Construction of plasmid-based expression and secretion vectors and study of the immobilization of proteins on the surface of *Bacillus subtilis* cells

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Zusammenfassung

Plasmide sind wichtige Werkzeuge, um die Expression von Genen und ihre Funktion zu untersuchen. Die meisten der bei *Bacillus subtilis* eingesetzten Plasmide zeigen allerdings strukturelle Instabilität als Folge ihres Rolling-circle Replikationsmechanismus. Im Rahmen dieser Arbeit wurden stabile Plasmide konstruiert, die eine Expression rekombinanter Proteine im Cytoplasma und ihre Sekretion in den Kulturüberstand erlauben. Weiterhin wurde ein experimentelles System etabliert, welches die Immobilisierung von Proteinen in der Zellwand ermöglicht. Außerdem wurde die Verankerung von *B. subtilis*-Proteinen in der Zellwand untersucht.

Eine erste Generation von Plasmiden, die pHCMC-Serie von Expressions-Vektoren, wurde konstruiert, die eine stabile intrazelluläre Expression rekombinanter Proteine in *B. subtilis*-Zellen erlauben. Diese Expressions-Vektoren basieren auf dem kürzlich beschriebenen *Escherichia coli - B. subtilis* Shuttle-Vektor pMTLBs72, der den Theta-Replikations-Mechanismus nutzt. Drei verschiedene kontrollierbare Promotoren wurden in diesen Vektor eingebaut: P_{gsiB}, der durch Hitze, Säureschock und Ethanol induziert werden kann, und P_{xylA} und P_{spac}, die durch Zugabe von Xylose bzw. IPTG aktiviert werden können. Die Anwendbarkeit dieser Expressions-Vektoren wurde durch die Fusion ihrer Promotoren an ein Reportergen und die Überproduction von HtpG, einem Hitzeschockprotein, nachgewiesen. Alle rekombinanten Vektoren zeigen volle strukturelle Stabilität.

Eine zweite Generation von Plasmiden, zwei weitere Expressions-Vektoren, wurden konstruiert. Während eines dieser beiden Plasmide die intrazelluläre Produktion von rekombinanten Proteinen erlaubt, veranlasst das zweite ihre Sekretion ins Kulturmedium. Beide Vektoren nutzen den starken Promotor, der für die Expression des *groESL*-Operons (codiert für die essentiellen Hitzeschock-Proteine GroES und GroEL) von *B. subtilis* verantwortlich ist, fusioniert an den IPTG-induzierbaren *lac*-Operator (P_{grac}). Während der Background-Level in Abwesenheit des Induktors sehr gering war, wurde nach Zugabe von IPTG ein Induktionsfaktor von etwa 1300 gemessen. Nach Fusion der Gene *htpG* und *pbpE* (codiert für ein Penicillin-Bindeprotein) an den *groE*-Promotor und ihrer Induktion mit IPTG betrug die Menge an rekombinantem Protein 10 bzw. 13% des Gesamtproteins. Um eine Sekretion der rekombinanten Proteine zu erreichen, wurde die codierende Region für das Signalpeptid des *amyQ*-Gens von *Bacillus amyloliquefaciens*, welches für eine α-Amylase

codiert, an den *groE*-Promotor fusioniert. Mit diesem Vektor wurde die Sekretion großer Mengen an α-Amylase und Cellulase A und B von *Clostridium thermocellum* nachgewiesen.

Gram-positive Bakterien codieren für ein oder mehrere Enzyme, die als Sortasen bezeichnet werden, und die die kovalente Verankerung von Substratproteinen in der Zellwand katalysieren. Sie erkennen eine Aminosäuresequenz, Sorting-Motiv genannt, welche nahe dem C-terminalen Ende der Substratproteine gelegen ist, spalten innerhalb des Motivs und katalysieren die Verankerung der Polypeptidkette an der Peptidbrücke, die die Glycanstränge verbindet, in einer Transpeptidierungsreaktion. Für *B. subtilis* wurden zwei potentielle Sortasen beschrieben, YhcS und YwpE genannt, aber die von ihnen erkannten Sorting-Motive sind unbekannt. Um Proteine auf der Oberfläche von *B. subtilis*-Zellen verankern zu können, wurde das *srtA*-Gen aus *Listeria monocytogenes*, welches ein bekanntes Sorting-Motiv erkennt, in *B. subtilis* kloniert. *L. monocytogenes* und *B. subtilis* enthalten identische Peptidbrücken.

Im nächsten Schritt wurde das α-Amylasegen an die C-terminale Region des Fibronectin-Bindeproteins B (FnBPB) aus *Staphylococcus aureus* fusioniert. Dieses Protein enthält eine Sorting-Sequenz mit dem Sorting-Motiv LPETG. Eine kovalente Bindung der α-Amylase an die Zellwand konnte durch Lysozym-Behandlung der Zellen und mittels Immunofluoreszenz-Mikroskopie nachgewiesen werden. Bis zu 240 000 α-Amylase Moleküle konnten pro Zelle immobilisiert werden, 24-mal mehr als bislang für andere Bakterien-Spezies publiziert. Um den Einfluss des Abstandes zwischen dem Sorting-Motiv und dem C-Terminus der α-Amylase auf die Enzymaktivität zu untersuchen, wurde die Länge des Spacers variiert. Die höchste Aktivität wurde mit einer Spacer-Länge von 123 Aminosäureresten gemessen.

Um herauszufinden, ob die beiden potentiellen Sortasen YhcS und YwpE von *B. subtilis* die beiden Substrate YfkN und YhcR in der Zellwand verankern können, wurden Knockout-Stämme konstruiert (Δ*yfkN*, Δ*yhcR* und eine Doppelknockout). In diese wurden Translations-Fusionen zwischen AmyQ und dem C-terminalen Ende von YfkN und YhcR mit einer Spacer-Region von 123 Aminosäureresten einkloniert. Eine anschließende Analyse der Stämme zeigte, dass YhcS AmyQ-YhcR123 an der Zellwand verankern kann und YwpE die Ausbeute erhöht. YhcS konnte das Sorting-Motiv LPDTS von YhcR erkennen, nicht aber LPETG von FnBPB. Daher könnte YhcR ein Substrat von YhcS sein. Ein Modell für die kovalente Verankerung von Proteinen in der Zellwand durch Sortasen wird präsentiert.

Summary

Plasmids are useful tools to study gene expression and their function. However, most of the available plasmids for *Bacillus subtilis* suffer from structural instability because of their rolling-circle replication mechanism. In this work, stable plasmids have been constructed allowing expression of recombinant proteins in the cytoplasm and their secretion into the culture supernatant. The author has also established an experimental system to immobilize proteins on the cell wall of *B. subtilis*. The sorting of surface proteins to the cell wall in *B. subtilis* has been investigated.

A first generation of plasmids, the series of plasmid-based expression vectors pHCMCs has been constructed allowing stable intracellular expression of recombinant proteins in *B. subtilis* cells. These expression vectors are based on the recently described *Escherichia coli - B. subtilis* shuttle vector pMTLBs72 that uses the theta mode of replication. Three different controlable promoters have been inserted into the shuttle vector: P_{gsiB} that can be induced by heat, acid shock, and by ethanol, and P_{xylA} and P_{spac} that respond to the addition of xylose and IPTG, respectively. The versatility of these expression vectors was demonstrated by fusing their promoters to a reporter gene and by overexpression the gene of the HtpG (a heat shock protein) protein with three of them. All recombinant vectors exhibited full structural stability.

A second generation of plasmids, two plasmid-based expression vectors have been constructed, where one plasmid allows intracellular production of recombinant proteins while the second directs the proteins into the culture medium. Both vectors use the strong promoter preceding the *groESL* operon (codes for the essential heat shock proteins GroES and GroEL) of *B. subtilis* fused to the *lac* operator allowing their induction by addition of IPTG. While the background level of expression of these expression cassettes was very low in the absence of the inducer, an induction factor of about 1300 was measured. When the genes *htpG* and *pbpE* (coding for a penicillin-binding protein) were fused to the *groE* promoter, the amount of recombinant protein produced after addition of IPTG represented 10 and 13%, respectively, of the total cellular protein. To obtain secretion of recombinant proteins, the coding region for the signal peptide of the *amyQ* gene encoding the α-amylase from *Bacillus amyloliquefaciens* was fused to the *groE* promoter. High-level secretion of *amyQ* α-amylase and cellulase A and B of *Clostridium thermocellum* was demonstrated.

Gram-positive bacteria code for one or more enzymes termed sortases that catalyze the covalent anchoring of substrate proteins on their cell wall. They recognize an amino acid sequence designated sorting motif, present close to the C-terminal end of the substrate proteins, cleave within this motif, and catalyze anchoring of the polypeptide chain to the peptide crossbridge linking the peptidoglycan strands in a transpeptidation reaction. *B. subtilis* has been reported to code for two putative sortases, YhcS and YwpE, but the sorting sequences recognized by them are yet unknown. To be able to immobilize proteins on the surface of *B. subtilis* cells, the *srtA* gene coding for sortase A of *Listeria monocytogenes* that recognizes a known sorting motif was introduced into *B. subtilis*. *L. monocytogenes* and *B. subtilis* share the same peptide crossbridge. Next, the coding region of the α-amylase gene was fused to the C-terminal region of *Staphylococcus aureus* fibronectin binding protein B (FnBPB) containing the sorting sequence including its sorting motif (LPETG). Covalent linkage could be proven by treatment of the cells with lysozyme and by immunofluorescence microscopy. Up to 240,000 molecules of α-amylase could be immobilized per cell, 24 times more than previously reported for other bacterial species. To study the influence of the distance between the sorting motif and the C-terminus of the α-amylase (AmyQ) on the activity of the enzyme, the length of the spacer was varied. It turned out that the highest activity was measured with a spacer length of 123 aa residues.

To elucidate whether the putative sortases YhcS and YwpE of *B. subtilis* can retain the two potential substrates of the sortase YfkN and YhcR, the *yhcS* and/or *ywpE* knockout strains were constructed and the translational fusions between AmyQ and N-terminal of YfkN or YhcR, both harbouring the 123-aa spacers, were generated resulting in AmyQ-YfkN123 and AmyQ-YhcR123, respectively. The results demonstrated that YhcS could retain the fusion AmyQ-YhcR123 on the cell wall and YwpE seems to assist YhcS to perform its functions. YhcS could recognize the sequence containing the sorting motif LPDTS from YhcR but not LPETG from FnBPB. YhcR could be a substrate of YhcS, while it is not clear whether YfkN is a cell wall protein. A model for the covalent anchoring of proteins on the cell wall by sortases is presented.

1 Introduction

Gram-positive bacteria are well known for their contributions to agricultural, medical, and food biotechnology and for the production of recombinant proteins. Among them, *Bacillus subtilis* has been developed as an attractive host because of several reasons: (i) it is non-pathogenic and is considered as a GRAS organism (generally regarded as safe); (ii) it has no significant bias in its codon usage; (iii) it is capable of secreting functional extracellular proteins directly into the culture medium (at present, about 60% of the commercially available enzymes are produced by *Bacillus* species); and (iv) a large body of information concerning transcription, translation, protein folding and secretion mechanisms, genetic manipulation, and large-scale fermentation has been acquired [54, 97, 167, 171]. Therefore, a lot of studies to increase the use of *B. subtilis*, many expression vectors and novel aspect of applications have been investigated and described. The present work is in this effort.

1.1 Expression systems and promoters used in *B. subtilis*

Two different systems are basically used for gene cloning and gene expression in *B. subtilis*. The first makes use of plasmids that replicate autonomously in *B. subtilis*, and the other allows integration of the expression cassettes into the host chromosome. In the following sections, these systems and promoters used are briefly summarized.

1.1.1 Plasmid-based systems

Plasmids are important tools for studying bacterial functions and protein expression. Attempts to use plasmids for the expression of recombinant proteins in the cytoplasm and further secretion into the culture medium in *B. subtilis* have not often been successful because of structural instability of the recombinant plasmids [13, 39]. Most available plasmids are derivatives of natural plasmids isolated from *Staphylococcus aureus* such as pC194 [38], pUB110 [77] and pE194 [62]. While these plasmids replicate stably in *B. subtilis*, addition of recombinant DNA can confer mainly structural and sometimes segregational instability, too. The molecular basis for the structural instability is related to their replication mode. These plasmids replicate as rolling circles producing single-stranded DNA as an intermediate, and short direct repeats within this single-stranded DNA may lead to the deletion of one of the two repeats and the intervening DNA [11].

This obstacle could be completely overcome by using plasmids using the θ-mode of replication. Cloning vectors without structural instability have already been described based on the natural plasmids pAMβ1 and pTB19 [65] and derivatives of pMTLBs72 [79, 153]. Plasmid pMTLBs72 is a derivative of a large plasmid from *B. subtilis* soil isolates. It is a low copy number plasmid (6 copies per chromosome) and stably inherited in *B. subtilis*. Using this plasmid, quite recently, we published the construction of different expression vectors based on the *E. coli - B. subtilis* shuttle vector pMTLBs72 exhibiting full structural stability [104, 114].

1.1.2 Chromosomal integration systems

The structural instability of plasmids led to the development of vectors that integrate into chromosome. Two systems are frequently used: the first concerning chromosomal integrations involving single-crossover events, and the second involving double-crossover events [97]. As illustrated in Fig. 1.1A, a single-crossover event will result in an integrated state in which the homologous part is duplicated. In addition, in between the duplicated fragment, the entire plasmid, including cloned DNA fragments, are incorporated. In many cases, further amplification of the integrated plasmid in the chromosome can occur, in particular in presence of antibiotic pressure, and copy numbers up to about 50 can be obtained [96]. A well-known vector of this kind is pMUTIN4 [161] that was used in this work as shown in Fig. 2.7.

The second type as illustrated in Fig. 1.1B exploits the integration by a double-crossover event that appears as a single copy in the cell. This process results in the replacement of the chromosomal DNA fragment between the sites of crossing-over by the "corresponding fragment" on the plasmid. Basically, any gene in the chromosome can be changed at any nucleotide position, either by a mutant base-pair or by any other DNA sequence, or gene(s). In a variant of this application, especially for gene expression, the integration vector contains the 5'- and 3'-ends of the *lacA* gene (coding for a β-galactosidase) at the Y and Z (Fig. 1.1B) positions of the integration vector. This enables the insertion of any gene of interest at the *lacA* locus [53, 141], which was used to construct the *B. subtilis* strain NDH03 in this work. Similar vectors have also been reported using different loci such as the *amyE* locus [71, 145], *thrC* [49], *pyrD*, *gltA*, and *sacA* [98].

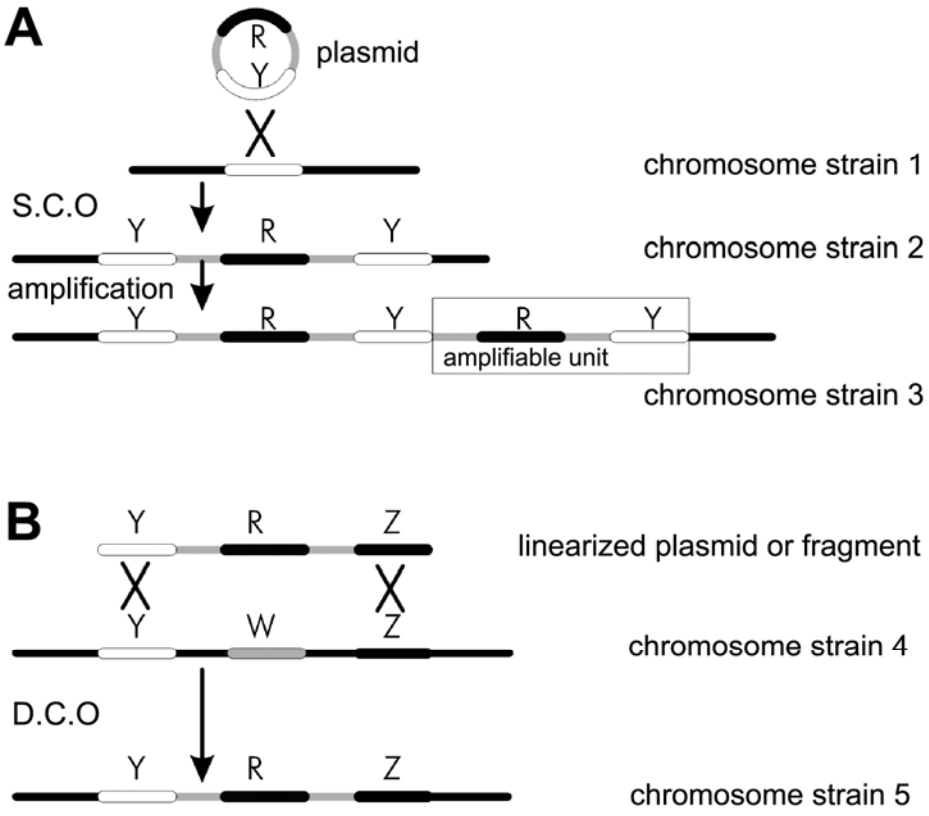


Fig. 1.1. A schematic representation of chromosomal integration systems [97]. A: single-crossover recombination (S.C.O.). A typical vector is an *E. coli* plasmid, such as a pUC derivative, which contains replication functions, an antibiotic resistance determinant ("R"), and a fragment of *B. subtilis* DNA ("Y"). Single-crossover recombination at the homologous site in the chromosome (strain 1) results in the integration of the entire plasmid and, consequently, duplication of the cloned homologous fragment (strain 2). This may be followed by further amplifications [96] (strain 3). B: Double-crossover recombination (D.C.O). The vector is usually an *E. coli* plasmid containing two regions of homology with the *B. subtilis* chromosome (indicated as Y and Z), which is interrupted by other DNA sequences (here indicated as "R"). The latter can be as small as one base-pair (mainly for the introduction of directed mutations in the chromosome). A double-crossover event in the flanking homologous regions (Y and Z) results in the replacement of the original part on the chromosome (W) by the new sequence(s), here called "R".

1.1.3 Promoters used for expression systems in *B. subtilis*

Promoters play a key role in the initiation of RNA synthesis leading to gene expression, which has been examined for a long time in *B. subtilis*. The temporal and compartmentalized

expression of genes is governed by a variety of sigma factors (σ), together with the RNA polymerase core enzyme constituting the RNA polymerase holoenzyme. The holoenzyme stimulates transcription of genes in a process that is initiated by sequence-specific interaction between the -35 and -10 promoter sequences and specific regions within the sigma factors. There are 17 sigma factors encoded in the *B. subtilis* chromosome. For instance, many housekeeping genes expressed during vegetative growth of Bacilli contain a typical σ^A-dependent promoter, which is characterized by a -35 TTGACA consensus sequence and -10 TATAAT hexanucleotide [97]. The σ^A-dependent promoter is illustrated in Fig. 2.2 for the P*grac* promoter, which contains a *lac* operator sequence.

Inducible gene expression in *B. subtilis* usually involves the promoters of six different groups. The first is a modified promoter region of the *E. coli lac* operon and is induced by IPTG, which includes the P*spac* promoter [53, 161, 177] and the T7 promoter [21]. The second makes use of the xylose-inducible promoter from *B. subtilis* [8, 44] or from *Bacillus megaterium* [71, 126]. The third is the promoter of *sacB*, the gene encoding extracellular levansucrase, which is induced by sucrose [21, 57]. The fourth exploits the promoter that is induced by phosphate-starvation [81]. The fifth uses the citrate-inducible promoter [43, 176]. The last consists of a group of promoters that direct low level expression in the lag and log phase, and much higher level in the stationary phase called "auto-inducible promoters". This group includes the promoter of *aprE* encoding for subtilisin E [64] and promoters belonging to σ^B-dependent promoters such as of *gsiB*, encoding for a general stress protein [88]. Auto-inducible promoters are not used in basic research but they are important for industrial purposes because they do not require inducers. Up to date, only IPTG-inducible and xylose-inducible promoters are often used, where the whole expression cassette is integrated into the chromosome.

1.2 The sorting mechanism in Gram-positive bacteria

Surface proteins of eubacteria play an important role in pathogenicity. While in Gram-negative bacteria, these proteins are predominantly anchored in the outer membrane [82], Gram-positive bacteria utilize their cell wall for anchoring and display of surface proteins [103]. One important mechanism of protein anchoring utilizes sortase, a transpeptidase that cleaves substrate proteins at a specific sorting motif (for recent reviews [85, 113, 157]).

1.2.1 The cell wall sorting pathway of surface proteins in Gram-positive bacteria

A cell wall sorting pathway of surface proteins in Gram-positive bacteria was initially proposed by Olaf Schneewind from his study of the surface protein A of *S. aureus* [140]. A genetic screen for *S. aureus* mutants that failed to anchor protein A to the bacterial cell wall resulted in the identification of the sortase gene named *srtA* (surface protein sorting A) that codes for sortase A (SrtA) [91]. Since then, anchoring of protein A by sortase A on the cell wall is the paradigm of covalently anchoring of proteins in Gram-positive bacteria (Fig. 1.2).

Table 1.1. C-terminal sorting signals

Organism	Protein	Sequence of C-terminal sorting signal[a]
L. monocytogenes	Internalin A	S***LPTTG***DSDNALYLLLGLLAVGTAMALTKKARASK
S. aureus	Protein A	A***LPETG***EENPFIGTTVFGGLSLALGAALLAGRRREL
S. aureus	FnBPB	E***LPETG***GEESTNKGMLFGGLFSILGLVLLRRNKKNHKA
B. subtilis	YfkN	L***LPDTA***TSMYSILLAGFLISALGTAMYLHQRRKQNRANQA
B. subtilis	YhcR	Q***LPDTS***AGYYNFMVIGAAVTLSGTYLYVRRKRSASRT

[a]The sorting motifs representing possible sortase cleavage sites are indicated in bold italics. The positively charged residues that constitute the charged C-terminal tail are underlined. Hydrophobic segments are indicated in grey shading and were predicted by the Membrane Protein Explorer (MPEx) V.2.2 from the Laboratory of Dr. Stephen White. This table was extracted partly from sortase substrate database [10].

The surface protein A of *S. aureus* carries two important elements for the anchoring: (i) the N-terminal signal peptide that directs protein A across the cytoplasmic membrane by the Sec pathway and (ii) the C-terminal cell wall sorting signal that encompasses a 35-residue peptide with an LPXTG sorting motif, followed by a hydrophobic domain and a positively charged tail (Table 1.1). This surface protein is synthesized as precursor in the bacterial cytoplasm bearing an N-terminal signal peptide and a C-terminal cell wall sorting signal. After translocation across the cytoplasmic membrane, the N-terminal signal peptide is removed by the signal peptidase, thereby generating the P2 precursor. The C-terminal sorting signal retains the P2 precursor species within the secretory pathway and permits substrate recognition at the LPXTG motif. The sortase, a membrane-anchored transpeptidase with an

active-site cysteine, cleaves surface proteins between the threonine (T) and the glycine (G) of the LPXTG motif generating an acyl-enzyme intermediate. The acyl-enzyme intermediate, with a thioester bond between the thiol of sortase and the carboxyl group of the threonine at the C-terminal end of surface proteins, is resolved by the nucleophilic attack of the amino group of the m-diaminopimelic acid (m-Dpm) cross-bridge of *L. monocytogenes* [28] or of the pentaglycine cross-brige of *S. aureus* [92, 158] within the lipid II precursor. Surface proteins linked to lipid II may be incorporated into the cell wall envelope by the transglycosylation and transpeptidation reactions that generate the mature cell wall (Fig. 1.2). This pathway is universal in many Gram-positive bacteria, and the functional elements, the cell wall cross bridges, sorting motif, sortase, and penicillin binding proteins are conserved.

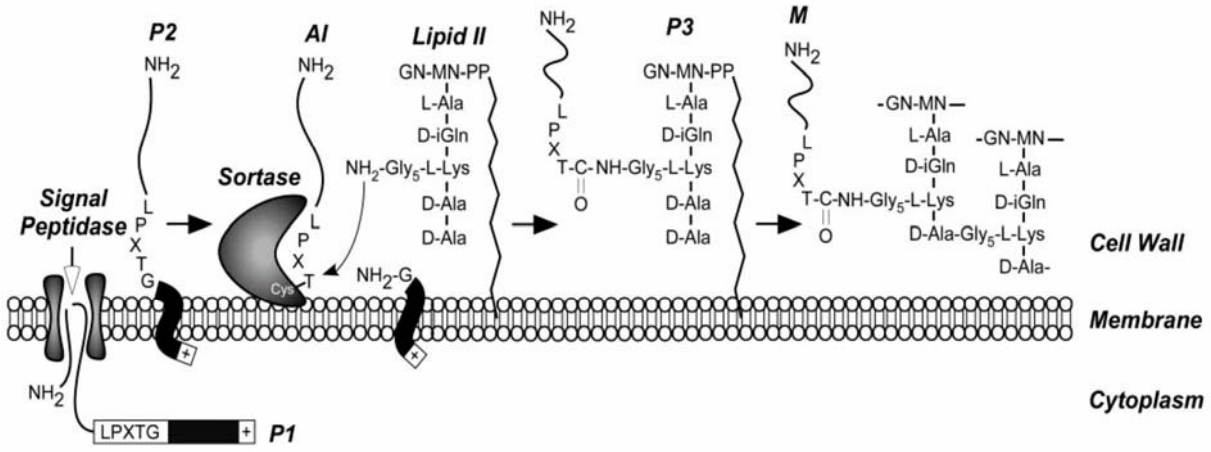


Fig. 1.2. The cell wall sorting pathway of surface proteins in *S. aureus* [85]. Surface proteins are first synthesized in the bacterial cytoplasm as full-length precursor (P1) containing an N-terminal signal sequence and a C-terminal sorting signal. The signal peptide directs the translocation of the polypeptide through the Sec system followed by removal of the signal peptide. The product of this reaction, the P2 precursor harbouring the C-terminal sorting signal, is retained in the cytoplasmic membrane via its C-terminal hydrophobic domain (black box) and positively charged tail (+). Sortase cleaves the peptide bond between the threonine (T) and the glycine (G) of the LPXTG motif, generating an acyl intermediate (AI). Lipid II, the peptidoglycan biosynthesis precursor, and its pentaglycine cross bridge (Gly5) amino group attacks the acyl intermediate, linking the C-terminal threonine of the surface protein to lipid II (P3 precursor) and regenerating the active site of sortase. The P3 precursor functions as a substrate for penicillin binding proteins and is incorporated into the cell wall envelope to generate mature anchored surface protein (M), which is also displayed on the bacterial surface.

Screening of sequenced genomes of Gram-positive bacteria revealed that typically more than one sortase homologue is present; and interestingly, in the majority of genomes where sortase enzyme genes have been identified, usually multiple sortases are encoded [20, 110]. Based on sequence alignments of sixty-one sortases from completely sequence Gram-positive genomes, four groups of sortases designated SrtA, SrtB, SrtC and SrtD were suggested [31]. Each subgroup is characterized by membrane topology, genome position, and preference for substrates with specific amino acids within the cell wall sorting signal pentapeptide motif (Table 1.2). The *S. aureus* genome, where sortases have been detected first, codes for two enzymes, sortase A and B [20, 91, 93, 110]. While sortase A recognizes the sorting sequence containing the LPXTG sorting motif and cleaves the peptide bond between the threonine and glycine residues [92, 158], sortase B recognizes the NPQTN motif, catalyzing cleavage of the peptide bond between T and N [180]. In *Streptococcus pyogenes*, one LPXTG containing protein is recognized by SrtC (Spy0135) which is characterized by the presence of an additional C-terminal hydrophobic domain [6].

Table 1.2. Sortase classifications

Sortase class[a]	Cleavage site[b]	Membrane anchor domain[c]	Bacterial taxa[d]	References
A	LPkT-Ge[e]	N terminus	*Bacillus*, *Listeria*, *Staphylococcus*, *Enterococcus*, *Lactobacillaceae*, *Streptococcaceae*	[20, 31, 85, 138, 156]
B	NPqt-nd[e]	N terminus	*Bacillus*, *Listeria*, *Staphylococcus*, *Streptococcaceae*, *Clostridia*	[20, 31, 85, 86]
C	lPkT-GG	N and C-terminus	*Actinobacteria*, *Bacillus*, *Enterococcus*, *Leuconostocaceae*, *Streptococcaceae*, *Clostridia*	[20, 31, 85]
D	LPnT-At	N terminus	*Bacillus*	[20, 31, 85]
	LAcT-Ga	N terminus	*Actinobacteria*	[20, 31, 85]

[a] Classification of sortases is based on sequence, membrane topology, genomic positioning, and preference for specific amino acids within the cell wall sorting signal pentapeptide motif region of their cognate substrates [20, 31, 85]. [b] Cell wall sorting signal is normally an pentapeptide motif, in which uppercase letters represent amino acids that are absolutely conserved. [c] Membrane anchor region is based on transmembrane predictions and regions of high hydrophobicity. [d] Bacterial taxa harbour one or more sortase genes belonging to the respective sortase classification. [d] The cleavage site symbolized by a hyphen is verified experimentally.

1.2.2 Putative sortases of *B. subtilis* and their potential substrates

So far, 732 putative sortase substrates encoded in 49 prokaryotic genomes were identified and strikingly species-specific variation for the LPXTG motif has been revealed *in silico* [10]. Despite being intensively studied as a model organism and possessing two putative sortase homologues, there is no direct evidence published that *B. subtilis* might decorate its surface with sortase-dependent proteins covalently linked to peptidoglycan [110]. However, it encodes two potential sortase substrates YfkN and YhcR, encoded by *yfkN* and *yhcR* [10, 20]. Instead of the LPXTG motif, YfkN contains the sequence LPDTA and YhcR the sequence LPDTS (Table 1.1).

Both YfkN and YhcR were previously detected in the extracellular proteome of *B. subtilis* [2]. It was proposed that these *B. subtilis* proteins were cleaved by unidentified signal peptidases and/or proteases that are active at the membrane - cell wall interface, resulting in their release into the growth medium. YfkN exhibits 2', 3' cyclic nucleotide phosphodiesterase and 2' (or 3') nucleotidase and 5' nucleotidase activities, a trifunctional nucleotide phosphoesterase [18]. YhcR is a sugar-nonspecific nuclease that cleaves endonucleolytically to yield nucleotide 3′-monophosphate products. The analysis identified six domains covering most of the sequence, plus an N-terminal signal peptide and a C-terminal gram-positive anchor. The N-terminal end of the sequence (residues 1 to 46) contains a signal peptide that is predicted to direct secretion by the twin-arginine translocation pathway [108]. However, two other lines of evidence support the identification of YhcR as the substrate of a sortase-like protein: *yhcR* is adjacent to *yhcS*, which encodes one of the two sortase-like proteins in *B. subtilis*, and YhcR appears to have 5′-nucleotidase activity, a property shared by LPXTG proteins from several other bacteria [110]. In addition, recent analysis has shown that YfkN and YhcR could accumulate in the culture medium when investigated in *B. subtilis* cells carrying null alleles in *yhcS* and *ywpE*. Therefore, YfkN and YhcR could, in principle, be sorted to the cell wall by the *B. subtilis* sortase homologues YwpE and/or YhcS [166].

