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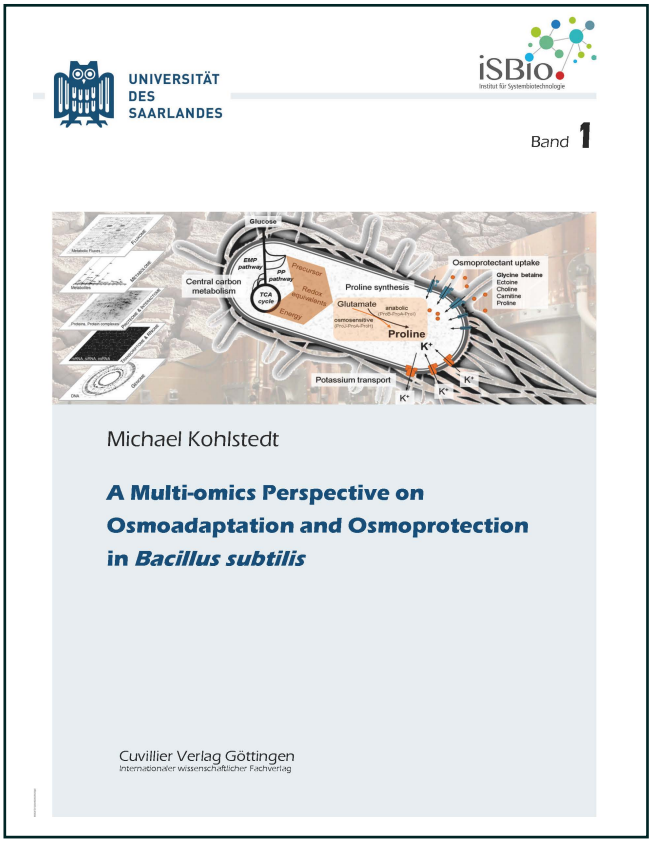
A Multi-omics Perspective on Osmoadaptation and Osmoprotection in Bacillus subtilis



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1 Introduction

1.1 *Bacillus subtilis* – paradigm of gram-positive bacteria and versatile cell factory

At present, *Bacillus subtilis* is Nature's best-characterized gram-positive bacterium. The rod-shaped, prokaryote is a member of the Firmicutes and inhabits all kinds of ecosystems including the upper layers of the soil, aqueous milieus, the rhizosphere of plants and even the human gastrointestinal tract (Earl *et al.*, 2008). The bacterium serves as paradigm to study cell physiology and cellular differentiation processes, e.g. metabolism, gene regulation, sporulation, competence, biofilm formation or responses to environmental stresses. Therefore, its genome was sequenced as one of the first in 1997 and has been re-sequenced in 2009, providing valuable insights into the genetic repertoire of the *Bacillus* clade (Kunst *et al.*, 1997; Barbe *et al.*, 2009). The *Bacillus* genome consists of 4,215 kb pairs coding for over 4,100 proteins, of which the half has yet to have a function assigned. Moreover, *B. subtilis* is a model system for the study of pathogens, such as *Bacillus anthracis*, *Clostridium perfringens*, *Staphylococcus aureus*, *Streptococcus pneumonia* and *Listeria monocytogenes* (Michna *et al.*, 2014). Apart from the scientific interest, a multitude of *Bacillus* species is used for the commercial production of fine chemicals, antibiotics and vitamins, as well as food, feed and technical enzymes (Harwood, 1992; Schallmey *et al.*, 2004; Papagianni, 2012). Among the most important biotechnological products are α-amylases, lipases, proteases (e.g. subtilisin), riboflavin (vitamin B2), the polypeptide antibiotic bacitracin, insecticidal toxins and flavor-enhancing nucleosides (e.g. inosine, guanosine). Today, modern recombination techniques furthermore allow biosynthesis of medically relevant antibody fragments (Wu *et al.*, 1993; Wu *et al.*, 2002), hyaluronic acid (Widner *et al.*, 2005; Chien and Lee, 2007) or even biocommodities (Romero *et al.*, 2007; Romero-Garcia *et al.*, 2009; Zhang *et al.*, 2011; Zhang and Zhang, 2011) using *B. subtilis*. In agriculture *B. subtilis* strains serve as biological control agents (BCAs), protecting plants against pathogens, thus promoting plant growth (Cazorla *et al.*, 2007; Galindo *et al.*, 2013). *B. subtilis* is free of exo- and endotoxins in such a way as the respective products can be generally regarded as safe (GRAS).

