-500 MCL System and the SXP Software was used for data analysis.

2.2.4.3 α-Amylase assay

α-Amylase activity was determined as described (Nicholson and Chambliss, 1985) with whole spores and with the supernatants and presented in units per OD_{600}. One unit is defined as a decrease in OD_{620} of 0.1. All experiments were repeated at least twice.

2.2.4.4 Cellulase assay

The cellulase assay was carried out as previously described using soluble dye-labelled substrate. First, carboxyl methylcellulose (CMC, low viscosity, Sigma) was labeled with Remazol Brilliant Blue (RBB) followed by the L. Fülöp and T. Ponyi's method (Fulop and Ponyi, 1997). A reaction buffer was prepared as 0.55 % ($^{w}/_{v}$) RBB-O-CMC in 0.1 M phosphate buffer pH 6.8.

The 0.25 ml cells/spores samples were transferred into 1.5-ml Eppendorf tubes and mixed with equal volume of reaction buffer then incubated for 1 h at 37 °C. The cell/spore pellets were gained by centrifugation. Two volumes of stop solution (ethanol: acetone 1:1 ($^{v}/_{v}$)) was then added to each supernatant. The precipitated substrate was removed by centrifugation at 3000 g for 2 min at room temperature, and the absorbance of the supernatants was measured at 590 nm against the substrate blank. The CMCase activities were determined with whole cells and spores and presented as Units per OD_{578} (for cells) or OD_{600} (for spores). One enzyme unit is defined as

the amount of enzyme which released one absorbance unit (590 nm) within 1 h from RBB-O-CMC under defined conditions (Fulop and Ponyi, 1997).

2.2.4.5 Determination of the number of α-amylase molecules on the spore surface

Spores from two different cultures were withdrawn, purified by the Renografin method as described in 2.2.1.4. The spore-coat proteins were then extracted by treatment with SDS and DTT as described in 2.2.2.3. Decoated spores were centrifuged and 12.5 µl of supernatant corresponding to 1.5 x 10^8 spores were applied per lane. Defined amounts of purified α-amylase (Sigma) from 22.5 to 60 ng (corresponding to 0.45 – 1.2 pmol) were run on the same gel. The collected pictures were analyzed by the Multi Gauge Ver3.1 Software (*Fujifilm*). Only the material in the upper band of the samples was quantified.

2.2.5 Construction of plasmids and strains

2.2.5.1 Construction of vectors for displaying recombinant proteins on the spore surface

For displaying recombinant proteins on the spore surface, the coat protein CotB and CotG were chosen as carrier proteins. The vectors were constructed in such a way that the fusions can be either integrated into or replicated independently of the *B. subtilis* chromosomal DNA.

First, to construct vectors which can allow integration of transcriptional fusions at the *amyE* locus, the integration vector pDG364 was chosen. The vector contains a multiple cloning site and a *cat* gene which encodes chloramphenicol acetyl transferase sandwiched between *amyE*-front and *amyE*-back (Cutting and Vander Horn, 1990) and was used as a backbone for construction of the pQAS03 and pQAS05 plasmids. The pQAS03 vector was a modified plasmid from pSW01 (Wendel, 2007) by addition of a flexible linker (G-G-G-G-S). The flexible linker was produced by using the two complementary oligonucleotides ON01 and ON02 (Table 2.3) and inserted downstream of *cotBΔ* gene at *Hin*dIII and *Eco*RI sites of pSW01 resulting in pQAS03. The pQAS05 vector contains *cotG* gene with its own promoter. The *cotBΔ* gene is a shortened version of *cotB* deleted of the three 27-amino-acids repeats. It has been shown that fusions of recombinant proteins to *cotBΔ* are correctly assembled and exposed on the spore surface (Isticato *et al.*, 2001).

The *cotG* cassette was received from a PCR reaction with the two primers ON03 and ON04 (Table 2.3) using *B. subtilis* 1012 chromosomal DNA as template. The ON04 was

designed with the flexible linker at 3' end. The amplicon was then cleaved by *Bam*HI and *Eco*RI and ligated into pDG364 treated with the same enzymes resulting in pQAS05.

Second, the *cotB* cassette contains the *cotBΔ* gene (written as *cotB* from now on in this thesis) and its own promoter was amplified by PCR with the ON05 and ON06 primers (Table 2.3) using *B. subtilis* 1012 chromosomal DNA as template. This cassette and the pHCMC01 (Nguyen *et al.*, 2005) plasmid were treated with *Sac*I and *Bam*HI enzymes and ligated together resulting in pQAS07.

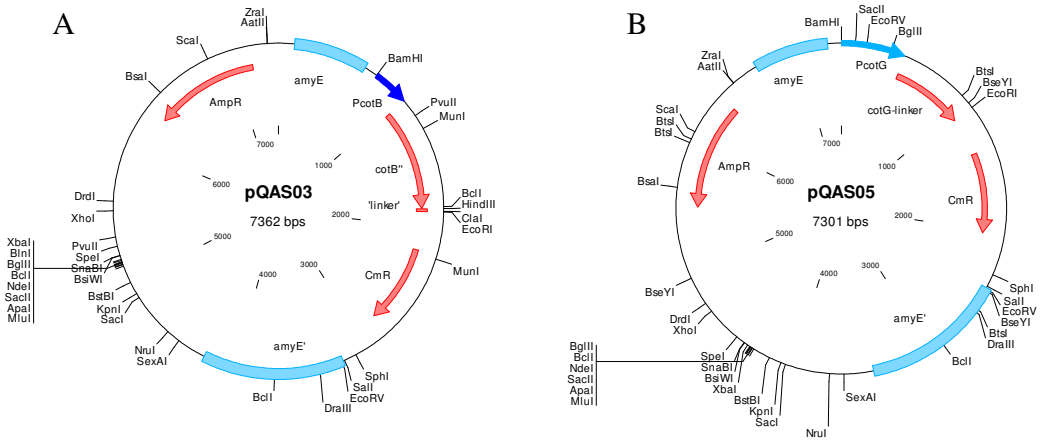

Fig 2.1 Genetics and restriction maps of the integration vectors for displaying recombinant proteins on the spore surface using native promoters. *(A): pQAS03 with the cotBΔ gene; (B): pQAS05 with the cotG gene.*

Third, the coding regions of CotB, CotC and CotG were amplified from the genome of *B. subtilis* 1012 using ON07 and ON06 (*cotB*), ON08 and ON09 (*cotC*), and ON10 and ON11 (*cotG*). All three amplicons were cleaved by *Bgl*II and *Bam*HI and inserted into the pHT01 (Nguyen *et al.*, 2007) plasmid treated with *Bam*HI. The orientation of the insertions was verified by PCR using ON12 and the forward primer of each gene and also tested with appropriate restriction enzymes. The plasmids were constructed so that the translation of *cotB*, *cotC* and *cotG* were put under control of the IPTG-inducible P_{grac} promoter, resulting in pQAS17, pQAS18 and pQAS20, respectively. The *Bgl*II- and *Bam*HI- treated *cotB* amplicon was also inserted into the

pBG01 vector, containning the strong IPTG-inducible P_{Sgrac} promoter, at *Bam*HI, resulting in pQAS19.

2.2.5.2 Construction of plasmids for the display of GFPuv and AmyQ on the spore surface

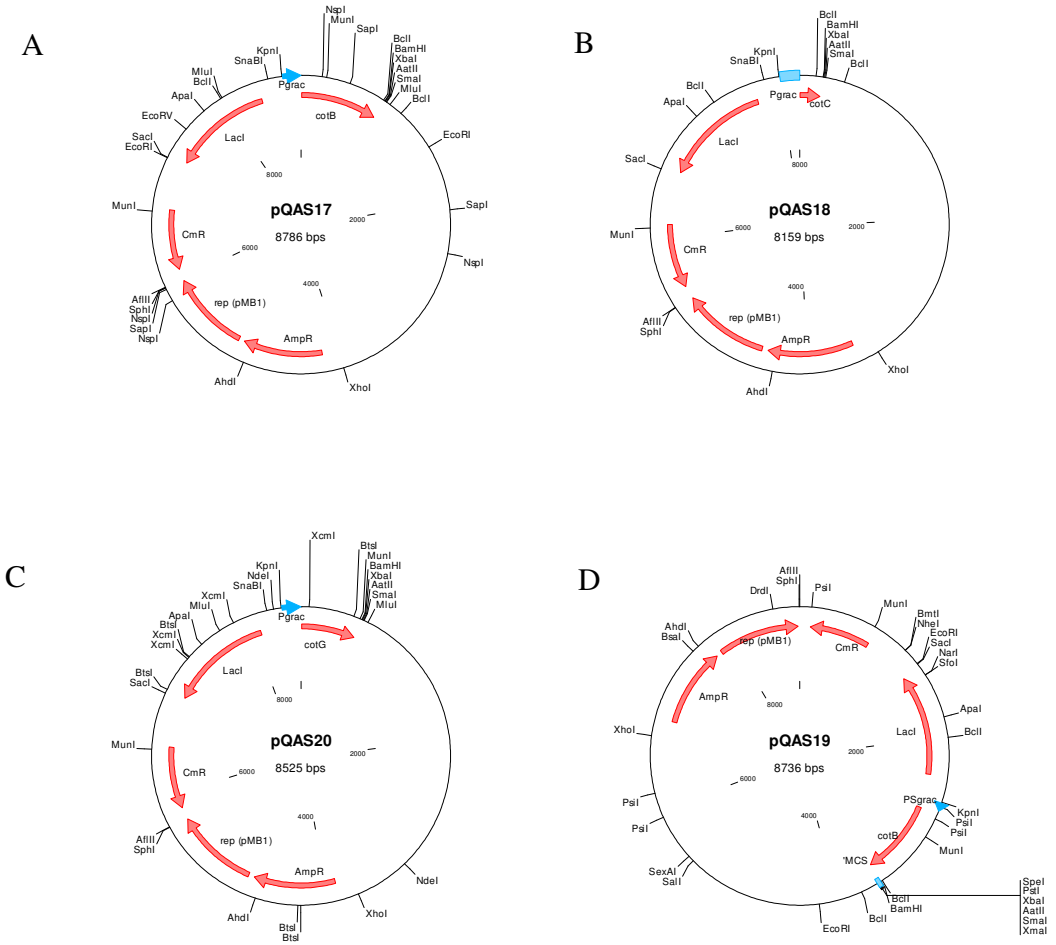

Fig 2.2 Genetics and restriction maps of the plasmid-based vectors for displaying recombinant proteins on the spore surface using IPTG-inducible promoters. *P_{grac} promoter: (A): pQAS17 with the cotB gene; (B): pQAS18 with the cotC gene; (C): pQAS20 with the cotG gene; P_{Sgrac} promoter: (D): pQAS19 with the cotB gene. All vectors replicate both in E. coli and in B. subtilis and contain the indicated cloning sites downstream of the cot gene.*

To allow display of the α-amylase *amyQ* of *B. amyloliquefaciens* and GFP_{uv}, an enhanced version from GFP protein of the jellyfish *Aequorea victoria* (Crameri *et al.*, 1996), on *B. subtilis*

spore surface, the coding region of these proteins were fused with the coat genes using the previously constructed vectors, followed by transformation into the *B. subtilis* WB800N strain. The pSDJH-cotG-GFP$_{uv}$ plasmid (Kim *et al.*, 2007) was used as a template for amplification of the *gfp$_{uv}$* gene via PCR. The *amyQ* gene was amplified using pKTH10 plasmid (Palva, 1982) as template. Constructions of the plasmids which allow display of GFPuv or AmyQ on the spore surface were listed below (***Fig 2.3*** and ***Fig 2.4***).

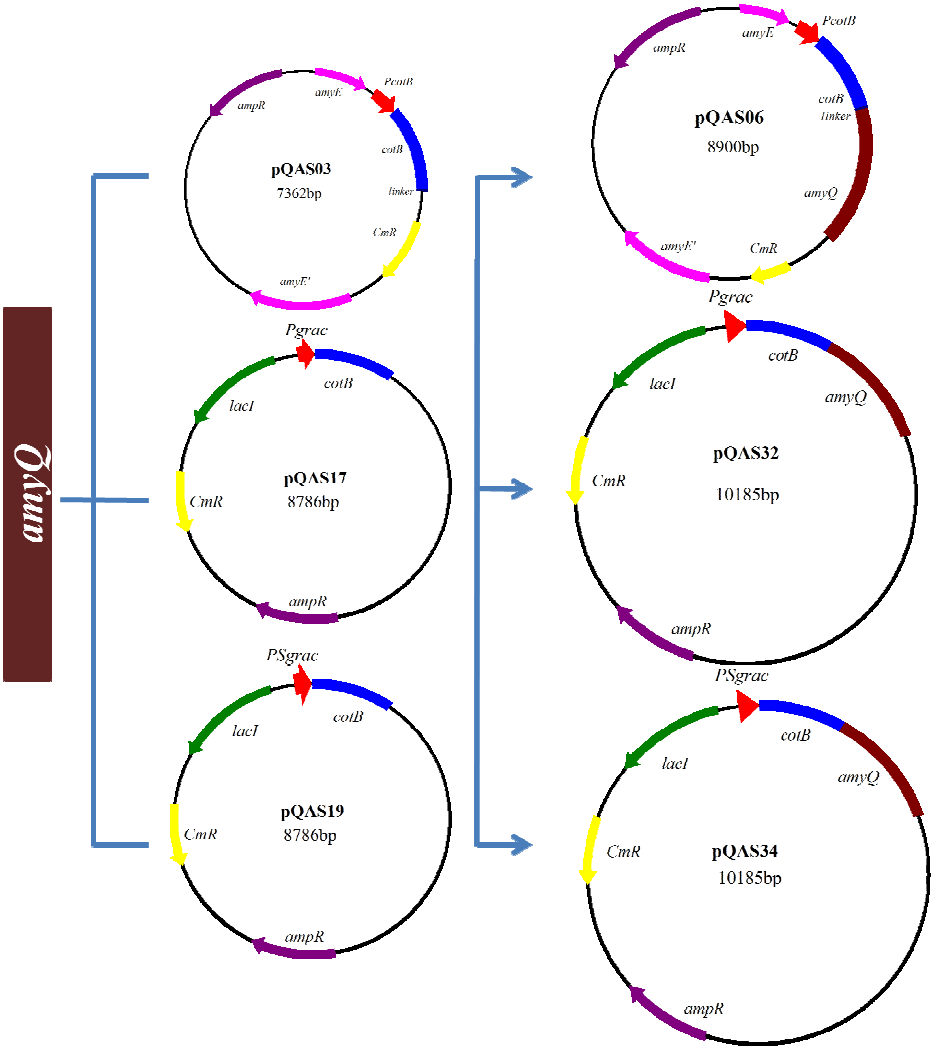

Fig 2.3 ***Construction of plasmids for display of AmyQ on the spore surface.*** *The amyQ gene was amplified by PCR and then inserted downstream of the cotB gene in the spore display vectors, under the control of the cotB native promoter, the P_{grac} promoter and the P_{Sgrac} promoter (pQAS03, pQAS17 and pQAS19) to generate pQAS06, pQAS32 and pQAS34, respectively.*

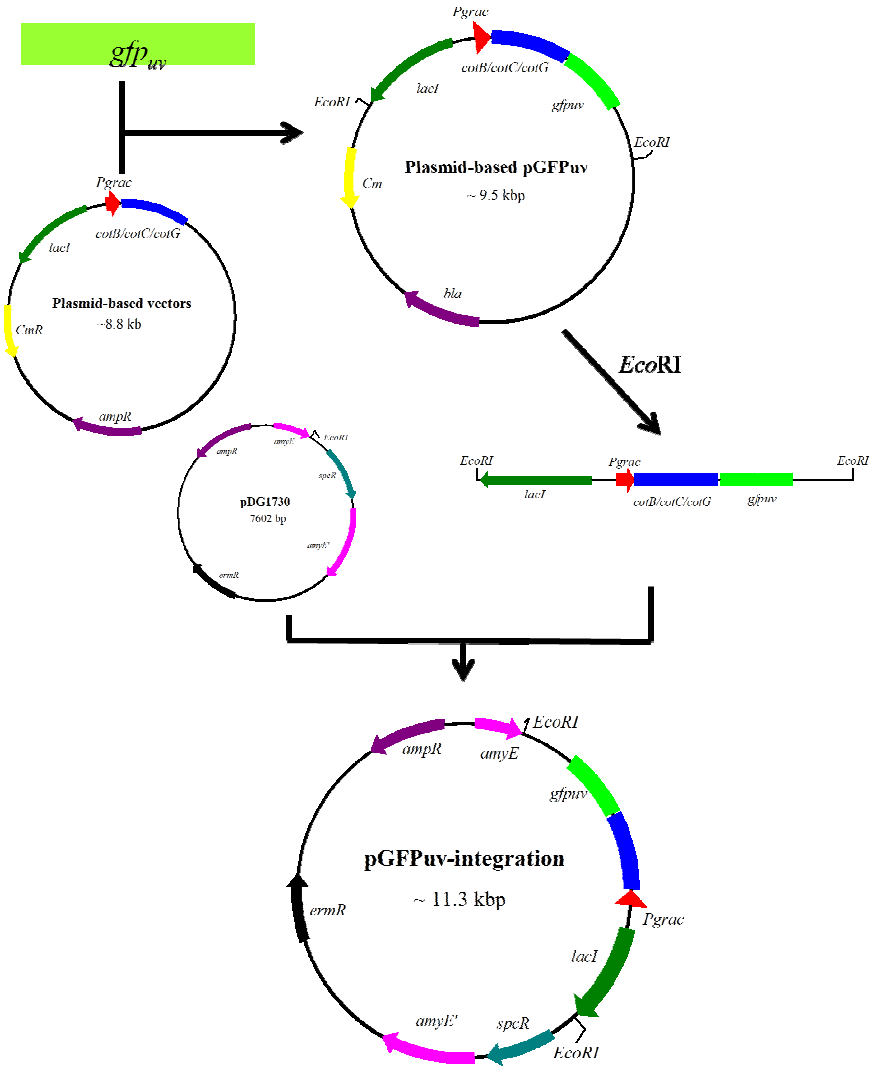

Fig 2.4 ***Construction of plasmids for the display of GFPuv on the spore surface.*** *The gfp_{uv} gene was amplified by PCR as mentioned above and inserted downstream of the cot gene in the spore display vectors, under the control of the P_{grac} promoter, resulting in plasmid-based pGFPuv (includes pSK01, pQAS26, pQAS23 which carry cotB, cotC and cotG respectively). The EcoRI fragment from the plasmid-based pGFPuv was then inserted at the EcoRI site of pDG1730 vector, resulting in pGFGuv-integration (includes pQAS43, pQAS48, pQAS49 which carry cotB, cotC and cotG, respectively).*

pQAS06: insertion of the *amyQ* fragment (ON17, ON18) at the *Cla*I site of plasmid pQAS03

pQAS32: insertion of the *amyQ* fragment (ON19, ON20) at the *Bam*HI-*Aat*II site of plasmid pQAS17

pQAS34: insertion of the *amyQ* gene (ON19, ON20) at the *Bam*HI-*Aat*II site of plasmid pQAS19

pQAS52: insertion of the gfp_{uv} fragment (ON13, ON14) at the *Eco*RI site of plasmid pQAS03

pQAS43: insertion of the 3703 bp *Eco*RI fragment which contains *cotB*-gfp_{uv} from pSK01 plasmid into plasmid pDG1730. The pSK01 plasmid was constructed by ligation of the gfp_{uv} fragment (ON15, ON16) at the *Aat*II site of plasmid pQAS17 (Krauß, 2009).

pQAS23: insertion of the gfp_{uv} fragment (ON15, ON16) at the *Aat*II site of plasmid pQAS20

pQAS49: insertion of the 3446 bp *Eco*RI fragment which contains *cotG*-gfp_{uv} from plasmid pQAS23 into plasmid pDG1730

pQAS26: insertion of the gfp_{uv} fragment (ON15, ON16) at the *Aat*II site of plasmid pQAS18

pQAS48: insertion of the 3080 bp *Eco*RI fragment which contains *cotC*-gfp_{uv} from plasmid pQAS26 into plasmid pDG1730

2.2.5.3 Construction of plasmids for analyzing the spore-based system for protein expression and purification

The 154 amino acids mini-intein, derived from the *dnaB* gene of *Synechocystis* sp. (Mathys, 1999) is a self-cleaving affinity tag which is commercially available in the Impact system of the New England Biolabs (NEB) company. Based on this mini-intein, we had constructed a system for protein expression and purification using *B. subtilis* spores as affinity matrix. First, the coding sequence of the 154 amino acids N-terminus of the Ssp DnaB mini-intein (*miniInt*) was amplified from pTWIN1 (NEB) and fused downstream of *cotB* in pSW01 plasmid. This work has been done by a co-worker and a new plasmid was named pMTB121. Second, to study the influence of the mini-intein, α-amylase Q was used as reporter. The *amyQ* gene without its signal sequence was amplified by PCR using the primer pair ON21 and ON22 and pKTH10 (Palva, 1982) as template. The amplicon was cleaved with *Not*I and inserted into pMTB121 treated with the same enzyme, resulting in plasmid pQAS40 (see ***Fig 2.5***).

