Genome-wide analysis of nutrient starvation responses of *Bacillus licheniformis*

Inauguraldissertation

zur

Erlangung des akademischen Grades

Doktor rerum naturalium (Dr. rer. nat.)

an der Mathematisch-Naturwissenschaftlichen Fakultät

der

Ernst-Moritz-Arndt-Universität Greifswald

2006

vorgelegt von Le Thi Hoi
geboren am 26. März 1978
in Thanh Hoa - Vietnam

Greifswald, 16.05.2006

Dekan: Prof. Dr. Klaus Fesser

1. Gutachter 1: Prof. Dr. Thomas Schweder (Greifswald)
2. Gutachter 2: Prof. Dr. Peter Neubauer (Oulu)

Tag der Promotion: 18.07.2006

TABLE OF CONTENTS

		Page
Chapter I	1. Introduction	1
	1.1. The physiology of *B. licheniformis*	1
	1.2. The general stress response of *B. subtilis*	4
	1.2.1. The general stress sigma factor σ^B	5
	1.2.2. The stringent response	6
	1.3. The specific stress response of *B. subtilis*	8
	1.3.1. The phosphate starvation response	8
	1.3.2. The glucose and nitrogen starvation response	10
	1.4. *Bacillus* expression systems	15
	2. Scope of the thesis	18
Chapter II	The phosphate starvation response of *Bacillus licheniformis*	21
Chapter III	Response of *Bacillus licheniformis* to glucose and nitrogen starvation	49
Chapter IV	The regulation of the phytase gene expression in *Bacillus licheniformis*	94
Chapter V	Summary and general discussion	115
References		122
List of publications and patents		132

Chapter I

1. Introduction

1.1. The physiology of *B. licheniformis*

Bacillus licheniformis DSM13 is a Gram-positive and saprophytic bacterium. It is capable of producing endospores that are resistant to adverse environmental conditions. *B. licheniformis* is widespread distributed in soil and other natural habitats. Its cells have a characteristic rod-shape and are motile (Rey et al. 2004; Veith et al. 2004).

B. licheniformis is closely related to *B. subtilis*, the Gram-positive model organism. The genome sequences of *B. licheniformis* (Veith et al. 2004) and other *Bacillus* species including *Bacillus anthracis* (Read et al. 2003), *Bacillus cereus* (Ivanova et al. 2003), the alkaliphilic specie *Bacillus halodurans* (Takami et al. 2000) and *B. subtilis* (Kunst et al. 1997) have been determined. Most of them are important industrial hosts.

B. licheniformis is one of the most important hosts used in the biotechnological industry for production of numerous extracellular industrial enzymes including proteases, amylases, phytases as well as antibiotics and special chemicals for a long period of time. The ability to produce and secrete high amounts of enzymes into the extracellular medium (20-25 g/L) makes *B. licheniformis* to an attractive organism in commercial processes (Schallmey et al. 2004). Several proteases from *B. licheniformis* are applied in household detergents and the leather industry (Tang et al. 2004 a, b). The subtilisin Carlsberg-type enzyme is produced annually in about 500 metric tonnes (Rao et al. 1998; Tye et al. 2002). Amylases have been used in industrial processes such as food, fermentation, textile and paper industries (Pandey et al. 2000). In addition, because of its good thermostability, the α-amylase from *B. licheniformis* is a unique industrial enzyme for liquefying starch, especially cornstarch. Due to a broad temperature and pH spectrum, the phytase from *B. licheniformis* has a potential application in animal feeding production (Tye et al. 2002). Bacitracins are antibiotics produced from *Bacillus* species. In this group, bacitracin A is a dominantly commercial product. These antibiotics are the secondary metabolites formed at the early stages of sporulation (Demain 1999). However, genes for bacitracin synthesis are absent in strain DSM13 but present in the other *B. licheniformis* isolates (Rey et al. 2004; Veith et al. 2004). Furthermore, *B. licheniformis* is a dominant producer of special substances including 5'-inosinic acid and inosine, citric acid, substituted L-tryptophan and pharmaceutical supplement poly-γ-glutamic acid (γ-PGA) (Shih and Van 2001; Schallmey et al. 2004). Together with the high potential for producing and secreting enzymes into the extracellular

medium, *B. licheniformis* is generally regarded as safe (GRAS) by the U.S. Food and Drug Administration (Gupta et al. 2002), and shows short development time.

Because of its important applications in the industry, the sequencing of the complete nucleotide sequence of *B. licheniformis* DSM13 genome was carried out recently (Veith et al. 2004). The genome of *B. licheniformis* DSM13 is a single circular chromosome consisting of 4 222 748 base pairs (bp) with a GC content of 46.2 %. It contains 4286 open reading frames (ORFs), 72 transfer-RNAs (tRNAs) and 7 ribosome-RNAs (rRNAs) operons. *B. licheniformis*, *B. subtilis* and *B. halodurans* belong to one taxonomical group (Veith et al. 2004). They are clearly distinct from the *B. cereus* and *B. anthracis* group (Rasko et al. 2004). Genome comparison among these three *Bacilli* revealed that they had a core genome of 2323 orthologous proteins. *B. licheniformis* DSM13 had its own 902 genes and shared with *B. subtilis* 872 genes and only 189 with *B. halodurans* (Veith et al. 2004).

The genome sequence of *B. licheniformis* DSM13 has also been analysed for potential sigma factors. A sigma factor (σ) is a protein component of RNA polymerase that determines the specific site on DNA where transcription begins. The sequence recognised by each σ factor at the promoter of genes is highly conserved (Helmann and Moran 2002). By searching the consensus sequence in regions of core genes, three sigma factors, σ^B, σ^D and σ^L have been identified in *B. licheniformis* DSM13 (Veith et al. 2004). σ^D regulates the expression of genes required for chemotaxis and motility. Conserved recognition sequences of σ^D were identified in the upstream sequence of the *hag*, *motA*, *cheV*, *mcpA-C yvyC-F* and *lytD* genes of *B. licheniformis*, whereas, σ^L regulates the expression of genes, which are involved in the catabolism of certain amino acids and in the acetoin metabolism. Consensus sequences were found in the upstream region of the *levD*, *rocD*, *ptb*, *yveP* and *acoA* genes (Veith et al. 2004). σ^B plays a major role in the regulation of the general stress response in *B. subtilis*. *B. licheniformis* genome possesses a homologue of the σ^B general stress operon, including the σ^B structural gene and seven *rsb* regulatory genes. The regulatory networks of the general stress response in *B. licheniformis* probably are similar to *B. subtilis* (Brody and Price 1998).

The genome analysis indicated that there is a high functional conservation between *B. subtilis* and *B. licheniformis* in the regulatory mechanisms of genes of the core genome. Analysis of the *B. licheniformis* genome sequence revealed that most of the genes involved in the central pathways of glycolysis, pentose phosphate cycle and the tricarboxylic acid cycle, which are described in detail for *B. subtilis*, were also found in *B. licheniformis* (Veith et al. 2004). Besides, the genome of *B. licheniformis* contains genes with strong homology to isocitrate

lyase and malate synthase (Veith et al. 2004). These genes allow this organism to grow on C-2 substrates e.g. acetate and 2,3-butanediol following the glyoxylate bypass. This possibility is not found in *B. subtilis*. The growth on C-2 substrates made a great advantage for *B. licheniformis* to better adapt in obtaining additional energy from incompletely oxidized products accumulated under insufficient oxygen supply.

The genes coding for proteins which are mainly responsible for the sporulation system have mostly been identified in *B. licheniformis* (Veith et al. 2004). Furthermore, *B. lichenifomis* also consists of a potential siderophore biosynthesis gene cluster as found in *B. subtilis*. Interestingly, the genome of *B. licheniformis* consists of a large number of genes encoding proteins required for a secretory pathway, so called Sec pathway (Veith et al. 2004). Proteins transported by Sec pathway are synthesised as pre-proteins with an N-terminal signal peptide. The N-terminal signal peptide plays an important role for targeting of the protein to the Sec machinery (Tjalsma et al. 2000; Antelmann et al. 2001).

The main difference between the sequence of *B. subtilis* and *B. licheniformis* is that *B. licheniformis* does not exhibits the presence of polyketide synthases, which occupy about 4% of the genome in *B. subtilis*. The polyketide synthases are multifunctional enzymes that are responsible for the synthesis of a number of secondary metabolites with diverse structure and functions e.g. vancomycin, erythromycin, equinocandin and rapamycin (Schwarzer and Marahiel 2001). In addition, Veith et al. (2004) also identified several genes of two operon structures included in type I restriction modification systems. This finding explains the reason why it is difficult to transform heterologous DNA into this organism.

The proteomic signature of a cell is an overview of a subset of proteins and its change in synthesis is characteristic for the response of the cell to physiological or genetic changes (VanBogelen et al. 1999; Hecker 2003). It provides information about the physiological state of the cell and supports the classification of proteins into functional groups as well as in stimulons and regulons. The identification of biomarkers by analysing specific transcriptomic and proteomic signatures is a suitable approach to enlighten the physiological capacity of the cells but also to monitor the cellular physiology during industrial bioprocesses (Voigt et al. 2004; Jürgen et al. 2005 a, b).

The genome sequence of *B. licheniformis* is now available (Veith et al. 2004, Rey et al. 2004). These data bear an important source of information to get new insights and tools for investigation of the physiology of the cells on the transcriptome and proteome level as well as in the application of this strain in industrial protein production. Voigt et al. (2004) gave a first overview of cell physiology of *B. licheniformis* based on the two-dimensional polyacrylamide

gel electrophoresis (2D-PAGE). The analysis of the cytoplasmic proteome of *B. licheniformis* cells leads to an identification of about 300 protein spots on the gels, either in samples taken from cells growing on minimal medium or on complex medium. Almost all glycolytic and TCA cycle enzymes, 50 enzymes involved in amino acid metabolism and 20 aminoacyl-tRNA synthetase and only 10 % of unknown proteins have been found. Additionally, translational proteins (elongation factor EF-G, EF-Tu), some ribosomal proteins, chaperones, flagellin (Hag) and enzymes of main metabolic pathways such as glycolytic and TCA cycle enzymes were among the most abundant proteins. In minimal medium, enzymes for amino acid biosynthesis were synthesised, whereas in Luria Broth (LB) medium enzymes for amino acid degradation (RocD, RocF and other) and a number of proteins involved in catabolic pathways like YcgN, IolS, AcoB and PurR have been found.

Furthermore, the study of Voigt et al. (2004) revealed that the most obvious changes of protein synthesis during stationary phase were subjected to a severe oxidative stress and a resulting protein stress. The strong induction of the katalase (KatA) and alkylhydroperoxide reductases (AhpC, F), the protease ClpC, and the chaperonin GroEL were found. In addition, many vegetative proteins in growing cells were no longer synthesised in non-growing cells. Different catabolic proteins necessary for the mobilization of alternative nutrient sources were induced. *B. licheniformis* also increased the synthesis of enzymes required for arginine and ornithine (RocD and F), acetate utilisation (AcsA) as well as the aldehyde dehydrogenase (DhaS) and the protease (Vpr).

1.2. The general stress response of *B. subtilis*

In their natural environment, bacteria are frequent subjects of a variety of stresses. In order to adapt to environmental changes such as physical stresses (e.g. heat, low temperature, osmotic shock, salt, oxygen limitation) and nutrient starvations (e.g. glucose, nitrogen, phosphate, amino acids), cells have evolved complex regulatory systems for their survival including a series of general and specific responses. The set of proteins usually synthesised under conditions of different stresses and nutrient starvations is called general stress proteins (GSPs) (Hecker and Völker 2001). Induction of these genes is essential to protect the cell against oxidative damage of DNA, membranes and proteins under these adverse conditions, e.g. the induction of Dps in the protection of DNA; ClpP, ClpC and ClpX in the degradation of misfolded or denatured proteins that are toxic for the cell (Hecker and Völker 2001). In addition, *B. subtilis* has also an adaptive stress response, so called stringent response. The

stringent response prevents wastage of nutrients during nutrient deprivation (Wendrich and Marahiel 1997; Eymann et al. 2002). It is the adaptive response to metabolic stress and promotes energy conservation by turning off most of the synthetic processes in non-growing cells, e.g. the down regulation of genes belonging to the translational machinery like rRNAs, tRNAs, ribosomal proteins, translational factor (*trig*, *rplL*, *infB*, *tsf*) and RNA synthesis (*nusA*) (Eymann et al. 2002). Besides these general stress responses, the cell produces a set of specific proteins that leads to specific protections against a defined stress condition, so called specific stress proteins. For example under phosphate starvation condition, the induction of *phoA* and *phoB*, both coding for alkaline phosphatases enables the cells to use limiting phosphate resources more efficiently. Furthermore, the induction of a phosphate ABC transporter (*pstS*) under these conditions is responsible for a high-affinity phosphate transport system (Eymann et al. 1996; Antelmann et al. 2000).

1.2.1. The general stress sigma factor σ^B

The *B. subtilis* sigma factor σ^A is the primary, vegetative σ factor (Helmann and Moran 2002). σ^B, as an alternative σ factor in *B. subtilis*, is also present in *B. licheniformis* (Brody and Price 1998; Veith et al. 2004). The general stress response of *B. subtilis* is mainly controlled by this transcription factor and a signal transduction network that regulates the activity of σ^B. The sigma factor σ^B regulates the expression of over 200 genes in *B. subtilis* (approximately 5% of the genes in the genome) (Price et al. 2001; Schweder and Hecker 2004; Hoper et al. 2005; Völker and Hecker 2005). σ^B is regulated by two partner-switching modules: RsbX-RsbS-RsbT and RsbU-RsbV-RsbW. Each module consists of a phosphatase (RsbX or RsbU), an antagonist protein (RsbS or RsbV), and a switch kinase (RsbT or RsbW) (Yang et al. 1996; Zhang and Haldenwang 2005).

The activation of σ^B is controlled by the phosphorylation state of the anti-σ factor antagonist RsbV. The phosphorylation state of RsbV is controlled by opposing kinase RsbW and phosphatase RsbU activities. Under non-stress conditions, anti-σ^B protein kinase RsbW uses adenosine triphosphate (ATP) to phosphorylate an antagonist protein RsbV. Phosphorylated RsbV is an inactive form and not able to form a complex with RsbW. RsbW therefore binds to σ^B and keeps it in an inactivated complex, RsbW-σ^B (Kang et al. 1996; Hecker and Völker 2001; Zhang and Haldenwang 2005). Under nutrient starvation conditions, which cause a low energy level, cellular ATP levels are decreased. This leads to an unphosphorylated RsbV. Unphosphorylated RsbV is an active form. It binds to RsbW in a RsbW-RsbV complex,

thereby releasing σ^B from its inhibition by RsbW and allowing σ^B to interact with the RNA polymerase, which finally promotes the transcription of σ^B dependent genes (Alper et al. 1996; Kang et al. 1996; Hecker and Völker 2001). In addition, two specific PP2C (protein phosphatases 2C) domain phosphatases, RsbU and RsbP also act on RsbV (Vijay et al. 2000). When *B. subtilis* is exposed to stress, one of two stress-specific phosphatases is activated to remove the serine phosphate from phosphorylated RsbV, thereby allowing RsbV to displace σ^B from the RsbW-σ^B complex. RsbP is activated upon energy stress, RsbU seems to be activated by physic stress. However, the mechanisms by which RsbP or RsbU becomes active are not elucidated yet.

1.2.2. The stringent response

The stringent response was at first observed in *Escherichia coli* in response to amino acid starvation (Stent and Brenner 1961). It has been shown that the adaptation of the cells depends on the synthesis of hyperphosphorylated guanosine tetraphosphate ((p)ppGpp), in which the *relA* gene is known to encode a ribosome bound (p)ppGpp synthetase. RelA catalyses the transfer of a pyrophosphoryl group of ATP to the 3´-hydroxyl group of guanosine triphosphate (GTP), this produces (p)ppGpp (Haseltine et al. 1972). Under conditions of excess of amino acids, most of the tRNAs are attached to amino acids and existed in a charged state. However, when cells starve for amino acids, the concentration of uncharged tRNAs increases and accumulates, and subsequently competes with charged tRNAs for binding to the A site on the ribosome. This activates RelA to produce (p)ppGpp. Then, (p)ppGpp binds to the β-subunit of RNA polymerase and inhibits transcription of rRNAs and tRNAs (Chatterji et al. 1998; Chatterji and Ojha 2001). The inhibition of rRNA synthesis is considered as a result of an accumulation of ppGpp. Moreover, the regulation of (p)ppGpp also depends on the product of a *spoT* gene coding for a ppGpp synthetase and hydrolase. However, it is not associated with ribosomes (Sarubbi et al. 1989). Activity of ppGpp hydrolyase is activated when the cells are grown in an amino acid rich medium. This does not lead to an accumulation of ppGpp. When cells are grown in amino acid starvation conditions, activity of ppGpp synthesis is activated, resulting in ppGpp accumulation, which causes the stringent response. On the other hand, rRNA synthesis is tightly related to the nutritional status of the cell. Changes of nucleotide triphosphate (NTP) and ppGpp concentrations also regulate rRNA promoter activities in *E. coli* (Gaal et al. 1997). The increase of (p)ppGpp concentration competes with GTP and guanosine diphosphate (GDP),

which is required for translation factors. Therefore, protein biosynthesis is reduced by the repression of stable RNA (rRNA, tRNA) synthesis.

The stringent response has been studied in more detail in *B. subtilis* (Hecker et al. 1987; Wendrich and Marahiel 1997; Eymann et al. 2002; Krasny and Gourse 2004). The *relA* gene plays also a key role in the stringent response of *B. subtilis*. The *B. subtilis relA* gene was cloned and characterized by Wendrich and Marahiel (1997). RelA revealed a significant homology to the *E. coli* RelA and SpoT coding genes. However, the *B. subtilis* RelA protein has only (p)ppGpp synthetase activity. The regulation of (p)ppGpp in *B. subtilis* has been shown to be more complicated. Krasny and Gourse (2004) found that the initiating nucleotide triphosphate (NTP) for the transcription of *B. subtilis* rRNA promoter was GTP. Changes in the intracellular GTP and ppGpp concentrations affected the activity of this promoter. Synthesis of ppGpp is a result of a significant drop in GDP/GTP level in *B. subtilis*. Similar to *E. coli*, the regulation of rRNA promoter activities also depends on changes of concentration of NTP and ppGpp. However, *B. subtilis* ppGpp probably inhibits rRNA transcription indirectly by decreasing GTP pools. The inhibition of ppGpp to transcription is also explained by the competition between binding of ppGpp and the substrate NTPs to the active site of the RNA polymerase. The concentration of the initiating NTPs plays a crucial role in this inhibition (Jores and Wagner 2003). The stringent response regulated genes of *B. subtilis* were divided into four distinct classes (Eymann et al. 2002). These classes have been characterised as the i) *relA*-dependent repression, ii) *relA*-dependent induction, iii) *relA*-independent repression and iv) *relA*-independent induction. Eymann et al. (2002) identified 20 proteins and around 40 transcriptional units which were under the negative control of the stringent response. They belong to the translational machinery including rRNAs, tRNAs, ribosomal proteins and translational factors (trigger factors, initiation and elongation factors) as well as RNA synthesis (*nusA*, *rpoA*, *rpoB*). In addition, the expression of genes involved in other processes typical for growing cells such as the tricarboxylic acid cycle, nucleotide biosynthesis, synthesis of lipids, energy metabolism, RNA and protein modification, cell wall synthesis and DNA replication was repressed. The down-regulation of these genes was also observed when *B. subtilis* was grown under conditions of peroxide, superoxide stresses and glucose starvation (Bernhardt et al. 2003; Mostertz et al. 2004; Voigt et al. 2004; Koburger et al. 2005). It was found that (p)ppGpp positively controls the expression of 13 proteins and 50 transcriptional units in *B. subtilis*. One interesting finding is the *relA*-dependent induction of isoleucine and leucine biosysthesis (*ilv-leu*) operon in norvaline-treated cells (Eymann et al. 2002). The regulation of the gene expression of the *ilv-leu* operon is controlled by a

transcriptional pleiotropic repressor (CodY), a global regulatory protein in the nitrogen metabolism (Shivers and Sonenshein 2004; Tojo et al. 2005). Thus, the stringent response is also involved in the derepression of CodY. However, the interaction between CodY and RelA is not elucidated. (p)ppGpp is involved in the regulation of developmental processes and antibiotic production of Gram-positive bacteria (Ikehara et al. 1985; Switzer et al. 1985). Interestingly, *relA* plays a role in nutritional stress activation of σ^B (Zhang and Haldenwang 2003). *B. subtilis relA* mutant prevents the activation of σ^B under nutritional starvation conditions. Whereas, the activation of σ^B is not affected under physical stresses. However, the stringent response is distinct from the σ^B-mediated response to amino acid starvation conditions (Wendrich and Marahiel 1997; Zhang and Haldenwang 2003).

1.3. The specific stress response of *B. subtilis*

1.3.1. The phosphate starvation response in *B. subtilis*

Phosphate is one of the most important nutrients for all organisms. It plays a crucial role in the formation of the phospholipid bilayers in the cell membranes, of biomolecules such as pyrophosphate, ATP, adenosine diphosphate (ADP) or adenosine monophosphate (AMP) and it is also important for the energy transfer and metabolic regulation. However, in nature, phosphate is the second nutrient after nitrogen, which is frequently limited for soil bacteria (del Pozo et al. 1999; Salisbury and Ross 1992).

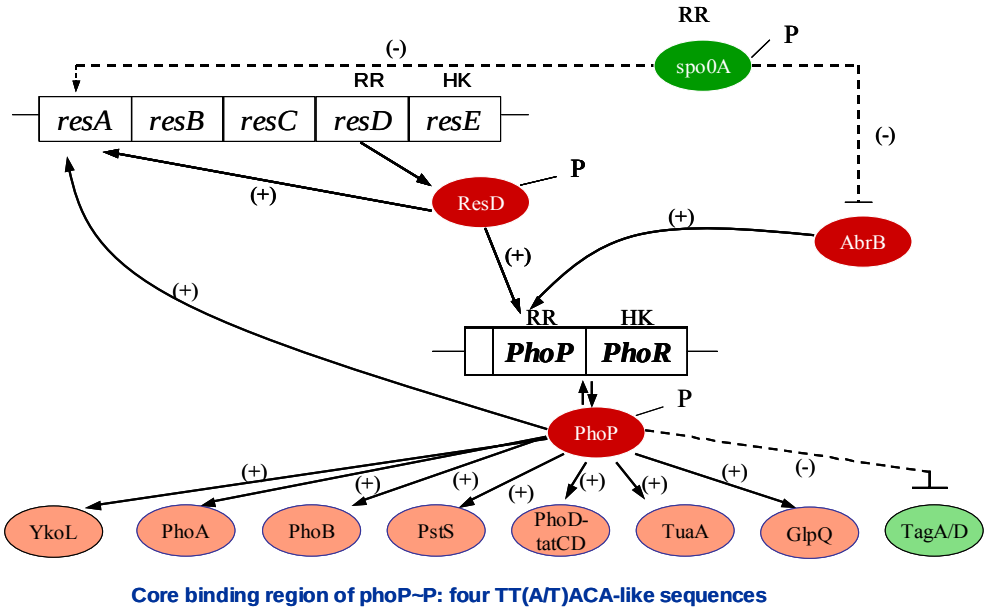


Figure 1: Schematic presentation of the phosphate starvation response in *B. subtilis*. (Adapted from Hulett, F.M 2002). PhoPR is the main regulator. It regulates the expression of the Pho regulon genes to allow the cell to use alternative organic phosphate sources. ResD is required for full expression of Pho genes. Spo0A~P represses the transcription of *phoPR* through inhibiting the expression of AbrB and ResDE. Detailed information is given in the text.

When exposed to phosphate starvation, the Gram-positive bacterium *B. subtilis* induces a specific response, resulting in the activation of the PhoPR regulon, Spo0 phosphorelay, ResD-ResE (Hulett 1996, 2002; Sun et al. 1996; Allenby et al. 2005), as well as the σ^B-dependent general stress response (Petersohn et al. 1999; Antelmann et al. 2000; Allenby et al. 2005). A third gene class induced under such conditions is regulated independently of either PhoPR or σ^B (Antelmann et al. 2000) (see also table 1).

PhoP–PhoR is one of the three two-component systems known to play a major role in the adaptation to the phosphate starvation response (Hulett 2002). The Pho regulon consists of more than forty genes (Allenby et al. 2005). It directly regulates the induction and repression of the Pho regulon genes in order to enable the cell to use and transfer limiting phosphate resources more efficiently. The Pho regulon contains genes that consist of PhoP binding regions in their upstream sequences regulated by phosphate starvation. In addition, down or up regulation of Pho dependent genes enable the cell to scavenge other forms of phosphates such as organic phosphate or to utilise other phosphate sources. PhoP and PhoR of *B. subtilis* have been considered to be homologous to PhoB and PhoR of *E. coli* (Seki et al. 1987, 1988). PhoR is a histidine kinase and a membrane-bound protein, which is sensitive to low phosphate concentrations at both the outer surface of the cell membrane and within the cytoplasm. When phosphate source is limited, PhoR is autophosphorylated. PhoR then transfers a phosphoryl group to PhoP thereby activating PhoP to PhoP~P. PhoP~P in turn activates the transcription of genes as shown in table 1. For example, proteins encoded by genes of the *tuaABCDEFGH* operon are responsible for replacing the polyglycerol phosphate cell wall polymer by a non-phosphate anionic cell wall polymer (Lang et al. 1982; Qi and Hulett 1998 b; Liu and Hulett 1998). PhoP~P also represses the expression of other Pho regulon genes such as *tagA/D* encoding the biosynthesis of teichoic acid (Liu et al. 1998 a). PhoP recognises and binds to four TT(A/T)ACA-like sequences repeated at intervals of 11 bp and separated by approximately 5 bp in the promotor regions of Pho regulon genes (Hulett 2002; Pragai et al. 2004).

Induction of the *resABCDE* operon genes results in increased expression levels of the ResD protein. *B. subtilis resD* mutants decreases in a range of approximately 20% the expression levels of Pho regulon genes. ResD therefore is required for full induction of the Pho regulon response (Sun et al. 1996; Schau et al. 2004). AbrB is a transition state regulator of gene expression in *B. subtilis*. It functions as an activator of ResD. A *resD/abrB* double mutant shows no induction of the Pho regulon gene. Thus, AbrB also is involved in activation of the Pho regulon (Hulett et al. 1994, Hulett 1996). In addition, the phosphate starvation response

of *B. subtilis* is also regulated by Spo0A, a transcriptional factor essential for the initiation of sporulation (Burbulys et al. 1991, Hulett 1996). A major role of Spo0A~P is the down regulation of *abrB* transcription, thereby repressing the expression of *phoPR*. In addition to its repression by AbrB, Spo0A~P also inhibits the response of the Pho regulon by repression of ResDE (Hulett 1996, 2002).

1.3.2. The glucose and nitrogen starvation response

The regulation of the carbon metabolism in *B. subtilis*

Bacteria are able to use a variety of carbohydrates as sources of carbon and energy. When grown under the abundance of nutrients, they prefer to use favorable carbon sources such as glucose for optimal growth rates. However, in the natural environment, nutrient stress conditions are common (Hulett 1996; Detsch and Stülke 2003; Koburger et al. 2005). The ability to rapidly adapt to environment changes is essential for cell survival. Complex regulatory systems are required for enabling the cells to adapt to new environmental conditions. By changing the synthesis of degradative enzymes, required for the catabolism of nutrients, and transporters, bacteria can maintain at slow growth rates under nutrient starvation conditions. When *B. subtilis* cells were grown in medium containing different carbohydrate sources, hundreds of carbohydrate catabolism genes have been induced, which are under the control of global regulatory networks (Moreno et al. 2001; Yoshida et al. 2001; Wacker et al. 2003) (see also table 1).

Carbon catabolite repression (CCR) is an important regulatory mechanism, controlling the expression of numerous genes in response to the availability of metabolisable carbon sources (Saier et al. 1996; Stulke and Hillen 2000; Bruckner and Titgemeyer 2002). CCR is mediated by a catabolite regulatory protein A (CcpA), a member of the LacI/GalR repressor family, its co-repressors (HPr, Crh, NADP), other agents and a bifunctional protein HPr kinase/phosphatase (HPrK/P) (Hueck and Hillen 1995; Saier et al. 1996; Stulke and Hillen 2000; Turinsky et al. 2000) (see also figure 2).

CcpA acts as a repressor or an activator of transcription in response to the availability of glucose and other carbohydrates in the growth medium. CcpA plays a major role as a repressor of genes, which are involved in the utilization of secondary carbon sources. It is also an activator of carbon excretion pathways, including those for production of acetate, acetoin and glycogen during cell growth in media containing glucose (Renna et al. 1993; Presecan-Siedel et al. 1999; Turinsky et al. 2000; Yoshida et al. 2001; Koburger et al. 2005).

Moreover, CcpA-mediated catabolite repression or activation also depends on so-called conserved CRE-sites (cis-acting catabolite-responsive element) located within, downstream or upstream in the promoter regions of its DNA target sequences (Miwa et al. 2000; Turinsky et al. 2000; Moreno et al. 2001).

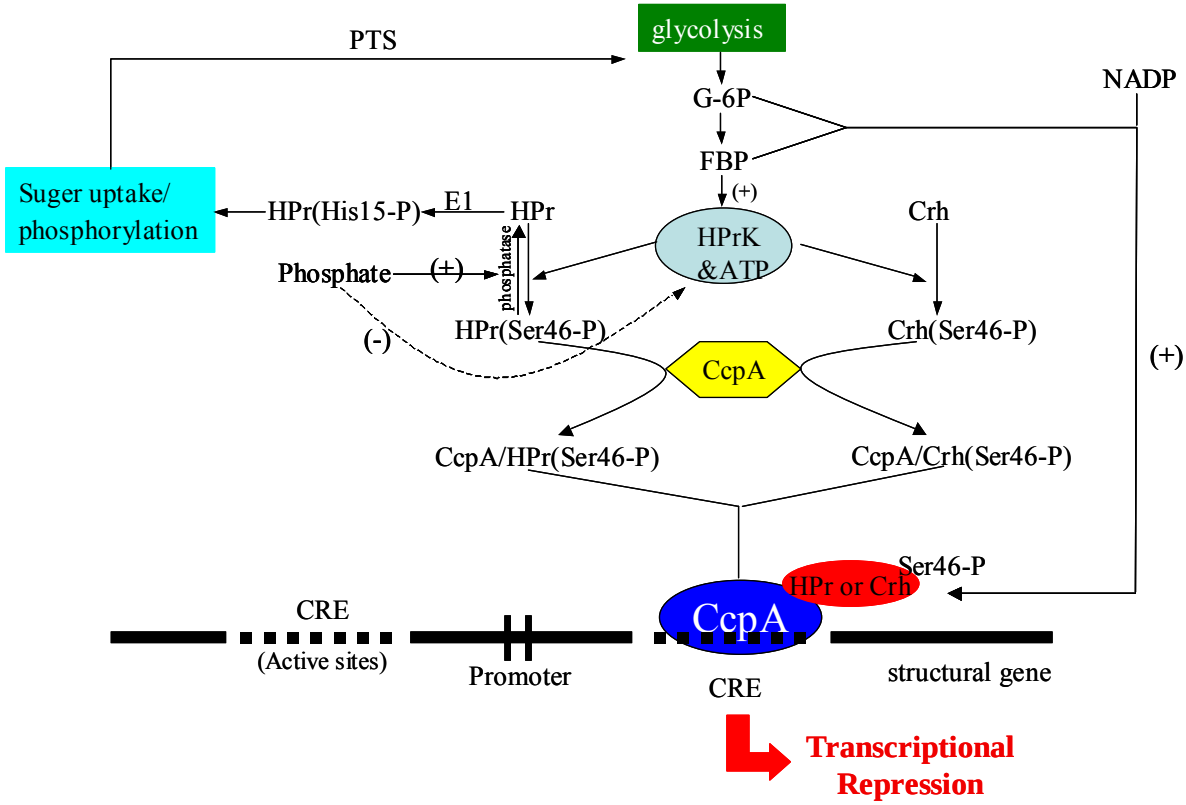


Figure 2: A schematic presentation of the carbon catabolite repression (CCR) in *B. subtilis*. (Adapted from Kim *et al*. 1998; Galinier *et al*.1998; Deutscher *et al*. 2002). CcpA is the main regulator of the CCR.An elevated uptake of carbohydrates leads to the increase of FBP. Then it stimulates the phosphorylation of HPr or Crh by HPrK. The phosphorylated HPr binds to CcpA to form a protein complex that promotes interaction of the CcpA-HPr-Ser~P complex to the DNA at specific CRE sites. Detailed information is given in the text.

The co-repressors of the CcpA, HPr and Crh, play a central role in the CCR (Galinier et al. 1997, 1999; Faires et al. 1999; Deutscher et al. 2002). They exhibit a high sequence identity of around 45% to each other. HPr, a phosphocarrier protein plays a key role in the uptake of carbohydrates via the phosphoenolpyruvate (PEP): sugar phosphotransferase system (PTS) as well as in the regulation of carbon metabolism. HPr is phosphorylated in two different ways either by an ATP-dependent phosphorylation at a regulatory serine (Ser-46) or by PEP-dependent phosphorylation at histidine 15 residue (His-15) (Deutscher et al. 2002).

The uptake of carbohydrates takes place when HPr is phosphorylated at His-15. The transport and phosphorylation of its sugar substrates is accomplished by using a phosphorylation cascade consisting of Enzyme I (EI), HPr and sugar-specific proteins II (EIIA) and (EIIB).

PEP is the source of energy for the transport step and also provides the phosphoryl group for the phosphorylation of carbohydrates. The proteins EII are multidomain proteins. EIIA and EIIB are hydrophilic proteins and involved in the transfer of phosphoryl groups, while EIIC is a membrane-bound protein and catalyses the sugar transport. The first step in the cascade is the phosphorylation of EI to EI~P by PEP and subsequently, the phosphoryl group is transferred from PEP to the phosphoryl-carrier protein HPr. Secondly, the phosphorylated HPr transfers the phosphoryl group to the sugar-specific EIIAs, each enzyme II consists of domains specific for a single carbohydrate. Thirdly, the corresponding EIIB transfers the phosphoryl group from EIIA~P to the membrane-bound protein EIIC. The phosphorylated carbohydrate is then released into the cell. The enzyme EII can exist as a single protein with three domains ABC or two to four distinct proteins (Postma et al. 1993).

The phosphorylation of HPr at Ser-46 is catalysed by an ATP-dependent fructose 1,6-bisphosphate-activated HPr kinase (HPrK), a bifunctional allosteric key enzyme in the main mechanism of carbon catabolite repression/activation (Deutscher et al. 1995; Deutscher et al. 2002; Fieulaine et al. 2002; Ramstrom et al. 2003). The ATP-dependent phosphorylation of HPr regulates the expression of catabolic genes in response to rapidly metabolisable carbon sources. The activity of the kinase is stimulated by glycolytic key intermediates such as fructose-1,6-bisphosphate (FBP) and glucose-6-phosphate (G6P) and repressed by inorganic phosphate (Pi). Thus, the intracellular concentrations of glycolytic intermediates can be regarded as an indicator of the metabolic state of the cell. When rapidly catabolisable carbon sources are available, the level of FBP will increase, whereas the Pi level will decrease. The increased level of FBP stimulates the activity of the kinase, leading to an increase in HPr-Ser46~P. Afterwards, HPr-Ser-46~P binds to CcpA in order to form a protein complex that promotes interaction of the CcpA-HPr-Ser~P complex to the specific CRE sites on the DNA of the respective gene. Depending on the location of the CRE sites on the sequence of a gene, this protein complex can either repress or activate its transcription (Jones et al. 1997; Reizer et al. 1998; Stulke and Hillen 2000; Turinsky et al. 2000). Low uptake of carbohydrates results in an increase of inorganic phosphate. Then it stimulates the phosphatase activity as well as represses the activity of the kinase, leading to a decrease of HPr-Ser~P and the release of catabolite repression. These phosphorylation reactions are the key steps in the mechanism of catabolite repression and inducer exclusion (Deutscher et al. 1994; Saier et al. 1996). Besides HPr and Crh, NADP also acts as a co-repressor of CcpA. When NADP was added to CcpA, it enhanced transcription inhibition of the *amyE* promoter

up to 120-fold (Kim et al. 1998).

However, there are some genes or operons the expression of which is under the control of CcpA-independent mechanisms or partially under CcpA-independent control or indirectly CcpA-dependent control (Moreno et al. 2001; Yoshida et al. 2001; Darbon et al. 2002). Yoshida et al. (2001) and Servant et al. (2005) showed that the expression of the *iol* operon, the *ctaCDEF* operon and some gluconeogenesis genes such as *gapB*, *pckA* were under the control of CcpA-independent or at least partially CcpA dependent.

Table 1: Summary of the main regulators involved in regulation of genes during glucose, nitrogen and phosphate limitation. Further details are given in the text

	Regulators	Genes regulated	References
Glucose starvation	CcpA	repressed by CcpA: *acoABCDL*, *acuABC*, *tdh*, *qcrABC*, *rbsABCDKR*, *kbl*, *bglHP*, *etfAB*, *citG*	Turinsky et al. 2000 Moreno et al. 2001 Belitsky and Sonenshein 2004 Presecan-Siedel et al. 1999 Renna et al. 1993 Koburger et al. 2005 Darbon et al. 2002 Tojo et al. 2005
		induced by CcpA: *pta*, *ackA*, *alsSD*, *ilv-leu*, *gltAB*	
		repressed partially by CcpA: *acoA*, *gapB*, *pckA*, *iol* operon	
Nitrogen starvation	TnrA	repressed by TnrA: *glnRA*, *gltAB*, *ilv-leu*, *pel*, *ywdIJK*, *ywlFG*, *yodF*, *alsT*	Wray et al. 1996, 1997 Belitsky et al. 2000 Brandenburg et al. 2002 Wacker et al. 2003 Yoshida et al. 2003 Tojo et al. 2004, 2005
		induced by TnrA: *tnrA*, *nasABCDEF*, *ureABC*, *nrgAB*, *gabP*, *asnZ*, *pucJKLM*, *glnQHMP*, *oppABCDF*, *yknA*, *ywrD*, *kipI*, *ykzB-ykoL*	
	GlnR	repressed by GlnR: *tnrA*, *glnA*, *ureABC*,	
	CodY	*dpp*, *gabP*, *acsA*, *ilv-leu*, *ureABC*, *hut*, *rocABC* and *rocDEF*, *bkd*, *comK*, *citB*, *hag*	
Phosphate starvation	PhoPR	*phoA*, *phoB-ydhF*, *pstS*, *glpQ*, *phoD-tatCD*, *tuaABCDEFGH*, *tagA/D*, *resABCDE*, *yurI*, *yfkN*, *vpr*, *yjdB*	Antelmann et al. 2000 Hulett 2002 Allenby et al. 2005

The regulation of the nitrogen metabolism in *B. subtilis*

CcpA is not only a central key regulator of the carbon metabolism but also of the nitrogen metabolism in *B. subtilis* and other Gram-positive bacteria (Wacker et al. 2003). It regulates the expression of glutamate synthase (*gltAB* operon), one of the most important enzymes which play a key role in ammonium assimilation (Wacker et al. 2003). Due to the lack of the expression of gltAB, *B. subtilis ccpA* mutants are not able to grow on minimal medium with ammonium and glucose as single sources of nitrogen and carbon (Faires et al. 1999). The expression of *gltAB* operon is induced by glucose and is also controlled by TnrA, a global regulator of nitrogen metabolism (Belitsky et al. 2000; Wacker et al. 2003; Belitsky and Sonenshein 2004). Moreover, nitrogen sources such as amino acids are necessary for full induction of glycolytic genes as well as for repression of genes in the Krebs citric acid cycle (Ludwig et al. 2001). In addition, the expression of the *ilv-leu* operon, which plays a role in protein and fatty acid metabolism by synthesis of branched-chain amino acids, is under the control of not only CcpA but also TnrA and CodY, both are global regulator proteins in the nitrogen regulation system (Ludwig et al. 2002; Tojo et al. 2004; Tojo et al. 2005). Consequently, there are tight interactions between carbon and nitrogen metabolism (see also table 1).

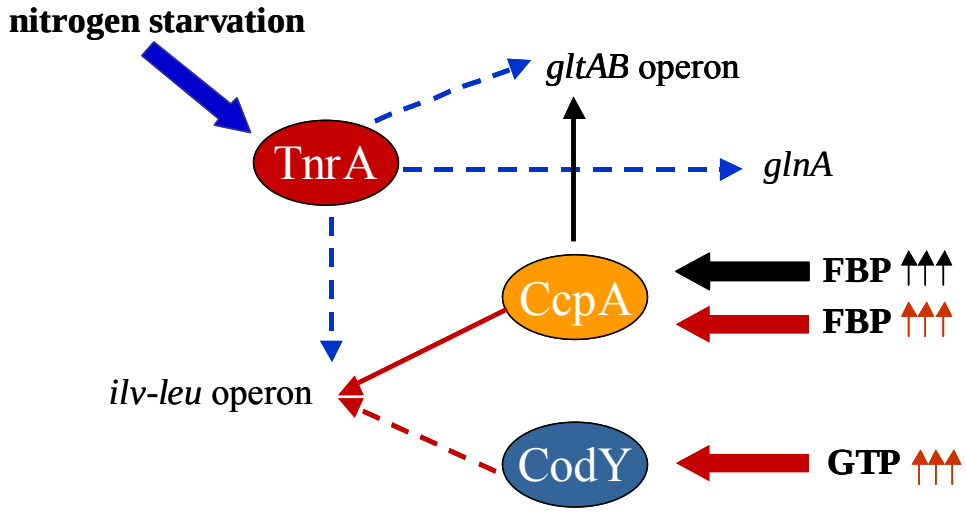


Figure 3: Schematic presentation of regulators involved in the carbon and nitrogen regulation in *B. subtilis*. CcpA is the regulator of not only glucose starvation response but also nitrogen starvation response in *B. subtilis*. Together with CodY and TnrA, it regulates the expression of *gltAB* and the *ilv-leu* operon. (Adapted from Wacker *et al*. 2003; Tojo *et al.* 2004). Detailed information is given in the text.

Nitrogen metabolism in *B. subtilis* is controlled by at least three global regulatory proteins TnrA, GlnR and CodY (Fisher and Sonenshein 1991; Wray et al. 1998; Fisher 1999; Wray et

al. 2001; Fisher and Debarbouille 2002) (see also table 1). These regulator proteins are active under different growth conditions.

TnrA functions as either an activator or repressor of gene expression only during nitrogen-limited conditions, whereas GlnR is active only during excess of nitrogen (Fisher and Debarbouille 2002). CodY controls the expression of several genes in cells growing rapidly with amino acids. GlnR and TnrA play important roles in the nitrogen regulatory systems which allow the cell to adapt to growth under different nutritional conditions. TnrA transcriptionally represses the expression of genes listed in table1 (Wray et al. 1996; Yoshida et al. 2003; Wacker et al. 2003). TnrA also positively activates its own promoter and the expression of genes which are responsible for the mobilization of alternative nitrogen sources during nitrogen-limited conditions (Schreier et al. 1991; Wray et al. 1994; Wray et al. 1997; Yoshida et al. 2003; Tojo et al. 2005). TnrA is known to be repressed by glutamine synthetase (GS) during nitrogen excess conditions via a protein-protein interaction. On the other hand, GlnR represses the transcription of *glnA* encoding GS, urease and *tnrA* during excess nitrogen conditions (Schreier et al. 1989; Wray et al. 1997; Brandenburg et al. 2002).

Although the nutrient conditions responsible for their activation are different, TnrA and GlnR bind to similar DNA sequences. TnrA and GlnR belong to the MerR family. The MerR family is a group of transcriptional activators. It has similar N-terminal "helix-turn-helix" DNA and C-terminal effector binding regions. These binding regions are specific DNA sequences recognised by the effector (Brown et al. 2003). The nitrogen signal regulating TnrA activity has been elucidated. Feedback-inhibited *B. subtilis* GS directly interacts with TnrA and controls DNA binding activity of TnrA by a protein-protein interaction. The binding of feedback inhibitors to GS alters the structure in the GS protein and increases the binding affinity of GS to TnrA to form a protein-protein complex and thereby blocks the DNA binding activity of TnrA (Wray et al. 2001; Fisher and Wray 2002; Wray and Fisher 2005). However, the role of GS in controlling GlnR activity is not identified yet. The regulation of TnrA activity by GS is a unique and important mechanism allowing the cell to flexibly response to different nitrogen sources.

1.4. *Bacillus* expression systems

Based on modern recombinant DNA techniques like mutation procedures, cloning and protein engineering processes new enzyme properties and biotechnological applications are arising (Schallmey et al. 2004). Furthermore, improved expression systems can result in increased

enzyme productivity. The knowledge of the complete genome sequence of *B. licheniformis* provides now the basis for a better understanding of its cell physiology, and information about the functions of its genes. Although, *B. licheniformis* has been used for a long time as an expression host in the industry only little information on expression systems are available. Up to now, only some expression systems have been published for *B. subtilis* (Harwood et al. 2002).

The essential elements for an expression vector are: an (i) expression cassette containing the suitable promoter, (ii) an efficient terminator, (iii) suitable signal sequence for the secretion of the protein, (iv) a selection marker and (v) a replication function for bacterial vectors which determines the copy number of the vector in a given host (Nordstrom and Uhlin 1992). Furthermore, a suitable expression plasmid requires a good segregational and structural stability during fermentation processes (Bron and Luxen 1985; Warnes and Stephenson 1986; Janniere et al. 1990; Ebersbach and Gerdes 2005).

One of the most important strategies for constructing an expression system is the selection of the suitable promoter and its regulators. The promoter is the specific DNA sequence recognised by a sigma factor and from which RNA polymerase initiates transcription (Haldenwang 1995). Promoter strength plays an important role because it determines the number of a given mRNA transcribed per unit time. This depends on composition of bases in the promoter sequence and critical regulators of gene expression like operators, enhancers, ribosomal binding site (RBS), spacer and start codon (Lisser and Margalit 1993; Jürgen et al. 1998; Rocha et al. 1999). Codon usage is also a critical factor in the heterologous gene expression. Because preferred codons of each organism correlate with the abundance of cognate tRNAs available within the cell (Karlin and Mrazek 2000). The tendency in the use of strong native promoters is being widely applied because the foreign gene promoters are often found with general inefficiency (Wang and Doi 1992). Moreover, specific characteristics of selected native promoters enable a control of the recombinant gene expression in response to the physiological status of the cell under defined conditions. These lead to the reduction of costs of the inducer and easy handling of the system (Yansura and Henner 1984, 1990). The efficiency of the expression system determined by the amount of functional protein is a result of a complex interplay among the promoter strength, the structural gene codon usage, the secretion efficiency and the host strain physiology.

The selection marker for plasmid maintenance used in laboratory studies is usually an antibiotic resistant gene. However, incorporating antibiotic into the large-scale production fermentation is frequently not accepted. Different approaches, for example auxotrophies, are

therefore preferable to avoid the formation of multi-resistant bacteria and problems in allergy for biopharmaceutical consumers. For instance the D-alanine racemase (*dal*) genes was tested as selection marker for plasmid maintenance. The plasmid containing the *dal* is introduced into a dal^{-} mutant. In the dal^{-} mutant strain, D, L-alanine racemase is not produced. This racemase is required for producing the D-alanine component of the cell wall (Brans et al. 2004).

For plasmid-bearing strains, multi copy expression vectors are constructed to ensure a high rate of protein production. However, there exist some disadvantages of high-copy expression vectors such as they often cause problems in either segregational or structural instability. Therefore, the insertion of DNA directly into the chromosome of the host organisms in one copy or in multiple copies has been applied to generate more stable clones (Gupta et al. 2002).

B. licheniformis is an interesting candidate not only for homologous gene expression but also for heterologous gene expression, because of its high capacity to secrete proteins into the medium. Whereas, Gram-negative bacteria like *E. coli* accumulate proteins in the cytoplasm or periplasmic space, probably causing toxicity to the organism (Schallmey et al. 2004). This frequently leads to the formation of insoluble protein inclusion bodies, incorrect protein folding or inefficient disulfide bond formation. The cost for protein recovery and purification are also very high. Furthermore, *E. coli* is regarded as an unsafe organism which produces endotoxins. This can be problematic in the down stream processing of recombinant proteins especially in the case of biopharmaceuticals (Schallmey et al. 2004). In Gram-positive bacteria such as *B. subtilis* and *B. licheniformis*, proteins can be released into the surrounding medium via translocators. This greatly simplifies the purification process and the production of soluble, intact and biologically active protein. Moreover, *B. licheniformis* and *B. subtilis* were classified as GRAS organisms by the U.S. Food and Drug Administration. However, production yields from *Bacillus* are sometimes lower than in the case of *E. coli*. For example, heterologous proteins are usually exposed to cell wall-associated proteases during the slow protein-folding process (Wang et al. 1988; Braun et al. 1999). Thus, many efforts have focused on the development of efficient expression systems by increasing the secretion of heterologous proteins as well as eliminating cell wall-associated proteases by constructions of specific mutant strains. *B. subtilis* strain WB 600 or 800, constructed by Wu et al. (1991, 2002), are already deficient in 6-8 extracellular proteases.

2. Scope of the thesis

B. licheniformis is an important industrial producer of extracellular enzymes. However, at the beginning of this PhD thesis little was known about the physiology of *B. licheniformis*. Therefore, this bacterium was chosen for an in depth analysis of the starvation response by means of transcriptome studies. The transcriptome data of this study have been compared with proteome data. The gram-positive model bacterium *B. subtilis*, as a close relative of *B. licheniformis*, was used as reference in order to analyse the results of this functional genome study.

Due to the ability of *B. licheniformis* to secrete large amounts of proteins into the medium, this bacterium is one of the most important hosts for industrial enzyme production. The study of the physiology of *B. licheniformis* under different environmental conditions is very important and necessary, in order to understand the function of genes and regulators and their interaction with each other. Such knowledge will help to better understand industrial fermentation processes. This has strong implications on the optimization of such bioprocesses in the future. Furthermore, the functional genome analysis of this bacterium will help to find biomarkers for the prediction of the physiological state of the cells. This information could be used for the monitoring of the cellular physiology of *B. licheniformis* cells during industrial bioprocesses.

Furthermore, the construction of expression systems for *B. licheniformis* is a interesting point. Most of the industrial relevant proteins produced by *B. licheniformis* so far are homologous proteins. The corresponding genes were set under the control of their own gene promoters. To the best of our knowledge, there is no expression vector published for *B. licheniformis* which is free for the scientific community. The few established expression systems of *B. licheniformis* are patented.

The aims of this thesis were:

1) Investigation of the cellular physiology of *B. licheniformis* in response to phosphate starvation, glucose and nitrogen conditions by means of transcriptome analyses based on full genome microarrays: In order to verify the data obtained by the microarray analysis and to analyse the expression of several, selected genes in detail, we additionally used the Northern-blot technique and the real-time reverse transcription polymerase chain reaction (real-time RT-PCR).

2) Identification of marker genes suitable for the monitoring of the physiological state of *B. licheniformis* cells during fermentation processes: The results of the transcriptome analyses of *B. licheniformis* under the three starvation conditions have been compared with proteome data of Voigt et al. (2006).

3) Investigation of the *phy* promoter for its use in a *B. licheniformis* expression system.

In chapter II the analysis of the phosphate starvation stimulon of *B. licheniformis* at the transcriptional and translational level is described. More than 100 genes were identified as significantly induced (higher than 4 fold) under phosphate starvation conditions. Induction of most of the genes enables the cell to use alternative organic phosphate sources such as *phoB*, *phoD* and ribonucleases (*rph, yurI*). In addition, the genes *pstS* also showed very strong induction. An interesting finding was the identification of the very strong induction of phytase at the mRNA and protein levels. Analysis of the phosphate starvation response of *B. licheniformis* identified potential marker genes e.g. *pstS*, *phy*, *phoB* or *yvnA* for a potential monitoring of critical phosphate limitation situations in *B. licheniformis* fermentation processes.

Chapter III describes the analysis of the transcriptome and proteome of *B. licheniformis* under glucose and nitrogen starvation conditions. More than 250 genes were identified to be induced (higher than 3 fold) under glucose starvation conditions and 200 genes were induced (higher than 3 fold) under nitrogen starvation conditions. There were about 80 genes, which show significant induction under both conditions. Most of the genes induced under glucose and nitrogen starvation conditions allowed *B. licheniformis* to use alternative carbon and nitrogen sources, e.g. high induction of the *acoABC* operon or genes for nitrate and nitrite assimilation, such as the *nasBCDEF* operon. Our analysis identified several stress specific genes like *acoA*, *mmgA*, *kbl* as biomarkers for glucose starvation and *glnA*, *nasE* for nitrogen starvation.

In chapter IV a detailed investigation of the expression of the *phy*-gene under different nutrient conditions using the combination of the Northern blot, Western blot and microarrays and the construction of a *phy*-promoter based expression vector is described. Northern blot analyses, using a *phy*-specific mRNA probe, showed a high induction of the 1.1-kb *phy*-specific transcript when *B. licheniformis* was grown under phosphate-limited conditions. Western blot analysis of the extracellular protein fraction using *B. licheniformis* phytase-specific antiserum indicated the similar results. Surprisingly, when *B. licheniformis* was grown under conditions with phytate as the sole phosphate, only a weak induction of

phytase was observed. Although the growth experiments indicate that the *B. licheniformis* DSM 13 cells use phytate as a good phosphate source. In parallel, to the analysis of the phytase expression the *phy* promoter sequence was analysed by reporter gene fusion. The different fusions using the *phy* promoter sequence and the alpha-amylase gene of *B. subtilis* were constructed as single copy on the chromosome and as multi copy on a plasmid. The strains containing recombinant plasmid pDH25 showed a significant induction of the α-amylase under the control of the phytase promoter in the phosphate starved cells. The strains bearing the P_{phy}-*amyE* inserted in the *amyE* locus indicated a lower induction of the α-amylase.

Chapter II

The phosphate starvation response of *Bacillus licheniformis*

Le Thi Hoi[1], Birgit Voigt[2], Britta Jürgen[1], Armin Ehrenreich[3], Gerhard Gottschalk[3], Stefan Evers[4], Jörg Feesche[4], Karl-Heinz Maurer[4], Michael Hecker[2], and Thomas Schweder[1]

[1]Institut für Pharmazie, Ernst-Moritz-Arndt Universität, Greifswald.
[2]Institut für Mikrobiologie, Ernst-Moritz-Arndt Universität, Greifswald.
[3]Institut für Mikrobiologie und Genetik, Georg-August-Universität, Göttingen.
[4]Henkel KGaA, VTB Enzymtechnologie, Düsseldorf.

Keywords: *Bacillus licheniformis*, phosphate starvation, proteome, transcriptome

ABSTRACT

The phosphate starvation stimulon of *B. licheniformis* was analysed at the transcriptional and translational level. The comparison of the transcriptome and the proteome demonstrated that this specific starvation response of *B. licheniformis* is partially similar to that of *B. subtilis*. But it is also shown that *B. licheniformis* has evolved its own strategies to cope with this nutrient limitation. By means of the secretome analysis the phytase was identified as the most abundant protein under phosphate starvation conditions. Data of this study indicate that, unlike in *B. subtilis*, phosphate starvation in *B. licheniformis* does not induce the SigmaB-dependent general stress response.

1. Introduction

Phosphate is one of the most important nutrients for all organisms and is frequently limiting in soil (del Pozo et al. 1999). Therefore phosphate-limited growth is probably a common phenomenon for soil bacteria, such as *B. subtilis* and *Bacillus licheniformis*. Hence, the ability to react suitably to such unfavourable environmental conditions is essential for ensuring the survival of the bacteria in their natural habitat.

When exposed to phosphate starvation the Gram-positive bacterium *B. subtilis* induces a specific response, the PhoPR regulon (Ogura et al. 2001; Hulett 2002; Pragai and Harwood 2002), as well as the δ^B-dependent general stress response (Eymann et al. 1996; Antelmann et al. 2000). A third gene class induced under such conditions is regulated independently of either PhoPR or δ^B (Antelmann et al. 2000). The specific Pho regulon of *B. subtilis* consists of more than 30 genes that are regulated in response to low concentrations of inorganic phosphate (P_i <0.1 mM) (Ogura et al. 2001; Hulett 2002). *B. subtilis* induces under these conditions genes that allow the cell to use alternative organic phosphate sources (e.g. *phoA, phoB*, *phoD*, and *glpQ*) (Hulett et al. 1991; Eder et al. 1996; Antelmann et al. 2000). In addition, there is a strong induction of genes (*pstSACBABB*) coding for a high-affinity ABC phosphate transporter (Eymann et al. 1996). The *tuaABCDEFGH* operon, which encodes enzymes necessary to replace the phosphate containing teichoic acids in the cell wall by phosphate free teichuronic acid, was also shown to be induced in a PhoPR dependent manner in *B. subtilis* (Liu and Hulett 1998). At the same time the teichoic acid biosynthesis operons (*tagAB* and *tagDEF*) are repressed (Liu et al. 1998 a). Furthermore, the Pho regulon includes the genes *tatAD*, which form an operon with *phoD*, and *tatCD*. These genes encode structural components of the twin-arginine translocation (Tat) secretion system (Pragai and Harwood 2002).

