“Identification and functions of type I signal peptidases of *Bacillus amyloliquefaciens*”

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Abbreviations

aa	amino acid(s)
Amp	ampicillin
bp	base pairs
BSA	Bovine serum albumin
Cm	chloramphenicol
CWBP	cell wall bound proteins
DNA	Desoxyribonucleic acid
EDTA	Ethylendiaminetetraacetic acid
Em	erythromycin
h	hour
IPTG	β-D-isopropylthiogalactopyranoside
kb	kilobase
kDa	kilodalton
MCS	multi-cloning site
min	minute
nt	nucleotides
OD	optical density
orf	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
Pm	phleomycin
PMSF	phenylmethylsulfonyl flourid
pre-OmpA	OmpA precursor protein
Rep	replication initiation protein
rpm	rounds per minute
RT	room temperature
SDS	sodium dodecyl sulphate
SPase I	signal peptidase I (leader peptidase I)
Sip	signal peptidase protein
sip	signal peptidase gene
TCA	trichloroacetic acid
ts	temperature sensitivity
X-gal	5-bromo-4-chloro-3-indolyl-β-galactoside

1. Introduction

1.1. General

In all living cells proteins are synthesised according to their genetic information (encoded in genes), but the information transfer process does not end with the biosynthesis of a polypeptide chain. The synthesised proteins can only fulfil their biological functions if they are in a correct conformation. A significant subset of proteins, about 20 to 30 % of the total proteom, must be transported to the place for native folding and functions, i.e. to a distinct subcellular or membrane-enclosed compartment. For this purpose, prokaryotic as well as eukaryotic cells have developed mechanisms to address and to transport proteins to various sub– or extracellular compartments.

The exported proteins of both prokaryotic and eukaryotic origin are usually synthesised as precursors with an amino-terminal extension, the signal peptide, that is recognised by the transport apparatus and often contains additional sequence motifs for adhesion or binding of cellular targets, i.e. cell wall, organelles, proteins etc. (Schneewind, *et al.*, 1992, 1993, 1995; Navarre & Schneewind, 1994; Navarre, *et al.,* 1996). The signal peptide distinguishes the exported proteins from the cytoplasmic ones and is needed for targeting of proteins to the export pathway (von Heijne, 1990a, 1990b, 1998). The signal peptide consists of short stretches of amino acids, which, after protein delivery to the correct subcellular compartment, are specifically removed by special signal peptidases (Dalbey, *et al.*, 1985; Briggs, *et al.*, 1986; Deshaies, 1989). In general, targeting occurs by binding of the signal peptide to the membrane through soluble cytoplasmic protein components. In bacteria the exported proteins must pass across the cytoplasmic membrane (CM), in eukaryotic cells across the endoplasmic reticulum (ER) membrane. A translocation motor, which binds and hydrolyses nucleoside triphosphates, is needed for driving the transport of a polypeptide chain through a proteinaceous channel. Finally, the signal peptide is removed and the protein is released from the translocase. If the protein is translocated in its unfolded conformation, it will fold into a native conformation shortly after release from the translocase with the assistance of specific chaperones. These principles of protein transport through membranes are basically similar in eukaryotic and prokaryotic organisms (Schatz & Dobberstein, 1996; Pohlschröder, *et al.*, 1997; Riezman, 1997; Economou, *et al.*, 1998).

In contrast to eukaryotic cells where proteins are transported to numerous destinations (the nucleus, the ER, the Golgi apparatus, lysozomes, chloroplasts, mitochondria, etc.), in eubacterial and archaeal cells protein transport is limited to only few compartments, such as to the cytoplasmic membrane, into the cell wall of Gram-positive eubacteria and archaea, the

periplasm and the outer membrane of Gram-negative eubacteria, or across the cell barriers into the culture media. For these, bacteria have developed multiple pathways such as the general secretory pathway (Sec), the twin-arginine pathway (Tat), the ATP-binding cassette (ABC) transporters, as well as the type IV pilin-like secretion pathway.

The following sections will provide a general overview about protein transport in *Bacillus* with most knowledge stemming from *B. subtilis* studies. Notably, the protein export machineries of Gram-negative eubacterium *Escherichia coli* and of certain eukarya, such as the yeast *Sacchromyces cerevisiae*, have in more detail been characterised than those of *B. subtilis*. For comparison, these machineries will be mentioned and discussed.

1.2. Cellular compartments in Bacillus

Bacillus cell is structurally less complicated compared to eukaryotic cell. In *Bacillus*, the cytoplasm is surrounded by the cytoplasmic membrane, which is covered by a thick cell wall (10 to 50 nm) composed mainly of chains of peptidoglycan and teichoic or teichuronic acid (Archibald, *et al.*, 1993). After synthesis, proteins can be either retained in the cytoplasm or targeted into the CM or transported across the CM into the cell wall, remained attachment or released from the cell wall into the external medium.

Cytoplasmic proteins

In general, proteins lacking transport signals are retained in the cytoplasm and folded into their native conformation with or without the aid of chaperones, such as GroEL-GroES and DnaK-DnaJ-GrpE (Ewalt, *et al.*, 1997; Hartl, 1998; Beissinger & Buchner, 1998; Netzer & Hartl, 1998).

Membrane proteins

Very little is known about the targeting of proteins into the membrane in *Bacillus* species. However, by analogy with protein export in *E. coli*, it is believed that some of the CM proteins are actively integrated into the membrane by the aid of export pathway, while some proteins might “spontaneously” insert as a result of ionic and hydrophobic interactions.

Cell wall proteins

The cell wall of *B. subtilis*, in analogy to the Gram-negative periplasm, defines a cellular compartment containing approximately 9 % of the total mass of the cellular protein content (Pooley, *et al.*, 1996). In *B. subtilis*, proteins retained in the cell wall include DNases, RNases (Merchante, *et al.*, 1995), proteases (Margot & Karamata, 1996; Msadek, *et al.*, 1998),

enzymes involved in the synthesis of peptidoglycan (penicillin-binding proteins) and cell wall hydrolases that are involved in cell wall turnover during cell growth, cell division, sporulation and germination (Kuroda, *et al.,* 1993; Margot, *et al.,* 1994, 1999; Smith, *et al.,* 1996, 2000; Blackman, *et al.,* 1998).

Extracellular proteins

Most proteins that are finally transported across the cytoplasmic membrane are synthesised with an amino-terminal signal peptide. Since *B. subtilis,* like other Gram-positive eubacteria, lacks an outer membrane, many of these proteins are directly secreted into the growth medium. In most cases, these secreted proteins are enzymes involved in the hydrolysis of natural polymers such as proteases, lipases, carbohydrases, DNases and RNases. Such degradative enzymes are usually synthesised as part of an adaptive response to changes in the environment, allowing the cell to optimally benefit from available resources (Simonen & Palva, 1993). A second group of secreted proteins consists of relatively small proteins, denoted PhrA to PhrK. They are members of the Phr family of phosphatase regulators and are associated each with a corresponding Rap phosphatase (Perego, *et al.,* 1996; Kunst, *et al.,* 1997). After removal of the signal peptide during secretion and proteolytically process into active form (pentapeptides) the Phr proteins are re-imported into the mother cell to fulfil their regulatory action by inhibiting phosphorylation activities of a certain cytoplasmic Rap phosphatase. The production of those active Phr peptapeptides was postulated to be a regulatory mechanism required for timing and co-ordination of alternative physiological events such as growth, competence and sporulation (Solomon, *et al.,* 1995; Perego, *et al.,* 1997; Lazzazera, *et al.,* 1997; Jiang, *et al.,* 2000).

1.3. Signal peptides and transport pathways in Bacillus

1.3.1. Signal peptides

The presence of a signal peptide is not the only, but the prominent feature that distinguishes the exported proteins from the cytoplasmic ones. Although the primary structures of different signal peptides are not conserved, three distinct domains can be recognised (von Heijne, 1990a, 1990b, 1998). The positively charged NH_2 terminus (N-region) contains at least one arginine or lysine residue, which has been suggested to interact with the translocase machinery as well as with negatively charged phospholipids in the lipid bilayer of the membrane during translocation (Jones, *et al.,* 1990; Akita, *et al.,* 1990). The hydrophobic region (H-region), following the N-region, is formed by a stretch of hydrophobic

residues that seem to adopt an α-helical conformation in the membrane. Helix-breaking glycine or proline residues are often present in the middle of the H-region; these residues might allow the signal peptide to form the hairpin-like structure that can insert into the membrane. The more polar C-region, following the H-region, contains the cleavage or recognition site for signal peptidase (SPase) processing which at the other side of the membrane removes the signal peptide from the mature part of the protein and thereby releases export proteins from the membrane, allowing proteins to fold into their native conformation. Although different amino-terminal signal peptides seem to have a rather similar structure, small differences among individual signal peptides will determine them to be cleavaged by a different SPase or to be exported through different pathways to different destinations (Ng, *et al.*, 1996; Weiner, *et al.*, 1998). At present, on the basis of the SPase recognition sequence and the targeting transport pathway, five major classes of amino-terminal signal peptides can be distinguished (Figure 1).

1.3.1.1. Secretory (Sec) signal peptides

The first class is defined to characterise "typical" signal peptides which are common in preproteins that are cleaved by type I SPases. Although in *Bacillus* most proteins having such a signal seem to be secreted into the extracellular environment, some of them are retained in the cell wall or sorted after membrane translocation specifically to the inner membrane space (IMS) of endospores via the Sec pathway. Despite these common features, statistically significant differences between signal peptides of various organisms can be found (von Heijne & Abrahmsen, 1989). For example, the N-regions of Gram-positive signal peptides are clearly more positively charged than those of *E. coli* or eukaryotes. The signal peptides of Gram-positive bacteria are often longer than those of other organisms. The different lengths of signal peptides may be related to either differences in recognition by the translocase, by the SPases or by other secretion components in Gram-positive and Gram-negative bacteria. It has indeed been observed that signal peptidases of *E. coli* and *Bacillus* species often cleave a given signal peptide at different sites, with *E. coli* favouring cleavage sites that produce shorter signal peptides than those of *Bacillus* species (Takase, *et al.*, 1988; Itoh, *et al.*, 1990). Thanks to the completion of the *B. subtilis* genome sequencing project (Kunst, *et al.*, 1997) and the assistance of computer analysis (Wallin & von Heijne, 1998; Nielsen, *et al.*, 1997), now we can have a closer look at signal peptides of all putative secretory proteins in this organism. About 166 proteins of the total *B. subtilis* proteom seem to possess a secretory signal peptide. The secretory signal peptides of *B. subtilis* vary in length from 19 to 44 residues, with an average of 28 residues. The N-region often contains 2 or 3 positively

charged lysine (K) or arginine (R) residues, however some may have as much as 5 to 11 positively charged residues. The H-region has an average length of 19, but a length of 17 or 18 residues seems to be preferred. About 60 % of signal peptides have a residue (mostly glycine) in the middle of the H-region, and 50 % have a helix-breaking residue (proline or glycine) at positions –7 to –4. The C-region of *B. subtilis* secretory signal peptides contains a common consensus sequence A-X-A at the positions –3 and –1 and serves as an SPase I cleavage site (Tjalsma, *et al.*, 2000).

1.3.1.2. Twin-arginine signal peptides

Twin-arginine signal peptides are a subgroup of secretory signal peptides, which contain an additional so-called twin-arginine motif (S/T-R-R-x-F-L-K) at the boundary between N-region and H-region (Berks, 1996). The twin-arginine signal peptides were believed to direct proteins into a distinct translocation pathway known as the Tat pathway (Berks, *et al.*, 2000). The H-region of twin-arginine signal peptides often has a helix-breaking proline at position -6 from the signal peptidase cleavage site (Cristobal, *et al.*, 1999). The C-region of twin-arginine signal peptides also contains basic amino acids (Brüser, *et al.*, 1998; Wexler, *et al.*, 1998; Cristobal, *et al.*, 1999), which were not preferred in Sec-pathway signal peptides (von Heijne, 1990b).

1.3.1.3. Lipoprotein signal peptides

The third major class of signal peptides is present in prelipoproteins which are cleaved by the lipoprotein-specific (type II) SPase of *B. subtilis* (Pragai, *et al.*, 1997; Tjlsma, *et al.*, 1999a). The major difference between signal peptides of lipoproteins and non-lipid proteins is the presence of a well-conserved lipo-box within the lipoprotein precursors (von Heijne, 1989). This lipo-box contains an invariable cysteine residue that is lipid-modified by the diacylglyceryl transferase prior to precursor cleavage by SPase II. After translocation across the cytoplasmic membrane, lipid-modified proteins remain anchored to the membrane by their amino-terminal lipid-modified cysteine.

1.3.1.4. Signal peptides and secretion of pilin-like proteins

The fourth major class is formed by signal peptides of prepilin-like proteins, which, in *B. subtilis,* are cleaved by the prepilin-specific SPase ComC (Chung & Dubnau, 1995). The recognition sequence for the prepilin SPase is, in contrast to that of secretory and lipoproteins, localised between the N- and H-domains, leaving the H-domain after cleavage attached to the mature pilin (Pugsley, 1993; Lory, 1994; Chung & Dubnau, 1998) (see Figure 1). In *B.*

subtilis, ComG or type IV pilin-like proteins (encoded by *com*GC, *com*GD, *com*GE, and *com*GG genes, which are involved in the development of genetic competence) are exported in a Sec-independent manner. They resemble type IV pilins of various Gram-negative bacteria that are synthesised as precursors with cleavable signal peptides. Although prepilin signal peptides show certain similarities to those of secretory proteins or of lipoproteins, the prepilin-like precursors are believed to bypass the Sec and Tat secretion pathways, as their translocation is dependent on a cleavage event at the cytoplasmic side of the membrane (Pugsley, 1993; Chung, *et al.*, 1998). The *B. subtilis* SPase ComC is an integral membrane protein, which contains eight (putative) transmembrane regions and has a high degree of similarity to prepilin peptidases of various other organisms (Lory, 1994). The processing of the ComG proteins seems to be required for the assembly and anchoring of pilin-like structures to the membrane, which in turn are required for DNA binding during transformation in *B. subtilis* (Dubnau, 1997; Chung, *et al.*, 1998).

1.3.1.5. Signal peptides and secretion of bacteriocins and pheromones

Several *Bacillus* species produce peptide antibiotics, which are synthesised through either a ribosomal or nonribosomal mechanism (Nakano & Zuber, 1990; Zuber, *et al.*, 1993). Some of the ribosomally synthesised antimicrobial peptides contain signal peptides for their translocation across the membrane by dedicated ABC transporters (Havarstein, *et al.*, 1995; Sahl, *et al.*, 1995). These signal peptides lack a hydrophobic H-domain and are removed from the mature protein by a subunit (transport protease) of the ABC transporter that is responsible for the export of a particular bacteriocin or pheromone (Figure 1). In *B. subtilis* 168 the *sun*S-*sun*T operon has recently been shown to encodethe lantibiotic sublancin 168 and the ABC-transporter SunT, respectively, the latter is required for sublancin production. Interestingly, ABC transporters such as SunT have a dual role in secretion as they are responsible both for removal of the signal peptide and for translocation of the mature lantibiotic across the cytoplasmic membrane (Havarstein, *et al.*, 1995; Paik, *et al.*, 1998; Franke, *et al.*, 1999). Some other antibacterial peptides like subtilin of the *B. subtilis* strain ATCC 6633 (Banerjee & Hansen, 1988; Chung, *et al.*, 1992), subtilosin or two newly discovered ericins of the *B. subtilis* strain A1/3 (Zheng, *et al.*, 1999; Stein, *et al.*, 2001) have different sequence motifs to sublancin in their signal peptide, but seem to be processed in the same manner.

Although not documented, it is believed that an extracellular pheromone ComX, which is involved in cell density-controlled onset of transcription of competence genes (Magnuson, 1994), is secreted via an ABC transporter. In addition, the identification of 77 (putative) ABC

transporters in *B. subtilis* suggested that a couple of unidentified proteins might be also transported via those systems (Kunst, *et al.*, 1997; Quentin, *et al.*, 1999).

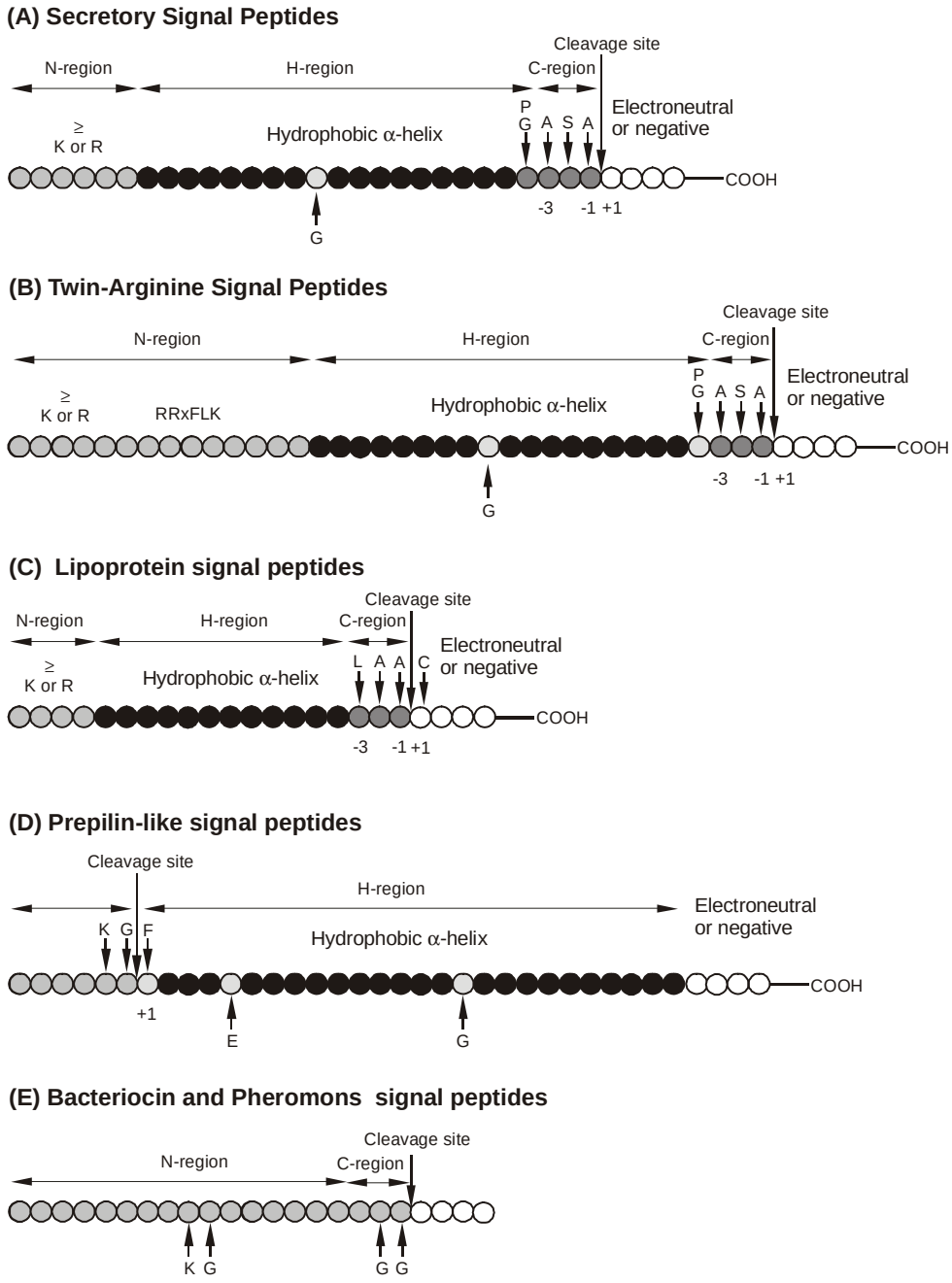

Figure 1. Schematic representation of the five main types of signal peptides found in precursors of exported *B. subtilis* proteins. Each amino acid within the functional elements of the signal peptides is indicated by a black or grey circle. Black circles represent hydrophobic or apolar residues, grey circles represent polar or charged residues and the white circles represent residues in the mature protein. The positions of conserved amino acids are indicated. (A), secretory (sec-type) signal peptides; (B), twin-arginine signal peptides; (C), lipoprotein signal peptides; (D), prepilin-like signal peptides; (E), bacteriocin and pheromone signal peptides.

1.3.2. Transport pathways

In *B. subtilis*, most proteins seem to be exported to or inserted into the cytoplasmic membrane via the Sec pathway, but apparently several alternative export pathways exist. First, the recently identified twin-arginine translocation (Tat) pathway seems to be present in *B. subtilis*, as judged from the identification of signal peptides with the RR-motif and conserved components of this pathway. Second, the assembly of extracellular prepilin-like structures depends on components which are, most likely, not involved in Sec-dependent protein secretion. Finally, some small prepeptides contain signal peptides lacking a hydrophobic domain. These peptides are transported across the membrane and cleaved by ABC transporters. This report will focus on the first two pathways relating to our main topics: "Type I SPases of *B. amyloliquefaciens* and their role in proteins secretion".

1.3.2.1. Twin-Arginine translocation pathway (Tat pathway)

Protein secretion via this pathway was shown to be independent of Sec components in *E. coli* and plant chloroplasts. Possibly, this pathway has evolved specifically for the export of folded preproteins (Dalbey & Robinson, 1999; Berks, *et al.,* 2000). The Tat pathway was first discovered in chloroplasts, in which it is involved in ΔpH-dependent protein import into the thylakoid lumen (Robinson, *et al.,* 1994; Chaddock, *et al.*, 1995; Settles, *et al.*, 1997). For the chloroplast system it was shown that, in contrast to Sec-dependent translocation, via this pathway proteins can be translocated in a folded conformation (Clark & Theg, 1997; Hynds, *et al.,* 1998). Furthermore, it was demonstrated that two adjacent arginines combined with a hydrophobic determinant (preferably leucine) at position +2 or +3, relative to the twin arginines, are needed in the N-domain of signal peptides in order to direct precursors into this pathway (Brink, *et al.*, 1997, 1998; Cristobal, *et al.,* 1999). Although the exact mechanism of protein export via the Tat pathway has not been yet unravelled, five components of the Tat pathway of *E. coli* have been identified. These are TatA (a putative membrane-bound receptor, homologous to the maize Hcf106 protein) (Settles, *et al.,* 1997), TatB (a TatA paralogue) (Sargent, *et al.*, 1998), TatC (the putative translocase), TatD (a predicted soluble protein) and TatE (a TatA paralogue). Interestingly, *B. subtilis* contains three homologues of TatA/B/E (encoded by the *ydi*I, *ycz*B and *ynz*A genes), two homologues of TatC (encoded by the *ydiJ* and *ycbT* genes) and another TatD homologue which is encoded by the *yab*D gene (Kunst, *et al.*, 1997).

1.3.2.2. General secretory pathway (Sec pathway)

The Sec pathway is responsible for the transport of most proteins in *B. subtilis*. The various components of the Sec-dependent secretion machinery can be divided into 5 groups: cytosolic chaperones, the translocation machinery (SecA, SecY, SecE, SecG, and SecDF-YajC), SPases, SPPases and folding factors that function at the *trans* side of the membrane (Figure 2). Moreover, the Sec pathway could be separated into three distinct, but sequential and interdependent stages.

The stage I of protein export in *E. coli* requires two secretion-specific chaperones: SecB and a ribonucleoprotein complex forming the signal recognition particle (SRP). SRP is composed of an Ffh protein and a 4.5S RNA species. SRP and SecB both recognize subsets of secreted preproteins, the SRP are most important for the targeting of membrane proteins, which have multiple-membrane-spanning domains (de Gier, *et al.*, 1998; Valent, *et al.*, 1998). In *B. subtilis* the Ffh protein and a small cytoplasmic RNA (functionally related to eukaryotic 7S RNA and *E. coli* 4.5S RNA) have been identified (Nakamura, *et al.*, 1994). SRP of *B. subtilis* seems to contain an additional component, HBsu (an Histone-like protein), which is not present in SRP of *E. coli* (Nakamura, *et al.*,1999). The SecB homologue is not present in *B. subtilis*, but a SecB analogue seems to exist. A candidate for a SecB analogue in *B. subtilis* is the CsaA protein (Müller, *et al.*, 1992, 2000a, b).

Stage II of protein transport in the Sec-pathway of *B. subtilis* involves the translocase complex in the membrane, which consists of at least 6 subunits: SecA (the translocation motor, an ATPase), SecE, SecDF, SecY, SecG and YajC. From electron-microscopy studies in *E. coli*, it has been estimated that up to three SecYE dimers can assemble in a quasipentagonal ring-shaped structure built around a putative pore (Meyer, *et al.*, 1999). The SecA ATPase, "translocation motor", is essential and unique to bacteria (Pohlschroder, *et al.*, 1997). SecA is a large, elongated, dimeric molecule comprising two primary domains: the ATPase (amino-terminal) domain and the dimerisation (carboxy-terminal) domain. It was shown in *E. coli* that the carboxy-terminal domain allows SecA to bind to SecYEG, leading to the functional translocase core (Economou & Wickner, 1994; Economou, *et al.*, 1995; Duong *et al.*, 1997). In *B. subtilis*, SecDF is a natural Siamese Twin protein (Bolhuis, *et al.*, 1998). SecDF was shown not to be essential for the cell viability, but to optimise and to maintain a high capacity for protein secretion (Bolhuis, *et al.*, 1998). In *E.coli*, SecD and SecF form a heterometric subcomplex with YajC (Duong, *et al.*, 1997). Disruption of the *yaj*C gene of *E. coli* did not have a clear effect on protein export, but overproduction of YajC suppresses the dominant negative phenotype of *sec*Y-d1 mutation, an internal inframe deletion in the *sec*Y gene (Taura, *et al.*, 1994). Finally, the energy for the translocase machinery comes from

chemical (ATP) as well as electrochemical (proton motive force, PMF) souces; ATP is essential, and PMF enhances translocation rates (Duong, *et al.*, 1997; Economou, 1998).

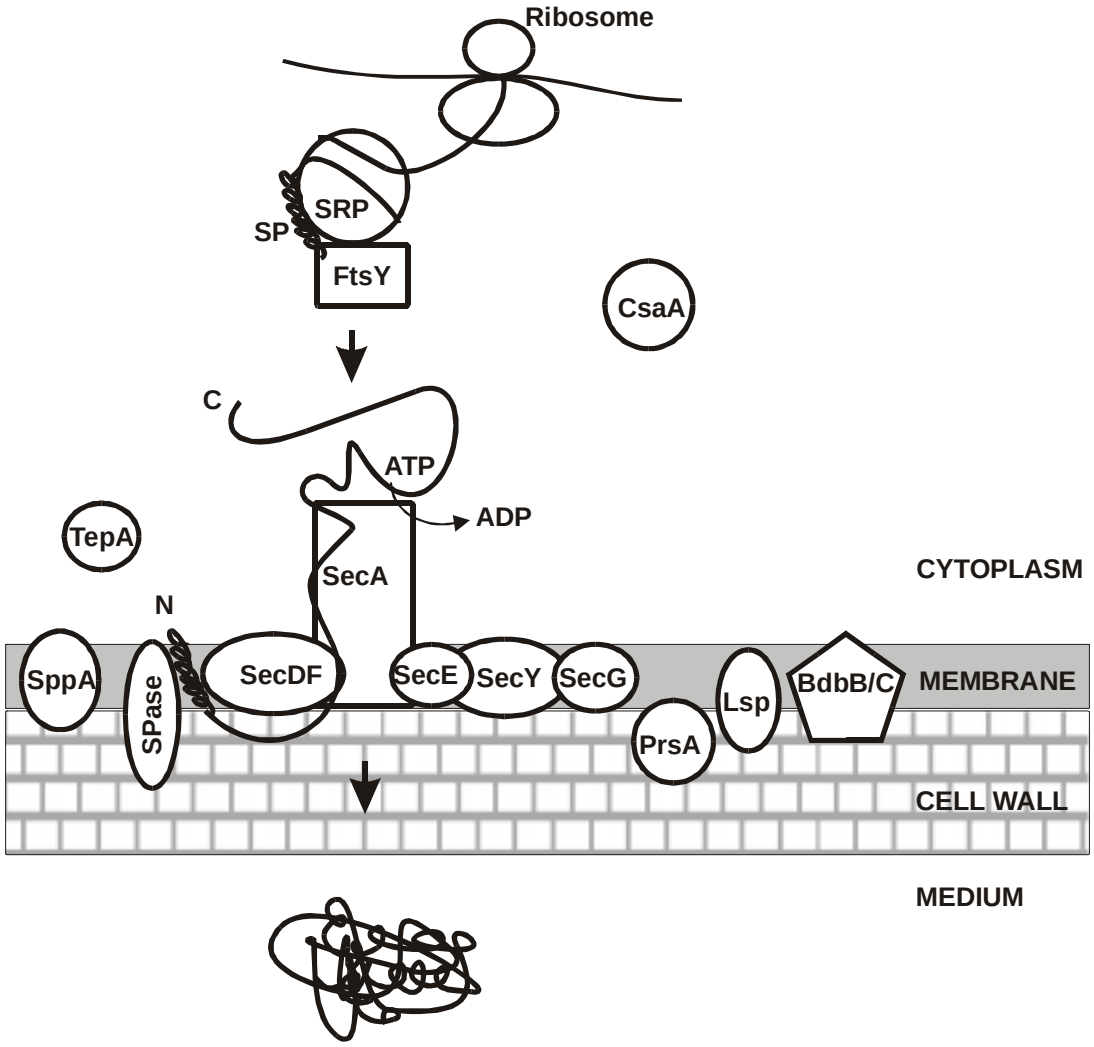

Figure 2. Schematic representation of components of the secretory machinery of *B. subtilis*. The secretory proteins are synthesized as precursors with an N-terminal signal peptide. Cytoplasmic chaperones, such as the **SRP** complex, **FtsY**, and **Csa** play a role in keeping these precursors in a translocation-competent form and targeting them to the translocase in the membrane. The *B. subtilis* translocase are contains **SecA**, **SecY**, **SecE**, **SecG**, and **SecDF**. Shortly after translocation, pre-proteins are processed either by one of the type I SPases (**SipS, SipT, SipU, SipV, SipW**) in case of secretory proteins or by type II SPases (**Lsp**) in case of lipoproteins. SppA and TepA are thought to be involved in the degradation of cleaved signal peptides. **PrsA** and/or **DdbB/C** take part in the folding of the mature protein. The protein is finally released into the medium after passing the cell wall.

Stage III involves the action of signal peptidase(s) (or leader peptidase(s)) and the extracytoplasmic folding catalysis (PrsA, BdbA/B/C, metal ion, etc.). Five distinct chromosomally encoded type I signal peptidases (SipS, SipT, SipU, SipV, and SipW), and

one type II signal peptidase (LspA), have been identified in *B. subtilis* (van Dijl, *et al.*, 1992; Tjalsma, *et al.*, 1997; Kunst, *et al.*, 1997). In contrast to *B. subtilis*, several other eubacteria, archaea and yeasts have only one type I signal peptidase (Dalbey & Wickner, 1985; Bult, *et al.*, 1996; Goffeau, *et al.*, 1997; Smith, *et al.*, 1997; Dalbey, *et al.*, 1997). However, it seems that most eukaryotic species contain two paralogous type I-SPases (Dalbey, *et al.*, 1997). The multiple type I-SPases have meanwhile been observed in many other bacteria and archaea, such as *Archaeoglobus fulgidus* (Klenk, *et al.*, 1997), *Streptomyces lividans* (Parro, *et al.*, 1999), *Bradyrhizobium japonicum* (Bairl & Müller, 1998) and *Bacillus amyloliquefaciens* (Hoang & Hofemeister, 1995). It was shown in *E. coli*, *S. serevisiae* and *B. subtilis* that type I-SPases are essential for cell viability (Dalbey & Wichne,r 1985; Böhni, *et al.*, 1988; Dalbey & von Heijne, 1992; Tjalsma, *et al.*, 1998). However in *B. subtilis*, only SipS and SipT seem to be of major importance for processing of preproteins as well as for cell viability. SipU, SipV, SipW seem to play a minor role in protein secretion (Tjalsma, *et al.*, 1998). In addition, some *B. subtilis* strains have an extra type I-SPase (SipP), which resides on plasmids. It was shown that SipP can functionally replace the major SPases SipS and SipT (Tjalsma, *et al.*, 1999). SipW apparently belongs to the subfamily of eukaryotic type (ER-type) SPases, while all other type I-SPases of *B. subtilis* belong to the prokaryotic type (P-type). X-ray crystallography studies of LepB of *E. coli* by Paetzel *et al.* (1998) proved that P-type SPases use serine-lysine catalytic dyad. The (eukaryotic) ER-type SPases seem to apply a Ser-His-Asp catalytic triad or Ser-His catalytic dyad, which is supported by site-directed mutant experiments and by the fact that the catalytic Lys residue of P-type SPases could replaced by His in ER-type SPases (Dalbey, *et al.*, 1997).

Several extracellular folding catalysts (foldases) mediate folding of export proteins at the trans side of the membrane. In *B. subtilis*, a lipoprotein PrsA (a PPIase), which catalyses the cis-trans isomerization of peptidyl-prolyl bonds, has been identified. PrsA is essential for viability and strains containing mutant PrsA were shown to be defect in secretion of proteins. This is probably due to the slowdown of folding, which might result in increased sensitivity of these exoproteins to proteolysis (Kontinen, *et al.*, 1991, 1993). Another foldase which has been identified in *B. subtilis* is thiol-disulfide oxidoreductase, which is responsible for the formation of disulfied bonds. Three genes (*bdb*A, *bdb*B, *bdb*C) coding for proteins with similarity to thiol-disulfide oxidoreductases have been found in the *B. subtilis* genome (Kunst, *et al.*, 1997; Bolhuis, *et al.*, 1999). In addition, metal ions can act as folding factors. For example, Ca^{2+} and Fe^{3+} can act as folding catalysts for levansucrase in *B. subtilis*.

1.4. Protein traffic in sporulation

A large number of the Gram-positive bacilli, including *B. subtilis*, have adopted sporulation as a means of survival when environmental conditions are less than optimal for growth, e.g. after nutrient starvation, drought, extreme cold or heat (Stragier & Losick, 1996). At the beginning of the sporulation process, a septum is formed that divides the cell into two unequally sized compartments. Subsequently, the larger compartment (the mother cell) engulfs the smaller compartment (the forespore), which ultimately becomes the spore. The forespore is surrounded by two membranes. The inter-membrane space (IMS) is the assembly site of two specialised peptidoglycan layers, called tile germ cell wall and the cortex. Finally about six different spore coat layers are formed (Henriques & Moran, 2000). Consequently, proteins residing in the germ cell wall or in the cortex must be sorted within the IMS towards the forespore and the mother cell.

One of the processes that require protein transport during sporulation is the communication between the mother cell and the forespore. Several proteins involved in stage II of sporulation, such as SpoIID, SpoIIP, SpoIIQ and SpoIIR (Kunst, *et al.*, 1997), contain a putative signal peptide. The SpoIIR protein, synthesised in the forespore prior to engulfment, was shown to be exported by the forespore and to interact with membrane proteins of the mother cell. SpoIIR directly or indirectly activates the receptor/protease SpoIIGA, which is required for pro-δ^E processing (Hofmeister, *et al.*, 1995, 1998). SpoIID is homologous to LytB, the modifier protein that enhances the activity of the major vegetative amidase LytC (Lazarevic, *et al.*, 1992; Kuroda, *et al.*, 1993). This suggested that SpoIID might play a role in activation of one or more autolysins, which are required for hydrolysis of the asymmetric septum permitting prespore engulfment (Illing & Errington, 1991). The lysostaphin-like SpoIIQ also has a role in prespore engulfment, although apparently not in septum hydrolysis, because a *spo*IIQ mutant is blocked after the septum has disappeared (Londono-Vallejo, *et al.*, 1997). The SpoIIP protein is proposed to be involved in dissolution of the peptidoglycan located in the sporulation septum. Disruption of *spo*IIP prevents complete degradation of the septal cell wall and leads to bulging of the forespore into the mother cell without further progression to engulfment (Frandsen & Stragier, 1995).

Other processes in sporulation that require transport of proteins, are the biogenesis of the germ wall and spore-cortex in the IMS of the forespore and the degradation of the spore peptidoglycan during germination. CwlD and DacB (also known as PBP5*) (Sekiguchi, *et al.*, 1995; Popham, *et al.*, 1995, 1999) are two export proteins that were reported to be involved in cortex synthesis. The germination-specific amidase SleB was found to be localized on the exterior side of the cortex in spores, while it is synthesised in the forespore compartment

(Moriyama, *et al.,* 1996; Boland, *et al.,* 2000). The fact that pre-SleB has to be transported across the forespore inner membrane and processed into its mature form to reach the IMS implies a functional protein translocation machinery and at least one of the type I SPases to be present in the forespore inner membrane. The recent finding that TasA (for translocated antibacterial spore-associated protein) with a broad spectrum of antibacterial activities is transported to *B. subtilis* endospores provides another example of spore-specific protein sorting. TasA is thought to confer a competitive advantage to the spore during the onset of sporulation and later, during germination, by inhibiting the growth of competing organisms (Stöver & Driks, 1999). In addition, TasA has been suggested to be required for proper spore coat assembly, and recent studies showed that SipW (signal peptidase) is specifically required for this process (Serano, *et al.,* 1999; Stöver & Driks, 1999; Tjalsma, *et al.,* 1999).

1.5. The aims of this study

Several *Bacillus* species are in use for industrial production of enzymes, of fine biochemicals, antibiotics, insecticides and also have been used in several traditional food fermentation processes (Harwood, 1992; Priest, 1993; Bron, *et al.,* 1999). Because of common apathogenicity (the GRAS status), high secretion capacity and the good knowledge about their fermentation technology, *Bacillus* species have been regarded as attractive production hosts, especially for the secretion of endogenous and heterologous proteins. Among those species, *B. amyloliquefaciens* is well known for its high capacity of extracellular enzyme production. In distinction from *B. subtilis, B. amyloliquefaciens* was named for the first time in 1943 by Fukomoto (Fukomoto, 1943) but was only recently recognised as an independent species (Priest, *et al.,* 1987). Despite the fact that *B. amyloliquefaciens* strains were widely used in industries, their usage is complicated due to difficulties in the genetic manipulation techniques and also due to the few studies concerning cell growth and physiology.

As reviewed, *B. subtilis* is known to contain five type I signal peptidases (SPases) and this multiplicity of SPases is proposed to correlate with high export capacities as well as highly specialised protein transportation during vegetative growth and cell differentiation leading to sporulation and germination (Kunst, *et al.,* 1997, Tjalsma, *et al.,* 1997, 2000b). The export capacity of *B. amyloliquefaciens* strains is even 10 times higher as documented by its use for production of enzymes for large-scale fermentation (Ingle & Boyer, 1976; Vehmaanpera, *et al.,* 1991). In continuation of initial studies (Hoang & Hofemeister, 1995) and based on the constitution of the genome of *B. subtilis* for multiple type I SPases, we decided to search for and to specify the peculiarities of *sip(Ba)* genes in *B. amyloliquefaciens* and to investigate the

specificity of Sip(*Ba*) enzymes with respect to their functions and subcellular localisation. The specific functions of each of the SPases of *B. amyloliquefaciens* was studied by construction of gene-specific (*sip*-) mutants, characterisation of the mutant phenotype, heterologous expression in *E. coli* and complementation of LepB, the type I-SPase of *E. coli* by wildtype and hybrid SPase enzymes. In additional, we decided to clone a gene encoding a new extracellular protein, of which the transport was thought to be affected in the *sipT* mutant. The chitin-binding properties of the protein will be characterised and its export will be investigated.