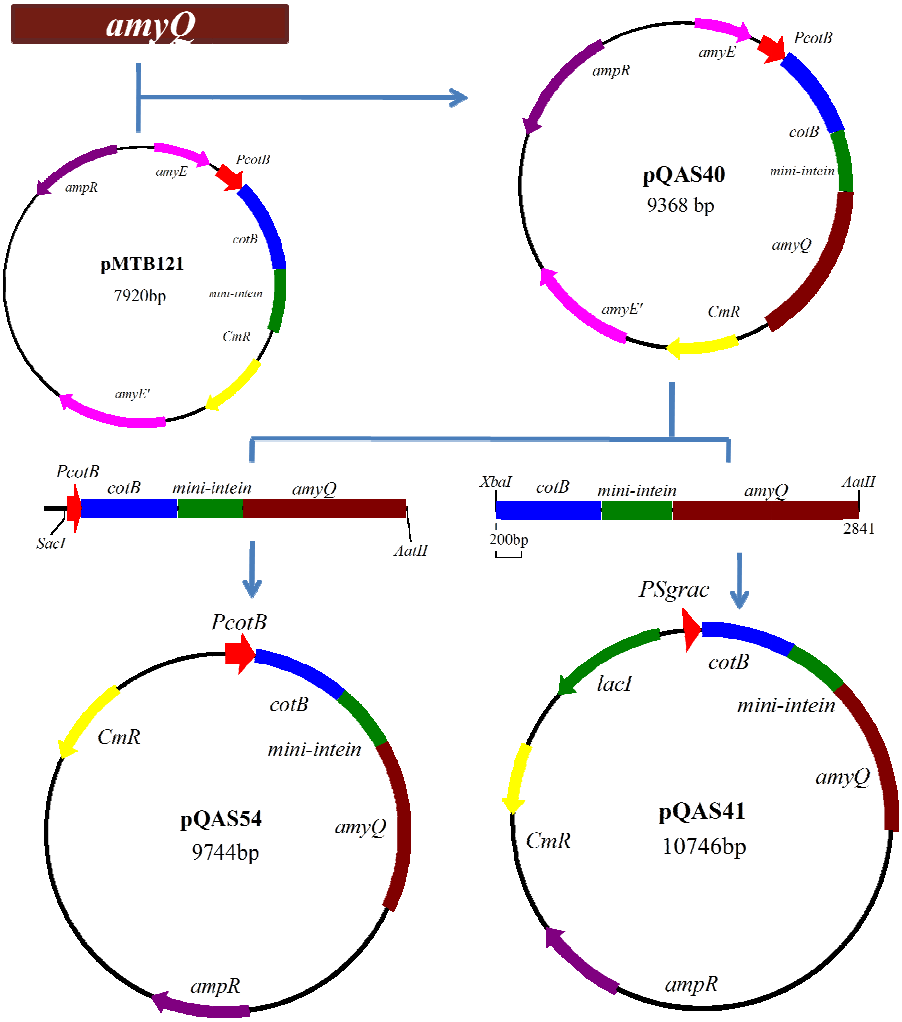

Fig 2.5 Construction of plasmids for analyzing the spore-based system for protein expression and purification. *The amyQ gene was amplified by PCR and then inserted downstream of the Ssp dnaB mini-intein in the pMTB121 vector, resulting in pQAS40. Using this plasmid as a template, two amplicons which contain either cotB-mini intein-amyQ or P_{cotB} cotB-mini intein-amyQ were obtained by PCR, treated with the indicated enzymes and inserted into either the pBG01 or the pHCMC01 vector to generate pQAS41 and pQAS54, respectively.*

The cassette containing *cotB-miniInt-amyQ* was also generated by PCR using the primer pair ON23 and ON18 and pQAS40 as template. This was cleaved with *Xba*I and *Aat*II and ligated

into pBG01 treated with the same enzymes, resulting in plasmid pQAS41. In addition, a cassette containing *cotB-miniInt-amyQ* with the *cotB* native promoter was produced by PCR using the ON05 and ON18 primers and pQAS40 as template. The amplicon was then treated with *Sac*I and *Aat*II enzymes and inserted at the *Sac*I and *Aat*II sites of pHCMC01, resulting in plasmid pQAS54 (***Fig 2.5***).

2.2.5.4 Construction of plasmids for the display of cellulase A on the spore surface

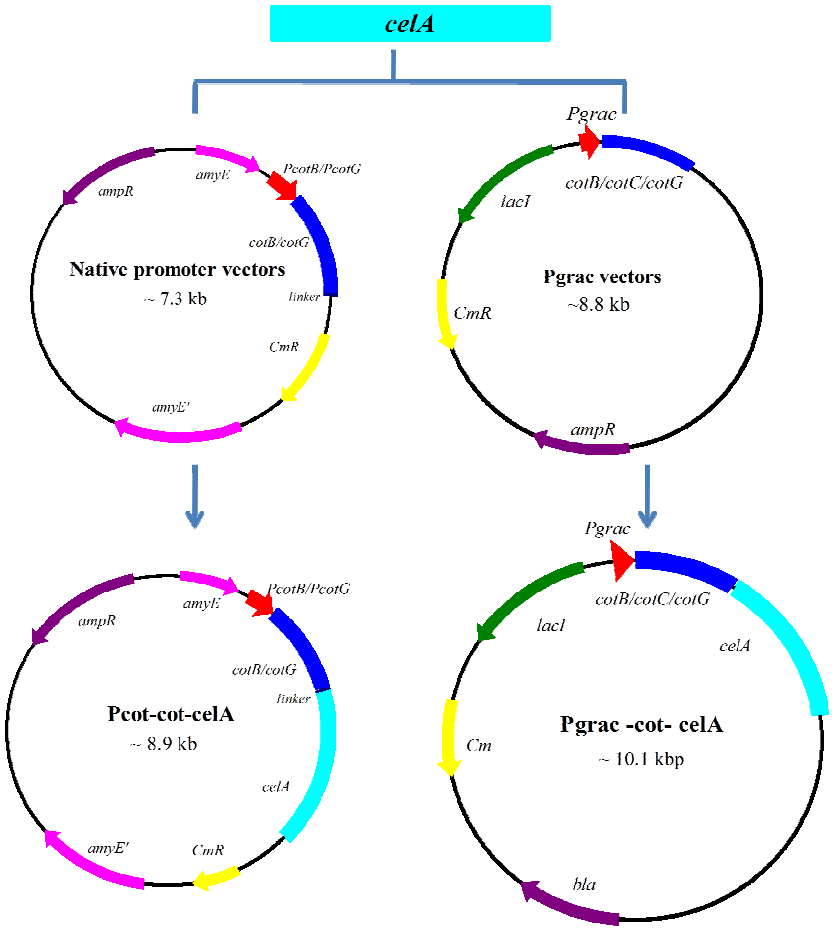

Fig 2.6 ***Construction of plasmids for the display of CelA on the spore surface.*** *The celA gene was amplified by PCR as mentioned above, treated with suitable enzymes and inserted downstream of the cot gene in the spore display vectors (the native promoters of the cot genes and the IPTG-inducible promoter P_{grac} were used for controlling expression of fusion Cot-CelA proteins). The P_{cot}-cot-celA plasmid includes pQAS15 and pQAS16, which carry cotB and cotG, respectively. The P_{grac}-cot-celA plasmid includes pQAS24, pQAS27 and pQAS25 which carry cotB, cotC and cotG, respectively.*

To allow expression of the *C. thermocellum* cellulase A on *B. subtilis* spore surface, the CelA coding region (*celA*) without its own signal sequence was amplified via PCR using pCT105 (Cornet *et al.*, 1983) as template. The primer pair ON24 and ON25 was used to obtain the first amplicon which was then cleaved with *Eco*RI and ligated into pQAS03 and pQAS05 treated with the same enzyme, resulting in pQAS15 and pQAS16, respectively. The second *celA* amplicon was generated using the two primers ON26 and ON27, cleaved by *Aat*II and inserted into pQAS17, pQAS18 and pQAS20 at the *Aat*II site, resulting in pQAS24, pQAS27 and pQAS25 respectively (***Fig 2.6***).

2.2.5.5 Construction of plasmids for display of CelA on the cell surface

To display the *C. thermocellum* CelA on *B. subtilis* the cell surface, the pNDH11 plasmid (Nguyen, 2006) was used as a backbone vector. This plasmid contains a xylose-inducible promoter, the signal sequence from the α-amylase of *B. amyloliquefaciens* and the 94-FnbB sorting sequence from *S. aureus,* which can be recognized by the sortase A enzyme to catalyze a covalent link between the protein and the peptidoglycan layer at the cell wall. The *Aat*II-treated *celA* amplicon was inserted into pNDH11 cleaved by the same enzyme, resulting in pQAC07 plasmid, in which fusion of the signal sequence, the *celA* gene and the sorting sequence was put under the control of the P_{xylA} promoter (***Fig 2.7***). When induced by xylose, the fusion of signal peptide-CelA-sorting signal would be produced, translocated through the cytoplasmic membrane and then anchored on the cell wall.

Fig 2.7 Construction of plasmids for displaying of CelA on the cell surface.

2.2.5.6 Construction of a *B. subtilis* strain which can produce sortase A

To allow the permanent expression of the sortase A gene (*srtA*) of *L. monocytogenes* in *B. subtilis*, the plasmid pDG1731 (Guérout-Fleury *et al.*, 1996) was chosen as a backbone vector. First, the 130 bp promoter region of the *dnaK* gene was amplified with the two primers ON28 and ON29 using *B. subtilis* 1012 chromosomal DNA as template and inserted into pDG1731 at

*Bam*HI and *Hin*dIII yielding pQAC01. Second, the sortase A coding sequence was amplified using ON30 and ON31 and chromosomal DNA of *L. monocytogenes* strain P14 (Ripio *et al.*, 1996) as template. The amplicon was then cleaved with *Hin*dIII and *Eco*RI and inserted into plasmid pQAC01 treated with the same enzymes resulting in pQAC02, in such a way that the transcription of the *srtA* gene was put under control of the house-keeping promoter P_{dnaK}. Next, pQAC02 was linearized by treatment with *Mlu*I and transformation into *B. subtilis* strain WB800N (***Fig 2.8***). Candidate colonies were identified as being resistant to spectinomycin and sensitive to erythromycin. Correct integration was confirmed by PCR using the primer pair ON28 and ON31, and one transformant (QAC04) was kept for further studies.

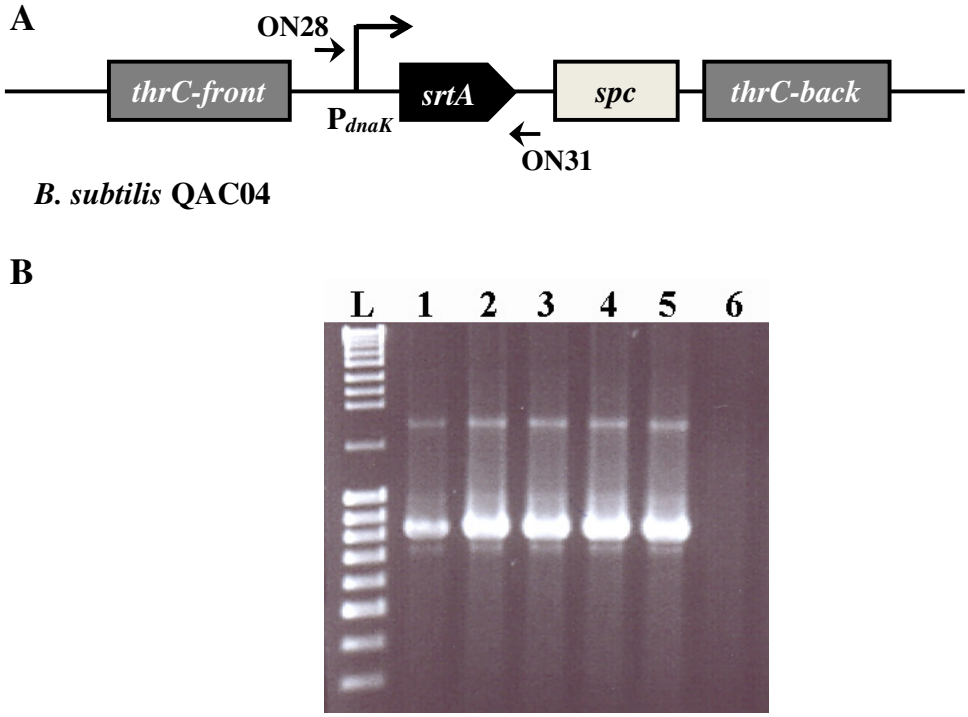

Fig 2.8 Integration of srtA in the chromosome of B. subtilis WB800N (A) and PCR for confirmation of correct insertion into the strain QAC04 (B). *L, DNA ladder (Fermentas)1- 5, samples from B. subtilis QAC04; 6, sample from B. subtilis WB800N (control).*

2.2.5.7 Construction of strain WB800N *amyE ::spec*

WB800N is an eight-fold protease-deficient *B. subtilis* strain which is derived from the WB800 strain and used for production of secreted heterologous proteins with plasmids carrying a chloramphenicol resistance gene. This strain can produce α-amylase enzyme from the *amyE* gene present in the chromosomal DNA. To use this strain for examination of the amylase Q reporter protein, *amyE* locus of this strain should be inactivated. The pDG1730 plasmid was used for this purpose. By transformation of this plasmid into WB800N, the *amyE* gene can be inactivated by the integration of the *spec* cassette, resulting in strain WB800Spec. The candidate colony was chosen as resistance to spectinomycin, sensitivity to erythromycin and no halo formation when plated on medium with 0.5 % starch (I_2/KI dyed). One correct transformant was kept for further studies.

3 Results

The main goal of this thesis was to construct a more effective system for displaying proteins on the *B. subtilis* spore surface. First, two inducible promoters were used to substitute the native promoter in spore display systems. The constructs were either integrated into or replicated independently from *B. subtilis* chromosomal DNA. The efficiency of each construct was evaluated and compared to each other through two reporter proteins, AmyQ and GFPuv. Secondly, a new *B. subtilis* spore-based system for protein expression and purification was then developed, taking advantage of a mini-intein of *Synechocystis* sp. (Mathys *et al.*, 1999). Finally, cellulose chips based on *B. subtilis* spores and cells were generated using the new spore display system and the cell display system as described (Nguyen and Schumann, 2006).

3.1 Anchoring recombinant proteins on the spore surface using inducible promoters

The conventional method for anchoring a protein on the *B. subtilis* spore surface is to fuse the coding region of the target protein in-frame to a gene encoding an outer spore coat protein, and expression of the fusion gene is controlled by the spore coat gene promoter (carrier gene) (Kim *et al.*, 2007; Ricca and Cutting, 2003). As a result, the target protein would be produced in the cytoplasm and anchored on the spore surface together with the carrier coat protein when cells form spores. When the mother cells lyse, spores will be released with the target protein displayed on their surface. This method guarantees the timing of fusion protein‘s synthesis for spore coat formation; however, the quantity of the produced fusion protein cannot be controlled. To address this issue, I tried several approaches to substitute the coat’s native promoters with IPTG-inducible promoters thereby allowing control of expression of the fusion genes.

3.1.1 Optimization of the expression conditions

B. subtilis cells produce a variety of both intra- and extracellular proteases (Schaeffer, 1967; Schaeffer, 1969; Kole *et al.*, 1988) which might interfere with the production of recombinant proteins. With the help from genetic manipulation techniques, major extracellular proteases could be deleted for stabilization of heterologous proteins (Wu *et al.*, 1991). However, due to the fact that proteases are required in certain processes, including sporulation (Mandelstam and Waites, 1968), not all proteases of *B. subtilis* can be inactivated.

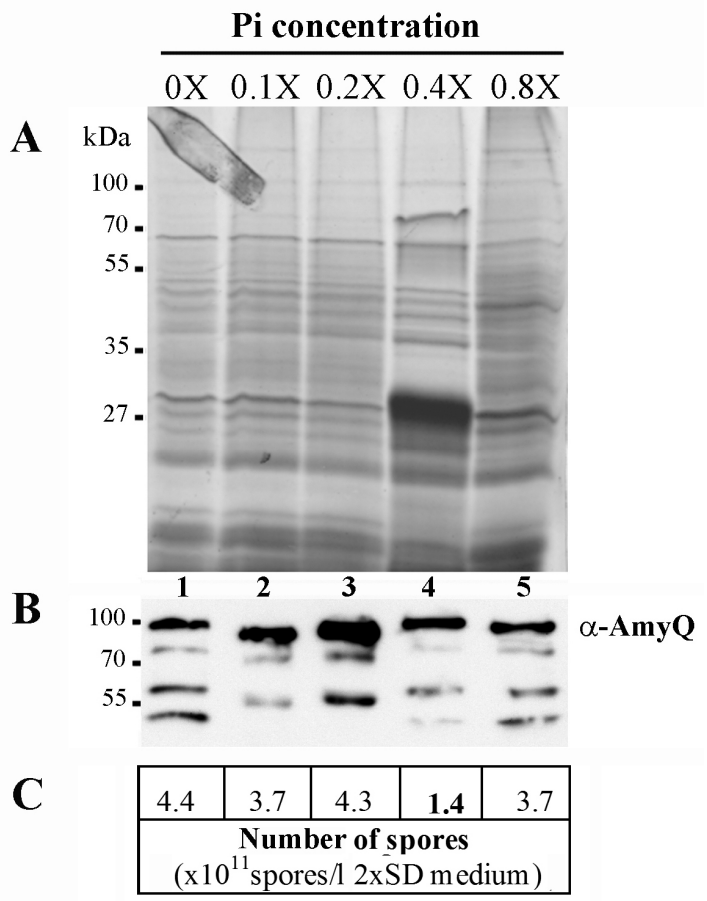

Fig 3.1 Stabilization of heterologous proteins on the spore surface*. The strain QAS06 (WB800N amyE::P_{cotB}-cotB-amyQ) was grown in 2xSG medium, well shaken at 37°C until t_2 of sporulation (2 h after entering the transition phase, about 6 h after inoculation). The culture was then divided into subcultures where one was further grown without adding any Pi, while others were supplemented with different concentrations of Pi (Protease inhibitors cocktail) (indicated at the top of each lane). The spores were purified at t_{18} (about 24 h after inoculation) and decoated as described in 2.2.2.3. The coat extracts from purified spores at an OD_{600} of 1 per sample were applied per well, fractionated on 12% SDS-PAGE and subjected to either Coomassie blue staining (A) or immunoblot using rabbit anti α-amylase antibodies (B). The number of spores obtained after purification was calculated by measuring the OD_{600} of the spore solution and direct counting with a Thoma counting chamber under an optical microscope and given as number of spores $x10^{11}$ per 1 liter of 2xSG medium (C).*

For all experiments in this thesis, the eight protease-deficient *B. subtilis* strain WB800N (Nguyen, 2006) was used. To improve the stability of fusion proteins for the spore surface display, different concentrations of protease inhibitor (Pi) cocktail (Roche Diagnostics) were examined. The Pi solution should be added on time to rescue the fusion proteins but not to negatively influence sporulation. It is known that proteases are released soon after the cells enter sporulation, the transition phase, which correlated with stage 0 (at t_{0-1}). Many spore coat proteins are synthesized at stage II (at t_{2-3}) of sporulation (Doi, 1989). Therefore, t_2 was chosen for adding the Pi cocktail.

B. subtilis strain QAS06 is derived from strain WB800N harboring the P_{cotB}-*cotB-amyQ* fusion at the *amyE* locus. In this construct, the *amyQ* gene was translationally fused to the *cotB* gene under control of its native promoter. Therefore, once the *cotB* promoter is activated during sporulation for the synthesis of native CotB protein in the mother cell, the heterologous fusion protein CotB-AmyQ would be produced, ready to be assembled on the spore surface. The QAS06 culture was challenged with 0.1X, 0.2X, 0.4X and 0.8X of Pi (1X is equal to 1 tablet of Pi cocktail in 50 ml cell culture). A culture in the absence of Pi was prepared as a control. To evaluate the effectivity of the Pi on the stability of α-amylase, an immunoblotting analysis was carried out. The molecular weight (MW) of the CotB-AmyQ fusion was estimated to be approximately 98 kDa (the MW of CotB used in this thesis is around 42.9 kDa and the MW of AmyQ is 55 kDa), therefore, the signal was expected at the position relatively close to the 100 kDa molecular weight marker.

Analysis of total coat proteins prepared from different cultures in the SDS-PAGE exhibited minor differences between the sample without Pi and with 0.1X, 0.2X and 0.8X Pi (***Fig 3.1A***, lane 1, 2, 3 and 5, respectively). With the 0.4X Pi sample, a significant increase in intensity of bands between 27 and 35 kDa was observed (***Fig 3.1A***, lane 4). This might indicate the overproduction of some unknown coat proteins in the presence of 0.4X Pi. Immunoblotting result gave the strongest signal with the sample from the 0.2X Pi culture (see ***Fig 3.1B***, lane 3). This band and that of the sample 0.1X Pi (***Fig 3.1 B***, lane 2) also showed less degradation product signals than the others. In addition, compared to the other cultures, fewer spores could be purified from the 0.4X Pi culture (***Fig 3.1C***) suggesting the negative influence of Pi on sporulation. The 0.1X and 0.8X samples showed less effect on the coat proteins but these concentrations were not

optimal for stabilization of heterologous proteins, presented as CotB-AmyQ. Therefore, the 0.2 X Pi concentration was chosen for further experiments.

3.1.2 Evaluation of an inducible promoter for display of heterologous proteins on the surface of spores

Next, the idea of using a controllable instead of the native promoter for display of heterologous proteins was examined, and CotB was chosen as the carrier protein. The α-amylase Q (AmyQ) from *B. amyloliquefaciens* was employed as a model protein for evaluating the system. Expression of the *cotB-amyQ* fusion gene was regulated by either the P_{grac}, an IPTG-inducible promoter, which consists of P_{groES} and the *lac* operator (pQAS32), or the promoter-up mutation P_{Sgrac} (pQAS34), in a series of plasmids which can be replicated independently of *B. subtilis* chromosomal DNA. As previously mentioned, many spore coat proteins were synthesized at stage II of sporulation (Doi, 1989), therefore IPTG was added at t_2. The QAS06 (*amyE::*P_{cotB}*-cotB-amyQ*) strain was used for comparison using the native *cotB* promoter.

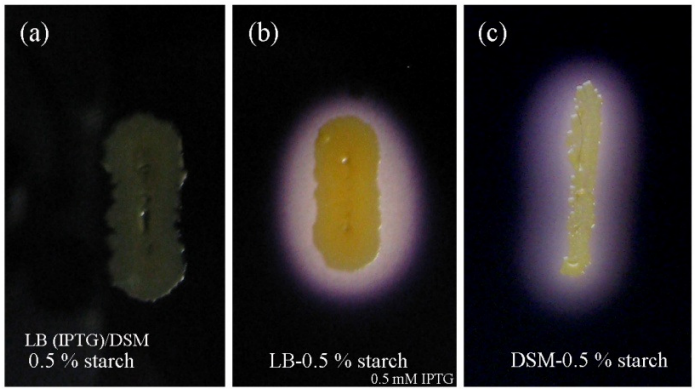

Fig 3.2 Visualization of α-amylase activity by plate assay. *The B. subtilis WB800Spec harboring either none (a) or the plasmid pQAS32 or pQAS34 (b) was grown on LB plates containing 0.5 mM IPTG and 0.5% insoluble starch for 24 h. The QAS06 strain (c) was also grown on a DSM plate containing 0.5% insoluble starch for the same time. The plates were then stained with I2/KI solution.*

The candidate colonies from each examined strain were screened by a plate assay using either LB plates supplement with 0.5 mM IPTG or DSM plates, both containing 0.5% insoluble starch. The colonies from the negative control, the WB800Spec strain, (***Fig 3.2a***) would form no

halo when grown in any medium. The colonies from strains carrying the construction with P_{grac} or P_{Sgrac} would show α-amylase activity, presented as halo-forming, on LB-0.5% starch supplemented with 0.5 mM IPTG (***Fig 3.2b***). Using the *cotB* native promoter, activated at stage II of sporulation, the colonies from QAS06 strain formed halos, too, when grown in DSM-0.5% starch (***Fig 3.2c***).

3.1.2.1 Determination of the optimal IPTG-concentration for expression of AmyQ on the spore surface

To identify the optimal inducer concentration for expression of the *cotB-amyQ* fusion, sporulation of *B. subtilis* strain WB800Spec harboring either plasmid pQAS32 (QAS32 strain) or pQAS34 (QAS34 strain) was induced by the exhaustion method in 2xSG medium, and then challenged with different IPTG-concentrations. Total coat proteins from different spore samples were analyzed by SDS-PAGE and by immunoblotting.

