Investigation of glycolysis in *Bacillus subtilis*

Dissertation

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I hereby declare that the doctoral thesis entitled, “Investigation of Glycolysis in *Bacillus subtilis*” has been written independently and with no other sources and aids than quoted.

Nico Pietack

Die beste und sicherste Tarnung ist immer noch die blanke und nackte Wahrheit. Die glaubt niemand!

(Max Frisch)

Content

List of abbreviations

% (v/v)	% (volume/volume)
% (w/v)	% (weight/volume)
Amp	ampicillin
APS	ammoniumperoxodisulfat
ATP	adenosine triphosphate
BSA	bovine serum albumin
C	carbon
CCR	carbon catabolite repression
DMSO	dimethylsulfoxide
DNA	desoxyribonucleic acid
dNTP	desoxyribonucleic triphosphate
DTT	dithiothreitol
EI	enzyme I
EII	enzyme II
EDTA	ethylenediaminetetra acetic acid
et al.	*et alia*
H_2O $_{deion.}$	deionized water
IPTG	isopropyl-β-D-thiogalactopyranoside
LB	Luria Bertani (medium)
LFH	long flanking homology
MES	2-(N-morpholino)ethanesulfonic acid
mM	millimolar
NAD^+ / $NADH_2$	β-nicotinamide adenine dinucleotide
$NADP^+$ / $NADPH_2$	β-nicotinamide adenine dinucleotide phosphat
Ni^{2+}-NTA	nickel-nitrilotri acid
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
pH	power of hydrogen
PTS	phosphoenolpyruvate: sugar phosphotransferase system
RT	room temperature
SDS	sodium dodecyl sulfate
SP	sporulation medium
Tab.	table

TCC	tricarboxylic acid cycle
TEMED	N,N,N´,N´-tetramethylethylendiamine
Tris	tris-(hydroxymethyl)-aminomethane
U	units
WT	wild type

Enzyme	**Gene**	
Eno	*eno*	enolase
FbaA	*fbaA*	fructose-1,6-bisphosphate aldolase
GapDH	*gapA*	glyceraldehyde-3-phosphate dehydrogenase
GapDH	*gapB*	glyceraldehyde-3-phosphate dehydrogenase
PfkA	*pfkA*	phosphofructokinase
Pgi	*pgi*	glucosephosphate isomerase
Pgk	*pgk*	phosphoglycerate kinase
Pgm	*pgm*	phosphoglycerate mutase
Pyk	*pykA*	pyruvate kinase
Tpi	*tpiA*	triosephosphate isomerase

Glycolytic intermediates

G6P	glucose-6-phosphate
F6P	fructose-6-phosphate
2PG	2-phosphoglycerate
3PG	3-phosphoglycerate
1,3PG	1,3-bisphosphoglycerate
DHAP	dihydroxyacetone phosphate
FBP	fructose-1,6-bisphosphate
GAP	glyceraldehyde-3-phosphate
Glc	glucose
PEP	phosphoenolpyruvate
PYR	pyruvate

Units

°C	degrees Celsius
A	Ampere
bp	base pair
Ci	Curie
cpm	counts per minute
Da	Dalton
g	gram
h	hour
l	litre
M	molar (mol/l)
min	minute
sec	second
rpm	rounds per minute
V	Volt
W	Watt

Prefixes

k	kilo	10^{3}
m	milli	10^{-3}
µ	micro	10^{-6}
n	nano	10^{-9}

Nucleotides

A	adenine
C	cytosine
G	guanine
T	thymine
U	uracile

Amino acids- nomenclatur: (IUPAC-IUB 1969)

A	Ala	alanine	M	Met	methionine
C	Cys	cysteine	N	Asn	asparagine
D	Asp	aspartate	P	Pro	proline
E	Glu	glutamate	Q	Gln	glutamine
F	Phe	phenylalanine	R	Arg	arginine
G	Gly	glycine	S	Ser	serine
H	His	histidine	T	Thr	threonine
I	Ile	isoleucine	T	Tyr	tyrosine
K	Lys	lysine	V	Val	valine
L	Leu	leucine	W	Trp	tryptophan

List of publications

Pietack, N., Becher, D., Hecker, M., Commichau F. M., Stülke, J. 2010. *In vitro* phosphorylation of key metabolic enzymes from *Bacillus subtilis*: PrkC phosphorylates enzymes from different branches of basic metabolism. *Journal of Molecular Microbiology and Biotechnology*.**18** (3):129-140.

Schmidl, S. R., Gronau, K., **Pietack, N.**, Hecker, M., Becher, D., Stülke, J. 2010. The phosphoproteome of the minimal bacterium *Mycoplasma pneumoniae*: Analysis of the complete known Ser/ Thr kinome suggests the existence of novel kinases. *Molecular & CellularProteomics*, *in press*

Lehnik-Habrink, M., Pförtner, H., **Pietack, N.**, Herzberg, C., Stülke, J., 2010. Identification of CshA as the major RNA helicase in the multi-protein complex. *in revision*

Summary

Bacillus subtilis is a model organism for Gram-positive bacteria and object for biotechnological applications. The extensive investigation of this bacterium makes it one of the best studied prokaryotes in terms of molecular and cell biology. However, a lot of issues are still open for the complete comprehension of this bacterium. The functions of various genes must be elucidated, as well as the role of posttranslational modifications. Among the posttranslation modifications the phosphorylation of proteins has a great regulatory potential. Recent studies revealed, that the phosphorylation of proteins on serine, threonine and tyrosine residues is widespread among bacteria, including *Bacillus subtilis*.

The aim of this work was to investigate the origin and function of phosphorylation events on serine, threonine and tyrosine residues in *B. subtilis*. For this purpose, multiple kinase mutants were constructed and their phosphoproteomes were analysed. However, the phosphoproteome of these mutants remained unchanged. Hence, it was assumed that unknown kinases or other mechanism of phosphorylation must be responsible. Several approaches revealed that certain proteins are autophosphorylated. The conserved GTP-binding proteins Obg and YdiB were found as autophosphorylated in the presence of their substrates GTP and ATP, respectively. Furthermore, the phosphosugar mutases phosphoglycerate mutase (Pgm) and phosphoglucosamine mutase (GlmM) are also autophosphorylated on conserved serine residues. The autophosphorylation of these residues is part of their enzymatic activity and thus kinase independent. These results indicate that apparently not all phosphorylation events in *B. subtilis* are kinase dependent. However, completely new kinases can not be excluded, but are obviously not responsible for the entirety of phosphorylation events in *B. subtilis*. Hence, it must be summarized that autophosphosphorylation of proteins can occur during interaction with energy rich phosphate carriers and it also can be part of enzymatic activity.

In *B. subtilis*, nearly all enzymes of glycolysis were found to be phosphorylated. In addition, previous studies described all genes that code for glycolytic enzymes as

essential. However, in this work it was discovered that each glycolytic gene can be deleted and that even strains with combinations of mutations are still viable. Growth tests revealed, that single mutants of glycolytic genes are able to grow on minimal medium with glucose and malate. Moreover, the phosphofructokinase (Δ*pfkA*) and glyceraldehyde-3-phosphate dehydrogenase mutant (Δ*gapA*) grow with glucose as sole carbon source.

For long time it was supposed that most of the glycolytic genes were essential for an unknown reason. This is the first time, that deletion mutants were constructed for each individual glycolytic gene of *B. subtilis*. Glycolytic mutants are a good basis for further studies in *B. subtilis*.

1. Introduction

Bacteria are a large group of unicellular microorganisms. Their metabolic diversity and their ability to adapt to nearly every habitat makes them an interesting and valuable source for science and biotechnological applications. Bacteria are distributed from the earth crust and hydrothermal vents in deep sea to the glaciers of antarctica. Living under these extreme conditions requires sophisticated adaptation mechanisms. All cellular and metabolic functions have to be tightly regulated to cope with these challenging environmental conditions. However, the cultivation of bacteria from extreme habitats is difficult and time-consuming. Therefore, the knowledge of basic biochemical and genetic processes must be achieved by studying model organisms like *Escherichia coli* or *Bacillus subtilis*. Furthermore, bacteria are also the main cause of infectious diseases worldwide. Working with pathogenic bacteria requires complex safety regulation and remains still risky. This disadvantage can be circumvented by studying non-pathogenic relatives of pathogenic bacteria. For example, the knowledge about the regulation of the basic metabolism gives on one hand the opportunities to modulate the metabolism for biotechnological demands. On the other hand, new targets for drugs against multi-resistant bacteria could be raised. This work focusses on glycolysis as one of the central metabolic pathways.

1.1. The Gram-positive model organism *B. subtilis*

B. subtilis is a Gram-positive rod-shaped soil bacterium with low GC-content. The natural habitat is the upper soil (rhizosphere). Thus, it is subject to rapidly changing environmental conditions. The availability of water, osmolarity, oxygen and nutrient supply and fluctuations in temperature are just a few examples that require elaborate adaptation mechanisms. Therefore, *B. subtilis* possesses different strategies that guarantee the survival under changing conditions. *B. subtilis* and other aerobic Gram-positives can adapt to stress condition, like energy depletion, cold and environmental shock by triggering the general stress response (Hecker *et al*., 2007). Furthermore,

during starvation of carbon and nitrogen sources, growing cultures of *B. subtilis* are able to form dormant, heat- and stress resistant endospores (Losick *et al*., 1986). Various pathogens are able to form endospores, too. For example *Bacillus anthracis* and *Clostridium botulinum* as important toxin producers. Therefore *B. subtilis* is a interesting model for the investigation of cell differentiation and division processes (Errington, 2003). In the rhizosphere, *B. subtilis* utilises different polymers as energy and carbon source. For their depolymerization several classes of enzymes are secreted. These skills, make *B. subtilis* an important source for biotechnological applications. Furthermore, it is used as workhorse for the production of vitamins and enzymes. As a non-pathogenic bacterium it is classified as GRAS-organism (GRAS = general recognized as safe) and can therefore be cultivated without extensive safety regulations. In addition, the natural competence in combination with a fully annotated genome make *B. subtilis* a meaningful toolbox for molecular biology (Kunst *et al*., 1997; Barbe *et al*., 2009).

1.2. Central carbon metabolism in *B. subtilis*

The central carbon metabolism in *B. subtilis* consists of glycolysis, gluconeogenesis, the pentose phosphate pathway and the citric acid cycle (Sauer & Eikmann, 2005). The main function of these metabolic pathways are the supply of energy and essential intermediates. Free energy that is released in metabolic processes is converted to high energy compounds, like ATP and reduction equivalents like NAD(P)H_2. Reduction equivalents are further necessary for anabolic enzymatic reactions. These central metabolic pathways are conserved in most organisms. Some pathogens display specific adaptation to their nutrition rich environment. For example *Mycoplasma* exhibits a reduced genome, but still requires the glycolytic pathway as the only source for energy generation (Himmelreich *et al*., 1996). Furthermore, nitrogen metabolism is of great importance for the production of amino acids. The central carbon metabolism generates carbon networks. These precursors are further converted in addition to a amino group

donor. Amino groups are mainly provided by the universal amino acids glutamate and glutamine.

1.2.1. Carbon sources and uptake mechanisms

Plants are the principle suppliers of biomass in the rhizosphere. This biomass provides polysaccharides like cellulose as the main nutrition source for *Bacillus*. These complex compounds must be broken down before transported into the cell. Therefore, *B. subtilis* secretes several enzymes to degrade the polymers, among them amylases, glucanases, lichenases, levanases (Stülke & Hillen, 2000).
Different mechanisms are used for the sugar transport across the membrane. Uptake occurs by facilitated diffusion, secondary transporters, ABC-transporters (ATP-binding cassette) and the PTS- system (phosphoenolpyruvate: phosphotransferase system) (Higgins, 1992; Postma *et al*., 1993).

1.2.2. Function and composition of the PTS

In bacteria the uptake of sugars occurs primarily by the PTS, resulting in the phosphorylation of monosaccharide or disaccharides. This modification produces a charged phosphosugar that cannot enter the membrane by diffusion. The trapped sugar is immediately converted in glycolysis. Besides its role in sugar uptake, the PTS has a function in signal transduction. In general the PTS consists of three basic components. These components are the enzyme I (EI), HPr and the enzyme II (EII). In *B. subtilis* the glucose specific enzyme II is organized in three domains. One of them is membrane associated (EIIC), whereas two are localized in the cytoplasm (EIIA and EIIB). The PTS dependent sugar uptake occurs via EIIC, where the sugar becomes phosphorylated. An energy rich intermediate of the glycolysis, phosphoenolpyruvate (PEP), serves as phosphate donor. The phosphate transfer from PEP to the sugar occurs through a phosphorylation cascade from EI to HPr-His15 and EIIA, EIIB (Fig. 1.1.). The general components of the PTS are encoded in the *ptsGHI*-operon that is glucose inducible

(Stülke *et al*., 1997). The HPr protein is the key element in the sugar uptake system. It can be phosphorylated by the ATP dependent HPr kinase at Ser-46 (Deutscher & Saier, 1983; Galinier *et al*., 1998; Reizer *et al*., 1998). The serine phosphorylation facilitates the binding of the transcriptional regulator CcpA (catabolite control protein A) (Schumacher *et al*., 2004). This complex regulates the expression of genes for the utilization of alternative carbon sources and is therefore important for carbon catabolite repression (Deutscher, 2008; Görke & Stülke, 2008; Fujita, 2009). In addition, a different phosphorylation site of HPr was identified at Ser-12 (Macek *et al*., 2007). However, the biological relevance of this phosphorylation event is unknown.

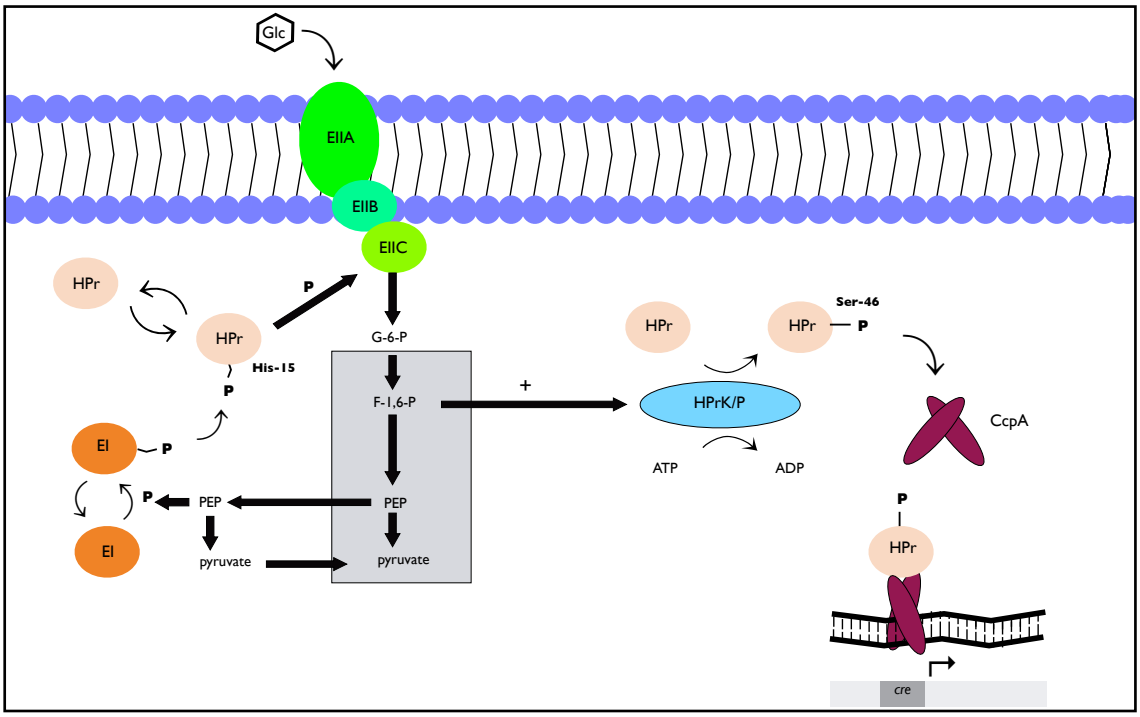

Figure 1.: Glucose transport via PTS and carbon catabolite repression in *B. subtilis*. The phosphate is transfered from PEP via EI and HPr protein (His-15), and EIIA and EIIB to the incoming sugar. The glucose uptake is mediated by the membrane spanning EIIC. In the presence of glucose HPr is phosphorylated by the HPrK/P on Ser-46. Serine phosphorylated HPr forms a complex with CcpA . This complex bind cre sequences of the DNA and thereby represses the transcription of catabolic operons. CM = cytoplasmatic membrane; *cre* = catabolite responsive elements.

1.2.3. Glycolysis

Glycolysis is a central metabolic pathway in nearly all organisms. Its function is the generation of ATP by substrate level phosphorylation and the production reduction equivalents. Furthermore it provides essential precursors for the amino acids- and cell wall biosynthesis. *B. subtilis* encodes all enzymes for the entire glycolysis (Ludwig *et al*., 2002).

Glucose is phosphorylated immediately when entering the cell by the PTS. Unphosphorylated hexoses must be phosphorylated by the ATP-dependent glucose kinase (Skarlatos & Dahl, 1998). Rearrangement of glucose-6-phosphate to fructose-6-phosphate is catalyzed by phosphoglucose isomerase encoded by *pgi*. Fructose-6-phosphate is then phosphorylated in a irreversible, ATP-dependent reaction by the phosphofructokinase (*pfkA*). The resulting fructose-1,6-diphosphate (FBP) is an important intermediate that indicates the metabolic status of the cell. It also acts as inducer and cofactor in regulatory processes (Jault *et al*., 2000; Zorilla *et al*., 2006). The FBP hexose ring is split by the fructose-1,6-diphosphate aldolase (*fbaA*) into two triose phosphates, dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (G-3-P). DHAP is rapidly interconverted to G-3-P by the triosephosphate isomerase (*tpiA*). The pay off phase in glycolysis starts with the oxidation of G-3-P to 1,3-bisphosphoglycerate by the glycolytic dehydrogenase (*gapA*), and reduction of NAD. In the reaction catalyzed by phosphoglycerate kinase (*pgk*) one molecule of ATP is obtained. 3-Phosphoglycerate (3-PG) is converted to 2-PG by the phosphoglycerate mutase (*pgm*), and further to PEP by enolase (*eno*). The pyruvate kinase (*pyk*) catalyses the final reaction in glycolysis, from PEP to pyruvate with the gain of one ATP. The reactions catalyzed by phosphofructokinase and the pyruvate kinase are irreversible. Interestingly, both genes are encoded in one operon. In the presence of glucose, the expression the *pfkA*-*pyk*-operon is induced twofold (Ludwig *et al*., 2001). The monocistronic *pgi* and *fbaA* genes are constitutively expressed. The remaining genes for the lower part of glycolysis are coded in the hexacistronic *gap* operon. This operon codes also for the own specific regulator (*cggR*) and the genes *gapA*, *pgk*, *tpiA*, *pgm* and

eno (Fillinger *et al*., 2000). The expression of the operon is repressed by CggR in the absence of glucose. A second promoter downstream of *gapA* guarantees the expression of the downstream genes, that are necessary for both glycolysis and gluconeogenesis.

1.2.4. Gluconeogenesis

Glycolysis provides energy, but more importantly metabolites for anabolic reactions. These metabolites are needed for amino acid and peptidoglycan biosynthesis. Three reactions in glycolysis are irreversible and must therefore be bypassed. Gluconeogenesis is mainly fed by the products of PEP carboxykinase and malic enzymes. PEP can be generated from oxaloacetate by the PEP carboxykinase (*pckA*) (Tännler *et al*., 2008). Malic enzymes, especially YtsJ, convert malate to pyruvate (Lerondel *et al*., 2008). The gained PEP is further converted by the reversible reactions of enolase, phosphoglycerate mutase and phosphoglycerate kinase to 1,3-bisphosphoglycerate. Conversion of 1,3-bisphosphoglycerate to glyceraldehyde-3-phosphate is catalyzed by the gluconegenetic glyceraldehyde-3-phosphate dehydrogenase (*gapB*). The reversible reaction of aldolase (*fbaA*) produces fructose-1,6-bisphosphate. The irreversible activity of the phosphofructokinase is bypassed by fructose-1,6-bisphosphatase (*fbpA*). Interestingly, Fujita and Freese have shown that the phosphatase activity is not strictly required for growth on gluconeogenetic substrates (Fujita & Freese, 1981). Recently, GlpX was described to be also active as fructose-1,6-bisphosphatase. A double mutant lacking both fructose-1,6-bisphosphatases (FbpA and GlpX) was unable to grow on gluconeogenetic carbon sources (Jules et al., 2009). The last reaction resulting in glucose-6-phosphate is reversible and catalyzed by phosphoglucose isomerase. Glucose-6-phosphate is an important precursor for the synthesis of polysaccharides. These polysaccharides are the basis for the peptidoglycan synthesis and therefore essential for cell wall synthesis. The enzymes PckA (*pckA*) and GapB (*gapB*) catalyse the irreversible and therefore essential reactions at junctions of glycolysis and gluconeogenesis. Their expression isrepressed during growth on glycolytic substrates by the transcriptional regulator CcpN (Servant *et al*., 2005).

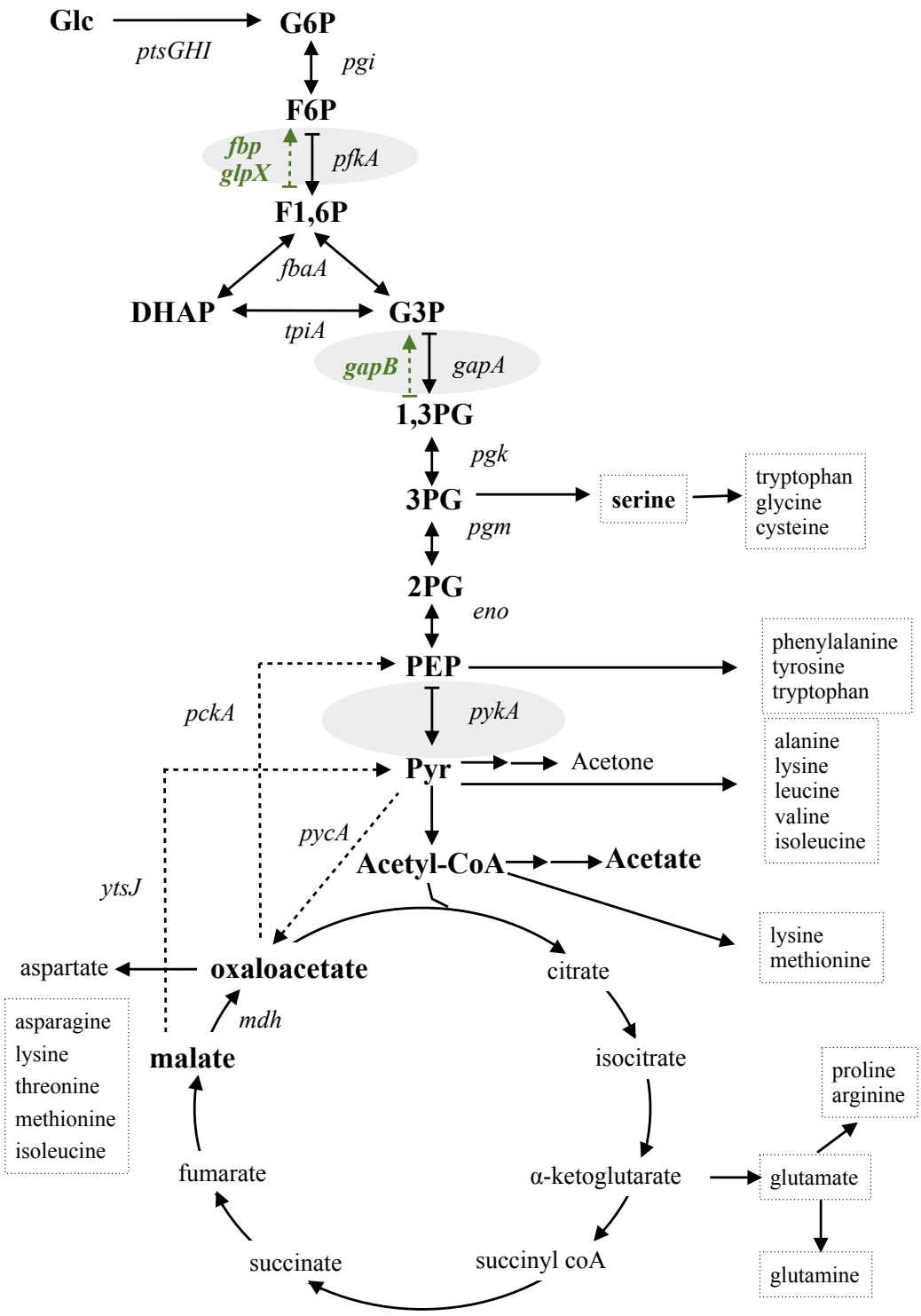

Figure 2.: Central carbon metabolism in *B. subtilis*. Intermediates of the main reactions in glycolysis and gluconeogenesis are highlighted in bold letters. Irreversible reactions in glycolysis are marked in grey. Reaction in gluconeogenesis are indicated with dashed lines. Connection between carbon metabolism and amino acids biosynthesis is indicated in dotted boxes.

1.2.5. Main routes of carbon utilization in *B. subtilis*

Bacteria evolved different pathways for the utilization of glucose. The anaerobic lactic acid bacteria metabolize glucose through homo- and heterofermentative metabolism. Homofermentative bacteria utilize glucose via glycolysis. Heterofermentative bacteria do not have an aldolase, therefore they can not utilize glucose via glycolysis. However, they can utilize glucose via the phosphoketolase pathway. When *B. subtilis* is grown aerobically on glucose, the main carbon flow passes glycolysis. About 40% of the carbon enters the pentose phosphate pathway (Furer *et al*., 2004; Kleijn *et al*., 2010). Only half of carbon passes the citric acid cycle. Most of the sugar is excreted by overflow metabolism. The main product of overflow metabolism in *B. subtilis* is acetic acid. Surprisingly, during growth on malate just 10% of the carbon is converted in gluconeogenesis, while excretion in overflow metabolism remained unchanged.

1.3. Posttranslational modifications

The posttranslation modification of proteins is an important mechanism for the regulation of cellular processes. Different kinds of protein modification have been described so far. The main types are acetylation, adenylation, glycosylation, methylation and phosphorylation (Amaro & Jerez, 1984; Merrick & Edwards, 1995; Cozzone, 1998; Abu-Qarn *et al*., 2008; Wang *et al*., 2010). These modifications occur at different amino acids residues of proteins that are related to different functional groups in the cell, like carbon metabolism and protein synthesis *etc*. Furthermore, phosphorylation of proteins plays an important role in the regulation of enzymatic activities.

1.3.1. Protein phosphorylation in bacteria

Reversible protein phosphorylation is an important mechanism of posttranslational modification. In *B. subtilis*, about 5% of all proteins are subject to phosphorylation. In general the phosphorylation of proteins is catalyzed by protein kinases that use ATP as

phosphate donor for the phosphorylation of specific amino acid residues. The dephosphorylation is catalyzed by phosphatases that remove the phosphate group. In 1978 the first phosphorylation of proteins on Ser- and Thr residues in bacteria was described (Wang & Koshland, 1978). Moreover, studies in *E. coli* revealed that phosphorylation of the isocitrate dehydrogenase on a Ser-residue regulates the carbon flow between citric acid cycle and glyoxylate pathway (Garnak & Reeves, 1979). However, it was long time assumed that phosphorylation of proteins is specific for eukaryotes and protein phosphorylation in bacteria is limited to the PTS and two-component regulatory systems (Deutscher & Saier, 2005).

In the last decades protein phosphorylation turned into focus of scientific research. The progress in the development of methods for analysis of phosphorylation events allows a rapid and sensitive investigation of phosphoproteomes. Phosphoproteome analysis in *B. subtilis* identified around 80 proteins phosphorylated on Ser/ Thr and Tyr residues (Levine *et al*., 2006 Macek *et al*., 2007; Eymann *et al*., 2007). However, the function and the origin of these phosphorylation events is mostly unknown. Further phosphoproteome studies were performed in *Campylobacter jejuni*, *Echerichia coli*, *Klebsiella pneumoniae*, *Lactococcus lactis*, *Mycoplasma pneumoniae, Pseudomonas aeruginosa* and *Streptococcus pneumoniae* (Li *et al*., 2007; Voisin *et al*., 2007; Macek *et al*., 2008; Ravichandran *et al*., 2008; Soufi *et al*., 2008; Sun *et al*., 2010; Schmidl *et al*., 2010). These studies revealed that phosphorylation on Ser-/ Thr and Tyr residues is wide spread among Gram-positive and Gram-negative bacteria and is therefore not specific for eukaryotes. In addition, conserved phosphoproteins were identified in all investigated species.

In general protein phosphorylations in bacteria can be summarized in three groups. First, phosphorylation of proteins during the sugar uptake by PTS; second, signal transduction by two-componenent sytems; third, phosphorylation of proteins on Ser-/Thr- and Tyr residues that is involved in a variety of cellular processes.

1.3.2. Two-component regulatory systems

Two-component regulatory systems are wide spread among bacteria and are important for sensing of and reacting to environmental changes. They consist of a membrane associated sensor kinase and a response regulator. The sensor kinase responds to specific stimuli with ATP-dependent autophosphorylation on a His-residue. The phosphate group is further passed to an aspartate residue of the cognate response regulator. The response regulator binds specific DNA-sequences and thereby regulates the expression of certain genes. *B. subtilis* possesses 36 histidine kinases and 34 response regulators, that are part of two-component regulatory systems (Fabret *et al*., 1999). Two-component systems are involved in regulation of diverse cellular processes for example, the development of competence, spore formation, citrate uptake and peptidoglycan synthesis (Dubnau, 1991; Trach *et al*., 1991; Yamamoto *et al*., 2000; Bisicchia *et al*., 2010).

1.3.3. Protein phosphorylation on serine/ threonine and tyrosine

In *B. subtilis* about 80 proteins were found to be phosphorylated on Ser-/ Thr-and Tyr-residues. Only a few of these phosphorylation events are well understood. Interestingly, nearly all glycolytic enzymes were identified as phosphorylated on Ser-/Thr- and partially Tyr-residues.

Protein phosphorylations on Ser/Thr and Tyr residues are the basis of differential regulatory mechanisms like the regulation of gene expression, regulation of enzymatic activities and they also may regulate protein-protein interactions. Studies in *B. subtilis* revealed that regulatory protein phosphorylation takes place in carbon catabolite repression, general stress response, peptidoglycan synthesis, replication and many more. Well studied examples for protein phosphorylation are Crh and HPr. These proteins are phosphorylated under certain conditions by the ATP-dependent HPr-kinase on Ser-46. The activity of the HPr kinase is stimulated by ATP and FBP. The phosphorylated forms

of Crh and HPr act as co-factors for the transcriptional regulator CcpA, that mediates carbon catabolite repression (Brückner & Titgemeyer 2002; Stülke & Hillen, 1999).

Besides, the activity of sigma factors is regulated by protein phosphorylation. The sigma factor B (SigB) is responsible for the transcription of genes under different stress conditions. SigB is regulated by a partner switching mechanism that is dependent on the phosphorylation status of the participating proteins (on serine and threonine) (Alper *et al*., 1996; Xiaofeng *et al*., 1996). Under standard growth conditions, SigB is sequestered by an anti-sigma factor that phosphorylates the anti-anti-sigma factor. The phosphorylated anti-anti-sigma factor is inactive. Under stress conditions different activation routes result in the dephosphorylation of the anti-anti-sigma factor that now binds and inactivates the anti-sigma factor. The released SigB can now associate to the RNA polymerase and the transcription of the stress genes occurs.

B. subtilis possesses also a protein kinase that is similar to eukaryotic Ser-/Thr-kinases. Blast analysis revealed that the kinase domain of PrkC is conserved among bacteria. In *B. subtilis* PrkC is required for germination in response to muropeptides that were sensed by extracellular PASTA-domain (Shah *et al*., 2008). Different proteins were identified as phosphorylated by PrkC; among them elongation factors (EF-Tu and G), the ribosome associated GTPase CpgA and the potential stressosome component YezB and proteins of central carbon metabolism (Gaidenko *et al*., 2002; Absalon *et al*., 2009; Pietack *et al*., 2010).

In contrast to phosphorylations on serine and threonine, phosphorylation on tyrosine is rare. In *B. subtilis* two bacterial tyrosine kinases (BY-kinase) are described, PtkA and EpsB. However, EpsB is just a potential tyrosine kinase (Olivares-Illana *et al*., 2008).