2. Materials and Methods

2.1. Enzymes and Chemicals

Amersham-Pharmacia, Braunschweig	ECL random primer and labelling kit, Hybond-N+, Hyperfilm ECL, SureClone ligation kit, Restriction enzymes, ^{35}S-Methionine *in vivo* labelling grade, Megaprimer DNA labelling systems, (α-^{32}P)dCTP.
Roche, Mannheim	Agarose, PCR-nucleotide mix, Expand long template PCR-kit, restriction enzymes, Taq-DNA polymerase, T4-DNA ligase, T4-DNA polymerase, protease-inhibitor set.
Carl Roth, Karlsruhe	Ampicillin, BSA, Chloramphenicol, EDTA, Acetic acid, Ethanol, Ethidiumbromid, Fructose, Glucose, Glycerin, HCl, IPTG, $MgCl_2$, Sodium acetate, NaCl, nButanol, Phenol, Phenol-Chloroform, Proteinase K, Sucrose, SDS, Tris-HCl, X-Gal, rotiphorese Gel30 (30% acrylamid, 0.8% bisacrylamid solutions).
Difco Laboratories, Augsburg	Agar-Agar, Trypton broth, Yeast extract.
Merck, Darmstadt	KH_2PO_4, K_2HPO_4.
Millipore, Eschborn	Nitrocellulose (0,025 µm) .
New England-Biolabs, Schwalbach/Ts.	Restriction enzymes, Shrimp alkali phosphatase.
Qiagen, Hilden	Plasmid Midi kit (50), Plasmid Maxi kit (10), QIAEx gel extraction kit, QIAquick PCR purification kit, QIAGEN Genomic-tip 100/G and 500/G, Rneasy Mini kit, Ni-NTA superflow (25), His Antibody (100).
Promega,	pGEM-T vector systems.
Sartorius, Göttingen	Sterilfilter (0.2 µm).
Sigma-Aldrich, Steinheim	ATP, $MnSO_4$, Papain, Pepsin, PMSF, amino acids.
Serva, Heidelberg	Succinat, Dextransulfat, Dimethylsulfoxid, Lysozyme, RNase, Triton X-100, Tween20.
Winthorp, Dublin, Ireland	Kanamycin.

2.2. Strains and growth conditions

2.2.1. Strains

Table 1: Bacterial strains

Bacteria	**Geno-/Phenotype & Reference**
Bacillus amyloliquefaciens GBA12	ALKO2718; *ΔnprE, ΔaprE*; Vehmaanperrä, et al., (1991)
GBA13	GBA12, but *sipS::*pEAS* [a]; This study
GBA14	GBA12, but *sipT*::pEAT*; This study
GBA15	GBA12, but *sipV*::pEAV*; This study
GBA16	GBA12, but *sipW::*pEAW*; This study
Bacillus amyloliquefaciens	ATCC 15841
Bacillus amyloliquefaciens	ATCC 23350 = DSM 7T
Bacillus amyloliquefaciens	ATCC 23842
Bacillus amyloliquefaciens	IAM 1523 DSM No. 1061
Bacillus amyloliquefaciens	IFO 3034 DSM No. 1062
Bacillus amyloliquefaciens	IFO 3037 DSM No. 1063
Bacillus amyloliquefaciens	KA 63 DSM No. 1060
Bacillus amyloliquefaciens	OUT 8419 DSM No. 1064
Bacillus amyloliquefaciens	OUT 8420 DSM No. 1065
Bacillus amyloliquefaciens	OUT 8421 DSM No. 1066
Bacillus amyloliquefaciens	OUT 8426 DSM No. 1067
Bacillus amyloliquefaciens N	strain collection of Ag BAG, IPK
Bacillus amyloliquefaciens P	strain collection of Ag BAG, IPK
Bacillus amyloliquefaciens SB I	strain collection of Ag BAG, IPK
Bacillus amyloliquefaciens T	strain collection of Ag BAG, IPK
Bacillus amyloliquefaciens ZFL 14/4	strain collection of Ag BAG, IPK
Bacillus amyloliquefaciens ZF 178	strain collection of Ag BAG, IPK
Bacillus brevis 475 Q	strain collection of Ag BAG, IPK
Bacillus circulans GB2	strain collection of Ag BAG, IPK
Bacillus lentus 3601 FZB	strain collection of Ag BAG, IPK
Bacillus licheniformis 41p	strain collection of Ag BAG, IPK
Bacillus macerans B30	strain collection of Ag BAG, IPK

Bacillus megaterium PV361	strain collection of Ag BAG, IPK
Bacillus polymyxa	ATCC 842
Bacillus sphaericus	ATCC 14577
Bacillus stearothermophilus	DSM No. 22 T = ATCC 12980
Bacillus subtilis GSB26	*arol906 metB6 sacA321 str6 amyE.* Derivative of QB1133; Steinmetz, *et al.*, (1976)
Bacillus subtilis 168	ATCC 6051
Bacillus thuringiensis 2046	strain collection of Ag BAG, IPK
Escherichia coli DH5α	F', ϕ80d/*lac*ZΔM15, *rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17(r_K-, m_K+), *sup*E44, *rel*A1, *deo*R, Δ(*lac*ZYA-*arg*F) U169; Hanahan (1983)
Escherichia coli XL1-Blue	*rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17, *sup*E44, *rel*A1, lac, [F *pro*AB, *lac*I^qZΔM15, Tn10(tet^R)]; Stratagene
Escherichia coli M15[pREP4]	Nal^S, Str^S, Rif^S, Thi^-, Lac^-, Ara^+, Gal^+, Mtl^-, F^-, $RecA^+$, Uvr^+, Lon^+; QIAGEN
Escherichia coli IT41	W3110, Lep-9*ts*; Tc^r; Inada, *et al.*, (1989)
Thermoactinomyces vulgaris 94-2A	Klingenberg, *et al.*, (1979)

2.2.2. Nutrient media

All the media listed here were sterilized for 20 min at 1atm/121°C. If it is not indicated otherwise, all the media were prepared with deionized water and the solid medium was prepared with the same ingredients as liquid medium, but with addition of agar–agar (1.5 %).

2.2.2.1. DM3 - Agar

- for regeneration of protoplast of *Bacillus*

Na-Succinate (2 M)	15 %
Saccharose (2 M)	5 %
K_2HPO_4 / KH_2PO_4	10 %
Casamino acids	0.25 %
Yeast extract	0.25 %
Glucose	1 %
Needed amino acids (2 mg/ml)	2.5 %
Agar solution (2%) + soluble starch (2%)	50 %

2.2.2.2. LBSP medium

- for preperation of *Bacillus* cells for electrotransformation

LBSP-Liquid medium:	Trypton	1 %
	Yeast extract	0.5 %
	NaCl	0.5 %
	Saccharose	250 mM
	K_2HPO_4 / KH_2PO_4	50 mM
	- pH = 7,2	
LBSPG-Liquid medium:	LBSP-Liquid medium + 10% (v/v) glycerol	
SHMG:	Sucrose	250 mM
	Hepes	1 mM
	$MgC1_2$	1 mM
	Glycerol	10% (v/v)
	- pH = 7.0	

2.2.2.3. M9 minimal medium

- For cultivation of *E. coli*

M9 1x salt

$Na_2HPO_4.7H_2O$	1.28 %
KH_2PO_4	0.3 %
NaCl	0.05 %
NH_4Cl	0.1 %
- pH = 7.4	

after autoclaving the following sterile solutions were added:

$MgSO_4$ (1M)	0.1 %
Glucose (20 %)	2 %
$CaCl_2$ (0.1 M)	1 %

M9 medium 1: like standard M9 with addition of:

all amino acids	2.5 mg/ml
Thiamine	1 µg/ml
Thymidine	2 µg/ml

M9 medium 2: similar to M9-1 but $MgSO_4.7H_2O$ was replaced by $MgCl_2$, and the amino acids solution contained all amino acids (2.5 mg/ml each) except methionine and cysteine.

2.2.2.4. PbS medium

- For preparation of protoplasts of *Bacillus*

$MgCl_2$ (1 M)	0.1 %
Glucose	0.1 %
Saccharose (2 M)	5 %
8 x Pbm	30 %

8x Pbm

Antibiotic medium 3	3.7 %
pH = 7.0	

SMMPA

BSA	0.3 %
Sucrose (2.0 M)	5.0 %
8x Pbm	25 %
2x SMM	50 %

2x SMM

Sucrose (2.0 M)	50 %
Sodium maleate (0.2 M, pH=6.5)	20 %
$MgCl_2$ (1 M)	2 %

2 M Sodium succinate solution

Succinic acid	23.6 %
NaOH	16 %
pH = 7.3	

2.2.2.4. SOB medium

Trypton	2 %
Yeast extract	0.5 %
NaCl	10 mM
KCl	2.5 mM
$MgCl_2$	10 mM
$MgSO_4$	10 mM
- pH = 6.8 – 7.0	

SOC mediumlike SOB-liquid medium but with addition of Glucose (0.2 %)

2.2.2.5. Schaeffer's sporulation medium (SSM)

- For sporulation test of *Bacillus*

Bacto-nutrient broth	0.8 %
KCl (w/v 10%)	1 %
$MgSO_4.7H_2O$ (w/v 1.2 %)	1 %
NaOH (1 M)	0.05 %

The following sterile solutions were added after autoclaving:

$Ca(NO_3)_4$ (1 M)	0.1 %
$MnCl_2$ (0.01 M)	0.1 %
$FeSO_4$ (1 mM)	0.1 %

2.2.2.6. Spi medium

- For preparation of *B. subtilis* competent cells

Spi I medium:

2 x SS	50 %
Glucose	0.5 %
Casamino acids	0.02 %
Yeast extract	0.1 %

Spi II medium:

Spi I +		
	$MgCl_2$ (0.1 M)	2.5 %
	$CaCl_2$ (0.05 M)	1 %

Spi III medium:

Spi II +		
	EGTA (0.1 M)	2%

2x SS solution:

KH_2PO_4	1.2 %
K_2HPO_4	2.8 %
NH_4SO_4	0.4 %
Sodium citrate	0.2 %
$MgSO_4$	0.04 %

2.2.2.7. Spizizen's minimal medium (MSM) (Anagnostopoulos & Spizizen, 1961)

- For cultivation of *Bacillus*

MSM-Agar:	Agar-Agar	1,75 %
	MSM-nutrient solution	10 %
MSM-nutrient solution:	K_2HPO_4	3 %
	KH_2PO_4	1 %
	NH_4Cl	0,5 %
	NH_4NO_3	0,1 %
	Na_2SO_4	0,1 %
	$MgSO_4$ x 7 H_2O	0,01 %
	$MnSO_4$ x 4 H_2O	0,001 %
	$FeSO_4$ x 7 H_2O	0,001 %
	$CaCl_2$	0,0005 %
	- pH = 7.2	

2.2.2.8. TBY medium

Trypton	1 %
Yeast extract	0.5 %
NaCl	0.5 %
- pH = 7.2	

Antibiotics were added as supplements at the final concentration listed below. In case of agar medium, the antibiotics were added after the medium had been cooled down to 50°C:

Ampicillin	50 µg/ml for selection of *E. coli*
Chloramphenicol	5 – 10 µg/ml for selection of *E. coli* and *Bacillus*
Erythromycin	3 µg/ml for selection of *Bacillus* and 50 µg/ml for *E. coli*
Kanamycin	25 µg/ml for selection of *E. coli*

200-700 µg/ml in DM3-agar for selection of *Bacillus*

7 µg/ml in all other media for selection of *Bacillus*

For blue-white selection of Lac-positive colonies, the respective agar media were supplemented with 40 µg/ml X-Gal and 40 µg/ml IPTG.

2.2.3. Swarming plate assay

The swarming experiments were done according to Blackman *et al.,* (1998). Swarming motility of wild type and mutants strains was measured using TBY or MSM soft agar (0.3%) plates. Samples (1 µl) from overnight (30^{o}C) liquid cultures were spotted onto swarm plates and incubated at 37^{o}C (TBY agar for 18-22 h, MSM agar for 44-48 h) or 25^{o}C (nutrient agar for 44-48 h, minimal agar for 68-72 h). The extent of swarming motility was measured as percentage of the diameter of growth colonies relative to the wild type strain control.

2.2.4. Na_3N induced cell autolysis assay

Azide induced cell autolysis experiment was carried out as described by Blackman *et al.,* (1998). Cultures of wild type and mutant strains of *B. amyloliquefaciens* were grown to the mid-exponential phase (OD_{600} 0.5-0.6) in TBY medium. After addition of 0.05 M sodium azide, lysis of cells was followed spectrophotometrically while continuing incubation at 37^{o}C and 200 rpm.

2.2.5. Sporulation test

The frequency of sporulation was estimated by the heat resistance test according to Nicholsen & Setlow (1990). Cultures of wild type and mutant strains of *B. amyloliquefaciens* were grown in the Schaeffer's sporulation medium (SSM). The samples were taken from the cultures after 12, 24, 36 h and diluted serially 10-fold in 10 mM potassium phosphate buffer (pH 7.4) containing 50 mM KCl and 1mM $MgSO_4$. 0.2 ml aliquots of the dilutions were plated on TBY agar plates before and after heat treatment at 80^{o}C for 10 min. The spore frequency was determined according to the proportion of the population which survived the heat treatment by counting colonies the next day.

2.2.6. Phase contrast and electron microscopy

Microscopical pictures of bacterial cultures were made with phase contrast microscope Nikon T120.

The electron microscopy picture were prepared using Zeiss CEM 920A transmission electron microscope. For primary fixation and embedding, *Bacillus amyliquefeciens* cells were kept in 50 mM cacodylate buffer (pH 7.2), containing 0.5% (v/v) glutaraldehyde and 2.0% (v/v) formaldehyde for 1 h at room temperature. After washing samples were kept for the secondary fixation 1 h in a solution of 1% (w/v) OsO_4 in 50 mM cacodylate buffer. Prior to dehydration the cells were washed and transferred into 1,5% agar. The dehydration of $1mm^3$ agar blocks was done stepwise by increasing the concentration of ethanol. The steps were performed as follows: 30% (v/v), 50% (v/v), 60% (v/v), 75% (v/v) and 90% (v/v) ethanol for 60 min each, 100% (v/v) ethanol two times for 1 h. After 1 h dehydration with propylene oxide the samples were infiltrated subsequently with Spurr (Plano GmbH, Marburg, Germany) as follows: 33% (v/v), 50% (v/v) and 66% (v/v) Spurr resin in propylene oxide for 2 h each and then 100% (v/v) Spurr overnight. Samples were transferred into embedding molds, kept there for 6 h in fresh resin and polymerised at 70 °C for 24 h. Thin sections with a thickness of approximately 70 nm were cut with a diamond knife and contrasted with a saturated methanolic solution of uranyl acetate and lead citrate prior to examination in a Zeiss CEM 920A transmission electron microscope at 80 kV.

2.3. Molecular biological methods

2.3.1. Vectors

pDG148

Vector pDG148 possess the replicon of pBR322 and the β-lactamase gene *amp*R for the replication and ampicilline selection in *E. coli* (Stragier, *et al.,* 1988). Moreover as a shuttle-vector, pDG148 possess the replicon from pUB110 for multiplication in *B. subtilis* (McKenzie, *et al.,* 1987) and also the *phl*R and *kan*R genes of pUB110 which permit a selection by phleomycin and/or kanamycin. The presence of the P*spac*-promoter with associated Lac-operator and the *lac*I encoding Lac-repressors from *E. coli* under control of the penicillinase promoter P*pen* of *B. licheniformis* allowed the IPTG-induction expression of a promoterless genes. The multiple cloning site (MCS) *Hind*III-*Sal*I-*Sph*I allowed to clone interested genes into the vector under the control of the P*spac*-promoter (Figure 3).

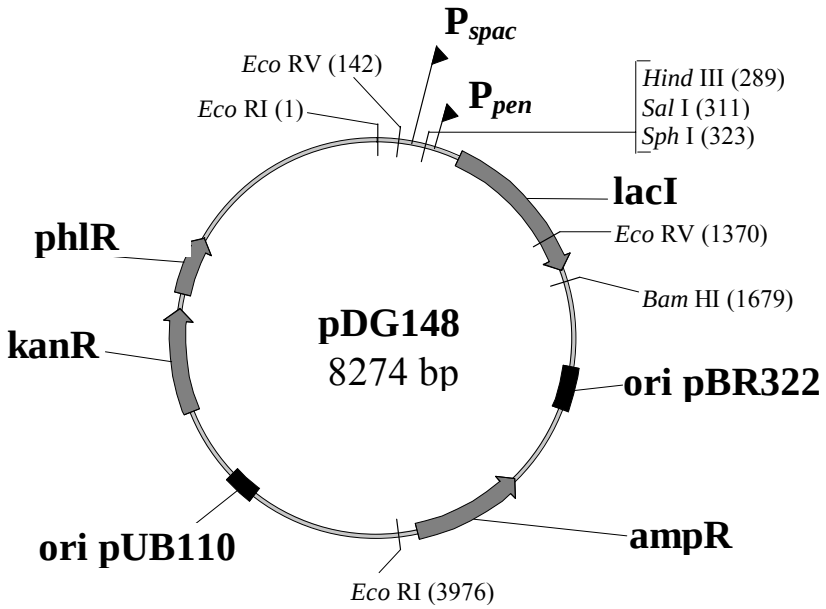

Figure 3. Physical map of Shuttle-Vector pDG148.

pE194*ts*

The thermo-sensitive vector pE194ts was used for construction of integrational gene disruption mutants in *Bacillus* species that lack natural transformation competence. The pE194*ts* (originally Staphylococcus) replicon is unable to sustain autonomous replication in *Bacillus* at temperature above 37°C (Youngman, 1990). The pE194*ts* plasmid contains the *ery*R gene that allowed for erythromycin selection. Moreover, the pE194*ts* could be easily accomplished with vector contained ColE1-derived replicon such as pUC18 at the *Pst*I site to form a shuttle-plasmid which can work both in *E. coli* and in *Bacillus*.

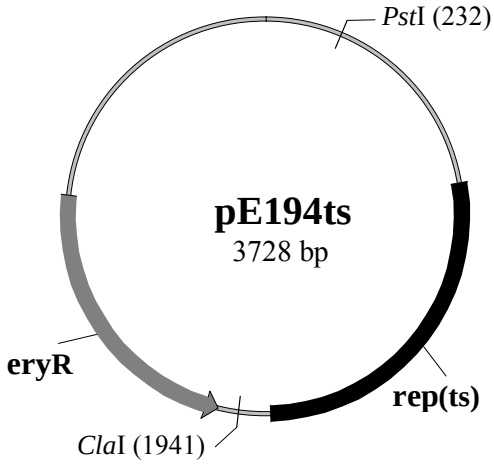

Figure 4. Physical map of the temperature-sensitive vector pE194*ts*.

pGEM-T

The pGEM-T vector system (Promega) was used for the cloning of PCR products. The vector was provided with added 3' terminal thymidine to both ends of the *Eco*RV-digested pGEM-5Zf. These single 3'-T overhangs at the insertion site allowed the efficient ligation of PCR products as several thermostable polymerases often add a single deoxyadenosine to the 5'-end of the amplified products.

The pGEM-T vector contains T7 and SP6 promoters flangking a multiple cloning region within the α-peptide coding region of the enzyme β-galactosidase. This allows recombinant clones to be directly identified by blue-white screening on indicator plates (with IPTG/X-Gal addition). The presence of the *amp*R gene coding for β-lactamase permits a selection by ampicilline.

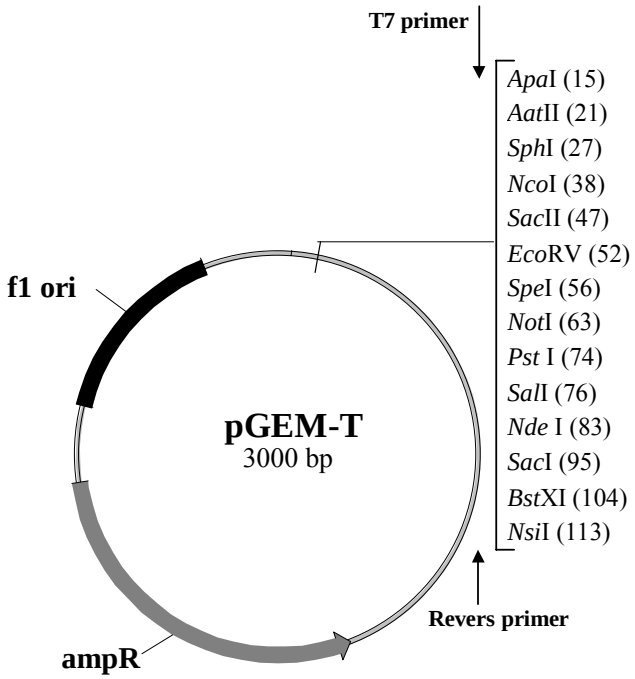

Figure 5. Physical map of pGEM-T vector for cloning of PCR products.

pHB201

The *Bacillus*/*E.coli* shuttle pHB201 plasmid (Bron *et al.,* 1998) carries the replication function of pUC19 (for *E. coli*) and pTA1060 (for *B. subtilis*) (Figure 6). The *lacZ*α gene was provided with promoter P59 of *Lactobacillus*. The extended polylinker site in *lacZ*α allows the a blue-white selection of recombinant clones on X-gal containing agar plates in *E. coli*.

The polykinker site in the *lacZα* was derived from pBluescript II. The T1 and T2 transcription termiators prevent read-through transcription from the *lacZα* region.

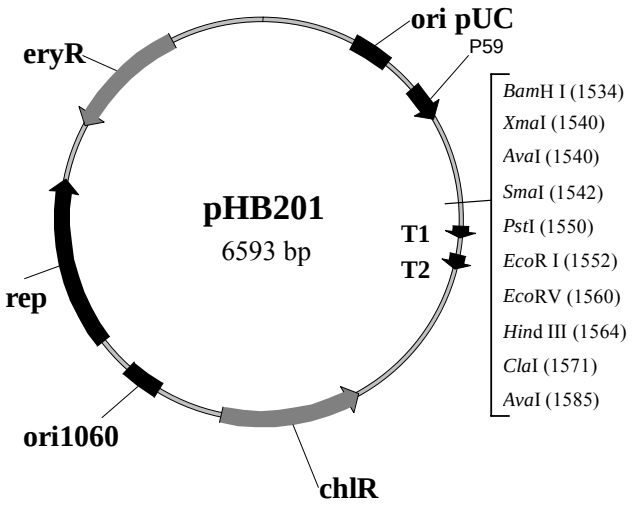

Figure 6. Physical map of shuttle vector pHB201.

pQE16

The pQE16 belongs to the QIAexpress pQE vector family of QIAGEN which are designed for overexpression of recombinant proteins in *E.coli*. The pQE plasmids were derived from plasmids pDS56/RBSII and pDS781/RBSII-DHFRS. The pQE plasmids possess phage promoter T5, two lac operator sequences at the T5 promoter, the ColE1 origin of replication, the β-lactamase gene for ampicillin resistance and the 6xHis-tag coding sequences.

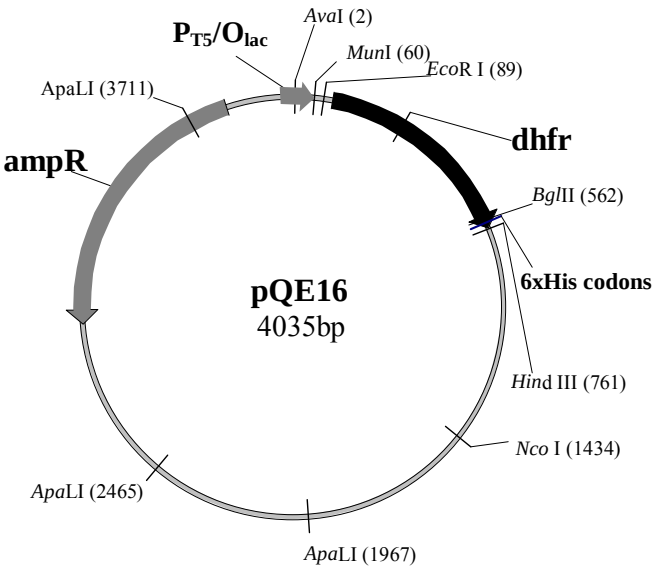

Figure 7. Physical map of vector pQE16.

2.3.2. Recombinant plasmids

Table 2. Recombinant palsmids

Plasmids	Description and references
pEAS*	pE194, Em^r::pUC18, Ap^r with core-DNA of *sipS*(Ba*) gene
pEAT*	pE194, Em^r::pUC18, Ap^r with core-DNA of *sipT*(Ba)* gene
pEAV*	pE194, Em^r::pUC18, Ap^r with core-DNA of *sipV*(Ba)* gene
pEAW*	pE194, Em^r::pUC18, Ap^r with core-DNA of *sipW*(Ba)* gene
POpacSh	pDG148 with expression cassettes P*spac-ompA- sipS(Ba)*His-tag
POpacTh	pDG148 with expression cassettes P*spac-ompA- sipT(Ba)*His-tag
POpacVh	pDG148 with expression cassettes P*spac-ompA- sipV(Ba)*His-tag
POpacWh	pDG148 with expression cassettes P*spac-ompA- sipW(Ba)*His-tag
POpacBh	pDG148 with expression cassette P*spac-ompA- lepB(Ec)*His-tag.
pOpacBTh	pDG148 with expression cassette P*spac-ompA- lepB-sipT* fusion His-tag.
pOpacTBh	pDG148 with expression cassette P*spac-ompA- sipT-lepB* fusion His-tag.
pTK99	pJQ501, Gm^r , *sipS(Bj)* in antisenese orientation
pTK100	pJQ501, Gm^r, *sipS(Bj)* in sense orientation
pQS	pQE with *sipS(Ba)* gene
pQT	pQE with *sipT(Ba)* gene
pQV	pQE with *sipV(Ba)* gene
pQW	pQE with *sipW(Ba)* gene
pQB	pQE with *lepB(Ec)* gene
pQC1	pQE with *chbB* gene
pQBT	pQE with *sipT-lepB* fusion gene
pQTB	pQE with *lepB-sipT* fusion gene
pHBC1	pHB201 with *chbB* gene
pGEMO	pGEM-T with *E. coli ompA* gene

2.3.3. PCR-primer and Protocol

PCR technique was used in several parts of this work for gene isolation, or construction of gene fusions e.t.c. The PCR was carried out using Taq polymerase or Expand long template PCR-Kit (Roche). If it is not indicated otherwise, all the PCR was performed following the suppliers' instructions.

Table 3. Oligonucleotide primers used for PCR.

Name	**5' → 3' Sequence** [a]	**Description**
A	CAYGGNTAYATAHTTKGARCCNGT	cloning of *chbB*
B	GTNWSNMGNGCNTAYATGGGNGC	cloning of *chbB*
C	CTACCATCCGGCGGACCTGCAGCCGG	cloning of *chbB*
D	TTGTCCAGATCCTCCGTTTGCAGACGC	cloning of *chbB*
E	ACTTGGCACTACACCGCACCTCATGCG	cloning of *chbB*
F	ACTTGGCACTACACCGCACCTCATGCG	cloning of *chbB*
G	CGTATGTCCGGTTACGGCAACCTTCAC	cloning of *chbB*
H	AAAATTCTTGTATTGCCTGTTCATTCG	cloning of *chbB*
I	CACCACGATTAACGCAAAGGAGCTACC	cloning of *chbB*
K	CCATATGATCTCACCTCCCTTAAGAGG	cloning of *chbB*
L	CAAAGAAGGGAGGATGACGTAGAGATG	construction of pQEC1
M	CATAGATCTTTTTGTGAGGTTTACATC	construction of pQEC1
CH1	CAYTTYGGNGCNGGNAAYATNGG	cloning of *sipU*
CH2	CAYGGNWSNGCNCCNGAYATNGCNGG	cloning of *sipU*
CH5	ATGATHGCNGCNYTNATHTTYACNAT	cloning of *sipU*
CH6	TTYTAYAARCCNTTYYTNATHGARGG	cloning of *sipU*
CH7	TCYTCNSWNGGCATNCCCATNCCRTT	cloning of *sipU*
CH8	TTNGCYTGNCKCATYTCNCCRAANGG	cloning of *sipU*
HV11	TTRTCNCCCATNACRAARTA	cloning of *sipU*
U1	TTGAAYGCNAARACNATHACNYTNAARAA	cloning of *sipU*
V1	TTGAARAARMGNTTYTGGTTYYTNGC	cloning of *sipV*
V2	GTNTTYATNGTYTAYAARGTNGARGG	cloning of *sipV*
V3	TCNGCRTCNSWNATNACNCCNACNAT	cloning of *sipV*
V4	GCCAAAACAACGATAAGCACGCC	cloning of *sipV*
V5	GGATTCATGCTGATTCCTTCGAC	cloning of *sipV*
V6	ACTTGGCACTACACCGCACCTCATGCG	cloning of *sipV*
V7	ATTTCGTGATTGGCGACAACCGC	cloning of *sipV*
V8	GAGAATTCCGGAGGGGGACAGGAATCTTG	construction of pOpacVh
V9	GCAGATCTC TTGGCGTATGATTCACTGAT	construction of pOpacVh
W1	GGNWSNATGGARCCNGARTTYAAYACNGG	cloning of *sipW*
W2	TCNGCNGCNGCRTTRTTRTCNCCYTTNGT	cloning of *sipW*
W7	TTGTGTAAAAGTGATGACATCGCC	cloning of *sipW*
W8	GTGATCCCGATTATTCTGTGTGTT	cloning of *sipW*
W9	GGCGATGTCATCACTTTTACACAA	cloning of *sipW*
W10	AACACACAGAATAATCGGGATCAC	cloning of *sipW*

W11	GAGAATTCAAAAGAAAGCGGGGAAGAA	construction of pOpacWh
W12	CGAGATCTTGTGGACATGGTCCCGTTTC	construction of pOpacWh
Lep1	CAGCAATTGACCCTTAGGAGTTGGCAT	construction of pOpacBh
Lep2	GATGGATCTATGGATGCCGCCAATG	construction of pOpacBh
Lep3	CGAGAAATGGCGCACAATCAATACGATAGC	for *sipT-lepB* fusion
Lep4	GCGCTGTTAATCCGTTCGTTTATTTATGAA	for *sipT-lepB* fusion
S1	CGGAATTCGCTAATGGGAGGAAATCAC	construction of pOpacSh
S2	TACAGATCTTTTCGTCTTGCGAATTTC	construction of pOpacSh
T1	CAGAATTCGTCTAGGAGGAACCACGTT	construction of pOpacTh
T2	GCGAGATCTTTTTGTCTGACGCATATC	construction of pOpacTh
T3	AATAAACGAACGGATTAACAGCGCAAGTGC	for *sipT-lepB* fusion
T4	GTATTGATTGTGCGCCATTTCCTGTTTGAA	for *sipT-lepB* fusion
Omp1	GCAAAGCTTATTTTGGATGATAACGAGGCG	for *OmpA* amplification
Omp2	GCGAATTCCTACCAGACGAGAACTTAAGCC	for *OmpA* amplification
Uni1	GTTTTCCCATGCACGAC	universal sequencing primer for pUC18
Uni2	GTAAAACGACGGCCAGT	universal sequencing primer for pUC18

[a] The IUPAC-code was used; N denotes an inosine residue.

2.3.4. Rapid amplification of genomic DNA ends (RAGE)

The RAGE protocol for highly specific amplification of unknown genomic DNA adjacent to a short stretch of known sequence (Mizobuchi & Frohman, 1993; Hoang & Hofemeister, 1995) was used to clone *sipV*, *sipW* and *chbB* genes of *B. amyloliquefaciens*. 5 µg of pUC18 plasmid and 5 µg of genomic DNA of *B. amyloliquefaciens* strain ALKO2718 were individually digested by a single restriction enzyme. After dephosphorylation, the prepared plasmid and digested genomic DNA were ligated overnight. A nested PCR, with two rounds of PCR, was performed using pairs of primers Uni1, Uni2 of pUC18 vector and the two primers located on the known region of the gene, using the ligation mixtures as templates. The PCR was performed in a volume of 50 µl using either the Taq polymerase or the Expand long template PCR-Kit (Roche). The reaction conditions were 94°C for 5 min, 30 times (94°C for 30 sec; 55°C for 30 sec; 72°C for 3 min) and 72°C for 8 min.

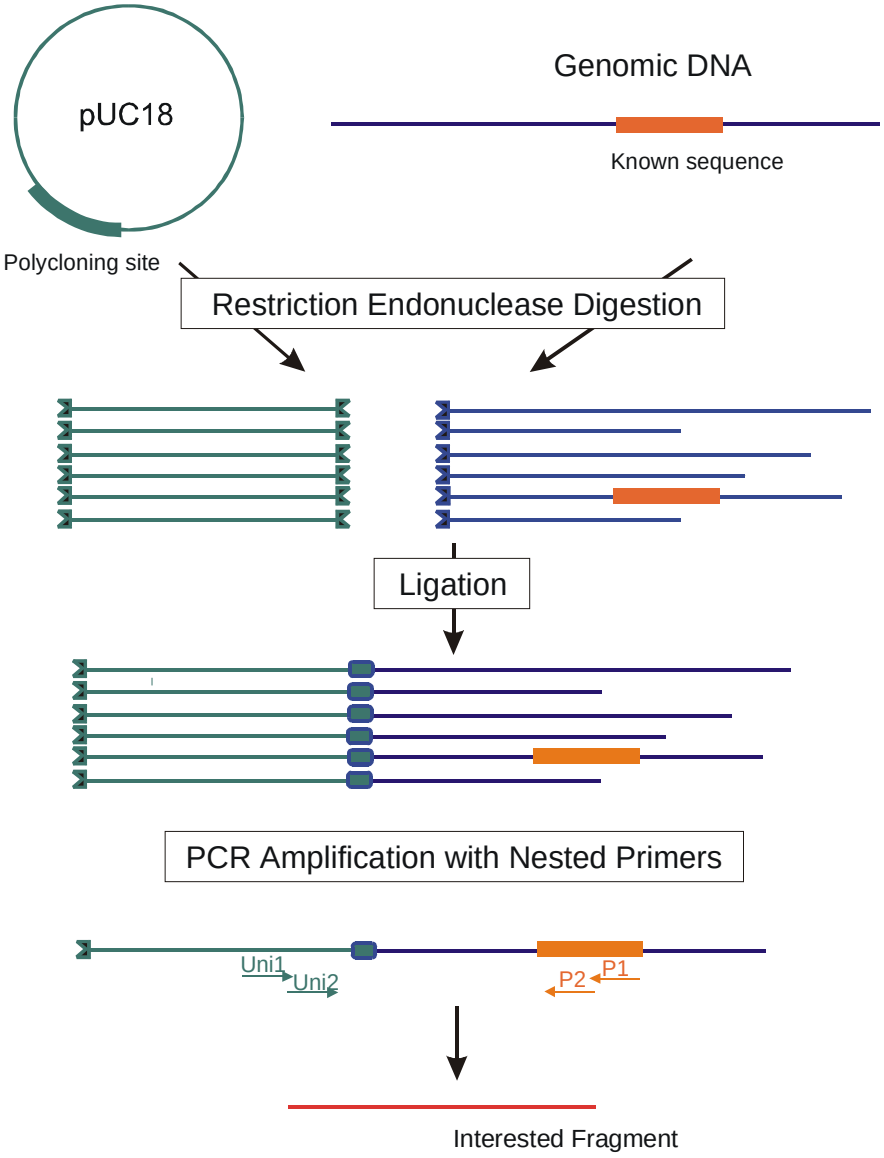

Figure 8. Diagram representation of the RAGE protocol. pUC18 vector and genomic DNA were digested using the same restriction enzyme and ligated. Two rounds of PCR amplification were performed using pairs of nested primer, which were located either on the pUC18 plasmid (Uni1 and Uni2) or on the know sequence of the interested gene (P1 and P2).

2.3.5. Agarose DNA gel electrophoresis

DNA samples were always separated by gel electrophoresis with agarose gels. Depending on the size of the DNA fragments, the agarose concentration differed from 0.8 % to 1.5 %. The gels were prepared by adding agarose to 1x TAE buffer and boiling for 20 min. Ethidiumbromid was added to the gel solution to a final concentration of 0.5 µg/ml. DNA samples were mixed with 1/10 volume of sample buffer. The electrophoreses were run in 1x TAE-buffer with the currency around 50-80 mA. DNA fragments were visualised under UV-light (λ=254nm).

TAE-Buffer:

Tris-acetate	0.04 M
EDTA	0.001 M
- pH = 8.0	

Sample buffer "Helsinki":

Glycerol	50 %
Tris-HCl	10 mM
SDS	0.05 %
Bromophenol blue	0.2 %
- pH = 8.0	

TE-Puffer:

Tris-HCl	10 mM
EDTA	1 mM
- pH = 8.0	

2.3.6. Isolation of chromosomal DNA

The mini preparations of chromosomal DNA were carried out following a standard procedure (Harwood, *et al.*, 1990). 5 ml overnight culture (TBY medium containing appropriate antibiotics) was grown in a 20 ml flask at 37°C in a waterbath shaker (200 rev/min). The culture was diluted 50-fold in 5 ml fresh medium and continued growth at 37°C for 2 to 3 h, until the OD_{650} was 0.8. Cells were harvested by centrifugation for 10 min at 9000 g (4°C). The cell pellet was resuspended in 1.5 ml precooled (0°C) buffer 1 and re-collected by centrifugation. The cell pellet was again resuspended in 0.7 ml lysis buffer, containing 8 mg/ml of lysozyme, and mixed by vortexing. After incubation for 10 min at 0°C and 10 min at 37°C, 25 µl Sarkosyl 30% and 5 µl proteinase K (10 mg/ml) were added. The cell suspension was mixed by vortexing and incubated for 30 min at 70°C. After vortexing for 1 min at maximum speed, the lysate was subjected to 3 times phenol extraction by adding 700 µl phenol, vortexing gently for 1 min and centrifugation to separate phases in a microfuge for 15 min at full speed. The upper (water) phase was transferred with a 1-ml micropipet tip into a fresh microtube. After addition of 5µl of RNase (10 mg/ml) and incubation for 15 min at 37°C, the lysate was extracted one time by phenol:chloroform (1:1) and one time by 600 µl chloroform:isoamyl alcohol (24:1). The upper phase was collected. The DNA was precipitated by adding 2.5 volume ice-cold ethanol and 1/10 volume sodium acetate (3M, pH 4.8), after keeping at –20°C for 20 min and centrifugation (14000 rpm) for 30 min at 4°C. The pellet was washed two times with 70 % ethanol and dried by leaving the tubes open for 15 min. The DNA was dissolved in 100 µl of TE.