A significant increase in the amount of the CotB-AmyQ fusion protein was observed when adding IPTG (***Fig 3.3***). The QAS32 sample without IPTG showed almost no signal of CotB-AmyQ (***Fig 3.3A***, lane 1), while stronger signals were observed after addition of 0.5 and 1.0 mM IPTG (***Fig 3.3A,*** lane 3 and 4). The signal from the 1.0 mM IPTG sample was better but analysis of the Comassive stained gel showed alterations (increase in intensity of some bands) in the composition of total coat proteins which remained an open question about this sample (***Fig 3.3A,*** lane 4). On the contrary, the QAS34 sample exhibited a weak signal of fusion protein in the absence of IPTG (***Fig 3.3B***, lane 1). The strongest signal was noticed when 0.25 mM IPTG was added (***Fig 3.3B,*** lane 2). Surprisingly, the intensity of the CotB-AmyQ signals was reduced with increase in the IPTG-concentration (***Fig 3.3B***, lane 3 and 4).

In addition to immunoblotting analysis, α-amylase assays were performed to determine the optimal IPTG-concentration for *cotB-amyQ* expression. The purified spores from the different cultures challenged with different IPTG-concentrations were used directly in the α-amylase assay. With the P_{grac} construct, less than 0.12 units were measured in the absence of inducer while addition of IPTG resulted in an increase of activity. Less than 2 units were measured in the culture induced with 0.25 mM IPTG and about 3 units with the spores in the cultures in presence of 0.5 and 1.0 mM IPTG (see ***Fig 3.4***).

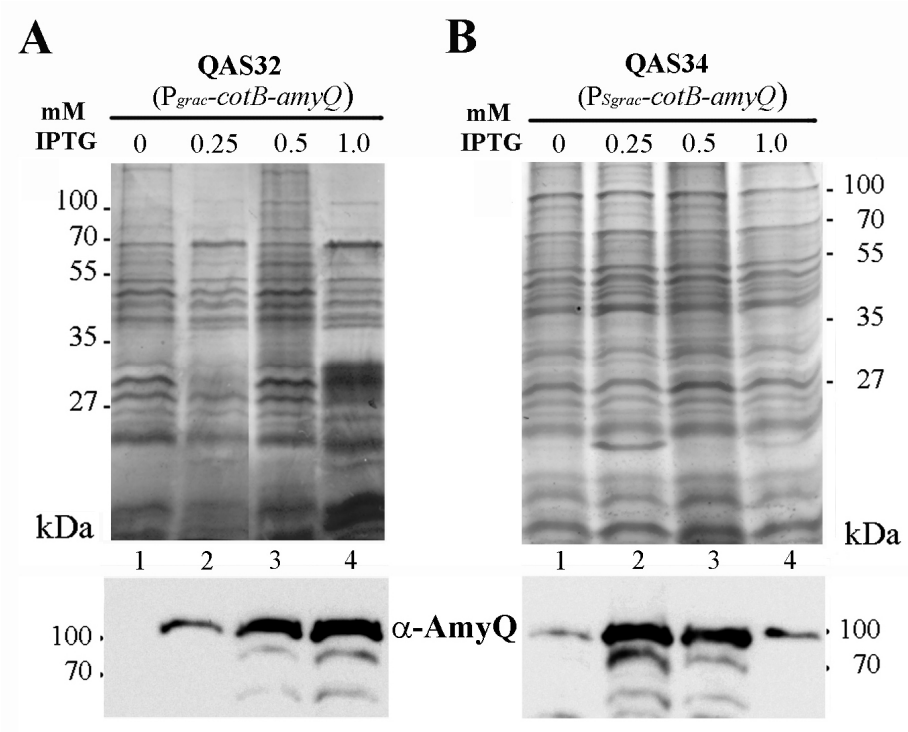

Fig 3.3 Identification of the optimal IPTG-concentration for cotB-amyQ expression. *Sporulation of B. subtilis strains WB800Spec carrying plasmid (A) pQAS32, with the P_{grac}-cotB-amyQ construction, and (B) pQAS34, with the P_{Sgrac}-cotB-amyQ construction, was induced by the exhaustion method with 2xSG medium. At t_2 of sporulation, 0.2X Pi was added, and the cultures were then divided into subcultures where one was further grown without induction while others were induced by different IPTG-concentrations (indicated at the top of each lane). The spores were purified at t_{18} (about 24 h after inoculation) and decoated as described in 2.2.2.3. The coat extracts from purified spores of an OD_{600} of 1 per sample were applied into each lane, fractionated on 12% SDS-PAGE and subjected to either Coomassie blue staining or immunoblotting.*

With the P_{Sgrac} construction, up to 0.4 units were measured in the absence of IPTG and the highest activity, 1.5 units per OD_{600} with the spores from the 0.25 mM IPTG culture. The α-amylase activity decreased when the IPTG-concentration increased. This result confirmed those obtained by the immunoblot analysis (compare ***Fig 3.3B*** and ***Fig 3.4***).

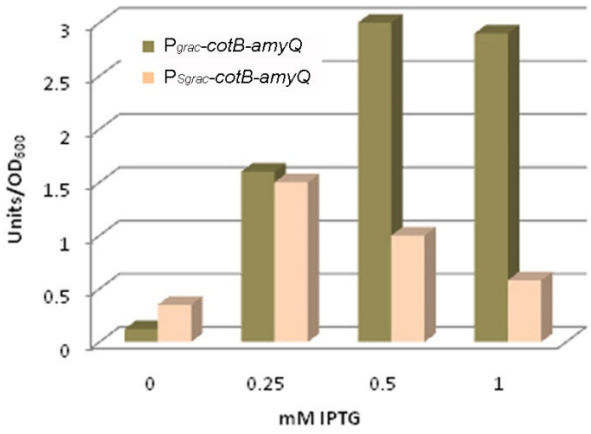

Fig 3.4 α-Amylase assay for the identification of the optimal IPTG-concentration for cotB-amyQ expression. *Purified spores from strains QAS32 (P_{grac}-cotB-amyQ) and QAS34 (P_{Sgrac}-cotB-amyQ) were prepared as described in the legend to Fig 3.3 and used directly for determination of the α-amylase activity. The result is presented in units per OD_{600} of spore.*

In summary, 0.25 mM was the optimal IPTG-concentration for expression of *cotB-amyQ* when using the P_{Sgrac} promoter. The two concentrations 0.5 and 1.0 mM were both suitable for activation of the P_{grac} promoter. However, due to the concern with the alteration of coat protein components and the fact that a lower induction is preferred, the 0.5 mM concentration was chosen for induction of the CotB-AmyQ using the P_{grac} promoter in further experiments.

3.1.2.2 P_{grac} promoter are more suitable for the expression of AmyQ on the spore surface

After selection of the optimal IPTG-concentration for the expression of CotB-AmyQ using IPTG-inducible promoters, the spores displaying AmyQ on the surface were purified using different strategies. The AmyQ immobilized on the surface of different spore samples should be visualized by fluorescence confocal microscopy. The spores were first incubated with antibodies raised against AmyQ and then with Alexa-conjugated secondary antibodies. The secondary antibodies bind to the primary antibodies raised against AmyQ and emit fluorescence light under the proper excitation wavelength of an immunofluorescence microscope. The microscopic result showed that AmyQ was exposed on the spore surface with fusion of its coding region downstream of the *cotB* gene using all three promoters for spore surface display (***Fig 3.5***). As anticipated, no fluorescence was observed with the control spores.

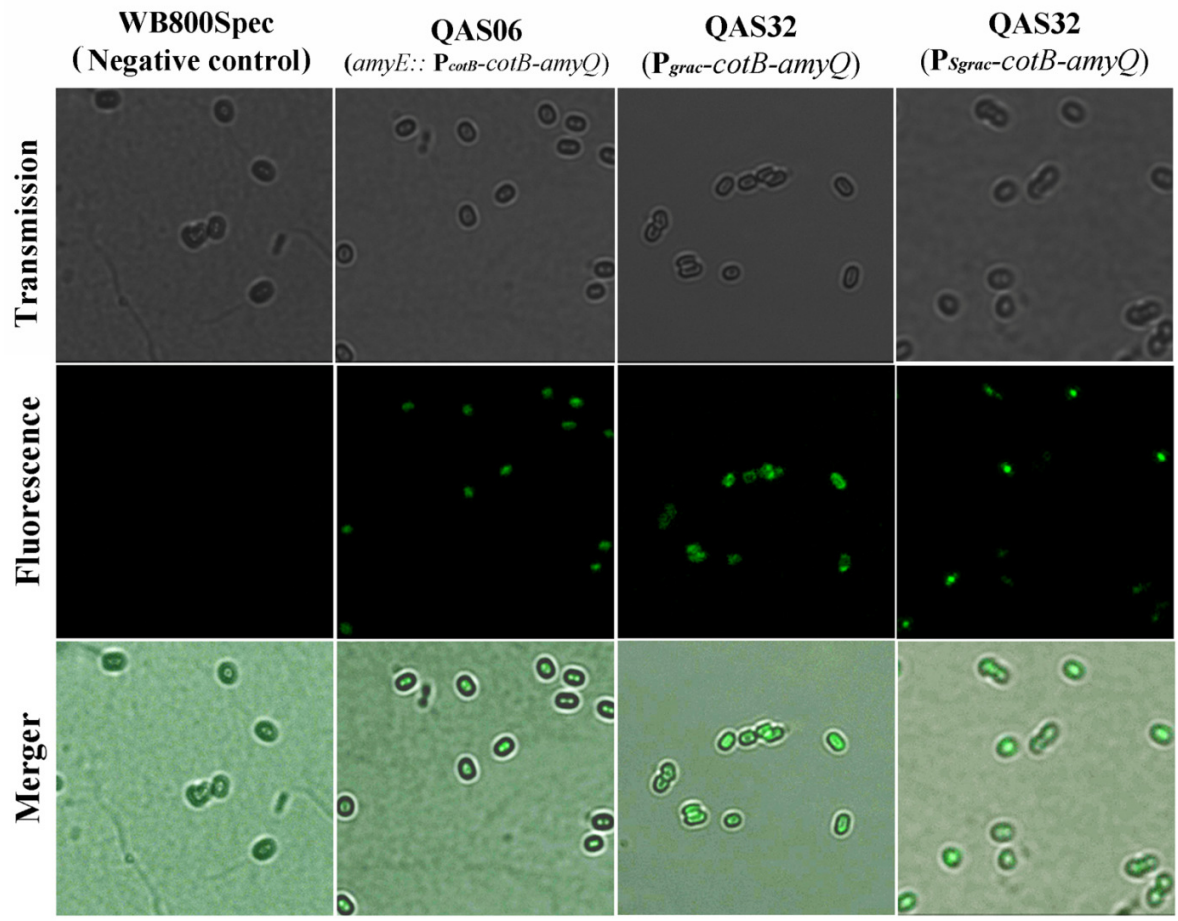

Fig 3.5 Visualization of the AmyQ on the spore surface. *The purified spores, prepared as described in the legend to Fig 3.6, from three different cultures (QAS06, QAS32-0.5 mM IPTG and QAS34-0.25 mM IPTG) were treated with the primary antibody against α-amylase, then with an Alexa 488-conjugated secondary antibody for immunofluorescence microscopy as described in 2.2.4.1. The AmyQ displayed on the spore surface was visualized by transmission and fluorescence Leica SP5 microscopy. The purified spore from WB800Spec were treated the same way and used as negative control.*

To evaluate the CotB-AmyQ expression level on the spore surface using different methods, immunoblotting analyses of the coat proteins and an α-amylase assay were carried out. First, the total coat extracts from an identical number of spores from different methods were prepared and applied into the same SDS-PAGE and subjected to immunoblotting. As we can see from the result of immunoblotting, the fusion CotB-AmyQ was present in the all coat extract

samples whenever its expression was induced (***Fig 3.6A***, lane 1, 3 and 5). The strongest signal was from the QAS34 sample induced with 0.25 mM IPTG (the P_{Sgrac} promoter) (***Fig 3.6A***, lane 5). The CotB-AmyQ signal was still observed in the absence of IPTG when using the P_{Sgrac} promoter (***Fig 3.6A***, lane 4), but not detectable with the P_{grac} promoter (***Fig 3.6A***, lane 2). This demonstrates the leakiness of the P_{Sgrac} promoter in controlling expression of the fusion gene.

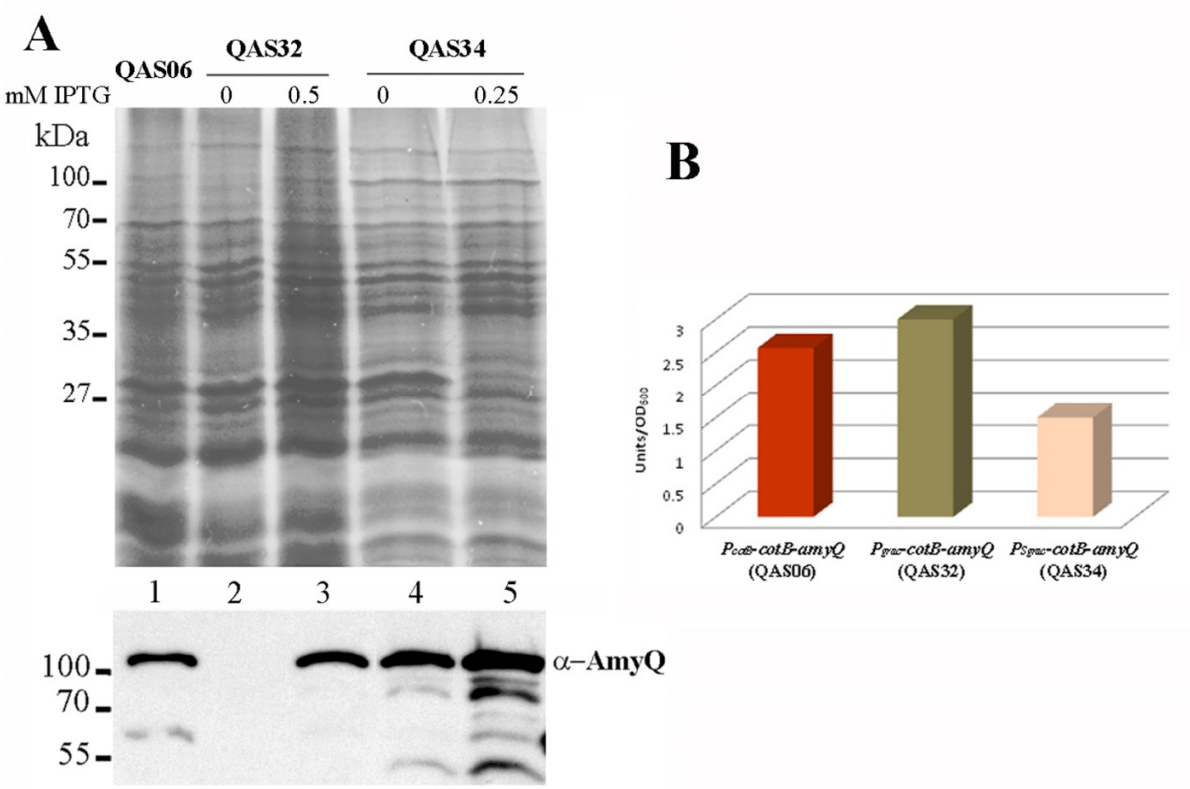

Fig 3.6 ***Comparison of the CotB-AmyQ expression.*** *The B. subtilis strain WB800Spec harboring one of the fusions P_{cotB}-cotB-amyQ (QAS06), P_{grac}-cotB-amyQ (QAS32) or P_{Sgrac}-cotB-amyQ (QAS34) was grown as mentioned in the legend to Fig 3.3. The IPTG-concentration from the result of part 3.1.2.1 was used for induction of the cotB-amyQ expression. Uninduced cultures of QAS06, QAS32 and QAS34 were used for comparison. Coat extracts from purified spores were subjected to immunoblot (A) using the rabbit anti α-amylase antibodies. The spores corresponding to an OD_{600} of 1 from each sample were used for determination of α-amylase activity (B).*

Second, the purified spores either from QAS06, QAS32-0.5mM IPTG or QAS34-0.25 mM IPTG were used directly in α-amylase assay. Spores from strain WB800Spec were used as a

reference in the activity tests. The activities, presented as units per OD_{600}, were calculated and compared among the samples. As shown in ***Fig 3.6B***, about 2.5 units were measured when using the spores from strain QAS06 (P_{cotB} promoter), while the activity of spores using the P_{grac} promoter for expression (strain QAS32) was increased to 3 units. An unexpected result was observed with P_{Sgrac} spores (from strain QAS34) when their activity was the lowest (about 1.5 units) although the amount of fusion protein on the spores was the highest as compared to other samples (***Fig 3.6***). I conclude that part of the α-amylase was present in its inactive form. The results suggest that the P_{grac} promoter is more suitable than the P_{Sgrac} promoter for increasing display of proteins on the spore surface.

3.1.2.3 Determination of the number of α-amylase molecules on the spore surface

The amount of α-amylase molecules displayed on the spore surface was determined by densitometrical analysis of immunoblots. The soluble coat fractions were extracted from a defined amount of purified spores (1.5×10^8) of strains QAS06 and QAS32, the coat proteins were separated by SDS-PAGE and CotB-AmyQ was identified by immunoblotting.

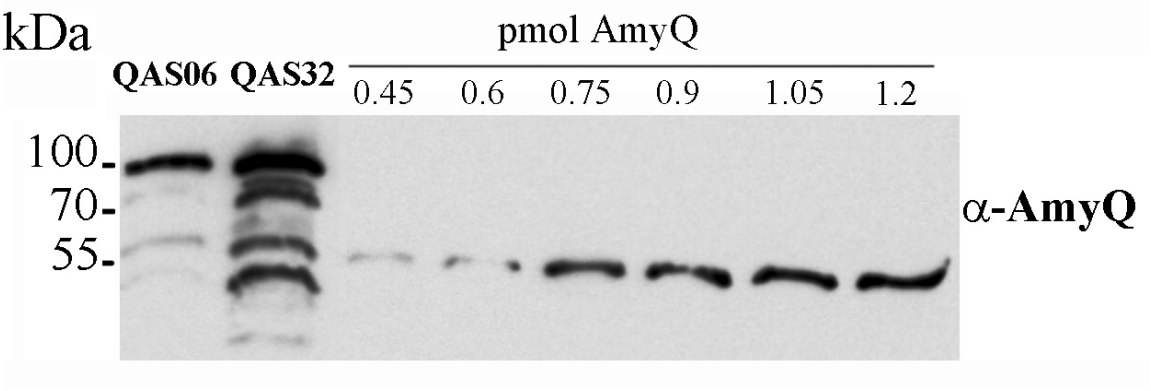

Fig 3.7 Determination of the number of α-amylase molecules on the spore surface. *Strains QAS06 and QAS32 were grown in 2xSG medium as described in the legend to Fig 3.5. The spore coat fraction from purified spores was prepared as described in 2.2.2.3 and the content of 1.5 x 10^8 spores were applied per lane. Defined amounts of purified α-amylase from 22.5 to 60 ng (corresponding to 0.45–1.2 pmol) were run on the same gel. Only the material in the upper band in two lanes of QAS06 and QAS32 was quantified.*

To calculate the amount of α-amylase, increasing amounts of purified enzyme were applied on the same gel (***Fig 3.7***). From the densitometric scanning of the different bands, the

numbers of α-amylase molecules were calculated to be 4.6 x 10^3 molecules per QAS06 spore and 9.73 x 10^3 molecules per QAS32 spore. This means that the amount of proteins displayed per spore can be increased twice by substitution of the P_{grac} promoter for the native promoter.

3.1.3 Evaluation of the new expression system using GFPuv as reporter protein

The second model protein used for evaluating the new idea is GFPuv (MW of 27 kDa), an enhanced version of the GFP protein of the jellyfish *Aequorea victoria,* which is 18-fold brighter and can be detected by the antibody against native GFP (Crameri *et al.*, 1996). In this part, the three Cot proteins, CotB, CotC (MW of 12 kDa) and CotG (MW of 43 kDa), were employed as carrier proteins. The P_{grac} promoter was used for controlling production of the fusion Cot-GFPuv proteins. The constructs of the P_{grac} promoter with *cot-gfp*$_{uv}$ fusions were designed in such a way that they could be inserted either in integration or plasmid-based vectors so that the effect of the gene copy number on the ability of display of heterologous proteins could be tested. The *gfp*$_{uv}$ was also fused downstream of the *cotB* gene in the integration vector pQAS03 under the native *cotB* promoter and used for comparison to the new constructs.

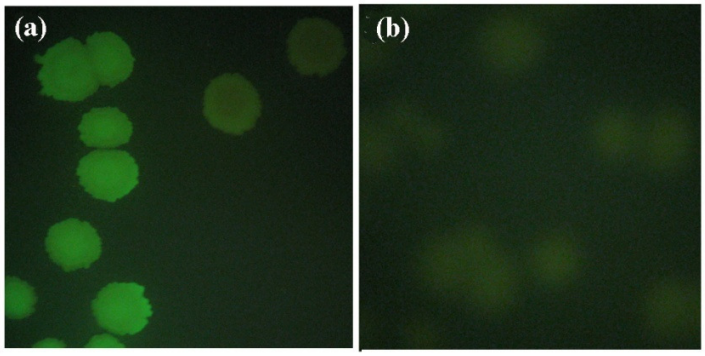

Fig 3.8 ***Visualization of the GFPuv expression by plate assay.*** *The B. subtilis strain WB800N carrying the P_{grac}-cot-gfp$_{uv}$ construct either integrated into chromosomal DNA or present on a plasmid was grown on LB plates containing 0.5 mM IPTG for 24 h. The colonies were analyzed using a fluorescence microscope with the GFP2 filter (a). The WB800N strain was grown under the same conditions as a negative control (b).*

Candidate colonies of each strain were first screened on DSM (when the native promoter was used) or LB plates containing 0.5 mM IPTG (***Fig 3.8***). The colonies from strains harboring the P_{grac}-*cot-gfp*$_{uv}$ construct showed fluorescence when observed under a microscope with GFP

filter (***Fig 3.8a***), and were used for further studies. No fluorescence signal could be visualized when studying colonies of the negative control strain WB800N (***Fig 3.8b***).

3.1.3.1 Determination of the optimal IPTG-concentration for the display of GFPuv on the spore surface

To determine the optimal IPTG-concentration for the diplay of GFPuv on the spore surface, the exhaustion method was used to induce the sporulation of *B. subtilis* WB800N carrying any of the P_{grac}-*cot-gfp*$_{uv}$ constructs. Different IPTG-concentrations were examined.