The UDP-glucose dehydrogenase (Ugd) is required for cell wall synthesis and its phosphorylation by PtkA is required for its enzymatic activity (Mijakovic *et al*., 2003; Petranovic *et al*., 2009). PtkA also phosphorylates single strand DNA binding proteins. Here the phosphorylation enhances the binding activity for DNA and is therefore required for efficient replication (Petranovic *et al*., 2007).

However, protein phosphorylation is not limited to His/Asp and Ser/Thr/Tyr. Recently it was shown that the transcription repressor for heat shock proteins, CtsR is

phosphorylated by McsB on an arginine residue. The arginine phosphorylation prevents CtsR from binding DNA (Fuhrmann *et al*., 2009).

1.4. Essential genes and metabolic skills

In the recent years a large number of complete genomic sequences became available for bacteria that are adapted to diverse habitats. The available genomes data are basis for for the detailed investigation of cellular functions. The gained knowledge can be used for specific modification of bacterial metabolism. This opportunity is an important aspect of biotechnology and allows purposeful construction of metabolic pathways.

1.4.1. Minimal genome and essential processes

Organisms must have certain properties to be classified alive. They must be capable of reproduction and cellular development. Furthermore, they can respond to stimuli and maintain homeostasis. Related to these general qualities, every organism must have a set of genes for DNA and RNA metabolism, protein processing and folding, for cellular processes and energy and intermediary metabolism.

Comparative genomics revealed a high conservation of genes in bacteria. This conservation can be ascribed to horizontal gene transfer, that served as dominant force of prokaryotic evolution (Koonin *et al*., 2008). Therefore, genes-products that catalyze essential reactions in the cell, are more conserved then species specific genes. The smallest genome of a self-replicating organism was found in *Mycoplasma genitalium.* This Gram-positive pathogen possesses only 468 protein-coding genes (Fraser *et al*., 1995). These small genomes are typical for *Mycoplasma* species and are the product of degenerative evolution. *Mycoplasma* species are obligate parasites that require the supply of essential metabolites. They possess a complete glycolysis, however the pentose phosphate pathway is incomplete and the tricarboxylic acid cycle is missing (Himmelreich *et al*., 1996). Because of these metabolic restrictions, they need complex nutrition for growth. However, complex medium did not make glycolysis dispensable

for their survival. In contrast to that, *B. subtilis* possesses a complete glycolysis, the pentose phosphate pathway and the tricarboxylic acid cycle. The genome of *B. subtilis* codes for around 4,100 protein-coding genes, from that 271 genes were described to be essential by single deletion (Kobayashi *et al*., 2003). However, bioinformatic analysis showed that around 2,000 genes are duplications. In contrast to that the *M. genitalium* genome with around 500 genes possesses only 90 duplications (Hannay *et al*., 2008).

1.4.2. Specific requirements and versatile adaptation

B. subtilis is a ubiquitous soil bacterium, that is exposed to rapidly changing environmental conditions and nutrition supply. Therefore, its genome contains specific genes for the utilization of various nutrients. The expression of genes enables the cell to grow on different carbon sources. As *B. subtilis* is not using all carbon sources at the same time, the expression of these genes has to be regulated to avoid futile expression. Thus, *B. subtilis* possesses different transcription regulators that coordinate the expression of genes for the efficient utilization of different carbon sources. However, metabolites of glycolysis serve as signals for the metabolic status of the cell. When *B. subtilis* is grown on glucose, FBP stimulates the phosphorylation of HPr and Crh that act as co-repressors for CcpA in carbon catabolite repression. CcpA regulates the transcription of around 300 genes by binding on *cre*-DNA sequences. In general the expression of genes involved in the overflow metabolism is activated by CcpA (Tobisch *et al*., 1999; Blencke *et al*., 2003).

Furthermore, the expression of glycolytic genes is regulated depending on the metabolic status. During growth under gluconeogenetic conditions, CggR represses the transcription of the *gapA* operon. However, under glycolytic condition the expression is derepressed. The derepression is also mediated by FBP (Doan & Aymerich, 2003).

The gluconeogenetic genes *pckA* and *gapB* are also regulated on the transcriptional level by CcpN, that represses their expression under glycolytic conditions (Servant *et al*., 2005).

1.5. Aim of this work

The posttranslational modification of proteins is an ubiquitary mechanism to regulate cellular processes in all domains of life. Protein phosphorylation is one of the most important and best studied forms of modification. Phosphorylation on serine, threonine and tyrosine residues was long time assumed to be unique to eukaryotes. Recent studies indicated that this modification does also occur in bacteria. However, the origin and relevance of these phosphorylation events are unknown to great extent.

The aim of this work was to investigate protein phosphorylation events in *B. subtilis*. Therefore, the phosphoproteome of multiple kinase mutants was under investigation.

2. Materials and Methods

2.1. Microbiological techniques

Materials: Chemicals, utilities, equipment, commercial systems, proteins, antibodies, enzymes and oligonucleotides are listed in the appendix.

2.1.1. Bacterial strains and plasmids

See appendix of this work, in chapter 6.

Growth media

Buffers, solutions and media were prepared with deionized water and autoclaved (20 min at 121°C and 2 bar). Thermolabile substances were dissolved and sterilized by filtration. Solutions are related to water, other solvents are indicated.

Bacterial growth media and optional additives

B. subtilis was grown in C-minimal medium, supplemented with specific additives as indicated. C- minimal medium was supplemented with glucose (Glc) and malate (0.5% w/v). CSE-Glc minimal medium contains beside glucose, sodium succinate (S) (final concentration 8 g/l) and potassium glutamate (E) (final concentration 6 g/l). Further variations of carbon sources are indicated. Basic media were supplemented with agar (16 g/l) for solidification.

5x C salts (1 l)	KH_2PO_4	20 g
	K_2HPO_4 x 3 H_2O	80 g
	$(NH_4)_2SO_4$	16.5 g

III` salts (1 l)	$MnSO_4$ x $3H_2O$	0.232 g
	$MgSO_4$ x $7H_2O$	12.3 g
10x MN medium	K_2HPO_4 x 3 H_2O	136 g
(1l)	KH_2PO_4	60 g
	Sodium citrate x 2 H_2O	10 g
1x C minimal medium	5 x C salts	20 ml
(100 ml)	Tryptophan (5 mg ml^{-1})	1 ml
	Ammonium iron citrate (2.2 mg ml^{-1})	1 ml
	III`salts	1 ml
	H_2O_{deion}	ad 100 ml
1x CSE medium	5 x C salts	20 ml
(100 ml)	Tryptophan (5 mg ml^{-1})	1 ml
	Ammonium iron citrate (2.2 mg ml^{-1})	1 ml
	III`salts	1 ml
	Potassium glutamate (40%)	2 ml
	Sodium succinate (30%)	2 ml
	H_2O_{deion}	ad 100 ml
SP medium	Nutrient Broth	0.8 g
(1 l)	$MgSO_4$ x $7H_2O$	0.25 g
	KCl	1.0 g
	H_2O_{deion}	ad 1 l
	autoclave, after cooling down addition of:	
	$CaCl_2$ (0.5 M)	1 ml
	$MnCl_2$ (10 mM)	1 ml
	Ammonium iron citrate (2.2 mg ml^{-1})	2 ml
MNGE medium	1 x MN medium	8.77 ml
(10ml)	Glucose (20%)	1 ml
	Potassium glutamate (40%)	50 µl
	Ammonium iron citrate (2.2 mg ml^{-1})	50 µl
	Tryptophan (5 mg ml^{-1})	100 µl

	$MgSO_4$ x $7H_2O$ (1 M)	30 µl
+/-	CAA (10%)	100 µl

X-Gal Stock solution: 40 mg/ml X-Gal in DMF, final concentration 40 µg/ml in medium.

Antibiotics

Antibiotics were prepared as 1000-fold concentrated stock solutions. Ampicillin, spectinomycin, lincomycin and kanamycin were dissolved in deion. water, chloramphenicol, erythromycin and tetracycline in 70% ethanol. All solutions were sterile filtrated and stored at -20°C. Autoclaved medium was chilled down to approximately 50°C, and antibiotics were added to their final concentration. For light sensitive additives such as tetracycline, incubation occurred in the dark.

Selection concentration for *E. coli*

Ampicillin	100 µg ml^{-1}
Spectinomycin	100 µg ml^{-1}
Kanamycin	50 µg ml^{-1}

Selection concentration for *B. subtilis*

Chloramphenicol	5 µg ml^{-1}
Erythromycin	2 µg ml^{-1}
Kanamycin	5 µg ml^{-1}
Lincomycin	25 µg ml^{-1}
Spectinomycin	100 µg ml^{-1}
Tetracycline	10 µg ml^{-1}

[1]For selection on *ermC* a mixture of erythromycin and lincomycin was used in their respective concentration, see above.

2.2. Methods

2.2.1. General methods

Some general methods used in this work that are described in the literature are listed in table 3.1.

Method	Reference
Absorption measurement	Sambrook *et al*., 1989
Ethidiumbromide staining	Sambrook *et al*., 1989
Precipitation of nucleic acids	Sambrook *et al*., 1989
Gel electrophoresis of DNA	Sambrook *et al*., 1989
Plasmid preparation from *E. coli*	Sambrook *et al*., 1989
Ligation of DNA fragments	Sambrook *et al*., 1989
Determination of protein amounts	Bradford, 1976
Gel electrophoresis of proteins (denaturating)	Laemmli, 1970
Sequencing according to the chain termination method	Sanger *et al*., 1977

Tab. 3.1. General methods

2.2.2. Cultivation of bacteria

Unless otherwise stated, *E. coli* was grown in LB medium at 37°C and 200 rpm in tubes and flasks. *B. subtilis* was grown in LB medium, CSE, C-Glc, malate and MNGE medium at 37°C or 28°C in tubes and Erlenmeyer flasks. Fresh colonies from plates were used for inoculation. Furthermore, overnight liquid cultures were used. Growth was measured at a wavelength of 600 nm. For the calculation of the generation time the optical density of cultures in the logarithmic growth phase was used. The generation time (g) was calculated with the following formula:

$$g = \frac{lg2 \times (t_2 - t_1)}{\log K_2 - \log K_1}$$

g= generation time (min)

t_1= time point 1 (min) K_1=OD_{600} at time point 1

t_2 =time point 2 (min) K_2=OD_{600} at time point 2

Storage of bacteria

E. coli was kept on LB medium agar plates up to 4 weeks at 4°C. For long-term storage glycerol or DMSO cultures were established. *B. subtilis* was cultured on LB medium agar plates not longer than 3 days. SP agar plates and tubes were used for the long-term storage of *B. subtilis*. Strains that require glucose or malate were stored in cryo cultures. For the storage of bacteria in glycerol, 900 µl of a fresh overnight culture was gently mixed with 600 µl of 50% glycerol (final concentration of 33.3% (w/v) glycerol). DMSO cultures were prepared with 900 µl of a fresh overnight culture mixed with 100 µl of DMSO (100%). Stocks were frozen and stored at -70°C.

2.2.3. Transformation of *E. coli*

Preparation of competent cells by the $RbCl_2$ method

A single colony of *E. coli* DH5α was used to inoculate a LB overnight culture (4 ml). This culture was used to inoculate 500 ml of LB medium in a dilution of 1 to 200. *E. coli* DH5α was incubated at 37°C and 200 rpm in baffled flasks for 4 h. Cultures were cooled down by shaking in ice water for 10 min. The cells were harvested by centrifugation (10 min; 3,000 rpm; 4°C) and suspended in 20 ml of ice-cold TfbI. After centrifugation (10 min; 3,000 rpm; 4°C), the cells were suspended in 4 ml of ice-cold TfbII. Aliquots of 200 µl were frozen in liquid nitrogen and stored at -70°C. *E. coli* was transformed by the heat shock method.

TfbI- Buffer

	Final concentration	g /100 ml
K-Acetate	30 mM	0.29
$MnCl_2$	50 mM	0.99
$RbCl_2$	100 mM	1.21
$CaCl_2$	10 mM	0.147
Glycerol	15%	(30 ml)

The chemicals were dissolved in 100 ml H_2O_{deion}. The pH value was adjusted to 5.8 with HCl. The buffer was sterile filtrated and stored on ice.

TfbII- Buffer

	Final concentration	g /100 ml
MOPS	10 mM	0.21
$CaCl_2$	75 mM	1.1
$RbCl_2$	10 mM	0.121
Glycerol	15%	(30 ml)

The chemicals were dissolved in 100 ml H_2O_{deion}. The pH value was adjusted to 6.8 with NaOH. The buffer was sterile filtrated and stored on ice.

Competent cells were thawed on ice, and 10-100 ng DNA were added to 200 µl cells. The suspension was mixed and incubated on ice for 30 minutes. The heat shock was performed at 42°C for 90 seconds. Afterwards, the samples were incubated for 5 minutes on ice. After addition of 600 µl LB medium, the samples were incubated 60 minutes at 37°C for (with shaking). One hundred microlitres and the concentrated rest were plated on LB selection plates (with ampicillin, spectinomycin and kanamycin, respectively).

2.2.4. Transformation of *B. subtilis*

Preparation of competent cells

Ten millilitres of MNGE medium containing 1% CAA were inoculated with an overnight culture of *B. subtilis* to an optical density of ~0.1. This culture was grown at 37°C with aeration until OD_{600} of ~1.3 was reached. Then the culture was diluted with 10 ml MNGE medium without CAA and incubated again for one hour. After the incubation step, the cells were harvested by centrifugation (5 min; 5,000 rpm; RT). The supernatant was retained in a sterile falcon tube. The pellet was resuspended in 1/8 of the supernatant and supplemented with glycerol to a final concentration of 10%. Aliquots of 300 µl were frozen in liquid nitrogen and stored at -70°C.

Transformation of competent cells

Three hundred microlitres of aliquoted competent cells were thawed at 37°C and supplemented with 100 µl of following solution:

MN (1x)	1.7 ml
Glucose (20%)	43 µl
$MgSO_4$ (1 M)	34 µl

400 microlitres of competent cells were incubated with 5 µg DNA for 30 min at 37°C with shaking. Afterwards, 100 µl of expression solution (50 µl yeast extract (5%), 25 µl CAA (10%), 25 µl deion. water and 5 µl tryptophan (5 mg/ml)) was added and the samples were incubated at 37°C for one hour. The cell suspensions were spread onto SP or LB plates containing the appropriate antibiotics for selection.

2.2.5. Preparation and detection of DNA

Preparation of plasmid DNA from *E. coli*

Plasmid DNA was prepared from *E. coli* carrying the desired plasmid. An overnight culture (4 ml) with cells carrying the desired plasmid was harvested (2 min; 13,000 rpm). The plasmid DNA was isolated using the Mini Prep Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. Deion. water was used for elution of the DNA from the columns. All steps were performed at room temperature.

Isolation of genomic DNA of *B. subtilis*

Genomic DNA of *B. subtilis* was isolated using the DNeasy Tissue Kit (Qiagen, Hilden, Germany). *B. subtilis* was grown overnight in LB medium. Cells of 1.5 ml culture volume were harvested (2 min; 13,000 rpm; RT). The pellet was resuspended in 180 µl lysis buffer and incubated at 37°C for 60 min. The further steps for the isolation of the genomic DNA were performed according to the manufacturers instructions.

Solutions for isolation of genomic DNA

Lysis buffer	Lysozyme	50 mg
	Tris-HCl pH 8.0 (1 M)	50 µl
	EDTA pH 8.0 (0.5 M)	10 µl
	H_2O_{deion}	2.5 ml

Agarose gel electrophoresis

For analytical and preparative separation of DNA fragments, agarose gels containing 1 to 2% (w/v) agarose (according to the expected fragment size) in TAE buffer were prepared. The DNA samples were mixed with 5 x DNA loading dye to facilitate loading and to indicate the migration of the samples in the gel. A voltage of 80–120 V was

applied until the color marker reached the last third of the gel. DNA fragments migrate towards the anode with a velocity that is proportional to the negative logarithm of their length. After electrophoresis, gels were incubated in ethidium bromide solution for 5 min and briefly rinsed with H_2O_{deion}. The DNA was detected and documented via its fluorescence under UV light (λ= 254 nm). For the estimation of the size of the DNA fragments, the GeneRuler™ DNA Ladder Mix and λ-DNA marker were used. For the isolation of DNA fragments from preparative gels, the bands were visualized at a wavelength of λ = 365 nm. The bands were cut out and led across a microcentrifuge tube. The purification of the DNA followed with the QIAquick PCR Purification Kit (250) (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Solutions for agarose gel electrophoresis

1% Agarose gel	1% (w/v) Agarose in 1 x TAE	1% (w/v)
50 x TAE buffer	Tris	242 g
	Tris-acetate	57.1 ml
	EDTA (0.5 M, pH 8.0)	100 ml
	H_2O_{deion}	ad 500 ml
5 x DNA loading dye	Glycerol	5 ml
	H_2O_{deion}	4.5 ml
	TAE (50 x)	200 µl
	Bromphenol blue	0.01 g
	Xylencyanol	0.01 g

Sequencing of DNA

Sequencing was done based on the chain termination method (Sanger) with fluorescence labeled dideoxynucleotides. The sequencing reactions were conducted by SeqLab (Göttingen, Germany) and the Laboratorium für Genomanalyse (G_2L) of the Georg-August-University Göttingen.

Digestion of DNA

The digestion of DNA with endonucleases was performed with buffers recommended by the manufacturer. Reaction buffers, concentration of enzymes and DNA as well as incubation temperatures were chosen according to the manufacturer's instructions. The digestion was allowed to proceed for up to 2 h and was, if possible, followed by heat inactivation of the restriction endonucleases (20 min; 65°C or 80°C). The DNA was purified using the PCR Purification Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

Dephosphorylation of DNA

To avoid re-circularization of a previously digested DNA vector, the 5' phosphate groups of the linearized vector were removed prior to the ligation reaction. The dephosphorylation of the 5`-prime end of DNA fragments was performed with the FastAP (alkaline phosphatase) (Fermentas, Lithuania) with buffers supplied by the manufacturer. Approximately 3-10 ng/µl DNA were mixed with 1 µl FastAP (1U/µl) and incubated at 37°C for 30 min. The FastAP was inactivated by boiling at 75°C for 10 min.

Ligation of DNA

DNA fragments were ligated using T4-DNA ligase (Fermentas, Lithuania) with buffers supplied by the manufacturer. The ligation reaction contained 20 - 200 ng of vector DNA and an excess of the DNA fragment (insert to vector molar ratio of 10:1 to 20:1). The reaction was started after addition of 5 U T4-DNA ligase in a final volume of 20 µl. The ligation occurred for 2 h at RT or overnight at 16°C.

Polymerase chain reaction (PCR)

DNA was amplified by PCR with specific oligonucleotide primers. For each DNA fragment of interest, primers were designed. For cloning of DNA fragments into plasmid vectors, recognition sequences for specific restriction endonucleases were added via primers at both ends of the amplified DNA fragments. Oligonucleotides were purchased from Eurofins MWG Operon (Ebersberg, Germany) and Sigma-Aldrich (Munich, Germany). All oligonucleotides used in this work are listed in the appendix.
The PCR reactions were performed in a total volume of 50 µl. For check PCR the *Taq* polymerase (own purification) was used. The *Taq* polymerase is known to create one mismatch in 1,000 base pairs.

Reaction conditions for the *Taq* polymerase		
template DNA	2.5 µl	10 ng plasmid DNA / 200 ng genomic DNA
10 x reaction buffer	5 µl	
forward primer (20 pmol)	5 µl	
reverse primer (20 pmol)	5 µl	
dNTPs 12.5 mM	2 µl	
Taq polymerase	1 µl (1 U)	
H_20_{deion}	30.5 µl	

The sample was mixed and placed in a thermocycler with a preheated lid. After an initial DNA denaturation step, a cycle consisting of three steps including denaturation, primer annealing and primer elongation was performed for 30 times. The reaction was terminated after a final elongation step by cooling down to 15°C. Time and temperature for denaturation, time of annealing and elongation temperature remained constant for each reaction. The annealing temperature (Tm) was dependent on the length of the oligonucleotide and its G+C content. It was calculated as follows:

$$Tm\ [°C] = 69.3 + 0.41\ (\%\ G+C) - 650/n$$

% G+C represents the G+C content in the primer; *n* represents the number of nucleotides.

Standard program for the *Taq* polymerase				
denaturation	95°C	300 s		
denaturation	95°C	30 s	}	30 x
annealing	Tm [°C] 1/- 10°C	45 s		
elongation	72°C	60s per 1 kb		
elongation	72°C	600 s		
break	15°C	∞		

For cloning of PCR fragments, the Phusion™ polymerase (Finnzymes, Espoo, Finland) was used, because of its proofreading activity that avoids mistakes during amplification.

Reaction conditions for the *Phusion*™ polymerase	
template DNA	2.5 µl (10 ng plasmid DNA /
5 x reaction buffer	10 µl
forward primer (20 pmol)	5 µl
reverse primer (20 pmol)	5 µl
Phusion™ dNTPs 12.5 mM	1µl
Phusion™ polymerase	0.5 µl (1 U)
H_2O_{deion}	26 µl

Standard program for the *Phusion*™ polymerase				
denaturation	98°C	60 s		
denaturation	98°C	10 s	}	30 x
annealing	Tm [°C] 1/- 10°C	30 s		
elongation	72°C	20s per 1 kb		
break	15°C	∞		

Combined chain reaction (CCR)

For site directed mutagenesis the combined chain reaction was used. The CCR is a PCR-based mutagenesis in which the mutagenic primers hybridize more strongly to the template then the flanking primer. The mutagenic primer is phosphorylated at its 5`end, and is ligated to the 3` OH-group of the extended upstream primer by the action of a thermostable DNA ligase. For the design of the mutagenic primer three guidelines were followed: First, the melting temperature of the mutagenic primer is 10°C above that of the external primers. Second, the number of mismatching nucleotides should be as low as possible. And third, the mutation must be localized in the middle of the primer.

Combined chain reaction (CCR): Reaction conditions for the *Accuzyme*™ polymerase	
plasmid DNA (200 ng)	1 µl
10 x CCR buffer	5 µl
forward primer (20 pmol)	2 µl
reverse primer (20 pmol)	2 µl
mutagenic primer (20 pmol)	4 µl
dNTPs (12.5 mM)	1µl
bovine serum albumine (10 mg/ml)	2 µl
Ampligase®	3 µl (15 U)
Accuzyme™ polymerase	1 µl (2.5 U)
H_2O_{deion}	24 µl

The CCR reaction was perfomed with 2.5 U of Accuzyme™ DNA polymerase (Bioline, Luckenwalde, Germany) and Ampligase® (Epicentre, Madison, USA) in CCR buffer (20 mM Tris-HCl [pH 8.5], 3 mM $MgCl_2$, 50 mM KCl, 0.5 mM NAD^+ and 0.4 mg/ml bovine serum albumin (BSA, New England BioLabs, Ipswich, USA)) in a total volume of 50 µl.

Standard program for CCR with *Accuzyme*™ polymerase				
denaturation	95°C	300 s		
denaturation	95°C	30 s	}	30 x
annealing	57°C	30 s		
elongation	65°C	180 s per 1 kb		
elongation	65°C	360 s		
break	15°C	∞		

Long flanking homology PCR (LFH-PCR)

Deletion of a gene in *B. subtilis* was performed with the long flanking homology PCR (LFH-PCR) technique (Wach, 1996). For this purpose, genes that mediate resistance against chloramphenicol, erythromycin, kanamycin and spectinomycin were amplified from the plasmids pDG646, pDG780, pDG1726 and pGEM-cat, respectively (Guerout-Fleury *et al*., 1995). DNA fragments of about 1,000 bp flanking the target gene at its 5' and 3' ends were amplified. The 3' end of the upstream fragment as well as the 5' end of the downstream fragment extended into the target gene in a way that all expression signals of genes up- and downstream of the gene remained intact. The joining of the two fragments to the resistance cassette was performed in a second PCR. Joining was allowed by complementary sequences of 25 bp that were attached to the single fragments by the respective primers. Thus, the 3` end of the upstream fragment was linked with the 5` end of the resistance cassette and the 3`end of the resistance with the 5`end of the downstream fragment. For the LFH joining reaction, 150 ng of the up- and downstream fragments and 300 ng of the resistance cassette were used. The fused fragment was amplified by PCR using the forward primer of the upstream fragment and the reverse primer of the downstream fragment. The PCR product was purified using the QIAquick PCR Purification Kit (Qiagen; Hilden; Germany). *B. subtilis* was transformed with the purified PCR products and transformants were selected on plates. Clones were examined by check PCR for the integrity of the resistance cassette (see appendix). The DNA sequence of the flanking regions was verified by sequencing.

Long flanking homology PCR (LFH-PCR)	
10 x buffer	10 µl
upstream fragment (150 ng)	5 µl
downstream fragment (150 ng)	5 µl
resistance cassette (300 ng)	3 µl
H_2O_{deion}	64 µl
After step 1 addition of primer, dNTPs and polymerase	
forward primer upstream fragment (20 pmol)	4 µl
reverse primer downstream fragment (20 pmol)	4 µl
dNTPs (12.5 mM)	4µl
Extender™ polymerase	1 µl (2.5 U)

The up- and downstream fragments and the resistance cassette were first incubated to allow joining. Afterwards, the respective primer pair, dNTPs and *Extender*™ polymerase were added. In step 2 the joint PCR product was amplified using the flanking primer.

LFH standard program for the *Extender*™ polymerase				
1. step	denaturation	94°C	120 s	
	denaturation	94°C	20 s	
	annealing	Tm [°C] 1/- 10°C	45 s	10 x
	elongation	68°C	480 s	
	break	15°C	∞	
	Addition of primer, dNTPs and polymerase.			
2. step	denaturation	94°C	20 s	
	annealing	Tm [°C] 1/- 10°C	45 s	30 x
	elongation	68°C	360 s	
	break	15°C	∞	

2.2.6. Preparation and analysis of proteins

Cell disruption by sonication

Cells were harvested by centrifugation (8,000 rpm; 10 min; 4°C) and the supernatant was completely removed. The pellet was resuspended in the cell disruption buffer. The disruption was performed with a ultrasonic probe, three times with 20 intervals of 0.5 seconds. The samples were stored on ice during the whole process. The samples were centrifuged (30 min; 13,000 rpm; 4°C) to remove remaining membrane compounds.

Cell disruption with the French press

The prechilled bomb was filled with the cell suspension and the remaining air was squeezed out before the bomb was locked. After closing the release valve the bomb was placed in the French press and set under pressure. The disruption took place with a pressure of 1,400 bar and was performed three times.

Cell disruption with One shot

The cell disruption with the One Shot was in principle equal to the French press. Here, 6 ml of the cell suspension were pipetted into the precooled cylinder and closed airtight. Internal pressure was established using the starter stick. The disruption of the cells was carried out at maximum pressure (2,500 psi; 2 to 3 passages)

Discontinuous SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were analyzed by SDS-PAGE as described by Laemmli (1970). Protein samples were denatured by boiling in SDS loading dye at 95°C for 10 min. The polyacrylamide concentration of the gels was chosen according to the expected proteins

sizes varying from 12 to 16% (v/v). Samples were loaded onto the prepared gel. Electrophoresis was performed at 120 V until the bromophenol blue had reached the lower end of the gel. During electrophoresis, proteins were first focused in the stacking gel and subsequently separated according to their molecular mass in the running gel. The self constructed protein marker *Page King* and the purchased Protein Molecular Weight Marker (Fermentas, Lithuania) were used as size standards. Gels were stained with Coomassie Brilliant Blue G-250 and destained until the protein bands were clearly visible. For analytic gels with small protein amounts silver staining was performed.

Solutions for SDS-PAGE

5 x SDS loading dye	Tris-HCl pH 7.0 (1 M)	1.4 ml
	Glycerol (100%)	3 ml
	SDS (20%)	2 ml
	β-Mercaptoethanol (100%)	1.6 ml
	Bromphenol blue	0.01 g
	H_2O_{deion}	2 ml
6 %Stacking gel (v/v)	Acrylamide-Bisacrylamide (39:1) (40%)	0.75 ml
	Tris-HCl pH 6.8 (1 M)	0.65 ml
	H_2O_{deion}	5.35 ml
	SDS (20%)	
	APS (10%)	200 µl
	TEMED	10 µl
12.5% Running gel (v/v)	Acrylamide-Bisacrylamide (39:1) (40%)	4,6 ml
	Tris-HCl pH 8.8 (1 M)	1.3 ml
	H_2O_{deion}	6.5 ml
	SDS (20%)	200 µl
	APS (10%)	200 µl
	TEMED	12.5 µl

Electrophoresis buffer	Glycine	1.92 M
	Tris	0.5 M
	SDS	1 %

Overexpression of proteins in *E. coli*

An overnight culture of *E. coli,* carrying the relevant plasmid, was used to inoculate one litre of LB medium to OD_{600} ~0.1. Cultures were grown with shaking at 16°C or 37°C until they had reached an optical density of 0.6-0.8. Expression of recombinant proteins was induced by the addition of isopropyl-β-D-thio-galactopyranoside (IPTG, final concentration: 1 mM) (PeqLab, Erlangen, Germany). The cultures were incubated for three hours. To test the expression, small aliquots (sample [µl] =100/OD_{600}) were taken before and after the induction. The samples were boiled in SDS loading dye and analyzed on a SDS gel. The main culture was harvested by centrifugation (10 min; 8,000 rpm; 4°C). After removing the supernatant the cells were washed in cold cell disruption buffer and the pellets were stored at -20°C.

Purification of His_6-tagged proteins

For protein purification, the frozen pellets were resuspended in cold cell disruption buffer, and the cells were disrupted by at least 3 passages through the French pressure cell (Spectonic Unicam) at 1,000 psi. Cell debris and other insoluble material was removed by ultracentrifugation (Beckmann, L7-55) (60 min; 27,000 rpm; 4°C). For purification of recombinant His_6-tagged proteins the supernatant fraction was loaded onto a 4 ml bed volume of Ni^{2+}-NTA resin (Qiagen) in a Poly-Prep Chromatography Column (Biorad, Munich, Germany). The Ni^{2+}-NTA resin had been pre-equilibrated with 10 ml cell disruption buffer. After extensive washing with 20 ml of cell disruption buffer containing 10 mM imidazole (Roth, Karlsruhe, Germany) and 20 ml with 20 mM imidazole the His_6-tagged proteins were eluted. The elution was performed in 10 ml steps with cell disruption buffer containing an increasing concentration of imidazole

(50 mM, 100 mM, 200 mM, and 500 mM). To analyze the purification success, 15 µl of each fraction was mixed with SDS loading dye and boiled at 95°C for 10 minutes. The samples were loaded onto a SDS gel and after electrophoresis stained with Coomassie brilliant blue. The relevant fractions were combined and dialyzed overnight. Protein concentration was determined using the Bio-rad dye-binding assay using Bovine serum albumin as the standard.

Purification of Strep-tagged proteins

Overexpression and cell disruption were performed as described above. The proteins were purified using 1 ml Strep-Tactin Sepharose (IBA, Göttingen, Germany) loaded on a Poly-Prep Chromatography Column (Biorad, Munich, Germany). The matrix specifically binds a sequence of eight amino acids (WSHPQFEK). D-desthiobiotin was used to elute the bound proteins. The matrix was equilibrated with 5 ml of buffer W. Afterwards the column was loaded with 10 ml of the crude extract. Washing steps were performed with 5 ml buffer W. The bound proteins were eluted with buffer E in 6 fractions of 500 µl. The fractions were analyzed by SDS-PAGE.

Buffer for purification of Strep-tagged proteins

Buffer W	Tris-HCl pH 8	100 mM
	NaCl	150 mM
	EDTA	1 mM
Buffer E	Tris-HCl pH 8	100 mM
	NaCl	150 mM
	EDTA	1 mM
	D-desthiobiotin	2.5 mM

Dialysis of proteins

Proteins purified by Ni^{2+}-NTA or Strep-Tactin Sepharose contain immidazole and D-desthiobiotin, respectively. These components can have negative effects on following experiments. Thus, the elution fractions were dialyzed against cell disruption buffer supplemented with 1 mM desthiothreitol **(**DTT) in a dialysis tube. The exclusion size of the dialysis tube was half the size of the protein. Protein solution was dialyzed against buffer, in relation 1 to1000.

