Vitamin B6 Production in *Bacillus subtilis*

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Interference of Heterologous and Host Pathways

Dissertation

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I hereby declare that the doctoral thesis entitled "Vitamin B6 Production in *Bacillus subtilis* – Interference of Heterologous and Host Pathways" has been written independently and with no other sources and aids than quoted.

Jonathan Rosenberg

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List of publications

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Rosenberg, J.; Dickmanns, A.; Neumann, P.; Gunka, K.; Arens, J.; Kaever, V. et al. (2015): Structural and biochemical analysis of the essential diadenylate cyclase CdaA from *Listeria monocytogenes*. In *The journal of biological chemistry* 290 (10), pp. 6596–6606. DOI: 10.1074/jbc.M114.630418

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Rosenberg, J.; Yeak, K. C.; Commichau, F. M. (2017b): A two-step evolutionary process establishes a non-native vitamin B6 pathway in *Bacillus subtilis*. In *Environmental microbiology. DOI:* 10.1111/1462-2920.13950

Rosenberg, J.; Commichau, F. M. (in preparation): Harnessing bacterial underground metabolism for pathway development

1. Introduction

The fermentative synthesis of valuable compounds is a sustainable and feasible alternative to chemical synthesis or extraction (Vandamme 1992; Revuelta et al. 2016; Revuelta et al. 2017; Rosenberg et al. 2017a). The importance to find sustainable ways to produce valuable goods like vitamin B6 is demonstrated by the current investment of industry in more environment friendly production. DSM nutritional products, currently market leader in vitamin B6 production, is moving its chemical B6 synthesis plant to a new, more sustainable site in China, investing approximately US$ 100 million (Gray 2016). However, fermentative production of many substances is hindered by host-heterologous interactions or the malicious effects of overexpression of pathways. To circumvent these problems, the regulation of the pathways of interest (POIs) and the regulatory cross-talk with the remaining metabolism must be understood. Many groups are characterizing the regulation of all known metabolic pathways and this growing knowledge is going to help to generate strains able to produce valuable substances. Furthermore, alternative synthesis pathways are present for many products as summarized in a recent review and in the discussion of this work (Dairi et al. 2011). These routes offer opportunities for the failing approaches to fermentatively produce valuable goods. The growing knowledge of genomic sequences from all branches of life is generating an even greater knowledge about the different variants of metabolic pathways and alternative routes for synthesis of valuable substances (Dairi et al. 2011). Although a plethora of different genomic sequences is already known, most of them are barely characterized. The functions of single genes and the existence of many pathways in these uncharacterized genomes can be inferred by homology from model organisms, which are far better characterized, but the exact metabolic impact remains unknown until experimental characterization has been achieved. This is because simple presence of the genes and enzymes comprising a pathway are not enough to state that the pathway actually exists or at least does the job it does in other organisms, which has been exemplified in *Escherichia coli*, where despite the presence of the conserved pathway, an alternative route dominates thiamine synthesis (Bazurto et al. 2016). In addition, promiscuous enzyme activities make the metabolic makeup of uncharacterized organisms even less predictable (Khersonsky, Tawfik 2010). Therefore, the characterization and harnessing of alternative pathways is key to improve fermentative production of valuable goods. As part of the effort to improve an existing vitamin B6 production strain, this work is proposing and applies a feasible, ubiquitously applicable approach to achieve this via heterologous reverse engineering of alternative pathways.

1.1. *Bacillus subtilis*

B. subtilis is a Gram-positive, rod-shaped, non-pathogenic bacterium, which has for decades served as a model organism in basic research and has been used in many applications for biotechnological production of valuable goods. Extensive knowledge about physiology, nutritional requirements and methods for genetic manipulation make this organism an ideal platform for genetic engineering. Furthermore, compared to other organisms, the functions and cross-relation of the encoded proteins are well understood and some groups are on the way to establish *B. subtilis* strains with a minimal gene set in which all genes are essential and have dedicated functions (Juhas et al. 2014). Most of the information concerning *B. subtilis* can be accessed through the comprehensive and convenient database SubtiWiki (Michna et al. 2016).

Many biotechnological applications used *B. subtilis* as a platform. Those applications include production of vaccines, riboflavin, terpenoids, and antibiotics (Craig et al. 1949; Hao et al. 2013; Rosales-Mendoza, Angulo 2015; Guan et al. 2015; Ozturk et al. 2016). Furthermore, *B. subtilis* has been engineered to produce vitamin B6 with intermediate success (Pflug, Lingens 1978; Commichau et al. 2014; Commichau et al. 2015a).

1.2. Vitamin B6

Vitamin B6 is the designation for the six compounds (so called vitamers) pyridoxamine (PM), pyridoxine (PN), pyridoxal (PL) and their respective 5'-phoshate esters (hereafter called phosphate) pyridoxamine phosphate (PMP), pyridoxine phosphate (PNP) and pyridoxal

phosphate (PLP) (Mukherjee et al. 2011; Rosenberg et al. 2017a). PLP is the essential and metabolically active form of vitamin B6 and is required for approximately 4% of all known enzymatic reactions, most of which are part of amino acid metabolism (Percudani, Peracchi 2003, 2009). A list of 67 known or predicted vitamin B6-dependent proteins in *B. subtilis* has recently been published (Rosenberg et al. 2017b). The enzymes and complexes requiring PLP are catalyzing amination, deamination, transamination, racemization, α, β and γ-elimination and replacement as well as decarboxylation by the common mechanism of aldimine formation and deprotonation of the α carbon under inclusion of an electron donor (Eliot, Kirsch 2004). In addition to the aldehyde group of PLP, the phosphate group can act as a proton donor e.g. in the glycogen phosphorylase reaction (Palm et al. 1990; Schneider et al. 2000). It has also been reported that PMP acts as a cofactor and substrate for transamination and deoxygenation in deoxy sugar reactions (Romo, Liu 2011). In addition to its function as a cofactor, there is evidence that vitamin B6 is also an antioxidant due to its redox quencher ability (Bilski et al. 2000).

1.2.1. Vitamin B6 biosynthesis

Vitamin B6 is biosynthesized by many bacteria, archaebacteria, fungi and plants, but animals and thus humans lack biosynthetic pathways and have to ingest it with their diet (Mittenhuber 2001; Tanaka et al. 2005; Fitzpatrick et al. 2007; Mukherjee et al. 2011; Rosenberg et al. 2017a). Two non-homologous biosynthesis pathways have been described for the synthesis of PLP (Figure 1).