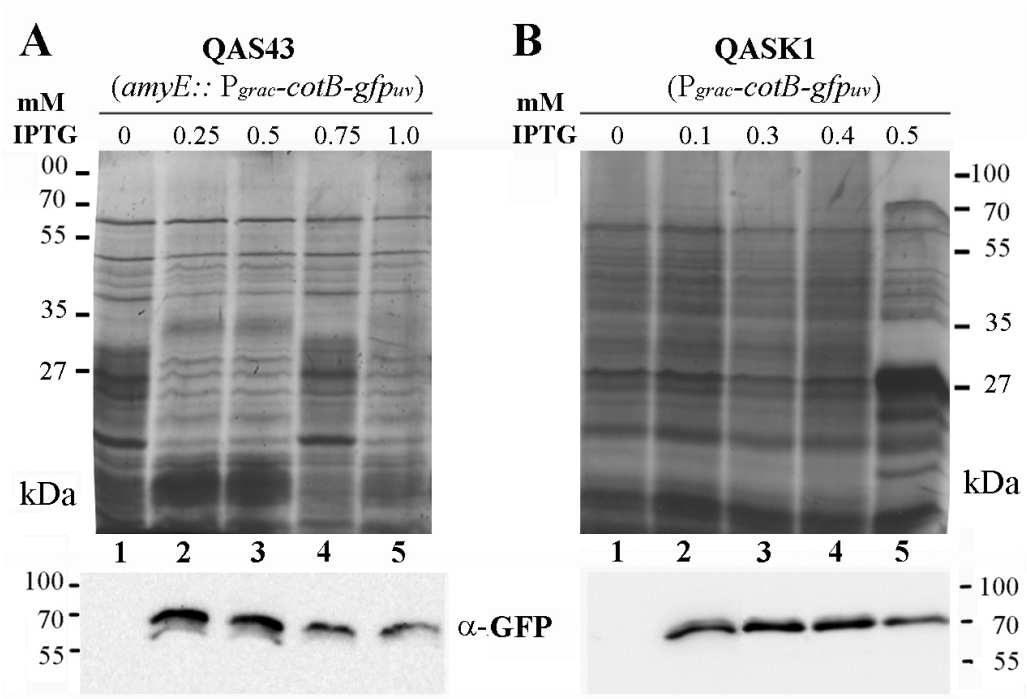

Fig 3.9 Identification of the optimal IPTG-concentration for CotB-GFPuv expression. *The B. subtilis strain WB800N harboring the construct P_{grac}-cotB-gfp$_{uv}$ either integrated into chromosomal DNA (QAS43) or in a plasmid (QASK1) was grown in 2xSG medium as described in the legend to Fig 3.3, divided into subcultures which were then induced with different IPTG-concentrations (indicated at the top of each lane). The spores of QAS43 (A) and QASK1 (B) were purified at t_{18} (about 24 h after inoculation) and decoated as described in 2.2.2.3. The coat extracts from spores of an OD_{600} of 1 per sample were applied per well, fractionated by 12% SDS-PAGE and subjected to immunoblot using rabbit anti GFP antibodies. Coomassie stained gels were prepared in parallel.*

Total coat proteins were extracted from the purified spores prepared from different cultures. Extracts of spores corresponding to an OD_{600} of 1 were loaded per lane. Immunoblot using primary antibodies against GFP was performed. With the CotB carrier protein, no fusion with GFPuv was detected in the absence of IPTG in both the integration and the plasmid-based constructs (***Fig 3.9*, *A*-**lane 1 and ***B*-**lane 1). This observation fits previous results described under **3.1.2**. Strain QAS43 harboring the integration construct was challenged with IPTG with up to a concentration of 1.0 mM. The strongest signal was detected with 0.25 mM IPTG (***Fig 3.9A*,** lane 2). A reduced intensity of the signal was observed when increasing concentrations of IPTG, and the highest IPTG-concentration gave the weakest signal on the immunoblot (***Fig 3.9A*,** lane 3-5). With the plasmid-based construct (strain QASK1), up to 0.5 mM IPTG resulted in induction of the expression of CotB-GFPuv. The signal of the fusion protein was detected at 0.1 mM IPTG (***Fig 3.9B,*** lane 2) and the intensity of the signal increased when the added IPTG was increased to 0.3 mM (***Fig 3.9B,*** lane 3). It remained constant at the concentration of 0.4 mM IPTG and started to decrease when more IPTG was added (0.5 mM) (***Fig 3.9B,*** lane 4 and 5).

Using CotC and CotG as carrier protein, no detectable signal in the absence of IPTG were observerd, too (***Fig 3.10A*** and ***B***, lane 1 and 6). The strongest signals were observed when using 0.35 mM IPTG for strain QAS48 (***Fig 3.10A,*** lane 4); 0.1 mM IPTG for strain QAS25 (***Fig 3.10A,*** lane 7); 0.5 mM IPTG for strain QAS49 (***Fig 3.10B,*** lane 5) and 0.1 mM IPTG for strain QAS23 (***Fig 3.10B,*** lane 7). Except for strain QAS49 (carrying the integrated P_{grac}-*cotG*-gfp_{uv} construct), more degradation signals were detected with all three strains QAS48, QAS26 and QAS23, when IPTG was added at higher concentrations.

Analysis of the Coomassie stained gel showed significant alterations in total coat proteins when different IPTG-concentrations were used. For example, notable differences in the intensity of bands between 27 and 35 kDa and/or between 55 and 70 kDa were observed in the gel with both fusion CotC-GFPuv and CotG-GFPuv (***Fig 3.10***).

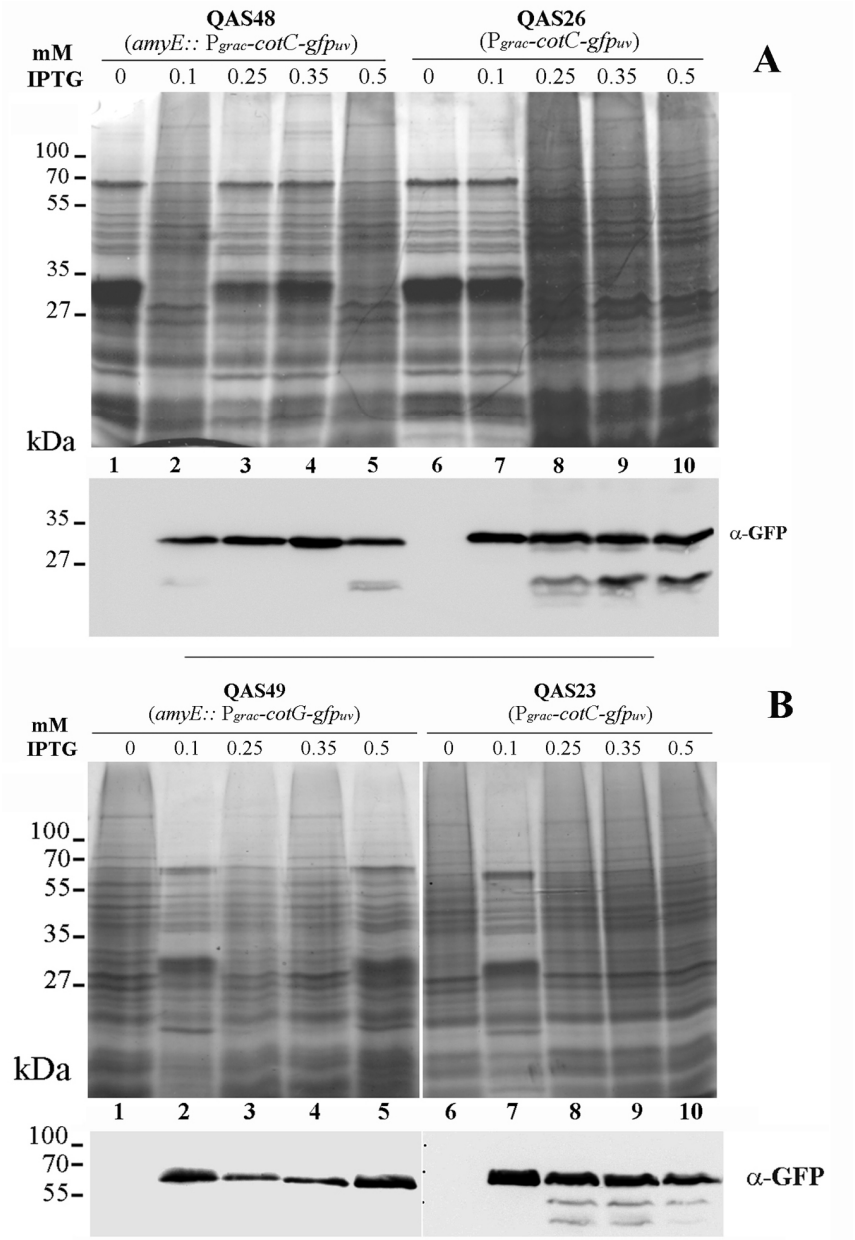

Fig 3.10 Identification of the optimal IPTG-concentration for the CotC-GFPuv (A) and CotG-GFPuv (B) expression. *The B. subtilis strains QAS46 (amyE:: P_{grac}-cotC-gfp_{uv}),QAS26 (P_{grac}-cotC-gfp_{uv} – plasmid),QAS49 (amyE:: P_{grac}-cotG-gfp_{uv}) and QAS23(P_{grac}-cotG-gfp_{uv} – plasmid) were grown in 2xSG medium, challenged with different IPTG-concentrations (indicated at the top of each lane) and prepared as described in the legend to Fig 3.9. The coat extracts from spores of an OD_{600} of 1 of were loaded per lane. Coomassie staining and immunoblot analysis of the fusion proteins CotC-GFPuv (A) and CotG-GFPuv (B) were performed using rabbit anti-GFP antibodies.*

In summary, it is obvious that the amount of the fusion protein Cot-GFPuv was increased when IPTG was added to induce expression. However, providing too much inducer could alter the composition of total coat proteins and might indirectly damage the fusion protein. The optimal IPTG for expression of Cot-GFPuv on the spore surface were 0.25 mM for QAS43 (CotB, integration), 0.3 mM for QASK1 (CotB, plasmid-based), 0.35 mM for QAS48 (CotC, integration), 0.1 mM for QAS26 (CotC, plasmid-based), 0.5 mM for QAS49 (CotG, integration) and 0.1 mM for QAS23 (CotG, plasmid-based).

3.1.3.2 The number of fusion gene copies did not influence expression of GFPuv on the spore surface significantly

The ability of transducing the blue chemiluminescence into green fluorescence of GFP and its analogs makes them visible under fluorescence microscope. To visualize the GFPuv displayed on the surface of different spore samples were imagined using Leica SP5 confocal microscopy. The microscopic result showed that GFPuv was observed around the shape of the examined spores which indicated that this protein is exposed on the surface of spores, but could not be detected with the reference spores (***Fig 3.11***). This observation had proven the success of using the P_{grac} promoter on management of the fusion *cot-gfp*$_{uv}$ genes for spore surface display purpose.

To analyze the amount of GFPuv displayed on the spore surface, spores from different cultures were purified then washed three times with ddH_2O. Dilutions of 10^5 spores per ml in ddH_2O were examined directly under a Beckman-Coulter FC-500 MCL System flow cytometer. The data were analyzed by using CXP Software and presented in ***Fig 3.12.*** Compared to WB800N spores (***Fig 3.12a***), the histogram of all seven constructs were shifted to the right, indicating that Cot-GFPuv fusions were expressed as active fusion proteins and embedded in the spore coat. In case of CotB-GFPuv constructs, percentage of gated spores were 18.06%, 59.06% and 62.37% for QAS52 (integration, native *cotB* promoter) (***Fig 3.12b***), QAS43 (integration, P_{grac} promoter) (***Fig 3.12c***) and QASK1 (plasmid, P_{grac} promoter) (***Fig 3.12d***), respectively.

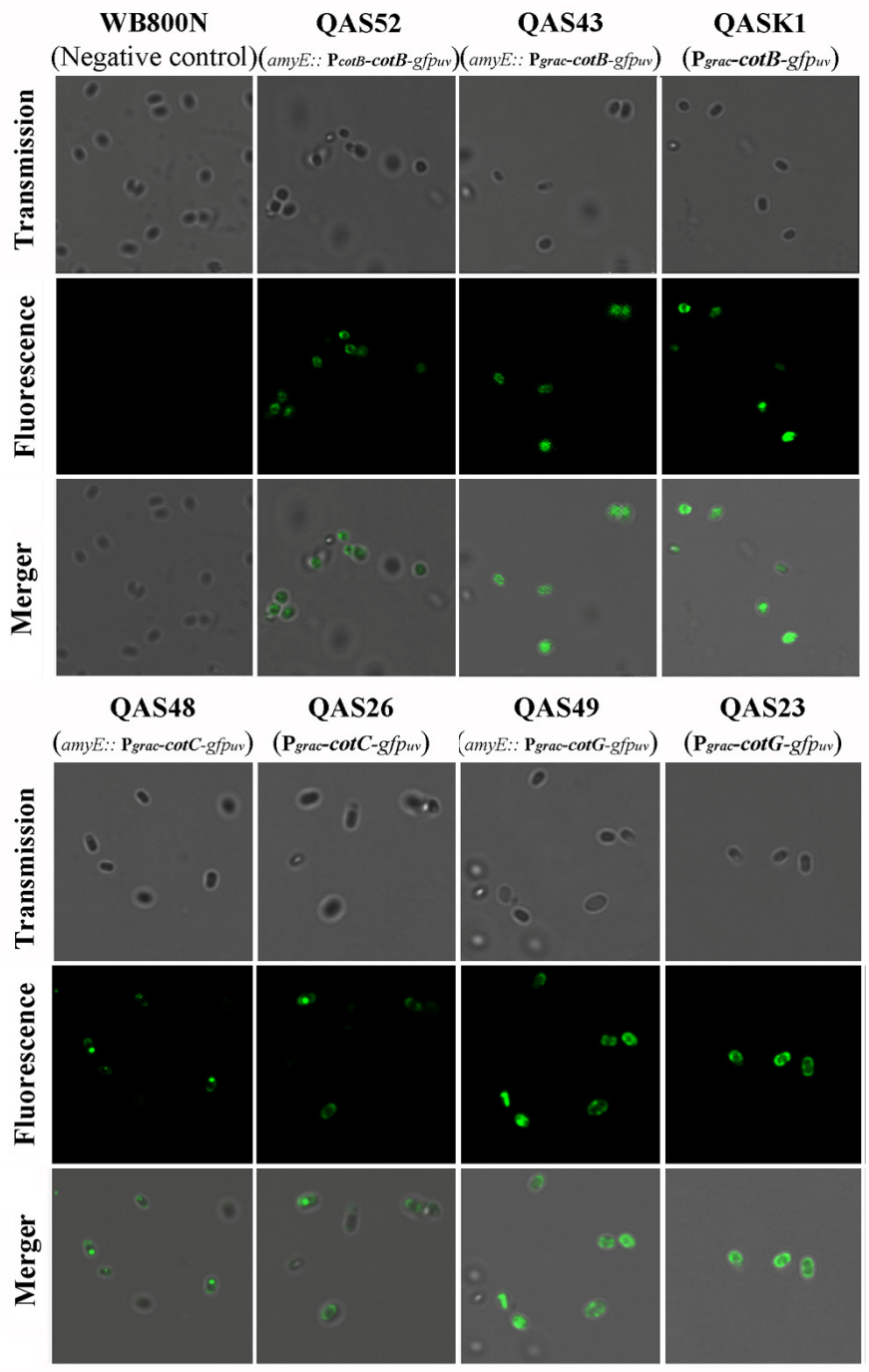

Fig 3.11 Visualization of the GFPuv on the spore surface using CotB, CotC and CotG as carrier proteins. *The spores from different cultures (QAS43-0.25 mM IPTG, QASK1-0.3 mM IPTG, QAS48-0.3 mM IPTG, QAS26-0.1 mM IPTG, QAS49-0.5 mM IPTG and QAS23-0.1 mM IPTG) were harvested as described in the legend to Fig 3.9 and then directly observed by transmission and fluorescence Leica SP5 microscopy. Purified spores from strain QAS52 not treated with IPTG were also examined. The WB800N spores were used as negative control.*

The significant increase of GFP intensity when using the P_{grac} promoter indicated that more protein could be indeed displayed on the spore surface when its level of expression was increased. This observation was comparable with previous results presented in **3.1.2**. Slight differences between the intensity of integration and plasmid-based constructions showed the minor effect of increasing the copy number of the fusion gene at the level of display protein. This result was also observed with the CotC-GFPuv constructs; even the plasmid-based construction gave less intensity than the integration construct did (57.35% of QAS48 as compared to 49.15% of QAS26) (***Fig 3.12 e*** and ***f***).

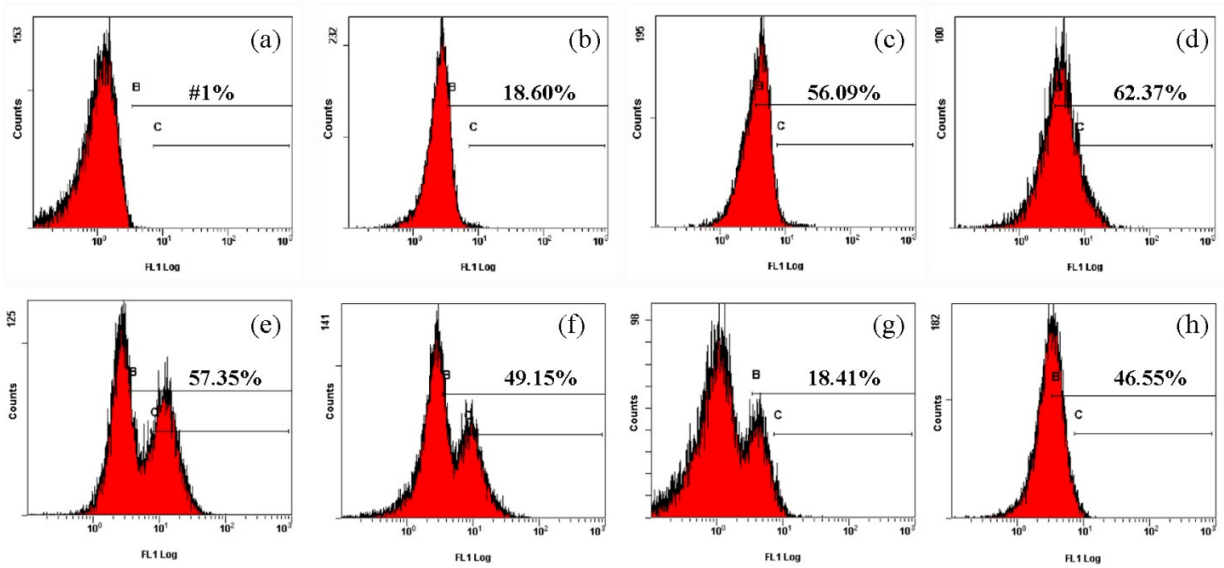

Fig 3.12 Flow cytometric analysis of GFPuv expression on the spore surface. *The purified spores from the following cultures were analyzed: QAS52 (b), QAS43-0.25 mM IPTG (c), QASK1-0.3 mM IPTG (d), QAS48-0.3 mM IPTG (e), QAS26-0.1 mM IPTG (f), QAS49-0.5 mM IPTG (g) and QAS23-0.1 mM IPTG (h). Spores were prepared as described in the legend to Fig 3.9 and directly used in FACS analysis. The WB800N spores (a) were used as negative control. FL1log indicates units of GFP fluorescence intensity; a total of 20000 spores per sample were counted. The percentage of positive fluorescence spores, gated by panel B, was used for comparison of the GFPuv expression on the spore surface from different samples.*

On the contrary, the CotG-GFPuv samples showed an increase in percentage of gated spores from 18.41% of QAS49 (integration, P_{grac} promoter) (***Fig 3.12g***) to 46.55% of QAS23

(plasmid, P_{grac} promoter) (***Fig 3.12h***). This result demonstrates the gene dosage effect of the translational fusion present on a plasmid (4 – 6 copies) versus the chromosome (one copy). The results suggested that each strategy has a different effect on displaying proteins on the spore surface when different carrier proteins were used.

Considering the histograms from different spore samples, there was another observation with the two CotC-GFPuv constructs and the CotG-GFPuv integration construct. The two peaks in the histogram represented for two populations of spores emitting different intensities in each construct. Still, no convincing explanation is possible for this event (Zhou *et al.*, 2008b).

3.1.3.3 Carrier protein is required for display of heterologous protein on the spore surface

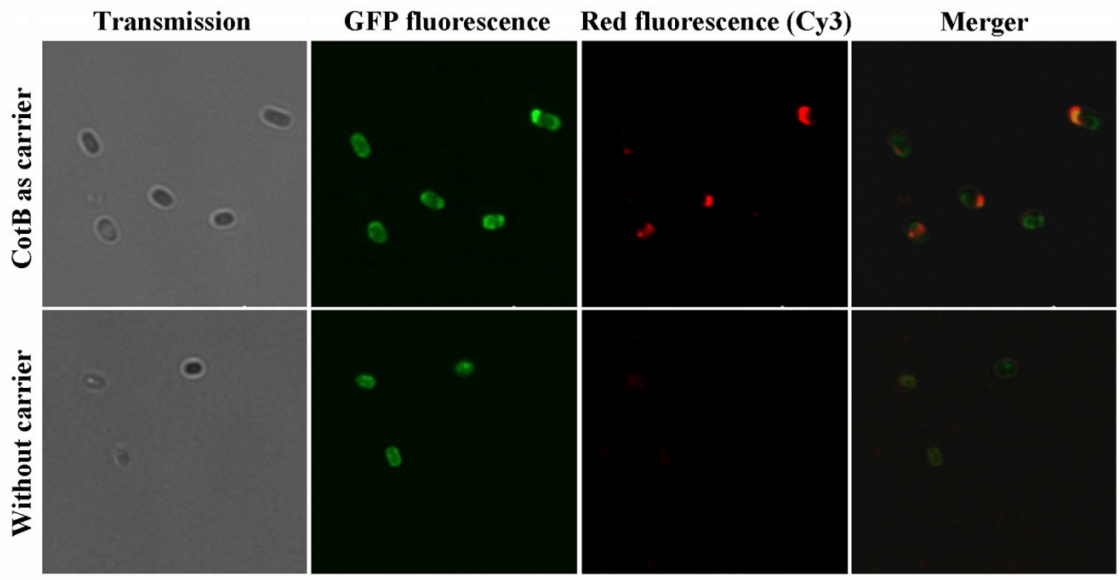

Fig 3.13 Expression of GFPuv with/without carrier protein for displaying on the spore surface. *The spores from WB800N strain harboring either plasmid pHT01-GFPuv (without carrier) or pQASK1 (CotB as carrier) were purified as described in the legend to Fig 3.9 (0.3 mM IPTG was used for induction). These spores were treated with the primary antibodies against GFP, followed by Cy3-conjugated secondary antibodies for immunofluorescence microscopy. The GFPuv displayed on the spore surface was visualized by Leica SP5 microscopy.*

It has been previously described that the P_{grac} promoter could be used in the absence of carrier and was able to display recombinant protein on the spore surface (Yim *et al.*, 2009).

Therefore, a construct with GFPuv without carrier protein was generated for testing. These spores exhibited the GFP fluorescence, however very weak signals were detected when stained with primary antibodies against GFP and Cy3-conjugated secondary antibodies (***Fig 3.13***). In addition, a similar construct was made with AmyQ which showed less activity compared to that with carrier protein. In both cases, the proteins were detected in the coat fraction by immunoblotting (data not shown). Taken together, those phenomena and the observations from Yim *et al.* might be the result from the overproduction of the recombinant protein in mother-cell compartment leading to the random assembly of the target protein in the spore coat. Hence, the carrier proteins still have a very important role in displaying heterologous protein on the spore surface.

