Journal of Molecular Microbiology and Biotechnology

J Mol Microbiol Biotechnol 2009;16:53–68
DOI: 10.1159/000142894

Published online: October 29, 2008

Cell Physiology and Protein Secretion of *Bacillus licheniformis* Compared to *Bacillus subtilis*

Birgit Voigt[a] Haike Antelmann[a] Dirk Albrecht[a] Armin Ehrenreich[b]
Karl-Heinz Maurer[c] Stefan Evers[c] Gerhard Gottschalk[b] Jan Maarten van Dijl[e]
Thomas Schweder[d] Michael Hecker[a]

[a]Institut für Mikrobiologie, Ernst-Moritz-Arndt-Universität, Greifswald, [b]Institut für Mikrobiologie und Genetik, Georg-August-Universität, Göttingen, [c]Henkel KGaA, VTB Enzymtechnologie, Düsseldorf, and [d]Institut für Pharmazie, Ernst-Moritz-Arndt Universität, Greifswald, Germany; [e]Department of Medical Microbiology, University Medical Centre Groningen and University of Groningen, Groningen, The Netherlands

Key Words
Bacillus licheniformis · *Bacillus subtilis* · Cell physiology · Extracellular proteome · Protein secretion

Abstract
The genome sequence of *Bacillus subtilis* was published in 1997 and since then many other bacterial genomes have been sequenced, among them *Bacillus licheniformis* in 2004. *B. subtilis* and *B. licheniformis* are closely related and feature similar saprophytic lifestyles in the soil. Both species can secrete numerous proteins into the surrounding medium enabling them to use high-molecular-weight substances, which are abundant in soils, as nutrient sources. The availability of complete genome sequences allows for the prediction of the proteins containing signals for secretion into the extracellular milieu and also of the proteins which form the secretion machinery needed for protein translocation through the cytoplasmic membrane. To confirm the predicted subcellular localization of proteins, proteomics is the best choice. The extracellular proteomes of *B. subtilis* and *B. licheniformis* have been analyzed under different growth conditions allowing comparisons of the extracellular proteomes and conclusions regarding similarities and differences of the protein secretion mechanisms between the two species.

Introduction

Bacillus subtilis and *Bacillus licheniformis* are closely related species that feature similar saprophytic lifestyles in soils. Both species are known for their ability to secrete numerous proteins into the surrounding medium, some in quite high amounts [Antelmann et al., 2001; Voigt et al., 2006]. This enables them to degrade a variety of high-molecular-weight substances found in soils and use the degradation products as nutrients to sustain growth or to ensure their survival under conditions of nutrient limitation. In addition, the ability to secrete large amounts of proteins has been exploited by the fermentation industry for a long period of time, especially for the production of industrial enzymes.

Bacillus cells can be divided into several subcellular compartments: the cytoplasm, the cytoplasmic mem-

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

1464–1801/09/0162–0053$26.00/0

Accessible online at:
www.karger.com/mmb

Dr. Birgit Voigt
Ernst-Moritz-Arndt-Universität, Institut für Mikrobiologie und Molekularbiologie
Friedrich Ludwig Jahn Strasse 15, DE–17487 Greifswald (Germany)
Tel. +49 3834 86 4237, Fax +49 3834 86 4202
E-Mail birgit.voigt@uni-greifswald.de

brane, the membrane-cell wall interface and the cell wall. Because Gram-positive bacteria lack an outer membrane, their thick cell wall is thought to perform a role similar to the periplasm of Gram-negative bacteria. Yet, the lack of an outer membrane enables the secretion of large amounts of proteins directly into the extracellular medium.

Since protein synthesis occurs in the cytoplasm, proteins which function in other cellular compartments or in the extracellular milieu have to be targeted for export and transported across the membrane. Knowledge of the genome sequence allows the prediction of the secreted proteins and the mechanisms of protein secretion can be deduced. By means of proteomics these predictions and the contribution of the different secretion pathways to the extracellular proteome can be verified. In *B. subtilis*, four protein secretion pathways are known – the secretory protein translocation (Sec) pathway, the twin-arginine translocation (Tat) pathway and the pseudopilin pathway and ABC transporters [van Dijl et al., 2002]. The main secretion pathway is the Sec pathway. Transport through the Sec machinery is only possible for unfolded preproteins. The twin-arginine translocation, or Tat pathway, is used for the transport of proteins already tightly folded in the cytoplasm, which cannot be transported through the Sec pathway. However, the Tat pathway, as well as the pseudopilin export pathway and ABC transporters, are specific pathways which are used for the translocation of only few proteins. Proteins targeted for export from the cytoplasm are synthesized as preproteins with amino-terminal signal peptides. These N-terminal signal peptides direct proteins specifically into the appropriate translocation pathways [van Dijl et al., 2002]. The preproteins are recognized by targeting factors in the cytoplasm and directed to the transport systems in the membrane. In an energy-dependent manner the protein is then transported through the membrane. During or shortly after the translocation of the protein the signal peptide is cleaved by specific signal peptidases and the protein is released into the extracellular medium if it does not contain an additional retention signal, e.g. for the cell wall or a lipid anchor that retains the corresponding proteins (lipoproteins) at the outer surface of the cytoplasmic membrane.

B. subtilis has become the model organism of Gram-positive bacteria, mainly because of the early availability of its genome sequence [Kunst et al., 1997] and due to the fact that it can be easily genetically modified. In 2004, the *B. licheniformis* genome sequence was published [Rey et al., 2004; Veith et al., 2004]. Comparison of the two genomes revealed that for 1,091 ORFs of *B. licheniformis* there is no ortholog in *B. subtilis* [Veith et al., 2004], showing that even closely related bacteria can have significant differences in their genome layout. Completion of the *B. licheniformis* genome sequence now allows the comparison of the physiology of both *Bacillus* species during growth and their responses to different growth conditions. In addition, we use a proteomic approach to compare the extracellular proteomes and the protein secretion mechanisms of both organisms.

General Metabolism of *B. licheniformis* Compared to *B. subtilis*

Cell physiology of *B. licheniformis* has been studied under different growth conditions [Voigt et al., 2004, 2007; Hoi et al., 2006]. It could be shown that many aspects of the general metabolism are similar in *B. licheniformis* and *B. subtilis*, but there are also obvious differences. Regulation of glycolysis and the TCA cycle in response to glucose, for instance, seem to be similar [Tobisch et al., 1999; Voigt et al., 2004]. The amounts of some glycolytic enzymes and of the pyruvate dehydrogenase were significantly increased in *B. licheniformis* cells grown in the presence of glucose. At the same time almost all TCA cycle enzymes and the ATP synthase were repressed. The same glucose-dependent regulation of glycolysis and TCA cycle enzymes was shown for *B. subtilis* [Tobisch et al., 1999]. Furthermore, in *B. licheniformis* cells grown in an abundance of glucose, the acetate kinase (AckA) and the phosphotransacetylase (Pta) are enhanced. These enzymes are known to be involved in the overflow metabolism in *B. subtilis*, where they are also induced when glucose is present in the growth medium in high concentrations [Tobisch et al., 1999]. The data show that in *B. licheniformis* a Crabtree effect occurs as in *B. subtilis*. The reaction of *B. licheniformis* to various nutrient starvation conditions is also partially similar to that of *B. subtilis*. *B. licheniformis* cells subjected to glucose, nitrogen or phosphate starvation employ similar mechanisms as *B. subtilis* to counteract and survive such unfavorable conditions [Hoi et al., 2006; Voigt et al., 2006, 2007]. For example, glucose-starving cells of both species induce genes/proteins which are aimed at the mobilization of carbon from alternative sources. Even if there are differences in the precise set of induced genes, the overall answer to all three starvation conditions is similar. In *B. subtilis*, in addition to the specific reaction to glucose and phosphate starvation, the general σ^B-dependent stress response is

induced. Such induction of σ^B-dependent genes was not observed in *B. licheniformis* which is probably due to the absence of the *rsbQP* operon in the *B. licheniformis* genome. In *B. subtilis*, RsbQ and RsbP form the energy-signaling branch of the σ^B regulon, which is responsible for σ^B induction under nutrient starvation conditions [Brody et al., 2001; Völker et al., 1995].

These differences in energy signaling could reflect a fundamental difference in the energy metabolism of *B. subtilis* and *B. licheniformis*, because the genome of the latter contains genes homologous to isocitrate lyase and malate synthase organized in an operon [Veith et al., 2004]. These enzymes form a glyoxylic acid shunt, which is required for the degradation of substrates that enter central metabolism as acetyl-CoA units such as longer chained fatty acids, acetate and 2,3-butanediol [Fründ et al., 1989; Gottschalk, 1986]. The last two mentioned substrates are of special importance as they are overflow metabolites, formed as a consequence of the Crabtree effect during growth on high glucose concentrations [Hecker and Völker, 2004]. In contrast to *B. subtilis*, *B. licheniformis* can grow on 2,3-butanediol or acetate as sole carbon source. It is interesting to note that both organisms contain the genes for acetate activation via acetyl-CoA synthetase (*acsA* gene) and for butanediol breakdown (*aco* and *acu* operons) [Grundy et al., 1993; Huang et al., 1999], but only *B. licheniformis* is able to use these substrates as sole carbon sources [Veith et al., 2004]. Therefore, this organism is able to completely oxidize the overflow metabolites accumulated during growth on glucose and as a result generate more energy from a given amount of this substrate.