Purification of *Taq* DNA polymerase

The modified DNA polymerase of *Thermus thermophilus* was overexpressed in *E. coli* (Nadicom *E. coli* stock 147). Therefor, 4 ml of a LB_{Amp} preculture was inoculated with one colony of this strain and incubated at 37°C overnight. The preculture was used to inoculate 100 ml LB_{Amp} medium, that was incubated at 37°C with shaking. At an OD_{600} of 0.3 the culture was supplemented with IPTG (500 mM) and incubated at 37°C for 24 h with shaking. Cells were harvested by centrifugation (15 min; 5,000 rpm; 4°C) in two falcon tubes. The supernatant was refused and the pellets were resuspended in buffer A and pooled. The suspension was supplemented with 12 mg lysozyme and incubated with shaking at room temperature for 15 min. Addition of 3 ml buffer B followed with reversion. The solutions were divided into 2 (2 ml) centrifuge cups and incubated at 75°C for 1 h under smooth shaking. Afterwards, the solutions were centrifuged (13,000 rpm; 10 min; RT) and the supernatant was pipetted in new centrifuge cups. The success of the overexpression of the DNA polymerase was tested by PCR. For this purpose, 40 µl of the suspensions was mixed with 40 µl of storage buffer and 40 µl glycerol. Test PCR‘s were performed with selected primer pairs for the amplification of *B. subtilis* genes from 0.5 to 2.1 kb. After confirmation of DNA polymerase activity, dialysis was prepared. Approx. 10 cm dialysis tube was shortly boiled with 2 mM EDTA in a microwave. The polymerase solution was filled in the dialyze tube and closed with clips. Dialysis occurred in 500 ml storage buffer for 12 h

under light stirring. Altogether, dialysis was proceeds two times and the solution was tested again on polymerase activity. Then, 10-fold storage buffer was added to a final dilution of 1:10. DNase solution was added and incubated at 37°C for 30 min. Afterwards, DNase was inactivated by boiling at 80°C for 10 min. The solution was dialyzed again two times. *Taq*-DNA polymerase solution was aliquoted (50 µl polymerase solution + 50 µl glycerol 100%) and tested once again before stored at -20°C.

Primer combinations for test PCR

Primer	**expected size**	**gene**
NP74 / NP75	500 bp	*ydiB*
NP70 / NP71	903 bp	*era*
NP78 / NP79	1208 bp	*yphC*
NP 94 / NP95	2115 bp	*pnpA*

Solutions for *Taq*-DNA polymerase purification

Buffer A	Tris	50 mM
	Glucose	20 mM
	EDTA (stock solution 100 mM)	1 mM
Buffer B	Tris	10 mM
	KCl	50 mM
	EDTA	1 mM
	Tween 20	5%
Storage buffer	Tris	50 mM
	NaCl	100 mM
	EDTA	0.1 mM
	DTT	0.5 mM
	Triton X100	1%
	PMSF	spatula point

10 x DNase storage buffer	Tris-HCl	1.75 g
pH 7.5	$CaCl_2$ x 2 H_2O	1.47 g
	$MgCl_2$ x 7 H_2O	2.03 g
	Glycerol	50 ml
	H_2O_{deion}	ad 100 ml
10 x Reaction-buffer DNase	Tris-HCl	100 mM
pH 8.8	KCl	500 mM
	$MgCl_2$	15 mM
	Nonidet P40	8%
DNase solution	DNase	70 mg
	Storage buffer	1 ml

Preparation of a protein marker (unstained)

A protein marker was prepared using existing purified proteins of good purity. These proteins were purified using the appropriate affinity tag (Tab. 3.2.). The protein solution was mixed with storage buffer to a final protein concentration of 0.2 µg/µl. The marker was stored at -20°C.

Protein	kDa	Plasmid	Affinity-Tag	Vector / Strain	Cloning
PnpA	77	pGP838	His_6	pWH844 / DH5α	NP94 / NP95
Obg	48	pGP843	His_6	pWH844 / DH5α	NP72 / NP73
LDH *M.p.*	35	pGP369	His_6	pWH844 / DH5α	SH52 / SH55
TpiA	27	pGP394	His_6	pWH844 / DH5α	EW5 / NP92
YwjH	23	pGP819	Strep	pGP172 / BL21	NP11 / NP12
HPr	9	pAG2	His_6	pQE30 / DH5α	Galinier *et al.*, 1997

Table 3.2. Proteins used for the protein marker.

Storage buffer for unstained protein marker ‚*Page King*‘	
Tris-HCl (pH 7.7)	62.5 mM
DTT	10 mM
SDS	2%
Glycerol	33%

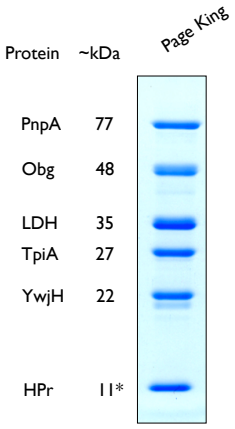

Fig. 1: Protein marker Page King covering 11 to 77 kDa. The protein concentration is 0.2 µg /µl. Prior to use, 5 µl of marker were boiled for 5 min at 95°C and then loaded on the gel.

Coomassie staining of polyacrylamide gels

Protein gels were stained with Coomassie Brilliant Blue. For this purpose, the gels were incubated in fixing solution for 10 minutes. Staining occurred with coomassie staining solution for about 5-15 minutes and the gels were destained until an optimal contrast between protein bands and background was reached.

Solutions for coomassie staining	
Fixing solution	50% Methanol
	10% Acetic acid
	40% H_2O_{deion}
Staining solution	2.5 g Coomassie Brillant Blue R250
	10% Acetic acid
	50% Methanol
	40% H_2O_{deion}

Destaining solution	10% Acetic acid 90% H_2O_{deion}

Silver staining of polyacrylamide gels

Silver staining is one of the most sensitive staining methods for polyacrylamide gels. It was used to verify the purity of protein purifications and for the analysis of protein-protein interactions. The advantage of this method is the high sensitivity (detection limit of approximately 5 ng protein). The disadvantage is that it is not exactly reproducible and quantifiable, due to the physics of the accumulation of the silver particles (Butcher and Tomkins, 1985). The silver staining of the protein band was performed as described by Nesterenko (1994). For staining, the polyacrylamide gels were incubated with the following solutions with shaking.

Step	**Reagent**	**Duration**
Fixing	Fixer	1 to 24 h
Washing	Ethanol 50 %	3 x 20 min
Reduction	Thiosulfate solution	90 s
Washing	H_2O_{deion}	3 x 20 s
Staining	Impregnating	25 min
Washing	H_2O_{deion}	2 x 20 s
Developing	Developer	until sufficiently stained
Washing	H_2O_{deion}	20 s
Stoping	Stop solution	5 min

Solutions for silver staining		
Fixing solution (100 ml)	Methanol (100%)	50 ml
	Acetic acid (100%)	12 ml
	Formaldehyde (37%)	100 µl
	H_2O_{deion}	ad 100 ml
Thiosulfate solution (100 ml)	$Na_2S_2O_3$ x 5 H_2O	20 mg
	H_2O_{deion}	ad 100 ml
Impregnater (100 ml)	$AgNO_3$	0.2 g
	Formaldehyde (37%)	37 µl
	H_2O_{deion}	ad 100 ml
Developer (100 ml)	$NaCO_3$	6 g
	Thiosulfate solution	2 ml
	Formaldehyde (37%)	50 µl
	H_2O_{deion}	ad 100 ml
Stop solution (100 ml)	EDTA	1.86
	H_2O_{deion}	ad 100 ml

Two-dimensional SDS polyacrylamide gel electrophoresis (SDS-PAGE)

For the separation of complex protein mixtures, two-dimensional gel electrophoresis was used. Briefly, the proteins were separated in two dimensions according to the characteristics of proteins, their charge and their size. In the first dimension, the separation is based on the isoelectric point of the proteins. In the second dimension, the separation is carried out according to the relative molecular masses.

For the separation in the first dimension, IPG strips with a linear pH range of 4–7, 4.5–5.5 or 6–11 were used (Immobiline™ DryStrip, Amersham Biosciences). The proteins were mixed with rehydration solutions A and B. The mixture was applied to the IPG

strips which were placed into a chamber with the gel coated side upside down. The dry gel on the strips swelled and the proteins were soaked into the gel. The swelling chamber was sealed with parafilm to protect the strips from evaporation. The strips were further incubated for 16 h at RT. The IEF was performed using the following program at RT and constant currency of 50 µA per gel.

Program for isoelectric focussing

Step	Voltage	Time
1	250 V	1 h
2	500 V	1 h
3	1000 V	1 h
4	5000 V	4 h
5	500 V	1 h
6	Break	

After the IEF, the pH-gradient strips were incubated for 15 min in equilibration buffer containing 15 mM DTT. Subsequently, an incubation of 15 min was carried out in equilibration buffer containing 150 mM of iodacetamide. All steps occurred at RT. The pH-gradient strips were placed on the top of the SDS-PAGE gel and fixed.

After electrophoresis, the gels were consecutively stained with Pro-Q Diamond (Invitrogen) and Flamingo™ fluorescent dye (BioRad) to visualize the phosphoproteins and the whole proteome, respectively. The determination of phosphorylated proteins was based on the Pro-Q/ Flamingo™ log ratio. Phosphoprotein spots were cut from the gel and identified by mass spectrometry.

Solutions for two-dimensional SDS polyacrylamid gel electrophoresis

Rehydration solution A	Urea	7 M
	Thio urea	2 M
	CHAPS	4 % (w/v)
	DTT	50 mM
	H_2O_{deion}	

Rehydration solution B	Rehydration solution A	2.625 ml
	Ampholyte solution (for pH 3-10)	125 µl
	Bromphenol blue	335 µl
SDS equilibration buffer	Tris-HCl (pH 8.8)	50 mM
	Urea	6 M
	SDS	2 % (w/v)
	Glycerol	35 % (w/v)

Protein identification by mass spectrometry

Gel pieces were washed twice with 200 µl 20 mM NH_4HCO_3; acetonitrile (ACN) (30% (v/v)) for 30 min, at 37°C and dried in a vacuum (Concentrator 5301 (Eppendorf, Hamurg, Germany)). Trypsin solution (10 ng/µl trypsin in 20 mM ammonium bicarbonate) was added until gel pieces stopped swelling and digestion was allowed to proceed for 16 to 18 hours at 37°C. Peptides were extracted from gel pieces by incubation in an ultrasonic bath for 15 min in 20 µl HPLC grade water and transferred into micro vials for mass spectrometric analysis. Peptides were separated by liquid chromatography and measured online by ESI-mass spectrometry using a nanoACQUITY UPLC™ system (Waters, Milford, USA) coupled to an LTQ Orbitrap™ mass spectrometer (Thermo Fisher Scientific, Waltham, USA). Peptides were desalted onto a trap column (Symmetry® C18, Waters). Elution was performed onto an analytical column (BEH130 C18, Waters) by a binary gradient of buffer A (0.1% (v/v) acetic acid) and B (100% (v/v) acetonitrile, 0.1% (v/v) acetic acid) over a period of 80 min with a flow rate of 400 nl/min. The LTQ Orbitrap™ was operated in data-dependent MS/MS mode for phospho-relevant masses. Proteins were identified by searching all MS/MS spectra in .dta format against all *B. subtilis* proteins (extracted from the NCBI database) using SEQUEST (Bioworks 3.3.1/Sequest v. 2.7 rev. 11, Thermo Electron). Initial mass tolerance for peptide identification on MS and MS/MS peaks were 10 ppm and 1 Da respectively. Up to two missed tryptic cleavages were allowed. Methionine oxidation

(+15.99492 Da) as well as carbamidomethylation (+57,021465 Da) and phosphorylation (+79,966331Da) of serine, threonine, and tyrosine were set as variable modifications. Proteins were identified by at least two peptides applying a stringent SEQUEST filter (Xcorr vs. charge state: 1.90 for singly, 2.2 for doubly and 3.3 for triply charged ions). Phosphorylated peptides which passed this filter were examined manually and accepted only, when b- or y- ions confirmed the phosphorylation site.

Assays for protein phosphorylation

Protein phosphorylation assays were carried out with purified proteins (10 µg) and crude extracts (20 µg) in assay buffer with [γ-^{32}P]ATP (480 Ci/mmol). Different cations and metabolites were tested. The assays were carried out at 37°C for 30 min followed by thermal inactivation of the proteins (95°C; 10 min). The assay mixtures were analyzed on 16% SDS-polyacrylamide gels. Proteins were visualized by Coomassie staining. Gels were dried (Gel Dryer585, Biorad, Munich, Gemany) and radioactive protein spots were detected using a Phosphoimager (STORM 860, Molecular Dynamics, USA).

Assay buffer for protein phosphorylation experiments (25 µl)

	Stock solution	Working concentration	Volume (µl)
Tris-HCl (pH 7.4)	250 mM	25 mM	2.5
$MgCl_2$	200 mM	5 mM	0.625
DTT	40 mM	1 mM	0.625

Strep-protein interaction experiment (SPINE) for the analysis of protein- protein interactions

The Strep-protein interaction experiment (SPINE) was performed according to Herzberg *et al*. 2007. This experiment was used to identify potential interaction partners of *B. subtilis* proteins *in vivo*. For this purpose, the gene of interest was cloned into

expression vectors for *B. subtilis*. The gene was fused to a N-terminal or C-terminal Strep-tag (pGP380, pGP382). The transcription of the fusion proteins was under the control of the constitutive *degQ* promoter, which assured a high expression of the proteins. For the experiment a preculture of *B. subtilis*, carrying the appropriate plasmid was grown for 10 hours at 37°C in LB medium containing erythromycin. This culture was used to inoculate 100 ml CSE medium (0.5% glucose) and was grown overnight at 37°C. One liter of the same medium was then inoculated with the second preculture to an OD_{600} of 0.1. When this culture had reached an OD_{600} of 1.0, 500 ml were supplemented with formaldehyde (4% in PBS) to a final concentration of 0.6%. This culture was and incubated for additional 20 minutes. After cross-linking, the cells of the formaldehyde treated and the untreated culture were harvested by centrifugation (10 min; 5,000 rpm; 4°C) (Sorvall RC 5B, SLA-3000 rotor). The cells were washed in buffer W and harvested again. The pellets were stored at -20°C. For the preparation of the crude extract, the cell pellet was thawed and resuspended in 5 ml buffer W. Cell disruption was carried out using a French press or OneShot.

2.2.7. Determination of enzyme activities

Determination of pyruvate kinase activity

The pyruvate kinase catalyzes the transfer of one phosphate group from phosphoenolpyruvate (PEP) to ADP. Pyruvate and ATP are the products of this reaction. Enzymatic activity was achieved by the lactate dehydrogenase (LDH) reaction. LDH reduces pyruvate to lactate under oxidation of $NADPH_2$. The decrease of $NADPH_2$ by oxidation was measured at wavelength of 340 nm. Furthermore, the pyruvate kinase require Mg^{2+} for its activity. It is important to note that the pyruvate kinase of *B. subtilis* is allosteric activated by ribose-5-phosphate. The assay conditions were examined by using commercial pyruvate kinase (lactate dehydrogenase) purchased from Roche.

Pyruvate kinase: $PEP + ADP \leftrightarrow pyruvate + ATP$

Lactate dehydrogenase: pyruvate + $NADPH_2$ ↔ lactate + $NADP^+$

Solutions for pyruvate kinase assay

	Stock solution	Assay approach
Potassium phosphate buffer	100 mM	200 µl
$MgSO_4$	100 mM	100 µl
ADP	10 mM	50 µl
$NADH_2$	1.5 mM	50 µl
Ribose-5-phosphate	100 mM	10 µl
Lactate dehydrogenase (5 mg/ml))	970 µl + 30 µl LDH	20 µl
Pyruvate kinase (10 mg/ml)	970 µl + 30 µl (Pyk)	20 µl
H_2O_{deion}		ad 1 ml

The absorption was monitored at OD_{340} for 3-5 min. The specific activity was calculated with the following formula.

$$\text{Units/mg} = \frac{\Delta A_{340}/\text{min}}{6.22 \times \text{mg enzyme/ml reaction mixture}}$$

Measurement of methylglyoxal concentrations

Methylglyoxal is a reactive dicarbonyl compound formed as a by-product of glycolysis. Due to the fact that methylglyoxal is toxic, the levels must be kept low in the cell. For this reason, living cells exhibit several glyoxalases for the detoxification of methylglyoxal. Measurement of methylglyoxal was performed by addition of 2,4 dinitrophenylhydrazin and results in a violet coloration. The methylglyoxal concentration was quantified colorimetrically at 550 nm. Factor for the calculation of the total amount of methylglyoxal: OD_{550}=16.4 corresponds to 1 µmol of methylglyoxal. Cells were grown in liquid medium. At defined time points, samples of

2 ml were taken and centrifuged (8 min; 8,000 rpm; RT). The supernatants were stored on ice. For the measurement 320 µl, 160 µl and 80 µl were mixed with 2,4-dinitrophenylhydrazin (10 mg/ml in 2 M HCl) and incubated at 30°C for 15 min. Afterwards, the samples were mixed with 560 µl of 10% NaOH and incubated for 10 min at RT. The samples were centrifuged again (10 min; 13,000 rpm; RT) and the supernatants were measured at OD_{550} against a blank without supernatant.

Disk diffusion assay (Cao *et al*., 2002)

The analysis of phenotypes of kinase mutants was carried out with a disk diffusion assay. LB cultures of the respective mutant and wild type strains were grown to OD_{600} 1.0. Twenty microliters of the culture was mixed with 3 ml of LB soft agar (0.7% agar) and poured onto the bottom agar. After cooling and drying of the plates (20 min at 37°C), filter paper disks (6-mm diameter) carrying 5 µl of stock solution (antibiotics at a concentration of 100 mg/ml each; lysozyme at 10 mg/ml; and uncouplers at 5 mg/ml) were placed on top of the agar. After incubation for 12 to 24 h at 37°C, the plates were scored for growth inhibition produced by the diffusion of the antibiotics from the filter disks .After incubation for 12 to 24 h, the plates were scored for growth inhibition produced by the diffusion of the antibiotics from the filter disks.

3. Results

3.1. Construction and analysis of multiple Ser/ Thr kinase mutants

3.1.1. Phosphoproteome analysis of kinase mutants

Analysis of the annotated genome sequence revealed the presence of three additional potential kinases, PrkD, YabT and YxaL. For PrkC autokinase activity was already shown (Madec *et al*., 2002). Sequence comparison revealed that PrkD is similar to the kinase domain of PrkC and is also autophosphorylated in the presence of ATP (Pietack *et al*., 2010). However, the functions and the contributions of these potential kinases to the phosphoproteome of *B. subtilis* were unknown. To investigate, whether these uncharacterised kinases are responsible for protein phosphorylation events in *B. subtilis*, deletion mutants of *prkC*, *prkD* and *yabT* were constructed. YxaL was excluded from this study, since it contains a signal peptide and is mainly part of the secretome (Voigt *et al*., 2009).

To avoid redundant kinase activity, the mutations were combined, resulting in the triple kinase mutant strain GP581 (*ΔprkC*::*spec ΔprkD*::*kan ΔyabT*::*erm*). This strain was used for further phosphoproteomic investigations. Therefore, GP581 was grown in LB medium to an optical density of 0.8, according to other phosphoproteomic studies (Eymann *et al*., 2007). Protein extracts were separated by 2D-SDS-PAGE and specifically stained as described in Material and Methods. No differences in the phosphoproteome were detected between the *B. subtilis* 168 wild type strain and the triple kinase mutant (data not shown). In further experiments, both strains were grown in CSE medium with glucose; however, no differences in the protein- and phosphorylation pattern were detected, too.

In conclusion it has to be stated that the annotated Ser/Thr kinases are not responsible for the phosphorylation events in *B. subtilis* under standard conditions. Perhaps, these kinases have specific targets or require a specific stimulus for their activation.

3.1.2. The phenotype of the triple kinase mutant

The triple kinase mutant GP581 showed no differences in the phosphorylation pattern compared to the *B. subtilis* wild type strain 168 under the tested conditions. Thus, it was of interest under which conditions the kinases are active. To address this question, the phenotype of GP581 was investigated. Growth tests were performed in rich medium (LB), sporulation medium (SP) and minimal medium (CSE-glucose). However, the mutant strain demonstrated no differences in growth compared to the wild type strain (data not shown).

The kinase mutant strain showed no significant phenotype under standard growth conditions. Additionally, the amount of the respective kinases in the cell was unclear. Thus, the single kinases were overexpressed in *B. subtilis*, to force potential effects on the phenotype. Growth tests were performed in different media, like described above. However, the overexpression of kinases had no effect on growth. A further approach, to identify potential interaction partners or substrates of the respective kinases by *in vivo* cross linking (SPINE) was not successful.

The deletion and overexpression of PrkC, PrkD and YabT had no effect on the phenotype. Thus it must be concluded that either the kinases do need a specific stimulus or the specific substrate(s) is only present under certain conditions. Furthermore, it is possible that these kinase are only active under specific conditions.

Consequently, conditions must be found were the kinase function is necessary. A starting point for this approach was the PASTA domain of PrkC (Fig. 3.1.). PASTA domains are described as binding domains for beta-lactam antibiotics and analogues of peptidoglycan (Yeats *et al*., 2002). For that reason, disk diffusion assays were performed with detergents and an extensive set of antibiotics that target cell wall synthesis (Jordan *et al*., 2008). Furthermore, the *B. subtilis* wild type and GP581 strains were treated with lysozyme during growth, but neither the disk diffusion assays nor the lysozyme treatments showed a significant difference between both strains (data not shown).

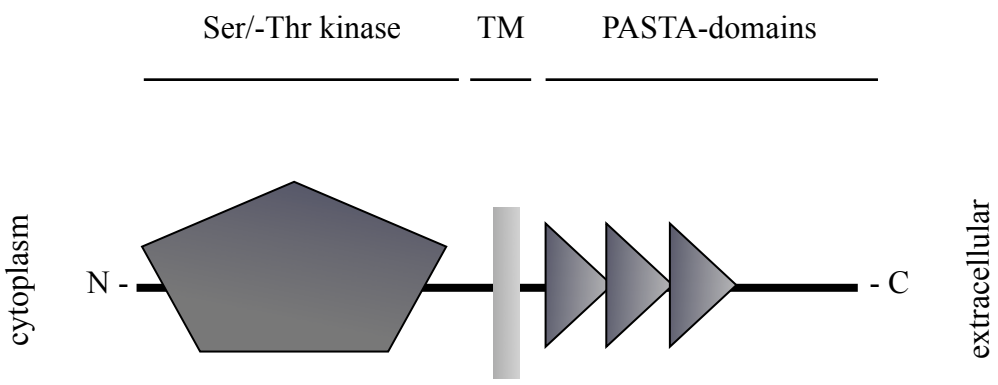

Figure 3.1.: Domain structure of PrkC (prediction by EMBL)

PrkC consists of N-terminal, extracellular PASTA domains (beta-lactam-binding domains) followed by a transmembrane domain (TM). The Ser/-Thr kinase domain is localized in the cytoplasm, at the C-terminal end.

3.2. Investigation of conserved GTPases

The investigation of the multiple kinase mutant revealed, that the annotated Ser/Thr kinases were not responsible for the bulk of phosphorylation events in *B. subtilis* under standard conditions. Therefore, it is tempting to speculate that an unknown Ser/Thr kinase is responsible for these phosphorylation events.

Phosphoproteome analysis in *B. subtilis*, *E. coli* and *Campylobacter jejuni* revealed a common set of proteins that are phosphorylated in these bacteria (Macek *et al*., 2007; Voisin *et al*., 2007 Macek *et al*., 2008). Hence, it seemed likely that the orthologous proteins were phosphorylated by a kinase that is conserved in all three organisms. The *B. subtilis* genome with a size of 4.2 Mb comprises 4,106 protein coding genes, similar to that of *E. coli* with 4.6 Mb, coding for 4,288 proteins (Barbe *et al*., 2009; Kunst *et al*., 1997; Blattner *et al*., 1997). In contrast to *B. subtilis* and *E. coli*, the genome of *Campylobacter jejuni* with a size of 1.6 Mb encodes just 1,654 proteins (Parkhill *et al*., 2000).

An *in silico* comparison of annotated proteins of *B. subtilis, E. coli* and *Campylobacter jejuni* revealed a common set of around 500 conserved proteins. Subsequently, all proteins with walker A and walker B motifs were summarized (Ramakrishnan *et al*.,

2002; Walker *et al*., 1982). Furthermore, proteins were selected where the coding genes were described as essential in *B. subtilis*(Kobayashi *et al*., 2003). These criteria resulted in a selection of six GTP-binding proteins (Era, Obg, YdiB, YphC, YyaF and YsxC). These proteins are present in all domains of life and were predicted to be essential (Mittenhuber, 2001). The selected genes were cloned into expression vectors for *B. subtilis*, and fused to an N-terminal Strep-tag. The expressed proteins were investigated in protein-protein interaction experiments, without success.

Furthermore, genes of the GTP-binding proteins were cloned into overexpression vectors for *E. coli* DH5α and purified via N-terminal His_6-tag. The purified proteins were investigated in *in vitro* phosphorylation experiments. Proteins were tested for autophosphorylation activity using [γ-^{32}P] ATP and [γ-^{32}P] GTP. As negative control [α-^{32}P] GTP was used to exclude non-specific binding of nucleotides. Moreover, all proteins were tested for their ability to phosphorylate purified proteins of different metabolic pathways of *B. subtilis*. However, none of the potential target proteins were phosphorylated by the selected proteins.

Interestingly, GTP-dependent autophosphorylation was observed for Obg, and ATP-dependent phosphorylation for YdiB (Fig. 3.2.). The GTP-dependent autophosphorylation of Obg is in good agreement with previous studies (Welsh *et al*., 1994). Obg is a ribosome-associated protein and the association is stabilised by GTP (Zhang & Haldenwang, 2004). Recently, it was shown that deletion of the `essential` *ydiB* gene yielded in reduced growth of a mutant, but the cells were still viable. The growth defect was dependent on the ATPase activity of YdiB (Karst *et al*., 2009; Hunt *et al*., 2006).

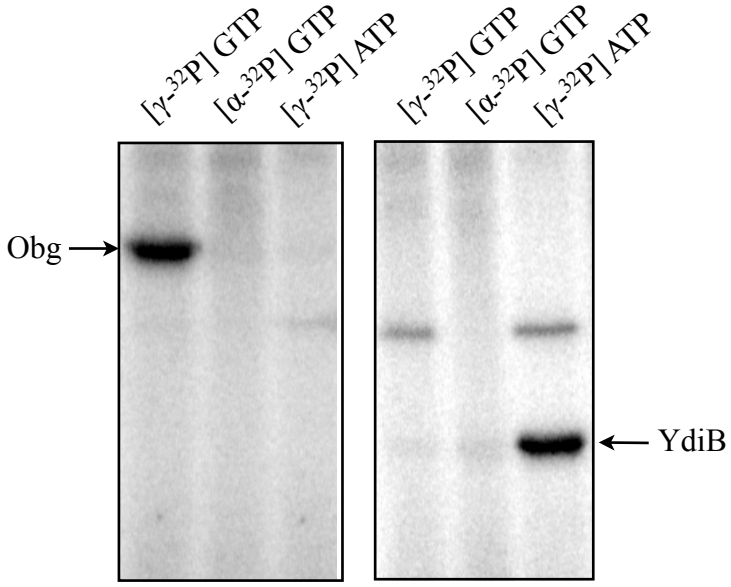

Figure 3.2.: Autophosphorylation of conserved GTP binding proteins

The *B. subtilis* proteins Obg and YdiB were overexpressed in *E. coli* and purified by making use of the His_6-tag. Ten microgram of dialysed proteins were incubated in phosphorylation buffer at 37°C for 15 min. Radiolabeled nucleotides were used to determine the substrate specificity for either ATP or GTP. To exclude non-specific binding of nucleotides, [α-^{32}P] GTP was used as control. Obg (approx. 47 kDa) showed GTP dependent autophosporylation, whereas YdiB (approx. 17 kDa) showed ATP dependent autophosphorylation.

3.3. Analysis of the phosphorylation of HPr

HPr (histidine containing protein) is a prime example for protein phosphorylation. It is phosphorylated on His-15 by enzyme I in the phosphotransferase system (PTS) and on Ser-46 by the HPrK/P (Reizer *et al*., 1998). A recent analysis of the *B. subtilis* phosphoproteome revealed a novel phosphorylation of the HPr protein on Ser-12 (Macek *et al*., 2007). However, the source of this phosphorylation was unknown. Phosphorylation experiments with purified kinases and HPr revealed, that PrkC phosphorylates HPr in the presence of [γ-^{32}P]ATP (Pietack *et al*., 2010). A HPr (Ser-46-Ala) mutant was used to exclude a phosphorylation on Ser-46. As expected, the mutant protein was not longer phosphorylated by the HPr kinase, thus

confirming that Ser-46 is the only phosphorylation site for HPr kinase. PrkC was still able to phosphorylate HPr (Ser-46-Ala), on Ser-12.

The biological relevance of the phosphorylation of HPr on Ser-12 was unknown. To investigate the function of the Ser-12 of HPr *in vivo* a HPr (Ser-12-Ala) mutant was constructed and expressed from plasmid in GP105. In GP105 the His-15 of HPr is replaced by an alanine, therefore HPr is not active in its function in the PTS. It was not possible to use a deletion mutant of HPr, because of the negative polar effect on the expression of the downstream gene *ptsI* (EI). GP105 carrying the empty vector (pBQ200) and derivates for expression of HPr (WT), HPr (His-15-Ala) and HPr (Ser-12-Ala) were analysed in growth tests in minimal medium with fructose as single carbon source (Fig. 3.3.). In contrast to glucose, fructose uptake only occurs over the PTS, thus active HPr is required.

Growth tests showed that the His-15 of HPr is absolutely required for the sugar uptake, whereas the Ser-12 phosphorylation is not necessary for HPr activity. Interestingly, recent studies with purified HPr and EI revealed that HPr is phosphorylated on Ser-12 after extended incubation in the presence of PEP(Sebastian Himmel, unpublished data). These results indicate that Ser-/Thr- phosphorylations are not strictly dependent on kinases. However, the function of this phosphorylation event is still unclear.

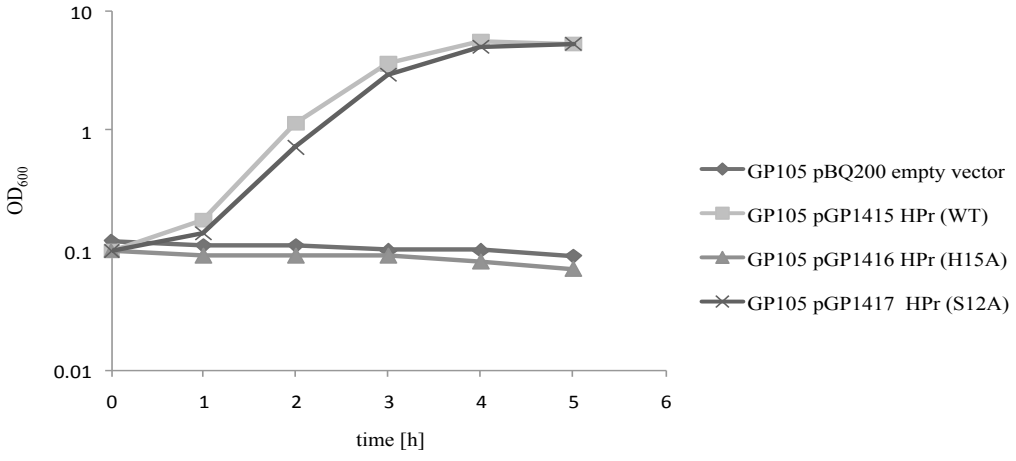

Figure 3.3.: Analysis of the implication of S12 of HPr in sugar uptake

Growth with different HPr variants was analysed. The strain GP105 (HPr; His-15-Ala) carrying the vectors pGP1415, pGP1416, pGP1417 and pBQ200 was grown in C-minimal medium with fructose as sole carbon source.

3.4. Investigation of essential glycolytic genes in *B. subtilis*

3.4.1. Conditions for the construction of mutants of glycolytic genes

The *B. subtilis* genome codes for around 4,106 proteins (Barbe *et al*., 2009). A study in *B. subtilis* revealed that 271 genes are indispensable for growth (Kobayshi *et al*., 2003; Thomaides *et al*., 2007). Among these essential genes are almost all genes that code for glycolytic enzymes. However, it was unknown why these genes should be essential, while other metabolic pathways might have the capability to compensate the loss of several glycolytic enzymes in rich media. Furthermore, rich medium contains substrates and products of the glycolysis that should allow growth in the absence of single enzymatic reactions.

During investigation of protein phosphorylation events, mutants of glycolytic enzymes were constructed. Before deletion, the mutants were complemented with expression vectors carrying the respective genes. Surprisingly, deletion strains were also viable when complementation occurred with enzymatic inactive enzymes. Therefore, growth in absence of the respective gene must be examined.For that reason, deletion mutants of glycolytic genes were constructed. The respective genes were replaced by antibiotic resistance cassettes, using LFH-PCR fragments (described in material and methods). It is important to note, that after the transformation the clones were selected on SP medium supplemented with glucose and malate.