1.2 Central metabolism of *Bacillus subtilis*

Carbon substrate uptake

B. subtilis uses glucose as its preferred carbon and energy source (Stülke and Hillen, 2000). The sugar is metabolized via three main pathways of carbon metabolism: glycolysis (or Embden-Meyerhof-Parnas (EMP) pathway), the tricarboxylic acid (TCA) cycle and the pentose phosphate (PP) pathway (Figure 1.1). Uptake and simultaneous phosphorylation of glucose and various other carbon sources is realized via a phosphoenolpyruvate (PEP):carbohydrate phosphotransferase system (PTS). Phosphotransferase systems can be found in numerous gram-positive and gram-negative bacteria and are composed of two general components: i) phosphotransferases enzyme I (EI) and histidine protein (HPr), and ii) several sugar-specific enzymes II (EII). In *B. subtilis* EII, HPr and EI are encoded by the operon *ptsGHI*, in which *ptsH* and *ptsI* are constitutively expressed. In the presence of glucose, a phoshoryl group is transferred from PEP, which serves as energy source and phosphate donor, to the sugar, eventually yielding glucose 6-phosphate. Other substrate uptake mechanisms active in *Bacilli*, are ATP-binding cassette (ABC) transporters, facilitated diffusion and secondary active transport (Stülke and Hillen, 2000; Deutscher *et al.*, 2003).

Central carbon metabolism

Glucose 6-phosphate is simultaneously metabolized via the EMP and the PP pathway (Figure 1.1). Under unperturbed growth conditions and in a large number of mutants the split ratio between the two catabolic pathways is about 2:1 (Fischer and Sauer, 2005). Both pathways fulfill different cellular functions. Whereas the EMP pathway yields 2 moles of pyruvate with concomitant formation of ATP and NADH, the PP pathway, on the other hand, is a major source of NADPH and pentose phosphates for anabolic purposes (Zamboni *et al.*, 2004; Commichau *et al.*, 2009). Through the EMP pathway, phosphorylated glucose is initially split into triose phosphates, which are further metabolized to pyruvate through the total action of eight enzymes (Figure 1.1) (Ludwig *et al.*, 2001; Commichau *et al.*, 2009).

In the oxidative part of the PP pathway, 2 moles of NADPH are generated by action of glucose 6-phosphate dehydrogenase and phosphogluconate dehydrogenase.