Another metabolic feature that, among bacilli, is unique to *B. licheniformis* is the capability to grow on propionic acid as sole carbon source [Sneath, 1986]. This is reflected in the genome by an operon coding for putative methylcitrate synthase *(mmgD)*, methylaconitate-hydratase *(mmgE)* and a methylisocitrate lyase *(yqiQ)*, as suggested by sequence homology to the enzymes of the methylcitric acid cycle in *S. typhimurium*. This operon could confer the ability to degrade propionyl-CoA units coming from activation of propionic acid, from degradation of branched chain fatty acids or from the degradation of certain amino acids [Horswill and Escalante-Semerena, 1999]. The genes needed for fatty acid degradation via β-oxidation are organized in a second operon in *B. licheniformis*. Interestingly, the genes for a putative methylcitric acid cycle also occur in *B. subtilis*, but as part of a large operon for β-oxidation of fatty acids. It comprises the genes for an acetyl-CoA acetyltransferase *(mmgA)*, hydroxybutyryl-CoA-dehydrogenase *(mmgB)* and an acyl-CoA dehydrogenase *(mmgC)* beside the enzymes for the methylcitric acid cycle. The operon is under control of a σ^E-specific promoter that is expressed specifically in the mother-cell compartment during sporulation [Bryan et al., 1996]. This situation might reflect that *B. subtilis* uses this operon to metabolize branched chain fatty acids or amino acids that are degraded via propionyl-CoA during sporulation [Koburger et al., 2005], but is unable to grow on propionate, whereas the separation of the operons with distinct putative regulators gives *B. licheniformis* a higher flexibility in regulation and application of this metabolic feature during vegetative growth [Claus, 1986].

An additional difference of the genome of *B. licheniformis* as compared to *B. subtilis* is that the former encodes a number of proteins that are characteristic for an anaerobic or facultative anaerobic mode of life. The most prominent of these is an anaerobic ribonucleotide reductase (BLi03824) with its associated activase (BLi03823), in addition to the aerobic ribonucleotide reductase isoenzyme encoded by the genomes of *B. subtilis* and *B. licheniformis* [Veith et al., 2004; Torrents et al., 2000]. The presence of the anaerobic isoenzyme suggests that *B. licheniformis* is a true facultative anaerobic organism that can grow during prolonged anaerobic periods [Nordlund and Reichard, 2006]. Altogether, the differences in the metabolism between the two *Bacillus* species as reflected by their genome sequences seem to suggest that *B. licheniformis* has a more flexible metabolism which is notably more adapted for good exploitation of its substrate. This might be the reason why *B. licheniformis* is better suited to industrial high-density fermentations than *B. subtilis* [Schallmey et al., 2004].

Protein Secretion by *B. licheniformis* and *B. subtilis*

Prediction of the Secretion Machinery Components

Protein secretion of *B. subtilis* has been studied for many years and the different secretion systems are well understood [Dubnau, 1999; Jongbloed et al., 2002; van Dijl et al., 2002]. Since the *B. licheniformis* genome sequence is now available, the existence of the components of the different protein secretion systems can be predicted based on the knowledge gained from the *B. subtilis* studies. All genes required for Sec-dependent protein secretion have been found in the *B. licheniformis* sequence [Rey et al., 2004; Veith et al., 2004]. Not only the genes for the Sec protein translocase complex itself are present in

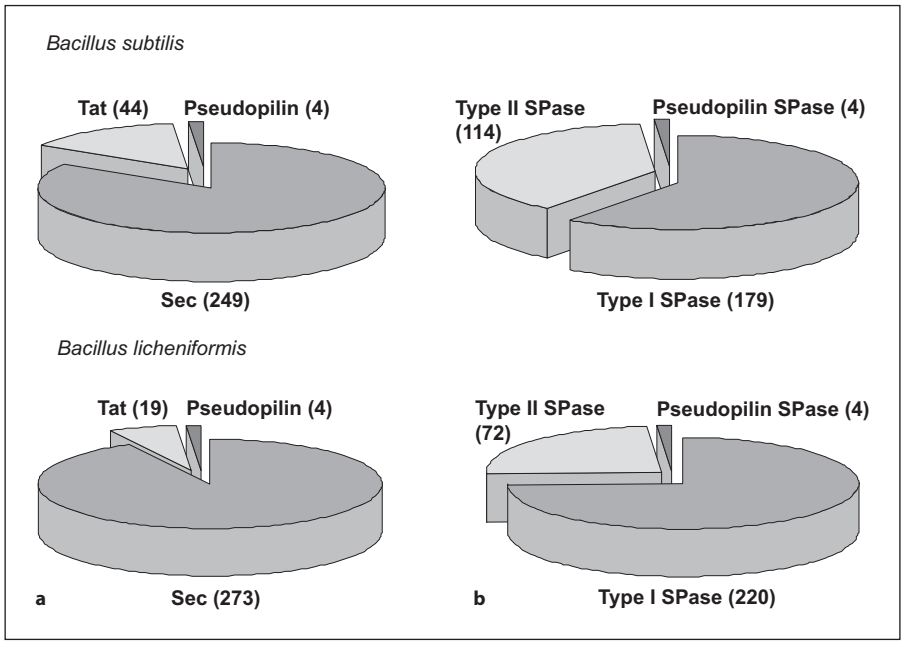

Fig. 1. Prediction of the proteins with signal peptides of *B. licheniformis* and *B. subtilis* [Tjalsma et al., 2000; van Dijl et al., 2002; Voigt et al., 2006]. **a** Contribution of the different secretion systems (except for the ABC transport). The numbers in parentheses are the number of proteins which have signal peptides that can potentially direct transport via the appropriate secretion system. **b** Fractions of the signal peptide exhibiting proteins with recognition sites for the different signal peptidases. The numbers in parentheses are the number of proteins with recognition sites for the appropriate signal peptidase (SPase). Note that the number of *B. subtilis* lipoproteins may be significantly lower than the originally estimated number of 114 as marked in this figure. Sec = Sec-dependent protein secretion; Tat = twin-arginine translocation pathway; Pseudopilin = pseudopilin export pathway.

B. licheniformis, but also orthologs for signal recognition particle components, for type I signal peptidases and for extracytoplasmic chaperone proteins involved in the folding and quality control of the exported proteins. However, instead of the five type I signal peptidase genes found in *B. subtilis*, in *B. licheniformis* only four such genes could be identified (*sipU* is absent). The presence of a prolipoprotein diacylglyceryl transferase gene *(lgt)* and of genes for type II signal peptidases (two homologs of *lspA*) suggests that a mechanism similar to that of *B. subtilis* is employed for the lipid modification of lipoproteins in *B. licheniformis*. In addition to the Sec pathway, the *B. licheniformis* genome encodes four genes for Tat pathway components *(tatAd, tatCd, tatAy, tatCy)*. In *B. subtilis* there is a fifth Tat gene, *tatAc*, for which no ortholog could be identified in *B. licheniformis*. Furthermore, the genes for the pseudopilin translocation pathway are present in the *B. licheniformis* genome sequence. Therefore, it can be suggested that the general principles of protein secretion are conserved in both *Bacillus* species.

Prediction of the Secreted Proteins

The genome sequencing of *B. subtilis* and *B. licheniformis* allowed the prediction of all proteins containing signals directing them to one of the so far known systems for protein secretion. From the genome data 4,107 ORFs were identified for *B. subtilis* and 4,286 ORFs for *B. licheniformis* [Kunst et al., 1997; Rey et al., 2004; Veith et al., 2004]. Based on signal peptide predictions, 297 proteins have the potential to be exported from the cytoplasm in *B. subtilis* [Tjalsma et al., 2000; van Dijl et al., 2002] and 296 in *B. licheniformis* [Voigt et al., 2006]. This prediction shows that the number of potentially secreted proteins is similar in both organisms. In both organisms, most of these signal peptides would direct proteins to the Sec secretion machinery (fig. 1). The number of potentially sec-dependently secreted proteins is slightly higher in *B. licheniformis* than in *B. subtilis* (273 and 249, respectively). A potential twin-arginine motif (RR or KR) was found in the signal peptides of 44 *B. subtilis* proteins, but only in 19 signal peptides of *B. licheniformis* proteins. Notably however, the *B. subtilis* Tat pathway probably exports significantly less proteins than the 44 predicted proteins with potential twin-arginine signal peptides. In fact, only two genuine Tat substrates of *B. subtilis* (i.e. PhoD and YwbN) have been identified to date [Jongbloed et al., 2004].