Surprisingly, it was possible to construct deletion mutants of all glycolytic genes of *B. subtilis*. Up to that, only a pyruvate kinase mutant was described in detail (Frey *et al*., 2000) However, the *pyk*-gene was not reported to be essential (Kobayashi *et al*., 2003). Interestingly, deletion mutants of *pgm*- and the *eno* were described previously (Leyva-Vazquez & Setlow, 1994). In addition, it was possible to construct mutant strains with multiple deletions (Fig. 3.4.). This was mainly du to the fact, that glycolytic genes are coded in operons. The *gapA*-operon codes the genes *gapA*, *pgk*, *tpiA*, *pgm* and *eno* which products are enzymes of the lower part of the glycolysis. It was possible to delete a maximum of four genes in one step, with exception of *gapA* (Fig. 3.4.C.).

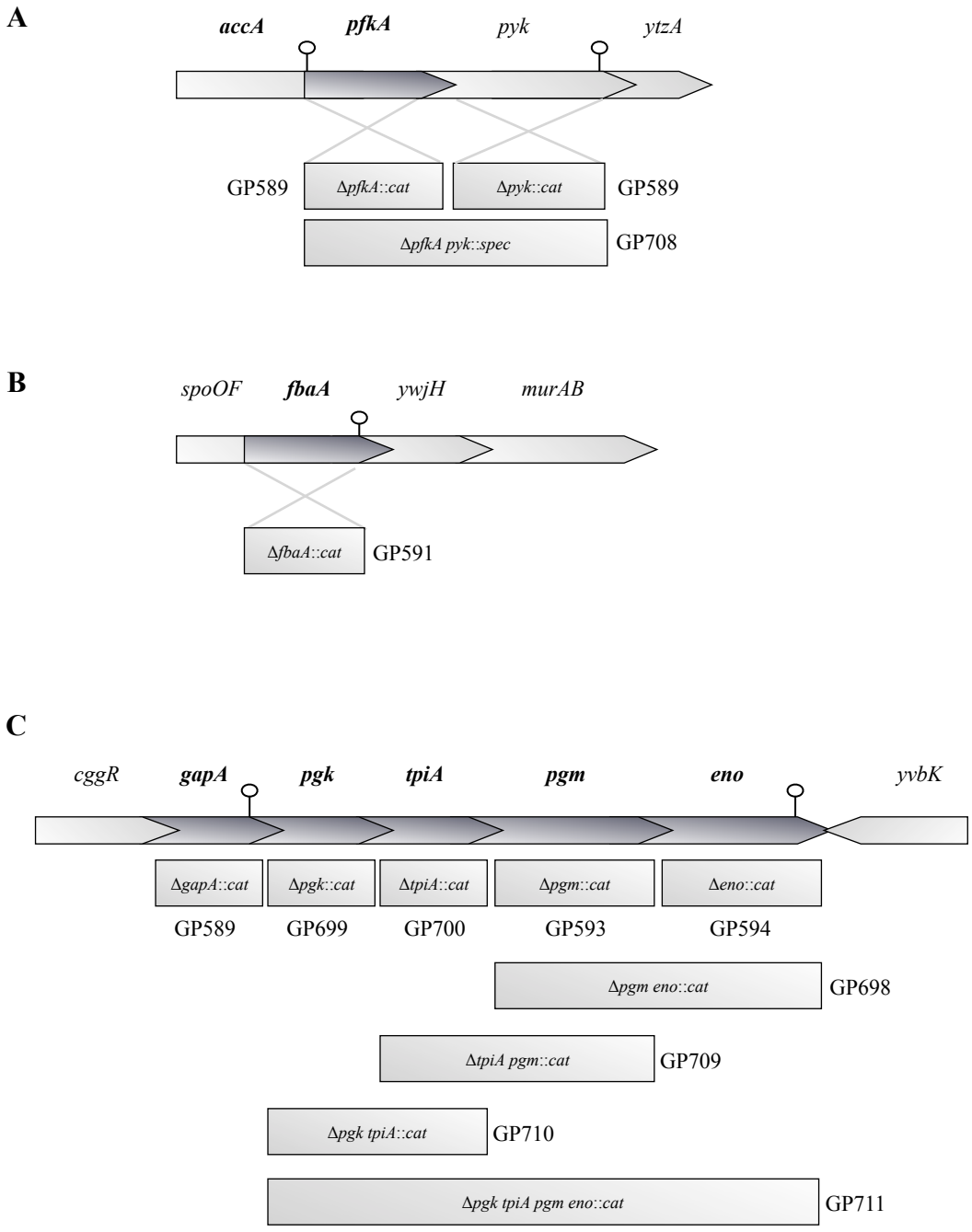

Figure 3.4.: Schematic overview about constructed multiple glycolytic mutants.

(A) Illustration of the genetic organisation of the phosphofructokinase (*pfkA*) and the pyruvate kinase (*pyk*). (**B**)The gene for fructose-1,6-bisphosphate aldolase (*fbaA*) is localised upstream of the transaldolase (*ywjH*). (**C**) Organisation of the *gapA* operon coding the genes for glyceraldehyde-3-phosphate dehydrogenase (*gapA*), phosphoglycerate kinase (*pgk*), triosephosphate isomerase (*tpiA*), phosphoglycerate mutase (*pgm*) and enolase (*eno*).

After the examination of the single mutant strains by PCR and sequencing, the isolated genomic DNA was used for the transformation of *B. subtilis* 168. Transformation approaches were used to decide the optimal supplementation of the growth medium (Tab. 3.1.). With the exception of Δ*pfkA* and Δ*pyk*, all mutants require malate for growth. This is plausible, while malate can be metabolised in the Krebs cycle and passed through glyconeogenesis by malic enzymes and PEP-caboxykinase (*pckA*). This need of malate is a disadvantage for storing of glycolysis mutants, as malate like glucose repress sporulation in *B. subtilis* (Ohné *et al*., 1976; Freese *et al*., 1970; Schaeffer *et al*., 1965;). Therefore, the strains were stored in DMSO cryo cultures.

Strain		LB	SP	SP- glucose	SP- malate	SP- glucose, malate	SP- glycerol, succinate
GP590	Δ*pfkA*	++	+	++	++	++	++
GP591	Δ*fbaA*	+	n.g.	-	+	+	-
GP700	Δ*tpiA*	+	-	+	+	+	-
GP592	Δ*gapA*	n.g.	n.g.	n.g.	++	+	n.g.
GP699	Δ*pgk*	+	-	-	+	+	-
GP593	Δ*pgm*	n.g.	n.g.	n.g.	+	+	n.g.
GP594	Δ*eno*	n.g.	n.g.	n.g.	+	+	-
GP589	Δ*pyk*	+++	+	+	+	+++	+

Tab. 3.1.: Determination of the optimal growth medium and supplementation.
B. subtilis wild type was transformed with genomic DNA of the respective mutant and stroke out on LB and SP plates, supplemented with glucose, malate, glycerol and succinate. The plates were incubated at 42°C for 24 h at. Evaluation occurred as followed: (n.g. no growth); (- very small colonies); (+ growth); (++ normal growth); (good growth).

Furthermore, optimal pH value was investigated by streaking out transformed cells on SP plates with glucose and malate. The pH value of the medium was adjusted at pH of 6 and 8, respectively.

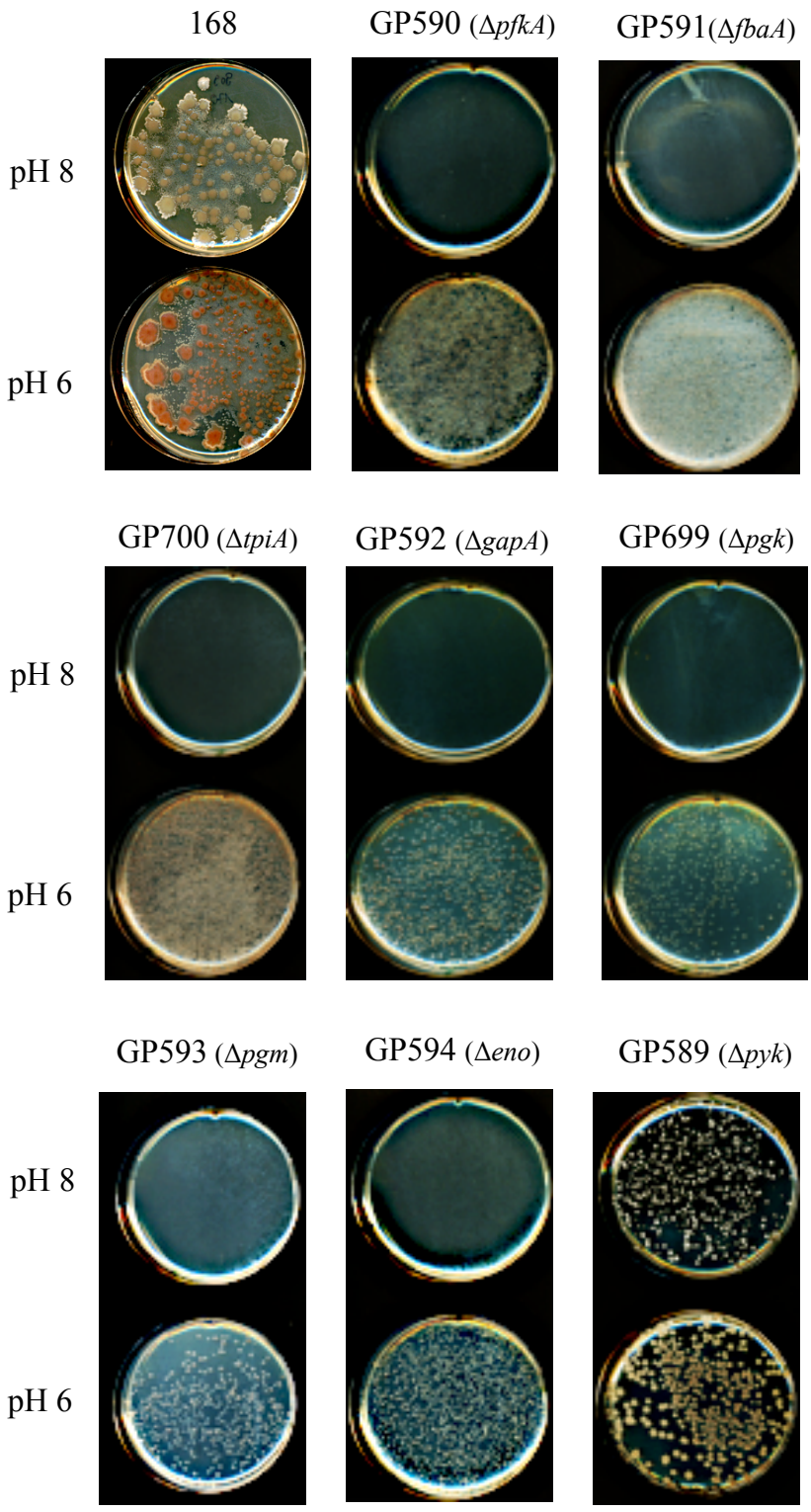

Figure 3.5.: Analysis of optimal pH value for construction of glycolytic mutants

B. subtilis was transformed with chromosomal DNA of the respective mutant strain. Selection occurred on SP medium with glucose and malate. The pH of the media was adjusted to 6 and 8 with MES and Tris-HCl. Plates were incubated at 42°C for 24 h.

The plates with a pH of 6.0 were adjusted with 0.5 M MES buffer (50 mM final concentration). For plates with a pH of 8.0, 0.5 M Tris-HCl was used (50 mM final concentration). The plates were incubated at 42°C for 24 h and documented (see fig. 3.5.). Transformants for all glycolysis mutants were obtained. The colonies showed a striking red coloration that could be due to expression of cytochromes at acidic conditions (Wilks *et al*., 2009). However, at pH 8 glycolytic mutants were unable to grow, with exception of the wild type and the pyruvate kinase mutant (see discussion).

3.4.2. Morphological phenotype of glycolytic mutants

B. subtilis is a rod shaped peritrichous flagellate with a cell length of 3-5 µm. The glycolysis mutants showed diminished growth on plates, therefore the morphological phenotype of the mutant strains was investigated. The mutants were grown in LB medium supplemented with glucose and malate to an optical density of 0.8. The mutants were investigated by light microscopy (Fig. 3.6.). The mutants displayed a normal cell length of around 3-5 µm, that is similar to the wild type. With the exception of the Δ*pyk* mutant, all mutants displayed abnormal cell morphology. Cells were mostly organized in chains and fixed. Only the Δ*pyk* mutant formed single motile cells.

The most severy effect of knock-out of glycolytic genes on morphology was observed for GP591 (Δ*fbaA*::*cat)* and GP700 (Δ*tpiA*::*cat)*. The Δ*fbaA* mutant displayed long cells and irregular morphology. In contrast, the Δ*tpiA*mutant showed abnormal long cell chains. When cultures of the Δ*tpiA* mutant were harvested, no typical pellet was received. The pellet was fluffy, sponge-like impossible to compress.

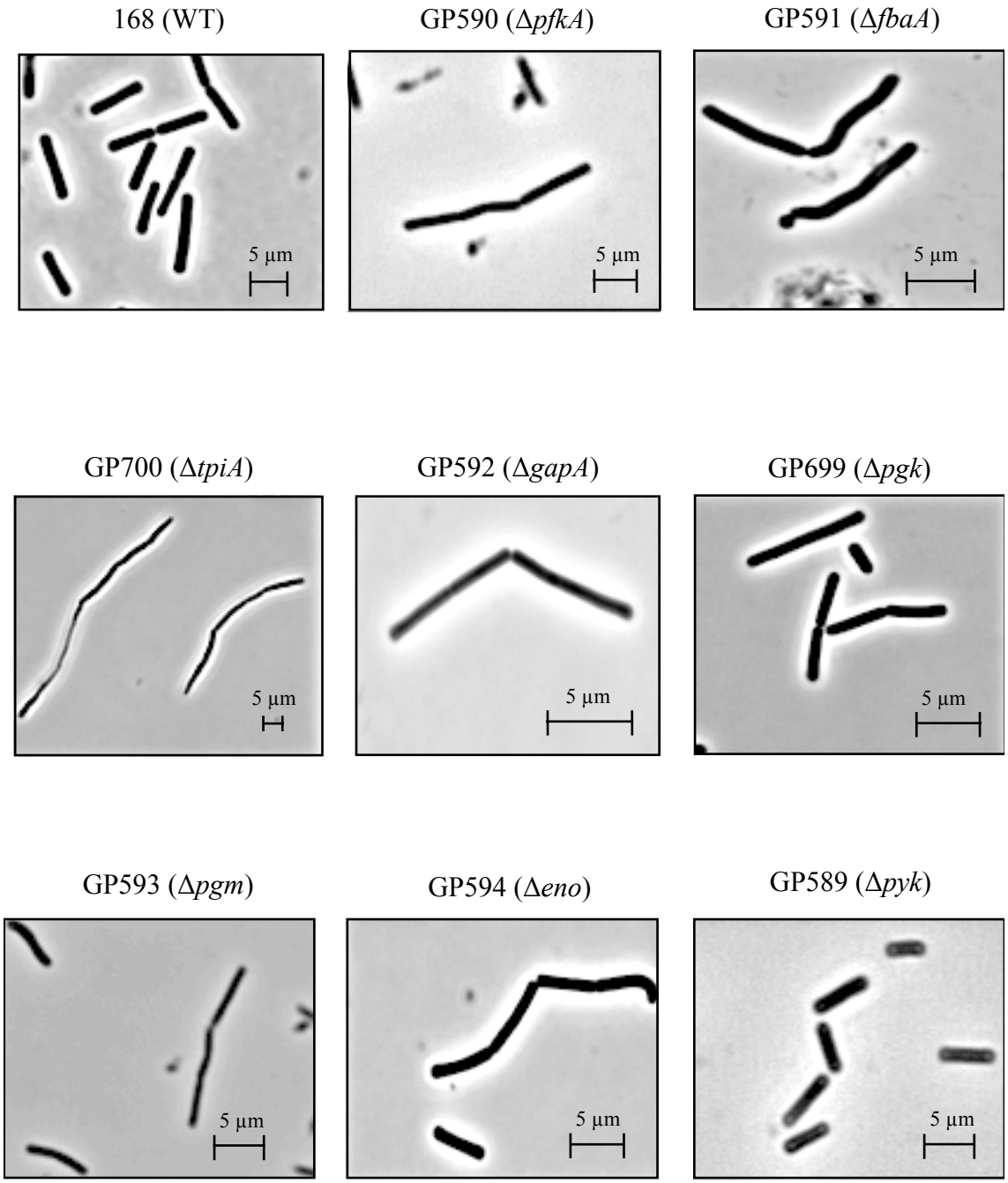

Figure 3.6.: Phase-contrast microscopy of glycolytic mutants.

Glycolysis mutants were grown in LB medium with glucose and malate to OD_{600} of around 0.8. Morphology of cells was examined by light microscopy at enlargement 100x (Zeiss).

3.4.3. Growth of glycolytic mutants on different carbon sources

Glycolysis can be summarized as a sequence of reactions that produce oxidizable, energy yielding substrates and essential biosynthetic precursors such as hexose monophosphates, dihydroxyacetone phosphate, glycerate-3-phosphate, phosphoenolpyruvate and pyruvate. A block in any of these enzymes should divide this pathway into two branches with opposite directions starting with glucose from the glycolytic direction and pyruvate from the gluconegenetic direction.

Extensive studies were made with mutants deficient in glycolytic enzymes in *S. cerevisiae* and *E. coli* (Irani & Maitra, 1977; Clifton *et al*., 1978). In *B. subtilis*, the glycolytic genes were described to be essential, thus little is known about the properties of mutants lacking enzymes of glycolysis.

As described before, mutants lacking single enzymes and multiple deletions of glycolytic genes were now available. On this basis the growth of each mutant on glycolytic and gluconeogenetic substrates was analyzed. For this purpose, the strains were precultured in LB medium with glucose and malate. The growth test was performed on C-minimal medium with glucose, with malate and both carbon sources (Fig. 3.7.). The plates were incubated for 3 days at 42°C. All strains were selected on chloramphenicol. GP909 carrying a cat-resistance gene in the *amyE* gene was used as positive control (WT). The results of the growth test are summarized in table 3.2..

Growth of glycolytic mutants on glucose

As expected, the wild type strain and strain GP589 (Δ*pyk*::*cat*) were able to grow with glucose. In addition, GP 590 (Δ*pfkA*::*cat*) and GP592 (Δ*gapA*::*cat*) grew with glucose as single carbon source. In *S. cerevisiae*, a phosphofructokinase mutant was described, that showed growth on glucose similar to the wild type under aerobic condition (Lobo *et al*., 1983). In contrast, growth of GP592 (Δ*gapA*::*cat*) with glucose was not described until now.

Growth of glycolytic mutants on malate

When malate was used as the single carbon source, growth of the `wild type` strain GP909 (*amyE*::[*hag-lacZ cat*]), of GP590 (Δ*pfkA*::*cat*), GP592 (Δ*gapA*::*cat*) and GP589 (Δ*pyk*::*cat*) was observed. Malate can be metabolized in gluconegenesis.
In glycolysis the enzymatic reactions of phosphofructokinase and pyruvate kinase are irreversible. In gluconeogenesis the reverse reaction of phosphofructokinase is accomplished by fructose-1,6-bisphosphatase (Fbp) (Fujita *et al*., 1979; 1981; 1998). To circumvent the pyruvate kinase reaction different reactions are possible. Malate can be converted to oxaloacetate by malate dehydrogenase and by PEP carboxykinase (PckA) to phosphoenolpyruvate. Besides, pyruvate can also be converted to oxaloacetate by pyruvate carboxylase and further to PEP. Growth was also observed for GP592 (Δ*gapA*::*cat*) with malate. *B. subtilis* possesses two glyceraldehyde-3-phosphate dehydrogenases with different roles in metabolism, *gapA* and *gapB* (Fillinger *et al*., 2000). Under glycolytic conditions GapA is expressed, whereas GapB is only expressed under gluconeogenetic conditions (Servant *et al*., 2005). Under glycolytic conditions the expression of *pckA* and *gapB* is repressed by transcription regulator CcpN (Tännler *et al*., 2008). Therefore, growth of GP592 (Δ*gapA*::*cat*) on glucose was surprising.

Growth of glycolytic mutants on glucose and malate

With glucose and malate, all strains were able to grow. Growth tests on C-glucose and on C-malate revealed no growth of GP591 (Δ*fbaA*::*cat*), GP700 (Δ*tpiA*::*cat*) , GP699 (Δ*pgk*::*cat*), GP593 (Δ*pgm*::*cat*) and GP594 (Δ*eno*::*cat*). Growth was possible for all mutants when a glycolytic and a gluconeogenetic carbon source were provided. Gluconeogenesis can supply metabolites that cannot be formed because of the missing glycolytic enzyme.

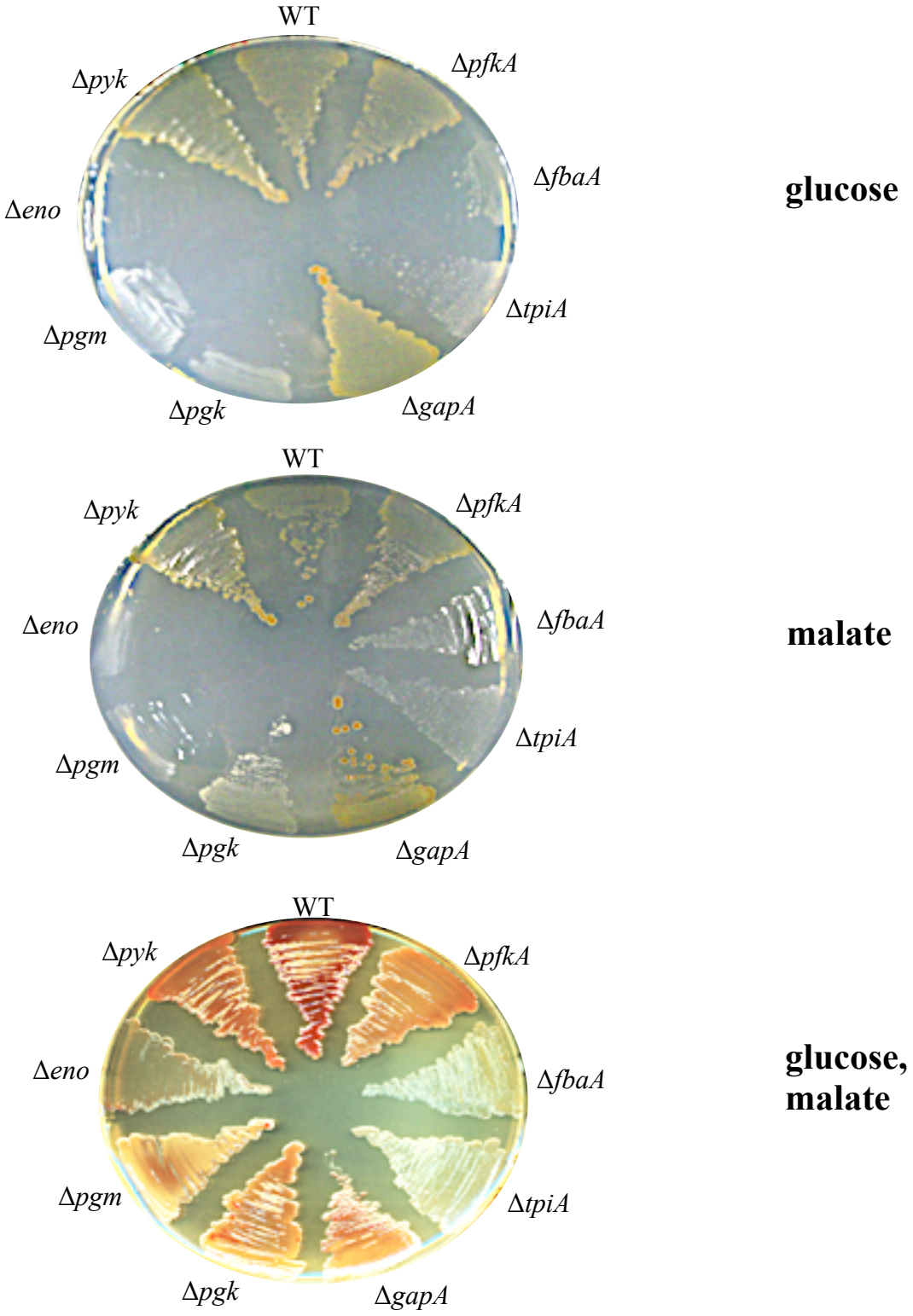

Figure 3.7.: Growth test of glycolysis mutants on minimal medium with glucose and malate. Strains were grown in C-medium with glucose, malate. Growing cultures were streaked out on C-glucose, C-malate and C-glucose, malate agar plates and incubated at 42°C for 3 days. Growth of mutants is summarized in table 3.2..

Strain		C-glucose	C-malate	C-glucose, malate
GP909	WT	✓	✓	✓
GP590	Δ*pfkA*	✓	✓	✓
GP591	Δ*fbaA*	-	-	✓
GP700	Δ*tpiA*	-	-	✓
GP592	Δ*gapA*	✓	✓	✓
GP699	Δ*pgk*	-	-	✓
GP593	Δ*pgm*	-	-	✓
GP594	Δ*eno*	-	-	✓
GP589	Δ*pyk*	✓	✓	✓

Table 3.2.: Growth glycolysis mutants on glucose and malate as carbon and energy source. All mutants strains were able to grew on C-minimal medium with glucose and malate agar plates. Growth on glucose and malate alone was only observed for GP909, GP590, GP592 and GP589. For the further description the deleted gene will be used instead of the strain number.

3.4.4. Elucidation of growth of a *gapA* mutant on glucose

Growth experiments with glycolysis mutants revealed, that the GP590 (Δ*pfkA*::*cat*), GP592 (Δ*gapA*::*cat*) and GP589 (Δ*pyk*::*cat*) are able to grow with either glucose or malate as sole carbon source. The growth of the pyruvate kinase mutant is dependent on the intracellular PEP level, whose concentration is connected to the phosphoenolpyruvate: sugar phosphotransferase system (PTS). The PTS is the only method to modulate the intracellular PEP level (Pan *et al*., 2006). Furthermore it was observed, that GP592 (Δ*gapA*::*cat*) was able to grow on glucose as sole carbon source. *gapA* codes for the NAD^+-dependent glyceraldehyde-3-phosphate dehydrogenase (GapDH), that converts glyceraldehyde-3-phosphate to D-glycerate-1,3-bisphosphate. The reverse reaction is catalyzed by GapB. The two GapDHs of *B. subtilis* have opposite roles in metabolism and are differentially expressed. *gapA* expression is induced under glycolytic conditions whereas *gapB* is repressed under glycolytic conditions and induced under gluconeogenetic conditions (Fillinger *et al*., 2000).

Since GapB is active in the opposite direction and not expressed with glucose, the question arised how the glycolytic flux is modified to allow growth of a Δ*gapA* mutant on glucose. The lower part of the glycolytic pathway can be bypassed by the methylglyoxal pathway (Fig. 3.8.). This pathway starts with dihydroxyacetone phosphate (DHAP) that is converted to methylglyoxal by the methylglyoxal synthase (MgsA). Methylgloxal is further metabolized to lactate and pyruvate that can be fed into overflow metabolism and the TCA cycle. This pathway produces a toxic intermediate, methylglyoxal.

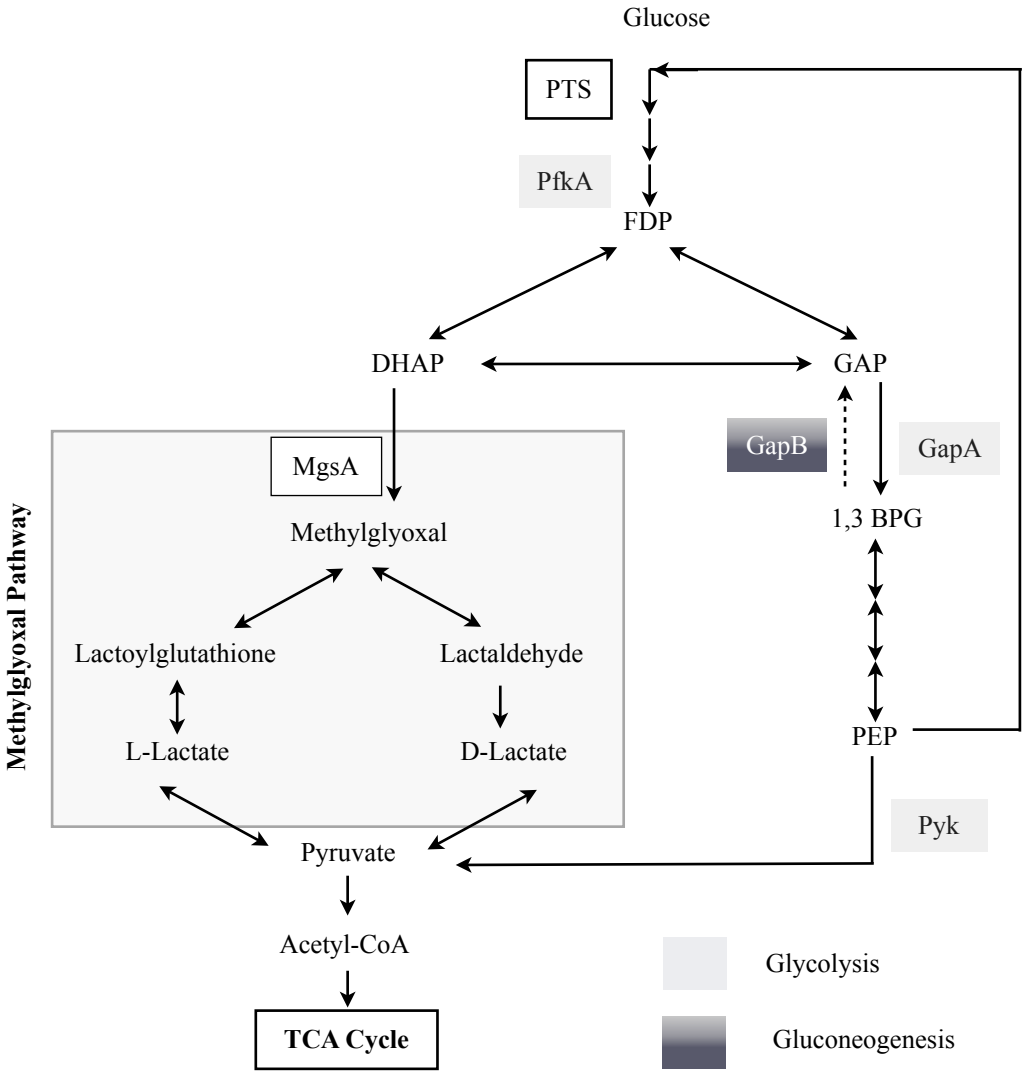

Figure 3.8: The methylglyoxal pathway integrated in glycolysis.
The network is shown in a simplified manner. Reversible reactions are indicated with double headed arrows. Glycolytic enzymes catalyzing irreversible reactions are labeled in grey boxes. Enzymes involved in gluconeogenesis are indicated in dark boxes.

To study whether the methylglyoxal pathway is responsible for growth of the Δ*gapA* mutant on glucose, a Δ*mgsA* Δ*gapA* double mutant (GP705) was constructed and tested for growth on glucose. The double mutant was still able to grow with glucose. Furthermore, measurement of methylglyoxal concentration of *B. subtilis* 168 compared with GP592 (Δ*gapA*::*cat*) grown with glucose revealed no elevated methylglyoxal level in the mutant (data not shown). Consequently, the methylglyoxal pathway is not responsible for the growth of the Δ*gapA* mutant on glucose.

Therefore, a Δ*gapA* Δ*gapB* double mutant (GP703) was constructed to determine whether GapB is able to replace the enzymatic activity of GapA under conditions when *gapB* expression is repressed by CcpN. Growth tests on C-glucose and C-glucose, malate were carried out with *B. subtilis* 168, GP592 (Δ*gapA*::*cat*), GP701 (Δ*gapB*::*spec*) and GP703 (Δ*gapA*::*cat* Δ*gapB*::*spec*) (Fig. 3.9.). In minimal medium with glucose and malate, the single mutants showed growth comparable to the *B. subtilis* 168. Only the double mutant displayed a significant growth defect (Fig. 3.9.A). In minimal medium with glucose, growth of GP701 (Δ*gapB*::*spec*) was similar to *B. subtilis* 168. GP592 (Δ*gapA*::*cat*) displayed growth after a lag-phase of around 5 hours, whereas GP703 (Δ*gapA*::*cat* Δ*gapB*::*spec*) was unable to grow with glucose alone (Fig. 3.9. B). This result confirmed that GapB can complement the enzymatic activity of GapA in glycolysis under the tested conditions. Tests on solid medium verified that GP703 (Δ*gapA*::*cat* Δ*gapB*::*spec*) does not grow with glucose or malate as sole carbon source.