Expression of the *B. subtilis* Pho regulon is regulated via the action of at least three signal transduction systems (Hulett et al. 1994; Hulett 1996; Sun et al. 1996). The main regulatory system is PhoPR, consisting of the sensor kinase PhoR and the transcriptional activator PhoP (Seki et al. 1987, 1988; Liu and Hulett 1998). Phosphorylated PhoP binds directly to specific sequences in the promoters of the Pho regulon genes and thereby positively regulates their transcription (Qi and Hulett 1998 a). Mutations in the *phoP* or *phoR* gene strongly reduce the phosphate starvation response, reflected by a 95 % reduction of the phosphatase activity in *B. subtilis* (Hulett et al. 1994). Full induction of the Pho regulon requires a second regulatory system, the ResDE two-component system, which activates the *phoPR* operon by an unknown

mechanism. Mutations in the *resD* or *resE* gene also reduce the phosphate starvation response, although to a lesser extent than mutations in *phoP* or *phoR* (Sun et al. 1996). The *phoPR* operon as well as the *resABCDE* operon are themselves members of the Pho regulon (Birkey et al. 1998). The PhoPR and the ResDE two-component systems regulate the Pho regulon in a positive way. Spo0A~P represses both of these activator pathways, thereby repressing the Pho regulon (Hulett et al. 1994; Sun et al. 1996). If the deprivation of phosphate continues despite the action of the Pho regulon genes, Spo0A initiates sporulation and terminates the phosphate starvation response by repressing the transcription of the *phoPR* operon via AbrB and ResD-ResE. It has been shown that mutations in the *spo0A* gene hyperinduce Pho regulon genes of *B. subtilis* (Hulett 1996).

The study presented here aims at exploring the phosphate starvation response of *B. licheniformis*, a Gram-positive soil bacterium that is closely related to *B. subtilis*. *B. licheniformis* has been used in industrial fermentation processes for many years, especially for the production of technical enzymes, like proteases and amylases. However, there is not much known in terms of the physiology of this important industrial microorganism. The recently finished sequencing of the genomes of the *B. licheniformis* type strains DSM13 (Veith et al. 2004) and ATCC 14580 (Rey et al. 2004) has now established the basis for detailed investigations of the cellular physiology of this bacterium and its adaptation to distinct environmental conditions by global expression profiling analysis. Hornbaek et al. (2004), for example, used a global transcriptome analysis for the investigation of the intracellular pH regulation in *B. licheniformis* upon external pH up-shifts. In a recent study we established protein master gels for *B. licheniformis* cells grown either in minimal medium or in a complex medium (Luria Broth) (Voigt et al. 2004). Based on these master gels we were able to study the regulation of major metabolic pathways of this bacterium. Furthermore, by proteomic studies we were able to determine the extracellular proteome of *B. licheniformis* under defined growth conditions with different media (Voigt et al. 2006).

In this study we investigated in detail the gene expression pattern of *B. licheniformis* cells at the transcriptional and translational level under phosphate starvation conditions. The comparison of the transcriptome and the proteome demonstrated that the specific starvation response of *B. licheniformis* is partially similar to that of *B. subtilis*. But it is also shown that *B. licheniformis* has evolved its own strategies to cope with this nutrient starvation condition.

2. Material and Methods

2.1. Strains and culture conditions

B. licheniformis DSM 13 (equivalent to ATCC 14580, type strain from the German Collection of Microorganisms and Cell Cultures, DSMZ GmbH, Braunschweig, Germany) was cultivated at 37°C under vigorous agitation in minimal medium (MM) containing 15 mM $(NH_4)_2SO_4$, 8 mM $MgSO_4$ x 7 H_2O, 27 mM KCl, 7 mM Na-citrate x 2 H_2O, 50 mM Tris-HCl (pH 7.5) supplemented with 0.6 mM KH_2PO_4, 2 mM $CaCl_2$ x 2 H_2O, 1 µM $FeSO_4$ x 7 H_2O, 10 µM $MnSO_4$ x 4 H_2O and 0.2 % (w/v) glucose. For the starvation experiments the concentration of phosphate was reduced to 0.15 mM. This concentration of phosphate allowed the cells to grow to a maximal optical density at 500 nm of about 1.2. Bacterial cells were harvested at different time points during the exponential growth phase, the transition phase, and 0.5, 1, and 2 h after the onset of the stationary growth phase.

2.2. RNA isolation

For RNA isolation cells were harvested in half of the sample volume ice cold killing buffer (20 mM NaN_3, 20 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$). Cell pellets were resuspended in Lysis buffer II (3 mM EDTA, 200 mM NaCl) and mechanically disrupted using the RiboLyser™ (Thermo Electron Corporation GmbH, Dreieich, Germany) as described by Jürgen et al. (2005b). Isolation and purification of total RNA was done with the KingFisher ml™ (Thermo Electron Corporation GmbH, Dreieich, Germany) according to Jürgen et al. (2005b).
Buffers and chemical reagents for the KingFisher ml™ were used from the MagNA Pure LC RNA Isolation Kit I (Roche Diagnostics, Penzberg, Germany). Concentration and quality of the isolated total RNA were determined with the Bioanalyzer 2100 (Agilent Technologies, Berlin, Germany) as recommended by the manufacturer.

2.3. Transcriptome analysis

Full genomic *B. licheniformis* DNA microarrays, representing more than 95% of the ORFs longer than 300 bp, were produced by spotting PCR products of 300 – 500 bp length in duplication on microarray slides using a Lucidea (Amersham Biosciences, Freiburg, Germany) contact printer. Library clones from shotgun sequencing of *B. licheniformis* were used as templates for the PCR. Correct clones for each gene were identified by custom-made

software. PCR products where purified using the QIAquick 96 PCR Purification Kit on a BioRobot 8000 (Qiagen, Hilden, Germany). Each spot contained 30 pg of probe DNA and was generated by placing 100 pL of a 50% aqueous DMSO solution on amino-silane coated GAPS II slides (Corning, Corning NY, USA). The probes were cross-linked with the slides by UV illumination (120 mJ/ cm^2) after total evaporation of the spotting solution.

Microarray analysis was carried out as described by Jürgen et al. (2005b). For the labelling of cDNA 25 µg of total RNA were used with random hexamer primers. Positive and negative mRNA controls for microarray analysis were added (Jürgen et al. 2005b) and the reaction mix was incubated at 65°C for 10 min. The mixture was cooled down for 10 min at room temperature and the following reagents were added and mixed: 10 µL 5x Superscript reaction buffer, 4 µL of a dNTP-master mix (0.5 mM dATP; 0.5 mM dGTP; 0.2 mM dTTP; 0.5 mM dCTP) (Roche Diagnostics, Penzberg, Germany), 0.1 mM Cy3 and 0.1 mM Cy5 dUTP (Amersham Biosciences Europe GmbH, Freiburg, Germany), 0.01 M DTT, 1 µL Superscript reverse transcriptase (Invitrogen Life Technologies, Karlsruhe, Germany). The whole mixture was incubated at 42°C for 2 h. Afterwards, 10 µL 1 M NaOH were added for hydrolysis of RNA and incubated at 65°C for 10 min. 10 µL 1 M HCl were added for neutralisation and the reaction mix was filled up with 200 µL TE buffer (pH 8.0). The labelled cDNA was purified by means of the CyScribe GFX Purification Kit (Amersham Biosciences, Freiburg, Germany) as described by the manufacturer. After purification the samples were combined in one Eppendorf tube and dried in a speed-vac. The labelled cDNA mix was resuspended in 50 µL hybridisation buffer (MWG AG Biotech, Ebersberg, Germany) and used for hybridisation.

The *B. licheniformis* full genomic DNA microarrays were prehybridised at 42°C for at least 45 min in 2x SSC, 0.05% SDS, 5% BSA. Afterwards the slides were washed 5 times with A. bidest and dried at 1500 rpm, room temperature for 5 min. The labelled cDNA mixture was denatured for 2 min at 95°C prior to hybridisation, which was performed for at least 16 h at 42°C by means of the Slide Booster device (Advalytix, Brunnthal, Germany). The hybridised slides were washed at room temperature for 5 min in 2x SSC, 0.1% SDS; for 5 min with 1x SSC, 0.1% SDS; for 5 min with 0.5x SSC and subsequently dried at room temperature at a speed of 1500 rpm for 5 min and finally scanned with the ScanArray® Express (PerkinElmer Life and Analytical Sciences, Rodgau-Jügesheim, Germany).

Images were processed and analysed with the ScanArray® Express software. The raw Cy3 and Cy5 intensity values were normalised in order to equalise the median values. Only genes, which were nearly 4-fold induced or repressed, were considered in this study. When located

in the same operon with a gene with such pronounced changes in the expression also genes with lower expression level changes were listed. The values and standard deviation, presented in table 2 and 4, were calculated from at least 2 biological samples, obtained from 2 different experiments. Furthermore, the RNA of one of the biological samples was analysed twice, whereas the transcriptome of the second biological sample was investigated by Dye Swap. Both the biological samples as well as the Dye Swap analysis were included in the standard deviation calculation.

2.4. Real-time-RT-PCR

Real-time-RT-PCR was carried out with the LightCycler from Roche Diagnostics (Penzberg, Germany). Gene specific primers were deduced with the program Array Designer 2.0 (PREMIER Biosoft International, Palo Alto, USA) and ordered from Invitrogen (Karlsruhe, Germany) (Tab. 1). For the amplification validation the Cyber Green Detection Kit from Roche Diagnostics (Penzberg, Germany) was used. Standard RNA probes for quantification of the mRNA were generated as described by Jürgen et al. (2005 a, b). Purity and concentration of the transcript were determined by the Bioanalyzer 2100 (Agilent Technologies, Berlin, Germany).

A standard curve with serial dilutions from 1 ng to 100 fg RNA of the standard RNA probes in RNase free H_2O mixed with MS2 RNA (final concentration 0.5 µg/µL; Roche Diagnostics, Penzberg, Germany) was generated. The protocol from the instruction manual of the RNA Master SYBR Green I Kit (Roche Diagnostics, Penzberg, Germany) was adapted to prepare standard curves for each gene of interest (amplification, segment 2, target temperature: 52°C; amplification, segment 3, incubation time: 12 s). The standard curves were defined after verification of the specificity and purity of the RT-PCR products by melting curve analyses and electrophoresis.

Serial dilutions from 500 to 5 ng of total RNA in RNase free H_2O and MS2 RNA (final concentration 0.5 µg/µL) were prepared. The LightCycler-RNA Master SYBR Green I Kit (Roche Diagnostics, Penzberg, Germany) was used for preparing the master mix following the instructions of the manufacturers. The same protocol as described for the standard curve was used. RNase free and PCR-grade H_2O mixed with MS2 RNA (final concentration 0.5 µg/µL) served as negative control. One dilution of the appropriate standard RNA probe served as standard. The amount of the respective mRNAs in different samples was determined by using the external standard curves for quantification with the second derivative maximum

method. The specificity of the RT-PCR products was checked by melting curve analyses. RT-PCR analysis of the genes was carried out in two individual experiments. Both experiments yielded similar induction ratios.

Table 1: List of primers used in this study

Target gene	Forward primers	Reverse primers
phoD	5`-ggagaacaactatgcgaatg-3`	5`-tcagaaggcggcttattg-3`
sigF	5`-atccttgatggcttccttc-3`	5`-gatgattatcggcgagattc-3`
yhdW	5`-cttacgccccagaaaatac-3`	5`-gctcattcatcgtatactcg-3`
rph	5`-ccgtccagttcacttcttg-3`	5`-gaaggagatgccgatcac-3`
spoIIAB	5`-gcttcttcaagattagggatg-3`	5`-ccgacgatggatgaactg-3`
phy	5`-aaggctcggtcatcaatatgtc-3`	5`-gcttgcttcttcggatgctag-3`
pstS	5`-aaggcttcaggttctttgacg-3`	5`-gctgccattccgacaacc-3`
phoB	5`-aaggctatgacacaacac-3`	5`-gttgtgaatccgatgttg-3`

2.5. 2-DE, protein identification and imaging

For the preparation of cytoplasmic proteins, bacteria were pulse labelled during exponential growth (OD_{500} 0.4), at the onset of the stationary phase (OD_{500} 0.9) and 30 min after transition into the stationary phase. Pulse-labelling was carried out with 556 Bq/mL L-[^{35}S]-methionine for 5 min. The L-[^{35}S]-methionine incorporation was stopped by adding unlabelled methionine (1 mM) and chloramphenicol (100 µg/mL). Cells were washed in TE buffer (10 mM Tris, pH 7.5, 1 mM EDTA), resuspended in TE buffer containing 1.4 µM PMSF and disrupted by sonication. For the preparation of unlabelled protein samples for preparative gels, cells were harvested and washed as described above and then disrupted by passage through a French Press Cell. Protein fractions were collected by centrifugation (20 000 x g, 4°C, twice 30 min). For the preparation of the extracellular protein fraction bacteria were harvested at the same time points. Extracellular proteins were prepared according to Voigt et al. (2004). Protein concentration was determined in all samples with the RotiNanoquant Kit (Roth, Karlsruhe, Germany).

Protein extracts (80 µg protein for radiolabelled samples, 500 µg protein for preparative gels) were loaded onto commercially available IPG-strips (pH 4-7 for cytoplasmic proteins, pH 3-10 for extracellular proteins, Amersham Bioscience, Freiburg, Germany) as described previously (Büttner et al. 2001). In the second dimension polyacrylamide gels of 12.5% acrylamide and 2.6% bisacrylamide were used. The resulting preparative 2-D gels and the extracellular protein gels were stained with colloidal Coomassie Brilliant Blue as described by Voigt et al. (2004). The gels with the radioactively labelled proteins were silver stained (Blum

et al. 1987) and then scanned with a Quato-Graphic x-finity ultra Scanner (Quatographic, Braunschweig, Germany). After drying on a heated vacuum dryer the gels were exposed to storage phosphor screens (Molecular Dynamics, Sunnyvale, USA). Screens were scanned using a Typhoon Scanner (Amersham Biosciences, Freiburg, Germany). Proteins, cut from the preparative Coomassie stained gels, were identified by mass spectrometry as was described by Voigt et al. (2004). For analysing the gels the Delta 2D software (Decodon, Greifswald, Germany) was used (Bernhardt et al. 1999). This software was also used for quantification of the gels and calculation of the synthesis rates. The labelling experiments were repeated twice and the synthesis rates given in table 5 were derived from these two independent experiments.

2.6. Bioinformatic motif search

To identify potential PhoP binding regions in the upstream area of genes regulated by phosphate starvation the pattern matching program PatScan (D'Souza et al. 1997) was compiled. A FASTA file with all ORFs of *B. licheniformis* together with a 150 bp upstream sequence was analysed using the patterns (given in the format required by the program) TTHACA[1,0,0] 4...6 TTHACA[1,0,0] for a single repeat constituting a potential dimer binding site and TTHACA[2,0,0] 4...6 TTHACA[2,0,0] 4...6 TTHACA[2,0,0] 4...6 TTHACA[2,0,0] for two repeats constituting a potential tetramer binding site of PhoP. The consensus sequence was derived from the literature (Hulett 2002) allowing for one or two mismatches per core binding region. The program output was filtered by shell scripts to include only potential binding sites completely upstream of the genes of interest. From the resulting potential PhoP binding sites in the genome of *B. licheniformis* only those were included that map in front of genes identified as induced by phosphate starvation in this study.

3. Results

3.1. Transcriptome analysis

Transcriptome analysis is an excellent tool for the comprehensive determination of gene expression profiles in response to changing environmental conditions. For the transcriptome analysis of *B. licheniformis* whole genome DNA microarrays were used that cover 95% of the genes longer than 300 bp identified during the annotation of the *B. licheniformis* genome.

The growth of *B. licheniformis* DSM13 in a phosphate-limited synthetic Belitsky minimal medium is shown in Figure 1. The cells reached in this medium the stationary phase at an optical density of about 1.2. Samples for the transcriptome and proteome analysis were taken throughout the different growth phases as indicated in Figure 1. For the transcriptome analysis the genomic *B. licheniformis* DNA microarrays were hybridised with an RNA sample from the exponential growth phase (OD 0.5) as a control in combination with RNA samples form the transient and stationary (30 min, 1 h and 2 h) growth phase.

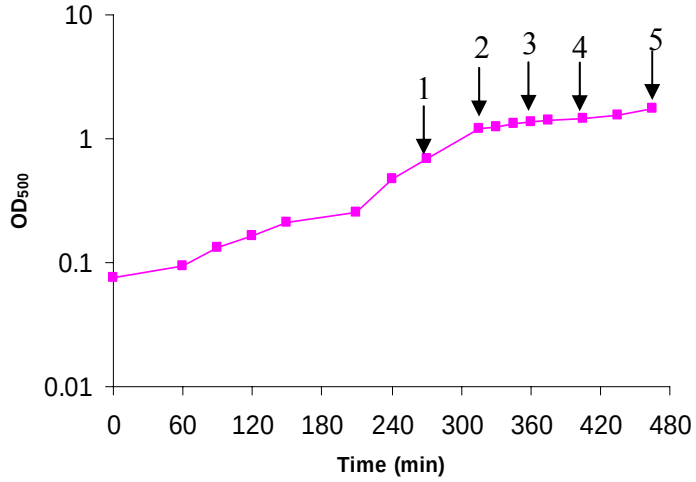


Figure 1: Growth curve of *B. licheniformis* in a phosphate limited MM medium. The arrows indicate the time points at which samples were taken (1: exp. growth phase (reference); 2: transition phase; 3, 4, 5: 0.5 h, 1 h and 2 h after onset of the stationary growth phase, respectively).

The genes *pstS, phoD*, *phoB, yfkN* and *phy* belong to the most strongly induced genes at the transcriptional level during the transient phase (Tab. 2). Based on a sequence analysis with the known conserved PhoP binding sequence of *B. subtilis* these genes could be affiliated to a potential Pho regulon of *B. licheniformis* (Tab. 3). Potential PhoP binding regions upstream of genes regulated by phosphate starvation were predicted by pattern matching. To identify a PhoP dimer binding site, two or four TTAACA-like sequences have to be separated by 4 to 6 nucleotides and this motif has to be within 150 bp upstream of the gene. To prevent false

positive predictions, because similar sequences occur frequently by chance, two restrictive searches were done. Firstly, motifs were identified, in which only one deviation from the consensus TT(A/T/C)ACA in the core binding region, that has been described for *B. subtilis,* occurs. Secondly, such motifs where identified that allowed for two deviations from the TT(A/T/C)ACA consensus, but where the consensus repeats 4 times. Please note that these two conditions may exclude many real binding sites because the consensus of PhoP binding is not very well conserved. Therefore, many sites might have escaped this prediction, but the data shown in table 3 clearly demonstrate that PhoP binding regions could be identified upstream of many of the genes induced by phosphate starvation conditions. More than four TTAACA-like sequences are located upstream of *pstS* and two TTAACA-like sequences are located in front of *phoB* and *phoD*. Further members of this potential Pho regulon of *B. licheniformis*, such as *phoPR*, *pstS-C-A-BA-BB*, and *tatCD* were about two to three fold induced during the transient phase but showed their strongest induction 30 min after the onset of the stationary phase (Tab. 2).

Besides the up-regulation of putative Pho regulon genes, we also found a strong induction of genes, which apparently play a role in the adaptation to phosphate starvation conditions, but which do not show binding regions for PhoP. To this group of genes belong for example *yurI* and *rph*, coding for ribonucleases involved in nucleic acid degradation (Tab. 2).

A set of genes, the function of which can not simply be affiliated to this specific nutrient limitation condition, showed a very strong induction during phosphate starvation. The genes *cypX*, coding for a cytochrome P450-like enzyme and *dhaS*, coding for a potential aldehyde dehydrogenase involved in siderophore 2,3-dihydroxybenzoate (DHB) synthesis belong to this group. Furthermore, the *alsSD* operon, coding for the alpha-acetolactate decarboxylase involved in acetoin biosynthesis (Turinsky et al. 2000), was strongly induced in the transient phase and kept the elevated mRNA levels up to 2 h into the stationary phase. Finally, several genes with unknown functions could be identified as phosphate starvation induced, e.g. *yvmC* and *yvnA*. These two genes revealed the strongest induction out of all genes in the microarray analysis of phosphate starving *B. licheniformis* cells.

Table 2: Kinetic of the phosphate starvation inducible genes of *B. licheniformis*. Listed are such genes, the mRNA level of which showed a more than fourfold induction during at least one sampling point throughout the growth curve. Trans. ph. = transient phase, SD = standard deviation

Gene Name		Trans. ph.	SD	30 min	SD	60 min	SD	120 min	SD	Transcriptional organisation	Function
Adaptation to atypical conditions											
clpP	BLi03710	0.1	± 0.2	4.1	± 0.1	3.3	± 0.3	4.3	± 0.8	*clpP*	ATP-dependent Clp protease
htpG	BLi04256	19.9	± 0.5	4.3	± 0.4	3.4	± 0.3	4.4	± 0.1	*htpG*	class III heat-shock protein
ywqC	BLi03855	3.7	± 0.2	3.8	± 0.3	8.2	± 3.2	7.1	± 2.1	*ywqE-C-B-A*	similar to capsular polysaccharide biosynthesis
ywqE	BLi03854	2.6	± 0.1	3.5	± 0.1	4.7	± 0.4	4.2	± 0.8	*ywqE-C-B-A*	similar to capsular polysaccharide biosynthesis
ywsC	BLi03839	6.0	± 0.6	3.4	± 0.3	4.6	± 1.5	3.3	± 0.2	*ywsC*	similar to capsular polysaccharide biosynthesis
Detoxification											
penP	BLi00280	3.7	± 0.3	3.3	± 0.1	4.1	± 0.2	5.1	± 0.6	*penP*	known beta-lactamase precursor
cypX	BLi03567	15.4	± 2.3	missing		37.3	± 3.6	41.2	± 2.2	*yvmA-yvmC-cypX-yvnA-BLi03570-03571*	cytochrome P450-like enzyme
DNA replication											
ywpH	BLi03869	3.2	± 0.1	1.4	± 0.1	5.3	± 0.4	4.9	± 0.1	*ywpH*	similar to single-strand DNA-binding protein
DNA restriction/modification and repair											
dinB	BLi02244	4.7	± 0.6	5.2	± 0.7	3.2	± 0.5	3.3	± 0.3	*dinB*	nuclease inhibitor
Electron transport chain and ATP synthase											
cydA	BLi04134	1.4	± 0.1	1.2	± 0.3	5.8	± 1.8	6.0	± 0.4	*cydA-B-C-D*	cytochrome bd ubiquinol oxidase (subunit I)
cydB	BLi04133	1.5	± 0.1	1.0	± 0.5	3.3	± 0.5	4.6	± 0.7	*cydA-B-C-D*	cytochrome bd ubiquinol oxidase (subunit II)
qcrA	BLi02391	2.0	± 0.1	1.6	± 0.2	5.0	± 0.5	5.7	± 0.2	*qcrA-B-C*	menaquinol:cytochrome c oxidoreductase
qcrB	BLi02390	2.1	± 0.1	2.0	± 0.6	5.6	± 1.4	7.4	± 1.9	*qcrA-B-C*	menaquinol:cytochrome c oxidoreductase
qcrC	BLi02389	1.4	± 0.1	1.5	± 0.1	4.4	± 1.6	7.1	± 0.5	*qcrA-B-C*	menaquinol:cytochrome c oxidoreductase
Metabolism of amino acids and related molecules											
bpr	BLi01748	3.6	± 0.3	3.6	± 0.5	6.2	± 0.6	6.9	± 1.1	*bpr*	bacillopeptidase F
ispA	BLi01423	1.5	± 0.3	4.4	± 0.2	4.3	± 0.1	4.2	± 0.1	*ispA*	major intracellular serine protease
nasC	BLi00483	1.2	± 0.2	1.7	± 0.1	3.2	± 0.3	3.2	± 0.3	*nasB-C-D-E-F*	assimilatory nitrate reductase (catalytic subunit)
nasD	BLi00484	1.7	± 0.2	6.2	± 0.3	6.0	± 1.4	7.4	± 0.6	*nasB-C-D-E-F*	assimilatory nitrite reductase (subunit)
nasE	BLi00485	1.9	± 0.1	6.2	± 0.2	10.0	± 2.0	11.5	± 1.7	*nasB-C-D-E-F*	assimilatory nitrite reductase (subunit)
nasF	BLi00486	3.5	± 0.4	3.2	± 0.1	9.3	± 1.7	8.0	± 1.3	*nasB-C-D-E-F*	uroporphyrin-III C-methyltransferase
BLi03989		3.1	± 0.2	6.7	± 0.2	5.5	± 0.1	5.4	± 0.3		putative hydroxybenzoate hydroxylase
BLi04163		2.3	± 0.1	2.2	± 0.1	4.3	± 0.2	4.2	± 0.3		known arginine deiminase
yrbE	BLi00809	11.7	± 0.1	8.8	± 1.2	10.1	± 0.5	19.6	± 0.1	*yrbE*	similar to dehydrogenase
ywfG	BLi04237	3.1	± 0.1	3.2	± 1.1	5.3	± 1.2	6.3	± 2.0	*ywf operon*	similar to aspartate aminotransferase
ywrD	BLi03850	4.1	± 1.5	3.5	± 0.8	5.3	± 0.6	4.4	± 0.9	*ywr operon*	similar to gamma-glutamyltransferase
Metabolism of nucleotides and nucleic acids											
yfkN	BLi00814	19.7	± 5.3	25.9	± 9.5	21.8	± 1.5	22.3	± 6.5	*yfkMNK*	similar to 2'.3'-cyclic-nucleotide2'-phosphodiesterase

Table 2: continued

Gene Name		Trans. ph.	SD	30 min	SD	60 min	SD	120 min	SD	Transcriptional organisation	Function
yhcR	BLi00982	5.6	± 0.7	8.3	± 1.6	5.7	± 0.1	10.0	± 2.2	*yhc operon*	similar to 5'-nucleotidase
yurI	BLi03441	4.8	± 0.5	9.5	± 1.6	12.6	± 0.9	12.8	± 1.2	*yurI*	similar to ribonuclease
BLi03718		8.0	± 1.1	11.0	± 2.1	13.7	± 3.7	19.0	± 2.5		putative ribonuclease
Metabolism of coenzymes and prosthetic groups											
BLi03994		11.7	± 1.3	19.8	± 3.2	30.7	± 2.2	20.8	± 0.1	*dhaS*	close homolog to DhaS aldehyde dehydrogenase
dhaS	BLi02249	4.0	± 1.1	6.6	± 0.5	9.1	± 2.4	19.4	± 0.7	*dhaS*	aldehyde dehydrogenase
nasF	BLi00486	3.5	± 0.1	3.2	± 0.1	9.3	± 1.7	8.0	± 1.3	*nasB-C-D-E-F*	uroporphyrin-III C-methyltransferase
Metabolism of phosphate											
phoB	BLi02565	61.7	± 8.5	77.2	± 5.2	88.3	± 10.7	101.9	± 15.2	*phoB-gdh*	alkaline phosphatase III
phy	BLi00448	22.1	± 8.9	32.4	± 13.1	40.5	± 13.6	20.4	± 0.6	*yclF-phy*	phytase
phoD	BLi00281	21.2	± 5.3	23.7	± 14.2	40.1	± 2.8	51.2	± 12.1	*phoD-tatCD*	phosphodiesterase/alkaline phosphatase
BLi02820		22.9	± 0.9	25.8	± 9.7	22.2	± 0.9	34.6	± 1.4	*BLi02820-BLi02821-cypA*	putative phosphatase
Protein secretion											
tatCD	BLi00283	3.4	± 0.5	3.2	± 0.1	4.8	± 0.9	10.8	± 3.52	*phoD-tatCD*	twin-arginine translocation pathway
Specific pathways											
alsD	BLi03847	18.1	± 3.1	13.1	± 1.5	14.4	± 6.2	23.1	± 7.5	*alsR-S-D*	alpha-acetolactate decarboxylase
alsS	BLi03848	40.5	± 1.7	19.2	± 8.3	30.1	± 9.7	42.4	± 7.5	*alsR-S-D*	alpha-acetolactate synthase
gdh	BLi02566	13.1	± 0.9	7.3	± 0.1	12.1	± 0.9	24.6	± 1.7	*gdh-phoB*	glucose 1-dehydrogenase
BLi00447		3.4	± 0.6	6.7	± 3.1	4.3	± 0.1	3.6	± 0.2	*lacA*	close homolog to LacA beta-galactosidase
Sporulation											
cotE	BLi01927	6.4	± 0.6	3.9	± 0.7	4.0	± 0.2	13.7	± 1.1	*cotE*	spore coat protein
BLi00802		9.5	± 1.2	6.7	± 3.1	6.3	± 0.2	7.9	± 0.5		putative spore coat polysaccharide
sigE	BLi01750	1.2	± 0.1	8.5	± 0.2	6.2	± 0.5	7.2	± 0.7	*spoIIGA-sigE-sigG*	RNA polymerase sigma factor
sigF	BLi02495	3.6	± 0.6	3.5	± 0.8	4.7	± 0.9	5.1	± 1.7	*dacF-spoIIAA-spoIIAB-sigF*	known RNA polymerase sigma factor
sigG	BLi01751	1.3	± 0.1	7.8	± 0.1	4.8	± 0.4	5.8	± 0.2	*spoIIGA-sigE-sigG*	RNA polymerase sigma factor
spoIIAA	BLi02497	4.1	± 0.1	5.6	± 1.6	8.7	± 0.2	12.9	± 1.4	*dacF-spoIIAA-spoIIAB-sigF*	anti-sigma F factor antagonist
spoIIAB	BLi02496	6.1	± 0.4	10.9	± 3.3	12.4	± 0.7	20.6	± 9.5	*dacF-spoIIAA-spoIIAB-sigF*	anti-sigma F factor
spoIIGA	BLi01749	1.8	± 0.2	3.6	± 0.1	11.4	± 1.0	11.2	± 0.1	*spoIIGA-sigE-sigG*	protease
spoIIIAA	BLi02614	1.0	± 0.1	5.6	± 0.5	3.1	± 0.1	3.0	± 0.1	*spoIIIAA-AB-AD-AE-AF-AG-AH*	mutants block sporulation after engulfment
spoIIIAE	BLi02610	1.2	± 0.1	5.1	± 0.6	2.9	± 0.1	3.2	± 0.5	*spoIIIAA-AB-AD-AE-AF-AG-AH*	mutants block sporulation after engulfment
spoIIIAF	BLi02609	0.7	± 0.1	22.7	± 0.8	4.5	± 0.1	3.2	± 0.3	*spoIIIAA-AB-AD-AE-AF-AG-AH*	mutants block sporulation after engulfment
spoIIIAG	BLi02608	0.8	± 0.1	17.9	± 0.2	7.2	± 0.5	6.6	± 1.9	*spoIIIAA-AB-AD-AE-AF-AG-AH*	mutants block sporulation after engulfment
spoIIIAH	BLi02607	0.7	± 0.1	16.7	± 0.4	7.1	± 0.1	4.3	± 0.7	*spoIIIAA-AB-AD-AE-AF-AG-AH*	mutants block sporulation after engulfment
spoIIIE	BLi01906	5.4	± 0.3	3.7	± 0.3	4.7	± 0.2	2.3	± 0.1	*tepA-spoIIIE*	DNA translocase
Transcription regulation											
BLi03995		3.2	± 0.1	3.4	± 0.2	6.7	± 0.8	7.7	± 0.6		putative transcriptional regulation

Table 2: continued

Gene Name		Trans. ph.	SD	30 min	SD	60 min	SD	120 min	SD	Transcriptional organisation	Function
sacT	BLi04018	1.4	± 0.1	5.4	± 0.5	3.8	± 0.5	5.1	± 1.4	*sacT*	transcriptional antiterminator
Transport/binding proteins and lipoproteins											
cydC	BLi04132	2.4	± 0.1	2.3	± 0.1	3.5	± 0.1	5.8	± 0.4	*cydA-B-C-D*	ABC transporter
dppA	BLi01392	1.6	± 0.1	3.5	± 0.5	3.3	± 0.6	0.1	± 0.3	*dppA-B-C-D-E*	dipeptide ABC transporter
dppB	BLi01393	1.4	± 0.1	3.4	± 0.1	4.9	± 0.8	2.0	± 0.1	*dppA-B-C-D-E*	dipeptide ABC transporter
dppC	BLi01394	1.6	± 0.1	4.3	± 0.3	3.0	± 0.1	3.3	± 0.1	*dppA-B-C-D-E*	dipeptide ABC transporter
dppE	BLi01396	1.5	± 0.1	4.3	± 0.1	5.5	± 0.8	5.0	± 0.2	*dppA-B-C-D-E*	dipeptide ABC transporter
pstA	BLi02674	2.4	± 0.2	11.8	± 0.6	4.3	± 0.7	3.7	± 0.7	*pstS-C-A-BA-BB*	phosphate ABC transporter
pstBA	BLi02673	3.2	± 0.4	28.7	± 8.9	6.0	± 2.5	5.3	± 1.6	*pstS-C-A-BA-BB*	phosphate ABC transporter
pstBB	BLi02672	2.1	± 0.2	16.1	± 3.2	4.4	± 2.0	4.1	± 0.5	*pstS-C-A-BA-BB*	phosphate ABC transporter
pstC	BLi02675	2.9	± 0.1	26.5	± 5.1	4.8	± 1.4	5.1	± 1.0	*pstS-C-A-BA-BB*	phosphate ABC transporter
pstS	BLi02676	16.8	± 1.7	52.4	± 26.6	21.8	± 5.4	16.7	± 0.8	*pstS-C-A-BA-BB*	phosphate ABC transporter
BLi03990		6.4	± 0.1	12.7	± 1.3	13.7	± 0.7	15.6	± 0.1		putative benzoate transport protein
BLi04241		1.9	± 0.1	3.5	± 0.2	4.3	± 0.1	4.7	± 0.1		putative transporter
yhdG	BLi00475	7.1	± 0.6	5.5	± 3.0	5.4	± 0.4	7.9	± 2.8	*yhdG*	similar to amino acid transporter
yvmA	BLi03565	7.3	± 1.8	missing		12.2	± 0.2	12.8	± 1.9	*yvmA-yvmC-cypX-yvnA-BLi03570-03571*	similar to multidrug transporter
ywrB	BLi03852	3.2	± 0.3	2.0	± 0.1	4.0	± 0.8	5.1	± 0.1	*ywrD-C-B-A*	similar to chromate transport protein
Hypothetical											
BLi02066		2.0	± 0.1	2.0	± 0.1	8.5	± 2.2	10.9	± 0.1		putative acetoin reductase
BLi03991		5.9	± 2.1	15.2	± 2.7	10.2	± 0.7	13.0	± 4.6		putative aromatic compounds specific dioxygenase
BLi03993		9.4	± 0.5	19.9	± 1.0	11.9	± 1.2	14.4	± 2.2		putative decarboxylase
BLi03996		3.0	± 0.3	3.7	± 0.3	5.2	± 0.2	4.3	± 0.2		conserved hypothetical
BLi01466		1.7	± 0.1	5.4	± 1.0	3.4	± 0.5	4.3	± 1.1		putative phage protein
BLi01465		3.5	± 0.5	4.1	± 0.5	4.8	± 0.3	4.3	± 0.5		putative portal protein
BLi00401		2.7	± 0.1	4.6	± 0.6	4.9	± 0.1	4.6	± 1.1		known lichenysin synthetase A
BLi03193		14.0	± 0.3	10.1	± 3.7	10.3	± 0.9	11.3	± 0.4		conserved hypothetical
BLi03194		4.1	± 0.4	4.2	± 0.2	3.8	± 0.1	3.4	± 0.1		putative glucosyl transferase
BLi04184		4.9	± 0.5	2.1	± 0.1	6.5	± 1.7	6.4	± 0.5		hypothetical
BLi02819		4.1	± 0.5	3.3	± 0.2	4.1	± 0.2	8.9	± 0.5		putative oxidoreductase protein
BLi02821		3.0	± 0.1	2.3	± 0.1	3.0	± 0.1	3.9	± 0.2	*BLi02820-BLi02821-cypA*	putative lipase/esterase
BLi04238		3.0	± 0.1	1.8	± 0.1	4.4	± 0.2	5.6	± 0.1		hypothetical
BLi03570		4.0	± 0.1	1.6	± 0.1	4.5	± 0.5	6.1	± 0.5	*yvmA-yvmC-cypX-yvnA-BLi03570-03571*	hypothetical
BLi03571		3.4	± 0.2	1.3	± 0.1	5.2	± 0.8	4.2	± 0.4	*yvmA-yvmC-cypX-yvnA-BLi03570-03571*	putative amidase
BLi04308		3.6	± 0.3	2.4	± 0.2	6.9	± 0.1	16.9	± 2.0		hypothetical
BLi02704		4.4	± 0.5	4.3	± 0.7	4.0	± 0.2	5.8	± 1.0		conserved hypothetical
BLi00235		6.9	± 1.5	7.1	± 1.2	8.2	± 0.6	13.5	± 2.7		hypothetical
Unknown function											
yfkH	BLi00820	15.0	± 0.3	4.4	± 1.2	10.9	± 1.5	20.0	± 8.5	*yfk operon*	unknown
yfmQ	BLi00629	3.2	± 0.1	1.5	± 0.1	19.4	± 8.7	26.6	± 3.7	*yfm operon*	unknown
yhbD	BLi00958	0.6	± 0.1	0.7	± 0.1	4.5	± 0.2	13.6	± 3.4	*yhbD-E-F*	unknown
yhbE	BLi00959	0.9	± 0.1	1.0	± 0.1	3.3	± 0.1	15.3	± 0.2	*yhbD-E-F*	unknown
yjoA	BLi02905	4.4	± 0.8	3.2	± 0.2	5.5	± 0.4	7.6	± 1.7	*yjoA-B*	unknown
yvnA	BLi03566	74.0	± 4.7	missing		103.1	± 36.9	149.6	± 4.5	*yvmA-yvmC-cypX-yvnA-BLi03570-03571*	unknown
yvmC	BLi03569	36.7	± 4.4	missing		118.9	± 15.0	112.2	± 12.6	*yvmA-yvmC-cypX-yvnA-BLi03570-03571*	unknown
ywfL	BLi03988	2.80.1	± 0.1	6.6	± 1.5	5.9	± 0.9	6.3	± 0.1	*ywfL*	unknown
ywiC	BLi02079	1.50.1	± 0.1	1.1	± 0.1	4.2	± 1.2	6.4	± 0.1	*ywiC*	unknown

Table 3: List of phosphate starvation regulated genes as identified by transcriptomic or proteomic analysis with probable PhoP binding boxes in the upstream region. PhoP binding boxes were identified by bioinformatic pattern searching, using the *B. subtilis* PhoP core binding region consensus and allowing one deviation in the TT(A/T/C)ACA motif when it appears in tandem or two deviations when the motif appears four times. The motifs are always spaced by 4 to 6 bases

Gene ID	Gene Annotation	Start	Stop	Sequence
BLi00281	PhoD: phosphodiesterase/alkaline phosphatase	-112	-97	**TTTGCA** ATAC **TTTACA**
BLi00448	Phy: phytase	-107	-91	**CTCACA** GCCCC **TTTAAA**
BLi00448	Phy: phytase	-81	-42	**TTTTAA** CAAAAG **TTTACA** TTTC **CTCAAA** TGATAG **TTTTCA**
BLi00484	NasD: assimilatory nitrite reductase (subunit)	-142	-105	**TTGACA** ATTTTG **TGAATA** AATT **GTCATA** AAAA **GTCATA**
BLi00814	YfkN: unknown; similar to 2',3'-cyclic-nucleotide 2'-phosphodiesterase	-64	-49	**TCTACA** AAAC **TTTACA**
BLi00982	YhcR: unknown; similar to 5'-nucleotidase	-95	-58	**TTCTCA** TTCTT **TTTTGA** AATT **TTTACA** AAAAA **ATCATA**
BLi01423	IspA: major intracellular serine protease	-112	-96	**TTTTCA** TAAAA **TTCATA**
BLi01423	IspA: major intracellular serine protease	-130	-94	**TTATCT** ATAT **TTTTTA** ACTT **TTCATA** AAATT **CATACA**
BLi01465	putative portal protein	-20	-3	**GTAACA** AGGAGG **TTTAGA**
BLi01465	putative portal protein	-111	-75	**TGAAGA** CGAA **TTCAAC** ATCGA **TCCACA** TACT **TTCTCG**
BLi01748	Bpr: bacillopeptidase F	-141	-125	**TAAACA** AGGCC **TTGACA**
BLi01749	SpoIIGA: protease (processing of pro-sigma-E to active sigma-E)	-103	-66	**TGTACG** ATTTAC **GCAACA** TTAA **TTGACA** GACT **TTACCT**
BLi01906	SpoIIIE: DNA translocase required for chromosome partitioning into the forespore	-35	-20	**TTTACA** TTTT **TTAACA**
BLi01906	SpoIIIE: DNA translocase required for chromosome partitioning into the forespore	-112	-75	**GTCTCA** AGGA **TAAATA** TGCT **ATAATA** GGGGAA **TCCAGA**
BLi01927	CotE: spore coat protein (outer)	-116	-78	**TGCATA** CACT **TAAACA** GATTC **ATTATA** TGGGAA **TTTATT**
BLi02066	putative acetoin reductase	-111	-96	**TTTAAA** CATA **TTTTCA**
BLi02066	putative acetoin reductase	-143	-106	**CTTTCA** AAAACT **ATTAAA** TTTT **TTCGAA** AAAT **TTTAAA**
BLi02495	SigF: known RNA polymerase sigma factor	-54	-37	**TTAACA** AAGCAC **TTATCA**
BLi02565	PhoB: alkaline phosphatase III	-96	-80	**TTTACA** TAAGA **TTAATA**
BLi02609	SpoIIIAF: mutants block sporulation after engulfment	-59	-42	**TTAACC** GTGATC **ATTACA**
BLi02609	SpoIIIAF: mutants block sporulation after engulfment	-81	-42	**TTCGCT** GATGT **TTTTCT** TAAGC **TTAACC** GTGATC **ATTACA**
BLi02673	PstBA: phosphate ABC transporter	-48	-32	**TTCATA** AAAAG **TTAACG**
BLi02676	PstS: phosphate ABC transporter	-98	-60	**TTTACA** AAACC **TTAATA** ATTCA **TTTAAA** CAAGA **TTAATA**
BLi02676	PstS: phosphate ABC transporter	-120	-82	**TCTATA** CAAAG **TTCAAG** CTTAA **TTTACA** AAACC **TTAATA**
BLi02820	putative phosphatase	-129	-113	**TTCCCA** AAATA **TAAACA**
BLi03193	conserved hypothetical	-80	-43	**TTTTCC** TCGGTA **TGAACA** GATT **ATTTCA** TCAA **AAAACA**
BLi03565	YvmA: unknown; similar to multidrug transporter	-147	-111	**GTAAAA** AATA **TGTAAA** AAAC **TTCAAA** TCTCA **ATAAGA**
BLi03847	AlsD: alpha-acetolactate decarboxylase	-89	-50	**GGAACA** TTTTAA **TAAAGA** AGCCTC **TAAGCA** ATCT **TGATCA**
BLi03848	AlsS: alpha-acetolactate synthase	-82	-43	**TTTCCA** CAAGA **TGTATA** TTTAAT **AATACA** ATAAT **TTTATT**
BLi03850	YwrD: unknown; similar to gamma-glutamyltransferase	-124	-108	**TTAACG** TTTTT **TTAATA**
BLi03993	putative decarboxylase	-41	-26	**TTAACC** AATA **TTTGCA**
BLi03993	putative decarboxylase	-52	-14	**TCTTCA** GCGAA **TTAACC** AATA **TTTGCA** TCAAGC **TGTAAA**
BLi03994	close homolog to DhaS: aldehyde dehydrogenase	-88	-50	**TCCGCA** TAGA **GGAACA** TTGCTG **TTTTCT** TGTCA **GTTAAA**
BLi04018	SacT: transcriptional antiterminator	-50	-12	**TTTACA** CGGGAA **CTTAAA** CAAG **GTAAAA** TCCCA **CTCATA**
BLi04163	known arginine deiminase	-149	-112	**TTTTCC** ATTT **ATCATA** ATTA **TTCATT** TTTGTT **TTTGCT**
BLi04184	hypothetical	-105	-66	**CTCACC** TAAAA **TTTAAC** TTATA **TTTCTA** GACAAA **CTCACC**
BLi04241	putative transporter	-149	-112	**TGTAAA** GATA **TTCTAA** AGGT **TCAAAA** GTATGA **TTGACT**

Already during the transient phase an upregulation of genes involved in sporulation could be determined (Tab. 2). This early induction could be detected for the *dacF-spoIIAA-spoIIAB-sigF* operon, *spoA* and *cotE*. The *spoIIGA-sigE-sigG* and the *spoIIIAA-AB-AD-AE-AF-AG-AH* operons showed an induction about 30 min after onset of phosphate starvation.

In order to verify these transcriptome data, levels of selected mRNAs were determined by real-time-RT-PCR (Fig. 2). Although the overall induction ratios of all tested genes were higher in the microarray analysis, the real-time–RT-PCR analyses showed similar trends in the induction patterns of the selected genes. For example, *phoB*, *phoD*, and *sigF* revealed an up to 10fold induction in the real-time-RT-PCR analysis. A probe for the *yhdW* mRNA was not present on the genomic *B. licheniformis* microarrays. Therefore, the mRNA level of this gene was detected only by means of the real-time-RT-PCR. It was shown that the *yhdW* mRNA is about 9fold increased under phosphate starvation conditions. The strongest induction of over 80fold was detected by this technique for the *pstS* gene.

Table 4: Kinetic of the phosphate starvation repressed genes of *B. licheniformis*. Listed are such genes, whose mRNA levels showed a more than fourfold repression during at least one sampling point throughout the growth curve. Trans. ph. = transient phase, SD = standard deviation

Gene name	ID	Trans.ph	SD	30 min	SD	60 min	SD	120 min	SD	Transcriptional organisation	Function
Adaptation to atypical conditions											
dnaJ	BLi02738	4.0	± 0.6	4.7	± 0.7	4.0	± 0.2	3.2	± 0.1	*dnaK-J*	heat-shock protein activation of DnaK
Cell wall											
dacA	BLi00015	4.5	± 1.1	3.7	± 0.5	4.5	± 0.4	2.8	± 0.1	*dacA-yaaDE*	penicillin-binding protein
tagA	BLi03819	2.2	± 0.2	2.9	± 0.2	2.0	± 0.2	2.0	± 0.1	*tagA-B*	involved in polyglycerol phosphate teichoic acid biosynthesis
tagB	BLi03820	3.1	± 0.2	3.1	± 0.2	3.1	± 0.1	2.9	± 0.1	*tagA-B*	involved in polyglycerol phosphate teichoic acid biosynthesis
tagD	BLi03818	3.3	± 0.2	4.0	± 0.1	3.3	± 0.2	3.2	± 0.4	*tagD-F*	involved in polyglycerol phosphate teichoic acid biosynthesis
tagF	BLi03817	3.2	± 0.3	3.2	± 0.1	3.6	± 0.1	3.2	± 0.3	*tagD-F*	involved in polyglycerol phosphate teichoic acid biosynthesis
tagH	BLi03814	3.7	± 0.1	3.6	± 0.1	3.4	± 0.1	5.0	± 0.1	*tagG-H*	involved in polyglycerol phosphate teichoic acid biosynthesis
DNA replication											
ssb	BLi04362	9.8	± 0.3	6.8	± 0.9	10.8	± 0.2	7.1	± 0.6	*rplKJ-rpsLG-ssb*	single-strand DNA-binding protein
Metabolism of amino acids and related molecules											
cysC	BLi01781	4.9	± 1.6	5.0	± 0.5	5.0	± 2.2	4.5	± 0.6	*cysH-sat-cysC-ylnDEF*	probable adenylylsulfate kinase
gcvPA	BLi02630	3.9	± 1.3	4.0	± 0.4	4.2	± 0.4	3.2	± 0.3	*gcvT-gcvPA-PB*	probable glycine decarboxylase
gcvPB	BLi02629	4.4	± 0.5	6.0	± 1.8	4.5	± 0.2	3.4	± 0.9	*gcvT-gcvPA-PB*	probable glycine decarboxylase
hisB	BLi03735	3.5	± 0.4	4.3	± 0.1	8.3	± 0.1	12.5	± 0.1	*hisZGDBHAFI*	imidazoleglycerol-phosphate dehydratase
hisD	BLi03736	3.2	± 0.2	3.8	± 0.3	12.5	± 0.2	10.0	± 0.2	*hisZGDBHAFI*	histidinol dehydrogenase

Table 4: continued

Gene name	ID	Trans.ph	SD	30 min	SD	60 min	SD	120 min	SD	Transcriptional organisation	Function
serA	BLi02446	4.6	0.7	10.1	0.2	3.6	0.1	2.4	0.1	*serA*	phosphoglycerate dehydrogenase
yjcI	BLi01289	8.5	0.3	14.2	6.3	9.5	2.3	10.0	2.6	*yjcI-J*	similar to cystathionine gamma-synthase
yjcJ	BLi01290	6.8	0.2	8.9	3.1	6.2	0.6	6.7	0.1	*yjcI-J*	similar to cystathionine beta-lyase
ykrV	BLi01514	5.7	1.1	4.9	2.2	4.5	2.1	4.3	0.7	*ykrV-W..*	similar to aspartate aminotransferase
yrhB	BLi02853	4.6	0.3	5.2	0.7	4.0	0.1	3.5	0.2	*yrhA-B*	similar to cystathionine gamma-synthase
Metabolism of lipids											
cdsA	BLi01875	5.1	0.3	4.8	0.5	4.3	0.5	2.9	0.2	*uppS-cdsA-dxr*	phosphatidate cytidylyltransferase
uppS	BLi01874	3.8	0.1	3.4	0.4	2.6	0.1	1.6	0.1	*uppS-cdsA-dxr*	undecaprenyl pyrophosphate synthetase
Metabolism of coenzymes and prosthetic groups											
bioA	BLi00767	3.5	0.2	6.7	0.2	14.2	1.2	12.3	0.5	*bioAFDBI*	biotin synthetase
bioB	BLi00770	4.0	0.4	1.5	0.5	17.8	0.6	14.5	0.1	*bioAFDBI*	biotin synthetase
bioF	BLi00768	3.0	0.5	1.6	0.1	12.5	0.5	16.4	0.9	*bioAFDBI*	8-amino-7-oxononanoate synthase
bioI	BLi00771	2.4	0.1	1.6	0.2	10.7	0.6	9.1	0.1	*bioAFDBI*	cytochrome P450 enzyme
nadA	BLi02912	12.5	1.5	9.0	4.3	7.0	2.4	5.5	0.5	*nadBCA*	quinolinate synthetase
nadB	BLi02914	8.6	0.5	4.8	1.3	4.7	1.0	6.0	1.2	*nadBCA*	L-aspartate oxidase
nadC	BLi02913	10.7	1.3	6.6	1.3	7.2	2.2	7.0	0.1	*nadBCA*	nicotinate-nucleotide pyrophosphorylase
nifS	BLi02915	8.1	1.4	5.4	0.3	4.8	0.2	4.4	0.1	*maf-nifS*	required for NAD biosynthesis
thiF	BLi01264	2.2	0.1	2.2	0.1	5.2	0.3	3.8	0.5	*tenAI-goxB-thiGF -yjbV*	hydroxyethylthiazole phosphate biosynthesis
thiG	BLi01263	2.2	0.2	1.7	0.1	4.8	1.5	4.5	0.9	*tenAI-goxB-thiGF -yjbV*	hydroxyethylthiazole phosphate biosynthesis
yjbV	BLi01265	2.2	0.1	2.2	0.1	4.5	0.2	4.8	0.1	*tenAI-goxB-thiGF -yjbV*	similar to phosphomethylpyrimidine kinase
ylnD	BLi01782	3.1	0.2	3.5	0.2	3.3	0.1	3.0	0.1	*cysH-sat-cysC -ylnDEF*	similar to uroporphyrin-III C-methyltransferase
ylnF	BLi01784	3.3	0.2	4.4	0.3	1.8	0.2	1.7	0.1	*cysH-sat-cysC -ylnDEF*	similar to uroporphyrin-III C-methyltransferase
Metabolism of sulfur										*cysH-sat-cysC-ylnDEF*	
sat	BLi01780	6.1	1.2	7.1	0.7	7.4	0.8	5.1	0.4	*cysH-sat-cysC-ylnDEF*	probable sulfate adenylyltransferase
yvgQ	BLi01303	9.1	0.1	8.0	0.9	7.1	0.3	5.0	0.2	*yvgR-Q*	similar to sulfite reductase
yvgR	BLi01302	6.0	1.6	5.7	0.1	3.8	0.2	5.2	0.1	*yvgR-Q*	similar to sulfite reductase
Detoxification											
ahpC	BLi04291	10.3	4.9	1.9	0.1	7.4	0.4	5.5	1.1	*ahpC-F*	alkyl hydroperoxide reductase
ahpF	BLi04292	8.8	2.0	1.8	0.1	6.6	0.1	5.4	0.1	*ahpC-F*	alkyl hydroperoxide reductase
Protein folding											
tig	BLi02953	4.8	1.2	4.1	0.6	5.2	0.5	3.8	0.5	*tig-clpX*	trigger factor
Protein modification											
map	BLi00155	9.7	0.5	12.9	5.0	11.3	5.0	10.4	4.9	*rplO-secY-adk-map*	methionine aminopeptidase
Metabolism of nucleotides and nucleic acids											
adk	BLi00217	8.8	2.3	14.8	4.8	18.8	4.8	10.2	1.5	*rplR-secY-adk-map*	adenylate kinase
guaA	BLi00686	7.6	1.6	5.8	0.9	6.0	0.6	4.5	0.2	*guaA*	GMP synthetase
purA	BLi04341	6.9	0.1	8.5	2.4	4.0	1.1	3.1	0.1	*purA*	adenylosuccinate synthetase
purB	BLi00695	16.0	5.2	21.0	4.6	9.8	0.6	9.7	1.4	*pur* operon	adenylosuccinate lyase
purK	BLi00694	14.1	1.0	16.5	3.1	11.8	1.0	7.6	0.3	*pur* operon	purine biosynthesis
purL	BLi00699	13.8	2.8	19.6	2.0	10.5	0.1	8.1	1.4	*pur* operon	purine biosynthesis
pyrC	BLi01770	3.7	0.4	3.5	0.4	4.1	0.5	3.7	0.6	*pyr operon*	dihydroorotase
pyrF	BLi01775	4.4	0.1	6.2	0.5	6.9	0.4	5.3	0.4	*pyr operon*	orotidine 5'-phosphate decarboxylase
pyrH	BLi01872	4.6	0.9	3.2	0.1	4.8	1.2	4.0	0.5	*pyrH-frr*	uridylate kinase
pyrAA	BLi01771	4.9	0.5	8.0	1.0	5.8	0.8	5.4	0.5	*pyr operon*	carbamoyl-phosphate synthetase
pyrK	BLi01773	5.6	0.9	9.7	0.5	9.1	0.3	5.4	0.6	*pyr operon*	dihydroorotate dehydrogenase

Table 4: continued

Gene name	ID	Trans.ph	SD	30 min	SD	60 min	SD	120 min	SD	Transcriptional organisation	Function
pyrK	BLi01773	5.6	± 0.9	9.7	± 0.5	9.1	± 0.3	5.4	± 0.6	*pyr operon*	dihydroorotate dehydrogenase
Protein secretion											
secY	BLi00153	8.5	± 2.1	11.4	± 2.7	11.1	± 0.3	15.5	± 1.7	*rplR-secY-adk-map*	preprotein translocase subunit
RNA modification											
rnpA	BLi04378	4.1	± 0.4	3.4	± 0.1	4.1	± 0.3	4.7	± 0.3	*rnpA-spoIIIJ*	protein component of ribonuclease P
truA	BLi00166	4.8	± 0.6	4.3	± 0.9	5.8	± 1.9	4.4	± 0.8	*truA*	pseudouridylate synthase
Signal transduction											
cheA	BLi01864	3.5	± 0.2	3.6	± 0.6	6.1	± 0.3	4.5	± 0.1	*che*operon	two-component sensor histidine kinase
Motility and chemotaxis											
cheB	BLi01863	3.1	± 0.1	3.1	± 0.1	3.3	± 0.3	4.6	± 0.1	*che*operon	MCP-glutamate methylesterase
cheC	BLi01866	4.4	± 0.2	2.9	± 0.1	5.9	± 0.2	6.7	± 0.1	*che*operon	inhibition of CheR
flgE	BLi01849	2.2	± 0.2	2.8	± 0.1	2.9	± 0.3	3.5	± 0.1	*flgB-fli* operon	flagellar hook protein
flhA	BLi01860	2.1	± 0.3	3.8	± 0.2	3.7	± 1.0	2.9	± 0.2	*flgB-fli* operon	flagella-associated protein
flhB	BLi01859	3.0	± 0.1	4.0	± 0.8	5.2	± 0.3	3.4	± 0.3	*flgB-fli* operon	flagella-associated protein
fliH	BLi01843	1.7	± 0.2	5.4	± 0.2	4.1	± 0.2	3.3	± 0.4	*flgB-fli* operon	flagellar assembly protein
fliR	BLi01858	3.2	± 0.2	3.0	± 0.1	4.7	± 0.8	4.5	± 0.2	*flgB-fli* operon	flagellar protein
Transcription regulation											
tenA	BLi01259	2.6	± 0.1	1.4	± 0.1	4.6	± 0.3	4.3	± 0.5	*tenAI-goxB-thiGF-yjbV*	transcriptional regulator
tenI	BLi01260	2.4	± 0.1	2.0	± 0.1	5.6	± 0.2	5.3	± 0.4	*tenAI-goxB-thiGF-yjbV*	transcriptional activator
Transcription termination											
nusA	BLi01885	8.0	± 2.1	5.2	± 0.2	5.3	± 0.1	3.6	± 0.2	*infB-nusA*	transcription termination
Ribosomal proteins											
rplB	BLi00136	20.2	± 5.6	17.7	± 3.2	23.6	± 1.9	15.8	± 1.2	*rpl* operon	ribosomal protein L2 (BL2)
rplN	BLi00143	19.1	± 1.2	20.0	± 0.1	27.8	± 7.8	25.0	± 3.5	*rpl* operon	ribosomal protein L14
rpoB	BLi00125	5.0	± 0.2	4.3	± 0.3	3.8	± 0.2	3.7	± 0.1	*rpoB-C*	RNA polymerase (beta subunit)
rpoC	BLi00126	5.9	± 0.1	4.5	± 0.6	6.5	± 1.1	5.7	± 0.2	*rpoB-C*	RNA polymerase (beta subunit)
rpsC	BLi00139	8.1	± 4.2	10.4	± 0.1	8.1	± 0.9	6.2	± 0.1	*rps* operon	ribosomal protein S3 (BS3)
rpsK	BLi00160	9.2	± 1.1	9.2	± 0.2	16.0	± 0.9	12.1	± 3	*rps* operon	ribosomal protein S11 (BS11)
Translation elongation											
tsf	BLi01871	6.4	± 1.0	4.4	± 0.3	3.8	± 1	3.2	± 0.2	*tsf*	elongation factor Ts
Translation initiation											
InfB	BLi01888	5.6	± 0.9	4.2	± 0.5	4.1	± 0.1	3.7	± 0.1	*infB-nusA*	initiation factor IF-2
rbfA	BLi01890	3.8	± 0.2	4.5	± 0.2	4.1	± 0.2	4.0	± 0.1	*rbfA*	ribosome-binding factor A
ykrS	BLi01511	5.2	± 0.8	5.2	± 0.6	4.8	± 0.2	3.8	± 0.2	*ykr operon*	similar to initiation factor eIF-2B
Transport/binding proteins and lipoproteins											
cysP	BLi01779	5.7	± 0.1	5.1	± 1.9	6.7	± 3.0	5.2	± 0.2	*cysHP-sat-cysC-ylnDEF*	sulfate permease
ptsG	BLi01598	7.7	± 0.7	8.1	± 0.9	12.4	± 2.6	6.8	± 0.1	*ptsI-G-glcT*	PTS glucose-specific enzyme
ycdH	BLi03213	32.5	± 10.6	20.3	± 0.5	22.2	± 1.9	20.0	± 0.3	*ycdFGHI*	similar to ABC transporter
yclP	BLi00465	5.6	± 0.2	8.9	± 0.5	3.0	± 0.1	4.0	± 1	*yclNOPQ*	similar to ferrichrome ABC transporter
ykoE	BLi04204	6.6	± 0.8	5.3	± 0.5	6.7	± 0.1	10.5	± 0.2	*ykoC-D-E*	similar to cation ABC transporter
yxdL	BLi04146	8.6	± 2.0	10.1	± 1.9	8.4	± 0.1	5.9	± 1.9	*yxdK-L-M*	similar to ABC transporter
yxdM	BLi04147	7.0	± 3.4	9.3	± 1.8	7.5	± 1.1	6.6	± 0.5	*yxdK-L-M*	similar to ABC transporter
Unknown function											
BLi04205		5.5	± 1.3	3.5	± 0.1	8.7	± 1.1	8.3	± 0.2		putative transcriptional regulation
BLi00764		6.6	± 2.1	2.5	± 0.3	7.4	± 1.3	7.5	± 2.3		hypothetical
BLi00763		8.4	± 1.0	1.8	± 0.2	8.7	± 0.1	8.4	± 1.2		putative oxidoreductase
BLi00156		7.8	± 2.4	10.6	± 0.8	14.2	± 0.2	12.6	± 0.3		conserved hypothetical

Table 4: continued

Gene name	ID	Trans.ph	SD	30 min	SD	60 min	SD	120 min	SD	Transcriptional organisation	Function
yciC	BLi00765	13.1	± 0.3	12.4	± 0.5	11.4	± 0.3	11.8	± 0.3	*yciC*	unknown
ykoC	BLi04202	5.5	± 0.5	5.2	± 0.5	6.1	± 0.3	6.0	± 0.3	*ykoC-D-E*	unknown
ykoE	BLi04204	6.0	± 2.3	4.8	± 0.4	11.3	± 1.9	10.5	± 3.9	*ykoC-D-E*	unknown
ykrT	BLi01512	9.4	± 0.4	9.7	± 0.5	6.8	± 0.1	5.0	± 0.3	*ykr* operon	unknown
ykrY	BLi01516	5.1	± 0.6	8.0	± 2.9	7.0	± 2.8	5.8	± 0.1	*ykr* operon	unknown
ykrZ	BLi01517	6.2	± 0.7	11.5	± 1.4	7.3	± 1.0	4.5	± 0.1	*ykr* operon	unknown
yrvM	BLi02879	10.5	± 0.3	5.9	± 0.1	9.1	± 0.1	8.5	± 0.9	*yrv* operon	unknown
yusB	BLi03456	13.3	± 4.7	11.3	± 1.8	10.6	± 0.6	6.3	± 0.1	*yusABCD*	unknown

In contrast, genes encoding proteins that are involved in basic metabolic pathways, such as amino acid biosynthesis, nucleotide or coenzyme metabolism and ribosomal protein genes were down-regulated during phosphate starvation conditions (Tab. 4), indicating a stringent response of *B. licheniformis* cells under this nutrient starvation conditions. It is interesting to note that the secretion specific gene *secY* revealed an about 10fold decreased mRNA level 30 min after onset of phosphate starvation. The strongest down regulation could be observed for genes encoding ribosomal proteins, such as L2 and L14. These genes showed an about 20fold lower mRNA level already 30 min after onset of the stationary phase in comparison to the exponential growth phase. Furthermore, chemotaxis related genes like *cheA*, *cheBC* or *flgE* showed an about 5fold lower mRNA level under phosphate starvation conditions. Finally, the expression of major genes of the teichoic acid synthesis pathway was about 4fold repressed.