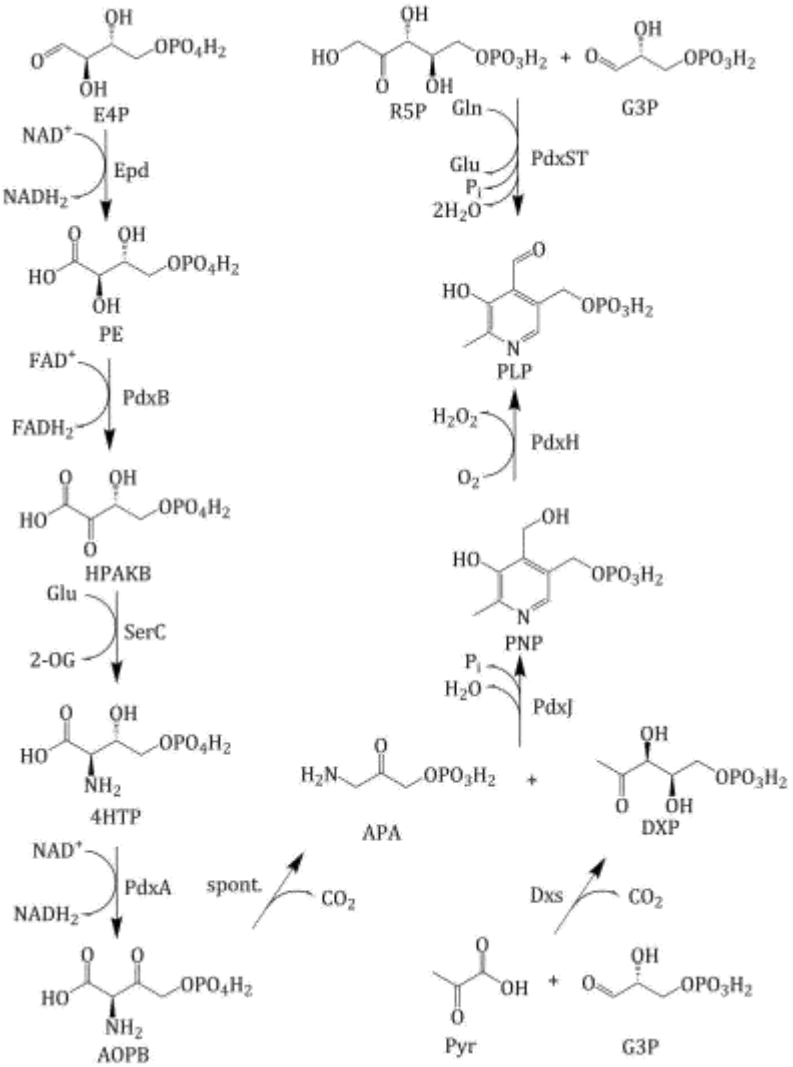

Figure 1: Vitamin B6 biosynthesis pathways. The DXP-dependent pathway synthesizes PLP in a seven-step semi-enzymatic process, while the DXP-independent pathway comprises only one step. E4P, erythrose-4-phosphate; PE, 4-phphoerythronate; HPAKB, 3-hydroxy-4-phospho-α-ketobutyrate; 4HTP, 4-hydroxy-L-threonine phosphate; AOPB, 2-amino-3-oxo-4-phosphohydroxybutyrate; APA, 1-amino-3-phosphohydroxy-acetone; R5P, ribulose-5-phosphate; G3P, glyceraldehyde-3-phosphate; PLP, pyridoxal phosphate; DXP, 1-deoxyxylulose-5-phosphate; Pyr, pyruvate; PNP, pyridoxine phosphate; PLP; pyridoxal phosphate; amino acids are three-letter code.

The evolutionary ancient DXP- independent and the newer, albeit earlier described DXP-dependent pathway (Fitzpatrick et al. 2007; Mukherjee et al. 2011; Rosenberg et al. 2017a).

The DXP-dependent pathway was found in *E. coli* and other γ-proteobacteria and a very similar pathway in α-proteobacteria. Here, the name giving compound 1-deoxy-D-xylulose-5-phosphate (DXP) together with 1-amino-3-phosphohydroxy acetone (APA) are substrates for the synthesis of PNP by activity of the PNP synthase PdxJ (Cane et al. 1999). Subsequently, PNP oxidase PdxH forms PLP, producing hydrogen peroxide as a byproduct (Zhao, Winkler 1995). The precursor DXP is formed by Dxs and is essential also due to involvement in isoprenoid and thiamine synthesis in most organisms (Kuzuyama et al. 2000; Cane et al. 2001). APA is the product of a four-enzyme pathway starting with erythrose-4-phosphate (E4P), which is dehydrated by E4P dehydrogenase Epd (Zhao et al. 1995; Boschi-Muller et al. 1997). 4-Phoshoerythronate (PE) dehydrogenases PdxB and PdxR (in γ and α-proteobacteria, respectively) form 3-hydroxy-4-phospho-α-ketobutyrate (HPAKB), which is subsequently aminated by 3-phosphoserine aminotransferase SerC yielding 4-hydroxy-L-threonine phosphate (4HTP) (Drewke et al. 1996; Rudolph et al. 2010; Tazoe et al. 2006). Dehydratase PdxA then forms 2-amino-3-oxo-4-phosphohydroxybutyrate (AOPB), which is non-enzymatically decarboxylated to APA (Cane et al. 1998; Cane et al. 1999; Laber et al. 1999; Sivaraman et al. 2003).

The DXP-independent pathway is present more organisms including species of bacteria, archaea, fungi and plants and consist of only two enzymes, PdxS and PdxT (or PDX1 and PDX2 in plants and fungi), which form a hetero-24-mer, which catalyzes the formation of PLP from glyceraldehyde-3-phosphate (G3P) or dihydroxyacetone phosphate (DHAP) and ribulose-5-phosphate (R5P) or ribose-5-phosphate (Ri5P) (Ehrenshaft et al. 1999; Ehrenshaft, Daub 2001; Belitsky 2004b; Burns et al. 2005; Raschle et al. 2005; Strohmeier et al. 2006). PdxST can use either two combinations of the four substrates due to an intrinsic sugar isomerase activity, thus the complex unifies triose isomerase, pentose isomerase and imine formation activity to synthesize PLP directly (Burns et al. 2005).

It is an interesting fact that PLP synthesis has been evolved twice using completely unrelated enzymes and several publications have discussed this fact. An ancestor of α and γ-proteobacteria seemingly has lost the ability to synthesize vitamin B6, possibly during adaptation to a niche providing this cofactor (Mittenhuber 2001; Tanaka et al. 2005). The DXP-dependent pathway then evolved from existing enzymatic functions from thiamine/isoprenoid (Dxs), serine (SerC) and vitamin B6 salvage (PdxH) pathways and α and γ-proteobacteria filled the last missing step with the novel PE dehydrogenase function of PdxR and PdxB, respectively. The two enzymes are not homologous but carry out the same function, demonstrating the plasticity of bacterial genomes.

1.2.2. Regulation of vitamin B6 metabolism

The vitamer PLP is highly reactive and can be toxic to cells, as demonstrated later in this work. Therefore, accumulation of free PLP is most likely circumvented by vitamin B6 producers by feedback inhibition, export or catabolism (Mukherjee et al. 2011; Rosenberg et al. 2017a). Only little is known about the regulation of vitamin B6 synthesis and metabolism. The DXP-dependent pathway has been shown to be feedback inhibited. PdxH is inhibited by PLP and can be acetylated leading to modulation of its activity and Dxs is inhibited by products of the isoprenoid synthesis pathway (Zhao, Winkler 1995; Banerjee et al. 2013; Kudoh et al. 2017; Gu et al. 2017a). In several Gram-positive organisms, the regulator $PdxR^{reg}$ (not the enzyme from α-proteobacteria) has been shown to inhibit transcription of *pdxST* in presence and activate it in absence of PLP (Bramucci et al. 2011; Jochmann et al. 2011; Belitsky 2014; Liao et al. 2015; Tramonti et al. 2015; Suvorova, Rodionov 2016). A regulator of the B6 salvage enzymes has been found in *Salmonella typhimurium* (Tramonti et al. 2017). However, similar mechanisms remain to be found in *B. subtilis*. Furthermore, the activator of the γ-aminobutyric acid (GABA) utilization *gabTD* operon GabR has been shown to be active only in presence of PLP and GABA, ensuring that the

enzymes are only transcribed when enough cofactor for GABA transaminase GabT is present together with the substrate (Belitsky 2004a; Wu et al. 2017).