The expression of *gapB* is repressed by CcpN in the presence of glucose, it can therefore be suggested that there is might be a mutation affecting CcpN that allows *gapB* expression in the *gapA* mutant. To test this, the *ccpN* gene was analysed by sequencing. Furthermore, the negative effector of CcpN activity, YqfL was examined by sequencing. However, no mutations were found in *ccpN* and *yqfL*. It was suspected, that CcpN is responsible for the delayed growth of the Δ*gapA* mutant on glucose. To test this hypothesis, the strain GP706 (Δ*gapA*::*erm* Δ*ccpN*::*cat*) was constructed and growth on glucose was tested. The strain GP706 was unable to grow on glucose as sole carbon source (data not shown). A possible explanation could be given by the expression of

pckA. Derepression of *pckA* under glycolytic conditions leads to a growth defect in a *ccpN* mutant (Tännler *et al*., 2008). Expression of PckA under glycolytic condition could result in block at the intersection between glycolysis and gluconeogenesis.

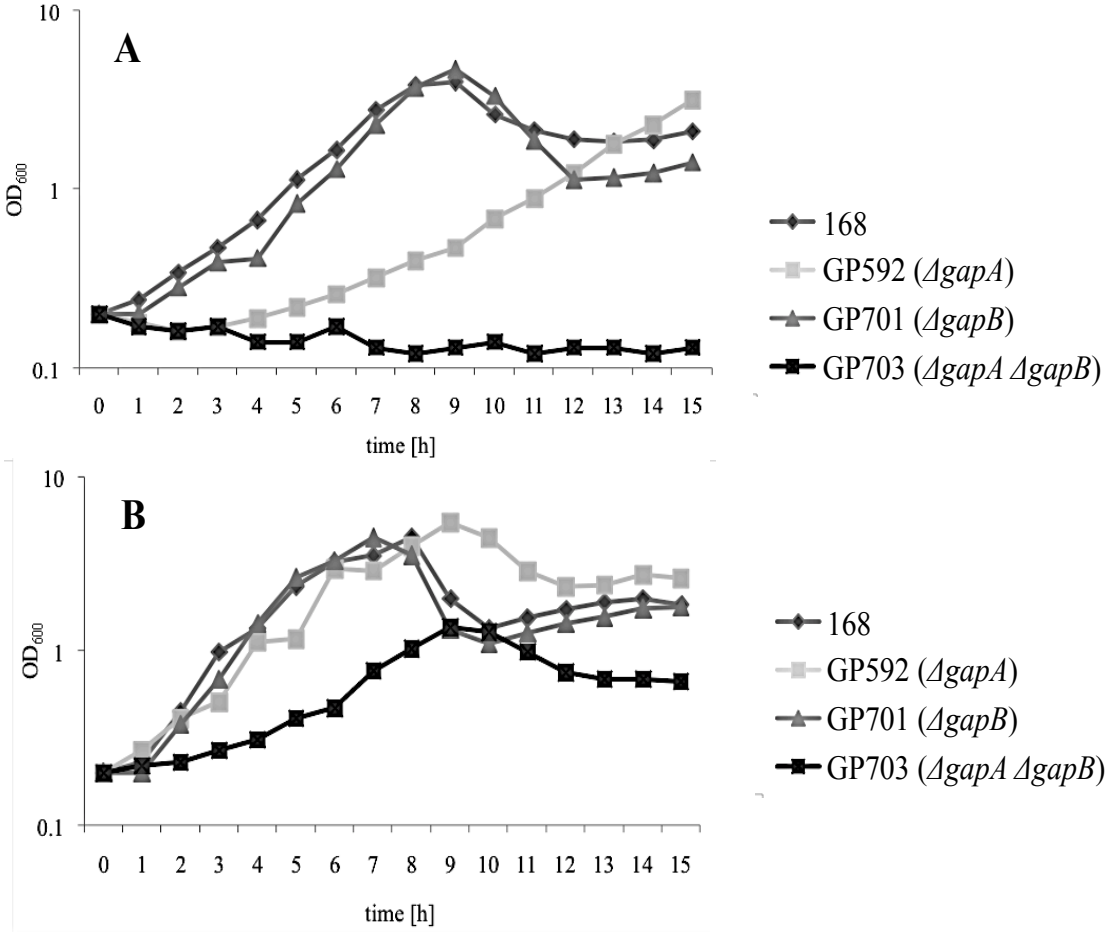

Figure 3.9.: Growth tests of glyceraldehyde-3-phosphate dehydrogenase mutants with glucose and glucose malate. Diagram **A** shows growth of WT, GP592 (Δ*gapA*::*cat*), GP701 (Δ*gapB*::*spec*) and GP703 (Δ*gapA*::*cat* Δ*gapB*::*spec*) in C-medium with glucose. Diagram **B** illustrates growth of these strains in C-medium with glucose and malate.

3.5. Phosphorylation of glycolytic enzymes

In *B. subtilis* almost all glycolytic enzymes were found to be phosphorylated on Ser/Thr and tyrosine residues (Levine *et al*., 2006; Macek *et al*., 2007; Eymann *et al*., 2007). The origin and relevance of their phosphorylation is unknown. Investigation of the phosphorylated residues showed that the glycolytic enzymes are mainly phosphorylated on residues in close proximity to their active centres or close to their substrate or cofactor binding sites.

3.5.1. ATP dependent phosphorylation events in crude extracts

The glycolytic enzymes are among the most abundant cytoplasmatic proteins in living cells (Tobisch *et al*., 1999). Recent phosphoproteome analysis revealed that phosphorylation of glycolytic enzymes is widespread in bacteria, archaea and eukaryotes (Soufi *et al*., 2008; Aivaliotes *et al*., 2009; Gnad *et al*., 2009).
In general, protein kinases use ATP as phosphate donor for the phosphorylation of their substrates. Therefore it was tested, if proteins in *B. subtilis* crude extracts were phosphorylated after addition of radioactively labeled [γ-^{32}P]ATP or GTP. Four distinct phosphorylation signals were detected after incubation with either nucleotide at approximately 70, 65, 45 and 30 kDa (Fig. 3.10.). Unfortunately, the identity of the phosphorylated proteins is unknown.

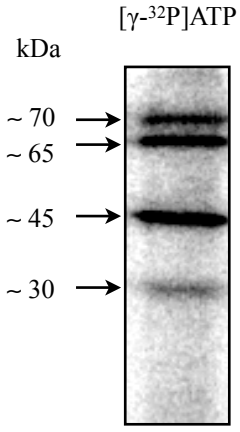

Figure 3.10.: Nucleotide dependent phosphorylation in *B. subtilis* crude extracts. *B. subtilis* was grown in LB medium to an optical density of 0.8. The cells were disrupted by sonification and remaining cell debits were removed by centrifugation. For the phosphorylation approach 20 µg of cytoplasmic proteins were incubated with [γ-^{32}P]ATP (480 Ci/ mmol) for 30 min at 37°C.

3.5.2. Autophosphorylation of the phosphoglycerate mutase

Nucleotide dependent protein phosphorylation in *B. subtilis* crude extracts was discovered. The main interest was to investigate the phosphorylation of glycolytic enzymes. Genes, encode glycolytic enzymes were cloned into the expression vector pWH844. Proteins were overexpressed in *E. coli*, and purified via the fused N-terminal His_6-tag.. The purified proteins were incubated with and without crude extracts of *B. subtilis* and analyzed on phosphorylation.

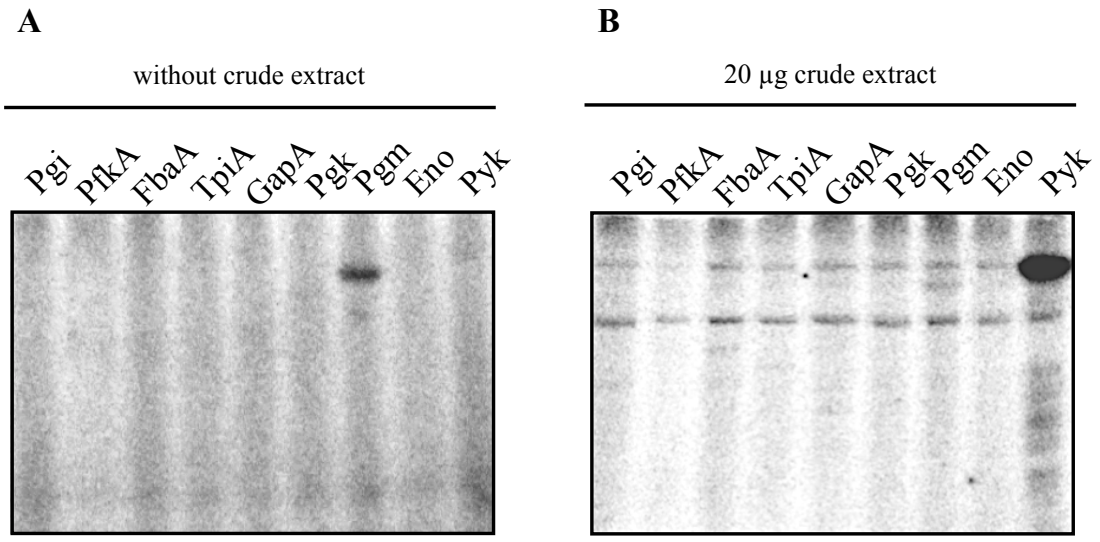

Figure 3.11.: Phosphorylation of glycolytic enzymes.

Autoradiogram A shows a phosphorylation experiment with 10 µg of purified proteins that were incubated with phosphorylation buffer including 10 mM $MgCl_2$ and 0.4 mM [γ-^{32}P]ATP (480 Ci/ mmol)). The assays were carried out at 37°C for 30 min followed by thermal inactivation of proteins (10 min at 95°C).

Glycolytic enzymes were tested for autophosphorylation. For this purpose, the proteins were incubated without addition of crude extract. The phosphoglycerate mutase (Pgm) was identified as autophosphorylated; however, no phosphorylation of further proteins was detected (Fig.3.11.A). After incubation of the glycolytic enzymes with crude extracts a strong phosphorylation signal for the pyruvate kinase was detected and was further investigated, see chapter 3.5.5. (Fig.3.11.B). Phosphoproteome analysis revealed the Pgm as phosphorylated on the Ser-62 residue (Eymann *et al*., 2007; Macek *et al*.,

2007). In previous works the Ser-62 residue of Pgm was identified as the catalytic active residue that forms a phosphoserine intermediate during the phosphotransferase part of the catalytic reaction (Chander *et al*., 1999; Jedrzejas *et al*., 2000). The autophosphorylation of *B*. *subtilis* Pgm has not been previously reported. However, autophosphorylation of phosphoglycerate mutase was also found in the archaeon *Sulfolobus solfataricus* (Potter *et al*., 2003).

3.5.3. Significance of phosphorylation of phosphoglycerate mutase

The phosphoglycerate mutase was identified as autophosphorylated. However, ATP is no known *in vivo* substrate of Pgm. Do determine the phosphorylation site an amino acid exchange of the catalytic Ser-62 residue to alanine was performed by CCR. The mutated gene was cloned into an overexpression vector. Both purified proteins (wild type and mutated) were analysed in phosphorylation experiments. Pgm (Ser-62-Ala) was unable to autophosphorylate (Fig. 3.12.). Pgm that catalyzes the interconversion 3-phosphoglyceric acid to 2-phosphoglyceric acid requires manganese ions for its enzymatic activity (Oh & Freese, 1976). When phosphorylation assays with Pgm were supplemented with manganese, increased phosphorylation signals for Pgm were obtained in contrast to incubation magnesium (data not shown).

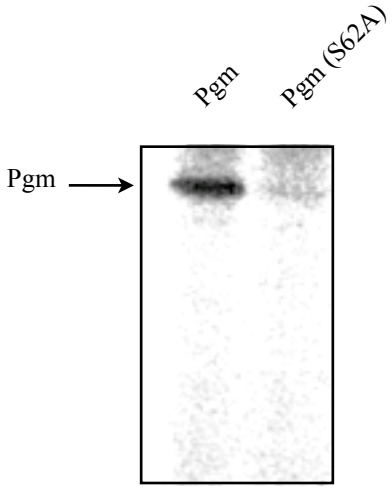

Figure 3.12.: Identification of the phosphorylated amino acid residue of phosphoglycerate mutase (Pgm).

The autoradiogram shows phosphorylation assays with 10 µg of purified Pgm and Pgm (S62A). Proteins were incubated with phosphorylation buffer including 10 mM $MnCl_2$ and 0.4 mM [γ-^{32}P]ATP (480 Ci/ mmol)). The assays were carried out at 37°C for 15 min followed by thermal inactivation of the proteins (10 min at 95°C).

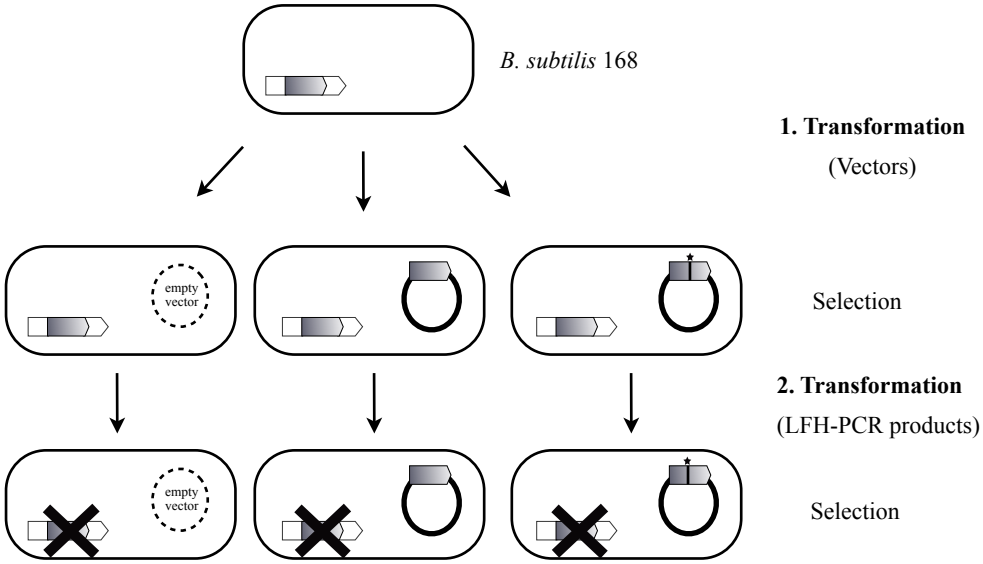

Figure 3.13.: General test procedure for determination of essential amino acid residues. *B. subtilis* 168 was transformed plasmids for complementation. Transformants were selected for erythromycin-resistance conferred by the plasmid. In the second step, the transformants were transformed with LFH-PCR products for the deletion of the chromosomal gene. Selection occurred on spectinomycin or chloramphenicol, for the genomic integration of the resistance cassette, and on erythromycin for the vector. Transformants were checked for the integrity of the resistance cassette and the vector-encoded gene by PCR and sequencing. The asterisk indicates the point mutation.

3.5.4. Relevance of the phosphorylation of triosephosphate isomerase

The triosephosphate isomerase (TpiA) catalyses the reversible interconversion of dihydroxyacetone phosphate and D-glyceraldehyde-3-phosphate in glycolysis. TpiA was identified as phosphorylated on Ser-213 residue (Macek *et al*., 2007). For Pgm it was shown that the phosphorylated Ser-62 residue is essential for its enzymatic activity. To test whether the Ser-213 residue is similarly essential for the enzymatic activity of TpiA, complementation tests were performed (Fig. 3.13.). As described previously, a Δ*tpiA* mutant is unable to grow on glucose or malate alone. If Ser-213 of TpiA is essential for the enzymatic activity, a TpiA-S213A mutant should be unable to grow on glucose alone. Therefore, the Ser-213 residue of TpiA was replaced by the nonphosphorylatable alanine. Overexpression vectors, carrying the native gene, the mutant variant as well as the empty vector were transformed into *B. subtilis* 168. GP700 (Δ*tpiA*::*cat*) showed diminished growth, thus the genomic *tpiA* gene was deleted after

transformation with the respective vectors. The strains were grown in C-minimal medium with glucose and malate (Fig. 3.14.A.). Here, all strains were able to grow. The Δ*tpiA* mutant carrying the empty vector displayed strongest growth defect followed by the mutant without vector. Best growth was observed for *B. subtilis* 168. The mutants complemented with wild type gene and the gene coding for the S213A mutation showed growth similar to *B. subtilis* 168. In C-medium with glucose, were the active form of TpiA is required, the Δ*tpiA* mutant displayed no growth (Fig. 3.14.B.). However, the growth of the complemented mutants was equal to the wild type. Thus, Ser-213 of TpiA and its phosphorylation are not essential for the enzymatic activity of TpiA. It is unknown, if the phosphorylated form would be affected. In contrast, it seems likely that the phosphorylation of TpiA is comparable to the phosphorylation of HPr on Ser-12.

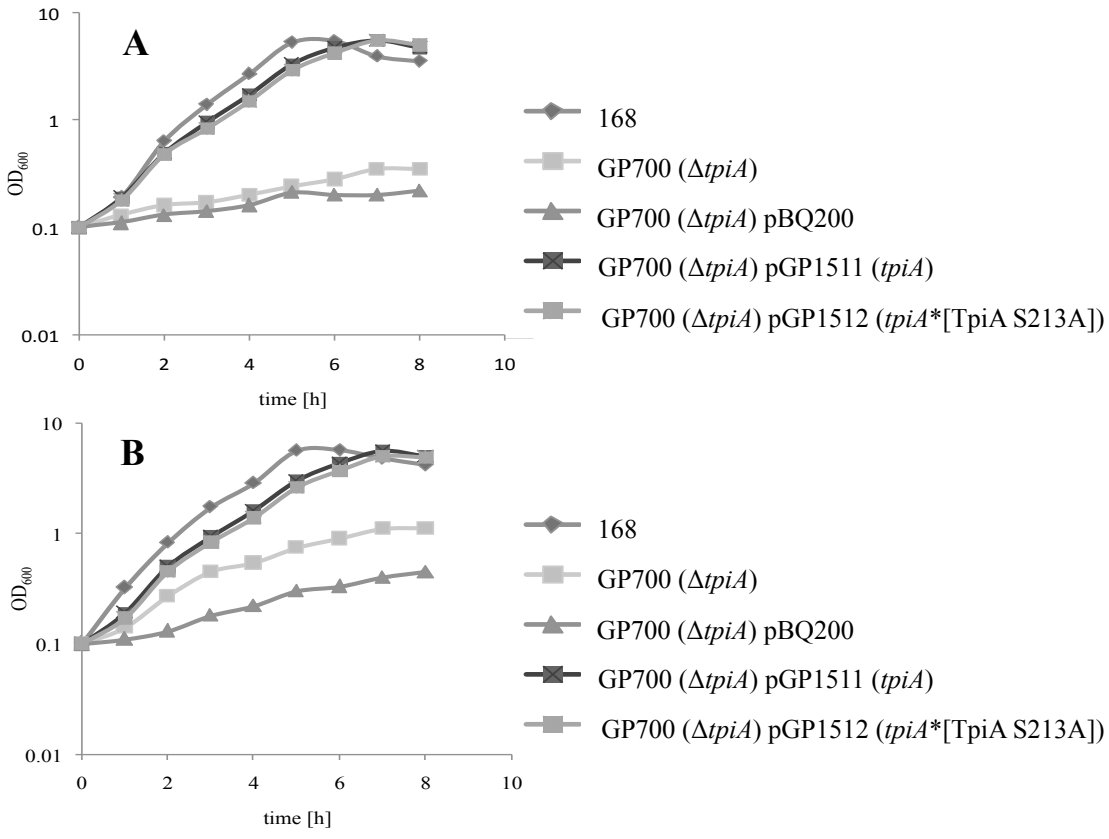

Figure 3.14.: Complementation tests of the Δ*tpiA* mutant.

Growth tests were carried out with GP700 (Δ*tpiA*) complemented with plasmids expressing TpiA (WT), mutated TpiA (S213A) and the empty vector pBQ200. (**A**) Growth tests was performed in C- minimal medium with glucose. (**B**) Growth tests in C- minimal medium with glucose and malate. *Complementations with mutated *tpiA* gene, expressing TpiA (Ser213Ala).

3.5.5. Identification of phosphorylation sites of the pyruvate kinase

The pyruvate kinase of *B. subtilis* catalyzes the last and irreversible step in glycolysis. Thus, it plays an important role in the transition from glycolysis to citric acid cycle during carbon utilization. The enzyme converts PEP to pyruvate, yielding one molecule of ATP. The gene *pyk* that codes for the pyruvate kinase is not essential. However, the generation of pyruvate by the pyruvate kinase is sufficient for growth on non PTS carbon sources (Frey *et al*., 2000).

Pyruvate kinase was identified as phosphoprotein in several bacteria including *B. subtilis*, *E. coli*, *C. jejuni*, and *Lactococcus lactis* (Schmidl *et al*., 2010; Voisin *et al*., 2008; Soufi *et al*., 2008; Macek *et al*., 2008, Macek *et al*., 2007; Eymann *et al*., 2007). One conserved phosphorylation site is known, serine-36 that is also phosphorylated in *E. coli* (Macek *et al*., 2008). In addition, the pyruvate kinase of *B. subtilis* is phosphorylated on a further serine residue, which has not exactly been identified (S536 or S538) (Eymann *et al*., 2007). As shown before, pyruvate kinase became phosphorylated when incubated with crude extracts (Fig. 3.11.B & 3.15.). The question appeared whether pyruvate kinase was phosphorylated in a kinase dependent manner or by autophosphorylation. Furthermore, protein kinase activity that is dependent on specific metabolites or ions was suspected. No phosphorylation of Pyk occurred when the proteins of crude extract were denaturated by boiling. This could indicate that phosphorylation of Pyk is dependent on other proteins in the crude extract.

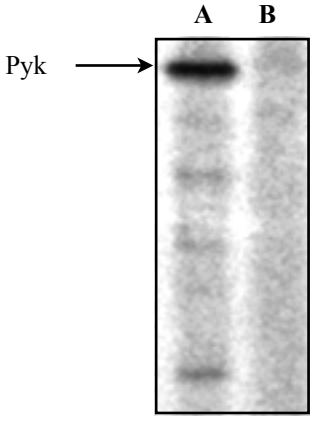

Figure 3.15.: Autoradigram: Phosphorylation of pyruvate kinase (Pyk) with crude extracts.
(**A**) Ten microgram of purified His_6-Pyk was incubated with 20 µg of *B. subtilis* crude extract. (**B**) Proteins in crude extracts were denatured by boiling for 10 min at 95°C and supernatant was then incubated with His_6-Pyk.

Phosphorylation experiments with crude extracts displayed a phosphorylation signal at the size of Pyk after incubation with labeled ATP (Fig. 3.15.). To check, whether Pyk is phosphorylated in crude extracts, strain GP589 (Δ*pyk*::*cat*) was analysed. When crude extracts of the wild type strain 168 were incubated with His_6-Pyk, a strong signal was detected. At the same height, a signal was found in the crude extract of wild type strain (Fig. 3.16). However, this signal was missing in GP589 (Δ*pyk*::*cat*), confirming Pyk as the suspected phosphoprotein. Furthermore, additional phosphorylation signals were detected when purified Pyk was phosphorylated with crude extracts. However, these phosphorylation signals could be the result of degradation of the supplemented, phosphorylated Pyk. Additional phosphorylation signals were also found in the crude extract of *B. subtilis* 168, in the lower part of the autoradigraph (Fig. 3.16.). These phosphosignals are missing in GP589 (Δ*pyk*::*cat*), suggesting that the pyruvate kinase is involved in further phosphorylation events in *B. subtilis*.

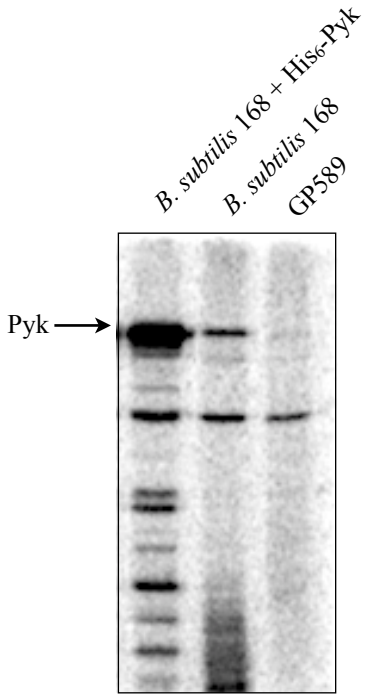

Figure 3.16: Autoradigraph: Identification of phosphoprotein in *B. subtilis* crude extracts. Crude extracts of *B. subtilis* WT (20 µg) was incubated alone and with His_6-Pyk (10 µg). Additionally, crude extract of the Δ*pyk* mutant (GP589) was applied.

Phosphorylation assays revealed that single amino acid replacement of the conserved Ser-36 residue did not prevent phosphorylation of Pyk (Fig. 3.17.). A second phosphorylation site was suspected in the PEP-binding domain of Pyk, around His-539. For the determination of the second phosphorylation site of pyruvate kinase, the

potential phosphorylated serine residues (S36; S538) and the catalytically active His-539; were mutated against alanine. The mutated His_6-tagged Pyk variants were purified and tested on phosphorylation with *B. subtilis* crude extracts (Fig. 3.17.). Only the exchange of both, the Ser-36 and Ser-538 residue against alanine prevented phosphorylation of Pyk in crude extracts (Fig.3.17.).

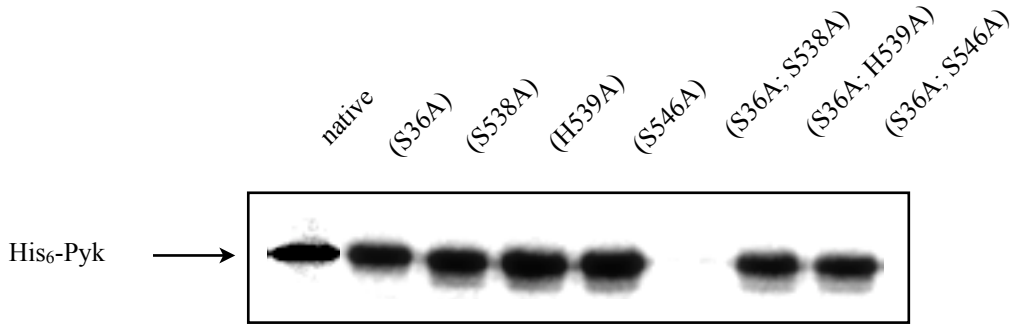

Figure 3.17.: Determination of phosphorylated residues of the pyruvate kinase.
Crude extracts of *B. subtilis* 168 (20 µg) were incubated with His_6-Pyk variants (10 µg) and analyzed by autoradiography.

3.6. Conservation of autophosphorylation of phosphosugar mutases

In *B. subtilis* five mutases (AroA, Drm, Pgm, GlmM and PgcM) were found to be phosphorylated at Ser-/Thr- and Tyr residues (Eymann *et al*., 2007; Macek *et al*., 2007). These mutases are involved in central carbon metabolism, amino acid synthesis and cell wall biosynthesis. In this work it was already shown that the phosphoglycerate mutase is autophosphorylated in the presence of ATP. In addition, several other proteins in *B. subtilis* crude extracts were phosphorylated after incubation with ATP. However, only Pyk was identified so far. For the identification of the remaining phosphoproteins 2D-gels were used.

3.6.1. Autophosphorylation of GlmM from *B. subtilis* and ManB from *M. pneumoniae*

In *B. subtilis* crude extract different proteins were phosphorylated. This observation gave a clue to investigate the origin of certain phosphorylation events. Furthermore, it was shown that phosphorylation of proteins could be stimulated by specific metal ions or intermediates of carbon metabolism.

Therefore, *B. subtilis* crude extracts were supplemented with different intermediates of glycolysis and changes in the phosphorylation pattern were observed (Fig. 3.18.). When crude extracts were dialyzed, the intensity of the phosphorylation signals was enhanced. The addition of pyruvate had a negative effect on the phosphorylation pattern, and addition of phosphoenolpyruvate (PEP) had no effect on phosphorylation. Supplementation with FBP leads to two significant signals. The phosphorylation signal in the lower part of the autoradiograph is HPr. HPr phosphorylation on Ser-46 by HPrK is known to be stimulated by FBP. The identity of the phosphorylated protein in the upper part of the autoradiograph, at around 45 kDa, was unknown.

To identify the corresponding proteins, the treated crude extracts were analyzed in 2D-PAGE (Fig.3.19.). Two dimensional gel analysis revealed YbbT, now renamed GlmM, as a significant phosphoprotein, when incubated with ATP and FBP. GlmM is a phosphoglucosamine mutase that catalyzes the conversion of α-D-glucosamine-1-phosphate to D-glucosamine-6-phosphate. Autophosphorylation was also shown for GlmM of *E. coli* (Jolly *et al*., 2000).

In *B. subtilis* the enzymatic activity of GlmM is essential for peptidoglycan synthesis. Protein alignments of phosphosugar mutases showed, that the active centers of these mutases are conserved across the domains of life (Jolly *et al*., 1999). It seems unambiguous that the enzymatic reaction mechanism by which the mutases are phosphorylate *in vivo* is conserved as well. It seems likely that autophosphorylation as result of the enzymatic activity is common.

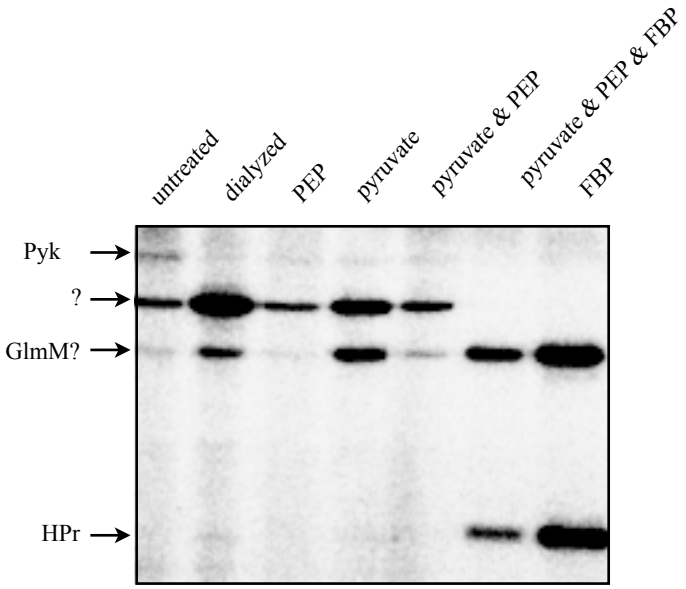

Figure 3.18.: Influence of glycolytic intermediates on phosphorylation events. Crude extracts of *B. subtilis* were dialyzed and following incubated in phosphorylation buffer with manganese and [γ-^{32}P]ATP. Additionally, the approaches were supplemented with 20 mM of PEP, pyruvate and FBP.

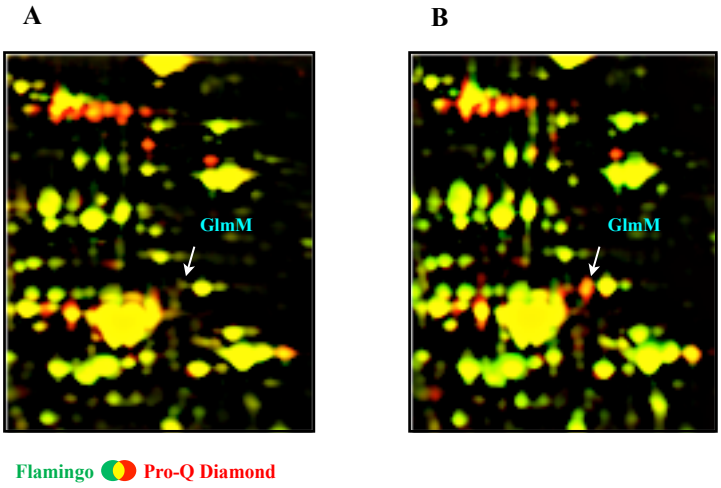

Figure 3.19.: Sector of 2D-PAGE analysis: (**A**) Dialyzed crude extract was incubated with phosphorylation buffer alone. (**B**) Dialyzed crude extract was incubated with phosphorylation buffer, ATP and FBP. Phosphorylation signal for GlmM is indicated by arrows.