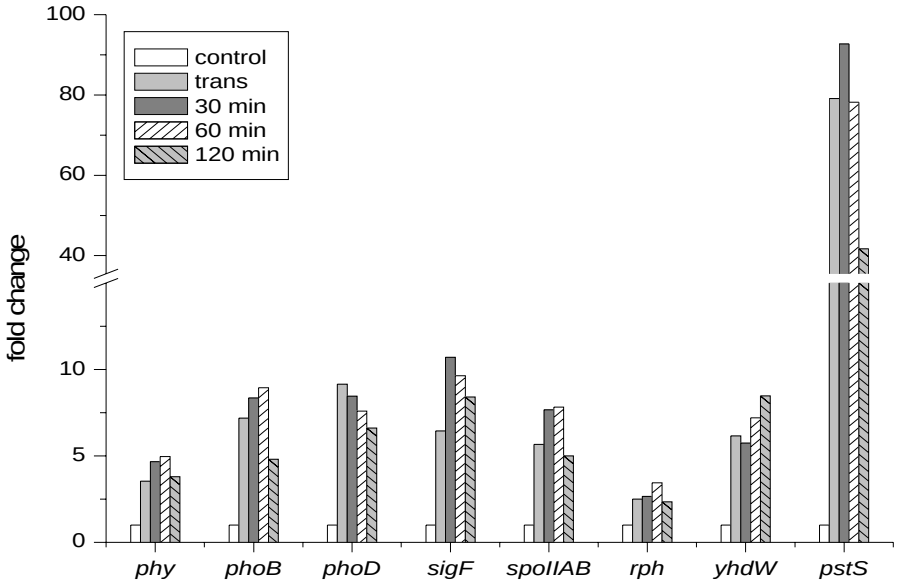


Figure 2: Verification of the induction of selected genes by phosphate starvation conditions via real-time-RT-PCR. Shown are averaged data of two independent experiments.

3.2. Proteome analysis

The transcriptome analysis was combined with the analysis of the cytoplasmic and extracellular proteome of *B. licheniformis* cells grown under phosphate-limited conditions. In both cellular compartments the induction of the synthesis of selected proteins belonging to the potential Pho regulon was observed. In the cytoplasmic proteome for example a transient induction of PhoP could be detected (Fig. 3, Tab. 5). PstS, the substrate binding protein of the high-affinity phosphate transporter, could also be detected in the cytoplasmic proteome during phosphate starvation. The synthesis rate of PstS was higher at the later starvation time point. PstBA, however, one of the two ATP-binding proteins of the high-affinity phosphate transporter, was only slightly induced. Both proteins are supposedly membrane associated; therefore the main amount of these proteins is very likely localised in the membrane and not found in the cytoplasmic protein fraction.

Besides the up-regulation of Pho regulon members, the starvation led to the induction of the sporulation related proteins Spo0A, SpoIIAA, SpoIVA and SigF in the cytoplasmic proteome (Fig. 3, Tab. 5). Furthermore, the synthesis of selected intracellular proteases and peptidases was induced (e.g. YjbG, YpwA, YusX and IspA). The intracellular serine protease IspA was the protein with the highest synthesis rate observed during our phosphate starvation experiments. Another group of proteins, the synthesis of which was enhanced, were enzymes involved in the synthesis of coenzymes (e.g. DhaS and NadE).

In consistence with the transcriptome analysis, the proteome analysis revealed that phosphate starvation led to the repression of most basic metabolic pathways, like glycolysis, amino acid synthesis and nucleotide synthesis. The TCA cycle enzymes were mostly not affected, i.e., their synthesis was only slightly changed. But CitG, the fumarate hydratase, was strongly repressed. There was also a strong repression of the translation machinery, as could be seen by the decreased synthesis of ribosomal proteins (e.g. RplJ), of the amino acyl-tRNA synthetases (e.g. ThrS) and of the elongation factors (FusA, Tsf, TufA). The elongation factor TufA was found to be fragmented, since TufA protein spots were identified in gel regions were the protein should not be found according to its pI and molecular mass.

B. licheniformis is able to efficiently secret a high number of proteins into the extracellular medium. In order to identify phosphate specific extracellular proteins the secretome of phosphate starving *B. licheniformis* cells was analysed. The two most prominent protein spots in the secretome of these cells were the phytase, Phy, and YhdW, a protein similar to glycerophosphoryl diester phosphodiesterase (Fig. 4).

Table 5: Synthesis rates of proteins from *B. licheniformis* cells during phosphate starvation conditions. Quantification of the proteins and calculation of the synthesis rates were done with the Delta 2D software (Decodon GmbH, Greifswald, Germany). Only proteins, the expression of which was induced in two independent experiments at least threefold are included in the table. The protein spots YpwA and BLi00416 could not be separated for quantification. Therefore the same synthesis ratio is given for both spots

Protein name	pI	MW	ID number	Synthesis ratio				Function
				OD 1 (gel 1)	OD 1 (gel 2)	OD 1+30min (gel 1)	OD 1+30min (gel 2)	
1 Cell envelope and cellular processes								
1.2 Transport/binding proteins and lipoproteins								
AppA	4.8	62.7	BLi01226	7.4	8.4	16.8	10.0	oligopeptide ABC transporter
AppA 2. spot	4.8	62.7	BLi01226	5.1	7.6	1.8	1.5	oligopeptide ABC transporter
OppD	5.8	39.5	BLi01235	4.3	7.2	7.7	10.6	oligopeptide ABC transporter
PstBA	5.8	31.0	BLi02673	1.4	0.9	1.0	1.3	phosphate ABC transporter
PstS	4.6	32.6	BLi02677	2.6	2.0	34.2	32.2	phosphate ABC transporter
1.3 Sensors (signal transduction)								
YhjL	5.4	48.4	BLi03946	19.2	9.5	14.9	15.5	similar to sensory transduction pleiotropic regulatory protein
1.7 Cell division								
DivIVA	4.8	19.4	BLi01761	4.1	18.8	2.0	2.4	cell-division initiation protein
1.8 Sporulation								
CotJC	5.1	21.8	BLi01147			8.6	6.9	polypeptide composition of the spore coat
SpoIVA	4.6	55.4	BLi02416	28.6	34.1	44.6	51.0	required for proper spore cortex formation and coat assembly
SpoIIAA	5.9	13.0	BLi02497	71.8	23.6	16.6	18.6	anti-sigma F factor antagonist
2 Intermediary metabolism								
2.1 Metabolism of carbohydrates and related molecules								
2.1.1 Specific pathways								
AlsD	4.6	28.9	BLi03847			13.0	33.6	alpha-acetolactate decarboxylase
YhcW	4.8	25.0	BLi00987			4.7	4.3	similar to phosphoglycolate phosphatase
YtoP	5.1	39.3	BLi03137			3.7	3.0	similar to endo-1,4-beta-glucanase
YvgN	5.2	31.8	BLi03528	2.6	3.0	3.0	4.5	similar to dehydrogenase
2.2 Metabolism of amino acids and related molecules								
Dat	5.3	31.3	BLi01040	6.2	5.4	1.3	1.1	D-alanine aminotransferase
Dat 2. spot	5.3	31.3	BLi01040	2.7	2.6			D-alanine aminotransferase
IspA	4.3	33.8	BLi01423	114.5	145.9	32.6	33.7	major intracellular serine protease
RocD	5.4	43.9	BLi00422	7.4	5.1			ornithine aminotransferase
YcgN	5.2	56.6	BLi00374	36.4	68.4			similar to 1-pyrroline-5-carboxylate dehydrogenase
YpwA	5.2	57.8	BLi02349			11.714 (BLi00416)	9.667 (BLi00416)	similar to carboxypeptidase
YqhS	5.2	16.3	BLi02618			6.6	6.5	similar to 3-dehydroquinate dehydratase
YusX	4.9	67.0	BLi03477			68.8	49.4	similar to oligoendopeptidase
YusX (2. spot)	4.9	67.0	BLi03477			6.1	8.0	similar to oligoendopeptidase
YusX (3. spot)	4.9	67.0	BLi03477			5.5	5.2	similar to oligoendopeptidase
YuxL	5.2	73.3	BLi03411	12.0	7.8			similar to acylaminoacyl-peptidase
BLi00977	5.0	67.8	BLi00977	4.5	8.7	3.1	3.1	putative acylamino-acid-releasing enzyme

Table 5: continued

Protein name	pI	MW	ID number	Synthesis ratio				Function
				OD 1 (gel 1)	OD 1 (gel 2)	OD 1+30min (gel 1)	OD 1+30min (gel 2)	
FabHB	5.8	36.2	BLi01097	5.4	5.0			beta-ketoacyl-acyl carrier protein synthase III
BLi00828	4.6	39.5	BLi00828	2.7	2.7	45.1	44.0	putative glycerol dehydrogenase
BLi00828 (2. spot)	4.6	39.5	BLi00828	2.7	2.7	1.7	2.0	putative glycerol dehydrogenase
2.5 Metabolism of coenzymes and prosthetic groups								
DhaS	5.2	54.4	BLi02249	15.7	28.9	8.1	8.3	aldehyde dehydrogenase
DhbC	4.9	42.8	BLi03901			6.1	6.0	isochorismate synthase
NadE	4.8	30.0	BLi00370	4.9	4.0	2.0	1.5	NH3-dependent NAD+ synthetase
BLi03994	5.2	53.8	BLi03994	19.2	29.4	24.1	9.8	2-hydroxymuconic semialdehyde dehydrogenase
2.6 Metabolism of phosphate								
YlaK	5.1	49.7	BLi01698	5.0	7.3	26.0	23.5	similar to phosphate starvation inducible protein
3 Information pathways								
3.1 DNA replication								
Ssb	5.0	18.4	BLi04362	23.1	8.3			single-strand DNA-binding protein
3.5 RNA synthesis								
3.5.1 Initiation								
SigF	5.2	29.6	BLi02495	29.1	29.1			RNA polymerase sigma-F factor
3.5.2 Regulation								
PhoP	5.1	27.7	BLi03059	4.3	3.5	1.4	1.5	two-component response regulator involved in phosphate regulation
Spo0A	6.2	29.5	BLi02593	17.1	15.1	4.0	8.6	two-component response regulator central for the initiation of sporulation
3.8 Protein modification								
PrkA	5.4	72.8	BLi00962	14.6	6.8	2.9	1.3	serine protein kinase
YqhT	5.0	38.3	BLi02617	4.2	4.0	0.7	0.9	similar to Xaa-Pro dipeptidase
4 Other functions								
4.1 Adaptation to atypical conditions								
BsaA	9.2	18.2	BLi02327	18.5	44.4	97.3	143.0	putative glutathione peroxidase
BsaA (2. spot)	9.2	18.2	BLi02327			18.7	28.1	putative glutathione peroxidase
ClpC	5.7	90.2	BLi00104	5.5	11.5	2.0	1.8	class III stress response-related ATPase
ClpC (2. spot)	5.7	90.2	BLi00104	5.0	8.4	1.9	1.7	class III stress response-related ATPase
ClpP	4.9	21.8	BLi03710	3.9	3.4	4.4	4.7	ATP-dependent Clp protease proteolytic subunit
ClpP (2. spot)	4.9	21.8	BLi03710			1.4	1.7	ATP-dependent Clp protease proteolytic subunit
YvtA	4.7	48.7	BLi03481	1.2	1.7	6.7	3.3	similar to HtrA-like serine protease
4.2 Detoxification								
CypA	5.1	47.3	BLi02822	4.3	3.8	10.1	9.3	cytochrome P450-like enzyme
MsrA	5.3	21.0	BLi02303	6.8	5.1			peptidyl methionine sulfoxide reductase
5. unknown								
YdhD	5.7	50.0	BLi00025			10.1	11.9	
YngK	5.3	45.4	BLi02129	7.4	7.1			
YtoQ	6.1	16.7	BLi03136			4.6	6.2	
YtoQ (2. spot)	6.1	16.7	BLi03136			0.8	1.3	
BLi00416	5.2	61.7	BLi00416			11.714 (YpwA)	9.667 (YpwA)	putative 2,4-diaminobutyrate decarboxylase
BLi02843	5.6	34.7	BLi02843			12.9	19.9	
BLi03379	4.5	11.3	BLi03379	3.2	1.9	5.8	11.5	
BLi03989	5.7	45.1	BLi03989			25.9	23.6	P-hydroxybenzoate hydroxylase
BLi04184	5.2	20.2	BLi04184	12.2	9.4	4.4	6.0	

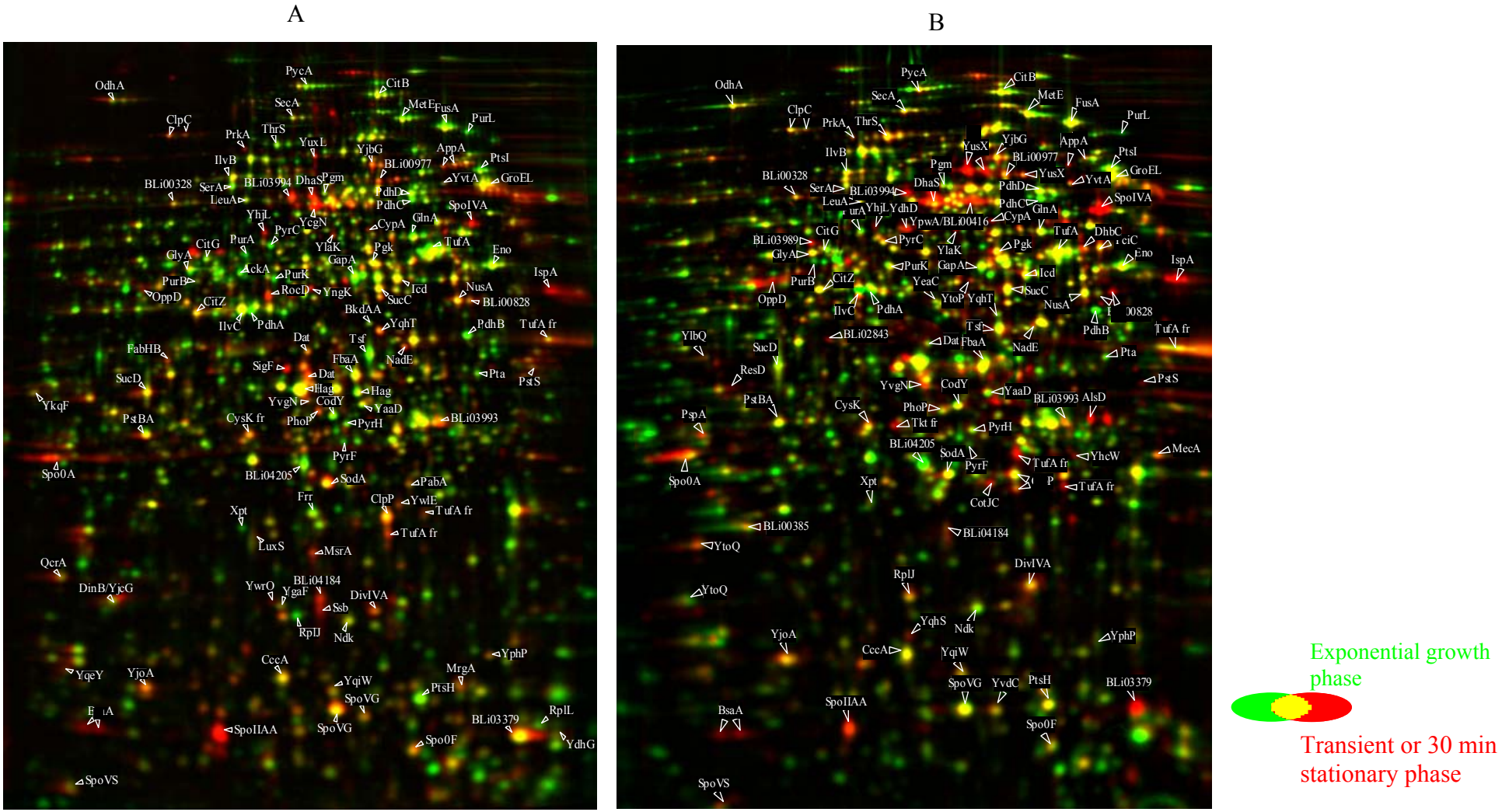


Figure 3: Changes in the cytoplasmic proteome of phosphate starving *B. licheniformis* cells. (A) Transient phase, (B) 30 min after entry into stationary phase. The dual channel images were created with the Delta 2D software (Decodon GmbH, Greifswald, Germany). Cell samples were labelled with L-[^{35}S]-methionine during the exponential growth phase (OD 0.4), at the transition phase (OD 0.9) and 30 min after entry into stationary growth phase. Proteins were separated in a pH gradient 4-7.

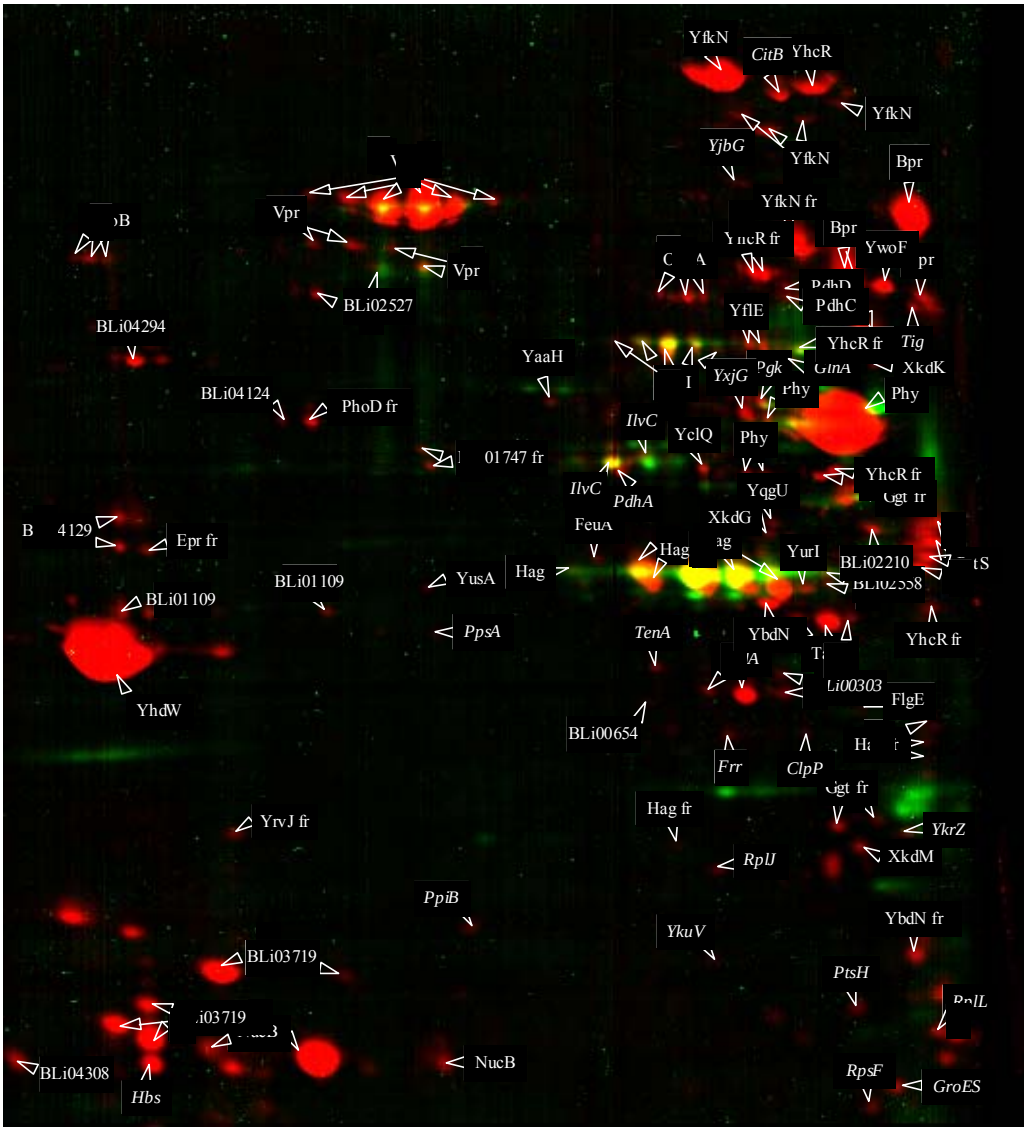


Figure 4: Dual channel image of the extracellular proteome from phosphate starving *B. licheniformis* cells created with the Delta 2D software (Decodon GmbH, Greifswald, Germany). Proteins were prepared during exponential growth and one hour after entry into stationary growth phase. Proteins were separated in a pH gradient 3-10 and stained with colloidal Coomassie Brilliant Blue. Spots labelled in italics are presumably intracellular proteins. fr: protein fragments.

Furthermore, some nucleic acid degrading enzymes (e.g. YfkN, YhcN, NucB and BLi03719) were secreted to a high amount. Such induction of nucleic acid degrading enzymes during phosphate starvation was also described for other organisms (Znamenskaya et al. 1995; Ishige et al. 2003). Although there was a 20-80fold induction of the phosphatase genes *phoB* and *phoD* at the transcriptional level (Table 2), both proteins formed only minor spots in the extracellular proteome of phosphate starving *B. licheniformis* cells. The ABC transporter protein PstS was also found in the extracellular proteome. As described above this protein is

very likely lipid-anchored in the membrane. But PstS also forms an abundant protein in the extracellular proteome of phosphate starving *B. subtilis* cells (Antelmann et al. 2000). The strong secretion of the proteases Vpr and Bpr was very likely not specifically induced by the phosphate starvation but rather due to the fact that the cultures were in the stationary growth phase, because the secretion of the proteases was also observed in cultures subjected to other starvation conditions (Voigt et al. 2004).

4. Discussion

The transcriptome and proteome analysis of *B. licheniformis* cells revealed more than 100 genes that were significantly induced (more than 4fold) under phosphate starvation conditions. In *B. subtilis* many of these genes have been addressed as crucial genes responsible for the cellular adaptation to phosphate starvation conditions. This includes for example the gene *phoB*, encoding an alkaline phosphatase (Hulett et al. 1991), which is able to mobilise phosphate from alternative organic phosphate sources, or the *pstS-C-A-BA-BB* operon, encoding a high-affinity phosphate transporter (Qi et al. 1997). The *phoB* and *pstS* genes belong to the most strongly induced genes under these conditions.

The putative glycerophosphoryl diester phosphodiesterase YhdW, an enzyme which plays a role in the hydrolysis of deacylated phospholipids, was found to be highly accumulated in the secretome of *B. licheniformis*. The *BLi02820* gene, coding for a putative phosphatase, was strongly induced at the transcriptional level. Under phosphate starvation conditions, this protein was also determined in the extracellular proteome (data not shown).

In addition, *phoD*, coding for a phosphodiesterase, which has a role in cell-wall teichoic acid turnover (Voigt et al. 2004), was strongly induced under phosphate starvation conditions in *B. licheniformis*. PhoD is an extracellular protein which is known to be secreted in *B. subtilis* via the Tat secretion system (Antelmann et al. 2000). In *B. licheniformis*, the *phoD* gene forms an operon with *tatCD*, which encode structural components of the Tat secretion system. This indicates that PhoD of *B. licheniformis* is secreted via the Tat secretion system, as it is in *B. subtilis*.

The strong induction of the nuclease encoding genes *yhcR*, *yfkN*, *nucB*, *yurI*, *rph*, and *BLi03719* indicates that *B. licheniformis* cells try to mobilise phosphate groups from intracellular and extracellular ribonucleic and desoxyribonucleic acids. The nucleases NucB, YfkN, and BLi03719 belong to the most dominant protein spots in the extracellular proteome under phosphate starvation conditions.

Another strongly induced gene under these conditions codes for CypX, a cytochrome P450-like enzyme. In addition, other cytochrome related oxidases (*cydAB*) and oxidoreductases (*qcrABC*) were induced. These proteins are presumably involved in energy metabolism. However, the relation of the increased expression of these proteins to the phosphate starvation condition is not clear.

It is important to stress that genes encoding proteins that are involved in basic metabolic pathways, such as amino acid biosynthesis, nucleotide or coenzyme metabolism and

ribosomal protein genes were down-regulated in an early stage of the stationary phase in a phosphate limited *B. licheniformis* growth culture. This indicates a stringent response of the cells under this nutrient starvation conditions.

One striking difference between the phosphate starvation responses of *B. licheniformis* DSM13 and *B. subtilis* 168 is represented by the massive accumulation of the phytase in the extracellular proteome of *B. licheniformis*. This indicates that phytate is an important alternative phosphate source in the natural environment of *B. licheniformis*. Phytic acid (*myo*-inositol hexakis dihydrogen phosphate) is the major storage form of phosphate in many plants like cereals or legumes. Although, *B. subtilis* 168 possesses a phytase encoding gene, this enzyme could not be detected in the extracellular medium during phosphate limitation conditions (Antelmann et al. 2000).

The mRNA levels of the *tagAB* and *tagDF* operons, involved in the synthesis of a phosphate-containing cell-wall polymer (Müller et al. 1997; Liu et al. 1998 a), polyglycerolteichoic acid, were about threefold decreased. In contrast the expression of the *tuaABCD-BLi03803-EFGH* operon of *B. licheniformis*, coding for proteins which are presumably responsible for the synthesis of teichuronic acid (Müller et al. 1997; Liu and Hulett 1998), a non-phosphate-containing polymer that could replace teichoic acid in the wall, was only about two- to threefold induced during the phosphate starvation conditions in this study (data not shown). Although, similar to *B. subtilis*, potential PhoP binding boxes could be detected in front of the *tuaABCD-BLi03803-EFGH* operon, the expression of this alternative pathway does obviously not play a crucial role in the phosphate starvation response of *B. licheniformis*.

Already in 1991 by Lee et al. (1991) it was shown that an alkaline phosphatase gene from *B. licheniformis* (MC 14) is expressed in a PhoPR dependent manner in *B. subtilis*. We could identify in this study potential PhoP consensus binding boxes for *B. licheniformis* genes by means of a bioinformatic pattern search using the *B. subtilis* PhoP core binding region. It turned out that the potential Pho regulon genes of *B. licheniformis*, identified by this bioinformatic analysis, belonged to the most strongly induced genes under phosphate starvation conditions. Furthermore, these genes were in all cases already induced in the transient phase. This result is not surprising since the phosphate specific kinase PhoR of the two-component system PhoPR should be the first cellular sensor to recognise the limitation of inorganic phosphate in the medium (Hulett et al. 1994; Hulett 1996, 2002; Sun et al. 1996).

Results of this study also demonstrate that *B. licheniformis* responds to phosphate limitation conditions by starting the sporulation cascade. Several genes involved in the sporulation

process are induced in a very early stage of the stationary phase under this nutrient limitation condition. For *B. subtilis* it was suggested that if the deprivation of phosphate continues despite the action of the Pho regulon genes, Spo0A initiates sporulation and terminates the phosphate starvation response by repressing the transcription of the *phoPR* operon via AbrB and ResD-ResE (Hulett 1996). Mutations in the *spo0A* gene hyperinduce Pho regulon genes of *B. subtilis*. In *B. licheniformis* the most strongly induced intracellular protein has been shown to be the serine protease IspA. It has been observed for *Bacillus thuringiensis* that the process of sporulation was delayed in an *ispA*-deficient mutant (Chen et al. 2004). The results of a proteomic analysis indicated that a sporulation-associated protein was upregulated, while two other sporulation-associated proteins, SpoVD and SpoVR, were downregulated in the *ispA*-deficient mutant. Based on these results it could be speculated that IspA of *B. licheniformis* also plays a role in the early regulation of the sporulation process under phosphate limitation conditions.

Beside the Pho regulon, phosphate starvation in *B. subtilis* also induces the σ^B-dependent general stress regulon (Hecker and Völker 1998; Hulett 2002). σ^B-dependent general stress proteins (Gsps) are thought to provide nongrowing cells with nonspecific, multiple stress resistances. These proteins seem to be involved in the protection of DNA, membranes, and proteins against oxidative stress and appear to contribute to the survival under extreme environmental conditions, such as heat or osmotic stress as well as acid or alkaline shock of starved cells (Hecker and Völker 2001). Although the ATP-dependent protease ClpP and its ATPase subunit ClpC showed elevated protein levels after onset of the stationary phase, no indication for an induction of the σ^B-regulon under phosphate limitation conditions in *B. licheniformis* could be detected. This is a surprising result and indicates that the general stress response in *B. licheniformis* could be different from that in *B. subtilis*. Brody and Price (1998) suggested that *B. licheniformis* possesses a general stress response system that is organised in a manner that is strikingly similar to the σ^B-system of *B. subtilis*.

It is worth emphasising that during the transient phase an upregulation of *sodA* could be observed at the transcriptional level. This led to a drastically higher level of SodA in the cytoplasmic and the extracellular proteome under these conditions. Furthermore, the putative superoxide-inducible protein YaaD and the putative glutathione peroxidase BsaA were strongly induced at the protein level in the stationary phase. The increased expression of these proteins could indicate that the cells experienced an oxidative stress under these nutrient limited growth conditions. However, the expression of typical oxidative stress marker genes

like *ahpC* and *ahpF* was strongly, and *katA* was weakly downregulated at the transcriptional level. Voigt et al. (2004) observed during the late growth phase of *B. licheniformis* in a complex medium a strong oxidative stress response. The monitoring of potential oxidative stress situations could be of relevance for the quality of overproduced proteins under bioprocess conditions. Oxidative stress conditions can cause protein modifications (Mostertz et al. 2004) and would thus negatively influence the functionality of the overproduced proteins in an industrial fermentation process.

Furthermore, 30 min after onset of phosphate starvation the elongation factor TufA and the cysteine synthetase A CysK were identified in gel regions were the proteins should not be found according to their pI and molecular mass. This indicates a fragmentation of these vegetative proteins of *B. licheniformis* at this time of the cultivation. This observation is consistent with results of Voigt et al. (2004), who found a fragmentation of crucial vegetative proteins of *B. licheniformis* in the late stationary phase during growth in a complex LB medium.

A comprehensive understanding of cell physiology and its adaptation to atypical conditions is a prerequisite for a directed optimisation of industrial bioprocesses based on microbial cells. Furthermore, so called marker genes specific for distinct stress or nutrient limitation conditions are useful indicators that will allow for a better monitoring of microbial fermentation processes in the future. By means of new techniques for the at-line analysis of process critical marker genes a fast and directed intervention into the fermentation process will be possible (Jürgen et al. 2005 a). The exploration of the phosphate starvation stimulon of *B. licheniformis* is one step toward a better understanding of the physiology of this industrially important bacterium. Selected genes, which were identified in this study as specifically induced (e.g. *pstS*, *phy*, *phoB* or *yvnA*), are potential marker genes for monitoring critical phosphate limitation situations in *B. licheniformis* fermentation processes.

Acknowledgments

This work was supported by a sholarship to Le Thi Hoi from the Ministry of Education and Training of Vietnam (MOET) and by funds of the Competence Network “Genome Research on Bacteria” financed by the German Federal Ministry of Education and Research (BMBF). This work was also supported by the Ministry of Culture and Education of Mecklenburg-Vorpommern. We thank Stefanie Leja and Anne Krause for excellent technical assistance. We are grateful to the Decodon GmbH (Greifswald, Germany) for their cooperation.

Chapter III

Response of *Bacillus licheniformis* to glucose and nitrogen starvation

Le Thi Hoi[1], Birgit Voigt[2], Britta Jürgen[1], Dirk Albrecht[2], Armin Ehrenreich[3], Gerhard Gottschalk[3], Stefan Evers[4], Jörg Feesche[4], Karl-Heinz Maurer[4], Michael Hecker[2], and Thomas Schweder[1]

[1]Institut für Pharmazie, Ernst-Moritz-Arndt Universität, Greifswald.
[2]Institut für Mikrobiologie, Ernst-Moritz-Arndt Universität, Greifswald.
[3]Institut für Mikrobiologie und Genetik, Georg-August-Universität, Göttingen.
[4]Henkel KGaA, VTB Enzymtechnologie, Düsseldorf.

Keywords: *Bacillus licheniformis*, glucose starvation, nitrogen starvation, proteome, transcriptome

ABSTRACT

The glucose and nitrogen starvation stimulons of *B. licheniformis* were determined by transcriptome and proteome analyses. The main response of *B. licheniformis* to glucose starvation was a switch to the usage of alternative carbon sources. In addition, *B. licheniformis* seems to be using other organic substances like amino acids and lipids as carbon sources when subjected to glucose starvation. This was indicated by the induction of a high number of genes the proteins of which are involved in amino acid and lipid degradation. During nitrogen starvation genes necessary for the recruitment of nitrogen from alternative sources were induced, e.g. genes for nitrate and nitrite assimilation, several proteases and peptidases. Both starvation conditions led to a down-regulation of the transcription of most vegetative genes and subsequently to a reduced synthesis of the corresponding proteins. Only a few genes were induced by both starvation conditions like *yvyD*, *citA* and the methylcitrate shunt genes *mmgD*, *mmgE* and *yqiQ*.

1. Introduction

The sequencing of the genome of the important industrial host bacterium *Bacillus licheniformis* and the following development of genomic DNA microarrays now allows for the transcriptional profiling of all genes expressed by the bacterium under defined environmental conditions. However, transcriptomic studies can not give information about protein synthesis, protein stability and localisation. Therefore complementation of transcriptomic data with proteomic data is desired to gain deeper insight into the physiology of cells under changing environmental conditions.

Bacteria are able to use a variety of substances as nutritional sources of carbon and nitrogen. When grown in an abundance of nutrients, they prefer to use their favoured nutrient sources, which allow the highest growth rates. However, in natural environments nutrient starvation conditions are common. The ability to rapidly adapt to changed environmental conditions is therefore essential for cell survival. Complex regulatory networks are required to allow such adaptations.

When subjected to glucose starvation *B. subtilis* responds with changes in the expression of many genes of the central carbon metabolism e.g. the glycolysis or the TCA cycle (Moreno et al. 2001; Yoshida et al. 2001; Bernhardt et al. 2003; Koburger et al. 2005). Furthermore, there is a switch to the utilisation of alternative carbon sources. Most of the genes for utilisation of such alternative carbon sources are regulated by carbon catabolite repression (CCR) in dependence on the availability of metabolisable carbon sources (Saier et al. 1996; Stülke and Hillen 2000; Bruckner and Titgemeyer 2002). In *B. subtilis* CCR is mediated by the catabolite regulatory protein A (CcpA), a member of the LacI/GalR repressor family, its co-repressors HPr, Crh, and the bifunctional protein HPr kinase/ phosphorylase (HPrK) (Henkin et al. 1991; Deutscher et al. 1995; Saier et al. 1996; Stülke and Hillen 2000; Turinsky et al. 2000; Deutscher et al. 2002; Lorca et al. 2005). If glucose is present in the medium the proteins HPr and Crh are phosphorylated by the HPr kinase/ phosphorylase (HPrK) and trigger the binding of CcpA to the catabolite-responsive elements (*cre* sites) in the promoter region of the target genes (Galinier et al. 1997, 1999). The binding of the *cre* site by CcpA results in general in a repression of transcription. But it has also been reported that CcpA can cause a transcriptional activation (Stülke and Hillen 2000). For example, for carbon excretion pathways, including those for production of acetate, acetoin, glycogen, CcpA acts as an activator (Grundy et al. 1993; Renna et al. 1993; Presecan-Siedel et al. 1999; Turinsky et al. 2000).

In *B. subtilis* and other Gram-positive bacteria, CcpA is a key regulator not only of carbon but also of nitrogen metabolism (Faires et al. 1999; Wacker et al. 2003). It regulates for example the expression of the glutamate synthase, a key enzyme in ammonium assimilation. A *B. subtilis ccpA* mutant is not able to grow in minimal medium with ammonium and glucose as single sources of nitrogen and carbon (Faires et al. 1999). Furthermore, expression of the *ilv-leu* operon, which plays an important role in protein metabolism through the synthesis of branched-chain amino acids, is under control not only of CcpA, but also TnrA and CodY, two global regulator proteins in the nitrogen regulation system (Ludwig et al. 2002; Tojo et al. 2004; 2005). Moreover, nitrogen sources such as amino acids, are necessary for the full induction of glycolytic genes as well as for the repression of the TCA cycle in the presence of glucose (Ludwig et al. 2001, 2002; Deutscher et al. 2002). The TCA cycle enzyme aconitase (*citB*) is regulated not only by the specific regulator CcpC, but also by CcpA and TnrA (Blencke et al. 2006). Consequently, there are tight interactions between carbon and nitrogen regulation.

Apart from the influence by CcpA, nitrogen metabolism of *B. subtilis* is controlled by at least three global regulatory proteins TnrA, GlnR and CodY in response to nutrient availability (Fisher 1999). These regulators are active under different growth conditions. The repressor protein CodY controls expression of several genes in response to the growth rate. CodY activity is regulated by the level of intracellular GTP, which serves as an indicator for sensing the nutritional state of the cell (Ratnayake-Lecamwasam et al. 2001). CodY repression is highest in cells growing rapidly with amino acids. This repression is relieved when nitrogen becomes limiting (Fisher 1999). TnrA functions either as an activator or repressor of gene expression but only during nitrogen-limited conditions, whereas GlnR is active only during excess nitrogen availability (Schreier et al. 1989; Wray et al. 1996, 1997). During excess nitrogen conditions the transcription of TnrA is repressed by GlnR (Wray et al. 1997). Thus, GlnR and TnrA respond directly to nitrogen availability, whereas CodY responds to the total nutritional state of the cell.

Although the conditions for their activity are different, TnrA and GlnR bind to similar DNA sequences and both of them belong to the MerR family of transcriptional regulators (Gutowski and Schreier 1992; Brown and Sonenshein 1996; Wray et al. 1998; Fisher 1999). The nitrogen signal regulating TnrA activity has been elucidated. During growth with excess nitrogen, feedback-inhibited *B. subtilis* glutamine synthetase (GS) directly interacts with TnrA and inhibits the DNA binding activity of TnrA by a protein-protein interaction, thereby preventing

expression of alternative nitrogen assimilation pathways (Wray et al. 2001; Fisher and Wray 2002; Wray and Fisher 2005). This inhibition of TnrA is removed when nitrogen becomes limiting. The role of GS in controlling GlnR activity, however, is not yet clearly identified. GS therefore plays a key role in the regulation of nitrogen metabolism.

This study aimed to comprehensively determine the glucose and nitrogen starvation stimulons of *B. licheniformis*. Potential links between both starvation responses were analysed by means of transcriptome and proteome analyses.

2. Material and Methods

2.1. Strains and culture conditions

Bacillus licheniformis DSM 13 (equivalent to ATCC 14580, type strain from the German Collection of Microorganisms and Cell Cultures, DSMZ GmbH, Braunschweig, Germany) was cultivated at 37°C under vigorous agitation in minimal medium (MM) containing 15 mM $(NH_4)_2SO_4$, 8 mM $MgSO_4$ x 7 H_2O, 27 mM KCl, 7 mM Na-citrate x 2 H_2O, 50 mM Tris-HCl (pH 7.5) supplemented with 0.6 mM KH_2PO_4, 2 mM $CaCl_2$ x 2 H_2O, 1 µM $FeSO_4$ x 7 H_2O, 10 µM $MnSO_4$ x 4 H_2O and 0.2 % (w/v) glucose. For the starvation experiments the concentration of glucose and nitrogen were reduced to 0.08 % and 0.001 mM, respectively. This concentration of the nutrients allowed the cultures to grow to a maximal optical density measured at 500 nm (OD_{500}) of about 1.2. Bacterial cells were harvested at different growth points during exponential growth phase, transition phase, and 0.5, 1, and 2 h after onset of the stationary growth phase.

2.2. RNA isolation

For RNA isolation cells were harvested in half of the sample volume ice cold killing buffer (20 mM NaN_3, 20 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$). Cell pellets were resuspended in Lysis buffer II (3 mM EDTA, 200 mM NaCl) and mechanically disrupted using the RiboLyser™ (Thermo Electron Corporation GmbH, Dreieich, Germany) as described by Jürgen et al. (2005b). Isolation and purification of total RNA was done with the KingFisher ml™ (Thermo Electron Corporation GmbH, Dreieich, Germany) according to Jürgen et al. (2005b).

Buffers and chemical reagents for the KingFisher ml™ were used from the MagNA Pure LC RNA Isolation Kit I (Roche Diagnostics, Penzberg, Germany). Concentration and quality of the isolated total RNA were determined with the Bioanalyzer 2100 (Agilent Technologies, Berlin, Germany) as recommended by the manufacturer.

2.3. Transcriptome analyses

Full genomic *B. licheniformis* DNA microarrays representing more than 95% of the ORFs longer than 300 bp were produced by spotting PCR products of 300 – 500 bp length in duplication on microarray slides using a Lucidea (Amersham Biosciences, Freiburg, Germany) contact printer according to Hoi et al. (2006). The microarray analysis was carried

out as described by Hoi et al. (2006). Only genes, which were nearly 5fold induced or repressed, were considered in this study (3fold induced or repressed genes are shown in supplementary material tables 1-4). When located in the same operon with a gene with such pronounced changes in the expression also genes with lower expression level changes were listed. The values and standard deviation (SD), presented in the tables, were calculated from at least 2 biological samples, obtained from 2 different experiments. Furthermore, the RNA of one of the biological samples was analysed twice, whereas the transcriptome of the second biological sample was investigated by Dye Swap. Both the biological samples as well as the Dye Swap analysis were included in the standard deviation calculation.

2.4. Real-time-RT-PCR

Real-time-RT-PCR was carried out with the LightCycler from Roche Diagnostics (Penzberg, Germany) as described by Hoi et al. (2006). Gene specific primers were deduced with the program Array Designer 2.0 (PREMIER Biosoft International, Palo Alto, USA) and ordered from Invitrogen (Karlsruhe, Germany) (Tab. 1). For the amplification validation the Cyber Green detection Kit from Roche Diagnostics (Penzberg, Germany) was used. Standard RNA probes for quantification of the mRNA were generated as described by Jürgen et al. (2005 a, b). The amount of the respective mRNAs in different samples was determined by using the external standard curves for quantification with the second derivative maximum method. The specificity of the RT-PCR products was checked by melting curve analyses. RT-PCR analysis of the genes was carried out in two individual experiments. Both experiments yielded similar induction ratios.

Table 1: List of primers used in this study.

Target gene	Forward primer	Reverse primer
etfA	ACGCTTTGCCCGAGTAAATC	AAGCCTTGTTGCCGATCATAG
mmgA	TCGTGATGAATGAAGACAAGG	CGTTGACATTGACCTTCTCC
acoA	AAAGGCGGATCGATGCATATTG	CTTCGCCATACCCGTTGTTTTC
glnA	CCTTGAGCGTACTTCTTG	AGTGTTCGAATAGATGGTC
trpE	CATTGCTTACATCGGATTTGAC	TCGTTCATTGCCGTCCTC
tnrA	ATTGGCATTGTCAGCGAG	GCCTTCGAGCATTTTCTTTC
nrgB	AGGGCATACTGAGCTTTACCG	GCCTGTGCGGATGTTGATG

2.5. 2-DE, protein identification and imaging

For preparation of cytoplasmic proteins, bacteria were pulse labelled with L-[^{35}S]-methionine during exponential growth (OD_{500} 0.4), at the onset of stationary phase (OD_{500} 1.0) and 60

min after transition into stationary phase as described by Hoi et al. (2006).

Protein extracts (80 µg protein for radiolabelled samples, 500 µg protein for preparative gels) were loaded onto commercially available IPG-strips (pH 4-7, Amersham Biosciences, Freiburg, Germany) as described previously (Büttner et al. 2001). In the second dimension polyacrylamide gels of 12.5% acrylamide and 2.6% bisacrylamide were used. Preparative 2-D gels were stained with colloidal Coomassie Brilliant Blue as described earlier (Voigt et al. 2004). Gels with radioactively labelled proteins were silver stained (Blum et al. 1987) and after drying exposed to storage phosphor screens (Molecular Dynamics, Sunnyvale, USA). Screens were scanned using a Typhoon Scanner (Amersham Biosciences, Freiburg, Germany). Proteins from preparative Coomassie stained gels were identified by mass spectrometry as described by Voigt et al. (2006). For analysis of the gels (including quantification and calculation of the protein synthesis rate) the Delta 2D software (Decodon, Greifswald, Germany) was used (Bernhardt et al. 1999). Labelling experiments were repeated twice and the synthesis rates given in supplementary tables 5 and 6 were derived from these two independent experiments. A threshold of 3 was used for analysis of the changes in protein synthesis.

2.6. Bioinformatic motif search

The pattern-matching program PatScan (D'Souza et al. 1997) was used to identify potential *cre*-boxes inside and in the upstream region of the *B. licheniformis* genes. A fasta file with the nucleotide sequence of all ORFs of the organism including 150 bp of the upstream region of each gene was analyzed by scanning for the pattern TGWNANCGNTNWCA. N stands for any nucleotide, whereas W indicates an A or T at the corresponding position. This pattern was described in the literature to be the consensus of *cre*-binding sites in *B. subtilis* (Deutscher et al. 2002).

3. Results

3.1. Growth of *B. licheniformis* under glucose and nitrogen starvation conditions

Growth of *B. licheniformis* under glucose and nitrogen starvation condition is shown in Figure 1. Under these conditions cells reached stationary phase at an OD of about 1.2. Throughout the growth, samples were taken in the exponential growth phase, in the transition phase and after entry into stationary phase (time points indicated by arrows in Fig.1) and used for transcriptome and proteome analysis. Gene expression and protein profiles during transient and stationary phase were compared to the profile in the exponential phase.

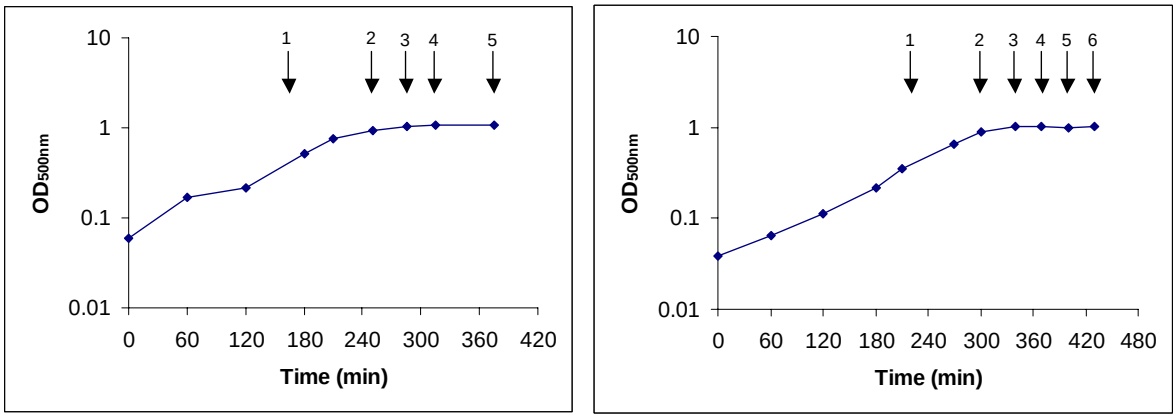


Figure 1: Growth curve of *B. licheniformis* in (A) glucose and (B) nitrogen limited MM medium. The arrows indicate the time points at which samples were taken (1: exp. growth phase (reference); 2: transition phase; 3: 0.5 h after onset of the stationary phase; 4: 1 h after onset of the stationary phase; 5: 2 h after onset of the stationary phase).

3.2. Glucose starvation

Most of the genes encoding vegetative proteins were strongly repressed at the transcriptional level after entry into the stationary phase due to glucose exhaustion. Synthesis of the corresponding proteins was down-regulated too. This down-regulation included genes encoding proteins for basic metabolic pathways such as amino acid biosynthesis, nucleotide or coenzyme metabolism and ribosomal proteins (Tab. 2, Fig. 2). An exception of the repression of such vegetative genes was the genes involved in tryptophan biosynthesis (*trpB-F*). These genes were induced after the onset of glucose starvation (Tab. 3, Fig. 2). In addition, there was a significant repression of most glycolysis enzymes (Eno, FbaA, GapA, PdhB-PdhD, Pgk, Pgm, TpiA) at the transcriptional as well as on the translational level. The glycolysis protein gene with the strongest repression at the transcriptional level was *gapA*

(9-18fold repressed). The *ackA* gene, encoding for the acetate kinase involved in overflow metabolism, was significantly repressed 10 min after onset of the stationary phase. Further down-regulated genes were those encoding for flagellum and chemotaxis proteins. Synthesis of the flagellum protein Hag was down-regulated during transition phase. One hour after entry into stationary phase synthesis of Hag was reinduced. Although a large number of vegetative genes were no longer transcribed and the corresponding proteins no longer synthesised after limitation of glucose, many of the proteins were still present in the cell and presumably also active. This was indicated by the fact that the proteins are visible on stained protein 2D gels even at late during stationary phase (data not shown).

A strong repression of almost all TCA cycle genes of *B. licheniformis* by glucose has been reported recently (Voigt et al. 2004). It is therefore not surprising that a significant induction of *citA*, *citB* and c*itG* was found under glucose starvation conditions (Tab. 3). On the 2D gels additionally a strong increase of the CitZ and SdhA protein level could be observed (Fig. 2).