Exporters for vitamin B6 have not been identified to date, although importers have been shown to exist in fungi and plants (described in more detail in the discussion) (Stolz, Vielreicher 2003; Stolz et al. 2005; Szydlowski et al. 2013; Kato et al. 2015). In order to be exported, the vitamers would have to be dephosphorylated according to the current model (Yamada et al. 1977; Yamada, Furukawa 1981). A PLP phosphatase has been found in *Sinorhizobium meliloti*, which is most likely involved in the export of PLP (Tazoe et al. 2005).

Furthermore, cells have been shown to contain very low amounts of free PLP, but rather bind the reactive cofactor to carriers (Fu et al. 2001). Much about these so called channeling mechanism remains unknown, but it has been shown that PdxH contains a PLP binding site in addition to its active site, proposing a 'parking position' for PLP until it is transferred to an enzyme utilizing it (Yang, Schirch 2000; Di Salvo et al. 2011). A similar mechanism has been proposed for PdxK, the PL kinase from *E. coli*, which is also feedback inhibited by PLP remaining bound to PdxK after phosphorylation (Ghatge et al. 2012; Di Salvo et al. 2015). In *B. subtilis*, PLP release has been shown to be facilitated in presence of a PLP-dependent enzyme, the aminotransferase BacF (Moccand et al. 2011). This way PLP would be prohibited from going into solution in the first place. Recently, a novel and highly conserved protein has been shown to be involved in B6 homeostasis. *E. coli* lacking YggS show a disturbed coenzyme A (CoA) metabolism and are vulnerable to PN, showing a curious growth phenotype in presence of this vitamer, allowing growth only in presence of intermediate concentrations and not above or under certain thresholds (Ito et al. 2013; Prunetti et al. 2016). Interestingly, some of the phenotypes can be rescued by external addition of amino acids, showing a connection to PLP-dependent pathways. It will be very interesting to further investigate this protein, which has a homolog in *B. subtilis*, called YlmE (Knockout and overexpression construct BP995 and pBP626, respectively, were constructed by the author and are available but not further mentioned in this work).

1.3. Approaches for fermentative vitamin B6 production

Vitamin B6 is currently being produced in large scale chemical synthesis utilizing expensive and toxic substrates and technically challenging chemical processes (Eggersdorfer et al. 2012). The state of the art Diels-Alder approach was invented in the 1960´s and uses substrates including toxic acetic anhydride, ammonia, formic acid and very expensive ethoxyoxazoles (Eggersdorfer et al. 2012). Several attempts to produce vitamin B6 by fermentation have been made in the meantime but were not considered competitive with the Diels-Alder approach as reviewed recently (Rosenberg et al. 2017a). Thereby it was tried to isolate natural overproducers, overexpress the endogenous pathways, single or several genes, or to overexpress the heterologous pathways. In the 60´s and 70´s of the last century, isolated strains of the genera *Bacillus*, *Klebsiella*, *Kluyveromyces*, *Saccharomyces*, *Achromobacter*, *Pichia*, *Vibrio* and *Flavobacterium* were tested for vitamin B6 production and it was found that *P. guilliermondii* NK-2 was able to produce up to 25 mg l^{-1} vitamin B6 within several days of fermentation in a complex medium with addition of a detergent (Scherr, Rafelson 1962; Pardini, Argoudelis 1968; Ishida, Shimura 1970; Suzue, Haruna 1970a, 1970b; Tani et al. 1972; Nishio et al. 1973; Pflug, Lingens 1978). Thirty years later, the first genetically engineered strains were tested. The overexpression of the heterologous PdxST complex in *E. coli* yielded approximately 60 mg l^{-1} within two days, while overexpression of the endogenous pathway with Epd, PdxJ and Dxs was more successful with about 78 mg l^{-1} within 31 hours (Yocum et al. 2004; Hoshino et al. 2006b). *S. meliloti* was shown to be a natural overproducer of vitamin B6 and strain IFO14782 already produced 103 mg l^{-1} within 168 hours without further optimization (Hoshino et al. 2006a). Overexpression of the endogenous phosphatase PdxP and PdxJ from *E. coli* lead to improved production of 149 mg l^{-1} within 216 hours (Nagahashi et al. 2008). Further improvement by isolation of mutants and overexpression of the endogenous PdxJ as well as Epd from *E. coli*

lead to a titer of 1.3 g l^{-1} within 168 hours (Hoshino et al. 2006a; Hoshino et al. 2006c).

Also *B. subtilis* was engineered to overexpress the complete DXP-dependent pathway with enzymes from *E. coli* (Epd) and *S. meliloti* (PdxR, SerC, PdxA, PdxJ) in two genomic cassettes (Figure 2, Commichau et al. 2014). This strain reached 41 mg l^{-1} within 72 hours of fermentation (Commichau et al. 2014). Most recently, a strain was engineered to convert the intermediate 4HT to PN by overexpression of PdxA from *E. coli* and PdxJ from *S. meliloti*, which reached a titer of 65 mg l^{-1} within 72 hours (Commichau et al. 2015a).

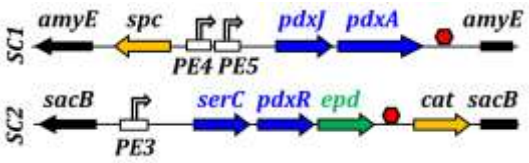

Figure 2: Scheme showing the two genetic constructs used by Commichau et al. 2014. Synthetic construct (SC) 1 encodes PdxJ and PdxA from *S. meliloti* (blue) under control of two constitutive promoters (PE4 and 5) and the spectinomycin resistance gene *spc*. SC2 encodes SerC and PdxR from *S. meliloti* and Epd from *E. coli* (green) under control of a constitutive promoter and the chloramphenicol resistance gene *cat*. SC1 and SC2 were integrated into the *amyE* and *sacB* loci of *B. subtilis* SP1 by homologous recombination, respectively. Red hexagons depict terminators.