Buffer 1:

Tris-HCl	10 mM
NaCl	150 mM
EDTA	10 mM

- pH = 8.0

Lysis Buffer:

Tris-HCl	20 mM
NaCl	50 mM
EDTA	10 mM
Lysozym	8 mg/ml

- pH = 8.0

QIAGEN genomic-tips 100/G and 500/G were used to isolate large amounts of chromosomal DNA following the supplier's instruction.

2.3.7. Isolation of plasmid DNA

Qiagen Plasmid Midi and Maxi Kits were used to prepare plasmid DNA for sequencing and to prepare more than 10 µg DNA. In case plasmids isolation from *Bacillus* 8 mg/ml lysozyme was added to buffer P1 and the incubation step was prolonged up to 30 min at RT after buffer P2 was added.

The minipreparation of Plasmid-DNA of *E. coli* and *Bacillus* was done following the method described by Birnboim & Doly (1979). 5 ml cultures in TBY or NBY were incubated overnight at 37°C. Depending on the copy number of the plasmids 1-5 ml cell cultures were collected by centrifugation for 5 min at 6000 rpm RT. The cell pellets were resuspended in 200 µl buffer P1 and incubated at 37°C for 10 min. For *Bacillus* cells, buffer P1 was supplied with 8 mg/ml lysozyme and the incubation was extended up to 30 min. 200 µl buffer P2 were added and the probes were incubated on ice for 10 min, then for another 10 min after adding 200 µl buffer P3. The lysates were subjected to two times phenol-chloroform (1:1) extraction by adding 500 µl phenol-chloroform, vortexing and centrifugation at 14000 rpm for 15 min. The upper phase was collected and transferred to a new tube. Two volumes of ice-cold ethanol were added to precipitate the DNA. The DNA-pellet was recovered by centrifugation at 14000 rpm 4^{o}C for 30 min. The pellet was washes with ice-cold 70% ethanol, dried by vacuum centrifugation and dissolved in 50 µl TE.

Buffer P1:

Tris-HCl	50 mM
EDTA	10 mM
RNase A	100 µg/ml

- pH = 8.0

Buffer P2:

NaOH	0.2 N
SDS	1 %

Buffer P3:

Sodium acetate	3 M
- pH = 5.5	

2.3.8. Extraction of DNA from agarose gels

DNA fragments were isolated from agarose gels after electrophoretic separation using QIAquick or QIAEX II gel extraction kit following the supplier's instructions. The latter was used when the DNA fragment was larger than 5 kb.

2.3.9. Restriction digestion and ligation of DNA

The DNAs were cleaved with various restriction enzymes following the supplier's instructions. Ligation was performed with T4 DNA ligase (Sambrook, *et al.*, 1989).

2.3.10. Methylation of restriction site

Transformation of plasmid DNA into *B. amyloliquefaciens* spec. which harboured the restriction endonuclease enzyme *Bam*HI, required the methylation of the *Bam*HI restriction site to protect the DNA from *Bam*HI digestion. The *Bam*HI methylase catalyzes the transfer of a methyl group from S-adenosylmethionine to the nucleotide N^4 (cytosine) of the recognition sequence of *Bam*HI (GGATCC). 5-10 units of the *Bam*HI methylase were used to methylate 1 µg DNA in 1x *Bam*HI methylase buffer containing 80 µM S-adenosylmethionine. Incubation was carried for 1 h at 37^{o}C, followed by 15 min at 65^{o}C for enzyme inactivation.

2.3.11. Filling of DNA ends

The "sticky ends" of DNA fragments, which were not suitable for ligation to other DNA molecules, were "filled" using T4 DNA-polymerase. T4 DNA-polymerase has 3'-5' exonuclease activity besides of 5'-3' DNA-dependent polymerase activities. The DNAs were incubated with T4 DNA-polymerase in a solution containing 1 mM dNTPs and 1x T4-polymerase-buffer for 30 min at 12°C and then for 30 min at 37°C. The enzyme was inactivated by incubation for 20 min at 70°C.

2.3.12. Dephosphorylation of linearized plasmid DNA

The vectors were linearized by restriction endonuclease and afterwards always dephosphorylated by Shrimp alkaline phosphatase in order to prevent religation of the linearized vector. One unit of shrimp alkaline phosphatase was used for dephosphorylation of 1 µg linearized vector in dephosphorylation buffer (50 mM TrisHCl, 0.1 mM EDTA pH 8.5) at 37°C for 60 min. The shrimp alkaline phosphatase was inactivated by heating at 65°C for 15 min.

2.3.13. Southernblot hybridization procedures

DNA transfer and fixation

For hybridization the chromosomal or plasmid DNA were digested by restriction enzymes and separated by electrophoresis on agarose gels (0.8%). After electrophoresis, the agarose gel was placed in 0.25N HCl for 15min. The gel was rinsed two times with distilled water, then placed in denaturation buffer for 30 min at room temprature with shaking. The gel was again rinsed with distilled water and placed in neutralization buffer for 30 min with shaking. The DNA was transferred onto a nylon membrane Hybond N^+ using a Vacuum-Blotter machine (Pharmacia). The Hybond N^+ membrane was irrigated for 5 min in H_2O and subsequently for 10 min in transfer buffer. In the vacuum plate, the gel was placed on top of the membrane and was then overlayed with transfer buffer. The transfer was carried out for 90 min. After transfer, the membrane was washed for 5 min in 2x SSC, air-dried and fixed for 2 h at 80°C. The dried blot was stored at RT or used immediately for a hybridization.

Denaturation buffer:

NaOH	0,5 M
NaCl	1,5 M

Neutralization buffer:

Tris-HCl	0,5 M
NaCl	1,5 M
- pH = 7,5	

20x SSC:

NaCl	3,0 M
Sodium citrate	0,3 M
Transfer solution:	10x SSC

DNA hybridization

DNA hybridizations were done using either the ECL system (Amersham-Pharmacia) or the Megaprimer DNA labelling system for ^{32}P radioactive labelling. Hybridization was carried out according to the supplier's protocols.

2.3.14. DNA sequencing and computer analysis

DNA sequencing was performed by an automated system (A.L.F. express, Pharmacia), using the recommended primers for the pGEM®-T vector and the AutoRead sequencing kit (Pharmacia). Sequencing of the pQE constructs was done using the primers recommended by Qiagen. Sequence analysis was performed with the PC/GENE software system of IntelliGenetics, Inc. (Mountain View, Calif.) and DNASTAR software of Lasergene Inc. (Madison, WI 53715, USA). The BLAST software (National Center for Biotechnology Information, Bethesda, Md.) was used for online database scanning. Phenetic and cladistic analyses of the amino acid alignment were performed in PAUP* 4.0b8 (Swofford, 2001). Mean character differences were used to calculate pairwise distances, which were clustered with the Neighbor-Joining algorithm. Fitch parsimony analysis was conducted with ACCTRAN character optimization, gaps treated as missing data and the heuristic search algorithm with 100 random sequence additions. To test the statistical support of the branches in phenetic and cladistic analyses, bootstrap resamples were conducted with 5000 and 500 replicates, respectively. Parsimony analysis resulted in two equally parsimonious trees of 1388 steps length (CI 0.7177, RI 0.6631), which are compatible with the tree topology obtained by the Neighbor-Joining analysis.

2.3.15. Transformation of plasmid DNA into E. coli

$CaCl_2$ – method

E. coli competent cells were prepared with the $CaCl_2$-method (Sambrook, *et al.,* 1989). The competent cells can be stored at - 80°C and used for up to 3 months. 2 ml of an overnight TBY culture was inoculated from a fresh colony. The culture was diluted 100 fold in fresh TBY and inoculated for about 3 h at 37°C, 200 rpm in a rotary shaker until the OD_{600} is 0.4 – 0.5. The culture was kept on ice for 30 min and pelleted by centrifugation at 4000 rpm for 10 min at 4°C in a Sorvall GS3 rotor. The cells were washed twice with 20 ml ice-cold 0.1 M $CaCl_2$ solution, then gently resuspended in 4 ml ice-cold 0.1 M $CaCl_2$ 15% Glycerol. 100 µl of each suspension of competent cells was transferred to a sterile pre-chilled microfuge tube. The competent cells were directly used or stored at – 80°C.

For transformation, plasmid DNA was mixed with 100 µl competent cells and incubated on ice for 20–40 min. The tubes were placed at 42°C in a water bath for 90 sec, and on ice for 1 min. 400 µl SOC medium was added and incubated at 37°C for 30 – 60 min. 150 µl of transformation culture was plated onto a agar TBY plate containing appropriate antibiotic(s).

Electrotransformation method

The electrotransformation was used in order to achieve high-efficiency transformation of plasmid DNA in *E. coli*. 200 ml TBY culture was inoculated from a single fresh colony with vigorous shaking (250 rpm) at 37°C until the OD_{600} reached 0.5 – 0.7. After chilling on ice for 15 min, the cells were collected by centrifugation 15 min 5000 g. The pellets were washed 2 times with 100 ml ice-cold sterile de-ionised water and resuspended in 4 ml ice-cold 10% glycerol. 100 µl of each cell suspension was transferred into a pre-chilled tubes. The cells can be directly used or stored at –80°C.

1 – 5 µl of solution containing 0.05-0.5 µg plasmid DNA were added to 100 µl fresh or thawed cells. The cells were mixed by pipeting and then placed on ice for 15 min. The DNA-cell mix was transferred into a pre-chilled cuvette. Electrotransformation was performed with 2500 V, 25 µF, and 200 Ohms. The transformed cells were diluted 10 fold with ice-cold SOC medium and incubated for 30 – 60 min at 37°C. 150 µl of transformation culture was plated onto a agar plate containing appropriate antibiotic(s) and incubated overnight at 37°C.

2.3.16. Transformation of plasmid DNA into Bacillus

Transformation of competent B. subtilis cells

Competent cells of *B. subtilis* were prepared as described by Cutting and Vander Horn (1990). 2 ml Spi I culture were inoculated overnight from a fresh colony at 30°C, with 200 rpm shaking. The following morning the culture was diluted with 18 ml fresh Spi I and continued incubation at 37°C with vigorous shaking (180 rpm). When the rate of cell growth reached stationary phase (i.e. no significant change in cell density over 20-30 min), the culture was mixed with 180 ml of prewarmed Spi II and continued incubation at 37°C for additional 90 min. The culture was kept on ice for 10 min and centrifugated (8000g) for 10 min at 4°C. 18 ml supernatant was saved and the remain was carefully decanted. The cell pellet was gently resuspended in 18 ml of the "saved" supernatant with addition of 2 ml of sterile glycerol. Aliquots of competent cells (0.2, 0.5 or 1.0 ml) were transferred into sterile tubes, frizzed rapidly in liquid nitrogen or in a dry-ice/ethanol bath and stored at –80°C.

The competent cells were thawed rapidly by immersing frozen tubes in a 37°C water bath. 200 µl of Spi III was added to 200 µl thawed cells, mixed gently with 10 µl DNA solution (0.5 – 5 µg) and the mixture was incubated at 30°C for 90 min or 37°C for 1 h with shaking (150 rpm). The transformation mixture was appropriately diluted and plated onto TBY agar plates with selective antibiotic(s).

Electrotransformation of Bacillus

Electrotransformation of *Bacillus* was performed as described by Vehmaanperä (1989). 2 ml of TBY culture were inoculated overnight from a single fresh colony. The overnight culture was diluted 50 fold with 100 ml LBSP medium and incubated at 37°C to late exponential phase (OD_{600} ~1.0) with vigorous shaking (200 rpm). After leaving on ice for 10 min, the cells were harvested by centrifugation (10000 g) for 5 min at 4°C using a pre-cooled rotor and tubes. The cell pellet was washed three times with 100 ml of ice-cold SHMG and resuspended in 3 ml of ice-cold SHMG. 0.1 ml aliquots of cell suspension were transferred into microfuge tubes and stored at –80°C. The cells remain transformable for at least several months.

100 µl portion of frozen cells were thawed quickly in a 37°C waterbath. 1-10 µl solution, containing 0.01-1 µg of DNA in SHMG, was added to the cell suspension. The mixture was transferred into a 2 mm pre-cooled electroporation cuvette and kept on ice for 30 min. The electroporation was carried out with a single pulse (2500 V = 12.5 kV/cm, 25 µF for *B. subtilis*; 1500 V = 7.5 kV/cm, 25 µF for *B. amyloliquefaciens*). The cell suspension was immediately diluted 10 fold with LBSPG medium and incubated for 60 min with vigorous aeration at 37°C (or 90 min at 30°C) to allow expression of antibiotic resistance markers. 150 µl of transformed cells were plated on a TBY agar plate containing appropriate antibiotic(s) and overnight incubated.

Bacillus protoplast transformation

The protoplast transformation of *Bacillus* was done following the method of Chang & Cohen (1979). A 5 ml of TBY culture was inoculated from a single colony for 5 h. The culture was diluted 10^2, 10^4, 10^6 fold in TBY and inoculated overnight at 37°C in a waterbath shaker (200 rpm). The overnight culture was diluted 20-fold into 10 ml of fresh PbS medium and grown at 37°C in a waterbath shaker (200 rpm) until the OD_{470} reach 1.7. The cells were harvested by centrifugation in glass tubes at 3500 rpm/RT for 10 min with a table centrifuge machine (HERMLE). After removed the supernatant, the cell pellet was resuspended in 10 ml of SMMPA containing 50 mg lysozyme. The cell suspension was incubated at 37°C in a waterbath shaker (90 rpm) and examined under the microscope after 30 min and thereafter in 15 min intervals. The incubation was continued until 99% of the cells are apparently converted into protoplasts (globular appearance). The protoplasts were harvested by centrifugation 4000 rpm at RT. After 2 times of gentle washing with 7 ml of SMMPA, the pellet was resuspended in 2.5 ml SMMPA.

1-10 µl of DNA (0.2 µg) were added into 0.5 ml protoplast suspension, gently mixed with 1.5 ml of PEG solution (40 % of PEG 6000 in 1x SMM solution) in a microtube, and placed for 2 min at RT. 5 ml of SMMPA was added to this mixture. The protoplasts were harvest by centrifugation (7 min, 4000 rpm). After complete removal of the supernatant, 1 ml of SMMPA were added to the pellet. The suspension was incubated for 60-90 min at 37°C in a waterbath shaker (100 rev/min) in order to allow the expression of antibiotic resistance markers (the protoplasts become sufficiently resuspended through the shaking action of the waterbath). The protoplasts were appropriately diluted in 1 x SSM and 0.1 ml of the dilution was plated onto a DM3 agar plate.

2.3.17. Pulse–chase protein labelling and immunoprecipitation

Pulse chase labelling

The method described by Edens (1982) was modified and used for pulse chase *in vivo* labelling of proteins in *E.coli*. An overnight culture was inoculated from a single colony in M9-1 medium at 30°C. The pre-culture was diluted 20 fold to start a 20 ml culture with M9-1 medium and grown at 30°C until OD_{600} is 0.5. The cells were collected by centrifugation (5000g) for 5 min, washed two times with M9-2 medium and resuspended in 10 ml M9-2 medium. The cultures were grown for 30 min at 30°C for methionine starvation. Aliquots of 0.5 ml culture were used for the pulse-chase experiment at either 30°C or 45°C. The pulse was done by adding "hot" ^{35}S-methionine into the culture to a final concentration of 50µCi / ml. After 1 min of labelling, "cold" non-radioactive methionine and cysteine 2.5 mg/ml were added. The samples were collected after 0, 1, 5, 10, 20 min. 0.5 ml ice-cold TCA 20% was added to each sample and subsequently left on ice for 30 min. The pellets were collected by centrifugation, washed three times with aceton and air-dried. The pellets were resuspended in 100 µl TES buffer and boiled for 5 min. For *Bacillus*, the M9 minimal medium was replaced with S7 medium (van Dijl *et al.,* 1991).

Immunoprecipitation

100 µl STD in 10 mM Tris-HCl pH 7.0 and 5 µl antiserum were added to the 100 µl sample (from upper part), then incubated for 16 h at 4°C. 200 µl of STDS in 10 mM Tris-HCl pH 8.5 were added together with 50 µl of a 10% (v/v) suspension of protein A-sepharose (Pharmacia) in STDS and incubated for 30 min at room temperature. The mixture was layered over 300 µl 10% sucrose in STDS / 10 mM Tris-HCl pH 8.2 and centrifuged to collect the A-sepharose beads. The pellets were washed 3 times with STDS in 10 mM Tris-HCl pH 8.2. The pellets were suspended in 50 µl sample buffer and heated for 5 min at 100°C.

SDS-PAGE and fluorography

The samples were loaded and separated onto 12% SDS-PAGE. Fluorography was performed as described by Skinner and Griswold (1983).

STD buffer: 0.9% NaCl, 1% Triton X-100, 0.5% sodium deoxycholate.
STDS buffer: 0.9% NaCl, 1% Triton X-100, 0.5% sodium deoxycholate and 0.5% SDS.
TES buffer: 50 mM Tris/HCl, 2 mM EDTA, 2% SDS, pH 8.0

2.4. Biochemical methods

2.4.1. Protein determination

The protein concentrations of the samples were determined using the methods established by Lowry et al., (1951) or Bradford (1976).

2.4.2. Purification of ChbB protein

Purification of the His-tag protein

The plasmid pQEC1 was transformed into *E. coli* M15 (pREP4), and overexpression was achieved following the procedure outlined in the QIAexpressionist handbook (Qiagen). The His-tag fusion protein was accumulated in the periplasm and was thus available by osmotic shock treatment. The washed cells were suspended in 30 mM Tris/HCl, pH 8.0, 20 % sucrose, incubated on ice for 10 min, sedimented (8,000 x g, 15 min, 4°C), resuspended in 5 mM of ice-cold $MgSO_4$ for 10 min, and centrifuged for 15 min at 4°C. The supernatant was collected, equilibrated to 50 mM Na_2HPO_4, pH 8.0, 300 mM NaCl, 10 mM imidazole, and applied to an Ni-NTA column (Qiagen). After five washes, corresponding to the volume of the column, in the same buffer supplemented with 40 mM imidazole, proteins were released by increasing the imidazole concentration. The fusion protein was found to be released in the presence of 200 mM imidazole. The purity of the protein was analyzed by SDS-PAGE and by immunodetection using *anti*-ChbB antibodies.

Purification of ChbB from *B. amyloliquefaciens* culture supernatant

The strain was streaked on LB agar medium and incubated at 37°C overnight. Cells were collected from 10 plates and transferred to 200 ml of MSM containing 1 % of ground crab shell chitin on a rotary shaker (200 rpm for 4 h at 37°C). After 30-fold dilution (final volume 6 l), cultivation was continued for 36 h. The bacteria and the insoluble substrate were released from the culture by centrifugation (8,000 x g, 30 min). The supernatant was cooled to 4°C,

and $(NH4)_2SO_4$ (70 % saturation) was added. After stirring for 2 h, the precipitate was obtained by centrifugation (18,000 x g, 30 min, 4°C), suspended in 50 ml of 20 mM Tris/HCl buffer (pH 9.0) and applied to a DEAE column (HR10/10, equilibrated with 20 mM Tris/HCl, pH 9.0). The eluate comprising the unbound proteins was dialysed overnight using 20 mM citrate/phosphate (pH 5.2) and applied to a MonoS column equilibrated to pH 5.2. Proteins were eluted in the same buffer with continuously increasing concentrations of NaCl (from 0 - 0.5 M) at flow rate of 0.6 ml min^{-1}. The fractions containing ChbB protein were collected and equilibrated to pH 8.0, 1 M $(NH_4)_2SO_4$, then applied to phenyl superose (HR 5/5) at a flow rate of 0.6 ml min^{-1}.

2.4.3. SDS-PAGE

The SDS polyacrylamide gel electrophoresis was performed according to Laemmli (1970).

2.4.4. Western blot

Proteins were separated by SDS-PAGE and blotted onto nitrocellulose (Satorius) or nylon membranes (Fluorotrans, Pall). The membrane was treated with blocking buffer (1 % BSA, 150 mM NaCl, 10 mM Tris/HCl, pH 7.5) for 1 h, and incubated in the same buffer in the presence of antiserum (dilution 1:1000) for 2 h. After two washes with TBS (20 mM Tris/HCl, pH 7.5, 500 mM NaCl containing 0.05 % (v/v) Tween 20 and 0.2 % (v/v) Triton X-100) and one wash with TBS only, the membrane was treated with alkaline phosphatase-conjugated goat *anti*-rabbit secondary antibodies (diluted 1:10,000) for 2 h. Colour development was performed in TBS-buffer after addition of 0.33 mg/ml NBT (Nitro blue tetrazolium chloride) and 0.165 mg/ml BCIP (5-bromo-4-chloro-3-indolyl phosphate) (Sigma).

2.4.5. Determination of amino-terminal amino acids

For N-terminal amino acid sequencing, proteins were separated by 12.5 % SDS-PAGE and transferred to a PVDF membrane (Immobilon P, Millipore). The interested band was subjected to Edman degradation using a model LF 3400 gas-phase sequencer (Beckman), followed by HPLC of the phenyl thiohydantoin amino acids.

2.4.6. Nuclease detection after SDS-PAGE

The method of Rosenthal (1977) was used to detect nuclease activity bands by enzymography after SDS-PAGE. Proteins were separated in a 12% SDS-PAGE using gels

contain 10 μg/ml calf thymus DNA. Following electrophoresis, the gel was rinsed with water and placed in 300 ml of 0.04 M Tris-HCI, pH 7.6, 2 mM $MgCl_2$, and 0.02% sodium azide (to prevent microbial growth). After gentle shaking at room temperature for 1 h, the gel was again rinsed with water and overnight incubated in the aforementioned buffer at 37°C. The next morning, the gel was transferred to fresh buffer containing 2 mM $CaCl_2$ ($CaCl_2$ cannot be added at earlier times because it precipitates with SDS in the gel). Incubation was continued at 37°C. Within several hours, ethidium bromide was added to the incubation buffer to yield approximately 1 μg/ml. After 30 min the gel was transferred to fresh buffer without ethidium bromide. The presence of ethidium bromide in the gel did not interfere with nuclease action in the course of further incubation. The gels were periodically placed on a long-wave UV light box and were photographed.

2.4.7. Preparation of cell-wall-binding protein (CWBP)

The cell wall binding proteins of vegetative *B. amyloliquefacines* cells were prepared according to Blackman et al. (1998). 1 litre cultures of *B. amyloliquefaciens* wild type or mutant strains (OD_{600} 1.0) were centrifuged (11000g, 4°C, 10 min) in order to harvest the cells. The cells were washed once with ice-cold 50 mM Tris/HCl, 0.5 mM PMSF (pH 7.5), and resuspended in 30 ml of the same buffer. The cells were broken by ultrasonication, subsequently centrifuged at 5000 g for 10 min to remove unbroken cells, then at 27000 g for 15 min at 4°C. The pellets were washed twice with ice-cold 50 mM Tris/HCl (pH 7.5), once with ice-cold 100 mM NaCl, 0.5 mM PMSF to remove any loosely associated proteins, then again two times with ice-cold 50 mM Tris/HCl, 0.5 mM PMSF (pH 7.5). Cell wall proteins were extracted (100°C, 3 min) with SDS sample buffer (62.5 mM Tris/HCl pH 6.8, 1mM EDTA, 1%(w/v) SDS, 5% mercaptoethanol, 0.0025% bromophenolblue, 10% (v/v) glycerol).

2.4.8. Preparation of purified cell-wall from B. amyloliquefaciens

For preparation of purified cell-wall material, the bacteria was grown, collected and broken in the same way as described for preparation of CWBP. The crude cell wall fraction was resuspended in a minimal volume of water at 0°C and poured with stirring into boiling 4% (w/v) sodium dodecyl sulphate (SDS) to a final concentration of about 200 mg (wet wt) per ml, then incubated at 100°C for 15 min. The suspension was cooled to RT and centrifuged to sediment the cell wall at 35000*g* for 30 min at 20°C. It is essential that, in all steps where SDS may be present, low temperature is avoided to prevent irreversible precipitation of some of the detergent in the cell wall pellet. The cell wall material was washed again two times with 4% SDS at 100°C for 15min, repeatedly washed by resuspension and centrifugation with 0.9%

NaCl, and then with water until no further SDS can be removed. The cell walls were dried by lyophilization and checked for being free of proteins and nucleic acids.

2.4.9. Autolysin activities detection after SDS-PAGE

The renaturing gel electrophoresis method to detect autolysin activity bands on SDS-PAGE was employed as previously described by Forster (1992). SDS-PAGE (11% w/v acrylamide) containing 0.05% (w/v) of purified *B. amyloliquefaciens* vegetative cell wall material were used for the detection of lytic activity. Following electrophoresis, gels were soaked for 30 min in 250 ml of distilled water at room temperature with gentle agitation. The gels were then transferred to 250 ml of renaturation solution, gently agitated for 30 min at RT, and then transferred to 250 ml of the same solution and incubated for 16 h at 37^{o}C. After incubation, the gels were rinsed in distilled water, stained in 0.1 % methylene blue in 0.01% KOH for 3 h, and destained in distilled water. Autolytic activity appeared as clearing zones in the blue background. Molecular masses were determined by comparison to standard proteins (Sigma) of known sizes which were run on the same gel and stained with Coomassie blue. The results are shown as representative photographs of gels.

Renaturation solution: 0.1% Triton X-100, 10 mM $MgCl_2$ and 25 mM Tris-HCI [pH 7.5]

2.4.10. Assay for ChbB protein binding properties

5 µg of the His-tag ChbB protein purified from *E. coli* or the purified ChbB protein secreted by *B. amyloliquefaciens* were mixed with 2 mg of substrate in 50 µl of the indicated buffer (see below) containing 1 % BSA and shaken for 16 h at 4°C or for 3 h at room temperature. The samples were centrifuged at 10,000 x g for 5 min, then each supernatant was collected. The pellet was washed twice in the same buffer and resuspended with 50 µl of buffer. Each sample (supernatant or resuspended pellet) was mixed with loading buffer, heated for 10 min at 100°C, and analysed using 12 % SDS-PAGE. The relative quantities of the protein were estimated after Coomassie staining or immunodetection (Zeltins & Schrempf, 1995) by scanning of corresponding bands and subsequent analysis using Scion Image software (Scion crop., Maryland 21701, USA). In order to study the effect of the pH, crab shell powder was mixed with ChbB protein in aliquots of various buffers adjusted to different pHs. Citrate-phosphate buffer (10 mM) was used for tests at pH 3 – pH 7, Tris/HCl buffer (10 mM) for pH 7 – pH 10. The effect of salt was tested after the addition of NaCl (0.5 M or 1 M) to citrate-phosphate buffer (pH 7, 10 mM, 1 % BSA) containing the chitinous sample. Various substrates, i.e. α-chitin (crab shells), β-chitin from Sepia and from

Siboglinum fjordicum, chitosan, β-glucan (yeast), β-glucan (barley), cellulose from cotton linters, and xylan (oat spells), were used to study the binding specificity of the ChbB protein in citrate-phosphate buffer (pH 7, 10 mM, 1 % BSA).

2.4.11. Test for chitinolytic activity

Cultures were grown in Spizizen minimal medium with 0.5 % colloidal chitin and 0.2 % yeast extract at 37°C for 24 h. The cell-free supernatant was used for enzyme testing. The test was performed using carboxymethyl chitin-Remazol Brilliant Violet (No. 04106, Loewe Biochimica GmbH) by mixing 0.1 ml of citrate-phosphate buffer (0.2 M, pH 6.0), 0.1 ml of the substrate (0.2 % in water) with 0.2 ml of respective culture supernatant or enzyme sample. The incubation was performed for 12 h at 37°C. The reaction was stopped by adding 0.1 ml of 1 N HCl, and absorption was estimated at 600 nm. The plate assay was performed by striking cells on TBY agar containing 2 % of colloidal chitin. The plates were incubated for 48 h at 37°C and rinsed with KJ/J_2 reagent. Chitin hydrolysis was recorded by halos in zones of colony growth.

3. Results

3.1. Cloning of sipV(Ba) and sipW(Ba) genes of B. amyloliquefaciens

Using a PCR-based cloning strategy with oligonucleotide primers that were designed according to amino acid sequence of *B. subtilis* SipS, we previously succeed in cloning two different type I SPase (*sip*) genes in *B. amyloliquefaciens*. The genes were named *sipS1* and *sipS2* (Hoang & Hofemeister, 1995), but have also been known as *sipS(Ba)* and *sipT(Ba)* following the names of their *B. subtilis* counterparts (Kunst, *et al.*, 1997; Tjalsma, *et al*., 1997), respectively. Surprisingly, after completion of the *B. subtilis* 168 genome sequencing project, three additional *sip* genes, *sipU, sipV* and *sipW* were found (Kunst, *et al.*, 1997). The proposed but not yet understood function of these multiple SPases induced us to search for their likely counterparts in *B. amyloliquefaciens* in order to prove their general existence as well as to search their (specific) functions in other closely related *Bacillus* species. As previously indicated (Meijer, *et al.*, 1995), the amino acid sequence alignment of type I SPases of *B. subtilis* and *B. amyloliquefaciens* (see Figure 16 & 17), demonstrated conserved regions and extensive sequence similarities among the group of type I SPases of *B. subtilis*. The similarity of the latter was significantly higher than that of different SPases of the same organism, as *B. subtilis* SipS was found closest homology with *B. amyloliquefaciens* SipS. This allowed us to use the amino acid sequences of the various *B. subtilis* SPases to design degenerated oligo primers for PCR amplification of a similar gene candidate in *B. amyloliquefaciens*.

3.1.1. Cloning of a sipV gene homologue

The regions MKKRFWFLA, VFIDYKVEG, and IVGVISDAE were chosen from the amino acid sequence of *B. subtilis* SipV to prepare the primers V1, V2, and V3 (Table 3). PCR reactions with genomic DNA of the strain ATCC 23843 yielded two PCR products of about 500 bps and 400 bps (see Figure 9). The fragments were cloned into pUC18 using the sure cloning kit (see Materials and methods). Sequencing data confirmed that both fragments overlap in one DNA region. The sequences were then used to make a BLAST search at the NCBI server (http://www.ncbi.nlm.nih.gov/BLAST), and the resulting amino acid sequence showed about 70% similarity to SipV of *B. subtilis*. The 500 bp *sipV(Ba)* DNA fragment was used as probe for Southern hybridization of either *Pst*I- or *Eco*RI-digested chromosomal DNA of the strain ATCC 23843. Only one single band gave a positive signal (data not shown)

indicating specificity to only one *sipV*-like gene [*sipV(Ba)*] in *B. amyloliquefaciens* genomic DNA.

In order to isolate the full length of the *sipV(Ba)* gene as well as flanking DNA regions, we decided to use the RAGE (rapid amplification of genomic DNA ends) protocol, as outlined in Figure 9C. *Pst*I- as well as *Eco*RI-digested chromosomal DNA of *B. amyloliquefaciens* was ligated to either *Pst*I- or *Eco*RI-cut vector DNA (pUC18) in order to get a suitable large template for PCR amplification of the whole gene region (Figure 9C). Two distinct PCR fragments were obtained, cloned and sequenced. The sequences were analysed and confirmed to overlap with the core DNA of the proposed *sipV(Ba)* gene region. The BLAST search using the nucleotide sequence of the amplified DNA fragment revealed the presence of three ORFs, and the deduced proteins to have 70, 77, and 67 percent of identity with *B. subtilis* proteins encoded by genes *yhjE-sipV-yhjG*, respectively (Figure 9C). Thus, flanking ORFs indicated that the *B. amyloliquefaciens sipV(Ba)* gene occupies a similar genomic position as compared to *sipV(Bs)* of *B. subtilis* (Kunst, *et al*., 1997). The sequence was submitted to the GenBank database (http://www.ncbi.nlm.nih.gov/) and given the accession number **AF084950.**

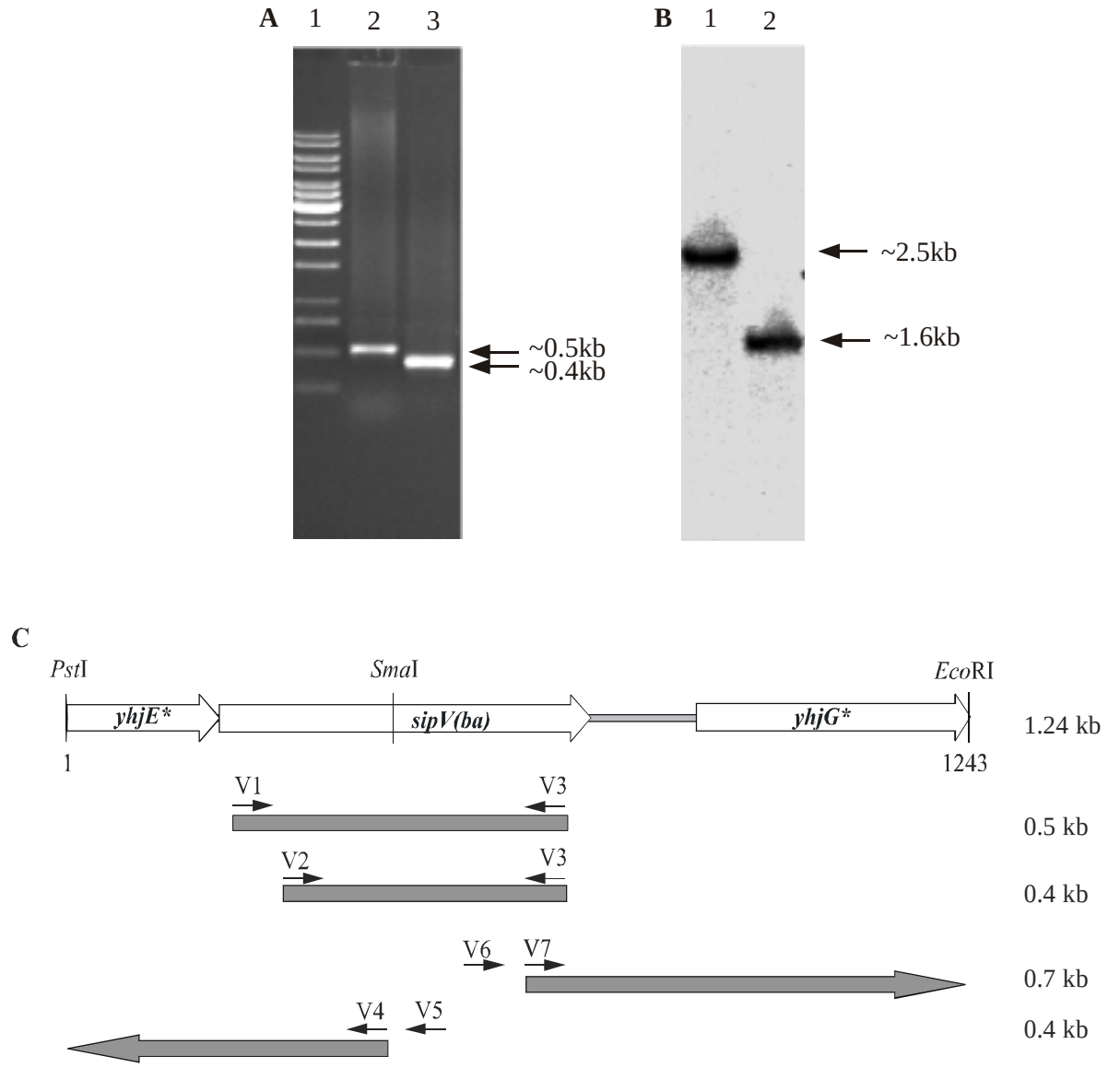

Figure 9. Identification of the *sipV*(*Ba*) gene region of *B. amyloliquefaciens*. The following PCR steps were performed. (A) PCR reactions with genomic DNA of *B. amyloliquefaciens* and the degenerative primers V1 and V3 and V2 and V3 led to the isolation of DNA fragments of about 500 bp (lane 2) and 400 bp (lane 3), respectively. These PCR fragments were subcloned and sequenced. (B) The 500 bp-DNA fragment was used as a probe for Southern hybridisation of either *Pst*I- or *Eco*RI-digested chromosomal DNA. Each digest indicated one specific signal and suggested the existence of a *sipV*-like gene in *B. amyloliquefaciens*. (C) Next, the previously described RAGE protocol (Hoang & Hofemeister, 1995) was used for PCR amplification with "ligated" genomic DNA of *B. amyloliquefaciens.* The DNA of *B. amyloliquefaciens* was cut with either *Pst*I or *Eco*RI and ligated into corresponding sites of pUC18 DNA. The "ligated" DNA was used for PCR with the oligonucleotides Uni1 or Uni2 and V6, V7 or V4, V5 (Table 2) as "forward" or "reverse" primers, respectively. The latter were chosen according the indicated regions within the 500 bp-PCR fragment which was amplified in the first PCR step. The PCR fragments of either 0.4 or 0.7 kb were cloned and sequenced. Ultimate PCR led to DNA covering a 1243 bp fragment.

```
        10        20        30        40        50        60
TTTATATCAGCGGCCGCTATGGGGAAAATGGTCATGATCTTTATGATCAGCTTTATCGGGTATGAT
 F  I  S  A  A  A  M  G  K  M  V  M  I  F  M  I  S  F  I  G  Y  D

  70        80        90       100       110       120       130
CTTCACGCACTCATTACACAGCCTTTCCGGATGGCGGGCGCCCTAACGGTGATCGCGGTGCTGTGG
 L  H  A  L  I  T  Q  P  F  R  M  A  G  A  L  T  V  I  A  V  L  W

     140       150       160       170       180       190    RBS
TATGCCGGAAAGCATATCGAACGGCGTTTACAGACGAGAATCAGTCAGCATGAGAAAGACGGAGGG
 Y  A  G  K  H  I  E  R  R  L  Q  T  R  I  S  Q  H  E  K  D  G  G

200       210       220       230       240       250       260
GGACAGGAATCTTGAAAAAACGATTTTGGTTTATAGCCGGCGTGCTTATCGTTGTTTTGGCCATAC
 G  Q  E  S  -

            M  K  K  R  F  W  F  I  A  G  V  L  I  V  V  L  A  I

   270       280       290       300       310       320       330
AATTAAAAAATGCCATTTTTATTGATTATAAAGTCGAAGGAATCAGCATGAATCCGACCTTTAAAC
Q  L  K  N  A  I  F  I  D  Y  K  V  E  G  I  S  M  N  P  T  F  K

       340       350       360       370       380       390
AAGGAAATGAGCTGATGGTCAACAAGTTTTCCCATCGTTACAAAACGATCCGCCGTTTCGATATTG
Q  G  N  E  L  M  V  N  K  F  S  H  R  Y  K  T  I  R  R  F  D  I

 400       410       420       430       440       450       460
TCCTGTTTAAAGGCCCGCACCACAAAGTGCTGATCAAACGTGTCATCGGGCTCCCGGGCGAGTCCA
V  L  F  K  G  P  H  H  K  V  L  I  K  R  V  I  G  L  P  G  E  S

     470       480       490       500       510       520
TCACTTACAGGGAGGACCAGCTGTTTGTGAATGGAAAACGGGTGGCGGAACCGTTTTTAAAACCGT
I  T  Y  R  E  D  Q  L  F  V  N  G  K  R  V  A  E  P  F  L  K  P

530       540       550       560       570       580       590
TAAAGTCATCATTGTCCGCCGCAAGCCATGTGACCGGTGATTATACGTTGAAGGAAACGACCGGTC
L  K  S  S  L  S  A  A  S  H  V  T  G  D  Y  T  L  K  E  T  T  G

   600       610       620       630       640       650       660
GCAAGAGGATACCGAAGGGACAATATTTCGTGATTGGCGACAACCGCATATACAGCCTCGACAGCC
R  K  R  I  P  K  G  Q  Y  F  V  I  G  D  N  R  I  Y  S  L  D  S

       670       680       690       700       710       720
GTCATTTCGGACCGATTAAGGATAAAGACATTGTCGGTGTTATCAGTGAATCATACGCCAAGTGAA
R  H  F  G  P  I  K  D  K  D  I  V  G  V  I  S  E  S  Y  A  K  -

 730       740       750       760       770       780       790
CGGCGTCTTTTTTAGAAATGATTGTCAAAAAGCAAAGGAAAGATTATAATAGAATTGTTACGCGCG

     800       810       820       830       840       850    RBS
AAACAAAAATCACGCGTTTTATTTTTAGTTATTTTGTTACGTGTGCAACAAAAAATGCGAAAGAGG

860       870       880       890       900       910       920
GCTGATAAGATGGAAACACAGGCTGCCATTATCGGGGGAGGTCCGGTCGGGCTCATGCTTGCATCA
          M  E  T  Q  A  A  I  I  G  G  G  P  V  G  L  M  L  A  S

   930       940       950       960       970       980       990
GAGCTTGCGCTTGCCGGAGTGAAAACGACTGTCATTGAACGATTGAWGAAGACGGTGCCATATTCT
 E  L  A  L  A  G  V  K  T  T  V  I  E  R  L  X  K  T  V  P  Y  S
```

```
      1000      1010      1020      1030      1040      1050
AAAGCGCTTATTATGCATCCGCGCACACTTGAACTTTTTGCAATGAGAGGCATCTTGGGACGCTTT
 K  A  L  I  M  H  P  R  T  L  E  L  F  A  M  R  G  I  L  G  R  F

1060      1070      1080      1090      1100      1110      1120
TTGGATAAAGGAACGAAGGTGTCTTCCGGCCATTTTTCGATGCTGGATACACGGCTGGATTTTTCT
 L  D  K  G  T  K  V  S  S  G  H  F  S  M  L  D  T  R  L  D  F  S

    1130      1140      1150      1160      1170      1180
CGGCTGGATTCTAAGCAGAATTATTCGTTAATGCTGCCTCAGGCAGAAACGGAGCGGCTTTTGGAG
 R  L  D  S  K  Q  N  Y  S  L  M  L  P  Q  A  E  T  E  R  L  L  E

1190      1200      1210      1220      1230      1240
GAGTATGCATCAAGTTTAGGCGCAGATATCATCCGCAGGGCAGAAGCAGTGGCTC
 E  Y  A  S  S  L  G  A  D  I  I  R  R  A  E  A  V  A
```

Figure 10. DNA sequence of the *sipV(Ba)* gene region of *B. amyloliquefaciens*. Three open reading frames and the translated amino acid sequences are shown. The first incomplete ORF shows similarity to ORF YhjE, the second to SipV and the following incomplete ORF to YhjG of *B. subtilis*.