Of the 297 predicted signal peptides of *B. subtilis*, 179 have recognition sites for cleavage by a type I signal peptidase (SipS, SipT, SipU, SipV or SipW), and lack a potential cleavage site for the lipoprotein-specific signal peptidase II (LspA). More recent predictions made for *B. licheniformis* [Voigt et al., 2006], based on insights derived from studies on the *B. subtilis* membrane proteome [Tjalsma and van Dijl, 2005], suggest that a considerably higher number of signal peptides, 220, have type I signal

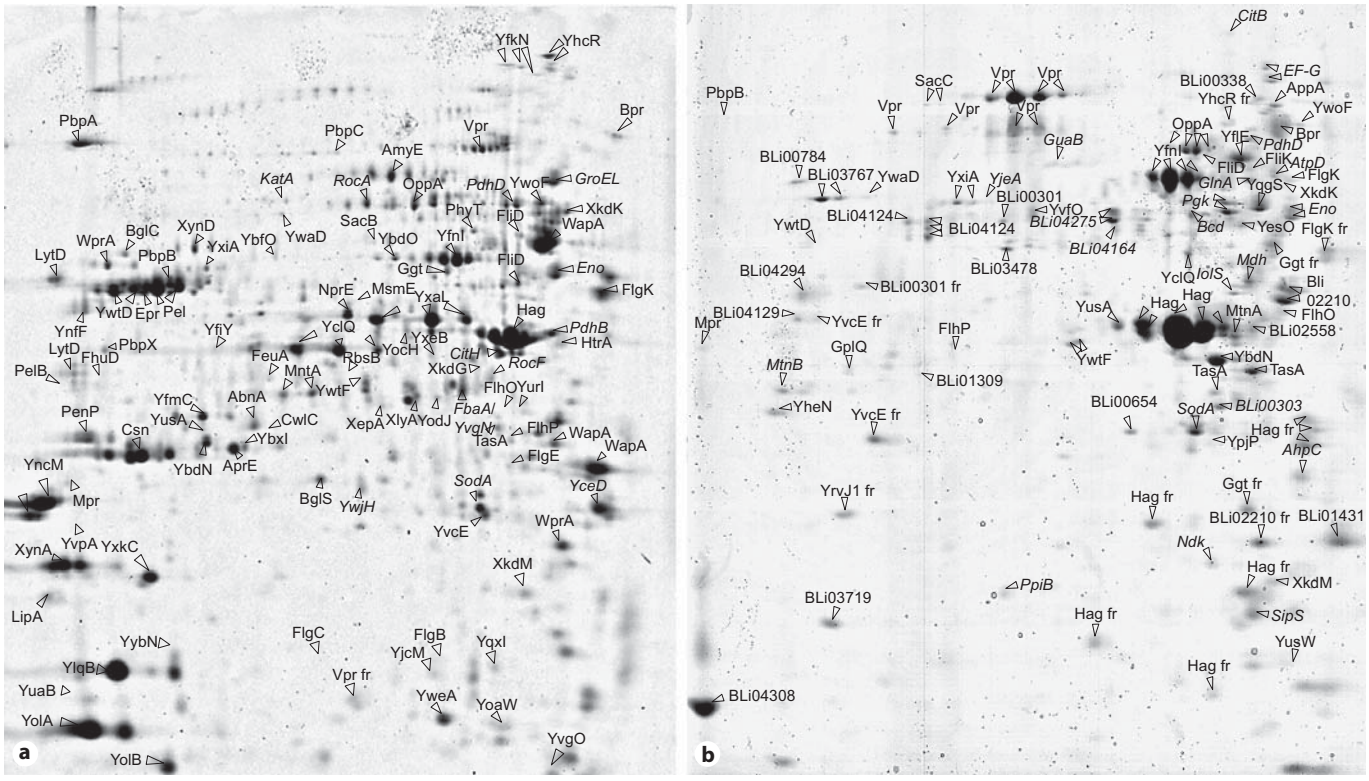

Fig. 2. The extracellular proteomes of *B. subtilis* (**a**) and *B. licheniformis* (**b**) grown in complete medium (LB). Extracellular proteins were prepared 1 h after entry into the stationary growth phase. After precipitation with TCA, the extracellular proteins were separated in a pH gradient 3–10 and stained with Sypro ruby (**a**) or with colloidal Coomassie brilliant blue (**b**). Spots labeled in italics are presumably intracellular proteins. fr = Fragment. *B. licheniformis* proteins with similarity to a *B. subtilis* protein were named accordingly. Proteins with no homolog in *B. subtilis* received the gene ID of the sequencing project.

peptidase recognition sites and lack signal peptidase II cleavage sites. Accordingly, the number of signal peptides with recognition sites for cleavage by a lipoprotein-specific signal peptidase II (LspA) appeared to be significantly lower in *B. licheniformis*. Only 72 proteins with lipoprotein signal peptides were predicted for *B. licheniformis* compared to 114 such proteins in *B. subtilis*. It should be noted, however, that application of the more stringent proteomics-based criteria for the prediction of lipoproteins has indicated that the actual number of lipoproteins in *B. subtilis* may be much lower than originally predicted [Tjalsma and van Dijl, 2005]. According to these predictions, *B. subtilis* would have the potential to produce 68 different lipoproteins, which would be in the range of the number of lipoproteins predicted for *B. licheniformis*. Thus, it seems that a full definition of the lipoproteomes of *B. subtilis* and *B. licheniformis* will require more research in which the actual lipid-modification of exported proteins is demonstrated.

Composition of the Extracellular Proteome of B. subtilis *and* B. licheniformis

Gram-positive bacteria are able to secrete large amounts of extracellular proteins directly into the growth medium because they lack an outer membrane. Cells of both *Bacillus* species discussed here secrete the highest amounts of protein in the stationary growth phase when grown in a rich medium (Luria broth, complete medium – LB).

About 200 protein spots are visible in the 2-D gels when extracellular proteins from both *Bacillus* species are analyzed. Some of the secreted proteins occur as multiple spots, like Vpr, Hag and YfnI. Altogether, 113 different proteins were identified in *B. subtilis* and 143 in *B. licheniformis*, although not all spots could be identified [Antelmann et al., 2006; Voigt et al., 2006] (tables 1–4; fig. 2). The overall composition of the extracellular proteome is similar in both bacteria, pointing to similar lifestyles in the soil. The proteins secreted by both *Bacillus*

Table 1. Extracellular proteins of *B. subtilis* with signal peptides from cells grown in LB, SNB medium (^{SNB}) and phosphate-limited minimal medium

		Retention signal[a]	% abundance LB[b]	% abundance phosphate[b]
Metabolism of carbohydrates				
AbnA	arabinan-endo 1,5-α-L-arabinase		0.304	
AmyE	α-amylase		0.386	0.139
BglC	endo-1,4-β-glucanase, cellulase		0.078	
BglS	endo-β-1,3-1,4-glucanase		0.209	0.201
Csn	chitosanase		1.886	0.87
Pel	pectate lyase		1.23	4.58
PelB	pectate lyase		0.23	0.637
SacB^{SNB}	levansucrase		0.002	
XynA	endo-1,4-β-xylanase		1.601	0.724
XynD	endo-1,4-β-xylanase		0.194	0.123
YdhT	mannan endo-1,4-β-mannosidase		0.001	
YnfF	endo-xylanase		0.214	
YvpA	pectate lyase		0.003	
YxiA	arabinan-endo 1,5-α-L-arabinase		0.057	
Metabolism of proteins and peptides				
AprE	serine alkaline protease (subtilisin E)		0.441	0.226
Bpr	bacillopeptidase F		0.133	0.221
Epr	minor extracellular serine protease		0.883	
Ggt	γ-glutamyltranspeptidase		0.055	
Mpr	extracellular metalloprotease		0.056	
NprE	extracellular neutral metalloprotease		0.466	0.141
Vpr	extracellular serine protease		0.405	0.398
YwaD	aminopeptidase		0.112	
Metabolism of nucleotides and nucleic acids				
YfkN^{pho}	2′,3′-cyclic-nucleotide 2′-phosphodiesterase	TM	0.041	1.582
YhcR	5′-nucleotidase	TM	0.142	
YurI	ribonuclease		0.054	
Metabolism of lipids				
GlpQ^{pho}	glycerophosphoryl diester phosphodiesterase		0.036	5.787
LipA	lipase		0.246	0.56
Metabolism of phosphate				
PhoA^{pho}	alkaline phosphatase A		0.001	0.54
PhoB^{pho}	alkaline phosphatase III		0.001	5.532
PhoD^{pho}	phosphodiesterase/alkaline phosphatase D		0.002	9.432
Phy^{SNB}	phytase		0.035	
Metabolism of the cell wall				
LytD	*N*-acetylglucosaminidase (major autolysin)	CWB	0.409	
PbpA	penicillin-binding protein 2A	TM	0.87	
PbpB	penicillin-binding protein 2B		0.95	
PbpC	penicillin-binding protein 3	Lipid	0.032	
PbpX	penicillin-binding protein		0.112	
WapA	Cell wall-associated protein precursor	CWB	4.45	0.536
WprA	Cell wall-associated protein precursor	CWB	0.606	0.112
YocH	cell wall-binding protein	CWB	0.012	
YodJ	D-alanyl-D-alanine carboxypeptidase	Lipid	0.06	
YvcE	cell wall-binding protein	CWB	0.354	
YwtD	DL-glutamyl hydrolase	CWB	0.821	

Table 1 (continued)

		Retention signal[a]	% abundance LB[b]	% abundance phosphate[b]
Transport/binding proteins and lipoproteins				
FeuA	iron-binding protein	Lipid	0.04	
FhuD	ferrichrome-binding protein	Lipid	0.042	
MntA	manganese-binding protein	Lipid	0.297	
MsmE	multiple sugar-binding protein		0.006	
OppA	oligopeptide-binding protein	Lipid	0.301	
OpuAC	glycine betaine-binding protein	Lipid	0.002	
PstS^{pho}	phosphate-binding protein	Lipid	0.001	7.871
RbsB	ribose-binding protein	Lipid	0.006	
YcdH	zinc-binding protein	Lipid	0.002	0.257
YclQ	ferrichrome-binding protein	Lipid	1.809	0.501
YfiY	iron(III)-binding protein	Lipid	0.001	
YflE	similar to anion-binding protein	TM	0.001	
YfmC	ferrichrome-binding protein	Lipid	0.318	
YfnI	probable transmembrane glycoprotein	TM	1.426	0.613
YqiX	amino acid-binding protein	Lipid	0.001	0.456
YxeB	putative binding protein	Lipid	0.112	
Sporulation				
TasA	antimicrobial spore component		0.107	0.224
RNA synthesis and regulation				
YwtF	transcriptional regulator		0.15	
Adaptation to atypical conditions/detoxification				
HtrA	serine protease	TM	0.183	
PenP	β-lactamase precursor		0.71	
YbfO^{SNB}	similar to erythromycin esterase		0.098	
YbxI	similar to β-lactamase		0.128	
Protein modification				
YxaL	similar to serine/threonine protein kinase		2.27	0.645
Unknown				
YbdN			0.337	0.134
YbdO			0.223	
YdhF^{pho}		Lipid	0.001	0.747
YjcM			0.005	
YjfA			0.001	
YlqB			2.086	2.018
YncM			2.33	1.535
YoaW			0.023	
YolA			3.427	0.226
YolB			0.908	
YqxI			0.115	
YrpD			0.45	
YrpE		Lipid	0.001	0.154
YuaB			0.002	
YusA		Lipid	0.076	
YvgO			0.231	
YweA			0.495	3.98
YwoF			0.223	0.633
YxkC			0.864	

[a] Retention signals include cell wall-binding domains (CWB), lipid modifications (Lipid) and transmembrane domains TM.
[b] Protein quantification was done with the Delta 2-D software and indicates the relative portion of an individual spot of the total protein present on the gel. [pho] Proteins belonging to the PhoPR regulon.