The two putative sortase homologues are YwpE and YhcS (Fig. 1.3) [20, 110]. YwpE encodes a small protein of 102 aa that contains the active site of the sortase enzyme, the TLXTC motif, at the C-terminus but it has no signal peptide at the N-terminus, so it looks like a truncated SrtA. YhcS encodes a protein of 198 aa carrying a transmembrane domain at its N-terminus and has been classified in group SrtD. There is no clear experimental evidence that SrtD sortases recognize and anchor proteins on the surface of Gram-positive bacteria

[31]. However, a recent study has indicated that YhcS appears to be involved in the cellular retention of YhcR and YfkN, the two potential sortase substrates of *B. subtilis*, while YwpE has no apparent function in protein export and retention [166].

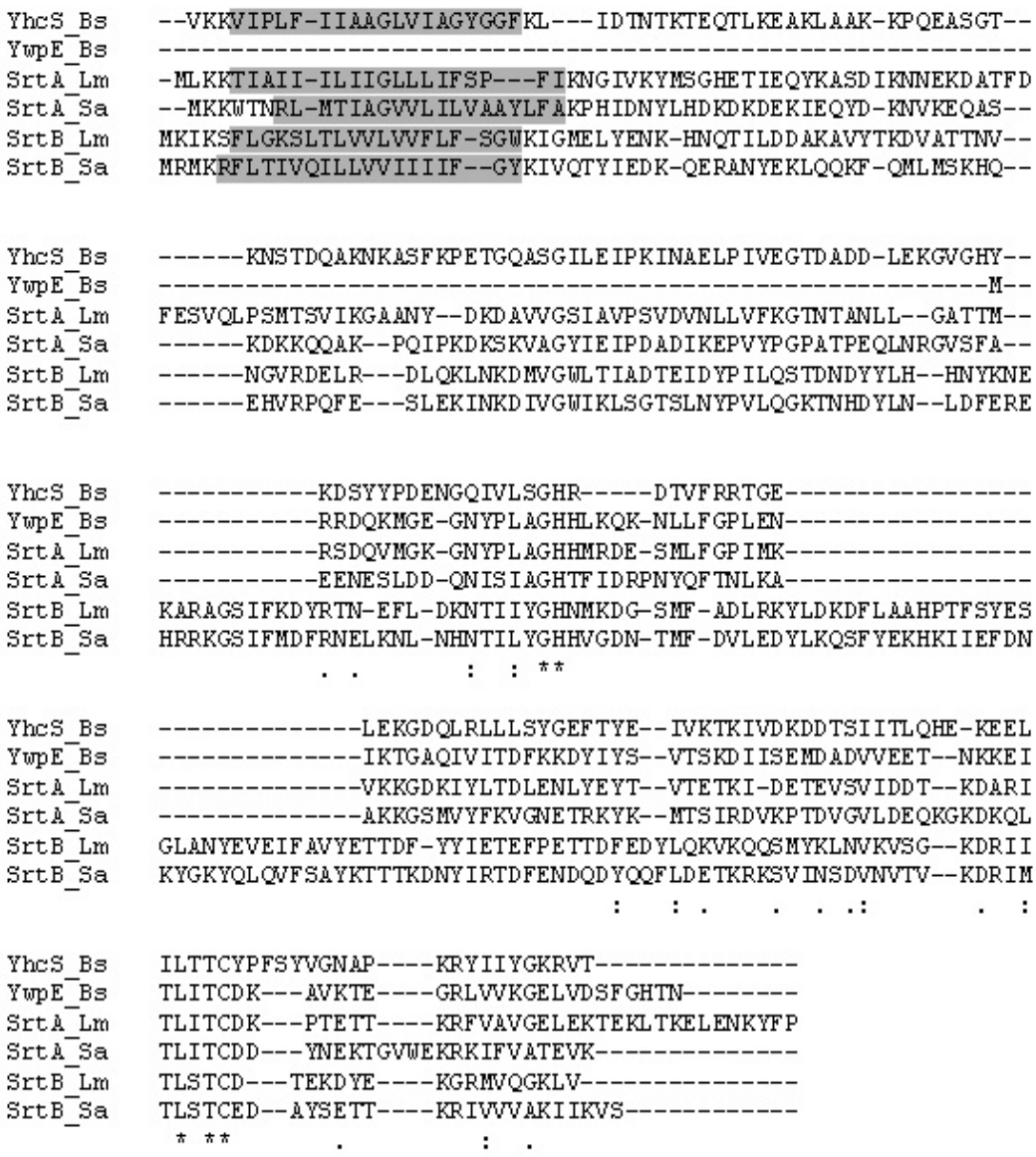


Fig. 1.3. Alignment of the *B. subtilis* sortase homologues with known sortases of different Gram-positive bacteria. The alignment includes the following proteins: YwpE and YhcS of *B. subtilis*, SrtA and SrtB of *L. monocytogenes* and *S. aureus*. Identical amino acids (*), conserved substitutions (:), or semi-conserved substitutions (.) are marked. Transmembrane segments are indicated in grey shading and were predicted by the Membrane Protein Explorer (MPEx) V.2.2 from the Laboratory of Dr. Stephen White, Department of Physiology and Biophysics, University of California, Irvine. The multiple sequence alignment was performed by using the T-COFFEE program [107].

The sortase D class includes 14 sortases originating from high and low GC% Gram-positive bacteria, and it can be divided into three subclusters reflecting host phylogeny (Bacilli, Clostridia and Actinomycetales). The genomes of high GC% Streptomycetes also display genes encoding sortases [110]. Two genes encoding SrtDs were found in the *Streptomyces coelicolor* genome. Interestingly, the genes encoding sortase D were adjacent on the chromosome. Four genes encoding SrtDs were found in the *Streptomyces avermitilis* genome. This group D contains the unique sortase encoded by *B. subtilis* as mentioned above and one in *Clostridium tetani*. It is noteworthy that, with the exception of *Corynebacterium diphtheriae*, all species encoding class D sortases display a morphological differentiation cycle and no SrtD has been functionally characterized to date [31].

1.3 Cell surface engineering

There are a lot of reports about cell surface display of heterologous proteins on bacterial cells, in which surface display of proteins is usually achieved through a translational fusion of the target protein to one of the naturally occurring surface proteins of the host cells. Display of proteins on the surface of microorganisms, enabled by means of recombinant DNA technology, has become an increasingly used strategy in various applications in microbiology, biotechnology and vaccination [133, 165], called cell surface engineering.

Sortases have also been used to anchor foreign proteins on the cell wall of different Gram-positive bacteria. The first heterologous protein to be immobilized was the alkaline phosphatase of *E. coli* [140]. When the coding region of this enzyme was sandwiched between that of the signal sequence and the sorting sequence of *S. aureus* Protein A, the hybrid protein was found equally distributed in the medium and on the cell wall. In the same year, it was reported that another hybrid protein consisting of the E7 protein of human papillomavirus type 16 and the M6 surface protein from *S. pyogenes* could be anchored on the surface of *S. gordinii* [117]. During the following years, these surface-display methods have been widely exploited for vaccine delivery, development of biocatalysts and whole cell adsorbents (reviewed in [164, 165]).

From a practical point of view, as described by Samuelson *et al.* [133], Gram-positive bacteria have certain properties that potentially make them more suitable for bacterial surface display applications. First, the surface proteins of Gram-positive bacteria seem to be more permissive for the insertion of extended sequences of foreign proteins (several hundreds of amino-acids),

as compared with the different Gram-negative surface proteins [42, 52]. A second, more obvious advantage with the Gram-positive systems is that translocation through only a single membrane is required to achieve proper surface exposure of the heterologous polypeptide, while in the Gram-negative systems both translocation through the cytoplasmic membrane and correct integration into the outer membrane are required for surface display. Finally, considering the practical handling of the bacteria, Gram-positive bacteria have the additional advantage of being more rigid, due to the thicker cell wall [109], which thus allows various laboratory procedures without extensive cell lysis.

1.4 Aims of the doctoral thesis

The major aim of this doctoral thesis is to develop novel plasmid-based expression vectors devoid of structural instabilities. First, vectors should be constructed allowing controlable overexpression of recombinant protein within the cytoplasm and into the culture medium, and experiments should be carried out to improve the transcription level. Second, an experimental system should be established allowing covalent anchoring of proteins on the cell wall of *B. subtilis* cells; experiments should be also done to identify target proteins for the two putative sortases of *B. subtilis*.

2 Materials and methods

2.1 Bacterial strains, plasmids, oligonucleotides, antibiotics and media

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. Summary of the bacterial strains used in this work

Name	Description	Reference
1012	*leuA8 metB5 trpC2 hsdRM1*	[131]
AS1	1012 *hrcA :: neo* (Neo^R)	[142]
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
IHA01-Spac-BgaB	1012 *lacA*::*erm* P*spac-bgaB*	[53]
IS58	*trpC2 lys-3*	[148]
LA01	1012 *xylAB* :: *tet* (Tet^R)	T.T.A. Le
NDH03	WW02 *lacA :: srtA* (Em^R)	*
NDH20	1012 P*yhcS-lacZ*, P*spac-yhcS* from pMUTIN4	*
NDH21	1012 P*ywpE-lacZ*, P*spac-ywpE* from pNUTIN4	*
NDH24	NIS6311 *cat::neo* (Neo^R), the *cat* cassette was replaced by *neo* using plasmid pB-*cat5-neo-cat3*	*
NDH30	1012 *yhcS* :: *neo* (Neo^R)	*
NDH31	1012 *yhcS* :: *neo*, *ywpE* :: *em*	*
NIS6311	*purA16, metB5, guaB, dnaN5, spoIIIJ*::pRK1 (a *dnaA*-null mutant, Cm^R)	[60]
SZ59	1012 *yhcS* :: *cat* (Cm^R)	S. Zellmeier
SZ60	1012 *ywpE* :: *em* (Em^R)	S. Zellmeier
WB800	*nprE aprE epr bpr mpr* :: *ble nprB* :: *bsr* Δ*vpr wprA* :: *hyg*; Cm^R	[173]

WB800N	*nprE aprE epr bpr mpr* :: *ble nprB* :: *bsr* Δ*vpr wprA* :: *hyg cm* :: *neo*; Neo^R (WB800 pB-*cat*5-*neo*-*cat*3)	*
WW02	1012 *amyE*::*neo* (Neo^R)	[163]
XL1 Blue (*E. coli*)	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q}*Z*Δ*M15* Tn*10* (Tet^R)]	Stratagene

Strains marked with an asterisk (*) were constructed during this work.

2.1.2 Plasmids

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

Table 2.2. Summary of plasmids used during this work

Name	Description	Reference
pAL01	carries an IPTG-inducible expression cassette, Em^R	[141]
pA-*spac*	template for *lacI* and *spac* promoter	[53]
pAX01	template for xylose cassette	[53]
pBluescript IIKS	*lacZ*, f1 *ori*, Amp^R, T7 and T3 promoter	Stratagene
pB-*cat*5-*neo*-*cat*3	*neo* in the middle of *cat*	* (as in *2.8.7*)
pCT105	pBR322 + *celA*, template for *celA*	[22]
pCT208	pBR322 + *celB*, template for *celB*	[22]
pHCMC01	pMTLBs72 with *trpA* transcriptional terminator, resistant to Cm (Cm^R)	*
pHCMC01-*bgaB*	pHCMC01 with *bgaB* reporter gene	*
pHCMC03	pHCMC01 with *gsiB* promoter, P*gsiB*	*
pHCMC03-*bgaB*	pHCMC03 with *bgaB* reporter gene	[104]
pHCMC03-*htpG*	pHCMC03 with *htpG* gene	*
pHCMC03-*lacZ*	pHCMC03 with *lacZ* reporter gene	*
pHCMC04	pHCMC01 with xylose-inducible cassette, *xylA* promoter, P*xylA*	*
pHCMC04-*bgaB*	pHCMC04 with *bgaB* reporter gene	*

pHCMC04-*htpG*	pHCMC04 with *htpG* gene	*
pHCMC05	pHCMC01 with IPTG-inducible cassette, P*spac* promoter	*
pHCMC05-*bgaB*	pHCMC05 with *bgaB* reporter gene	*
pHCMC05-*htpG*	pHCMC05 with *htpG* gene	*
pHSG575	replicon pSC101, P*lac*, *lacZ*α, (Cm^R)	[151]
pHT01	the derivative of pNDH33, removal of the deleted region	T.P.P. Phan
pKTH10	pUB110 with *amyQ*	[111]
pMTLBs72	*E. coli*/*B. subtilis* shuttle vector, the backbone for the series pHCMC plasmids	[153]
pMUTIN4	Amp^R, Em^R, template for *lacZ* gene	[161]
pMUTIN-*gfp*+	template for *gfp*+	[69]
pNDH09	pAL01 with *srtA* of *L.monocytogenes*	*
pNDH10	pHCMC04 with *fnbB94*	*
pNDH13	pNDH10 carrying signal sequence of *phoD*	*
pNDH15	pHCMC04 carrying *amyQ*	*
pNDH16	*amyQ* translationally fused to *fnbB94* in pNDH10	*
pNDH18	*gfp*+ translational fused to signal sequence of *phoD* and *fnbB94* in pNDH13	*
pNDH19	*fnbB123* translationally fused to *amyQ* in pNDH16	*
pNDH20	*fnbB162* translationally fused to *amyQ* in pNDH16	*
pNDH21	*fnbB196* translationally fused to *amyQ* in pNDH16	*
pNDH22	*fnbB234* translationally fused to *amyQ* in pNDH16	*
pNDH26	pMUTIN4 carrying 5’ end of *yhcS*	*
pNDH27	pMUTIN4 carrying 5’ end of *ywpE*	*
pNDH33	pHCMC05 with strong IPTG-inducible promoter, P*grac*	*
pNDH33-*bgaB*	pNDH33 with *bgaB* reporter gene	*
pNDH33-*htpG*	pNDH33 with *htpG* gene	*
pNDH33-*pbpE*	pNDH33 with *pbpE* gene	*

pNDH37	pNDH33 with signal sequence of *amyQ*	*
pNDH37-*amyQ*	pNDH33 with full *amyQ* gene	*
pNDH37-*celA*	pNDH37 with mature part of *celA* gene	*
pNDH37-*celB*	pNDH37 with mature part of *celB* gene	*
pNDH33-*yhcS*	pNDH33 carrying *yhcS*, P*grac-yhcS*	*
pNDH33-*ywpE*	pNDH33 carrying *ywpE*, P*grac-ywpE*	*
pNDH33-*ywpE-yhcS*	pNDH33 carrying *ywpE*, P*grac-ywpE-yhcS*	*
pNDH88	pHT01 with *amyQ*	*
pNDH89	*yhcR123* translationally fused to *amyQ* in pNDH88	*
pNDH90	*yfkN123* translationally fused to *amyQ* in pNDH88	*
pX-*bgaB*	using as template for *bgaB* gene	[71]
pXylR01	pHSG575 with *xylR* containing the fusion P*lac-xylR*	*

The name, descriptions and references are given. Plasmids marked with an asterisk (*) were constructed during this work.

2.1.3 Oligonucleotides

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

Table 2.3. Summary of oligonucleotides used in the course of this work

Name	Sequence (5' to 3')	Description
ON01	CCCCGGGGCAGCCCGCCTAATGAGCGGGCTTTTTTCACGT	*trpA* transcriptional terminator
ON02	GAAAAAAGCCCGCTCATTAGGCGGGCTGCCCCGGGGACGT	*trpA* transcriptional terminator
ON03	GGCCAT GAGCTC CTA TCG AGA CAC GTT TGG CTG	5' end of *gsiB* promoter
ON04	GGCCATGGATCC TTC CTC CTT TAA TTG GTG TTG GT	3' end of *gsiB* promoter
ON05	GGCCATGAGCTCCTAACTTATAGGGGTAACACTTAA	5' end of xylose cassette
ON06	GGCCATAGATCTGGATCCCATTTCCCCCTTTGATTT	3' end of xylose cassette
ON07	GGCCATGGTACCAGGCCTTACACAGCCCAGTCCAG	5' end of *spac* promoter

ON08	GGCCATGTCGACGGATCCTCACCTCCTTAAGCTTAATTGTTATCC	3' end of *spac* promoter
ON09	GGCCATGGTACCAAGCTAATTCCGGTGGAAACGAG	5' end of *lacI* gene
ON10	GGCCATGAGCTCAGGCCTTAACTCACATTAATTGCG	3' end of *lacI* gene
ON11	GCCTCTTCGCTATTACGCCAG	5′ MCS of pMTLBs72
ON12	TGTTTCAACCATTTGTTCCAGGT	3′ MCS of pMTLBs72
ON13	GAATTGTGAGCGGGAATACAAC	5′ end of *gsiB* promoter for sequence
ON14	GGGGAAATGACAAATGGTCCA	5′ end of *xylA* promoter for sequence
ON15	GAACAACCTCTGCTAAAATTCCTG	5′ end of *spac* promoter for sequence
ON16	GGCCATGGATCCATGAATGTGTTATCCTC	5' end of *bgaB* gene
ON17	GGCCATGGATCCCTAAACCTTCCCGGCTTCATCA	3' end of *bgaB* gene
ON18	GGCCATGGATCCACCATGATTACGGATTCACTGGC	5' end of *lacZ* gene
ON19	GGCCATTCTAGATTATTTTTGACACCAGACCAACTGG	3' end of *lacZ* gene
ON20	GGCCATGGATCCATGGCGAAAAAAGAGTTTAAAGCAGAGTC	5' end of *htpG* gene
ON21	GGCCATGGATCCTTACACCATGACCTTGCAAATATTGTTCG	3' end of *htpG* gene
ON22	TCGTTCGGTACCAGCTATTGTAACATAATCGGTACG	5' end of the *groE* promoter of *B. subtilis*
ON23	GGAATTGTTATCCGCTCACAATTCCACAATTCTTATAATAAAGAATCTCC	3' end of the *groE* promoter of *B. subtilis*
ON24	GGCCATGGATCCTTCCTCCTTTAATTGGGAATTGTTATCCGCTCACA	ON containing the *lacO* and the SD sequence of *gsiB*
ON25	GGCCATTGATCAATGATTCAAAAACGAAAGCGGACAG	5' end of *amyQ* gene
ON26	GGCCATGGATCCTACGGCTGATGTTTTTGTAATCGG	3' end of the coding region for the signal peptide of *amyQ*
ON27	GGCCATGGATCCATGAAGCAGAATAAAAGAAAGCATC	5' end of *pbpE* gene
ON28	GGCCATGGATCCTTACTACTTCGTACGGACCGCTTCT	3' end of *pbpE* gene
ON29	GGCCATGGATCCATGATTCAAAAACGAAAGCGGACAG	5' end of *amyQ* gene
ON30	GGCCATGACGTCTTATTTCTGAACATAAATGGAGACG	3' end of *amyQ* gene
ON31	GGCCATAGATCTGCAAACACTGTGTCAGCGGCA	5' end of *celA* gene
ON32	GGCCATGACGTCTTAATAAGGTAGGTGGGGTATGCTC	3' end of *celA* gene

ON33	GGCCATGGATCCGAAGGGTCATATGCTGATTTGGCAG	5' end of *celB* gene
ON34	GGCCATGACGTCTTATTTATACGGCAACTCACTTATGC	3' end of *celB* gene
ON35	GGCCATGACGTCGGAGGTACCCCAACGCCACCGACACCAGAAG	5' end of *fnbB94* of *S. aureus*
ON36	GCCATGACGTCCCGCGGAGTGGTCATAATGAAGGTCAACAAAC	5' end of *fnbB123* of *S. aureus*
ON37	GGCCATGACGTCCCGCGGCACGGATTCAATAAGCACACTGA	5' end of *fnbB162* of *S. aureus*
ON38	GGCCATGACGTCCCGCGGAATGGTAACCAATCATTCGAAGAAG	5' end of *fnbB196* of *S. aureus*
ON39	GGCCATGACGTCCCGCGGAGCGGTAATCAGTCATTTGAGG	5' end of *fnbB234* of *S. aureus*
ON40	GGCCATCCCGGGTTATGCTTTGTGATTCTTTTTATTTCTGCG	3' end of *fnbB* of *S. aureus*
ON41	GGCCATAGGCCTTTATGCTTTGTGATTCTTTTTATTTCTGC	3' end of *fnbB* of *S. aureus*
ON42	GGCCATGACGTCTTTCTGAACATAAATGGAGACGGAC	3' end of *amyQ* gene
ON43	GGCCATTGATCAATGGCATACGACAGTCGTTT	5' end of *phoD* gene
ON44	GGCCATGGATCCAAAGGCCCCAACCGACTG	3' end of the coding region for the signal peptide of *phoD*
ON45	GGCCATAGATCTATGGCTAGCAAAGGAGAAGAACT	5' end of *gfp+* gene
ON46	GGCCATAGATCTTTTGTAGAGCTCATCCATGCCA	3' end of *gfp+* gene
ON47	GGCCATGACGTCTTGGAAGCGACAGTTGAGTACG	5' end of *yhcR* gene
ON48	GAATAAGATATCTCACGTTCTGGAGGCGCTCCT	3' end of *yhcR* gene
ON49	GGCCATGACGTCCGCATGTTTGATATTGAAGAAGC	5' end of *yfkN* gene
ON50	AGCAGCGATATCTTATGCCTGATTCGCTCTATTCTG	3' end of *yfkN* gene
ON51	GCCATGGATCCATGTTAAAGAAAACAATTGCAATAATAATT	5' end of *srtA* gene
ON52	GGCCATGCATGCTCATTATTTACTAGGGAAATATTTATTCTC	3' end of *srtA* gene
ON53	AGCAATAACGCTGGATACATCTGC	3’ end of *yvfO* gene
ON54	GGCCATTTCGAAGACCTCTTTAGCTCCTTGGAAGC	3' end of *erm* gene
ON55	GACCTGAATGTGGAACGAGTGGAC	5' end of *ywpF* gene
ON56	GGCCATTTCGAACCGACTGTAAAAAGTACAGTCGGCA	3' end of *cat* gene
ON57	CGTCTTGATCAGGATACATCTGGC	5' end of *yhcT* gene
ON58	GAGAGCCATAAACACCAATAGCCTT	5' end of *neo* gene

ON59	GGCCATGAATTCAAAGGAGGAACTCCAGAACGTGAAAAAA GTTATTC	5' end of *yhcS* gene
ON60	CTAATACGACTCACTATAGGGAGAGGATCCCGACACCTTTTT CTAAATCA	3' end of *yhcS* gene
ON61	GGCCATGAATTCAAAGGAGGAACAACAATGCGCCGGGATCA	5' end of *ywpE* gene
ON62	CTAATACGACTCACTATAGGGAGAGGATCCTCTTCGTGCTTC ACTCTTGC	3' end of *ywpE* gene
ON63	TCTACATCCAGAACAACCTCTGC	5' end of P*spac* promoter
ON64	GGCCATAGATCTATGCGCCGGGATCAAAAAATG	5' end of *ywpE* gene
ON65	GGCCATAGATCTATGAAAAAAGTTATTCCACTATTCATCATT GC	5' end of *yhcS* gene
ON66	GGCCATAGATCTAGAATGAAGAAAAGCCGCAGGCACT	3' end of *yhcS* gene
ON67	CCAGAGATCTCAAAGGAGGAACTCCAGAACGTGA AAAAAGTTATTC	5' end of *yhcS* gene
ON68	AGTAAAGTTATCGGAATCGACTTAG	5' end of *dnaK* gene
ON69	CTAATACGACTCACTATAGGGAGAAAAGTATGCAGGAACTG TGAT	3' end of *dnaK* gene

ON, oligonucleotide; the DNA sequences recognized by restriction enzyme are underlined

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. Summary of antibiotic solutions used in this work

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

2.1.5 Media

Glucose free NAPS medium [71]

LB medium (1 % (w/v) trypton, 0.5 % (w/v) yeast extract, 1 % (w/v) NaCl)

Calcium caseinate agar (OXOID, CM639)

Antibiotics (Table 2.4), 2 % (w/v) insoluble starch and 0.5 % CMC (w/v) were added when necessary.

Agar was added to 1.5 % (w/v) to prepare plates

2.2 Enzymes, antibodies, biochemichals, chemicals and kits

2.2.1 Enzymes

Roche: alkaline phosphatase, T7 RNA polymerase, DNase I,

Merk: proteinase K

Sigma: RNase A, lysozyme

Fermentas: restriction enzymes

2.2.2 Antibodies

Table 2.5. Antibodies were used in this work

Name	From organism	Dilution	Reference
α-AmyQ	*Bacillus amiloliquefaciens*	1 : 15 000	V. Kontinen
α-CelA	*Clostridium thermocellum*	1 : 10 000	P. Béguin
α-CelB	*Clostridium thermocellum*	1: 10 000	
α-GFP	*Aequoria victoria*	1 : 5000	Clontech
α-HtpG	*Bacillus subtilis* 1012	1 : 10 000	S. Schwab
α-PBP*	*Bacillus subtilis* 1012	1 : 10 000	[179]
α-SrtA	*Listeria monocytogenes*	1 : 10 000	O. Schneewind

Information is given in Table 2.5 concerning the antibodies with their working dilutions which were used in the course of this work.

2.2.3 Biochemicals and chemicals

Amersham: Amonium persulphate, hyperfilm ECL

Fermentas: DNA ladder, RNA ladder and protein ladder

Pierce: Luminol substrate

Roche: blocking reagent, chemiluminescent substrate CPD-Star, protease inhibitor, RNAase inhibitor, Xgal, ONPG

Roth: acetic acid, agar, agarose, aqua phenol, chloroform, diethylpyrocarbonate (DEPC), ehtidium bromide, isopropanol, pepton, potasium acetate, potasium phosphate, polyacrylamide, sodium phosphate, sodium chloride, starch, MOPS, sodium dodecyl sulphate, sucrose, Tris, xylose, yeat extract.

2.2.4 Kits

Epicentre: Fast-Link™ DNA Ligation Kit

Qiagen: PCR purification kit, gel-extraction kit, midi purification kit

2.3 General methods

2.3.1 PCR and colony 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 for cloning [130]. This became effective by the isolation of a thermostable DNA polymerase from *Thermus aquaticus* [129]. During the PCR, DNA is denatured at high temperature and specific oligonucleotide primers are annealed and elongated at lower temperature in a cyclic manner.

Colony PCR based on standard PCR using total DNA from a colony as template allows rapid detection of the transformation success when primers are available to allow determination of correct ligation products by size or hybridization.

2.3.2 Cloning

All the steps necessary for cloning were carried out as described by using standard methods [132]. Preparation of competent *E. coli* cells and transformation were carried out as standard heat shock transformation [61] and electroporation [30]; PCR for screening of plasmids and preparation of plasmid DNA by the alkaline lysis method with SDS have been described [9, 63]. 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.

2.3.3 Growth and collection of samples

During this work, *B. subtilis* strains were grown in LB medium with the appropriate antibiotic(s) when necessary in a waterbath shaker (~200 rpm) at 37 ^{0}C. Overnight cultures in 5 ml medium in glass tubes were transferred partially to Erlenmeyer flasks containing medium to an OD_{578} of 0.05-0.08. When an OD_{578} of 0.8 was reached (set as t = 0), the culture was divided into two subcultures where one was further grown in the absence and the other in the presence of the inducer, 0.1 mM or 1 mM for IPTG and/or 0.5% or 1% of xylose. 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}.

2.3.4 Determination of structural and segregational stability of plasmids

Structural stability was measured by growth of *B. subtilis* cells harbouring 8 different plasmids: the four pHCMC plasmids with the *bgaB* reporter gene, pNDH33-*bgaB*, pNDH37-*amyQ*, pNDH37-*celA* and pNDH37-*celB* in LB medium in the presence of Cm (10 μg/ml) for about 100 generations involving several subcultures. This procedure involved growth of the four cultures into stationary phase, dilution to about 1000 cfu/ml which were grown again into stationary phase, etc. Finally, cells were plated on LB X-gal medium for plasmids harbouring *bgaB*, on LB 2% insoluble starch for pNDH33-*amyQ* and LB 0.5% CMC for pNDH37-*celA* and pNDH37-*celB* in the presence of the appropriate inducer and the number of blue/white colonies or colonies with and without a halo was counted.

Segregational instability was determined for the two plasmids pHCMC04-*bgaB* and pHCMC05-*bgaB*, in a similar way, but cells were grown in the absence of Cm. Aliquots were withdrawn at the number of generations elapsed as shown in the result, plated on LB plates, and replica plated on LB plates containing Cm. This allowed the calculation of antibiotic-resistant colonies remaining after the indicated number of generations.

2.4 Work with RNA: Northern blot analysis

Northern blot analysis was carried out to confirm the qualitative and quantitative determination of specific RNA molecules in the RNA mix. Northern blot analysis was performed as described [58], [124].

2.4.1 Isolation of total RNA from *B. subtilis*

B. subtilis cells were grown and induced as described in 2.3.3. Especially, strains NDH31/pNDH33-*yhcS* and NDH31-*ywpE-yhcS* were induced by 0.1 mM IPTG. The cells were then killed by addition of "killing buffer" (5 mM $MgCl_2$, 20 mM NaN_3, 20 mM Tris-HCl; pH7.5). Total RNA was extracted using the protocol for isolation of RNA from yeast with modification [123]. The cell walls of the cells were digested by lysozyme (1 mg/ml) on ice for 5 min before extraction of RNA. The samples were heated at 95 ^{0}C for 5 min before addition of phenol.

2.4.2 Electrophoresis of RNA and vacuum blot transfer to membranes

RNA samples were separated on 1.2% agarose gels and the transfer occurred onto Nylon-membranes. The transfer was carried out with the help of the Vacuum-Blot-Annex (VacuGene^{TM}X1, Pharmacia).

2.4.3 Transcriptional labelling of RNA probes

Pairs of primers ON59/ON60 and ON61/ON62 were used to amplify a part of the *yhcS* and the *ywpE* gene, respectively; ON68/ON69 were used to amplify *dnaK* used as a loading control [59]. These amplicons harbour a T7 promoter at the 3' end were used as templates for *in vitro* transcription according to the instructions of the manufacture Roche [124].

2.4.4 Cleaning of DIG-labelling RNA probes

When the DIG-labelled-antisense-RNA used at the beginning for hybridization experiments, a very strong background was detected on the X-ray-film. The more RNA probe was used, the more the background was decreased. While this phenomenon is known, its reasons are not known. Therefore, the RNA probes were purified routinely before using them in hybridization experiments. All the steps were carried as in the hybridization procedures, but using the blank membranes.

2.4.5 Hybridization of membrane-bound RNA with RNA probes

This experiment was carried out as described [124].

2.4.6 Stripping of RNA probes

This experiment was carried out as described [124].

2.5 Work with protein: SDS-PAGE and Western blot analysis

2.5.1 Preparation of soluble and insoluble cell extracts from *B. subtilis*

To examine the solubility of HtpG and PBP4* produced in the cytoplasm, cells prepared as described in 2.3.3 were disrupted by ultrasonication (12 W, 6 x 15 pulses with 15 sec intervals) in 1.5 ml Eppendorf tube containing 1 ml of cell suspension (lysozyme 250 µg/µl) on ice; 100 µl of the preparations were taken for the first total protein sample (T1). Then, the preparations were centrifuged at 430 g for 10 min to remove cell debris; 100 µl of supernatants were taken for the second total protein sample (T2). Subsequent centrifugation at 8,200 g for 10 min separated the insoluble (I) and soluble (S) protein fractions. The amount of proteins corresponding to 0.025 of OD_{578} was separated by SDS-PAGE, followed by immunoblotting with specific antiserum.