3.1.3.4 Cot proteins are accumulated at different positions on the surface of spores

As GFPuv emits a bright, green fluorescent color that can be easily visualized, it has been used to pinpoint locations of various intracellular proteins and thereby acts as a localization tag. A closer look to the position of Cot proteins on the spore surface, once overproduced, via fusion of the Cot proteins with GFPuv was performed using confocal microscopy. When the *cotB* promoter was used, GFPuv was visualized around the shape of the spore, indicating the presence of CotB protein on all over the surface of the spore (***Fig 3.14a***). Using the P_{grac} promoter for overexpression, the CotB protein was more directed to two poles of the spore (***Fig 3.14b***), the CotC protein accumulated into one spot on spore surface (***Fig 3.14c***) and CotG protein tended to arrange at the median place of spore.

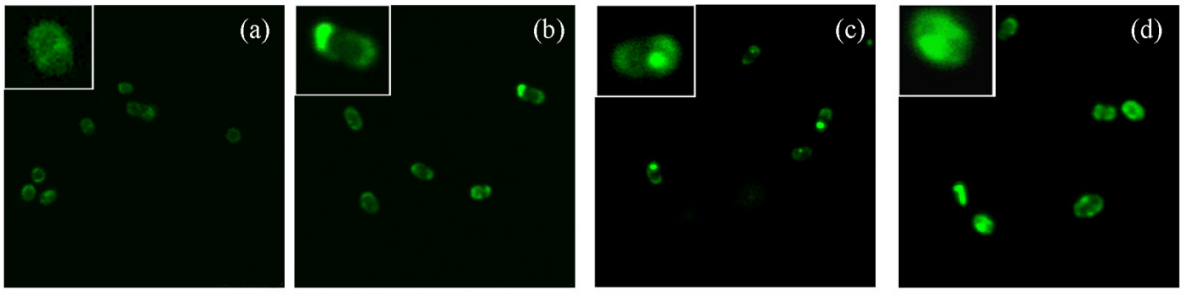

Fig 3.14 Localization of Cot proteins on the spore surface. *The spores from different strains QAS52 (a), QASK1(b), QAS26 (c) and QAS23(d) were prepared as described in the legend to Fig 3.11 and directly observed by fluorescence Leica SP5 microscopy.*

This result suggests self-interaction within each species of coat protein and their tendency of localization on the spore coat. Further studies should be carried out to understand more about this interesting phenomenon.

3.2 *B. subtilis* spore-based expression and purification system for recombinant proteins

Overexpression and purification of recombinant proteins is an important task in molecular biology (Marino, 1989; Baneyx, 2004; Terpe, 2006). Often, heterologous proteins form aggregates, so-called inclusion bodies, which are more complicated for purification (Baneyx and Mujacic, 2004). To circumvent this problem, we decided to anchor proteins prone to form inclusion bodies on the spore surface, separated by a mini-intein. Details on inteins and protein splicing mechanism are mentioned in the Introduction section **1.3**.

In this part, the 154 amino acids mini-intein, derived from the *dnaB* gene of *Synechocystis* sp. (Mathys *et al*., 1999) was utilized to develop a *B. subtilis* spore-based system for protein expression and purification. Because the DnaB mini-intein is a self-cleaving protein that can become active under optimal pH conditions, no additional protease or reducer is needed and the structure of the target protein will be less influenced. The idea was to use *B. subtilis* spores as an affinity matrix to express the fusion of this mini-intein (miniInt) with the target protein on the surface, purify the spores (which is a relatively simple process), activate the mini-intein to release the target protein and recover it in the supernatant.

3.2.1 Verification of the fusion *miniInt-amyQ* expression on the spore surface

To create the spore-based system for expression and purification of recombinant proteins, the conventional method, in which the Cot protein under its native promoter in an integration construct, was applied. In particular, the CotB protein was used as carrier and AmyQ was the model protein. A construct was designed so that the coding region of this mini-intein was flanked by the *cotB* and *amyQ* genes. The whole fusion was put under control of the *cotB* native promoter and integrated into chromosomal DNA of *B. subtilis* at the *amyE* locus. Expression of the fusion miniInt-AmyQ was confirmed by immunoblot, an α-amylase activity assay and immunofluorescence microscopy.

3.2.1.1 The conventional method gave less miniInt-AmyQ displayed on the spore surface

The strains used for the experiments in this part were first screened by plate assay performed as previously described in ***3.1.2***. A candidate colony from strain QAS40 (harboring the *cotB-miniInt-amyQ* construct) was grown in 2xSG medium to induce sporulation. The QAS40 spores were prepared as described in the legend to ***Fig 3.1*** for QAS06 using 0.2X Pi. During the spore purification process, the intein washing buffer (see 2.2.2.7) was used instead of ddH_2O for washing the pellets and spores in each step. The two strains QAS06 and MTB121 (carrying *cotB-miniInt* construct) were treated the same and used as control for comparison in immunoblot analysis and α-amylase activity assay.

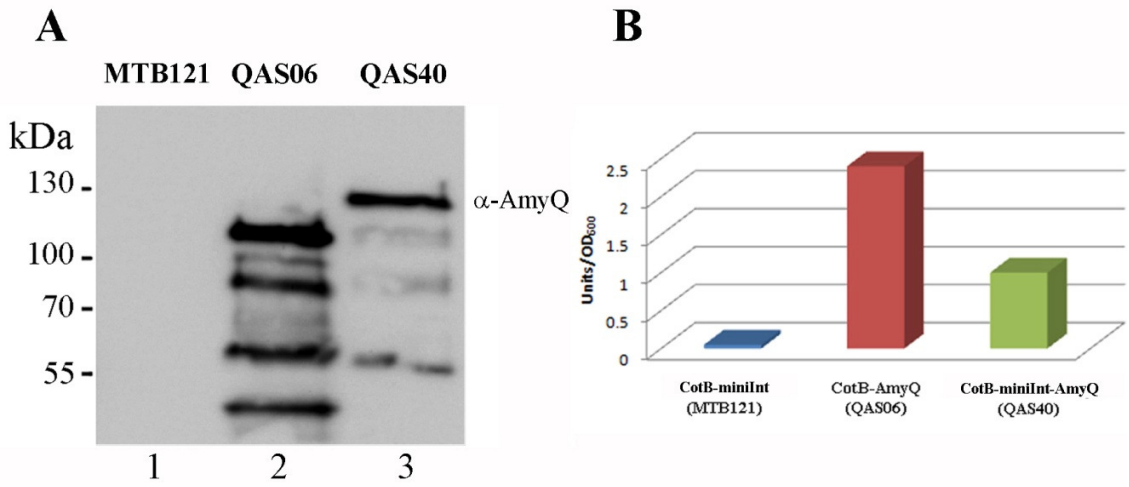

Fig 3.15 Detection of the fusion miniInt-AmyQ on the spore surface by immunoblotting (A) and by the whole spore α-amylase activity assay (B). *Spores of the three strains MTB121 (amyE::P_{cotB}-miniInt), QAS06 (amyE::P_{cotB}-amyQ) and QAS40 (amyE::P_{cotB}-miniInt-amyQ) were prepared as described in Fig 3.1, using 0.2X Pi. The coat extracts of spores corresponding to an OD_{600} of 1 were loaded per lane, fractionated on 10% SDS-PAGE and subjected to immunoblot using antibodies against AmyQ (A). The spores corresponding to an OD_{600} of 1 from each sample were also used for determination of α-amylase activity (B).*

First, the existence of the fusion protein CotB-miniInt-AmyQ in the spore coat was verified by the appearance of a signal being 22 kDa larger in molecular weight (***Fig 3.15A***, lane 3) as compared to the signal of the CotB-AmyQ fusion protein from QAS06 (***Fig 3.15A***, lane 2). No AmyQ was detected from the sample of MTB121 (***Fig 3.15A***, lane 1) as anticipated.

However, the weaker signal detected from the QAS40 sample demonstrated the suppression in the expression of CotB-miniInt-AmyQ in this strain.

Second, the α-amylase activities of the spores from the three strains were determined (***Fig 3.15B***). In the absence of AmyQ (MTB121), no α-amylase activity was shown because the α-amylase gene from the WB800N was inactivated by the insertion of *cotB-miniInt* at the *amyE* locus. While up to 3 units were measured with the QAS06 spores, the QAS40 spores gave less than 1 unit per OD_{600}.

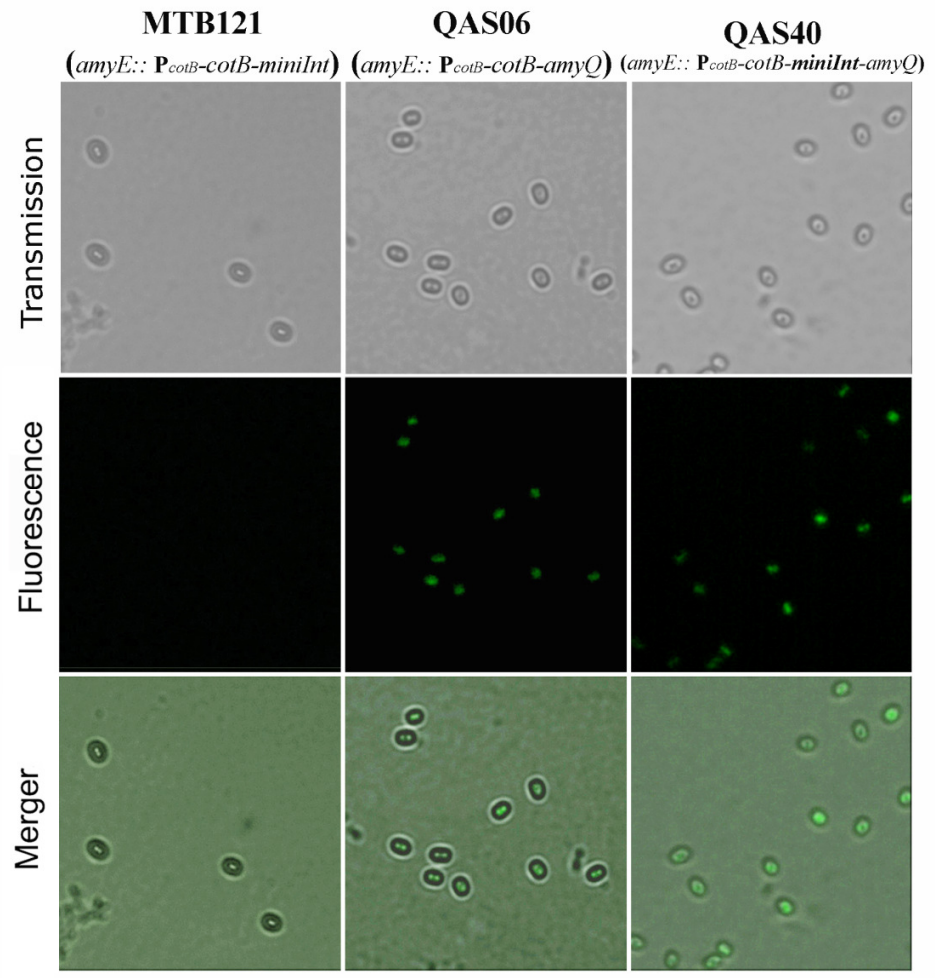

Fig 3.16 Visualization of the miniInt-AmyQ on the spore surface. *The purified spores (QAS06 and QAS40), prepared as described in the legend to Fig 3.15, were treated with the primary antibody against α-amylase, then with an Alexa 488-conjugated secondary antibody for immunofluorescence microscopy as described in 2.2.4.1. The fusion miniInt-AmyQ display on spore surface was visualized by transmission and fluorescence Leica SP5 microscopy. The purified spore from strain MTB121 was treated identically and used as a negative control.*

In parallel to the immunoblot experiment, the purified spores from the three strains were prepared for immunofluorescence visualization of the miniInt-AmyQ displayed on the surface via the anti-AmyQ antibodies. The spores were first incubated with antibodies raised against AmyQ and then with Alexa-conjugated secondary antibodies. The microscopic result shows that fluorescence indicated the presence of AmyQ with both QAS06 and QAS40 spores but not with the MTB121 spores (***Fig 3.16***). Therefore, the presence of miniInt-AmyQ on the spore surface was verified. However, the low α-amylase activity together with the weak signal that could be detected from the immunoblot, indicate that the less protein is anchored. With too less protein expressed on the spore surface, further studies to increase the efficiency of the system are necessary.

3.2.1.2 Optimization of the expression of *miniInt-amyQ* on the spore surface

Because of the low-level expression of CotB-miniInt-AmyQ, the two new constructs for displaying miniInt-AmyQ on spore surface were designed using the native *cotB* and the IPTG-inducible promoter P_{Sgrac}, both present in a plasmid-based vector.

The optimal IPTG-concentration for expression of the CotB-miniInt-AmyQ using P_{Sgrac} (QAS41 strain) was first determined using immunoblot analysis of the total coat proteins and whole spore α-amylase assay. Surprisingly, no IPTG was needed for the optimal expression of CotB-miniInt-AmyQ (***Fig 3.17A***, lane 2). The more IPTG was added, the weaker signals of the fusion protein were detected (***Fig 3.17A,*** lane 3 to 6). The signal obtained from the QAS41 sample in the absence of IPTG was stronger than the one from QAS40 sample which was applied in the same blot for comparison (***Fig 3.17A***, lane 7). The α-amylase assay is in correlation with the immunoblot result. Up to 2.5 units were measured with the QAS41 spores prepared in the absence of IPTG representing the highest activity. The activity was reduced to 1 unit when the IPTG concentration was increased to 0.25 mM, and no activity was observed when more IPTG was added (***Fig 3.17B***). Therefore, no IPTG was used for this construct.

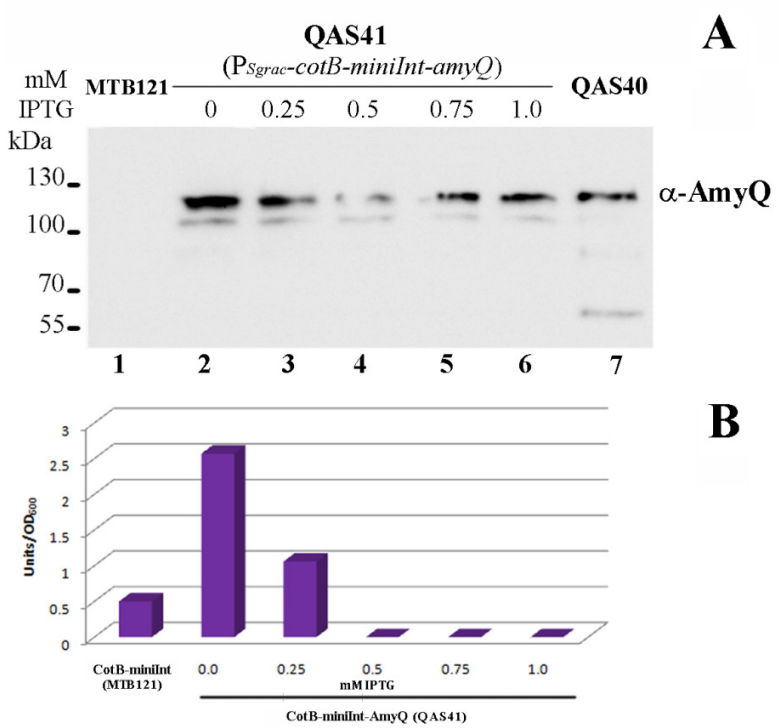

Fig 3.17 Identification of the optimal IPTG-concentration for the cotB-miniInt-amyQ expression. *The spores from strain WB800Spec harboring the plasmid pQAS41 (P$_{Sgrac}$-cotB-miniInt-amyQ – strain QAS41) were prepared as described in the legend to Fig 3.3 (the IPTG-concentration is indicated at the top of each lane). The coat extracts from spores corresponding to an OD$_{600}$ of 1 were loaded per lane, fractionated on 10% SDS-PAGE and subjected to immunoblot; the coat extracts from identical amounts of MTB121 and QAS40 spores were applied in the same gel for comparison (A). The QAS41 and MTB121 spores of an OD$_{600}$ of 1 per sample were also used directly for determination of α-amylase activity (B).*

Strains QAS40, QAS41 and QAS54 (carrying the native *cotB* promoter in a plasmid-based vector construct) were induced for sporulation under the same conditions. Immunoblot analysis of the coat extracts and α-amylase assay with whole spores were carried out to compare the expression levels of the fusion protein from different constructs.

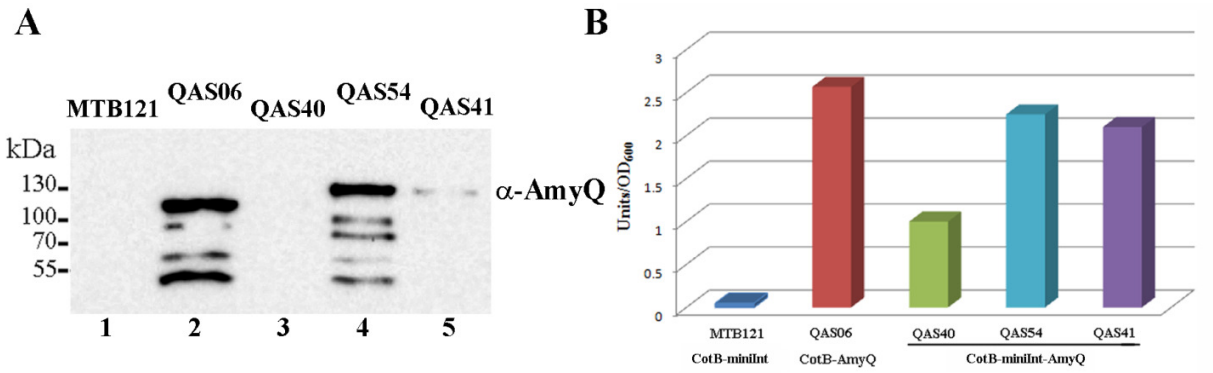

Fig 3.18 ***Comparison of the fusion with AmyQ expression on the spore surface.*** *The spores from strains MTB121 (amyE::P_{cotB}-miniInt), QAS06 (amyE::P_{cotB}-amyQ), QAS40 (amyE::P_{cotB}-miniInt-amyQ), QAS54(P_{cotB}-miniInt-amyQ) and QAS41(P_{Sgrac}-cotB-miniInt-amyQ) were prepared as previously described in the legends to Fig 3.15 and Fig 3.17 (no IPTG was added into the culture of QAS41). The coat fractions of the five strains' spores of an OD_{600} of 1 were applied per lane, fractionated on 10% SDS-PAGE and examined by immunoblot (A). The purified spores from each strain were used for determination of α-amylase activity (B).*

Among the *cotB-miniInt-amyQ* constructs, only QAS54 (***Fig 3.18A***, lane 4) gave a signal comparable in intensity to the signal from QAS06 (***Fig 3.18A***, lane 2). The CotB-miniInt-AmyQ produced by QAS41 (***Fig 3.18A***, lane 5) was clearer than that by QAS40, which is unable to be seen (***Fig 3.18A***, lane 3). But less protein from the QAS41 sample was obtained as compared to the one from the QAS54 sample. When the α-amylase activities were compared, the highest activities were measured with the QAS54 spores, about 2.3 units, while QAS41 spore gave about 2 units (***Fig 3.18B***).

In addition, immunostaining for visualization of the fusion miniInt with AmyQ was performed. Spores from the three strains including QAS40, QAS54 and QAS41 were analyzed using fluorescence microcopy. The microscopic result in ***Fig 3.19*** again confirmed the existence of the fusion miniInt-AmyQ on the spore surface.

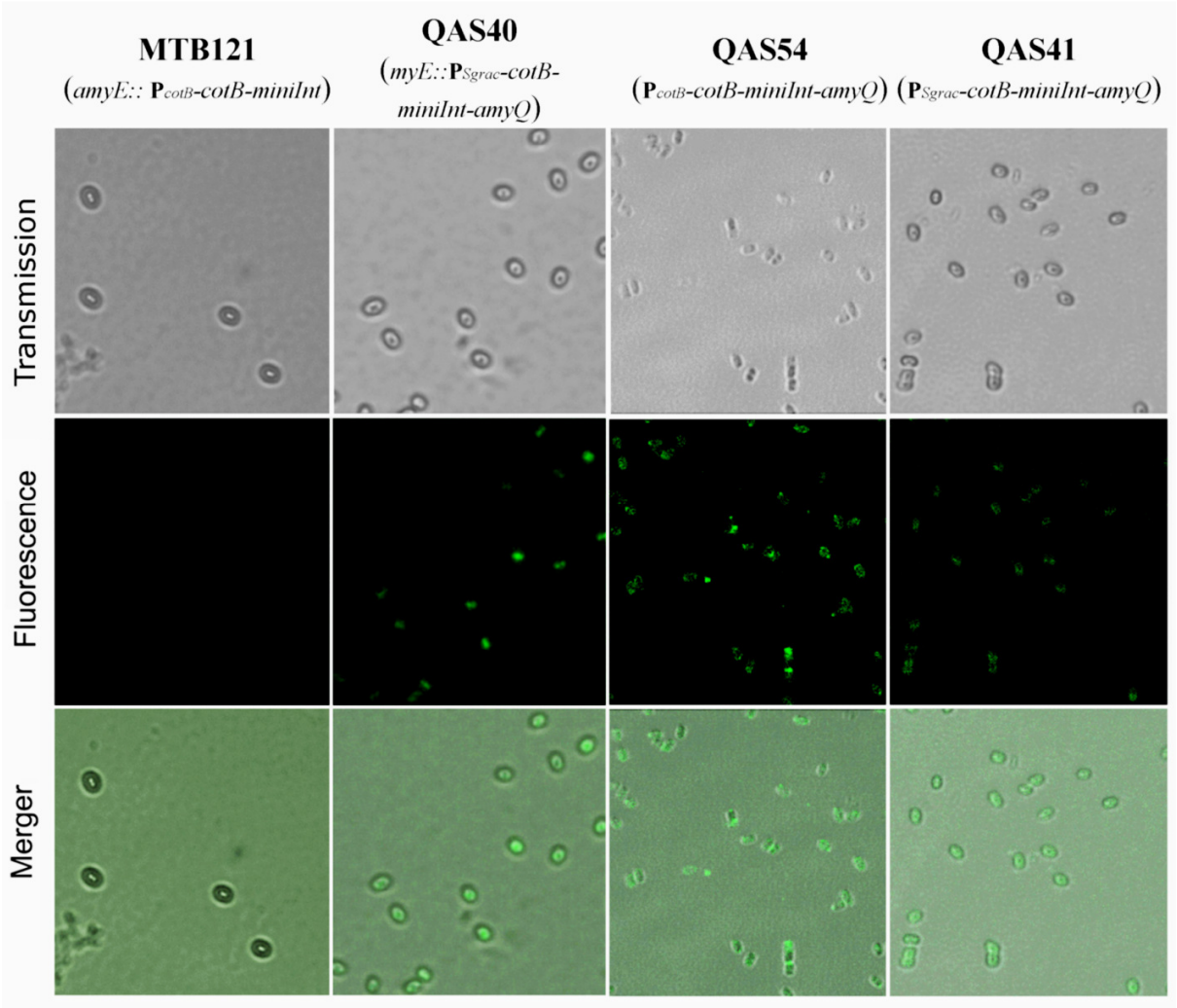

Fig 3.19 Visualization of the miniInt-AmyQ on the spore surface. *Purified spores from strains QAS40, QAS54 and QAS41, prepared as described in the legend to Fig 3.18, were treated with the primary antibodies against α-amylase, then with Alexa 488-conjugated secondary antibodies and visualized under immunofluorescence microscopy as described in 2.2.4.1. The fusion protein miniInt-AmyQ displayed on the spore surface was visualized by transmission and fluorescence Leica SP5 microscopy. Purified spores from MTB121 were treated identically and used as a negative control.*

From the correlation of the immunoblot analysis, α-amylase activity and immunofluorescence microscopic results, the QAS54 strain, harboring the plasmid-based vector with the P_{cotB}-*cotB-miniInt-amyQ* construct, was chosen for further studies.