3.1.2. Search for a sipU gene homologue

Several attempts were made in order to identify a *sipU* homologue gene of *B. amyloliquefaciens*. First, the regions MNAKTITLKK, MIAALIFTI, FKPFLIEG, YFVMGDN, NGMGMPSED, and PFGEMRQAK of the *B. subtilis* SipU protein aa sequence were chosen to design the degenerated oligo primers U1, CH5, CH6, HV11, CH7, and CH8 (Table 3). These aa sequence regions appeared to be most specific for SipU type SPase compared to other Sip proteins. Nine different combinations of primers were used for PCR amplification (Figure 11B). Amplification reactions with genomic DNA of *B. subtilis* 168 always resulted in the fragment of the expected size, while parallel PCR with genomic DNA of *B. amyloliquefaciens* repeatedly failed (data not shown). In a next step, we used two highly conserved regions of aa sequences of ORFs which are upstream of *B. subtilis sipU*. The conserved aa regions HFGAGNIG, and HGSAPDIAG of deduced *B. subtilis* protein MtlD and YcsA, respectively, were used to design the oligo primers CH1 and CH2 (Table 3). However, like in the upper amplification reactions, no specific amplification products were obtained (data not shown). In addition, a *sipU*-core DNA fragment of about 400 bp, which was amplified from *B. subtilis* DNA with the oligo primers CH5 and CH7, was used as probe for Southern hybridisation of genomic DNA of *B. amyloliquefaciens* and *B. subtilis*. The chromosomal DNA of the strains ATCC 23843 and BE71, as well as of *B. subtilis* 168 as a control was *Bcl*I-digested, blotted and the filter hybridised with the *sipU(Bs)*-probe at low

stringency (see Material and methods). As shown in Figure 11, no DNA band exhibited specificity.

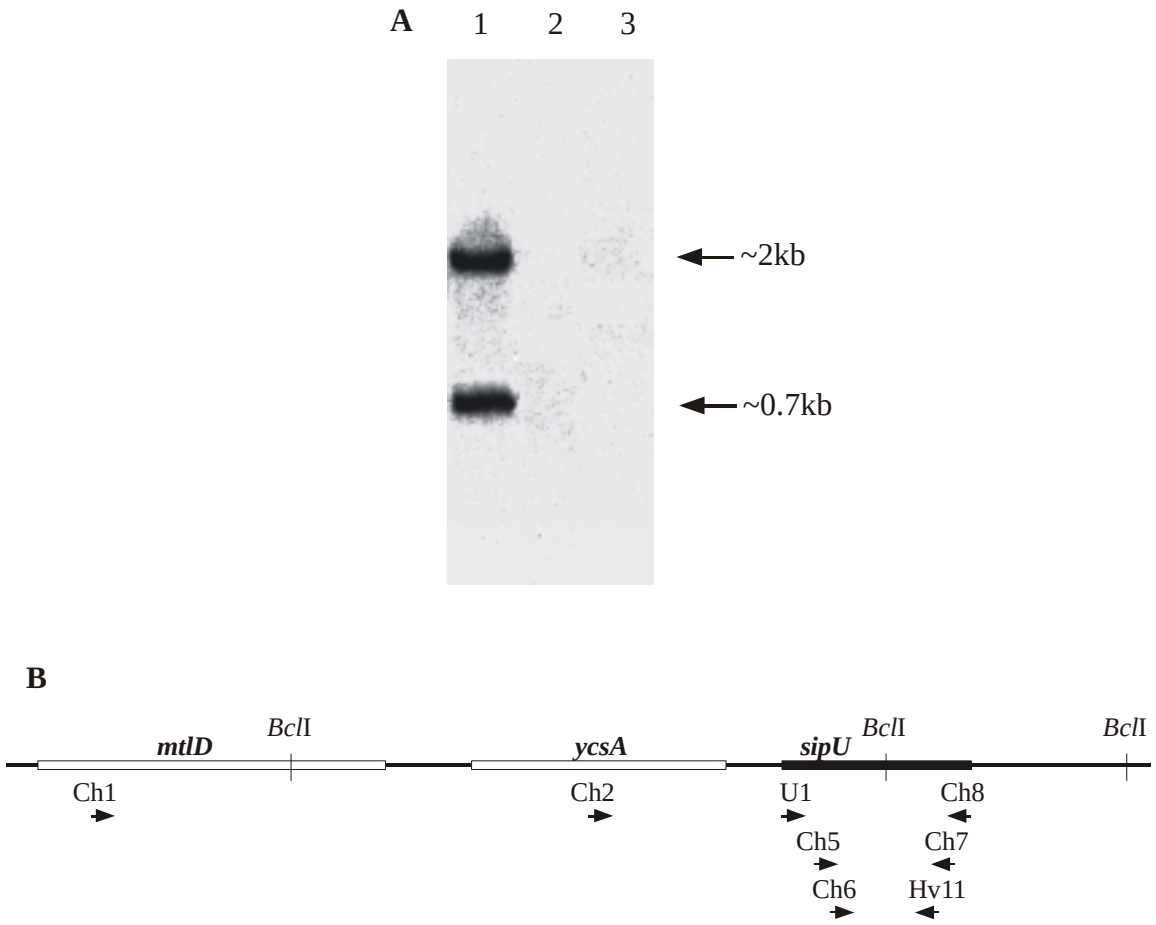

Figure 11. **Strategy for identification of the *sipU* homologue gene of *B. amyloliquefaciens***. A: Southern hybridization using the *sipU(Bs)* DNA core fragment as probe with *Bcl*I-digested chromosomal DNA of *B. amyloliquefaciens* ATCC 23843 (1), BE71 (2), and *B. subtilis* 168 (3). B: positions of oligo primers of the *B. subtilis* 168 *sipU* gene region used for PCR identification of the *sipU* homologue gene of *B. amyloliquefaciens.*

3.1.3. Cloning of a sipW gene homologue

From an amino acid sequence alignment using deduced proteins deposited in the Genbank database (http://www.ncbi.nlm. nih.gov/BLAST), it was surprising to learn that SipW*(Bs)* of *B. subtilis* seems to be closer to type I SPases of eucaryotes than to other type I SPases of bacterial origins. It was of interest to us to identify a *sipW* homologue of *B. amyloliquefaciens* as well as to search for its existence in other *Bacillus* species (see 3.1.3.). Two highly conserved regions (box 1 and 2, see Figure 6) of different so-called eucakyotic (ER) type I

SPases were chosen to design the degenerative oligo primers W1 and W2 (Table 3) for box 1 and box 2, respectively (Figure 6).

Box 1

```
SipW_Bs    VLSGSMEPEFNTG
Spc18_Hs   VLSGSMEPAFHRG
Spc18_Rn   VLSGSMEPAFHRG
Sec11_Sce  VLSGSMEPAFQRG
           ********.*..*
```

Box 2

```
SipW_Bs    TKGDNNAAAD
Spc18_Hs   TKGDNNAVDD
Spc18_Rn   TKGDNNAVDD
Sec11_Sce  TKGDNNAGND
           *******..*
```

Figure 12. Conserved regions of several ER- (SipW-) type I SPases. The alignment was done using aa sequences of SipW*(Bs)* (B69708) from *B. subtilis*; the Spc18*(Hs)* 18 kDa subunit of the Human (*Homo sapiens*) signal peptidase complex (M 00772/NM 014300); the Spc18*(Rn)* 18 kDa subunit of the Rat (*Ratus norvegicus*) signal peptidase complex (NM 031723); the Sec11*(Sce)* signal peptidase (NP 012288) of yeast (*Saccharomyces cerevisiae*). Stars indicate identical, dots similar aa positions.

Using chromosomal DNA of the *B. amyloliquefaciens* strain ATCC 23843 and the primers W1, and W2, we succeeded in amplifying a 0.2 kb core DNA fragment as shown in Figure 7A. The fragment was cloned into the pUC18 vector and sequenced. The sequence was subjected to a BLAST search. The search results showed similarity to several type I SPases, but highest to SipW of *B. subtilis*. Southern hybridization with chromosomal DNA of the strain ATCC 23843 using the 0.2 kb (core) PCR fragment as probe confirmed the presence of corresponding DNA in *B. amyloliquefaciens*.

As in the case of *sipV(Ba)*, the RAGE protocol was a simple and fast method for obtaining the *sipW(Ba)* gene region as outlined in Figure 7. In this protocol two pairs of the oligo primers W7, W8 and W9, W10, respectively, were designed (Table 3) and two different PCR products were generated as described in Figure 7(C). The two PCR fragments were cloned into the vector pUC18 and sequencing data indeed confirmed the cloning of a *sipW* like gene region of *B. amyloliquefaciens*.

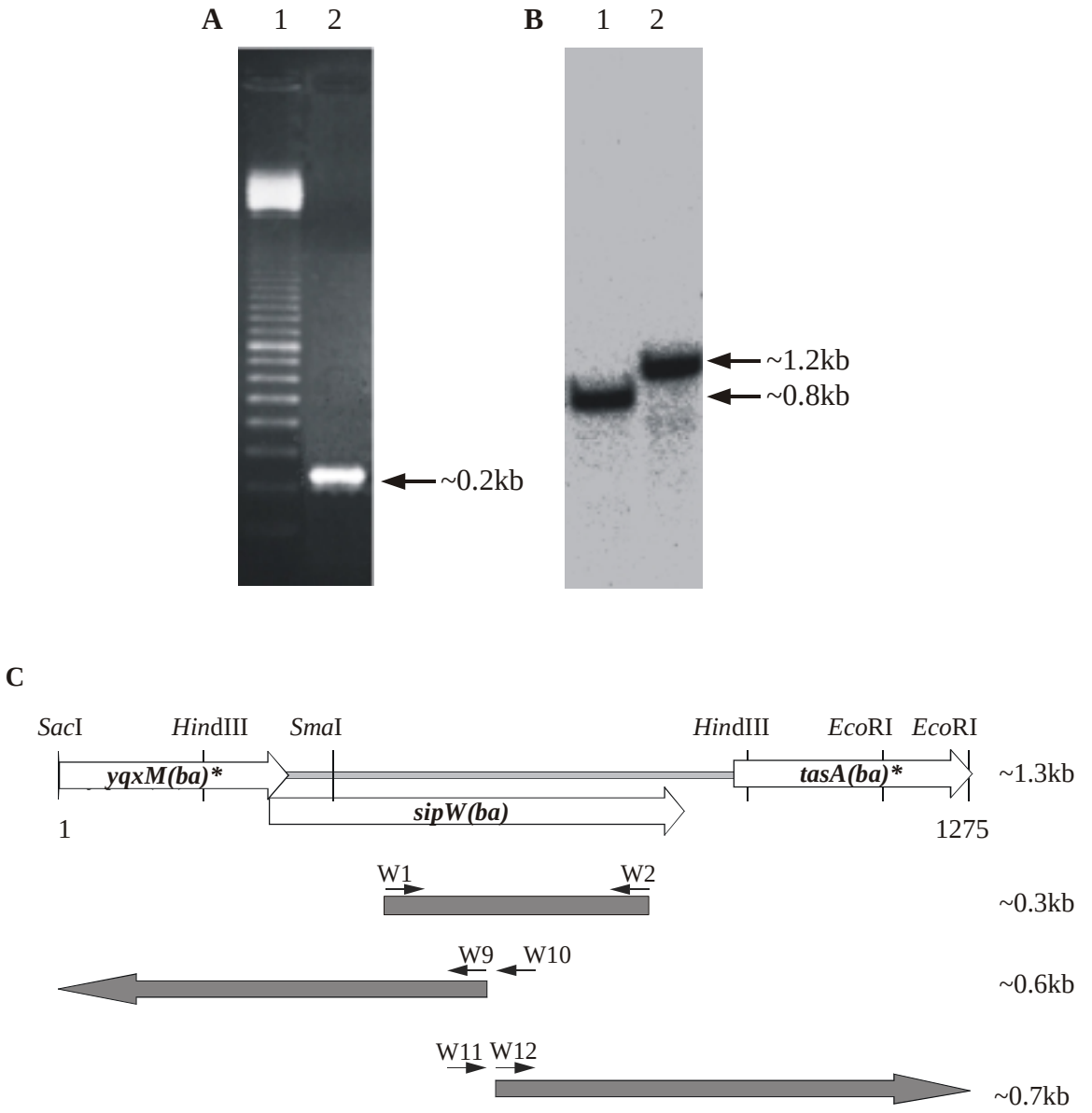

Figure 13. Identification of a *sipW(Ba)* gene region of *B. amyloliquefaciens*. (A) Two highly conserved regions (box 1 and box 2) of ER type-SPases (shown in Figure 2) were chosen for the degenerative primers W1 and W2 (Table 2). With these primers, a 0.2 kb DNA fragment was amplified from genomic DNA (lane 2), cloned and sequenced. (B) Southern hybridisation with either *Hind*III- (lane 1) or *Eco*RI-*Sac*I- (lane 2) digested genomic DNA indicated that the core fragment from step one hybridises with *B. amyloliquefaciens* DNA fragments of about 0.8 and 1.2 kb, respectively. (C) The *B. amyloliquefaciens* genomic DNA was *Eco*RI/*Sac*I-digested, and ligated to the corresponding sites of the plasmid pUC18, amplified using the primers Uni1 or Uni2, and the nested primers W9, W10 or W11, W12 (Table 2) as "forward" or "reverse" primers, respectively, were used. The PCR fragments of about 0.6 and 0.7 kb were cloned and sequenced. Ultimate PCR led to DNA covering a 1.3 kb fragment. The nucleotide sequence was submitted to GenBank and given the accession number AF085497.

```
        10        20        30        40        50        60
CTCCATAAAATTGCCTCTCAGCAACAGCCTTTACAAAACGGCAATGTCGTGGATAAAGGGCTTATT
 L  H  K  I  A  S  Q  Q  Q  P  L  Q  N  G  N  V  V  D  K  G  L  I

  70        80        90       100       110       120       130
ACTGATAAAAAAAGCAAAACGATTTATAAAATGGAGTCAAAGCGGGCCTTGCAGCCGGGAATTTAT
 T  D  K  K  S  K  T  I  Y  K  M  E  S  K  R  A  L  Q  P  G  I  Y

     140       150       160       170       180       190
GCATTCAAGGTTTACAAGCCTGAAGGATATCCTGCGAACGAAGAAAAATTTGAGTGGTCAAAACCG
 A  F  K  V  Y  K  P  E  G  Y  P  A  N  E  E  K  F  E  W  S  K  P

200       210       220       230       240       250       260
ATGAAGCTTGTTAAGTGCCAAGAACAGGCGACGGCTTCCGATCAGAAGAAAACAGAGAAAAAACCG
 M  K  L  V  K  C  Q  E  Q  A  T  A  S  D  Q  K  K  T  E  K  K  P

   270       280       RBS       300       310       320       330
GCTGAGCCGGCAAAAGAAAGCGGGGAAGAACATGAAAAAAACACTGAAACTGATCAGTAATATTTT
 A  E  P  A  K  E  S  G  E  E  H  E  K  N  T  E  T  D  Q  -

                                  M  K  K  T  L  K  L  I  S  N  I  L

       340       350       360       370       380       390
GTATGCCGTAATCTTCAGCTTGATTATTGTGCTGACATTGACGGTAATCTCGACCCGGGCTTCCGG
  Y  A  V  I  F  S  L  I  I  V  L  T  L  T  V  I  S  T  R  A  S  G

 400       410       420       430       440       450       460
CGGTGAGCCGGCCATCTTCGGCTATACGCTGAAATCCGTGCTGTCGGGTTCAATGGACCCGGAATT
  G  E  P  A  I  F  G  Y  T  L  K  S  V  L  S  G  S  M  D  P  E  F

     470       480       490       500       510       520
TAAGACGGGTTCACTCATAGCGGTAAAGAAAATTTCTGATGTGAATGACTTAAAAAAAGGCGATGT
  K  T  G  S  L  I  A  V  K  K  I  S  D  V  N  D  L  K  K  G  D  V

530       540       550       560       570       580       590
CATCACTTTTACACAAGATGACGGGTCAGTCGTAACACACAGAATAATCGGGATCACAAAAAAGGG
  I  T  F  T  Q  D  D  G  S  V  V  T  H  R  I  I  G  I  T  K  K  G

   600       610       620       630       640       650       660
CGGCCGCCTGTTGTTTGAGACAAAAGGAGACCATAATGCCGCTCCGGATGCCGCACCCGTTCAAGC
  G  R  L  L  F  E  T  K  G  D  H  N  A  A  P  D  A  A  P  V  Q  A

       670       680       690       700       710       720
TGAAAAAGTGGCGGCCCAGTATACGGGATATCAGCTCCCGTATGCGGGATATGTAATACATTTGGC
  E  K  V  A  A  Q  Y  T  G  Y  Q  L  P  Y  A  G  Y  V  I  H  L  A

 730       740       750       760       770       780       790
CAGCCAGCCGATCGGAACGGCGATTCTGTTAATCGTTCCCGGCGTCATGCTTTTAATTTACTCAAT
  S  Q  P  I  G  T  A  I  L  L  I  V  P  G  V  M  L  L  I  Y  S  I

     800       810       820       830       840       850
AACAGTCATTGCAAGCGCTCTCCGCGATATCGAGCGGAAGACGAAAGCGCTGGAGGAACATGAGAG
  T  V  I  A  S  A  L  R  D  I  E  R  K  T  K  A  L  E  E  H  E  R

860       870       880       890       900       910       920
AAACGGGACCATGTCCACATGAATACGGTTGCAACCTGGCGACAGGATTTGATAACAAAAAATGAA
  N  G  T  M  S  T  -
```

```
   930  RBS  940       950       960       970       980       990
AAAGTAAGGGGAGTACAAACATGGGTATGAAAAAGAAATTAAGCTTGGGCGTTGCCTCAGCCGCAC
                    M  G  M  K  K  K  L  S  L  G  V  A  S  A  A

      1000      1010      1020      1030      1040      1050
TCGGTTTAGCATTAGTAGGAGGAGGCACATGGGCCGCATTTAATGATGTGAAGTCCACGGACGCCA
L  G  L  A  L  V  G  G  G  T  W  A  A  F  N  D  V  K  S  T  D  A

1060      1070      1080      1090      1100      1110      1120
CATTTGCGTCAGGAACACTTGATTTATCGGCTAAAGAACAATCAGCCAATGTCAATTTGTCAAACT
T  F  A  S  G  T  L  D  L  S  A  K  E  Q  S  A  N  V  N  L  S  N

    1130      1140      1150      1160      1170      1180
TAAAACCAGGCGACAAATTGACAAAAGATTTTGAATTCAGAAACAACGGTTCACTTGCCATTAAAG
L  K  P  G  D  K  L  T  K  D  F  E  F  R  N  N  G  S  L  A  I  K

1190      1200      1210      1220      1230      1240      1250
AAGTGCTGATGGCTTTGAACTTTACTGGACTTCAAAGGAGCAAGAAAGGGAACGAATCTGCGGAGG
E  V  L  M  A  L  N  F  T  G  L  Q  R  S  K  K  G  N  E  S  A  E

  1260      1270
ATTTCTCAGCCAGTTTGAAAT
D  F  S  A  S  L  K
```

Figure 14. DNA sequence of the *sipW(Ba)* gene region of *B. amyloliquefaciens*. Three open reading frames and the translated amino acid sequences are shown. The first incomplete ORF shows similarity to ORF YqxM, the second to SipW and the following incomplete ORF to TasA of *B. subtilis.*

3.1.4. Existence of sipW-like genes in different Bacillus groups

After the presence of SipW type SPases had been confirmed for *B. subtilis* (Tjalsma, *et al*., 1998), *B. amyloliquefaciens* (this study), *B. halodurans* (from genome sequencing project of *B. halodurans*) and *B. anthracis* (from genome sequencing project of *B. anthracis*), it was therefore of interest to know whether the gene is also presented in other, distantly related *Bacillus* species. The primers W1 and W2, which were successful for PCR amplification of a *sipW* gene homologue from *B. amyloliquefaciens* (Figure 13), were applied to search for the abundance of similar genes in other, distantly related *Bacillus* species. Genomic DNA of several species, including at least one representative out of each *Bacillus* 16S rRNA-phylogenetic group (according to Ash, *et al*., 1991), was used to carry out the above-mentioned PCR approach.

PCR fragments of the expected size were generated from all tested strains, but not in the case of *B. stearothermophilus* and *Thermoactinomyces vulgaris*. This indicated the abundance of *sipW*-like genes in distantly related *Bacillus* species (Figure 15).

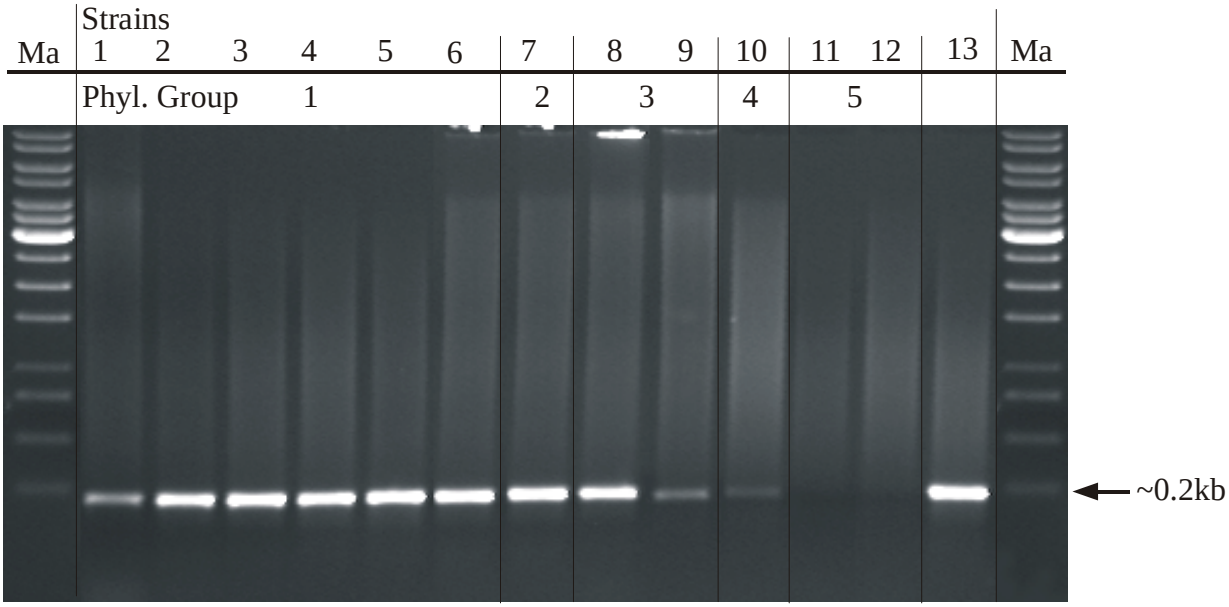

Figure 15. Abundance of *sipW*-like DNA in several *Bacillus* species representing different 16S rRNA phylogenetic groups (Ash, *et al.*, 1991; Priest, 1993): Group 1, *B. subtilis* (13), *B. amyloliquefaciens* (1), *B. circulans* (2), *B. lentus* (3), *B. licheniformis* (4), *B. megaterium* (5), *B. thuringiensis* (6) ; From group 2, *B. spaericus* (7); From group 3, *B. macerans* (8), *B. polymyxa* (9); From group 4, *B. brevis* (10); From group 5 etc., *B. stearothermophilus* (11); *Thermoactinomyces vulgaris* (12). PCR was under standard conditions using about 2 µg of genomic DNA and the primers W1 and W2 (Table 2). The brightness of DNA bands correlates with the amount of PCR product per run.

3.1.5. Sequence analysis of type I signal peptidases

The amino acid sequence of SipV*(Ba)* was aligned with as much as 18 *Bacillus* P-type SPases proteins known from *B. subtilis, B. amyloliquefaciens, B. licheniformis, B. stearothermophilus, B. caldolyticus, B. halodurans* and *B. anthracis* (Figure 16). The deduced amino acid sequence of SipV*(Ba)* shares 40% to 77% of identical amino acid with the known P-type Sip deduced proteins from *Bacillus* (Figure 16). Several conserved regions were found within all the deduced Sip proteins of *Bacillus* such as the transmembrane domain A and

domains B, C, D and E with the Serine in domain B and the Lysine in domain D both of which were shown to be essential for the catalytic centre of the enzyme (Sung & Dalbey, 1992; van Dijl, *et al*., 1995) (Figure 16).

Similar to *B. subtilis* SipW, the deduced amino acid sequence of *B. amyloliquefaciens* showed similarity to other known ER-type SPases (Figure 17). SipW also showed some similarity to the P-type SPases, however this was limited to the conserved domains B-E, which are present in all known type I SPases. Like other ER-type SPases, SipW of *B. subtilis* as well as SipW of *B. amyloliquefaciens* contains a conserved histidine residue in domain D instead of the lysine residue which was observed in domain D of P-type SPases.

Similar to SipW*(Bs)*, SipW*(Ba)* has one amino-terminal (A1) and one carboxyl-terminal transmembrane domains (A2). This seems to be common for ER-type SPases of *Bacillus* including the deduced amino acid sequences of SipW of *B. halodurans* and *B. anthracis*, which have also the same motifs (Figure 17). *B. amyloliquefaciens* SipS, SipT, SipV, like other known *Bacillus* P-type SPases have only the amino-terminal transmembrane domain (Figure 16 & 18). The P-type and ER-type SPases, however, were not only differed in the number of transmembrane domains but also in the distances between conserved domains. As shown in Figure 17, the domains B and C of ER-type SPases are separated by only one residue and domain D and E are separated by 10-11 residues, while P-type SPases are separated by 11-42 and 23-118 residues, respectively (Figure 16) (Dalbey, *et al*., 1997).

```
                    10        20        30   A    40        50        60   B    70
                     |         |         |        |         |         |         |
Sip1(Bst)    ----MTKQK----------EKRGRRWP--WFVAVCVVATLRLFVFSNYVVEGKSMMPTLESGNLLIVNK
Sip2(Ban)    ----MKKTL----------KKEGIEWIRTILIGVLLAVFFRTFFFSTYVVEGKSMMPTLQDGNMLVVNK
SipV(Ba)     ----MKKR----------------FWFIAGVLIVVLAIQLKNAIFIDYKVEGISMNPTFKQGNELMVNK
SipV(Bs)     ----MKKR----------------FWFLAGVVSVVLAIQVKNAVFIDYKVEGVSMNPTFQEGNELLVNK
Sip(Bc)      ----MEQ-K----------KSEWREWMKAIVVAVLLAGGIRYFIFAPIIVDGYSMMPTLHNHERMIVNK
Sip2(Bst)    ----MEQ-K----------KSEWREWMKAIVVAVLLAGGIRYFIFAPIIVDGYSMMPTLHNHERMIVNK
Sip1(Ban)    ----MKK-E----------KSSLWEWIKAILIAVVLAGVIRQFFFAPILVDGVSMASTLHDRDRMIVNK
Sip4(Ban)    ----MKENT----------KKELFSWAKTIGFTLVLIAIIRGVLFTPSLVQGESMMPTLENNERVLVNK
SipV(Bh)     MEALLVDTK----------KSDINEWIKAILIALLLAVLIRSFVFVSYEVRGESMEPTAYEGEMFIVNK
SipT(Ba)     ----MTEEQKPTSEKSVKRKSNTYWEWGKAIIIAVALALLIRHFLFEPYLVEGSSMYPTLHDGERLFVNK
SipT(Bs)     ----MTEEKNTNTEKTAKKKTNTYLEWGKAIVIAVLLALLIRHFLFEPYLVEGSSMYPTLHDGERLFVNK
Sip(Bl)      ----MTEEKSTN-----KK--NSLFEWVKAIIIAVVLALLIRAFLFEPYLVEGTSMDPTLHDGERLFVYK
SipS(Ba)     ----MKSEKE----K--TSKKSAVLDWAKAIIIAVVLAVLIRNFLFAPYVVDGESMEPTLHDRERIFVNM
SipS(Bs)     ----MKSEN--------VSKKKSILEWAKAIVIAVVLALLIRNFIFAPYVVDGDSMYPTLHNRERVFVNM
SipP1015     ----MTKEKV-------FKKKSSILEWGKAIVIAVILALLIRNFLFEPYVVEGKSMDPTLVDSERLFVNK
SipP1040     ---MFDKEK---------RKKSNIIDWIKAILIALILVFLVRTFLFEPYIVQGESMKPTLFNSERLFVNK
SipU(Bs)     ----MNAKTIT-----LKKKRKIKTIVVLSIIMIAALIFTIRLVFYKPFLIEGSSMAPTLKDSERILVDK
Sip3(Ban)    ---MMQKKK----------RWREFFGTIAIACLLVFLAKIFVFFPTTVKGASMKPTLQDGDKVIVNK
Sip5(Ban)    ----MDMKQ----------EIKRGWG-KYILFVFVLVVAYHSFTLCKVEGKSMQPTLYEEDYVFVNK
```

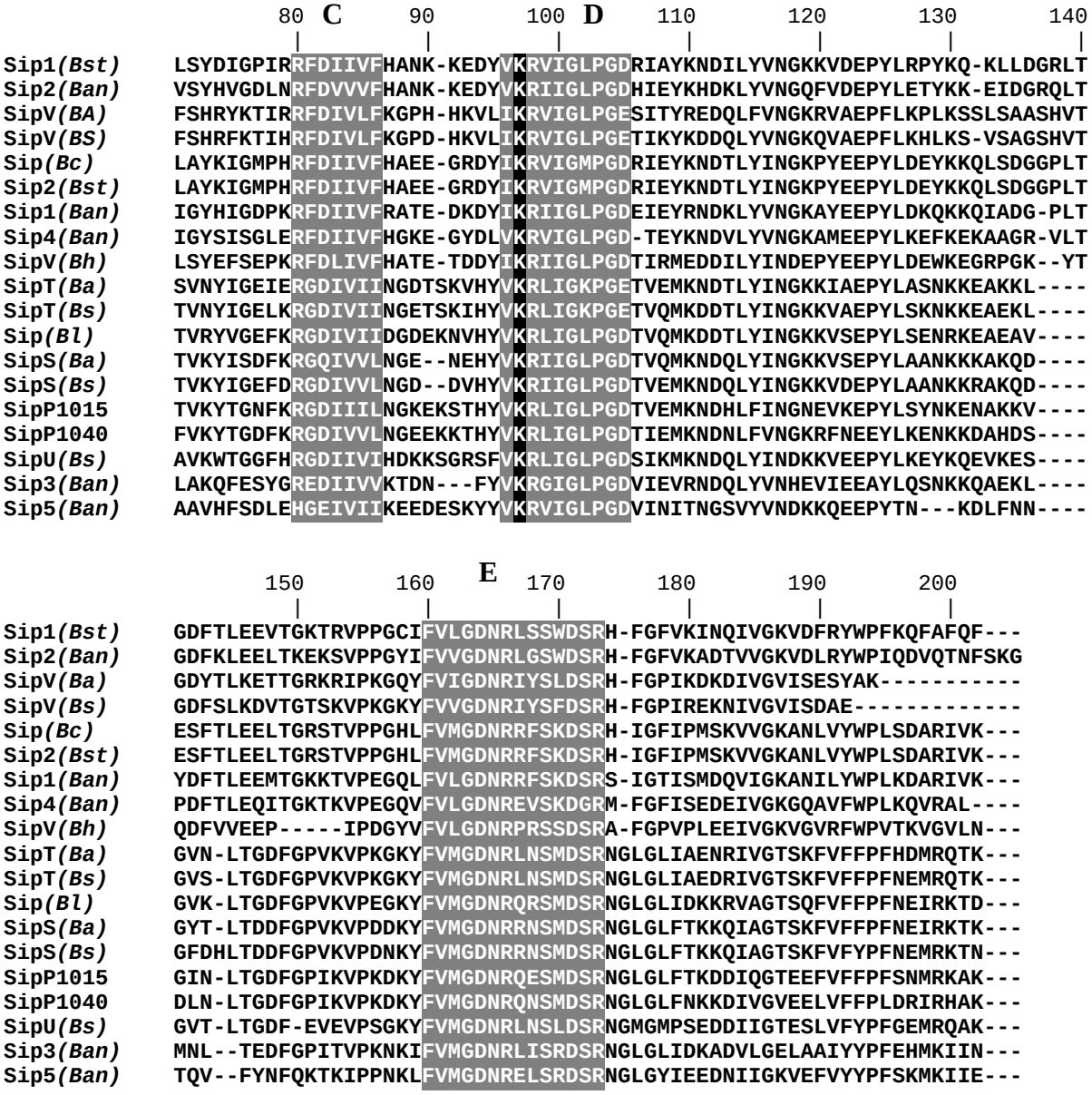

Figure 16. Multiple alignments of known *Bacillus* P-type Sip proteins. The analysed *Bacillus* Sip proteins are: *B. amyloliquefaciens* SipS*(Ba)* (P41026), SipT*(Ba)* (P41025), SipV*(Ba)*; *B. subtilis* SipS*(Bs)*, SipT*(Bs)*, SipU*(Bs)*, SipV*(Bs)* (Tjalsma *et al*., 1997), SipP1015 (I40470), SipP1040 (I40552); *B. halodurans* SipV*(Bh)* (BAB04749); *B. licheniformis* Sip*(Bl)* (CAA53272); *B. caldolyticus* Sip*(Bc)* (I40175); *B. anthracis* Sip1*(Ban)*, Sip2*(Ban)*, Sip3*(Ban)*, Sip4*(Ban)*, Sip5*(Ban)*; *B. stearothermophilus* Sip1*(Bst)*, Sip2*(Bst)* (preliminary sequence data from the website http://www.tigr.org). The transmembrane domains A and conserved domains B-E are shaded in grey. The serine and lysine residues that may be involved in catalysis are shaded in black.