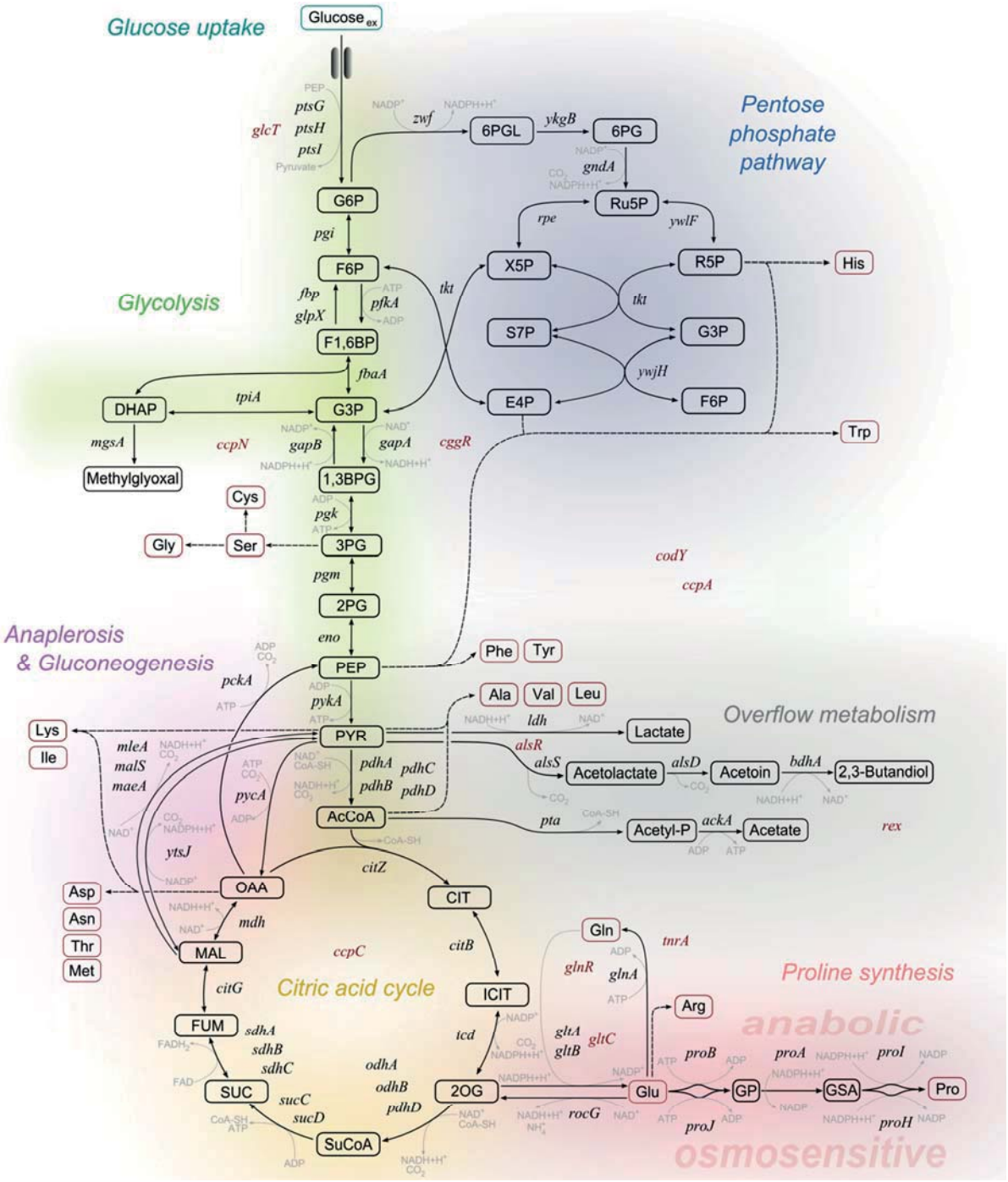

Figure 1.1: Central carbon metabolism and proline biosynthesis in *B. subtilis*. Metabolites are displayed in CAPITALS and encoding genes are displayed in *italics*. Dark red gene names indicate regulators of particular pathways. Metabolite abbreviations: G6P, Glucose 6-phosphate; F6P, Fructose 6-phosphate; F1,6BP, Fructose 1,6-bisphosphate; DHAP, Dihydroxyacetone phosphate; G3P, Glyceraldehyde 3-phosphate; 1,3BPG, 1,3-bisphosphoglycerate; 3PG, 3-phosphoglycerate; 2PG, 2-phosphoglycerate; PEP, Phosphoenolpyruvate; PYR, Pyruvate; AcCoA, Acetyl-CoA; CIT, Citrate; ICIT, Isocitrate; 2OG, 2-oxoglutarate; SuCoA, Succinyl-CoA; SUC, Succinate; FUM, Fumarate; MAL, Malate; OAA, Oxaloacetate; 6PGL, 6-phosphogluconolactone; 6PG, 6-phosphogluconate; Ru5P, Ribulose 5-phosphate; X5P, Xylulose 5-phosphate; R5P, Ribose 5-phosphate; S7P, Sedoheptulose 7-phosphate; E4P, Erythrose 4-phosphate; GP, Glutamate 5-phosphate; GSA, Glutamate 5-semialdehyde.