Table 2. Extracellular proteins of *B. subtilis* without typical signal peptides from cells grown in LB

		% abundance LB
Flagella-related proteins		
FlgB	flagellar basal-body rod protein	0.067
FlgC	flagellar basal-body rod protein	0.006
FlgE	flagellar hook protein	0.108
FlgK	flagellar hook-associated protein 1 (HAP1)	0.778
FlhO	flagellar basal-body rod protein	0.057
FlhP	flagellar hook basal-body protein	0.048
FliD	flagellar hook-associated protein 2 (HAP2)	0.224
Hag	flagellin protein	3.921
Phage-related proteins		
XepA	PBSX prophage lytic exoenzyme	0.065
XkdG	PBSX prophage gene	0.127
XkdK	PBSX prophage gene	0.161
XkdM	PBSX prophage gene	0.157
XlyA	*N*-acetylmuramoyl-L-alanine amidase	0.205

species include carbohydrate-degrading enzymes, several proteases and peptidases, enzymes involved in nucleic acid degradation and lipid metabolism, phosphodiesterases and phosphatases, enzymes involved in cell wall turnover, transport-related proteins, flagellum and phage-related proteins, and proteins involved in sporulation (tables 1–4). A considerable number of the secreted proteins have not yet a function assigned to them (24 in *B. subtilis*, 20 in *B. licheniformis*). Even though the composition of the extracellular proteomes of both species is similar, there are clear variations especially in the relative amounts of proteins belonging to different functional groups (tables 1–4). In the extracellular proteome of *B. licheniformis* grown in complete medium (LB), Hag is the main protein comprising about 30% of the relative spot volume (representing the relative portion of individual spots of the total protein present on the gel). The Hag protein represents only 3.9% of the relative spot volume in *B. subtilis*. The main protein in the *B. subtilis* LB extracellular proteome is the cell wall protein WapA, a protein for which no ortholog exists in *B. licheniformis*. Several processing products of WapA are found in the extracellular proteome which account for 4.4% of the relative spot volume. Another major difference in the composition of the extracellular proteome between the two bacteria is the number and the relative amounts of proteins involved in the utilization of alternative carbohydrates including amylases, pectate lyases, xylanases, arabinases, chitosanases, glucanases. *B. subtilis* secretes a higher number of such proteins (14 compared to only 10 in *B. licheniformis*). In *B. licheniformis*, the protein spots representing carbohydrate-degrading enzymes amount to only 1.05% of the relative spot volume, whereas in *B. subtilis* these proteins amount to 6.4% of the relative spot volume, with the most prominent spot (Csn, chitosanase) comprising already about 1.9% of the relative spot volume. On the other hand, *B. licheniformis* secretes proteases and peptidases to a much higher amount (7.7% relative amount to 2.6% in *B. subtilis*), although the number of such secreted proteins is similar in both organisms (9 in *B. licheniformis*, 8 in *B. subtilis*).

Extracellular Proteomes during Phosphate Starvation

The extracellular proteomes of *B. subtilis* and *B. licheniformis* cells under different starvation conditions were analyzed [Antelmann et al., 2000; Voigt et al., 2006] (fig. 3, 4). In response to such conditions, specific proteins are secreted enabling the cells to counteract the starvation.

The proteins secreted by both *Bacillus* species in response to phosphate starvation are mainly involved in the uptake of phosphate and in the utilization of alternative phosphate sources such as phospholipids, phosphoproteins and phosphonucleotides. Most *B. subtilis* proteins strongly induced in response to phosphate starvation are regulated by the PhoPR two-component system [Hulett et al., 2002]. Although there is not yet any experimental evidence, it can be suggested that the regulation in *B. licheniformis* is similar, because the genes for the two-component system PhoPR are present in the genome and PhoP-binding boxes have been found in the promoter regions of many genes induced by phosphate starvation conditions [Hoi et al., 2006].

There are major differences in the extracellular proteomes of *B. subtilis* and *B. licheniformis* in response to phosphate starvation (tables 1, 3; fig. 3). In *B. subtilis* the main proteins secreted from phosphate-starving cells are the alkaline phosphatases PhoA and PhoB and the phosphodiesterase PhoD (0.54, 5.5 and 9.4% of the relative spot volume, respectively). These phosphatases are secreted only in small amounts by *B. licheniformis* (PhoB and PhoD accounting for 0.3 and 0.2% of the relative spot volume, respectively). There is no homolog of the *phoA* gene encoded in the *B. licheniformis* genome. In *B. subtilis*, PhoD is secreted via the TatAdCd translocase. The proteins TatAd and TatCd are coregulated with PhoD in response to phosphate starvation [Jongbloed et al., 2000]. In *B. licheniformis*, *phoD* forms an operon with *tatAdtCd* sug-

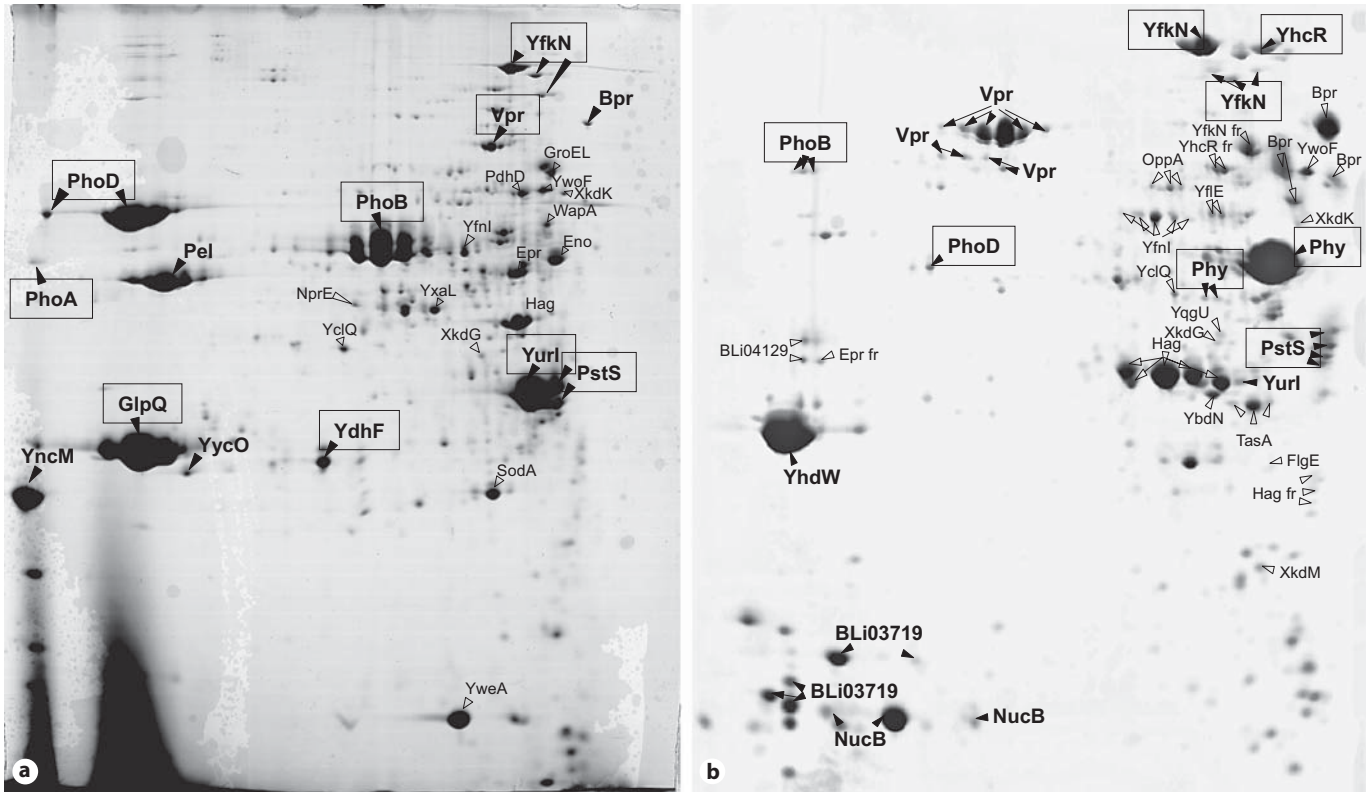

Fig. 3. The extracellular proteomes of *B. subtilis* (**a**) and *B. licheniformis* (**b**) grown under phosphate starvation conditions. Extracellular proteins were prepared 1 h after entry into the stationary growth phase as described for figure 2. Boxed proteins are (presumably) PhoPR-dependent (in *B. licheniformis* a PhoP-binding box has been predicted in the promotor region of the corresponding genes, but PhoPR dependency of the expression has not yet been shown). Proteins in bold letters are induced by phosphate starvation but are (presumably) PhoPR independent (in *B. licheniformis* no PhoP-binding box has been predicted in the promotor region of the corresponding genes).