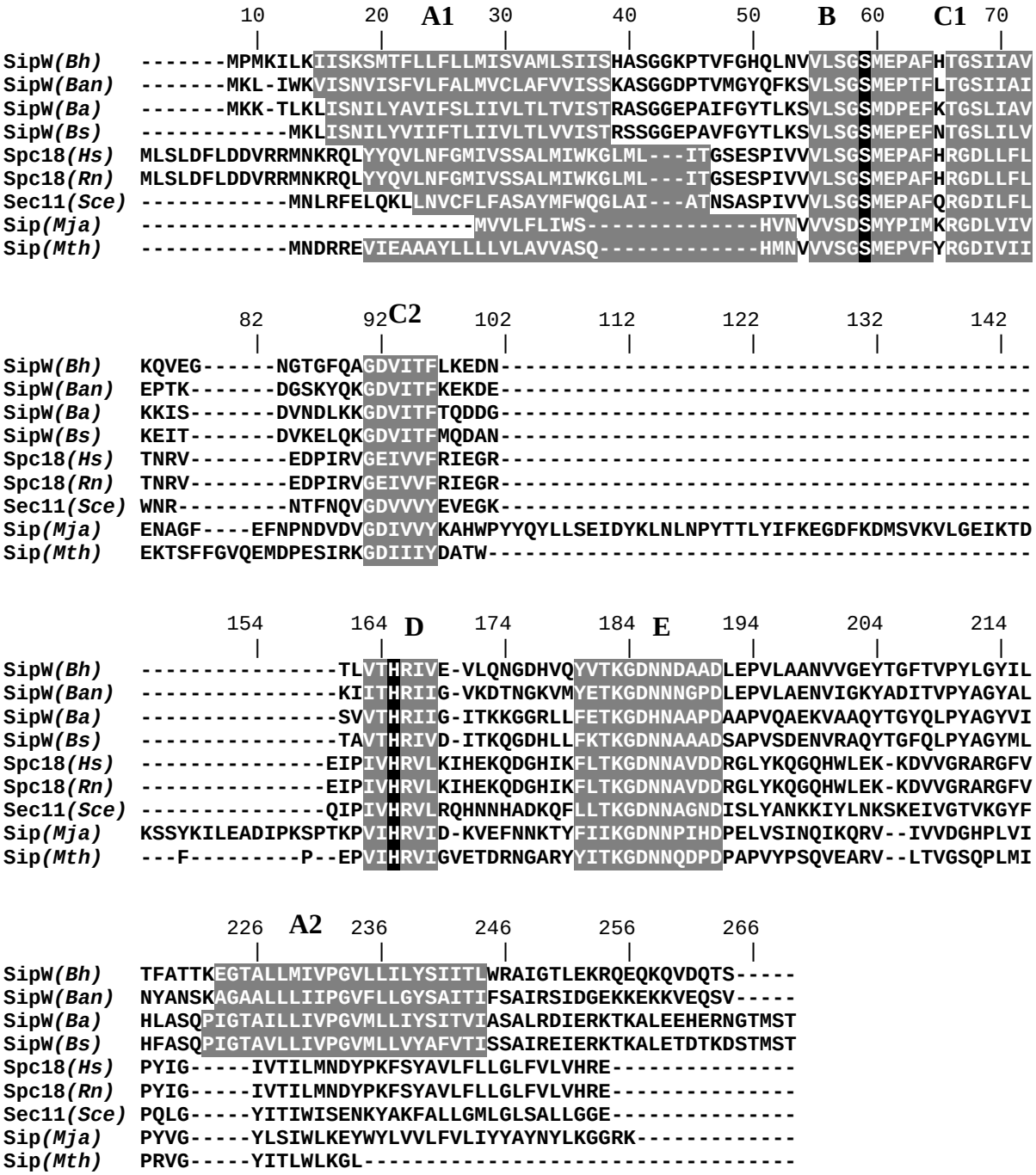

Figure 17. Multiple alignments of ER-type Sip proteins. The deduced amino acid sequence of the *sipW(Ba)* gene was aligned with: *B. subtilis* SipW*(Bs)* (B69708); *B. halodurans* SipW*(Bh)* (BAB05849); *B. anthracis* SipW*(Ban)* (was obtained as preliminary sequence data from the Institute for Genomic Research website at http://www.tigr.org); *Methanothermobacter thermautotrophicus* Sip*(Mth)* (AAB85923); *Methanococcus jannaschii* Sip*(Mja)* (E64332); *Saccharomyces cerevisiae* Sec11*(Sce)* (NP 012288); Human Spc18*(Hs)* (*Homo sapiens,* M 00772); Rat Spc18*(Rn)* (*Ratus norvegicus,* NM 031723). The transmembrane domains A1, A2, and the conserved domains B-E were shaded in grey. The serine and histidine residues that may be involved in catalysis are shaded in black.

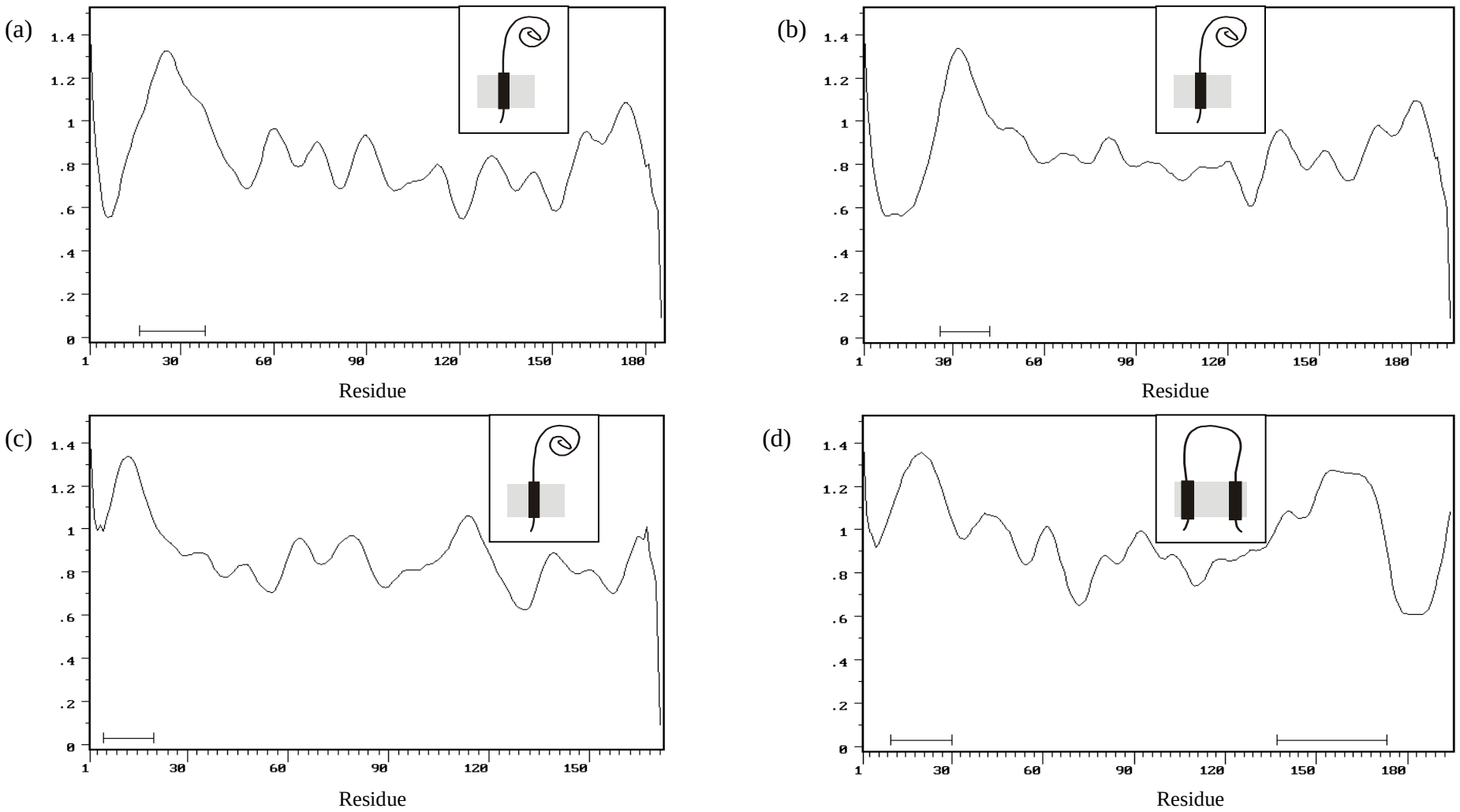

Figure 18. Hydrophobicity plots and topological models for type I signal peptidases of *B. amyloliquefaciens*. (a) SipS(*Ba*), (b) SipT(*Ba*), (c) SipV(*Ba*), (d) SipW(*Ba*). The transmembrane helices of SPases of *B. amyloliquefaciens* were detected using RAOARGOS program (PCGENE). The integral membrane domains were predicted according to a conformational preference parameter, called the membrane buried helix parameter. For a particular aa, this parameter is defined as the ratio of the composition of that aa in transmembrane helices region to its composition in all sequence region of the protein. The transmembrane are at least 12 aa long at a predefined base-line value (1.05) and which are associated with a peak above a cut-off value (minimal peak height is 1.13).

3.2. Function of B. amyloliquefaciens type I SPases in the LepBts mutant E.coli IT41 strain

3.2.1. Plasmid construction for expression and processing with Sip(Ba)s in E. coli

The *ompA* gene was PCR-amplified from the plasmid pJM20 (Meens, *et al*., 1993) using primers omp1 and omp2 with the artificial restriction sites *Hind*III and *Eco*RI, respectively. The PCR *ompA* fragment was then cloned into the pGEM-T vector. The correctness of the resulting construct was verified by mapping with restriction enzymes as well as by sequencing the cloned insert. The plasmid was named pGEM-O. In parallel, the genes encoding for SipS, SipT, SipV, SipW of *B. amyloliquefaciens* and LepB of *E. coli* were PCR-amplified from genomic DNA of the *B. amyloliquefaciens* strain ATCC 23843 and *E. coli* strain XL1-blue, respectively. The primers used for PCR amplification of *sip* genes or the *lepB* gene are listed in Table 3 (see Materials & Methods). The PCR fragments were cloned into the pQE16 vector in the same reading frame with the 6 histidine codons in order to fuse the His-tag to the COOH-end of each protein. After checking by restricted digestion and sequencing, the success of the cloning and the presence of *sip* or the *lepB* genes in sense orientation was confirmed. The plasmids were named pQS, pQT, pQV, pQW, and pQB (Table 2).

The *ompA* gene was extracted from the pGEM-O plasmid after *Hin*dIII and *Eco*RI restriction. The DNA-fragment containing the *sipS, sipT, sipV, sipW,* or *lepB* genes was isolated from pQS, pQT, pQV, pQW, or pQB plasmids after *Hin*dIII digestion and subsequently blunted and restricted by *Mun*I. The pDG148 vector was first cut by *Sal*I, and after blunt end treatment by *Klenow* was subsequently digested by *Hin*dIII. The *ompA* gene was together with each of the *sip* gene or with *lepB* DNA fragment cloned into the treated pDG148 vector, resulting in pOpacSh, pOpacTh, pOpacVh, pOpacWh, or pOpacBh, respectively. The *ompA* gene was also cloned into pDG148 to create the pOpac plasmid for a *sip* gene-less control (see Figure 19).

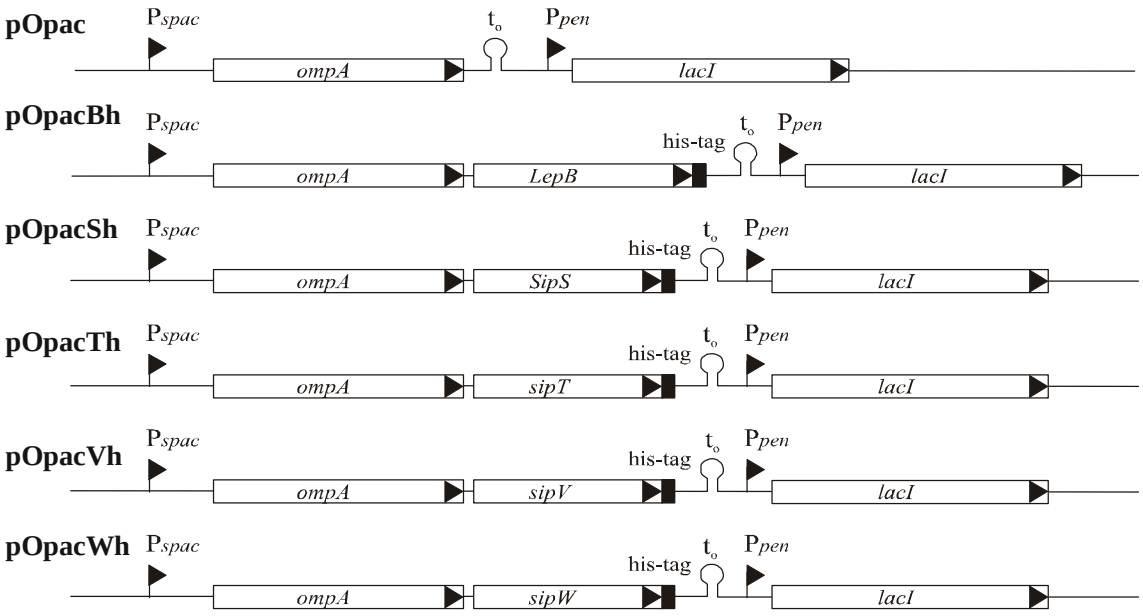

Figure 19. Construction of plasmids. Cloning of the *ompA* gene (from pGEM-O) in pDG148 resulted in the formation of pOpac; cloning of *ompA* together with *lepB* or *sipS* or *sipT* or *sipV* or *sipW* in pDG148 resulted in pOpacBh, pOpacSh, pOpacTh, pOpacVh, pOpacWh, respectively.

3.2.2. Complementation of the E. coli LepBts mutant IT41

Contrary to *B. subtilis* and *B. amyloliquefaciens*, *E. coli* has only one type I SPase, LepB (Blattner, *et al*., 1997). The enzyme is essential for cell viability in *E. coli* (Date, 1983). Although many mutations in the *lepB* gene of *E. coli* yield an inviable phenotype, an amber mutation in strain IT41 resulted in a temperature-sensitive (*ts*) phenotype, with normal growth at 30°C (Inada, *et al*., 1989). As shown previously, the mutant IT41 resumes growth at high temperature after transformation with *lepB*-like genes of Gram-negative as well as Gram-positive bacterial origin, i.e. of *Bradyrhizobium japonicum* (Müller, *et al*., 1995), *Staphylococcus aureus* (Cregg, *et al*., 1996), *Streptococcus pneumoniae* (Zhang, *et al*., 1997), and *Streptomyces lividans* TK21 (Parro, *et al*., 1999).

Therefore, the plasmids pOpacSh, pOpacTh, pOpacVh, pOpacWh, and pOpacBh (carrying the above described SPase genes of *B. amyloliquefaciens*) were transformed into this *E. coli* IT41 strain and grown at a permissive temperature (30°C). For additional control, we also used the plasmids pTK100 and pTK99 which carries the *sipS* gene of *Bradyrhizobium japonicum* in sense or anti-sense orientation, respectively (Müller, *et al*., 1995). Colony growth at 42°C was only observed for transformants containing the plasmids pOpacBh, pOpacSh or pK100, not with the plasmids pOpacTh, pOpacVh, pOpacWh or pOpac and

pKT99, respectively (data not shown). Three transformed colonies of each plasmid were chosen for testing growth complementation. The test bacteria were overnight grown at 30°C, then diluted 1:100 and shifted to grow at 42°C. Growth was followed by spectrophotometer measurement. The result in liquid cultures at 42°C was similar, i.e. transformants with the plasmids pOpacBh, pOpacSh or pK100 grew faster than transformants with the plasmids pOpacTh, pOpacVh, pOpacWh, pOpac or pKT99, respectively (Figure 20). Expression of Sip*(Ba)*(s) as well as of the LepB protein was monitored by immuno-detection using His-tag antibodies (Figure 21B). The expression level of all Sip*(Ba)* shown to be equal with and without IPTG induction, where induction resulted in the increase of expression of Sip*(Ba)*(s). Nevertheless, only SipS showed growth complementation with LepB*ts* and induction of other Sip*(Ba)* proteins by IPTG did not improve, but significantly slow down the growth of the latter cultures (data not shown).

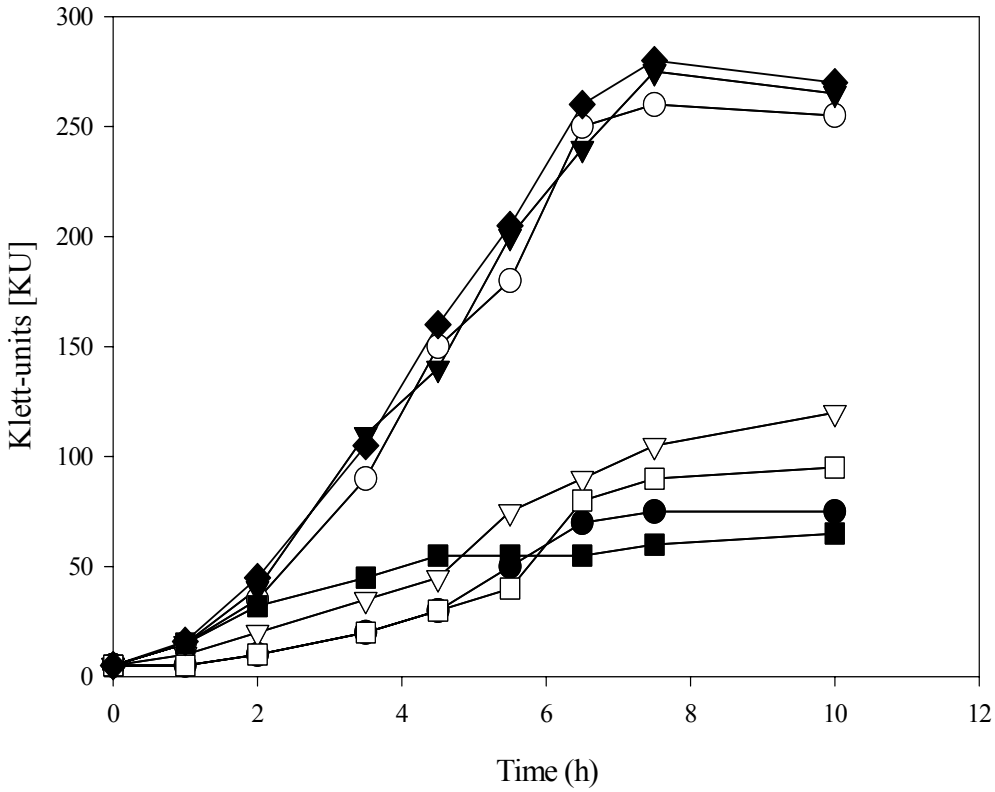

Figure 20. **Growth of the *E. coli* LepB*ts* transformants. Growth of *E.coli* IT41 transformants containing the following plasmids**: -O-, pTK100; -♦-, pOpacBh; - ▼-, pOpacSh; -●-, pTK99; -∇-, pOpacTh; -■-, pOpacVh; - □-, pOpacWh. The cultures were grown in TBY medium at 45°C.

3.2.3. Processing studies with pre-OmpA in LepBts E. coli IT41

Processing of the pre-OmpA precursor in the transformed LepB*ts E. coli* was done in order to verify in more detail the complementation of LepB*ts* after Sip*(Ba)* protein expressions. The *E. coli* IT41 strains, which carried the plasmids pOpac, or pOpacSh, or pOpacTh, or pOpacVh, or pOpacWh, or pOpacBh, were used for the pulse chase radioactive ^{35}S labelling at either 30°C or 42°C. After immunoprecipitation by OmpA antiserum, the samples were separated by SDS-PAGE and autoradiograms were obtained by the Phospho-Imager system (see Material & Methods). The results are shown in Figure 21A. While the processing of pre-OmpA was enhanced in cells with expression of either *B. amyloliquefaciens* SipS*(Ba)*, SipT*(Ba)*, or of LepB, the two other *B. amyloliquefaciens* Sip proteins SipV*(Ba)* and SipW*(Ba)* did not significantly improve processing of pre-OmpA compared to the mutant control culture. The two latter cases could be explained either by the limited activities of the enzymes or by the improper membrane integration of the two proteins, resulting in degradation as shown on the Western blot (see Figure 21B) for Sip(*Ba*) protein expression using His-tag antiserum. No such degradation forms were visible with SipS(*Ba*) and SipT(*Ba*) in *E. coli*.

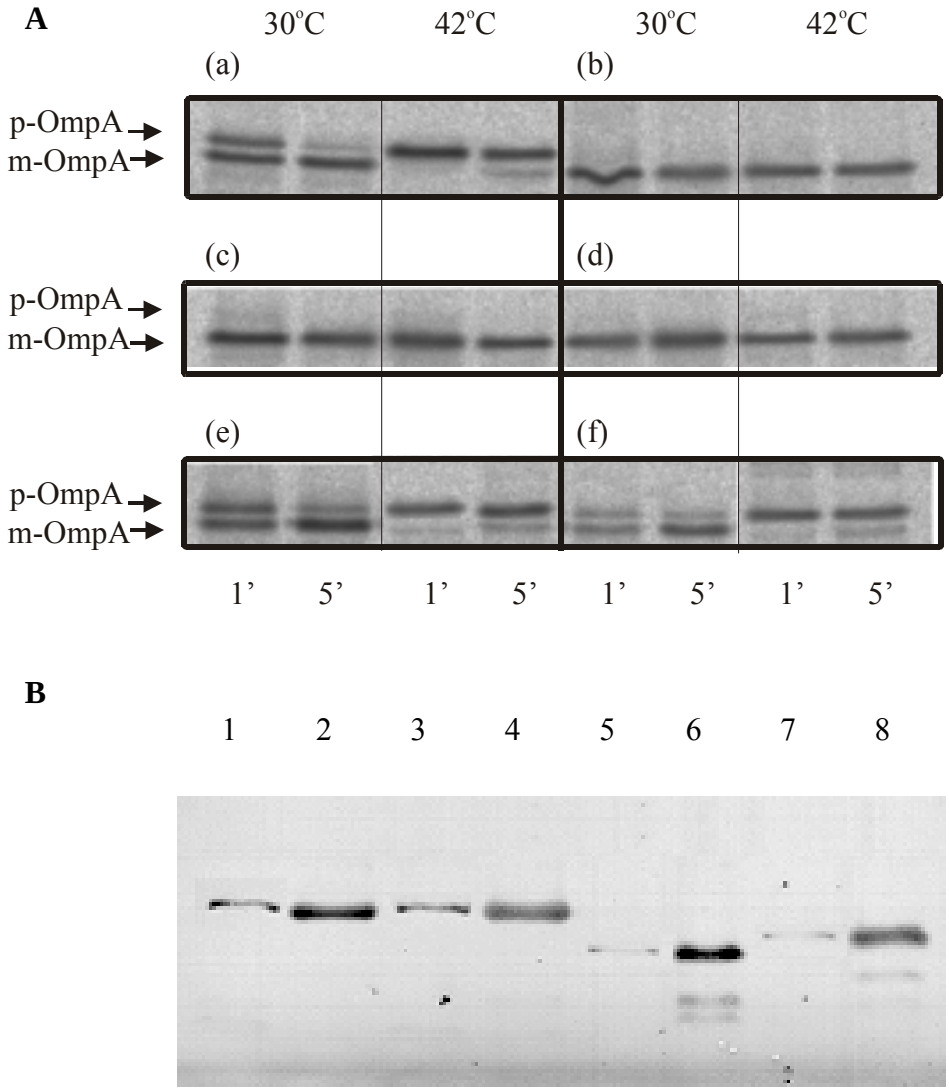

Figure 21. Processing of pre-OmpA in *E. coli* IT41. Processing of pre-OmpA in transformants and parent *E. coli* IT41 at 30°C and 42°C, A. Samples are from *E. coli* IT41 transformants containing the plasmids: (a), pOpac; (b), pOpacBh; (c), pOpacSh; (d), pOpacTh; (e), pOpacVh or (f), pOpacWh, respectively. The samples were collected after S^{35} labelling for 1 or 5 minutes (1’ or 5’). Expression of Sip(ba)s was detected by a Western blot using the penta-his antibody, B. Samples are from *E. coli* IT41 transformants containing the plasmids: lanes 1&2, pOpacSh; lanes 3&4, pOpacTh; lanes 5&6, pOpacVh; lanes 7&8, pOpacWh. With or without induction by IPTG, lanes 2, 4, 6, 8 or lanes 1, 3, 5, 7.

3.2.4. Study SipT – LepB fusions

The amino acid sequence alignment of *B. amyloliquefaciens* type I signal peptidase SipT*(Ba)* and *E. coli* LepB as shown in Figure 22a verified the separation of transmembrane and catalytic regions. The *E. coli* LepB with 323 amino acid has two transmembrane segment, while SipT*(Ba)* with 193 amino acid contains only one (Figure 22a). These structural differences and the non-functionality of SipT*(Ba)* in *E.coli* inclined us to ask whether the intermolecular exchange of the transmembrane domains would mediate and therefore apparently cause functionality/non-fuctionality between these SPases. The SipT*(Ba)* and LepB had been exchanged their N-terminal transmembrane domains in order to construct hybrid-proteins between LepB and SipT*(Ba)*. In a first step the DNA region encoding transmembrane or catalytic domains of *sipT(Ba)* or *LepB* gene were PCR-amplified using "hybrid" primers (Table 3), the PCR products were then purified. The PCR product corresponding to transmembrane domain of LepB or SipT(*Ba*) was mixed with the PCR fragment of catalytic domain of SipT(*Ba*) or LepB, respectively. The final PCR amplification step was done using the 5'-primer of *lepB* and the 3'-primer of *sipT(Ba)* or 5'-primer of *sipT(Ba)* and the 3'-primer of *lepB* to achieve the *sipB-T* (encoding a fusion protein with catalytic region of SipT and transmembrane region of LepB) or *lepT-B* (encoding a fusion protein with catalytic region of LepB and transmembrane region of SipT). The PCR-DNA fragments corresponding to hybrid genes *sipB-T* and *lepT-B* were cloned into vector pDG148 in a similar way with genes encoding Sip*(Ba)*(s) and LepB as was described above (see section 3.2.1). The pDG148 vector which carried the *ompA* gene in a tandem with hybrid genes *sipB-T* or *lepT-B* were named pOpacBTh or pOpacTBh (Table 2), respectively.

The plasmids pOpacTBh and pOacBTh were transformed into *E. coli* IT41. Three transformed colonies of each plasmid were chosen to examine growth at 42^{o}C. The plasmids pOpacTh and pOpacBh which carried *sipT(Ba)* and *lepB* genes, were used as controls. The results with "hybrid SPase" pOpacTBh similar to pOpacBh plasmids, however, exhibited much faster growth than the ones carrying "hybrid SPase" pOpacBT, pOpacTh or pOpac (Figure 24). This data indicated that replace of transmembrane domain of SipT(*Ba*) by transmembrane domain of LepB did not improve the complementation activity of SipT(*Ba*) in *E. coli* mutant strain. On other hand, exchange of transmembrane domain of LepB with the one of SipT(*Ba*) did not disturb the functional complementation of LepB protein. This data suggested that the exchange of transmembrane domains of LepB and SipT(*Ba*) did not change their growth complementation properties in the *E. coli* LepB*ts* mutant.

(a)

```
                    A1                                                                  A2
SipT(Ba) ------------------------------------MTEEQKPTSEKSVKRKSN---TYWEWGKAIIIA  30
LepB(Ec) MANMFALILVIATLVTGILWCVDKFFFAPKRRERQAAAQAAAGDSLDKATLKKVAPKPGWLETGASVFPV  70
                                              : :*:. :*      : * * ::: .

                                  B                                   C
SipT(Ba) VALALLIRHFLFEPYLVEGSSMYPTLHDGERLFVNK-----------SVNYIGEIERGDIVIING-DTS  87
LepB(Ec) LAIVLIVRSFIYEPFQIPSGSMMPTLLIGDFILVEKFAYGIKDPIYQKTLIETGHPKRGDIVVFKYPEDP 140
         :*:.*::* *::**: : ..** ***  *: ::*:*            ::   *. :*****:::  : .

              D
SipT(Ba) KVHYVKRLIGKPG---------------------------------ETVEMKNDTLYINGKKIAEPYL 122
LepB(Ec) KLDYIKRAVGLPGDKVTYDPVSKELTIQPGCSSGQACENALPVTYSNVEPSDFVQTFSRRNGGEATSGFF 210
         *:.*:** :* **                                 *. :: :     ** : :. ::

                                                                          E
SipT(Ba) ASNKKEAKKLGVNLTG-----------------------------DFGPVKVPKGKYFVMGDNRLNSM 161
LepB(Ec) EVPKNETKENGIRLSERKETLGDVTHRILTVPIAQDQVGMYYQQPGQQLATWIVPPGQYFMMGDNRDNSA 280
            *:*:*: *:.*:                              ::..  ** *:**:***** **

SipT(Ba) DSRNGLGLIAENRIVGTSKFVFFPFHD--------MRQTK----- 193
LepB(Ec) DSR-YWGFVPEANLVGRATAIWMSFDKQEGEWPTGLRLSRIGGIH 324
         ***   *::.* .:** :. :::.*..        :* ::
```

(b)

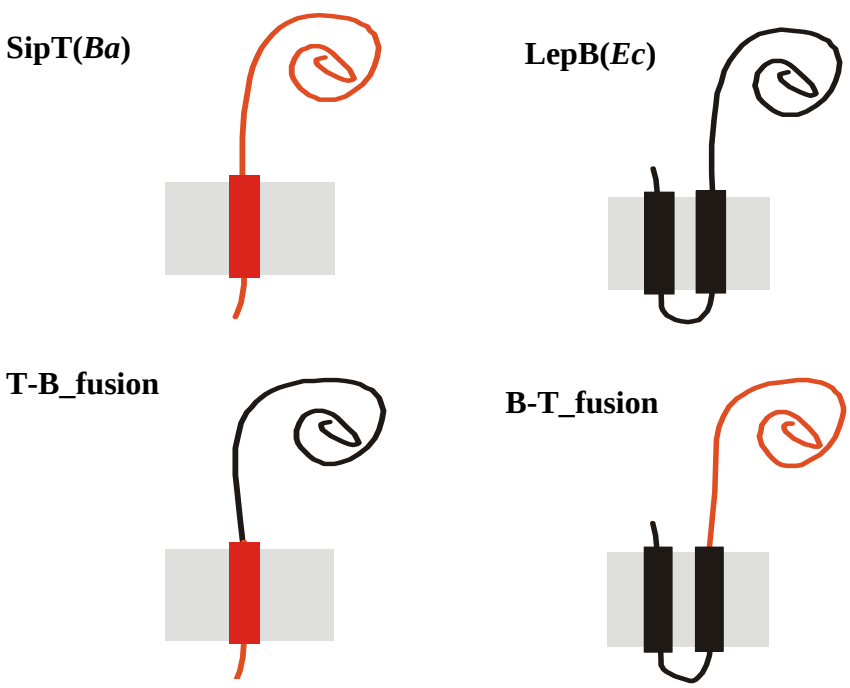

Figure 22. **Amino acid sequence alignment and topological model of *B. amyloliquefaciens* SipT*(Ba)*, *E. coli* LepB and two hybrid SPases SipB-T and LepT-B**. Alignment of SipT*(Ba)* and *E. coli* LepB amino acid sequences, (a). The transmembrane domains A1, A2 and conserved domains B, C, D, E were shaded in grey. Serine and Lysine residues, which are involved in active site, were in black. The identical residues and similar residues were marked by asterisks and (**:**) or (**.**). Topological model of SipT*(Ba)*, LepB, and two hybrids, (b). Sip*(Ba)* and LepB portion are given in red and black, while the red or black parts in the hybrids are represented their origins.

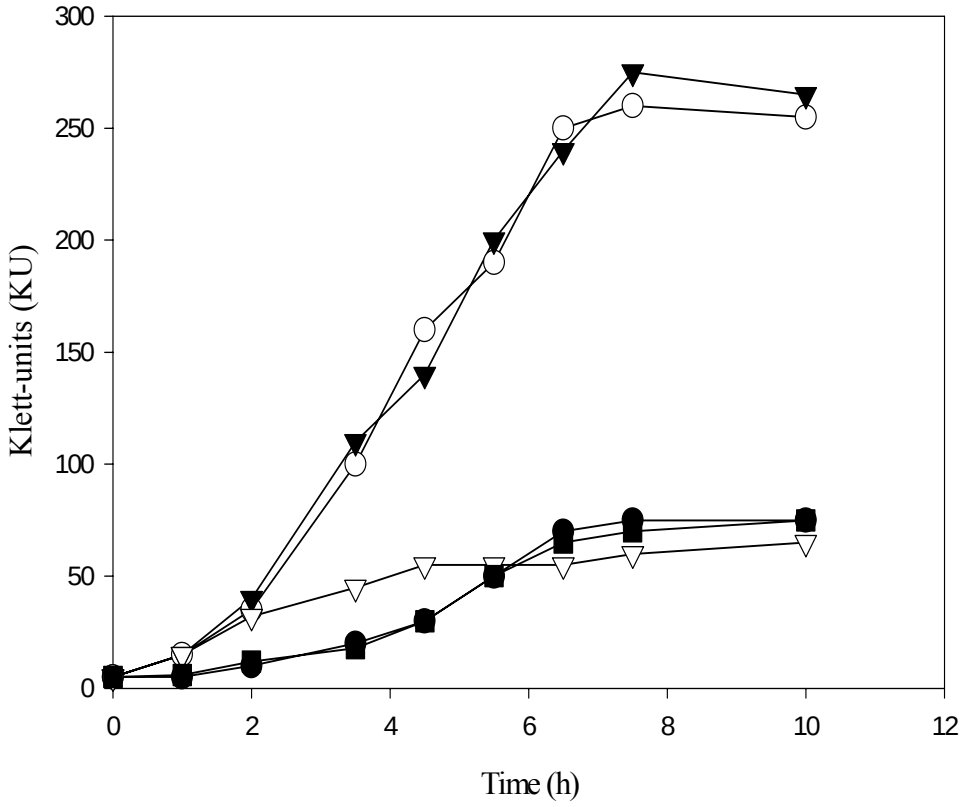

Figure 23. **Growth of the *E. coli* LepB*ts* after transformation of plasmids carrying *lepB, sipT, sipB-T* and *lepT-B* hybrid genes**. Growth of *E.coli* IT41 transformants containing the following plasmids: -O-, pOpacBh; -●-, pOpacTh; -∇-, pOpacB-Th; -▼-, pOpacT-Bh; -■-, pOpac. The cultures were grown in TBY medium at 42°C.

3.3. Phenotype of sip disruption mutants

3.3.1. Construction sip disruption mutants

In order to study phenotypic deficiencies of the *sip(Ba)* genes of *B. amyloliquefaciens*, four disruption mutants were constructed. Since *B. amyloliquefaciens* did not develop natural transformation competence, we used the thermo-sensitive vector pE194 and the integrational gene disruption procedure (Youngman, 1990) in order to construct mutants of each *sip(Ba)* gene. The plasmids pEAS*, pEAT*, pEAV* and pEAW* with pUC18 and respective core DNA of each *sip(Ba)* gene were transformed and maintained in *B. amyloliquefaciens* GBA12 at 30°C. Integrative recombination at homologous chromosomal *sip* gene regions was forced after growth at 45°C in the presence of erythromycin (3µg/ml) in liquid cultures (Hofemeister, *et al*., 1983). After several rounds of selection at 45°C cultures were plated. Colonies growing on selective TBY agar at 45°C were proven to be plasmid-free and to contain the disruption within the desired *sip(Ba)* gene. The mutants were confirmed by southern hybridization and PCR amplification using genomic DNA of potent mutant colonies. Hence, the following mutants GBA13 (*sipS*::pEAS*), GBA14 (*sipT*::pEAT*), GBA15 (*sipV*::pEAV*) and GBA16 (*sipW*::pEAW*) contain about 6.3 kb-DNA components of the vectors pUC18 and pE194 which basically disrupted the target to for a mutant front- and back-portion of the *sip(Ba)* genes. Since only internal DNA lacking 5'- as well as 3'-terminal portions of the respective gene was used, the front portion could theoretically become translated and form a truncated protein, while the back portion should even not be transcribed (see Figure 24). The mutants GBA13 (*sipS*::pEAS*), GBA14 (*sipT*::pEAT*), GBA15 (*sipV*::pEAV*) and GBA16 (*sipW*::pEAW*) were tested for changed properties with respect to growth, protein secretion, sporulation, autolysis and cell motility.

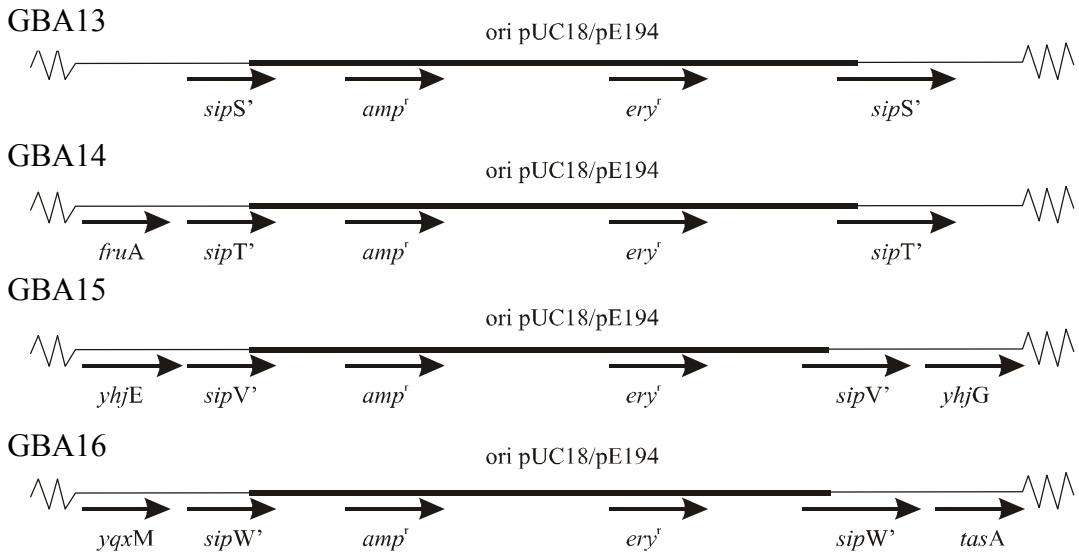

Figure 24. **Schematic presentation of the *sipS, sipT, sipV* and *sipW* regions on the chromosomes *of B. amyloliquefaciens*** GBA13 (*sipS*::pEAS*), GBA14 (*sipT*::pEAT*), GBA15 (*sipV*::pEAV*) and GBA16 (*sipW*::pEAW*).

3.3.2. Growth and protein secretion

The growth of *sip(Ba)* mutant and the parental strains were compared at either 37 or 45 °C in TBY (Figure 25) and MSM medium (data not shown). Under each condition, strain GBA15 (*sipV*::pEAV*) exhibited exceptionally slower growth rate, compared to the wild type and the other *sip(Ba)* mutant strains. The yields of protein secretion after 24 h of growth in TBY medium were compared as total extra cellular protein as well as after SDS-PAGE of extracellular proteins, which were TCA precipitated from supernatant of respective mutant cultures. Although the total protein of GBA13 (*sipS*::pEAS*) and GBA14 (*sipT*::pEAT*) mutant cultures was about 30 percent lower, compared to wild type cultures, the resolution of extracellular proteins after SDS PAGE was not sufficient to demonstrate more than vague differences in the protein pattern of these *sip(Ba)* mutants (data not shown).