3.2.2 Activation of mini-intein for the release of AmyQ into the supernatant

The proteolytic activity of the Ssp DnaB mini-intein was induced by shifting the pH in the incubation buffer from rather basic (pH 8.5) to neutral (pH 7.0) (Mathys *et al.*, 1999). Purified spores from strain QAS54 were prepared as previously described with strain QAS40 in the *3.2.1.1.* The amount of spores corresponding to an OD_{600} of 5 per ml were washed and incubated either with intein cleavage buffer, for activation of the mini-intein, or intein washing buffer as a control, well shaken at 25°C. Samples were collected at different times, supernatants and spores were separated by centrifugation. These supernatant and spore samples were then examined for α-amylase activity.

As shown in the ***Fig 3.20A***, the α-amylase activity of the supernatant was increased over time when the spores were incubated in intein cleavage buffer. After 48 h, more than 6 units were measured and it could be raised up to 8 units when the sample was taken at 54 h. The result represented the release of AmyQ from spores, which indicated the activation of the mini-intein. In contrast, the α-amylase activity from the supernatants incubated in washing buffer was just changed a little and did not depend on the time of incubation. This result verified that the activity of Ssp DnaB mini-intein was only induced at the appropriate pH. The residual α-amylase activity of spores over the incubation time was also analyzed. The activity measured with the spores before incubation was set at 100% (***Fig 3.20B***). With the spores incubated in cleavage buffer, we could see a slow decrease of activity over time of incubation. As to the spores incubated in washing buffer, after 48 h, only 20% retained activity. However, the reduction of these spores' activity showed no relation with the time of incubation. So, activation of the mini-intein at the proper pH buffer was confirmed.

The AmyQ released into the supernatant was then verified by immunoblot. The QAS54 spores were incubated in different buffers (cleavage buffer, washing buffer and cleavage buffer supplement with 0.1X Pi) for 24 h. The supenantans were withdrawn, concentrated by Amicon Ultra Centrifugal Filters, applied in 12% SDS-PAGE and subjected to Western-blot. The supernatant from QAS06 spores incubated in intein cleavage buffer was identically treated and used for comparison.

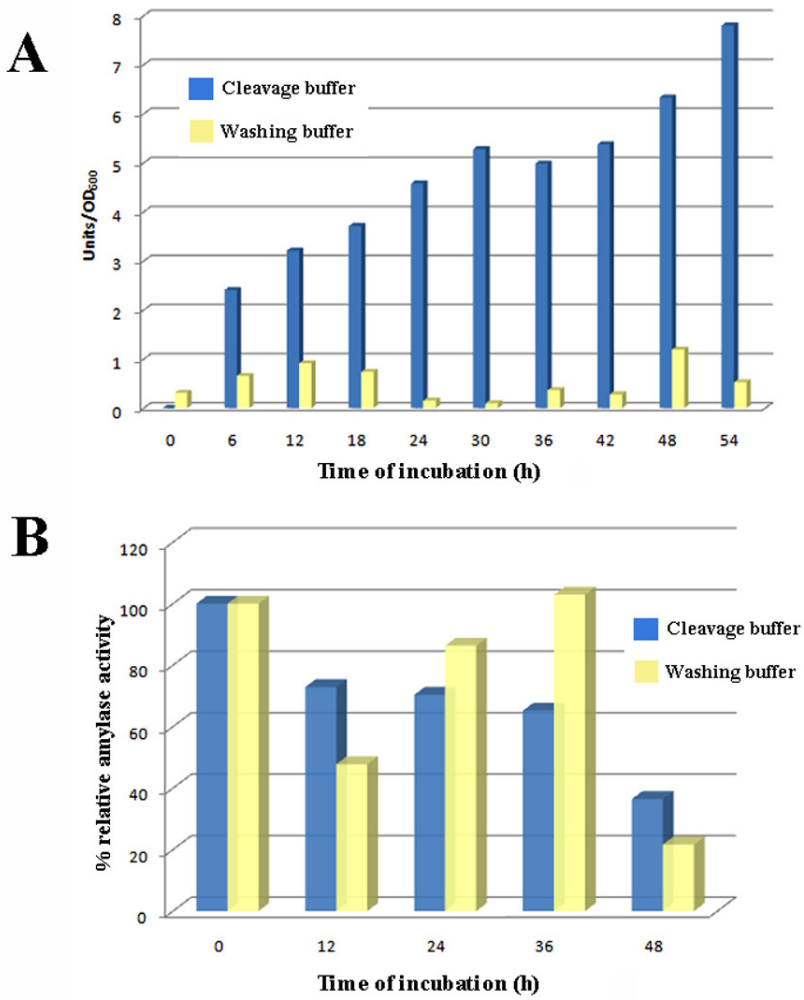

Fig 3.20 Kinetics of released α-amylase in supernatants (A) and relative α-amylase activity (B) of spores after activation of the intein. *Spores of the strain QAS54 were prepared as described in Fig 3.15. The purified spores of an OD_{600} of 5 per ml were incubated either in intein cleavage buffer, for activation of the mini-intein, or intein washing buffer as a control, well shaken at 25°C. 0.25 ml per sample was collected at different times, separated into supernatants and spores by centrifugation. These supernatants and spores were then examined in α-amylase assay. The release of AmyQ was represented as the alteration of α-amylase activity of supernatant over the incubation time (A). The retained activity of spores over the incubation time was also presented with the α-amylase activity of spores right before the incubation (per sample) was set as 100% for calculation (B).*

The immunoblot analysis of the concentrated supernatant from spores representing an OD_{600} of 1 showed the strongest signal with QAS54 spores incubated in cleavage buffer (***Fig 3.21***, lane 2). The decrease of the AmyQ signal when 0.1X Pi was added (***Fig 3.21,*** lane 4) indicated inhibition of the intein activity in the presence of the Pi. No signal was detected with the concentrated supernatant from QAS54 spores incubated in washing buffer, which was expected when the mini-intein was activated at neutral pH of the cleavage buffer (***Fig 3.21***, lane 3). However, the slight signal detected with the concentrated supernatant from QAS06 spores incubated in cleavage buffer gave an open question about the auto-release of the protein into supernatant due to unclear factors.

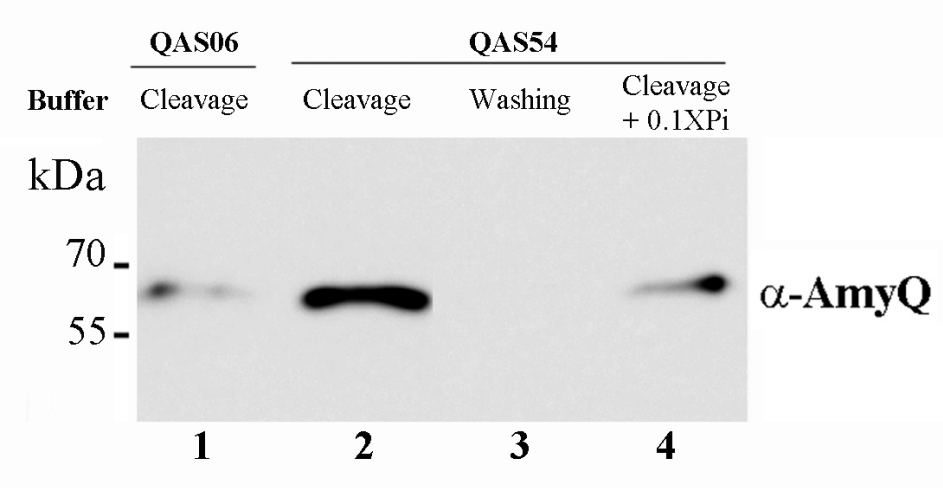

Fig 3.21 Detection of α-amylase from supernatants after intein activation. *QAS54 spores (OD_{600} of 5 per ml) were incubated in different buffers (cleavage buffer, washing buffer and cleavage buffer with 0.1X Pi), well shaken at 25°C for 24 h. 700 µl of each supernatant was taken out and concentrated by Amicon Ultra Centrifugal Filters 3K (Millipore) to 70 µl. 10 µl per sample were loaded on each lane of 12% SDS-PAGE gel and subjected to immunoblot using rabbit anti α-amylase antibody as primary antibody. Supernatant from QAS06 spore incubated in cleavage buffer, prepared as the same way, was also applied on the same gel for comparison.*

In summary, the *B. subtilis* spore-based expression and purification system, using the DnaB mini-intein from *Synechocystis* sp., was constructed and improved. The efficiency of the system was also validated. However, some problems had appeared during the work: (i) the presence of Ssp DnaB mini-intein seemed to interfere with the expression level of the fusion

protein; (ii) unknown factors (probably proteases) caused the uncontrollable release the displayed protein into the supernatant. To solve these problems, additional experiments have to be carried out to improve the system before its proper application.

3.3 Construction of cellulase chips using *B. subtilis* cells and spores

As previously mentioned, cellulases are being used in many industrial fields (Bhat, 2000). Biotechnological cellulase chips, in which these enzymes were immobilized on the surface of any carrier such as microorganism cells or spores, once created, could be applied in many fields such as food or textile industries to convert cellulosic material into glucose and other fermentable sugars. This part of the thesis focused on the construction of cellulase chips based on *B. subtilis*. The *C. thermocellum* endoglucanase A gene (*celA*) which codes for a highly potent and thermostable cellulase A (1,4-ß-D-glucan glucanohydrolase EC 3.2.1.4.) with a M.W. of 56 kDa (Beguin *et al.*, 1985) was chosen to be immobilized on the surface of *B. subtilis* cells and spores.

3.3.1 Display of Cellulase A from *C. thermocellum* on the *B. subtilis* cell surface

The first task of this part was creation of a cell-based cellulase chip using the cell display system which was constructed by Nguyen and Schumann (Nguyen and Schumann, 2006). For anchoring the protein on the *B. subtilis* surface, the sortase A from *L. monocytogenes* was employed for the catalysis of the covalent link between the cell wall and the polypeptides containing the sorting sequence. The sorting sequence using in here was from *S. aureus* FnbB with the sorting motif LPETG.

3.3.1.1 Permanent expression of sortase A (SrtA) from *L. monocytogenes* in *B. subtilis*

In the original cell surface display system, the SrtA enzyme was controllably produced by addition of IPTG. Therefore, the protein anchoring process needed the use of this costly inducer. Because of this, I constructed a new *B. subtilis* strain which can permanently produce SrtA from *L. monocytogenes* in the cytoplasm.

The *srtA* gene of *L. monocytogenes* was fused to the P_{dnaK} promoter and integrated at the *thrC* locus of *B. subtilis* strain WB800N resulting in strain QAC04. To demonstrate expression of the *srtA* gene, *B. subtilis* strains WB800N (control, no *srtA* gene), NHD03 (control, IPTG-inducible construct) and QAC04 were grown to the mid-logarithmic growth phase and NDH03 culture was submitted to IPTG-induction. The cultures were further grown for 2 h. Aliquots were

fractionated in SDS-PAGE and subjected to immunoblot probed with polyclonal antibodies against sortase A. The anti-DnaK antibodies were used for loading control. Analysis of the extraction from strain QAC04 showed a clear signal appeared at the molecular mass of sortase A (estimated 30 kDa) (***Fig 3.22***, lane 3). This signal was comparable with the one from the extract from strain NDH03 (***Fig 3.22***, lane 1). However, due to the lower amount of loading sample from NDH03 extraction (see ***Fig 3.22***, lane 1-probed with α-DnaK), the production of SrtA in QAC04 was reduced. No unexpected band was detected with the negative SrtA strain (***Fig 3.22***, lane 2). It can be concluded that strain QAC04 is able to produce SrtA in the cytoplasm constitutively. The amount of expressed SrtA in QAC04 was lower when compared to IPTG-inducible NDH03 strain. However, because SrtA is an enzyme needed for anchoring the main target protein, even a small amount of it would be sufficient for this purpose. Therefore, the QAC04 strain was used for further studies.

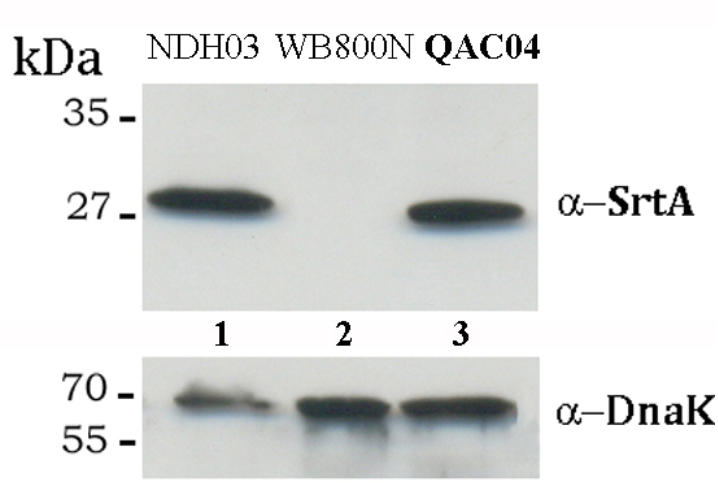

Fig 3.22 Detection of SrtA of L. monocytogenes in extracts of B. subtilis. *B. subtilis strains NHD03, WB800N and QAC04 were grown in LB medium at 37 °C to the mid-exponential growth phase (OD_{578} ~ 0.8), 0.5 mM IPTG was added into the culture of strain NDH03 to induce expression of SrtA, while the other two cultures were further grown without induction for 2 h. Samples were taken and treated as described in 2.2.2.1, then processed for immunoblotting using rabbit anti L. monocytogenes SrtA antibodies. The anti-DnaK antibodies were used for loading control.*

3.3.1.2 Less CelA can be anchored on the *B. subtilis* cell surface

To display cellulase A (CelA) on the cell surface, the *celA* gene was inserted into the pNDH11 plasmid resulting in pQAC07, so that a fusion of Secretion Signal-CelA-Sorting Signal would be produced upon induction using xylose.

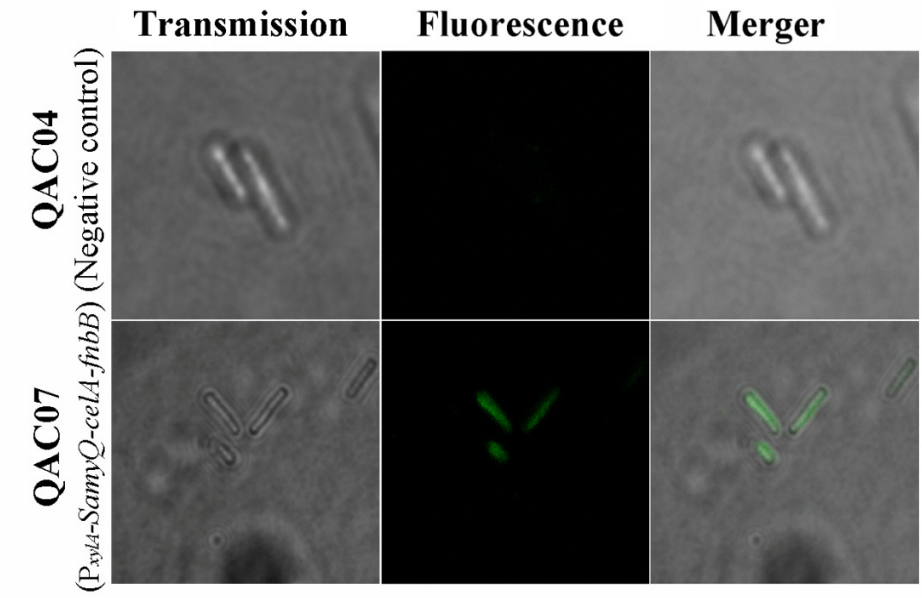

Fig 3.23 Visualization of CelA on the B. subtilis cell surface. *The B. subtilis strains QAC04 (thrC::P_{dnaK}-srtA) and QAC07 (P_{xylA}-SamyQ-celA-fnbB) were grown in LB medium at 37 °C to the mid-exponential growth phase (OD_{578} ~ 0.8), 0.5% xylose was added to induce the expression of celA. Further growth for 2 h was performed. Then, aliquots were collected; the cells were separated from the growth medium by centrifugation and processed for immunofluorescence microscopy as described in 2.2.4.1. The immobilization of CelA on the cell wall of B. subtilis was visualized by transmission and fluorescence Leica SP2 microscopy.*

The strain QAC04 harboring the plasmid pQAC07 (P_{xylA}-*SamyQ-celA-fnbB* – strain QAC07) was grown in LB medium to the mid-logarithmic growth phase, then 0.5% xylose was added. Further growth for 2 h was performed. The cells were withdrawn, gently washed twice with AP buffer and used directly for immunofluorescence staining using primary antibodies against CelA and Alexa-conjugated secondary antibodies (see 2.2.4.1). The QAC04 strain itself was grown under similar conditions, then treated with the same antibodies and used as a negative

control. The microscopic analysis showed the presence of CelA on the cell surface with the QAC07 cells. Little unspecific fluorescence was detected with the negative control (***Fig 3.23***).

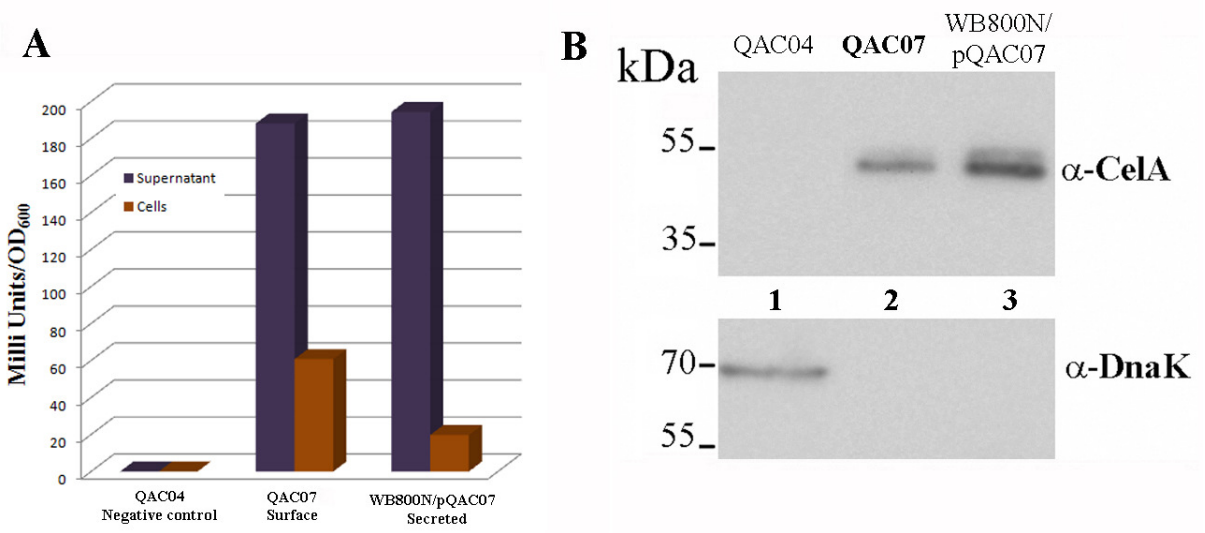

Fig 3.24 Detection of celA expression on the cell wall by cellulase activity (A) and immunoblotting (B). *The B. subtilis strains QAC07 and WB800N harboring plasmid pQAC07 and QAC04 were grown in LB medium and equal amounts of cells were washed as described in the legend to Fig 3.23. Cellulase activities were determined with whole cells and within the supernatant and presented in units per OD_{578} (A). The cell wall fractions from an identical number of cells from each strain were prepared as described in 2.2.2.2, loaded on 12% SDS-PAGE and subjected to immunoblot using rabbit anti CelA antibodies (B). The anti-DnaK antibodies were used for controlling the leakiness of the cytoplasmic membrane per sample.*

To obtain a more precise confirmation for anchoring of CelA on the cell wall by SrtA transpeptidase, plasmid pQAC07 was transformed into the strain WB800N (does not contain SrtA). This strain was expected to secrete the fusion CelA-sorting signal into the medium. Next, this strain and strain QAC07 were grown in LB and induced by xylose. Whole cells were separated from the growth medium by centrifugation and analyzed separately for cellulase activity. The QAC04 was used in this experiment as a negative control. While a background level in the absence of any CelA was minor, the activities were increased with the cells from strain WB800N carrying plasmid pQAC07. The strongest activities from cells were measured with strain QAC07, about 65 milli units (***Fig 3.24A***). The cellulase activities measured from

supernatant of the two strains QAC07 and WB800N/pQAC07 were equivalent. However, as compared to activities from cells, they were significantly higher (***Fig 3.24A***). This indicated that more CelA was secreted to supernatant than anchored on cell wall.

In addition, the cell wall fractions from the three strains were prepared as described in 2.2.2.2 and subjected to immunoblot analysis using primary antibodies against CelA. The polyclonal antibodies against DnaK were used for controlling the leakiness of the cytoplasmic membrane in each sample. As anticipated, no CelA band was detected with the QAC04 sample (***Fig 3.24B***, lane 1). The signals from the WB800N/pQAC07 and QAC07 samples were detected with comparable amount (***Fig 3.24B***, lane 2 and 3). The result made it difficult to demonstrate the anchoring of CelA on the cell wall.

In summary, the CelA could not be effectively anchored on the *B. subtilis* cell wall using the *L. monocytogenes* SrtA transpeptidase.

3.3.2 Display of CelA on the *B. subtilis* spore surface

The second task of this part was the display of CelA on the spore surface. The spore surface systems constructed in part **3.1** were utilized.

3.3.2.1 Verification of the CelA immobilization on the spore surface

To achieve the goal of this task, the three Cot proteins, CotB, CotC and CotG were used as carriers. The *celA* gene was translationally fused downstream of the *cot* genes. The fusions were then managed by either the IPTG-inducible P_{grac} promoter in a plasmid-based vector or the native promoters of *cotB* or *cotG* as part of an integration vector.

For the activation of P_{grac}, the optimal IPTG-concentration was defined for each construct with CotB, CotC and CotG. The spores from strains QAS24, QAS25 and QAS27 (related to CotB, CotG and CotC, respectively) were prepared as with GFPuv spores described in the legend to ***Fig 3.9***. The purified spores from different cultures induced with different IPTG-concentrations were directly used for measurement of cellulase activity.