The reasons for the inability to reach considerably higher titers that 1 g l^{-1} are widely unknown. It is however known that vitamin B6 is required in very low concentrations of approximately 0.1 µM for growth of *B. subtilis*. It was furthermore described above, that vitamin B6 metabolism is strictly regulated (by mostly unknown mechanisms) and that only a small fraction of the cofactor is present as free PLP, due to its reactivity (Fu et al. 2001). 1 g l^{-1} PLP, which is equivalent to 4 mM, is at minimum 400.000x higher than the physiological concentration of this cofactor. The hypothetically required titer to compete with chemical production of 10 g l^{-1} would equal 4.000.000x this concentration, suggesting that the bacteria will have to adapt or will need to be engineered to withstand such high amounts of vitamin B6 in addition to the ability to produce such amounts (Rosenberg et al. 2017a). It was previously determined that *B. subtilis* could withstand up to 2 g l^{-1} PL and up to 40 g l^{-1} of PN externally added to the medium (Commichau et al. 2014). However, it was reported that the recombinant DXP-dependent pathway expressed in *B. subtilis* was genetically instable and fractions of the fermentation population inactivated it by mutation despite a much lower titer of PN produced in the experiment (Commichau et al. 2014). In part, this fact might be due to the toxicity of intermediates, which have also been shown to be toxic to the host cells. 4HTP, the product of SerC in the DXP-dependent pathway has a detrimental effect on growth of *B. subtilis* and strains grown in presence of toxic concentrations develop resistance by mutation of the branched-chain amino acid transporter encoding gene *bcaP* and mutations leading to the deregulation of the threonine synthesis operon, indicating interference with threonine metabolism (Commichau et al. 2015a; Rosenberg et al. 2016). It was furthermore observed that *B. subtilis* strains overexpressing the DXP-dependent pathway accumulated intermediates of the aromatic amino acid synthesis pathway, indicating even more unknown cross-talk with the host metabolism (Fabian Commichau, personal communication). The brute force overexpression of the unregulated heterologous pathway in *B. subtilis* might lead to inefficient catalysis and accumulation of intermediates, which might lead to toxic effects in the host and therefore limited yield and inactivation of the pathway as described above (Rosenberg et al. 2017a).

The story of vitamin B6 production is in part like that of vitamin B2 riboflavin. B2 is today produced by fermentation using different organisms, including *B. subtilis*, but was until the 90´s chemically produced using toxic and expensive agents like barbiturate and xylidine, which is why fermentation was chosen to be more feasible (Revuelta et al. 2016; Revuelta et al. 2017). Possibly, this work helps to establish a successful and competitive fermentative production of vitamin B6.

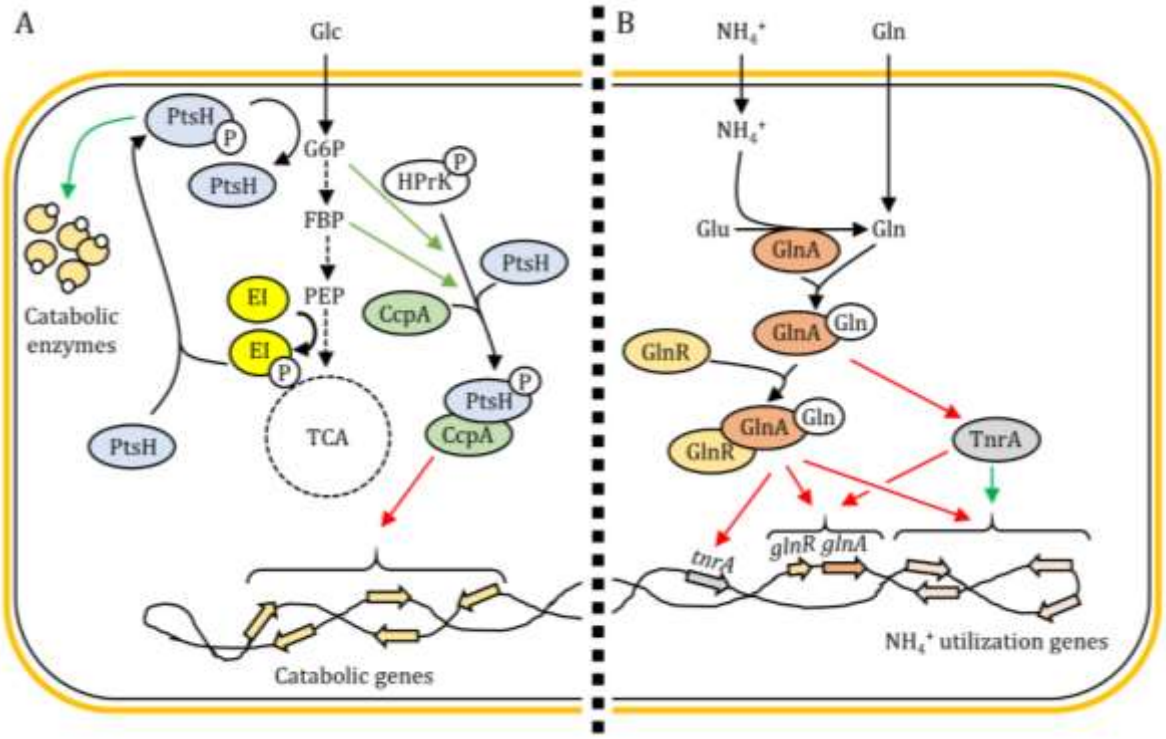

Figure 3: Scheme of the regulation of central metabolism in *B. subtilis*. (A) CCR and (B) nitrogen utilization. Glc, glucose; G6P, glucose-6-phosphate; FBP, fructose-1,6-bisphosphate; PEP, phosphoenolpyruvate; TCA, citric acid cycle; amino acids are three-letter code.

1.4. Regulation of central metabolism in *B. subtilis*

The overexpression of heterologous pathways can be detrimental for the host as mentioned above. The interferences causing this effect can be related to central metabolism and the following section will summarize some relevant mechanisms from *B. subtilis* (Figure 3).

1.4.1. Carbon catabolite repression

B. subtilis prefers glucose as primary carbon (C) and energy source and when several sugars are present, glucose is consumed before all others. This preference for glucose is conveyed mainly by three proteins, catabolite control protein A CcpA, PtsH and the bifunctional kinase and phosphorylase HPrK (Görke, Stülke 2008; Fujita 2009). PtsH has a phosphorylation site at S46, which can be phosphorylated by activity of HPrK and serves as a switch. When S46 is phosphorylated, PtsH can interact with the regulator CcpA and the complex can bind to conserved catabolite responsive elements (*cre*) repressing catabolic enzymes (Henkin et al. 1991; Deutscher et al. 1995; Jones et al. 1997). However, HPrK is only active when fructose-1,6-bisphosphate (FBP) is present, which is an intermediate of glycolysis and whose abundance is proportional to glycolytic activity, ensuring CCR is only triggered, when glucose is abundant (Deutscher et al. 1995; Galinier et al. 1998; Jault et al. 2000). Interestingly, HPrK also dephosphorylates S46, when high concentrations of inorganic phosphate (P_i) and/or low concentrations of FBP are present, which is why it is considered a bifunctional protein (Mijakovic et al. 2002). Furthermore, FBP and glucose-6-phosphate (G6P) enhance the interaction between CcpA and phosphorylated PtsH, facilitating this regulatory connection (Seidel et al. 2005; Schumacher et al. 2007). The target *cre* elements are often located in the promoter regions or the 3'-regions of target genes and lead to a transcriptional roadblock for RNA polymerase. Targets of the CcpA PtsH complex are uptake systems and catabolic pathways for alternative C sources, as for example the ribose utilization operon, the N-acetyl glucosamine (GlcNAc) utilization operon, or the Glycerol uptake factor (Blencke et al. 2003). But also lipid metabolism, respiration and sporulation-related genes and operons as

well as peptide transporters are negatively regulated by CCR (Blencke et al. 2003).