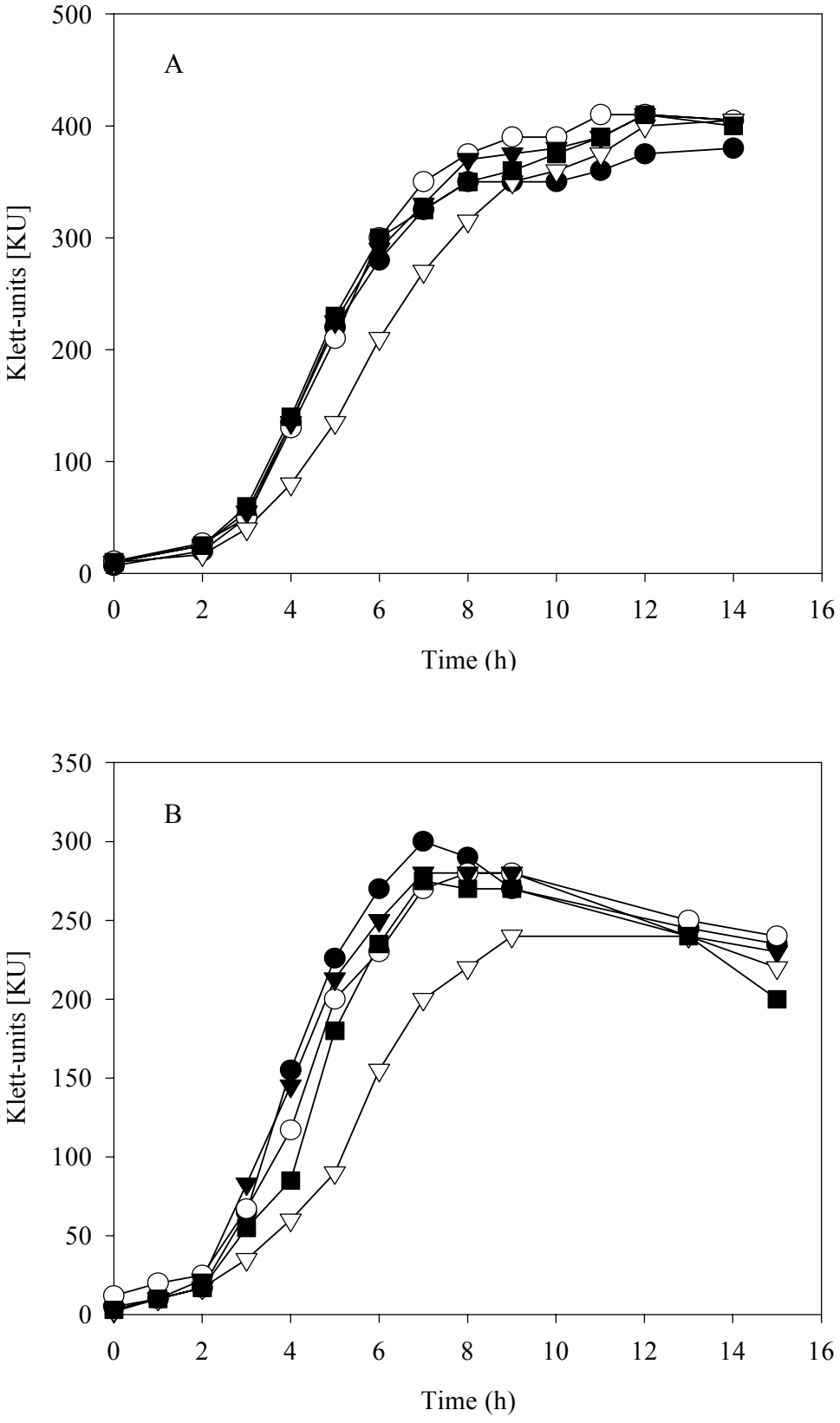

Figure 25. **Growth of *B. amyloliquefaciens sip(Ba)* mutants and parental strains** in TBY medium at 37°C (A) and 45°C (B). -●-, ALKO 2718; -▼-, GBA13 (*sipS* mutant); -■-, GBA14 (*sipT* mutant); -o-, GBA15 (*sipV* mutant); -∇-, GBA16 (*sipW* mutant).

3.3.3. Sporulation

In order to study the effect of *sip(Ba)* gene disruption on sporulation, strains were grown for 8 to 48 h at 37°C in Schaeffer's sporulation medium (SSM) and respective samples were heat-treated (10 min, 80°C). Under these conditions, wild type GBA12 cultures after 24 and 48 h contained about 25 and 43 percent of spores, respectively. No significant differences were observed for *sipS(Ba), sipV(Ba)* and *sipW(Ba)* mutant cultures, whereas in case of the *sipT(Ba)* mutant strain GBA14 spores were rarely found at frequencies of about 0.001 percent. It was questionable whether or not *sipT*-gene disruption only frequently or strictly correlated with sporulation deficiency. These disruption experiment were several times repeated and gene disruption after plasmid pEAT* integration was always found to correlate with reduced formation of heat resistant spores (less than 0.1 percent) (see Figure 26). This deficiency did not affected the growth character of mutant cultures in TBY, but in SSM medium the mutant cultures rapidly lysed after 8 h of growth (data not shown). The few sporulating cells from *sipT*::pE* (GBA14) mutant cultures at this incubation period exhibited stage III prespore-like structures which obviously differed from pre-spore images of sporulating wild type cells (Figure 26) and similar to structure found in cells which were blocked in stage III of sporulation (A. Driks, personal communication). Restoration of this apparent sporulation deficiency of GBA14 mutant cells was tested after wild type SipT*(Ba)* expression. After transformation of plasmid pSpacT expression of SipT*(Ba)* was monitored by immuno-detection after Western blotting experiments (data not shown). Neither induced nor non-induced SipT*(Ba)* expression, however, yielded in enhanced sporulation frequencies of GBA14(pSpacT) cultures. Moreover, GBA14 cultures grown in SSM medium exhibited increased spore frequencies as well as enhanced frequencies of Em sensitive colonies (data not shown). This correlation with growth without antibiotic selection strongly indicated (precise) excision of the mutant insert to reverse the sporulation deficiency. The letter was also supported by the fact that progenies of spores from GBA14 culture were about 80 percent Em sensitive, i.e. apparently lost the mutant insert.

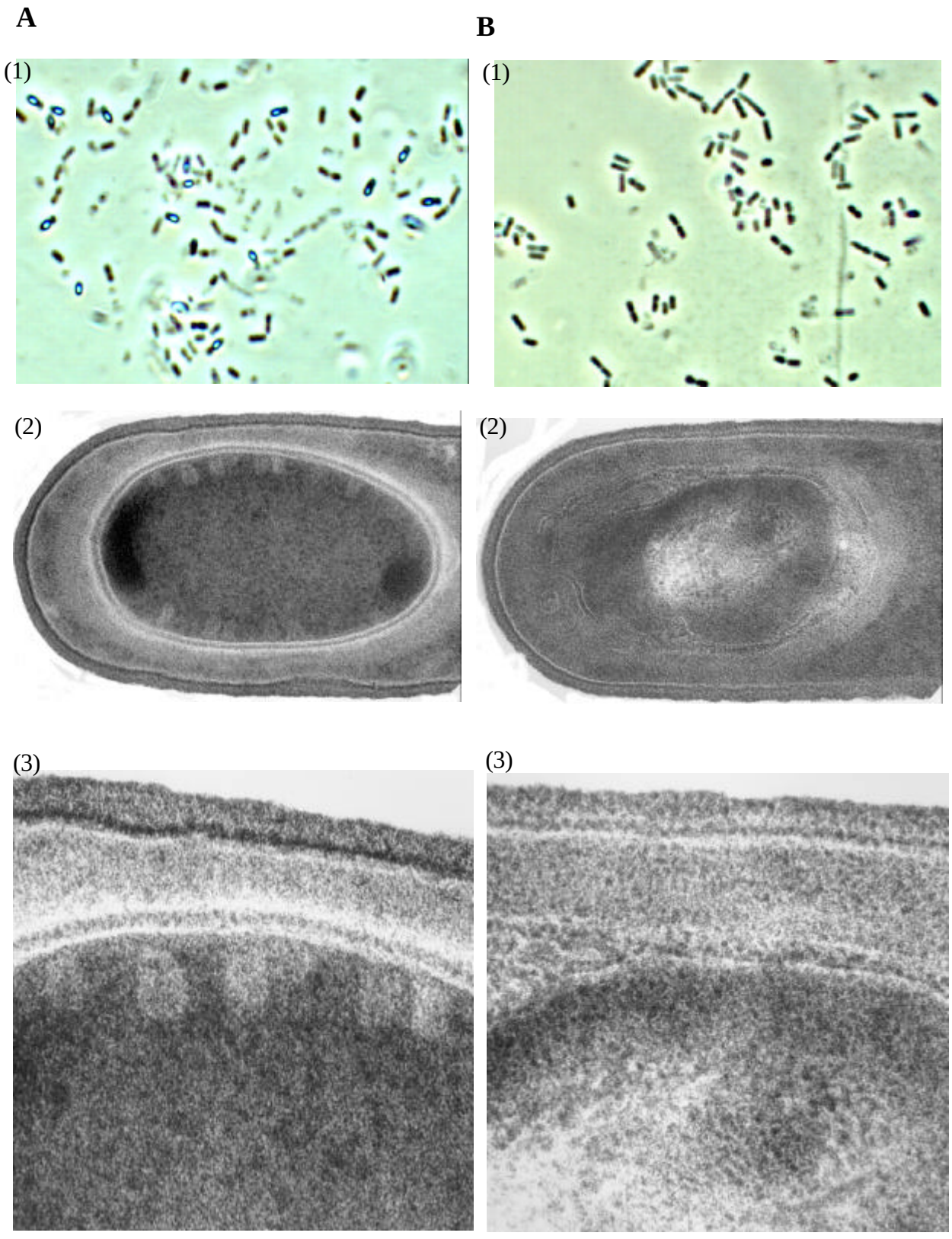

Figure26. **Microscopypictures of *B.amyloliquefaciens* wild typeand *sipT* disruptionmutant strains**. *Bacillus* strainsweregrowninSSMmediumfor 8h.Cellswere collectedandprepared asdescribedinMaterials& Methods. A: wildtype;B: *sipT* disruptionmutant;(1)phasecontrastmicroscopy picture;(2)and (3),electromicroscopy pictures ofpartofthecellwithspore,andsectionofsporemembrane,cortexorprecoatstructure..

3.3.4. Cell autolysis and cell motility

Microscopical inspection of *sip(Ba)* mutant cultures indicated that cells of the mutant strain GBA15 (*sipV::*pEAV*) in TBY grew in filament form, while cells of the other mutants like the wild type culture were rod-shaped. This observation indicated a defect of the *sipV* mutant in either cell division or cell wall formation. We therefore compared the *sip(Ba)* mutants for cell autolysis, cell motility and autolysin activities using CWBPs (cell wall bound proteins) after SDS-PAGE.

Cellular lysis of mutant and parental strains was measured by adding sodium azide (at the final concentration of 0.05 M) to exponentially growing cells and following lysis spectrophotometrically (Figure 28). Cultures from the mutant strain GBA15 were found to contain intact cells, even after 100 min of exponential growth, while under the same conditions wild type GBA12 cultures progressively lysed from OD_{600} 0.6 down to zero. The lysis of other *sip(Ba)* mutants was only slightly affected (Figure 27). Changed cell autolysis was predicted to correlate with changed cell motility (Blackman, *et al*., 1998). The swarming mobility of colonies of wild type as well as *sip(Ba)* mutant cultures were therefore also compared after plating on soft agar and growth at 25 or 37°C. The results were expressed as the percentage colony diameter relative to the parental strain. The mutant GBA15 (*sipV::*pEAV*) colonies in the average had the halo diameters of about 49 percent compared to the diameter of wild type, while other mutant colonies had the same, except that of GBA16 (*sipW::*pEAW*), which were reduced to about 70 percent (Table 4).

In the further experiment, the CWBP pattern and autolysin activities of vegetative growing cells of sip mutants was compared to the wild type by SDS-PAGE and enzymographically analysed for autolysin activities after renature of SDS-PAGE (Figure 28). The CWBP pattern of mutant GBA15 (*sipV::*pEAV*) cells was indeed found to differ to the wild type with respect to the presence and the relative amount of protein as well as autolysin activity bands. At least one (about 35kDa) out of the five major autolysin activity bands were indicated to appear in wild type cells, which run in a double band was apparently missing in the CWBP fraction of mutant GBA15 (Figure 28). Other mutants did not show any significant changes neither in CWBPs nor in the autolysin profile compared to the wild type (data not shown).

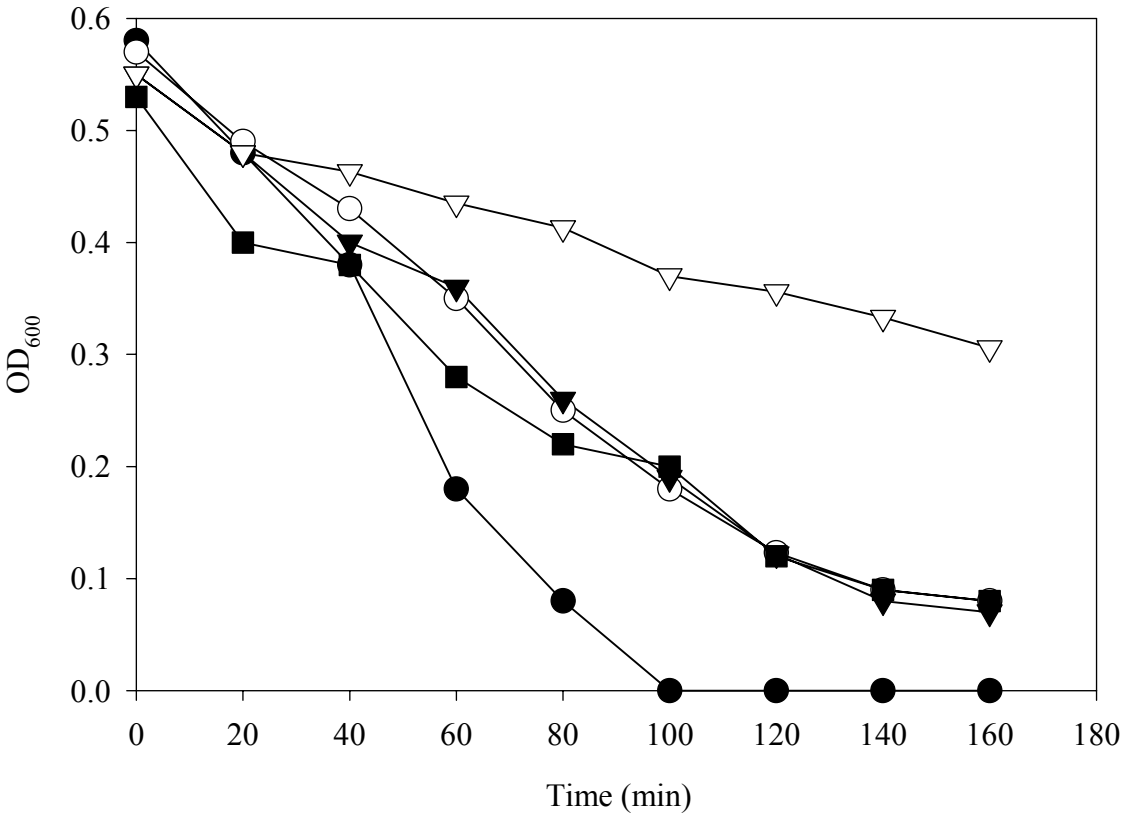

Figure 27. **Cell autolysis of *B. amyloliquefaciens* of *sip(Ba)* mutant and parental strains**. Sodium azide (0.05 M) was added to exponential-phase TBY-cultures (OD_{600} = 0.5-0.6). Cell lysis was followed spectrophotometrically at 600 nm. -●-, GBA 12/ wild type; -O-, GBA 13(*sipS*::pEAS*); -▼-, GBA 14 (*sipT*::pEAT*); -∇-, GBA 15 (*sipV*::pEAV*); -■-, GBA16 (*sipW*::pEAW*).

Table 4. Motility assay of *B. amyloliquefaciens* wild-type and *sip* mutants

Strain	*Swarm diameter (%)*			
	MSM, 37°C	MSM, 25°C	TBY, 37°C	TBY, 25°C
GBA13 (*sipS* mutant)	100	110	97.5	99
GBA14 (*sipT* mutant)	105	98	103	110
GBA15 (*sipV* mutant)	**49**	**54**	**50**	**61**
GBA16 (*sipW* mutant)	70	65	75	70

* Swarm diameters were measured on nutrient agar (TBY) or minimal agar (MSM) plates at 37 or 25°C. Samples (1 µl) from overnight (37°C) liquid cultures were spotted onto swarm plates (0.3% agar) and incubated at 37°C (TBY agar for 14 - 16 h, MSM agar for 32 - 35 h) or 25°C (TBY agar for 28 - 35 h, MSM agar for 55 - 60 h). Results are shown as a percentage of the wild-type strain (ALKO 2718).

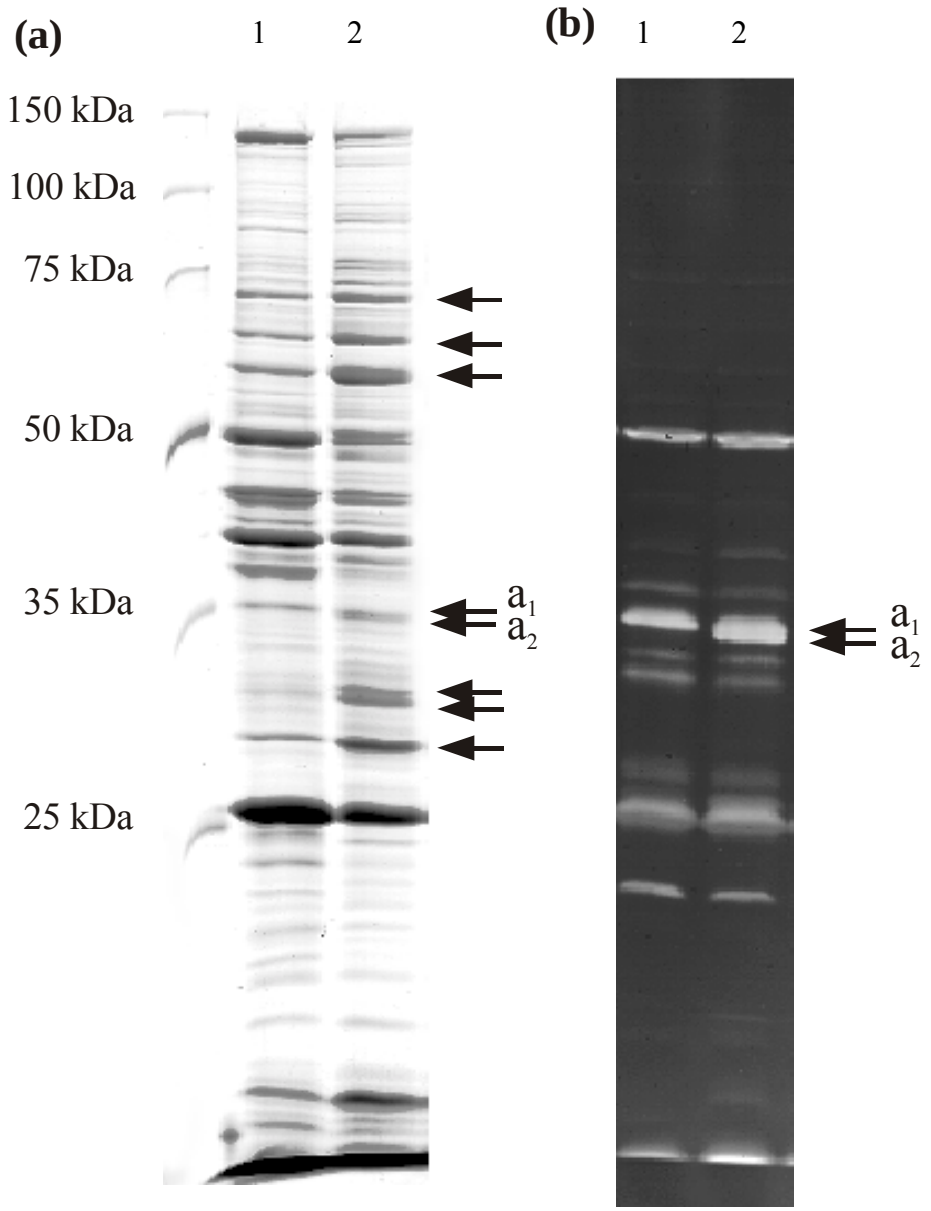

Figure 28. **Cell wall protein and autolysin activities pattern of *B. amyloliquefaciens sipV*(*Ba*) mutants and wild type strains**. SDS PAGE separation of the CWBP fraction of *B. amyloliquefaciens* wild type and GBA15 (*sipV*::pEAV*) mutant (a) and enzymography of autolysin activities after renaturation of SDS PAGE gels containing purified *B. amyloliquefaciens* cell wall material as substrate (b). Sample preparation is described in Methods and renaturation of the SDS-PAGE gel was according to Foster (1992). 1, GBA15 (*sipV*::pEAV*); 2, GBA12 (wild type).

3.3.5. Nuclease activities

Occasionally we observed that higher yields of plasmid DNA could be isolated from distinct *sip(Ba)* mutant cultures. The respective *sipS(Ba)* and *sipT(Ba)* gene disruption mutants were therefore suspected to have either changed content of plasmid DNA or reduced DNA degradation due to loss or reduction of nuclease activities. A pronounced zone of nuclease activity was after SDS-PAGE and enzymography found in the area of 30 kDa-proteins of supernatant fractions of wild type and several *sip(Ba)* mutant cultures. This nuclease activities were reduced in the *sipT(Ba)* and nearly lacking in the supernatant of *sipS(Ba)* mutant cultures (Figure 29). In spite of numerous attempts to purify that suspected nuclease, likely due to low protein concentration, it could not be isolated from wild type cultures. Consequently, the identity of the suspected nuclease, even its extracellular localisation, remains questionable.

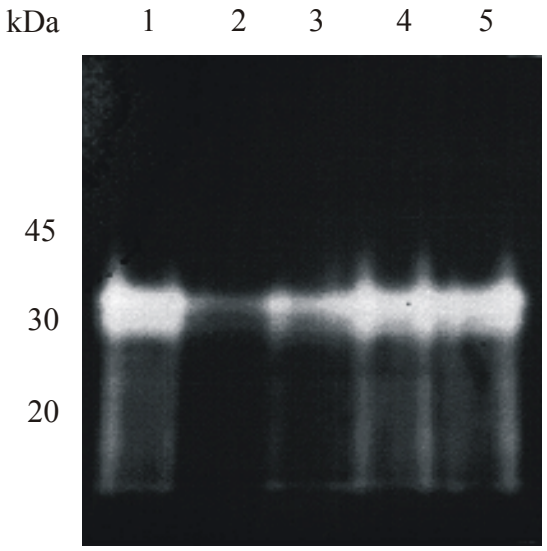

Figure 29. Extracellular nuclease activity of culture supernatant proteins from *B. amyloliquefaciens sip(Ba)* mutants. 50 µl aliquots of cell-free supernatant from 24 h TBY-cultures of the wild type GBA12 (1) mutant strains GBA13 (2), GBA14 (3), GBA 15 (4) and GBA 16 (5) were separated after 12% SDS-PAGE containing calf thymus DNA (10 µg/ml). The gel was renatured and overnight incubated in buffer (40 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 2 mM $MnCl_2$) at 37 °C. The gels were stained with ethidium bromide according to Rosenthal *et al.* (1977).

3.4. Identification of a new B. amyloliquefaciens exported protein

On the basis of the results of earlier studies, which were initiated by V. Hoang & B. Hofemeister, extracellular protein profiles of TBY cultures of a strain carrying the "antisense" *sipT* in a plasmid and the *sipS* gene disruption mutant strain were compared to the wild type *B. amyloliquefaciens* ALKO 2718. Some protein bands were significantly reduced or absent especially in case of *sipT* antisense expression compared to the parental strain (Hoang, *et al*., unpublished data). One of these protein bands had an apparent molecular weight of about 20 kDa and its N-terminal amino acids were HGYIKEPVSRAYMGA. This sequence was initially not found any homologues in protein database, but was lately detected to share significant identities with the mature chitin-binding proteins CHB1 and CHB2 of streptomycetes (Schnellmann, *et al*., 1994; Kolbe, *et al*., 1998). While several chitin-binding proteins were found in streptomycetes, a homologue has not yet been reported from bacilli. This induced us to characterize the protein and the corresponding gene and to study its secretion in relation to the activities of different signal peptidases in *B. amyloliquefaciens*. In additional to this protein, we also searched for a physiological rule, i.e. the presence of chitinolytic system in various *B. amyloliquefaciens* strains. The data (see section 3.4.6 & 3.4.7) indicated *B. amyloliquefaciens* strains did not only possess the newly detected chitin binding protein, but also various moderate chitinase activities.

3.4.1. Cloning and sequence analysis of the chbB gene region

Using total DNA of *B. amyloliquefaciens* ALKO 2718, the initial PCR amplification was performed with the primers A and B designed according to the determined N-terminal amino acids (see first paragraph) from the mature 20 kDa protein (Figure 30). Subsequent steps of the RAGE (rapid amplification of genomic ends) procedure were performed using the primer pairs EF or GH and CD or IK, respectively (Figure 30, Table 3). Restriction fragments of total *B. amyloliquefaciens* genomic DNA (cut with *Hin*dII, *Hin*dIII or *Eco*RI) were found to hybridise with the labelled PCR fragment, which corresponded to those predicted from the restriction map of the amplified PCR product. Finally, as outline in Figure 30 a fragment of about 4.5 kb was generated after PCR cloning and sequencing. This DNA region shown to contain 3 complete and 2 incomplete open reading frames (Figure 30a). The experimentally determined N-terminal amino acid sequence of the unknown 20 kDa protein (see first paragraph) corresponded to the amino acid residues No. 28 – 43 of the protein deduced from *orf3* (with a predicted signal peptide, No. 1 – 27) (Figure 31). The predicted mature 19.8 kDa protein deduced from *orf3* shares 39, 37, and 45 % of identical amino acids with the

previously identified chitin-binding protein CHB1 from *Streptomyces olivaceoviridis* (Schnellmann, *et al*., 1994), CHB2 from *Streptomyces reticuli* (Kolbe, *et al*., 1998), and CBP21 from *Serratia marcescens* (Suzuki *et al*., 1998), respectively. The *B. amyloliquefaciens* gene was named *chbB* (Figure 31), as it was proposed also to have a chitin binding properties. According to homologous search this was the first chitin-binding protein found from a *Bacillus* species.

About 140 nt upstream of *orf3*, a putative (Sigma A) promoter could be deduced, and palindromic sequences downstream of the gene are likely to represent transcription terminators. Three motifs, TGAAAGCGTTTTCA (box 1), TGGAGGCGCTTTCT (box 2) and AGAGCCCGTCAGCA (box 3) were found in the vicinity of the proposed promoter, as well as about 50 and 90 nt downstream of the translation initiation codon ATG within the gene (Figure 30c). These motifs match the consensus WGNAASCGNWWNCA of the DNA binding (*cre*) site for the catabolite control protein CcpA (Hueck, *et al*., 1994). These boxes share different conservative degrees with the consensus structure, with similarity decreasing gradually from box 1 to box 3 (Figure 30d).

The proteins deduced from additional *orf*s (Figure 30a) share a high degree of similarity with proteins of unknown functions, i.e. incomplete Orf1 to YnaE, Orf4 to YvgO or incomplete Orf5 to YobB, the encoding genes of which are scattered in the chromosome of *B. subtilis* 168 (Kunst, *et al*., 1997). The protein deduced from the *orf*2 (Figure 30a) is similar to N5,N10-methylene-tetrahydromethanopterin reductase from *Staphylococcus aureus* (accession No. U96107). It remains to be shown whether the *B. amyloliquefaciens* Orf2 protein corresponds to a functional reductase catalyzing the reversible reduction of N5,N10-methylenetetrahydromethanopterin with the reduced F420 as electron donor, a characteristic feature of methanogenic archaebacteria (Vaupel & Thauer, 1995), or whether it corresponds to another type of F420-dependent reductase.

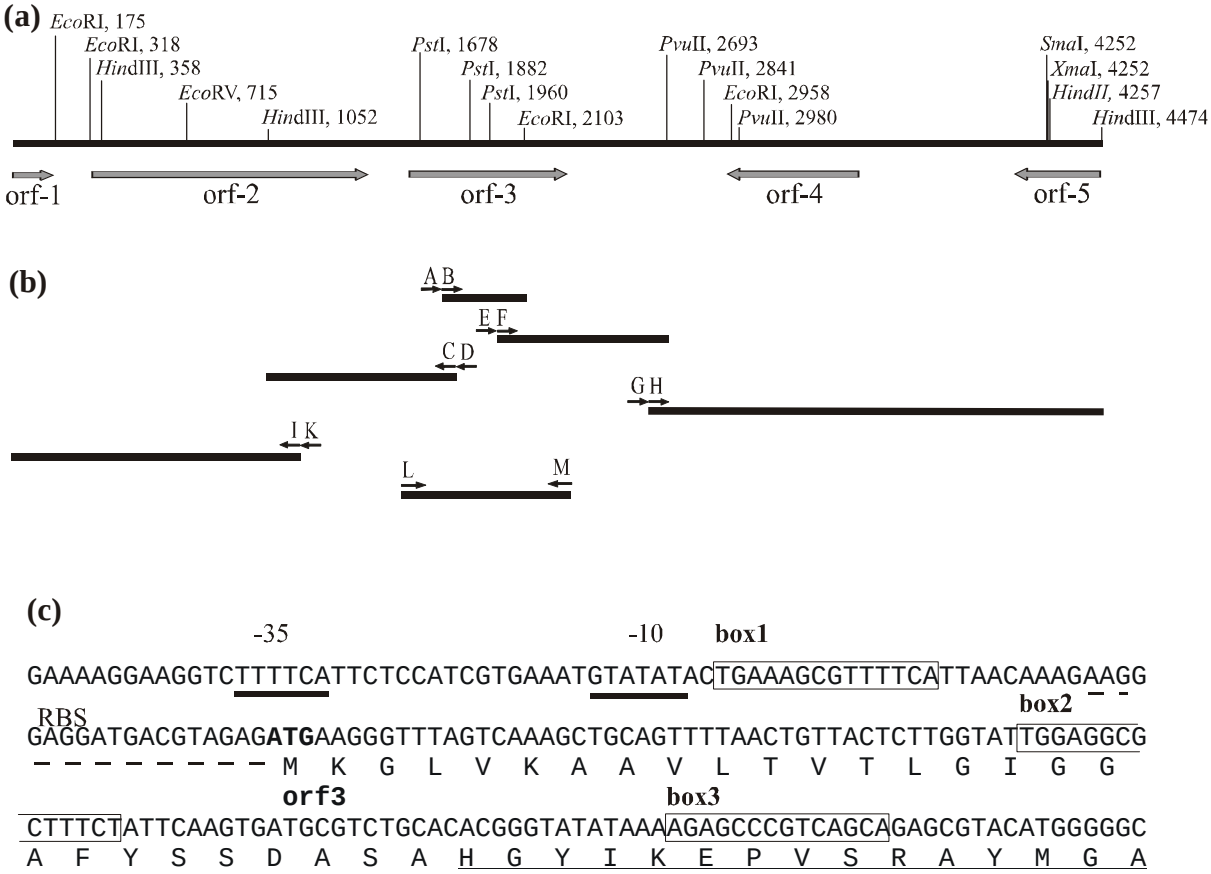

Figure 30. Cloning strategy and map of the cloned DNA region. The restriction map and the orientation of the *orf*s are indicated. *orf*3 encodes the ChbB protein (a). Sets of PCR primers (see Table 1) were used for stepwise PCR cloning of stretches (black lines) comprising a 4.5 kb chromosomal DNA region (b). The predicted promoter, ribosome binding site (RBS), translation initiation codon as well as the motifs (box 1, 2, and 3) of *orf3* matching the Cre binding site for the catabolite control protein CcpA are indicated (c). The amino acids, which matches with the sequence obtaining by N-terminal sequencing, are underlined. The sequence has been deposited under the accession number AF181997. Three possible Cre motifs (box 1, 2, and 3) were aligned with the Cre consensus sequence (Hueck, *et al*., 1994) (d), the conserved nucleotides were shaded in black.

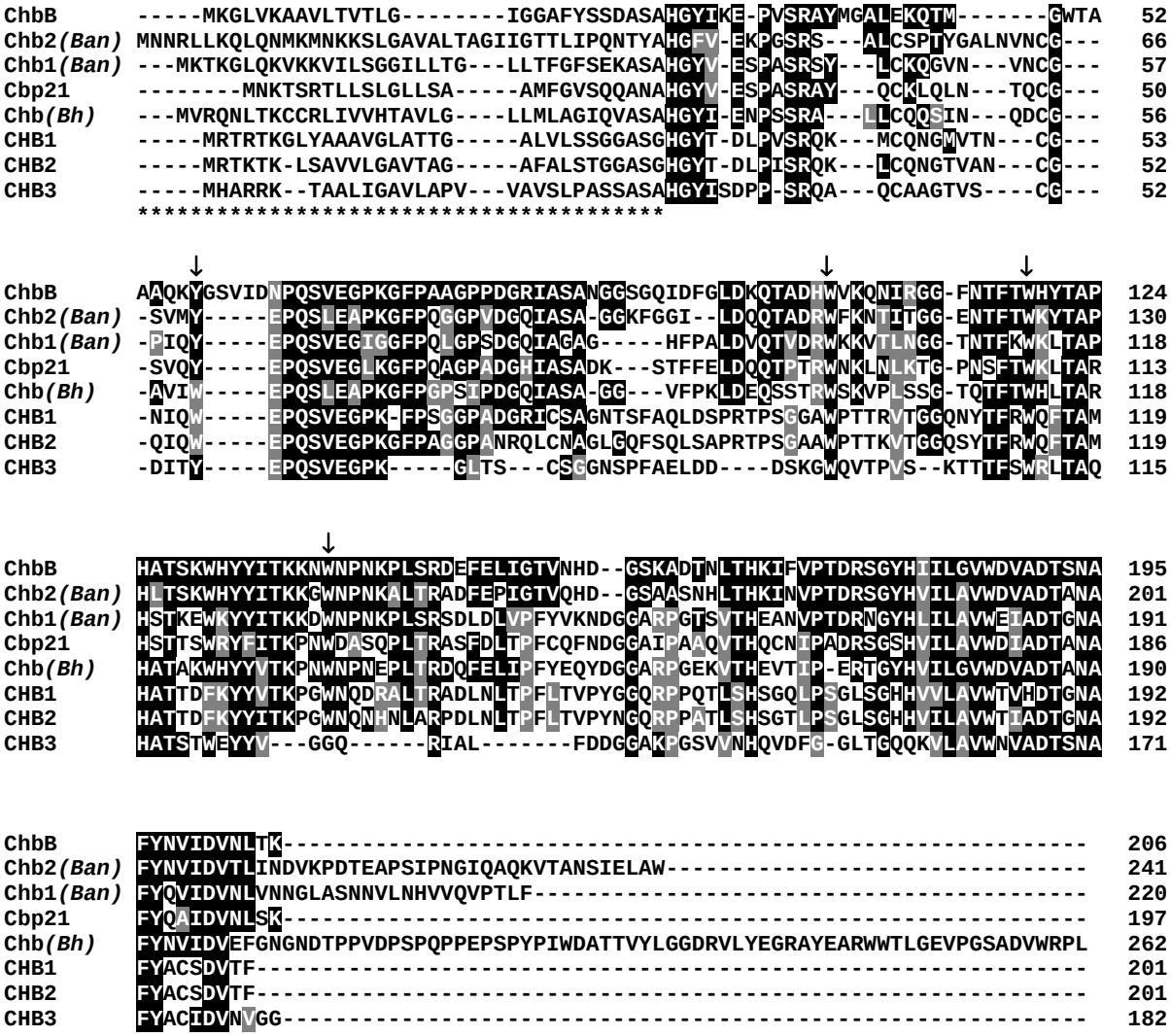

Figure 31. Alignment of deduced chitin-binding proteins. The amino acid sequence of ChbB from *Bacillus amyloliquefaciens*, the Cbp21 protein from *Serratia marcescens* (Suzuki, *et al*., 1998), CHB1 from *Streptomyces olivaceoviridis* (Schnellmann, *et al*., 1994), CHB2 from *Streptomyces reticuli* (Kolbe, *et al*., 1998), CHB3 from *Streptomyces coelicolor* A3(2) (Saito *et al*., 2001), the putative chitin binding protein Chb(Bh) from *B. halodurans* (Takami, *et al*., 2000), and two deduced amino acid sequences {Chb1*(Ban)* & Chb2*(Ban)* from *B. anthracis* (preliminary sequence data from the website http://www.tigr.org) were aligned. Identical amino acids are shaded in black, conserved exchanges are given in grey. Tryptophan (W) residues referred to in the Discussion are labelled by arrows. The predicted amino acids of the signal peptide are marked by asterisks.

3.4.2. Induction and glucose repression of the putative chitin-binding protein

B. amyloliquefaciens ALKO 2718 was grown in MSM in the presence of various substrates (glucose, yeast extract, starch, cellulose (Avicel) or chitin). The 20 kDa protein was synthesized in each of these media, except if glucose served as carbon source (Figure 32a). Further analyses revealed that production of the 20 kDa protein in the medium with yeast extract was strongly reduced when glucose (1%) was added (Figure 32b). In case of growing

in MSM with the presence of chitin (0.5 %), the 20 kDa protein was mainly found in the supernatant (80 %), but also adhering to chitin (about 20 %), from which it could be detached by 3 M of guanidium hydrochloride. This indicated the chitin-binding properties of ChbB protein.

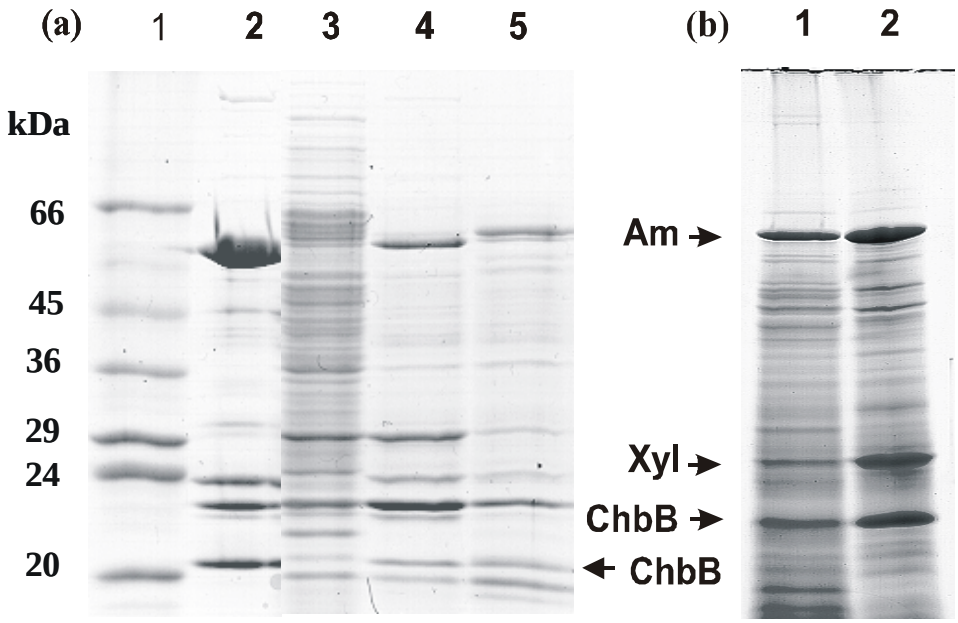

Figure 32. Synthesis of a 20 kDa protein dependent on the carbon source. The supernatant proteins of *B. amyloliquefaciens* cultures (16 h, 37°C) in Spizizen's minimal medium were separated by SDS-PAGE and stained with Coomassie blue. (a) Lane 1, reference proteins; cultures with: lane 2, 1 % chitin (ground crab shells); lane 3, 1 % glucose; lane 4, 1 % starch 1; lane 5, 1 % Avicel. (b) Cultures with: lane 1, 0.5 % yeast extract and 1 % glucose; lane 2, 0.5 % yeast extract. The positions of the amylase (Am) and the xylanase (Xyl) are also marked in addition to ChbB for internal reference proteins.