As expected during glucose starvation there was a strong induction of three genes involved in gluconeogenesis (*gapB, pckA* and *fbaB*) (Tab. 3). Transcription of the *gapB* and *pckA* genes was induced at a high level from transition phase to stationary phase. Expression of the gluconeogenic gene *fbp*, encoding for a fructose-1,6-bisphosphatase, remained however at a low level at all time points.

When *B. licheniformis* cells were subjected to glucose starvation they induced a high number of genes involved in metabolism of alternative carbon sources. Genes from many different metabolic pathways were found to be induced, for example the *aco* and *acu* operons involved in acetoin usage, *glpDFT* involved in glycerol metabolism and 10 genes involved in myo-inositol catabolism (Tab. 3, Fig. 2). Some of the corresponding proteins showed the highest synthesis rate during glucose starvation conditions. Furthermore, there was a high induction of the isocitrate lyase (BLi04207) and the malate synthase (BLi04208), proteins of the glyoxylate bypass, a metabolic pathway that is not encoded in the genome of *B. subtilis*. This pathway allows *B. licheniformis* the usage of C-2 substrates (Gottschalk 1996, Veith et al. 2004). Yet, *B. licheniformis* cells seem to be able to use other organic substances than carbohydrates as carbon sources. Response to glucose starvation includes up-regulation of a number of genes necessary for catabolism of amino acids (like *rocD-F*, *rocG*, *tdh*, *ycgM*, *ycgN*) and also some proteases (e.g. *bpr*, *yjbG*). A second functional group of induced genes that are possibly responsible for carbon recruitment from non-carbohydrates was those involved in lipid metabolism (e.g. *acdA*, *mmgABCDE*). Synthesis of the corresponding proteins was also highly induced.

Furthermore, there was also a strong induction of many transport related proteins, which are very likely used to take up substances used as alternative carbon sources into the cells. Among the strongest induced genes of this functional group was the *yvd* operon (Tab. 3). The *yvdG* and *yvdH* genes, coding for potential maltose/maltodextrin transporter proteins were more than 40fold induced 30 min after onset of the stationary phase. Furthermore, the mRNA-level of genes encoding components of the electron transport chain (like *etfAB*) was strongly up-regulated under glucose starvation conditions. The corresponding proteins belong to the strongest synthesised proteins at the beginning of the stationary phase. The gene *yvyD*, encoding a protein with similarity to σ^{54} modulation factors, was induced at the transcriptional as well as at the translational level during glucose starvation.

There was only a slight induction of a few genes related to sporulation after two hours of glucose starvation. In contrast to *B. subtilis*, no induction of the σ^{B} regulon could be detected in glucose starving *B. licheniformis* cells.

Although the *B. Iicheniformis* genome encodes numerous extracellular enzymes that hydrolyse carbohydrates, proteins or lipids (Rey et al. 2004; Veith et al. 2004), which could serve as alternative carbon sources, only a few of these genes were specifically glucose starvation induced (Tab. 3, Fig. 3). Most of these potential extracellular enzymes probably need the presence of an inducer compound to be expressed.

In order to verify the transcriptome data, levels of selected mRNAs were determined by real-time-RT-PCR (Fig. 4). Although the overall induction ratios of all tested genes were higher in the microarray analysis, the real-time–RT-PCR analyses showed similar trends in the induction patterns of the selected genes. For example, *mmgA* revealed an about 4fold induction and the *acoA* gene showed an over 12fold induction during glucose starvation conditions by this technique. The *etfA* mRNA was found to be 9fold increased 60 min after the onset of the stationary phase. One hour later a decrease of the mRNA level could be detected. A similar expression kinetic of *etfA* was observed by the transcriptome analysis.

Table 2: Kinetic of the glucose starvation repressed genes of *B. licheniformis*. Listed are such genes, whose mRNA levels showed a more than fivefold repression during at least one sampling point throughout the growth curve. Genes the expresion of which is also repressed under nitrogen starvation conditions are labelled with "*". Tr.ph = transient phase, Transcript. = Transcriptional , SD = standard deviation

Gene Name	Tr.ph.	SD	10 min	SD	20 min	SD	30 min	SD	60 min	SD	120 min	SD	Transcript. organisation	Function
Cell wall														
tuaB	1.2	0.1	3.9	0.1	3.1	0.2	3.1	0.5	5.0	0.1	4.2	0.8	*tuaABCDEFGH*	biosynthesis of teichuronic acid
tuaC	1.2	0.1	4.4	0.2	3.8	0.1	4.0	0.2	4.4	0.1	3.5	0.8	*tuaABCDEFGH*	biosynthesis of teichuronic acid
tuaD	1.2	0.3	4.9	0.8	3.6	0.4	3.4	0.7	5.9	0.1	6.3	2.0	*tuaABCDEFGH*	biosynthesis of teichuronic acid
tuaG	1.2	0.1	4.3	0.1	4.0	0.1	3.6	0.1	7.3	1.3	7.1	3.5	*tuaABCDEFGH*	biosynthesis of teichuronic acid
BLi03767	1.2	0.1	5.7	1.1	3.9	0.1	4.1	0.1	10.8	2.4	9.1	1.4		putative cell wall-binding protein
Transport/binding proteins and lipoproteins														
yclN	1.0	0.1	12.9	0.6	7.7	2.9	10.8	1.0	11.3	3.7	9.3	1.7	*yclNOPQ*	similar to ferrichrome ABC transporter
yclO	1.0	0.2	6.0	1.2	6.9	2.2	11.8	3.9	4.5	0.4	3.1	1.2	*yclNOPQ*	similar to ferrichrome ABC transporter
yclP	1.2	0.2	9.1	3.2	7.5	1.7	5.9	0.1	7.3	3.8	7.2	1.3	*yclNOPQ*	similar to ferrichrome ABC transporter
yhcJ	2.4	0.3	6.5	1.8	5.9	1.2	6.7	0.1	5.9	3.3	5.5	0.1	*yhcJ-N*	similar to ABC transporter
yhcL	1.9	0.1	8.2	1.0	4.7	0.6	7.3	1.5	5.4	0.5	4.9	0.2	*yhc operon*	similar to sodium-glutamate symporter
yxdL	3.3	0.2	9.3	1.2	9.9	1.4	12.5	0.1	12.5	5.6	7.7	0.1	*yxdK-L-M*	similar to ABC transporter
yxdM	4.8	0.5	5.3	1.2	4.5	1.9	10.6	0.8	5.6	0.1	7.9	2.6	*yxdK-L-M*	similar to ABC transporter
cysP	1.7	0.3	4.1	1.0	5.0	1.2	7.3	2.2	4.7	0.8	5.8	0.6	*cysP*	sulfate permease
pbuX	1.8	0.1	5.4	0.4	4.2	0.2	5.1	0.3	4.2	1.7	3.0	0.5	*pbuX*	xanthine permease
yqiX	1.1	0.2	8.8	1.0	7.5	1.5	12.6	1.6	4.8	1.0	2.8	0.8	*yqi operon*	probable amino-acid ABC transporter
yqiY	1.1	0.0	6.1	1.8	6.5	1.0	8.5	3.9	5.3	1.3	3.1	1.6	*yqi operon*	probable amino-acid ABC transporter
yvrG	1.2	0.3	2.9	0.7	1.7	0.4	1.9	0.3	8.1	2.2	8.2	1.8	*yvrH-G*	similar to two-component sensor histidine kinase
yvrH	1.2	0.1	2.9	0.9	2.1	0.3	2.3	0.5	5.2	1.3	5.3	2.4	*yvrH-G*	similar to two-component sensor histidine kinase
BLi00972	3.3	0.7	9.5	1.7	12.9	2.8	8.3	0.1	9.5	2.3	7.3	1.0		putative ABC transporter
BLi00973	2.6	0.4	6.9	0.5	6.3	1.8	7.7	0.1	5.5	1.8	4.6	0.9		putative ABC transporter permease
BLi03767	1.2	0.1	5.7	1.1	3.3	0.8	4.1	0.5	10.8	3.9	8.5	1.4		putative cell wall-binding protein
Motility and chemotaxis														
ylxH	1.4	0.1	5.6	0.1	6.5	2.3	3.7	0.1	6.1	0.1	7.3	0.5	*..flg..fli*	similar to flagellar biosynthesis switch protein
flgE	1.0	0.1	3.3	1.3	2.8	0.5	3.0	0.2	8.2	2.3	8.0	2.1	*..flg..fli*	flagellar hook protein
flhB	1.1	0.1	4.9	0.1	3.6	0.7	4.7	0.5	9.1	2.1	6.7	2.2	*..flg..fli*	flagellar biosynthetic protein
fliD	1.0	0.1	1.3	0.1	1.7	0.1	1.9	0.2	5.3	0.7	5.8	2.0	*fliD-S-T*	flagellar hook-associated protein 2
fliE	1.3	0.2	1.8	0.3	1.5	0.3	1.9	0.6	5.7	2.3	4.4	1.2	*..flg..fli*	flagellar hook-basal body complex protein

Table 2: continued

Gene Name	Tr.ph.	SD	10 min	SD	20 min	SD	30 min	SD	60 min	SD	120 min	SD	Transcript. organisation	Function
fliG	1.3	0.1	2.3	0.8	2.8	0.4	2.3	0.7	6.2	0.9	5.7	2.2	*..flg..fli*	flagellar motor switch protein
fliH	0.9	0.3	2.6	0.6	2.3	0.6	2.7	0.7	1.1	1.7	7.3	2.4	*..flg..fli*	frobable flagellar assembly protein
fliI	1.0	0.1	3.4	1.4	2.1	0.4	2.3	1.4	4.9	1.0	6.2	0.8	*..flg..fli*	flagellum-specific ATP synthase
fliJ	0.8	0.2	3.7	0.6	1.7	0.6	3.1	0.5	11.1	2.8	6.7	0.6	*..flg..fli*	flagellar protein
fliK	1.1	0.6	3.7	1.2	2.4	0.1	3.0	0.9	5.3	1.9	4.5	0.8	*..flg..fli*	flagellar hook-length control protein
fliM	1.0	0.2	3.9	0.8	2.0	0.2	3.1	0.5	6.4	2.5	5.3	0.6	*..flg..fli*	flagellar motor switch protein
fliP	1.0	0.1	3.4	0.8	3.3	0.9	3.1	0.7	5.0	1.2	5.3	1.5	*..flg..fli*	flagellar biosynthetic protein
fliR	1.0	0.1	5.3	1.5	4.3	1.7	4.8	1.3	5.3	0.2	6.3	2.9	*..flg..fli*	flagellar biosynthetic protein
fliZ	1.0	0.1	4.2	0.8	4.1	1.3	3.7	1.4	7.0	1.0	10.3	4.6	*..flg..fli*	flagellar biosynthetic protein
Protein secretion														
*secY**	1.1	0.1	8.4	2.1	9.9	2.7	5.4	1.7	10.6	2.7	17.7	3.2	*rplR-secY-adk-map*	preprotein translocase subunit
Specific pathways														
*ackA**	1.0	0.1	5.9	1.7	6.5	1.0	6.7	1.3	4.8	0.7	4.2	1.4	*ackA*	acetate kinase
Main glycolytic pathways														
*eno**	1.1	0.2	3.7	0.7	3.9	1.0	3.4	0.3	5.5	0.3	6.1	0.5	*gapA-pgk-tpiA-pgm-eno*	enolase
*gapA**	1.4	0.1	8.9	0.3	16.2	2.7	18.3	2.3	11.4	1.2	9.2	2.5	*gapA-pgk-tpiA-pgm-eno*	glyceraldehyde-3-phosphat
*pdhC**	0.4	0.1	3.8	0.8	3.8	0.1	4.1	0.8	4.3	0.3	4.6	0.9	*pdhABCD*	pyruvate dehydrogenase
*pdhD**	0.5	0.1	4.1	0.1	2.6	0.1	2.7	0.1	12.6	3.7	7.1	0.1	*pdhABCD*	pyruvate dehydrogenase/2-oxoglutarate dehydrogenase
*pgk**	1.1	0.1	5.6	0.6	4.6	1.1	4.2	1.1	11.0	3.2	11.0	2.3	*gapA-pgk-tpiA-pgm-eno*	phosphoglycerate kinase
*pgm**	1.1	0.1	5.4	0.8	4.6	0.1	3.8	0.5	7.3	0.1	9.5	3.2	*gapA-pgk-tpiA-pgm-eno*	phosphoglycerate mutase
ptsG	0.7	0.2	5.3	1.6	10.0	0.1	8.7	0.1	8.3	2.0	9.5	2.1	*ptsG-I*	PTS glucose-specific enzyme IICBA component
Metabolism of amino acids and related molecules														
argB	1.2	0.1	7.8	0.3	5.4	0.1	3.0	0.1	0.9	0.1	0.8	0.1	*..arg..*	N-acetylglutamate 5-phosphotransferase
argC	0.8	0.1	6.2	1.0	5.8	0.1	5.6	0.1	3.6	0.7	2.6	0.2	*..arg..*	N-acetylglutamate gamma-semialdehyde dehydrogenase
argD	0.9	0.1	5.8	1.2	4.5	0.8	9.8	2.5	3.3	0.1	1.9	0.1	*..arg..*	N-acetylornithine aminotransferase
argF	1.1	0.1	7.9	0.2	11.1	3.7	7.9	0.7	5.9	0.0	7.1	0.1	*..arg..*	ornithine carbamoyltransferase
argG	1.2	0.1	12.5	4.1	7.6	2.6	16.3	8.7	5.8	0.9	3.5	0.6	*..arg..*	argininosuccinate synthase
argH	1.4	0.4	14.2	7.9	13.3	0.1	14.3	0.2	5.1	0.6	3.4	0.7	*..arg..*	argininosuccinate lyase

Table 2: continued

Gene Name	Tr.ph.	SD	10 min	SD	20 min	SD	30 min	SD	60 min	SD	120 min	SD	Transcript. organisation	Function
argJ	0.8	0.1	7.6	1.0	8.2	1.4	12.5	0.2	3.7	0.1	4.4	0.1	*..arg..*	ornithine acetyltransferase
carA	1.0	0.1	15.0	2.3	9.5	3.0	6.1	0.1	4.3	2.0	2.9	0.1	*..arg..*	carbamoyl-phosphate transferase-arginine
carB	1.0	0.1	7.5	1.2	6.9	0.1	13.4	2.8	3.7	0.7	4.8	0.1	*..arg..*	carbamoyl-phosphate transferase-arginine
leuA	1.1	0.1	4.5	1.2	3.6	0.1	4.0	0.1	9.6	1.8	9.9	0.5	*ilvBHC-leuABCD*	2-isopropylmalate synthase
leuB	1.1	0.1	4.2	0.3	3.4	0.8	3.2	0.1	7.9	4.3	10.2	2.2	*ilvBHC-leuABCD*	3-isopropylmalate dehydrogenase
leuD	1.2	0.1	4.9	0.1	4.0	0.1	3.5	0.5	4.9	0.4	4.8	0.7	*ilvBHC-leuABCD*	3-isopropylmalate dehydrase
yjcI	2.3	0.1	6.6	1.6	9.0	2.2	12.5	1.1	13.8	2.3	9.8	1.5	*yjcI-J*	similar to cystathionine gamma-synthase
yjcJ	2.2	0.2	5.5	0.5	6.9	0.1	10.8	2.4	5.8	1.3	5.4	1.1	*yjcI-J*	similar to cystathionine beta-lyase
hisB	1.2	0.2	1.8	0.2	5.2	2.0	4.7	0.3	4.1	1.7	5.0	1.0	*his..*	imidazoleglycerol-phospha
hisD	1.0	0.1	2.0	0.1	4.0	0.4	5.1	0.9	6.6	2.5	5.2	1.8	*his..*	histidinol dehydrogenase
ykrV	1.1	0.4	3.8	0.9	5.5	2.3	5.3	0.4	5.1	1.1	5.5	0.9	*ykrV*	similar to aspartate aminotransferase
yrhB	1.2	0.1	4.1	0.5	5.0	0.7	6.1	1.1	4.5	1.0	3.3	0.1	*yrhB*	similar to cystathionine beta-lyase or gamma-lyase
Metabolism of nucleotides and nucleic acids														
adk	1.3	0.3	11.1	0.9	6.6	1.3	7.5	2.3	10.0	0.7	9.6	3.8	*rplR-secY-adk-map*	adenylate kinase
guaA	1.3	0.2	7.7	2.0	4.7	1.5	5.1	0.3	11.8	0.1	11.1	2.3	*guaA*	GMP synthetase
nrdE	1.5	0.3	4.7	0.8	3.1	0.1	2.9	0.1	9.0	2.3	6.0	1.0	*nrdE-nrdF*	ribonucleoside-diphosphate reductase
nrdF	1.5	0.3	5.0	0.4	3.9	1.2	3.2	0.1	4.8	0.6	4.3	1.1	*nrdE-nrdF*	ribonucleoside-diphosphate reductase
purA	0.6	0.1	1.8	0.1	4.2	1.3	3.1	0.1	6.1	1.4	5.3	0.6	*purA*	adenylosuccinate synthetase
purB	0.8	0.1	6.7	2.7	8.3	1.2	6.4	0.1	8.0	0.8	7.5	0.2	*pur* operon	adenylosuccinate lyase
purC	0.8	0.1	9.5	3.4	10.4	4.2	15.6	6.3	12.9	0.6	10.0	0.2	*pur* operon	phosphoribosylaminoimidazole succinocarboxamide synthetase
purD	0.8	0.1	6.8	1.7	5.1	0.6	19.4	3.9	5.4	1.3	5.3	0.1	*pur* operon	phosphoribosylglycinamide synthetase
purE	0.8	0.1	4.3	0.5	3.9	0.0	9.3	2.5	2.9	0.1	3.8	0.1	*pur* operon	phosphoribosylaminoimidazole carboxylase I
purF	0.9	0.2	7.7	1.7	6.6	0.2	8.3	0.1	9.6	1.3	7.9	4.5	*pur* operon	glutamine phosphoribosylpyrophosphate amidotransferase

Table 2: continued

Gene Name	Tr.ph.	SD	10 min	SD	20 min	SD	30 min	SD	60 min	SD	120 min	SD	Transcript. organisation	Function
purH	1.0	± 0.2	11.8	± 5.3	8.0	± 1.9	15.5	± 1.7	10.3	± 1.1	7.9	± 1.5	*pur* operon	phosphoribosyl aminoimidazole carboxy formyl formyltransferase
purK	0.9	± 0.1	7.1	± 1.5	7.0	± 3.5	14.3	± 0.1	8.8	± 2.4	7.6	± 1.3	*pur* operon	phosphoribosyl aminoimidazole carboxylase II
purL	0.8	± 0.2	8.0	± 1.8	5.6	± 1.5	16.0	± 0.9	7.8	± 1.3	6.3	± 1.1	*pur* operon	phosphoribosyl formylglycinamidine synthetase II
purM	0.8	± 0.2	5.6	± 1.0	4.0	± 0.5	12.1	± 3.0	4.7	± 0.1	3.3	± 0.4	*pur* operon	phosphoribosyl aminoimidazole synthetase
purN	1.1	± 0.2	10.5	± 2.4	10.6	± 0.8	16.0	± 0.9	10.6	± 5.2	9.2	± 0.2	*pur* operon	phosphoribosyl glycinamide formyltransferase
purQ	0.9	± 0.1	10.2	± 4.3	8.7	± 3.4	12.1	± 1.5	10.5	± 0.1	8.4	± 2.8	*pur* operon	phosphoribosylfo rmylglycinamidine synthetase I
pyrAA	0.5	± 0.1	6.3	± 3.0	5.7	± 0.5	6.3	± 0.1	5.1	± 0.5	4.3	± 0.1	*pyr* operon	carbamoyl-phosphate synthetase
pyrAB	0.6	± 0.2	4.5	± 0.7	4.3	± 1.0	5.8	± 0.7	3.5	± 0.4	3.2	± 0.2	*pyr* operon	carbamoyl-phosphate synthetase
pyrC	0.5	± 0.1	4.1	± 0.6	3.4	± 0.7	5.4	± 0.9	3.0	± 0.1	3.1	± 0.1	*pyr* operon	dihydroorotase
pyrD	0.8	± 0.2	5.7	± 0.7	3.8	± 0.1	5.6	± 0.2	3.1	± 0.1	3.4	± 0.1	*pyr* operon	dihydroorotate dehydrogenase
pyrE	0.7	± 0.2	4.2	± 0.8	6.1	± 0.1	8.4	± 1.0	4.1	± 0.3	3.0	± 0.3	*pyr* operon	orotate phosphoribosyltransferase
pyrF	0.8	± 0.1	5.7	± 1.9	7.7	± 0.1	7.0	± 1.0	5.2	± 1.1	3.9	± 0.1	*pyr* operon	orotidine 5'-phosphate decarboxylase
pyrK	0.6	± 0.1	7.5	± 2.9	6.1	± 1.2	8.4	± 3.8	4.5	± 0.7	3.3	± 0.1	*pyr* operon	dihydroorotate dehydrogenase
pyrP	0.6	± 0.2	4.4	± 1.6	3.5	± 0.2	5.8	± 0.7	1.8	± 0.1	2.2	± 0.1	*pyr* operon	uracil permease
Metabolism of lipids														
cdsA	1.0	± 0.1	5.6	± 1.2	3.1	± 0.2	3.6	± 1.2	7.8	± 1.9	7.4	± 0.3	*uppS-cdsA-dxr*	phosphatidate cytidylyltransferase
fabD	1.1	± 0.2	6.2	± 1.0	5.1	± 1.3	4.3	± 0.9	7.4	± 0.9	5.9	± 0.8	*plsX-fabD-fabG*	strong similarity to malonyl CoA-acyl carrier protein transacylase
Metabolism of coenzymes and prosthetic groups														
bioA	1.1	± 0.4	3.5	± 0.8	4.4	± 0.5	3.6	± 0.1	4.1	± 0.6	4.5	± 2.0	*bioAFDBI*	adenosylmethionine-8-amino-7-oxononanoate aminotransferase
bioB	1.1	± 0.2	4.6	± 0.1	3.4	± 0.6	5.6	± 0.1	7.4	± 1.3	6.6	± 3.3	*bioAFDBI*	biotin synthetase
bioD	1.1	± 0.2	5.6	± 0.4	5.1	± 2.1	6.0	± 0.4	18.2	± 2.9	33.3	± 16.0	*bioAFDBI*	dethiobiotin synthetase
bioF	1.2	± 0.3	5.7	± 0.1	5.3	± 0.1	7.8	± 4.5	11.1	± 2.9	29.8	± 18.3	*bioAFDBI*	8-amino-7-oxononanoate sythase
bioI	1.1	± 0.1	4.1	± 1.1	4.6	± 0.1	3.9	± 0.2	8.7	± 3.5	8.3	± 0.9	*bioAFDBI*	cytochrome P450 enzyme
nadA	1.2	± 0.3	8.4	± 0.4	10.5	± 2.6	10.8	± 3.5	4.8	± 2.2	7.4	± 1.3	*nadBCA*	quinolinate synthetase
nadB	1.2	± 0.1	6.7	± 0.4	7.0	± 1.8	9.4	± 2.4	4.7	± 1.0	4.2	± 0.3	*nadBCA*	L-aspartate oxidase
nadC	1.2	± 0.3	7.7	± 0.5	11.5	± 1.4	9.8	± 1.8	6.0	± 2.8	8.1	± 0.1	*nadBCA*	nicotinate-nucleotide pyrophosphorylase
nifS	1.0	± 0.2	7.1	± 1.7	6.5	± 0.9	6.2	± 2.4	5.5	± 1.4	5.1	± 1.2	*nifS*	required for NAD biosynthesis

Table 2: continued

Gene Name	Tr.ph.	SD	10 min	SD	20 min	SD	30 min	SD	60 min	SD	120 min	SD	Transcript. organisation	Function
DNA replication														
ssb	1.1	0.1	9.3	0.9	5.3	0.1	6.0	1.2	15.1	6.1	11.4	0.1	*ssb*	single-strand DNA-binding protein
Protein modification														
map	1.2	0.1	9.3	2.6	10.3	5.0	6.3	1.6	12.9	1.8	13.3	2.6	*rplR-secY-adk-map*	methionine aminopeptidase
Translation initiation														
ykrS	1.1	0.1	6.3	1.1	6.3	2.0	6.0	1.7	5.8	0.5	5.0	0.1	*ykr* operon	similar to initiation factor eIF-2B
Translation elongation														
tsf	1.0	0.1	8.2	2.7	4.7	1.5	3.8	0.1	8.2	1.8	7.7	0.1	*tsf*	elongation factor Ts
Ribosomal proteins														
rplA	1.2	0.1	8.2	0.2	4.9	0.3	4.2	0.1	10.0	2.4	10.6	0.8	*rpl* operon	ribosomal protein L1 (BL1)
rplB	1.1	0.2	12.1	3.0	7.3	2.0	6.8	1.3	12.3	0.8	12.9	0.1	*rpl* operon	ribosomal protein L2 (BL2)
rplC	1.1	0.1	8.9	0.3	5.5	0.7	4.7	0.8	6.8	1.5	6.7	0.1	*rpl* operon	ribosomal protein L3 (BL3)
rplD	1.2	0.3	13.4	1.3	8.9	0.3	6.1	1.4	10.9	4.1	13.7	1.6	*rpl* operon	ribosomal protein L4
rplE	1.3	0.2	16.0	0.9	13.2	3.0	7.5	1.7	13.9	3.0	20.0	0.1	*rpl* operon	ribosomal protein L5 (BL6)
rplF	1.0	0.1	9.3	0.3	7.7	0.1	6.2	1.3	7.0	4.6	10.9	2.3	*rpl* operon	ribosomal protein L6 (BL8)
rplJ	1.3	0.1	25.0	12	21.4	10	10.4	2.9	31.8	12	54.1	31.0	*rpl* operon	ribosomal protein L10 (BL5)
rplK	1.3	0.1	5.6	1.0	3.9	0.2	5.7	0.1	6.3	1.5	2.4	1.2	*rpl* operon	ribosomal protein L11 (BL11)
rplL	1.3	0.2	17.7	3.3	8.9	0.8	8.9	0.3	15.4	0.1	12.6	2.9	*rpl* operon	ribosomal protein L12 (BL9)
rplM	1.2	0.0	4.2	0.7	4.4	0.3	3.8	0.4	4.7	0.8	6.3	0.2	*rpl* operon	ribosomal protein L13
rplN	0.9	0.3	16.7	0.1	11.8	0.1	7.7	0.1	14.2	1.2	1.9	0.1	*rpl* operon	ribosomal protein L14
rplO	1.1	0.1	8.0	4.4	7.1	0.0	8.0	5.3	6.5	1.3	12.7	7.7	*rpl* operon	ribosomal protein L15
rplP	1.0	0.9	13.3	0.0	10.0	2.4	6.3	1.9	12.5	1.1	13.5	0.2	*rpl* operon	ribosomal protein L16
rplQ	1.1	0.2	4.6	1.2	6.7	0.3	4.2	0.8	6.0	1.6	7.3	0.9	*rpl* operon	ribosomal protein L17 (BL15)
rplR	1.3	0.2	14.4	5.4	6.9	0.3	5.7	0.5	8.8	3.7	11.8	0.1	*rpl* operon	ribosomal protein L18
rplT	1.2	0.1	6.2	0.4	5.1	0.2	5.0	0.9	8.7	0.5	12.1	0.1	*rpl* operon	ribosomal protein L20
rplV	1.0	0.2	7.9	1.1	5.8	2.6	5.5	1.3	6.8	0.7	6.0	2.4	*rpl* operon	ribosomal protein L22 (BL17)
rpoA	1.3	0.1	1.3	0.1	6.3	0.3	5.0	0.4	8.1	1.3	13.8	0.9	*rpoA*	RNA polymerase (alpha subunit)
rpoB	1.3	0.2	4.1	0.4	3.0	0.1	3.2	0.1	4.7	0.1	5.1	1.4	*rpoB-C*	RNA polymerase (beta subunit)
rpoC	1.3	0.2	4.8	0.3	3.0	0.1	3.4	0.1	6.5	0.3	5.3	0.1	*rpoB-C*	RNA polymerase (beta' subunit)
rpsB	1.1	0.2	10.6	0.8	5.3	0.1	5.1	1.6	8.7	0.1	6.3	0.1	*rps* operon	ribosomal protein S2
rpsC	1.3	0.1	5.4	0.1	3.6	0.1	5.1	1.6	5.7	0.2	3.6	1.4	*rps* operon	ribosomal protein S3 (BS3)
rpsE	1.2	0.3	10.6	0.8	8.5	2.2	7.0	1.9	8.2	2.5	11.7	2.1	*rps* operon	ribosomal protein S5

Table 2: continued

Gene Name	Tr.ph.	SD	10 min	SD	20 min	SD	30 min	SD	60 min	SD	120 min	SD	Transcript. organisation	Function
rpsG	1.1	± 0.1	5.6	± 1.3	4.1	± 0.1	3.3	± 0.1	5.4	± 1.0	5.8	± 2.2	*rps* operon	ribosomal protein S7 (BS7)
rpsI	1.1	± 0.1	4.1	± 0.4	4.4	± 1.3	3.8	± 0.4	4.9	± 0.3	5.1	± 0.6	*rps* operon	ribosomal protein S9
rpsK	1.0	± 0.2	9.3	± 2.6	8.7	± 0.1	5.8	± 0.9	10.4	± 4.1	12.1	± 3.0	*rps* operon	ribosomal protein S11 (BS11)
rpsL	1.2	± 0.2	6.3	± 2.8	3.8	± 0.1	3.5	± 0.7	5.0	± 0.4	4.1	± 1.5	*rps* operon	ribosomal protein S12 (BS12)
rpsM	1.2	± 0.2	8.7	± 0.1	8.6	± 2.0	5.8	± 0.1	13.4	± 1.3	13.3	± 2.7	*rps* operon	ribosomal protein S13
Aminoacyl-tRNA synthetases														
asnS	1.0	± 0.1	3.3	± 0.1	2.1	± 0.1	1.9	± 0.1	9.5	± 0.5	7.8	± 0.1	*asnS*	asparaginyl-tRNA synthetase
Unknown function														
BLi00156	1.0	± 0.2	8.9	± 0.8	7.9	± 1.4	6.2	± 0.3	12.4	± 3.9	9.1	± 2.2		conserved hypothetical
ybxB	0.9	± 0.2	5.8	± 0.6	3.6	± 0.6	4.9	± 1.2	5.2	± 0.8	4.6	± 0.7	*ybxB*	unknown
yjlC	1.1	± 0.2	5.3	± 1.4	3.1	± 1.0	3.4	± 0.5	7.2	± 2.4	3.1	± 1.2	*yjl* operon	unknown
ykrT	0.9	± 0.3	5.6	± 0.6	5.8	± 1.2	6.9	± 1.9	4.2	± 0.8	2.5	± 1.1	*ykr* operon	unknown
ykrY	0.9	± 0.6	4.2	± 1.5	5.3	± 2.2	8.3	± 1.6	6.1	± 2.6	6.0	± 1.3	*ykr* operon	unknown
ykrZ	0.9	± 0.2	4.7	± 0.7	6.3	± 1.0	6.6	± 1.9	6.1	± 2.3	6.0	± 0.9	*ykr* operon	unknown
ylxF	1.1	± 0.1	3.6	± 0.6	3.1	± 0.8	4.1	± 0.1	10.1	± 3.6	7.1	± 4.7	*..ylxF..*	unknown
yuiI	0.9	± 0.2	7.5	± 2.4	6.3	± 0.2	8.3	± 2.7	9.3	± 3.0	5.4	± 0.8	*yui* operon	unknown
yusA	1.1	± 0.1	5.5	± 0.9	3.8	± 0.1	5.3	± 0.1	5.0	± 1.5	3.7	± 0.5	*yus* operon	unknown
yusB	2.9	± 0.1	4.8	± 1.5	5.6	± 0.1	11.1	± 2.6	5.5	± 1.0	6.7	± 1.9	*yus* operon	unknown
yxjG	1.1	± 0.1	5.5	± 0.4	5.6	± 1.2	7.0	± 0.9	3.6	± 0.7	4.2	± 1.2	*yxj* operon	unknown

Table 3: Kinetic of the glucose starvation inducible genes of *B. licheniformis*. Listed are such genes, the mRNA level of which showed a more than fivefold induction during at least one sampling point throughout the growth curve. Genes the expresion of which is also induced under nitrogen starvation conditions are labelled with "*".Trans. ph. = transient phase, SD = standard deviation

Gene name	Tr.ph	SD	10 min	SD	20 min	SD	30 min	SD	60 min	SD	120min	SD	Transcript. organisation	Function
Cell wall														
dltE	0.8	± 0.1	1.5	± 0.1	1.2	± 0.1	5.3	± 0.3	5.2	± 1.1	5.2	± 0.8	*dltE*	involved in lipoteichoic acid biosynthesis
BLi00494	1.0	± 0.1	7.8	± 0.1	10.8	± 2.2	36.3	± 8.8	28.5	± 14	21.7	± 4.7		putative membrane protein
Transport/binding proteins and lipoproteins														
bglP	1.5	± 0.1	6.0	± 1.6	2.6	± 0.1	4.5	± 1.5	6.3	± 1.1	6.4	± 0.6	*bglP-H*	PTS beta-glucoside-specific enzyme IIBCA component
fruA	1.1	± 0.2	6.1	± 0.7	2.6	± 0.1	2.8	± 0.1	5.1	± 1.3	6.2	± 0.7	*fruA-K-R*	PTS fructose-specific enzyme IIABC component
licA	1.0	± 0.1	12.1	± 0.3	8.6	± 1.1	6.2	± 0.2	3.3	± 0.1	3.6	± 0.4	*licA*	PTS lichenan-specific enzyme IIA component
licC	1.1	± 0.1	8.1	± 0.4	7.2	± 0.3	6.2	± 1.6	3.3	± 0.2	3.5	± 0.6	*licH-C-R*	PTS lichenan-specific enzyme IIC component
manP	1.1	± 0.1	6.1	± 0.2	4.3	± 0.1	6.0	± 0.3	5.3	± 0.8	4.2	± 0.1	*manA-P-R*	phosphotransferase system (PTS) mannose-specific enzyme IIBCA component
yvdG	1.2	± 0.1	6.1	± 0.1	16.1	± 0.2	41.2	± 12	41.5	± 16	28.2	± 6.3	*yvd operon*	maltose/maltodextrin-binding protein
yvdH	1.1	± 0.1	3.4	± 0.2	4.1	± 1.0	6.8	± 0.9	11.1	± 1.1	10.4	± 0.5	*yvd operon*	similar to maltodextrin transport system permease
yvdI	1.4	± 0.3	5.9	± 1.2	12.1	± 2.6	27.7	± 5.2	50.6	± 4.9	27.1	± 4.3	*yvd operon*	similar to maltodextrin transport system permease
yvfK	1.2	± 0.1	9.6	± 0.1	6.3	± 1.2	4.7	± 0.1	6.6	± 1.7	7.3	± 0.2	*yvf operon*	similar to maltose/maltodextrin-binding protein
*BLi03990**	1.3	± 0.1	8.3	± 1.4	18.2	± 3.5	35.3	± 1.2	13.8	± 2.2	12.5	± 1.4		putative benzoate transport protein
BLi02118	1.0	± 0.1	3.5	± 0.1	3.4	± 0.2	4.6	± 0.1	6.3	± 1.4	5.3	± 0.4		putative C4-dicarboxylate transport protein
*BLi04117**	1.2	± 0.2	4.3	± 0.2	4.7	± 1.6	4.2	± 0.1	6.2	± 0.7	13.8	± 2.7		putative ABC transporter ATP-binding protein
BLi04125	0.9	± 0.2	3.2	± 0.1	3.5	± 0.1	4.2	± 0.8	7.8	± 1.3	6.7	± 0.7		putative ABC-transporter
BLi03551	1.6	± 0.1	2.4	± 0.2	2.1	± 0.1	2.5	± 0.1	8.5	± 3.0	9.7	± 0.7		putative phosphotransferase system enzyme II
BLi03917	1.0	± 0.1	4.2	± 1.6	3.2	± 0.1	4.4	± 0.2	5.2	± 0.6	5.2	± 2.1		putative oxalateformate antiporter
Electron transport chain and ATP synthase														
ctaB	0.9	± 0.1	3.2	± 0.1	3.7	± 0.3	3.2	± 0.1	2.2	± 0.1	1.9	± 0.1	*ctaABCDEFG*	cytochrome caa3 oxidase(assembly factor)
ctaC	1.1	± 0.2	5.8	± 1.0	7.9	± 0.3	7.6	± 1.0	4.0	± 0.3	3.4	± 0.1	*ctaABCDEFG*	cytochrome caa3 oxidase(subunit II)
ctaD	1.0	± 0.2	3.4	± 0.1	4.9	± 1.2	4.0	± 0.1	3.2	± 0.1	2.5	± 0.1	*ctaABCDEFG*	cytochrome caa3 oxidase(subunit I)
ctaE	1.0	± 0.1	3.6	± 0.3	5.4	± 0.6	6.2	± 0.9	3.2	± 0.1	2.6	± 0.1	*ctaABCDEFG*	cytochrome caa3 oxidase(subunit III)
ctaF	1.0	± 0.1	4.0	± 0.1	2.9	± 0.1	4.7	± 0.8	1.9	± 0.1	1.7	± 0.1	*ctaABCDEFG*	cytochrome caa3 oxidase(subunit IV)
ctaG	1.0	± 0.1	3.7	± 0.1	5.6	± 0.9	6.1	± 2.2	3.3	± 0.1	2.4	± 0.1	*ctaABCDEFG*	function unknown

Table 3: continued

Gene name	Tr.Ph	SD	10 min	SD	20 min	SD	30 min	SD	60 min	SD	120min	SD	Transcript. organisation	Function
etfA	1.0	± 0.1	10.7	± 0.9	10.3	± 2.6	10.3	± 1.0	42.5	± 4.6	20.4	± 1.8	*etfA-B*	electron transfer flavoprotein
etfB	0.8	± 0.2	12.3	± 2.7	10.3	± 0.3	13.5	± 1.2	44.2	± 0.5	17.2	± 1.5	*etfA-B*	electron transfer flavoprotein
ndhF	1.4	± 0.2	1.2	± 0.1	1.4	± 0.2	3.8	± 0.3	7.7	± 1.5	6.1	± 0.4	*ndhF*	NADH dehydrogenase
ywjF	1.0	± 0.1	14.6	± 4.1	11.2	± 1.5	9.7	± 3.1	44.3	± 2.2	20.1	± 1.0	*ywjE-F*	similar to iron-sulphur-binding reductase
BLi03918	0.9	± 0.1	11.2	± 0.4	10.6	± 2.2	3.8	± 0.2	15.5	± 2.6	15.7	± 0.7		putative formate dehydrogenase alpha chain
Sporulation														
*spoIIAA**	1.1	± 0.1	3.5	± 0.1	2.4	± 0.1	3.4	± 0.1	3.5	± 0.1	1.7	± 0.1	*dacF-spoIIAA-spoIIAB-sigF*	anti-sigma F factor antagonist
*spoIIAB**	1.2	± 0.1	5.3	± 0.2	4.6	± 1.3	5.1	± 0.8	4.6	± 1.1	4.3	± 0.8	*dacF-spoIIAA-spoIIAB-sigF*	known anti-sigma F factor
Specific pathway														
acoA	1.1	± 0.1	2.0	± 0.2	6.7	± 1.9	37.3	± 6.6	88.8	± 12	84.0	± 15.6	*acoABCLR*	acetoin dehydrogenase E1
acoB	1.2	± 0.1	2.3	± 0.3	6.6	± 0.9	47.1	± 25	104	± 39	88.5	± 16.3	*acoABCLR*	acetoin dehydrogenase E1
acoC	1.0	± 0.2	0.9	± 0.1	3.4	± 0.4	8.4	± 0.2	51.0	± 5.7	41.2	± 8.6	*acoABCLR*	acetoin dehydrogenase E2
acoL	1.0	± 0.1	2.6	± 0.2	3.7	± 0.1	18.4	± 1.0	51.7	± 14	39.7	± 5.7	*acoABCLR*	acetoin dehydrogenase E3
acoR	1.1	± 0.1	3.9	± 0.8	4.1	± 0.2	9.1	± 1.5	22.2	± 14	25.1	± 3.7	*acoABCLR*	transcriptional activator
acsA	1.1	± 0.1	21.7	± 2.3	21.1	± 4.8	19.3	± 0.1	14.6	± 3.8	17.0	± 1.2	*acuC-A-acsA*	acetyl-CoA synthetase
acuA	1.2	± 0.2	21.7	± 0.7	17.9	± 1.0	17.1	± 2.7	15.8	± 2.1	11.6	± 2.7	*acuC-A-acsA*	acetoin dehydrogenase
acuC	0.8	± 0.2	6.1	± 2.0	4.9	± 0.7	5.8	± 0.4	4.6	± 1.1	2.9	± 0.1	*acuC-A-acsA*	acetoin dehydrogenase
alsD	9.2	± 1.6	4.7	± 1.5	2.0	± 0.1	1.6	± 0.1	1.7	± 0.1	1.4	± 0.1	*alsR-S-D*	alpha-acetolactate decarboxylase
alsR	1.6	± 0.1	2.3	± 0.2	1.2	± 0.1	1.1	± 0.1	1.3	± 0.1	1.3	± 0.1	*alsR-S-D*	transcriptional regulator
alsS	13.0	± 1.5	5.9	± 0.5	3.4	± 1.1	2.0	± 0.1	2.3	± 0.1	2.4	± 0.1	*alsR-S-D*	alpha-acetolactate synthase
fruK	1.1	± 0.1	9.7	± 4.6	3.9	± 0.6	4.1	± 0.1	8.3	± 2.6	10.3	± 1.8	*fruA-K-R*	fructose 1-phosphate kinase
glpD	1.1	± 0.1	20.2	± 5.2	18.5	± 3.3	42.4	± 3.0	4.1	± 0.3	3.6	± 0.1	*glpD*	glycerol-3-phosphate dehydrogenase
glpF	1.1	± 0.1	9.2	± 2.5	8.4	± 1.9	16.4	± 3.0	3.5	± 0.1	3.3	± 0.1	*glpF-P*	glycerol uptake facilitator
hxlA	2.6	± 0.4	11.0	± 2.3	8.8	± 0.4	7.5	± 0.9	9.9	± 2.9	6.9	± 0.8	*hxlA-B*	3-hexulose-6-phosphate synthesase
hxlB	1.7	± 0.2	6.4	± 1.4	4.4	± 1.7	5.6	± 0.8	6.0	± 1.5	3.4	± 0.1	*hxlA-B*	6-phospho-3-hexuloisomerase
iolB	1.1	± 0.1	4.4	± 0.9	3.1	± 0.1	2.9	± 0.1	3.3	± 0.1	1.9	± 0.1	*IolBCDEF-idh-iolHI-fbaB*	myo-inositol catabolism
iolC	0.9	± 0.2	7.2	± 0.2	9.0	± 0.8	8.2	± 2.9	4.6	± 0.5	4.5	± 0.1	*IolBCDEF-idh-iolHI-fbaB*	myo-inositol catabolism
iolD	0.9	± 0.1	15.8	± 0.3	8.7	± 0.2	12.8	± 2.6	5.3	± 0.1	5.4	± 0.1	*IolBCDEF-idh-iolHI-fbaB*	myo-inositol catabolism

Table 3: continued

Gene name	Tr.Ph	SD	10 min	SD	20 min	SD	30 min	SD	60 min	SD	120min	SD	Transcript. organisation	Function
iolE	0.9	± 0.1	13.6	± 6.5	8.5	± 0.1	8.0	± 1.0	5.1	± 1.5	4.5	± 1.3	*IolBCDEF-idh-iolHI-fbaB*	myo-inositol catabolism
iolF	1.1	± 0.1	5.3	± 0.2	2.3	± 0.1	2.8	± 0.1	2.3	± 0.1	1.3	± 0.1	*IolBCDEF-idh-iolHI-fbaB*	inositol transport protein
iolH	0.7	± 0.2	5.1	± 0.3	5.5	± 0.2	7.8	± 2.8	3.4	± 0.1	4.0	± 0.1	*IolBCDEF-idh-iolHI-fbaB*	myo-inositol catabolism
iolI	0.8	± 0.1	3.4	± 0.1	2.6	± 0.1	2.3	± 0.1	1.7	± 0.1	1.7	± 0.1	*IolBCDEF-idh-iolHI-fbaB*	myo-inositol catabolism
iolR	1.1	± 0.1	5.2	± 1.2	3.5	± 0.2	4.3	± 0.2	3.3	± 0.1	3.0	± 0.1	*IolBCDEF-idh-iolHI-fbaB*	transcriptional repressor
iolS	1.1	± 0.1	3.5	± 0.1	3.3	± 0.1	3.9	± 0.1	1.8	± 0.1	1.7	± 0.1	*IolBCDEF-idh-iolHI-fbaB*	myo-inositol catabolism
idh	0.8	± 0.1	9.5	± 6.5	3.6	± 0.1	6.9	± 0.2	4.3	± 0.3	3.4	± 0.2	*IolBCDEF-idh-iolHI-fbaB*	myo-inositol 2-dehydrogenase
malA	1.3	± 0.2	1.5	± 0.1	2.0	± 0.1	2.3	± 0.1	39.7	± 20	41.2	± 0.4	*malA-yfiA-malP*	6-phospho-alpha-glucosidase
malL	1.0	± 0.1	7.0	± 0.7	15.7	± 4.6	43.0	± 7.1	65.6	± 7.4	80.8	± 21.6	*malL-pgcM*	maltose-inducible alpha-glucosidase
pgcM	1.0	± 0.1	3.7	± 0.7	5.8	± 1.7	16.0	± 3.3	22.4	± 9.2	18.9	± 0.1	*malL-pgcM*	beta-phosphoglucomutase
malP	1.0	± 0.1	1.9	± 0.1	1.4	± 0.1	4.5	± 1.1	24.2	± 4.9	38.0	± 2.3	*malA-yfiA-malP*	PTS maltose-specific enzyme
yqiQ	1.1	± 0.1	11.5	± 0.1	10.3	± 2.7	12.8	± 1.8	12.4	± 2.9	12.7	± 2.1	*mmgD-E-yqiQ*	similar to phosphoenolpyruvate mutase
sacX	1.2	± 0.1	1.2	± 0.1	1.0	± 0.1	1.8	± 0.1	6.0	± 2.4	7.1	± 0.6	*sacX-Y*	negative regulatory protein
uxuA	1.0	± 0.1	5.8	± 3.0	3.5	± 0.1	5.0	± 1.5	3.4	± 0.7	3.1	± 0.1	*yjmD-uxuA-yjmF*	D-mannonate hydrolase
BLi04208	1.0	± 0.1	20.4	± 3.6	19.8	± 9.0	19.4	± 3.7	76.7	± 18	95.2	± 7.3		putative malate synthase
BLi02105	1.5	± 0.2	20.6	± 0.3	25.6	± 1.3	27.1	± 0.5	55.6	± 12	41.5	± 11.9		putative 2-hydroxy-3-oxopropionate reductase
BLi02116	0.7	± 0.1	3.7	± 0.1	3.9	± 0.1	5.0	± 0.1	6.1	± 1.1	6.0	± 0.5		putative D-mannonate dehydratase
BLi01109	0.8	± 0.1	7.2	± 1.1	5.6	± 1.6	5.9	± 2.4	14.2	± 1.1	20.3	± 1.8		known subtilisin carlsberg precursor
BLi00658	1.0	± 0.1	3.2	± 0.1	4.6	± 1.2	6.3	± 0.2	11.4	± 1.6	9.4	± 0.2		known maltogenic alpha-amylase
*BLi00828**	1.4	± 0.1	5.0	± 2.0	3.6	± 0.1	3.8	± 0.5	6.4	± 1.0	5.1	± 0.6		putative glycerol dehydrogenase
BLi03549	1.4	± 0.1	1.8	± 0.1	1.8	± 0.1	2.3	± 0.1	6.1	± 1.1	6.3	± 0.4		putative fructose 1-phosphate kinase
BLi00656	1.3	± 0.1	3.3	± 0.3	3.8	± 0.5	4.4	± 1.3	9.0	± 1.4	8.3	± 1.0		known alpha-amylase precursor
BLi04207	1.6	± 0.1	7.4	± 1.7	6.5	± 1.0	4.3	± 0.4	24.5	± 2.3	33.0	± 3.6		putative isocitrate lyase
Main glycolytic pathways														
gapB	1.2	± 0.1	8.3	± 1.8	7.4	± 1.7	4.5	± 1.2	11.6	± 1.7	9.2	± 0.9	*gapB-speD*	glyceraldehyde-3-phosphate dehydrogenase
*pckA**	1.0	± 0.1	24.9	± 8.2	16.3	± 3.9	11.7	± 1.2	21.9	± 3.4	18.2	± 1.9	*pfkA-pyk*	6-phosphofructokinase
BLi00496	1.3	± 0.1	5.3	± 0.2	25.0	± 0.5	112	± 11	57.8	± 7.2	55.9	± 8.1		putative transketolase
BLi00495	1.1	± 0.3	3.7	± 0.2	8.7	± 1.0	27.9	± 3.9	18.5	± 5.5	24.8	± 0.6		putative transketolase

Table 3: continued

Gene name	Tr.Ph	SD	10 min	SD	20 min	SD	30 min	SD	60 min	SD	120min	SD	Transcript. organisation	Function
TCA cycles														
*citA**	0.9	± 0.1	3.9	± 0.1	3.8	± 0.1	1.9	± 0.1	7.8	± 1.4	9.5	± 3.2	*citA-R*	citrate synthase I
mmgD	1.2	± 0.1	17.0	± 6.0	11.8	± 0.4	28.0	± 6.5	28.1	± 6.0	30.0	± 3.9	*mmgD-E-yqiQ*	citrate synthase III
Metabolism of amino acids														
*bpr**	1.1	± 0.1	4.7	± 0.3	7.8	± 1.5	12.9	± 6.3	19.9	± 2.5	16.5	± 0.6	*bpr*	bacillopeptidase F
*ispA**	1.1	± 0.3	3.8	± 0.1	3.0	± 0.1	4.1	± 0.1	7.4	± 0.2	11.4	± 4.3	*ispA*	major intracellular serine protease
mmsA	1.2	± 0.1	25.6	± 4.9	24.4	± 1.8	41.8	± 10	51.8	± 29	27.3	± 0.9	*mmsA*	methylmalonate-semialdehyde dehydrogenase
BLi00198	1.1	± 0.2	5.77	± 0.7	6.31	± 0.3	6.27	± 0.2	6.42	± 0.5	3.81	± 0.1		close homolog to RocF: arginase
rocD	1.3	± 0.1	7.8	± 2.3	6.3	± 1.6	12.7	± 0.9	4.7	± 0.9	1.5	± 0.1	*rocRDEF*	ornithine aminotransferase
rocE	1.2	± 0.1	5.1	± 2.3	4.1	± 0.3	8.8	± 0.8	3.5	± 0.1	1.2	± 0.1	*rocRDEF*	amino acid permease
rocF	1.1	± 0.1	3.9	± 0.7	3.6	± 0.4	5.7	± 0.2	5.0	± 0.5	3.1	± 0.1	*rocRDEF*	arginase
kbl	1.1	± 0.2	50.3	± 12	45.8	± 0.3	56.8	± 13	18.1	± 2.1	14.3	± 1.6	*tdh-kbl*	2-amino-3-ketobutyrate CoA ligase
tdh	1.2	± 0.2	23.6	± 1.1	23.5	± 3.1	27.5	± 6.0	8.7	± 0.2	9.3	± 1.2	*tdh-kbl*	threonine 3-dehydrogenase
*ggt**	0.8	± 0.1	3.8	± 0.1	4.1	± 0.1	4.2	± 1.0	5.2	± 0.2	5.2	± 0.1	*ggt*	gamma-glutamyltranspeptidase
*ycgM**	1.2	± 0.1	16.4	± 3.5	10.1	± 2.8	22.1	± 1.7	51.7	± 20	14.7	± 0.2	*ycgLMNOPQR*	similar to proline oxidase
*ycgN**	1.2	± 0.1	12.4	± 3.4	12.3	± 2.6	19.8	± 10	55.3	± 4.7	24.0	± 5.4	*ycgLMNOPQR*	similar to 1-pyrroline-5-carboxylate dehydrogenase
BLi01747	1.1	± 0.1	3.0	± 0.2	3.5	± 0.1	3.4	± 0.1	5.2	± 0.4	5.8	± 0.5		putative bacillopeptidase F
*BLi00301**	0.9	± 0.1	9.5	± 0.8	5.8	± 0.3	9.8	± 2.8	19.2	± 4.1	20.0	± 2.0		putative serine protease
BLi04251	1.3	± 0.2	10.7	± 3.9	6.4	± 0.5	9.5	± 1.5	4.9	± 0.2	3.5	± 0.1		putative methylmalonate-semialdehyde dehydrogenase
Metabolism of lipids														
acdA	0.9	± 0.1	3.9	± 0.3	5.5	± 0.1	7.9	± 1.3	19.6	± 3.8	11.7	± 1.9	*acdA-mmgABC*	acyl-CoA dehydrogenase
mmgA	1.5	± 0.1	7.0	± 0.6	8.1	± 2.8	8.7	± 3.0	35.8	± 6.3	23.0	± 3.4	*acdA-mmgABC*	acetyl-CoA acetyltransferase
mmgB	1.0	± 0.1	5.0	± 0.7	6.3	± 0.9	7.6	± 3.0	27.5	± 4.3	14.5	± 0.1	*acdA-mmgABC*	3-hydroxybutyryl-CoA dehydrogenase
mmgC	1.0	± 0.1	4.3	± 0.1	7.8	± 0.3	7.2	± 2.0	30.4	± 7.9	11.4	± 3.4	*acdA-mmgABC*	acyl-CoA dehydrogenase
mmgE	1.0	± 0.2	11.5	± 0.4	11.1	± 0.2	9.7	± 4.0	12.8	± 1.5	14.0	± 0.8	*mmgD-E-yqiQ*	function unknown
yhaR	1.0	± 0.1	7.7	± 1.7	4.7	± 0.4	7.7	± 1.1	6.8	± 0.6	4.8	± 0.2	*yha operon*	similar to enoyl CoA hydratase
yusJ	1.0	± 0.1	8.3	± 1.8	12.4	± 3.1	7.1	± 0.6	40.2	± 7.1	16.3	± 1.4	*yusJ-K-L*	similar to butyryl-CoA dehydrogenase
yusK	0.9	± 0.1	6.7	± 0.6	5.2	± 0.6	6.1	± 0.9	21.3	± 2.0	10.0	± 0.7	*yusJ-K-L*	similar to acetyl-CoA C-acyltransferase
yusL	1.1	± 0.1	18.7	± 8.0	13.0	± 2.9	15.3	± 7.3	51.2	± 3.7	14.0	± 4.2	*yusJ-K-L*	similar to 3-hydroxyacyl-CoA dehydrogenase