In the non-oxidative part, different pentoses are interconverted and the enzymes transketolase (Tkt) and transaldolase (YwjH) transfer 2-carbon and 3-carbon groups to form fructose 6-phosphate and glyceraldehyde 3-phosphate, thereby linking PP and EMP pathway (Figure 1.1) (de Wulf, 1998; Zamboni *et al.*, 2004). NADH mainly serves for cell respiration to build ATP via the electron transport chain, whereas NADPH from PP pathway is needed for biosynthetic purposes in anabolism. Both routes, EMP and PP pathway, additionally provide intermediates which serve as precursors for the synthesis of numerous biomass building blocks, especially amino acids, cell wall components and nucleotides (Table 1.1). The alternative Entner-Doudoroff pathway is not present in *B. subtilis* (Stülke and Hillen, 2000).

In case of glucose excess, *B. subtilis* converts a significant portion of glucose into pyruvate and acetyl-CoA, respectively, and drains the respective carbon flux towards overflow metabolism; i.e. the synthesis and secretion of lactate, acetate, acetoin and 2,3-butanediol (Sonenshein, 2007). The synthesis of overflow metabolites quickly regenerates NAD^+ for continued glycolysis and additional ATP is gained via substrate-level phosphorylation when for instance acetate is formed. The pyruvate dehydrogenase complex (PdhABCD), whose action leads to formation of acetyl-CoA, links EMP pathway, overflow metabolism and the TCA cycle (Stülke and Hillen, 2000). Acetyl-CoA can be converted to acetate through phosphotransacetylase and acetate kinase, or serves as precursor for the biosynthesis of fatty acids.

In the TCA cycle, pyruvate is completely oxidized into carbon dioxide. Enzymes of the TCA cycle generate ATP, reducing power in the form of NADH, $FADH_2$ and NADPH for oxidative phosphorylation and anabolic biosyntheses, respectively, as well as different biomass precursors (Table 1.1). At the same time, the membrane-bound TCA cycle enzyme succinate dehydrogenase (SdhABC) is involved in electron transport in the respiratory chain (Sonenshein, 2003; von Wachenfeldt and Hederstedt, 2003). *B. subtilis* has no glyoxylate shunt and is thus unable to grow on acetate or fatty acids as sole carbon source (Sonenshein, 2003).

In the absence of glucose, *B. subtilis* is able to perform gluconeogenesis to generate glucose from TCA cycle intermediates by the action of phosphoenolpyruvate carboxykinase (PckA) and glyceraldehyde 3-phosphate dehydrogenase (GapB). All remaining steps are reversible reactions of glycolysis.

Table 1.1: Major biomass building blocks of a *B. subtilis* cell, their corresponding precursor metabolites and the involved metabolic pathways.

Precursor metabolite	**Building blocks**	**Biomass component**	**Pathway**
Glucose 6-phosphate	UDP-glucose, glucosyldiacylglycerols, lipoteichoic acids, teichoic acids	cell wall, lipids	EMP
Fructose 6-phosphate	*N*-acetylglucosamine, lipoteichoic acids, minor teichoic acids	cell wall, lipids	EMP
Glyceraldehyde 3-phosphate	fatty acids, lipoteichoic acids, teichoic acids	cell wall, lipids	EMP
3-phosphoglycerate	serine, cysteine, glycine, nucleotides	protein, DNA, RNA	EMP
Phosphoenolpyruvate	phenylalanine, tryptophan, tyrosine	protein	EMP
Pyruvate	alanine, lysine, isoleucine, valine, leucine, branched-chain fatty acids	protein, lipids	EMP
Ribose 5-phosphate	histidine, tryptophan, nucleotides, ATP, GTP	protein, DNA, RNA	PP
Erythrose 4-phosphate	phenylalanine, tryptophan, tyrosine	protein	PP
Acetyl-CoA	fatty acids, lipoteichoic acids, minor teichoic acids	cell wall, lipids	TCA
2-oxoglutarate	arginine, glutamate, glutamine, proline	protein, hemes	TCA
Oxaloacetate	aspartate, asparagine, isoleucine, lysine, methionine, threonine, nucleotides	protein, DNA, RNA	TCA