For the CotB-CelA expression (strain QAS24), the highest activity, 99 milli units, were measured when the culture was induced by 0.1 mM IPTG. This result was also observed with spores displaying the fusion protein CotG-CelA (strain QAS25); here 112 milli units were measured. Up to 80 milli units were quantified in the absence of IPTG with QAS25 spores, most

probably due to the leakiness of the P_{grac} promoter. 0.5 mM IPTG was needed to achieve the highest expression of the CotC-CelA fusion, 17 milli units for strain QAS27 (see ***Fig 3.25)***. Therefore, these concentrations determined to be optimal for the expression of the fusion Cot-CelA and were used for further experiments.

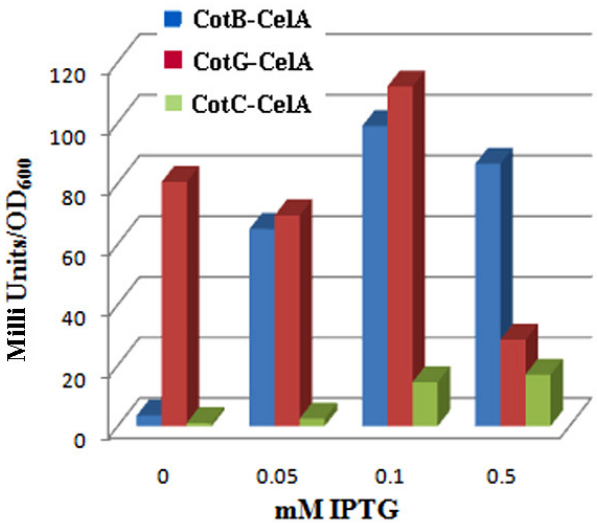

Fig 3.25 Identification of the optimal IPTG-concentration for the anchoring of CelA on the spore surface. *Spores from B. subtilis strains QAS24 (P_{grac}-cotB-celA), QAS25 (P_{grac}-cotG-celA) and QAS27 (P_{grac}-cotC-celA) were prepared as previously described in the legend to Fig 3.3. An identical number of spores per sample were used to perform cellulase assays, and the results were presented in milli units per OD_{600}. The IPTG-concentrations used in the experiment were indicated.*

The spores from the five strains QAS15 (carrying *amyE::*P_{cotB}*-cotB-celA* construct), QAS16 (*amyE::*P_{cotG}*-cotG-celA* construct), QAS24 (P_{grac}*-cotB-celA*), QAS25 (P_{grac}*-cotG-celA*) and QAS27 (P_{grac}*-cotC-celA*) were purified and prepared for the visualization of the CelA on the surface by fluorescence microscopy. The antibodies against CelA were used as primary antibodies and the secondary antibodies were conjugated with Alexa staining material for localization of the primary antibody through fluorescence microscopy using Leica SP2 camera. There was no detectable fluorescence with QAS27 spores (data not shown), together with very low enzymatic activity as compared to other samples (***Fig 3.25***), suggesting the unsuccessfulness in displaying CelA on the spore surface using CotC as carrier. The CelA was present on the surface of spores whenever CotB or CotG was utilized (in both native *cot* promoter and P_{grac}

promoter constructs) (see ***Fig 3.26***). It can be concluded here that CotB and CotG are more suitable for the display of CelA on the spore surface.

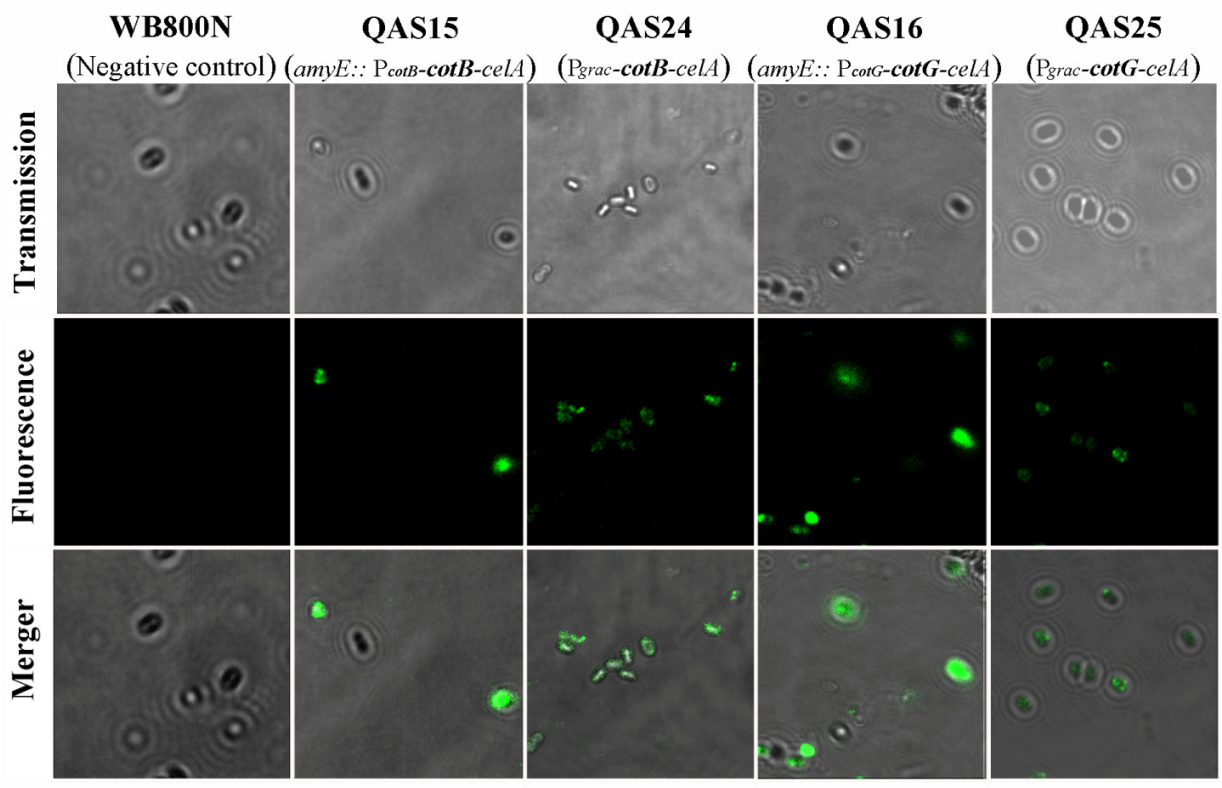

Fig 3.26 Detection of CelA on the surface of spores by confocal microscopy. *Spores of strains QAS15 (amyE::P_{cotB}-cotB-celA) and QAS16 (amyE::P_{cotG}-cotG-celA) were prepared as described in Fig 3.15, and spores from strains QAS24, QAS25 and QAS27 were prepared as described in Fig 3.25 with the optimal IPTG-concentration. The purified spores were then processed for immunofluorescence microscopy as described in 2.2.4.1. Purified spores from WB800N were treated identically and used as reference. The expression of CelA on the cell wall of B. subtilis was visualized by transmission and fluorescence Leica SP2 microscopy.*

3.3.2.2 Comparison of cellulase activities measured with spores displaying CelA on the surface

Purified spores from the five strains, including the one with the P_{grac}-*cotC-celA* construct (strain QAS27), were measured for cellulase activity and compared to each other (***Fig 3.27***). It is obvious that the new method with an IPTG-inducible P_{grac} promoter was more effective in displaying CelA on the spore surface, an about 2-fold increase in activity was observed, using

both CotB and CotG as carriers. CotC protein, on the contrary, is not appropriate as a carrier for anchoring CelA on the spore surface due to the low activities compared to others.

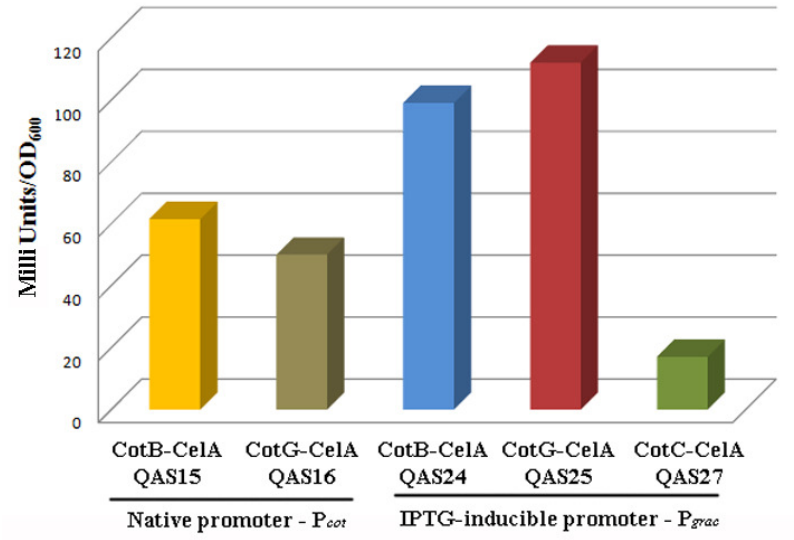

Fig 3.27 Cellulase activity of spores displaying CelA on the surface by different carrier proteins. *The spores from the five different strains QAS15, QAS16, QAS24, QAS25 and QAS27 were prepared as described in Fig 3.26 and their cellulase activities were measured.*

The cellulase activities measured in this thesis are somewhat comparable to the activities of CelA previously mentioned with *S. cerevisiae* (Chung *et al*., 1997). In this previous study, up to 280 units per liter of culture were obtained with *S. cerevisiae* secreting CelA, while the highest activities measured here are 195 milli units per OD_{578} (equivalent to 600 units/l) association with CelA in the supernatant. In both studies, a large volume of culture (up to liter) has to be prepared to assure sufficient CMC degradation. This demonstrates the insufficient activity of CelA. Further studies should be carried out in selection of a stronger cellulase and improvement of the systems to generate a more effective cellulase chips.

4 Discussion

Since the first surface expression system was published by G. P. Smith in 1985 using gene III of the filamentous phage M13 for display (Smith, 1985), many other systems have been studied and developed due to their wide range of biotechnological and industrial applications (Lee *et al.*, 2000; Lee *et al.*, 2003; Benhar, 2001).

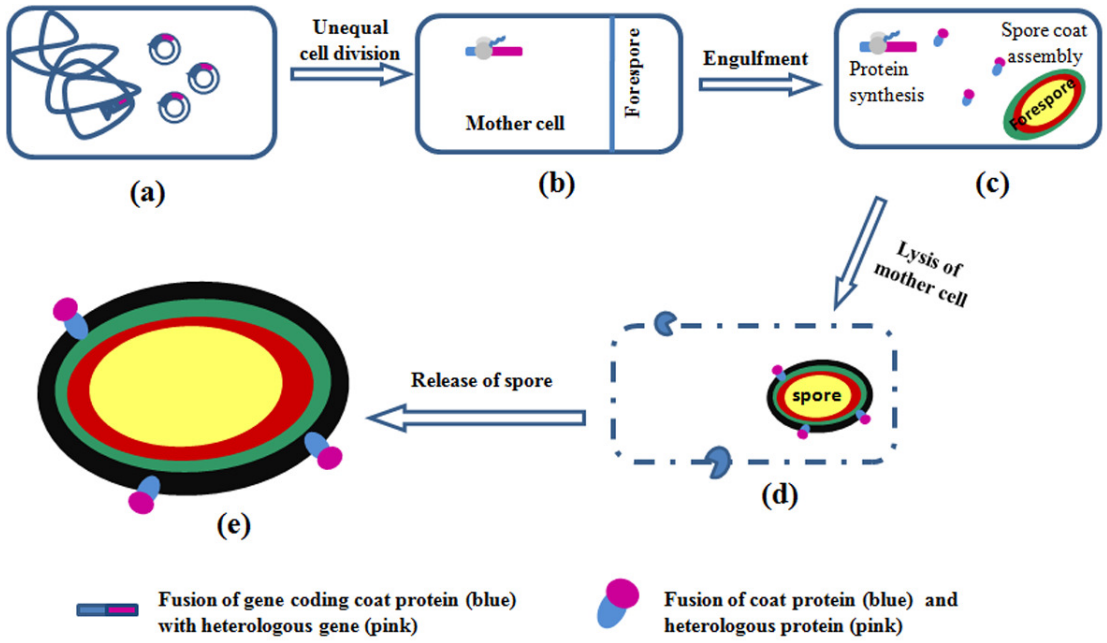

Fig 4.1 ***The spore surface display system****. The fusion between a gene coding for a coat protein and the heterologous gene is introduced into cells (a). When cells enter sporulation, unequal cell division take place to separate the cell into the forespore and the mother cell compartments. The expression of spore coat-related genes occurs within the mother cell compartment (b). After the completion of engulfment of the forespore by the mother cell, the coat proteins and the fusion with the coat proteins produced inside the mother cell assemble on the forespore (c). Cell lysis (d) occurs at the final step to release the mature spore displaying the heterologous protein on the surface.*

A major disadvantage of phage-based systems is the limited size of the recombinant proteins which can be anchored (Benhar, 2001; Li, 2000). In bacterial cell-based systems, since some fast-folding or hydrophobic patches containing proteins may be obstructed at the

cytoplasmic membrane (Kim and Schumann, 2009), the transportation of heterologous proteins through the cell membrane followed by anchoring on the surface is not always effective. Using endospores for this purpose may help to overcome these problems. Because the endospore is formed within the sporulating cell, all components of the spore including anchored proteins are produced inside the cytoplasm and then directed to the spore surface (***Fig 4.1***). Therefore, the heterologous proteins do not have to cross the cytoplasmic membrane. In addition, ß-galactosidase, a very large protein (116 kDa per monomer) and active only as a tetramer, has been successfully anchored on the *B. subtilis* surface with its full activity (Kwon *et al.*, 2007). This result suggests the advantage of spore over cell and phage systems in displaying of heterologous proteins with no size-limitation.

4.1 Stabilization of the expressed protein for the spore surface display

B. subtilis cells generate a variety of both intra- and extracellular proteases (Schaeffer, 1967; Schaeffer, 1969; Kole *et al.*, 1988). As a result, the heterologous proteins produced by this organism may face a degradation possibility before assembling on the spore surface. To improve the stability of extracellular heterologous proteins, genetic manipulation techniques were applied to remove genes coding for major extracellular proteases (Wu *et al.*, 1991; Wu *et al.*, 1993; Wu *et al.*, 2002). However, not all the proteases of *B. subtilis* could be inactivated because they are required in certain processes, especially in sporulation (Mandelstam and Waites, 1968; Schaeffer, 1969). Hence, for all the experiments in this thesis, the eight-fold protease-deficient *B. subtilis* strain WB800N (Nguyen, 2006) was used. In addition, the Pi cocktail solution (Roche Diagnostics), prepared as described in section 2.2.1.3, was directly added into the cell cultures to stabilize the recombinant proteins.

The Pi cocktail was known to inhibit a multitude of protease classes, including serine proteases, cysteine proteases, and metalloproteases (Roche manual). Addition of the Pi solution into the culture should directly help to protect the proteins displayed on the free spores. Normally, the Pi is usually added into the cell lysate, in which the proteins have already been released from cells. It is unclear whether the Pi solution can be taken up by *B. subtilis* cells. Here, I have shown that when the cultures were supplemented with 0.4X Pi concentration, alterations of the spore coat composition and sporulation suppression appeared. When 0.1X, 0.2X or 0.8X Pi concentrations were added, an improvement of the amount of recombinant proteins occurred (***Fig***

3.1). These data suggested that Pi indeed exerts an effect inside the intact cells. The result also demonstrated the possibility of using externally added protease inhibitors to optimize the stability of proteins displayed on the spore surface.

In addition, previous studies described that the amount of expressed fusion proteins on the spore surface seemed independent of the nature of the heterologous part, and the number of recombinant protein molecules was approximately 1.1 x 10^3 per spore when CotB was used as carrier (Isticato *et al.*, 2001; Hinc *et al.*, 2010). However, using the same strategy in this study resulted in 4.6 x10^3 AmyQ molecules displayed per QAS06 spore surface, which is four times higher than the results from previous reports. That indeed suggested an effect of the passenger protein itself on the amount of recombinant protein. The difficulties in attempting to display the entire UreA subunit (Hinc *et al.*, 2010) also demonstrated the effect of the heterologous protein on the whole fusion, that supported my hypothesis.

4.2 Anchoring recombinant proteins on the spore surface using inducible promoters

4.2.1 The IPTG-inducible P_{grac} promoter is able to increase the amount of heterologous proteins displayed on the spore surface

B. subtilis endospores showed some advantages over host strains for displaying heterologous proteins on the surface (reviewed recently in (Kim and Schumann, 2009; Ricca and Cutting, 2003)). The first strategy applied to engineer *B. subtilis* spores is to display heterologous proteins on the surface using CotB, an outer spore coat protein, with its native transcriptional and translational signals as carrier (Isticato *et al.*, 2001). Since then, CotC, CotG and even OxdD, an inner-coat protein, were successfully used for displaying proteins of interest on the *B. subtilis* spore surface (Potot *et al.*, 2010; Kim *et al.*, 2005a; Kwon *et al.*, 2007; Mauriello *et al.*, 2004; Hinc *et al.*, 2010). Using this strategy for spore surface display, the expression level of displayed proteins relies on the expression signals of the carriers and therefore cannot be controlled. In an attempt to improve the expression level of heterologous proteins on the *B. subtilis* spore surface, systems using the IPTG-dependent promoters P_{grac} and P_{Sgrac} were designed in this doctoral thesis. These promoters were chosen for their ability to produce high levels of recombinant proteins in *B. subtilis* (Phan *et al.*, 2006).

For approaching the new strategy, all three previously used coat proteins, CotB, CotC and CotG, were utilized as carriers. Among them, only CotG was used for displaying an enzyme so

far (Kim *et al.*, 2005a; Kwon *et al.*, 2007). In my thesis, for the first time, CotB was exploited to express an enzyme, α-amylase from *B. amyloliquefaciens* (AmyQ), on the spore surface. The GFPuv, fused to all three coat proteins, was used as the second model protein. As a significant increase in the amount of the fusions was observed when adding IPTG for induction (***Fig 3.3***, ***Fig 3.9*** and ***Fig 3.10***), it could be concluded that the production of the fusion proteins were controllable and the fusions were directed to the spore coat since their presence were identified in the coat extracts. In addition, the measurable α-amylase activity along with immunofluorescence experiments using purified spores had indicated the existence of AmyQ in its active form on the spore surface (***Fig 3.4*** and ***Fig 3.5***). Under control of a stronger promoter, the amount of recombinant proteins displayed on the spore surface was indeed increased, approximately two- to three-fold with the P_{grac} promoter and AmyQ as compared to one which used the native promoter for display.

4.2.2 A certain amount of fusion protein is required for optimal display of the heterologous proteins on the spore surface

Although it is obvious that the strong, inducible P_{grac} promoter can help improving the amount of displayed protein on the spore surface, using the stronger P_{Sgrac} promoter does not give the same influence. While the amount of fusion protein observed with the P_{Sgrac} promoter was the highest compared to other samples, the lowest activity was measured with this sample in an α-amylase assay (***Fig 3.6***). The P_{Sgrac} promoter is stronger than the P_{grac} promoter in controlling expression of recombinant genes (BayGenetics), hence higher amounts of heterologous protein was extracted from the spores using this promoter. The lower activity observed with this sample suggested a possibility that fewer protein molecules were exhibited on the spore surface. In addition, the P_{Sgrac} promoter also showed leakiness in controlling production of the fusion proteins. These results suggest the unsuitability of the P_{Sgrac} promoter for expression of recombinant proteins on the spore surface.

Moreover, when the inducer IPTG was added at a higher than optimal concentration, the fusion proteins became unstable, shown through decrease of the fusion proteins together with an increase in degraded products, and an alteration appeared in the coat composition. During experimental progress, the spores which exhibited a very high expression level of the fusion proteins were sometimes harvested (data not shown). But these spores were poorly purified,

which indicated that sporulation was suppressed. From the results with overproducing heterologous proteins for display on the spore surface, it was clear that just a certain amount of proteins was needed, and the unnecessary production of recombinant proteins might cause the coat assembly disorder or even suppression of sporulation.

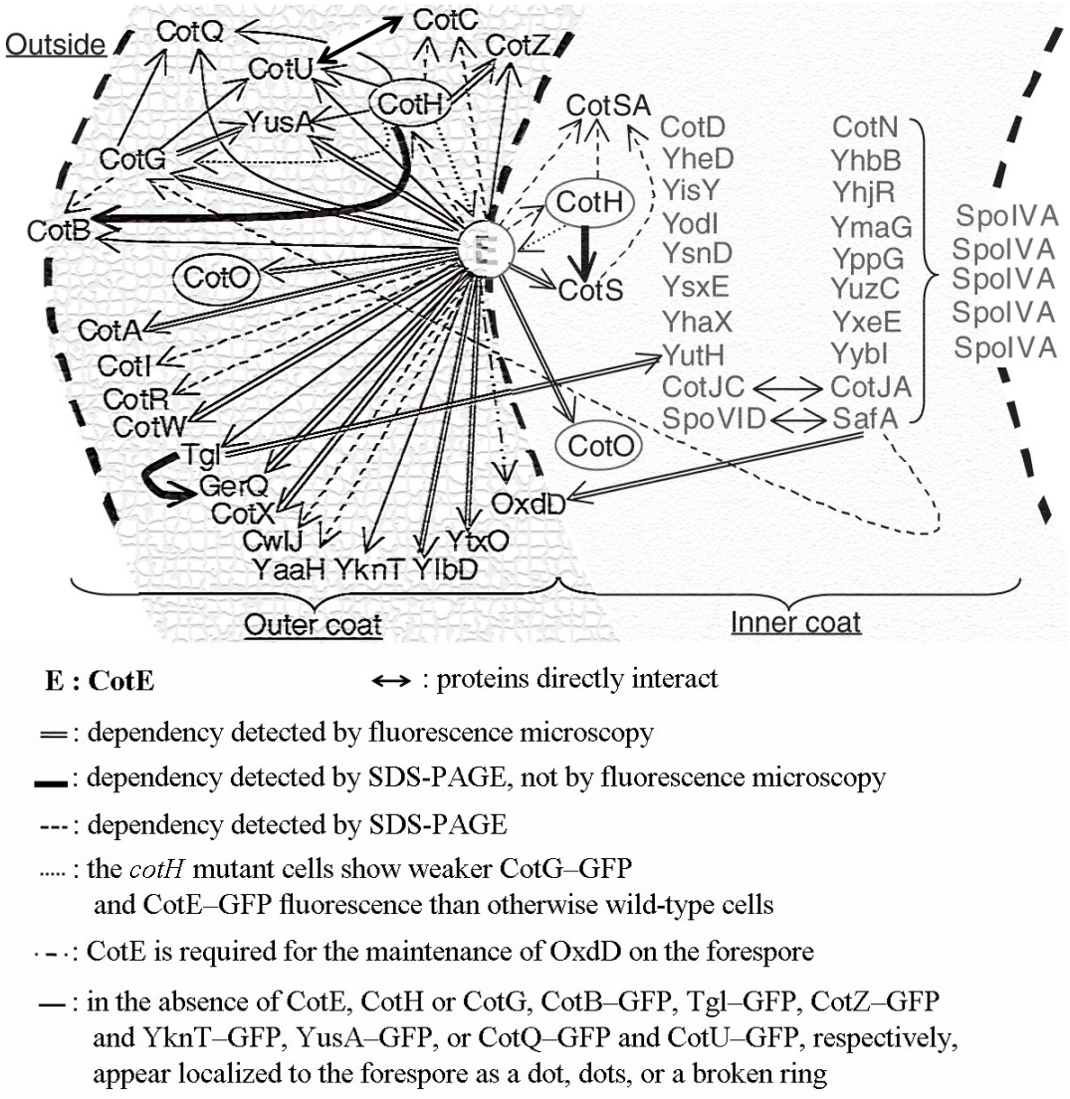

Fig 4.2 ***Model of the coat protein interaction network.*** *Directions of the arrows indicate the assembly dependencies. In some cases, the dependencies are partial. The layer in which a coat protein resides is known definitively in only a few cases (including CotB, CotC, CotE, CotG and CotS; (Driks et al., 1994; Takamatsu et al., 1998; Isticato et al., 2001; Mauriello et al., 2004; Kim et al., 2005a)). In the others, assignment to a layer in the diagram is provisional and based largely on control by CotE. Adaption from (Kim et al., 2006).*

The copy number of the fusion between *cot* and heterologous genes was shown to have an influence on the production of the fusion proteins. In general, more IPTG inducer was required for optimal expression when the fusion genes were present on the chromosome (***Fig 3.9*** and ***Fig 3.10***). However, depended on the Cot protein, the different gene dosage effect of the translational fusion present on a plasmid (4 – 6 copies) versus the chromosome (one copy) were demonstrated (***Fig 3.12***).