Table 3: continued

Gene name	Tr.Ph	SD	10 min	SD	20 min	SD	30 min	SD	60 min	SD	120min	SD	Transcript. organisation	Function
ywjE	1.0	± 0.1	3.1	± 0.2	2.8	± 0.1	2.1	± 0.1	7.5	± 1.9	4.9	± 0.4	*ywj operon*	similar to cardiolipin synthetase
BLi02102	0.9	± 0.1	8.9	± 1.2	10.3	± 0.6	15.9	± 0.5	17.5	± 5.8	16.0	± 0.8		putative enoyl (3-hydroxyisobutyrate dehydrogenase)
BLi02103	0.9	± 0.1	15.0	± 6.0	16.9	± 2.3	34.2	± 8.6	29.2	± 8.4	22.8	± 2.0		putative enoyl-CoA hydratase
BLi02106	1.0	± 0.1	48.4	± 5.9	50.8	± 8.7	60.1	± 12	90.6	± 34	78.3	± 4.0		putative butyryl-CoA dehydrogenase
Metabolism of coenzymes														
*BLi03994**	1.7	± 0.3	11.3	± 0.8	23.9	± 0.5	31.2	± 11	12.0	± 0.2	9.7	± 0.3		close homolog to DhaS: aldehyde dehydrogenase
*dhaS**	1.2	± 0.3	7.0	± 0.7	10.8	± 1.2	14.3	± 1.5	16.5	± 2.1	9.9	± 1.3	*dhaS*	aldehyde dehydrogenase
DNA restriction/modification and repair														
yxlJ	1.0	± 0.1	5.7	± 1.0	4.3	± 0.5	5.0	± 1.1	4.7	± 0.8	5.0	± 0.6	*yxlJ*	similar to DNA-3-methyladenine glycosidase
Adaptation to atypical conditions														
cstA	1.1	± 0.1	8.9	± 1.1	6.1	± 0.5	6.5	± 0.4	6.2	± 1.2	5.8	± 0.6	*cstA*	carbon starvation-induced protein
*BLi04126**	0.8	± 0.1	3.4	± 0.1	3.3	± 0.1	3.7	± 0.7	5.2	± 0.4	6.8	± 0.1		putative bacteriocin formation
Detoxification														
yflM	1.0	± 0.2	1.3	± 0.1	2.4	± 0.1	6.3	± 1.4	5.0	± 0.8	6.9	± 3.2	*yfl operon*	similar to nitric-oxide synthase
yxeI	1.1	± 0.1	5.4	± 0.7	4.7	± 1.0	4.7	± 0.1	10.1	± 3.1	10.8	± 1.2	*yxe operon*	unknown; similar to penicillin amidase
Transcription regulation														
BLi01063	0.9	± 0.1	6.2	± 1.5	6.6	± 2.0	7.4	± 1.8	6.2	± 1.1	4.4	± 0.1		putative response regulator aspartate phosphatase A
ydhQ	1.2	± 0.1	2.3	± 0.2	2.5	± 0.1	1.6	± 0.1	5.2	± 0.1	5.4	± 0.5	*ydhP-Q*	similar to transcriptional regulator
*ykoM**	1.0	± 0.1	6.7	± 1.6	6.5	± 0.6	7.2	± 2.0	5.0	± 0.3	3.6	± 0.1	*yko operon*	similar to transcriptional regulator
ysiA	0.9	± 0.1	10.3	± 0.6	8.9	± 2.4	8.7	± 1.4	25.7	± 1.6	4.4	± 0.1	*lcfA-ysiA*	similar to transcriptional regulator
yvdE	1.1	± 0.1	4.1	± 1.0	4.3	± 0.5	9.5	± 0.2	10.8	± 2.3	8.3	± 1.6	*yvd operon*	similar to transcriptional regulator
BLi00492	1.6	± 0.1	7.2	± 2.3	18.3	± 3.2	33.0	± 8.3	18.8	± 3.5	20.6	± 3.7		putative transcriptional regulation
BLi04093	1.6	± 0.1	6.1	± 1.4	6.9	± 1.6	10.4	± 6.7	6.7	± 0.6	8.4	± 0.1		putative transcriptional regulation
Transcription initiation														
*yvyD**	2.4	± 0.3	11.4	± 1.9	6.8	± 1.7	3.8	± 0.3	9.1	± 0.6	8.5	± 2.9	*yvyD*	similar to sigma-54
*sigX**	1.2	± 0.1	4.4	± 1.0	5.7	± 1.1	5.6	± 0.9	9.6	± 0.4	12.7	± 1.7	*sigX*	RNA polymerase ECF-type sigma factor
Unknown function														
*BLi00235**	1.0	± 0.1	8.5	± 0.8	11.7	± 5.3	9.3	± 1.6	8.7	± 1.2	6.6	± 0.6		hypothetical
*BLi00236**	0.9	± 0.1	5.9	± 0.1	7.6	± 1.9	8.2	± 1.7	4.7	± 1.2	3.8	± 0.7		hypothetical
BLi00303	1.1	± 0.1	3.9	± 0.2	3.0	± 0.1	5.4	± 0.2	7.8	± 2.5	6.2	± 1.4		hypothetical
BLi00654	0.9	± 0.1	5.2	± 0.7	5.7	± 2.6	3.6	± 0.1	4.0	± 1.0	6.8	± 0.6		conserved hypothetical

Table 3: continued

Gene name	Tr.Ph	SD	10 min	SD	20 min	SD	30 min	SD	60 min	SD	120min	SD	Transcript. organisation	Function
BLi00858	1.0	± 0.2	1.4	± 0.1	1.8	± 0.1	2.4	± 0.1	7.6	± 2.4	8.6	± 0.6		conserved hypothetical
BLi01466	1.0	± 0.1	4.0	± 0.3	6.6	± 1.0	9.1	± 0.2	3.7	± 0.6	3.4	± 0.1		putative phage protein
BLi02101	0.8	± 0.1	3.6	± 0.1	4.4	± 0.2	7.6	± 0.5	8.6	± 2.0	5.9	± 0.6		hypothetical
BLi03498	1.0	± 0.1	14.1	± 1.4	10.0	± 1.7	15.7	± 7.7	11.8	± 4.2	10.2	± 1.9		putative pectin methylesterase
*BLi03989**	1.0	± 0.1	5.0	± 0.1	10.7	± 1.6	15.8	± 4.7	6.8	± 0.8	8.5	± 1.3		putative hydroxybenzoate hydroxylase
*BLi03991**	1.0	± 0.1	11.2	± 1.0	19.3	± 4.8	40.4	± 6.2	12.2	± 3.5	13.2	± 5.0		putative aromatic compounds specific dioxygenase
*BLi03993**	0.9	± 0.1	14.2	± 1.6	30.4	± 2.6	45.2	± 8.6	16.5	± 1.7	13.4	± 1.5		putative decarboxylase
BLi04116	1.8	± 0.1	3.6	± 0.8	4.1	± 0.6	4.2	± 0.4	7.8	± 1.0	14.3	± 5.0		hypothetical
BLi04118	1.0	± 0.1	3.6	± 0.1	3.9	± 0.1	4.5	± 0.1	6.6	± 2.2	6.9	± 3.0		hypothetical
BLi04119	0.7	± 0.1	4.9	± 0.2	4.1	± 0.2	3.7	± 0.7	6.8	± 2.3	7.7	± 1.7		hypothetical
BLi04120	1.1	± 0.1	3.5	± 0.2	3.8	± 0.1	3.9	± 1.2	5.4	± 1.0	7.2	± 2.2		conserved hypothetical
BLi04124	1.2	± 0.1	3.0	± 0.1	3.7	± 0.1	4.4	± 0.5	6.6	± 0.5	5.9	± 1.4		conserved hypothetical
BLi04185	0.9	± 0.1	5.2	± 0.8	5.9	± 1.8	5.3	± 0.1	10.6	± 3.8	11.1	± 1.9		hypothetical
ybdN	0.9	± 0.1	4.0	± 0.1	4.5	± 0.1	4.6	± 0.1	10.3	± 0.3	6.2	± 2.3	*ybdN*	unknown
ycsE	1.3	± 0.2	1.8	± 0.1	3.5	± 0.2	6.3	± 1.2	5.9	± 0.4	5.8	± 0.5	*ycsE*	unknown
yeaA	1.8	± 0.3	2.6	± 0.1	2.8	± 0.1	2.6	± 0.1	4.6	± 0.4	4.3	± 1.1	*yeaA*	unknown
yesL	1.6	± 0.1	5.5	± 1.4	4.4	± 1.1	6.4	± 0.5	7.3	± 2.0	5.3	± 1.6	*yes operon*	unknown
yfiA	1.1	± 0.2	2.3	± 0.1	2.3	± 0.1	5.4	± 0.1	68.1	± 38	53.9	± 11.9	*malA-yfiA-malP*	unknown
yfmQ	0.8	± 0.1	9.0	± 0.5	8.4	± 0.7	3.9	± 0.8	2.7	± 0.1	2.5	± 0.1	*yfmQ*	unknown
ylbP	1.1	± 0.1	7.3	± 0.4	6.4	± 0.7	6.9	± 1.4	7.9	± 0.1	7.6	± 0.6	*ylbO-P*	unknown
ymcB	1.0	± 0.2	6.7	± 0.2	6.4	± 0.4	8.3	± 1.4	2.0	± 0.1	2.1	± 0.1	*ymcA-B*	unknown
yoeB	1.0	± 0.1	29.2	± 4.4	30.6	± 9.2	15.6	± 1.9	37.1	± 3.9	60.0	± 0.4	*yoeB*	unknown
*ypiB**	1.6	± 0.1	8.3	± 1.1	4.3	± 0.6	5.2	± 0.6	5.0	± 1.2	3.3	± 0.2	*ypiA-B-F*	unknown
ysbA	0.6	± 0.1	17.8	± 0.1	7.9	± 0.4	2.1	± 0.1	1.7	± 0.1	1.2	± 0.1	*ysbA-B*	unknown
yvdJ	1.3	± 0.1	7.3	± 0.2	13.0	± 2.2	43.5	± 15	63.4	± 22	47.5	± 7.5	*yvd operon*	unknown
yvdK	2.2	± 0.3	9.5	± 0.5	17.1	± 2.4	36.5	± 12	65.6	± 17	65.0	± 13.9	*yvd operon*	unknown
yvoA	1.0	± 0.1	1.9	± 0.1	1.9	± 0.1	9.0	± 3.1	12.5	± 1.6	19.6	± 0.3	*yvoA*	unknown
*yvqH**	1.2	± 0.1	4.2	± 0.1	6.5	± 0.8	8.3	± 2.1	18.0	± 5.6	17.4	± 5.5	*yvq operon*	unknown
*yvqI**	0.9	± 0.1	4.6	± 0.3	6.5	± 1.1	9.4	± 2.1	16.0	± 6.9	15.1	± 3.2	*yvq operon*	unknown
ywfL	0.9	± 0.1	3.6	± 0.1	7.5	± 0.7	11.2	± 1.8	5.7	± 0.2	6.1	± 0.2	*ywfL*	unknown
yycA	0.8	± 0.1	3.6	± 0.1	3.4	± 0.1	3.4	± 0.1	5.1	± 0.2	6.2	± 0.1	*yycA*	unknown

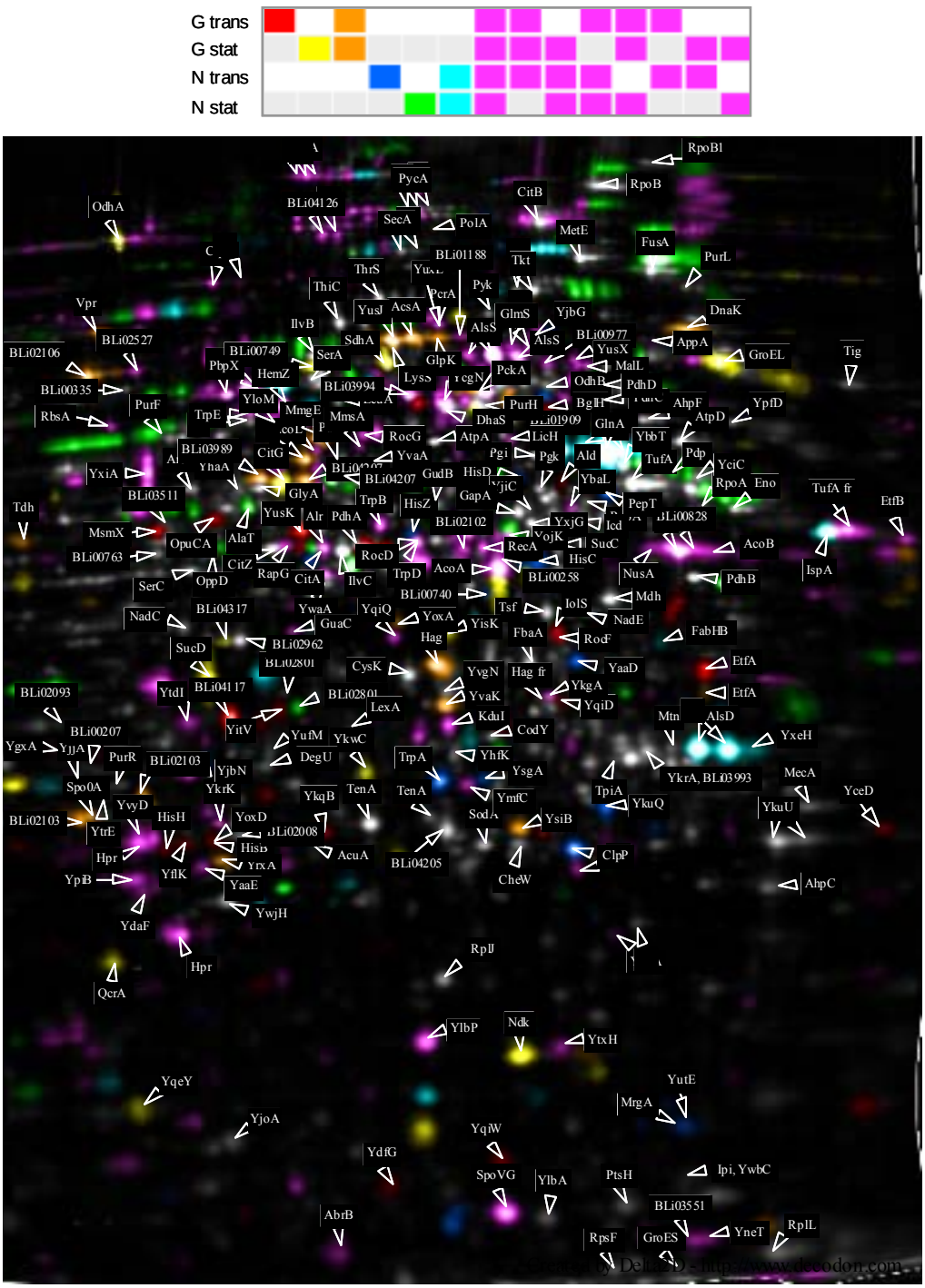


Figure 2: Changes in the cytoplasmic proteome of glucose and nitrogen starving *B. licheniformis* cells. Cell samples were labelled with L-[^{35}S]-methionine during the exponential growth phase (OD 0.4), at the transition phase (OD 1.0) and 60 min after entry into stationary growth phase. Proteins were separated in a pH gradient 4-7. A fusion image containing the proteome images of the two time points of the starvation conditions and the exponential growth phase proteome image was created using the "union fusion" method of the Delta 2D software (Decodon GmbH, Greifswald). Colour coding of the protein spots 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 combinations of starvation conditions received a defined, subset specific colour (see colour scheme). Only proteins induced more than 2.0 fold compared to the exponential growth phase were included in the colour coding. Quantification, gel fusion and colour coding was done with the Delta 2D software (Decodon GmbH Greifswald). (G: glucose starvation, N: nitrogen starvation, trans: transition phase, stat: stationary phase).

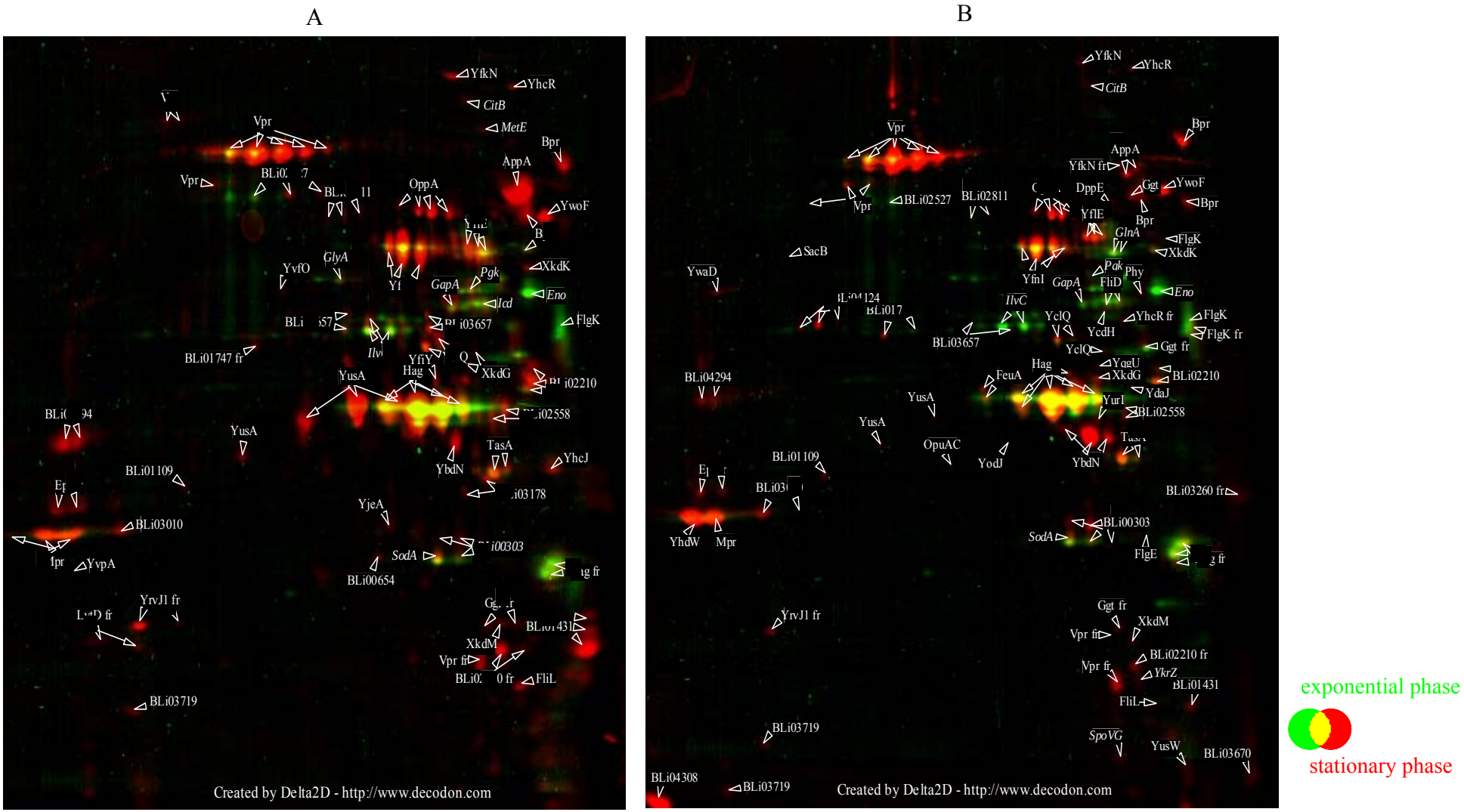


Figure 3: The extracellular proteome of (A) glucose and (B) nitrogen starving *B. licheniformis* cells. The dual channel images were created with the Delta 2D software (Decodon GmbH, Greifswald, Germany). Proteins were prepared during exponential growth (OD 0.4) and one hour after entry into stationary growth. Proteins were separated in a pH gradient 3-10 and stained with colloidal CBB. Spots labelled in italics are presumably intracellular proteins. fr: Protein fragments.

3.3. Nitrogen starvation

Similar to glucose starvation conditions most vegetative proteins of *B. licheniformis* were negatively controlled by nitrogen starvation. Many genes involved in basic cellular processes like translation, glycolysis, TCA cycle, coenzyme and lipid synthesis, nucleotide and nucleic acid synthesis were repressed (Tab. 4, Fig. 2). This probably caused the cell division arrest indicated by the growth curve (Fig. 1) and the down-regulation of genes like *ftsX*, *gid* and *maf*, and several genes encoding proteins contributing to cell wall synthesis. Furthermore, motility and chemotaxis were limited because transcription of a lot of genes involved in flagellum formation and chemotaxis was restricted. The transcriptional regulator CodY, controlling the expression of nitrogen metabolism genes, was also down-regulated at the transcriptional (Tab. 4) as well as at the protein level (Fig. 2).

Genes/ proteins required in amino acid synthesis were differently regulated. The synthesis of most amino acids was down-regulated. For instance, the *arg* and the *ilv-leu* operons were strongly repressed (Tab. 4). There were, however, some exceptions. First, there was an induction of the transcription and the protein synthesis of the glutamine synthetase (GS), GlnA (Tab. 5, Fig. 2). Surprisingly, *glnR*, encoding the transcriptional repressor of the GS, was also induced. Furthermore, genes required for the synthesis of tryptophan and histidin were up-regulated. There was a strong induction of the *trpA-F* genes (5-26fold), which was also detectable at the protein level. A weak induction (5-2.3 fold) of some enzymes involved in the synthesis of histidin (HisB-D and HisH) was seen only at the protein level during the transition phase. At the transcriptional level only *hisC* was induced. Beside this, a few genes from other amino acid biosynthesis pathways were induced (e.g. *aroE*, *tyrA*, *proG*). The physiological significance of the induction of single genes from distinct pathways is not clear. On the other hand, the amino acid proline could even become degraded, since *ycgM* and *ycgN*, genes encoding enzymes for the degradation of proline to glutamate were induced (Tab. 5).

Nitrogen starvation led to an induction of different processes, which are aimed at the mobilisation of alternative nutrient sources. First, the *nasA-F* operon was induced, which encodes a nitrate transport protein (*nasA*) and the assimilatory nitrate and nitrite reductase (*nasB-F*). Furthermore, the synthesis of some protease and peptidases was up-regulated, which might be involved in the mobilisation of nitrogen from proteins (e.g. *bpr*, *BLi00301*, *BLi01109*). In accordance with this, several peptide and amino acid ABC transporter genes were induced, which could be used for the uptake of peptides and amino acids resulting from

the action of extracellular proteases. Genes encoding ammonium transporter proteins were up-regulated too (e.g. *nrgAB*, *BLi01175*). The strongest induction of about 140fold showed the *BLi01488* gene 90 min after the onset of nitrogen starvation at the transcriptional level. For this gene no function could be assigned so far.

The shortcoming of amino acids caused by nitrogen starvation probably led to mistranslation of proteins, indicated by an induction of the ATP-dependent protease ClpP and its ATPase subunit ClpC, required for the recycling of such mistranslated proteins.

There was also a remarkably strong induction of *alsS* and *alsD* at the transcriptional as well as at the translational level. These genes are involved in acetoin production. However, an induction of acetoin metabolising enzymes could not be detected.

Table 4: Kinetic of the nitrogen starvation repressed genes of *B. licheniformis*. Listed are such genes, whose mRNA levels showed a more than fivefold repression during at least one sampling point throughout the growth curve. Genes the expresion of which is also repressed under glucose starvation conditions are labelled with "*".Tr.ph = transient phase, Transcript. = Transcriptional , SD = standard deviation

Gene Name	Tr. ph	SD	30 min	SD	60 min	SD	90 min	SD	120 min	SD	Trancript. organisation	Gene Name
Cell wall												
tuaA	1.7	± 0.1	6.8	± 0.6	6.7	± 1.8	7.1	± 0.7	6.9	± 1.3	*tuaABCDEFGH*	biosynthesis of teichuronic acid
tuaB	2.1	± 0.2	5.0	± 0.4	6.7	± 1.9	6.9	± 1.1	5.4	± 1.2	*tuaABCDEFGH*	biosynthesis of teichuronic acid
tuaC	2.2	± 0.1	6.3	± 0.5	6.7	± 1.5	7.1	± 1.1	6.0	± 1.1	*tuaABCDEFGH*	biosynthesis of teichuronic acid
tuaD	2.1	± 0.2	6.3	± 1.4	6.7	± 1.8	7.1	± 2.1	6.2	± 1.2	*tuaABCDEFGH*	biosynthesis of teichuronic acid
tuaE	2.2	± 0.1	4.7	± 0.7	4.6	± 0.8	5.5	± 0.9	4.5	± 1.0	*tuaABCDEFGH*	biosynthesis of teichuronic acid
BLi03767	1.7	± 0.2	5.4	± 0.7	7.3	± 2.0	9.3	± 0.7	9.1	± 1.6		putative cell wall-binding protein
Transport/binding proteins and lipoproteins												
feuA	0.6	± 0.1	2.6	± 0.1	5.1	± 1.2	4.9	± 1.7	6.0	± 1.3	*feuA-B-C*	iron-uptake system
opuAB	1.5	± 0.4	6.3	± 0.7	7.6	± 1.2	9.5	± 2.9	6.1	± 1.5	*opuAA-AB-AC*	glycine betaine ABC transporter
opuAC	1.8	± 0.1	6.6	± 1.5	8.4	± 0.9	11.3	± 1.8	7.0	± 1.3	*opuAA-AB-AC*	glycine betaine ABC transporter
ptsG	2.0	± 0.2	23.3	± 6.7	26.1	± 6.7	27.1	± 4.2	24.6	± 6.3	*ptsI-G-glcT*	PTS glucose-specific enzyme IICBA component
ptsI	1.9	± 0.1	4.2	± 0.9	10.4	± 2.4	12.9	± 2.2	11.6	± 2.8	*ptsI-G-glcT*	PTS enzyme I
BLi00972	3.1	± 0.1	4.9	± 0.7	7.1	± 1.0	10.3	± 2.1	6.0	± 1.0		putative ABC transporter ATP-binding protein
yhcL	3.5	± 0.3	3.6	± 1.0	5.0	± 0.8	4.8	± 0.3	5.5	± 1.0	*yhc operon*	similar to sodium-glutamate symporter
ykoD	1.7	± 0.1	6.8	± 1.0	4.1	± 0.8	7.1	± 1..4	6.8	± 1.4	*ykoC-D-E*	similar to cation ABC transporter
ytrB	0.5	± 0.2	1.1	± 0.1	5.4	± 0.6	10.5	± 1.5	6.7	± 2.2	*ytrABCEF*	similar to ABC transporter
ytrE	0.3	± 0.1	1.6	± 0.1	4.1	± 0.7	8.3	± 2.6	6.3	± 0.7	*ytrABCEF*	similar to ABC transporter
yxdL	7.7	± 0.2	8.0	± 1.5	8.3	± 1.2	11.1	± 2.8	8.7	± 1.7	*yxdK-L-M*	similar to ABC transporter

Table 4: continued

Gene Name	Tr. ph	SD	30 min	SD	60 min	SD	90 min	SD	120 min	SD	Trancript. organisation	Gene Name
Electron transport chain and ATP synthase												
atpA	1.4	± 0.3	4.2	± 0.8	5.6	± 1.6	6.0	± 1.4	7.5	± 1.4	*atpBFHAGDC*	ATP synthase
atpB	1.6	± 0.2	7.4	± 0.7	8.3	± 2.3	7.4	± 0.8	9.1	± 0.7	*atpBFHAGDC*	ATP synthase
atpD	1.2	± 0.2	5.3	± 0.3	3.7	± 1.8	5.3	± 1.3	5.9	± 1.5	*atpBFHAGDC*	ATP synthase
atpF	1.3	± 0.1	7.4	± 1.2	7.8	± 2.6	8.4	± 0.7	8.6	± 1.1	*atpBFHAGDC*	ATP synthase
atpG	1.4	± 0.2	7.5	± 1.5	11.0	± 4.3	7.7	± 1.2	12.4	± 2.2	*atpBFHAGDC*	ATP synthase
Motility and chemotaxis												
cheC	1.8	± 0.1	7.6	± 0.8	6.3	± 0.7	7.0	± 1.2	6.6	± 0.8	*flg* operon	inhibition of CheR-mediated methylation of MCPs
BLi03295	1.0	± 0.6	1.3	± 0.1	4.8	± 1.2	10.4	± 1.2	5.5	± 1.1		close homolog to McpA methyl-accepting chemotaxis protein
flgB	0.8	± 0.2	1.9	± 0.1	4.2	± 0.2	6.6	± 1.1	6.8	± 0.9	*flgB-fliR*	flagellar basal-body rod protein
flgE	1.2	± 0.1	4.8	± 0.7	7.2	± 1.6	10.9	± 1.7	11.1	± 2.0	*flg* operon	flagellar hook protein
flhB	1.5	± 0.5	7.7	± 1.1	8.6	± 1.1	11.4	± 2.2	10.1	± 0.8	*flg* operon	flagella-associated protein
flhP	1.1	± 0.1	1.6	± 0.2	5.4	± 1.6	7.5	± 0.8	6.3	± 1.1	*flhO-P*	flagellar hook-basal body protein
fliD	1.0	± 0.1	1.8	± 0.1	5.3	± 1.1	6.8	± 1.3	6.9	± 1.2	*hag-fliD*	flagellar hook-associated protein 2
fliG	1.1	± 0.1	3.0	± 0.5	5.0	± 1.2	5.0	± 0.7	5.3	± 0.9	*fli*operon	flagellar motor switch protein
fliH	1.2	± 0.2	3.3	± 1.3	4.8	± 0.9	6.7	± 1.4	7.8	± 0.9	*flg* operon	flagellar assembly protein
fliI	1.1	± 0.2	4.2	± 0.7	5.4	± 1.1	4.8	± 1.0	6.5	± 0.9	*flg* operon	flagellar-specific ATP synthase
fliJ	1.1	± 0.2	3.8	± 0.8	7.4	± 1.4	10.0	± 2.2	10.1	± 1.2	*flg* operon	flagellar protein required for formation of basal body
fliK	1.1	± 0.2	5.0	± 0.5	5.8	± 1.0	7.7	± 1.5	7.3	± 1.1	*flg* operon	flagellar hook-length control
fliM	1.3	± 0.1	4.1	± 0.9	6.5	± 1.3	9.8	± 1.8	7.3	± 1.4	*flg* operon	flagellar motor switch protein
fliP	1.5	± 0.1	4.9	± 1.6	8.9	± 2.7	5.9	± 1.0	9.8	± 2.8	*flg* operon	flagellar protein required for flagellar formation
fliR	1.7	± 0.2	6.1	± 0.7	7.8	± 2.3	6.2	± 1.9	5.9	± 1.3	*flg* operon	flagellar protein required for flagellar formation
fliZ	1.6	± 0.2	5.2	± 1.5	8.2	± 0.9	11.0	± 1.7	12.1	± 4.0	*flg* operon	flagellar protein required for flagellar formation
hag	0.9	± 0.1	1.5	± 0.1	7.4	± 1.1	12.3	± 2.2	20.2	± 4.8	*hag-fliD*	flagellin protein
ylxG	1.1	± 0.1	2.6	± 0.1	4.9	± 1.0	6.0	± 0.2	6.7	± 1.4	*ylxG*	similar to flagellar hook assembly protein
ylxH	1.6	± 0.3	8.9	± 1.2	5.6	± 1.3	5.3	± 1.2	4.9	± 0.6	*ylxH*	similar to flagellar biosynthesis switch protein
signal transduction												
cheA	1.5	± 0.1	6.2	± 1.3	7.9	± 1.1	8.7	± 1.4	9.3	± 0.7	*cheA*	two-component sensor histidine kinase chemotactic signal modulator

Table 4: continued

Gene Name	Tr. ph	SD	30 min	SD	60 min	SD	90 min	SD	120 min	SD	Trancript. organisation	Gene Name
Protein secretion												
secY	1.8	± 0.4	16.7	± 2.3	14.8	± 3.4	25.0	± 0.3	15.7	± 4.3	*rplR-secY-adk-map*	preprotein translocase subunit
Cell division												
ftsX	1.8	± 0.1	3.1	± 0.3	4.3	± 1.1	6.5	± 0.8	5.2	± 0.6	*ftsE-X*	cell-division protein
Sporulation												
safA	1.4	± 0.1	5.4	± 1.1	1.2	± 0.3	9.8	± 1.4	1.5	± 0.2	*safA*	morphogenetic protein associated with SpoVID
Specific pathways												
yfmT	1.1	± 0.3	3.8	± 0.7	6.2	± 0.7	6.5	± 1.3	3.9	± 0.4	*yfmS-T*	similar to benzaldehyde dehydrogenase
Main glycolytic pathways												
*eno**	1.2	± 0.2	16.7	± 3.9	20.3	± 3.0	25.0	± 0.1	22.5	± 2.7	*gapA-pgk-tpiA-pgm-eno*	enolase
*gapA**	1.2	± 0.1	10.5	± 1.8	26.7	± 4.9	26.1	± 6.0	25.7	± 4.8	*gapA-pgk-tpiA-pgm-eno*	glyceraldehyde-3-phosphate dehydrogenase
pdhA	1.6	± 0.1	18.0	± 8.1	11.8	± 2.2	11.3	± 1.8	12.0	± 2.9	*pdhABCD*	pyruvate dehydrogenase
pdhB	1.6	± 0.1	7.9	± 2.5	11.2	± 2.0	9.5	± 1.4	11.2	± 1.9	*pdhABCD*	pyruvate dehydrogenase
*pdhC**	1.3	± 0.2	5.1	± 0.7	7.0	± 1.8	10.7	± 2.7	13.5	± 1.6	*pdhABCD*	pyruvate dehydrogenase
*pdhD**	1.2	± 0.3	6.0	± 0.6	7.1	± 1.1	5.9	± 0.6	8.8	± 0.8	*pdhABCD*	pyruvate dehydrogenase
*pgk**	1.1	± 0.2	25.8	± 8.8	26.3	± 6.7	33.3	± 0.2	25.7	± 4.8	*gapA-pgk-tpiA-pgm-eno*	phosphoglycerate kinase
*pgm**	1.4	± 0.1	24.6	± 6.3	19.2	± 1.7	23.3	± 6.7	18.7	± 5.0	*gapA-pgk-tpiA-pgm-eno*	phosphoglycerate mutase
tkt	0.6	± 0.1	3.7	± 0.4	4.0	± 0.3	5.1	± 0.2	5.4	± 0.7	*tkt*	transketolase
tpiA	1.2	± 0.2	13.6	± 3.6	14.8	± 1.6	21.7	± 2.9	18.2	± 7.5	*gapA-pgk-tpiA-pgm-eno*	triose phosphate isomerase
Metabolism of amino acids and related molecules												
ampS	0.6	± 0.1	4.3	± 0.9	4.0	± 1.0	6.3	± 1.0	5.7	± 0.7	*ampS*	aminopeptidase
argB	4.8	± 0.6	4.5	± 1.2	5.5	± 0.9	5.0	± 1.9	0.6	± 0.2	*arg* operon	N-acetylglutamate 5-phosphotransferase
argD	3.6	± 0.7	5.1	± 1.6	5.1	± 1.2	4.0	± 1.1	0.8	± 0.4	*arg* operon	N-acetylornithine aminotransferase
argF	4.9	± 0.2	3.2	± 0.2	4.8	± 0.6	3.8	± 0.8	1.1	± 0.4	*arg* operon	ornithine carbamoyltransferase
argG	5.0	± 1.0	6.1	± 0.8	6.8	± 1.7	2.1	± 0.7	0.2	± 0.2	*arg* operon	argininosuccinate synthase
argH	6.0	± 0.5	12.1	± 2.2	10.4	± 2.8	3.3	± 0.4	0.3	± 0.1	*arg* operon	argininosuccinate lyase
argJ	5.6	± 1.1	8.7	± 2.2	5.0	± 0.9	5.4	± 1.2	0.6	± 0.2	*arg* operon	ornithine acetyltransferase
carA	6.3	± 0.7	8.7	± 1.1	9.4	± 2.0	9.1	± 1.2	1.2	± 0.1	*arg* operon	carbamoyl-phosphate transferase-arginine
carB	4.7	± 0.8	11.8	± 1.1	10.5	± 1.8	9.5	± 2.3	1.5	± 0.3	*arg* operon	carbamoyl-phosphate transferase-arginine
gcvPB	1.6	± 0.1	2.7	± 0.6	4.0	± 1.8	5.2	± 0.6	7.3	± 1.1	*gcvT-gcvPA-PB*	probable glycine decarboxylase
goxB	1.0	± 0.1	2.9	± 0.4	1.7	± 0.1	5.2	± 1.0	3.7	± 1.0	*goxB*	glycine oxidase

Table 4: continued

Gene Name	Tr. ph	SD	30 min	SD	60 min	SD	90 min	SD	120 min	SD	Trancript. organisation	Gene Name
ilvB	1.9	± 0.3	8.5	± 0.5	7.4	± 1.4	9.8	± 0.5	9.4	± 1.9	*ilvBHC-leuABCD*	acetolactate synthase
ilvC	1.4	± 0.1	6.5	± 1.5	6.6	± 1.1	9.5	± 1.4	8.3	± 1.7	*ilvBHC-leuABCD*	ketol-acid reductoisomerase
ilvH	1.8	± 0.2	7.0	± 1.6	7.6	± 1.6	7.7	± 0.1	6.7	± 1.3	*ilvBHC-leuABCD*	acetolactate synthase
leuA	1.8	± 0.1	8.5	± 0.4	9.3	± 2.0	15.1	± 2.4	3.6	± 2.7	*ilvBHC-leuABCD*	2-isopropylmalate synthase
leuB	1.9	± 0.3	8.9	± 0.8	9.8	± 1.1	14.3	± 2.1	11.0	± 1.9	*ilvBHC-leuABCD*	3-isopropylmalate dehydrogenase
leuC	1.9	± 0.2	2.0	± 0.4	4.7	± 0.9	2.2	± 0.7	5.2	± 1.3	*ilvBHC-leuABCD*	3-isopropylmalate dehydratase
leuD	2.5	± 0.1	4.7	± 0.3	6.2	± 0.2	6.0	± 1.7	3.1	± 1.2	*ilvBHC-leuABCD*	3-isopropylmalate dehydratase
yjcI	6.5	± 1.2	6.8	± 0.8	12.5	± 1.5	17.8	± 1.9	9.1	± 0.5	*yjcI-J-L*	similar to cystathionine gamma-synthase
yjcJ	5.6	± 0.3	5.0	± 0.6	6.5	± 1.1	7.4	± 1.5	6.0	± 1.1	*yjcI-J-L*	similar to cystathionine beta-lyase
Metabolism of nucleotides and nucleic acids												
adk	2.7	± 0.3	25.8	± 5.5	31.9	± 3.4	29.2	± 4.8	25.6	± 4.3	*rplR-secY-adk-map*	adenylate kinase
guaA	1.4	± 0.1	5.3	± 0.6	5.3	± 1.3	8.7	± 1.3	4.8	± 1.0	*guaA*	GMP synthetase
purB	0.9	± 0.1	10.1	± 3.6	4.9	± 1.5	6.4	± 1.0	4.8	± 0.4	*pur* operon	adenylosuccinate lyase
purC	0.7	± 0.1	7.7	± 1.4	8.0	± 0.7	8.3	± 2.0	9.3	± 2.1	*pur* operon	phosphoribosylaminoimidazole succinocarboxamide synthetase
purD	1.0	± 0.1	11.6	± 2.1	5.2	± 1.8	10.5	± 1.8	6.4	± 1.2	*pur* operon	phosphoribosylglycinamide synthetase
purE	1.0	± 0.2	4.5	± 1.6	2.9	± 0.6	2.7	± 0.3	2.3	± 0.6	*pur* operon	phosphoribosylaminoimidazole carboxylase I
purF	1.0	± 0.2	11.7	± 1.4	11.7	± 1.4	10.3	± 0.6	11.4	± 1.0	*pur* operon	glutamine phosphoribosyl pyrophosphate amidotransferase
purH	1.0	± 0.2	15.2	± 4.2	10.3	± 3.0	13.3	± 1.0	14.8	± 1.5	*pur* operon	phosphoribosylaminoimidazole carboxy formyl formyltransferase
purK	0.8	± 0.2	5.6	± 0.7	3.9	± 0.5	3.0	± 0.1	3.9	± 0.6	*pur* operon	phosphoribosylaminoimidazole carboxylase II
purL	1.1	± 0.1	12.9	± 2.5	9.5	± 1.4	8.2	± 0.3	10.3	± 0.6	*pur* operon	phosphoribosylformylglycinamidine synthetase II
purM	1.0	± 0.1	12.5	± 3.6	6.9	± 0.5	5.6	± 0.6	5.5	± 0.9	*pur* operon	phosphoribosylaminoimidazole synthetase
purN	1.1	± 0.3	15.9	± 4.8	9.7	± 1.4	11.4	± 2.2	12.3	± 2.7	*pur* operon	phosphoribosylglycinamide formyltransferase
purQ	1.0	± 0.1	6.6	± 1.7	7.8	± 1.7	6.1	± 1.1	8.2	± 1.1	*pur* operon	phosphoribosyl formylglycinamidine synthetase I
pyrAA	0.9	± 0.1	8.9	± 0.4	6.0	± 2.4	7.3	± 1.5	7.0	± 0.7	*pyr* operon	carbamoyl-phosphate synthetase
pyrAB	1.1	± 0.1	5.4	± 1.4	4.0	± 1.3	5.5	± 1.7	3.8	± 0.8	*pyr* operon	carbamoyl-phosphate synthetase

Table 4: continued

Gene Name	Tr. ph	SD	30 min	SD	60 min	SD	90 min	SD	120 min	SD	Trancript. organisation	Gene Name
pyrB	0.9	± 0.2	4.2	± 0.4	5.4	± 0.2	4.7	± 2.1	3.8	± 1.0	*pyr* operon	aspartate carbamoyltransferase
pyrC	0.9	± 0.1	5.2	± 1.0	3.1	± 0.3	4.3	± 0.8	3.4	± 0.8	*pyr* operon	dihydroorotase
pyrD	1.2	± 0.1	4.3	± 1.0	3.5	± 0.2	3.7	± 1.0	3.7	± 0.3	*pyr* operon	dihydroorotate dehydrogenase
pyrE	0.9	± 0.1	6.9	± 0.3	4.2	± 0.1	8.9	± 1.2	5.4	± 0.9	*pyr* operon	orotate phosphoribosyltransferase
pyrF	1.0	± 0.1	5.6	± 0.7	3.9	± 0.2	7.5	± 2.1	4.1	± 0.6	*pyr* operon	orotidine 5'-phosphate decarboxylase
pyrH	1.6	± 0.2	6.1	± 1.6	7.7	± 1.8	3.8	± 0.6	8.7	± 1.7	*pyrH-frr*	uridylate kinase
pyrK	0.9	± 0.2	11.5	± 1.2	5.6	± 0.9	10.9	± 1.2	9.4	± 0.5	*pyr* operon	dihydroorotate dehydrogenase
pyrP	1.1	± 0.3	5.4	± 0.7	3.6	± 0.1	4.1	± 1.1	3.6	± 0.3	*pyr* operon	uracil permease
Metabolism of lipids												
cdsA	1.6	± 0.2	6.3	± 1.7	6.8	± 1.7	5.3	± 1.7	6.1	± 1.5	*uppS-cdsA-dxr*	phosphatidate cytidylyltransferase
fabD	2.8	± 0.1	4.8	± 0.7	6.7	± 0.5	8.2	± 0.3	6.3	± 1.3	*plsX-fabD-G*	malonyl CoA-acyl carrier protein transacylase
fabG	2.7	± 0.2	4.6	± 0.2	6.3	± 0.6	5.6	± 1.1	6.0	± 0.6	*plsX-fabD-G*	beta-ketoacyl-acyl carrier protein reductase
Metabolism of coenzymes and prosthetic groups												
bioA	1.9	± 0.4	8.5	± 1.2	11.7	± 3.6	11.1	± 3.9	11.4	± 0.9	*bioABDFI*	adenosylmethionine-8-amino-7-oxononanoate aminotransferase
bioB	1.7	± 0.4	20.0	± 0.3	16.5	± 3.1	21.7	± 2.9	12.9	± 2.8	*bioABDFI*	biotin synthetase
bioD	1.8	± 0.2	28.9	± 7.7	25.7	± 7.6	25.8	± 5.5	30.8	± 5.5	*bioABDFI*	dethiobiotin synthetase
bioF	1.9	± 0.1	12.1	± 4.8	10.9	± 1.2	10.1	± 1.0	12.5	± 1.5	*bioABDFI*	8-amino-7-oxononanoate sythase
bioI	2.2	± 0.3	9.6	± 2.4	10.0	± 1.8	12.5	± 1.8	10.5	± 2.3	*bioABDFI*	cytochrome P450 enzyme
Metabolism of sulfur												
yvgQ	6.4	± 0.4	8.9	± 2.6	10.5	± 2.7	13.5	± 2.7	10.3	± 1.6	*yvgQ-R*	similar to sulfite reductase
yvgR	6.1	± 0.6	8.3	± 0.1	8.2	± 1.4	15.3	± 5.5	9.1	± 2.2	*yvgQ-R*	similar to sulfite reductase
DNA replication												
ssb	2.3	± 0.1	12.1	± 1.5	13.3	± 3.2	16.9	± 2.3	18.3	± 1.9	*ssb*	single-strand DNA-binding protein
DNA packaging and segregation												
parC	0.9	± 0.1	5.2	± 0.1	4.0	± 0.7	6.9	± 0.6	2.6	± 0.1	*parC*	subunit of DNA topoisomerase IV
RNA modification												
rnc	2.2	± 0.2	4.5	± 1.0	5.7	± 1.0	4.3	± 1.0	5.3	± 0.5	*rnc*	ribonuclease III
rnpA	1.8	± 0.2	8.7	± 1.5	6.2	± 0.5	7.0	± 1.1	5.3	± 0.9	*rnpA-spoIIIJ-jag*	protein component of ribonuclease P (RNase P)
Transcription elongation												
rpoA	2.8	± 0.2	8.0	± 1.0	11.6	± 1.8	10.4	± 0.6	11.5	± 2.2	*rpoA*	RNA polymerase
rpoB	1.7	± 0.1	6.3	± 1.2	3.5	± 0.6	4.3	± 0.2	3.8	± 0.7	*rpoB-C*	RNA polymerase
rpoC	1.8	± 0.2	8.7	± 1.8	5.0	± 0.5	5.8	± 0.2	4.7	± 1.2	*rpoB-C*	RNA polymerase
Transcription regulation												
cggR	1.8	± 0.1	26.7	± 3.6	21.7	± 2.9	30.6	± 4.8	17.0	± 5.0	*cggR*	transcriptional repressor of gapA
cheY	1.5	± 0.2	4.0	± 0.3	5.6	± 0.8	8.1	± 0.4	11.5	± 2.1	*cheY*	modulation of flagellar switch bias
BLi04205	1.4	± 0.1	5.4	± 0.5	3.8	± 0.8	4.8	± 0.7	5.0	± 0.8		putative transcriptional regulation
ykvE	0.8	± 0.1	4.6	± 0.4	5.4	± 1.0	6.2	± 0.7	5.6	± 0.5	*ykvE*	similar to transcriptional regulator

Table 4: continued

Gene Name	Tr. ph	SD	30 min	SD	60 min	SD	90 min	SD	120 min	SD	Trancript. organisation	Gene Name
ytrA	0.4	± 0.1	2.1	± 0.2	4.5	± 0.5	5.4	± 1.2	5.1	± 1.0	*ytrABCEF*	similar to transcriptional regulator
yvrG	1.0	± 0.1	5.2	± 1.1	4.6	± 0.3	6.5	± 0.7	3.1	± 0.3	*yvrG-H*	similar to two-component sensor histidine kinase
transcriptional terminator												
nusA	1.8	± 0.1	10.3	± 1.5	7.8	± 0.8	9.1	± 1.6	6.7	± 1.6	*infB-nusA*	transcription termination
Translation elongation												
tsf	1.6	± 0.2	10.3	± 1.0	7.5	± 0.8	8.3	± 1.3	7.2	± 0.4	*tsf*	elongation factor Ts
Translation initiation												
infB	2.0	± 0.2	5.6	± 1.0	6.2	± 1.5	6.7	± 0.7	7.8	± 1.4	*infB-nusA*	initiation factor IF-2
ykrS	4.8	± 0.1	5.0	± 0.4	5.4	± 0.7	5.6	± 1.6	5.8	± 0.5	*ykr* operon	similar to initiation factor eIF-2B (alpha subunit)
Translation termination												
frr	1.6	± 0.2	4.6	± 0.9	5.1	± 0.8	4.2	± 0.4	6.5	± 0.7	*pyrH-frr*	ribosome recycling factor
Ribosomal proteins												
rplA	2.0	± 0.1	8.0	± 0.4	9.9	± 1.1	10.4	± 1.2	9.6	± 0.8	*rpl* operon	ribosomal protein L1 (BL1)
rplB	2.2	± 0.2	27.1	± 8.0	20.0	± 0.1	25.0	± 0.2	18.6	± 2.9	*rpl* operon	ribosomal protein L2 (BL2)
rplC	1.9	± 0.1	6.0	± 0.6	8.3	± 1.9	5.7	± 0.2	6.5	± 1.2	*rpl* operon	ribosomal protein L3 (BL3)
rplD	2.1	± 0.1	20.2	± 5.6	17.6	± 1.5	17.3	± 3.6	14.5	± 1.9	*rpl* operon	ribosomal protein L4
rplE	2.7	± 0.2	27.6	± 5.8	21.0	± 2.2	23.6	± 6.3	25.2	± 5.5	*rpl* operon	ribosomal protein L5 (BL6)
rplF	2.5	± 0.1	21.1	± 2.5	17.2	± 1.4	14.9	± 1.2	14.9	± 2.6	*rpl* operon	ribosomal protein L6 (BL8)
rplJ	2.4	± 0.1	33.3	± 0.1	27.8	± 4.3	41.7	± 11.8	24.7	± 5.5	*rpl* operon	ribosomal protein L10 (BL5)
rplK	2.3	± 0.1	8.5	± 0.7	8.5	± 1.4	6.9	± 0.6	7.6	± 0.6	*rpl* operon	ribosomal protein L11 (BL11)
rplL	2.8	± 0.1	10.0	± 1.2	7.4	± 1.8	13.0	± 3.0	13.6	± 2.9	*rpl* operon	ribosomal protein L12 (BL9)
rplM	2.1	± 0.2	5.3	± 0.9	5.8	± 1.0	7.3	± 0.7	5.5	± 0.8	*rpl* operon	ribosomal protein L13
rplN	2.7	± 0.3	25.8	± 5.5	25.6	± 4.3	31.3	± 4.2	25.5	± 3.9	*rpl* operon	ribosomal protein L14
rplO	2.0	± 0.1	18.2	± 1.9	18.3	± 1.9	17.6	± 6.2	15.6	± 2.9	*rpl* operon	ribosomal protein L15
rplP	2.1	± 0.2	21.1	± 4.1	13.8	± 1.7	17.6	± 2.8	13.6	± 2.5	*rpl* operon	ribosomal protein L16
rplQ	2.2	± 0.1	7.1	± 1.0	8.3	± 0.9	9.3	± 2.2	9.0	± 1.8	*rpl* operon	ribosomal protein L17
rplR	2.3	± 0.3	29.2	± 4.8	25.0	± 0.1	30.6	± 4.8	33.3	± 8.3	*rpl* operon	ribosomal protein L18
rplS	3.0	± 0.5	2.6	± 4.9	3.2	± 0.6	3.7	± 2.3	2.3	± 1.2	*rpl* operon	ribosomal protein L19
rplT	2.7	± 0.1	3.8	± 0.9	8.1	± 1.8	6.4	± 2.7	6.7	± 1.3	*rpl* operon	ribosomal protein L20
rplV	2.4	± 0.3	18.2	± 1.9	12.0	± 3.3	12.6	± 1.3	14.4	± 0.8	*rpl* operon	ribosomal protein L22 (BL17)
rpsB	1.8	± 0.1	14.3	± 0.1	13.3	± 2.3	12.9	± 1.3	12.1	± 2.2	*rps* operon	ribosomal protein S2
rpsC	2.2	± 0.3	12.1	± 2.4	8.3	± 2.2	12.1	± 2.8	9.6	± 1.7	*rps* operon	ribosomal protein S3 (BS3)
rpsD	2.5	± 0.5	4.3	± 1.4	4.4	± 1.2	9.2	± 2.5	6.3	± 1.5	*rps* operon	ribosomal protein S4 (BS4)
rpsG	1.5	± 0.3	10.3	± 0.6	8.2	± 1.4	8.2	± 0.4	8.3	± 1.5	*rps* operon	ribosomal protein S7 (BS7)
rpsI	2.2	± 0.2	5.4	± 1.1	4.8	± 1.2	5.6	± 0.5	5.5	± 0.5	*rps* operon	ribosomal protein S9
rpsK	2.5	± 0.1	18.2	± 2.9	15.4	± 1.4	12.9	± 6.3	16.1	± 1.2	*rps* operon	ribosomal protein S11 (BS11)
rpsL	1.7	± 0.2	8.5	± 1.8	7.4	± 1.8	5.4	± 1.0	6.7	± 1.5	*rps* operon	ribosomal protein S12 (BS12)
rpsM	2.5	± 0.3	15.4	± 2.1	15.6	± 1.8	16.7	± 3.9	16.8	± 1.8	*rps* operon	ribosomal protein S13
Protein folding												
tig	1.8	± 0.1	11.4	± 2.1	13.3	± 1.9	16.7	± 1.2	12.5	± 1.7	*tig-clpX*	trigger factor
Protein modification												
map	2.5	± 0.1	12.5	± 0.6	17.1	± 2.9	17.6	± 6.2	15.9	± 5.0	*rplR-secY-adk-map*	methionine aminopeptidase

Table 4: continued

Gene Name	Tr. ph	SD	30 min	SD	60 min	SD	90 min	SD	120 min	SD	Trancript. organisation	Gene Name
unknown function												
BLi00156	2.5	0.1	22.2	2.9	22.2	2.7	22.2	2.9	20.0	2.9		conserved hypothetical
yitJ	5.6	0.2	3.7	0.7	4.9	0.8	5.5	0.7	4.1	0.8	*yitK-J*	unknown
ykoC	1.9	0.1	4.8	1.9	5.0	1.0	5.1	1.1	6.4	2.1	*ykoC-D-E*	unknown
ykoE	1.5	0.1	8.2	1.5	6.1	0.7	8.3	1.2	5.6	1.5	*ykoC-D-E*	unknown
ykrY	5.2	0.6	3.9	0.3	7.9	2.1	9.1	2.2	9.6	1.7	*ykr operon*	unknown
ylxF	1.4	0.2	5.0	1.5	9.2	2.4	14.3	2.3	11.8	0.1	*ylx operon*	unknown
yoaZ	1.2	0.2	8.4	2.2	5.0	0.5	4.7	0.4	4.9	0.7	*yoaZ*	unknown
ysoA	2.1	0.1	5.9	0.5	5.3	0.9	5.5	0.1	5.1	1.1	*ysoA*	unknown
ytrF	0.5	0.5	0.9	0.1	5.0	1.1	5.5	1.1	5.3	1.4	*ytrABCEF*	unknown
yuiI	0.4	0.1	5.4	2.5	4.5	0.6	6.6	0.4	4.9	0.9	*yuiI-dhbABCDEF*	unknown
yusA	4.7	0.3	6.2	1.9	6.0	0.8	7.8	1.0	9.3	2.3	*yusABCD*	unknown
yusB	5.9	0.1	8.0	0.8	12.0	1.4	13.3	2.8	17.4	3.2	*yusABCD*	unknown

Table 5: Kinetic of the nitrogen starvation inducible genes of *B. licheniformis*. Listed are such genes, the mRNA level of which showed a more than fivefold induction during at least one sampling point throughout the growth curve. Genes the expresion of which is also induced under glucose starvation conditions are labelled with "*".Tr.ph = transient phase, SD = standard deviation

Gene name	Tr.ph	SD	30 min	SD	60 min	SD	90 min	SD	120 min	SD	Transcript. organisation	Function
Transport/binding proteins and lipoproteins												
dppE	2.6	0.3	8.1	2.5	10.8	1.2	5.6	0.9	7.2	1.2	*dppABCDE*	dipeptide ABC transporter
nrgA	3.0	0.3	5.7	0.4	5.4	1.1	6.6	1.2	7.3	1.3	*nrgA-B*	ammonium transporter
*BLi03990**	4.1	0.4	4.7	0.4	6.1	0.9	13.7	2.6	11.9	1.9		putative benzoate transport protein
*BLi04117**	2.1	0.2	2.9	0.3	6.1	0.6	9.3	4.0	10.8	2.6		putative ABC transporter ATP-binding protein
BLi01175	6.2	0.7	20.0	3.0	19.5	1.5	21.0	2.1	22.8	2.9		putative ammonium transporter
BLi03212	8.1	0.4	4.4	0.7	5.3	0.2	4.6	1.4	1.4	0.3		putative ABC transporter
BLi00817	3.9	0.5	6.4	1.5	12.4	1.2	12.5	1.3	17.1	2.4		putative Na(+)-linked D-alanine glycine permease
ycnJ	4.0	0.6	5.3	0.5	4.6	1.1	9.4	2.4	5.5	0.5	*ycnI-J*	similar to copper export protein
Sporulation												
*spoIIAA**	1.4	0.2	4.6	0.9	3.7	0.9	3.2	0.4	3.0	0.6	*dacF-spoIIAA-spoIIAB-sigF*	known RNA polymerase sigma factor
*spoIIAB**	2.4	0.5	7.1	0.7	5.6	1.5	4.9	1.5	1.8	0.4	*dacF-spoIIAA-spoIIAB-sigF*	known anti-sigma F factor
Specific pathways												
alsD	5.8	0.4	22.2	1.8	7.4	0.8	38.1	2.0	9.8	1.5	*alsR-S-D*	alpha-acetolactate decarboxylase
alsR	1.7	0.1	8.8	4.5	7.8	1.2	7.4	0.7	3.9	0.8	*alsR-S-D*	transcriptional regulator
alsS	17.7	1.5	76.8	8.0	9.7	2.5	143.5	29	22.3	6.3	*alsR-S-D*	alpha-acetolactate synthase
*BLi00828**	2.0	0.1	5.2	0.8	5.7	1.0	5.7	0.1	6.5	1.1		putative glycerol dehydrogenase
BLi04208	1.0	0.1	6.0	1.7	5.1	1.0	17.1	4.4	3.3	1		putative malate synthase
Main glycolytic pathways												
*pckA**	0.9	0.1	5.9	0.7	16.9	6.3	28.6	4.6	14.7	1.8	*pfkA-pyk*	phosphoenolpyruvate carboxykinase
TCA cycle												
*mmgD**	1.3	0.1	5.3	1.5	6.4	0.3	6.9	1.0	3.5	0.9	*mmgD-E-yqiQ*	citrate synthase III
*citA**	1.1	0.1	1.8	0.1	5.8	1.7	7.4	0.6	10.4	1	*citA-R*	citrate synthase I (minor)