PtsH can also be transiently phosphorylated at H15 by activity of enzyme I (EI) PtsI, which itself acquires its phosphate from phosphoenol pyruvate (PEP). When glucose is abundant and taken up by the phosphotransferase system (PTS), the phosphate is transferred to the sugar, but when glucose is scarce, PtsH can phosphorylate catabolic enzymes specific for other PTS substrates, which contain PEP-carbohydrate PTS-regulatory domains (PRDs) and are thereby activated (Stülke et al. 1998).

1.4.2. Regulation of nitrogen utilization

Nitrogen (N) metabolism in *B. subtilis* is regulated mainly by three proteins, the global regulator TnrA, the transcriptional repressor GlnR and the glutamine synthase (GS) GlnA (Fisher 1999; Gunka, Commichau 2012). *B. subtilis* preferentially utilizes glutamine or ammonium as N sources and the pleiotropic regulator TnrA is the major molecular switch. In absence of glutamine, TnrA activates the genes necessary for ammonium transport and assimilation as well as the transporters und utilization operons for other N sources and at the same time TnrA represses GS and GlnR and genes and operons that are responsible for reactions consuming ammonium (like amino acid synthesis) as well as several unknown genes (Atkinson, Fisher 1991; Wray, jr. et al. 1996; Yoshida et al. 2003; Zalieckas et al. 2006; Khademi, Stroud 2006; Mirouze et al. 2015). In presence of the preferred N source glutamine, the DNA-binding capability of TnrA is strongly impaired by interaction with feedback inhibited (FBI)-GS and additionally, the complex of FBI-GS and GlnR represses transcription of *glnRA*, of *tnrA* and of genes activated by TnrA, like the urea utilization operon (Wray, jr. et al. 1997; Brandenburg et al. 2002; Fisher, Wray, jr. 2008; Hauf et al. 2016).

1.4.3. Stringent response

In addition to the regulation of N and C source utilization, a second major N-related regulatory mechanism exists in *B. subtilis*, which is switching the molecular programing between a rich, amino acid containing and minimal, amino acid limited environments. This mechanism is called stringent response and includes two major pleiotropic regulatory mechanisms, one of them being conveyed by the global branched-chain amino acid-responsive regulator CodY (Sonenshein 2005; Geiger, Wolz 2014). During growth in presence of amino acids, CodY acts as a transcriptional repressor for operons and genes responsible for acquisition and synthesis of amino acids like the peptide transporter operons *opp* and *dpp*, the branched-chain amino acid transporter gene *bcaP*, as well as the aspartokinase III gene *thrD* and the threonine, isoleucine and leucine synthetic *ilv-leu* and *hom* operons among many others (Slack et al. 1995; Molle et al. 2003; Belitsky, Sonenshein 2008; Kriel et al. 2014; Belitsky et al. 2015a; Belitsky et al. 2015b). The DNA binding activity of CodY is enhanced in presence of branched-chain amino acids and this way ensures that those amino acids are not produced under rich medium conditions (Shivers, Sonenshein 2004). However, CodY is also activated by GTP, which conveys the second major stringent response mechanism, 'stringent transcription control' (Ratnayake-Lecamwasam et al. 2001; Handke et al. 2008; Tojo et al. 2010; Brinsmade, Sonenshein 2011; Steinchen, Bange 2016). During the transition from rich medium to an amino acid starvation condition, from exponential to stationary phase, and at initiation of sporulation, GTP is depleted by action of (p)ppGpp synthase RelA (Lopez et al. 1979; Ochi et al. 1981; Tojo et al. 2008). In *S. aureus* and *E. coli*, RelA has been shown to be activated by the presence of uncharged tRNA molecules, signaling amino acid limitation (Pedersen et al. 1973; Rojiani et al. 1989; Geiger et al. 2010; Brown et al. 2016). There are also two minor, so called small (p)ppGpp synthases, RelP and RelQ, but their exact functions are still unknown (Geiger, Wolz 2014). The stringent response messenger (p)ppGpp furthermore inhibits the GTP synthetic enzymes GuaAB, Gmk and HprT, facilitating GTP depletion and at the same time rerouting flux of the purine precursor IMP in the direction of ATP synthesis (Lopez et al. 1981; Beaman et al. 1983; Kriel et al. 2012). Besides the inactivation of CodY due to absence of GTP, the lower GTP level and concomitantly rising ATP level have an additional, transcriptional effect on transcripts starting with adenines or guanines (Krasny et al. 2008; Tojo et al. 2008; Tojo et al. 2010). It was shown that the guanine or adenine bases at positions +1 and +2 have an

influence on transcription depending on the ATP and GTP (and correspondingly (p)ppGpp) levels. During exponential growth in a nutrient rich medium, high concentrations of GTP are present and transcripts initiated with a guanine are produced normally, while transcripts initiated with an adenine are less efficiently produced due to (relatively) low ATP concentrations. During stringent response, GTP gets depleted and ATP is produced in higher amounts than usual, which leads to the opposite situation (Lopez et al. 1979; Ochi et al. 1981; Tojo et al. 2008; Tojo et al. 2010). This kind of regulation has been shown in detail for the *ilv-leu* operon and several operons of glucose and pyruvate metabolism. Moreover, stringent response as very recently also been shown to be important for the adaptation to fatty acid starvation in *B. subtilis* (Pulschen et al. 2017).

1.4.4. Regulation of the aspartate-derived amino acids

The amino acids asparagine, lysine, threonine, isoleucine and methionine have aspartate as a common precursor in *B. subtilis*. The synthesis of the aspartate-derived amino acids lysine, threonine, isoleucine and methionine is initiated by the activity of three aspartokinases that feed into a branching pathway, which is very differentially regulated by action of allosteric feedback inhibition as well as transcriptional repression and activation (Figure 4). Already the initial step is highly regulated, as the aspartokinases I, II and III are under control of different feedback inhibitory and activating interactions. Essential aspartokinase I DapG earns its name, because it is feedback inhibited by diaminopimelate (DAP), an intermediate of the branch leading to peptidoglycan (PG) and lysine (Graves, Switzer 1990). Aspartokinase II LyC, a non-essential paralog of DapG, is transcriptionally inhibited by lysine by a lysine-dependent riboswitch (Grundy et al. 2003; Wilson-Mitchell et al. 2012). Aspartokinase III ThrD is transcriptionally activated by lysine, synergistically feedback inhibited by lysine and threonine and repressed by CodY (Graves, Switzer 1990; Kobashi et al. 2001). Essential L-aspartate-4-semialdehyde (ASA) dehydrogenase Asd uses the product of aspartokinase, aspartyl-phosphate (Asp-P) and forms ASA (Daniel, Errington 1993).