GlmM was analyzed for autophosphorylation (Fig. 3.20.). The previously identified phosphorylation site, Ser-100 was replaced by alanine (Macek *et al*., 2007; Eymann *et al*., 2007). Phosphorylation assays revealed that GlmM is autophosphorylated in the presence of manganese and ATP (Fig. 3.20.). Furthermore, autophosphorylation on Ser-100 was confirmed by amino acid exchange against alanine.

Phosphoproteome analysis of Ser- and Thr phosphorylated proteins in *Mycoplasma pneumoniae* revealed a phosphorylated paralog of GlmM. ManB is a potential phosphosugar mutase in *M. pneumoniae* with high similarity to GlmM. Phosphorylation was found at the conserved Ser-149 residue. Phosphorylation assays revealed manganese dependent autophosphorylation of ManB on Ser-149. The phosphorylation site was confirmed by alanine replacement (Fig.3.20.). GlmM of *B. subtilis* and ManB of *M. pneumoniae* showed enhanced autophosphorylation signals when incubated with manganese (Fig.3.21.).

Consequently, autophosphorylation of phosphosugarmutases, like Pgm and GlmM is not exclusively conserved in *B. subtilis*. Investigation of ManB of *M. pneumoniae* revealed that autophosphorylation also occurs on conserved residues of paralogous proteins in other species like *M. pneumoniae* (Schmidl *et al*., 2010).

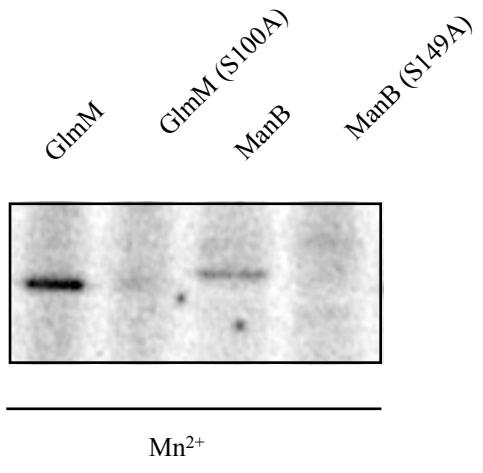

Figure 3.20.: Autophosphorylation of GlmM (*B. subtilis*) and ManB (*M. pneumoniae*).
Proteins were analyzed for autophosphorylation. Autoradiograph of His_6-tagged GlmM and ManB and the respective point mutants.

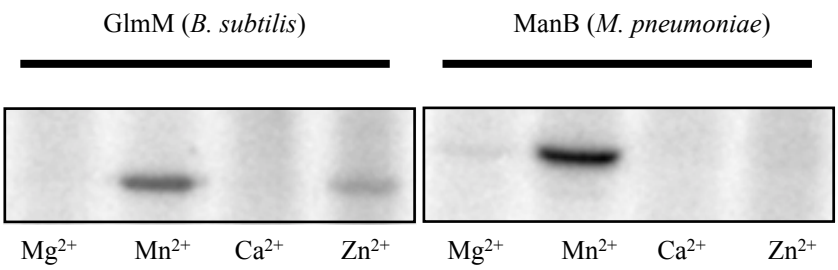

Figure 3.21: Specificity of metal ions for autophosphorylation of GlmM and ManB. Ten microgram of GlmM and ManB were incubated with 10 mM of magnesium (Mg^{2+}), manganese (Mn^{2+}), calcium (Ca^{2+}) and zinc (Zn^{2+}).

3.6.2. Importance of the conserved Ser-100 of GlmM for the viability of *B. subtilis*

In previous experiments it was shown that GlmM is able to perform autophosphorylation. This raised the question, whether Ser-100 is essential for the enzymatic activity, as for Pgm. The test procedure for the analysis of essential residues is shown in Fig. 3.13. Overexpression plasmids for *B. subtilis* were constructed. The *glmM* wild type gene and the mutated *glmM* gene (Ser-100-Ala) were cloned into vectors (pBQ200) for expression in *B. subtilis*. *B. subtilis* 168 was transformed with the respective plasmids (pBQ200; pGP400; pGP1403) and the gained transformants were transformed again with LFH-PCR products for the deletion of the genomic copy of *glmM*. Transformation of the wild type strain carrying the empty vector (pBQ200) gave no clones. Previous studies already showed that *ybbT* (*glmM*) is essential under standard conditions (Kobayashi *et al*., 2003). *B. subtilis* was able to complement the loss of *glmM* when expression of the wild type gene occurred from plasmid (pGP400). Expression of the mutated GlmM (S100A) variant from pGP1403 was not able to complement the loss of the chromosomal *glmM* gene. Therefore, it can be concluded that the S100 of GlmM is essential for the enzymatic activity. Furthermore, GlmM was found as phosphorylated on its conserved serine residue in all tested organisms so far.

4. Discussion

4.1 Mutants of *essential* glycolytic genes

The natural habitat of *B. subtilis* is the soil. In this habitat it is exposed to various kinds of environmental stress conditions. Furthermore, it needs to maintain against other competitors for nutritions. Therefore, a rapid uptake and utilization of carbon and nitrogen sources is essential for survival. The preferred carbon sources of *B. subtilis* are glucose and malate. Glucose is mainly metabolised in glycolysis, followed by incomplete oxidation in overflow metabolism. The main products of overflow metabolism, acetic acid is secreted. When glucose is exhausted, acetic acid can be again taken up and further oxidized in tricarboxylic acid cycle. The incomplete but rapid oxidation of hexoses gives *B. subtilis* an advantage towards other soil bacteria.

This shows that glycolysis is indispensable for rapid carbon sugar utilization. Studies with the aim to identify essential genes in *B. subtilis* revealed that nearly all genes coding for enzymes of glycolysis are indispensable for survival (Kobayashi *et al*., 2003; Thomaides *et al*., 2007). It was not obvious why these genes should be essential, when cells were grown in rich medium. Because, precursors that are required for amino acid and cell wall biosynthesis are available in rich medium. Moreover, in other organisms like *E. coli* and *Saccharomyces cerevisiae* mutants in glycolysis were already described (Irani & Maitra, 1974; Lam & Marmur, 1976).

In this work it was shown that single and multiple mutants of glycolytic genes can be generated and that these mutants are still viable. From this, the question appeared how Kobayashi and colleagues analyzed *B. subtilis* genes for essentiality. In this work, the mutants were generated by replacement of the specific gene by a terminator-less antibiotic resistance cassette, that was introduced via double homologous recombination of a LFH-PCR product into the specific gene locus. In contrast to that, Kobayashi and co-workers used the pMUTIN vector for their deletion experiments. Negative effects of the integrated vector on the expression of the downstream genes is avoided by IPTG- dependent expression (Vagner *et al*., 1998). It could be possible that an enhanced expression of downstream genes has a negative effect on the viability of the glycolytic

mutants. Moreover, it must be noted that not every essential gene can be deleted with LFH-PCR products, as shown for *glmM* in this work. Similarly, other genes that were previously described as essential were later found to be not essential, as in the case of *ydiB*, *yloQ*, *yqeL* and *ywlC* (Kobayashi *et al*., 2003; Hunt *et al*., 2006; Karts *et al*., 2009).

In general, the construction of glycolytic mutants was limited by two factors, the pH value and for certain mutants the presence of a gluconeogenetic carbon source. Mutants were only obtained when the pH value of the medium was below 7.5 and addition of malate supported growth of the mutants. In contrast to malate, supplementation with succinate was not sufficient. This could be explained by the preference of *B. subtilis* for malate as carbon source (Kleijn *et al*., 2009). Furthermore, mutants of the *eno* and *pgm* genes were already described sixteen years ago and that growth of these mutants is enhanced by supplementation with malate (Leyva-Vazquez and Setlow, 1994). The malate uptake and utilization could give an clue for the viability defect at pH 8. In *B. subtilis* malate uptake is driven by the transporters, MaeN, CimH, MleN and YflS (Wei *et al*., 2000; Krom *et al*., 2001). However, only MaeN is indispensible for growth on malate (Tanaka *et al*., 2003). Transcriptome analysis revealed that alkaline stress results in downregulation of *maeN* (Kitko *et al*., 2009; Wilks *et al*., 2009). Moreover, the enzymatic activity of the malate dehydrogenase, catalyzing the conversion of malate to oxaloacetate in the citric acid cycle, is inhibited at pH above 7.5 (Yoshida, 1965).

It was shown that growth of the mutants (Δ*gapA*, Δ*pgk*, Δ*pgm* and Δ*eno*), that miss the enzymes of the lower part of glycolysis is supported by malate. Growth of the Δ*fbaA* and Δ*tpiA* mutant was not affected by malate. Furthermore, the Δ*pfkA*, Δ*gapA* and Δ*pyk* mutants were able to grow with glucose as sole carbon source. Studies with a transaldolase mutant of *E. coli* revealed that the transaldolase reaction, that is located in the pentose phosphate pathway can be taken over by the activity of the phosphofructokinase and aldolase (Nakahigashi *et al*., 2009). When *B. subtilis* is grown on glucose as the main carbon source, about 40% of the carbon is utilized via the pentose phosphate pathway (Kleijn *et al*., 2010). Thus, it can be suggested that the

pentose phosphate pathway alone allows growth of a phosphofructokinase mutant on glucose. To test this hypothesis, the pentosephosphate pathway should be interrupted in the Δ*pfkA* mutant. The *B. subtilis* genome encodes a fructose-1-phosphate kinase (*fruK*) that might complement the missing phosphofructokinase combined with activity of a hexosephosphate mutase (Reizer *et al*., 1999).

The glyceraldehyde-3-phosphate dehydrogenase mutant was also able to grow with glucose as sole carbon source, but displaying a growth delay of around five hours in liquid medium. It was assumed that the methylglyoxal pathway is able to bypass the block in glycolysis. However, it displayed no capacity for complementation of the lack of *gapA*. Certain bacteria possess a NAD independent glyceraldehyde-3-phosphate dehydrogenases (GapN) that does not phosphorylate its substrate. Interestingly, this enzyme can substitute the reactions of two glycolytic enzymes, the glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase. GapN was proven, in addition to the GapDH, in *Neisseria meningitidis*, *Streptococcus mutans* and *Sulfolobus solfataricus* (Pailot *et al*., 2006; Ettema *et al*., 2006; Fourrat *et al*., 2007. However, the *B. subtilis* genome does not code for GapN. Therefore GapB came into the focus of this study. In the literature, the *gapA gapB* gene pair was proposed as essential, because of the essentiality of *gapA* (Thomaides *et al*., 2007). However, in this work a double mutant of the *gapA* and *gapB* gene was constructed. This shows, that disruption of both genes is possible. Growth tests revealed that the double mutant can not grow on glucose as sole carbon source. Therefore, it is suggested that GapB is able to complement the function of GapA. However, *gapA* and *gapB* are differently expressed dependent on the carbon source. The expression of the glycolytic GapDH (*gapA*) is repressed under gluconeogenetic conditions by CggR and the expression of the gluconeogenetic GapDH (*gapB*) is repressed under glycolytic conditions by CcpN (Doan & Aymerich, 2003; Servant *et al*., 2005). Therefore, it can be assumed that the growth delay of a *gapA* mutant on glucose is a result of CcpN dependent repression. However, sequencing of genes for CcpN, and its negative regulator regulator YqfL, revealed no mutations. Additionally, the formation of suppressor mutants can be excluded, because, the growth delay also occurred when growing cells were used to

inoculate fresh medium. These results lead to the suggestion that CcpN dependent repression is further modulated. It seems reasonable, that glycolytic intermediates influence the DNA binding activity of CcpN. However, Licht and co-workers showed, that glycolytic metabolites and nucleotides have no negative effect on CcpN activity *in vitro* (Licht *et al*., 2008). In addition, in this work it was tested, whether the absence of CcpN supports growth of the *gapA* mutant on glucose. Unlike the assumption, the double mutant was unable to grow with either glucose or glucose and malate as sole carbon source. It is known that the disruption of *ccpN* results in a drastically reduced growth rate and in alteration of the metabolic flux, that is mainly due to the pyruvate carboxykinase (Pck) activity (Tännler *et al*., 2008). Perhaps, derepression of Pck prevents growth of the Δ*ccpN ΔgapA* double mutant. However, in this case the *ΔgapA* mutants should be unable to grow, if the growth delay would be dependent on CcpN repression. Therefore, other mechanism of regulation must be suspected.

The remaining mutants (Δ*fbaA*, Δ*tpiA*, Δ*pgk*, Δ*pgm and* Δ*eno*) required both a glycolytic and gluconeogenetic carbon source for growth on minimal medium. This addiction is due to the deletion of these genes that divide the amphibolic sequence into two branches, starting from glucose and pyruvate or PEP, respectively. Both branches are functioning in opposite directions and provide biosynthetic precursors for the cell. However, accumulation of phosphorylated intermediates can be assumed and should be investigated by metabolome analysis.

Fructose-1,6-bisphosphate aldolase is required for the cleavage and synthesis of fructose-1,6-bisphosphate in glycolysis and gluconeogenesis. Mutants lacking the *fbaA* gene showed diminished growth and impaired cell morphology. When Δ*fbaA* mutants were grown with hexose supplementation, FBP accumulates. FBP displays the metabolic status of the cell and regulates the activity of transcription factors like CcpA and CggR. It was tried to eliminate the effect of FBP on carbon catabolite repression via CcpA by constructing a Δ*fbaA* Δ*ccpA* double mutant. However, it was not possible to construct such a mutant, suggesting that the lack of CcpA dependent regulation prevents efficient carbon utilisation.

For the triosephosphate isomerase mutant, growth was only observed in the presence of both glycolytic and glyconeogenetic carbon sources. In *E. coli* a metabolic bypass of this enzymatic reaction was found. Growth of mutants, lacking the *tpiA* gene on gluconeogenetic carbon sources is enabled by an enzyme (YghZ), that reduces L-glyceraldehyde-3-phosphate to dihydroxyacetone phosphate in a NADPH dependent manner (Desai & Miller, 2008). This enzyme is unable to catalyze the reverse direction to allow growth on glycolytic substrates. However, Blast analysis revealed that *B. subtilis* does not possess this enzyme.

Around forty years ago a mutant of *B. subtilis* was described, that lacks phosphglycerate kinase activity. In this mutant initiation of spore germination by alanine was extremely diminished (Prasad *et al*., 1972). The relevance of glycolytic enzymes for cell differentiation processes was also shown for further enzymes. About the phophoglycerate mutase it is known that activity is inhibited when the intracellular pH becomes more acidic during sporulation (Magill *et al*., 1996; Chander *et al*., 1998). Moreover, mutants lacking phosphoglycerate mutase or enolase are unable to sporulate (Leyva-Vazquez & Setlow, 1994). In general, phosphoglycerate mutases are subdivided into two classes, depending on their catalytical mechanism. For one class of Pgm, the reaction is dependent on 2,3-bisphosphoglycerate (dPgm), whereas the enzymes of the other class are independent from this metabolite (iPgm) (Pearson *et al*., 2000). In *B. subtilis* only the iPgm is the common mutase, whereas in *E. coli* the dPgm is the common (Fraser *et al*., 1999).

Growth of a Δ*pyk* mutant on glucose as sole carbon source was not unexpected, thus PEP is converted to pyruvate during glucose uptake by the PTS (Fry *et al*., 2000).

Glycolytic enzymes from different organisms were identified to have secondary roles in the cell. For enolase various secondary functions were found in different organisms. It was shown that this enzyme is involved in the regulation of transcription, the transport of proteins, the regulation of DNA and RNA synthesis and several other cellular processes (Feo *et al.,* 2000; Decker & Wickner, 2006; Tovy *et al*., 2010). Studies in *B. subtilis* revealed that glycolytic enzymes interact with each other and with other essential proteins. Therefore, they were assumed to be involved multienzyme

complexes, like glycosome and RNA-degradosome (Commichau *et al*., 2009). It was suggested that glycolytic enzymes are *essential* because of their interaction with other essential enzymes. However, on the basis of this work this hypothesis must be reconsidered.

4.2. Protein phosphorylation in *B. subtilis:* Of conservation and coincidence

Protein phosphorylation is one of the most important forms of modification, but the origin and function of phosphorylation events are unknown to great extent. In this work the phosphorylation of proteins on Ser/Thr and Tyr residues in *B. subtilis* was investigated. For this purpose multiple protein kinase mutants were analyzed. The well-known kinases were not required for the majority of phosphorylation events in *B. subtilis*. Moreover, the annotated protein kinases seem to have specific target proteins and consequently specific functions in the cell, for example as shown for PrkC in spore germination (Shah *et al*., 2008; Shah *et al*., 2010). In conclusion, it was suspected that other unknown kinases or mechanism are responsible for these phosphorylations events. The comparison of the phosphorylation sites in *B. subtilis* displayed no generally conserved motif. However, individual phosphorylation sites are conserved among species like *E. coli* and *B. subtilis* (Macek *et al*., 2007; Macek *et al*., 2008).

In general, kinases do phosphorylate specific targets on defined residues. For example the HPr kinase specifically phosphorylates HPr and its ortholog Crh on Ser-46 (Galinier *et al*., 1997). Moreover, the number and diversity of phosphorylation peptides in *B. subtilis* would lead to the assumption that *B. subtilis* possesses a great number of uncharacterized kinases or that some of these kinases phosphorylate promiscuous substrates. In previous studies, the knowledge about phosphorylation sites in eukaryotes was used to predict phosphorylation sites in bacteria, with the aim to identify substrate kinase couples. However, only around 14% of the experimentally verified phosphorylation sites were confirmed in these bioinformatic approaches (Soufi *et al*., 2007). Therefore, phosphorylation events in bacteria seem to be more different then in eukaryotes.

In this work it was shown that the GTPase Obg and YdiB are autophosphorylated as well as the phosphosugar mutases Pgm and GlmM. Besides, the HPr protein and glycolytic enzymes were investigated. Previous studies identified a new phosphorylation of HPr at the Ser-12 residue (Macek *et al*., 2007). The known phosphorylation sites of HPr, His-15 and Ser-46 are sufficient for sugar uptake via PTS and carbon catabolite repression. An attempt for the identification of targets of protein kinases in *B. subtilis* revealed, that PrkC is able to phosphorylate HPr on Ser-12 *in vitro* (Pietack *et al*., 2010). However, the relevance of this phosphorylation is unclear. It was assumed that phosphorylation on Ser-12 influences the conformation of the neighbouring amino acids including His-15 and, hence the sugar uptake is influenced. However, growth tests revealed that the Ser-12 residue is not sufficient for the function of HPr in the PTS. Moreover, experiments with purified HPr and enzyme I revealed that HPr is phosphorylated on Ser-12, during phosphate transfer through His-15. This leads to the assumption that Ser-12 phosphorylation of HPr is just an coincidence. In contrast to His-15 and Ser-46, the Ser-12 residue is not conserved among *Firmicutes* and other bacteria (Deutscher *et al*., 2006). Interestingly, in *E. coli* HPr was also found to be phosphorylated on an additional residue, the Thr-12 (Macek *et al*., 2008). However, it is not clear, if phosphorylation of HPr on Ser-12 or Thr-12 is of biological relevance for the common functions of HPr. It could be speculated, that the Ser-12 phosphorylation is associated with a unknown function. In the literature, several interaction partners of HPr were described. Recently, interaction of HPr (His-P) with the transcriptional regulator YesS was found and was described as a novel form of carbon catabolite repression (Poncet *et al*., 2009). Perhaps, HPr (Ser-12-P) mimics histidine phosphorylated HPr and is therefore associated in YesS interaction. A further interaction of HPr was described for GapA. It was shown that only HPr (Ser-46-P) is able to interact with GapA, and that the interaction inhibits the activity of GapA (Pompeo *et al*., 2007). To determine the function of the Ser-12 phosphorylation, the amount of HPr (Ser-12-P) in the cell must be elucidated. And in addition the influence of permanent phosphorylation of Ser-12 on the HPr activity must be clarified.

A further phosphorylation event that was analysed in this work was the phosphorylation of TpiA on Ser-213 (Macek *et al*., 2007). The relevance of the Ser-213 for the enzymatic activity was tested in complementation assays with the result that Ser-213 of TpiA is not required for the enzymatic activity *in vivo*. However, in contrast to Ser-12 of HPr, the Ser-213 residue of TpiA is conserved among other species (Fig.:6.1).

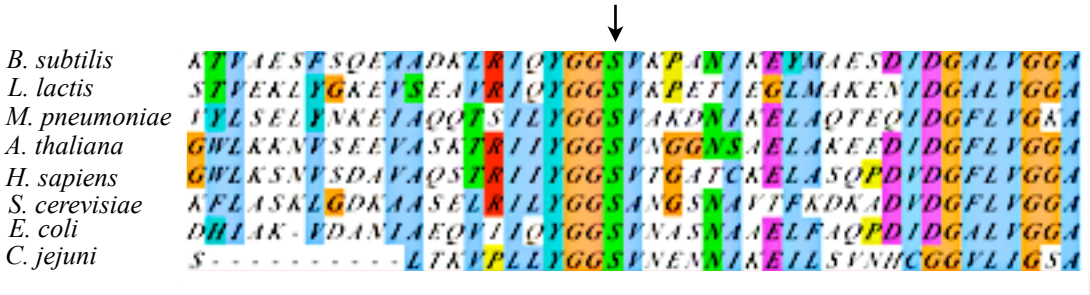

Figure 6.1.: Multiple sequence alignment of triosephosphate isomerase of different organism. The protein sequences of triosephosphate isomerases of *B. subtilis*, *Lactococcus lactis*, *Mycoplasma pneumoniae*, *Arabidopsis thaliana*, *Homo sapiens*, *Saccharomyces cerevisiae*, *Escherichia coli and Campylobacter jejuni* were aligned with ClustalW2. The alignment displays conservation of the Ser-213 (indicated by arrow) residue that is phosphorylated in *B. subtilis*.

Moreover, TpiA was not found as phosphorylated in all species that were investigated so far. In *M. pneumoniae* and *C. jejunii,* TpiA was identified as phosphoprotein, but the phosphorylated residues are unknown (Voisin *et al*., 2007; Schmidl *et al*., 2010). Studies on the reaction mechanism of TpiA in *Trypanosoma* revealed that the conserved Ser-213 residue is part of peptide flip, a structural element of the enzyme. The peptide bond of the flip provides a hydrogen bounding partner for the phosphate of the substrate (Casteleijn *et al*., 2006). The direct binding of the phosphate on Ser-213 suggests, that phosphorylation of TpiA on Ser-213 is just a result of enzymatic activity. For the Pgm it was already described that phosphorylation on the conserved Ser-62 occurs during the enzymatic reaction. However, in the case of Pgm the enzymatic activity gets lost when the catalytic serine was replaced against a nonphosphorylateable amino acid (Rigden *et al*., 2003). Perhaps phosphorylation of TpiA on Ser-213 influences its activity, but it is questionable if the amount of phosphorylated TpiA in the cells has an effect on growth.

The first regulatory phosphorylation of an enzyme in prokaryotes was identified for the isocitrate dehydrogenase of *E. coli* (Garnak & Reeves, 1979). This phosphorylation is catalyzed by a specific kinase and leads to a change of the carbon flow from the citric acid cycle to the glyoxylate pathway. In *B. subtilis* the isocitrate dehydrogenase is also phosphorylated on a different residue; however, *B. subtilis* possesses no glyoxylate pathway and no counterpart of the *E. coli* kinase. This leads to the assumption that phosphorylation of enzymes is not strictly associated with the regulation of enzymatic activity. The phosphorylation of proteins can be required to regulate the formation of multimers and protein-protein interaction in general. Moreover, phosphorylation can regulate the localization of proteins in the cell. For example, tyrosine phosphorylation of *B. subtilis* enolase by PtkA leads to migration to the cell poles (Mijakovic, unpublished). In *S. cerevisiae* it was described that the phosphorylation of the hexokinase depends on extracellular glucose concentration. The phosphorylation results in the dissociation of the dimeric to the monomeric form, that has a higher affinity for the substrate (Vojtek & Fraenkel, 1990).

Another subject of this study was the pyruvate kinase. Phosphorylation of pyruvate kinase was described on the conserved Ser-36 residue and in the PEP-binding domain (Macek *et al*., 2007; Eymann *et al*., 2007). Phosphorylation of this enzyme was also found in *C. jejuni*, *L. lactis* and in *E. coli* on Ser-36 (Macek *et al*., 2008; Voisin *et al*., 2008; Soufi *et al*., 2008). The identified phosphorylation site (Ser-36) is located at the interface between the edge of the catalytic domain A and the domain B, so it seems obvious that it could be an important feature in regulating the enzymatic activity of Pyk. This kind of regulation can be found for example in the mammalian pyruvate kinase isoform L, where the enzymatic activity is inhibited upon phosphorylation (Pilkis & Claus, 1991). The second phosphorylation site, was identified at Ser-538 and is located in the PEP-binding domain of the enzyme, next to the catalytically active His-539 residue. Pyk was phosphorylated when incubated with crude extracts of *B. subtilis*. In addition, further phosphorylation signals were detected in crude extracts together with Pyk phosphorylation. Only replacement of both phosphorylation sites prevents phosphorylation of Pyk and of the additional proteins.

For the human pyruvate kinase 2 it was shown that it binds phosphotyrosine peptides. Binding of these peptides result in the release of the activator fructose-1,6-bisphosphate and consequently in loss of enzymatic activity (Christofk *et al*., 2008). However, the Pyk in *Bacilli* is not activated by FBP as was shown for the Pyk of *B. stearothermophilus* (Sakai *et al.,* 1986). Phosphorylation of Pyk in *B. subtilis* could have a regulatory influence on enzymatic activity and moreover could be a part of suspected protein kinase activity. This is not unlikely, because protein kinase activity was previous reported for the glyceraldehyde-3-phosphate dehydrogenase from rabbit (Duclos-Vallee *et al*., 1998). In this example, the enzyme forms a high-energy acetyl complex that behaves as a protein kinase. Protein kinase activity of conserved glycolytic enzymes could explain the absence of other protein kinases in *B. subtilis*.

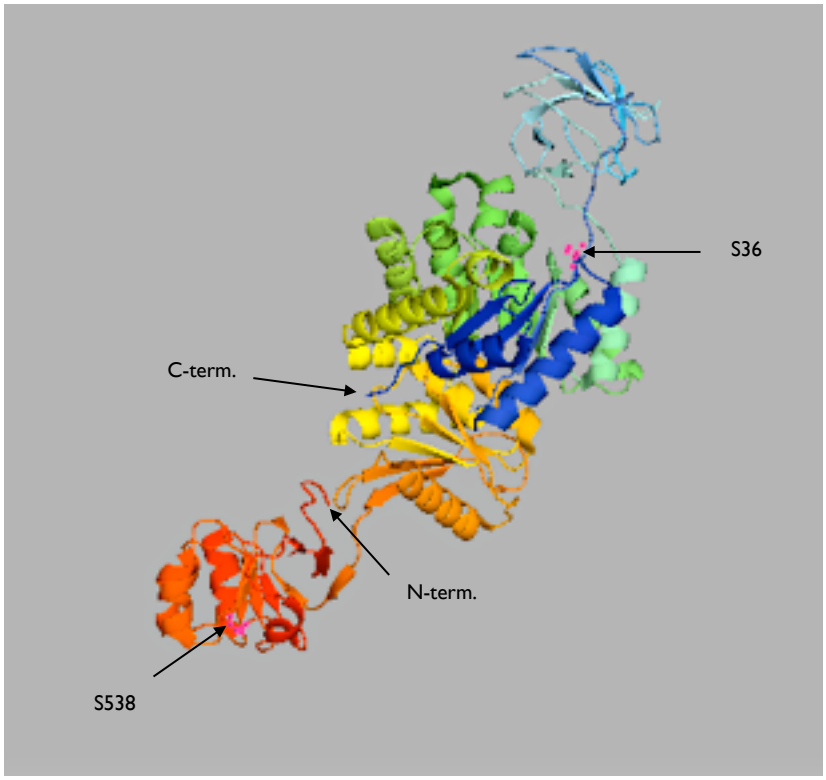

Figure 6.2.: Model of the pyruvate kinase from *B. subtilis* based on the Pyk structure from *Geobacillus. stearothermophilus*.

The overall structure of the enzyme. Following the nomenclature used by Suzuki et al. (2008) the following colors represent: cyan - domain A (catalytic domain), magenta - domain C (capped domain), green - domain C (containing the allosteric effector binding site) and red - domain C' (PEP-binding domain). The locations of the N-terminus, the C-terminus, Ser- 36 and S538 are indicated by arrows.

4.3. Autophosphorylation: The answer for kinase independent protein phosphorylation?

The identification of around 80 proteins that are phosphorylated on Ser/Thr/Tyr-residues in *B. subtilis* leads to the question of the biological origin of these modifications. Since the known kinases were not responsible for these phosphorylation events, other origins came into focus. Moreover, the question about the biological relevance of these phosphorylation is important. For several proteins, phosphorylation must be interpreted as snapshot of their catalytic activity. The results of this work revealed that several proteins in *B. subtilis* are autophosphorylated. Furthermore, autophosphorylation of proteins would explain, why certain phosphorylation events are conserved among the domains of life.

In this work mainly two groups of proteins were identified to perform autophosphorylation, GTP-binding proteins and phosphosugar mutases. Genome comparisons revealed that GTP-binding proteins are conserved in prokaryotes and eukaryotes. These proteins are involved in essential cellular processes working as molecular switches (Koonin, 1998). They bind and hydrolyse GTP resulting in a conformational change of the protein (Bourne *et al*., 1991). It was shown that Obg and YdiB are autophosphorylated in the presence of their substrates GTP and ATP respectively. Autophosphorylation was also identified for the GTP-binding protein Era in *E. coli* (Sood *et al*., 1994). However, the biological sense of these autophosphorylations is questionable and just seems to be a snapshot of the switched-on or switched-off configuration (Welsh *et al*., 1994). This underlines the hypothesis that phosphoproteomes partially display the short-term state of autophosphorylation during catalytic activity of proteins. The brief phosphorylation of proteins stands in contrast to autophosphorylations that are required for enzymatic activity. For example most protein kinases must be autophosphorylated to be active as protein kinase.

The phosphosugar mutases Pgm and GlmM were also identified as autophosphorylated. GlmM is an enzyme that is required for the interconversion of glucosamine-6-phosphate and glucosamine-1-phosphate, an essential reaction for peptidoglycan and

lipopolysaccharide biosynthesis. For the ortholog of GlmM in *E. coli* it was shown that the protein is only active in the phosphorylated form (Jolly *et al*., 1999; Jolly *et al*., 2000). However, it is not clear if ATP or another molecule is required for the initial autophosphorylation *in vivo*. Within this work it was also shown that the conserved Ser-100 residue of GlmM is required for the viability of *B. subtilis*. In *Streptococcus gordonii* and *Pseudomonas aeruginosa* mutants lacking GlmM were constructed. However, these mutants displayed impaired growth, elongated cells, reduced biofilm formation and sensitivity to lysozyme (Shimazu *et al*., 2008; Tavares *et al*., 2000).

Furthermore, autophosphorylation of Pgm was shown in this work. It is already known that Pgm forms a phosphoserine intermediate during the enzymatic reaction (Rigden *et al*., 2003). In addition it is known that the conserved Ser-62 is required for the enzymatic activity *in vitro*. The phosphorylation of this glycolytic enzyme is therefore, a further example for reaction dependent phosphorylation. Mutases are also conserved in other organisms to great extent. In consequence, the reaction mechanisms are conserved, too. Therefore, it was not unexpected that autophosphorylation of mutases was also found in other organisms, like in the archaeon *Sulfolobus solfataricus* and in mouse (Potter *et al*., 2003; Gururaj *et al*., 2004). Different studies indicated that also other glycolytic enzymes do perform autophosphorylation, like the mammalian glyceraldehyde-3-phosphate dehydrogenase (Kawamoto & Caswell, 1986).

However, autophosphorylation is also known for other classes of enzymes, like phosphatases and chaperones. Phosphatases also form phosphoserine intermediates as consequence of their activity (Jedrzejas & Setlow, 2001; Rigden *et al*., 2003). Heat shock proteins are required for folding of misfolded proteins. For the chaperones DnaK and GroEL of *E. coli*, it was described that autophosphorylation is a result of ATPase activity. The autophosphorylation of these chaperones is increased after temperature shift, as consequence of enhanced ATPase activity. Replacement of phosphorylated Thr residues results in the loss of activity, too (Żylicz *et a*l., 1983; McCarty and Walker, 1991). The same was shown for the heat shock protein Hsp70 of *Mycobacterium tuberculosis* (Preneta *et al*., 2004). Orthologs of these proteins are also phosphorylated

in *B. subtilis* and phosphorylation was found to be increased after heat shock exposure (Lévine *et al*., 2006; Eymann *et al*., 2007).