2.5.2 Extraction of denatured total cell lysate from *B. subtilis*

For the extraction of denatured cell lysate from *B. subtilis* cells (2.5 of OD_{578}) prepared as in 2.3.3 were resuspended in 100 µl of lysis buffer (15% (w/v) sucrose, 50 mM Tris/HCl; pH 7.2) containing 2.5 mg/ml lysozyme and incubated at 37 ^{0}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) were added to the suspension and frozen until use. Before use the samples were heated for 5 min at 95 ^{0}C and 15 µl of each sample were used for SDS-PAGE.

2.5.3 Preparation of proteins released from the cell wall

B. subtilis cultures corresponding to an OD_{578} of 10 were taken and the cells were collected by centrifugation; the pellets were washed twice in 0.1 M sodium phosphate buffer (PB, pH7.0). To release the α-amylase anchored on the cell wall, whole cells were treated with lysozyme. Cells were resuspended 100 µl of the PB and 50 µl lysozyme (5 mg/ml dissolved in water) or mutanolysin (Sigma, M9901), followed by incubation for 15 min. Then the samples were centrifuged at 12 000 rpm and 4 ^{0}C for 10 min, and the supernatant was collected. The supernatant was then used for SDS-PAGE by addition of loading buffer or for determination of α-amylase activity.

It is observed that proteins from the cytoplasm were released together with cell wall proteins. To eliminate the cytoplasmic protein, the following method was used. The ***B. subtilis*** strains NDH31, SZ60, NDH30, 1012, and NDH31 carrying plasmid pNDH33, pNDH33-*yhcS*, pNDH33-*ywpE*, pNDH33-*ywpE*-*yhcS*, and strain WB800N harbouring plasmid pNDH33-*ywpE*-yhcS were grown as described in 2.3.3. After 1 hour inoculation, 0.1 mM IPTG was added to induce the expression of *yhcS* and/or *ywpE* and amounts of cells corresponding to 200 of OD_{578} were collected at 8 later. The cells were suspended in 1.5 ml water (final volume) containing a cocktail of protease inhibitors (Roche Diagnostics), 2 mM EDTA and 100 mg/ml Dnase I and disrupted by sonicator (12 W, 10 x 30 pulses with 30 sec intervals) on ice. The unbroken cells were removed by low-speed centrifugation 3 000 *rpm* (980 g) at 4 ^{0}C for 10 min. The supernatants were centrifuged at higher speed 14 000 *rpm* (21 000 g), at 4 ^{0}C for 15 min to obtain a pellet containing the envelope materials. These materials were washed three times in water containing protease inhibitors. The pellets containing peptidoglycan with cell wall proteins were suspended in 100 µl of lysozyme 1mg/ml lysozyme, and incubate at 37 ^{0}C for 45 min, mix occasionally. Fifty microlites of 3x loading buffer was added and boiled for 5 min; 15 µl of each sample were applied for SDS-PAGE on 8% or 12% later (Fig. 3.22).

2.5.4 Measurement of protein concentrations

The method of Bradford was used for the measurement of the protein concentrations from cell extracts [76].

2.5.5 Precipitation of proteins from culture supernatant

Protein from cultured supernatant was collected by the TCA method. One volume of 40% TCA was mixed with 3 volumes of culture supernatant, incubated on ice for 10 min and centrifuged (12000 rpm at 4 ^{0}C for 10 min). The pellet was then washed twice with ice-cold acetone and dried at room temperature. The pellet was dissolved in water and loading buffer for SDS-PAGE.

2.5.6 Protein electrophoresis using discontinuous SDS-PAGE

The electrophoretic separation of proteins according to their molecular mass was performed as first described by Laemmli [78].

2.5.7 Immunoblot analysis

In order to immunochemically detect proteins using antibodies, the proteins were transferred after their electrophoretic separation onto a nitrocellulose membrane using electroblotting [159]. 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 Biociences).

To corroborate the versatilities of the expression vectors pHCMC for the overproduction of proteins, *B. subtilis* 1012 harbouring plasmids with the heat shock gene *htpG* [143] pHCMC03-*htpG*, pHCMC04-*htpG* and pHCMC05-*htpG* were analysis by immunoblot, while 1012 strain harbouring the empty vector served as a control. *B. subtilis* strain 1012 carrying one of these three and the empty vector pHCMC01 as a control were grown either in LB medium (pHCMC01, pHCMC04-*htpG* and pHCMC05-*htpG*) or LB medium supplemented with 0.5% glucose and 1.5 mM KH_2PO_4 [101] at 37°C to the mid-exponential growth phase. Then, the cultures were divided into subcultures where one was further grown untreated while the others were either challenged with different stress factors (acid shock, pH 5.8; 4% ethanol

and heat shock, 48 ^{0}C) for plasmid pHCMC03-*htpG* or induced with 1% xylose or 1 mM IPTG. These cells were further grown and samples were collected as described in 2.3.3 and prepared (see under *2.5.2*). Equal amounts of proteins were applied per lane (3.5 µg in Fig. 3.1A and 4.7 µg in B), and the blots were probed with α-HtpG.

To confirm the expression of α-amylase, cellulase A and cellulase B in the culture medium, cells of *B. subtilis* 1012 containing the plasmids pNDH37-*amyQ*, pNDH37-*celB* and pNDH37-*celA* were grown as described in 2.3.3. Aliquots were taken after 3 h (3*) without addition of IPTG and immediately before addition of 1 mM IPTG (0) and 1 to 7 h after induction. The aliquots were centrifuged and the amounts corresponding to either 5 µl (α-amylase) or 60 µl (cellulase B), 1.5 µl (cellulase A) of the supernatant were analysed for the presence of the secreted exoenzyme as indicated in Fig. 3.8.

All other cells were grown as described under 2.3.3, the samples were applied on the polyacrylamide gels and transferred to membranes for immunoblot as described in this section.

2.6 Visualization and measurement of reporter gene expression

2.6.1 Visualization of extracellular enzyme activity on plates

2.6.1.1 α-Amylase (AmyQ)

Single colonies of the *B. subtilis* strains carrying plasmid pHCMC04, pNDH15, pNDH16, pNDH19, pNDH20, pNDH21, pNDH22, pNDH37-*amyQ*, pNDH89, pNDH90 and pNDH91 were grown for 24 h on LB plates containing 1 mM IPTG or 1% xylose and 2% insoluble starch and stained with I2/KI solution [105]. Pictures were recorded using a digital camera.

2.6.1.2 Cellulase (CelA and CelB)

Single colonies of the *B. subtilis* strains carrying plasmid pNDH37-*celA* or pNDH37-*celB* were grown for 24 h on LB plates containing 1 mM IPTG and 0.5% CMC and stained with 1% congo red solution [22]. Pictures were taken by a digital camera.

2.6.2 Measurement of the β-galactosidase activity

2.6.2.1 β-Galactosidase BgaB

Blue colonies from LB-Xgal plates were used for determination of β-galactosidase activity (white colonies for the strains with pHCMC01-*bgaB*). *B. subtilis* strains IHA01-Spac-BgaB and 1012 carrying pHCMC01-*bgaB*, pHCMC02-*bgaB*, pHCMC03-*bgaB*, pHCMC04-*bgaB*, pHCMC05-*bgaB* and pNDH33-*bgaB* were grown and samples collected as described under 2.3.3 and 2.5.7. The activity was determined at 55 ^{0}C as described [99] with the exception that the BgaB activity was measured in a microtiterplate reader (VersaMax, Molecular Devices). One unit is defined as $\Delta A_{420}*OD_{578}^{-1}*min^{-1}$ and displayed as units/OD_{578} for all the results except for the result using pHCMCs plasmid series which were displayed as units/mg protein, in which one OD_{578} is defined as the optical density of the samples used in the assay, A_{420} is the absorbance of the samples measured by the microtiterplate reader and min indicates the incubation time of the plate at 55 ^{0}C.

2.6.2.2 β-Galactosidase LacZ

B. subtilis strain 1012 carrying pHCMC03-*lacZ* and NDH20, NDH21 containing the transcriptional fusions P*yhcS*-*lacZ* and P*ywpE*-*lacZ* were grown and samples collected according to section 2.3.3. β-Galactosidase activity were measured at 405 nm at 28 ^{0}C as describe elsewhere [168]. One unit is defined as $V_{max}*OD_{578}^{-1}$, in which OD_{578} is defined as the optical density of the samples used in the assay and Vmax is the maximum kinetic rate reported as miliOD/minute. The data were displayed as units/mg protein for pHCMC03-*lacZ* and units/OD578 for strains NDH20 and NDH21.

2.6.3 Measurement of the α-amylase activity

Strains NDH03 with different plasmids (pHCMC04, pNDH15, pNDH16, pNDH19, pNDH20, pNDH21, pNDH22) were grown in LB medium at 37 ^{0}C as described under 2.3.3. Cells were grown to an OD_{578} 0.1 - 0.2 at 37 ^{0}C, then 1 mM of IPTG was added to induce production of sortase A. When the cultures reached an OD_{578} of 0.8, 0.5% xylose was added to induce production of wild-type or hybrid α-amylase. All cultures were further grown for 2 h. Then, aliquots were collected and the cells were separated from the growth medium by

centrifugation. Samples were kept at - 20 ^{0}C until determination of the enzymatic activity. Proteins released from the cell walls were prepared as described in section 2.5.3.

Strain 1012 carrying plasmid pNDH37-*amyQ* was grown in LB medium with Cm at 37 ^{0}C and samples were collected as described under 2.3.3. Culture supernatants were withdrawn after centrifugation step. Samples were kept at - 20 ^{0}C.

α-Amylase activity was determined as described [105]. Samples of cells were transferred into 1.5-ml Eppendorf tubes and samples from supernatants into microtiterplates. α-Amylase activities were determined with whole cells and with the supernatants and presented in units per OD_{578}. One unit is defined as a decrease in OD_{620} of 0.1. All experiments were repeated at least twice.

2.6.4 Determination of covalent anchor-reporters on the cell wall

Colonies exhibiting halos on plate containing insoluble starch were used for this experiment. Strains WW02 and NDH03 harbouring pNDH15 or pNDH16 were grown as described in section 2.6.3 and preparation of proteins released from the cell wall was carried out as described in section 2.5.3. Protein loading buffer was added directly to the culture supernatant. These samples were applied for immunoblot analysis using the antibodies against α-amylase (AmyQ). The same protocol was applied for other *B. subtilis* strains carrying plasmids pNDH15, pNDH16, pNDH19, pNDH20, pNDH21, pNDH22, pNDH89 and pNDH90.

Colonies from strain NDH03/pNDH18 and WW02/pNDH18 exhibiting green fluorescence under the microscope (2.7.2) on LB plates containing 1% xylose were used for this experiment. These strains were grown as described in section 2.6.3 and preparation of proteins released from the cell wall according to section 2.5.3. Protein loading buffer was added directly to the culture supernatant. These samples were applied for immunoblot analysis using the antibodies against GFP.

2.6.5 Determination of the number of α-amylase molecules on the cell surface

Cells from two different cultures were withdrawn, sedimented by centrifugation, washed twice with the PB and treated with lysozyme to release the α-amylase as described in 2.5.3. Cells were centrifuged, and 5 µl of supernatant corresponding to 850,000 cells were applied

per lane. α-Amylase from two different cultures was analysed. Defined amounts of purified α-amylase (Sigma) from 2.5 to 20 ng (corresponding to 0.05 - 0.4 pmol) were run on the same gel. The collected pictures were analysed by Quantity One (BioRad). Only the material in the upper band of the samples was quantified (Fig. 3.15).

2.6.6 Kinetics of α-amylase immobilization on the cell wall

Strain NDH03 with a plasmids able to express α-amylase with a 94-aa and 123-aa spacer (pNDH16 and pNDH19, respectively) were prepared and the α-amylase activity was determined as in section 2.6.3. Cells were withdrawn for the determination of the α-amylase activity at the time points indicated in Fig. 3.18. Before collecting the samples, tetracycline was added at a final concentration of 20 μg/ ml to inhibit protein synthesis, and the cultures were shaken for another 10 min and then stored in ice. Proteins released from the cell walls were prepared as described in section 2.5.3.

2.7 Visualisation of *B. subtilis* cells under the microscope

2.7.1 Confocal microscope

All steps of this procedure were carried out at room temperature. Alive cells were washed in PBS (80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl; pH 7.5) and resuspended in PBS containing the primary antibody raised in rabbits against *B. amyloliquefaciens* α-amylase at a dilution of 1:3000 for 1 h [150]. Then, the cells were washed and incubated with anti-rabbit IgG Alexa conjugate (Alexa Fluor®568 goat anti-rabbit IgG; Molecular Probes) at a dilution of 1:2000. A 3 μl aliquot of this cell suspension was spread onto a slide, air dried and observed under a confocal microscope (Leica).

2.7.2 Fluorescence stereomicroscope

In order to make sure the plasmid pNDH18 was successfully transformed into the *B. subtilis*, single colonies of the strains WW02 and NDH03 carrying plasmid pNDH18 were grown for 24 h on LB plates containing 1% xylose. These plates were then observed under MZFLIII microscope (Leica) using the GFP2 filter. The pictures were recorded by the camera and the computer connected to this device.

2.8 Construction of plasmids and strains

2.8.1 Construction of the plasmid-based expression vectors pHCMC series

The author started from the *E. coli - B. subtilis* shuttle vector pMTLBs72 [153] and inserted first the efficient *trpA* transcriptional terminator at the *Aat*II restriction site to ensure efficient termination of transcription immediately downstream of the recombinant genes using the two complementary oligonucleotides (ON) ON01 and ON02 (Table 2.3) as described [69] resulting in pHCMC01. This plasmid served as a backbone into which four different expression cassettes were inserted.

First, the *gsiB* promoter was chosen. This promoter is recognized by the alternative sigma factor σ^B and has been reported to be expressed at a very low level under physiological conditions and being induced by different stresses such as heat and acid stress and ethanol [88, 162], and the strong RBS of *gsiB* has been reported to be crucial for increased stability of the *gsiB* transcript [68]. The promoter region of *gsiB* including the DNA sequence coding for the strong ribosome-binding site were amplified with two primers ON03 and ON04 (Table 2.3) using *B. subtilis* chromosomal DNA as template and inserted into pHCMC01 yielding pHCMC03 (Fig. 2.1).

Second, the complete xylose-inducible cassette was amplified using ON05 and ON06 and plasmid pAX01 [53] as template resulting in pHCMC04 (Fig. 2.1). Third, the last expression vector contains the IPTG-inducible P*spac* promoter and was assembled by first inserting P*spac* using ON07 and ON08 followed by the *lacI* gene (ON09 and ON10) using pA-*spac* as template [53] resulting in pHCMC05 (Fig. 2.1). All inserts were verified by DNA sequencing using ON11 and ON12. The cloning sites are indicated immediately downstream of the promoters.

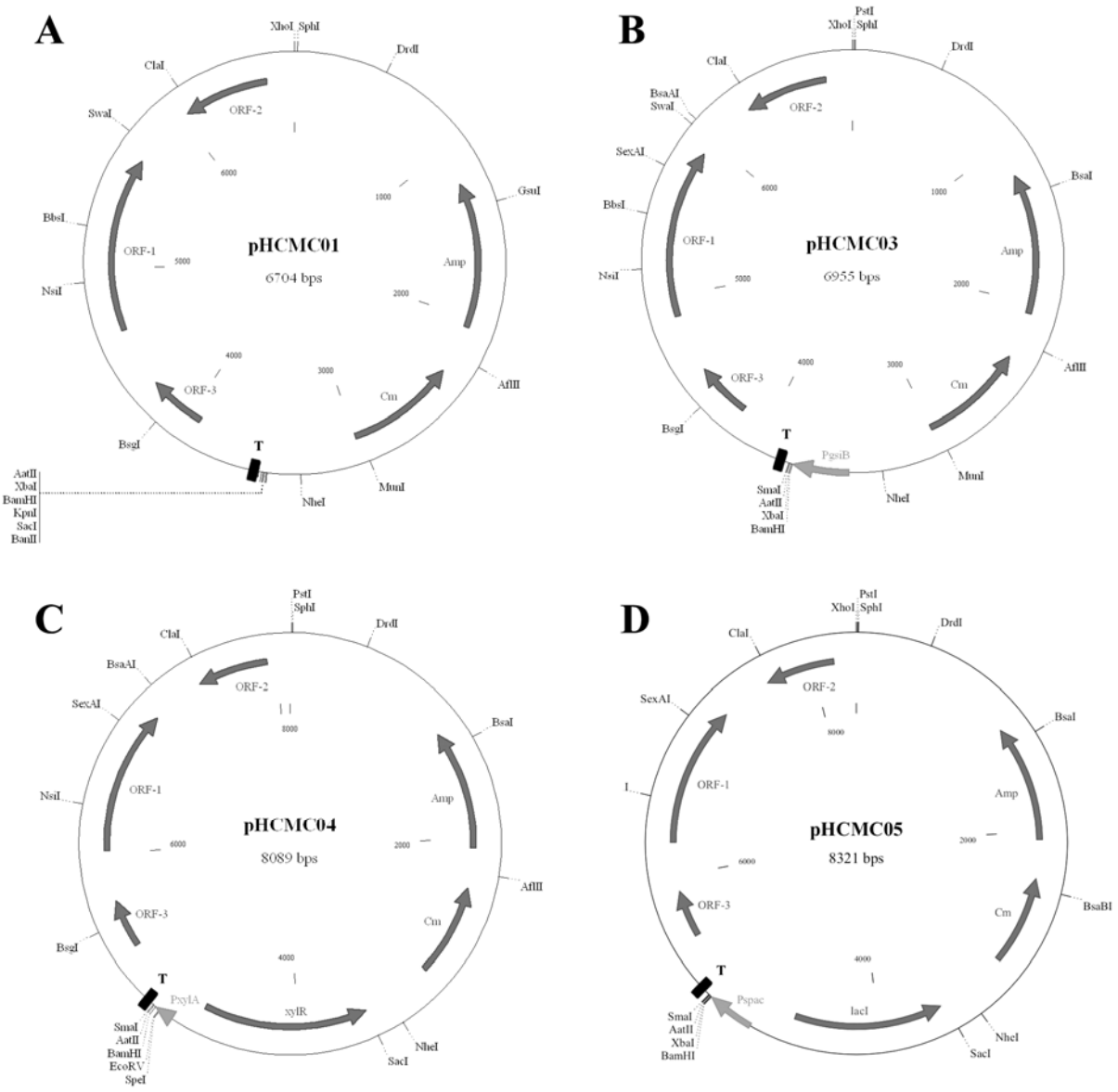


Fig. 2.1. Genetic and restriction map of the pHCMC vectors. (A): backbone plasmid pHCMC01; (B): pHCMC03 with the *gsiB* promoter; (C): pHCMC04 with the xylose-inducible *xylA* promoter; and (D): pHCMC05 with the IPTG-inducible *spac* promoter. All vectors replicate both in *E. coli* and in *B. subtilis*, and contain the efficient transcriptional terminator *trpA* immediately downstream from the promoters as indicated by a black solid bar. The cloning sites immediately downstream of the promoters are indicated.

To measure the strength and inducibility of three promoters, these were transcriptionally fused to either the reporter gene *lacZ* or/and *bgaB*, both coding for β-galactosidase. Two different reporter genes were chosen since the β-galactosidase activity encoded by *lacZ* disappears

within a few minutes after heat or ethanol challenge, while that coded for by *bgaB* is heat-stable [99]. On the other hand, when the cells were challenged to acid stress, the β-galactosidase activity encoded by *bgaB* turned out to be unstable. To comply with these observations, the *bgaB* reporter gene was fused to all three promoters and *lacZ* to P*gsiB* in addition. The b*gaB* gene was amplified by PCR with primers ON16 and ON17 using pX-*bgaB* as template [71]; this PCR product was then treated with *Bam*HI and ligated to those four vectors at *Bam*HI resulting pHCMC01-*bgaB*, pHCMC03-*bgaB*, pHCMC04-*bgaB* and pHCMC05-*bgaB*. The l*acZ* gene was amplified by PCR with primers ON18 and ON19 using pMUTIN4 as template [161]; this PCR product was then treated with *Bam*HI and *Xba*I and ligated into pHCMC03 at *Bam*HI and *Xba*I resulting pHCMC03-*lacZ*. Beside *bgaB* and *lacZ*, *htpG* encoding for a class III heat shock protein [143] was also amplified with primers ON20 and ON21 using *B. subtilis* 1012 chromosomal DNA as template and cloned into those vectors at *Bam*HI resulting in pHCMC03-*htpG*, pHCM04-*htpG* and pHCMC05-*htpG*. These correct plasmids were then transformed into *B. subtilis* 1012 and the expression level of β-galactosidase and HtpG was measured in the absence and presence of inducer.

2.8.2 Construction of novel expression and secretion vectors

To construct the expression vector pNDH33, pHCMC05 [104] was used as a backbone. The P*spac* promoter of this vector was replaced by the *groE* promoter. This was accomplished by cutting pHCMC05 with *Kpn*I and *Bam*HI and replacing the small 186-bp fragment by the *groE-lacO* (*lac* operator) sequence. The *groE* promoter was generated by PCR using ON22 and ON23 (see Table 2.3) and chromosomal DNA of *B. subtilis* strain 1012. This amplicon was then used as templates with ON22 and ON24 to amplify by PCR, in which *lacO* and the SD sequence of the *gsiB* gene of *B. subtilis* was added which has been reported to be very efficient [68]. The double-stranded product was cleaved with *Kpn*I and *Bam*HI and inserted into pHCMC05 treated with the same enzymes resulting in pNDH33 (Fig. 2.2). The DNA sequence of the regulatory region was verified by DNA sequencing. To obtain a vector allowing secretion of exoenzymes, the coding region for the signal sequence of the α-amylase encoded by *amyQ* was amplified using ON25 and ON26 and pNDH16 as template. The amplicon was cut with *Bcl*I and *Bam*HI, inserted into *Bam*HI-linearized pNDH33, and the correct orientation was determined using PCR with ON22 and ON26 resulting in the expression-secretion vector pNDH37. Here, too, the regulatory region including the *amyQ* signal sequence was verified by DNA sequencing.

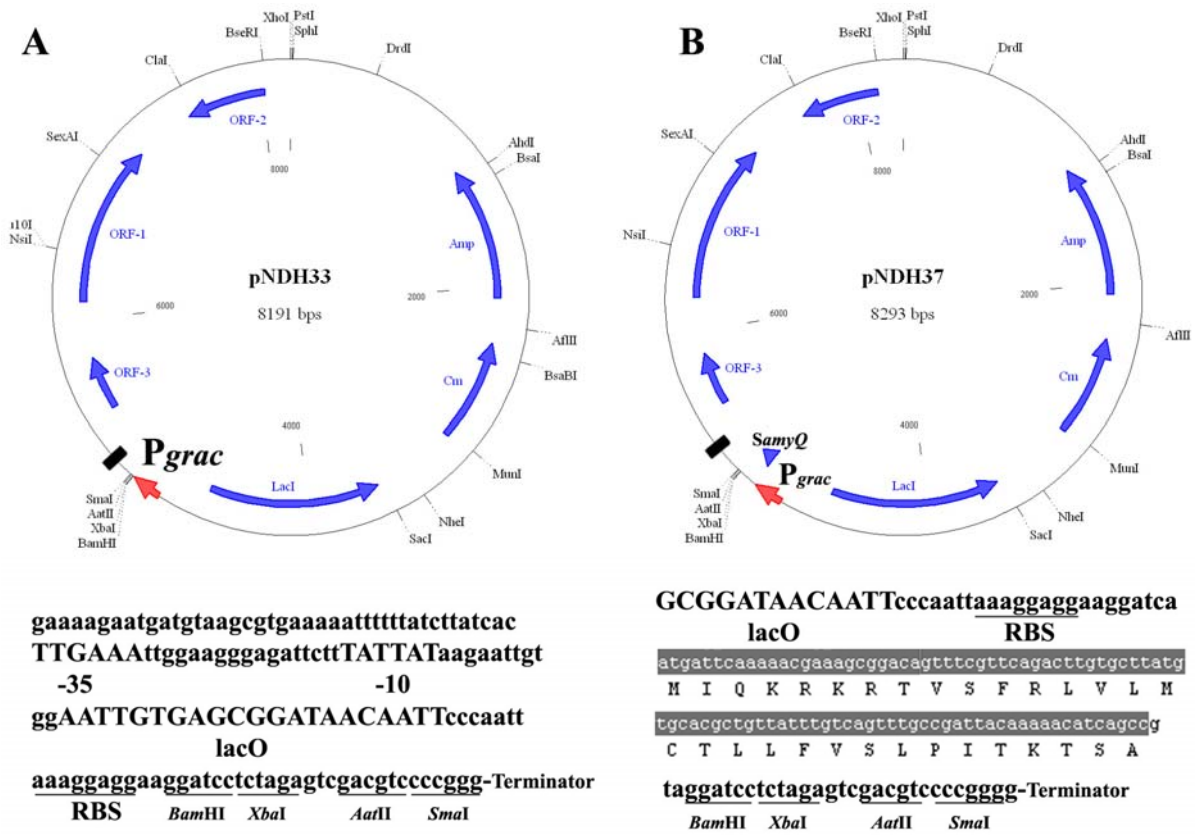


Fig. 2.2. Genetic and restriction map of the expression vector pNDH33 and the expression-secretion vector pNDH37. (A) pNDH33 and the DNA sequence of the P*grac* promoter (in capital letters at -35 and -10 regions) including the upstream AT-rich UP element, the *lac* operator (*lacO*; in capital letters) and the ribosome-binding sequence (RBS, underlined); (B) pNDH37 and the DNA sequence starting with *lacO*, the RBS (underlined) and the coding region for the signal sequence (highlighted in grey). Unique restriction sites which can be used for insertion of recombinant genes are also given.

The *bgaB* reporter gene and the *htpG* and *pbpE* genes were inserted into pNDH33 using the oligonucleotide pairs ON16/ON17, ON20/ON21 and ON27/ON28 respectively (Table 2.3), which resulted in the new recombinant plasmids pNDH33-*bgaB*, pNDH33-*htpG* and pNDH33-*pbpE*. The *amyQ* gene including its coding region for the signal sequence and mature part was amplified using pNDH16 and ON29 and ON30 and ligated into pNDH33 resulting in pNDH37-*amyQ*. The genes *celA* and *celB* (both code for cellulases and are derived from *Clostridium thermocellum*) [22, 23] were amplified using ON31/ON32 and ON33/ON34, respectively and ligated into *Bam*HI/*Aat*II-linearized pNDH37 resulting in pNDH37-*celA* and pNDH37-*celB*.

2.8.3 Construction of plasmids for anchor of AmyQ and GFP on the cell surface

To allow covalent anchoring of the coding region of the α-amylase gene *amyQ* of *B. amyloliquefaciens* and any other gene on the *B. subtilis* cell wall, a sorting vector was constructed. This sorting vector contains part of the coding region of the fibronectin binding protein B gene (*fnbB*) of *S. aureus* [67] inserted into the expression vector pHCMC04. This expression vector contains a xylose cassette consisting of the *xylR* repressor and the promoter/operator region of the *xylAB* operon [71] which can be induced by the addition of xylose. The coding region of the 3' end of fibronectin B consisting of the immediate 3' including the sorting motif (Table 1.1) and additional 94 codons, the spacer region (Fig. 2.3), was amplified using ON35 and ON40, the amplicon was cleaved with *Aat*II and *Sma*I and inserted into pHCMC04 treated with the same enzymes resulting in pNHD10. Next, the *amyQ* gene was generated by PCR using pKTH10 [111] and ON29 and ON42, the amplicon treated with *Bam*HI and *Aat*II and ligated into pNHD10 cut with the same enzymes resulting in pNDH16. In addition, *amyQ* was inserted into pHCMC04 resulting in pNHD15. Furthermore, the spacer length was varied from 94 aa (FnBPB94) to 234 (FnBPB234). To obtain this goal, oligos ON36 through ON39 were used together with ON41 to amplify the appropriate regions of *fnbB*. Then, these amplicons cut by *Aat*II and *Stu*I were used to replace the *Aat*II/*Sma*I fragment in pNHD16 resulting in the new plasmids pNDH19 (FnBPB123), pNDH20 (FnBPB162), pNDH21 (FnBPB196) and pNDH22 (FnBPB234) (Fig. 2.3).

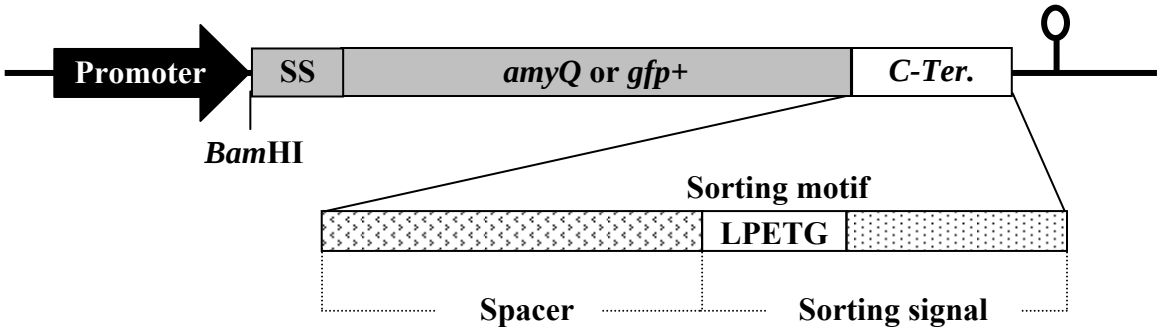


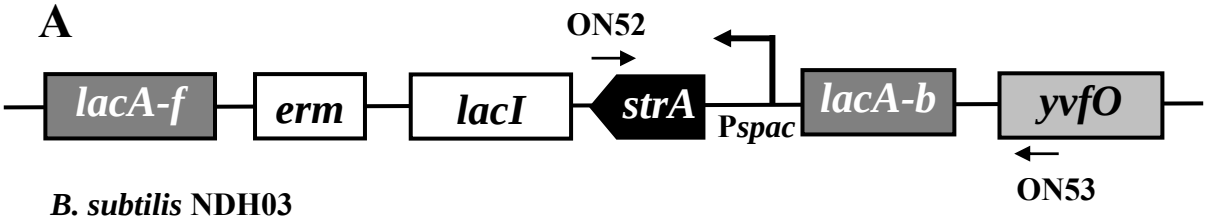
Fig. 2.3. Construction of the anchoring structure for surface proteins. SS, signal sequence coding for signal peptide (SP) of AmyQ (Sec pathway) or PhoD (Tat pathway)**;** N-Ter., N-terminal of the sorting signal with its spacer of FnBPB, YhcR and YfkN.

To study whether proteins could be secreted via TAT pathway and subsequently anchored on the cell wall, the anchor vector pNDH13 was constructed. The coding region for the signal peptide of the alkaline phosphatase encoded by *phoD* was amplified using ON43 and ON44

and *B. subtilis* 1012 chromosomal DNA as template. The amplicon was cut with *Bcl*I and *Bam*HI, inserted into *Bam*HI-linearized pNDH10, and the correct orientation was determined using PCR with ON12 and ON43 resulting in the anchor vector pNDH13. Here, the regulatory region including the *phoD* signal sequence was verified by DNA sequencing using ON12. To evaluate whether pNDH13 could anchor proteins on the cell wall, the GFP+ was chosen as model reporter gene. The *gfp*+ gene was generated by PCR using pMUTIN-*gfp*+ [69] as template and ON43 and ON44, the amplicon treated with *Bgl*II and ligated into pNHD13 cut with *Bam*HI resulting in pNDH18 (Fig. 2.3).