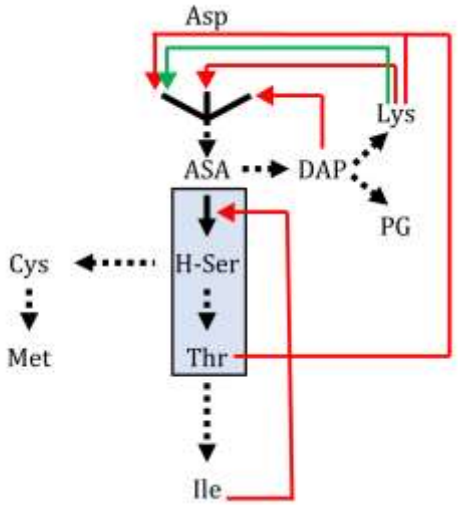

Figure 4: Scheme showing the regulation of the aspartate-derived amino acids. Amino acids are three-letter-code; ASA, L-aspartate-4-semialdehyde, H-Ser, homoserine; PG, peptidoglycan; DAP, diaminopimelate. Blue box, part of the Thr pathway encoded in the *hom* operon. Red and green lines show inhibitory and activating regulation, respectively. Dotted lines show metabolic pathways containing more than one enzyme.

At this point the pathway divides into the DAP and the homoserine branches. The DAP branch starts with the formation of (4S)-4-hydroxy-2,3,4,5-tetrahydro-(2S)-dipicolinate from ASA and pyruvate by the also essential 4-hydroxy-tetrahydrodipicolinate synthase DapA (Grant Pearce et al. 2017; Daniel, Errington 1993). Further down this branch divides again into the lysine and PG synthesis pathways. The homoserine branch starts with activity of the homoserine dehydrogenase Hom (Parsot, Cohen 1988). Here, the pathway divides again. Homoserine is consumed by activity of homoserine O-succinyltransferase MetA feeding into methionine synthesis and by homoserine kinase ThrB feeding into threonine and isoleucine synthesis (Rowbury, Woods 1964; Zubieta et al. 2007). The genes encoding the pathway leading from homoserine to threonine, Hom, ThrB and threonine synthase ThrC are part of the *hom* operon, which is under transcriptional control of CodY and TnrA (Kriel et al. 2014; Mirouze et al. 2015). This pathway is furthermore feedback inhibited by sensitivity of Hom to threonine (Parsot, Cohen 1988). Threonine can either be catabolized to glycine by threonine dehydrogenase Tdh and 2-amino-3-ketobutyrate CoA ligase Kbl, or it enters the

isoleucine synthesis pathway by activity of threonine dehydratase IlvA, which forms 2-oxobutanoate (2-OB) (Hatfield et al. 1970; Hatfield, Umbarger 1970a, 1970b). IlvA is also under transcriptional control of CodY (Molle et al. 2003). Subsequently, the enzymes encoded in the *ilv-leu* operon acetolactate synthase IlvBH and ketol-acid reductoisomerase IlvC as well as dihydroxy-acid dehydratase IlvD and the branched-chain amino acid aminotransferases YwaA and YbgE produce isoleucine (Ward, jr, Zahler 1973; Berger et al. 2003). The synthesis pathways of isoleucine, leucine and valine share these last steps although with differing substrates. The *ilv-leu* operon is tightly regulated through CcpA, TnrA, CodY, stringent transcription control and a tRNA-dependent riboswitch-conveyed antitermination in absence of branched-chain amino acids (Grundy, Henkin 1994; Ludwig et al. 2002; Molle et al. 2003; Tojo et al. 2004; Belitsky, Sonenshein 2008). IlvD and the ubiquitous aminotransferases YwaA and YbgE are also under transcriptional control of CodY (Molle et al. 2003; Kriel et al. 2014). Many enzymes in amino acid metabolism, including all three aspartokinases, Hom and IlvH contain regulatory aspartate kinase-chorismate mutase-tyrA (ACT) domains, which are known to regulate proteins dependent on binding to small molecule effectors, which are mostly amino acids (Grant 2006; Curien et al. 2008; Lo et al. 2009).

1.4.5. Oxidative stress response

Oxidative stress originates mainly (as the name says) from the intrinsic nature of oxygen, which is one of the most important elements for most living organisms due to redox chemistry and respiration. Oxygen in solution has the capability to oxidize amino acids, metals and cofactors and cleave bonds. Redox chemistry leads to the formation reactive oxygen species (ROS) like superoxide (O_2^-) or hydrogen peroxide (H_2O_2) and reactive nitrogen species (RNS) like nitric oxide (NO). Together, these reactive species can lead to inhibition of central metabolic functions and DNA damage, eventually causing cell death. Bacteria possess many mechanisms by which they can deal with this natural but harmful problem of their chemistry and under normal conditions, this is well managed. During conditions where additional oxidative potential is present, bacteria activate the so called oxidative stress response, which protects vulnerable targets in the cell and activates detoxification and repair pathways. This can happen, when pathogenic bacteria encounter the immune system, where host cells secrete NO and other reactive species to defend themselves. *B. subtilis*, which is not a pathogen, but a soil bacterium encounters this condition either on substrates that have oxidative properties or due to the secretion of reactive species by competing organisms. The targets of ROS in *B. subtilis* are diverse. Thiols can be oxidized irreversibly by ROS, which is especially the case for those found in side chains of cysteine and homocysteine (demethylated methionine) residues within proteins (Di Simplicio et al. 2003; Lee et al. 2007; Winterbourn, Hampton 2008). When these residues are exposed to the solvent and located in crucial parts of enzymes or are involved in catalysis, this oxidation can lead to irreversible inactivation (Hondorp, Matthews 2004; Hochgrafe et al. 2005; Winterbourn, Hampton 2008). Furthermore, solvent exposed $[4Fe\text{-}4S]^+$ clusters (FeS clusters), which are often present in dehydratases, are vulnerable to iron oxidation leading to loss of one iron atom, inactivating the enzymes containing these clusters (Beinert, Kiley 1999; Imlay 2006). The released iron cations themselves exhibit toxic Fenton chemistry, leading to the production of highly reactive hydroxyl species in reaction with hydrogen peroxide (Rogers, Ding 2001; Woodmansee, Imlay 2003; Imlay 2006; Spiro 2007). ROS also interfere with iron homeostasis, because the pleiotropic metal homeostasis repressor Fur is prone to oxidation (Bsat et al. 1998). Fur represses genes encoding proteins responsible for iron uptake and metabolism and oxidation of the bound iron leads to the inability to bind DNA and therefore uncontrolled uptake of iron, leading to even more harmful Fenton chemistry and DNA damage (Imlay, Linn 1988; Varghese et al. 2007). NO can directly harm the respiratory chain of *B. subtilis* by inhibition of cytochrome oxidases and together with ROS forms other RNS like dinitrogen trioxide (N_2O_3) and can modify cysteine and tyrosine residues as well as FeS clusters, also leading to release of reactive iron cations (Brown et al. 1997; Rogers, Ding 2001; Di Simplicio et al. 2003; Fang 2004; Spiro 2007; Winterbourn, Hampton 2008).

Interestingly, *B. subtilis* even encodes an NO synthase, which is thought to be important for the activation of oxidative stress response (Gusarov, Nudler 2005).