3.4.3. Purification of the ChbB protein

B. amyloliquefaciens was pregrown (see Methods) and after washing transferred to MSM supplemented with ground crab shell chitin (1 %). Since the predominant part of the desired protein was found in the supernatant of the culture, the latter served as isolation source. Like the previously described CHB1 and CHB2 proteins (Schnellmann, *et al*., 1994; Kolbe, *et al*., 1998), the *Bacillus* protein did not bind to DEAE at a range of various pH values, including pH 9. The concentrated run-through of the DEAE column contained two dominant proteins of 20 kDa and 24 kDa. The 20 kDa protein crossreacted with *anti*-CHB1 antibodies and could be purified to near homogeneity by rechromatography using MonoS material and

citrate/phosphate at pH 5.2 (Figure 34b). About 1 mg of ChbB protein was gained per 1 l-culture.

3.4.4. Overproduction of ChbB protein in E. coli

In order to gain larger amounts of the protein, the *chbB* gene was fused in frame with six histidine codons. Using chromosomal DNA of *B. amyloliquefaciens* as well as the primer L (corresponding to the Shine Dalgarno Sequence AAAGAAGGGAG) and the primer M (which replaces the stop codon of *orf3* by a *Bgl*II site), the complete *orf3* (about 650 bp) was amplified and the DNA fragment was cloned into the pQE16 vector, resulting in the plasmid pQEC1 (Figure 33). The primers used are listed in Table 3.

After induction with ITPG, an *E. coli* transformant carrying pQEC1 produced larger quantities (2 mg/ 500 ml culture) of the His-tag ChbB fusion protein (Figure 34a). The His-tag fusion protein was accumulated in the periplasm and was thus available by osmotic shock treatment. Using the Ni-NTA sepharose column (Qiagen), the ChbB protein was purified. Purified recombinant protein was checked by N-terminal protein sequencing, the data confirmed that the protein produced by *E. coli* was identical with the native one and that its signal peptide had been processed in the same position as in *Bacillus*. The purified *E. coli* produced ChbB was used to raise *anti*-ChbB antibodies, as well as for binding studies.

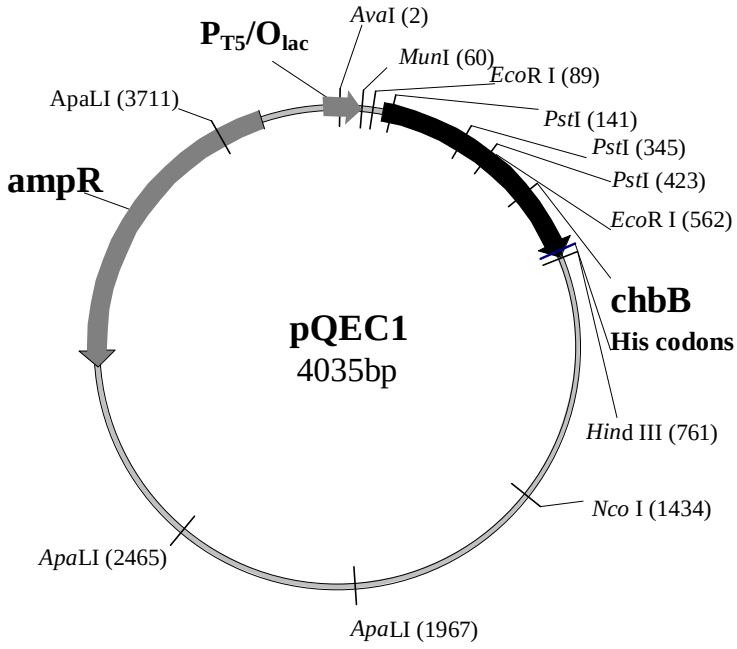

Figure 33. **pQEC1 vector for overexpression of the ChbB protein in *E. coli***. The *chbB* gene with its RBS was cloned into the pQE16 vector under control of the T5 promotor/lac operator.

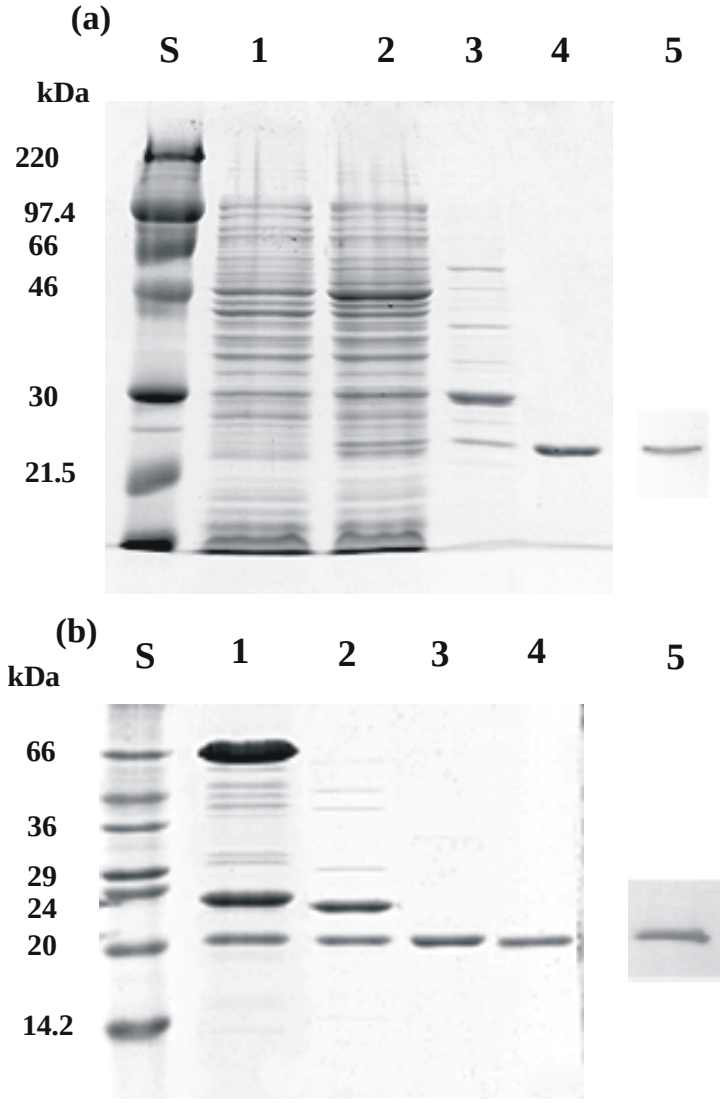

Figure 34. Purification of His-tag ChbB and ChbB. (a) The his-tag ChbB fusion protein was purified from *E. coli* XL-1 blue containing the plasmid pQEC1 after induction with IPTG as described under Methods. Lane 1, total cell protein of uninduced cells; lane 2, total protein of induced cells; lane 3, an aliquot of the periplasmic proteins (obtained from the induced cells by osmotic shock); lane 4, a portion of the pure His-tag ChbB protein (obtained by affinity chromatography with NTA agarose and 200 mM of imidiazole); lane 5, the protein from lane 4 was transferred to a nylon membrane and tested for crossreaction with antibodies raised against the CHB1 protein. (b) Lane S, sizes of reference proteins; lane 1, the proteins from the supernatant of a *B. amyloliquefaciens* culture were precipitated by $(NH_4)_2SO_4$; lane 2, an aliquot of the pooled fractions obtained after FPLC chromatography using DEAE; lane 3, an aliquot of pooled proteins obtained by use of a MonoS column; lane 4, an aliquot of the protein obtained after use of Phenylsepharose. Aliquots of the peak fractions and of reference proteins (lane S) were investigated by SDS-PAGE and stained with Coomassie blue. Lane 5, the purified protein from lane 4 was transferred to a nylon membrane and tested for reaction with antibodies raised against the His-tag ChbB protein.

3.4.5. Binding properties of the ChbB protein

The proposed chitin-binding properties of the purified mature *Bacillus amyloliquefaciens* ChbB protein were studied (Figure 35). The quantities of the proteins, which were bound and/or unbound, were analyzed by SDS-PAGE and, if necessary, the proteins were immunodetected. For initial studies *anti*-CHB1 antibodies were used; however, as their affinity was comparatively low, they were substituted by antibodies newly raised against the His-tag ChbB protein (gained from *E. coli*, see above). For binding tests, CHB1 (5 µg) was mixed with 2 mg of each substrate. The maximally bound CHB1 was set as 100 %. The pH optimum was established as 7 (Figure 35a), salt reduced binding to about 30 % (0.5 M NaCl) or 50 % (1 M NaCl) (Figure 35b). ChbB showed a preference for β-chitin, less for α-chitin, however, weak binding of *β*-glucans from yeast and barley and of crystalline cellulose was also ascertainable (Figure 35c). For visualization (detection of fluorescence) *α*- and *β*-chitin were treated with ChbB. Its binding was detected with primary *anti*-ChbB antibodies, followed by secondary fluorescein-labelled antibodies. Fluorescence was most intense for β-chitin and reduced for α-chitin. No fluorescence was scored on the control chitin sample which had not been treated with ChbB (Chu, *et al*., 2001). After purification, the His-tag fusion protein ChbB obtained from the heterologous *E. coli* host was found to have identical binding characteristics.

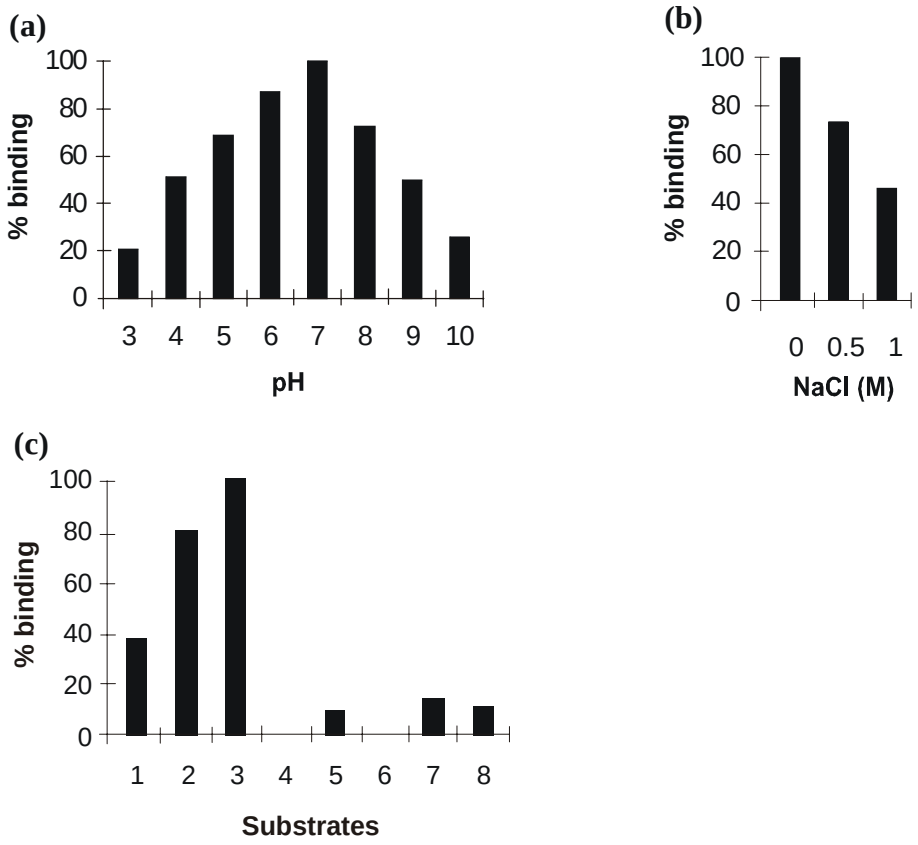

Figure 35. Binding studies. 2 mg of substrates were mixed with 5 µg of ChbB protein purified from *B. amyloliquefaciens* in 50 µl of the buffers as described (see Methods). After removal of the supernatant and two washes, the pellet from each sample was resuspended in 40 µl loading buffer, separated by SDS-PAGE and either stained with Coomassie blue or subjected to immunodetection after transfer onto a nylon membrane. Binding of ChbB to α-chitin (purified crab shell powder) was determined at different pHs (a), and at different salt concentrations (b). In addition, binding to different substrates was studied at pH 7: lane 1, α-chitin (crab shells); lane 2, β-chitin from Sepia; lane 3, β-chitin from *Siboglinum fjordicum*; lane 4, chitosan; lane 5, cellulose from cotton linters; lane 6, xylan from oat spells; lane 7, β-glucan from yeast or lane 8, β-glucan from barley, respectively. The relative amount of protein present in each lane was calculated after scanning and the amount of ChbB bound to the substrate was compared setting the sample with highest binding activity as 100 %. In these samples 100 % binding corresponded for (a) and (b) to 0.85 µg and for (c) 2.17 µg ChbB per mg substrate.

3.4.6. Abundance of homologues of the chbB gene and of the ChbB *protein*

To assess the abundance of the *chbB* gene, total DNA was isolated from several *B. amyloliquefaciens* strains and the primers L and M were used for PCR detection. The PCR product of 12 out of 17 strains yielded a DNA fragment corresponding in size to ALKO 2718 (Figure 7a). The DNA of some strains (IFO 3034, IFO 3037, and OUT 8421) induced the formation of a fragment of the same size, however in lower quantities. The DNA of the strains OUT 8420 and OUT 8426 did not yield a PCR product under standard conditions. All strains showed a hybridizing band which was either in the same or different size to ALKO 2718. Neither a PCR product nor a hybridizing fragment was obtained with DNA of *B. subtilis* strains 168 and GB72. Moreover, using the *anti*-ChbB antibodies a protein similar in size to the ChbB protein of ALKO 2718 was detected among proteins of several *B. amyloliquefaciens* strains grown in the presence of chitin. A corresponding protein was missing in cultures of *B. subtilis* 168 (data not shown).

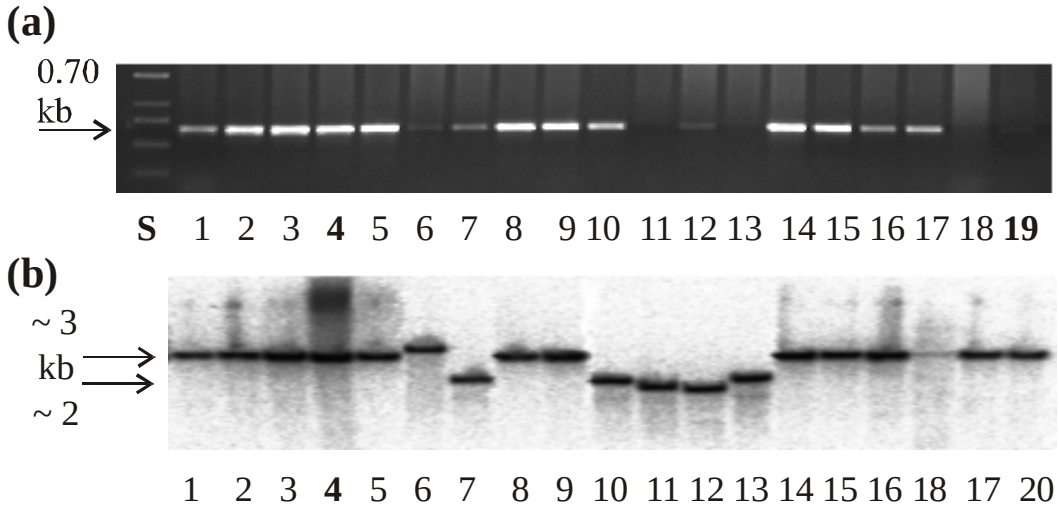

Figure 36. Detection of *chbB*-like DNA in various *B. amyloliquefaciens* strains.

(a) PCR amplification of *chbB* DNA (~0.7 kb) of *B. amyloliquefaciens* strains (#1 to 18) compared to ALKO 2718 (#4) and of *B. subtilis* 168 (#19) for a (negative) control. The oligo-nucleotide primers L and M were used under identical PCR conditions. The lanes are: S, 2 kb-ladder; 1 to 18 are the following *B. amyloliquefaciens* strains: Lane 1, ATCC 15841; lane 2, ATCC 23350; lane 3, ATCC 23842; lane 4, ATCC 23844 (**ALKO 2718**); lane 5, IAM 1523; lane 6, IFO 3034; lane 7, IFO 3037; lane 8, KA 63; lane 9, N; lane 10, OUT 8419; lane 11, OUT 8420; lane 12, OUT 8421; lane 13, OUT 8426; lane 14, P; lane 15, SB I; lane 16, T; lane 17, ZFL 14/4; lane 18, GB 72/ SK 1590; lane 19, *B. subtilis* 168.

(b) Southern hybridization of genomic DNA of several *B. amyloliquefaciens* strains after *Hind*II digestion. The blot was probed with a 0.7 kb-DNA fragment encoding the *chbB* gene without promoter region. The lanes are numbered as under (a) except number 20 shows in addition strain ZF178.

3.4.7. Chitinolytic activity of B. amyloliquefaciens strains

Growing on chitin-containing plates, the *B. amyloliquefaciens* strains OUT 8419, OUT 8420, OUT 8421, OUT 8426, IFO 3034 and IFO 3037 degraded chitin (clear zones), like the strain *B. licheniformis* 41p known to have chitinolytic activity. Using the dye Remazol brilliant violet coupled with carboxymethyl-chitin, the relative chitinolytic activities were compared. The strains ATCC 23842, KA63 and N showed only low activities, others (including ALKO 2718 and ATCC 15841, ATCC 23350, IAM 1523, P, SBI, T, ZFL 14/4) had moderate activities, while the strains IFO 3034, IFO 3037, OUT 8419, OUT 8420, OUT 8421 and OUT 8426 showed increased activities (Figure 33); these were however lower than those of *B. licheniformis* 41p (Hofemeister *et al.*, unpublished).

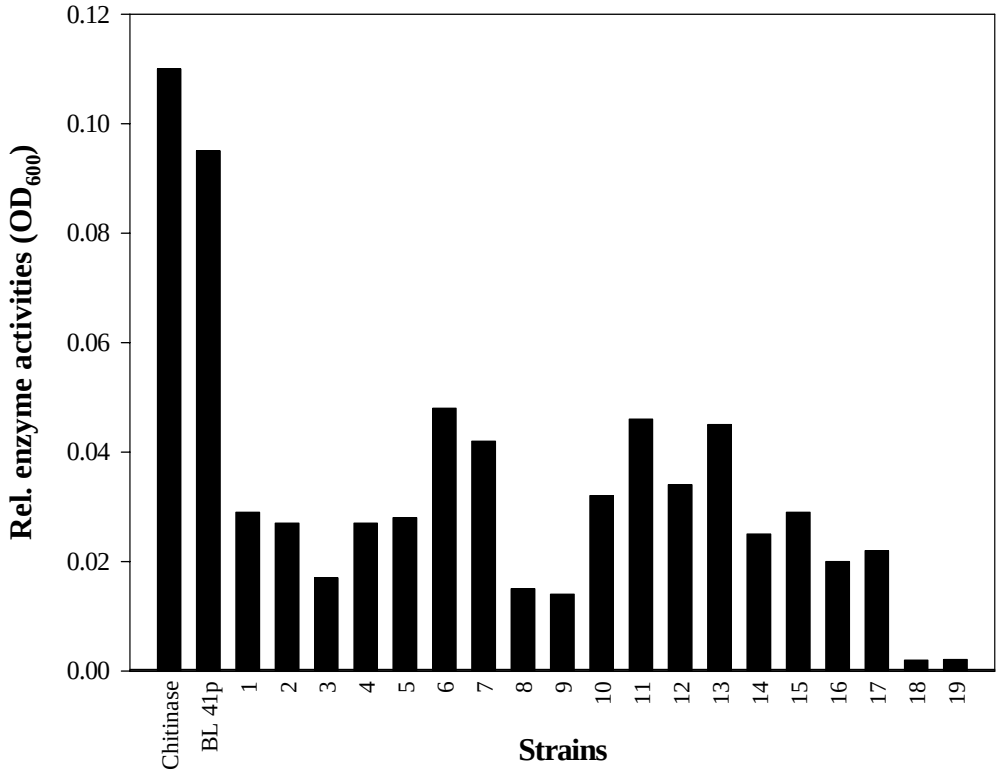

Figure 37. **Detection of chitinolytic activity**. Hydrolysis of carboxymethyl chitin-Remazol brilliant violet by an aliquot of the culture supernatant of several *B. amyloliquefaciens* strains (#1 to 17) compared to *B. licheniformis* (BL 41p) and to 0.007 units of *Serratia marcescens* chitinase (Sigma). Numbers 1 to 17 indicate *B. amyloliquefaciens* strains: bar 1, ATCC 15841; bar 2, ATCC 23350; bar 3, ATCC 23842; bar 4, ATCC 23844 (**ALKO 2718**); bar 5, IAM 1523; bar 6, IFO 3034; bar 7, IFO 3037; bar 8, KA 63; bar 9, N; bar 10, OUT 8419; bar 11, OUT 8420; bar 12, OUT 8421; bar 13, OUT 8426; bar 14, P; bar 15, SB I; bar 16, T; bar 17, ZFL 14/4. Numbers 18 and 19 show the activities of *B. subtilis* 168 with either plasmid pHB201 (without the *chbB* gene) and plasmid pHBC1 (with the *chbB* gene).

3.4.8. Export of ChbB in sip disruption mutants

In order to study the effect of signal peptidase mutants on the secretion of ChbB protein in *Bacillus*, the shuttle plasmid pHBC1 was constructed using vector pHB201. The *chbB* DNA-fragment was isolated from pQEC1 after *Hind*III digestion and after blunting and *Mun*I digestion ligated into *Eco*RI and *Eco*RV digested pHB201 vector DNA (Figure 38).

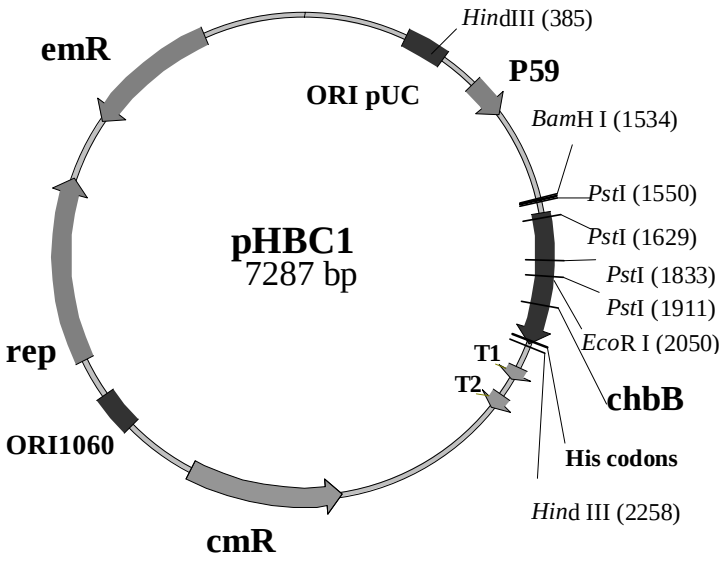

Figure 38. ***Bacillus/E. coli* shuttle vector pHBC1**. The *chbB* gene including 6xHis tag codons from pQC1 vector was cloned into pHB201 (Bron, *et al*., 1998) under the control of P59 promoter.

The production of the ChbB protein was investigated after transformation of pHBC1 plasmid DNA into various *sip* disruption mutants and parental strain (Figure 39). The amount of mature ChbB protein was slightly reduced in the supernatant *sipT* mutant compared to *sipS*, *sipV*, *sipW*, or parent strains (see Figure 39a). In addition, the accumulation of the ChbB precursor was slightly higher in the total protein fraction of the *sipT* mutant culture (see Figure 39b&c).

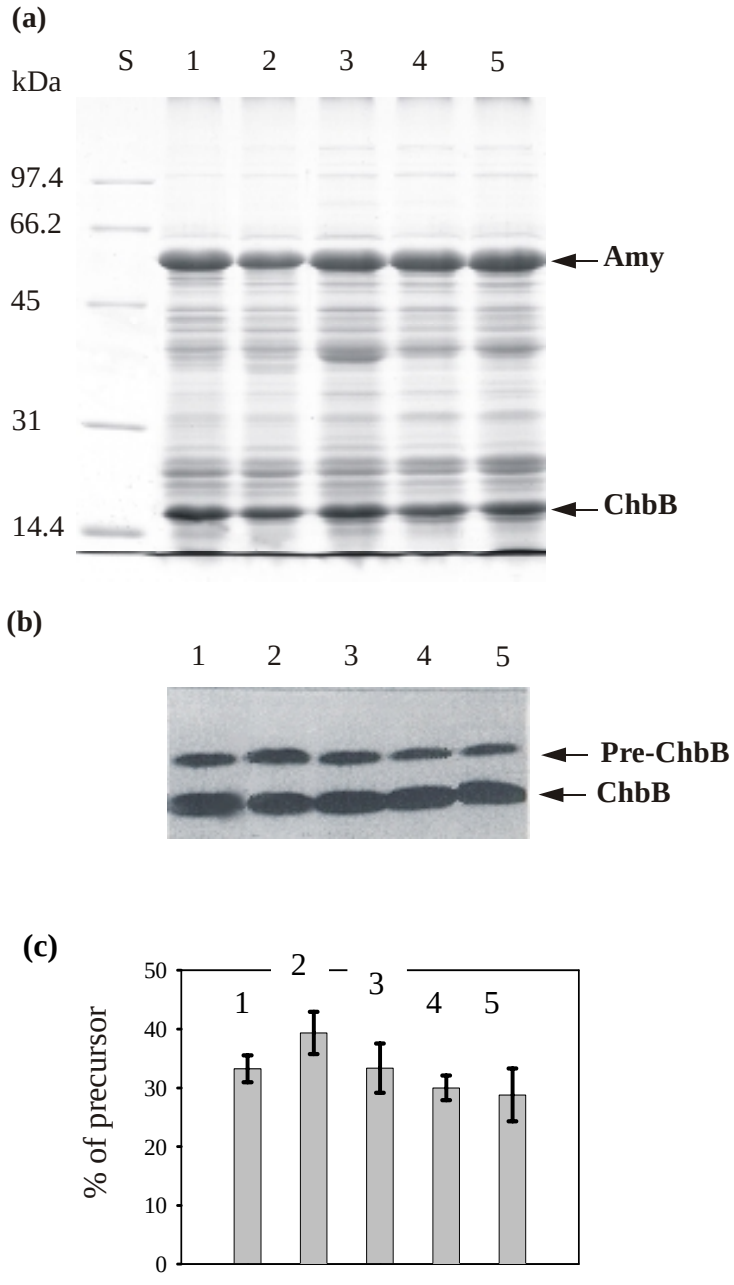

Figure 39. Production of the ChbB protein in *B. amyloliquefaciens sip* disruption mutants and parental strain. SDS-PAGE of supernatant proteins of overnight MSM containing yeast extract cultures **(a)**. Western blot of total proteins of pHBC1 transformed strains (cell proteins and supernatant proteins) using penta-His antiserum **(b)**. The column bars represent the percentage of remaining ChbB precursor (pre-protein)**(c)**, as calculated according to the intensity of bands of the western blot shown in part (b).

4. Discussion

4.1. General remarks

The main question behind the present studies was the mystery of the presence of multiple signal peptidases in various eubacterial species, notable in bacilli (see the Introduction). As prokaryotic genomes are extremely "economical", i.e. are usually devoid of non essential DNA or genes and the most Gram-negative bacteria indeed live with only one single type I signal peptidase gene in their genome, it was reasonable to speculate about the "necessity" of so many Sip- like proteins. This question may be addressed i) by isolation of the enzymes and characterisation of enzymatic properties, ii) by construction and characterisation of gene knockout mutants, iii) by genetic (functional) substitution of one type of one Sip by another Sip-like protein, iv) by comparing the processing activity of a distinct Sip enzyme using a special, likely specific export protein for a substrate, etc. In this studies we attempted to analyse *B. amyloliquefaciens* in comparison to *B. subtilis* 168, where the genome project convincingly proved the existence of two major (SipS, SipT) and three minor Sip proteins (SipU, SipV, SipW) in *B. subtilis* 168 (Kunst, *et al.*, 1997; van Roosmalen, *et al*., 2001). If evolutionary constrains within the genus *Bacillus* caused the branching of species, these should be also reflected on the genetic level, i.e. by changed, preserved or missing genes. Especially protein export is one ostentatious property of *B. amyloliquefaciens*, as it is basically about 10 to 20 times more efficient compared to *B. subtilis* (Ingle & Boyer, 1976; Vehmaanpera, *et al.*, 1991). The export apparatus of these two (closely related) species might have been adapted, i.e. by changed bottleneck functions. We therefore proposed Sip proteins to differ either in their capacity or processing specificity. Any change in the presence or in the specificity of Sip proteins from *B. subtilis* to *B. amyloliquefaciens* could then elucidate the selective value of those export components. However, this strategy was not without complications mainly due to the inert genetic manageability of *B. amyloliquefaciens* in comparison to *B. subtilis*. With the methods ad hands, the transformation efficiencies are about 10^3-10^5 times lower and the nucleotide sequence diversity compared to *B. subtilis* did not allow the use of DNA sequence information from *B. subtilis* for reverse genetical aims, as for cloning of proposed genes. The latter affected mainly the characterisation of *sip* insertion mutants with respect to the proposed target proteins as well as the stringency of the claimed number of *sip* genes in *B. amyloliquefaciens*. The detailed analysis of a proposed new export target protein of *B. amyloliquefaciens*, which seemed to have processing specificity towards a distinct Sip proteins, i.e. a chitin-binding protein ChbB, was thus "a leap in the dark" which

finally was disappointed as a processing target. On the other hand, however, it was welcome as a new export protein of this species.

4.2. Cloning and similarity of Sip-like signal peptidases of B. amyloliquefaciens

In addition to *sipS* and *sipT* (Hoang & Hofemeister, 1995; Meijer, *et al.,* 1995), here we present evidences for the existence of two more *sip*-like genes in chromosomal DNA of *B. amyloliquefaciens* strain ATCC 23843, which are *sipV* and *sipW*. As in case of *sipS* and *sipT* genes, which were defined according to their sequence similarity and similar map positions with their counterparts *sipS* and *SipT* of *B. subtilis* (Hoang & Hofemeister, 1995; Meijer, *et al.,* 1995; Tjalsma, *et al.,* 1997), *sipV* and *sipW* of *B. amyloliquefaciens* were also classified with respect to sequence similarity as well as the map position to their *B. subtilis* counterparts. A *sipU*-like gene was not found. Although, the absence of a *sipU* gene could have been overlooked due to sequence diversity, one of these two species might have gained or lost a *sipU*-type gene after evolutionary constrains, likely after plasmid mediated transmission. This hypothesis was supported by the presence of plasmids in certain *B. subtilis* (natto) strains which carry the so-called *sipP* genes (Meijer, *et al.,* 1995; Bron, *et al.,* 1998). Consequently, our data would suggest that *B. amyloliquefaciens* differs from *B. subtilis* with respect to the number of *sip*-like genes, i.e. to contain in the genome *sipS, sipT, sipV* and *sipW,* but likely to miss a *sipU*-like gene candidate.

Many bacteria contain paralogous type I Spases in multiplicity (isoforms). The presence of multiple type I SPases seems to be a common feature of Gram-positive eubacteria. For example, *Staphylococcus aureus* contains at least two type I SPases, whereas *Streptomyces lividans* seems to contain four (Parro, *et al.,* 1999). Data obtained from genome sequencing projects of *B. subtilis* (Kunst *et al.,* 1997), *B. halodurans* (Takami, *et al.,* 2000) and *B. anthracis* (preliminary data from the Institute for Genomic Research website at http://www.tigr.org), provided a precise estimation of the number of genes encoding type I SPases. While the *B. subtilis* genome harbours five *sip* genes (Kunst, *et al.,* 1997), only two are found in *B. halodurans*. The largest number of *sip* genes was found in *B. antharacis,* where at least six genes encoding for type I SPases could be identified. These findings would rise questions about the phylogenetic diversity and the mode of conservation of structural and enzymatic properties among these SPase isoforms in bacilli. The alignment of as much as 18 *Bacillus* P-type SPases proteins from *B. subtilis, B. amyloliquefaciens, B. licheniformis, B. stearothermophilus, B. caldolyticus, B. halodurans* and *B. anthracis* is shown in Figure 16. For example, the amino acid sequence of SipV*(Ba)* shares 40% to 77% of identical amino

acid residues with others known *Bacillus* Sip-like proteins (Figure 16). Among Sip-like proteins, however, this distinct SipV protein shares highest sequence similarity within one branch of Sip proteins, which apparently form a cluster of closely related proteins. Most striking with respect to the multiplicity of Sip proteins in *Bacillus*, however, is the distinction of P- and ER-type SPases. These were only recently discovered after the SipW of *B. subtilis* was characterised (Tjalsma, *et al.,* 1998, 2000). The cloning and sequence diversity of a SipW-like protein in *B. amyloliquefaciens* strengthen these conclusion as these proteins have similar characters of conserved sequence motifs in domains B and C (Table 5), as well as with respect to the exchange of the catalytic amino acid residue histidine for lysine within domain D (Table 5, Figure 17). The latter agreed to recent findings which indicated that P-type SPases make use of a serine-lysine catalytic dyad (Sung & Dalbey, 1992; Tschantz, et al., 1993; van Dijl *et al.,* 1995; Dalbey, *et al.,* 1997; Paetzel, *et al.,* 1998), while the ER-type SPase of *B. subtilis* instead seems to employ a Ser-His-Asp triad or Ser-His catalytic dyad (Tjalsma, *et al.,* 2000). The P-type and ER-type SPases also differ in the distance of the conserved domains (Dalbey, *et al.*, 1997). As shown in Figure 12 the domains B and C of ER-type SPases are separated by only one and domain D and E by 10-11 residues, while these domains of P-type SPases are separated by 11 to 42 and 23 to 118 residues, respectively (Figure 16). While SipW-like SPases share significant sequence as well as domain similarity to P-type, the similarities are mostly limited to the conserved domains B, C, D and E (Table 5).

With respect to the exceptional properties of SipW-like SPases it was interesting to find SipW-like DNA also in the genomic DNA of 10 out of 12 strains representing four distant 16S rRNA groups of the phylogenetic tree of bacilli (Ash, *et al.,* 1991; Priest, 1993), but not in group 5 strains *B. stearothermophilus* and *Thermoactinomyces vulgaris*. Although, the PCR approach could have been failed in these two strains of remote Gram-positive spore-forming bacilli (Stackebrandt, *et al.,* 1987), the absence could correlate with the fact that *sip*W gene disruption mutants neither in *B. subtilis* (Serrano, *et al.,* 1999) nor in *B. amyloliquefaciens* (this study) had any detectable deficiency in cell viability or sporulation. Consequently, SipW functions could be completely replaced by other Sip candidates (Tjalsma, *et al.,* 1998). As already mentioned, *B. halodurans* (Takami, *et al.,* 2000) and *B. anthracis* also contain genes encoding SipW-like proteins. Moreover, a similar protein was indicated for *Clostridium perfringens* (Tjalsma, *et al.,* 1998). The absence of SipW-like genes in group 5 of the *Bacillus* phylogenetic groups, however, needs further confirmation.

Considering the diversity among P- and ER-type SPases of bacilli and of other Gram-positive bacteria, it is likely that the two groups of enzymes have different functions. In general, SPases are integral membrane proteins with their active site of the enzymes located at the outer side of the membrane. Thus, one character of Sip proteins reflects the position and number of transmembrane domains. As far this concerns SipS, SipT, and SipV-like proteins of *B. amyloliquefaciens, B. subtilis* and of other *Bacillus* species, these P-type SPases have only one amino-terminal transmembrane domain (Figure 16 and 18), while known SipW-like proteins, i.e. of *B. subtilis* (Tjalsma, *et al.*, 1998), *B. amyloliquefaciens, B. halodurans* and *B. anthracis* (Figure 17 and 18), have two transmembrane domains, one amino-terminal domain A1 and a carboxyl-terminal domain A2. However, this domain arrangement seems to be preserved among P- and ER-type SPases of bacilli, but are not a general distinction between P- and ER-type SPases.

	B	C
P-type	**v-G-SM-pTl**	**R(f/g)d(i/v)(i/v)(i/v)**
ER-type	**VlSgSMeP-f**	**TGS#### [C1]** **GDVITF [C2]**
P-type*	**--sgSM-ptl**	**r-gd-i###**
ER-type*	**vlsgSMeP-f**	**r-Gd-l###**
	D	E
P-type	**y(i/v)KR#IglPG(d/e)**	**FV-GDNR-S-DsR**
ER-type	**##HR##**	**f-TKGDnN---D**
P-type*	**-##-KR##**	**----GDnr---D**
ER-type*	**p#v-HRv#**	**f-tKGDnn---d**

Table 5. Consensus sequences of the conserved domains B-E of the P- and ER-type SPases. The conserved amino acid sequences were taken from the alignments of all known P- and ER-type SPases from *Bacillus*. * indicate the consensus sequences of domain B-E of P- and ER-type SPases according to Tjalsma *et al.*, (1998). The strictly conserved residues are indicated by upper case letters, conservative substitutions by lower case letters, conserved hydrophobic residues by a number sign (#) and (i/v) indicate the residues that can be substituted by either isoleusine (i) or valine (v).

Diversity of paralogous Sip proteins is also indicated from phenetic distance analysis as illustrated in Figure 40, where as much as 23 *Bacillus* Sip proteins known from *B. subtilis, B. amyloliquefaciens, B. licheniformis, B. stearothermophilus, B. caldolyticus, B. halodurans*

and B. anthracis were included. The Neighbour-Joining algorism was used to compare the *Bacillus* sequences with *E. coli* LepB and yeast Sec11 ER-type SPase. The phylogenetic tree strengthen the distinction between P- and ER- type SPases as previously proposed (Tjalsma, *et al.*, 1998), but also the clustering of P-type Sip proteins into at least three subgroups represented by *B. subtilis* SipV-, SipS,T,U- and *B. anthracis* Sip3,5-like SPases, respectively. This analysis showed close relationship between Sip proteins of *B. amyloliquefaciens* and *B. subtilis* as well as their relatedness to other SPases. Basically these data are similar to those of van Roosmalen et al. (2001), where 15 different SPases were included and the authors claimed the distinction between major and minor SPases upon similar phylogenetic analyses. According to our data, which include additional SPases from *B. halodurans,* as well as from *B. anthracis*, the given criteria for major and minor SPases might differ from one species to another. For instance, SipV(*Bha*) of *B. halodurans*, apparently plays the role of a major SPase, but according to its phylogenetic character would not belong to the group of major SPases.