These recombinant plasmids pNDH15, pNDH16, pNDH18, pNDH19, pNDH20, pNDH21 and pNDH22 were then transformed into *B. subtilis* 1012 and NDH03 to check the ability to anchor the reporters on the cell wall as described in *2.6.4*.

2.8.4 Construction of the *B. subtilis* strain NDH03



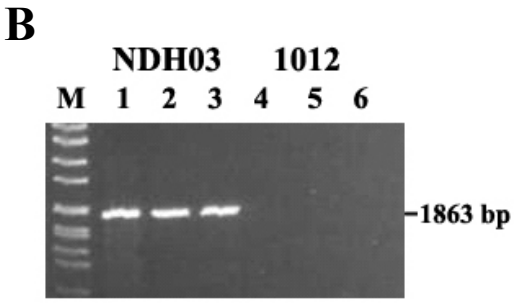


Fig. 2.4. Integration of *srtA* in the chromosome of *B. subtilis* 1012 and PCR for confirmation of correct insertion into the strain NDH03. 1, 2, 3, sample from *B. subtilis* NDH03; 4, 5 and 6, sample from *B. subtilis* 1012 (control).

To allow controlable expression of the sortase A gene (*srtA*) of *L. monocytogenes* [122] in *B. subtilis*, its coding sequence was amplified using ON51 and ON52 (Table 2.3) and chromosomal DNA of *L. monocytogenes* strain P14 [122] as template. The amplicon was cleaved with *Bam*HI and *Sph*I and inserted into plasmid pAL01 treated with the same enzymes resulting in pNDH09; in this plasmid, *srtA* was fused to the IPTG-inducible promoter P*spac*. Next, pNDH09 was linearized using *Pvu*I and transformed into *B. subtilis* strain WW02 (this strain was chosen because it carries a null mutation in its α-amylase gene

amyE), recombinants were selected on LB plates containing Erm. Correct integration at the *lacA* locus [53] was confirmed by PCR using ON52 and ON53 (Table 2.3), and one transformant (NHD03) was kept for further studies.

2.8.5 Construction of the anchoring vectors

In order to construct plasmids that allow anchor any other proteins on surface of *B. subtilis* cells, plasmid pNDH12 with a spacer of 94 aa residues and pNDH14 with a spacer of 123 aa residues were constructed as following. The coding region for the signal peptide of the α-amylase encoded by *amyQ* was amplified using ON25 and ON26 and pNDH16 as template. The amplicon was cut with *Bcl*I and *Bam*HI, inserted into *Bam*HI-linearized pNDH10 resulting in pNDH12. The coding region of the 3' end of fibronectin B consisting of the immediate 3' including the sorting motif and additional 123 codons, the spacer region (Fig. 2.3), was amplified using ON36 and ON40, the amplicon was cleaved with *Aat*II and *Sma*I and inserted into pNDH14 treated with the same enzymes resulting in pNHD14. The anchoring structure of the plasmids is illustrated as in Fig. 4.2.

2.8.6 Construction of plasmids using potential sorting sequences of *B. subtilis*

In order to study whether the putative *B. subtilis* sortases could recognize the potential sorting sequences, two plasmids that allow anchoring of AmyQ on the cell wall were constructed. In the previous report suggesting that the 123 aa-spacer between AmyQ and sorting sequence is optimal to anchor AmyQ on the cell wall. Plasmids were also generated, in which AmyQ was translationally fused to the putative sorting sequences with the 123 aa-spacers of YhcR123 and YfkN123 under the control of IPTG-inducible promoter P*grac*. First, the *amyQ* gene was generated by PCR using pKTH10 [111] as template together with ON29 and ON42, the amplicon was treated with *Bam*HI and *Aat*II and ligated into pHT01 (a derivative of pNDH33, see more detail in the section *4.1.2*) cut with the same enzymes resulting in pNDH88. Next, the coding regions of the 3' end of *yhcR* and *yfkN* consisting of the immediate 3' including the sorting motif and additional 123 codons, the spacer regions (Fig. 2.3) were amplified using ON47/ON48 and ON49/ON50 with *B. subtilis* 1012 chromosomal DNA as template, the amplicons were cleaved with *Aat*II and *Eco*RV and inserted into pNDH88 treated with *Aat*II and *Sma*I resulting in pNHD89 and pNDH90, respectively.

2.8.7 Construction of the knockout strains *yhcS* and *ywpE*

Two strains *B. subtilis* SZ59 (*yhcS* :: *cat*) and *B. subtilis* SZ60 (*ywpE* :: *erm*) used in this study are derivatives of *B. subtilis* 1012. The two putative sortase gene y*hcS* and *ywpE* were replaced by antibiotic markers as shown in Fig. 2.5.

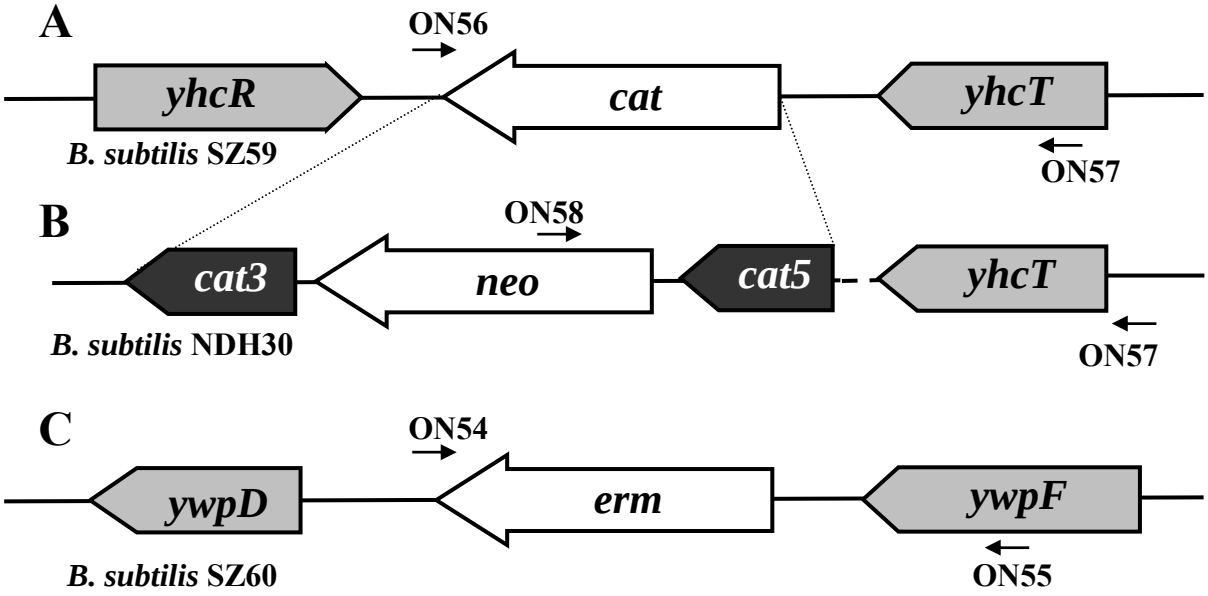


Fig. 2.5. Display of the chromosomal regions of the knockout strains SZ59 (A) and SZ60 (C) and construction of strain NDH30 (B). The positions of ONs used for verification of the null alleles by PCR are indicated.

To use these knockout strains with plasmids that carry a chloramphenicol resistant gene, the *cat* cassette in the strain SZ59 was replaced by a *neo* cassette, and then combines the two knockout stains in one. First, the plasmid *cat*5-*neo*-*cat*3 cassette was cloned into plasmid pBluescript II KS resulting plasmid pB-*cat*5-*neo*-*cat*3. This plasmid was treated with *Pvu*II and then transformed into the *B. subtilis* strain SZ59, Cm-sensitive and Neo-resistant colonies were selected; correct integration at the *cat* cassette was confirmed by PCR using ON57 and ON58, and one transformant (NHD30) was kept for further studies (Fig. 2.6). Second, chromosomal DNA of *B. subtilis* SZ60 was transformed into the strains NDH30, recombinants were selected on LB plates containing Erm and Neo. Correct integration at the *ywpE* locus was confirmed by PCR using ON54 and ON55, and one transformant (NDH31) was kept for further studies (Fig. 2.6).

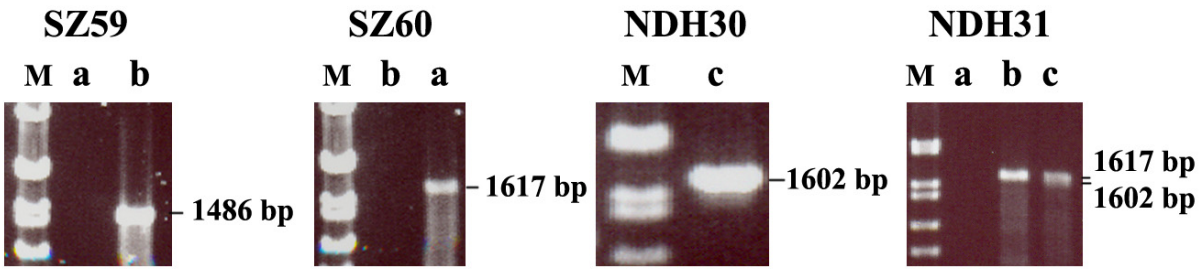


Fig. 2.6. Confirmation of the null alleles of the strains SZ59, SZ60, NDH30 and NDH31 by PCR. Three pairs of primers have been used: lane a, ON54 and ON55, specifically recognize chromosomal DNA of strain SZ60 (1617-bp PCR products); lane b, ON56 and ON57, strain SZ59 (1486-bp PCR products); lane c, ON57 and ON58 strain NDH30 (1602-bp PCR products). Strain NDH31 carries *ywpE* and *yhcS* double knockout. Using these tree pairs of primers and *B. subtilis* strain 1012 as negative control, no PCR product could be observed (data not shown).

2.8.8 Construction of the transcriptional fusions P*yhcS-lacZ* and P*ywpE-lacZ*

In order to measure expression of the *yhcS* and *ywpE* genes, the two strains NDH20 fusing the promoter *yhcS* with *lacZ* (P*yhcS-lacZ*) and P*spac* with *yhcS* (P*spac-yhcS*) (Fig. 2.7A) and NDH21 fusing the promoter *ywpE* with *lacZ* (P*ywpE-lacZ*) and P*spac* with *ywpE* (P*spac-ywpE*) (Fig. 2.7B) were constructed as follow. Part of *yhcS* containing the start codon and the complete *ywpE* gene were amplified using ON59/ON60 and ON61/ON62 with *B. subtilis* 1012 chromosomal DNA as template. The PCR products were cleaved by *Eco*RI and *Bam*HI and inserted into plasmid pMUTIN4 [161] treated with the same enzyme resulting in pNDH26 and pNDH27, respectively. Next, pNDH26 and pNDH27 were transformed into *B. subtilis* 1012, recombinants were selected on LB plates containing Erm. Correct integration at the *yhcS* locus was confirmed by PCR using ON57 and ON63 (which binds to P*spac* promoter) and at the *ywpE* locus by PCR using ON55 and ON63 (Fig. 2.8). These PCR products containing P*spac-yhcS* and P*spac-ywpE* were verified by sequencing using ON63. One correct transformant each was kept for further studies.

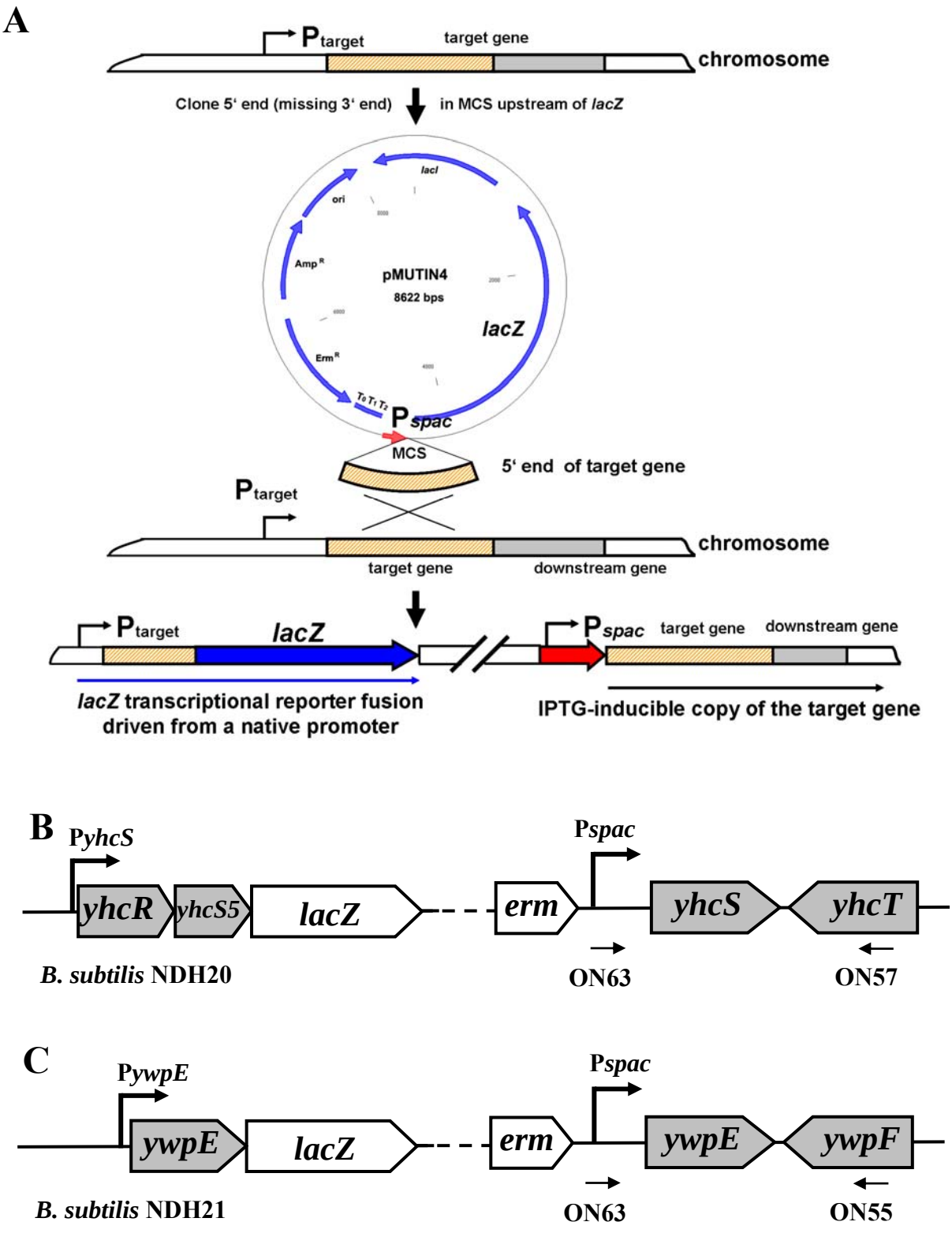


Fig. 2.7. Schematic representation of the chromosomal arrangement at the *yhcS* (B) and ywpE (C) locus. (A) Schematic representation of the principle to use pMUTIN4. Positions of *lacZ*, *yhcS*, *ywpE*, ONs and promoter *yhcS*, *spac* and of are indicated.

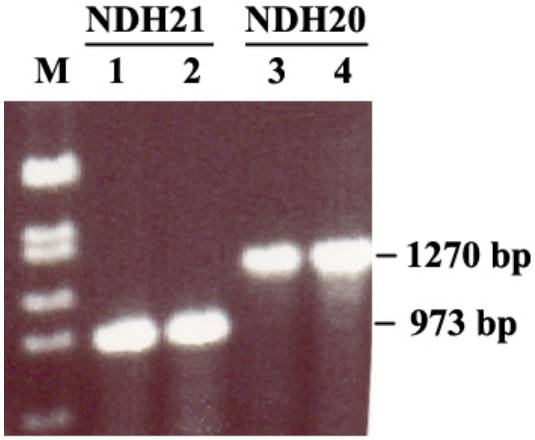


Fig. 2.8. Confirmation of *B. subtilis* strains NDH20 and NDH21 by PCR. The correct size of the PCR products using ON57 and ON63 with chromosomal DNA of the strain NDH20 is 1270 bp; using ON55 and ON63 with DNA of strain NDH21 is 973 bp. Two transformants of each strain were verified: NDH21 (lane 1, 2) and NDH20 (lane 3, 4).

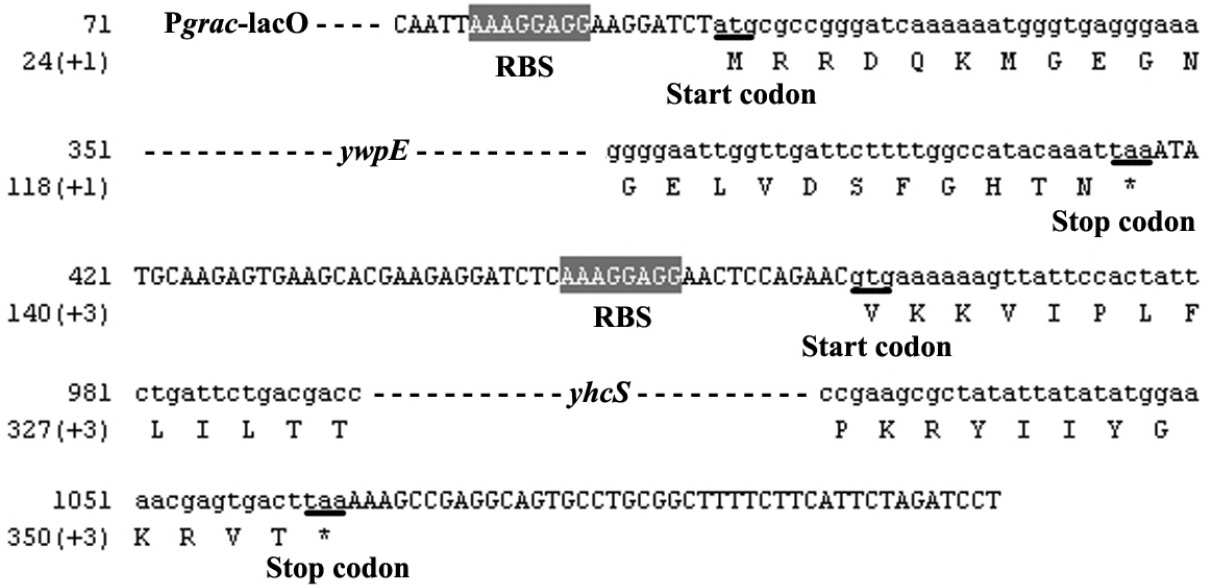


Fig. 2.9. DNA sequence around the start and stop codons of *ywpE* and *yhcS* as part of pNDH33-*ywpE-yhcS*. Both genes *ywpE* and *yhcS* are under the control of the IPTG-inducible promoter (P*grac-lacO*). RBS indicated in grey shading; start and stop codons of *ywpE* gene and *yhcS* gene are in small letters and underlined, which is marked by start symbol.

2.8.9 Construction of plasmids that allows overexpression *ywpE* and/or *yhcS*

To overexpress *yhcS* and/or *ywpE* in *B. subtilis* under the control of an IPTG-inducible promoter, the following plasmids were constructed. First, the coding sequence of the *ywpE* gene including its start codon was amplified by PCR using ON64 and ON62 with *B. subtilis* 1012 chromosomal DNA as template, the amplicon was cleaved with *Bam*HI and *Bgl*II and ligated into pNDH33 at its unique site *Bam*HI resulting in pNDH33-*ywpE*. Next, the gene

yhcS was amplified using ON65 and ON67 containing its RBS with *B. subtilis* 1012 chromosomal DNA as template; the PCR product was then cleaved by *Bgl*II and ligated into pNDH33-*ywpE* resulting pNDH33-*ywpE*-*yhcS* (Fig. 2.9). And the gene *yhcS* was amplified using ON65 and ON66, and the amplicon was cleaved by *Bgl*II and ligated into pNDH33 at *Bam*HI resulting pNDH33-*yhcS*.

2.8.10 Construction of strain WB800N

WB800 is an eight protease-deficient *B. subtilis* strain that is used for production of secreted heterologous proteins. This strain is resistant to Cm. To use this strain with the plasmid pNDH33, the *neo* cassette was inserted in the middle of *cat* cassette resulting strain WB800N. The *Pvu*II-treated plasmid pB-*cat*5-*neo*-*cat*3 was transformed into WB800 and plated on the indicator medium, calcium caseinate (Oxoid) plates with Neo. Colonies without halos (compared with the strain 1012) were checked for the sensitive of Cm and resistant to Neo. One transformant was kept for further study.

3 Results

This work was focused on the construction of plasmids using the theta mode of replication to express recombinant proteins in different cellular compartments including the cytoplasm, the culture supernatant and the cell wall of *B. subtilis*. First of all, the series pHCMCs of pHCMC plasmids were constructed allowing protein synthesis in the cytoplasm. Next, one of these plasmids was improved using the strong P*grac* promoter allowing both intra- and extracellular production of recombinant proteins. At the end, a system to anchor proteins on the cell wall was established and the pathway to sort proteins to the cell wall of *B. subtilis* was studied.

3.1 Construction of plasmid-based expression and secretion vectors for *B. subtilis*

This part describes the construction of the series of expression vectors pHCMC03-pHCMC05 exhibiting structural stability in *B. subtilis* and the construction of pNDH33 and pNDH37 using pHCMC05 as backbone allowing high level of proteins expression in the cytoplasm and in the culture supernatant, respectively.

3.1.1 Analysis of plasmid-based expression vectors for *B. subtilis* exhibiting full structural stability

This chapter describes the analysis of expression vectors containing three different inducible promoters. The stress-inducible P*gsiB* in pHCMC03, the xylose-inducible P*xylA* in pHCMC04 and the IPTG-inducible P*spac* in pHCMC05 have been fused to either *lacZ* and/or *bgaB* allowing determination of their inducibility and promoter strength by measuring the β-galactosidase activity. In addition, expression of *htpG* as an additional reporter was followed by Western blot.

3.1.1.1 Analysis of transcriptional fusions between the three promoters and the reporter genes

To analyse the versatility of the three different expression vectors, the *lacZ* and/or *bgaB* reporter gene was fused to the three promoters, and β-galactosidase activities were measured. It should be emphasised that the enzymatic activities obtained with *lacZ* and *bgaB* can not be

directly compared due to the different enzymatic characteristics. A plasmid without any promoter and the *bgaB* reporter gene (pHCMC01-*bgaB*) served as a control whether a promoter located on the plasmid could activate *bgaB* (Table 3.1). No activity could be measured indicating that activation of any gene inserted into any pHCMC plasmid by a plasmid-driven promoter does not take place. With P*gsiB*, between 12 and 20 units were measured in the absence of any stress factor, which increased about 13-fold after challenge with acid shock (Table 3.1). In contrast, ethanol and heat shock resulted only in an about 3-fold induction. When the β-galactosidase activities were measured in strain IS58, the background activity was reduced to 3–7 units, and increased about 7-fold after acid shock and 8- and 12-fold after heat and ethanol shock, respectively. This strain was chosen because it has been reported to express σ^B-dependent genes at a low basal level in the absence of stress factors known to induce this regulon (M. Hecker, personal communication). This observation could be confirmed here, but the highest activity was lower as compared to strain 1012 during growth under acid and heat shock conditions.

Plasmid pHCMC04 carries a xylose-inducible promoter and does not produce any detectable β-galactosidase activity in the absence of the inducer (Table 3.1). After addition of 0.5% xylose, up to 9 units were measured. The *B. subtilis* 168 genome codes for genes involved in catabolism of xylose (*xylAB*), and these two genes are under the transcriptional control of the XylR repressor [74], but this strain cannot actively transport xylose into the cell [137]. It raises the question whether the xylose, which must be taken up by the cell to induce the xylose expression cassette derived from *B. megaterium* [127], can be degraded, affecting production of the recombinant proteins. To accomplish this goal, β-galactosidase activities produced in the wild-type strain and its isogenic *xylAB* knockout strain were compared during the induction with 0.1% xylose for 5 h. Since only a minor effect was measured, degradation of xylose by the *xylAB* operon under these experimental conditions does not play any significant role (data not shown). Since it has been published that glucose acts as an anti-inducer by binding to XylR [26], it was asked whether increased expression could be observed in the glucose-free NAPS medium. As shown in Table 3.1, the measured β-galactosidase activities with plasmid pHCMC04 (given in parentheses) do not differ significantly from those where growth occurred in LB medium. It suggests that the LB medium contains only traces of glucose if any.

Table 3.1. β-Galactosidase activities from plasmids pHCMCs series

Plasmid	Promoter	Inducer	Reporter gene	Recipient strain	Time [min] after induction					
					0	5	10	20	30	60
pHCMC01	-	-	*bgaB*	1012	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
pHCMC03	P_{gsiB}	acid shock	*lacZ*	1012	19.8 ± 3.5	19.2 ± 3.7	39.3 ± 5.4	74.4 ± 5.5	188 ± 63	255 ± 48
pHCMC03	P_{gsiB}	acid shock	*lacZ*	IS58	7.7 ± 0.3	11.1 ± 2.0	24.3 ± 2.6	33.6 ±4.2	32.5 ± 3.0	56.5 ± 5.2
pHCMC03	P_{gsiB}	ethanol	*bgaB*	1012	12.0 ± 5.3	15.7 ± 3.4	19.1 ± 5.9	32.2 ± 8.2	20.9 ± 1.7	32.8 ±1.3
pHCMC03	P_{gsiB}	ethanol	*bgaB*	IS58	3.1 ± 0.5	20.8 ± 5.1	19.2 ± 3.7	30.6 ± 7.5	32.8 ± 9.2	36.7 ± 5.7
pHCMC03	P_{gsiB}	heat shock	*bgaB*	1012	12 ± 5.3	13 ± 1.4	21.9 ± 3.4	23.7 ± 10.2	20.4 ± 3.0	33.7 ± 1.1
pHCMC03	P_{gsiB}	heat shock	*bgaB*	IS58	3.1 ± 0.5	18.6 ± 9.0	22.8 ± 6.8	20.3 ± 3.2	19.1 ± 2.1	23.4 ± 3.9
pHCMC04	P_{xylA}	0.5% xylose	*bgaB*	1012	< 0.1 (0.17 ± 0.01)	4.7 ± 1.7 (1.01 ± 0.31)	7.9 ± 1.4 (2.54 ± 0.35)	9.6 ± 1.6 (4.91 ± 0.79)	9.4 ± 2.2 (6.49 ± 0.82)	8.9± 0.8 (9.28 ± 1.23)
pHCMC05	P_{spac}	0.1 mM IPTG	*bgaB*	1012	< 0.1	2.5 ± 0.4	2.6 ± 0.1	3.5 ± 0.7	4.2 ± 0.5	3.9 ± 0.9
pHCMC05	P_{spac}	0.5 mM IPTG	*bgaB*	1012	< 0.1	2.9 ± 0.9	4.5 ± 1.7	5.9 ± 1.7	6.9 ± 1.7	6.3 ± 1.5

Cells were grown in LB medium at 37 °C to the mid-logarithmic growth phase and then induced as indicated. The data given in parentheses have been obtained by growth in NAPS medium [71]. Data relating to pHCMC03-*bgaB* were obtained from our co-worker [104]. The β-galactosidase activities are given in units/mg protein. Each experiment has been carried out at least three times and the standard deviations are given.

Plasmid pHCMC05 carries the IPTG-inducible promoter P*spac*. As already observed with pHCMC04, no detectable β-galactosidase activity was measured in the absence of IPTG, and the activity reached 4.2 units after addition of 0.1 mM and 6.9 units after addition of 0.5 mM IPTG (Table 3.1).

In summary, the vectors pHCMC03 to pHCMC05 can be induced either by a stress factor or by addition of an inducer.

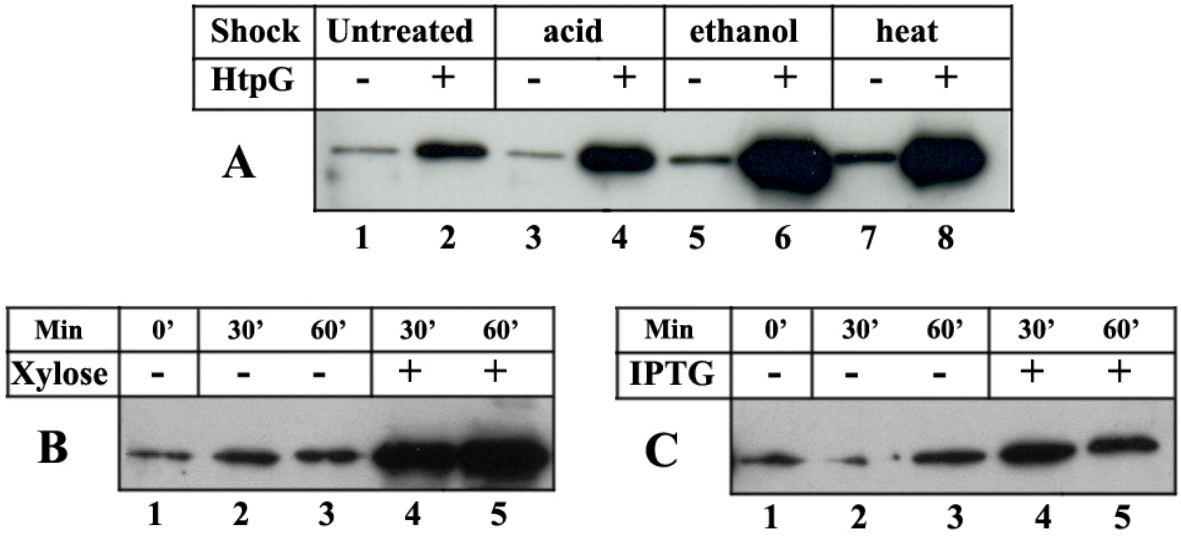


Fig. 3.1. Dectection of the HtpG protein by immunoblot analysis. *B. subtilis* strain 1012 carrying one of the plasmids pHCMC01, pHCMC03-*htpG*, pHCMC04-*htpG*, or pHCMC05-*htpG* was grown either in LB medium with appropriate supplements at 37 °C to the mid-exponential growth phase (OD_{578} ~ 0.8). Then, the cultures were divided into subcultures where one was further grown without induction, while the others were either challenged with different stress conditions (A), or induced with 1% xylose (B) or 1 mM IPTG (C). Aliquots were withdrawn immediately before treatment (t = 0) and 30 (t = 30), and 60 min (t = 60) after treatment. All samples were prepared for SDS–PAGE and for immunoblot analysis. Equal amounts of protein were applied per lane, and the blots were probed with α-HtpG. (A) pHCMC01 (lane 1, 3, 5 and 7) and pHCMC03-*htpG* (lane 2, 4, 6, 8), induced by acid, ethanol and heat for 30 min as indicated. (B) pHCMC04-*htpG* uninduced t = 0 (lane 1), t = 30 (lane 2) and t = 60 (lane 3); pHCMC04-*htpG* induced with 1% xylose, t = 30 (lane 4) and t = 60 (lane 5). (C) pHCMC05-*htpG* uninduced, t = 0 (lane 1), t = 30 (lane 2) and t = 60 (lane 3); pHCMC05-*htpG* induced with 1 mM IPTG, t = 30 (lane 4) and t = 60 (lane 5).