The 2-oxoglutarate-glutamate-glutamine hub

The 2-oxoglutarate pool is from outmost importance for the cell. It represents the hub between carbon and nitrogen metabolism as it is the direct precursor of glutamate, the most abundant metabolite in the cytosol with about 800 µmol g_{DCW}^{-1} (Bolten *et al.*, 2007; Commichau *et al.*, 2008). Glutamate and glutamine serve as the major amino group donors for most nitrogen-containing metabolites (e.g. amino acids, RNA, DNA) and they are direct precursor metabolites for *de novo* synthesis of the amino acid proline, an important compatible solute involved in protection against osmotic stress (Brill *et al.*, 2011; Gunka and Commichau, 2012). It is thus evident, that the intracellular glutamate pool has to be kept sufficiently high and that flux through this metabolic hub has to be tightly controlled. In *B. subtilis*, this is realized by the concerted cyclic action of the glutamate synthase complex (GltAB, also GOGAT) and glutamine synthetase (GlnA, also GS) (Figure 1.1).

The PEP-pyruvate-oxaloacetate node

Continued operation of the TCA cycle depends solely on the action of pyruvate carboxylase. This anaplerotic reaction replenishes the TCA cycle with oxaloacetate. Together with pyruvate kinase (PykA) and the corresponding gluconeogenic enzyme PEP carboxykinase (PckA), the metabolite triangle PEP-pyruvate-oxaloacetate forms a flexible node that links anabolism, catabolism and energetic cellular needs (Figure 1.2) (Sauer and Eikmanns, 2005).

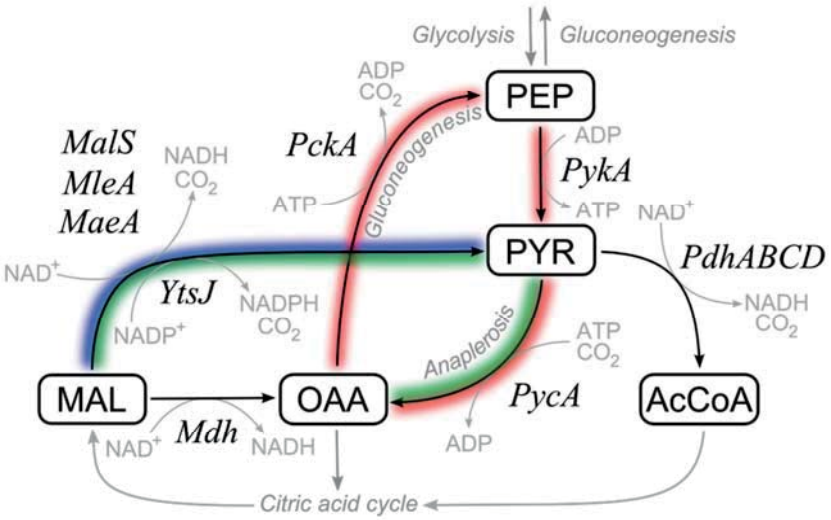

Figure 1.2: The PEP-pyruvate-oxaloacetate node in *B. subtilis* as switch point between anabolism and catabolism; including a transhydrogenation cycle to interconvert NADPH and NADH via 4 different malic enzymes (blue) (Lerondel *et al.*, 2006; Rühl *et al.*, 2012), an ATP-dissipating futile cycle (red) (Dauner *et al.*, 2001a; Dauner *et al.*, 2002) and the pyruvate shunt as bypass to malate dehydrogenase (Mdh) which provides additional OAA at the cost of one mole of ATP (green) (Diesterhaft and Freese, 1973; Sauer and Eikmanns, 2005).