4.4. Outlook

The results of this study show, that the glycolytic genes are not essential in *B. subtilis*. The glycolytic mutants build a perfect basis for the further investigation of the central metabolism in this model organism. In addition, the possibility to delete glycolytic genes allows the modulation of the carbon flow in *B. subtilis*, and is in consequence interesting for biotechnological applications. Metabolome analysis will provide further insights into the metabolic characteristics of these mutants. Besides, these mutants can be used to investigate the function of multienzyme complexes and additional functions in the cell.

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6. Appendix

6.1. Materials

6.1.1. Chemicals

[α- ^{32}P]-ATP (SCP-207)	Hartmann Analytic, Braunschweig
[γ- ^{32}P]-ATP (SCP-501)	Hartmann Analytic, Braunschweig
[γ- ^{32}P]-GTP (SCP-402)	Hartmann Analytic, Braunschweig
Acrylamide	Roth, Karlsruhe
Adenosine 5′-triphosphate disodium salt solution	Sigma, Taufkirchen
Agar	Roth, Karlsruhe
Agarose	Peqlab, Erlangen
Ammonium iron (III) citrate	Sigma, Taufkirchen
Ammonium Peroxydisulfate	Roth, Karlsruhe
Antibiotics	Applichem, Darmstadt
Bromphenol blue	Roth, Karlsruhe
Coomassie Brilliant Blue, G250	Roth, Karlsruhe
Desthiobiotin	IBA, Göttingen
DL-Dithiothreitol	Sigma, Taufkirchen
dNTPs	Fermentas, Lithuania
Ethidium bromide	Roth, Karlsruhe
D-Fructose-1,6-bisphosphate	Sigma, Taufkirchen
D-Glucose	Roth, Karlsruhe
Glycerine	Merck, Darmstadt
Immidazole	Sigma, Taufkirchen
Isopropyl ß-D-1- thiogalactopyranoside	Sigma, Taufkirchen

DL-Malate	Applichem, Darmstadt
ß-Mercaptoethanol	Roth, Karlsruhe
NaF	Sigma, Taufkirchen
Ni^{2+}-nitrilotriacetic acid superflow	Qiagen, Hilden
Nutrient Broth	Roth, Karlsruhe
Na-Orthovanadate	Sigma, Taufkirchen
Pefabloc	Roth, Karlsruhe
Sodium Dodecyl Sulfate	Roth, Karlsruhe
Strep-Tactin Sepharose	IBA, Göttingen
Tetramethylethylenediamine (TEMED)	Roth, Karlsruhe
Tris(hydroxymethyl)aminomethane	Roth, Karlsruhe
Yeast extract	Oxoid, Hampshire, U.K.

Other chemicals were purchased from Merck, Serva, Sigma or Roth.

6.1.2. Auxiliary materia

Dialysis tube	Roth, Karlsruhe
Cuvettes (microlitre, plastic)	Greiner, Nürtingen
Gene amp reaction tubes	Applied Biosystems, USA
Glas pipette	Ochs, Bovenden
Greiner tubes	Greiner, Nürtingen
Microlitre pipettes (2 µl, 200 µl, 1000 µl)	Gilson, Düsseldorf
non-returnable syringe	Becton Dickinson, Heidelberg
Poly-Prep Chromatography columns	Bio-Rad, Munich
Centrifuge cups	Beckmann, Munich

6.1.3. Instrumentation

Steam autoclave	Zirbus technology, Bad Grund
Biofuge fresco	Heraeus Christ, Osterode
Contamination meter	Berthold, Bad Wildbad
Frensh pressure cell press	SLM Aminco, Lorch
Gel electrophoresis apparatus	PeqLab, Erlangen
Ice maschine	Ziegra, Isernhagen
Image eraser	Molecular Dynamics, USA
Horizontal shaker VXR basic	IKA, Staufen
Hydro tech vacuum pump	Bio-Rad, Munich
Mini-Protean III System	Bio-Rad, Munich
Orbital shaker G10	New Brunswick (Eppendorf), USA
Phosphor imagerStorm 860	Molecular Dynamics, USA
pH meter	Knick Calimatic, Berlin
Refrigerated centrifuge	Kendro, Hanau
Scale	Sartorius, Göttingen
Special accuracy weighing machine	Sartorius, Göttingen
Spectral photometer	Amersham, Feiburg
Standard power pack	Bio-Rad, Munich
Thermocycler	Biometra, Göttingen
Ultra centrifuge, Sorvall Ultra Pro 80	Thermo scientific, USA
Ultrasonic device	Dr. Hielscher, Teltow
UV Transilluminator 2000	Bio-Rad, Munich
Vortex	Bender & Hobein, Bruchsal
Water-bath incubation system	GFL, Burgwedel
Water desalination plant	Millepore, Schwalbach

6.1.4. Commercial systems / software

1kb DNA ladder	NEB Biolabs, Frankfurt / Main
DNeasy Tissue Kit (50)	Qiagen, Hilden
Nucleospin Plasmid	Machery-Nagel, Düren
Qiaquick PCR Purification Kit	Qiagen, Hilden

Internet programs and software

http://genolist.pasteur.fr/SubtiList/	Institute Pasteur, Paris	sequence analysis, *Bacillus subtilis*
http://genolist.pasteur.fr/Colibri/	Institute Pasteur, Paris	sequence analysis, *Escherichia coli*
http://www.ncbi.nlm.nih.gov/	Natinal Institutes of Health, Berthesa, USA	literature enquiry
http://tools.neb.com/NEBcutter2/	NEB, USA	restriction sites analysis, DNA
http://www.expasy.ch/	Swiss Institute of Bioinformatics	protein structures
http://www.ebi.ac.uk/Tools/clustalw2/index.html	European Bioinformatics Institute	creation of alignments
http://www.subtiwiki.uni-goettingen.de/	University of Göttingen	database for *Bacillus subtilis*
http://subtiwiki.uni-goettingen.de/subtipathways.html	University of Göttingen	database for metabolic pathways of *Bacillus subtilis*

iWorks	Apple, USA
Microsoft Office 2007	Windows

6.2. Oligonucleotides

Oligonuleotides constructed in this work

Name	Sequence 5`→3`[#]	Description
NP28	CTGCGGCATGACATTAATACACCTT TAACGGCTC	LFH-PCR (*prkD*), fwd, up-fragment
NP29	TATCGATACAACAATACTGGAAGCT GGTCAAGATGAA	LFH-PCR (*prkD*), rev, up-fragment
NP30	TATCTACCTTTACGTATTCGGCATCA GCTGTCGG	LFH-PCR (*prkD*), rev, down-fragment; sequencing
NP31	GTCAATTTGTTAATTGAACGGACG ACATATCGTGAACTG	LFH-PCR (*prkD*), fwd, up-fragment; sequencing
NP32	CCTATCACCTCAAATGGTTCGCTGC ACCGCTTCCATTCGCGAGGATCTA	LFH-PCR (*prkD*), rev, down-fragment
NP33	CGAGCGCCTACGAGGAATTTGTAT CGTTCTTTAGAGGGCGGTCAAATA GCA	LFH-PCR (*prkD*), fwd, down-fragment; sequencing
NP34	ATTGAGGACGATATGACTGTTGTTG TCGTCCGGAT	LFH-PCR (*yabT*), fwd, up-fragment
NP35	ATGACCCGCAGGAGATTGCCGATT TGCTTAT	LFH-PCR (*yabT*), fwd, up-fragment, sequencing
NP36	CTGTAAGAATTGCTCATCTTCCGTC AGTGCT	LFH-PCR (*yabT*), rev, down-fragment
NP37	TCCGCCCAATCGAGAAACTGCTGA ATGTG	LFH-PCR (*yabT*), rev, down-fragment, sequencing
NP38	CCTATCACCTCAAATGGTTCGCTGC ATGCCAAACTCGTCAAAGCGTCGT	LFH-PCR (*yabT*), rev, up-fragment
NP39	CGAGCGCCTACGAGGAATTTGTAT CGTGAATGGTGCAAACTGCAGAGC CTA	LFH-PCR (*yabT*), fwd, down-fragment
NP40	AAAGAATTCGTTTCTGTTGCCCATA TCGGAGACAG	*prkC-lacZ* fusion (*EcoR*I) fwd
NP41	AAAGGATCCATTCCCCCGCCGCCT ATGACGCG	*prkC-lacZ* fusion (*BamH*I) rev

Name	Sequence 5`→3`[#]	Description
NP42	AAAGAATTCGAAGACCGAATCAAA TCAGGCAGCGGAA	*prkD-lacZ* fusion (*EcoR*I) fwd
NP43	AAAGGATCCATACCGAGGCACTCT TCGATTTTGTATTG	*prkD-lacZ* fusion (*BamH*I) rev
NP44	AAAGAATTCGTGGAAATGGAAGAG CTTTCTCCGG	*yabT-lacZ* fusion (*EcoR*I) fwd
NP45	AAAGGATCCACATGTCCATCTGATG TTTCTGCCAAATACAC	*yabT-lacZ* fusion (*BamH*I) rev
NP46	AAAAGATCTGTGCTAATCGGCAAG CGGATCAGCG	cloning of *prkC* into pGP380 (*Bgl*II) fwd
NP47	TTTCTGCAGTC*ATTATT*CATCTTTCG GATACTCAATGGTTTTGTAGC	cloning of *prkC* into pGP380 (*Pst*I) rev
NP48	TTTCTGCAGTCATTACCACTTTTTT CTTTTGCCGTTCTTCTTTGT	cloning of *prkC*c (kinase domain) into pGP380 (*Pst*I) rev
NP49	AAAGGATCCATGGCATTAAAACTT CTAAAAAAACTGCTATTTGACCGC	cloning of *prkD* into pGP380 (*BamH*I) fwd
NP50	TTTCTGCAG*TTATCA*TGTGACCGAT TGAATGGCCCGGTT	cloning of *prkD* into pGP380 (*Pst*I) rev
NP51	AAAAGATCTATGATGAACGACGCT TTGACGAGTTTGGCATGTA	cloning of *yabT* into pGP380 (*Bgl*II) fwd
NP52	TTTCTGCAG*TTATCA*GATTAAGAAA AAGATAATATAGGCGAAATAAAGC	cloning of *yabT* into pGP380 (*Pst*I) rev
NP53	AAAGGATCCATGGTCAAGTCATTT CGTATGAAAGCTTTGATTG	cloning of *yxaL* into pGP380 (*Bam*HI) fwd
NP54	TTTCTGCAG*CTATCA*TTTCCCAAAA GCCATCAGCTTCGTA	cloning of *yxaL* into pGP380 (*Pst*I) rev
NP55	**P**-GGCAAAACAGTTAACCTTAAA**T** **GT**ATTATGGGTGTTATGTCTTTA GGT	mutagenesis of *ptsH* (S46C); 5‘-phosphate
NP56	GCGTTCATGGCCTCCACCCAGATCT CATC	LFH-PCR (*ymdB*), fwd, up-fragment
NP57	CGATGACTATATTCGTGAGATGGGT GAGCAAACGACA	LFH-PCR (*ymdB*), fwd, up-fragment, sequencing

Name	Sequence 5`→3`[#]	Description
NP58	CCTATCACCTCAAATGGTTCGCTGG CCCGGTGAACCGACAACATCTCCG	LFH-PCR (*ymdB*), rev, up-fragment
NP59	CCGAGCGCCTACGAGGAATTTGTA TCGGACATTGACGATCAAACGAAA AAAG	LFH-PCR (*ymdB*), fwd, down-fragment
NP60	GCAGACACATACTCTCCCACTTTTA CACTGCTGACAT	LFH-PCR (*ymdB*), rev, down-fragment
NP61	AGTATTGGTACACACATGAGATTTT CCTGTTAG	LFH-PCR (*ymdB*), rev, down-fragment sequencing
NP62	CAGCGAACCATTTGAGGTGATAGG ATAGCTAGGGTAAGTAAATTGAGTA	Spc-cassette, without terminator from pGP961 (spec-fwd-kan)
NP63	CGATACAAATTCCTCGTAGGCGCTC GGCTACTAATTGAGAGAAGTTTCTA TAGA	Spc-cassette, without terminator from pGP961 (spec-rev-kan)
NP64	AGCAGGAAGCCATCCGTTATTTCA GCAATTTGCGG	LFH-PCR (*pnpA*), fwd, up-fragment
NP65	GAATGGTACAAACGGATTCGGAGC GAGCGGAA	LFH-PCR (*pnpA*), fwd, up-fragment, sequencing
NP66	CCTATCACCTCAAATGGTTCGCTGT AAAGACATGTTTTTCTTGTCCCAT	LFH-PCR (*pnpA*), rev, up-fragment
NP67	CCGAGCGCCTACGAGGAATTTGTA TCGGATAAACAAGGACGAGTGAAT TTAT	LFH-PCR (*pnpA*), fwd, down-fragment
NP68	GTTCATTAGTCTTGTCGAGCTGCTC GGAAATTCTTCC	LFH-PCR (*pnpA*), rev, down-fragment
NP69	GAGCGAACCACTTTGGCTTAACGC CGATCG	LFH-PCR (*pnpA*), rev, down-fragment, sequencing
NP70	AAAGGATCCATGACGAACGAAAGC TTTAAATCAGGATTTGTATCCATT	cloning of *era* into pGP380 (*Bam*HI) fwd
NP71	TTTCTGCAG*TCATTA*ATATTCGTCCT CTTTAAAGCCAAAATCGCG	cloning of *era* into pGP380 (*Pst*I) rev
NP72	AAAGGATCCATGTTTGTAGATCAG GTCAAAGTATATGTAAAAGGC	cloning of *obg* into pGP380 (*Bam*HI) fwd

Name	Sequence 5`→3`[#]	Description
NP73	TTTCTGCAG*TCATTA*ATCAATAAATT CAAATTCAAATTCCAGAAGCCTG	cloning of *obg* into pGP380 (*Pst*I) rev
NP74	AAAGGATCCGTGAAGCAATTAAAA TGGAGAACTGTAAATCCAGAAGA	cloning of *ydiB* into pGP380 (*Bam*HI) fwd
NP75	TTTCTGCAG*TCATTA*ATTGCTAATAT TGTCATGTCTACTTAACTCCTCAC	cloning of *ydiB* into pGP380 (*Pst*I) rev
NP76	AAATCTAGAATGAAAGTCACAAAG TCAGAAATCGTGATCAGTG	cloning of *ysxC* into pGP380 (*Xba*I) fwd
NP77	TTTCTGCAG*TCATTA*CCGGTTTATCA TTTTTTTGATCGCTCCC	cloning of *ysxC* into pGP380 (*Pst*I) rev
NP78	AAAAGATCTATGGGTAAACCTGTC GTAGCCATTGTCGG	cloning of *yphC* into pGP380 (*Bgl*II) fwd
NP79	TTTGTCGAC*TCATTA*TTTTCTAGCTC TTGCAAATATTTTGATTGGTGTCC	cloning of *yphC* into pGP380 (*Sal*I) rev
NP80	AAAGGATCCATGGCTTTAACAGCT GGAATTGTTGGTTTGC	cloning of *yyaF* into pGP380 (*Bam*HI) fwd
NP81	TTTGTCGAC*TCATTA*TACATTAAATC GGAAATGAATAACATCTCCGT	cloning of *yyaF* into pGP380 (*Sal*I) rev
NP82	AAAGGATCCATGGCAAGAGAGTTC TCCTTAGAAAAAACTCGTAATAT	cloning of *fusA* into pGP380 (*Bam*HI) fwd
NP83	TTTCTGCAG*TCATTA*TTCGCCTTTAT TTTTTTTGATAATTTCTTCTGC	cloning of *fusA* into pGP380 (*Pst*I) rev
NP84	AAAGGATCCATGGCTAAAGAAAAA TTCGACCGTTCCAAATCACATG	cloning of *tufA* into pGP380 (*Bam*HI) fwd
NP85	TTTCTGCAG*TCATTA*CTCAGTGATT GTAGAAACAACGCCTGAAC	cloning of *tufA* into pGP380 (*Pst*I) rev
NP86	CATTGACTCAAGGATAACAAGATC CTTCTC	sequencing of *fusA*, check rev
NP87	GCGGCTGCTGTAGGTCTTAAAGAT ACA	sequencing of *fusA*, check fwd
NP88	AAAGGATCCATGAAAGTCACAAAG TCAGAAATCGTGA	cloning of *ysxC* into pWH844 (*Bam*HI) fwd

Name	Sequence 5`→3`[#]	Description
NP89	AAAGGATCCATGACGCATGTACGC TTGACTACTC	cloning of *pgi* into pWH844 (*Bam*HI) fwd
NP90	TTTCTGCAG*TCATTA*ATCTTCCAGA CGTTTTTCAAGCTC	cloning of *pgi* into pWH844 (*Pst*I) rev
NP91	TTTCTGCAG*TCATTA*GATAGACAGT TCTTTTGAAAGCTGATACATGTTTT G	cloning of *pfkA* into pWH844 (*Bam*HI) rev
NP92	TTTGTGCAC*TCATTA*CTCATATTGAC CTTCCTCCAATAATTGAACGAATG	cloning of *tpiA* into pWH844 (*Sdu*I ⇔*Bsp*1286I) rev
NP93	AAACTGCAGTCATTAAGCTTGGTTT GAAGAACCAAATTCACGC	cloning of *fbaA* into pWH844 (*Pst*I) fwd
NP94	AAAGGATCCATGGGACAAGAAAA ACATGTCTTTACCAT	cloning of *pnpA* into pWH844 (*Bam*HI) fwd
NP95	TTTCTGCAG*TCATTA*AGATTGTTGTT CTTCTTTTTCTTTC	cloning of *pnpA* into pWH844 (*Pst*I)
NP96	AAAGGTACCGATGAAACGAATAGG GGTATTAACGAGCGG	cloning of *fbaA* into pGP172 (*Kpn*I) fwd
NP97	TTTGGATCC*TCATTA*GATAGACAGTT CTTTTGAAAGCTGATACATGTTTTG	cloning of *fbaA* into pGP172 (*Bam*HI) rev
NP98	**P**-GAACGTGGCTCGATTAAACTTT**G** **CT**CACGGAGATTTTGAGGAGCA CGG	mutagenesis of *pyk* (S36A)
NP99	**P**- CCTGAAGGGCAAATGGGGAAC **GCC**GAAGTAGGTCACTTAAATA TC	mutagenesis of *pgm* (S36A)
NP100	GACCGTGTAGAAAAAGCGTACCGC GCAATG	LFH-PCR (*eno*), fwd, up-fragment
NP101	ACGGAGGCGTGCACAGCCATATCA ATCATT	LFH-PCR (*eno*), fwd, up-fragment, sequencing
NP102	CCTATCACCTCAAATGGTTCGCTGT ACTTCAACTTCAACTGTTGGGTTG	LFH-PCR (*eno*), rev, up-fragment
NP103	CCGAGCGCCTACGAGGAATTTGTA TCGTTCTTCGCATCGAAGATCAGTT GGC	LFH-PCR (*eno*), fwd, down-fragment

Name	Sequence 5`→3`[#]	Description
NP104	ACCGGTGTAAGCAGAGTCGCGATT TGGTCC	LFH-PCR (*eno*), rev, down-fragment
NP105	TAGATCGGTATTTTTATAATTGCTGC TCACTT	LFH-PCR (*eno*), rev, down-fragment, sequencing
NP106	AAAGGATCCCTCACTTATTTAA***AGG AGG***AAACAATCATGTCCAAAATCG TAAAAATCATCGGTCGT	cloning of *eno* (*E. coli*) into pBQ200 (RBS_{gapA}) (*Bam*HI) fwd
NP107	TTTGTCGAC*TGATTA*TGCCTGGCCT TTGATCTCTTTACG	cloning of *eno* (*E.coli*) into pBQ200 (*Sal*I) rev
NP108	AAAGGATCCCTCACTTATTTAA***AGG AGG***AAACAATCATGGGCAAGTATT TTGGAACAGACG	cloning of *glmM* into pBQ200 (RBS_{gapA}) (*Bam*HI) fwd; Schmidl *et al*., 2010
NP109	TTTGTCGAC*CTATCA*CTCTAATCCCA TTTCTGACCGG	cloning of *glmM* into pWH844/ pBQ200 (*Sal*I) rev; Schmidl *et al*., 2010
NP110	GGCTATTCTCCGGAGCAGCCGATT GTCA	LFH-PCR (*glmM*), fwd, up-fragment; Schmidl *et al*., 2010
NP111	CAGGAACGGACGACCAAAAGTTTT CCCG	LFH-PCR (*glmM*), fwd, up-fragment, sequencing; Schmidl *et al*., 2010
NP112	CCTATCACCTCAAATGGTTCGCTGT AAAGGCCAGCTCAGGTGTAAGCTC	LFH-PCR (*glmM*), rev, up-fragment; Schmidl *et al*., 2010
NP113	CCGAGCGCCTACGAGGAATTTGTA TCGAGCGAAGACGAAAGAGCTGT GCGAT	LFH-PCR (*glmM*), fwd, down- fragment; Schmidl *et al*., 2010
NP114	CATCGCCATCGCATCAGATGCTACG ACGT	LFH-PCR (*glmM*), rev, down-fragment; Schmidl *et al*., 2010
NP115	TATAAGACGCACGTGTAATCACGTC ACCATC	LFH-PCR (*glmM*), rev, down – fragment, sequencing; Schmidl *et al*., 2010
NP116	**P**-GCAGAGGCGGGCGTCATGATTTC CGCT**GCC**CATAACCCAGTGCAG GATAACGGCATCAA	mutagenesis of *glmM*(S100A); Schmidl *et al*., 2010
NP117	AAAGGATCCATGGGCAAGTATTTT GGAACAGACG	cloning of *glmM* into pWH844 (*Bam*HI) fwd; Schmidl *et al*., 2010

Name	Sequence 5`→3`[#]	Description
NP118	**P**-GATATTTAAGTGACCTACTT**CGG** CGTTCCCCATTTGCCCTTCAGG	mutagenesis of *pgm* (S62A) rev
NP119	TTTCTGCAGAAGAACGCTCGCACG GCCTTGATAGAC	cloning of *pyk* into pGP382 rev (*Pst*I) (without stop-codon)
NP120	AAAGGATCCCTCACTTATTTAAAGG AGGAAACAATCATGAGAAAAACTA AAATTGTT	cloning of *pyk* into pBQ200/ pGP382 (*Bam*HI) fwd
NP121	GGAGTAGACTTCATCGCACCATCTT TC	sequencing of *pyk* (from 600b) fwd
NP122	GGTAAGGTTTCATAGGGAGGATGG AGATCC	LFH-PCR (*pyk*), fwd, up-fragment, sequencing
NP123	GGAATGAACGCAGCAGTTCGCGCA GTAG	LFH-PCR (*pyk*), fwd, up-fragment
NP124	CCTATCACCTCAAATGGTTCGCTGG TACAAACAATTTTAGTTTTTCTCAT TTGGTTCAC	LFH-PCR (*pyk*), rev, up-fragment
NP125	CCGAGCGCCTACGAGGAATTTGTA TCGGCGTCTGCTCTTATTACAGAAG AAGG	LFH-PCR (*pyk*), fwd, down-fragment
NP126	CTCTTAAAGGTTGAACACGCCGCA ACAATTGC	LFH-PCR (*pyk*), rev, down-fragment
NP127	GCTGTTAGCTGTCACACGATCCGG	LFH-PCR (*pyk*), rev, down-fragment, sequencing
NP128	AAAGGATCCCTCACTTATTTAA***AGG AGG***AAACAATCATATGGCACAAAA AACATTTAAAGTAACT	cloning of *ptsH* into pBQ200 (RBS_{gapA}) (*Bam*HI) fwd
NP129	**P**-ATTACAGAAGAAGGCGGTTTGA CT**GCC**CATGCTGCGGTAGTCGG ATTA	mutagenesis of *pyk* (S538A)
NP130	**P**-GAAGAAGGCGGTTTGACTAGC **GCT**GCTGCGGTAGTCGGATTA	mutagenesis of *pyk*(H539A)
NP131	**P**-GCTGCGGTAGTCGGATTA**GCC**C TTGGCATCCCGGTTATCGT	mutagenesis of *pyk* (S546A)
NP132	GTACTGGCGAATTTGTTTTAATGTG TTATAC	sequencing of *gapB* fwd (with NP168, rev)

Name	Sequence 5`→3`[#]	Description
NP133	TTTAAGCTT*TCATTA*CTCGCCGAGT CCTTCGCT	cloning of *ptsH* into pBQ200 (*Hind*III) rev
NP134	CCTATCACCTCAAATGGTTCGCTGC CGTTAATACCGACTTTTACTGCCAT	LFH-PCR (*gapA*), rev, up-fragment
NP135	GCGAGCGTGTGCTGAGGGGCGAG	LFH-PCR (*gapA*), fwd, up-fragment
NP136	CAGGCTGACAGAACCCATCGGGCG	LFH-PCR (*gapA*), fwd, up-fragment, sequencing
NP137	CCGAGCGCCTACGAGGAATTTGTA TCGCTCTTGGTACGATAACGAAAG CGGC	LFH-PCR (*gapA*), fwd, down-fragment
NP138	ACGAAAGTATAAGCAAGACCTCCG CCGATG	LFH-PCR (*gapA*), rev, down-fragment, sequencing
NP139	AAAGGATCCCTCACTTATTTAA***AGG AGG***AAACAATCATGGCAGTAAAAG TCGGTATTAACG	cloning of *gapA* into pBQ200 (RBS_{gapA}) (*Bam*HI) fwd
NP140	AAAGGATCCCTCACTTATTTAA***AGG AGG***AAACAATCATGCCTTTAGTTTC TATGACGGAAATGTTGAATAC	cloning of *fbaA* into pBQ200 (RBS_{gapA}) (*Bam*HI) fwd
NP141	CTGTGCCCGTCCCTGAGCAAAACA TCAG	LFH-PCR (*fbaA*), fwd, up-fragment, sequencing
NP142	GACAGATGGGAAGGGTTCTTTGCT TTCGG	LFH-PCR (*fbaA*), fwd, up-fragment
NP143	CCTATCACCTCAAATGGTTCGCTGC ATTTCCGTCATAGAAACTAAAGGC AT	LFH-PCR (*fbaA*), rev, up-fragment
NP144	CCGAGCGCCTACGAGGAATTTGTA TCGCCTTGGACCAGCTCGTGAAGC GATC	LFH-PCR (*fbaA*), fwd, down-fragment
NP145	CGGCTCGAGAGACATCGTCGTCAT CCGGC	LFH-PCR (*fbaA*), rev, down-fragment, sequencing
NP146	CAATGATTCCTTCTCTGTAAAGCGG CGTCAG	LFH-PCR (*fbaA*), rev, down-fragment
NP147	CCGAGCGCCTACGAGGAATTTGTA TCGCTTGCACCAACGTTATTAGACC TTC	LFH-PCR (*pgm*), fwd, down-fragment

Name	Sequence 5`→3`[#]	Description
NP148	CCAAGACGCTCAGTAAGAAGCTTG TGGCC	LFH-PCR (*pgm*), rev, down-fragment, sequencing
NP149	GGGTATTTAGAAACAAGCTCTTCAT ACCAGTC	LFH-PCR (*pgm*), rev, down-fragment
NP150	GGAAACCGCTTTTCCAAGTACATC AAGCTC	LFH-PCR (*gapA*), rev, down-fragment
NP151	GAAGCCGTGGGACCGGGTTCAAAT CGC	LFH-PCR (*pfkA*), fwd, up-fragment, sequencing
NP152	AATGTCACGGAGACAGAGCGTACG GGGA	LFH-PCR (*pfkA*), fwd, up-fragment
NP153	CCTATCACCTCAAATGGTTCGCTGC CGCTCGTTAATACCCCTATTCGTTT CAT	LFH-PCR (*pfkA*), rev, up-fragment
NP154	CCGAGCGCCTACGAGGAATTTGTA TCGGCTTGTAGACCATGATATTATA GAAATACTTG	LFH-PCR (*pfkA*), fwd, down-fragment
NP155	GCTTCCGCACGAGTCGGACGCGGG	LFH-PCR (*pfkA*), rev, down-fragment
NP156	CGGGTAACTTCCGGCAGCAGTTTC ACCAG	LFH-PCR (*pfkA*), rev, down-fragment, sequencing
NP157	AAAGGATCCCTCACTTATTTAA***AGG AGG***AAACAATCATGAAACGAATAG GGGTATTAACGAGCGG	cloning of *pfkA* into pBQ200 (RBS_{gapA}) (*Bam*HI) fwd
NP158	AAAGGATCCCTCACTTATTTAA***AGG AGG***AAACAATCATGCCATACATTGT TGATGTTTATGCACGCGAAG	cloning of *eno* into pBQ200 (RBS_{gapA}) (*Bam*HI) fwd
NP159	GCGGTTGCTGTGTGATCGG	sequencing of *ccpN,* fwd
NP160	GCCGTTTCACCGACGGAATCCG	sequencing of *ccpN,* rev
NP161	ATTATAATACAAACTTCACAAAGAT GCTA	sequencing of *yqfL,* rev
NP162	GCGGTTGCTGCCATCGTATTACCGG CA	LFH-PCR (*gapB*), fwd, up-fragment, sequencing
NP163	GAGGTCAATGAAGTTAATATAGGTG CCCCAAATG	LFH-PCR (*gapB*), fwd, up-fragment
NP164	CCTATCACCTCAAATGGTTCGCTGC GTTGATCGCTACTTTTACCTTCAT	LFH-PCR (*gapB*), rev,up-fragment

Name	Sequence 5`→3`[#]	Description
NP165	CCGAGCGCCTACGAGGAATTTGTATCGGAATGGGGCTACTCCTGCAGAGTTG	LFH-PCR (*gapB*), fwd, down-fragment
NP166	AATCGACTTTCCGTCATCCACAGGCCG	LFH-PCR (*gapB*), rev, down-fragment
NP167	CACAACGATGAGCGGGGTTTCCTCCAC	LFH-PCR (*gapB*), rev, down-fragment sequencing
NP168	CTGTGTTTAAGTCTAATTTGCAAGGTGCG	sequencing of *gapB* rev (with NP132 rev)
cat-fwd (kan)	CAGCGAACCATTTGAGGTGATAGGCGGCAATAGTTACCCTTATTATCAAG	construction of mutants by LFH-PCR
cat-rev (kan)	CGATACAAATTCCTCGTAGGCGCTCGGCCAGCGTGGACCGGCGAGGCTAGTTACCC	construction of mutants by LFH-PCR
cat-check fwd	CCTTCTACCCATTATTACAGCAGG	sequencing of mutants; LFH-PCR
cat-check rev	GTCTGCTTTCTTCATTAGAATCAATCC	sequencing of mutants; LFH-PCR
mls-fwd (kan)	CAGCGAACCATTTGAGGTGATAGGGATCCTTTAACTCTGGCAACCCTC	construction of mutants by LFH-PCR
mls-rev (kan)	CGATACAAATTCCTCGTAGGCGCTCGGGCCGACTGCGCAAAAGACATAATCG	construction of mutants by LFH-PCR
mls-check rev	GTTTTGGTCGTAGAGCACACGG	sequencing of mutants; LFH-PCR
mls-check fwd	CCTTAAAACATGCAGGAATTGACG	sequencing of mutants; LFH-PCR
spec-fwd (kan)	CAGCGAACCATTTGAGGTGATAGGGACTGGCTCGCTAATAACGTAACGTGACTGGCAAGAG	construction of mutants by LFH-PCR

Name	Sequence 5`→3`#	Description
spec-rev (kan)	CGATACAAATTCCTCGTAGGCGCTCGGCGTAGCGAGGGCAAGGGTTTATTGTTTTCTAAAATCTG	construction of mutants by LFH-PCR
spec check fwd	GTTATCTTGGAGAGAATATTGAATGGAC	sequencing of mutants; LFH-PCR
spec check rev	CGTATGTATTCAAATATATCCTCCTCAC	sequencing of mutants; LFH-PCR

restriction sites are underlined, introduced mutations are bold, promoters are italic,