Table 5: continued

Gene name	Tr.ph	SD	30 min	SD	60 min	SD	90 min	SD	120 min	SD	Transcript. organisation	Function
Metabolism of amino acids and related molecules												
asnO	2.2	0.1	5.6	0.1	5.2	1.3	5.5	1.1	6.1	0.8	*asnO*	asparagine synthetase
*bpr**	1.4	0.1	6.3	1.2	6.0	1.1	7.6	1.2	12.4	1.8	*bpr*	bacillopeptidase F
glnA	5.7	0.4	9.5	0.1	8.1	0.5	9.1	0.9	8.6	1.6	*glnR-A*	glutamine synthetase
*ggt**	1.8	0.1	8.6	2.0	5.3	0.8	8.8	3.6	6.8	1	*ggt*	gamma-glutamyltranspeptidase
hisC	1.6	0.2	6.3	0.3	5.2	0.9	5.1	1.0	5.0	1.7	*tyrA-hisC-trpABFCDE*	histidinol-phosphate aminotransferase
*ispA**	1.1	0.1	7.0	0.7	10.2	2.6	19.3	4.7	4.6	1	*ispA*	major intracellular serine protease
nasB	18.2	1.6	22.6	2.9	23.8	2.9	25.2	5.9	20.0	3.7	*nasBCDEF*	assimilatory nitrate reductase
nasC	11.1	1.4	22.1	1.4	22.4	2.8	21.0	1.9	20.5	3.3	*nasBCDEF*	assimilatory nitrate reductase
nasD	6.6	0.5	10.6	0.6	14.6	3.7	20.6	1.8	13.0	2.3	*nasBCDEF*	assimilatory nitrate reductase
nasE	10.6	1.9	23.5	6.7	26.8	8.8	43.0	8.5	23.2	3.8	*nasBCDEF*	assimilatory nitrate reductase
nasF	8.1	0.8	16.6	0.2	22.4	5.0	40.2	3.5	21.0	2.7	*nasBCDEF*	uroporphyrin-III C-methyltransferase
nrgB	3.6	0.6	6.9	0.7	7.0	1.0	10.1	1.6	6.4	0.9	*nrgA-B*	nitrogen-regulated PII-like protein
*BLi00301**	2.3	0.1	13.4	1.1	6.6	0.8	16.1	1.7	13.2	1.4		putative serine protease
trpA	1.0	0.1	6.0	0.5	6.3	1.5	5.3	0.8	6.8	2.8	*tyrA-hisC-trpABFCDE*	tryptophan synthase
trpB	1.5	0.2	9.2	1.4	8.2	2.6	11.4	3.4	11.9	4.2	*tyrA-hisC-trpABFCDE*	tryptophan synthase
trpC	1.5	0.1	12.1	0.7	10.2	2.6	11.2	2.5	14.3	5.3	*tyrA-hisC-trpABFCDE*	indol-3-glycerol phosphate synthase
trpD	1.5	0.3	10.2	2.9	11.7	4.0	11.9	2.5	11.8	3.8	*tyrA-hisC-trpABFCDE*	anthranilate phosphoribosyltransferase
trpE	2.0	0.4	26.1	5.5	22.3	2.0	19.7	2.3	17.8	4	*tyrA-hisC-trpABFCDE*	anthranilate synthase
trpF	1.4	0.1	12.0	0.4	9.6	1.9	11.6	3.3	18.9	2.1	*tyrA-hisC-trpABFCDE*	phosphoribosyl anthranilate isomerase
*ycgM**	1.2	0.1	10.7	1.7	21.5	1.3	18.0	3.1	3.1	0.5	*ycgLMNOPQR*	similar to proline oxidase
*ycgN**	1.2	0.3	7.6	1.0	16.8	2.5	12.1	4.0	1.9	0.6	*ycgLMNOPQR*	similar to 1-pyrroline-5-carboxylate dehydrogenase
yqjN	1.6	0.1	2.7	0.2	4.5	0.6	8.5	0.5	8.0	1.4	*yqj* operon	similar to amino acid degradation
BLi01109	1.7	0.3	13.8	3.2	6.5	1.1	10.9	0.6	7.7	0.8		known subtilisin carlsberg precursor
Metabolism of coenzymes and prosthetic groups												
*BLi03994**	5.3	0.9	9.1	1.2	9.5	1.8	22.4	3.5	21.1	3.9		close homolog to DhaS: aldehyde dehydrogenase
*dhaS**	1.6	0.1	4.3	0.8	6.4	1.7	5.6	0.6	5.5	0.6	*dhaS*	aldehyde dehydrogenase
DNA replication												
ykoU	1.9	0.4	5.3	1.4	5.2	1.3	6.3	1.1	6.0	0.2	*yko* operon	similar to ATP-dependent DNA ligase
DNA restriction/modification and repair												
dinB	1.4	0.1	5.2	0.3	6.4	1.7	5.8	0.4	5.7	0.5	*dinB*	nuclease inhibitor
Transcription initiation												
*yvyD**	1.6	0.1	10.8	0.2	9.5	1.7	11.5	2.5	9.3	1.3	*yvyD*	similar to sigma-54 modulating factor
*sigX**	2.2	0.2	5.7	0.4	2.8	0.4	5.4	0.9	4.3	0.4	*sigX*	RNA polymerase ECF-type sigma factor

Table 5: continued

Gene name	Tr.ph	SD	30 min	SD	60 min	SD	90 min	SD	120 min	SD	Transcript. organisation	Function
Transcription regulation												
glnR	4.4	± 0.2	11.7	± 4.4	8.3	± 1.2	8.4	± 3.4	10.3	± 2.3	*glnR-A*	transcriptional repressor of the glutamine synthetase gene
gltC	1.2	± 0.2	3.5	± 1.0	3.9	± 0.7	5.2	± 0.4	4.8	± 1.1	*gltC*	transcriptional activator of the glutamate synthase operon
BLi03995	1.8	± 0.1	5.0	± 0.8	6.8	± 0.8	13.7	± 2.9	16.9	± 1.9		putative transcriptional regulation
BLi01176	8.3	± 1.1	31.2	± 7.0	29.9	± 2.9	33.7	± 5.5	30.8	± 8.3		putative nitrogen regulatory protein
rsiX	2.1	± 0.1	5.0	± 0.5	2.6	± 0.4	5.4	± 0.7	6.1	± 0.7	*sigX-rsiX*	negative regulator of sigma-X activity
ycnK	4.6	± 0.9	10.1	± 1.5	9.7	± 1.5	14.5	± 2.1	8.9	± 1.6	*ycnI-J-K*	similar to transcriptional regulator (DeoR family)
*ykoM**	2.1	± 0.1	5.8	± 0.9	6.7	± 1.0	7.2	± 2.0	6.2	± 0.9	*yko* operon	similar to transcriptional regulator (MarR family)
ywrC	2.2	± 0.2	5.2	± 1.1	3.1	± 0.4	6.5	± 0.8	4.4	± 0.9	*ywrA-B-C*	similar to transcriptional regulator (Lrp/AsnC family)
Adaptation to atypical conditions												
htrA	7.9	± 1.4	8.1	± 0.8	2.0	± 0.4	1.9	± 0.1	1.7	± 0.2	*htrA*	serine protease Do (heat-shock protein)
*BLi04126**	1.6	± 0.2	3.7	± 1.3	6.7	± 1.0	6.9	± 0.7	6.4	± 1.2		putative bacteriocin formation
Aminoacyl-tRNA synthetases												
thrZ	2.3	±	4.7	± 1.4	4.4	± 1.5	5.6	± 0.1	5.5	± 1.5	*thrZ*	threonyl-tRNA synthetase
Detoxification												
yceD	2.4	± 0.4	3.9	± 0.9	5.3	± 0.4	4.1	± 1.3	5.0	± 0.7	*yceC-D-E-F*	similar to tellurium resistance protein
yceE	2.1	± 0.1	3.6	± 0.8	5.9	± 1.3	5.4	± 1.0	7.4	± 0.7	*yceC-D-E-F*	similar to tellurium resistance protein
Unknown												
*BLi03989**	2.2	± 0.3	2.8	± 0.1	3.9	± 1.0	6.4	± 0.6	7.2	± 0.8		putative hydroxybenzoate hydroxylase
*BLi03991**	3.3	± 0.6	4.3	± 0.9	6.0	± 2.1	17.6	± 2.1	14.7	± 3.2		putative aromatic compounds specific dioxygenase
*BLi03993**	3.8	± 0.2	5.2	± 0.2	10.5	± 2.3	22.9	± 1.0	23.1	± 4.4		putative decarboxylase
BLi03996	1.5	± 0.1	2.3	± 0.3	4.2	± 1.0	8.8	± 1.5	9.3	± 0.5		conserved hypothetical
BLi01470	3.2	± 0.6	14.0	± 1.5	9.4	± 0.9	9.1	± 0.5	5.6	± 0.9		putative phage capsid protein
BLi02233	1.3	± 0.1	3.4	± 0.6	9.7	± 1.3	10.2	± 2.7	9.3	± 1.3		putative hydrolase
BLi01933	6.4	± 3.9	5.5	± 0.8	4.1	± 0.2	4.3	± 1.2	1.0	± 0.1		conserved hypothetical
BLi04120	1.7	± 0.1	2.3	± 0.6	2.9	± 1.0	7.6	± 2.3	6.2	± 0.9		conserved hypothetical
BLi01486	2.2	± 0.6	8.6	± 1.1	15.0	± 3.5	16.5	± 3.2	13.5	± 1.9		putative glycosyl hydrolase
*BLi00236**	3.0	± 0.8	5.2	± 0.6	7.4	± 2.2	7.7	± 2.8	7.7	± 2.8		hypothetical
BLi04184	2.0	± 0.2	5.2	± 0.1	5.1	± 0.9	4.7	± 0.2	4.4	± 0.5		hypothetical
BLi04116	1.7	± 0.2	2.3	± 0.5	1.8	± 0.2	7.4	± 3.3	7.9	± 1.7		hypothetical
BLi01488	7.9	± 1.1	73.9	± 16	93.2	± 18	147.4	± 12	51.7	± 11.3		hypothetical
BLi00719	6.5	± 0.3	17.7	± 9.9	15.0	± 3.4	18.2	± 2.2	15.9	± 4.8		hypothetical
*BLi00235**	5.0	± 0.8	10.8	± 1.3	8.0	± 1.2	11.4	± 2.3	9.3	± 1.9		hypothetical
*ypiB**	1.7	± 0.1	5.0	± 1.3	5.6	± 0.7	6.2	± 0.3	6.4	± 0.9	*ypiA-B-F*	unknown
yrkC	3.0	± 1.5	38.7	± 6.9	32.3	± 4.8	23.5	± 1.5	15.7	± 1.2	*yrkC*	unknown
yvlA	1.3	± 0.1	4.6	± 0.2	10.3	± 2.5	10.5	± 2.3	11.6	± 0.9	*yvlA*	unknown
yvlB	1.5	± 0.1	3.9	± 0.5	8.0	± 1.2	10.2	± 0.4	10.3	± 1	*yvlB-D*	unknown
*yvqH**	1.0	± 0.1	2.8	± 0.9	6.2	± 1.2	13.1	± 1.0	22.1	± 3.3	*yvq* operon	unknown
*yvqI**	1.0	± 0.1	2.8	± 0.6	5.2	± 1.0	10.4	± 1.9	21.6	± 3.1	*yvq* operon	unknown
yxkH	0.8	± 0.1	1.7	± 0.1	7.1	± 0.3	6.0	± 0.8	5.8	± 0.3	*yxkH*	unknown

Under nitrogen starvation conditions several genes involved in the regulation of transcription were induced; e.g. genes coding for the sigma factors σ^W and σ^X, two ECF-type sigma factors. Furthermore, *yvyD*, a gene that shows similarity to sigma-54 modulating factors from Gram-negative bacteria, was up-regulated. The gene of the important regulator of most nitrogen starvation genes, *tnrA*, was also induced. The *tnrA* mRNA level was found to be more than 14fold increased one hour after the onset of nitrogen starvation (Fig. 4). The *tnrA* gene was not spotted on the array, therefore its transcription was investigated by means of real-time-PCR. Beside this, GltC, the transcriptional activator of the glutamate synthase was induced. The mRNA level of *pucR*, the regulator of the *puc* genes involved in purine degradation, was also increased, although the *puc* genes themselves were not induced.

Nitrogen starvation led to an increased secretion of different proteases and peptidases in *B. licheniformis* when the cells reached the stationary phase (Fig. 3). Furthermore, the substrate binding proteins of several peptide transporter systems were found in the extracellular proteome of nitrogen starving cells.

The verification of selected mRNA levels by real-time–RT-PCR analysis showed similar trends in the induction patterns of the genes as determined by the transcriptome analysis (Fig. 4). For example, *glnA* revealed an about 12fold induction in the real-time-RT-PCR analysis, which was pretty consistent with the data of the transcriptome analysis. Although the overall induction ratios of the tested *trpE* gene were lower than in the microarray analysis, the real time RT PCR analysis did also show a slight increase of its mRNA level 30 min after the onset of the stationary phase. Finally, the *nrgB* mRNA level was found to be about 14fold increased by this expression analysis technique.

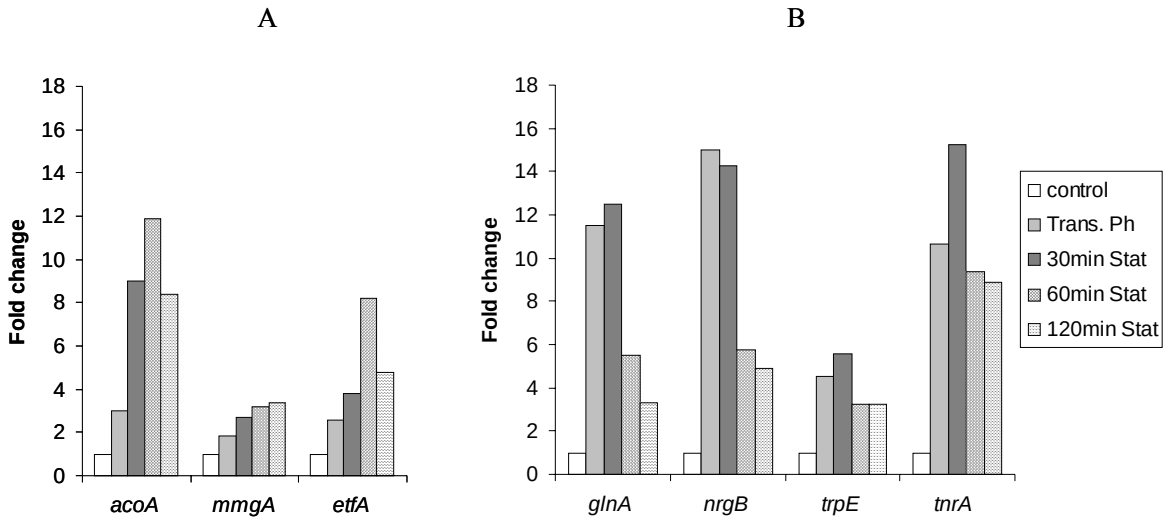


Figure 4: Verification of the induction of selected genes by glucose (A) and nitrogen (B) starvation conditions via real-time-RT-PCR. Shown are averaged data of two independent experiments.

Table 6: List of *B. licheniformis* genes with a potential *cre* binding box upstream or within the coding region. The *cre* binding boxes where identified by bioinformatic pattern searching, using the pattern TGWNANCGNTNWCA. This pattern identifies *cre* boxes in *B. subtilis* [44]. The positions indicate the location of the *cre* binding boxes relative to the first base of translation start. The column "induced/repressed" indicates by a '+' that the corresponding gene has been identified as induced by glucose limitation in this study. A '-' indicates, that the corresponding gene is experimentally identified as repressed by glucose in this study

Gene	Function	*cre* position start / stop		sequence	induced/ repressed
AdhB	alcohol dehydrogenase	77	90	TGATATCGTTGTCA	
AmyD	sugar transport	343	356	TGAAAGCGCTGACA	+
BglH	beta-glucosidase	-83	-70	TGAAAGCGTTGACA	+
BglP	PTS beta-glucoside-specific enzyme IIBCA component	1802	1815	TGAAAGCGTTGACA	-
BLi00198	hypothetical protein close homolog to RocF arginase	217	230	TGAAAGCGATTACA	+
BLi00491	conserved hypothetical protein	359	372	TGTAATCGATATCA	
BLi00639	putative transporter	-107	-94	TGATAACGCTTACA	
BLi00656	alpha-amylase precursor (EC 3.2.1.1)	1039	1052	TGAAAGCGGTTACA	+
BLi00658	maltogenic alpha-amylase glucan 1,4-alpha-maltohydrolase(EC 3.2.1.133)	-103	-90	TGTTACCGTTAACA	+
BLi00817	putative Na(+)-linked D-alanine glycine permease	991	1004	TGACATCGATGACA	
BLi01241	conserved hypothetical protein	-32	-19	TGAAAGCGATTACA	
BLi02062	putative sugar kinase, possible xylulose kinase	-28	-15	TGAAATCGCTATCA	
BLi02091	hypothetical protein	-143	-130	TGTTAGCGATCTCA	
BLi02115	putative glycerophosphodiester phosphodiesterase	476	489	TGTGATCGTTAACA	
BLi02331	hypothetical protein	-111	-98	TGAAATCGTTTTCA	
BLi03371	putative lipase precursor(EC 3.1.1.3)	-37	-24	TGAAAACGCTTACA	
BLi03503	protein putative dihydrodipicolinate synthase	31	44	TGACACCGCTTACA	
BLi03702	putative dihydrodipicolinate synthase	37	50	TGACACCGCTCACA	
BLi03865	putative PTS system, glucitol/sorbitol-specific IIBC component	554	567	TGAAACCGTTATCA	
BLi04069	putative galactonate transporter	-58	-45	TGATACCGTTTTCA	
BLi04163	arginine deiminase (Arginine dihydrolase) (EC 3.5.3.6)	-75	-62	TGTTATCGCTTTCA	
BLi04274	putative amino acid symporter	-106	-93	TGTAAGCGATTACA	
DnaA	initiation of chromosome replication	500	513	TGTCATCGATCACA	
Dra	deoxyribose-phosphate aldolase	527	540	TGACATCGGTGTCA	
FliF	flagellar basal-body M-ring protein	815	828	TGACATCGATTTCA	
GltP	H+/glutamate symport protein	-134	-121	TGTAAGCGATATCA	

Table 6: continued

Gene	Function	*cre* position start	*cre* position stop	sequence	induced/ repressed
GntZ	6-phosphogluconate dehydrogenase, decarboxylating(EC 1.1.1.44)	632	645	TGAAATCGCTGACA	
KdgR	transcriptional repressor of the pectin utilization operon	-40	-27	TGAAATCGGTTTCA	
KinD	two-component sensor histidine kinase involved in initiation of sporulation	713	726	TGAAATCGTTCACA	
LcfA	long chain acyl-CoA synthetase	412	425	TGAAAGCGCTAACA	+
LicB	PTS lichenan-specific enzyme IIB component	-73	-60	TGAAAACGGTTTCA	
LplA	lipoprotein	935	948	TGTGATCGATGTCA	
MaeN	Na+/malate symporter	19	32	TGAAACCGATTACA	
ManP	putative PTS mannose-specific enzyme IIBCA componen	7	20	TGTTAGCGATTACA	+
MmgC	acyl-CoA dehydrogenase	638	651	TGATAACGCTGTCA	+
NagA	N-acetylglucosamine-6-phosphate deacetylase	-31	-18	TGATAACGTTTTCA	
RbsR	transcriptional repressor of the ribose operon	20	33	TGTAAACGGTTACA	
SigL	RNA polymerase sigma factor	1177	1190	TGAAACCGCTTTCA	
UxaC	glucuronate isomerase	-42	-29	TGTTAACGTTAACA	
UxaC	glucuronate isomerase	-74	-61	TGTTAACGTTCACA	
UxuA	D-mannonate hydrolase	191	204	TGAAAGCGTTAACA	+
YcdC	hypothetical protein	722	735	TGATATCGATGACA	
YfiY	hypothetical protein; similar to iron(III) dicitrate transport permease	34	47	TGTTAGCGATTTCA	
YhfL	hypothetical protein; similar to long-chain fatty-acid-CoA ligase	436	449	TGAAATCGTTTACA	
YisL	hypothetical protein; similar to hypothetical proteins	-79	-66	TGACAGCGTTCACA	
YkoW	hypothetical protein; similar to hypothetical proteins	611	624	TGACAGCGTTGACA	
YndE	hypothetical protein; similar to spore germination protein	736	749	TGACAGCGATGACA	
YtcP	hypothetical protein; similar to ABC transporter (permease)	-96	-83	TGACAGCGCTTACA	
YtdP	hypothetical protein; similar to transcriptional regulator (AraC/XylS family)	919	932	TGTTAGCGCTTTCA	
YtsA	hypothetical protein; similar to two-component response regulator YtsB	563	576	TGTCAGCGATAACA	
YtsB	hypothetical protein; similar to two-component sensor histidine kinase YtsA	-129	-116	TGTCAGCGATAACA	
YvdG	hypothetical protein; similar to maltose/maltodextrin-binding protein	-90	-77	TGTTACCGGTAACA	+
YveN	hypothetical protein; similar to capsular polysaccharide biosynthesis	149	162	TGATATCGATATCA	
YvfH	hypothetical protein; similar to L-lactate permease	358	371	TGATAGCGTTTTCA	+
YvkB	hypothetical protein; similar to transcriptional regulator (TetR/AcrR family)	203	216	TGAAAACGATATCA	
YwcE	hypothetical protein; similar to hypothetical proteins	-37	-24	TGACAGCGCTATCA	

4. Discussion

4.1. Glucose starvation

The expression profiling experiments demonstrated that after exhaustion of glucose the expression of most glycolytic genes in *B. licheniformis* was repressed. The transcriptional down-regulation of glycolytic genes during glucose starvation was also reported by Koburger et al. (2005) for *B. subtilis*. It is known from *B. subtilis* that gene expression of glycolysis enzymes, which catalyse irreversible reactions (*pfkA*, *gapA*, *pykA*), is induced by glycolytic carbon sources (Tobisch et al. 1999). In *B. subtilis* the expression of the gylcolytic *gapA* operon is dependent on CcpA by an indirect mechanism (Ludwig et al. 2002b). Furthermore, *gapA* expression is repressed by the specific regulator CcgR as long as there are no glycolytic sugars present (Ludwig et al. 2001).

Glucose starvation in *B. licheniformis* led to the induction of genes encoding TCA cycle enzymes, which could be detected at the mRNA level for *citA*, *citB*, and *citG* and additionally at the protein level for CitZ and SdhA. CCR of genes encoding TCA cycle enzymes in *B. subtilis* is mediated by CcpC, a LysR-type transcriptional regulator (Jourlin-Castelli et al. 2000). CcpC represses the transcription of TCA cycle enzymes (*citZ*, *citB*) in the presence of glucose (Jourlin-Castelli et al. 2000). *B. licheniformis* cells growing in the presence of excess glucose show an increase in the amount of many glycolytic enzymes and simultaneously a repression of most TCA cycle enzymes (Voigt et al. 2004). This indicates that ATP is synthesised mainly via substrate phosphorylation if a sufficient amount of the preferred carbon source glucose is available. Under these conditions the TCA cycle fulfils primarily anabolic functions.

After onset of glucose starvation gluconeogenesis was switched on in *B. licheniformis* cells, indicated by the induction of *gapB, fbaB* and *pckA*. Transcription of *pckA* and *gapB* of *B. subtilis* is repressed by glucose via CcpN (Koburger et al. 2005; Servant et al. 2005).

There was also a quite strong induction of two genes encoding putative transketolases enzymes, which play a role in the pentose phosphate pathway. This is surprising because genes of the pentose phosphate pathway are expressed constitutively in *B. subtilis* and no regulation has been found in reaction to glucose concentration (Blencke et al. 2003). It is supposed that the reason for the constitutive expression of this pathway might originate from its major function in providing precursors and NADPH for anabolic reactions.

The genes for the pleiotropic regulator CcpA as well as for the specific regulators CggR,

CcpC and CcpN have been identified in the *B. licheniformis* genome sequence suggesting that similar complex regulatory mechanisms may be employed.

Under glucose starvation conditions the TCA cycle is also required to remetabolise secreted overflow metabolites such as acetoin or weak organic acids, which serve as alternative carbon and energy sources. The utilisation of such overflow metabolites by *B. licheniformis* cells during glucose starvation conditions was reflected by the induction of the *aco* and *acu* operons or the *acsA* gene. It is interesting to note that the *acu* operon had its induction peak early in the stationary phase (after 10 min), while the *aco* operon was fully induced not before 60 min after onset of the stationary phase. This sequential activation of the *acu* and the *aco* genes was also detectable at the protein level. The *aco* operon is δ^L dependent in *B. subtilis* (Ali et al. 2001). δ^L expression has been shown to be a subject of CCR mediated by CcpA (Choi and Saier 2005). In *B. licheniformis* a δ^L consensus sequence has been found upstream of the *acoA* gene (Veith et al. 2004). Furthermore, transcription of the *B. subtilis aco* operon is induced by AcoR, a protein that has similarities to transcriptional activators of δ^{54}-dependent promoters, and is itself negatively controlled by CcpA (Ali et al. 2001). In *B. licheniformis acoR* was among the glucose starvation induced genes suggesting a similar regulation. Since *B. licheniformis* possesses the enzymes of the glyoxylate cycle the overflow metabolites could be used during glucose starvation via this pathway. The two enzymes forming the glyoxylate cycle, isocitrate lyase and malate synthase were induced in glucose starving *B. licheniformis* cells.

The *alsRDS* operon encoding enzymes for acetoin formation was up-regulated in *B. licheniformis* during the transition phase and kept a slightly elevated mRNA level early in the stationary phase. The activation of these genes at a time very close to the induction of the acetoin metabolising enzymes (e.g. the *acu* operon) is because of the beginning exhaustion of glucose difficult to understand. Renna et al. (1993) observed a similar early induction of these genes in *B. subtilis*. AlsD and AlsS were synthesized in detectable amounts only in *B. subtilis* cells that reached the stationary phase. Although *ccpA* deficient cells do not produce acetoin, no *cre* site was found in the *alsSD* operon of *B. subtilis* (Turinsky et al. 2000). It is interesting to note that the *alsR* gene of *B. licheniformis*, which encodes a LysR family transcriptional regulator and which has been suggested to be an activator of *alsSD* transcription, is in contrast to *B. subtilis* not transcribed divergently from *alsSD* but is part of this operon.

Although no additional carbon sources except glucose or the already mentioned overflow metabolites were present in our medium, there were some pathways induced, which are responsible for the metabolisation of specific carbon sources. This includes genes required for

the utilisation of glycerol (e.g. *glpD*), myo-inositol (e.g *iolB-F*) and maltose (e.g. *malA*, *malL*). Furthermore, several sugar specific enzyme II components of the phosphotransferase system for sugar uptake were induced. Various other carbohydrate transport related genes (e.g. *yvd* operon, *amyC*, *amyD*) were among the glucose starvation induced genes too. In *B. subtilis* most genes involved in usage of alternative carbon sources are under the negative control of CcpA repressing them as long as glucose is present (Moreno et al. 2001; Yoshida et al. 2001; Blencke et al. 2003). A bioinformatic motif search for potential *cre* box sequences indicated a similar regulatory principle for selected genes of *B. licheniformis* by CcpA. For the genes *bglH*, *amyD* and the *yvd* operon putative *cre* boxes could be identified (Tab. 6). However, several other genes, for which potential *cre* boxes could be identified, showed only a weak (with an induction ratio under the threshold of five) or even no induction during glucose starvation.

In addition to switching to the usage of alternative carbohydrate sources *B. licheniformis* cells seem to be able to counteract carbon starvation by using other macromolecules, like proteins and lipids, as carbon sources. There was an induction of some intra- and extracellular proteases (e.g. *bpr*, *ispA*, *BLi00301*) indicating that the cells degrade proteins to use the resulting amino acids as alternative energy sources. Laishley and Bernlohr (1968) described the glucose repression of an extracellular protease of *B. licheniformis* indicating that this protease was subjected to CCR. In accordance with this is the induction of the *dpp* operon encoding a dipeptide ABC transporter during glucose starvation. Furthermore, there was a transient but strong induction of some genes involved in arginine, proline and glutamate degradation (*ycgM*, *ycgN* for proline, *rocF*, *rocD*, *ycgN* for arginine, *rocG* for glutamate degradation). In the *B. licheniformis* genome *ycgN* is the only gene encoding a 1-pyrroline-5-carboxylate dehydrogenase, there is no *rocA* gene. The proline permease gene *ycgO* and the putative arginine permease gene *rocE* were also up-regulated after onset of glucose starvation. For *B. licheniformis* Laishley and Bernlohr (1968) found a repression of the entire arginine degradation pathway by glucose, suggesting its control by CCR. In *B. subtilis* arginine degradation is also regulated by CCR in a CcpA dependent manner (Yoshida et al. 2001; Belitsky and Sonenshein 2004). In front of the *roc* genes δ^L promoters have been found in *B. subtilis* as well as in *B. licheniformis* (Belitsky and Sonenshein 1999; Veith et al. 2004). In addition, genes of the threonine degradation pathway were induced in glucose starving *B. licheniformis* cells (*tdh*, *kbl*). For both genes a CcpA dependently repression by glucose has been shown in *B. subtilis* (Moreno et al. 2001).

Furthermore, *B. licheniformis* cells induced in response to glucose starvation some genes involved in lipid degradation by β-oxidation. Induction of lipid degradation related genes was reported by Koburger et al. (2005) also for *B. subtilis* cells during glucose starvation. Some of the genes involved in β-oxidation have been found to be repressed by glucose in *B. subtilis*, some in a CcpA independent, some in a CcpA dependent manner (Blencke et al. 2003). In addition, Koburger et al. (2005) found the methylcitrate pathway induced by glucose starvation in *B. subtilis*. This pathway is necessary to feed the propionyl-CoA resulting from fatty acid degradation into the TCA cycle. The methylcitrate pathway genes (*mmgD*, *mmgE*, *yqiQ*) are as well among the glucose starvation induced genes in *B. licheniformis*.

Glucose starvation led to a strong induction of genes encoding electron transport chain proteins in *B. licheniformis*. Genes of the electron transfer pathway from menaquinol to oxygen (*qcr*ABC, *cta*CDEF) are glucose repressed in *B. subtilis* and this repression is directly mediated by CcpA (Moreno et al. 2001; Yoshida et al. 2001; Blencke et al. 2003). In *B. subtilis cre* sites have been found in front of these operons.

4.2. Nitrogen starvation

B. subtilis prefers glutamine, arginine and ammonium as nitrogen source (Fisher 1999). For *B. licheniformis* no studies have been published in which the preference to distinct nitrogen sources was addressed. In the present study no amino acids or ammonium were supplied as nitrogen source but only inorganic nitrogen sulfate. Assimilation of ammonium is mediated in *B. subtilis* by the glutamine synthetase (GS), which catalyses the formation of glutamine from ammonium and glutamate. In *B. subtilis glnA*, encoding the GS, forms an operon with the regulatory protein gene *glnR*. As long as nitrogen is present in the environment in abundance, GlnR represses transcription of the *glnRA* operon (Schreier et al. 1989; Brown and Sonenshein 1996). When *B. subtilis* cells become nitrogen starving, the synthesis of GS is drastically raised. The transcriptome and proteome analysis of this study demonstrates that *B. licheniformis* responses in a similar way to nitrogen limitation. The induced level of the *glnA* mRNA and protein synthesis in *B. licheniformis* was maintained throughout the stationary phase for at least 2 hours. In *B. subtilis* this raised synthesis of GS is due to the relief from repression by GlnR (Wray et al. 1996). TnrA, on the other hand, transcriptionally represses the expression of the GS and is therefore responsible for the fine-tuning of the *glnA* expression in *B. subtilis* (Wray et al. 1996; Belitsky et al. 2000; Wacker et al. 2003). However, the *glnR* and *tnrA* mRNA levels of *B. licheniformis* were also induced under

nitrogen starvation conditions and showed a similar expression pattern as *glnA*.

The second important enzyme in nitrogen metabolism beside the GS GlnA is the glutamate synthase, encoded by the *gltAB* operon. In *B. subtilis* expression of the *gltAB* operon is positively regulated by GltC, a member of the LysR family of transcriptional regulators, and negatively regulated by TnrA, which can repress transcription of the *gltAB* operon even when GltC is fully active (Belitsky and Sonenshein 1995; Belitsky et al. 2000). Nitrogen starvation in *B. licheniformis* caused an elevated transcription of *gltC*. There was also a slightly elevated synthesis of the GltA protein detected by the proteome analysis; however, the transcription of the corresponding gene was not increased. The GltB protein, which has a pI of about 7.7, could not be detected on our gels.

During nitrogen starvation conditions *B. licheniformis* cells induce genes the expression of which enable the mobilisation of alternative nitrogen sources. For example, the genes *nasABCDEF*, which are involved in the assimilation of nitrate and nitrite (Ogawa et al. 1995), were induced. NasA is a nitrate transport protein. NasB and NasC are subunits of the assimilatory nitrate reductase. NasD and NasE are subunits of the nitrite reductase. NasF is required for the synthesis of the siroheme cofactor of the nitrite reductase. Two of the genes up-regulated in nitrogen starving *B. licheniformis* cells were *nrgA* and *nrgB*. In *B. subtilis* NrgA, a homolog of the *E. coli* ammonium transporter AmtB (Wray et al. 1994; Coutts et al. 2002), is required for ammonium transport at low concentrations. A functional NrgB protein is necessary for full-level expression of the *nrgAB* operon in response to nitrogen starvation (Detsch and Stülke 2003). The gene *nrgB* encodes a homolog of the *E. coli* protein GlnK, a PII protein that prevents ammonium uptake at high ammonium concentrations by binding to AmtB (Wray et al. 1994; Coutts et al. 2002). Since there was another gene induced encoding a putative ammonium transporter (*BLi01175*), *B. licheniformis* might possess more than one system for ammonium uptake, as it has been suggested for other bacteria like *Corynebacterium glutamicum* (Meier-Wagner et al. 2001). Interestingly, the glutamine ABC transporter (*glnQHMP*) is absent in *B. licheniformis*.

It is interesting to note that only a limited number of the TnrA activated genes known from *B. subtilis* could be identified as induced by nitrogen starvation in *B. licheniformis*. This includes above all the *nasABCDEF* and *nrgAB* operons (Nakano et al. 1995; Wray et al. 1996). In contrast to *B. subtilis* the TnrA-dependent genes *pucJKLM* (genes of the purine catabolism, Beier et al. 2002) and *oppABCDF* (oligopeptide transporter genes, Yoshida et al. 2003) were not or only weakly induced under nitrogen starvation conditions.

In *B. subtilis* CodY is another important regulator, which controls the expression of several nitrogen metabolism genes (e.g. *hut* operon, *bdk* operon, *ureABC*, *gabP*, *ilv-leu* operon and *dpp* operon) but also of some genes in involved in acetate metabolism and in competence (e.g. *acsA*, *comK* and *srfA* operon) (Ferson et al. 1996; Fisher et al. 1996; Serror and Sonenshein 1996 a, b; Wray et al. 1997; Molle et al. 2003; Tojo et al. 2005). These genes are repressed by CodY when cells are grown in a medium rich in amino acids. In our experiments with *B. licheniformis* only an increased transcription of the *dpp* operon could be detected in the stationary phase, but none of the other potential CodY regulated genes showed an elevated expression under these conditions (the *hut* genes, *ureABC* and *gabP* are not present in the *B. licheniformis* genome). Expression of the *codY* gene itself was down-regulated in nitrogen starving *B. licheniformis* cells. Shivers and Sonenshein (2004) found in *B. subtilis* that the binding affinity of CodY for its target gene promoters is increased in the presence of branched-chain amino acids. Therefore, repression of the CodY regulated genes might have been not very strong during the exponential growth phase in our experiment since we used a minimal medium without amino acids in which CodY would not have been very active.

Among the genes repressed by nitrogen starvation in *B. licheniformis* were genes of different amino acid biosynthesis pathways, e.g the *ilv-leu* and the *arg* operons. These genes are also in *B. subtilis* (TnrA dependently) down-regulated under the conditions of nitrogen limitation (Tojo et al. 2004). The expression of genes involved in tryptophan (*trpA-F*) and histidin (*hisC*) synthesis, however, was up-regulated in *B. licheniformis*. Beside the induction of these genes also *tyrA*, encoding the prephenate dehydrogenase, was induced. In *B. subtilis* a co-regulation of the *trp* genes with *hisC* and *tyrA* has been found (Weigent and Nester 1976). Expression of the genes involved in tryptophan biosynthesis is regulated in *B. subtilis* in response to the tryptophan availability by the *trp* RNA-binding attenuation protein (TRAP) (Gollnick et al. 2005). The TRAP encoding gene *mtrB* has been found also in the genome sequence of *B. licheniformis* suggesting a similar regulation mechanism. On the other hand, genes of the degradation pathway of proline to glutamate have been found to be induced in *B. licheniformis* in response to nitrogen starvation. The proline permease gene *ycgO* was also up-regulated. Arginine seems to be not a preferred nitrogen source of *B. licheniformis* since the corresponding degradation enzymes (RocD and RocF) were induced during carbon but not during nitrogen starvation. *B. licheniformis* possesses an alternative arginine degradation pathway involving the arginine deiminase, an enzyme which degrades arginine to citrulline and ammonium but mainly during anaerobic growth (Broman et al. 1978). This enzyme (BLi04163) was neither induced during nitrogen starvation nor during carbon starvation.

In *B. subtilis hag* and other genes involved in motility and chemotaxis are σ^D controlled. Potential σ^D recognition sequences were also identified upstream of such genes in *B. licheniformis* (Veith et al. 2004). These genes are regulated in *B. subtilis* also by the repressor CodY (Bergara et al. 2003). This repression is relieved when nutrients, like carbon or nitrogen, become limiting, allowing expression of the genes. However, in *B. licheniformis* genes for motility and chemotaxis were repressed during carbon starvation as well as during nitrogen starvation conditions. The major flagellum protein Hag was even found to be fragmented during the glucose starvation induced transition phase (Voigt et al. 2006).

In *B. subtilis* and other Gram-positive bacteria a link between the carbon and nitrogen metabolism has been demonstrated (Faires et al. 1999, Wacker et al. 2003). It was found that this link is mainly mediated by the key regulator CcpA. The expression profiling of *B. licheniformis* cells of this study suggests that such a link also exists in the metabolism of this bacterium. In *B. subtilis* the expression of the TCA cycle enzyme aconitase (*citB*) is repressed by CcpA, although in an indirect manner, and activated by TnrA in addition to the regulation by the specific repressor CcpC (Blencke et al. 2006). Induction of *citB* was only observed when cells were grown with a slowly metabolisable carbon source and with limited nitrogen concentrations. Such a regulation seems be in place also in *B. licheniformis* cells, because *citB* expression is induced, although only weakly (1.5-4fold), during glucose starvation as well as during nitrogen starvation. Furthermore, the *yvyD* gene encoding a protein with similarities to σ^{54} modulation factors is induced by both starvation conditions. The *E. coli* YvyD homologue protein YfiA has been shown to bind to ribosomes and to inhibit the formation of translationally inactive 100S ribosomes (Ueta et al. 2005). Another indication is the increased expression of the genes *mmgDE* and *yqiQ*, encoding proteins of the methylcitrate shunt, under both starvation conditions.

The genome wide mRNA profiling analyses of this study allowed the elucidation of glucose and nitrogen stimulons of *B. licheniformis*. The induction and repression of more than 150 genes (higher than 5 fold) during glucose starvation and more than 80 genes (higher than 5 fold) under nitrogen limited growth demonstrates the drastic reprogramming of the metabolism and the adaptation of *B. licheniformis* cells to these specific adverse environmental conditions. By this approach we were able to identify transcriptome/proteome signatures and marker genes/ proteins which are specific for both nutrient starvation conditions. The knowledge of these maker genes and gene expression signatures can be used to better simulate and monitor the physiological state and productivity of *B. licheniformis* cell populations in industrial bioprocesses.

Acknowledgments

Le Thi Hoi and Birgit Voigt contibuted equally to this work. This work was supported by a sholarship to Le Thi Hoi from the Ministry of Education and Training of Vietnam (MOET) and by funds of the Competence Network "Genome Research on Bacteria" financed by the German Federal Ministry of Education and Research (BMBF). This work was also supported by the Ministry of Culture and Education of Mecklenburg-Vorpommern. We thank Stefanie Leja and Anne Krause for excellent technical assistance. We are grateful to the Decodon GmbH (Greifswald, Germany) for their cooperation.

Chapter IV

The regulation of the phytase gene expression in *Bacillus licheniformis*

Le Thi Hoi[1], Birgit Voigt[2], Britta Jürgen[1], Michael Hecker[2], and Thomas Schweder[1]

[1]Institut für Pharmazie, Ernst-Moritz-Arndt Universität, Greifswald.
[2]Institut für Mikrobiologie, Ernst-Moritz-Arndt Universität, Greifswald.

Keywords: *Bacillus licheniformis*, phytase, regulation, expression system

ABSTRACT

The phytase of *B. licheniformis* belongs to the most abundant proteins in the extracellular proteome during phosphate limited growth conditions. It is shown that the *phy* gene is strongly induced under phosphate starvation conditions at the transcriptional level. Furthermore, it is demonstrated that *B. licheniformis* is able to grow with phytate as the only phosphate source. However, no induction of the phytase gene expression in *B. licheniformis* cells under these conditions could be detected. In a glucose-limited culture with phytate as the only phosphate source only a very weak *phy* induction could be observed. These data indicate that the synthesis of the phytase is not regulated in dependence on the presence or absence of phytate. Finally, the *phy* promoter sequence was analysed by reporter gene fusion. For this purpose different fusions using the *phy* promoter sequence and the alpha-amylase gene of *B. subtilis* were constructed as single copy on the chromosome and as multi copy on a plasmid. The multi copy fusions showed a strong expression of the α-amylase gene under the control of the *phy* promoter in phosphate starving cells. Whereas, the α-amylase was hardly detcetable in the *B. licheniformis* strain bearing the single chromosomal fusion.

1. Introduction

Phytases are *myo*-inositol hexakisphosphate phosphohydrolases, which degrade phytate to release phosphate (Pi) and a series of lower phosphate esters of *myo*-inositol (Powar and Jagannathan 1982; Wodzinski and Ullah 1996; Kerovuo et al. 2000). Phytate, the salt of phytic acid, is the major storage form of phosphate in most mature plant seeds (Reddy et al. 1982). It is widespread distributed in nature such as in animals, plants, particularly fungi and microorganisms (Wodzinski and Ullah 1996; Dvorakova 1998).

The phytase belongs to a subgroup of phosphatases, which are a diverse group of enzymes. The hydrolysis of substrates by phosphatases is an important metabolic process in biological systems. It is related to energy metabolism, gene regulation and a wide variety of cellular signaling pathways (Vincent et al. 1992).

The enzymes with phytate hydrolysing activity from plants, bacteria, yeast and fungi are divided into three groups (Tye et al. 2002). Group 1 includes acid phosphatases or histidine acid phosphatases (EC 3.1.3.2), group 2 comprises the regular phytases (EC 3.1.3.8), and the purple acid phosphatases (EC 3.1.3.2) belong to group 3. Fungi and *Escherichia coli* phytases belong to the first group. The group 2 contains mainly *Bacillus* phytases, their activities depend on calcium as a cofactor (Powar and Jagannathan 1982). Finally, phytases of plant species belong to group 3. Based on the carbon atom of the inositol ring, where the phosphate is split off, phytases of microbes and plants are named as 3-phytases and 6-phytases, respectively, except for fungi and *E. coli* (Greiner et al. 1993; Lassen et al. 2001; Lei and Porres 2003).

The phytase plays different roles in the cellular metabolism. In plants, the hydrolysis of phytase provides an important phosphate source for the growth of seedlings during germination. In addition, inositol trisphosphate is an important secondary messenger in transport and cellular signal transduction in plants (Berridge 1984; Berridge and Irvine 1984).

In microorganisms, the phytase gene expression is most frequently induced in response to inorganic phosphate starvation conditions in order to supply an alternative phosphate source for the cells (van Hartingsveldt et al. 1993; Idriss et al. 2002; Vohra and Satyanarayana 2003; Haros et al. 2005). In *B. subtilis*, phytase hydrolyses phytate to yield Pi and myo-inositol trisphosphate. Ca^{2+} is also released from this reaction because it is bound to the phytate and is required for the activity of the phytase (Powar and Jagannathan 1982). The phytase activity of microorganisms contributes also significantly to an increase of the phosphate availability during plant growth (Idriss et al. 2002).

In contrast, the role of the phytase in animals is not clear. Some animals possess a gene encoding multiple inositol polyphosphate phosphatase (MIPP) which evolved as a distinct group within the histidine acid phosphatases family. MIPP regulates the hydrolysis of inositol 1,3,4,5,6-pentakisphosphate and generates the secondary messengers inositol 1,4,5-trisphosphate and inositol 1,3,4,5- tetrakisphosphate in animals (Ali et al. 1993; Van Dijken et al. 1995; Craxton et al. 1997; Chi et al. 1999). Monogastric animals, which have low levels of phytate-degrading enzymes excrete large amounts of phosphate into the environment. This causes environmental pollution and serious ecological problems. Therefore, phytase is frequently added to animal feed to facilitate optimal growth as well as to reduce environmental phosphate pollution (Simons et al. 1990; Lei and Porres 2003).

For *B. licheniformis*, the phytase was identified as a major extracellular protein during the stationary phase under phosphate starvation conditions (Voigt et al. 2006). Transcriptome analysis of this organism also showed a very strong induction of the phytase gene under these conditions (Hoi et al. 2006, in press). Phytate therefore might be an important alternative phosphate source for *B. licheniformis*. Potential PhoP binding sites located upstream of the phytase gene have been identified.

In the present study, we investigated the expression and regulation of the phytase gene of *B. licheniformis* under different nutrient starvation conditions. Furthermore, we verified the application of the *phy* promoter for a potential alternative expression system.

2. Materials and methods

2.1. Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 1. Specific primers used for PCR amplification were synthesised by Invitrogen (Karlsruhe, Germany) and listed in Table 2.

Table 1: Bacterial strains and plasmids used in this study

	Strain or plasmid	Genotype or antibiotic resistance	Source or reference
Strains			
	E. coli DH10B	F⁻ *mcrA* △(*mrr-hsd*RMS-*mcr*BC) △*lac*X74 ϕ80d*lacZ*△M15 *deo*R *rec*A1 *nup*G *end*A1 *ara*D139 △(*ara*, *leu*)7697 *gal*U *gal*K λ⁻ *rps*L	Grant et al. 1990
	B. subtilis 168	TrpC2	Anagnostopoulos and Spizizen 1961
	B. licheniformis DSM13	Wild type	Veith et al. 2004
Plasmids			
	pUC18	Amp^r (2.66), cloning vector *E. coli*	Fermentas
	pBC16	Tet^r (4.63), cloning vector *Bacillus*	Tang and Wilson 1996
	pE194	Ery^r (3.73) plasmid containing the temperature sensitive *Bacillus* origin	Horinouchi and Weisblum 1982
	pAMYEJSE	plasmid containing a erythromycin resistance gene	Silbersack et al. 2006
	pDH1	pUC18 bearing 540-bp fragment of back region of *amyE* from *B. licheniformis* DSM13	this study
	pDH2	pDH1 bearing 1.2-kb fragment of *ery* from pAMYEJSE	this study
	pDH3	pDH2 bearing 550-bp fragment of front region of *amyE* from *B. licheniformis* DSM13	this study
	pDH26	pDH3 bearing the promoter *phy* from *B. licheniformis* DSM13 fused with reporter *amyE* from *B. subtilis* 168	this study
	pDH79	pDH26 bearing the temparature sensitive *Bacillus* origin from pE194	this study
	pDH25	pBC16 bearing the promoter *phy* from *B. licheniformis* DSM13 fused with reporter *amyE* from *B. subtilis* 168	this study

Table 2: List of primers used in this study

name	sequence
*Xba*I/*Kpn*I-amyF-rev	GCGATCTAGAGGTACCTTGCTTCGGAAGCGGAACAG
*Hind*III-amyF-fw	AGTCAAGCTTTTGATGACGGTCCGAATCCC
amyE-fw	ATGTTTGCAAAACGATTCAA
*Bam*HI-*amyE*-rev	CACTGGATCCTCATTAATCATCCTTGCAGGGTAT
*Kpn*I-*amyE*-rev	CACTGGTACCTCATTAATCATCCTTGCAGGGTAT
*Sac*I-amyB-fw	TTGCGGATCCCAGAACACAGGTCGTCAAGC
*Bam*HI-amyB-rev	CTTGGAGCTCCCGAGCGCGATTCGGTCCGA
*Afl*III-Repts-rev	GAGTACATGTATAAGTAAGTTAAGGGATGC
*Afl*III-Repts-fw	ACTGGAGCTCCAAGAAACAATCTCTTCTGC
*Xba*I-*phy* fw	TGCATCTAGATCGTATGCTAAATCTCACAG
phy-rev	TTGAATCGTTTTGCAAACATATATTAACCTCCTTTTGATA
phy fw for Northern	AAATCTGGCTTGATCGTTTAC
phy revT7 for Northern	CTAATACGACTCACTATAGGGAGAACCATACACCTCCTCAATATC
yclF fw for Northern	GCTTGCCAATCCAATGTG
yclF revT7 for Northern	CTAATACGACTCACTATAGGGAGACGATGATGCCGAAGTAGG
amyE fw for Northern and realtime RT-PCR	TGCCAATGATGATGAAGAGTC
amyE rev for Northern and realtime RT-PCR	TGATAGAGACAGAGGATGAACC
amyE revT7 for Northern and realtime RT-PCR	CTAATACGACTCACTATAGGGAGATGATAGAGACAGAGGATGAACC

2.2. Growth conditions

E. coli DH10B was cultivated in Luria Broth medium (LB). For maintenance of plasmids, either ampicillin (100 µg/ml final concentration), streptomycine (100 µg/ml final concentration) or kanamycin (30 µg/ml final concentration) was added to the growth medium. The *B. subtilis* 168 strain carrying the plasmid pBC16 was grown in LB medium in the presence of tetracyline (15 µg/ml final concentration). Precultures of *B. licheniformis* DMS13 were grown in LB medium. All strains were cultivated at 37°C.

For the investigation of the expression of the phytase promotor, *B. licheniformis* DSM 13 (equivalent to ATCC 14580, type strain from the German Collection of Microorganisms and

Cell Cultures, DSMZ GmbH, Braunschweig, Germany) was cultivated under vigorous agitation in minimal medium (MM) containing 15 mM $(NH_4)_2SO_4$, 8 mM $MgSO_4$ x 7 H_2O, 27 mM KCl, 7 mM Na-citrate x 2 H_2O, 50 mM Tris-HCl (pH 7.5) supplemented with 0.6 mM KH_2PO_4, 2 mM $CaCl_2$ x 2 H_2O, 1 µM $FeSO_4$ x 7 H_2O, 10 µM $MnSO_4$ x 4 H_2O and 0.2 % (w/v) glucose. For the starvation experiments the concentration of phosphate was reduced to 0.15 mM and the concentration of glucose was reduced to 0.08 % (w/v).

2.3. DNA manipulations

For the isolation of chromosomal DNA the PCR-Template Preparation Kit (Roche Diagnostics; Penzberg, Germany) was used according to the manufacturer's instructions. The High Pure Plasmid Isolation Kit from Roche Diagnostics (Penzberg, Germany) was used to isolate plasmid DNA. For the plasmid DNA isolation from *Bacillus* strains the addition of 10 mg/ml lysozyme to the suspension buffer was required and the incubation step was prolonged up to 20 min at room temperature (RT) after lysis buffer was added. Extraction of DNA from agarose gels was done using the gel extraction kit (Qiagen; Hilden, Germany) following the manufacturer's instructions. Restriction digestion and ligation of DNA were carried out following the manufacturer's instructions.

2.4. Transformation of *E. coli, B. subtilis* and *B. licheniformis*

The protocol described by Sambrook et al. (1989) was used for the transformation of electroporation-competent *E. coli* cells. Protoplast transformation of *B. subtilis* 168 was performed according to the procedures described by Chang and Cohen (1979).
In order to transform DNA into *B. licheniformis*, the DNA had to be methylated prior to the transformation. To obtain the methylase, a *B. licheniformis* cell extract was prepared. A single fresh colony was inoculated in 10 ml 416 medium (2 % trypton, 1 % yeast-extract, 1 % NaCl and 0.2 M sucrose) overnight at 37°C at 200 rpm. For the main culture, 1 ml of the overnight culture was diluted in 100 ml of the medium 416. The cells were incubated until the OD_{600nm} reached 0.6-0.9. To stop the growth, the culture was placed on ice for 10 min and the cell pellet was collected by centrifugation for 15 min at 6000 rpm, 4°C. Afterwards, the cell pellet was washed twice in 100 ml 0.9 % NaCl and resuspensed in 10 ml cell extraction buffer (20 mM Tris acetate pH 7,9; 1 mM DTT; 5 mM EDTA pH 8,0; 50 mM KAC and 1 ml/4g cell of protease inhibitor cocktail set II (Calbiochem; Darmstadt, Germany)). Disruption of cells was carried out with the High Intensity Ultrasonic Liquid Processor. Afterwards the supernatant

containing the methylase was collected by centrifugation for 5 min at 13000 rpm, 4°C. In order to perform the methylase reaction 10-15 µg plasmid DNA (250 µl) and 250 µl of the cell extract were mixed with 1.25 µl of 32 mM S-Adenosylmethionin (SAM, New England Biolabs; Frankfurt am Main, Germany) and incubated at 37°C for 1 hour. The methylated plasmid DNA was twice purified by phenol and subsequently by chloroform:isoamylalcohol treatment. To precipitate the DNA, 2.5 volumes of 100% ice-cold ethanol and 1/10 volume of sodium acetate (pH 5.2) were added. The DNA-pellet was harvested by centrifugation at 14000 rpm at 4°C for 20 min. The pellet was washed twice with ice-cold 70% ethanol and dried in the Speed-Vac before it was dissolved in 25 µl of 10 mM Tris-HCl pH 8.5. The methylated DNA was then transformed into the cells by the polyethylene glycol-induced protoplast transformation method of Chang and Cohen with the modifications described by Quax (1990).