Additionally, *B. subtilis* possesses four different malic enzyme isoforms which decarboxylate malate to pyruvate. Thereby MalS, MleA and MaeA use NAD^+ as cofactor, whereas the YtsJ isoform uses $NADP^+$ and plays a major physiological role under most growth conditions (Lerondel *et al.*, 2006). Fuhrer *et al.* detected *in vitro* activity of a not yet identified transhydrogenase working in *B. subtilis* (Fuhrer and Sauer, 2009), which can directly transfer electrons from NADH to $NADP^+$ and the other way around. Together with transhydrogenation cycles, i.e. enzyme pairs that operate in reverse directions and in this way interconvert the two redox equivalents NADH and NADPH, the cell is able to balance its intracellular demand for redox equivalents (Lerondel *et al.*, 2006; Rühl *et al.*, 2012). Especially at lower growth rates, a significant portion of carbon is withdrawn from the TCA cycle via

gluconeogenic PEP carboxykinase or malic enzyme, even in the presence of glucose. However, carbon is reintroduced into the TCA cycle via pyruvate kinase (PykA) and pyruvate carboxylase (PycA) resulting in net loss of one mole of ATP (Dauner *et al.*, 2001a; Dauner *et al.*, 2001b). The latter pathway via malic enzyme and pyruvate carboxylase is also known as pyruvate shunt and serves as bypass to malate dehydrogenase (Mdh) (Figure 1.2). The utility of ATP-futile cycles is not fully understood, but the dissipation of excess ATP may be necessary for metabolic control and energy homeostasis (Katz and Rognstad, 1978; Dauner *et al.*, 2001b).

Cell respiration

B. subtilis generates between 0.5 and 1 ATP per mole NADH, which corresponds to a maximal P-to-O ratio of 1 (Sauer and Bailey, 1999; Zamboni and Sauer, 2003). Under aerobic conditions, the terminal electron acceptor is oxygen (von Wachenfeldt and Hederstedt, 2003). Under anaerobic conditions, *B. subtilis* is still able to grow by respiration with nitrate as electron acceptor or fermentatively, resulting in formation of various anaerobic fermentation byproducts (Nakano *et al.*, 1997; Cruz Ramos *et al.*, 2000).

Catabolite repression and global regulators of carbon and nitrogen metabolism

During evolution, bacteria developed mechanisms that enable selective uptake and degradation of different carbon sources. In the presence of a preferred carbon source, genes encoding for metabolism of other carbon substrates are not expressed. This mechanism is called 'carbon catabolite repression' (Singh *et al.*, 2008). In *B. subtilis*, several specific and global regulators are in charge to maintain an optimal flux distribution in central metabolism depending on the environmental conditions. Carbon catabolite control protein A (CcpA) is responsible for repression of hundreds of catabolic genes, when sufficient amounts of glucose are available (Sonenshein, 2007). The gluconeogenic genes *pckA* and *gapB* are additionally controlled by another carbon catabolite control protein, CcpN (Servant *et al.*, 2005). Transcription of several TCA cycle enzymes, namely citrate synthase (CitZ), aconitase (CitB) and isocitrate dehydrogenase (Icd), is controlled by CcpC. Genes encoding for these enzymes are also under regulation of CcpA and are furthermore controlled by the global regulator CodY (Sonenshein, 2007), altogether resulting in a complex regulatory network. As for CcpA and CodY, the cell senses intracellular levels of key metabolites such as fructose 1,6-

bisphosphate, branched-chain amino acids and GTP. Similar control mechanisms can be found in nitrogen metabolism as well (Fisher, 1999; Commichau *et al.*, 2006). TnrA, the global regulatory protein of nitrogen metabolism, senses the glutamine level, which is the preferred nitrogen source for *Bacillus* (Fisher, 1999). It activates operons that are responsible for the uptake of ammonium and the utilization of other nitrogen sources, when glutamine is not available.

1.3 Osmoregulation in *Bacillus subtilis*

In its environment, the soil-inhabiting bacterium *B. subtilis* is permanently exposed to fluctuations in the concentration of solutes as a consequence of raining and desiccation phases across the upper soil layers. This leads to substantial changes in external osmolarity caused by all kinds of compounds, such as ions and organic solutes. The cell may dehydrate under hypertonic conditions or burst under hypotonic conditions due to the semi-permeable cytoplasmic membrane. Also during industrial fermentation processes, production organisms are confronted with high substrate and salt concentrations in the growth medium, accumulating product titers and gradients thereof. Knowing the impact of osmotic shifts on cell physiology, regulatory network operation and production performance is thus of great importance for the design of a cell factory and the associated bioprocess.