gesting that PhoD translocation might also be Tat-dependent, although this has not yet been shown [Hoi et al., 2006]. The main protein in the extracellular phosphate starvation proteome of *B. licheniformis* is the phytase Phy (13.9% of the relative spot volume). Phytate is the major storage form of phosphorous in plants and accounts for up to 50% of the organic phosphorus in soils. In contrast to *B. licheniformis*, the phytase of *B. subtilis* is not induced in the extracellular proteome after phosphate starvation since it is not controlled by the PhoPR system. For the *B. licheniformis* phytase, a PhoP-binding box has been predicted, but PhoPR dependency has not yet been shown. However, for another *Bacillus* strain, *B. amyloliquefaciens*, PhoPR dependency of the phytase expression has been established [Makarewicz et al., 2006]. Thus, the regulation of the phytase is one of the major differences in the phosphate starvation response between both *B. subtilis* and *B. licheniformis*. In the extracellular proteome of both organisms the phosphate-binding component of a high-affinity phosphate uptake system (PstS) was found to be induced. Another protein, the glycerophosphodiester phosphodiesterase, was secreted at high amounts by both species. It is interesting to note that phosphate-starving *B. subtilis* cells secrete GlpQ, whereas phosphate-starving *B. licheniformis* cells secrete YhdW, a protein with similarity to GlpQ. The GlpQ protein itself was only found in the proteome of *B. licheniformis* cells grown in LB. Furthermore, a strong secretion of some proteins involved in the metabolism of nucleic acids was observed in *B. licheniformis* (YfkN, YhcR, NucB and BLi03719). In *B. subtilis*, the nucleotidase YfkN and the ribonuclease YurI were identified as strongly induced members of the PhoPR regulon in the proteome of phosphate-starving cells [Wolff et al., 2006]. Induction of nucleic acid-degrading enzymes in response to phosphate starvation was also described for *Corynebacterium glutamicum* [Ishige et al., 2003]. Further-

Table 3. Extracellular proteins of *B. licheniformis* with signal peptides from cells grown in LB and phosphate-limited minimal medium

		Retention signal[a]	% abundance LB[b]	% abundance phosphate[b]
Metabolism of carbohydrates				
Pel	pectate lyase (EC 4.2.2.2)			0.39
SacC	levanase (EC 3.2.1.65)		0.05	
YheN	similar to endo-1,4-β-xylanase		0.25	
YvfO	similar to arabinogalactan endo-1,4-β-galactosidase		0.37	
YxiA	similar to arabinan endo-1,5-α-L-arabinosidase		0.19	
BLi00338	putative chitinase (EC 3.2.1.14)		0.09	
BLi03029	close homolog to AbnA arabinan-endo 1,5-α-L-arabinase		LB 72 h	
BLi04129	putative pectate lyase (EC 2.1.3.3)		0.06	
Metabolism of proteins and peptides				
Bpr	bacillopeptidase F (EC 3.4.21.-)		0.81	6.44
Epr	minor extracellular serine protease (EC 3.4.21.-)			0.12
Ggt	γ-glutamyltranspeptidase (EC 2.3.2.2)	TM	0.87	0.38
Mpr	glutamyl endopeptidase precursor (EC 3.4.21.19)		0.08	
Vpr	extracellular serine protease (EC 3.4.21.-)		5.70	4.75
YwaD	similar to aminopeptidase		0.06	
BLi00301	putative serine protease		0.22	
BLi01109	subtilisin Carlsberg precursor (EC 3.4.21.62)			0.32
BLi01747	putative bacillopeptidase F			0.08
Metabolism of nucleotides and nucleic acids				
NucB	sporulation-specific extracellular nuclease (EC 3.-.-.-)			5.12
YfkN	similar to 2′,3′-cyclic-nucleotide 2′-phosphodiesterase (EC 3.1.4.16)	TM		5.21
YhcR	similar to 5′-nucleotidase	TM	0.09	1.89
YurI	similar to ribonuclease			0.22
BLi03719	putative ribonuclease (EC 3.1.27.-)		0.35	5.55
Metabolism of lipids				
GlpQ	glycerophosphoryl diester phosphodiesterase (EC 3.1.4.46)		0.04	
YhdW	similar to glycerophosphodiester phosphodiesterase (EC 3.1.4.46)			11.51
Metabolism of phosphate				
PhoB	alkaline phosphatase III (EC 3.1.3.1)			0.29
PhoD	phosphodiesterase/alkaline phosphatase (EC 3.1.3.1)			0.22
Phy	3-phytase (EC 3.1.3.8)/6-phytase (EC 3.1.3.26)			13.91
Metabolism of the cell wall				
PbpB	penicillin-binding protein 2B (cell-division septum)		0.09	
YrvJ1	similar to *N*-acetylmuramoyl-L-alanine amidase		0.28	0.12
YvcE	similar to cell wall-binding protein	CWB	0.69	
YwtD	similar to murein hydrolase	TM	0.04	
BLi01309	putative cell wall-binding protein	CWB	0.04	
BLi03478	putative penicillin-binding protein 5* (D-alanyl-D-alanine carboxy-peptidase) (EC 3.4.16.4)	TM	0.03	
BLi03767	putative cell wall-binding protein	CWB	0.44	
Transport/binding proteins and lipoproteins				
AppA	oligopeptide ABC transporter (oligopeptide-binding protein)	Lipid	0.28	
FeuA	iron-uptake system (binding protein)	Lipid		0.06
MntA	manganese ABC transporter (membrane protein)		0.83	
OppA	oligopeptide ABC transporter (binding protein)	Lipid	1.34	0.46
PstS	phosphate ABC transporter (binding protein)	Lipid		2.23
YclQ	similar to ferrichrome ABC transporter (binding protein)	Lipid	0.08	0.18
YesO	similar to sugar-binding protein	Lipid	0.13	
YflE	similar to anion-binding protein	TM	0.66	0.50

Table 3 (continued)

		Retention signal[a]	% abundance LB[b]	% abundance phosphate[b]
YfnI	similar to anion-binding protein	TM	8.59	1.28
YqgS	similar to putative molybdate-binding protein	TM	1.14	
BLi02527	putative ABC transporter			0.10
Sporulation				
TasA	translocation-dependent antimicrobial spore component		1.11	1.23
RNA synthesis and regulation				
YwtF	similar to transcriptional regulator	TM	0.25	
Unknown				
YbdN			1.31	0.41
YpjP			0.05	
YqgU		Lipid		0.10
YusA		Lipid	0.41	0.03
YusW		Lipid	0.09	
YwoF			0.08	0.71
BLi00654			0.13	0.01
BLi00784			0.12	
BLi01431			1.17	
BLi02210			2.28	0.35
BLi02558			0.78	0.60
BLi04124			0.74	0.05
BLi04294			0.41	0.31
BLi04308			2.86	0.39

[a] Retention signals include cell wall-binding domains (CWB), lipid modifications (Lipid) and transmembrane domains TM.
[b] Protein quantification was done with the Delta 2-D software and indicates the relative portion of an individual spot of the total protein present on the gel.

Table 4. Extracellular proteins of *B. licheniformis* without typical signal peptides from cells grown in LB

		% abundance LB
Flagella-related proteins		
FlgK	flagellar hook-associated protein 1 (HAP1)	0.6
FlhO	flagellar basal-body rod protein	0.1
FlhP	flagellar hook-basal body protein	0.02
FliD	flagellar hook-associated protein 2 (HAP2)	0.2
FliK	flagellar hook-length control	0.2
Hag	flagellin protein	30.5
Phage-related proteins		
XkdK	PBSX prophage	0.1
XkdM	PBSX prophage	0.2

more, in *B. subtilis* the serine protease Vpr belongs to the strongly induced PhoPR-dependent proteins in the extracellular proteome of phosphate-starving cells. This protein is also strongly secreted by phosphate-starving *B. licheniformis* cells, but high Vpr secretion was also noticed under other growth conditions.

Additionally, the composition of the extracellular proteome in response to glucose and nitrogen starvation was analyzed in *B. licheniformis* [Voigt et al., 2006] (fig. 4; table 5). The main protein secreted under both conditions was the flagellin protein Hag. Both starvation conditions also led to a high secretion of proteases and peptidases, although secretion of such enzymes was higher during nitrogen limitation. Surprisingly, only few proteins involved in carbohydrate degradation were found in the extracellular proteome of cells subjected to glucose starvation, although *B. licheniformis* has the genetic potential for the production of a high number of such proteins [Veith et al., 2004].