P indicates 5'-phosphorylation;

stop-codons are italic and underlined

LFH-PCR: long flanking homology PCR

Oligonucleotides used in this work

Name	Sequence 5`→3`	Reference
EW1	AAAGGATCCATGAAACGAATAGGGGTATTAACGAGCGG	Commichau *et al*., 2009
EW2	TTTGTCGAC*TCATTA*GATAGACAGTTCTTTTGAAAGCTGAT	Commichau *et al*., 2009
EW3	AAAGGATCCATGCCTTTAGTTTCTATGACGGAAATGTTGAATAC	Commichau *et a*l., 2009
EW4	TTTGTCGAC*TCATTA*AGCTTGGTTTGAAGAACCAAATTCACGC	Commichau *et a*l., 2009
EW5	AAAGGATCCATGAGAAAACCAATTATCGCCGGTAACTGG	Commichau *et a*l., 2009
EW8	TTTGTCGAC*TCATTA*AAGACCTTTTTTTGCGATGTAAGCTGCAAGGTC	Commichau *et a*l., 2009
EW9	AAAGGATCCATGAATAAAAAAACTCTCAAAGACATCGACGTAAAAGGC	Commichau *et a*l., 2009
EW12	TTTGTCGAC*TCATTA*TTTTTGAATTAAAGATGTTCCTGTCATTTC	Commichau *et a*l., 2009
EW14	TTTGTCGAC*TCATTA*CTTGTTTAAGTTGTAGAAAGAGTTGATACCGTGG	Commichau *et a*l., 2009
MA4	AAAGGATCCCTCACTTATTTAA***AGGAGG***AAACAATCATGAGTAAAAAACCAGCTGCACTC	Arnold, 2009

6.3. Plasmids

Plasmids constructed in this work

Name	Construction	Reference
pGP390	pGP380 + *prkD* gene (NP49-NP50) via *Bam*HI/*Pst*I	-
pGP391	pGP380 (*BamH*I / *Pst*I) + *yabT* (NP51/NP52) via *Bgl*II/*Pst*I	-
pGP392	pGP380 + *yxaL* (NP53/NP54) via *Bam*HI / *Pst*I	-
pGP393	pWH844 + *pfkA* (EW1-NP91) via *Bam*HI / *Pst*I	-
pGP394	pWH844 (*BamH*I / *Pst*I) + *tpiA* (EW5/NP92) via *Bam*HI/*Sdu*I	-
pGP395	pWH844 + *fbaA* (EW3/NP93) via *Bam*HI/*Pst*I	-
pGP396	pWH844 + *pgm* (S62A), (pgmfwd/pgmrev M:NP99) via *BamH*I/*Sal*I	-
pGP397	pWH844 + *pyk* (S36A) (pykfwd/pykrev/M:NP98) via *Bam*HI/*Pst*I	-
pGP398	pWH844 + *pgi* (NP89/NP90) via *Bam*HI/*Pst*I	-
pGP399	pBQ200 + *eno* (*E. coli*); (NP106/NP107) via *Bam*HI/*Sal*I	-
pGP400	pBQ200 + *glmM* (NP108/NP109) via *Bam*HI/*Sal*I	Schmidl *et al*., 2010
pGP826	pWH844 + *ptsH* (NP27/SH83) via *Bam*HI/*Hind*III (HPr-S12A)	-
pGP827	pWH844 + *ptsH1* (NP27/SH83) via *Bam*HI / *Hind*III (from pGP371) (HPr-S12A/S46A)	-
pGP828	pGP184 Cm^R(*EcoR*I)→ pGP821 (*prkD*) (*Mfe*I)	-
pGP829	pAC7 + *prkC-lacZ* fusion (NP40/NP41) via *EcoR*I/*Bam*HI	-
pGP830	pAC7 + *prkD-lacZ* fusion (NP42/NP43) via *EcoR*I/*Bam*HI	-
pGP831	pAC7 + *yabT-lacZ* fusion (NP44/NP45) via *EcoR*I/*Bam*HI	-
pGP832	pGP380 + *prkC* (NP46/NP47) via *Bgl*II/*Pst*I	-
pGP833	pGP380 + *ydiB* (NP74/NP75) via *Bam*HI/*Pst*I	-
pGP834	pGP380 + *ysxC* (NP76/NP77) via *Xba*I/*Pst*I	-

Name	Construction	Reference
pGP835	pGP380 + *obg* (NP72/NP73) via *Bam*HI/*Pst*I	-
pGP836	pGP380 + *yyaF* (NP80/NP81) via *Bam*HI/*Sal*I	-
pGP837	pGP380 + *era* (NP70/NP71)via *Bam*HI/*Pst*I	-
pGP838	pWH844 + *pnpA* (NP94/NP95) via *Bam*HI/*Pst*I	-
pGP839	pGP380 + *tufA* (NP84/NP85) via *Bam*HI/*Pst*I	-
pGP840	pGP380 + *fusA* (NP82/NP83) via *Bam*HI/*Pst*I	-
pGP841	pWH844 + *ydiB* (gel extraction, pGP833) via *Bam*HI/*Pst*I	-
pGP842	pWH844 + *ysxC* (NP88/NP77) via *Bam*HI/*Pst*I	-
pGP843	pWH844 + *obg* (gel extraction, pGP835) via *Bam*HI/*Pst*I	-
pGP844	pWH844 + *yyaF* (gel extraction, pGP836) via *Bam*HI/*Sal*I	-
pGP845	pWH844 + *era* (gel extraction, pGP837) via *Bam*HI/*Pst*I	-
pGP846	pWH844 + *yphC* (NP78/NP79) via *Bam*HI/*Sal*I	-
pGP847	pWH844 + *tufA* (gel extractionGP839) via *Bam*HI/*Pst*I	-
pGP848	pWH844 + *fusA* (gel extraction, pGP840) via *Bam*HI/*Pst*I	-
pGP849	pGP380 + *prkC*-kinase domain, (NP46/NP48) via *Bgl*II/*Pst*I	-
pGP1401	pWH844 + *glmM* (NP117/NP109) via *Bam*HI/*Sal*I	Schmidl *et al*., 2010
pGP1402	pBlueskript KS (+) + *glmM* (GlmM/S100A) (NP108/NP109 /M:NP116) via *Bam*HI/*Sal*I	-
pGP1403	pBQ200 + *glmM* (GlmM/S100A) (gel extraction, pGP1402) via *Bam*HI/*Sal*I	Schmidl *et al*., 2010
pGP1404	pBlueskript KS (+) + *glmM* (GlmM/S100A) (NP117/NP109/M:NP116) via *Bam*HI/*Sal*I	-
pGP1405	pWH844 + *glmM* (GlmM/S100A) (gel extraction, pGP1404 via *Bam*HI/*Sal*I	Schmidl *et al*., 2010
pGP1406	pWH844 + *prkC*-kinase domain (NP46/NP48) via *Bgl*II/*Pst*I	-

Name	Construction	Reference
pGP1407	pWH844 + *prkD* (NP49/NP50) via *Bam*HI/*Pst*I	-
pGP1408	pWH844 + *yabT* (NP51/NP52) via *Bgl*II/*Pst*I	-
pGP1409	pGP380 + *pyk* (pykfwd/pykrev) via *Bam*HI/*PstI*	-
pGP1410	pGP382 + *pyk* (NP119/NP120) via *Bam*HI/*Pst*I	-
pGP1411	pBQ200 + *pyk* (NP120/pykrev) via *Bam*HI/*PstI*	-
pGP1412	pGP380 + *pyk* (Pyk/S36A) (pykfwd/pykrev/ (M:NP98)) via *Bam*HI/*Sal*I	-
pGP1413	pGP382 + *pyk* (Pyk/S36A) (NP119/NP120/(M:NP98)) via *Bam*HI/*Pst*I;	-
pGP1414	pBQ200 + *pyk* (Pyk/S36A) (NP120/pykrev(M:NP98)) via *Bam*HI/*Pst*I	-
pGP1415	pBQ200 + *ptsH* (NP128/NP133) via *Bam*HI/*Hind*III	-
pGP1416	pBQ200 + *ptsH* (HPr/H15A) (NP128/NP133 from QB5250) via *Bam*HI/*Hind*III	-
pGP1417	pBQ200 + *ptsH* (NP128/NP133 from pGP826) via *Bam*HI/*Hind*III	-
pGP1418	pWH844 + *tpiA* (Tpi/S213A) (*Bam*HI/*Sal*I) (EW5/NP92 from pGP1512) via *Bam*HI/*Sdu*I	-
pGP1419	pWH844 + *pgk* (Pgk/S183A) (EW9/EW10 from pG1514) via *Bam*HI/*Sal*I	-
pGP1420	pWH844 + *pgk* (Pgk/T299A) (EW9/EW10 from pG1515) via *Bam*HI/*Sal*I	-
pGP1421	pWH844 + *pgk* (Pgk/S183A / T299A) (EW9/EW10 from pGP1516) *Bam*HI/*Sal*I	-
pGP1422	pBQ200 +. *pfk* (NP157/EW2) via *Bam*HI/*Sal*I	-
pGP1423	pBQ200 + *fbaA* (NP140/EW4) via *Bam*HI/*Sal*I	-
pGP1424	pBQ200 + *gapA* (NP139/EW8) via *Bam*HI/*Sal*I	-
pGP1425	pBQ200 + *pgm* (MA4/EW12) via *Bam*HI/*Sal*I	-
pGP1426	pBQ200 + *eno* (NP158/EW14) via *Bam*HI/*Sal*I	-
pGP1427[#]	pWH844 + *pyk* (Pyk/S538A) (M13 puc fwd/M13 puc rev/ (M:NP129) from pGP1410) via *Bam*HI/*Pst*I	-

Name	Construction	Reference
pGP1428 #	pWH844 + *pyk* (Pyk/H539A) (M13 puc fwd/M13 puc rev/ (M:NP130) from pGP1410) via *Bam*HI/*Pst*I	-
pGP1429 #	pWH844 + *pyk* (Pyk/S546A) (M13 puc fwd/M13 puc rev/(M:NP131) from pGP1410) via *Bam*HI/*Pst*I	-
pGP1430 #	pWH844 + *pyk* (Pyk/S36A/S538A) (M13 puc fwd/M13 puc rev/ (M:NP129) from pGP1430) via *Bam*HI/*Pst*I	-
pGP1431 #	pBQ200 + *pyk* (Pyk/S36A/ H539A) (M13 puc fwd/M13 puc rev/ (M:NP130) from pGP1413) via *Bam*HI/*Pst*I	-
pGP1432	pWH844 +*pyk* (Pyk/S538A) (pykfwd/pykrev from pGP1427) via *Bam*HI/*Pst*I	-
pGP1433	pWH844 + *pyk* (Pyk/H539A) (pykfwd/pykrev from pGP1428) via *Bam*HI/*Pst*I (mutation T266C, valine→alanine	-
pGP1434	pWH844 + *pyk* (Pyk/S546A) (pykfwd/pykrev from pGP1429) via *Bam*HI/*Pst*I	-
pGP1435	pWH844 + *pyk* (Pyk/S36A/S538A) (pykfwd/pykrev from pGP1428) via *Bam*HI/*Pst*I	-
pGP1436	pWH844 + *pyk* (Pyk/S36A/H539A) (pykfwd/pykrev from pGP1431) via *Bam*HI/*Pst*I	-
pGP1437	pWH844 + *pyk* (Pyk/S36A/S546A) (pykfwd/pykrev from pGP1430) via *Bam*HI/*Pst*I	-

not suitable for expression.

Plasmids used in this work

Plasmid	Resistance	Description	Referenz
pAC7	ampicillin/ chloram-phenicol	construction of LacZ-fusion	Weinrauch *et al*., 1991
pAG2	ampicillin	overexpression of His_6-HPr in *E. coli*	Galinier *et al.*, 1997
pBlue-skript KS (+)	ampicillin	cloning	Stratagene, USA
pDG646	ampicillin	PCR template for *ery*-cassette	Guérout-Fleury *et al*., 1995

Plasmid	Resistance	Description	Referenz
pDG780	ampicillin	PCR template for *kan*-cassette	Guérout-Fleury *et al*., 1995
pDG1726	ampicillin	PCR template for *spec*-cassette	Guérout-Fleury *et al*., 1995
pGEM-cat	ampicillin	PCR template for *cat*-cassette	Guérout-Fleury *et al*., 1995
pBQ200	ampicillin/ erythromycin	expression in *B. subtilis*	Martin-Verstraete *et al*., 1994
pGP371	ampicillin	*ptsH1* in pWH844	Halbedel, 2006
pGP380	ampicillin/ erythromycin	expression in *B. subtilis* with N-terminal Strep-tag	Herzberg *et al*., 2007
pGP382	ampicillin/ erythromycin	expression in *B. subtilis* with N-terminal Strep-tag	Herzberg *et al*., 2007
pGP821	ampicillin	construction of *prkD* mutant	Pietack, 2007
pGP826	ampicillin	*ptsH* (HPr S12A) in pWH844 (expression with N-terminal His_6-tag in *E. coli*)	Pietack, 2007
pGP1100	ampicillin	*pyk* in pWH844 (expression with N-terminal His_6-tag in *E. coli*)	Schilling, 2007
pGP1101	ampicillin	*pgm* in pWH844 (expression with N-terminal His_6-tag in *E. coli*)	Schilling, 2007
pGP1102	ampicillin	*pgk* in pWH844 (expression with N-terminal His_6-tag in *E. coli*)	Schilling, 2007
pGP1500	ampicillin/ erythromycin	*pgm/eno* in pBQ200 (expression in *B. subtilis*)	Arnold, 2009
pGP1501	ampicillin/ erythromycin	*pgm* (S62A)*/eno* in pBQ200 (expression in *B. subtilis*)	Arnold, 2009
pGP1511	ampicillin/ erythromycin	*tpi*A in pBQ200 (expression in *B. subtilis*)	Arnold, 2009
pGP1512	ampicillin/ erythromycin	*tpi*A (S213A) in pBQ200 (expression in *B. subtilis*)	Arnold, 2009
pGP1513	ampicillin/ erythromycin	*pgk* in pBQ200 (expression in *B. subtilis*)	Arnold, 2009
pGP1514	ampicillin/ erythromycin	*pgk* (S183A) in pBQ200 (expression in *B. subtilis*)	Arnold, 2009

Plasmid	Resistance	Description	Referenz
pGP1515	ampicillin/ erythromycin	*pgk* (S183A/T299A) in pBQ200 (expression in *B. subtilis*)	Arnold, 2009
pGP1516	ampicillin/ erythromycin	*pgk* (S183A/T299A) in pBQ200 (expression in *B. subtilis*)	Arnold, 2009
pWH844	ampicillin	overexpression vector	Schirmer *et al*., 1997

6.4. Strains

Strains constructed in this work

Strain	Genotype	Construction[a]	Reference
GP576	*trpC2* Δ*prkC*::*spec*	LFH → 168	
GP577	*trpC2* Δ*yabT*::*mls*	LFH → 168	
GP578	*trpC2* Δ*prkD*::*kan*	LFH → 168	
GP579	*trpC2* Δ*prkC*::*spec* Δ*yabT*::*kan*	LFH → GP576	
GP580	*trpC2* Δ*prkC*::*spec* Δ*prkD*::*kan*	GP578 → GP576	
GP581	*trpC2* Δ*prkC*::*spec* Δ*prkD*::*kan* Δ*yabT*::*erm*	GP577 → GP578	
GP582	*trpC2* Δ*prkC*::*spec* Δ*prkD*::*kan* Δ*yabT*::*mls* Δ*liaF*::*cat*	TMB293 → GP581	
GP583	*trpC2* Δ*ymdB*::*spec*	LFH → 168	
GP584	*trpC2* Δ*pnpA*::*kan*	LFH → 168	Lehnik-Habrink *et al*., *subm.*
GP585	*trpC2 amyE*::(*yabT-lacZ kan*)	pGP831 → 168	-
GP586	*trpC2 amyE*::(*prkC-lacZ kan*)	pGP829 → 168	-
GP587	*trpC2 amyE*::(*prkD-lacZ kan*)	pGP830 → 168	-
GP588	*trpC2* Δ*glmM*:: *spec;* complemented with pGP400	LFH → 168	Schmidl *et al*., 2010

Strain	Genotype	Construction[a]	Reference
GP589	*trpC2* Δ*pyk*::*cat*	LFH → 168	-
GP590*	*trpC2* Δ*pfkA*::*cat*	LFH → 168	-
GP591*	*trpC2* Δ*fbaA*::*cat*	LFH → 168	-
GP592*	*trpC2* Δ*gapA*::*cat*	LFH → 168 0,5% glucose 0,5% malate; pH≤7	-
GP593*	*trpC2* Δ*pgm*::*cat*	LFH → 168 0,5% glucose 0,5% malate; pH≤7	-
GP594*	*trpC2* Δ*eno*::*cat*	LFH → 168 0,5% glucose 0,5% malate; pH≤7	-
GP595*	*trpC2* Δ*pfk*::*erm*	LFH -> 168 0,5% glucose 0,5% malate; pH≤7	-
GP596*	*trpC2* Δ*fbaA*::*erm*	LFH -> 168 0,5% glucose 0,5% malate; pH≤7	-
GP597*	*trpC2* Δ*gapA*::*erm*	LFH → 168 0,5% glucose 0,5% malate; pH≤7	-
GP598*	*trpC2* Δ*pgm*::*erm*	LFH → 168 0,5% glucose 0,5% malate; pH≤7	-
GP599*	*trpC2* Δ*eno*::*erm*	LFH → 168 0,5% glucose 0,5% malate; pH≤7	-
GP600	*trpC2* Δ*pyk*::*erm*	LFH → 168	-
GP701	*trpC2* Δ*gapB*::*spec*	LFH → 168	-
GP702	*trpC2* Δ*gapB*::*spec* Δ*mgsA*::*tet*	GP701 → GP67	-
GP703*	*trpC2* Δ*gapA*::*cat* Δ*gapB*::*spec*	GP592 → GP701 0,5% glucose 0,5% malate; pH≤7	-

Strain	Genotype	Construction[a]	Reference
GP704*	*trpC2* Δ*pfk::erm* Δ(*pgm eno*)::*cat*	GP698 → GP595 0,5% glucose 0,5% malate; pH≤7	-
GP705*	*trpC2* Δ*gapA*::*cat* Δ*mgsA*::*tet*	GP592 → GP67 0,5% glucose 0,5% malate; pH≤7	-
GP706*	*trpC2* Δ*gapA*::*erm* Δ*ccpN*::*cat*	GP592 → DB104 0,5% glucose 0,5% malate; pH≤7	-
GP707*	*trpC2* Δ*pgk*::*erm*	LFH → 168 0,5% glucose 0,5% malate; pH≤7	-
GP708*	*trpC2* Δ(*pfkA pyk*)::*spec*	LFH → 168 0,5% glucose 0,5% malate; pH≤7	-
GP709*	*trpC2* Δ(*tpiA pgm*)::*spec*	LFH → 168 0,5% glucose 0,5% malate; pH≤7	-
GP710*	*trpC2* Δ(*pgk tpiA*)::*spec*	LFH → 168 0,5% glucose 0,5% malate; pH≤7	-
GP711*	*trpC2* Δ(*pgk tpiA pgm eno*)::*spec*	LFH → 168 0,5% glucose 0,5% malate; pH≤7	-
GP712*	*trpC2* Δ*fbaA*::*cat* Δ*pfk::erm*	GP591 → GP595 0,5% glucose 0,5% malate; pH≤7	-
GP713*	*trpC2* Δ*gapB::spec* Δ*pgk*::*erm*	GP707 → GP701 0,5% glucose 0,5% malate; pH≤7	-

[a] Arrows indicate construction by transformation.

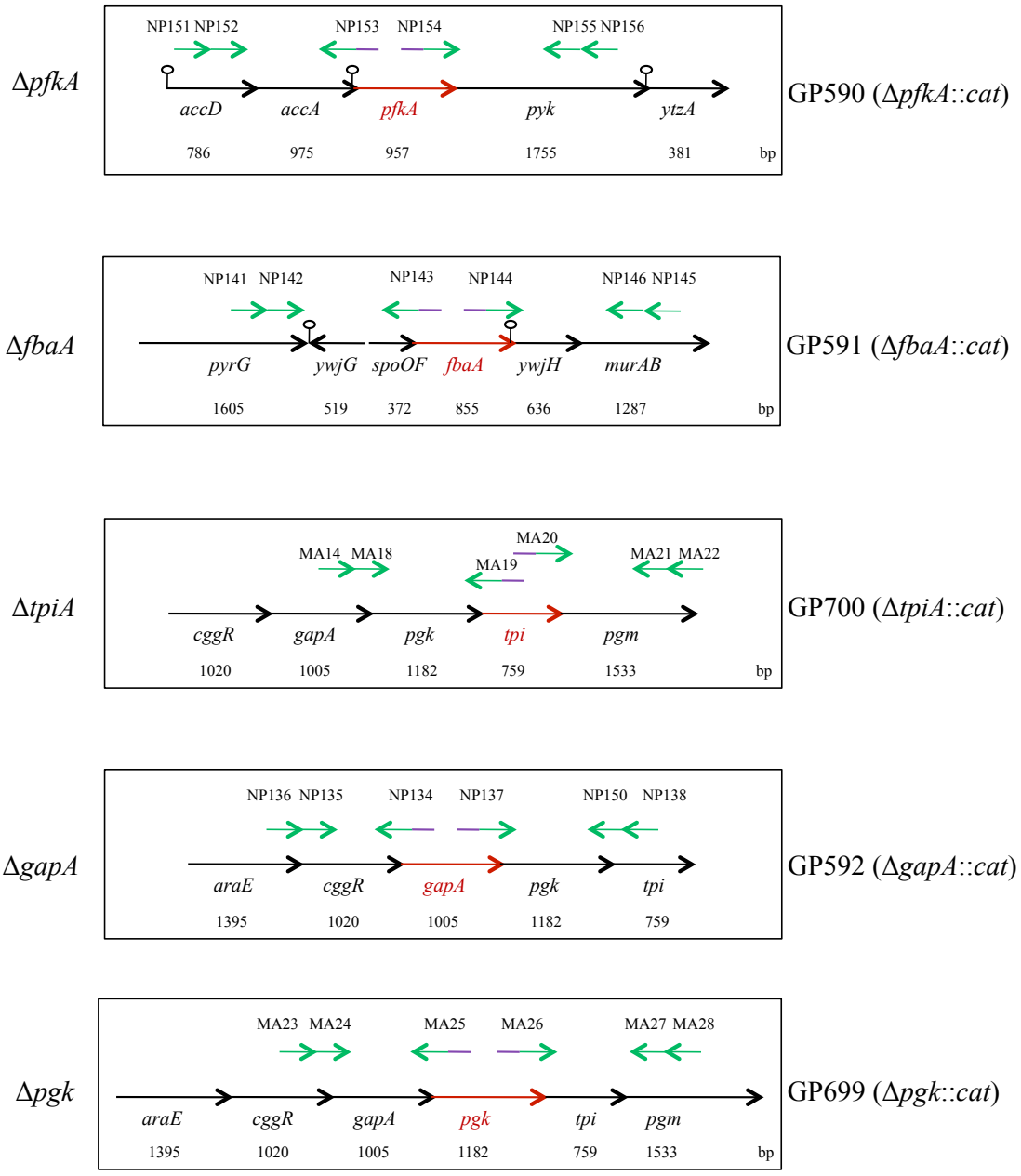
* strains are unable to sporulate, genomic DNA is saved. Transformants require supplementation with glucose and malate. pH value of the media must be keept below 7.

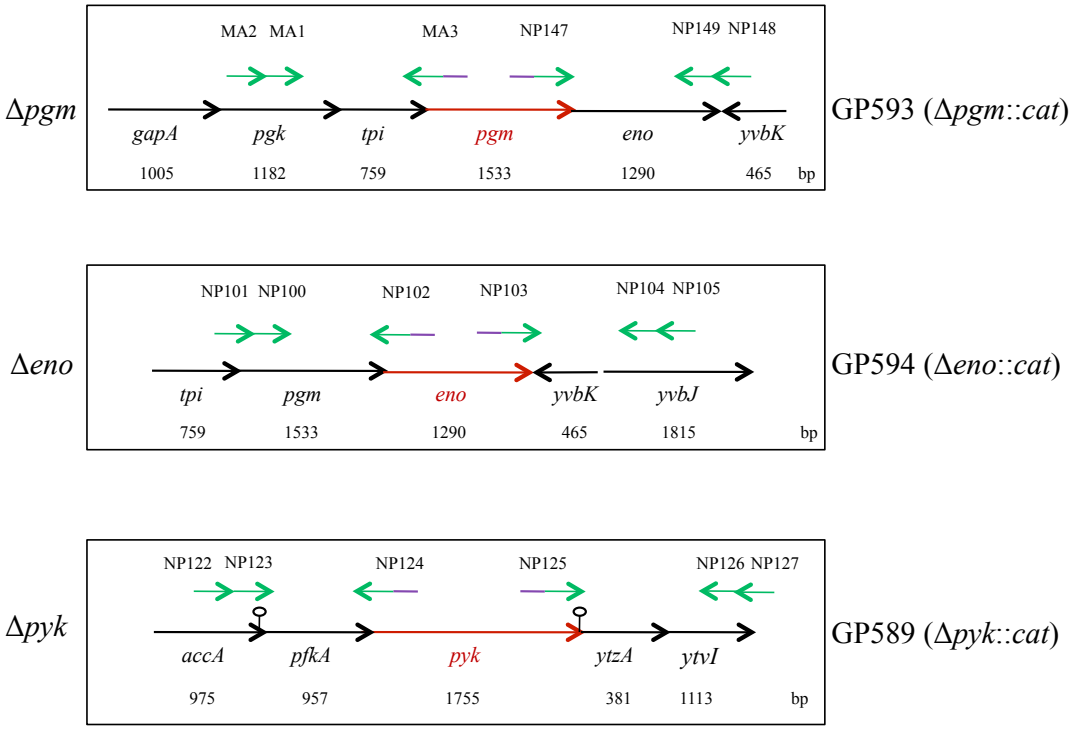
Strains used in this work

Strain	Genotype	Reference
Escherichia coli		
DH5α	*recA1 endA1 gyrA96 thi hsdR17*rK-mK+*relA1 supE44* Φ80Δ*lacZ*Δ*M15* Δ(*lacZYA-argF*)U169	Sambrook et al., 1989
Bacillus subtilis		
168	*trpC2*	Laboratory collection
DB104	*ccpN*::cat	Licht *et al*., 2005
GP67	*trpC2* Δ*mgsA*::*tet*	Landmann, Promo
GP698	*trpC2* Δ(*pgm-eno*)::*cat*	Arnold, 2009
GP699	*trpC2* Δ*pgk*::*cat*	Arnold, 2009
GP700	*trpC2* Δ*tpiA*::*cat*	Arnold, 2009
GP909	*trpC2 amyE*::(*hag-lacZ cat*)	Dietmaier, Promo
TMB293	*ΔliaF*::*cat*	Mascher, unpublished
E. coli stock 147	*Taq*-polymerase	Nadicom
QB5223	*trpC*2 *ptsH1*	Martin-Verstraete *et al*.,1995

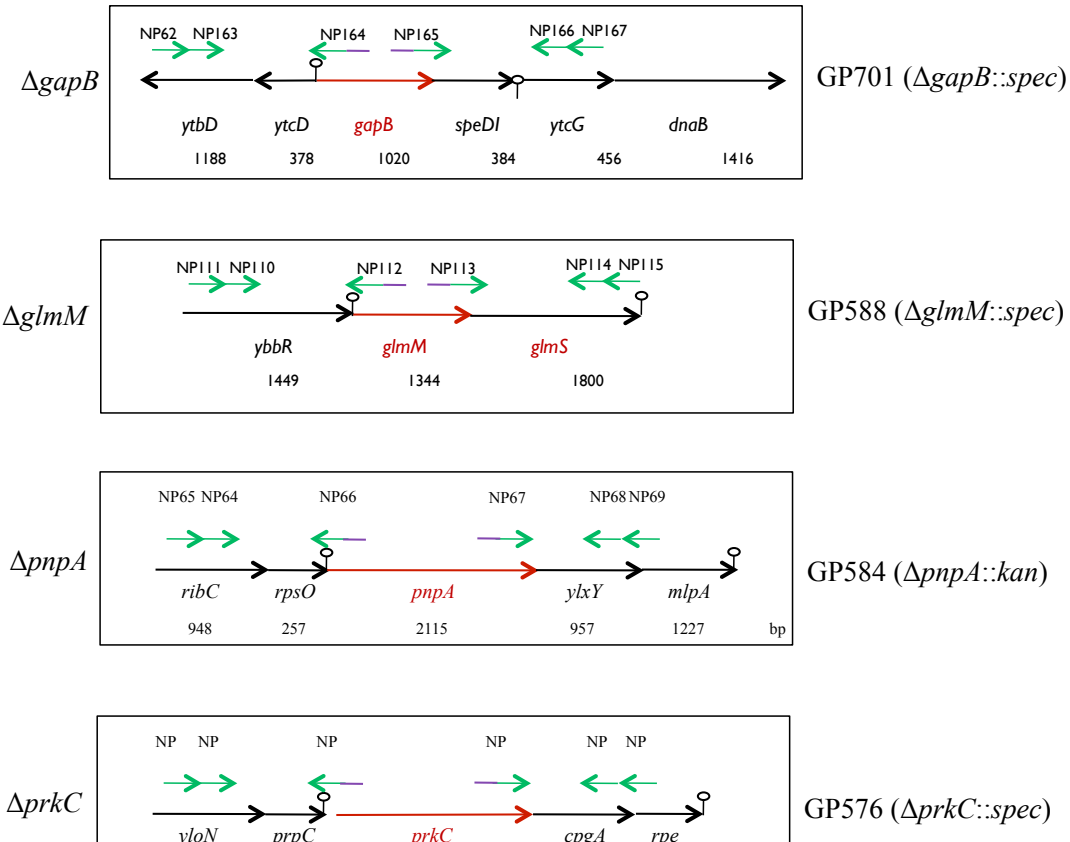
6.5. Construction of mutants

Mutants of glycolytic genes

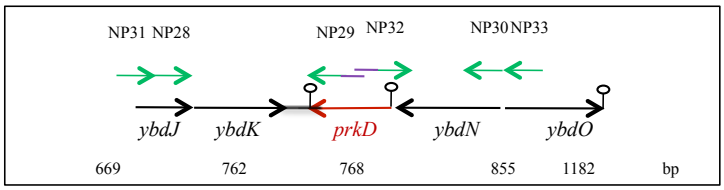




Additional mutants

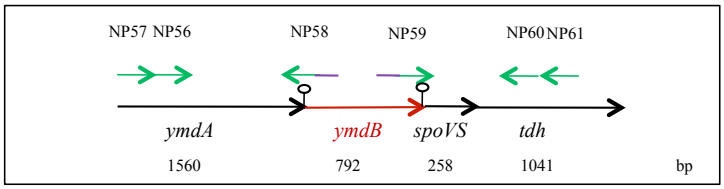


Δ*prkD*



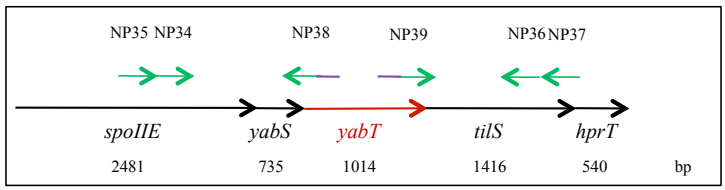
GP578 (Δ*prkD*::*kan*)

Δ*ymdB*



GP583 (Δ*ymdB*::*spec*)

Δ*yabT*



GP577 (Δ*yabT*::*mls*)

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8. *Curriculum vitae*

Personal Information

Name:	Nico Pietack
Date of birth:	July 28th, 1981
Place of birth:	Frankfurt (Oder), Germany
Parents:	Olaf Pietack and Gabriele Pietack, born Hannemann
Siblings:	Vanessa Pietack, Mandy Pietack
Nationality:	German

School Education

1988 - 1991	Allgemeine polytechnische Oberschule Fürstenwalde
1991 - 1994	Theodor-Fontane Grundschule Fürstenwalde
1994 - 2001	Geschwister-Scholl-Gymnasium Fürstenwalde
06/2001	General university qualification (Abitur)

Military service

2001 - 2002	basic military service in Strausberg

Scientific education

10/2002 - 04/2007	study of biology, University of Göttingen diploma thesis in the group of Prof. Dr. Jörg Stülke, University of Göttingen
thesis title:	"Protein phosphorylation in *Bacillus subtilis*"
05/2007 - 05/2010	PhD project: "Investigation of glycolysis in *Bacillus subtilis*" Dept. of General Microbiology (Prof. Dr. Jörg Stülke) University of Göttingen

Göttingen, 19.03.2010