Cellular response to osmotic stress

When *B. subtilis* is exposed to an upshift in osmotic pressure, it uses a two-step strategy to counteract dehydration of the cytosol (plasmolysis) and loss of cellular functioning (Kempf and Bremer, 1998), especially respiration, membrane processes and cell proliferation (Figure 1.3). Since dehydration occurs within seconds and the cell has to maintain its turgor pressure, *Bacillus* instantly imports potassium from its environment via two K^+ transport systems with different affinity (Whatmore *et al.*, 1990): KtrAB (high-affinity) and KtrCD (low-affinity) (Figure 1.3). Hereby, intracellular potassium levels increase up to three fold compared to basal levels of 300 mM (Whatmore *et al.*, 1990; Holtmann *et al.*, 2003), depending on the strength of the osmotic stress. High concentrations of potassium ions, however, interfere with many cellular processes and become thus toxic for the cell. Consequently, *B. subtilis* accumulates compatible solutes, which are highly water-soluble without net charge and do not impair with cellular physiology (Kempf and Bremer, 1998).

Rapid solute release via mechanosensitive channels (Msc) serves as rescue valve in case of a down-shift in osmolarity (Hoffmann *et al.*, 2008).

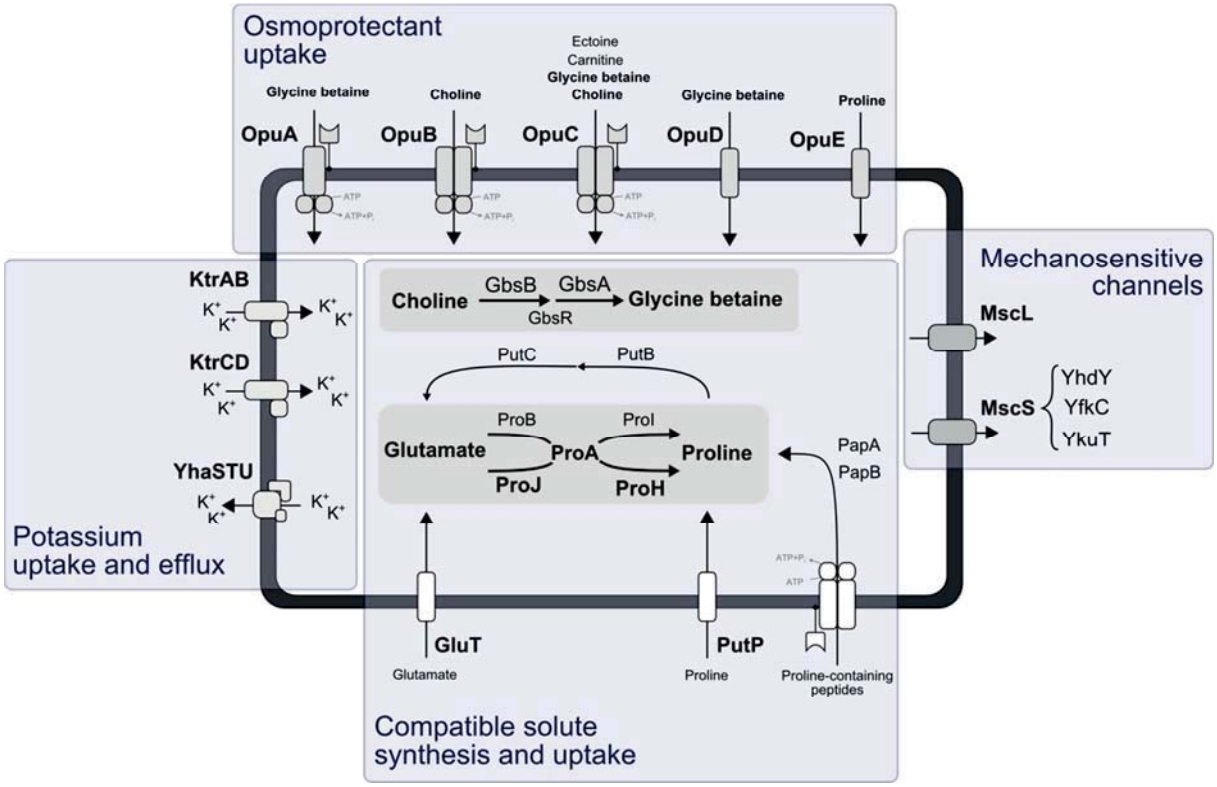

Figure 1.3: Overall cellular response of *B. subtilis* to cope with osmotic stress.