2.5. Construction of the single and multi copy P_{phy}-*amyE* translational fusions

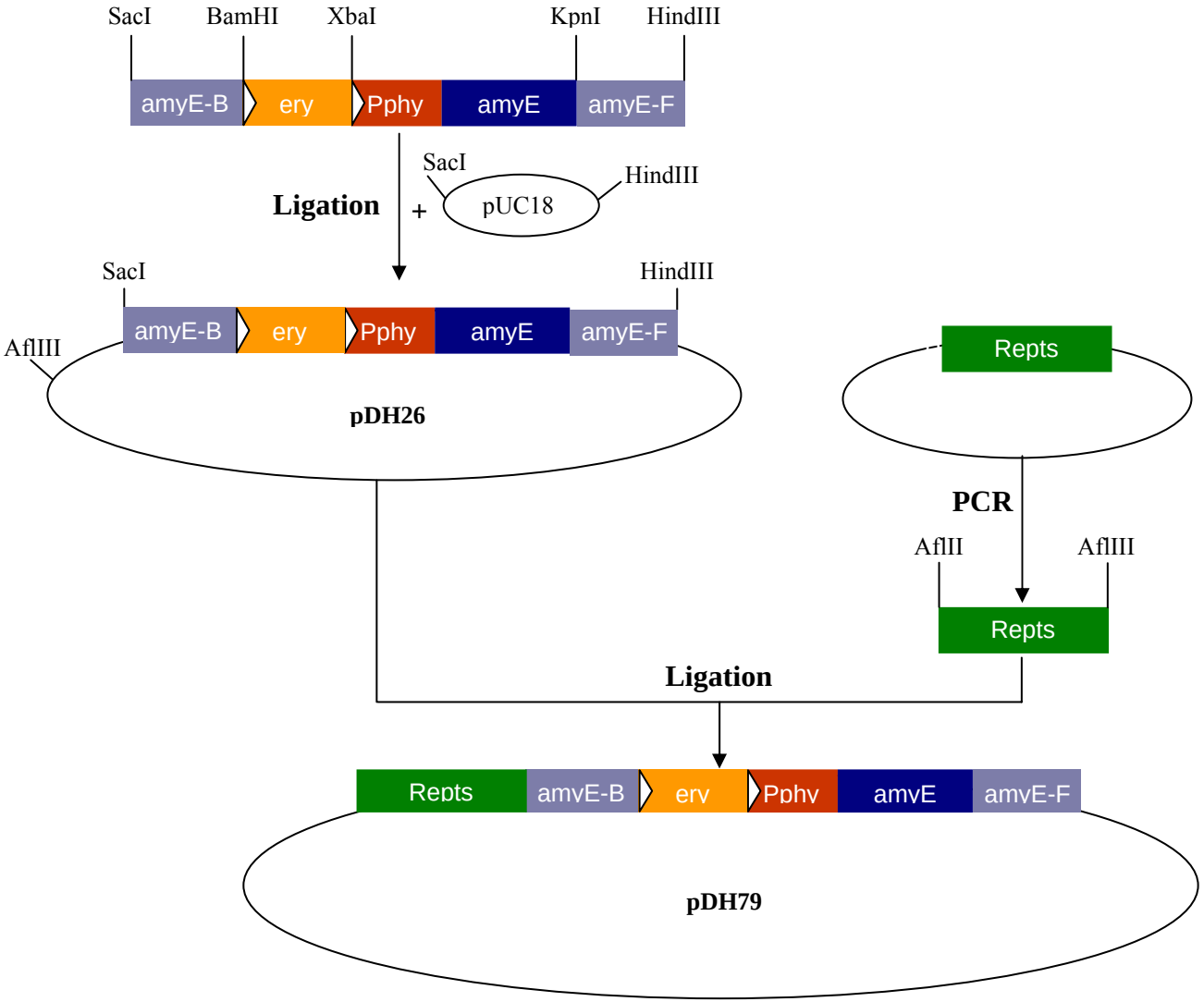
In order to construct the P_{phy}-*amyE* fusion on the multi copy plasmid pBC16, the promoter region of the *phy* gene containing the Pho box binding sites and the ribosome binding site was amplified using the primers *Xba*I-*phy*-fw and *phy*-rev containing a 20 bp repeated sequence from the start codon of *amyE*. The entire *amyE* gene including the signal sequence was amplified using the primers *amyE*-fw and *Bam*HI-*amyE*-rev. The PCR directed fusion was performed using the 120-bp *phy* and 2189-bp *amyE* PCR products as templates together with the forward primer *Xba*I-*phy*-fw and the reverse primer *Bam*HI-*amyE*-rev. The PCR product of the P_{phy}-*amyE* fusion was subcloned into the *E. coli* pCR-XL TOPO vector. The P_{phy}-*amyE* fusion was then isolated from this host vector by digesting it with *Xba*I and *Bam*HI. The digested fragment was ligated to plasmid pBC16 restricted with the same enzymes, resulting in plasmid pDH25. The recombinant plasmid pDH25 was used to transform *B. licheniformis* cells as described above. Positive transformants were selected on DM3 medium (Quax 1990) containing 10 µg/µl of tetracyline. The sequence of the recombinant plasmid bearing P_{phy}-*amyE* was verified by the Agowa sequencing service (Berlin, Germany).

A *B. licheniformis* DSM13 strain carrying a chromosomal P_{phy}-*amyE* fusion in the *amyE* locus was constructed based on the plasmid pUC18 (Figure 1). The entire *amyE* reporter gene containing also its signal peptide sequence was amplified by using the primers *amyE*-fw and *Kpn*I-*amyE*-rev. The PCR directed fusion of the promoter P_{phy} and *amyE* was performed as described above. The 5'-front region of the *amyE* gene of *B. licheniformis* was amplified

using the *Hin*dIII-amyF-fw and *Xba*I/*Kpn*I-amyF-rev primers. The 3'-back region of *amyE* of *B. licheniformis* was amplified by using the *Sac*I-amyB-fw and *Bam*HI-amyB-rev primers. The erythromycine-resistance gene from the plasmid pAMYEJSE (Silbersack et al. 2006, submitted) was obtained by digestion of the plasmid pAMYEJSE at the *Xba*I and *Bam*HI restriction enzyme sites. The temperature sensitive *Bacillus* origin (Repts) (Horinouchi and Weisblum 1982) was amplified by PCR, using the pE194 vector (Horinouchi and Weisblum 1982) as the DNA template and primers *Afl*III-Repts-fw, *Afl*III-Repts-rev. The 1788-bp amplified fragment was subcloned into pCR-XL TOPO vector and then isolated from this vector by digestion with *Afl*III.

Subsequently, the plasmid pUC18 was digested by *Sac*I and *Bam*HI. The 540-bp PCR product of the back region of *amyE* of *B. licheniformis* was inserted into pUC18 between the appropriate restriction enzyme sites, resulting in the first intermediate plasmid pDH1. The erythromycine-resistance gene from pAMYEJSE was inserted into pDH1 between the *Bam*HI and *Xba*I sites, resulting in the second intermediate plasmid pDH2. The 550-bp amplified fragment of the front region of *amyE* of *B. licheniformis* was obtained by digesting the pCR-XL TOPO vector at the *Hin*dIII and *Kpn*I sites. The digested fragment was inserted into pDH2 between these restriction sites, resulting in the third intermediate plasmid pDH3. The fragment of the P_{phy}-*amyE* fusion was inserted into pDH3 at the *Xba*I and *Kpn*I sites, resulting in the fourth intermediate plasmid pDH26. Finally, the temperature sensitive *Bacillus* origin was inserted into plasmid pDH26 at the *Afl*III sites to generate plasmid pDH79. Plasmid pDH79 was the final result of the construction. It was transformed into *B. licheniformis* protoplasts as described above. In order to inactivate the Repts and to enforce an integrative double-crossover recombination event at the *amyE* locus of the *B. licheniformis* chromosome, transformants were cultivated in 20 ml LB containing 1 µg/ml erythromycine until the cells reached an OD_{500nm} of 0.5. Afterwards, the cells were diluted 1:100 in new LB medium containing 1 µg/ml erythromycine, followed by a heat-shift treatment at 48°C overnight as described by Youngman (1990). The chromosomal DNA of the obtained clones was isolated and used as the template for the control PCR. To verify the sequence, the PCR product was sent to a sequencing service company (Agowa, Berlin, Germany).

Figure 1: Schematic presentation of the cloning procedure for the construction of the translational P_{phy}-*amyE* fusion plasmid used for the insertion of the fusion into the *amyE* locus of *B. licheniformis*.



2.6. Determination of the α-amylase activity

In order to analyse the α-amylase activity of *B. licheniformis* strains carrying the translational P_{phy}-*amyE* fusion the cells were grown in BMM with a limiting phosphate-concentration (0.15 mM). For the multi copy plasmid located fusions cells were harvested at the logarithmic growth phase (OD_{500nm} 0.4), transition phase (OD_{500nm} 1.0) and 1, 2, 3 hours after entry into stationary phase. To selected cultures phytate was added when the cells reached an OD_{500nm} of 1.0 in order to investigate whether phytate is an inducer of the *phy*-promoter. Samples from these cultures were harvested at the logarithmic phase (OD_{500nm} 0.4) before addition of phytate (OD_{500nm} of 1.0) and 1, 2, 3 hours after addition of phytate. The supernatant was used for the determination of the α-amylase activity. The α-amylase activity was determined using the α-amylase kit from Roche Diagnostics (Penzberg, Germany) following the manufacturer's instructions.

2.7. RNA isolation and Northern blot analysis

In order to investigate the phytase gene expression and to elucidate whether phytate is an inducer of the *phy*-promoter, *B. licheniformis* cells were cultivated in glucose-limited cultures and in cultures with a concentrated glucose containing minimal medium either supplemented with 1% phytate (final concentration) as the only phosphate source or with inorganic phosphate. The samples were taken during the logarithmic growth phase, and further samples were taken subsequently when the cells reached OD_{500} of 1 and 1and 2 hours later. For cells supplemented with 1% phytate at an OD_{500nm} of 1.0, the samples were taken at an OD_{500nm} of 0.4, before addition of phytate (OD_{500nm} of 1.0) and further samples were taken after addition of phytate at time intervals of 1 hour.

For the analysis of the α-amylase gene expression at the transcriptional level the *B. licheniformis* strains carrying the translational fusion of P_{phy}-*amyE* were cultivated under phosphate starvation conditions. The samples were taken during the logarithmic growth phase, OD_{500nm} of 1 and after the cells entered the stationary phase. In order to analyse whether phytate is an inducer, the cells were also supplemented in the late logarithmic growth phase (OD_{500nm} of 1.0) with 1% phytate (final concentration). The samples were taken at an OD_{500} of 0.4, before (OD_{500} of 1.0) and after addition of phytate at time intervals of 1 hour.

Total RNA was isolated using the RiboLyser for the cell disruption and the KingFisher for the purification of total RNA as described in detail by Jürgen et al. (2005b). Northern blot analyses were performed as described by Wetzstein et al. (1992). Hybridization, specific for

amyE, *phy* and *yclF*, were performed with appropriate digoxigenin-labelled RNA probes synthesized in vitro with a T7 RNA polymerase from the T7 promoter-containing internal PCR products of the respective genes using the primers listed in Table 2.

2.8. Real-time-RT-PCR

Real-time-RT-PCR was carried out with the LightCycler from Roche Diagnostics (Penzberg, Germany). Gene specific primers were deduced with the program Array Designer 2.0 (PREMIER Biosoft International, Palo Alto, USA) and ordered from Invitrogen (Karlsruhe, Germany) (Table 3). For the amplification validation the Cyber Green detection Kit from Roche Diagnostics (Penzberg, Germany) was used. Analysis and evaluation of the real-time-RT-PCR experiments was performed as described by Jürgen et al. (2005 a, b).

2.9. Western Blot analysis

In order to investigate the phytase protein expression in the extracellular medium, *B. licheniformis* was grown in glucose-limited minimal medium with phytate as the sole phosphate source or in glucose-limited minimal medium with phytate as an additional phosphate source. The control condition was phosphate-limited minimal medium. Cells were harvested during the exponential growth phase and 1 hour after the cells reached an OD_{500nm} of 1.0. The supernatant was collected and extracellular proteins prepared as described by Voigt et al. (2006).

An amount of 20 µg extracellular protein was loaded per lane in the 1D SDS-PAGE and the proteins were separated in the gel with 120 volt for 90 min. The gel was then blotted in a Mini-Trans-Blot cell (Bio-Rad, München, Germany) for 90 min at 200 mA whereas the proteins were transferred onto a polyvinylidene difluoride membrane. After blocking the membrane was incubated overnight with a polyclonal *B. licheniformis* phytase-specific antiserum (dilution 1:10000). Afterwards, the secondary antibody was added to the membrane for 4 hours. The secondary antibody (goat anti-rabbit immunoglobulin G) conjugated with alkaline phosphatase was used at a 1:200000 dilution (Sigma, Rödermark, Germany). Immunoblots were detected using nitroblue tetrazolium-5-bromo-4-chloro-3-indolylphosphate (0.033 % NTB, final concentration-0.017 % BCIP, final concentration), as a colour substrate (Sambrook et al. 1989).

2.10. Transcriptome analyses

Full genomic *B. licheniformis* DNA microarrays representing more than 95% of the ORFs longer than 300 bp were produced by spotting PCR products of 300 – 500 bp length in duplication on microarray slides (Corning Incorporated Life Sciences, Acton, USA) using a Lucidea contact printer (Amersham Biosciences, Freiburg, Germany) according to Hoi et al. (2006). The microarray analysis was carried out as described by Hoi et al. (2006). Only genes, which were at least 3-fold induced or repressed, were considered in this study. The values were calculated from at least 2 biological independent samples, obtained from 2 different experiments. Furthermore, the RNA of one of the biological samples was analysed twice, whereas the transcriptome of the second biological sample was investigated by Dye Swap. Both the biological samples as well as the Dye Swap analysis were included in the standard deviation calculation.

3. Results

3.1. Phytase gene expression under different growth conditions

Transcriptome and proteome analyses showed that the phytase gene is strongly induced under phosphate starvation conditions (Voigt et al. 2006; Hoi et al. 2006). In order to elucidate whether phytate is an inducer of the *phy*-promoter, we analysed *B. licheniformis* cells grown under conditions of glucose starvation and excess of glucose with phytate as the sole phosphate source. The growth curves of *B. licheniformis* under these conditions are shown in Figure 2. No significant differences in the growth rates of these different *B. licheniformis* cultivations could be detected.

Samples for the Northern blot analysis were taken throughout the different growth phases as indicated by arrows in the figure. Northern hybridisation analyses using a *phy*-specific mRNA probe showed very strong induction of the 1.1-kb *phy*-specific transcript under phosphate starvation conditions. However, only a slight induction of the *phy*-specific transcript could be detected in the presence of phytate under conditions of glucose starvation or in cultures with an excess of glucose (Figure 3).

Western blot analysis of the extracellular protein fraction using a polyclonal antiserum raised against *B. licheniformis* phytase showed that the phytase is strongly secreted only under phosphate starvation conditions (Figure 4). However, there was only a very low level of phytase observed under conditions of glucose starvation with phytate as the sole phosphate source or during glucose limitation conditions with addition of phytate. These results were consistent with the results of the Northern blot analyses as shown in Figure 3.

In *B. licheniformis* the *yclF* gene, coding for a protein similar to di-tripeptide ABC transporter membrane proteins, is located upstream of the phytase gene. Northern blot analysis using a probe specific for *yclF* indicated a significant induction of the 1.7-kb *yclF*-specific transcript under phosphate excess conditions. The transcript was not found in the phosphate-limited cells with additional phytate (data not shown).

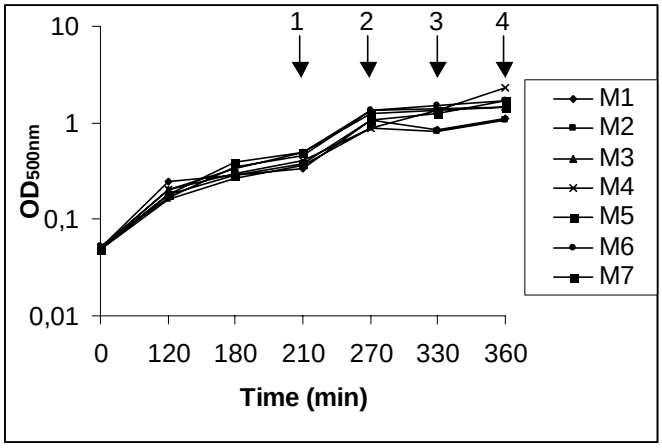


Figure 2: Growth curves of *B. licheniformis* under the conditions of 1: glucose-limited MM with 1% phytate as the sole phosphate source (M1); 2: glucose-limited MM with 1% phytate as an additional phosphate source (M2); 3: glucose-limited MM with 1% of phytate added when the cells reached an OD_{500nm} of 1.0 (M3); 4: excess glucose MM with 1% phytate as the sole phosphate source (M4); 5: excess glucose MM with 1% of phytate as an additional phosphate source (M5); 6: excess glucose MM with 1% phytate added when the cells reached an OD_{500nm} of 1.0 (M6); 7: phosphate-limited MM (M7)). The arrows indicate the time points at which samples were taken (1: exp. growth phase; 2: OD_{500nm} of 1; 3, 4: 1h and 2h after OD_{500nm} of 1.0, respectively).

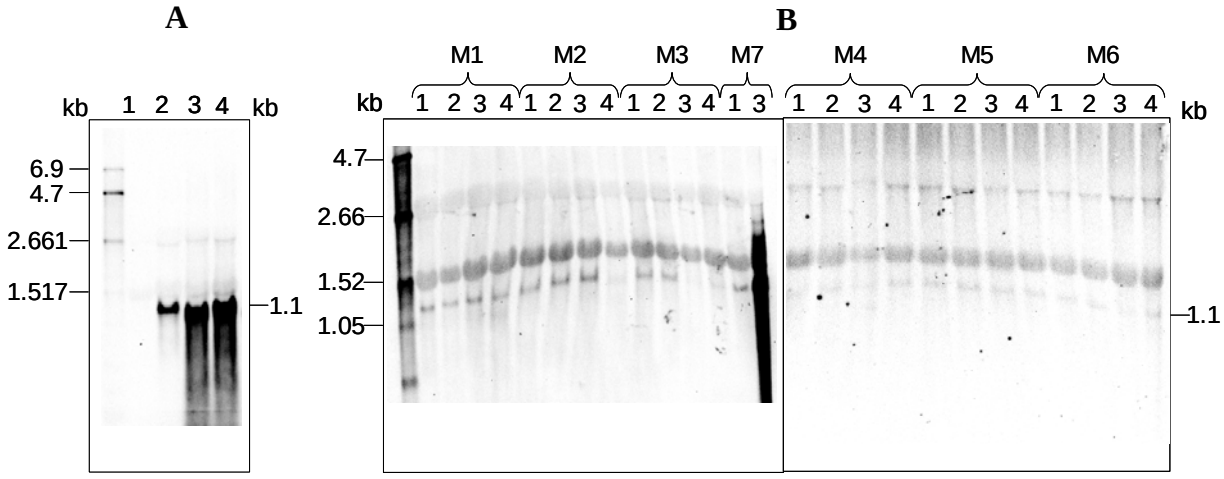


Figure 3: Northern blot analysis of the expression of the phytase in *B. licheniformis* cells grown under phosphate starvation conditions (A) and 1: glucose-limited MM with 1% phytate as the sole phosphate source (M1); 2: glucose-limited MM with 1% phytate as an additional phosphate source (M2); 3: glucose-limited MM with 1% of phytate added when the cells reached an OD_{500nm} of 1.0 (M3); 4: excess glucose MM with 1% phytate as the sole phosphate source (M4); 5: excess glucose MM with 1% of phytate as an additional phosphate source (M5); 6: excess glucose MM with 1% phytate added when the cells reached an OD_{500nm} of 1.0 (M6); 7: phosphate-limited MM (M7) (B). RNA samples were isolated at the time points indicated by arrows in Figure 3. 10 µg of total RNA was used.

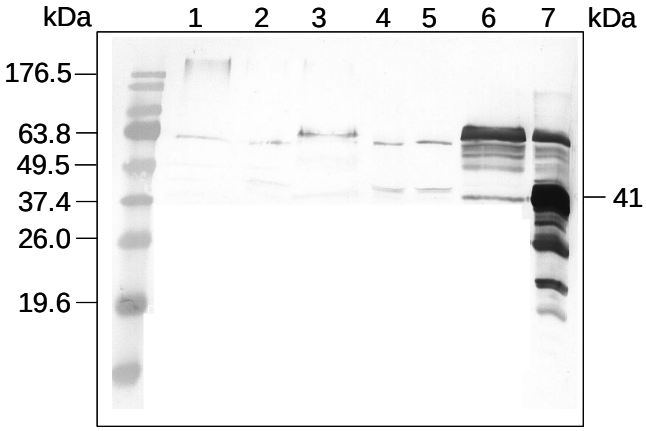


Figure 4: Western blot analysis of the extracellular phytase protein. Lane 1, 2, 3 total extracellular protein prepared from exponentially growing *B. licheniformis* cells under conditions of glucose-limitation with phytate as the sole phosphate source, glucose-limitation with phytate as the additional supplement and phosphate-limited minimal medium, respectively. Lane 4, 5, 6, 7 total extracellular protein prepared from growing *B. licheniformis* cells at 1 hour after OD_{500nm} of 1.0 under conditions of glucose-limitation with phytate as the sole phosphate source, glucose-limitation with phytate as the additional supplement, glucose-limited and phosphate-limited minimal medium, respectively. 20 µg of total extracellular protein were analyzed using the phytase-specific antibody.

For a global transcriptional profiling of the physiological state of *B. licheniformis* cells grown with phytate as the only phosphate source DNA microarray experiments were performed. For the control total RNA, isolated from exponentially growing *B. licheniformis* cells under phosphate excess conditions, was used. The investigated samples were total RNA isolated at the exponential growth phase (OD_{500nm} of 0.4), the transition phase (OD_{500nm} of 1.0) and 1 hour after entry into stationary phase from *B. licheniformis* cells growing with phytate as the only phosphate source. The microarray data revealed that only three genes (*yjcB*, *alsD*, *alsS*) were induced threefold in exponentially growing cells under these conditions. Some additional genes, including *dltA*, *dltB*, *yfmQ* and a gene encoding a putative serine protease (Bli00301), were about three to fourfold higher expressed when the cells reached an OD of 1.0 or 1 hour later.

According to a study of Kuwana et al. (2003) the *yjcB* gene together with *yjcA* and *yjcC* are involved in sporulation of *B. subtilis* cells. The transcription of *yjcB* depends on the σ^K RNA polymerase sigma factor. However, although the gene product is supposed to be involved in sporulation a clear function of *yjcB* in *B. licheniformis* is still not elucidated.

In *B. subtilis*, acetoin is a metabolite produced by overflow metabolism during growth on glucose (Ali et al. 2001). Genes required for the biosynthesis of acetoin are *alsS* and *alsD* (Renna et al. 1993). AlsS (α-acetolactate synthase) is involved in the production of acetolactate from pyruvate. Then, acetolactate is converted into acetoin by spontaneous

decarboxylation catalysed by the acetolactate decarboxylase encoded by the *alsD* gene.
DltA and *dltB* in *B. subtilis* are known to be involved in the D-alanine esterification of lipoteichoic acids and cell wall teichoic acids (Hyyrylainen et al. 2000). D-Alanine esterification decreases the negative charge of the cell wall as well as the binding capacity of cations to teichoic acid (Lambert et al. 1975). Mutants in the *B. subtilis dlt* operon are not able to alanylate teichoic acids. The absence of D-alanine leads to an increase of the rate of post-translocational folding of exported proteins because of the increased negative charge of the cell wall (Hyyrylainen et al. 2000). *YfmQ* is a gene of yet unknown function in *B. subtilis*.

3.2. The α-amylase expression of the different P_{phy}-*amyE* fusion strains

B. licheniformis possesses a number of restriction endonuclease enzymes. Therefore, it is difficult to transform foreign DNA into *B. licheniformis* cells. Foreign DNA must be methylated before transformation. The methylase catalyses the transfer of a methyl group from S-adenosylmethionine (SAM) to cytosine in CpG dinucleotides. For a successful transformation of foreign DNA into *B. licheniformis*, the polyethylene glycol-induced protoplast transformation method was applied (Quax 1990). Moreover, in case of the construction of the integration vector, a special strategy has been applied to increase the integration efficiency. A temparature sensitive *Bacillus* origin (Repts) was cloned into the integration vector (Neumuller et al. 2001).
The recombinant multi copy plasmid pDH25 bearing the P_{phy}-*amyE* fusion was transformed into *B. licheniformis* cells. To express the α-amylase gene from the *phy*-promoter, transformants were grown in BMM with limiting phosphate concentrations (0.15 mM). The determination of the α-amylase activity revealed very low level (data not shown). Therefore, we performed Northern hybridisation experiments using an *amyE*-specific probe in order to investigate the expression of *amyE* at the mRNA level. The results indicated a significant expression of the 2.3-kb *amyE*-specific transcript in the stationary growth phase under phosphate starvation conditions. The expression of *amyE* was switched off when phytate was added to the growth medium (Figure 5).
A plasmid carrying the P_{phy}-*amyE* translational fusion inserted in the *amyE* locus of *B. licheniformis* was also used to study the phytase promoter expression. Analysis of the α-amylase activity from theses strains showed very low expression levels during phosphate starvation (data not shown). The Northern blot experiments using the *amyE*-specific mRNA probe indicated a weak expression of the 2.3-kb *amyE*-specific transcript under phosphate

starvation conditions (Figure 6). The *amyE* mRNA levels of these strains were much lower than in the strains carrying the P_{phy}-*amyE* fusion on a multi copy plasmid.

Besides the analysis of the *amyE* expression by Northern-blot experiments, the sensitive real-time-RT-PCR technique was applied to more precisely evaluate the expression levels of the *amyE* mrNA in the strain carrying the multi copy plasmid and the strain bearing the P_{phy}-*amyE* inserted in the *amyE* locus. The results indicated that *amyE* expressed from the strain bearing P_{phy}-*amyE* inserted in the *amyE* locus was induced maximally threefold during the stationary phase under phosphate starvation conditions. In the multi copy plasmid carrying strain, the mRNA level of *amyE* were approximately tenfold induction (Figure 7).

From the results presented above, we suggest that the *amyE* transcript in the strains bearing the P_{phy}-*amyE* inserted in the *amyE* locus may be not very stable and for this reason we have relatively low mRNA levels.

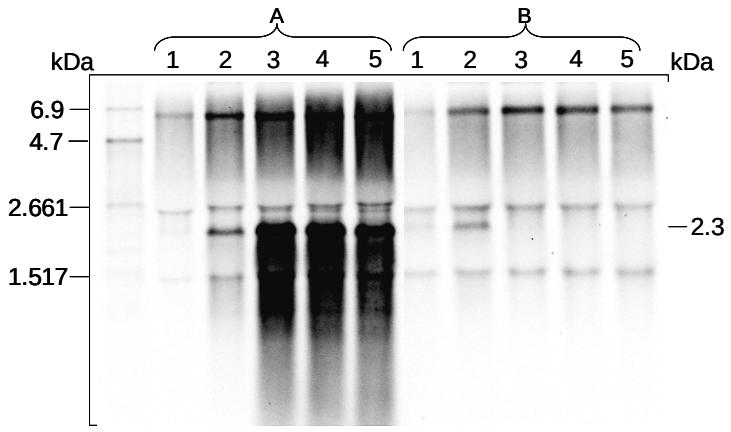


Figure 5: Northern-blot analysis of the *amyE* transcription from *B. licheniformis* cells containing the multi copy plasmid pDH25 grown under phosphate limited conditions (A) without phytate and (B) with addition of phytate (at an OD_{500nm} of 1.0). RNA samples were isolated from exponentially growing cells (OD_{500nm} of 0.4) (1), at an OD_{500nm} of 1.0 (2) and 1, 2, 3 hours after an OD_{500nm} of 1.0 or after addition of phytate (3, 4, 5). 10 µg of total RNA was used.

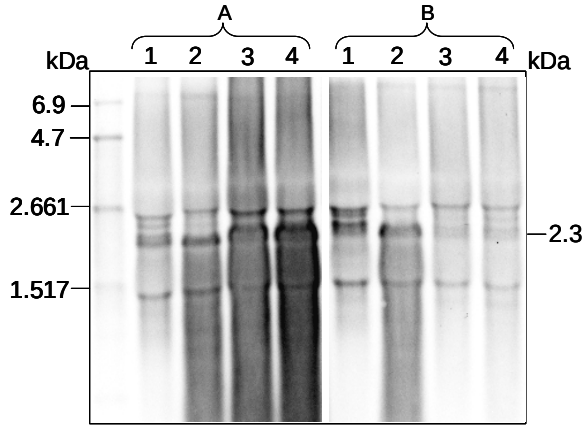


Figure 6: Northern-blot analysis of the *amyE* expression from *B. licheniformis* cells containing the single translational fusion P_{phy}-*amyE* in the chromosomal *amyE* locus grown under phosphate limited conditions (A) no phytate and (B) with addition of phytate (added at an OD_{500nm} of 1.0). RNA samples were isolated from exponentially growing cells (OD_{500nm} of 0.4) (1), at an OD_{500nm} of 1.0 (2) and 1, 2 hours after an OD_{500nm} of 1.0 or after adding of the phytate (3, 4). 10 µg of total RNA was used.

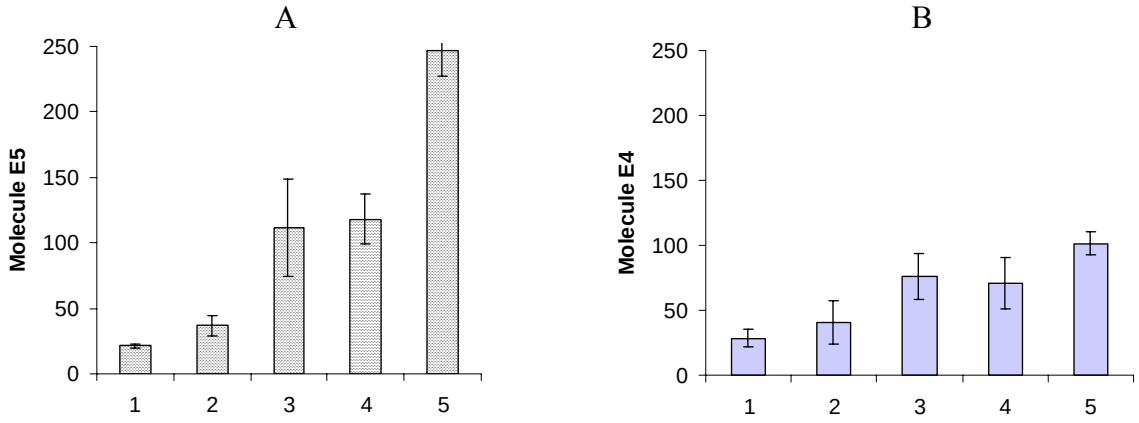


Figure 7: Verification of the *amyE* expression by phosphate starvation via real-time-RT-PCR.. Molecule number of the *amyE* transcript (A) in the multi copy strains, (B) in the chromosomal integration strains. RNA samples were isolated from exponentially growing cells (OD_{500nm} of 0.4) (1), at an OD_{500nm} of 1.0 (2) and 1, 2 and 3 hours after an OD_{500nm} of 1.0 or after adding of the phytate (3, 4, 5). 10 µg of total RNA was used.

4. Discussion

In this study we have analysed the expression of the phytase promoter under different growth conditions. The results indicate that the phytase is strongly synthesised when the cells grow under phosphate starvation conditions. However, when phytate was used as the sole phosphate source only a weak induction of the phytase gene both at the transcriptional and at the protein level was observed. Analysis of the microarray data suggest that a weak transcription of the *phy* gene is probably sufficient to ensure a basal level of the phytase, which ensures the hydrolysis of phytate and the release of inorganic phosphate for the growth of *B. licheniformis*. Our results strongly suggest that phytate is not an inducer of the *phy* expression. Nevertheless, the growth experiments indicate that phytate is a good alternative phosphate source for *B. licheniformis* cells.

The Northern-blot analyses of the *phy* and *yclF* gene, which is located upstream of the phytase promoter, demonstrate that the *phy* and *yclF* genes are transcribed monocistronically in a divergent manner. The *yclF* gene was in contrast to *phy* only active under phosphate excess conditions. The expression of this gene is turned off when phosphate sources become limiting.

The potential PhoP consensus binding boxes for the *phy* gene were identified by Hoi et al. (2006). The sequence of the phytase gene contains six potential binding sites for PhoP. This indicates that the expression of the phytase is probably regulated by the PhoPR two component system under phosphate starvation conditions.

The induction of the phytase expression by the substrate phytate varies in different organisms. For *E. coli*, the expression of the phytase was only observed when the cells starved for inorganic phosphate (Dassa et al. 1982; Touati et al. 1987; Greiner et al. 1993). Phytate had no influence on the synthesis of the phytase. Furthermore, under other nutrients starvation conditions such as carbon or nitrogen starvation the expression of the phytase was unaffected ((Dassa et al. 1982; Touati et al. 1987; Greiner et al. 1993)). Whereas, in *Klebsiella terrigena* the phytase was synthesised only in the presence of phytate and under carbon starvation conditions (Greiner et al. 1997). Phytase production of *Mitsuokella jalaludinii* was also induced by sodium phytate but repressed by the presence of glucose. Therefore, phytate was considered as an inducer of phytase in *M. jalaludinii* (Lan et al. 2002). In the presence of myo-inositol as the sole carbon source, the phytase expression was strongly induced in *Pseudomonas* sp. (Irving and Cosgrove 1971). In *Bacillus* species, the phytase was expressed under different nutrient conditions. The phytase of *B. subtilis* VTT E-68013 was induced

when the cells were grown in a defined medium containing phytate as the only phosphate source or in wheat bran medium (Kerovuo et al. 1998). The study of Powar and Jagannathan (1982) showed that *B. subtilis* 2712 produced a high amount of phytase in a medium containing phytate and yeast extract or wheat bran extract with addition of phytate. However, inositol had no effect on the induction of this enzyme. A strong induction of the phytase gene was also found in *B. amyloliquefaciens* growing in wheat bran medium (Kerovuo et al. 1998; Idriss et al. 2002). In contrast, for *Bacillus* sp. KHU-10 conditions used for the synthesis of phytase in *B. subtilis* 2712 gave unexpected results. *Bacillus* sp. KHU-10 produced an extracellular phytase at a high level in a medium containing maltose, peptone and beef extract (Choi et al. 2001). Inorganic phosphate is known as a repressor of phytase expression in most of the microorganisms producing phytases. However, repression of the phytase synthesis by inorganic phosphate was not observed in ruminal bacteria, since the levels of free phosphate are normally high in the rumen. Therefore, the regulation of phytase expression in these bacteria still remains obscure (Yanke et al. 1998).

Our findings suggest that the substrate induction of the phytase expression in *B. licheniformis* seems to be similar to that in *E. coli*. The phytase expression does not depend on the presence or absence of phytate and is therefore not substrate controlled.

Most of the *Bacillus* species analysed so far produce extracellular phytases (Shimizu 1992; Kerovuo et al. 1998; Kim et al. 1998; Choi et al. 2001; Idriss et al. 2002; Voigt et al. 2006). The activity of the phytase enzymes from most of these strains showed high specificity to phytate. *Bacillus* phytases degrade phytate to yield Pi and myo-inositol triphosphate as end products (Kerovuo et al. 2000; Idriss et al. 2002). Calcium is a cofactor required for the enzymatic activity of these enzymes. The biochemical characterisation of *Bacillus* phytases indicated that they do not belong to a subfamily of the histidine acid phosphatases (Kerovuo et al. 1998; Tye et al. 2002).

In parallel to the investigation of the phytase expression, we constructed an expression vector carrying a P_{phy}-*amyE* fusion. The strain containing the recombinant plasmid pDH25 showed a significant expression of the α-amylase under the control of the phytase promoter in phosphate starving cells. No expression of *amyE* was detected in the logarithmic growth phase. However, the α-amylase activity in the extracellular medium was very low. As indicated by Voigt et al. (2006), *B. licheniformis* secreted a number of proteases in response to different nutrient starvation conditions into the medium. It is possible that the *B. subtilis* α-amylase produced from our strains was cleaved by the action of some of these proteases

secreted by *B. licheniformis* during phosphate starvation conditions. For *B. subtilis* industrial production strains, genes encoding extracellular proteases were knocked out to prevent degradation of the overproduced proteins (Wu et al. 1991, 2002). The higher α-amylase expression in the strains containing the multicopy plasmid pDH25 compared to strains containing the chromosomally integrated α-amylase gene could be explained by the different copy number of the P_{phy}-*amyE* fusion in these strains. Moreover, the low expression level of the α-amylase in strains bearing the P_{phy}-*amyE* inserted in the *amyE* locus was likely due to a low *amyE* mRNA stability indicated by Northern-blot analyses.

Outlook

We are currently performing further experiments in order to investigate the influence of components of the Belitzky minimal medium on the expression of the phytase when phytate is present. Normally, the pH of the Belitzky minimal medium is 7.5. After the addition of phytate, the pH of the medium decreases to 5.5. We also started to analyse changes in the extracellular protein patterns of *B. licheniformis* cells when grown with phytate as the sole phosphate source by two-dimensional (2-D) polyacrylamide gel electrophoresis.

Chapter V

Summary and general discussion

So far there was only limited information available regarding the physiology and the regulation of the metabolism of *B. licheniformis*. Therefore, the aim of this thesis was to get a deeper insight into the physiology of *B. licheniformis* under changing environmental conditions, which might be also relevant for the industrial application of this bacterium.
The combination of the DNA microarray and the 2D-PAGE technology has been known to be a powerful tool for the global investigation of the cellular physiology of bacteria under various growth conditions. In this study, the physiology of *B. licheniformis* DSM13 was analysed using the DNA microarray and the 2D-PAGE technology under nutrient starvation conditions like phosphate, glucose and nitrogen starvation. In addition, the regulation of the expression of the phytase gene was investigated in detail, in order to test the use of the *phy* promoter sequence for an alternative expression system of *B. licheniformis*.

In Chapter II the phosphate starvation response of *B. licheniformis* was investigated in detail. The results of this study showed that a number of genes were induced or repressed under these conditions. More than 100 genes were identified as significantly induced (higher than 4 fold) under these conditions. Most of the strongly induced genes belong to the putative phosphate starvation specific Pho regulon (Hoi et al. 2006, in press). It is known from other organisms that the increased expression levels of these genes enable the cells to efficiently scavenge alternative organic phosphate sources from the environment in response to phosphate starvation. For example similar to *B. subtilis*, *B. licheniformis* increases the production of a high-affinity phosphate uptake system reflected by the induction of the *pstS-C-A-BA-BB* operon. In *B. licheniformis*, the genes *phoB* and *phoD* were shown to exhibit a very strong induction in phosphate starved cells. The *phoB* and *phoD* genes of *B. subtilis*, encoding an alkaline phosphatase and phosphodiesterase respectively, were also identified as the most prominent extracellular proteins under phosphate starvation conditions (Eymann et al. 1996; Antelmann et al. 2000). These genes are involved in the mobilisation of phosphate from alternative organic phosphate sources and cell wall teichoic acid turnover, respectively.
In addition, the high mRNA expression levels of the genes encoding nucleases such as *yhcR*, *yfkN*, *nucB*, *yurI*, *rph*, and BLi03719 were found in *B. licheniformis*. Some of them were identified as new members of the Pho regulon in *B. subtilis* (Allenby et al. 2005). The strong induction of these genes reveals that the cells try to mobilize phosphate groups from intracellular and extracellular ribonucleic and desoxyribonucleic acids. In *B. subtilis* it was

found that the *glpQ* gene, encoding a glycerophosphoryl diester phosphodiesterase, is also strong inducible member of the Pho regulon. GlpQ of *B. subtilis* plays a role in the hydrolysis of deacylated phospholipids to glycerol 3-phosphate. The glycerol 3-phosphate produced by GlpQ is a substrate for alkaline phosphatases (Antelmann et al. 2000). The *glpQ* gene could be also found on the *B. licheniformis* genome. However, this gene was not induced at the transcriptional level under phosphate starvation conditions. Our results indicate that in the case of *B. licheniformis*, the *yhdW* gene could have this function. The *yhdW* gene of *B. licheniformis* encodes a putative glycerophosphoryl diester phosphodiesterase and its expression was strongly activated during phosphate starvation conditions.

One of the most interesting findings of the study presented in Chapter II is a very high level of the phytase of *B. licheniformis* in the extracellular proteome and a strong induction at the transcriptional level of its gene after onset of phosphate starvation. Phytases hydrolyse phytate to release phosphate. This data indicate that phytate is probably an important alternative phosphate source in the natural environment of *B. licheniformis*. *B. subtilis* also possesses a phytase gene, however no induction of *phy* has been found in phosphate starving cells.

In parallel to the increased expression of genes, *B. licheniformis* also represses the transcription of over 100 genes (lower than 4 fold). Most of them are involved in the stringent response (like *nusA*, *rplN*, *tsf)*, a general adaptation of cells to adverse growth conditions.

Besides the study of the phosphate starvation response, a genome-wide expression profiling of *B. licheniformis* responses to glucose and nitrogen starvation was performed (Chapter III). In response to glucose starvation, *B. licheniformis* induces the expression of over 250 genes. Most of these genes seem to play a role in the mobilization of alternative carbon sources. The regulation of the gene expression involved in carbon metabolism in *B. subtilis* is under the control of a complex regulatory network including the main regulators CcpA, CcpN, CcpC and CggR (Jourlin-Castelli et al. 2000; Stülke and Hillen 2000; Ludwig et al. 2001; Servant et al. 2005). CcpA plays a key role in carbon catabolite repression. It acts as a repressor of genes involved in the utilisation of secondary carbon sources or as an activator of carbon excretion pathways under conditions of excess of glucose. In our study we could identify several glucose induced genes of *B. licheniformis* which carry a potential *cre* sequence. The *cre* sequence functions as the CcpA binding site.

Similar to *B. subtilis*, when *B. licheniformis* cells are starved for glucose, they activate the expression of genes responsible for the utilisation of alternative carbon sources and simultaneously they repress the synthesis of most glycolytic enzymes. At the same time, the

induction of gluconeogenesis (*gapB, fbaB* and *pckA*) and some TCA cycle enzymes was observed. The use of a variety of carbon sources and amino acids as sole carbon sources is common in bacteria. As expected, our results also show that *B. licheniformis* promotes the expression of a number of genes involved in the catabolism of glycerol (*glpD, F, Q*), acetoin and acetate utilisation (*acoABCLR, acuAC* and *acsA*), inositol (*iol* operon and *idh*, *mmsA*) as well as arginine, ornithine (*rocDEF* and close homolog to RocF) and threonine (*tdh, kbl*) under glucose starvation conditions. In *B. subtilis* most of these genes possess a *cre* box and are thus controlled by catabolite repression. In the case of *B. lichenniformis* for some of these genes such a potential *cre* box could be identified, which indicates also a catabolite repression of these genes. During growth on glucose, overflow metabolite like acetoin is secreted into the extracellular medium by *B. subtilis* (Ali et al. 2001). When glucose becomes exhausted, acetoin in turn serves as an alternative carbon and energy source for the cellular metabolism. This is reflected by the strong induction of the *acoABCL* operon of *B. licheniformis* under glucose starvation conditions. Degradation of threonine, arginine and ornithine in *B. licheniformis* may supply precursors for the biosynthesis of new glucose via gluconeogenesis. Glycerol also is a metabolisable glycolytic carbon source and it may serve as a secondary carbon source in glucose-limited cells. Similar to *B. subtilis*, *myo*-inositol is also an alternative carbon source for *B. licheniformis* under these conditions. The expression of the *B. subtilis iol* operon is a subject of CcpA-dependent regulation. In the case of the *iol* operon of *B. licheniformis* no *cre* box could be identified. However, glucose repression of the *iol* operon in *B. subtilis* is partially also CcpA-independently regulated (Miwa et al 2001; Yoshida et al. 2001).

Besides the increase of the expression of genes involved in the catabolism of various carbon sources and amino acids, the degradation of lipids probably also provides a significant energy source for *B. licheniformis* cells for a maintenance metabolism under glucose starvation conditions. It may produce additional sources for the generation of acetyl-CoA, a precursor of TCA cycle and gluconeogenesis. In glucose-limited *B. subtilis* cells, the products of genes which are involved in lipid degradation by β-oxidation, showed very strong induction. Expression of these genes is required for converting acetoacetate and propionyl-CoA to succinate and pyruvate via the methylcitrate pathway. Pyruvate then is utilised in the biosynthesis of new glucose (Koburger et al. 2005).

In addition to a detailed analysis of the glucose starvation response, a global view on gene expression patterns of *B. licheniformis* in response to nitrogen starvation was presented

(Chapter III). Nitrogen is an essential component of nearly all complex macromolecules in living cells. Therefore, in order to cope with nitrogen starvation situations, microorganisms have developed sophisticated response mechanisms that permit them to adapt to this detrimental environmental condition (Fisher 1999). The response of *B. licheniformis* to nitrogen limited conditions results in an increased expression of over 200 genes. Most of them are required for the recovery of nitrogen from secondary nitrogen sources.

One of the most prominent induced genes under nitrogen starvation conditions of *B. licheniformis* cells was similar to *B. subtilis* the *glnA* gene, coding for the glutamine synthetase. Furthermore, the mRNA level of *B. licheniformis* genes coding for proteins which are responsible for nitrate and nitrite assimilation was significantly increased in nitrogen starving cells. This is in agreement with the utilisation of nitrate and nitrite as sole nitrogen sources for growth of *B. subtilis* (Nakano et al. 1995; Ogawa et al. 1995). In addition, we also found a strong induction of *nrgA* and *nrgB* genes which are responsible for the uptake of ammonium at low ammonium concentrations. An interesting finding is that another gene encoding a putative ammonium transporter (BLi01175) of *B. licheniformis* was strongly induced. Probably *B. licheniformis* possesses two ammonium transporters as found in *Corynebacterium glutamicum* (Meier-Wagner et al. 2001). In C. *glutamicum*, the *amt* gene encodes an ammonium/methylammonium transporter, whereas the AmtB protein is highly specific for ammonium. An increased synthesis of *tnrA* was also observed in *B. licheniformis* during nitrogen starvation. However, it is interesting to note that unlike *B. subtilis*, the *gabP* gene encoding for gamma-aminobutyrate permease and *ureABC* genes involved in urea utilisation were not found in the genome of *B. licheniformis*.

In contrast to similar *B. subtilis* studies we could not detect an elevated synthesis of several CodY-dependent genes during nitrogen limited conditions. CodY is a GTP-binding repressor and active under conditions with high amino acid concentrations. In our experiments, *B. licheniformis* was cultivated in a defined salt minimal medium. Under these conditions the CodY is not full active during the exponential growth phase (Serror and Sonenshein 1996 a; Mirel et al. 2000). Furthermore, we also found a down regulation of *codY* and an up regulation of the known target of CodY, *dppABCDE* genes in nitrogen starved *B. licheniformis* cells.

Several previous studies have shown that there is a regulatory link between carbon and nitrogen metabolism in *B. subtilis* (Rosenkrantz et al. 1985; Ludwig et al. 2001, 2002 a; Sonenshein 2002; Wacker et al. 2003; Tojo et al. 2005). By the comparison of the gene

expression patterns under both starvation conditions we could demonstrate that there is also an interplay between carbon and nitrogen regulation in *B. licheniformis*. In *B. subtilis*, the transcription of the *citB* gene, encoding an aconitase enzyme in the TCA cycle, is such an example (Kim et al. 2003; Blencke et al. 2006). CcpA, CcpC and also the global repressors AbrB and CodY represses the expression of *citB* in the presence of glucose and amino acid availability (Fisher and Debarbouille 2002; Blencke et al. 2006). Similar to *B. subtilis*, we also found a slightly increased mRNA level of *citB* in glucose and nitrogen starved *B. licheniformis* cells. However, a significant repression of glycolytic enzymes was identified under both conditions in *B. licheniformis*. It was also reported for *B. subtilis* that glucose and a good nitrogen source are required for the full induction of glycolysis (Ludwig et al. 2001). Furthermore, the *yvyD* gene encoding a protein with similarities to σ^{54} modulation factors is induced by both starvation conditions. The *E. coli* YvyD homologue protein YfiA has been shown to bind to ribosomes and to inhibit the formation of translationally inactive 100S ribosomes (Ueta et al. 2005). An increased mRNA expression of the genes *mmgDE* and *yqiQ*, encoding proteins of the methylcitrate shunt, could be determined under both starvation conditions.

Besides the induction of starvation specific responses, microorganisms also produce the general stress response as well as the stringent response in order to cope with nutrient starvation conditions. The alternative sigma factor σ^B is known to be a major regulator of the general stress response in *B. subtilis* (Hecker and Völker 2001). It controls the transcription of a large number of genes involved in non-specific and prospective protection to non-growing cells against stress. However, our results indicate that unlike *B. subtilis*, *B. licheniformis* does not induce the σ^B-dependent general stress response, although *B. licheniformis* possesses the genes for the general stress response system (Price et al. 2001). In respect to the stringent response, our results clearly show that *B. licheniformis* switches on the stringent response under the three nutrient starvation conditions (phosphate, glucose and nitrogen starvation) tested.

The global transcriptional profiling expression by means of a combination of transcriptome and proteome analyses of *B. licheniformis* cells under different nutrient starvation conditions provides comprehensive insights into the cellular physiology. Based on this expression profiling, we were able to identify marker genes, which are specific indicators for distinct nutrient limitation conditions, e.g. *pstS*, *phy*, *phoB* or *yvnA* for a potential monitoring of

critical phosphate limitation situations; *acoA*, *mmgA*, *kbl* as biomarkers for glucose starvation and *glnA*, *nasE* for nitrogen starvation conditions. These marker genes could be useful indicators for monitoring of microbial fermentation processes in the future.

In Chapter IV a detailed analysis of the expression of the *B. licheniformis* phosphate starvation marker gene *phy*, coding for a phytase, is presented. The phytase was identified as one of the most prominent extracellular proteins when *B. licheniformis* was grown under phosphate starvation conditions (Voigt et al. 2006). This exorbitantly strong protein level of the phytase encouraged us to study the regulation of the expression of the corresponding gene in order to verify whether the *phy* promoter could be applied in an alternative expression system for *B. licheniformis* (Chapter IV). Phytase is an enzyme which is capable of degrading phytate to release phosphate and a series of lower phosphate esters like *myo*-inositol (Powar and Jagannathan 1982; Wodzinski and Ullah 1996; Kerovuo et al. 2000).

Our results indicated that the phytase promotor is strongly induced under phosphate starvation conditions. In the growth experiments, where phytate was added as the sole phosphate source, *B. licheniformis* DSM 13 cells were able to grow and seemed to utilize phytate as a good phosphate source. Despite the obvious good growth of the cells on phytate as the sole phosphate source there was only a low induction of the phytase gene expression in *B. licheniformis* cells under these conditions. A microarray analyses revealed that a selected genes (*yjcB*, *alsD*, *alsS*, *dltA*, *dltB*, and *yfmQ*) showed a three to fourfold induction under these growth conditions. The *yjcB* gene product is involved in sporulation of *B. subtilis* and its transcription depends on σ^K RNA polymerase (Kuwana et al. 2003). The genes *alsS* and *alsD* are required for the acetoin biosynthesis (Renna et al. 1993). The genes *dltA* and *dltB* are included in the D-alanine esterification of lipoteichoic acid and wall teichoic acid in *B. subtilis* (Hyyrylainen et al. 2000). The function of YfmQ is still unknown. Surprisingly, none of the genes, which were found to be upregulated during growth on phytate as the sole phosphate source, is somehow involved in the metabolism of phytate or any other phosphate sources.

The analysis of the *B. licheniformis phy* gene sequence showed that there were six potential Pho boxes. Furthermore, according to our results, its transcription seems to be strongly regulated by the PhoPR two component system under phosphate starvation.

Phytase is widespread in nature including microorganisms, plants and animal tissues (Wodzinski and Ullah 1996; Dvorakova 1998). Furthermore, microbial phytases play an important biotechnological role (Haefner et al. 2005). The substrate induction of the phytase

expression has been described for *Klebsiella terrigena*, *Mitsuokella jalaludinii*, *B. subtilis* VTT E-68013 and *B. subtilis* 2712 (Powar and Jagannathan 1982; Greiner et al. 1997; Kerovuo et al. 1998; Lan et al. 2002). The phytase expression was repressed by inorganic phosphate in almost all microorganisms tested except for ruminal bacteria (Yanke et al. 1998). In contrast to other organisms, our transcriptional analysis showed that the expression of the phytase gene in *B. licheniformis* is not induced by phytate. Based on these results, the utilization of phytate as phosphate source remains to be obscure. In order to elucidate this contradictory results, we investigated a potential influence of components in the Belitzky minimal medium on *phy* expression when phytate was added. Furthermore, we tried to identify the accumulation of the phytase under growth conditions with phytate as the sole phosphate source by two-dimensional (2-D) polyacrylamine gel electrophoresis.

Since the expression of the *phy* gene during phosphate starvation is remarkably high, the phytase promoter was tested for the construction of an alternative phosphate regulated expression system for *B. licheniformis*. The phytase promoter sequence was fused with the α-amylase gene from *B. subtilis* as a model reporter gene in order to perform expression studies. A multicopy plasmid (pBC16) bearing P_{phy}-*amyE* showed a strong expression of the α-amylase in *B. licheniformis* cells under phosphate starvation conditions. Whereas, the expression of the α-amylase was very low in *B. licheniformis* cells if the P_{phy}-*amyE* fusion was inserted into the chromosomal *amyE* locus.

References

Ali N., Craxton A. and Shears SB. (1993) Hepatic Ins(1,3,4,5)P4 3-phosphatase is compartmentalized inside endoplasmic reticulum. J Biol Chem 268:6161-7.

Ali NO., Bignon J., Rapoport G. and Debarbouille M. (2001) Regulation of the acetoin catabolic pathway is controlled by sigma L in *B. subtilis*. J Bacteriol 183:2497-504.

Allenby NE., O'Connor N., Pragai Z., Ward AC., Wipat A. and Harwood CR. (2005) Genome-wide transcriptional analysis of the phosphate starvation stimulon of *B. subtilis*. J Bacteriol 187:8063-80.

Alper S., Dufour A., Garsin DA., Duncan L. and Losick R. (1996) Role of adenosine nucleotides in the regulation of a stress-response transcription factor in *B. subtilis*. J Mol Biol 260:165-77.

Anagnostopoulos C and Spizizen J (1961) Requirements for Transformation in Bacillus Subtilis. J Bacteriol 81:741-746.

Antelmann H, Scharf C, Hecker M. (2000) Phosphate starvation-inducible proteins of *B. subtilis*: proteomics and transcriptional analysis. J Bacteriol. 182: 4478-90.

Antelmann H., Tjalsma H., Voigt B., Ohlmeier S., Bron S., van Dijl JM. and Hecker M. (2001) A proteomic view on genome-based signal peptide predictions. Genome Res 11:1484-502.

Beier L., Nygaard P., Jarmer H. and Saxild HH. (2002) Transcription analysis of the *B. subtilis* PucR regulon and identification of a cis-acting sequence required for PucR-regulated expression of genes involved in purine catabolism. J. Bacteriol. 184: 3232-3241.

Belitsky BR. and Sonenshein AL. (1995) Mutations in GltC that increase *B. subtilis* gltA expression. J Bacteriol 177:5696-700.

Belitsky BR. and Sonenshein AL. (1999) An enhancer element located downstream of the major glutamate dehydrogenase gene of *B. subtilis*. Proc Natl Acad Sci U S A 96:10290-5.

Belitsky BR. and Sonenshein AL. (2004) Modulation of activity of *B. subtilis* regulatory proteins GltC and TnrA by glutamate dehydrogenase. J Bacteriol 186:3399-407.

Belitsky BR., Wray LV Jr., Fisher SH., Bohannon DE. and Sonenshein A. L. (2000) Role of TnrA in nitrogen source-dependent repression of *B. subtilis* glutamate synthase gene expression. J Bacteriol 182:5939-47.

Bergara F., Ibarra C., Iwamasa J., Patarroyo JC., Aguilera R. and Marquez-Magana LM. (2003) CodY is a nutritional repressor of flagellar gene expression in *B. subtilis*. J Bacteriol 185:3118-26.

Bernhardt J., Buttner K., Scharf C. and Hecker M. (1999) Dual channel imaging of two-dimensional electropherograms in *B. subtilis*. Electrophoresis 20:2225-40.

Bernhardt J., Weibezahn J., Scharf C. and Hecker M. (2003) *B. subtilis* during feast and famine: visualization of the overall regulation of protein synthesis during glucose starvation by proteome analysis. Genome Res 13:224-37.

Berridge MJ. (1984) Inositol trisphosphate and diacylglycerol as second messengers. Biochem J 220:345-60.

Berridge MJ. and Irvine RF. (1984) Inositol trisphosphate, a novel second messenger in cellular signal transduction. Nature 312:315-21.

Birkey SM., Liu W., Zhang X., Duggan MF. and Hulett FM. (1998) Pho signal transduction network reveals direct transcriptional regulation of one two-component system by another two-component regulator: *B. subtilis* PhoP directly regulates production of ResD. Mol Microbiol 30:943-53.

Blencke HM., Homuth G., Ludwig H. and Mäder U. (2003) Transcriptional profiling of gene expression in response to glucose in *B. subtilis*: regulation of the central metabolic pathways. Metabolic Engin 5: 133-149.

Blencke HM., Reif I., Commichau, FM. and Detsch C. (2006) Regulation of citB expression in *B. subtilis*: integration of multiple metabolic signals in the citrate pool and by the general nitrogen regulatory system. Arch. Microbiol. 185: 136-46.

Blum H., Beier H and Gross HJ (1987) Improved silver staining of proteins, RNA and DNA. Electrophoresis 8:93-99.

Bohannon DE, Rosenkrantz MS and Sonenshein AL (1985) Regulation of Bacillus subtilis glutamate synthase genes by the nitrogen source. J Bacteriol 163:957-64.