In this thesis, all the fusion proteins were produced in the presence of the endogenous Cot proteins. The existence of both wild-type Cot proteins and the fusion with Cot proteins may cause competition for expression and display of fusion proteins on the spore surface. It was reported that deletion of the CotB protein might alter the ridge formation on the spore surface (Driks, 2004), CotC protein is shown to interact with the CotU protein (Isticato *et al.*, 2008) and CotG protein is required for the incorporation of CotB into the coat (Henriques *et al.*, 1998). *cotB* and *cotC* mutants have no significant effect on sporulation, germination or resistance (Donovan *et al.*, 1987). However, it was previously described that the *B. subtilis* surface display system based on the CotB protein required both endogenous CotB and CotG proteins' expression (Isticato *et al.*, 2001). Therefore, the two proteins have to be present for the proper display of the fusion proteins with CotB. On a contrary, the fusion proteins with CotG still can be exhibited on the surface of spores bearing *cotG* null mutant. The same result also occurred with a display system based on the CotC protein (Hinc *et al.*, 2010). This suggests the possibility for improving expression of the fusion with CotC or CotG proteins on the spore surface in the absence of either endogenous CotC or CotG.

A previous study had described significant alterations in spore coat and cortex composition caused by changing the sporulation temperature (Melly *et al.*, 2002). The clear alterations observed from the component of coat fractions together with the possibility for spore suppression suggested an influence of spore coat overexpression on the spore characteristics. It was shown in this thesis that the spore accommodates an appropriate amount of recombinant protein for optimal display on the surface. Further experiments should be performed to study the resistance and germination ability of spores with a suitable amount of heterologous protein expressed on the surface.

4.2.3 Localization of CotB, CotC and CotG proteins on the spore surface

An interesting phenomenon dealing with the localization of the coat proteins once overproduced was observed when using GFPuv as a reporter protein. According to my results (as shown in ***Fig 3.14***), each coat protein used in this study tends to localize and displays a very different pattern on the spore surface. The interaction of the proteins during coat assembly has been previously reported (also shown in ***Fig 4.2***) (Kim *et al.*, 2006; Driks, 2004; Driks *et al.*, 1994; Isticato *et al.*, 2008; Isticato *et al.*, 2010). My result might suggest a self-interaction within each species of coat protein and their tendency to accumulate at specific locations on the spore coat. This observation reported here is to my knowledge the first to deal with the localization of CotB, CotC and CotG proteins.

4.3 *B. subtilis* spore-based expression and purification system for recombinant proteins

4.3.1 Possibility of using the *B. subtilis* spore to develop an effective and low cost tool for protein expression and purification

Production of the recombinant proteins in an active and highly purified form is an important task in molecular biology and biotechnology. To achieve the final product, many steps dealing with overexpression and purification of proteins are required. In addition, heterologous proteins are often found to form inclusion bodies, which are more complicated for purification and require many additional steps to get the natural structure of the protein (Baneyx and Mujacic, 2004; Baneyx, 2004). Hence, many systems and methods for protein overproduction and purification have been constructed and are commercially available, which creates more options for the users to select the most suitable one for their purposes (Baneyx, 1999; Schumann and Ferreira, 2004; Schumann, 2007). With the development of a wide range of purification tags, a target protein can be simply purified by being genetically fused and expressed as a single fusion protein with a purification tag. (Arnau *et al.*, 2006; Lichty *et al.*, 2005). The fusion of the target protein with different purification tags can also improve the solubility and folding of the target protein (Terpe, 2003; Waugh, 2005). Combination of affinity tag systems with site-specific processing modules, based on engineered inteins, has created self-cleaving affinity tag-based systems allowing one-step purification (Chong *et al.*, 1997; Mao, 2004). The potential role and application of self-cleaving purification tags have been recently reviewed (Fong *et al.*, 2010; Elleuche and Poggeler, 2010).

By exploiting the previous concept to express a protein along with a tag, yet applying it to spores by using them as a matrix, a novel and unique system for expression and purification of recombinant proteins had been constructed. The system was based on the 154 amino acids mini-intein, derived from the *dnaB* gene of *Synechocystis* sp. (Mathys *et al.*, 1999). The idea was to express the target protein on the *B. subtilis* spore surface, followed by purification of spores (which is a relatively simple process), and activation of the intein to release the target protein and recover it in the supernatant. Because the *Ssp* DnaB mini-intein is a self-cleaving protein that can become active at the optimal pH value, no additional protease or reducer is needed and the structure of the target protein will be less influenced. Besides, display of the target protein on the spore surface might help preventing the formation of inclusion bodies, which relates to many additional steps of purification. After changing the pH of the buffer to induce the mini-intein self-cleavage process, we could see release of the reporter protein, AmyQ, into the supernatant with its activity (***Fig 3.20A***). In addition, a decrease of the α-amylase activity related to the incubation time was also observed with the spores incubated in the cleavage buffer, but not with those in washing buffer (***Fig 3.20B***). These results, together with a detectable signal from immunoblot analysis (***Fig 3.21***) demonstrate the possibility of using *B. subtilis* spores as an effective and low cost tool for expression and purification of proteins that likely to form aggregates.

4.3.2 Stability of the fusion protein with pH-dependent *SSp* DnaB mini-intein on the spore surface

In my initial construct, the native *cotB* promoter and the CotB protein was used for displaying fusion of heterologous proteins with the mini-intein. The fusion of the target gene with the *cotB* and *miniInt* genes was then integrated at the *amyE* locus of *B. subtilis*. This construct was shown to be ineffective because of the reduction of proteins expressed on the spore surface (shown in ***Fig 3.15***). The decrease in the expression level of the fusion of AmQ with the mini-intein compared to that of the fusion without it suggested an influence of the mini-intein on the stability of the whole fusion protein. In an attempt to improve expression of the whole fusion CotB-miniInt-AmyQ, other constructs using either a plasmid-based construction and/or IPTG-inducible promoters were made. Unfortunately, although the P_{grac} promoter was shown to be suitable in controlling the expression of protein followed by displaying on the spore surface, the construct with the P_{grac} promoter in a plasmid-based vector did not work; no α-amylase activity was observed using this construct. Expression of the fusion protein using the P_{Sgrac} promoter

(QAS41 strain) was shown to negatively correlate with the IPTG concentration (see ***Fig 3.17***). Surprisingly, the highest expression was achieved with the construct in which the copy number of the fusion gene, under control of the *cotB* native promoter, was increased by using a pBS72-derived plasmid vector (pQAS54). This vector has a low copy number (about 6 copies per chromosome) and is stably inherited in *B. subtilis* (Titok *et al.*, 2003). The expression level of the fusion protein with mini-intein using this construct was comparable with the one from the control without the mini-intein integrated into the chromosome (***Fig 3.18***). Therefore, it can be concluded that the mini-intein actually reduced the expression level of the fusion protein about six-fold and the *cotB* promoter is sufficient for display of the fusion protein with mini-intein tag on the spore surface.

Another aspect of working with the *SSp* DnaB mini-intein is the premature intein cleavage *in vivo.* The optimal pH for this mini-intein activation is neutral (pH 6.0 – 7.5) (Mathys *et al.*, 1999), and this is also the optimal range pH for the growth of *B. subtilis.* The pH of the sporulation medium was reported between 6 and 7 at stage 0 and changing afterwards, even could increase to 8.5 during sporulation (Yazdany and Lashkari, 1975). The pH of sporulation media at the time of spore collection was also measured (data not shown), and shown to be in agreement with a previous report. Therefore, there is a possibility that premature cleavage of the mini-intein happened at the time when the fusion proteins were produced. However, if the fusion proteins with the mini-intein were produced in sufficient amount to withdraw the effect of pH at the growth phase, they could be further processed for displaying on the spore surface. These events might suggest what has happened in the *B. subtilis* strain with the pQAS54 plasmid. In the case of the P_{Sgrac} promoter construct (pQAS41), due to the leakiness of this promoter, the fusion was synthesized during sporulation at optimal pH for mini-intein activation, so that the self-cleavage occurred all at the same time. Until the pH of the sporulation medium became more basic, the remaining undamaged fusion protein would be displayed on the spore surface. The inducer was added at the time when the pH of the medium was optimal for mini-intein activation, therefore the fusion proteins were overproduced but not remained stable enough to be displayed on the spore surface. Instead, the negative correlation with the IPTG dosage suggests that the disorder happened due to coat protein interactions. Moreover, the Pi addition would also help reducing the mini-intein activity (***Fig 3.21,*** lane 4). As it was shown in the previous part, Pi could be taken up and has an effect inside the intact cells. Supplement of the Pi solution into cultures would

suppress the premature intein cleavage. However, it is possible that activation of the CotB-miniInt-AmyQ is partly inhibited by the remaining Pi after washing. Therefore, cautious washing is needed for the suitable activity of the mini-intein.

The target protein release in the absence of mini-intein was observed in the case of the control sample – QAS06 (***Fig 3.21***, lane 1). This indicated a protease activity from an unknown factor on the spore coat. The possibility of protease contamination was excluded due to the fact that all buffers were autoclaved after preparation and no protease was supplemented during the experimental process. There are some coat proteins, e.g., YabG, known to have protease activity (Takamatsu *et al.*, 2000) and also more coat proteins with unknown functions. Therefore, there is a possibility for the coat protease to cut the target protein out of the spore surface. This might lead to the question whether the mini-intein or any other cleavage factor is essential here to release the target protein to the supernatant. It remains unclear how the process of coat protein discharging has happened, and where the active site for those proteases is. Hence, the answer is 'yes, it is needed' because the cleavage reaction of intein or any other factors was indeed performed as anticipated.

To overcome the protein instability due to the pH sensitivity, a pH control strategy could be applied. As the cells are growing, the pH should be kept optimal (pH 7) until stage I – II of sporulation. The basic pH can be adjusted afterwards for suppressing the mini-intein activity and stabilization of the fusion protein. The whole process can be easily performed using a bioreactor with pH control. Another possibility to overcome this problem is to use a tag containing an amino acid sequence recognized by an endoprotease inserted between the recombinant protein and the Cot protein. Using this strategy, a protease is needed to release the target protein into supernatant. Endoproteases that cleave proteins at specific sequences actually are the most controlled because cleavage does not occur until protease addition. However, highly specific endoproteases are typically expensive and unavailable in quantities required for large-scale manufacturing. In addition, the use of a protease requires extra purification steps, such as chromatography, dialysis or absorption on an affinity column, to get the completely purified target protein (Arnau *et al.*, 2006; Smith and Johnson, 1988). Hence, this is the most costly strategy. As the purpose of using spores as the matrix to express and purify recombinant proteins is to reduce the final product's expense, the bioreactor control strategy is more promising. A thiol-inducible intein could also be

utilized. Yet, thiol addition creates a dilemma. Its addition would lead to more steps afterward to purify the protein and might disrupt the disulfide bond(s) if existed in the target protein.

4.4 Construction of cellulase chips using *B. subtilis* cells and spores

Cellulases have many applications in several industrial fields such as food and textile industries to convert cellulosic material into glucose and other fermentable sugars (as reviewed in Introduction section **1.4.2** and (Bhat, 2000)). However, cellulosic bioconversion is a multi-step process requiring a multi-enzyme complex for efficient bioconversion into fermentable sugars with no known single organism capable of producing all the necessary enzymes in sufficient quantities. Biotechnological cellulase chips, in which these enzymes were immobilized of on the surface of microorganism cells or spores, once created, can be very useful.

C. thermocellum was known for its ability to form a cellulosome, an extracellular enzyme complex consisting of scaffolding proteins and cellulosomal enzymes that are capable of degrading plant cell walls. The endoglucanase A gene (*celA*) is one of the main cellulases of the *C. thermocellum* cellulosome (Beguin *et al.*, 1985). It was previously chosen in other studies with cellulases due to its highly potency and thermostable property. As the systems for displaying heterologous proteins on the surface of *B. subtlis* cells (Nguyen and Schumann, 2006) and spores (this thesis) have already been constructed, my goal was to apply the systems for creating cellulase chips based on this microorganism and to use them as whole-cell biocatalysts.

However, the cell-based cellulase chip could not be successfully constructed. The ineffective covalent anchoring of CelA on the *B. subtilis* cell wall was indicated by several experiments. First, the amount of CelA detected on the cell wall with the strain supposed to display this enzyme on the surface by immunoblotting was even less than one from the strain supposed to secrete this enzyme (***Fig 3.24***). Second, although the cellulase activity measured with the cells from a strain supposed to exhibit CelA on the surface was clearly higher than with the cells from the strain supposed to secrete this enzyme, both examined strains represented low activities as compared to those from the supernatant. The detected CelA of the immunofluorescence experiment properly showed the amount of trapped protein on the cell wall while being transported to the supernatant. This result may indicate insufficient activity of SrtA because the system is SrtA-dependent. There was the possibility of not enough SrtA for catalyzing the covalently linkage between CelA and the peptide cross-bridge of the cell wall

because the production of SrtA from the examined strain was lower than from the strain of the original paper (***Fig 3.22***). In addition, the spacer region, the amount of amino acid residues located between the C-terminal end of the target protein and the sorting motif is known to have a significant influence on the amount of protein molecules anchored per cell and the enzymatic activity (Nguyen and Schumann, 2006). A spacer length of 94 aa residues utilized in this thesis might be insufficient for anchoring of the CelA on the cell wall. Different lengths of the spacer region should be examined to find out the most suitable one to anchor CelA on the cell wall.

It was reported that there are exceptions in which the target protein, although being fused to an anchoring motif, cannot be anchored by SrtA due to the influence from the N-terminus of a target protein itself (Barnett *et al.*, 2004; Schneewind *et al.*, 1993). Maybe in this case, the CelA protein fused with the signal peptide from AmyQ (Nguyen and Schumann, 2006) had formed a conformation that could not be recognized and processed by SrtA. Recently, two substrate proteins of the *B. subtilis* putative sortase YhcS have been identified. The YhcS protein is demonstrated to belong to the group SrtD sortases (Nguyen *et al.*, 2010, submitted). A more effective *B. subtilis* cell surface system could be developed based on these new discoveries. Hence, a stronger cell-based cellulase chip could be created.

The spore-based cellulase chips were created using three different coat proteins, CotB, CotC and CotG, as carriers. When using the CotC protein, the cellulase activity measured with the spores was very low compared to spores using other Cot proteins. And the existence of CelA on the spore surface could not be verified by immunofluorescence microscopy. The presence of CelA on the spore surface using CotG or CotB as carrier was confirmed by both immunofluorescence analysis and cellulase activity assay. Therefore, it was obvious that CotB and CotG were more suitable than CotC for display of CelA on the spore surface. A flexible linker G-G-G-G-S was previously inserted between the CotG protein and streptavidin to improve the activity of the displayed enzyme on the spore surface (Kim *et al.*, 2005a). This linker should also be introduced between CelA and the carrier Cot protein to increase the activity of the cellulase on the spore surface.

In general, a lot of spores (e.g. OD_{600} of 8.3) and cells (e.g. OD_{578} of 15.4) must be prepared to measure one unit of cellulase activity. This means a large volume of culture has to be prepared to assure sufficient CMC degradation. The cellulase assay used in this thesis is based on the remaining labeled substrate (Fulop and Ponyi, 1997), but not on the formation of the reaction

product, which is a reducing sugar (or glucose). This method might yield lower measurable cellulase activity. However, the L. Fülöp and T. Ponyi's assay is more precise, so using this was the right decision. In a previous report where CelA was expressed and secreted using different *S. cerevisiae* strains, the cellulase activity was measured as unit per liter of culture (U/l) with the cellulase assay based on the release of reducing sugars mentioned above, and the highest activity was 280 U/l (Chung *et al.*, 1997). The result from my study together with this study might suggest the low activity of CelA. Therefore, in order to validate whether the cellulase chip works or not, it is advisable to have another enzyme for further testing.

Moreover, the ability for a contact between substrate and cellulase is limited when the enzyme has been immobilized. To overcome this problem, a cellulase binding domain could be co-displayed with the cellulase to enhance the reaction with the CMC substrate.

In conclusion, the cellulase chips based on *B. subtlis* cells and spores were generated but exhibited inappropriate activity on the CMC substrate. Further studies on the selection of a stronger enzyme and improvement of the system should be carried out to generate a more effective cellulase chip.

4.5 Outlook

B. subtilis spores displaying different heterologous proteins have been generated and used for oral antigen delivery for antibodies production (Isticato *et al.*, 2001; Ciabattini *et al.*, 2004; Hinc *et al.*, 2010) and whole-cell biocatalysts by immobilizing enzymes (Kwon *et al.*, 2007). My result with enhancing the amount of heterologous protein exhibited on the spore surface using the IPTG-inducible P_{grac} promoter provides a more effective tool for application of *B. subtilis* spores in biotechnology. A series of plasmids that allow the improvement of protein production for display on the spore surface was constructed in this study. Appropriate host strains with removal of endogenous CotC or CotG could also be generated to improve the amount of the fusion proteins with these Cot proteins on the spore surface. In addition, the observation with the localization of different Cot proteins might open another field for applying *B. subtilis* spores as bionano-chips and also in basic research on understanding the interaction among spore coat proteins.

To achieve a usable *B. subtilis* spore-based expression and purification system, a pH control strategy should be applied. The adjustment of the pH-value of the cultures during the

growth and sporulation can be easily performed by using a bioreactor with pH control. Further investigations are needed to make the proper use of this system. Moreover, another self-cleaving tag, such as thiol- or temperature-inducible inteins, could be utilized to avoid the premature cleavage that occurred with the pH-inducible intein. In this thesis, only the CotB protein was used for development of *B. subtilis* spore-based expression and purification system. Due to the fact that each Cot protein behaves differently when fused to a recombinant protein (Hinc *et al.*, 2010), it should be possible to design more spore-based systems for protein expression and purification.

For the construction of cellulase chips, since the CelA exhibited a low activity on the CMC substrate when immobilized on the spore and cell surface, another stronger cellulase should be utilized for generation of a more effective chip. In addition, the limitation of contact between substrate and immobilized cellulase can be overcome by co-display of a cellulase binding domain. With the development of a more efficient surface display system, stronger cellulase chip could be produced.

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List of abbreviations and symbols

Abbreviation	Denotation
2xSG	Modified Schaeffer' sporulation medium
Amp^R	Resistant to ampicillin
amyQ	Gene coding for α-amylase (AmyQ)
B. amyloliquefaciens	*Bacillus amyloliquefaciens*
bp	Base pairs
B. subtilis	*Bacillus subtilis*
cat	Gene coding for chloraphenicol-acetytransferase
celA	Gene coding for cellulase A (CelA) from C. thermocellum
Cm^R	Resistant to chloramphenicol
CMC	Carboxy methyl cellulose
cot	Gene coding for the used spore coat protein
cotB	Gene coding for CotB, an outer spore coat protein
cotC	Gene coding for CotC, an outer spore coat protein
cotG	Gene coding for CotG, an outer spore coat protein
C. thermocellum	*Clostridium thermocellum*
°C	Degrees centigrade
ddH_2O	Double distilled water
DNA	Deoxyribonucleic acid
Δ	Deletion
DSM	Difco Sporulation *Medium*
E. coli	*Escherichia coli* (used as host strain for cloning)

Erm	Erythromycin
Erm^R	Resistant to erythromycin
et al.	et alteri
gfp_{uv}	Gene coding for GFPuv , an enhanced GFP protein from the jellyfish *Aequorea victoria*
g	Gram
kb	Kilobase
kDa	Kilo-Dalton
LB	Luria-Bertani (growth medium)
h	Hour(s)
HCl	Hydrocloride acid
IAA	Isoamylalkohol
IPTG	Isopropyl-ß-D-thiogalactoside
l	Liter
L. monocytogenes	*Listeria monocytogenes*
min	Minute(s)
mg	Milligram
ml	Mililiter
mM	Milimole
µg	Microgram
µl	Microliter
Neo	Neomycin
nm	Nanometer
$OD_{578\ (600)}$	Optical Density at a wavelength of 578 (or 600) nm

P_{cot}	Native promoter of any *cot* genes
P_{cotB}	Native promoter of *cotB* gene
P_{cotC}	Native promoter of *cotC* gene
P_{cotG}	Native promoter of *cotG* gene
P_{dnaK}	Native promoter of *dnaK* gene
P_{grac}	An IPTG inducible promoter,which consists promoter of P_{groES} and *lac* operator
pmol	Picomole
P_{Sgrac}	An IPTG inducible, promoter-up mutation
P_{xylA}	Promoter of *xylA* gene, an xylose-inducible promoter
rpm	Revolution or round per minute
sec	Second
SDS	Sodium dodecyl sulphate
Spec	Spectinomycin
$Spec^R$	Resistant to spectinomycin
srtA	Gene coding for SrtA of *L. monocytogenes*
TEMED	N,N,N`,N`-tetramethylenethylendiamide
Tris	Tri-(hydroxymethyl)-aminomethane
Tween-20	Polyoxyethylensorbitane monlaurate
v/v	Volume/volume
w/v	Weight/volume

Erklärung

Hiermit erkläre ich, die vorliegende Arbeit selbstständig verfasst zu haben und keine anderen als die von mir angegebenen Quellen oder Hilfsmittel verwendet zu haben.

Ferner habe ich weder an der Universität Bayreuth, noch an einer anderen Hochschule versucht eine Dissertation einzureichen, oder mich einer Promotionsprüfung zu unterziehen.

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Bayreuth, Dezember 2010