The “biological sense” of the existence of multiple *sip* genes in a distinct group of bacteria could be viewed to provide them an advance to selectively control the export of proteins using the different specific processing functions of SPases or their alternatively regulated expression. Under natural conditions, the availability of multiple and paralogous SPases could provide alternatives for the flexible responses of the bacteria to environmental changes by export of different proteins into the surrounding media to initiate the switch from vegetative growth to sporulation or from spore to germination in case of bacilli. The characterisation of proposed functions of these multiple enzymes is still pending. Recent studies were mostly carried out with *B. subtilis* 168 – the “genome-proteome”-type strain of *B. subtilis*. Known peculiarities of Sip-enzymes of *B. subtilis* can be summarised as follows:

- The *sipW* gene maps in a gene cluster with *tasA* and *yqxM*. SipW was shown to have specificity for processing of TasA and YqxM protein (Stover & Driks, 1999a, b, c).
- SipS and SipT are major enzymes, hence they are highly expressed and both are essential for cell viability. The transcription of *sipS* and *sipT* is temporally controlled via the DegS-DegU two-component regulatory system, in concert with the transcription of the genes of most secretory proteins (Bolhuis, *et al.,* 1996; Tjalsma, *et al.,* 1998).
- The SipS, SipT, SipU, SipV and SipP of *B. subtilis* are proposed to have overlapping subtrate specificities, but have proposed different specific processing activities for a given substrate (amylase) (Tjalsma, *et al.,* 1997, 1999).

Here we made the attempt to answer the question about specificity of *B. amyloliquefaciens* Sip proteins by two ways: a) after expression in a *lepB* mutant of *E. coli* and testing of genetic complementation and b) after construction of *sip* gene disruption mutants and characterisation of mutant deficiencies.

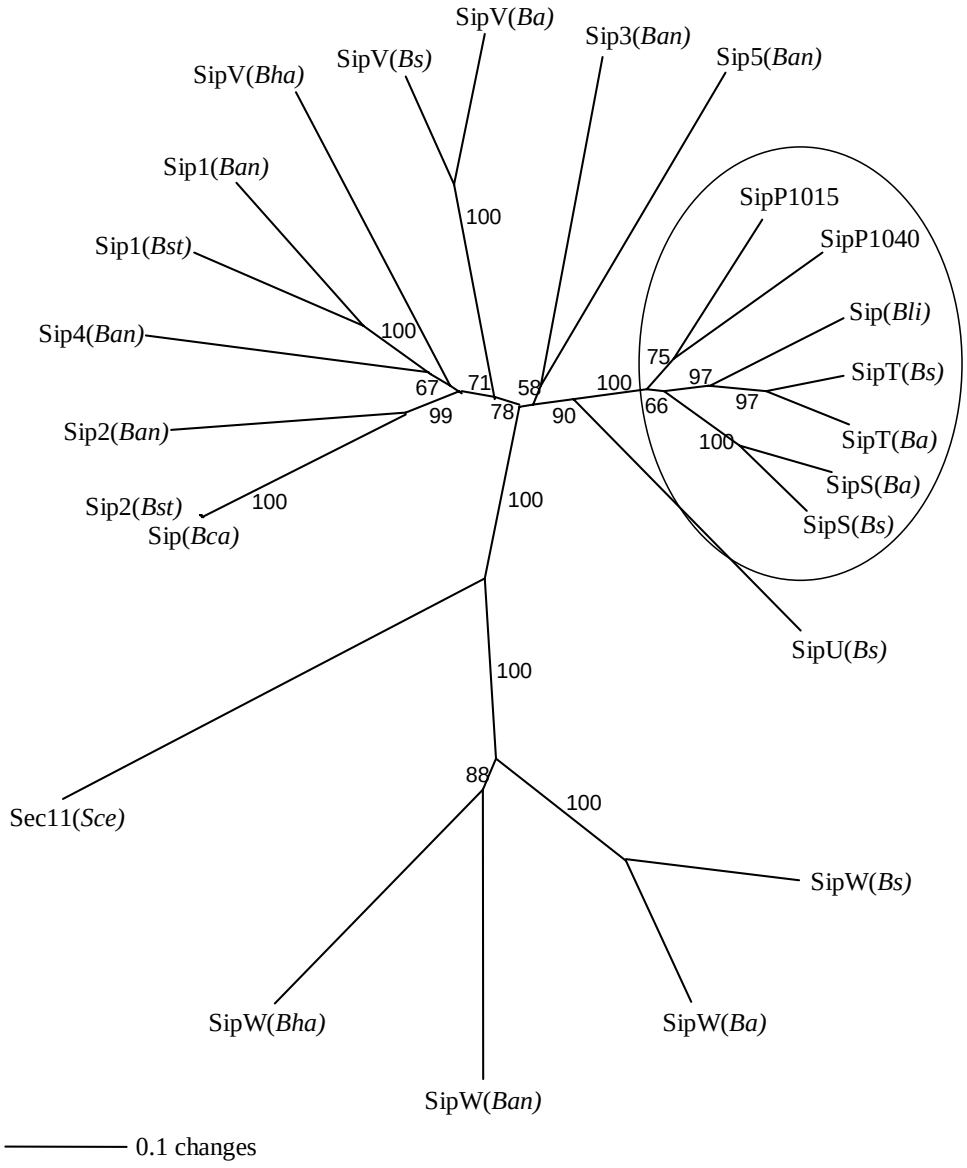

Figure 40. **Phylogenetic tree based on multiple sequence alignments of known *Bacillus* Sip proteins including** *Saccharomyces cerevisiae* Sec11*(Sce)* (NP_012288). The analysed *Bacillus* Sip proteins were: *B. amyloliquefaciens* SipS*(Ba)* (P41026), SipT*(Ba)* (P41025), SipV*(Ba)* (AAF02219), SipW*(Ba)* (AAF02220); *B. subtilis* SipS*(Ba)* (P28628), SipT*(Ba)* (G69707), SipU*(Ba)* (I39890), SipV*(Ba)* (A69708), SipW*(Ba)* (B69708), SipP1015 (I40470), SipP1040 (I40552); *B. halodurans* SipV*(Bh)* (BAB04749), SipW*(Bh)* (BAB05849); *B. licheniformis* Sip*(Bl)* (CAA53272); *B. caldolyticus* Sip*(Bc)* (I40175); *B. anthracis* Sip1*(Ban)*, Sip2*(Ban)*, Sip3*(Ban)*, Sip4*(Ban)*, Sip5*(Ban)*, SipW*(Ban)*; *B. stearothermophilus* Sip1*(Bst)*, Sip2*(Bst)* (preliminary sequence data from the website http://www.tigr.org). The length of each pair of branches represents the distance between sequence pairs, while the units at the bottom of the tree indicate similarity by the number of substitution events. The dotted lines indicate negative branch length, a product of averaging.

4.3. Functional complementation of an E. coli LepBts mutant

In conclusion of the data discussed in the previous section, one might assume that SPases with close sequence similarity are likely to complement each other more efficiently than those from another subgroup of the SPases. The difficulties of genetic manipulation in *B. amyloliquefaciens*, especially its inaccessibility to construct multiple *sip* knockout mutants, inclined us to address the question in *E. coli*, and to ask if *B. amyloliquefaciens* SPases substitute the *E. coli* LepB functions with respect to growth and processing.

The *E. coli* strain IT41, which carried an TAG amber terminator codon mutant at nucleotide position 115 in the *lepB* coding sequence (Inada, *et al.*, 1988; Cregg, *et al.*, 1996), has been previously proved to be a useful tool to study functions of SPases from *Bradyrhizobium japonicum* (Müller, *et al.*, 1995), *Staphylococcus aureus* (Cregg, *et al.*, 1996), *Streptococcus pneumoniae* (Zhang, *et al.*, 1997) and *Streptomyces lividans* TK21 (Parro *et al.*, 1999). Therefore, in this studies each of the four *B. amyloliquefaciens* Sip proteins were expressed in this *E. coli* LepB*ts* mutant strain and tested for processing of pre-OmpA and growth restoration at the non-permissive temperature. In summary, only SipS*(Ba)* and SipT*(Ba)*, but neither SipV*(Ba)* nor SipW*(Ba)*, were active in processing pre-OmpA in *E. coli*. In contrast, the growth of the LepB*ts* mutant was only restored after SipS*(Ba)* but not after SipT*(Ba)* expression. These likely reflects differences in processing specificity or capacities of SipT*(Ba)* compared to SipS*(Ba)* in order to cope with growth-limiting LepB processing functions. (Figure 20 and 21). In *B. subtilis*, SipS, SipT, as well as the plasmid encoded SipP, were shown to functionally replace each other and to be the “major” SPase as the cell need at least one of them to survive, while SipU, SipV and SipW seem to play “minor” roles. The latter were proposed to have limited substrate specificity for a subset of about 166 predicted export proteins (Tjalsma, *et al.*, 2000). When expressed in *E. coli*, only SipS, SipT and SipU were active in processing of the pre(A13i)-β-lactamase precursor, while SipV was inactive (Tjalsma, *et al.*, 1997). In turn, *B. amylolioquefaciens* SipV*(Ba)* as well as SipW*(Ba)* were not active in processing of the pre-OmpA in *E. coli*. The lack of processing in *E. coli* was not caused by differences in production levels of these Sip*(Ba)* proteins, but likely correlated with enhanced degradation and inactivation, as indicated by additional protein bands in SipV*(Ba)* and SipW*(Ba)* producing *E. coli* cells after SDS-PAGE and immuno-detection (Figure 21B). These observation could reflect less efficient membrane insertion of these Sip*(Ba)* proteins in *E. coli*, since soluble (truncated) forms of *B. amyloliquefaciens* SipS*(Ba)*, lacking their unique N-terminal membrane anchor, were also found to become inactivated in *E. coli* by self-cleavage (van Roosmalen, *et al.*, 2000). In contrast, SipT*(Ba)*

was apparently quite stable and efficient in processing of pre-OmpA, but was nevertheless unable to restore growth of LepB*ts E. coli* cultures. This distinction was likely due to non-efficiency in processing of some *E. coli* export proteins by SipT*(Ba)*. Hence, SipT*(Ba)* apparently did not support the processing of unknown but essential proteins, which were, however, efficiently processed by LepB or by SipS*(Ba)*.

The data indicated that heterologous function of Sip protein in *E. coli* can be affected by two factors: a) the protein stability due to membrane insertion and b) the range of substrate specificity of individual SPases. Therefore, we designed hybrid *sip*/*lep*-genes by exchanging the transmembrane and catalytic regions of *E. coli* LepB and *B. amyloliquefaciens* SipT*(Ba)*. The schema of the two hybrids LepT-B and SipB-T are shown in Figure 22. After expression in the *E. coli* LepBts mutant strain only the hybrid LepT-B was found to support growth at the non-permissive temperature, while the hybrid SipB-T did not. This indicated that the transmembrane portion of SipT*(Ba)*, in fusion with the LepB catalytic domain, efficiently restored the LepBts deficiency, but that the catalytic domain of SipT*(Ba)* in fusion with the transmembrane domain of LepB did not. This can be explained by the fact that both the transmembrane as well as the catalytic domain of SipT functioned, but the latter, however, was not able to complement the whole range of LepB processing functions. This suggested that complementation or non-complementation of LepB*ts* by any *B. amyloliquefaciens* SPase was most likely due to processing specificity and not or to an lesser extend due to inefficiency of their membrane insertion. This promising “hybrid” studies did not solve the problem completely, but indicated a genetic route to answer questions related to the limits of function or specificity of individual *B. amyloliquefaciens* SPases.

4.4. Mutant studies

Without processing, i.e. after inactivation of essential SPase genes, export proteins remain anchored at the membrane and could not finally target to their proper locations. Consequently, *E. coli* mutants lacking LepB are non-viable (Date, 1983; Inada, *et al.,* 1989). However, *B. subtilis* mutants lacking as many as four SPases, i.e. SipT,-U,-V,-W, are viable, but heat inactivation of SipS*ts* in a SipT mutant background, i.e. the SipS-SipT double deficiency, had lethal consequences (Bron, *et al.,* 1998; Tjalsma, *et al.,* 1998). All of the *B. amyloliquefaciens* Sip*(Ba)* disruption mutants were viable, but some had impaired growth, sporulation and changed cell division properties. Similar to *B. subtilis* (Bron, *et al.,* 1998), inactivation of either SipS or SipT decreased the total yields of export proteins compared to the wild type by about 30 percent, as was the quantity of extracellular amylase also significantly enhanced in

SipS*(Ba)* mutant cultures (data not shown). These two observation were also made with *B. subtilis* mutants and the former explained to reflect the predominant character of SipS and SipT enzymes (Tjalsm,a *et al.,* 1997; Bron, *et al.,* 1998). The other deficiencies of *B. amyloliquefaciens sip(Ba)* disruption mutants have not been reviewed for *B. subtilis*. However, strict correlation of a distinct mutant phenotype with that distinct *sip::*pEA* integration event, as well as restoration of the mutant phenotype after spontaneous excision of the insertion cassette from *B. amyloliquefaciens sipT*::pEAT* mutants, strongly indicated gene disruption to correlate with that distinct deficiencies. The recent finding about SipW activities in pre-TasA processing and transport into *B. subtilis* endospores provided a first example of specification of a SPase for spore-specific protein sorting (Tjalsma, *et al.,* 1998; Serrano, *et al.,* 1999; Stöver & Driks, 1999). In *B. amyloliquefaciens*, disruption of *sipT(Ba)* but not of *sipW(Ba)* correlated with a drastic reduction of heat resistant spores. The rare cells with spore structures after electronmicroscopical inspection exhibited forespore structures in stage III development with apparent changes in cortex or coat structures (Figure 26). These inhibition likely resulted from missing spore-specific export proteins. Sporulation-related export protein is suggested, but yet nearly unknown (Stragier & Losick, 1996; Dricks, 1999). In *B. subtilis*, sporulation deficiency was reported to correlate with *sipT-sipV* double deletion and suggested to indicate processing specificity of these two SPases for sporulation-related export proteins (Jiang, *et al.,* 2000). In this concern, it should be considered that SipS as well as SipT of *B. subtilis* are temporally controlled during transient growth in correlation with export of the mass of Sec proteins (Tjalsma, *et al.,* 1997) as well as in correlation with the initiation of sporulation. In analogy, SipT*(Ba)* in *B. amyloliquefaciens* might exclusively cope with processing of either a single or a couple of respective sporulation-specific export proteins (Stragier & Losick, 1996) either by growth phase specific expression and/or processing specificity. The same might be true for export of a not yet defined nuclease in *B. amyloliquefaciens*, which was most affected by *sipS(Ba)*, but to a lesser extend also by *sipT(Ba)* gene disruption. The respective nuclease of *B. amyloliquefaciens* is not likely to be a homologue of the 12 kDa-extracellular NucB of *B. subtilis* (van Sinderen, *et al.,* 1995), as the size of the protein was about 30 kDa and all attempt to amplify respective NucB-like DNA by reverse genetics from *B. amyloliquefaciens* were unsuccessful (data not shown). Moreover, impaired growth, inhibited cell autolysis and reduced cell motility specified *sipV*::pEAV* mutants of *B. amyloliquefaciens* to have changed autolysin activities. Indeed, changed pattern of CWBP's as well as loss of at least one (major) 35-kDa autolysin correlated with the SipV*(Ba)* deficiency. In *B. subtilis*, LytC (50 kDa amidase), LytD (90 kDa glucosaminidase)

or a *sigD* controlled (minor) 49-kDa autolysins are shown to change cell wall turnover, septation, cell lysis as well as swarming motility (Blackman, *et al.,* 1998). Processing of these unknown autolysins of *B. amyloliquefaciens* might correlate with the distinct SipV*(Ba)* mutant phenotype, but other autolysins are not excluded, as *B. subtilis* was suspected to contain several autolysins (Foster, 1992; Kunst, *et al.,* 1997).

In summary, more detailed studies are required to explain the mystery of multiple Sip proteins with more or less different characters in various *Bacillus* species. Genetic and biochemical data ad hands already indicated that the multiple SPases of *Bacillus* might be an prerequisite for adaptation of the secretion apparatus either due to their gene expression mode (Bolhuis, *et al.,* 1996; Tjalsma, *et al.,* 1997) or their different processing specificity (Bron, *et al.,* 1998; Tjalsma, *et al.,* 2000). Moreover, different localisation of SPases either in the cell- or membrane-specific compartment of the mother cell or forespore seems to be possible (Serrano, *et al.,* 1999; Stöver & Driks, 1999; Tjalsma, *et al.,* 2000). The presence of different Sip proteins might be interpreted in the same way as the obvious multiplicity of two-component signal transducer proteins in *B. subtilis*. In this case, 34 different candidates were found (Kunst, *et al.,* 1997) and each has regulatory functions for a distinct biosynthesis or pathway. We are therefore inclined to propose that multiple SPases are likewise serving for "specific" functions. Our mutant studies would need more precise definition of casual relationship between the Sip deficiencies and the mutant phenotype, but already indicated a specific and essential function of this distinct SPases in cell growth, cell division and spore formation. Since *B. amyloliquefaciens* strains were only recently recognised as an independent species (Stackebrandt, *et al.,* 1987; Priest, 1993), the differences of *B. amyloliquefaciens* Sip candidates with respect to the presence and specification of SPases in comparison to *B. subtilis* homologues could indicate that these closely related species already have slightly varied characters in their processing apparatus.

4.5. A new export protein of B. amyloliquefaciens

In conclusion of the data discussed in the previous sections, the presence of multiple paralogous SPases could be interpreted as a particular important feature for flexible adaptation of *Bacillus* and others Gram-positive bacteria in responses to environmental changes. In *B. subtilis*, 166 proteins had been predicted to be secretory proteins. While up to date only two proteins TasA and YqxM (Stöver & Driks, 1999a, b) were reported to be SipW specific, there are likely more undiscovered proteins, of which export requires one or another SPase. On the basis of earlier studies in *B. amyloliquefaciens* one distinct extracellular protein

band was reported to be absent in case of *sipT*-gene antisense expression strain compared to the parental strain (Hoang, *et al.,* unpublished data). The corresponding protein band was isolated from SDS-PAGE gels and after amino acid sequencing found to have the N-terminal sequence HGYIKEPVSRAYMGA. This sequence could not be identified at that time. However, recent BLAST search indicated that it is significantly similar to the N-terminus of the mature chitin-binding proteins CHB1 and CHB2 from *Streptomyces olivaceoviridis* and *Streptomyces reticuli* (Schnellmann, *et al.,* 1994; Kolbe, *et al.,* 1998). This inclined us to clone the correspondent gene and to analyse this export protein. The reverse genetic cloning approach was done using the known N-terminal amino acid sequence and earlier experience with the RAGE technique for PCR cloning (Hoang & Hofemeister, 1995). The gene was successfully cloned and analysed. Sequence analysis confirmed this export protein of *B. amyloliquefaicens* to be a not yet known protein with a signal peptide of 27 amino acids. As the ChbB protein was an export protein which was the first time isolated from *B. amyloliquefaciens* and a the first candidate of a chitin-binding protein of bacilli, we decided to analyse its fuctional characters in relation to growth in the presence of chitin, to chitin binding and export properties. The ChbB protein (Figure 26) shares 39, 37 and 45 % of identical amino acids with the proteins CHB1 from *S. olivaceoviridis* (Schnellmann, *et al.,* 1994), CHB2 from *S. reticuli* (Kolbe, *et al.,* 1998) and CBP21 from *Serratia marcescens* (Suzuki, *et al.,* 1998), respectively. Interestingly, our resent BLAST search for ChbB-like proteins indicated two deduced proteins of *B. anthracis* with high degree of sequence similarity to ChbB *of B. amyloliquefaciens*. The first one contains 221 amino acids and shares 52% identical residues with ChbB of *B. amyloliquefaciens* (Figure 26). The second contains 455 amino acids and is divided into two nearly equal portions, where the N-terminal part of the deduced protein has 61% of identical amino acids with ChbB and the C-terminal part seems to be a chitinase (Figure 31). This would be the first example of a chitinase containing a binding domain, which are similar to this newly discovered chitin-binding protein family (Schnellmann, *et al.,* 1994; Schrempf, 1999).

The ChbB protein adheres to chitin and moderately crossreacts with antibodies previously raised against the *Streptomyces olivaceoviridis* chitin binding protein CHB1 (Schnellmann, *et al.,* 1994). Like the *Streptomyces* CHB1 and CHB2 proteins, ChbB interacts with α-chitin, but, in difference to them, in addition with β-chitin, a feature characteristic for the CBP21 protein from *S. marcescens* which has highest affinity for β-chitin (Squid). The deduced ChbB protein shares several motifs with the *Streptomyces* CHBs and the *S. marcescens* CBP21 (Figure 31). The *Bacillus* ChbB carries a tyrosine residue (Y) (see Figure 31, row 1)

corresponding in its location to the W57 residue within the *Streptomyes* CHBs. The latter has been shown to be directly involved in the interaction with α-chitin (Zeltins & Schrempf, 1997). Its replacement by a leucine or a tyrosine residue also leads to nearly complete cessation of binding to α-chitin (Zeltins & Schrempf, 1997). The two *Serratia* proteins (CBP21 and Chi) also carry a tyrosine residue in this position corresponding to that of W57 in CHB1 (Figure 31) (Shin, *et al.,* 1996). It is interesting that the four additional W residues within the CHBs correspond in their relative positions to those present in the ChbB and the *Serratia* CBP21 protein (W99, W114, W134, W184, see Figure 31). Strikingly, ChbB lacks all the cysteine (C) residues which are found in the CHBs and CBP21. We have shown (Svergun, *et al.,* 2000) that within CHB1 S-S bridges are formed. Since ChbB lacks cysteine residues, S-S bridges stabilising the topology can be formed and it is expected that the shape of ChbB is more flexible than that of CHB1.

ChbB does not display relevant amino acid identities with various types of accessory chitin binding domains within chitinases from different organisms, including those from streptomycetes (Blaak & Schrempf, 1995; Saito, *et al.,* 1999) and *Bacillus circulans* (Watanabe, *et al.,* 1992). Neither does ChbB share relevant common motifs with a recently discovered *Streptomyces tendae* protein (9.8 kDa), targeting chitin within various fungi (Bormann, *et al.,* 1999), with the *Vibrio parahemolyticus* chitovibrin (134 kDa) (Montgomery & Kirchman, 1994). The latter is assumed to mediate adhesion to chitin-containing organisms, or with a small chitin binding polypeptide (of 73 residues) from the hemocyte of horseshoe crab (Suetake, *et al.,* 2000). In nature chitin is very diverse in its organisation (i.e. parallel [β] or anti-parallel [α] arrangement of N-acetylglucosamine chains, variable length, different degrees of crystallinity) and its associated compounds (i.e. protein, anorganic substances or glucan). It is therefore not surprising that a number of proteins have evolved determining subtle differences in recognition.

In contrast to *B. subtilis* strains 168 and GB72, all investigated *B. amyloliquefaciens* strains displayed varying levels of low and moderate chitinolytic activity and also secreted a protein of about similar size-range crossreacting with *anti*-ChbB antibodies. All *B. amyloliquefaciens* strains secreting a ChbB homologue share a DNA region which hybridised with the *chbB* gene. The different sizes of the hybridising DNA fragments as well as the varying efficiency of PCR amplification (using only one set of primers and the same conditions) reflected some evolutionary divergence of homologues to the *chbB* gene. Our sequence data showed that the *B. amyloliquefaciens chbB* gene is situated next to genes of so far unknown function, which have counterparts in the *B. subtilis* 168 genome. In *B. subtilis* they are found scattered (Kunst,

et al., 1997), while a *chbB* homologue could not be identified. It thus appears likely that the aquisition of the *chbB* gene by *B. amyloliquefaciens* leads to ChbB-mediated interaction with chitin-containing substrates (i.e. certain fungi and a number of chitin-containing organisms) which are subsequently degraded by their chitinolytic activity. Therefore, *B. amyloliquefaciens* strains are, in contrast to *B. subtilis*, expected to have a selective advantage in colonising and hydrolysing chitin-comprising substrates in their natural habitats, i.e. soil and marine environments (Gooday, 1990).

Finally, the main question was whether export of the ChbB protein indeed specifically or at least preferentially required one of the different type I SPases of *B. amyloliquefaciens*. Therefore, the export of this protein was studied in all of our *sip*-gene disruption strains. The results indicated only minor changes of the ChbB export (the ratio of pre- and mature ChbB) in the *sipT* mutant compared to the *B. amyloliquefaciens* parental strain. Thus, the previous observation of a strongly reduced ChbB export level in the *sip*T-antisense strain was not satisfactorily explained by this finding. We supposed that *sipT*-antisense expression caused or correlated with changed regulation of this protein due to culture conditions. The ChbB protein synthesis was indeed shown to be induced by the presence of substrates (chitin, glucan, avicel) in the culture medium (MSM) and to be repressed by the addition of glucose (tested for *B. amyloliquefaciens* ALKO 2718). These results indicate that the expression of the *chbB* gene is under substrate induction on one side and under catabolite control on the other. The latter assumption was strongly supported by the identification of *cre* boxes in the vicinity of a putative (Sigma A) promoter of the *chbB* gene (Hueck, *et al.*, 1994; Gösseringer, *et al.*, 1997; Stülke & Hillen 2000). The earlier observation of ChbB protein missing in *sipT* antisense culture might thus be due to repression or due to a secondary effect of the antisense RNA production.

The ChbB protein studies also indicated the complexity of environmental factors, which finally affect the quantity of an export protein. This nearly excluded any strategy to determine the export protein target of a distinct SPase after analysis of the export protein pattern of distinct *sip* mutant cultures. This was indeed one approach when we started these “Sip” studies. By this aim we made 1-D as well as 2-D electrophoresis analysis of the exported protein pattern of *B. amyloliquefaciens* wild type and *sip* mutant strains. However, we soon realised that the lacking of genome sequence information had made very difficult for precise identification of each export protein after micro-amino acid sequencing missing protein spot. Similar studies were recently carried out in *B. subtilis*. The results showed about 110 spots on an 2D-gel, of which over 90% were secretory protein candidates because they disappeared in

the absence of SecA (Hirose, *et al.*, 2000). Indeed, the genome and proteome analysis, which are more advanced in *B. subtilis* 168, would make this species more preferable for studies and rank *B. amyloliquefaciens* in a secondary position.

Nevertheless, it remains worth asking, which peculiarities of the export machinery or other properties make *B. amyloliquefaciens* to be about 10 to 20 times more efficient in protein secretion than *B. subtilis*, a question was already asked 25 years ago by Ingle and Boyer (1976). This study was aimed towards an answer.

5. Summary

- The main question behind the present studies was the mystery of the presence of multiple signal peptidases in various eubacterial species, notable in bacilli. The “biological sense” of multiple *sip* genes in distinct groups of bacteria are proposed to guarantee an advance by modulating the capacity of protein secretion under various conditions during vegetative growth and cellular differentiation of the bacterial cells. Therefore we proposed Sip proteins to have “bottleneck” functions under certain growth conditions. In this work, we attempted to analyse *B. amyloliquefaciens* in comparison to *B. subtilis* 168, where the completed genome project (Kunst, et al., 1997) convincingly proved the existence of two major (SipS, SipT) and three minor Sip proteins (SipU, SipV, SipW).

- In addition to *sipS* and *sipT*, which were previously isolated (Hoang & Hofemeister, 1995; Meijer, *et al*., 1995), here we present evidences for the existence of two more *sip*-like genes in chromosomal DNA of *B. amyloliquefaciens* strain ATCC 23843, which are *sipV* and *sipW*. The deduced amino acid sequences of SipV*(Ba)* and SipW*(Ba)* share 77% and 73% amino acid identities with their *B. subtilis* counterparts, respectively. A *sipU*-like gene was not found. Consequently, our data would suggest that *B. amyloliquefaciens* differs from *B. subtilis* with respect to the number of *sip*-like genes, i.e. its genome contains *sipS*, *sipT*, *sipV* and *sipW*, but likely misses a *sipU*-like gene candidate.
- With respect to the exceptional properties of SipW-like SPases, it was interesting to find SipW-like DNA also in the genomic DNA of 10 strains from four distant 16S rRNA- groups of the phylogenetic tree of bacilli, but not in group 5 strains *B. stearothermophilus* and *Thermoactinomyces vulgaris*. The proposed absence of SipW-like genes in group 5 of the *Bacillus* phylogenetic tree, however, needs further confirmation.
- The alignment of as much as 23 *Bacillus* type I SPases proteins did not only strengthen a basic distinction between P- and ER- type SPases, but also further the branching of P-type Sip proteins. The latter group likely divides into at least three subgroups, where two groups are represented by SipV and SipS/SipT/SipU of *B. subtilis* and a third group by Sip3/Sip5-SPases of *B. anthracis*.

- The question about processing specificities or distinctions between *B. amyloliquefaciens* Sip proteins was addressed by expression of each of the four *B. amyloliquefaciens* Sip proteins in a *E. coli* LepB*ts* mutant strain after testing the processing of pre-OmpA and growth restoration of the mutant at the non-permissive temperature. In summary, only

SipS*(Ba)* and SipT*(Ba)*, but neither SipV*(Ba)* nor SipW*(Ba)* were active in processing pre-OmpA in *E. coli*. In contrast, the growth of the LepB*ts* mutant was only restored after SipS*(Ba)* but not after SipT*(Ba)* expression. These likely reflects differences in processing specificities, since different production levels of these Sip*(Ba)* proteins were excluded. The lack of processing activities of SipV*(Ba)* and SipW*(Ba)* likely correlated with enhanced degradation.

- The complementation data indicated either the N-terminal transmembrane or the C-terminal catalytic portion of Sip proteins to limit processing functions in *E. coli*. This hypothesis was tested after construction of *sipT(Ba)-lepB* hybrid genes. The hybrid studies indicated that in case of SipT*(Ba)* lacking of processing capacity rather than membrane insertion properties causes non-complementation.

- All of the *B. amyloliquefaciens* Sip*(Ba)* disruption mutants were viable, but some had impaired growth, sporulation and changed cell division properties. The strict correlation of a distinct mutant phenotype with that distinct *sip::*pEA* integration event as well as restoration of the mutant phenotype after spontaneous excision of the insertion cassette from *B. amyloliquefaciens sipT*::pEAT* mutants, strongly indicated gene disruption to correlate with that distinct deficiencies.
- Disruption of *sipT(Ba)* resulted in a drastic reduction of heat resistant spores and apparent changes in cortex or pre-coat structures.
- SipV deficiency of *B. amyloliquefaciens* correlated with impaired growth, inhibited cell autolysis, reduced motillity specified and changed autolysin activities. The change of the CWBP's pattern correlated with the loss of at least one (major) 35-kDa autolysin, which could cause that distinct SipV*(Ba)* mutant phenotype.
- A 30 kDa extracellular DNase was strongly affected in *sipS* disruption mutants, but to a lesser extent also seen in *sipT* mutants. The respective nuclease of *B. amyloliquefaciens* was not identified.

- In an effort to search in *B. amyloliquefaciens* for export proteins, which require the function of a distinct SPases, a new export protein was identified. The correspondent gene was cloned. Its sequence similarity indicated ChbB to be a homologue of chitin-binding proteins CHB1, CHB2 and CBP21 from *S. olivaceoviridis*, *S. reticuli* or *Serratia marcescens*, respectively. This ChbB protein of *B. amyloliquefaciens* is the first of its type isolated from *Bacillus*. Binding studies indicated that ChbB protein targets β-chitin best, then α-chitin, but barely any other polysaccharide.

- The export of ChbB was studied in the four *sip*-gene disruption strains. The results indicated only minor changes of the ChbB export in the *sipT* mutant comparing to the parental strain. However, this finding did not satisfactorily explain the previous observation, where ChbB export was found to be strongly reduced in a *sipT*-antisense strain. Beside of a slight "bottle neck" function of SipT*(Ba)*, it is highlight that ChbB protein synthesis is affected by substrate induction and catabolite repression.

- Since *B. amyloliquefaciens* strains were only recently ranked as an independent species (Stackebrandt, *et al.*, 1987; Priest, 1993), the differences of *B. amyloliquefaciens* Sip candidates with respect to the presence and specificity of SPases in comparision to *B. subtilis* homologues could indicate that these closely related species already have slightly varied characters in their processing apparatus. More detailed studies are still required to explain the mystery of multiple Sip proteins. However, in addition to processing specificity our results suggest that SPases of *Bacillus* may also differ in their interaction with preprotein translocases or their localisation within the membrane such as the septa of dividing cells or the membrane of pre-spore compartments.

Zusammenfassung

Den vorliegenden Untersuchungen liegt die Vermutung zu Grunde, dass multiple (paraloge) Gene bei Prokaryoten im Prinzip nur dann vorkommen, wenn sie dem Wirt einen selektiven Vorteil bieten, sich innerhalb einer taxonomischen Gruppe sowohl hinsichtlich Anzahl, als auch Typus unterscheiden könnten und diese Unterschiede wiederum Aufschluss über die Funktion isoformer Enzyme geben könnten.

Gegenstand der Arbeit sind Typ-I Signalpeptidase-Isoformen bei *B. amyloliquefaciens*. Die Arbeit gliedert sich in drei Komplexe: 1. Nachweis von Signalpeptidase (*sip*-) Genen, Konstruktion, die Charakterisierung von Gendisruptionsmutanten, 2. die funktionelle Analyse von Sip-Isoformen und erster Sip-LepB-Hybridenzyme zur Komplementation einer *E. coli* LepB*ts*- Mutante bezüglich Processing und Wachstum und 3. die Isolierung und Analyse eines vermutlich Sip- spezifischen Exportproteins, dessen N- terminale Aminosäuresequenz den Ausgangspunkt für die Klonierung bot.

Der Nachweis von fünf paralogen *sip*- Genen (*sip*S, *sip*T, *sip*U, *sip*V und *sip*W) bei *B. subtilis* beruht auf der 1997 abgeschlossenen Genomsequenzierung [Kunst et al. (1997) Nature 390, pp249]. Bei *B. amyloliquefaciens* haben Hoang, V. & Hofemeister, J. [(1995) BBA **1269**, 64-68] durch reverse- genetische Methoden, den Nachweis von SipS und SipT erbracht. In vorliegender Arbeit, ist die Isolierung zweier weiterer *sip*- Gene, *sip*V*(Ba)* und *sip*W*(Ba)*, auf ähnlicher Grundlage gelungen. Ein zu *sip*U- ähnliches Gen wurde nicht gefunden. **Obwohl die Nachweismethode einen endgültigen Beweis nicht geben kann, wird spekuliert, das darin Unterschiede zu *B. subtilis* bezüglich der *sip*- Genausstattung erkennbar werden**, wobei die vorhandenen *sip*- Gene in einer zu *B. subtilis* übereinstimmenden (konservierten) Genomposition vorgefunden wurden.

In einer vergleichende PCR-Studie, wird das Fehlen von SipW, einer zu eukaryotischen (ER-Membran-) SPasen verwandten Sip- Isoform, bei 2 von 12 *Bacillus*- Stämmen, und zwar in der (Extremophilen-) Gruppe V (bei *B. stearothermophilus* und *Thermoactinomyces vurlgaris*), festgestellt. **Nach diesen Ergebnisse variieren Art und Anzahl von Sip-Isoformen bei *Bacillus* sowohl bei Gruppen mit phylogenetischer Distanz aber auch zwischen nahe verwandten Arten.**

Eine phylogenetische Analyse der 23 bekannten *Bacillus*- Sip- Sequenzen unterstreicht die bereits früher getroffene Unterscheidung von P- und ER- Typ SPasen und lässt eine weitere Einteilung der P-Typ SPasen in SipV-, SipS-T-U- und Sip3-Sip5- ähnliche Untergruppen sinnvoll erscheinen. Es wird postuliert, dass sich Sip- Proteine einer (phylogenetischen) Gruppe effektiver, als Isoformen einer anderen Subgruppe funktionell ersetzen können.

Komplementationsstudien belegen, dass phylogenetisch manifeste Unterschiede zwischen SipV und SipS-T-U SPasen auch funktionelle Konsequenzen haben, d.h. mit Unterschieden hinsichtlich der Membraninsertion bei *E. coli* korrelieren. Darüber hinaus sind aus einem Vergleich der „Ersetzbarkeit" von *E. coli* Signalpeptidase LepB durch SipS*(Ba)* bzw. SipT*(Ba)*, auch Unterschiede zwischen SPasen einer phylogenetischen Gruppe hinsichtlich Erkennen- und/oder Processing von Exportproteinen in *E. coli* abzuleiten. Durch Testung erster SipT- LepB bzw. LepB- SipT- Hybrid-SPasen in *E. coli* wird für SipT*(Ba)* eine im Vergleich zu LepB und SipS*(Ba)* eingeschränkte Processing- Spezifiät der katalytischen Domäne vermutet.

Ausfallfunktionen von *sip*- Gendisruptionsmutanten von *B. amyloliquefaciens* unterstreichen Unterschiede in der Funktion einzelner Sip- Isoformen bei *B. amyloliquefaciens,* d.h. konkret für SipT*(Ba)* hinsichtlich des Exportes von Sporulations-spezifischen Proteinen, für SipV*(Ba)* in Beziehung zur Lokalisierung Zellteilungs-beteiligter Autolysine und für SipS*(Ba)* und SipT*(Ba)* bezüglich des Exportes einer (unbekannten) 35 kDa-Deoxyribonuklease. Das Fehlen vergleichbarer Ergebnisse bei *B. subtilis* könnte auf Unterschieden in der Spezialisierung vorhandener Sip- Isoformen, der Eigenart der konstruierten (Disruptions-) Mutanten oder dem Fehlen vergleichbarer Analysen zurückzuführen sein. Die Mutationsergebnisse zeigen einen gangbaren Weg, um die noch unbekannte Spezifität von Sip-Isoformen herauszufinden und nach Isolierung betroffener Komponenten im Detail zu untersuchen.

Mit dem in dieser Arbeit als potentiell spezifisches Sip-Target isolierten Chitin-Bindeprotein (ChbB) wird ein für *B. amyloliquefaciens* bisher unbekanntes Exportprotein beschrieben, welches bei *B. subtilis* fehlt aber entgegen der Annahme, nicht Sip-spezifisch exportiert wird.

Eine hervorstechende Besonderheit von *B. amyloliquefaciens* gegenüber *B. subtilis* ist eine 100 bis 1000fach höhere Exkretion von Amylase und (alkalische und neutrale) Protease, was

zur Nutzung für die industrielle Enzymproduktion geführt hat. Dabei handelt es sich um nahe verwandte Arten, wobei *B. amyloliquefaciens* noch vor einigen Jahren als Unterart von *B. subtilis* betrachtet wurde, die Übereinstimmung homologer Proteine im Durchschnitt ca. 85 % beträgt und die Transformierbarkeit von DNA (in *B. subtilis*) um ca. drei Zehnerpotenzen verringert ist. **Vorliegende Ergebnisse lassen vermuten, dass die veränderte Anzahl und Spezifität der vorhandenen Signalpeptidasen (Sip- Proteine, *sip*- Gene) in einem noch nicht durchschaubaren Zusammenhang zur unterschiedlichen Proteinexportleistung dieser beiden *Bacillus*- Arten steht.**

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List of publications

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Statement

I declare that I wrote this thesis myself. I did not use other auxiliary material than indicated. Other work is always cited.

I have not tried to get a Ph.D. degree before.

Gatersleben, August, 2001.

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