De novo synthesis and uptake of compatible solutes

Compatible solutes are termed 'chemical chaperones', since they are able to stabilize the structure of proteins, i.e. they exclude solutes from the hydration shell around the protein because of unfavorable interactions with the protein surface (Arakawa and Timasheff, 1985). By minimizing the volume, from which the solutes have to be excluded, the protein is stabilized in its native form, since it has a smaller surface than the denatured form. This phenomenon is called 'preferential exclusion' (Arakawa and Timasheff, 1985). Compatible solutes are either synthesized *de novo* or are taken up from the environment as osmoprotectants.

The only compatible solute that *B. subtilis* can synthesize *de novo* is the amino acid proline. In addition to an anabolic route via glutamate 5-kinase (ProB), glutamate 5-semialdehyde dehydrogenase (ProA) and pyrroline 5-carboxylate reductase (ProI), *B. subtilis* possesses a second, osmosensitive route via ProJ (orthologous to ProB), ProA and ProH (orthologous to

ProI) for proline biosynthesis (Brill *et al.*, 2011). Only ProA is commonly used by both routes. The accumulated proline level correlates linearly with the osmotic burden.

Another, even more effective osmolyte is the methylamine glycine betaine (Hoffmann *et al.*, 2013). In *B. subtilis* it can be synthesized only when the precursor choline is exogenously provided (Kempf and Bremer, 1998). Uptake of usable osmoprotectants from the environment can occur via five compound-specific osmoprotectant uptake systems (OpuA to OpuE, Figure 1.3). Among them are three ATP binding cassette transporters (OpuA to OpuC). Glycine betaine can be imported via three high-affinity and osmotically inducible transport systems (OpuA, OpuC and OpuD) underlining the special importance of this osmoprotectant for *B. subtilis* (Hoffmann *et al.*, 2013). During fine-tuning of the intracellular pool, OpuE, a solute symporter, additionally recaptures continuously released proline. All Opu systems are induced in cells grown at high salinity. Additionally, the proline precursor glutamate, proline itself or proline-containing peptides can be imported via specific transport systems (Hoffmann *et al.*, 2013; Zaprasis *et al.*, 2013).

In general, alterations in osmolarity do not only influence metabolic processes, but rather affect the overall cell physiology including the activation of a general stress response mediated via the transcription factor σ_B (Hecker and Völker, 2001; Höper *et al.*, 2006; Hahne *et al.*, 2010), a high-salinity induced iron limitation (Hoffmann *et al.*, 2002; Höper *et al.*, 2006), an altered cell envelope composition (López *et al.*, 1998; López *et al.*, 2006), synthesis of degradative enzymes and expression of genetic competence (Kunst and Rapoport, 1995; Dartois *et al.*, 1998), an impeded sporulation (Ruzal *et al.*, 1998; Ruzal and Sanchez-Rivas, 1998), a repression of chemotaxis and cell motility genes (Steil *et al.*, 2003), as well as an impact on the supercoiling behavior of DNA (Krispin and Allmansberger, 1995; Alice and Sanchez-Rivas, 1997).

Industrial interest in the production of compatible solutes

Over the past decades, increasing knowledge about osmotic stress responses and concomitant release of compatible solutes in halotolerant and halophilic microorganisms, initiated the development of industrial bioprocesses for their commercial production (Sauer and Galinski, 1998; Schubert *et al.*, 2007; Pastor *et al.*, 2010; Becker *et al.*, 2013). The obtained products find broad applications in pharmaceutical, nutritional and cosmetic industries (Roberts, 2005; Lentzen and Schwarz, 2006; Pastor *et al.*, 2010).