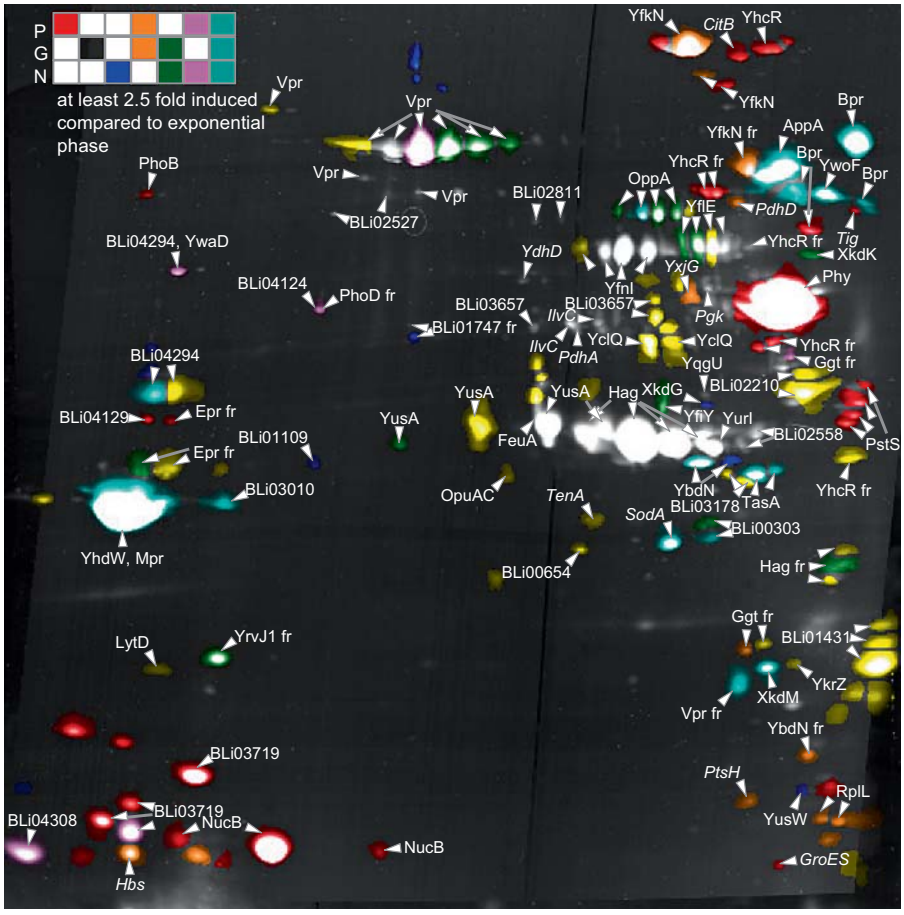

Fig. 4. Color-coded extracellular proteome map of *B. licheniformis* cells grown in minimal medium under phosphate, glucose and nitrogen starvation conditions. Proteome images of the three starvation conditions were fused with an exponential growth phase proteome image to create a proteome map, which contains all protein spots from the four individual images. Color coding was done in such a way that all proteins belonging to a spot subset, i.e. all proteins induced in response to one starvation condition or a certain combination of starvation conditions received a defined, subset specific color (color scheme see upper left corner; P = phosphate starvation, G = glucose starvation, N = nitrogen starvation). Only proteins induced more than 2.5-fold compared to the exponential growth phase were included in the color coding. Quantification, gel fusion and color coding was done with the Delta 2-D software (Decodon GmbH, Greifswald, Germany).

Comparison of the Predicted to the Real Extracellular Proteome

A considerable number of the proteins found in the extracellular medium of both bacteria species (about 50%) were not predicted to be secreted, because they either lack known export signals or possess an additional retention signal. Among the proteins found in the extracellular proteome lacking known export signals are cytoplasmic proteins [Antelmann et al., 2001; Voigt et al., 2006]. In the extracellular proteome of *B. subtilis,* 17 presumably cytoplasmic proteins were identified and in the extracellular proteome of *B. licheniformis* 54 presumably cytoplasmic proteins were identified. Among them are proteins from a range of physiological pathways and cellular functions. Other proteins found in the extracellular medium without N-terminal signal peptides include flagellum-related and phage proteins. Comparable numbers of such proteins were found in the extracellular proteome of *B. subtilis* and *B. licheniformis* (5 and 2 phage proteins, respectively, 7 flagellum proteins in both species; tables 2, 4). Another group of proteins not expected in the extracellular medium were cell wall-related proteins (tables 1, 3, 5). For *B. subtilis* it has been suggested that such proteins are retained in the cell wall, because they contain specific wall-binding domains in addition to the signal peptide [Tjalsma et al., 2000]. In both species, similar numbers of such proteins were found in the extracellular proteome (6 in *B. subtilis*, 9 in *B. licheniformis*). Cell wall proteins were always present to a higher extent in the extracellular proteome of cells in the exponential growth phase than of cells in the stationary growth phase [Antelmann et al., 2001; Voigt et al., 2006]. A considerable number of proteins involved in transport processes, among them several ABC transporter-binding proteins, were found to be secreted into the extracellular medium by both *Bacillus* species (tables 1, 3, 5). Many of these proteins contain a conserved lipobox that can be lipid-modified by the lipoprotein diacylglyceryl

Table 5. Extracellular proteins of *B. licheniformis* containing signal peptides additionally found when cells grow under nitrogen and glucose starvation conditions

		Retention signal[a]	N	G
Metabolism of carbohydrates				
SacB	levansucrase (EC 2.4.1.10)		x	
YvpA	similar to pectate lyase			x
Metabolism of the cell wall				
LytD	*N*-acetylglucosaminidase (major autolysin) (EC 3.2.1.96)	CWB		x
YodJ	similar to D-alanyl-D-alanine carboxypeptidase (EC 3.4.16.4)	Lipid	x	
Transport/binding proteins and lipoproteins				
DppE	dipeptide ABC transporter (dipeptide-binding protein) (sporulation)	Lipid	x	
OpuAC	glycine betaine ABC transporter (glycine betaine-binding protein)	Lipid	x	
YcdH	similar to ABC transporter (binding protein)	Lipid	x	
YfiY	similar to iron(III) dicitrate transport permease	Lipid		x
YhcJ	similar to ABC transporter (binding lipoprotein)	Lipid		x
BLi02811	putative oligopeptide transporter (putative substrate binding domain)		x	x
BLi03657	putative iron(III) transporter binding protein		x	x
Mobility and chemotaxis				
FliL	flagellar protein required for flagellar formation			x
Unknown				
YdaJ			x	
YkwD			x	
BLi03010			x	x
BLi03178				x
BLi03260		TM	x	
BLi03670			x	

[a] Retention signals include cell wall-binding domains (CWB), lipid modifications (Lipid) and transmembrane domains (TM).

transferase (Lgt) prior to cleavage by SPase II. These proteins should be retained in the membrane by the lipid anchor.

Proteomics of Protein Secretion Mechanisms in Bacillus

Studies of protein secretion mechanisms in *Bacillus* species were so far mainly done with *B. subtilis* [van Dijl et al., 2002]. For *B. subtilis* a comprehensive collection of mutants exists which can be used to analyze the contribution of the different secretion systems to the extracellular proteome [Antelmann et al., 2001, 2003; Hirose et al., 2000; Jongbloed et al., 2002; Tjalsma et al., 2000]. The definition of the extracellular proteome and the cell wall proteome in *B. subtilis* was the basis for a comparative proteome analysis between the *B. subtilis* wild type and mutants impaired in the targeting, export, processing, modification, folding or stability of extracytoplasmic proteins. The comparative proteome analyses shed new light on the protein secretion mechanisms of *Bacillus* (fig. 5) [Tjalsma et al., 2004].

Of the 113 identified extracellular proteins, only 54 were predicted to be secreted because of the presence of a signal peptide with an SPase I cleavage site and the lack of additional retention signals (fig. 5) [Tjalsma et al., 2000, 2004]. The secretion of most of these predicted secreted proteins is dependent on SecA and both components of the bacterial signal recognition particle (Ffh and FtsY) in *B. subtilis* and requires the major folding catalyst PrsA for proper folding [Hirose et al., 2000; Jongbloed et al., 2002; Vitikainen et al., 2004; Zanen et al., 2006]. SecA dependency of most of the secreted proteins has also been shown in *B. licheniformis*. Inhibition of SecA activity by sodium azide prevents secretion of these proteins (data not shown). The third signal recognition particle-GTPase of *B. subtilis* (FlhF) was shown to be dispensable for protein secretion in *B. subtilis* [Zanen et al., 2004].

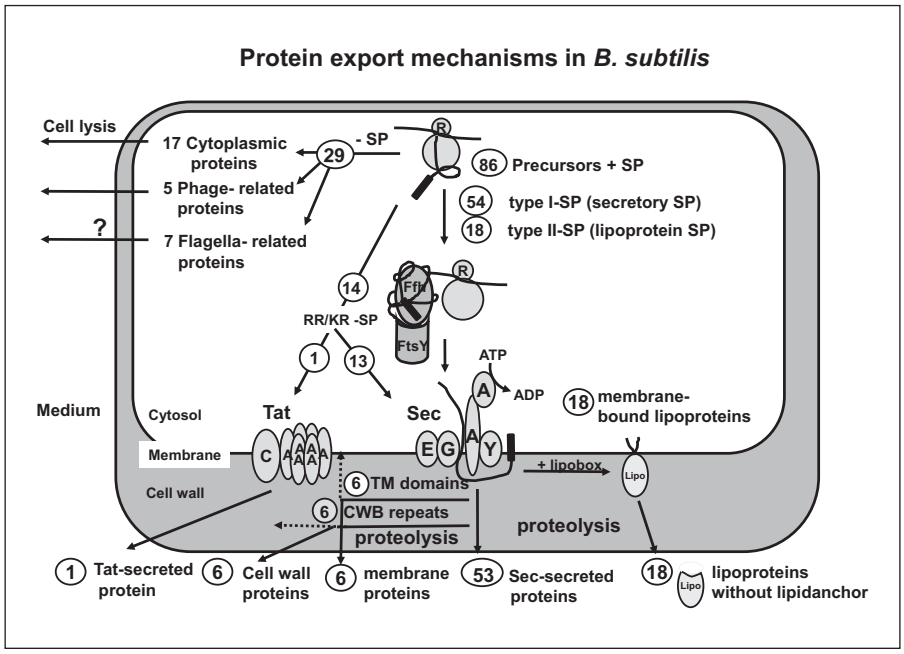

Fig. 5. Contribution of different secretion mechanisms to the extracellular proteome of *B. subtilis*. Altogether 113 proteins were identified in the extracellular proteome of *B. subtilis*. 86 of the secreted proteins contain a signal peptide, but 30 of these have additional retention signals and were not expected to be found in the extracellular medium (18 lipoproteins, 6 cell wall proteins, 6 proteins with transmembrane domains) [Antelmann et al., 2001]. From the 14 proteins for which a Tat signal peptide was predicted, only one was shown through proteomics to be Tat-dependently secreted [Jongbloed et al., 2002]. A significant portion of the proteins identified in the extracellular proteome were not predicted to be secreted because they do not contain signal peptides (17 cytoplasmic proteins, 5 phage-related and 7 flagella-related proteins). SP = Signal peptide; TM domains = transmembrane domains; CWB repeats = cell wall-binding repeats.