3.1.1.2 Immunoblot analysis to measure the strength of the P*gsiB*, P*xylA* and P*spac* promoters

While measurement of β-galactosidase activities just yields an indication of the promoter strength, straight visualization of a recombinant protein by Western blot provides a more direct measure of protein production levels. To achieve this goal, the heat shock gene *htpG* [143] was fused to all three promoters, while the empty vector served as control. *B. subtilis* strain 1012 carrying either pHCMC03-*htpG* or pHCMC01 was grown in supplemented LB medium (see legends to Fig. 3.1) and challenged with the three stresses described above. Due to the presence of the wild-type copy of *htpG* in the chromosome, there is a background level of HtpG protein (Fig. 3.1A, lanes 1, 3, 5, and 7) which increased after addition of the second copy on the plasmid (lane 2). After challenge with acid stress, the amount of HtpG protein was enhanced while that in the control strain remained constant (compare lanes 3 and 4). A significant stronger increase was observed after ethanol and heat treatment (lanes 6, 8).

When the strain carrying *htpG* fused to the xylose-inducible promoter (pHCMC04-*htpG*) was analysed after addition of 1% xylose, a significant amount of HtpG protein accumulated within 60 min (Fig. 3.1B, lane 5). In contrast, lower amounts of HtpG accumulated after induction of the strain carrying pHCMC05-*htpG* with IPTG (lanes 7–10). In summary, all three expression vectors allow increased expression of *htpG* after challenge with the appropriate inducer, where the strain carrying P*gsiB* yielded the highest amount of recombinant protein after ethanol and heat stress, followed by the strain carrying the xylose cassette. To our surprise, the IPTG-inducible cassette produced only modest amounts of recombinant protein.

3.1.1.3 Structural and segregational stability of the pHCMC plasmids

To check for structural stability of the pHCMC plasmids, all three vectors containing the *bgaB* reporter gene were grown in LB medium containing chloramphenicol for about 100 generations (involving several subcultures) and then plated on LB plates containing Xgal. It turned out that with all four recombinant plasmids, all colonies exhibited a blue phenotype, demonstrating structural stability of these four recombinant plasmids (data not shown).

We also checked for segregational stability using pHCMC04-*bgaB* and pHCMC05-*bgaB*. Again, *B. subtilis* 1012 carrying these plasmids was grown in LB medium this time in the

absence of chloramphenicol for up to 100 generations, aliquots were taken after different generations, plated on LB plates, and checked for the presence of the plasmid by replica plating on LB plates containing Cm. While pHCMC04-*bgaB* turned out to be fully stable over at least 100 generations (Fig. 3.2), which is in full agreement with published data for the original plasmid pMTLBs72 [153], the second plasmid, pHCMC05-*bgaB*, suffered from segregational instability (Fig. 3.2). After 60 generations, about 60% of the cells had lost the plasmid. The reason for this difference is not known, but this result indicates that cells carrying certain recombinant pHCMC plasmids should be grown in the presence of Cm to avoid loss of the plasmids.

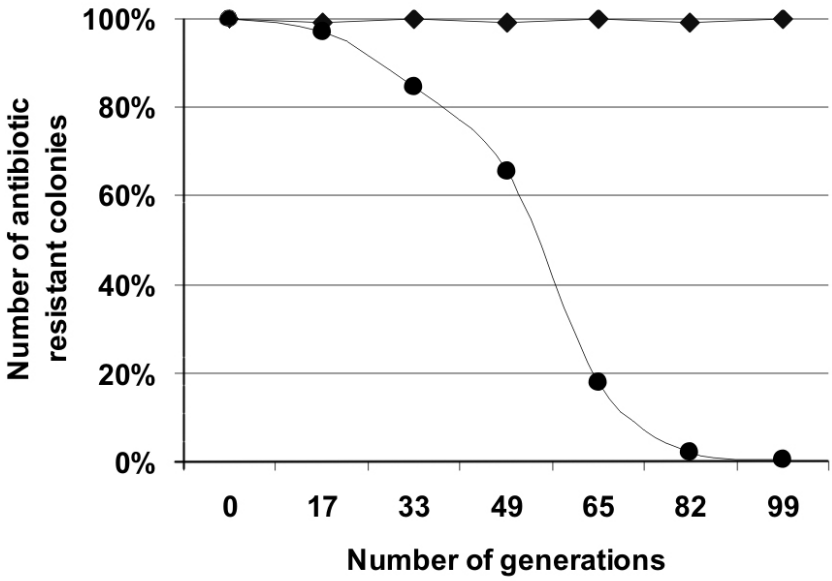


Fig. 3.2. Segregational stability of two recombinant plasmids. *B. subtilis* 1012 carrying pHCMC04-*bgaB* (♦) and pHCMC05-*bgaB* (●) was analysed as described under Materials and Methods. The number of Cm^R colonies is given.

For the first time, three different plasmid-based expression systems have been constructed and shown to exhibit structural stability for at least 100 generations of growth. Next, experiments were carried out to improve this expression system to obtain a lower background level in the absence of inducer, and a higher induction factor by increasing the promoter and ribosome-binding strength, and the stability of the transcript. The improved version of plasmid pHCMC05 is described in the next chapter. Furthermore, plasmid pHCMC04 was used to construct an anchoring vector to immobilize proteins on the cell wall as described in section 3.2.1.

3.1.2 Novel plasmid-based expression vectors for intra- and extracellular production of recombinant proteins in *B. subtilis*

The above chapter showed that pHCMC05 is able to control the expression of the reporter gene only in a modest amount. Here, the improvement of this vector is reported using the stronger P*grac* promoter leading to pNDH33. This plasmid allows expression of proteins in the cytoplasm while its derivative pNDH37 allows to secret protein into the culture medium. The versatility of this plasmid will be described by using different reporter proteins.

3.1.2.1 Construction of the expression vector pNDH33 and secretion vector pNDH37

We and others observed that the *groE* promoter of *B. subtilis* is a strong promoter most probably due to the presence of an UP element [56, 95]. Based on these observations, this promoter was used to study whether it can drive expression of recombinant genes. Since the *groE* promoter is constitutive and high-level production of many recombinant proteins can be deleterious to the cells, a regulatory element had to be added. The *lac* operator (*lacO*) of *E. coli* K12 was chosen, which had already been successfully used in combination with different promoters such as P*spac* [177]. High-level expression is not only dependent upon a strong regulatable promoter and 5' and 3' mRNA stabilizers, but also on an efficient ribosome binding site (RBS) sequence. Here, that of the *gsiB* gene was chosen, which had been shown to act as a strong RBS and to enhance the half-life of the original transcript [68].

To obtain the expression vector pNDH33 (Fig. 2.2A), the P*spac* promoter of pHCMC05 was replaced by the new expression cassette P*grac* consisting of the *groE* promoter, the *lacO* operator with the addition of GG before and CC after *lacO* to increase ΔG of the putative stem-loop structure, and the *gsiB* RBS sequence as described under Materials and Methods. The unique restriction sites *Bam*HI, *Xba*I, *Aat*II, and *Sma*I can be used to insert recombinant genes into this expression vector. The expression-secretion vector pNDH37 (Fig. 2.2B) was obtained by fusing the coding region for the signal sequence of the *amyQ* gene [111] to the RBS sequence of pNDH33. Here, the unique restriction sites *Bam*HI, *Xba*I, *Aat*II, and *Sma*I can be also used for the integration of recombinant genes to allow secretion of their products.

3.1.2.2 The *bgaB* reporter gene can be induced about 1300-fold

To assay for the functionality of the expression vector pNDH33, the *bgaB* reporter gene was fused to the IPTG-inducible *groE* promoter resulting in the recombinant plasmid pNDH33-

bgaB. Next, this plasmid was transformed in the *B. subtilis* strain 1012 and the β-galactosidase activity was measured before and after addition of IPTG (Fig. 3.3). While the activity was extremely low in the absence of IPTG (about 0.7 units), it increased to 946 units 4 h after induction (Fig. 3.3) representing an induction factor of 1350. Induction occurred in a biphasic way with a slow induction rate during the first hour followed by a fast induction rate within the next 60 min which further increased slowly over the next two hours. The behaviour for the induction pattern is unknown. If the induction factor measured here was compared to those reported, it was 30-fold higher than the one measured with pHCMC05-*bgaB* and 60-fold higher than the one measured with pA-spac-*bgaB* in the strain IHA01-Spac-BgaB (data not shown).

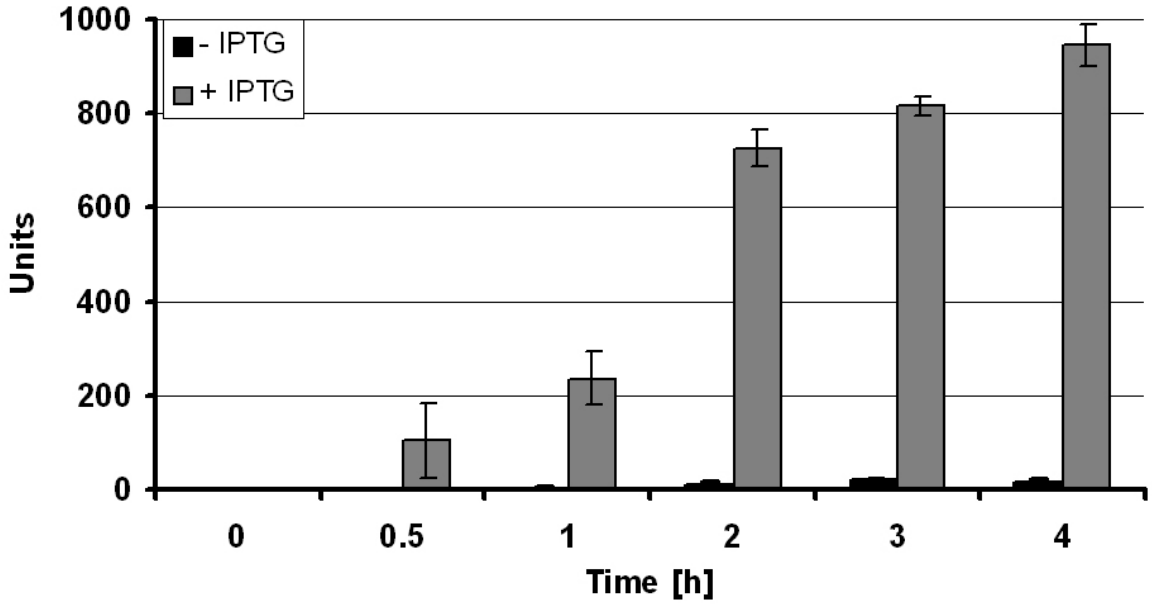


Fig. 3.3. Induction of β-galactosidase activity. *B. subtilis* strain 1012 harbouring pNDH33-*bgaB* was grown in LB to the mid-log phase. Then, the culture was divided into two subcultures where one was further grown in the absence (black columns) and the other in the presence of 1 mM IPTG (grey columns). Aliquots were removed for determination of β-galactosidase activities before addition of IPTG (t = 0) and at the time points indicated. The complete experiment was repeated three times and the standard deviations are indicated.

The production of the *bgaB* enzyme was also monitored directly by using the three different recombinant expression vectors by SDS–PAGE and Coomassie blue staining. As shown in Fig. 3.4, induction of both pHCMC05-*bgaB* and pA-spac-*bgaB* for 2 and 3 h did not result in the appearance of any additional band with a molecular mass of BgaB (78 kDa), while

pNDH33-*bgaB* led to the production of a distinct band (Fig. 3.4), thus corroborating the measurements of the enzymatic activities. In summary, the newly constructed expression cassette P*grac* is superior to those published and used so far.

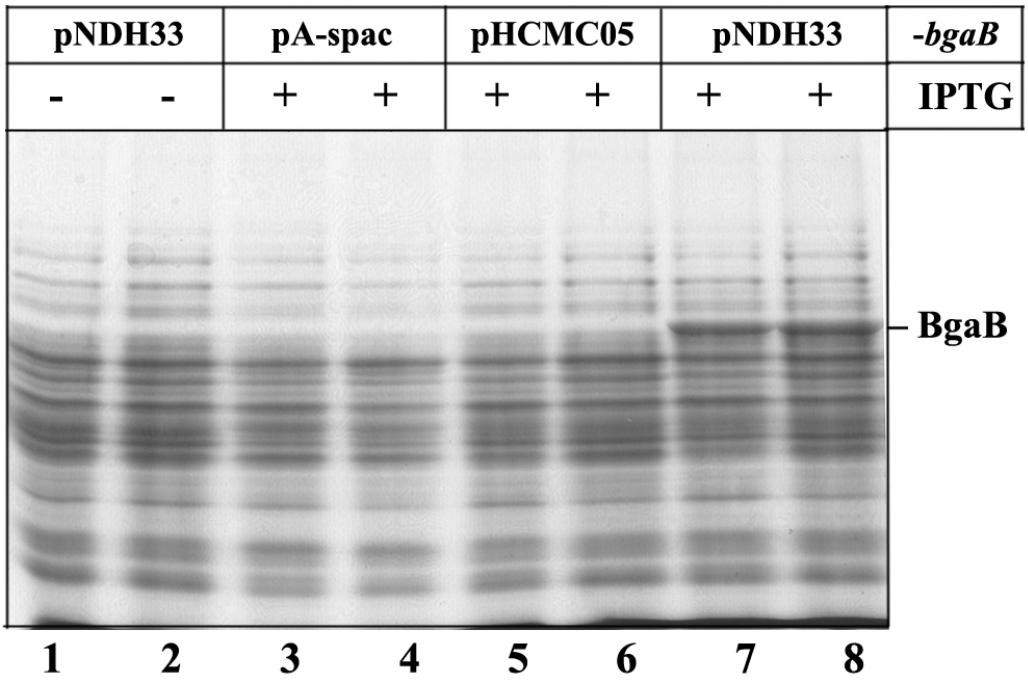


Fig. 3.4. Identification of the *bgaB* gene product. *B. subtilis* 1012 strains carrying either pNDH33-*bgaB* or pA-spac-*bgaB* or pHCMC05-*bgaB* were grown as described in the legend to Fig. 3.1, separated by SDS-PAGE and stained with Coomassie blue. All samples were taken after 2 h (lane 1, 3, 5, 7) or 3 h (lane 2, 4, 6, 8) after induction. Lane 1 and 2 contain samples without IPTG and lane 3-8 with 1 mM IPTG. The position of BgaB is marked.

3.1.2.3 The expression vector pNDH33 allows production of recombinant proteins up to 16% of the total cellular protein

To further evaluate the efficiency of the pNDH33 expression vector, the two genes *htpG* and *pbpE* were fused separately to the P*grac* promoter. Production of both proteins was followed by SDS–PAGE as described before. The quantity of protein was calculated by Quantity One (BioRad). The amount of HtpG protein 1 h after IPTG induction represented 10.8% of the total cellular proteins and slightly decreased thereafter to 9.1% (Fig. 3.5, HtpG). When the production of another protein, PBP4* was analysed, this accumulated to even 14.1% 1 h after induction with a further slight increase to 16.4% after 2 h (Fig. 3.5, PBP4*).

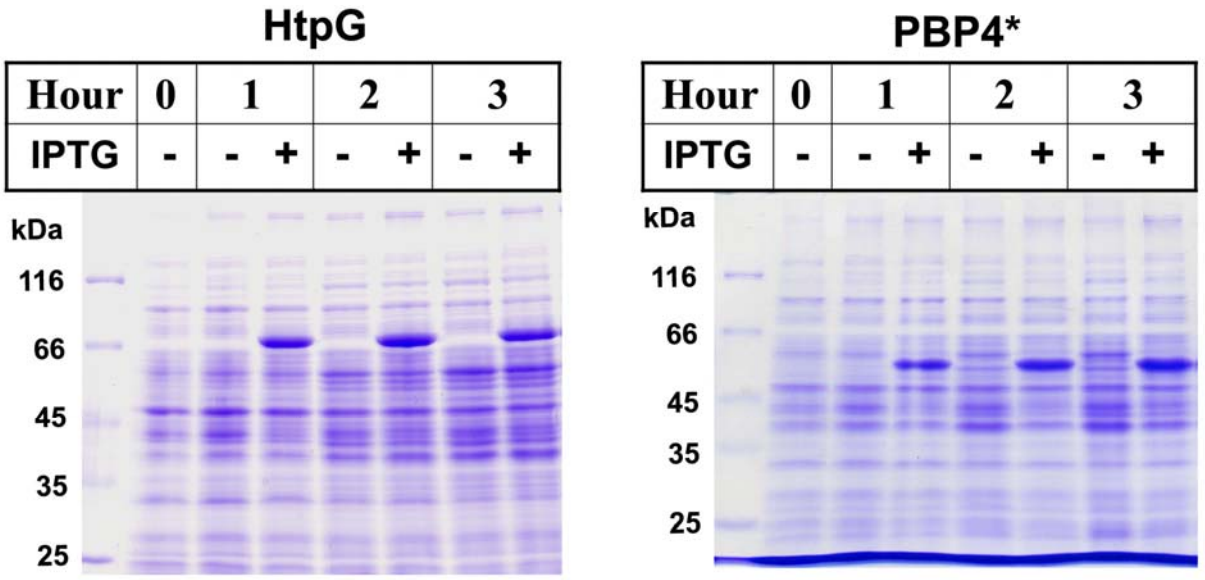


Fig. 3.5. Identification of the *htpG* and *pbpE* gene products. *B. subtilis* 1012 carrying either pNDH33-*htpG* (HtpG) or pNDH33-*pbpE* (PBP4*) were grown as described in the legend to Fig. 3.1, separated by SDS-PAGE and stained with Coomassie blue.

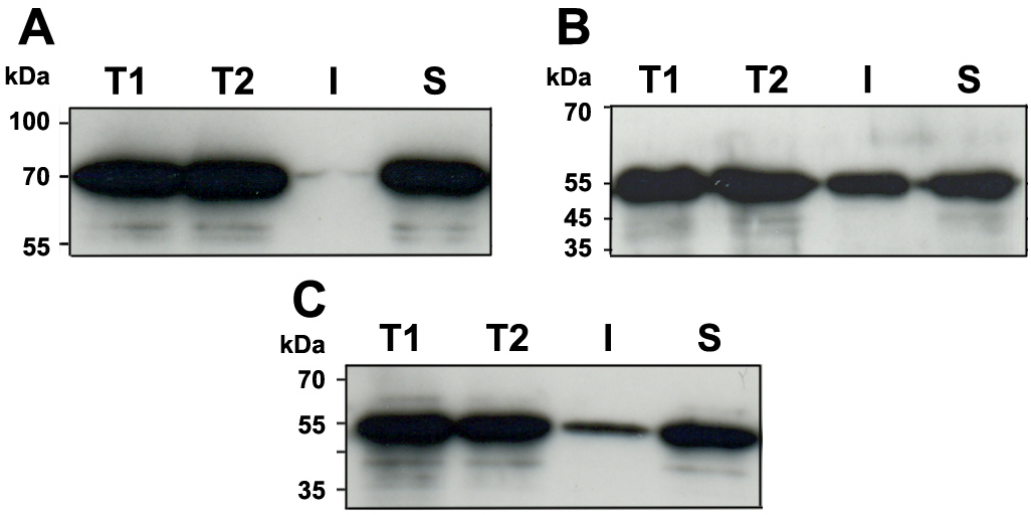


Fig. 3.6. Immunoblot analysis of HtpG and PBP4*. *B. subtilis* strain 1012 carrying pNDH33-*htpG* (A), 1012 with pNDH33-*pbpE* (B) and AS1 (1012 Δ*hrcA*) with pNDH33-*pbpE* (C) were grown as described in legend to Fig. 3.1. Cells were lysed by sonification, and the cellular lysate was applied either directly (T1) or after a centrifugation step to remove cellular debris (T2); I, insoluble proteins present within the pellet; S, soluble proteins from the supernatant. Molecular weight markers are indicated.

Next, it was examined whether all the recombinant proteins were present in a soluble form. Cells were lysed by sonification and the recombinant proteins were analysed by Western blot after a centrifugation step to separate soluble from insoluble proteins. As can be seen in Fig. 3.6A, all the HtpG protein produced under overexpressing conditions remained soluble. In contrast, part of the PBP4* protein turned out to be insoluble (Fig. 3.6B). The reason for the different behaviour of these two proteins could reside in the fact that PBP4* is attached to the membrane via hydrophobic patches [116]. It is widely recognized that coexpression of molecular chaperones can assist protein folding and that in at least some cases this leads to increased production of soluble and active protein [106]. The most abundant and physiologically important chaperones are the DnaK and the GroE teams whose synthesis is under the negative control by the HrcA transcriptional repressor in *B. subtilis* [142, 178]. It comes into question whether the amount of insoluble PBP4* protein can be reduced by increasing the amount of the major chaperones DnaK and GroEL by making use of an *hrcA* knockout where both chaperones are expressed at a higher level. Indeed, overexpression of *pbpE* in the *hrcA* null mutant significantly reduced the amount of insoluble PBP4* (Fig. 3.6C). This result is in agreement with an earlier finding that constitutive expression of both chaperone operons reduced the amount of single-chain antibody fragments [172].

3.1.2.4 Expression-secretion vector pNDH37 allows regulated secretion of exoproteins

Since the P*grac* promoter allows high-level production of recombinant proteins intracellularly, it was asked whether this expression cassette can also be used to obtain high-level secretion of exoproteins. First, the *amyQ* gene of *B. amyloliquefaciens* coding for an α-amylase and the *celA* and *celB* genes of *C. thermocellum* coding for cellulases were fused to the P*grac* promoter. To screen for the production of the respective exoenzyme, cells were plated on indicator plates containing either soluble starch or CMC in the absence and in the presence of the inducer IPTG. After about 24 h of growth, the production of exoenzyme was visualized by staining the plates. One representative colony each is shown in Fig. 3.7. While this colony is surrounded by a wide halo after induction with IPTG, a very faint halo became apparent in the absence of the inducer (Fig. 3.7, AmyQ). Comparable pictures were obtained with cellulase A (Fig. 3.7, CelA) and cellulase B (Fig. 3.7, CelB). In summary, this plate assay unequivocally demonstrates that the P*grac* promoter can promote regulated secretion of three different exoenzymes.

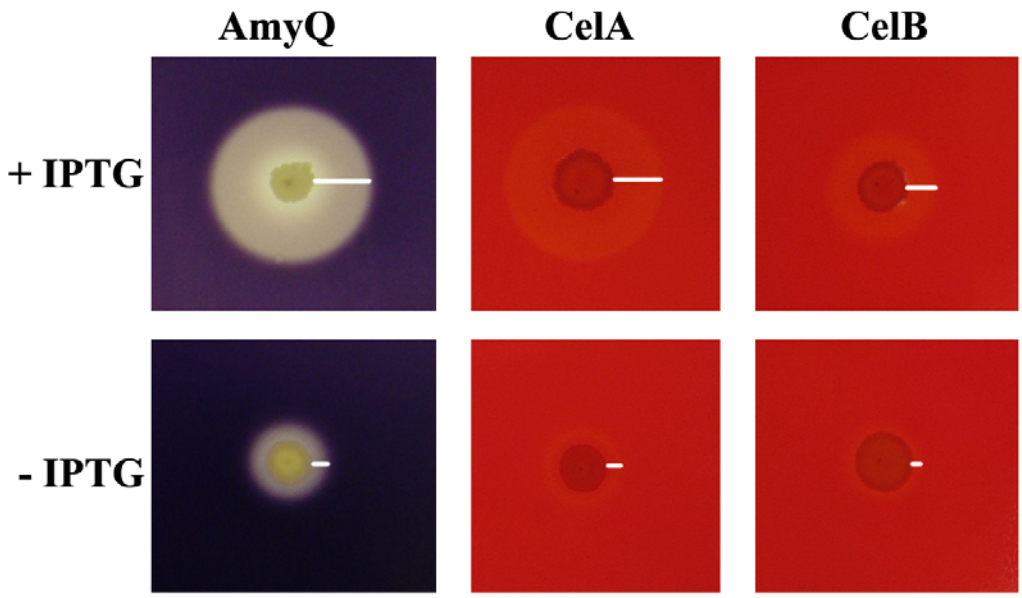


Fig. 3.7. Visualization of extracellular enzyme activity. Single colonies of the *B. subtilis* strains indicated grown for 24 h on indicator plates containing either 2% insoluble starch (AmyQ) or 0.5% CMC (CelA and CelB) and stained with I_2/KI (AmyQ) or congo red solution (CelA and CelB). White bars indicate the haloes around the colonies.

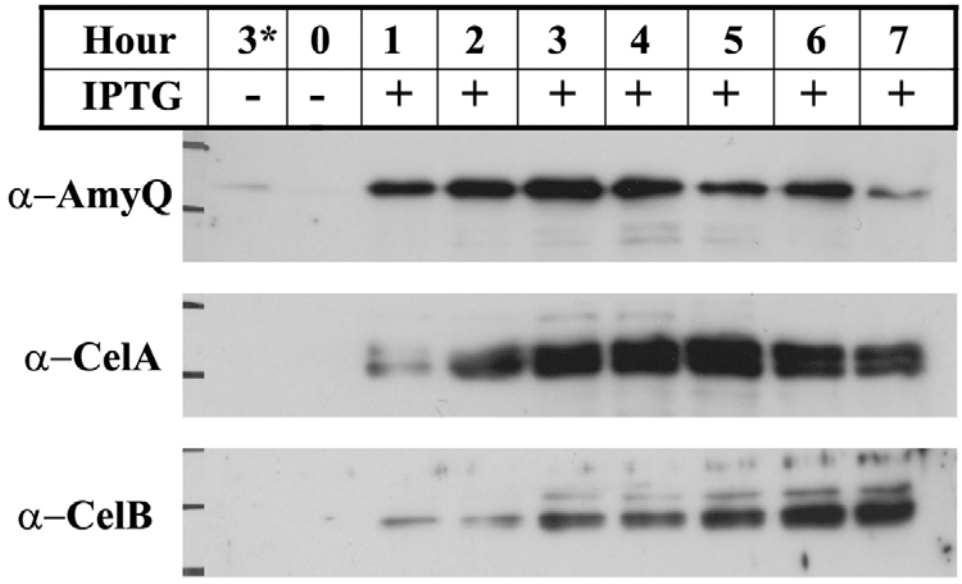


Fig. 3.8. Detection of extracellular enzymes by immunoblotting. Strains *B. subtilis* 1012 containing the plasmids pNDH37-*amyQ*, pNDH37-*celB* and pNDH37-*celA* were grown as described in legend to Fig. 3.1. 3*, 3 h culture in the absence of IPTG; 0, immediately before addition of 1 mM IPTG at an OD_{578} of 0.8; 1 to 7 h after induction. The antibodies used are indicated on the left side.

Next, the amount of exoenzyme secreted into the culture medium after IPTG induction was assayed directly by Western blot. As they can be seen from Fig. 3.8, upper panel, α-amylase started to accumulate 1 h after IPTG addition, reached a peak after 3 h, and thereafter declined, while cellulase A reached its highest amount about 5 h after induction (middle panel, α-CelA). In contrast, cellulase B accumulated late after induction (lower panel, α-CelB). This result shows that though the expression signals are identical for all three genes, secretion follows different kinetics. The α-amylase activity within the culture medium was also measured and showed a constant increase up to about 4 h post-induction (Fig. 3.9). Thereafter, the α-amylase activity remained constant up to at least 7 h.

The amount of α-amylase expressed from pNDH37-*amyQ* was also analysed and compared to the amounts expressed from pKTH10. In the latter case, *amyQ* is expressed from its own promoter [111]. First, no decrease was seen after IPTG induction for at least 7 h (Fig. 3.9), and second, the amounts produced were comparable to those produced from pKTH10 (Fig. 3.10). The amount of cellulase A and B within the supernatant were also analysed using the same technique. Both proteins appeared 1 h post-induction and increased up to about 4 h (Fig. 3.11).

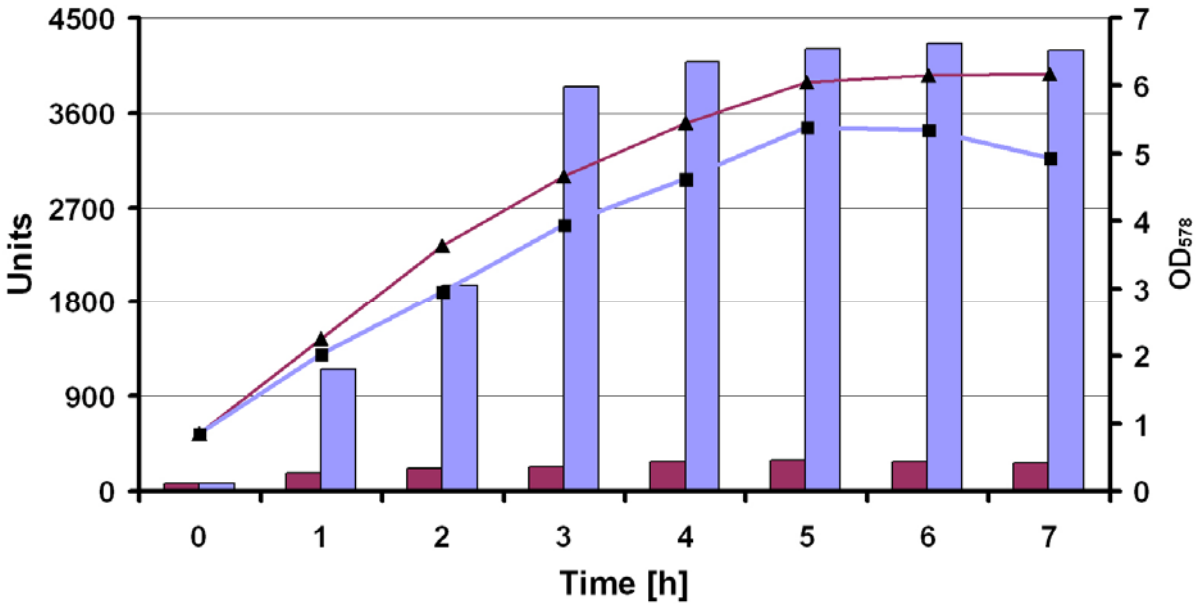


Fig. 3.9. α-Amylase activities in the culture supernatant. *B. subtilis* strain 1012 harbouring pNDH37-*amyQ* was grown in LB either in the absence (red line with ▲ and red column) or presence of 1 mM IPTG (blue line with ■ and blue column). Aliquots were removed from the two cultures at the time points indicated, centrifuged, and the supernatants were analysed for α-amylase activities.