Oxidative stress response is conveyed by five major players, the general stress sigma factor σ^B and the oxidative stress-responsive regulators PerR, OhrR and Spx as well as the novel low molecular weight (LMW) thiol bacillithiol (BSH) (Zuber 2009; Chandrangsu et al. 2017). The sigma factor σ^B is promoting the general stress response by activation of transcription of about 150 general stress-related genes under many conditions like envelope, heat, alkaline, acid, and oxidative stress (Hecker, Völker 2001; Hecker et al. 2007). The repressor PerR is more specifically responding to peroxide stress and its DNA binding is inhibited by structural modification through peroxide, allowing expression of the DNA protecting iron resistance protein MrgA, catalase KatA, alkylhydroperoxide reductase AhpCF, heme biosynthetic genes and Spx (Bsat et al. 1998; Herbig, Helmann 2001; Lee, Helmann 2006; Leelakriangsak et al. 2007; Traore et al. 2009).

The transcriptional repressor OhrR received its name because it responds specifically to organic hydroperoxides (OHPs) (Fuangthong et al. 2001; Fuangthong, Helmann 2002). Oxidation of a regulatory cysteine residue by OHP and subsequently by BSH, coenzyme A (CoA) or cysteine leads to release from the DNA and expression of the peroxiredoxin OhrA (Fuangthong, Helmann 2002; Lee et al. 2007). The Spx-mediated stress response involves many different genes not only related to oxidative, but also heat stress. Within the oxidative stress response, Spx mediates resistance to thiol-specific and paraquat stress (Zuber 2009; Antelmann, Helmann 2011). Spx carries a SsrA tag and is under physiological conditions rapidly proteolyzed by ClpXP under mediation of the adaptor protein YjbH (Nakano et al. 2003b; Garg et al. 2009). During oxidative stress, a regulatory motif containing two cysteine residues undergoes oxidation to a disulfide bond, allowing transcriptional regulation (Nakano et al. 2003a; Nakano et al. 2005). According to the current model, under the same condition, ClpX and the adaptor protein YjbH become inactive due to their own disulfide switches, leading to lower efficiency of Spx proteolysis (Zuber 2009). When present in high concentrations, Spx inhibits all genes requiring activation by a positive regulator because it binds to RNA polymerase alpha subunit, interfering with other regulators (Nakano et al. 2003b; Zuber 2004; Rochat et al. 2012). In addition to this pleiotropic regulatory mechanism, Spx regulates dozens of specific loci by activation and repression conferred by a conserved Spx *cis* element (Zuber 2004; Erwin et al. 2005; Reyes, Zuber 2008; Zuber et al. 2011; Rochat et al. 2012). Spx activates the thioredoxin system TrxAB, cysteine/methionine biosynthetic and repair enzymes like YrrT, MccAB, MtnN, MsrAB and CysK, and other redox-related proteins like the synthesis pathway for the LMW thiol BSH (Nakano et al. 2003a; Choi et al. 2006; You et al. 2008; Gaballa et al. 2013). In addition to activation, Spx also represses several genes, as for example surfactin production enzymes and *comS* (Nakano et al. 2003b).

A novel LMW thiol, BSH was identified in bacilli, which is also present in *staphylococci*, *streptococci* and *deinococci* (Newton et al. 2009). It is involved in oxidative stress resistance by e.g. the protection of proteins from irreversible oxidation and detoxification of methylglyoxal as well as in the resistance to fosfomycin (van Loi et al. 2015; Chandrangsu et al. 2017). BSH is produced from the substrate UDP-GlcNAc by three enzymes, glycosyltransferase BshA, deacetylase BshB and the putative cysteine ligase BshC (Gaballa et al. 2010; VanDuinen et al. 2015). The reactions of BshA and BshB have been demonstrated *in vitro*, but the reaction of BshC has not been closer characterized to date, probably due to an unknown cofactor to the reaction (VanDuinen et al. 2015). *B. subtilis* encodes the first two genes *bshA* and *bshB1* in one operon with the methylglyoxal synthase encoding gene *mgsA* and the biotin ligase *birA* which is under control of Spx as earlier mentioned. The gene encoding BshC is encoded separately in a bicistronic operon together with *ylbQ*, which is also under control of Spx (Gaballa et al. 2013). Additionally, there is a Spx regulated paralog of *bshB1*, *bshB2*, which is encoded in a tricistronic operon with two unknown genes and is thought to have a minor role in the synthesis of BSH (Fang et al. 2013).

BSH is thought to be the most important redox buffer in *B. subtilis* and is present in relatively high concentrations of 1-5 mM in the cytosol which is considerably higher than the concentrations of other thiols in this organism (Sharma et al. 2013). It can be present as a thiol or as a disulfide of two BSH molecules (BSSB) and the fact that most of it is measurable as the reduced form BSH has given reason to assume the existence of BSSB reductases, which to date remain to be found with YpdA, YqiW and YphP as hot candidates (Sharma et al. 2013; Chandrangsu et al. 2017). A function as a metal ion buffer has been shown by the capability of BSH to sequester zinc and copper ions (Ma et al. 2014; Kay et al. 2016). Furthermore, BSH deficient mutants have been shown to be susceptible to hypochlorite, diamide, ROS, osmotic, acid and alkaline stress (Gaballa et al. 2010; Chi et al. 2011). Other studies showed that BSH is also important for resistance to fosfomycin, because BSH deficient cells are more susceptible to this antibiotic and the fosfomycin resistance conveying epoxide hydrolase FosB requires BSH as a cofactor (Gaballa et al. 2010; Lamers et al. 2012; Thompson et al. 2014). BSH probably also conveys resistance to reactive electrophile species (RES) by either sequestration and direct S-conjugation or as a cofactor for the putative S-transferase YfiT, which is a homolog of BstA from *Staphylococcus aureus*. BstA has been shown to detoxify RES by S-conjugation and subsequent export of mercapturic acids (Rajan et al. 2004; Newton et al. 2011; Perera et al. 2014). BSH is also important for the detoxification of MG. It binds to MG forming BSH-hemithioacetal, which is catabolized to lactate by glyoxalases I and II GlxA and GlxB, respectively (Chandrangsu et al. 2014). One intermediate of this pathway, S-lactoyl-BSH is a messenger molecule that activates the three-component potassium/proton antiporter KhtSTU, which imports H^+ into the cytosol, leading to acidification and thus preventing nucleophilic damage by MG (Chandrangsu et al. 2014). There is also evidence that BSH is involved in formaldehyde detoxification in other bacilli (Harms et al. 1996; Nguyen et al. 2009; Chandrangsu et al. 2017). Strains deficient in BSH also show lower activity of FeS cluster containing enzymes leading to lower concentrations of branched-chain amino acids leucine and isoleucine (Fang, Dos Santos 2015; Rosario-Cruz et al. 2015; Chandrangsu et al. 2017). It is however unknown, by what mechanism BSH is involved in FeS cluster synthesis.