In the secretome of *B. subtilis,* 44 proteins have been predicted to contain twin-arginine signal peptides which could direct these proteins into the alternative Tat pathway [Jongbloed et al., 2002, 2004]. However, only the phosphate starvation-induced alkaline phosphodiesterase, PhoD, is specifically transported via the coregulated TatAdCd translocase [Jongbloed et al., 2000, 2002, 2004]. In *B. licheniformis* transport of PhoD via the TatAdCd translocase has not yet been shown, but both genes form an operon and are coregulated, suggesting that PhoD translocation might also be Tat-dependent [Hoi et al., 2006]. In contrast, the second Tat translocase (TatAyCy) is specifically involved in the transport of the iron starvation-induced Fur-dependent YwbN protein in *B. subtilis* [Jongbloed et al., 2004].

The extracellular proteome of *B. subtilis* also includes numerous unpredicted secreted proteins which either possess signal peptides and retention signals (18 lipoproteins, 6 cell wall-binding proteins and 6 membrane proteins), or lack signal peptides (17 cytoplasmic proteins, 5 phage-related proteins and 7 flagella-related proteins) (fig. 5) [Antelmann et al., 2001; Tjalsma et al., 2004]. Since the cytoplasmic proteins that accumulate in the extracellular medium are also abundant in the cytoplasmic proteome, the release of the majority of these proteins is most probably mediated by partial cell lysis in *B. subtilis*. This is supported by the fact that all conditional mutants in essential secretion genes *(ffh, ftsY, secA, prsA)* are sensitive to lysis and show strongly increased amounts of these cytoplasmic proteins in the extracellular proteome [Tjalsma et al., 2004; Vitikainen et al., 2004; Zanen et al., 2006]. It was initially assumed that cytoplasmic proteins identified in the extracellular proteome of *B. subtilis* might be secreted via prophage-encoded functions, such as holins. These holins have the potential to oligomerize in the membrane, forming membrane pores through which the lytic phage enzymes gain access to the bacterial cell wall [Young and Bläsi, 1995]. Notably however, a *B. subtilis* strain lacking all known holins still contained all 17 cytoplasmic proteins and 7 flagella-related proteins in the extracellular proteome, suggesting that holins have no role in the 'secretion' of these proteins [Westers et al., 2003; Tjalsma et al., 2004]. The 5 phage-related proteins could however be secreted via the holins of *B. subtilis*, but this still awaits experimental verification. The flagella-related proteins could be exported via a flagella assembly machinery related to the type III secretion machinery of Gram-negative bacteria [Tjalsma et al., 2004].

Lipoproteins are secreted Sec-dependently, lipid-modified by the diacylglyceryl transferase Lgt and cleaved by the type II signal peptidase LspA in *B. subtilis* [Leskelä et al., 1999]. Lipoproteins are usually retained in the cytoplasmic membrane via the lipid anchor [Tjalsma et al., 2000]. Since the lipoproteins in the extracellular proteome are lacking the N-terminal lipid-modified cysteine residue, they are probably released from the membrane

by proteolytic shedding [Antelmann et al., 2001]. The comparison of the extracellular proteome and lipoproteome of *B. subtilis* wild type and *lgt* mutant cells revealed that 9 lipoproteins identified in the lipoproteome fraction of the wild type are absent in the lipoproteome but accumulate in the extracellular proteome of the *lgt* mutant [Antelmann et al., 2001, 2006]. This demonstrates a redistribution of lipoproteins from the cytoplasmic membrane to the extracellular medium in the *lgt* mutant due to the lack of the lipid anchor [Antelmann et al., 2001, 2006; Tjalsma et al., 2004].

Cell wall proteins are secreted Sec-dependently in *B. subtilis* and typically retained in the cell wall by specific cell wall-binding domains. In contrast to predicted secretory proteins, like proteases which are secreted at higher levels during the stationary phase in *B. subtilis*, cell wall proteins are secreted predominantly during exponential growth while their production is repressed during the transition phase [Antelmann et al., 2002]. Comparative extracellular and cell wall proteome analyses with a multiple extracellular protease mutant and a σ^D mutant which is impaired in motility and cell wall turnover showed a stabilization of abundant cell wall proteins (e.g. WapA, YvcE) during the stationary phase in *B. subtilis* [Antelmann et al., 2002]. These results revealed that cell wall proteins are substrates for degradation by extracellular proteases during the stationary phase in *B. subtilis*. In addition, cell wall proteins might be released due to high cell wall turnover predominantly during exponential growth in *B. subtilis* which is mediated by the activity of the autolysins [Antelmann et al., 2002].

Proteins which were not predicted to be secreted are also found in the extracellular proteome of *B. licheniformis*. The similar composition of this part of the secretome in both *Bacilli* suggests that similar mechanisms are employed leading to the accumulation of these proteins in the extracellular medium.

Outlook

The growing number of available genome sequences for bacteria enables comparative studies of the cell physiology of more or less closely related species. These genome sequences are also a prerequisite for high throughput proteome analyses allowing rapid protein identification by large-scale mass spectrometry such as MALDI-TOF-MS and MS/MS techniques. Comprehensive studies of protein secretion using proteomics have expanded our understanding of the mechanisms of this process in *Bacillus*. Protein secretion is not only crucial for cell survival in changing natural environments, but also of key importance from a biotechnological viewpoint. Since *Bacillus* species are used as host organisms in industrial fermentation processes, especially for the production of enzymes, analyzing protein secretion in such strains yields valuable information that can be used for the improvement of industrial fermentation processes. The accumulated data on cell physiology of both *Bacillus* species under different growth conditions, including the information on protein secretion, now make it possible to dissect the influence the differences in the genome sequence exert on the cell performance under these conditions.

Acknowledgements

We thank Karin Binder, Anne Krause, Susanne Paprotny and Sebastian Grund for excellent technical assistance. We are grateful to the Decodon GmbH (Greifswald, Germany) for their cooperation. B.V., H.A. and M.H. received financial support from the 'Deutsche Forschungsgemeinschaft', the 'Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie' (0312707, 031U214B), the Henkel KGaA, the Bildungsministerium of the country Mecklenburg-Vorpommern, the Fund of the Chemical Industry of Germany and the EU (LSHC-CT-2004-503468/005257). H.A., M.H. and J.M.v.D. were supported in part by European Union Grants LSHG-CT-2004-503468, LSHG-CT-2004-005257, LSHM-CT-2006-019064 and LSHG-CT-2006-037469.

References

Antelmann H, Darmon E, Noone D, Veening JW, Westers H, Bron S, Kuipers O, Devine K, Hecker M, van Dijl JM: The extracellular proteome of *Bacillus subtilis* under secretion stress conditions. Mol Microbiol 2003;49:143–156.

Antelmann H, Scharf C, Hecker M: Phosphate starvation-inducible proteins in *Bacillus subtilis*: proteomics and transcriptional analysis. J Bacteriol 2000;182:4478–4490.

Antelmann H, Tjalsma H, Voigt B, Ohlmeier S, Bron S, van Dijl JM, Hecker M: A proteomic view on genome-based signal peptide predictions. Gen Res 2001;11:1484–1502.

Antelmann H, van Dijl JM, Bron S, Hecker M: Proteomic survey through the secretome of *Bacillus subtilis*. Methods Biochem Anal 2006;49:179–208.

Antelmann H, Yamamoto H, Sekiguchi J, Hecker M: Stabilization of cell wall proteins in *Bacillus subtilis*: a proteomic approach. Proteomics 2002;2:591–602.

Brody MS, Vijay K, Price C: Catalytic function of an α/β hydrolase is required for energy stress activation of the sigma(B) transcription factor in *Bacillus subtilis*. J Bacteriol 2001;183:6422–6428.

Bryan EM, Beall BW, Moran CP: A σ^E-dependent operon subject to catabolite repression during sporulation in *Bacillus subtilis*. J Bacteriol 1996;178:4778–4786.

Claus D, Berkeley RCW: Genus Bacillus; in Sneath PHA, Mair NS, Sharpe ME, Holt JG (eds): Bergey's Manual of Systematic Bacteriology. Baltimore, Williams & Wilkins, 1986, pp 1105–1139.

Dubnau D: DNA uptake in bacteria. Annu Rev Microbiol 1999;53:217–244.

Fründ C, Priefert H, Steinbüchel A, Schlegel HG: Biochemical and genetic analyses of acetoin catabolism in *Alcaligenes eutrophus*. J Bacteriol 1989;171: 6539–6548.