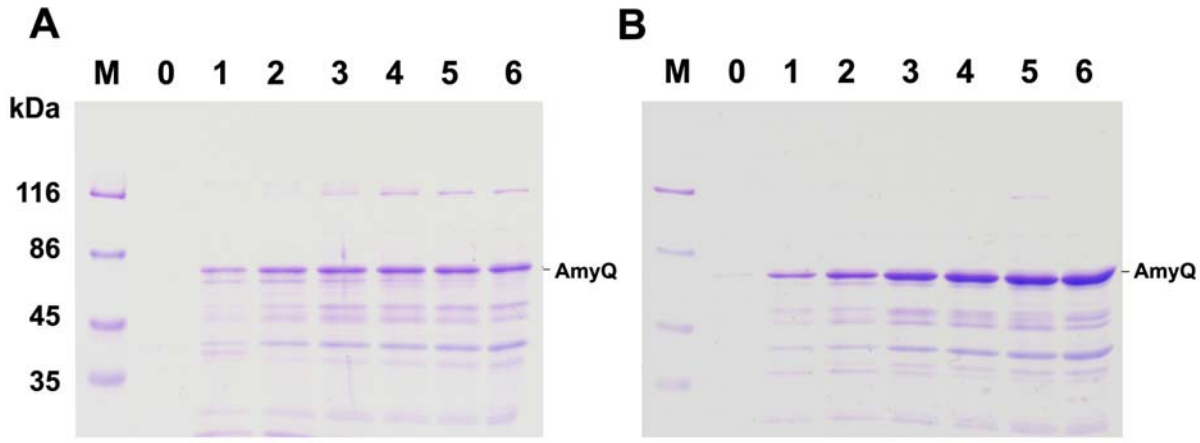


Fig. 3.10 Detection of the α-amylase expressed from two different plasmids. *B. subtilis* strains 1012 carrying either pNDH37-*amyQ* (A) or pKTH10 (B) were grown in LB to 0.8 of OD_{578}, set at 0 h. Cells were induced with 1 mM IPTG; aliquots were taken immediately before addition of 1 mM IPTG (0) and 1 to 6 h after induction as indicated. The aliquots were centrifuged and 300 µl of the supernatant were analysed for the presence of the secreted exoenzyme by SDS-PAGE and Coomassie blue staining.

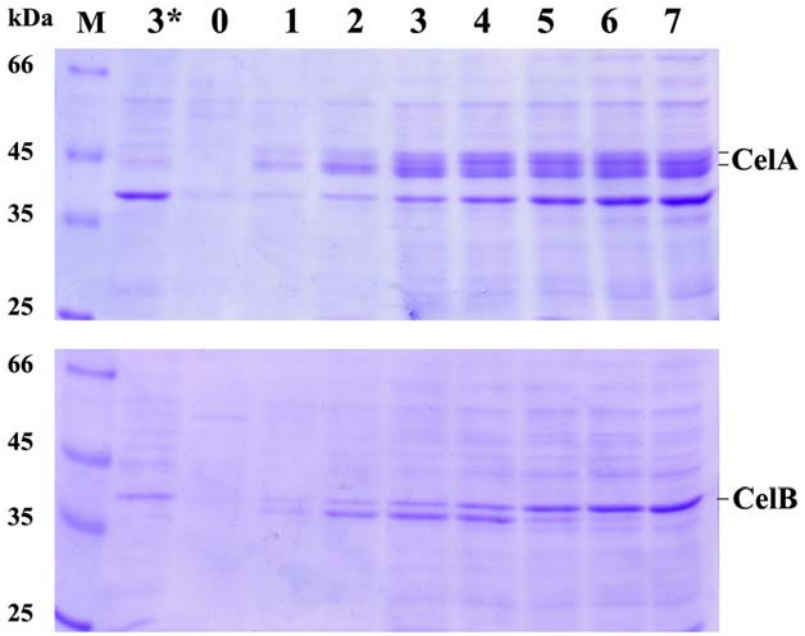


Fig. 3.11. Detection of cellulose A and B within the supernatants. *B. subtilis* strains 1012 carrying either pNDH37-*celA* (upper panel) or pNDH37-*celB* (lower panel) were grown and samples were collected as described in the legend to Fig. 3.8. Culture supernatants were collected and analysed for the presence of the secreted exoenzyme by SDS-PAGE and Coomassie blue staining as described in legend to Fig. 3.10. The positions of CelB and CelA are marked.

3.2 Study of immobilization of proteins on the surface of *B. subtilis* cells

By using the plasmids pHCMC04 and pNDH33, immobilization of proteins on the surface of *B. subtilis* was studied in which AmyQ was employed as model protein. In addition, the function of the two putative sortases YhcS and YwpE to anchor their potential substrates YhcR and YfkN on the cell wall was investigated.

3.2.1 Immobilization of proteins on the surface of *B. subtilis* cells

Next, an experimental system will be described allowing the immobilization of proteins on the surface of *B. subtilis* cells using AmyQ as model protein, the *L. monocatogenes* sortase A and the sorting sequence of *S. aureus* FnBPB containing the sorting motif LPETG.

3.2.1.1 Expression of the sortase A of *L. monocytogenes* in *B. subtilis*

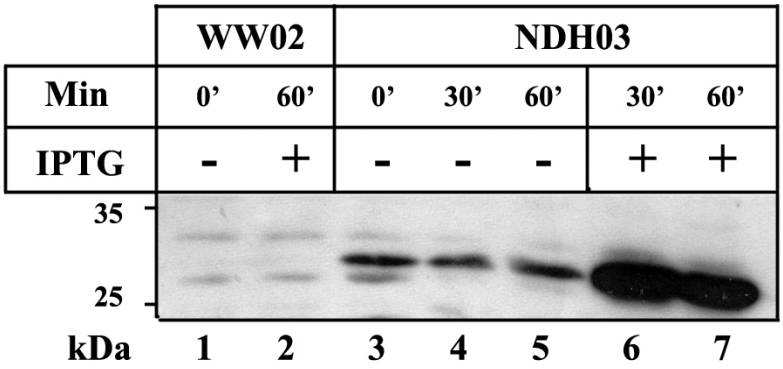


Fig. 3.12. Detection of sortase A of *L. monocytogenes* in extracts of *B. subtilis*. *B. subtilis* strains WW02 and NHD03 were grown as described in the legend to Fig. 3.1. Aliquots were taken at t = 0, 30 and 60 min and processed for immunoblotting. Strain WW02 uninduced (lane 1) and IPTG-induced for 60 min (lane 2); strain NDH03 uninduced at t = 0 (lane 3), t = 30 (lane 4) and t = 60 (lane 5); strain NDH03 induced with IPTG at t = 30 (lane 6) and *t* = 60 (lane 7). Antibodies against *L. monocytogenes* sortase A were used. Molecular weight markers as indicated on the left margin.

The *srtA* gene of *L. monocytogenes* was fused to the IPTG-inducible P*spac* promoter and integrated ectopically at the *lacA* locus resulting in strain NDH03. To demonstrate regulatable expression of the *srtA* gene, *B. subtilis* strains WW02 (control, no *srtA* gene) and NHD03 were first grown in the absence of IPTG to the mid-logarithmic growth phase and then submitted to IPTG-induction. Aliquots were subjected to SDS-PAGE, blotted and probed

with polyclonal antibodies raised against sortase A. When extracts from WW02 were analysed, two bands became visible most probably unknown proteins of *B. subtilis* cross-reacting with the antibodies (Fig. 3.12, lanes 1 and 2). When extracts from strain NHD03 were probed, a new band of the molar mass of sortase A (estimated molecular mass: 30 kDa) became apparent even in the absence of IPTG which dramatically increased after addition of 1 mM IPTG (lanes 3 to 7). It can be concluded that strain NHD03 is able to produce a low amount of sortase A in the absence of inducer, most probably due to the leakiness of the P*spac* promoter, which can be significantly enhanced after addition of IPTG. Next, it was analysed whether the sortase is able to anchor α-amylase covalently on the cell wall of *B. subtilis*.

3.2.1.2 The α-amylase-FnBPB fusion protein can be anchored on the cell wall

First, an anchoring vector was constructed by inserting the coding region of the 3' terminal part of the *fnbB* gene (FnBPB94) of *S. aureus* coding for fibronection binding protein B [67] including the sorting motif followed by another 94 codons called the spacer region (in total 131 aa) into pHCMC04 carrying a xylose-inducible expression cassette [104] resulting in pNHD10. Next, the *amyQ* gene of *B. amyloliquefaciens* [111] coding for α-amylase was translationally fused to FnBPB94 (pNHD16). α-Amylase was chosen as a model protein since this protein is naturally secreted and its enzymatic activity can be easily measured. Plasmids pNDH15 (devoid of the sorting motif) and pNHD16 were transformed into *B. subtilis* strains WW02 and NHD03. Cells of these strains were grown in the absence or presence of sortase A or/and hybrid α-amylase. Next, whole cells were separated from the growth medium by centrifugation, and both cells and supernatants were analysed separately for α-amylase activity.

While a background level in the absence of any α-amylase of 2-6 units were measured, this activity increased significantly in the supernatant in the presence of *amyQ* (134 units; Table 3). Expression of the hybrid α-amylase in the absence of sortase A reduced its activity to 98 units indicating impairment by the C-terminal extension of 94 aa residues. When both sortase A and the hybrid α-amylase were synthesized, there was an increase in the enzymatic activity to 11 units with a spacer length of 94 aa (pNDH16) which doubled with a spacer length of 123 (Table 3.2) measured with whole cells. In the latter case, 15.8% of the total α-amylase activity was associated with the cells.

Table 3.2. α-Amylase activities measured with different strains

Strain	Plasmid	Sortase present	α-amylase present	LPETG present	Units	
					Supernatant	Cells
NDH03	pHCMC04	+	-	-	6.1 ± 1.9	1.9 ± 0.5
NDH03	pNDH15	+	+	-	133.7 ± 22.4	4.3 ± 2.2
WW02	pNDH16	-	+	+	98.0 ± 12.1	5.2 ± 1.9
NDH03	pNDH16	+	+	+	93.7 ± 11.3	11.0 ± 2.5
NDH03	pNDH19	+	+	+	124.1± 21.6	23.3 ± 4.6

Cells were grown to an OD_{578} of 0.1-0.2 at 37 °C. Then, 1 mM IPTG was added to induce production of sortase A in all five cultures. When the cultures reached an OD_{578} of 0.8, 0.5% xylose was added to induce production of wild-type (pNDH15) and hybrid α-amylase (from pNDH16 and pNDH19). All five cultures were further grown for 2 h. Then, aliquots were collected, the cells were separated from the growth medium by centrifugation and α-amylase activities were determined with whole cells and within the supernatants and presented in units per OD_{578}.

Next, it is important to demonstrate directly that the α-amylase is indeed anchored on the cell wall. To accomplish this goal, cells of strains WW02 and NDH03 carrying pNDH15 and pNDH16, respectively, were grown as described under Materials and Methods. Aliquots of supernatants or cells treated with lysozyme were prepared for immunoblotting, and the results are presented in Fig. 3.13C. Figs. 3.13A and 3.13B show the schematic structure of the hybrid surface protein AmyQ-FnBPB and part of the structure of the *B. subtilis* cell wall-anchored surface proteins. While strain NDH03 carrying pNDH15 produced only authentic α-amylase (molecular mass: 55 kDa) as to be expected (lane 1), hybrid α-amylase (69 kDa) could be recovered from supernatants of strains WW02 and NDH03 (lanes 2 and 3). When cells of strain WW02 carrying pNDH16 were treated with lysozyme, no α-amylase could be released (lane 4) in contrast to strain NDH03 expressing sortase A (lane 5). The bigger molecular weight of the sample that presents the sortase A and the hybrid protein (lane 5 of Fig. 3.13) could be the result of the peptidoglycan residues which remain in the hybrid protein after lysozyme treatment. This result unequivocally demonstrates that sortase A of *L. monocytogenes* expressed in a heterologous host can covalently anchor α-amylase on the cell wall of *B. subtilis*.

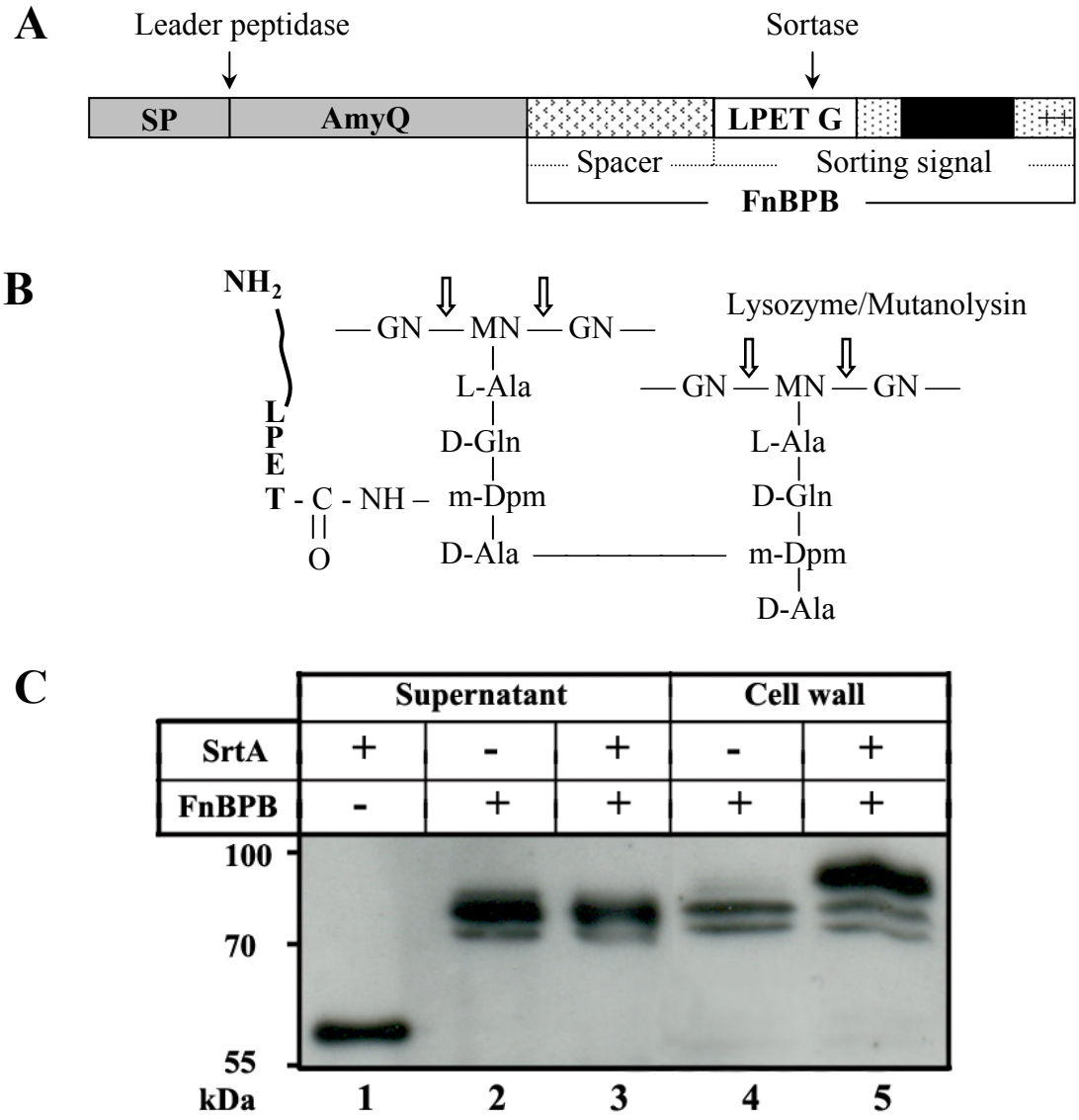


Fig. 3.13. Structure of the hybrid protein, of the cell wall-anchored surface protein and detection of α-amylase by immunoblotting. (A) Structure of the hybrid protein AmyQ-FnBPB; sites recognized by the leader peptidase and the sortase are indicated (not drawn to scale); (B) Schematic drawing of part of the *B. subtilis* cell wall showing the anchored protein (modified after Dhar et al. [28]); recognition sites for lysozyme or mutanolysin are indicated. (C) Detection of α-amylase by immunoblotting. Strains WW02 and NDH03 harbouring pNDH15 or pNDH16 were grown in LB medium and equal amounts of cells were washed as described in the legend to Table 3.2. After separation by centrifugation, the α-amylase present in the supernatants was detected by immunoblotting. Culture supernatants were analysed from strains NDH03/pNDH15 (lane 1), WW02/pNDH16 (lane 2), and NDH03/pNDH16 (lane 3). Cells from strain WW02 and NDH03 carrying pNDH16 were treated with lysozyme to release anchored α-amylase (cell wall, lane 4 and 5).

3.2.1.3 Immunofluorescence detection of α-amylase

In an independent experiment, the α-amylase bound covalently to the wall and exposed on the surface of *B. subtilis* cells should be visualized directly. Cell were first incubated with antibodies against α-amylase and then with an Alexa-conjugated secondary antibodies. The second binds to the antibodies against α-amylase and emit the fluorescent light under immunofluorescence microscopy. The microscopy showed that the α-amylase is indeed present on those cells expressing both sortase A and the hybrid α-amylase (Fig. 3.14). Most interestingly, the α-amylase is not distributed evenly on the surface but accumulated in patches. Treatment of the cells with trypsin dramatically reduced the fluorescence, which demonstrated that the α-amylase is exposed on the surface of *B. subtilis* NDH03.

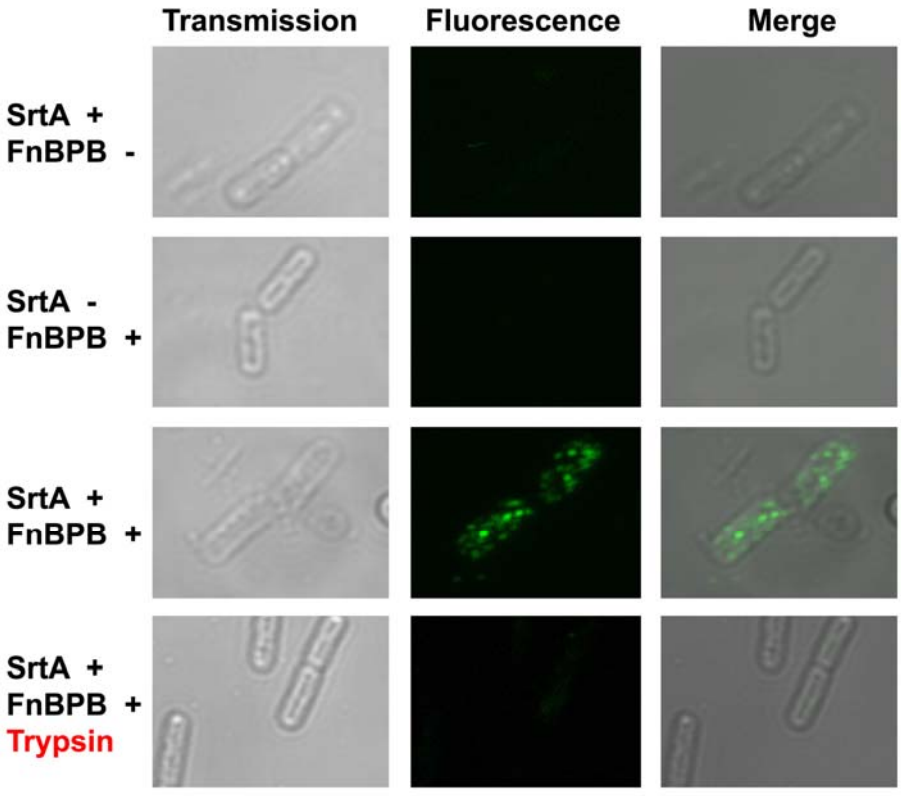


Fig. 3.14. Visualization of α-amylase on the surface of *B. subtilis* cells. Cells from three different strains (SrtA +, FnBPB - = NDH03/pNDH15; SrtA -, FnBPB + = WW02/pNDH16; SrtA +, FnBPB + = NDH03/pNDH16) were prepared for immunofluorescence microscopy as described in the legend to Table 3.2. Cells were visualized by transmission and fluorescence microscopy (Leica). The primary antibody against α-amylase and an Alexa-conjugated secondary antibody were used to visualize localization of the enzyme.

3.2.1.4 Up to 50,000 molecules of α-amylase are anchored on the cell wall with a spacer length of 94 aa

The amount of α-amylase molecules anchored on the cell wall was determined by densitometrical analysis of immunoblots. A defined amount of cells (850,000 cells) of strain NDH03 carrying pNDH16 were treated with lysozyme, and the liberated α-amylase separated by SDS-PAGE and subjected to immunoblotting. To calculate the amount of α-amylase, increasing amounts of purified enzyme were applied on the same gel (Fig. 3.15). From densitometric scanning of the different bands, the number of α-amylase molecules were calculated to $5.0 \pm 1.5 \times 10^4$ molecules per *B. subtilis* cell.

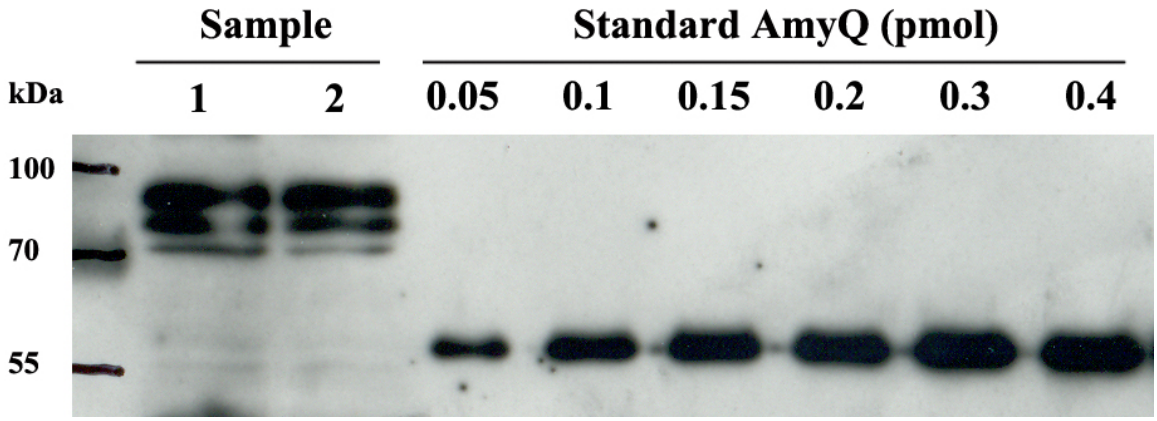


Fig. 3.15. Determination of the number of α-amylase molecules per *B. subtilis* cell. NDH03/pNDH16 was grown in LB medium at 37 °C as described in the legend to Table 3.2. Then, cells from two different cultures were withdrawn, sedimented by centrifugation, washed twice with PBS (pH 7.0) and treated with lysozyme to release the α-amylase. Cells were centrifuged, and 5 µl of the supernatant corresponding to 850,000 cells were applied per slot. α-Amylase from two different cultures was analysed (lanes 1 and 2). Defined amounts of purified α-amylase from 2.5 to 20 ng (corresponding to 0.05–0.4 pmol) were run on the same gel. Only the material in the upper band in lanes 1 and 2 was quantified.

3.2.1.5 Influence of the spacer length on the number of α-amylase molecules immobilized on the cell surface and on the activity

To study the influence of the spacer length on the activity and number of immobilized hybrid molecules, the length of the spacer was varied. When the distance between the cell wall sorting motif and the α-amylase was successively increased from 94 to 234 aa, the amount of

the hybrid protein molecules immobilized on the cell surface first increased (Fig. 3.16, F123) and then decreased to about the same amount (F162 and F196) and finally dropped dramatically (F234). These results indicated that the cell wall-spanning region must exceed a critical length to allow efficient anchoring. In the present case, a spacer region of about 123 aa seems optimal. The number of α-amylase molecules with the optimal spacer was determined to be $2.4 \pm 0.8 \times 10^5$.

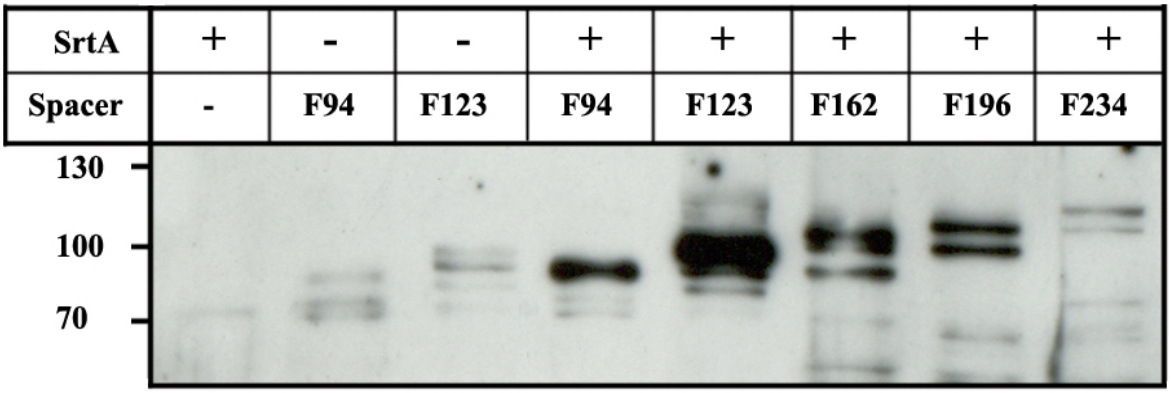


Fig. 3.16. Influence of the distance between the sorting motif and the C-terminus of the α-amylase on the amount of immobilized protein molecules. NDH03 cells harbouring pNHD16 (F94) and derivatives of this plasmid coding for an extended C-terminal region of FnBPB, spacer (from 123 to 234 aa residues) were analysed as described in the legend to Fig. 3.13.

Next, the enzymatic activity using whole cells and the supernatant of cells treated with lysozyme was analysed. While only background levels were measured in the absence of α-amylase as to be expected, small amounts were present in the cell suspension and supernatant of lysozyme treatment, even in the absence of sortase A using a 123-amino-acid spacer (Fig. 3.17). The activity increased about 5-fold upon induction of the *srtA* gene, and the activity measured with the 123-amino-acid spacer (F123) was set as 100%. In this case, the activity found with whole cells and the lysozyme-released one was comparable (Fig. 3.17). In the case of F94, the activity dropped to about 30% with whole cells and 50% after lysozyme treatment. 50% activities were also measured with both whole cells and lysozyme-released materials with F162. Then, the activity dropped to 30% (F196), and 20% (F234). These results indicated that there is an optimal spacer length, in the case of the α-amylase about 123 residues.

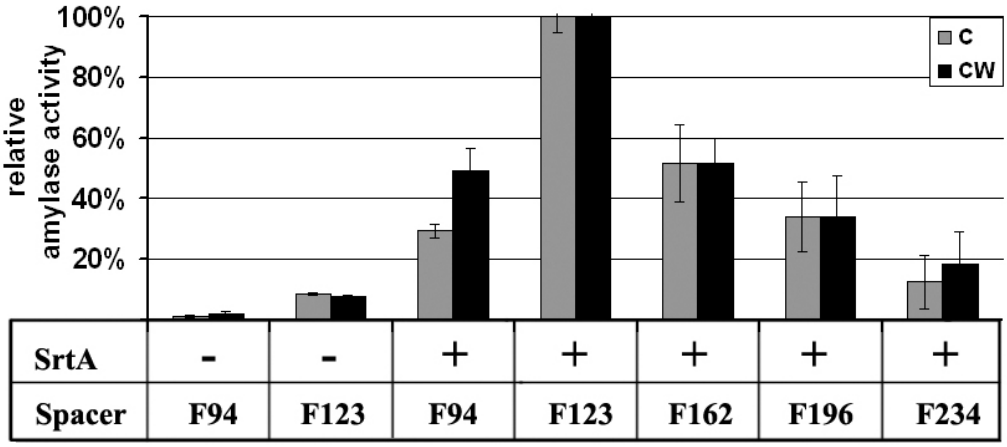


Fig. 3.17. α-Amylase activity in relation to the spacer length. The spacer length of the anchoring vector was increased from 94 to 234 aa residues as indicated. Cells of strain WW02 (without sortase) or NDH03 (with sortase) carrying the different vectors were grown and treated as described in the legend to Table 3.2. α-Amylase activity was measured with whole cells (grey columns, C) and in the supernatant obtained from the same amount of cells treated with lysozyme (black columns, CW). The highest enzymatic activity obtained with F123 was set as 100%.

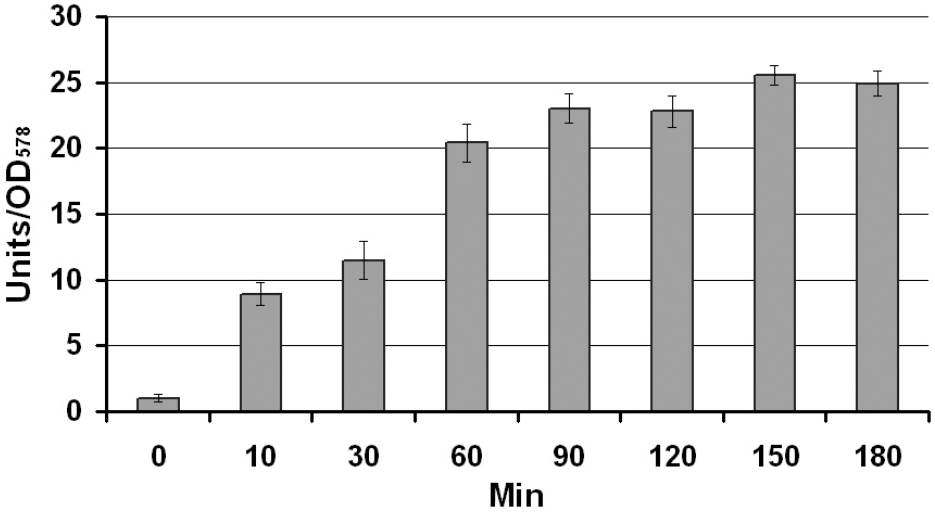


Fig. 3.18. Kinetics of α-amylase immobilization on the cell wall. Strain NDH03 with a plasmid able to express α-amylase with a 123 residue spacer (pNDH19) was treated with 0.5% xylose in the mid-exponential growth phase. Cells were withdrawn for the determination of the α-amylase activity at the time points indicated. Before collecting the samples, tetracycline was added (20 μg/ml) to stop protein synthesis; cultures were shaken for another 10 min and then stored on ice. Time-points are indicated in min after induction with xylose.

In the last experiment, it was asked what time it will take to saturate the cell wall with α-amylase after induction. The plasmid producing α-amylase was fused with the optimal spacer length (F123), its production was induced and the α-amylase activity was measured up to 180 min using whole cells. As can be seen in Fig. 3.18, the enzymatic activity increased up to 60 min after induction to about 20 units with a further slight increase up to about 25 units after 150 min. The same pattern was obtained with cells carrying α-amylase anchored with a 94-aa spacer (F94, data not shown). In conclusion, 60 min seem to be sufficient to saturate the cell wall with α-amylase molecules.

3.2.2 Analysis of two putative sortases of *B. subtilis*

The chromosome of *B. subtilis* codes for the two putative sortases YhcS and YwpE and two potential sortase substrates YhcR and YfkN. This chapter is focused on the question whether these sortases are expressed in the *B. subtilis* 1012 wild type cells and whether these sortases are able to anchor the foreign protein AmyQ on the cell wall. A primary attempt to identify cell wall sortase dependent proteins is also described.