Brandenburg JL., Wray LV Jr., Beier L., Jarmer H., Saxild HH. and Fisher SH. (2002) Roles of PucR, GlnR, and TnrA in regulating expression of the *B. subtilis* ure P3 promoter. J Bacteriol 184:6060-4.

Brans A, Filee P, Chevigne A, Claessens A. and Joris Ba-b (2004) New integrative method to generate *B. subtilis* recombinant strains free of selection markers. Appl Environ Microbiol 70:7241-50.

Braun P., Gerriste G., van Dijl JM. and Quax WJ. (1999) Improving protein secretion by engineering components of the bacterial translocation machinery. Curr. Opin. Biotechnol 10: 376-381.

Brody MS. and Price CW. (1998) Bacillus licheniformis sigB operon encoding the general stress transcription factor sigma B. Gene 212:111-8.

Broman K., Lauwers N., Stalon V. and Wiame JM. (1978) Oxygen and nitrate in utilization by Bacillus licheniformis of the arginase and arginine deiminase routes of arginine catabolism and other factors affecting their syntheses. J Bacteriol 135:920-7.

Bron S. and Luxen E. (1985) Segregational instability of pUB110-derived recombinant plasmids in *B. subtilis*. Plasmid 14:235-44.

Brown NL., Stoyanov JV., Kidd SP. and Hobman JL. (2003) The MerR family of transcriptional regulators. FEMS Microbiol Rev 27:145-63.

Brown SW. and Sonenshein AL. (1996) Autogenous regulation of the *B. subtilis* glnRA operon. J Bacteriol 178:2450-4.

Bruckner R. and Titgemeyer F. (2002) Carbon catabolite repression in bacteria: choice of the carbon source and autoregulatory limitation of sugar utilization. FEMS Microbiol Lett 209:141-8.

Burbulys D., Trach KA. and Hoch JA. (1991) Initiation of sporulation in *B. subtilis* is controlled by a multicomponent phosphorelay. Cell 64:545-52.

Büttner K, Bernhardt J, Scharf C, Schmid R, Mader U, Eymann C, Antelmann H, Volker A, Volker U, Hecker M. (2001) A comprehensive two-dimensional map of cytosolic proteins of *B. subtilis*. Electrophoresis 22: 2908-35.

Chang S. and Cohen SN. (1979) High frequency transformation of *B. subtilis* protoplasts by plasmid DNA. Mol Gen Genet 168:111-5.

Chatterji D., Fujita N. and Ishihama A. (1998) The mediator for stringent control, ppGpp, binds to the beta-subunit of Escherichia coli RNA polymerase. Genes Cells 3:279-87.

Chatterji D. and Ojha A. K. (2001) Revisiting the stringent response, ppGpp and starvation signaling. Curr Opin Microbiol 4:160-5.

Chen CC., Yudkin MD. and Delumeau O. (2004) Phosphorylation and RsbX-dependent dephosphorylation of RsbR in the RsbR-RsbS complex of *B. subtilis*. J Bacteriol 186:6830-6.

Chi H, Tiller GE, Dasouki MJ, Romano PR, Wang J, O'Keefe R J, Puzas JE, Rosier RN and Reynolds PR (1999) Multiple inositol polyphosphate phosphatase: evolution as a distinct group within the histidine phosphatase family and chromosomal localization of the human and mouse genes to chromosomes 10q23 and 19. Genomics 56:324-36.

Cho JS, LeeCW, Kang SH, Lee JC, Bok JD, Moon YS, Lee HG, Kim SC, Choi YJ (2003) Purification and characterization of a phytase from Pseudomonas syringae MOK1. Curr Microbiol 47:290–294.

Choi SK. and Saier MH Jr. (2005) Regulation of sigL expression by the catabolite control protein CcpA involves a roadblock mechanism in *B. subtilis*: potential connection between carbon and nitrogen metabolism. J Bacteriol 187:6856-61.

Choi YM., Suh HJ. and Kim JM. (2001) Purification and properties of extracellular phytase from Bacillus sp. KHU-10. J Protein Chem 20:287-92.

Coutts G., Thomas G., Blakey D. and Merrick M. (2002) Membrane sequestration of the signal transduction protein GlnK by the ammonium transporter AmtB. Embo J 21:536-45.

Craxton A., Caffrey JJ., Burkhart W., Safrany ST. and Shears SB. (1997) Molecular cloning and expression of a rat hepatic multiple inositol polyphosphate phosphatase. Biochem J 328 (Pt 1):75-81.

D'Souza M., Larsen N and Overbeek R (1997) Searching for patterns in genomic data. Trends Genet 13:597–498

Darbon E., Servant P., Poncet S. and Deutscher J. (2002) Antitermination by GlpP, catabolite repression via CcpA and inducer exclusion triggered by P-GlpK dephosphorylation control *B. subtilis* glpFK expression. Mol Microbiol 43:1039-52.

Dassa E., Cahu M., Desjoyaux-Cherel B. and Boquet PL. (1982) The acid phosphatase with optimum pH of 2.5 of Escherichia coli. Physiological and Biochemical study. J Biol Chem 257(12): 6669-76.

del Pozo JC., Allona I., Rubio V., Leyva A., de la Pena A., Aragoncillo C. and Paz-Ares J. (1999) A type 5 acid phosphatase gene from Arabidopsis thaliana is induced by phosphate starvation and by some other types of phosphate mobilising/oxidative stress conditions. Plant J 19:579-89.

Demain AL. (1999) Pharmaceutically active secondary metabolites of microorganisms. Appl Microbiol Biotechnol 52:455-63.

Detsch C. and Stülke J. (2003) Ammonium utilization in *B. subtilis*: transport and regulatory functions of NrgA and NrgB. Microbiology 149:3289-97.

Deutscher J, Galinier A and Martin-Verstraete I (2002) Carbon Uptake and Metabolism in: Sonenshein, A.L., Hoch , J.A., Losick, R.J. (ed.). *B. subtilis* and its closest relatives: from genes to cells. ASM Press, Washington, D.C pp.129-150.

Deutscher J., Kuster E., Bergstedt U., Charrier V. and Hillen W. (1995) Protein kinase-dependent HPr/CcpA interaction links glycolytic activity to carbon catabolite repression in gram-positive bacteria. Mol Microbiol 15:1049-53.

Deutscher J., Reizer J., Fischer C., Galinier A., Saier MH Jr. and Steinmetz M. (1994) Loss of protein kinase-catalyzed phosphorylation of HPr, a phosphocarrier protein of the phosphotransferase system, by mutation of the ptsH gene confers catabolite repression resistance to several catabolic genes of *B. subtilis*. J Bacteriol 176:3336-44.

Drzewiecki K., Eymann C., Mittenhuber G. and Hecker M. (1998) The yvyD gene of *B. subtilis* is under dual control of sigmaB and sigmaH. J Bacteriol 180:6674-80.

Dvorakova J. (1998) Phytase: sources, preparation and exploitation. Folia Microbiol (Praha) 43:323-38.

Ebersbach G. and Gerdes K. (2005) Plasmid segregation mechanisms. Annu Rev Genet 39:453-79.

Eymann C., Homuth G., Scharf C. and Hecker M. (2002) *B. subtilis* functional genomics: global characterization of the stringent response by proteome and transcriptome analysis. J Bacteriol 184:2500-20.

Eymann C., Mach H., Harwood CR. and Hecker M. (1996) Phosphate-starvation-inducible proteins in *B. subtilis*: a two-dimensional gel electrophoresis study. Microbiology 142 (Pt 11):3163-70.

Eymann C., Mittenhuber G. and Hecker M. (2001) The stringent response, sigmaH-dependent gene expression and sporulation in *B. subtilis*. Mol Gen Genet 264:913-23.

Faires N., Tobisch S., Bachem S., Martin-Verstraete I., Hecker M. and Stülke J. (1999) The catabolite control protein CcpA controls ammonium assimilation in *B. subtilis*. J Mol Microbiol Biotechnol 1:141-8.

Ferson AE., Wray LV Jr. and Fisher SH. (1996) Expression of the *B. subtilis* gabP gene is regulated independently in response to nitrogen and amino acid availability. Mol Microbiol 22:693-701.

Fieulaine S., Morera S., Poncet S., Mijakovic I., Galinier A., Janin J., Deutscher J. and Nessler S. (2002) X-ray structure of a bifunctional protein kinase in complex with its protein substrate HPr. Proc Natl Acad Sci U S A 99:13437-41.

Fisher SH. (1999) Regulation of nitrogen metabolism in *B. subtilis*: vive la difference! Mol Microbiol 32:223-32.

Fisher SH., Rohrer K. and Ferson A. E. (1996) Role of CodY in regulation of the *B. subtilis* hut operon. J Bacteriol 178:3779-84.

Fisher SH. and Sonenshein AL. (1991) Control of carbon and nitrogen metabolism in *B. subtilis*. Annu Rev Microbiol 45:107-35.

Fisher SH. and Wray LV Jr. (2002) Mutations in the *B. subtilis* glnRA operon that cause nitrogen source-dependent defects in regulation of TnrA activity. J Bacteriol 184:4636-9.

Fisher SH. and Debarbouille M (2002). Nitrogen source utilization and its regulation. In: A. L. Sonenshein, J. A.Hoch, and R. Losick (ed.), *B. subtilis* and its closest relatives: from genes to cells. ASM Press, Washington, D.C. pp.181-192.

Gaal T., Bartlett MS., Ross W., Turnbough CL Jr. and Gourse RL. (1997) Transcription regulation by initiating NTP concentration: rRNA synthesis in bacteria. Science 278:2092-7.

Galinier A, Deutscher J and Martin-Verstraete I (1999) Phosphorylation of either crh or HPr mediates binding of CcpA to the *B. subtilis* xyn cre and catabolite repression of the xyn operon. J Mol Biol 286:307-14.

Galinier A, Haiech J, Kilhoffer MC, Jaquinod M, Stülke J, Deutscher J and Martin-Verstraete I (1997) The *B. subtilis* crh gene encodes a HPr-like protein involved in carbon catabolite repression. Proc Natl Acad Sci U S A 94:8439-44.

Gollnick P., Babitzke P., Antson A. and Yanofsky C. (2005) Complexity in regulation of tryptophan biosynthesis in *B. subtilis*. Annu Rev Genet 39:47-68.

Gottschalk, G., *Bacterial metabolism*, Springer, New York 1986.

Greiner R., Haller E., Konietzny U. and Jany KD. (1997) Purification and characterization of a phytase from Klebsiella terrigena. Arch Biochem Biophys 341:201-6.

Greiner R., Konietzny U. and Jany KD. (1993) Purification and characterization of two phytases from Escherichia coli. Arch Biochem Biophys 303:107-13.

Grant SG, Jessee J, Bloom FR and Hanahan D (1990) Differential plasmid rescue from transgenic mouse DNAs into Escherichia coli methylation-restriction mutants. Proc Natl Acad Sci U S A 87:4645-9.

Grundy FJ., Waters DA., Allen SH., Henkin TM. (1993) Regulation of the *B. subtilis* acetate kinase gene by CcpA. J. Bacteriol. 175: 7348-7355.

Gupta R., Beg Q. K., Khan S. and Chauhan B. (2002) An overview on fermentation, downstream processing and properties of microbial alkaline proteases. Appl Microbiol Biotechnol 60:381-95.

Gutowski JC. and Schreier HJ. (1992) Interaction of the *B. subtilis* glnRA repressor with operator and promoter sequences in vivo. J Bacteriol 174:671-81.

Haefner S., Knietsch A., Scholten E., Braun J., Lohscheidt M. and Zelder O. (2005) Biotechnological production and applications of phytases. Appl Microbiol Biotechnol 68:588-97.

Haldenwang WG. (1995) The sigma factors of *B. subtilis*. Microbiol Rev 59:1-30.

Haros M., Bielecka M. and Sanz Y. (2005) Phytase activity as a novel metabolic feature in Bifidobacterium. FEMS Microbiol Lett 247:231-9.

Harwood CR., Wipat A and Pragai Z . (2002) Functional analysis of the *B. subtilis* genome. Methods Microbiol 33: 336-367.

Haseltine WA., Block R., Gilbert W. and Weber K. (1972) MSI and MSII made on ribosome in idling step of protein synthesis. Nature 238:381-4.

Hecker M., Richter A., Schroeter A., Wolfel L. and Mach F. (1987) [Synthesis of heat shock proteins following amino acid or oxygen limitation in *B. subtilis* relA+ and relA strains]. Z Naturforsch [C] 42:941-7.

Hecker M. and Völker U. (1998) Non-specific, general and multiple stress resistance of growth-restricted *B. subtilis* cells by the expression of the sigmaB regulon. Mol Microbiol. 29 :1129-36

Hecker M. and Völker U. (2001) General stress response of *B. subtilis* and other bacteria. Adv Microb Physiol 44:35-91.

Helmann JD and Moran CP (2002) RNA polymerase and sigma factors. In: Sonenshein AL, Losick R (eds) *B. subtilis* and its Closest Relatives. from genes to cells. ASM Press, Washington, D.C pp. 289-312.

Hoi LT, Voigt B, Jürgen B, Ehrenreich A, Gottschalk G, Evers S, Feesche J, Maurer KH, Hecker M and Schweder T (2006) The phosphate starvation response of Bacillus licheniformis. Proteomics.

Hoper D., Volker U. and Hecker M. (2005) Comprehensive characterization of the contribution of individual SigB-dependent general stress genes to stress resistance of *B. subtilis*. J Bacteriol 187:2810-26.

Horinouchi S. and Weisblum B. (1982) Nucleotide sequence and functional map of pE194, a plasmid that specifies inducible resistance to macrolide, lincosamide, and streptogramin type B antibodies. J Bacteriol 150:804-14.

Hornbaek T., Jakobsen M., Dynesen J. and Nielsen A. K. (2004) Global transcription profiles and intracellular pH regulation measured in Bacillus licheniformis upon external pH upshifts. Arch Microbiol 182:467-74.

Hueck CJ. and Hillen W. (1995) Catabolite repression in *B. subtilis*: a global regulatory mechanism for the gram-positive bacteria? Mol Microbiol 15:395-401.

Hulett FM. (1996) The signal-transduction network for Pho regulation in *B. subtilis*. Mol Microbiol 19:933-9.

Hulett FM., Kim EE., Bookstein C., Kapp NV., Edwards CW. and Wyckoff HW. (1991) *B. subtilis* alkaline phosphatases III and IV. Cloning, sequencing, and comparisons of deduced amino acid sequence with Escherichia coli alkaline phosphatase three-dimensional structure. J Biol Chem 266:1077-84.

Hulett FM., Lee J., Shi L., Sun G., Chesnut R., Sharkova E., Duggan MF. and Kapp N. (1994) Sequential action of two-component genetic switches regulates the PHO regulon in *B. subtilis*. J Bacteriol 176:1348-58.

Hulett FM. (2002) The Pho Regulon. In: Sonenshein AL, Losick R (eds) *B. subtilis* and its Closest Relatives. from Genes to Cells. ASM Press, Washington DC pp. 193-201.

Hyyrylainen HL., Vitikainen M., Thwaite J., Wu H., Sarvas M., Harwood CR., Kontinen V. P. and Stephenson K. (2000) D-Alanine substitution of teichoic acids as a modulator of protein folding and stability at the cytoplasmic membrane/cell wall interface of *B. subtilis*. J Biol Chem 275:26696-703.

Idriss EE., Makarewicz O., Farouk A., Rosner K., Greiner R., Bochow H., Richter T. and Borriss R. (2002) Extracellular phytase activity of Bacillus amyloliquefaciens FZB45 contributes to its plant-growth-promoting effect. Microbiology 148:2097-109.

Ikehara K., Kamitani E., Koarata C. and Ogura A. (1985) Induction of stringent response by streptomycin in *B. subtilis* cells. J Biochem (Tokyo) 97:697-700.

Irving GC. and Cosgrove DJ. (1971) Inositol phosphate phosphatases of microbiological origin. Some properties of a partially purified bacterial (Pseudomonas sp.) phytase. Aust J Biol Sci 24:547-57.

Ishige T., Krause M., Bott M., Wendisch VF. and Sahm H. (2003) The phosphate starvation stimulon of Corynebacterium glutamicum determined by DNA microarray analyses. J Bacteriol 185:4519-29.

Ivanova N., Sorokin A., Anderson I., Galleron N., Candelon B., Kapatral V., Bhattacharyya A., Reznik G., Mikhailova N., Lapidus A., Chu L., Mazur M., Goltsman E., Larsen N., D'Souza M., Walunas T., Grechkin Y., Pusch G., Haselkorn R., Fonstein M., Ehrlich SD., Overbeek R. and Kyrpides N. (2003) Genome sequence of Bacillus cereus and comparative analysis with Bacillus anthracis. Nature 423:87-91.

Janniere L., Bruand C. and Ehrlich SD. (1990) Structurally stable *B. subtilis* cloning vectors. Gene 87:53-61.

Jones BE., Dossonnet V., Kuster E., Hillen W., Deutscher J. and Klevit RE. (1997) Binding of the catabolite repressor protein CcpA to its DNA target is regulated by phosphorylation of its corepressor HPr. J Biol Chem 272:26530-5.

Jores L. and Wagner R. (2003) Essential steps in the ppGpp-dependent regulation of bacterial ribosomal RNA promoters can be explained by substrate competition. J Biol Chem 278:16834-43.

Jourlin-Castelli C., Mani N., Nakano MM. and Sonenshein AL. (2000) CcpC, a novel regulator of the LysR family required for glucose repression of the citB gene in *B. subtilis*. J Mol Biol 295:865-78.

Jürgen B., Barken KB., Tobisch S., Pioch D., Wumpelmann M., Hecker M. and Schweder T. (2005 a) Application of an electric DNA-chip for the expression analysis of bioprocess-relevant marker genes of *B. subtilis*. Biotechnol Bioeng 92:299-307.

Jürgen B., Schweder T. and Hecker M. (1998) The stability of mRNA from the gsiB gene of *B. subtilis* is dependent on the presence of a strong ribosome binding site. Mol Gen Genet 258:538-45.

Jürgen B., Tobisch S., Wumpelmann M., Gordes D., Koch A., Thurow K., Albrecht D., Hecker M. and Schweder T. (2005b) Global expression profiling of *B. subtilis* cells during industrial-close fed-batch fermentations with different nitrogen sources. Biotechnol Bioeng 92:277-98.

Kang CM., Brody MS., Akbar S., Yang X. and Price CW. (1996) Homologous pairs of regulatory proteins control activity of *B. subtilis* transcription factor sigma(b) in response to environmental stress. J Bacteriol 178:3846-53.

Karlin S. and Mrazek J. (2000) Predicted highly expressed genes of diverse prokaryotic genomes. J Bacteriol 182:5238-50.

Kim JH, Voskuil MI and Chambliss GH (1998) NADP, corepressor for the Bacillus catabolite control protein CcpA. Proc Natl Acad Sci U S A 95:9590-5.

Kim HJ, Kim SI, Ratnayake-Lecamwasam M, Tachikawa K, Sonenshein AL, Strauch M. (2003) Complex regulation of the Bacillus subtilis aconitase gene. J Bacteriol 185:1672-80.

Kim YO., Kim HK., Bae KS., Yu JH and Oh TK. (1998) Purification and properties of a thermostable phytase from *Bacillus* sp. DS11. Enzyme Microbial Technol. 22: 2-7.

Kim HW., Kim YO., Lee JH., Kim KK. and Kim YJ. (2003) Isolation and characterization of a phytase with improved properties from *Citrobacter braakii*. Biotechnol. Lett 25: 1231-1234.

Kerovuo J., Lauraeus M., Nurminen P., Kalkkinen N. and Apajalahti J. (1998) Isolation, characterization, molecular gene cloning, and sequencing of a novel phytase from *B. subtilis*. Appl Environ Microbiol 64:2079-85.

Kerovuo J., Rouvinen J. and Hatzack F. (2000) Analysis of myo-inositol hexakisphosphate hydrolysis by Bacillus phytase: indication of a novel reaction mechanism. Biochem J 352 Pt 3:623-8.

Koburger T., Weibezahn J., Bernhardt J., Homuth G. and Hecker M. (2005) Genome-wide mRNA profiling in glucose starved *B. subtilis* cells. Mol Genet Genomics 274:1-12.

Krasny L. and Gourse RL. (2004) An alternative strategy for bacterial ribosome synthesis: *B. subtilis* rRNA transcription regulation. Embo J 23:4473-83.

Kunst F., Ogasawara N., Moszer I., Albertini AM., Alloni G., Azevedo V., Bertero MG., Bessieres P., Bolotin A., Borchert S., Borriss R., Boursier L., Brans A., Braun M., Brignell SC., Bron S., Brouillet S., Bruschi CV., Caldwell B., Capuano V., Carter NM., Choi SK., Codani JJ., Connerton IF., Danchin A. and et al. (1997) The complete genome sequence of the gram-positive bacterium *B. subtilis*. Nature 390:249-56.

Kuwana R., Yamamura S., Ikejiri H., Kobayashi K., Ogasawara N., Asai K., Sadaie Y., Takamatsu H. and Watabe K. (2003) *B. subtilis* spoVIF (yjcC) gene, involved in coat assembly and spore resistance. Microbiology 149:3011-21.

Laishley EJ. and Bernlohr RW. (1968) Regulation of arginine and proline catabolism in Bacillus licheniformis. J Bacteriol 96:322-9.

Lambert PA., Hancock IC. and Baddiley J. (1975) Influence of alanyl ester residues on the binding of magnesium ions to teichoic acids. Biochem J 151:671-6.

Lan GQ., Abdullah N., Jalaludin S. and Ho YW. (2002) Culture conditions influencing phytase production of *Mitsuokella jalaludinii*, a new bacterial species from the rumen of cattle. *J. Appl. Microbiol.*, 93: 668-674.

Lang WK, Glassey K and Archibald AR (1982) Influence of phosphate supply on teichoic acid and teichuronic acid content of *B. subtilis* cell walls. J Bacteriol 151:367-75.

Lassen SF., Breinholt J., Ostergaard PR., Brugger R., Bischoff A., Wyss M. and Fuglsang CC. (2001) Expression, gene cloning, and characterization of five novel phytases from four basidiomycete fungi: Peniophora lycii, Agrocybe pediades, a Ceriporia sp., and Trametes pubescens. Appl Environ Microbiol 67:4701-7.

Le JK, Edwards CW and Hulett FM. (1991) Bacillus licheniformis APase I gene promoter: a strong well-regulated promoter in *B. subtilis*. J. Gen. Microbiol. 137: 1127-1133.

Lei XG and Porres JM (2003) Phytase enzymology, applications, and biotechnology. Biotechnol Lett 25:1787-94.

Lisser S. and Margalit H. (1993) Compilation of E. coli mRNA promoter sequences. Nucleic Acids Res 21:1507-16.

Liu W., Eder S. and Hulett FM. (1998 a) Analysis of *B. subtilis* tagAB and tagDEF expression during phosphate starvation identifies a repressor role for PhoP-P. J Bacteriol 180:753-8.

Liu W., Qi Y. and Hulett FM. (1998 b) Sites internal to the coding regions of phoA and pstS bind PhoP and are required for full promoter activity. Mol Microbiol 28:119-30.

Liu W. and Hulett FM. (1998) Comparison of PhoP binding to the tuaA promoter with PhoP binding to other Pho-regulon promoters establishes a *B. subtilis* Pho core binding site. Microbiology 144 (Pt 5):1443-50.

Lorca GL., Chung YJ., Barabote RD., Weyler W., Schilling CH. and Saier MH Jr. (2005) Catabolite repression and activation in *B. subtilis*: dependency on CcpA, HPr, and HprK. J Bacteriol 187:7826-39.

Ludwig H., Homuth G., Schmalisch M., Dyka FM., Hecker M. and Stülke J. (2001) Transcription of glycolytic genes and operons in *B. subtilis*: evidence for the presence of multiple levels of control of the gapA operon. Mol Microbiol 41:409-22.

Ludwig H., Meinken C., Matin A. and Stülke J. (2002 a) Insufficient expression of the ilv-leu operon encoding enzymes of branched-chain amino acid biosynthesis limits growth of a *B. subtilis* ccpA mutant. J Bacteriol 184:5174-8.

Ludwig H., Rebhan, N., Blencke HM and Merzbacher, M.(2002 b) Control of the glycolytic gapA operon by the catabolite control protein A in *B. subtilis*: a novel mechanism of CcpA-mediated regulation. Mol. Microbiol 45: 543-553.

Meier-Wagner J., Nolden L., Jakoby M., Siewe R., Kramer R. and Burkovski A. (2001) Multiplicity of ammonium uptake systems in Corynebacterium glutamicum: role of Amt and AmtB. Microbiology 147:135-43.

Mirel DB., Estacio WF., Mathieu M., Olmsted E., Ramirez J. and Marquez-Magana LM. (2000) Environmental regulation of Bacillus subtilis sigma(D)-dependent gene expression. J Bacteriol 182:3055-62.

Miwa Y., Nakata A., Ogiwara A., Yamamoto M. and Fujita Y. (2000) Evaluation and characterization of catabolite-responsive elements (cre) of *B. subtilis*. Nucleic Acids Res 28:1206-10.

Molle V., Nakaura Y., Shivers RP., Yamaguchi H., Losick R., Fujita Y. and Sonenshein AL. (2003) Additional targets of the *B. subtilis* global regulator CodY identified by chromatin immunoprecipitation and genome-wide transcript analysis. J Bacteriol 185:1911-22.

Moreno MS., Schneider BL., Maile RR., Weyler W. and Saier MH Jr. (2001) Catabolite repression mediated by the CcpA protein in *B. subtilis*: novel modes of regulation revealed by whole-genome analyses. Mol Microbiol 39:1366-81.

Mostertz J., Scharf C., Hecker M. and Homuth G. (2004) Transcriptome and proteome analysis of *B. subtilis* gene expression in response to superoxide and peroxide stress. Microbiology 150:497-512.

Müller JP., An Z., Merad T., Hancock IC. and Harwood CR. (1997) Influence of *B. subtilis* phoR on cell wall anionic polymers. Microbiology 143: 947-56.

Mullaney EJ., Daly CB., Sethumadhavan K., Rodriquez E., Lei XG. and Ullah AH. (2000) Phytase activity in Aspergillus fumigatus isolates. Biochem Biophys Res Commun 275:759-63.

Nakamura Y., Fukuhara H. and Sano K. (2000) Secreted phytase activities of yeasts. Biosci Biotechnol Biochem 64:841-4.

Nakano MM., Yang F., Hardin P. and Zuber P. (1995) Nitrogen regulation of nasA and the nasB operon, which encode genes required for nitrate assimilation in *B. subtilis*. J Bacteriol 177:573-9.

Neumuller AM., Konz D. and Marahiel MA. (2001) The two-component regulatory system BacRS is associated with bacitracin 'self-resistance' of Bacillus licheniformis ATCC 10716. Eur J Biochem 268:3180-9.

Nordstrom K. and Uhlin BE. (1992) Runaway-replication plasmids as tools to produce large quantities of proteins from cloned genes in bacteria. Biotechnology (N Y) 10:661-6.

Ogawa K., Akagawa E., Yamane K., Sun ZW., LaCelle M., Zuber P. and Nakano MM. (1995) The nasB operon and nasA gene are required for nitrate/nitrite assimilation in *B. subtilis*. J Bacteriol 177:1409-13.

Ogura M., Yamaguchi H., Yoshida Ki, Fujita Y. and Tanaka T. (2001) DNA microarray analysis of *B. subtilis* DegU, ComA and PhoP regulons: an approach to comprehensive analysis of B.subtilis two-component regulatory systems. Nucleic Acids Res 29:3804-13.

Pandey A., Nigam P., Soccol CR., Soccol VT., Singh D. and Mohan R. (2000) Advances in microbial amylases. Biotechnol Appl Biochem 31 (Pt 2):135-52.

Petersohn A., Antelmann H., Gerth U. and Hecker M. (1999) Identification and transcriptional analysis of new members of the sigmaB regulon in *B. subtilis*. Microbiology 145 (Pt 4):869-80.

Postma PW., Lengeler JW. and Jacobson GR. (1993) Phosphoenolpyruvate:carbohydrate phosphotransferase systems of bacteria. Microbiol Rev 57:543-94.

Powar VK. and Jagannathan V. (1982) Purification and properties of phytate-specific phosphatase from *B. subtilis*. J Bacteriol 151:1102-8.

Pragai Z. and Harwood CR. (2002) Regulatory interactions between the Pho and sigma(B)-dependent general stress regulons of *B. subtilis*. Microbiology 148:1593-602.

Pragai Z, Allenby NE, O'Connor N, Dubrac S, Rapoport G, Msadek T and Harwood CR (2004) Transcriptional regulation of the phoPR operon in *B. subtilis*. J Bacteriol 186:1182-90.

Presecan-Siedel E., Galinier A., Longin R., Deutscher J., Danchin A., Glaser P. and Martin-Verstraete I. (1999) Catabolite regulation of the pta gene as part of carbon flow pathways in *B. subtilis*. J Bacteriol 181:6889-97.

Price CW., Fawcett P., Ceremonie H., Su N., Murphy CK. and Youngman P. (2001) Genome-wide analysis of the general stress response in *B. subtilis*. Mol Microbiol 41:757-74.

Qi Y. and Hulett FM. (1998 a) PhoP-P and RNA polymerase sigmaA holoenzyme are sufficient for transcription of Pho regulon promoters in *B. subtilis*: PhoP-P activator sites within the coding region stimulate transcription in vitro. Mol Microbiol 28:1187-97.

Qi Y. and Hulett FM. (1998 b) Role of Pho-P in transcriptional regulation of genes involved in cell wall anionic polymer biosynthesis in *B. subtilis*. J Bacteriol 180:4007-10.

Qi Y., Kobayashi Y. and Hulett FM. (1997) The pst operon of *B. subtilis* has a phosphate-regulated promoter and is involved in phosphate transport but not in regulation of the pho regulon. J Bacteriol 179:2534-9.

Quan CS., Tian WJ., Fan SD. and Kikuchi J. (2004) Purification and properties of a low-molecular-weight phytase from Cladosporium sp. FP-1. J Biosci Bioeng 97:260-6.

Quax W (1990) Bacillus licheniformis protoplast transformation. In Molecular Biological Methods for Bacillus. (Cutting, S.M. & Harwood, C.R., eds). John Wiley & Sons Ltd, New York. pp. 152. .

Ramstrom H., Sanglier S., Leize-Wagner E., Philippe C., Van Dorsselaer A. and Haiech J. (2003) Properties and regulation of the bifunctional enzyme HPr kinase/phosphatase in *B. subtilis*. J Biol Chem 278:1174-85.

Rao MB., Tanksale AM., Ghatge MS. and Deshpande VV. (1998) Molecular and biotechnological aspects of microbial proteases. Microbiol Mol Biol Rev 62:597-635.

Rasko D. A., Ravel J., Okstad O. A., Helgason E., Cer R. Z., Jiang L., Shores K. A., Fouts D. E., Tourasse N. J., Angiuoli S. V., Kolonay J., Nelson W. C., Kolsto A. B., Fraser C. M. and Read T. D. (2004) The genome sequence of Bacillus cereus ATCC 10987 reveals metabolic adaptations and a large plasmid related to Bacillus anthracis pXO1. Nucleic Acids Res 32:977-88.

Ratnayake-Lecamwasam M., Serror P., Wong KW. and Sonenshein AL. (2001) *B. subtilis* CodY represses early-stationary-phase genes by sensing GTP levels. Genes Dev 15:1093-103.

Read TD., Peterson SN., Tourasse N., Baillie LW., Paulsen IT., Nelson KE., Tettelin H., Fouts DE., Eisen JA., Gill SR., Holtzapple EK., Okstad OA., Helgason E., Rilstone J., Wu M., Kolonay JF., Beanan MJ., Dodson RJ., Brinkac LM., Gwinn M., DeBoy RT., Madpu R., Daugherty SC., Durkin AS., Haft DH., Nelson W. C., Peterson J. D., Pop M., Khouri H. M., Radune D., Benton J. L., Mahamoud Y., Jiang L., Hance IR., Weidman JF., Berry KJ., Plaut RD., Wolf AM., Watkins KL., Nierman WC., Hazen A., Cline R., Redmond C., Thwaite J. E., White O., Salzberg S. L., Thomason B., Friedlander A. M., Koehler TM., Hanna PC., Kolsto AB. and Fraser CM. (2003) The genome sequence of Bacillus anthracis Ames and comparison to closely related bacteria. Nature 423:81-6.

Reddy NR., Sathe SK. and Salunkhe DK. (1982) Phytates in legumes and cereals. Adv Food Res 28:1-92.

Reizer J., Hoischen C., Titgemeyer F., Rivolta C., Rabus R., Stülke J., Karamata D., Saier MH Jr. and Hillen W. (1998) A novel protein kinase that controls carbon catabolite repression in bacteria. Mol Microbiol 27:1157-69.

Reizer J., Sutrina S. L., Wu LF., Deutscher J., Reddy P. and Saier MH Jr. (1992) Functional interactions between proteins of the phosphoenolpyruvate:sugar phosphotransferase systems of *B. subtilis* and Escherichia coli. J Biol Chem 267:9158-69.

Renna MC., Najimudin N., Winik LR. and Zahler SA. (1993) Regulation of the *B. subtilis* alsS, alsD, and alsR genes involved in post-exponential-phase production of acetoin. J Bacteriol 175:3863-75.

Rey MW., Ramaiya P., Nelson BA., Brody-Karpin SD., Zaretsky EJ., Tang M., Lopez de Leon A., Xiang H., Gusti V., Clausen IG., Olsen PB., Rasmussen MD., Andersen JT., Jorgensen PL., Larsen TS., Sorokin A., Bolotin A., Lapidus A., Galleron N., Ehrlich SD. and Berka RM. (2004) Complete genome sequence of the industrial bacterium Bacillus licheniformis and comparisons with closely related Bacillus species. Genome Biol 5:R77.

Rhaese H. J., Dichtelmuller H. and Grade R. (1975) Studies on the control of development. Accumulation of guanosine tetraphosphate and pentaphosphate in response to inhibition of protein synthesis in *B. subtilis*. Eur J Biochem 56:385-92.

Robichon D., Arnaud M., Gardan R., Pragai Z., O'Reilly M., Rapoport G. and Debarbouille M. (2000) Expression of a new operon from *B. subtilis*, ykzB-ykoL, under the control of the TnrA and PhoP-phoR global regulators. J Bacteriol 182:1226-31.

Rocha EP., Danchin A. and Viari A. (1999) Translation in *B. subtilis*: roles and trends of initiation and termination, insights from a genome analysis. Nucleic Acids Res 27:3567-76.

Rosenkrantz MS, Dingman DW, Sonenshein AL. (1985) Bacillus subtilis citB gene is regulated synergistically by glucose and glutamine. J Bacteriol 164:155-64.

Saier MH Jr., Chauvaux S., Cook GM., Deutscher J., Paulsen IT., Reizer J. and Ye JJ. (1996) Catabolite repression and inducer control in Gram-positive bacteria. Microbiology 142 (Pt 2):217-30.

Sajidan A., Farouk A., Greiner R., Jungblut P., Muller EC. and Borriss R. (2004) Molecular and physiological characterisation of a 3-phytase from soil bacterium Klebsiella sp. ASR1. Appl Microbiol Biotechnol 65:110-8.

Sambrook J, Fritsch EF. and Maniatis T. (1989) Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Salisbury FB and Ross CW (1992) Plant physiology, 4th Edn. Belmont, CA: Wadsworth Publishing Company.

Sanden AM., Prytz I., Tubulekas I., Forberg C., Le H., Hektor A., Neubauer P., Pragai Z., Harwood C., Ward A., Picon A., De Mattos JT., Postma P., Farewell A., Nystrom T., Reeh S., Pedersen S. and Larsson G. (2003) Limiting factors in Escherichia coli fed-batch production of recombinant proteins. Biotechnol Bioeng 81:158-66.

Sarubbi E., Rudd KE., Xiao H., Ikehara K., Kalman M. and Cashel M. (1989) Characterization of the spoT gene of Escherichia coli. J Biol Chem 264:15074-82.

Schallmey M., Singh A. and Ward OP. (2004) Developments in the use of Bacillus species for industrial production. Can J Microbiol 50:1-17.

Schau M., Eldakak A. and Hulett FM. (2004) Terminal oxidases are essential to bypass the requirement for ResD for full Pho induction in *B. subtilis*. J Bacteriol 186:8424-32.

Schreier HJ., Brown SW., Hirschi KD., Nomellini JF. and Sonenshein AL. (1989) Regulation of *B. subtilis* glutamine synthetase gene expression by the product of the glnR gene. J Mol Biol 210:51-63.

Schreier HJ., Rostkowski CA., Nomellini JF. and Hirschi KD. (1991) Identification of DNA sequences involved in regulating *B. subtilis* glnRA expression by the nitrogen source. J Mol Biol 220:241-53.

Schwarzer D. and Marahiel MA. (2001) Multimodular biocatalysts for natural product assembly. Naturwissenschaften 88:93-101.

Schweder T., Lee KH., Lomovskaya O. and Matin A. (1996) Regulation of Escherichia coli starvation sigma factor (sigma s) by ClpXP protease. J Bacteriol 178:470-6.

Schweder T., Lin HY., Jürgen B., Breitenstein A., Riemschneider S., Khalameyzer V., Gupta A., Buttner K. and Neubauer P. (2002) Role of the general stress response during strong overexpression of a heterologous gene in Escherichia coli. Appl Microbiol Biotechnol 58:330-7.

Schweder T., Skaug HJ., Langaas M. and Dimakos XK. (1999) Simulated likelihood methods for complex double-platform line transect surveys. Biometrics 55:678-87.

Schweder T and Hecker M (2004) Monitoring of stress responses. Adv Biochem Eng Biotechnol 89:47-71.

Seki T., Yoshikawa H., Takahashi H. and Saito H. (1987) Cloning and nucleotide sequence of phoP, the regulatory gene for alkaline phosphatase and phosphodiesterase in *B. subtilis*. J Bacteriol 169:2913-6.

Seki T., Yoshikawa H., Takahashi H. and Saito H. (1988) Nucleotide sequence of the *B. subtilis* phoR gene. J Bacteriol 170:5935-8.

Serror P. and Sonenshein AL. (1996 a) CodY is required for nutritional repression of *B. subtilis* genetic competence. J Bacteriol 178:5910-5.

Serror P. and Sonenshein AL. (1996 b) Interaction of CodY, a novel *B. subtilis* DNA-binding protein, with the dpp promoter region. Mol Microbiol 20:843-52.

Servant P, Le Coq D, Aymerich S. (2005) CcpN (YqzB), a novel regulator for CcpA-independent catabolite repression of *B. subtilis* gluconeogenic genes. Mol Microbiol 55: 1435-51.

Shih IL. and Van YT. (2001) The production of poly-(gamma-glutamic acid) from microorganisms and its various applications. Bioresour Technol 79:207-25.

Shimizu M (1992) Purification and characterization of a phytase from *B. subtilis* (natto) N-77. Biosci. Biotechnol. Biochem 56:1266-1269.

Shivers RP. and Sonenshein AL. (2004) Activation of the *B. subtilis* global regulator CodY by direct interaction with branched-chain amino acids. Mol Microbiol 53:599-611.

Silbersack J., Jürgen B., Hecker M., Schneidinger B., Schmuck R. and Schweder T. (2006) An acetoin-regulated expression system of *Bacillus subtilis*. Appl. Microbiol. Biotech. submitted.

Simons PC., Versteegh HA., Jongbloed AW., Kemme PA., Slump P., Bos KD., Wolters MG., Beudeker RF. and Verschoor GJ. (1990) Improvement of phosphorus availability by microbial phytase in broilers and pigs. Br J Nutr 64:525-40.

Sonenshein AL The Krebs Citric Acid Cycle. In: Sonenshein AL, Losick R (eds) *B. subtilis* and its Closest Relatives. from Genes to Cells. ASM Press, Washington DC pp. 151-162.

Stent GS. and Brenner S. (1961) A genetic locus for the regulation of ribonucleic acid synthesis. Proc Natl Acad Sci U S A 47:2005-14.

Stülke J. and Hillen W. (2000) Regulation of carbon catabolism in Bacillus species. Annu Rev Microbiol 54:849-80.

Sun G., Birkey SM. and Hulett FM. (1996) Three two-component signal-transduction systems interact for Pho regulation in *B. subtilis*. Mol Microbiol 19:941-8.

Switzer RL., Bond RW., Ruppen ME. and Rosenzweig S. (1985) Involvement of the stringent response in regulation of protein degradation in *B. subtilis*. Curr Top Cell Regul 27:373-86.

Takami H., Nakasone K., Takaki Y., Maeno G., Sasaki R., Masui N., Fuji F., Hirama C., Nakamura Y., Ogasawara N., Kuhara S. and Horikoshi K. (2000) Complete genome sequence of the alkaliphilic bacterium Bacillus halodurans and genomic sequence comparison with *B. subtilis*. Nucleic Acids Res 28:4317-31.

Tang XM., Lakay FM., Shen W., Shao WL., Fang HY., Prior BA., Wang ZX. and Zhuge J. (2004 a) Purification and characterisation of an alkaline protease used in tannery industry from Bacillus licheniformis. Biotechnol Lett 26:1421-4.

Tang XM., Shen W., Lakay FM., Shao WL., Wang ZX., Prior BA. and Zhuge J. (2004b) Cloning and over-expression of an alkaline protease from Bacillus licheniformis. Biotechnol Lett 26:975-9.

Tang MR and Wilson CR. (1996) The nucleotide sequence of plasmid pBC16 and its relationship to plasmid pUB110. GenBank accession no. U32369

Tjalsma H., Bolhuis A., Jongbloed JD., Bron S. and van Dijl JM. (2000) Signal peptide-dependent protein transport in *B. subtilis*: a genome-based survey of the secretome. Microbiol Mol Biol Rev 64:515-47.

Tojo S., Satomura T., Morisaki K., Deutscher J., Hirooka K. and Fujita Y. (2005) Elaborate transcription regulation of the *B. subtilis* ilv-leu operon involved in the biosynthesis of branched-chain amino acids through global regulators of CcpA, CodY and TnrA. Mol Microbiol 56:1560-73.

Tojo S., Satomura T., Morisaki K., Yoshida K., Hirooka K. and Fujita Y. (2004) Negative transcriptional regulation of the ilv-leu operon for biosynthesis of branched-chain amino acids through the *B. subtilis* global regulator TnrA. J Bacteriol 186:7971-9.

Touati E., Dassa E., Dassa J. and Boquet PL. (1987) Acid phosphatase (pH 2.5) of *Escherichia coli*: regulatory characteristics. In: Torriani-Gorini A., Rothman FG., Silver S., Wright A. and Yagil E. (eds). Phosphate metabolism and cellular regulation in microorganisms. American Society for Microbiology, Washington D.C. pp. 31-40.

Turinsky AJ., Moir-Blais TR., Grundy FJ. and Henkin TM. (2000) *B. subtilis* ccpA gene mutants specifically defective in activation of acetoin biosynthesis. J Bacteriol 182:5611-4.

Tye AJ., Siu FK., Leung TY. and Lim BL. (2002) Molecular cloning and the biochemical characterization of two novel phytases from *B. subtilis* 168 and *B. licheniformis*. Appl Microbiol Biotechnol 59:190-7.

Ueta M., Yoshida H., Wada C., Baba T., Mori H. and Wada A. (2005) Ribosome binding proteins YhbH and YfiA have opposite functions during 100S formation in the stationary phase of Escherichia coli. Genes Cells 10:1103-12.

Van Dijken P., de Haas JR., Craxton A., Erneux C., Shears SB. and Van Haastert PJ. (1995) A novel, phospholipase C-independent pathway of inositol 1,4,5-trisphosphate formation in Dictyostelium and rat liver. J Biol Chem 270:29724-31.

van Hartingsveldt W., van Zeijl CM., Harteveld GM., Gouka RJ., Suykerbuyk ME., Luiten RG., van Paridon P. A., Selten GC., Veenstra AE., van Gorcom RF. and et al. (1993) Cloning, characterization and overexpression of the phytase-encoding gene (phyA) of Aspergillus niger. Gene 127:87-94.

VanBogelen RA., Schiller EE., Thomas JD. and Neidhardt FC. (1999) Diagnosis of cellular states of microbial organisms using proteomics. Electrophoresis 20:2149-59.

Veith B., Herzberg C., Steckel S., Feesche J., Maurer K. H., Ehrenreich P., Baumer S., Henne A., Liesegang H., Merkl R., Ehrenreich A. and Gottschalk G. (2004) The complete genome sequence of Bacillus licheniformis DSM13, an organism with great industrial potential. J Mol Microbiol Biotechnol 7:204-11.

Vijay K., Brody MS., Fredlund E. and Price CW. (2000) A PP2C phosphatase containing a PAS domain is required to convey signals of energy stress to the sigmaB transcription factor of *B. subtilis*. Mol Microbiol 35:180-8.

Vincent JB., Crowder MW. and Averill BA. (1992) Hydrolysis of phosphate monoesters: a biological problem with multiple chemical solutions. Trends Biochem Sci 17:105-10.

Vohra A. and Satyanarayana T. (2003) Phytases: microbial sources, production, purification, and potential biotechnological applications. Crit Rev Biotechnol 23:29-60.

Voigt B., Schweder T., Becher D., Ehrenreich A., Gottschalk G., Feesche J., Maurer KH. and Hecker M. (2004) A proteomic view of cell physiology of Bacillus licheniformis. Proteomics 4:1465-90.

Völker U. and Hecker M. (2005) From genomics via proteomics to cellular physiology of the Gram-positive model organism *B. subtilis*. Cell Microbiol 7:1077-85.

Wacker I., Ludwig H., Reif I., Blencke HM., Detsch C. and Stülke J. (2003) The regulatory link between carbon and nitrogen metabolism in *B. subtilis*: regulation of the gltAB operon by the catabolite control protein CcpA. Microbiology 149:3001-9.

Wang LF, Wong SL, Lee SG, Kalyan NK, Hung PP, Hilliker S and Doi RH (1988) Expression and secretion of human atrial natriuretic alpha-factor in *B. subtilis* using the subtilisin signal peptide. Gene 69:39-47.

Wang LF and Doi RH (1992) Heterologous gene expression in *B. subtilis*. Biotechnology 22:63-104.

Warnes A. and Stephenson J. R. (1986) The insertion of large pieces of foreign genetic material reduces the stability of bacterial plasmids. Plasmid 16:116-23.

Weigent DA. and Nester EW. (1976) Regulation of histidinol phosphate aminotransferase synthesis by tryptophan in *B. subtilis*. J Bacteriol 128:202-11.

Wendrich TM. and Marahiel MA. (1997) Cloning and characterization of a relA/spoT homologue from *B. subtilis*. Mol Microbiol 26:65-79.

Wetzstein M., Volker U., Dedio J., Lobau S., Zuber U., Schiesswohl M., Herget C., Hecker M. and Schumann W. (1992) Cloning, sequencing, and molecular analysis of the dnaK locus from *B. subtilis*. J Bacteriol 174:3300-10.

Wodzinski RJ. and Ullah AH. (1996) Phytase. Adv Appl Microbiol 42:263-302.

Wray LV Jr., Atkinson MR. and Fisher SH. (1994) The nitrogen-regulated *B. subtilis* nrgAB operon encodes a membrane protein and a protein highly similar to the Escherichia coli glnB-encoded PII protein. J Bacteriol 176:108-14.

Wray LV Jr., Ferson AE. and Fisher SH. (1997) Expression of the *B. subtilis* ureABC operon is controlled by multiple regulatory factors including CodY, GlnR, TnrA, and Spo0H. J Bacteriol 179:5494-501.

Wray LV Jr., Ferson A. E., Rohrer K. and Fisher SH. (1996) TnrA, a transcription factor required for global nitrogen regulation in *B. subtilis*. Proc Natl Acad Sci U S A 93:8841-5.

Wray LV Jr. and Fisher SH. (2005) A Feedback-resistant Mutant of *B. subtilis* Glutamine Synthetase with Pleiotropic Defects in Nitrogen-regulated Gene Expression. J Biol Chem 280:33298-304.

Wray LV Jr., Zalieckas J. M., Ferson A. E. and Fisher SH. (1998) Mutational analysis of the TnrA-binding sites in the *B. subtilis* nrgAB and gabP promoter regions. J Bacteriol 180:2943-9.

Wray LV Jr., Zalieckas JM. and Fisher SH. (2001) *B. subtilis* glutamine synthetase controls gene expression through a protein-protein interaction with transcription factor TnrA. Cell 107:427-35.

Wu SC, Yeung JC, Duan Y, Ye R, Szarka SJ, Habibi HR and Wong SL (2002) Functional production and characterization of a fibrin-specific single-chain antibody fragment from *B. subtilis*: effects of molecular chaperones and a wall-bound protease on antibody fragment production. Appl Environ Microbiol 68:3261-9.

Wu XC, Lee W, Tran L and Wong SL (1991) Engineering a *B. subtilis* expression-secretion system with a strain deficient in six extracellular proteases. J Bacteriol 173:4952-8.

Yang X., Kang CM., Brody MS. and Price CW. (1996) Opposing pairs of serine protein kinases and phosphatases transmit signals of environmental stress to activate a bacterial transcription factor. Genes Dev 10:2265-75.

Yanke LJ., Bae HD., Selinger LB. and Cheng KJ. (1998) Phytase activity of anaerobic ruminal bacteria. Microbiology 144 (Pt 6):1565-73.

Yansura DG. and Henner DJ. (1984) Use of the Escherichia coli lac repressor and operator to control gene expression in *B. subtilis*. Proc Natl Acad Sci U S A 81:439-43.

Yansura DG. and Henner DJ. (1990) Use of Escherichia coli trp promoter for direct expression of proteins. Methods Enzymol 185:54-60.

Yoshida K, Kobayashi K, Miwa Y, Kang CM, Matsunaga M, Yamaguchi H, Tojo S, Yamamoto M, Nishi R, Ogasawara N, Nakayama T and Fujita Y (2001) Combined transcriptome and proteome analysis as a powerful approach to study genes under glucose repression in *B. subtilis*. Nucleic Acids Res 29:683-92.

Yoshida K, Yamaguchi H, Kinehara M, Ohki YH, Nakaura Y and Fujita Y (2003) Identification of additional TnrA-regulated genes of *B. subtilis* associated with a TnrA box. Mol Microbiol 49:157-65.

Yip W., Wang L., Cheng C., Wu W., Lung S. and Lim BL. (2003) The introduction of a phytase gene from *B. subtilis* improved the growth performance of transgenic tobacco. Biochem Biophys Res Commun 310:1148-54.

Youngman P. (1990) Use of transposons and integral vectors. In: Molecular Biological Methods for Bacillus. (Cutting, S.M. & Harwood, C.R., eds). John Wiley & Sons Ltd, New York. pp. 221-266.

Zhang S. and Haldenwang WG. (2003) RelA is a component of the nutritional stress activation pathway of the *B. subtilis* transcription factor sigma B. J Bacteriol 185:5714-21.

Zhang S. and Haldenwang WG. (2005) Contributions of ATP, GTP, and redox state to nutritional stress activation of the *B. subtilis* sigmaB transcription factor. J Bacteriol 187:7554-60.

Znamenskaya LV., Gabdrakhmanova LA., Chernokalskaya EB., Leshchinskaya IB. and Hartley RW. (1995) Phosphate regulation of biosynthesis of extracellular RNases of endospore-forming bacteria. FEBS Lett 357:16-8.

List of publications and patents

Hoi LT, Voigt B, Jürgen B, Ehrenreich A, Gottschalk G, Evers S, Feesche J, Maurer KH, Hecker M and Schweder T. (2006) The phosphate-starvation response of Bacillus licheniformis. Proteomics, in press.

Hoi LT, Voigt B, Jürgen B, Albrecht D, Ehrenreich A, Gottschalk G, Evers S, Feesche J, Maurer KH, Hecker M and Schweder T. (2006) Response of *Bacillus licheniformis* to glucose and nitrogen starvation. Proteomics, submitted.

Hoi LT, Voigt B, Jürgen B, Hecker M and Schweder T. (2006) The regulation of the phytase gene expression in B.licheniformis (in preparation).

Patents

DE 10 2004 061 664.7 (H 06514), Nukleinsäure-bindende Chips zur Detektion von Phosphatmangelzuständen im Rahmen der Bioprozeßkontrolle.

DE 10 2005 022 145.9 (H 06627), Nukleinsäure-bindende Chips zur Detektion von Glucosemangelzuständen im Rahmen der Bioprozeßkontrolle.

DE 10 2005 042 572.0 (H 06803), Nukleinsäure-bindende Chips zur Detektion von Stickstroffmangelzuständen im Rahmen der Bioprozeßkontrolle.

CURRICULUM VITAE

Le Thi Hoi 8-A6, Dam Trau, Bach Dang, Hai Ba Trung, Hanoi, Vietnam

Personal information:

Date of birth	26.03.1978
Place of birth	Thanh Hoa, Vietnam
Parents	Le Ba Khoat and Le Thi Diem
Marital status	Married
Nationality	Vietnamese
Religion	None

Education and work experience:

1984-1993	Primary and secondary school in Thanh Hoa
1993-1996	High school in Thanh Hoa
1996-2000	B.Sc. in Biology, Faculty of Biology, Hanoi National University, Vietnam
2000-2002	Researcher of the Institute of Biotechnology, Vietnam Academy of Science and Technology
2002-2003	Student of Diploma Equivalent course through Joint Graduate Educational Program held by Hanoi University of Science, Institute of Biotechnology, Vietnam and University of Greifswald, Germany
2003-2006	Ph.D. student of the Institute of Pharmacy and the Institute of Microbiology, Ernst-Moritz-Arndt University of Greifswald, Germany

Others

Language	English and German
Computer skills	Winword, Excel, Powerpoint

Acknowledgements

I wish to express my sincere gratitude to everyone who has helped and supported me while working on my dissertation. I would especially like to thank:

Prof. Dr. Thomas Schweder – my supervisor, for accepting me as your student and giving me a very interesting topic for my Ph.D work. Thank you very much for your continuous support and invaluable comments, guiding me through the thesis, and providing me with the great chance to attend annual conferences. It all helps broaden the expertise and knowledge in my field of study.

Dr. Britta Jürgen – my direct adviser, for instructing me to perform the new experiments when I first came to Greifswald. You are willing to spend time for discussing, reading, and correcting the draft of my dissertation. Thank you for sharing your thoughts about the life, work, people during my study here in Greifswald.

Prof. Dr. Michael Hecker – Director of the Institute of Microbiology, for allowing me to work in a very good working atmosphere with modern equipments and friendly staff.

Dr. Birgit Voigt – co-author, for giving me critical comments, discussing, and writing the scientific papers that were published in well-known journals.

Dr. Karl-Heinz Maurer, **Dr. Stefan Evers and Dr. Johannes Bongaerts** from Henkel company for very fruitful and productive collaborations and as co-authors.

Dr. Armin Ehrenreich in the Genomics Laboratory and Competence Centre for Genome Research on Bacteria Institute of Microbiology and Genetics, University of Göttingen, who designed genomic *B. licheniformis* DNA microarrays and identified the potential Pho binding boxes and the potential *cre* binding boxes by bioinformatic pattern searching in the *B. licheniformis* genome. **Prof. Dr. Gerhard Gottschalk**, Head of the central project of the competence network Genome Research project.

My friends, **Norma Welsch, Stefanie Leja, Stephanie Markert, Daniel Pioch, Johannes Kabisch, Sandra Beyer and Boris Wilmes** for helping me so much whatever I need, organising the dinner parties, and spending time talking about the work and daily life in Germany.

I would also like to thank the members of the Joint Graduate Education Program, the members of the 322 Project – Ministry of Education and Training of Vietnam, especially **Prof. Dr. Le Tran Binh**, **Prof. Dr. Maria- Theresia Schafmeister**, **Dr. Jörn Kasbohm**, **Dr. Le Thi Lai**, **Dr. Luu Lan Huong** for their full responsibility in this program.

Nguyen Quang Dung – my dear husband, to whom I owe my health, my perseverance and clear vision. You inspired me and shared your thoughts with me to write this work.

Finally, my thanks go to my parents, my older brother and his family, for always giving me confidence and supporting me all the way with care and love.

Eidesstattliche Erklärung

Hiermit erkläre ich, daß diese Arbeit bisher von mir weder an der Mathematisch-Naturwissenschaftlichen Fakultät der Ernst-Moritz-Arndt-Universität Greifswald noch einer anderen wissenschaftlichen Einrichtung zum Zwecke der Promotion eingereicht wurde.

Ferner erkläre ich, daß ich diese Arbeit selbständig verfaßt und keine anderen als die darin angegebenen Hilfsmittel benutzt habe.

Greifswald, den 16 Mai 2006 Unterschrift

Le Thi Hoi