Gottschalk G: Bacterial Metabolism, ed 2. New York, Springer, 1986.

Grundy FJ, Waters DA, Takova TY, Henkin TM: Identification of genes involved in utilization of acetate and acetoin in *Bacillus subtilis*. Mol Microbiol 1993;10:259–271.

Hirose I, Sano K, Shioda I, Kumano, M, Nalamura K, Yamane K: Proteome analysis of *Bacillus subtilis* extracellular proteins: a two-dimensional protein electrophoretic study. Microbiology 2000;146:65–75.

Hoi LT, Voigt B, Jürgen B, Ehrenreich A, Gottschalk G, Evers S, Feesche J, Maurer KH, Hecker M, Schweder T: The phosphate-starvation response of *Bacillus licheniformis*. Proteomics 2006;6:3582–3601.

Hecker M, Völker U: Towards a comprehensive understanding of *Bacillus subtilis* cell physiology by physiological proteomics. Proteomics 2004;4:3727–3750.

Horswill AR, Escalante-Semerena JC: *Salmonella typhimurium* LT2 catabolizes propionate via the 2-methylcitric acid cycle. J Bacteriol 1999;181:5615–5623.

Huang M, Oppermann-Sanio FB, Steinbüchel A: Biochemical and molecular characterization of the *Bacillus subtilis* acetoin catabolic pathway. J Bacteriol 1999;181:3837–3841.

Hulett FM: The Pho Regulon; in Sonenshein AL, Hoch JA, Losick R (eds): *Bacillus subtilis* and Its Closest Relatives. Washington, ASM Press, 2002, pp 193–201.

Ishige T, Krause M, Bott M, Wendisch VF, Sahm H: The phosphate starvation stimulon of *Corynebacterium glutamicum* determined by DNA microarray analyses. J Bacteriol 2003; 185:4519–4529.

Jongbloed JD, Martin U, Antelmann H, Hecker M, Tjalsma H, Venema G, Bron S, van Dijl JM, Muller J: TatC is a specificity determinant for protein secretion *via* the twin-arginine translocation pathway. J Biol Chem 2000;275:41350–41357.

Jongbloed JDH, Antelmann H, Hecker M, Nijland R, Bron S, Airaksinen U, Pries F, Quax WJ, van Dijl JM: Selective contribution of the twin-arginine translocation pathway to protein secretion in *Bacillus subtilis*. J Biol Chem 2002;277:44068–44078.

Jongbloed JD, Grieger U, Antelmann H, Hecker M, Nijland R, Bron S, van Dijl JM: Two minimal Tat translocases in *Bacillus*. Mol Microbiol 2004;54:1319–1325.

Koburger T, Weibezahn J, Bernhardt J, Homuth G, Hecker M: Genome-wide mRNA profiling in glucose starved *Bacillus subtilis* cells. Mol Gen Genomics 2005;274:1–12.

Krogh S, Joergensen ST, Devine KM: Lysis genes of the *Bacillus subtilis* defective prophage PBSX. J Bacteriol 1998;180:2110–2117.

Kunst F, Ogasawara N, Moszer I, Albertini AM, Alloni G, et al: The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis*. Nature 1997;390:249–256.

Leskelä S, Wahlström E, Kontinen VP, Sarvas M: Lipid modification of prelipoproteins is dispensable for growth but essential for efficient protein secretion in *Bacillus subtilis*: characterization of the *lgt* gene. Mol Microbiol 1999;31:1075–1085.

Makarewicz O, Dubrac S, Msadek T, Borriss R: Dual role of the PhoP~P response regulator: *Bacillus amyloliquefaciens* FZB45 phytase gene transcription is directed by positive and negative interactions with the *phyC* promoter. J Bacteriol 2006;188:6953–6965.

Nordlund P, Reichard P: Ribonucleotid reductases. Annu Rev Biochem 2006;75:681–706.

Rey MW, Ramaiya P, Nelson BA, Brody-Karpin SD, et al: Complete genome sequence of the industrial bacterium *Bacillus licheniformis* and comparisons with closely related *Bacillus* species. Genome Biol 2004;5:R77.

Schallmey M, Singh A, Ward OP: Developments in the use of *Bacillus* species for industrial production. Can J Microbiol 2004;50:1–17.

Sneath PHA: Endospore-forming Gram-positive rods and cocci; in Sneath PHA, Mair NS, Sharpe ME, Holt JG (eds): Bergey's Manual of Systematic Bacteriology. Baltimore, Williams & Wilkins, 1986, vol 2, pp 1104–1207.

Tjalsma H, Antelmann H, Jongbloed JD, Braun PG, Darmon E, Dorenbos R, Dubois JY, Westers H, Zanen G, Quax WJ, Kuipers OP, Bron S, Hecker M, van Dijl JM: Proteomics of protein secretion by *Bacillus subtilis*: separating the 'secrets' of the secretome. Microbiol Mol Biol Rev 2004;68:207–233.

Tjalsma H, Bolhuis A, Jongbloed JDH, Bron S, van Dijl JM: Signal peptide-dependent protein transport in *Bacillus subtilis*: a genome-based survey of the secretome. Microbiol Mol Biol Rev 2000;64:515–547.

Tjalsma H, van Dijl JM: Proteomics-based consensus prediction of protein retention in a bacterial membrane. Proteomics 2005;5: 4472–4482.

Tobisch S, Zühlke D, Bernhardt J, Stülke J, Hecker M: Role of CcpA in regulation of the central pathways of carbon catabolism in *Bacillus subtilis*: J Bacteriol 1999;181:6996–7004.

Torrents E, Buist G, Liu A, Eliasson R, Kok J, Gibert I, Graslund A, Reichard P: The anaerobic (class III) ribonucleotide reductase from *Lactococcus lactis*. Catalytic properties and allosteric regulation of the pure enzyme system. J Biol Chem 2000;275:2463–2471.

Van Dijl JM, Bolhuis A, Tjalsma H, Jongbloed JDH, de Jong A, Bron S: Protein transport pathways in *Bacillus subtilis*: a genome-based road map; in Sonenshein AL, Hoch JA, Losick R (eds): *Bacillus subtilis* and Its Closest Relatives. Washington, ASM Press, 2002, pp 337–355.

Veith B, Herzberg C, Steckel S, Feesche J, Maurer KH, Ehrenreich P, Bäumer S, Henne A, Liesegang H, Merkl R, Ehrenreich A, Gottschalk G: The complete genome sequence of *Bacillus licheniformis* DSM13, an organism with great industrial potential. J Mol Microbiol Biotech 2004;4:204–211.

Vitikainen M, Lappalainen I, Seppala R, Antelmann H, Boer H, Taira S, Savilahti H, Hecker M, Vihinen M, Sarvas M, Kontinen VP: Structure-function analysis of PrsA reveals roles for the parvulin-like and flanking N- and C-terminal domains in protein folding and secretion in *Bacillus subtilis*. J Biol Chem 2004;279:19302–19314.

Voigt B, Hoi LT, Jürgen B, Albrecht D, Ehrenreich A, Veith B, Evers S, Maurer KH, Hecker M, Schweder T: The glucose and nitrogen starvation response of *Bacillus licheniformis*. Proteomics 2007;7:413–423.

Voigt B, Schweder T, Becher D, Ehrenreich A, Gottschalk G, Feesche J, Maurer KH, Hecker M: A proteomic view of cell physiology of *Bacillus licheniformis*. Proteomics 2004;4: 1465–1490.

Voigt B, Schweder T, Sibbald MJJB, Albrecht D, Ehrenreich A, Bernhardt J, Feesche J, Maurer KH, Gottschalk G, van Dijl JM, Hecker M: The extracellular proteome of *Bacillus licheniformis* grown in different media and under different nutrient starvation conditions. Proteomics 2006;6:3582–3601.

Völker U, Völker A, Maul B, Hecker M, Dufour A, Haldenwang WG: Separate mechanisms activate sigma B of *Bacillus subtilis* in response to environmental and metabolic stresses. J Bacteriol 1995;177:3771–3780.

Westers H, Dorenbos R, van Dijl JM, Kabel J, Flanagan T, Devine KM, Jude F, Seror SJ, Beekman AC, Darmon E, Eschevins C, de Jong A, Bron S, Kuipers OP, Albertini AM, Antelmann H, Hecker M, Zamboni N, Sauer U, Bruand C, Ehrlich DS, Alonso JC, Salas M, Quax WJ: Genome engineering reveals large dispensable regions in *Bacillus subtilis*. Mol Biol Evol 2003;20:2076–2090.

Wolff S, Antelmann H, Albrecht D, Becher D, Bernhardt J, Bron S, Buttner K, van Dijl JM, Eymann C, Otto A, Tam LT, Hecker M: Towards the entire proteome of the model bacterium *Bacillus subtilis* by gel-based and gel-free approaches. J Chromatogr B Analyt Technol Biomed Life Sci 2007;849:129–140.

Young R, Bläsi U: Holins: form and function in bacteriophage lysis. FEMS Microbiol Rev 1995;17:191–205.

Zanen G, Antelmann H, Westers H, Hecker M, van Dijl JM, Quax WJ: FlhF, the third signal recognition particle-GTPase of *Bacillus subtilis*, is dispensable for protein secretion. J Bacteriol 2004;186:5956–5960.

Zanen G, Antelmann H, Meima R, Jongbloed JD, Kolkman M, Hecker M, van Dijl JM, Quax WJ: Proteomics dissection of potential signal recognition particle dependence in protein secretion by *Bacillus subtilis*. Proteomics 2006;6:3636–3648.