3.2.2.1 Expression of *yhcS* and *ywpE*

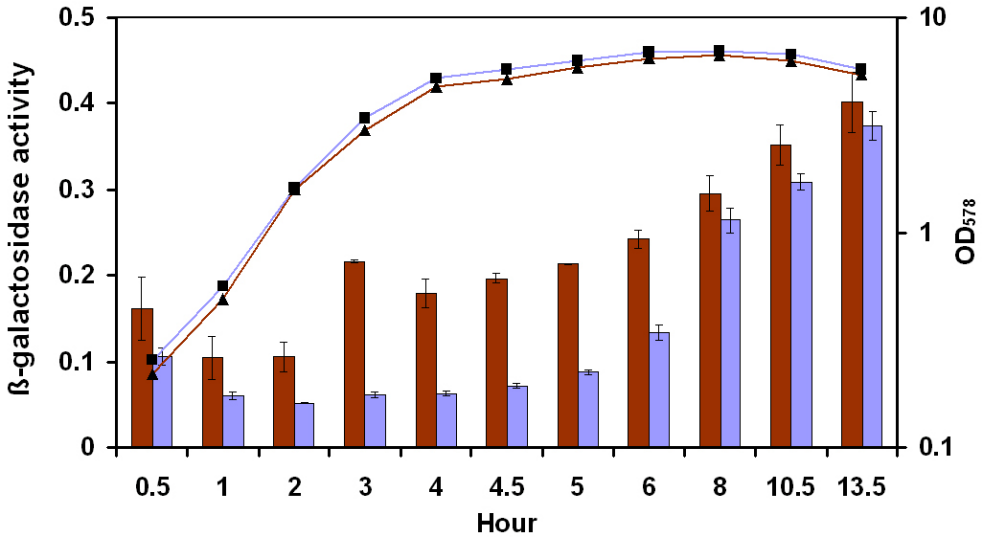


Fig. 3.19. Measurement of the expression of *yhcS* and *ywpE*. *B. subtilis* 1012 strains NDH20 (P*yhcS-lacZ*) and NDH21 (P*ywpE-lacZ*) were grown in LB medium, samples were collected for β-galactosidase activity measurements during the growth curve as indicated. Blue line: growth curve (■) and blue column: LacZ activity from NDH20 cells; red line (▲): growth curve and red column: LacZ activity from NDH21 cells.

B. subtilis YhcS is a putative secreted protein of 198 aa with one predicted transmembrane domain and *B. subtilis* YwpE is a predicted cytoplasmic protein of only 102 aa [20]. The latter protein has 23 % sequence identity with the C-terminal domain of SrtA (see Fig. 1.2). The transcriptional fusions between their promoters and the reporter gene, P*yhcS-lacZ* and P*ywpE-lacZ*, were constructed to study expression of *yhcS* and *ywpE* (Fig. 2.7). The β-galactosidase activity of these fusions was measured during the growth curve. As can be seen from Fig. 3.19, both genes exhibited increased expression during the late stationary phase.

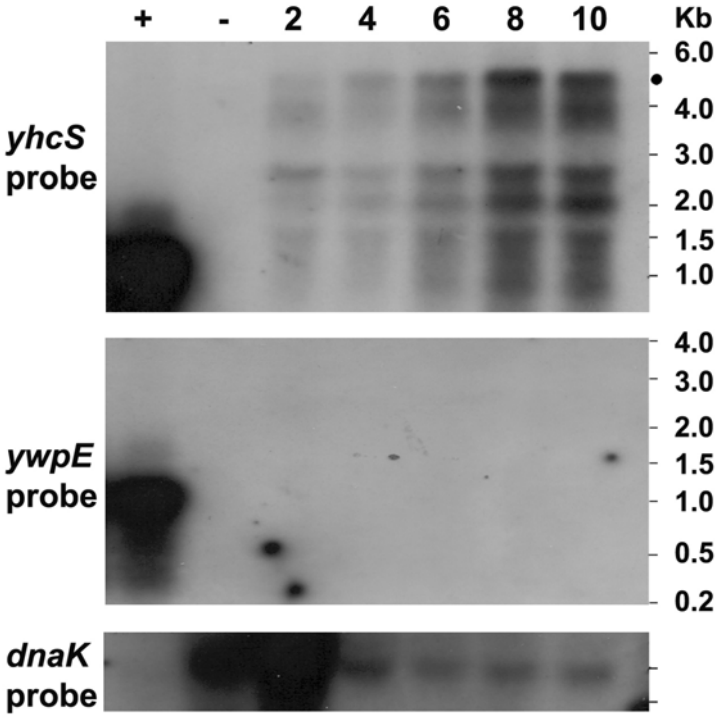


Fig. 3.20. Detection of the expression of *yhcS* and *ywpE* by Northern blot. Three different *B. subtilis* strains were grown in LB medium and aliquots were analysed by Northern blot using either *yhcS* (upper panel), *ywpE* (middle panel) or *dnaK* antisense RNA (lower panel, loading control). +: strain NDH31/pNDH33-*ywpE-yhcS* where both genes coding for putative sortases are artificially expressed from an IPTG-dependent promoter. -: strain NDH31 where both potential sortase genes have been deleted. Lanes 2 to10, aliquots from wild type strain 1012 were withdrawn 2, 4, 6, 8 and 10 h after inoculation.

When analysing the expression of both genes by Northern blot, a major signal was detected with *yhcS* antisense-RNA at around 4.5 kb which increased over time and peaked after 8 h when cells entered the late stationary phase (Fig. 3.20, black dot). This major band with a size of about 4.5 kb corresponds by size to the bicistronic operon *yhcR-yhcS* predicted by

Microbes Online Operon Predictions [120]; other smaller bands probably resulted from processing or degradation. This result indicates that *yhcS* is expressed in the late stationary phase and corroborates the data obtained by β-galactosidase assay. In the case of *ywpE* predicted to form a moncistronic operon, there was no signal corresponding to the *ywpE* transcript (~ 350 bases) even using a large amount of RNA (30 µg) for the experiment (Fig. 3.20, *ywpE* antisense probe). A possible explanation for this discrepancy (β-galactosidase activity in one experiment, but no transcript in a second one) could be that the *ywpE* transcript is rather unstable. If both genes were artificially expressed from a plasmid by addition of IPTG, the bicistronic transcript could be demonstrated (Fig. 3.20, positive control).

In conclusion, *yhcS* and *ywpE* were expressed at increased levels in the late stationary phase in *B. subtilis* wild type cells, in which the transcript of *yhcS* was clearly higher than that of *ywpE*.

3.2.2.2 YhcS and/or YwpE-dependent substrate proteins

In an attempt to identify YhcS and/or YwpE-dependent substrate proteins anchored on the cell wall, the double knockout strain NDH31 (Δ*yhcS* and Δ*ywpE*) was generated and three plasmids that are able to express *yhcS* and/or *ywpE* under the control of the IPTG-inducible promoter P*grac* were constructed. Plasmids pNDH33-*ywpE*, pNDH33-*yhcS* produce YwpE and YhcS respectively. Plasmid pNDH33-*ywpE-yhcS* contains a synthetic two-gene operon, and each gene has its own strong ribosome binding site (Fig. 2.9). Those plasmids and the pNDH33 empty vector (as negative control) were then transformed into the strain NDH31 resulting in NDH31/pNDH33, NDH31/pNDH33-*yhcS*, NDH31/pNDH33-*ywpE* and NDH31/pNDH33-*ywpE-yhcS*.

First, expression of *yhcS* and/or *ywpE* under control of IPTG was evaluated by Northern blot. *YhcS-ywpE* (about 1100 nt) including the size of *yhcS* (737 nt) and *ywpE* (436 nt), *yhcS* and *ywpE* were highly expressed when induced by IPTG (Fig. 3.21, lane 4, 6 and 8). All of these transcripts were equipped with the strong ribosome binding site AAAGGAGG, so that those constructs were expected to be translated to YhcS (pNDH33-*yhcS*), YwpE (pNDH33-*ywpE*), YhcS and YwpE (pNDH33-*ywpE-yhcS*). The strains NDH31 (Δ*yhcS* and Δ*ywpE*), SZ60 (Δ*ywpE*), NDH31 (Δ*yhcS*) and 1012 were also investigated; *yhcS* could be expressed in the strains SZ60 and 1012 after 8 h inoculation while *ywpE* could not be expressed in any strain (data not shown).

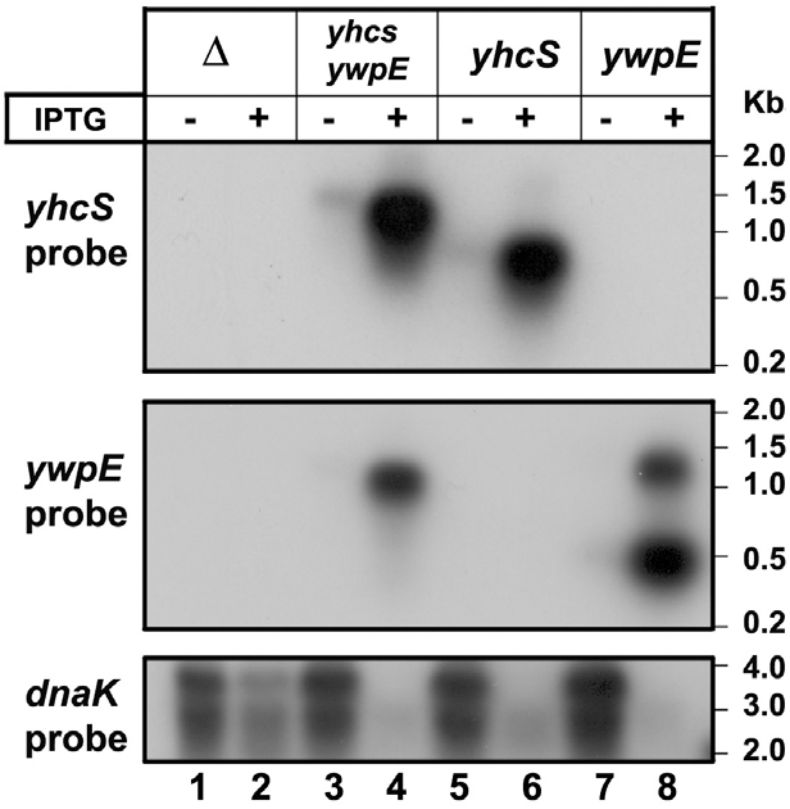


Fig. 3.21. Confirmation of the expression of *yhcS* and/or *ywpE* in strain NDH31 (Δ*yhcS* and Δ*ywpE*) from plasmids by Northern blot. Δ, strain NDH31/pNDH33; *yhcS*, strain NDH31/pNDH33-*yhcS*; *ywpE*, strains NDH31/pNDH33-*ywpE*; *yhcS*-*ywpE*, strain NDH31/pNDH33-*ywpE*-*yhcS*. Lanes 4, 6 and 8 (0.25 μg of total RNA) and lanes 1, 2, 3, 5, 7 (5 μg of total RNA) were loaded. RNA markers are indicated.

Next, strains NDH31, SZ60, NDH30, 1012 were inoculated for 8 h corresponding to late stationary phase and strains carrying the different plasmids were induced with IPTG for 8 h. The cells were harvested. To release proteins possibly anchored on the cell wall, the cells were sonicated and washed to partially remove the cytoplasmic proteins before lysozyme treatment. The supernatants containing cell wall released proteins were analysed by SDS-PAGE followed by Coomassie blue staining. The intensity of cytoplasmic proteins was dramatically decreased and the differences in the intensity of those bands and the molecular weights of candidates to be anchored to the cell wall are indicated (Fig. 3.22). There is no difference in the protein patterns between strains NDH31, SZ60, NDH30 and 1012 (Fig. 3.22, lane 1, 2, 3, 4). Interestingly, this preliminary experiment from strains that restored the expression of YwpE and/or YhcS exhibited two YhcS-dependent proteins with molecular weights about 27 and 55 kDa (Fig. 3.22, red arrows) which appeared in the strains that

express YhcS (Fig. 3.22, lane 6) or YhcS and YwpE (Fig. 3.22, lane 8); and two proteins with molecular weight about 35 and 45 kDa that appeared in the absence of YwpE (Fig. 3.22, green arrows). In addition, when the strain WB800N, deficient for eight different proteases, carrying plasmid pNDH33-*ywpE-yhcS* was investigated, eight bands became visible (Fig. 3.22, lane 9). Among them, bands of about 27 and 55 kDa corresponding to the two bands detected from the strains that produced YhcS (Fig. 3.22, red arrows) are present. It is worth to note that the band running with a molecular mass of 140 kDa seems to be a doublet. This became visible when performed longer run on the 8% polyacrylamide gel (data not shown).

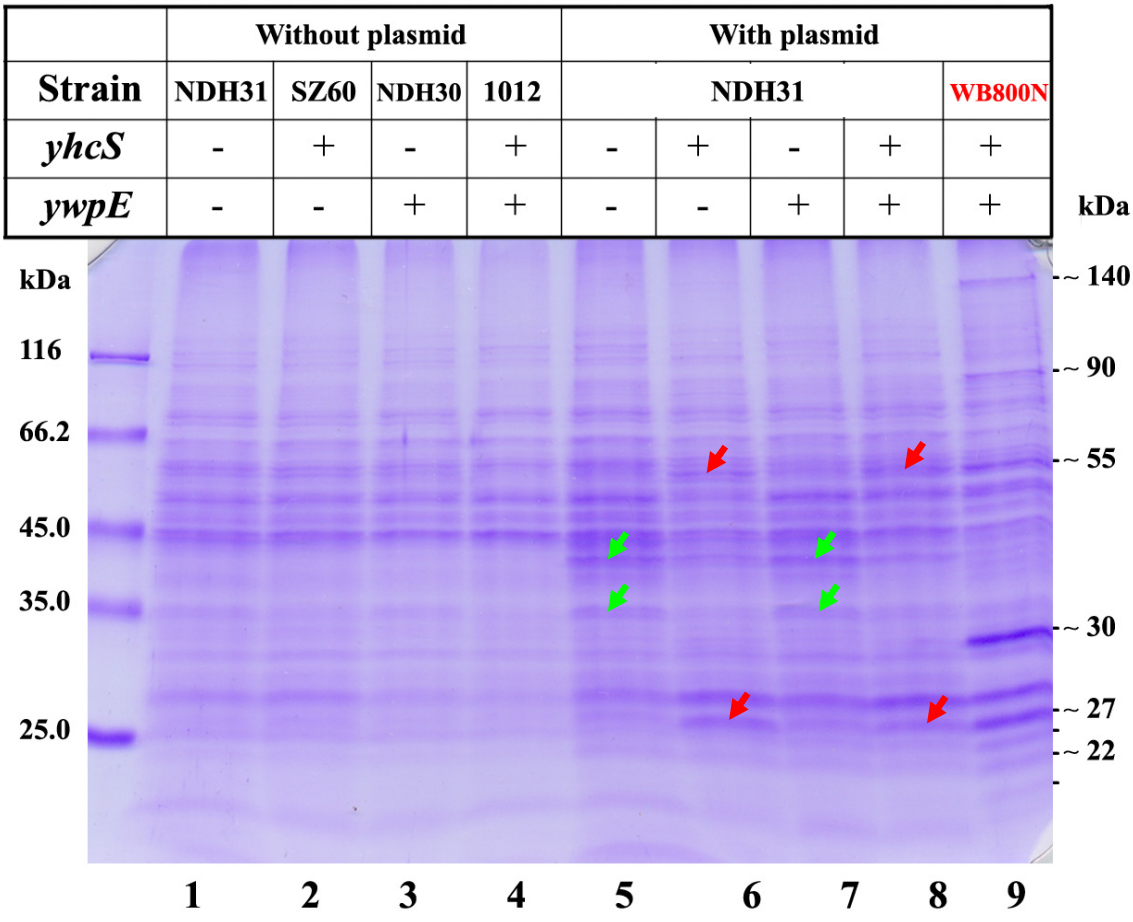


Fig. 3.22. Protein patterns of the putative sortase knockout strains. Samples were collected after 8 h of induction. The cells were sonicated, followed by intensive washing and lysozyme treatment. Samples for this SDS-PAGE and Coomassie blue staining were prepared as described in the Materials and Methods. Lane 1, 2, 3, 4 are from strains NDH31 (Δ*yhcS* Δ*ywpE*), SZ60 (Δ*ywpE*), NDH30 (Δ*yhcS*) and 1012; lane 5, NDH31/pNDH33; lane 6, NDH31/pNDH33-*yhcS*; lane 7, NDH31/pNDH33-*ywpE*; lane 8, NDH31/pNDH33-*ywpE-yhcS*; lane 9, WB800N/pNDH33-*ywpE-yhcS*. The size of molecular weight standards is indicated on the left margin and that of proteins anchored on the cell wall at the right margin.

In conclusion, the 27- and 55-kDa proteins seem to be YhcS-dependent proteins in *B. subtilis*. This result also indicates that the strain WB800N would be a better candidate for identification of YhcS- and/or YwpE-dependent target proteins.

3.2.2.3 Substrates of the putative sortase YhcS

Based on her results, Westers [166] suggested that YfkN and YhcR are substrates of one of the two putative sortases. To corroborate this assumption, the *amyQ*-encoded α-amylase was fused to the potential sorting sequences of the two proteins resulting in pNDH89 (AmyQ-YhcR123) and pNDH90 (AmyQ-YfkN123); AmyQ-FnBPB123 (pNDH19) was also included. The size of the hybrid proteins are 69 - 72 kDa including 55 kDa of AmyQ and 14 - 17 kDa of FnBPB123, YfkN123 or YhcR123. The structure of the hybrid proteins is shown in Fig. 3.13A and the sorting sequences are presented in Table 1.1. All three plasmids were transformed into strains SZ60 (Δ*ywpE*), NDH30 (Δ*yhcS*) and NDH31 (Δ*yhcS* and Δ*ywpE*). Cells of these strains were grown as described under Materials and Methods and the proteins were released from the cell wall by lysozyme treatment. The ability to anchor these fusion proteins was evaluated by Western blot and the results are presented in Fig. 3.23 and Fig. 3.24.

The strain with the double knockout was unable to retain the hybrid AmyQ-FnBPB123 (Fig. 3.23, lanes 4 and 5) on the cell wall when compared with the positive control (Fig. 3.23, lane 1 and 2), while in the strains carrying the putative sortases three bands appeared (Fig. 3.23, lanes 6, 7 and 8) as compared to the positive control (Fig. 3.22, lane 1, 2). Similar patterns of AmyQ-FnBPB123 were also observed in the case of AmyQ-YfkN123 (Fig. 3.24, YfkN), which is not clear whether the sortase will immobilize these fusion proteins on the cell wall. Interestingly, if one assumes that the knockout presents the background where the hybrid AmyQ-YhcR123 protein is not anchored on the cell wall (Fig. 3.24, lane 5), the hybrid protein could be retained on the cell wall in the strains that are able to express YhcS (Fig. 3.24, YhcR, lane 7 and 8) but in the *yhcS* knockout strain (Fig. 3.24, lane 6). Moreover, a higher level of the fusion protein AmyQ-YhcR123 always appeared in the wild type strain expressing both putative sortases (lane 8) as compare to the *ywpE* knockout strain (lane 7). The band with a molecular mass of about 90 kDa could be the anchor-fusion protein AmyQ-YhcR123 (Fig. 3.24, YhcR123) and is comparable with the size of AmyQ-FnBPB in the strains NDH03 (Fig. 3.13C and 3.16).

In conclusion, YhcS was able to anchor the fusion protein AmyQ-YhcR123 on the cell wall, while YwpE seem to assist YhcS to perform its functions.

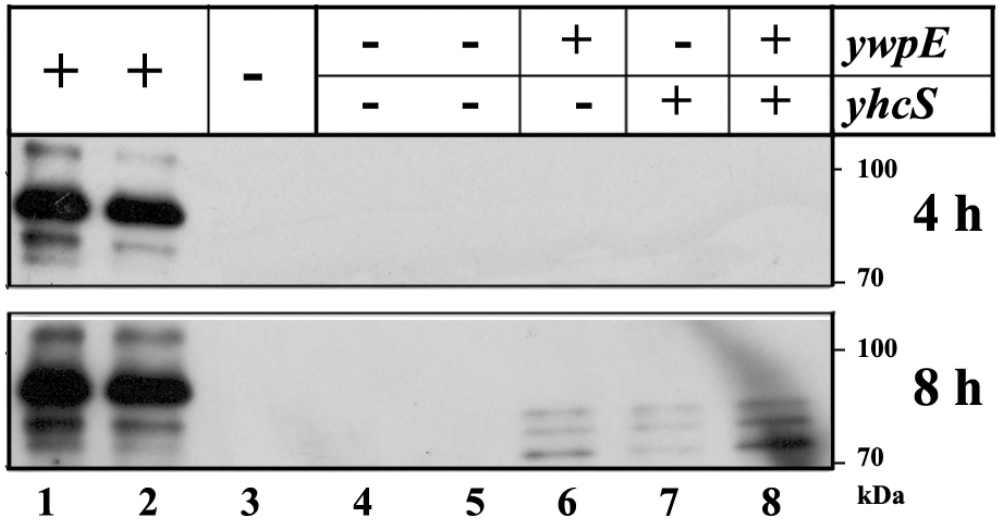


Fig. 3.23. Detection of α-amylase anchored on the cell wall of different strains using FnBPB123 by Western blot. Lanes 1 and 2, positive controls from the strain NDH03/pNDH19 (AmyQ-FnBPB123) and lane 3, negative control from the strain NDH03/pNDH15 (AmyQ); cells of these controls were grown as described in the legend to Table 3.2. Lanes 4 and 5, strain NDH31/pNDH19; lane 6, strain NDH30/pNDH19; lane 7, the strain SZ60/pNDH19; 8, strain 1012/pNDH19; cells of these samples were induced by 1 mM IPTG and collected after 4 and 8 h of induction.

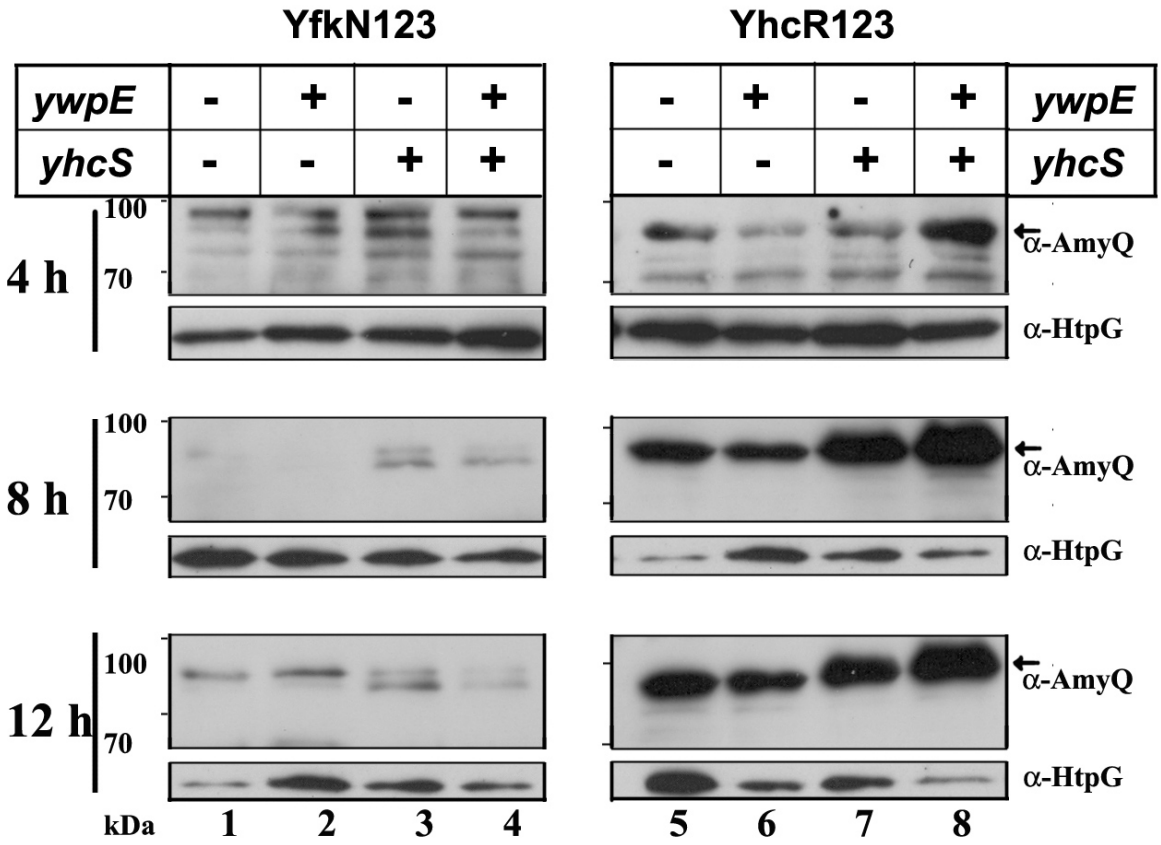


Fig. 3.24. Detection of α-amylase on the cell wall of four different strains using either the YhcR123 or the YfkN123 sorting sequence with the 123-aa spacer by Western blot. Lanes 1, 2, 3 and 4: strains NDH31 (*ywpE* $^{-}$, *yhcS* $^{-}$), NDH30 (*ywpE* $^{+}$, *yhcS* $^{-}$), SZ60 (*ywpE* $^{-}$, *yhcS* $^{+}$) and 1012 (*ywpE* $^{+}$, *yhcS* $^{+}$), all of them carrying the plasmid pNDH90 (AmyQ-YfkN123); Lanes 5, 6, 7 and 8: strains NDH31, NDH30, SZ60 and 1012 carrying plasmid pNDH89 (AmyQ-YhcR123). HtpG, a cytoplasmic protein, was used as loading control for the proteins released from cytoplasm.

4 Discussion

4.1 Construction of plasmid-based expression and secretion vectors for *B. subtilis*

4.1.1 Possibility to construct high-protein-production strain

B. subtilis is used for the high-level production and, most importantly, secretion of a variety of proteins by the industry [97, 166]. To optimise the use of the *B. subtilis* cell factory, three strategies are commonly used. The first strategy involves regulated gene expression from the chromosome [49, 53, 99, 145] or use of structurally stable multi-copy plasmids such as those based on pMTLBs72 [104, 153]. The second strategy identifies new promoters or/and optimization of existing promoters allowing high level of expression and optimal secretion, e.g. P*grac* [114] and P*aprE* [64]. The last involves optimization of *B. subtilis* host strains for protein production which includes removal of genes coding for extracellular proteases [173-175], asporogenous cells to prolong the expression phase [70, 115], relating to cell division in which the expression of cell-division initiation protein FtsZ (septum formation) is controlable in *B. subtilis* [112] and also in *E. coli* [4] and hypersecretion strain [25]. In an attempt to limit the expression of unnecessary proteins, the regulatable expression of *dnaA* has been constructed (data not shown). DnaA encoded by *dnaA* is a protein that is responsible in intitiation of chromosome replication [75, 100]. My idea is to minimize the expression proteins relating to DNA replication so that energy can be used only for production of heterologous protein during induction stage. This idea is applied for *E. coli*, in which a plasmid-encoded protein is expressed in nongrowing but metabolically active cells leading to high expression of recombinant protein [102].

4.1.2 Construction of plasmid-based expression vectors

While most plasmids use the theta-mode of replication, some produce single-stranded DNA as an intermediate via rolling-circle replication [13, 39, 48]. Most vector plasmids used in *B. subtilis* belong to the latter type. While the vector plasmids by themselves are structurally stable, insertion of restriction fragments may cause instability due to deletion formation involving both the vector and the insert. It has been experimentally demonstrated that small direct repeats are sufficient to cause deletion formation involving one repeat element and the

DNA sequence located in between the two repeats. Deletion formation occurs within the single-stranded DNA [11]. There are two possibilities to overcome this problem: (i) integration of the expression cassette into the *B. subtilis* chromosome at ectopic sites, and (ii) use of plasmid vectors using the theta-mode of replication.

The first strategy makes use of so-called delivery plasmids unable to replicate in *B. subtilis* carrying the expression cassette and antibiotic resistance marker both sandwiched between the first half and the second half of an nonessential *B. subtilis* gene. After transformation of the delivery plasmid into *B. subtilis*, the expression cassette together with the recombinant gene and the antibiotic marker are integrated into the bacterial chromosome in some of the transformants via a double crossing-over event. The most prominent and widely used chromosomal gene for ectopic integration is the *amyE* locus, coding for a nonessential α-amylase; this system has been developed by Shimotsu and Henner [145]. Two other systems make use of the *thrC* and the *lacA* locus [49, 53].

Only a few plasmids have been identified so far using the theta-mode of replication namely pAMβ1 and pTB19 [65, 146], pTA1060 [12] and very recently pMTLBs72 [122], but none of them has been developed into an expression vector allowing both intra- and extracellular production of recombinant proteins. Therefore, the major part of the doctoral thesis was devoted to convert pMTLBs72 into expression vectors using different promoters. Based on the *E. coli - B. subtilis* shuttle vector pMTLBs72, the pHCMC (for Ho Chi Minh City) series of vectors was developed. The first step was to insert a strong transcriptional terminator into this vector at downstream of the promoters. The *trpA* terminator of *E. coli* was chosen, which efficiently blocks transcription beyond this sequence [69]. Next, three different regulatable promoters were inserted upstream of this terminator (P*gsiB*, P*xylA* and P*spac*).

The first promoter, P*gsiB*, is found upstream of the *gsiB* gene in the *B. subtilis* chromosome, which codes for a glucose starvation-inducible protein [101]. This gene belongs to the sigma-B regulon comprising between 150 and 200 genes [119]. The sigma-B regulon is induced by different stresses which can be classified into two groups called energy and environmental stresses [118]. Both stresses result in the activation of two different phosphatases (RsbU in the case of environmental (physical) stress and RsbP in the case of energy stress). Both phosphatases have the same target, the anti-anti sigma factor RsbV. When this protein becomes dephosphorylated, it will attack a binary complex consisting of the anti-sigma factor RsbV and the alternative sigma factor σ^B. This attack will cause release of σ^B which in turn

will bind to the core RNA polymerase leading to the transcription of all the genes belonging to the sigma-B regulon. Physical stresses include starvation for glucose, reduced oxygen tension, heat and acid stress and addition of ethanol to the growth medium. Several of these stresses have been shown here to induce P*gsiB*. To improve the amount of the recombinant protein, the Shine-Dalgarno sequence of *gsiB* has been included. This DNA sequence has been shown not only to increase initiation of translation, but also the stability of the transcript [68]. The *gsiB* promoter has already been successfully used to express two different antigens in the mouse gut after oral administration of vegetative *B. subtilis* cells and spores [31]. Under these adverse conditions, induction of P*gsiB* occurs through acid shock in the stomach and through the absence of oxygen in the gut.

The second controlable promoter, P*xylA*, has been developed in our laboratory [71]. It consists of the xylose repressor gene *xylR* which binds to the *xylA* promoter in the absence of xylose [127]. This promoter can be induced by the addition of xylose. The third promoter, P*spac*, was developed by Yansura and Henner [29]. This expression cassette consists of the *lacI* gene coding for the Lac repressor and the artificial *spac* promoter, in which a promoter from a *B. subtilis* phage (sp) is fused to the *lac* operator (ac), and the Lac repressor can be inactivated by addition of IPTG to the growth medium. While P*xylA* turned out to be a strong promoter, P*spac* exhibited only weak activity for unknown reasons. This disappointing result prompted the construction of new vectors based on IPTG control.

4.1.3 Novel plasmid-based expression vectors

There are several ways to improve the expression level of a plasmid by (i) using a strong promoter, (ii) enhancing the stability of mRNA by using 5' and 3’ stabilizers [1, 17, 50] and/or a strong ribosome binding site [68], and (iii) increasing the copy number of the plasmid. To obtain a more efficient IPTG-regulatable promoter, the strong P*groE* promoter was fused to the Shine-Dalgarno sequence of the *gsiB* gene (see above). This promoter precedes the *groESL* operon coding for the GroEL chaperonin [136] and is powerful because of a potential UP element. UP elements are AT-rich sequences located upstream of the –35 element of some promoters (for reviews see [14, 46]). They act as binding sites for the αCTD of the α subunit of the RNA polymerase [19, 149]. In the wild-type situation, the P*groE* promoter is under the negative control of the HrcA repressor which is transiently inactivated by a heat shock [142]. To convert this heat-inducible promoter into an IPTG-inducible one,

the binding site for the HrcA repressor, the CIRCE element [181] was replaced by the *lac* operator *lacO* (Table 4.1), and the new promoter was termed P*grac* (acronym of ***gr****oE* and *l****ac***). Assuming that *lacO* is part of the transcript and located close to its 5' end, the stability of the stem-loop structure was enhanced by flanking it with two G and two complementary C residues as shown in Table 4.1. In addition, it has been shown that the Lac repressor binds more tightly to a symmetric *lacO* [128, 147], which should reduce the background expression level. P*grac* replaced P*spac* in pHCMC05 resulting in the new expression vector pNDH33. In addition, this expression vector was converted into a secretion vector by introducing the signal sequence of the *amyQ* gene downstream of P*grac* resulting in pNDH37.

Table 4.1 Minimum free energy of the stem-loop within P*grac* promoter

	RNA sequence of *lacO* region	minimum free energy (kcal/mol)
wild-type *lacO* region	AAUUGUGAGCGGAUAACAAUU <<<<<<<< < > >>>>>>	-1.70
mutant *lacO* region	U**GG**AAUUGUGAGCGGAUAACAAUU**CC**C . <<<<<<<<< < > >>>>>>>>>>	-8.70

The table shows the wild-type *lacO* operator from *E. coli* K12 and a mutant version. Symmetric nucleotides are marked by arrowheads; underlined letters in bold are the mutated bases, GG and CC, to reduce the free energy of the stem-loop. The data are calculated using the RNAfold program [87].

The strength of the new hybrid promoter could be demonstrated by measuring the activity and amount of several reporter proteins. While the β-galactosidase activity encoded by the *bgaB* gene could be induced about 1300-fold, the level of the *htpG* and *pbpE* gene products accumulated up to 10% and 16% of the total cellular protein, respectively. The secretion capability of the vector plasmid pNDH37 was analysed by following the appearance of three different exoenzymes in the growth medium. The high expression level of proteins could be explained as the result of the mRNA stability. The half-life of *bgaB* transcript under control of the P*grac* promoter was about 20 min (data not shown) while 80% of *B. subtilis* mRNAs has a half-life of less than 7 min [51].

The experimental data showed derivatives of plasmid pNDH33 were structurally stable in *B. subtilis* (data not shown). However, during the use of these plasmids and its derivatives, structural instability was detected after transformation into *E. coli* leading to the appearance of small and large colonies on the plates. Analysis of the plasmids from both colony types revealed the expected size, while those from large colonies carried smaller plasmids. A close inspection of the DNA sequence of these vectors revealed a 117-bp direct repeat present in both plasmids. Deletions occurred between these two repeats removing one copy of the repeat and the intervening DNA sequence. There are numerous reports in the literature that plasmids with direct repeats tend to be unstable. This could also be shown experimentally with small direct repeats inserted into a plasmid [90].

Very often, production of recombinant proteins intracellularly results in the formation of inclusion bodies, large spherical particles which are clearly separated from the cytoplasm and visible in the light microscope. The reason for the formation of these protein aggregates is the result of an increase in the concentration of nascent polypeptide chains unable to fold correctly. There are several possibilities to prevent or at least to reduce the formation of inclusion bodies including carrying out overexpression at low temperature, using a solubilizing partner or coexpressing molecular chaperones during the expression phase [5, 83, 144]. In the case of *B. subtilis*, the two major chaperone machines, the DnaK and the GroEL machine, are under the negative regulation by the HrcA repressor [142, 178]. Deletion of the *hrcA* gene results in a high constitutive expression of both chaperone teams. Such a *hrcA* knockout has already been used to reduce the amount of aggregated single-chain antibodies [172] and worked also in the case of the PBP4*. Therefore, it can be concluded that a *hrcA* null mutant will largely prevent formation of other intracellular protein aggregates as well.

There are still further possibilities to increase the amount of recombinant proteins: (i) to enhance the stability of the transcript by adding 5' and 3' stabilizers; (ii) to increase the copy number of the vector plasmid either by constitution or induction. While several 5’ stabilizers for *E. coli* [16, 40] and *B. subtilis* [50] transcripts have been described, just one 3' stabilizer has been identified in *Bacillus* [170]. This *cryIIIA* terminator is derived from *B. thuringiensis* and increases the half-life of certain mRNA molecules. As to the copy number of the plasmid vectors, their increase is possible by changing the amount of that protein which regulates initiation of replication. This can be done by either enhancing its activity which leads to a constitutive increase in the copy number or increasing the copy number on demand, e.g.,

during the expression phase. This can be accomplished by fusing the gene coding for the initiation protein to a controlable promoter [169]. In the case of the pMTLBs72-derived vectors, ORF-1 [80, 154, 154], coding for the initiator protein RepA, is the target gene to be placed under a regulatable promoter.

In summary, all the expression vectors constructed here are extremely useful for the regulatable expression and secretion of various recombinant proteins in conjunction with the *hrcA* knockout in those cases where the overproduced proteins tend to form aggregates.

4.2 Immobilization of proteins on the surface of *B. subtilis* cells

4.2.1 The sortase A could immobilize AmyQ on the surface of *B. subtilis* cells

Microbial cell-surface display, also called cell surface engineering, has a wide range of biotechnological and industrial applications including live vaccine development, screening of displayed peptide libraries, bioadsorbents for removal of harmful chemicals and heavy metals and biosensor development [82, 164]. In Gram-negative bacteria, several cell-surface display systems have been described including flagella, pili, outer membrane proteins such as OmpA and S-layer proteins [82]. In contrast, the number of cell-surface displayed systems in Gram-positives is less advanced; four major types of cell surface displayed proteins are currently recognized: (i) proteins anchored to the cytoplasmic membrane by hydrophobic transmembrane domain(s), (ii) lipoproteins which are covalently attached to membrane lipids after cleavage by signal peptidase II, (iii) proteins containing C-terminal LPXTG-like motif and covalently attached to peptidoglycan by sortase and (iv) proteins recognizing some cell wall components by specific domains, i.e. some cell wall binding domains (CWBDs) that remain stuck onto the cell wall after translocation [24, 27]. Fusing lipases with CWBDs, Kobayashi and his colleagues demonstrate that lipases accumulated on *B. subtilis* cell surface [72, 73, 160]. Among those, the sortase system is the most widely used cell-surface display system with a variety of applications using mainly *Staphylococcus carnosus* and *S. xylosus* as host bacteria [165]. Therefore, it is quite surprising that *B. subtilis*, the working horse of the Gram-positives with its sophisticated genetics and biotechnology, has never been used for immobilizing proteins on its surface using the sortase system. The major reason for this observation relies on the fact that, though there are two potential candidates for sortases [20], neither substrate proteins nor sorting motifs have been identified experimentally so far.

To circumvent the problem of trying one of the two potential *B. subtilis* sortases, the *srtA* gene of *L. monocytogenes* was introduced into the *B. subtilis* genome. Choosing this enzyme was based on two important observations: first, this sortase recognizes a known sorting motif, the LPXTG motif, and second, it recognizes a peptide cross-bridge which is identical to that found in the *B. subtilis* cell wall. The glycan chains of the *B. subtilis* peptidoglycan are cross-linked by the short cell wall peptide NH_2-L-Ala-D-Gln-m-Dpm-D-Ala-COOH [135] and this cell wall peptide is also found in *L. monocytogenes* [28]. α-Amylase was used as a reporter enzyme and shown to be anchored on the cell wall because of the ease of detection and identification of the enzymatic activity. The model for sorting proteins to the surface of *B. subtilis* cells is presented in Fig. 4.1.

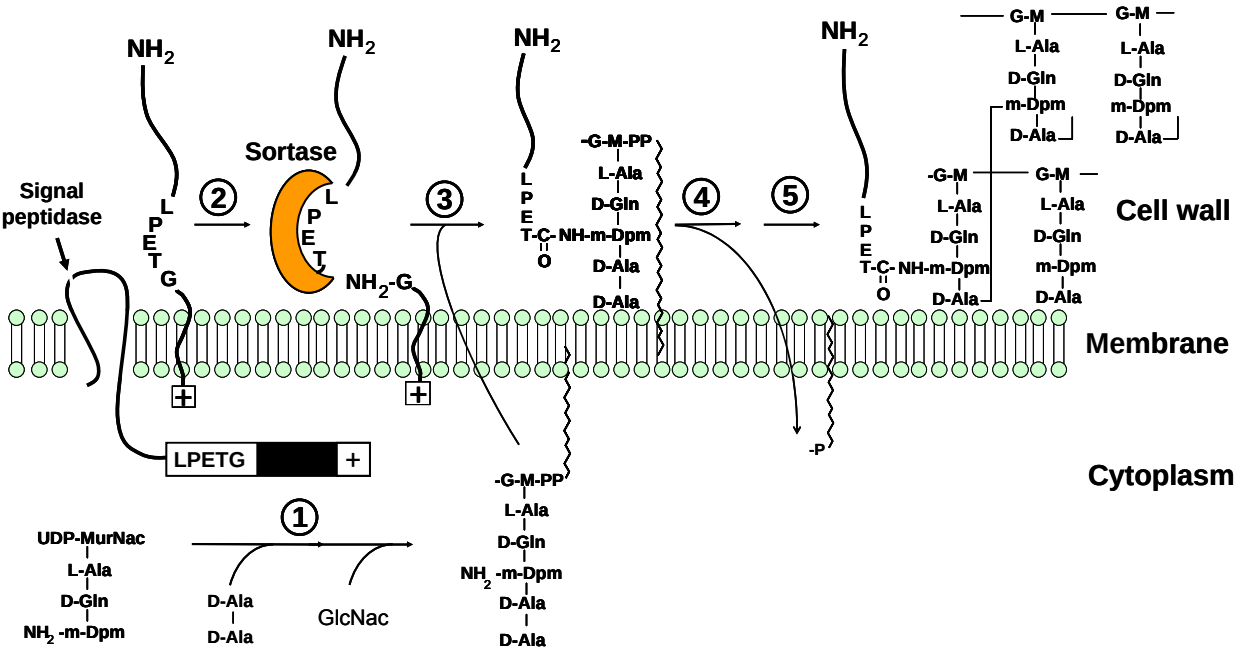


Fig. 4.1. Model for sorting surface proteins to the cell wall of *B. subtilis* modified after Dhar et al. [28] and Ton-That et al. [157]. Peptidoglycan precursor molecules (lipid II) are synthesized in the bacterial cytoplasm and translocated across the membrane (1). Precursors of surface proteins contain a signal sequence and a sorting signal. The sorting signal is comprised here of an LPXTG sequence motif, followed by a hydrophobic domain (black box) and a tail of positively charged residues (boxed +) (for more details, see Table 1.1). Surface proteins are first cleaved (2) between the threonine (T) and the glycine (G) of the LPETG motif and subsequently linked to the lipid II molecule (3). Surface proteins linked to lipid II may be incorporated into the cell wall envelope by the transglycosylation and transpeptidation reactions that generate mature cell wall (4, 5).

This is the first report that the sortase from one bacterial species works successfully in another species. In addition, the spacer region, the amount of amino acid residues located between the C-terminal end of the reporter protein and the sorting motif, strongly influenced both the amount of protein molecules anchored per cell and the enzymatic activity. In the present case, a spacer length of 123 aa residues turned out to be optimal for the α-amylase. This is in contrast to a lipase, where a spacer length from 92 to 223 residues yielded identical amounts and activity [150]. These two examples demonstrate that in the case of the α-amylase there is an optimal spacer length whereas in the case of the lipase the spacer length can vary in a wide range to yield a high enzymatic activity. It can be assumed that the spacer length affects folding of the protein anchored in those cases where its C-terminal end is part of the active structure. If, on the other hand, the C-terminal end is only loosely or not structured at all, the spacer length should not be important if a minimum length will be provided. It has also to be taken into consideration that the spacer itself may fold thereby impairing folding of the passenger protein. To solve all these folding problems, a spacer should be created of a length of about 130 amino acid residues.

Successful covalent anchoring of active α-amylase could be shown by two different experimental approaches: (i) treatment of whole cells by lysozyme, and (ii) by fluorescence microscopy. Lysozyme, an *N*-acetyl-muramidase [55], cuts the glycan strands and is able to release cell wall anchored proteins still bound to cell wall components [139]. These components did not inhibit the α-amylase activity. The same result was obtained when mutanolysin (a muramidase) was used instead of lysozyme (data not shown).

Visible inspection of whole cells with anchored α-amylase revealed that the enzyme is not equally distributed on the cell wall, but occurs in patches (clusters). This is reminiscent to the Sec machinery of *B. subtilis* which has been shown recently to be organized in spiral-like structures along the length of the cell, with most of the translocases organized in specific clusters [15]. In contrast, the secretory proteins and the motor protein SecA specifically localize to a microdomain distal to the cell poles of *S. pyogenes* [125]. It is conceivable that membrane-anchored sortase may be positioned in immediate vicinity of protein translocation sites as these enzymes are expected to scan polypeptide sequences for the presence of sorting motifs. If this assumption is indeed correct, sortase-dependent proteins of *S. pyogenes* should colocalize to the microdomain. When *B. subtilis* cells were treated with trypsin, the fluorescence signals were dramatically decreased (Fig. 3.14). It can be inferred that α-amylase

was exposed on the surface of *B. subtilis* cells. This result is similar to the one which has been described for lipase anchored on the cell wall of *S. carnosus* cells [150].

Two anchoring vectors have been constructed namely plasmids pNDH12 and pNDH14, both allowing insertion of the passenger gene between the *Bam*HI and *Aat*II sites where one will fuse a spacer of 94 and the second of 123 aa residues to the C-terminal end of the recombinant protein as schematically shown in Fig. 4.2.

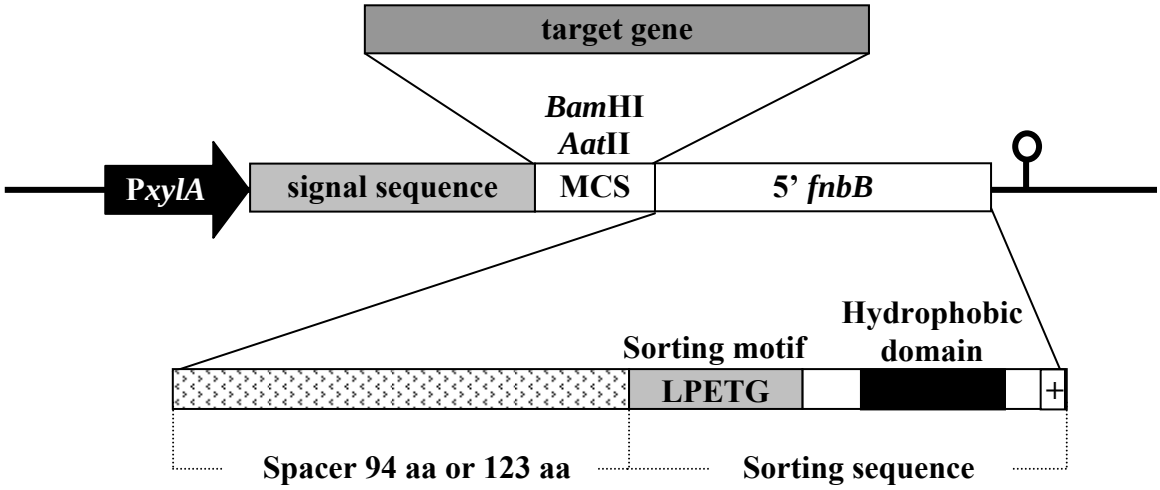


Fig. 4.2. Schematic representation of the anchoring vectors. Both vectors contain P*xylA*, a xylose-inducible promoter and a signal sequence encoded by the *amyQ* gene. A spacer length of 94 aa is present in plasmid pNDH12 and one of 123 aa in pNDH14.

Another important aspect is the number of molecules which can be immobilized per cell. Up to about 240,000 molecules of α-amylase could be anchored per cell while in previous studies approximately 10,000 molecules of lipase and alkaline phosphatase could be immobilized on the cell wall of *S. carnosus* and *S. aureus*, respectively [140, 150]. Is there an upper limit as to the amount of protein molecules which can be immobilized per cell? What component acts as bottleneck? The number of sortase molecules? The number of Sec machineries? The pace at which α-amylase is synthesized or/and secreted? It will be interesting to analyse these parameters in detail aiming to immobilize the maximum number of heterologous proteins on the cell wall of *B. subtilis*. Here, the excellent genetics developed with *B. subtilis* will possibly optimize the system and qualify *B. subtilis* cells as cellular chips.

4.2.2 The signal peptide of PhoD could not direct GFP secretion

In Gram-positive bacteria, most proteins are secreted into the culture medium using the Sec pathway. This pathway translocates only unfolded proteins through the cytoplasmic membrane [155]. Proteins are either kept unfolded by interaction with molecular chaperones such as SecB in *E. coli* [121] or are translocated co-translationally. But some proteins either fold very rapidly in the cytoplasm, or can fold correctly only in the cytoplasm such as GFP or need a co-factor which has to be incorporated into the folded structure of the protein which takes place in the cytoplasm. A second pathway has been described, the Tat pathway, which translocates folded proteins through the cytoplasmic membrane [7]. Therefore, an attempt has been made to find out whether proteins secreted via the Tat pathway can be anchored on the cell wall as well.

GFP, which is known to fold rapidly after its synthesis, was chosen. GFP originates from the jellyfish *Aequoria victoria* and is widely used as a tag to localise proteins within cells [84]. When GFP is equipped with a signal sequence for the Sec pathway, it is successfully secreted to the periplasm of *E. coli* cells. But it turned out that the protein is largely inactive most probably because it is not folded correctly [41]. Correct folding seems to occur only in the cytoplasm, but these protein molecules fail to be secreted through the cytoplasmic membrane. As mentioned above, folded proteins can be secreted using the Tat pathway. Proteins secreted via this pathway also carry a signal sequence at their N-terminal end which is slightly different from the Sec signal peptide. Most importantly, it carries two consecutive arginine residues which are essential for recognition by a so-far unknown protein factor(s). Quite recently, it could be shown that GFP equipped with a Tat signal sequence is secreted into the *E. coli* periplasm in its active form [152]. With the benefits: (i) easy to monitor the expression, (ii) emit the fluorescence under folded state, GFP is the first choice for studying the expression of secreted protein by the Tat pathway and subsequently anchoring on the cell wall of *B. subtilis*.

It has been shown that the twin-arginine signal peptide of *B. subtilis* phosphodiesterase PhoD (S*phoD*) can mediate transport of *E. coli* phytase AppA and direct its efficient secretion into the culture medium via Tat pathway in *B. subtilis* [45]. To answer the question whether GFP can be secreted and anchored on the cell wall of *B. subtilis* cell via the Tat pathway, the gfp^+ allele was fused with the N-terminal S*phoD* and the C-terminal sorting sequence *fnbB94*. The construct was then transformed into the strain NDH03 which expresses *L. monocytogenes*

sortase A when induced by IPTG. The cells exhibited GFP fluorescence under the fluorescence stereomicroscope and were analysed for covalent anchoring of GFP on the cell wall by Western blot. However, GFP could neither be found in the culture medium nor could be released by lysozyme treatment from the cell wall (data not shown). The fluorescence signal might come from GFP remaining in the cytoplasm. This failure might be explained in such a way that the S*phoD* is incompatible with GFP leading to inefficient secretion. In order to address this question, one can use the second Tat signal sequence already known, which is from the *ywbN* gene [66] or *yhcR* gene (from *in silico* analysis) [108] of *B. subtilis*. In addition, one should try anchoring the *phoD*-encoded phosphatase on the cell wall.

4.2.3 Putative sortase of *B. subtilis* could anchor protein on the cell wall

An *in silico* analysis identified multiple sortase gene homologs in the genomes of most Gram-positive bacteria [20, 110]. The housekeeping sortase (SrtA) seems to anchor most cell wall proteins while a limited number of proteins are anchored by accessory sortases [31]. The *B. subtilis* genome encodes two putative sortase, YhcS and YwpE [20]. The *yhcS* gene is located downstream of the *yhcR* gene encoding a potential sortase substrate. The gene for a second possible sortase substrate, the phosphodiesterase YfkN, is not genetically linked to a sortase-encoding gene [10]. In this study it could be demonstrated that YhcS could retain independently the hybrid protein AmyQ-YhcR123 on the cell wall and YwpE could assist YhcS to execute its function.

First of all, the expression of *yhcS* and *ywpE* was studied by two different methods, indirectly by a transcriptional fusion to a reporter gene and directly by a Northern blot. In the transcriptional fusion experiments, the promoter of *yhcS* and *ywpE* was fused to *lacZ* to allow quantification of the promoter during a growth curve by measuring the β-galactosidase activity. The results showed that both promoters tended to become active in the late stationary phase (Fig. 3.19). From this result, it can be inferred that target proteins will be anchored to the cell wall most probably late during the stationary phase. What might be the function of these proteins? Are they involved in survival of the cells during these adverse conditions? In the Northern blot experiment, the riboprobes for *yhcS* and *ywpE* were used to measure directly the expression of the two genes (Fig. 3.20). While *yhcS* was expressed in the late stationary phase thereby confirming the β-galactosidase assays, the y*wpE* gene did not exhibit any expression, even when a large amount of RNA (30 µg) have been applied per slot. How to

explain the positive result with the β-galactosidase assay and the negative one with the Northern blot? One possibility is that the *ywpE* transcript is extremely unstable as compared to the *lacZ* mRNA. Other possibilities not investigated here might be that the sortase genes are expressed only under specific conditions. These could occur when the cells are challenged with specific stress factors or under conditions of sporulation.

In a second step, sortase-dependent proteins should be identified. Since expression of the two sortases is rather low under the conditions analysed, both genes were fused independently and together forming a bicistronic synthetic operon to the IPTG-inducible promoter. A preliminary experiment to identify sortase-dependent proteins using SDS-PAGE from the lysozyme-treated cell wall revealed several bands present in cells expressing *yhcS* and the artificial *yhcS-ywpE* operon with a higher intensity than in other samples (Fig. 3.22). These protein bands could also be seen when the cells entered the stationary phase about 12 h after inoculation (data not shown). As mentioned above, YfkN and YhcR which have molecular weights of 159 and 132 kDa, respectively are potential cell wall proteins, but they can only detected in the medium [18, 108]. One possible reason is that YfkN and YhcR are removed by one or more extracellular proteases. To test this hypothesis, the strain WB800N carrying eight knockouts of extracellular protease genes [173] was utilized. Several distinct bands could be detected from the strain WB800N. A doublet band of about 140 kDa (Fig. 3.22) might be YfkN and YhcR anchored on the cell wall. To identify these proteins, total cell wall released proteins have to be separated by the 2-D gel technique followed by their analysis by mass spectrometry.

In another experiment, it was evaluated whether YhcR and YfkN could be anchored on the cell wall as expected by *in silico* analysis [10, 20]. To achieve this goal, the *fnbpB* part in the *amyQ-fnbpB123* fusion was replaced by either the C-terminal part of *yhcR* or *yfkN*. Both plasmids were transformed into the *B. subtilis yhcS* and/or *ywpE* knockout or wild type strain. The proteins released after lysozyme treatment of the cell wall identified YhcR123 when YhcS was present. It is worth noting that YhcS seems to recognize only the sorting signal of YhcR (LPDTS; (Table 1.1) and neither LPDTA nor LPETG. This indicates that YhcS belongs to class SrtD which is different from SrtA, SrtB and SrtC as described by Dramsi *et al* [31]. In addition, it might be "shave" by unknown protease(s) because the anchored YfkN123-AmyQ harbours the recognition site (YTNHFSEY) for unknown protease(s) within the spacer [18] as proposed by Westers [166]. This "shave" symptom might be also the case for FnBPB with the

longer spacers consisting of 162, 196 and 234 aa residues; they might be shaved by such proteases residing at the membrane-cell wall interface and/or in the culture medium.

Interestingly, the amount of AmyQ-YhcR123 was always higher in wild type cells harbouring both *yhcS* and *ywpE* than others. This result suggests that YwpE may assist YhcS. However, there is a localization problem with the putative YwpE sortase. All sortases analysed so far are anchored in the cytoplasmic membrane where the active side is on the outside. Anchoring occurs through a hydrophobic segment close to the N-terminus of the secreted sortase. The YwpE protein is missing such a hydrophobic segment and, as a consequence, should reside within the cytoplasm. How can a cytoplasmic sortase anchor target proteins on the cell wall? One possibility, though highly speculative, might be that this potential sortase anchors proteins on lipid II before it is translocated through the membrane. Alternatively, the *ywpE* gene does not encode a sortase, but a protein with a completely different function.

There is at least one conceptual problem concerning sortase functioning in *B. subtilis* involving the biogenesis of the cell wall turnover during cell growth and division [3, 134]. Cell wall biogenesis takes place at the membrane surface and, continuously during growth, layers of peptidoglycan move from the membrane side of the wall to the outside. Eventually, the outer layers of the wall are shed into the environment, which makes it uneasy to retain proteins in the cell by covalent anchoring to peptidoglycan. This is apparently no problem in cocci, such as *S. aureus*, which conserve one half of their wall together with the covalently attached proteins during cell division. Anyway, this work was demonstrated that the active α-amylase could be anchored on the surface of *B. subtilis* cells and a model for sorting surface protein to the cell wall in *B. subtilis* has been proposed for both sortase SrtA of *L. monocytogenes* and YhcS of *B. subtilis* (Fig. 4.1).

4.3 Outlook

The ongoing discovery of new vaccines and therapeutics asks for the development of efficient systems for the production of pharmaceutical proteins. The choice of an appropriate host and suitable expression systems is crucial for the down-stream processing of a pharmaceutical-grade product. *E. coli* and *Bacillus* species are the most frequently used prokaryotes for the industrial production of recombinant proteins. These organisms are above all favoured because their cultivation in large-scale production systems at high cell densities is easy and usually inexpensive. A series of plasmids that allow proteins expression in the cytoplasm and

culture medium or to be immobilized on the cell surface in *B. subtilis* described in this work will be considerable tools not only in industry but also in basic research in the future. These plasmids might also be improved for specific purposes.

B. subtilis is not regarded as a pathogen and is classified as novel food that is currently being used as a probiotic for both human and animal consumption [34, 47, 94]. The single, distinguishing feature of this microorganism is that it produces an endospore as part of its developmental life cycle when cells are starved for nutrients. The mature spore, when released from its mother cell, can survive in a metabolically dormant form for hundreds if not thousands of years [32]. It is probable to germinate in the gastrointestinal tract [35, 37] and can be employed as vaccine vehicles [33, 36]. Recently, *B. subtilis* has been employed to display antigens on spores coat to use them as oral vaccine [89]. However, by using this technique each spore can display only 1000 to 3000 molecules [89], which could reduce the immune response of the host animal. Combination of all these benefits for *B. subtilis* with the newly developed method to anchor up to 240 000 molecules per vegetative cell would be an excellent alternative to use *B. subtilis* as an antigen vehicle.

5 References

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6 List of abbreviations and symbols

Abbreviation	Denotation
Aa	amino acid(s)
A_{260}	absorption at a wavelength of 260 nm
A_{280}	absorption at a wavelength of 280 nm
Amp^R	resistant to ampicillin
amyQ	gene coding for protein α-amylase (AmyQ)
APS	ammoniumperoxodisulfate
α-	alpha, indicating antibodies against something (except α-amylase) e.g, α-AmyQ means antibodies against AmyQ
B. amyloliquefaciens	*Bacillus amyloliquefaciens*
bgaB	coding for the heat stable reporter β-gaclactosidase (BgaB) in *G. stearothermophilus*
bp	base pairs
B. subtilis	*Bacillus subtilis*
cat	gene coding for chloraphenicol-acetytransferase
celA	gene coding for cellulase A (CelA) from *C. thermocellum*
celB	gene coding for cellulase B (CelB) from *C. thermocellum*
Cm^R	resistant to chloramphenicol
CMC	carboxy methyl cellulose
C. thermocellum	*Clostridium thermocellum*
^0C	degrees centigrade
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
Δ	deletion
E. coli	*Escherichia coli* (used as host strain for cloning)
Erm	Erythromycin
Erm^R	resistant to erythromycin
et al.	et alteri
fnbB	gene coding for finbronectin binding protein B (FnBPB) in *S. aureus*
g	gram
G. stearothermophilus	*Geobacillus stearothermophilus*
kb	kilobase

kDa	kilo-Dalton
LB	Luria-Bertani (growth medium)
m-Dpm	m-diaminopimelic acid
h	hour(s)
HCl	hydrocloride acid
htpG	gene coding for hight temperature protein G (HtpG)
IAA	isoamylalkohol
IPTG	isopropyl-ß-D-thiogalactoside
lacA	gene coding for β-galactosidase in *B. subtilis*
lacZ	gene coding for the reporter β-gaclactosidase (LacZ) in *E. coli*
l	liter
L. monocytogenes	*Listeria monocytogenes*
min	minute(s)
mg	milligram
ml	mililiter
mM	milimole
MOPS	morpholiopropanesulfonic acid
mRNA	messenger RNA
µg	microgram
µl	microliter
Neo	neomycin
nm	nanometer
nt	nucleotide(s)
OD_{578}	optical Density at a wavelength of 578 nm
PBS	phosphate-buffer saline
pbpE	gene coding for penicillin-binding protein PBP4* in *B. subtilis*
P*grac*	an IPTG inducble promoter, a hybrid promoter of P*groES* and *lac* operator
pmol	picromole
P*spac*	an IPTG-inducible promoter, a hybrid promoter of the phage SPO-1 and the *lacO*
P*xylA*	promoter of *xylA* gene, an xylose-inducible promoter
PVDF	polyvinylidene difluoride
RBS	ribosome binding site
RNA	ribonucleic acid
rpm	revolution or round per minute

RT	room temperature
S. aureus	*Staphylococcus aureus*
sec	second
SDS	sodium dodecyl sulphate
srtA	gene coding for SrtA (e.i. of *L. monocytogenes*)
TEMED	N,N,N`,N`-tetramethylenethylendiamide
tet	gene coding for Tet protein
Tet^R	resistant to tetracycline
Tris	tri-(hydroxymethyl)-aminomethane
Tween-20	polyoxyethylensorbitane monlaurate
U	units (enzyme activity)
UDP	undecaprenylpyrophosphate
v/v	volume/volume
w/v	weight/volume
yfkN	gene coding for protein YfkN in *B. subtilis*
yhcR	gene coding for protein YhcR in *B. subtilis*
yhcS	gene coding for protein YhcS in *B. subtilis*
ywpE	gene coding for protein YwpE in *B. subtilis*
xylA	gene coding for xylose isomerase
xylB	gene coding for xylulose kinase

Erklärung

Hiermit erkläre ich, dass ich die Arbeit selbständig verfasst und keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt habe.

Ferner erkläre ich, dass ich anderweitig mit oder ohne Erfolg nicht versucht habe, diese Dissertation einzureichen. Ich habe keine gleichartige Doktorprüfung an einer aderen Hochschule endgültig nicht bestanden.

Bayreuth, Juli 2006

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