Another major role of BSH is reversible bacillithiylation of cysteines prone to irreversible oxidation (Chi et al. 2011; Chi et al. 2013). There have been studies identifying the bacillithiylation targets (Chi et al. 2011; Chi et al. 2013; van Loi et al. 2015). The best studied examples so far are the methionine synthase MetE and the previously mentioned organic hydroxide resistance regulator OhrR. Bacillithiylation leads to methionine auxotrophy under oxidative stress, which could be relieved by addition of the amino acid, demonstrating the inactivation of target enzymes. Further bacillithiylation targets under investigation are the translation factor TufA, AroA, GuaB and ribosomal protein RpsM among many others (Chi et al. 2011; Chi et al. 2014). In other firmicutes, glyceraldehyde-3-phosphate dehydrogenase Gap is considered a major target for bacillithiylation, but in *B. subtilis*, Gap is protecting itself from oxidative damage by an internal disulfide switch (Chi et al. 2011; Imber et al. 2017). Very interestingly, bacillithiylation is not observed under H_2O_2 stress indicating a very specific regulation of this mechanism (Chi et al. 2013). The reversibility of bacillithiylation is due to the enzymatic function of bacilliredoxins. So far two have been identified, BrxA, BrxB and the candidate YtxJ is under investigation (Gaballa et al. 2010; Chi et al. 2013; Gaballa et al. 2014). However, other than the thioredoxin system TrxAB, BrxAB are not essential and it is hypothesized that they exhibit redundant functions concerning bacillithiylated proteins (Gaballa et al. 2010; Gaballa et al. 2014; Chandrangsu et al. 2017).

1.5. Underground metabolism

In addition to cellular metabolism, there is a growing notion of the so called underground metabolism. The word underground describes the major property of what could also be called alternative or invisible metabolism. Most of the known enzymatic functions of *E. coli* can actually be carried out by a minority of the enzymes. 37% of the identified enzymes can use more than one substrate and are able to catalyze 65% of the known reactions in this organism

(Nam et al. 2012). The implicit redundancy is thought to be an indicator for a, to a certain degree proceeding, specialization of enzymes, with contemporary enzymes being specialists and their ancestors generalists (Khersonsky, Tawfik 2010; Innan, Kondrashov 2010; Nam et al. 2012). As a byproduct of selection for more specific reactions, generalist traits are retained although not selected for. When the ability to carry out side reactions does not cause toxicity, or is inefficient enough not to be selected against, then it can be carried along (Khersonsky, Tawfik 2010). The ability to carry out side reactions can also be a collateral effect of selection for another trait (Khersonsky, Tawfik 2010). It is important to note, what effect the realization of underground metabolism has: Pathways are not necessarily linear, branched or circular, but might in reality be an endless, interconnected network (Khersonsky, Tawfik 2010). This fact is, of course, of great interest for the development of novel synthesis pathways for biotechnological application (Notebaart et al. 2017). Several works have been dedicated to the harnessing of this new reservoir. Examples with relation to vitamin B6 have brought up exciting alternative pathways. One group established a bioinformatical platform, which can predict promiscuous enzyme activity, called PROPER (Promiscuity Predictor) (Oberhardt et al. 2016). By a permissive homology search and comparison of functions and reaction mechanisms, PROPER was able to predict a promiscuous PLP synthesis activity of ThiG in *E. coli*, which could also be shown in a multi-copy replacement experiment (Oberhardt et al. 2016). Another group applied a genetic approach by roadblocking *E. coli´*s DXP-dependent B6 synthesis pathway and overexpression of a *E. coli* ORF plasmid library (Kim et al. 2010). By overexpression of the genes encoded in the ORFs, promiscuous activities became visible and it was possible to identify several enzymes rescuing growth and comprising three serendipitous pathways bypassing the PE dehydrogenase PdxB (in more detail in the discussion) (Kim et al. 2010). These successful approaches demonstrate the potential of underground metabolism. And this potential might be further facilitated by combination of underground metabolism with the knowledge about alternative pathways from other organisms to form novel, hybrid pathways comprising reactions of underground metabolism and heterologous enzymes (Rosenberg, Commichau in preparation).

1.6. Aims and scope of this work

This work aims to add to the knowledge required for an economically competitive and ecologically sustainable, fermentative production of the valuable substance vitamin B6. To achieve this, the deleterious cross-talk between the heterologous pathway and host metabolism is investigated to allow the development of strategies to circumvent this obstacle in the future. Furthermore, genetic engineering and targeted evolution approaches are applied to optimize an existing *B. subtilis* vitamin B6 production strain. Also, a reverse engineering approach to generate a novel, hybrid pathway is applied exploiting rewiring of underground metabolism with the heterologous pathway and its application in other systems is proposed. Lastly, the blue-print for an optimized and streamlined vitamin B6 production strain based on the biochemical and kinetic characteristics of the synthetic enzymes is presented.

2. Material and Methods

2.1. Material

Materials used in this work are listed in the appendix.

2.2. Bacterial strains and plasmids

Bacterial strains and plasmids used in this work are listed in the appendix

2.3. Media and solutions

All media, buffers and solutions were prepared with deionized water (ddH_2O) unless stated otherwise. Sterilization was done by autoclavation for 20 minutes at 121°C and 2 bar. Temperature sensitive solutions and reagents were sterilized by filtration through a 0.22 µm nominal pore diameter filter membrane. Concentrations depicted as per cent are w/v unless otherwise stated. For solidification, media were supplemented with 1.5% Agar-Agar (Commichau et al. 2015b). Chemicals were commercially acquired from either Sigma Aldrich (Merck KGaA, Darmstadt) or Carl Roth GmbH + Co. KG (Karlsruhe).

2.3.1. Stocks and additives

5x C-salts
20 g KH_2PO_4
80 g K_2HPO_4 x 3 H_2O
16.5 g $(NH_4)_2SO_4$
Ad 1000 ml ddH_2O

III´ salts
0.232 g $MnSO_4$ x 4 H_2O
12.3 g $MgSO_4$ x 7 H_2O
Ad 1000 ml ddH_2O

10x MN
136 g K_2HPO_4 x 3 H_2O
60 g KH_2PO_4
10 g Sodium Citrate x 2 H_2O

IPTG
1000x Stock solution: 1 M in ddH_2O
Final concentration: 1 mM in medium

X-Gal
1000x Stock solution: 40 mg ml^{-1} in DMF
Final concentration: 40 µg ml^{-1} in medium
Plates and media containing X-Gal were stored in the dark.

2.3.2. Antibiotics

All used antibiotics were prepared as 1000x stock solutions in ddH_2O or 70% (v/v) ethanol (EtOH). For the selection of *ermC*, erythromycin and lincomycin were used in combination. In Table 1 the selective concentrations are shown. Antibiotics were sterilized by filtration and added to the media after autoclaving. Media and plates containing tetracycline were stored in the dark.

Table 1: Selective concentrations of antibiotics used in this work.

Antibiotic	Solvent	Selective concentration [µg ml^{-1}]	
		B. subtilis	*E. coli*
Ampicillin	ddH_2O	-	100
Chloramphenicol	EtOH	5	15
Erythromycin	EtOH	2	-
Kanamycin	ddH_2O	10	50
Lincomycin	ddH_2O	25	-
Phleomycin	ddH_2O	35	35
Spectinomycin	ddH_2O	150	-
Tetracycline	EtOH	12.5	-

2.3.3. Complex media

SOB medium
20 g Tryptone
5 g Yeast Extract
0.58 g NaCl
0.186 g KCl
Ad 980 ml ddH_2O
After autoclaving, the following, sterile compounds were added:
10 ml 1 M $MgCl_2$
10 ml 1 M $MgSO_4$

LB medium
10 g Tryptone
5 g Yeast Extract
10 g NaCl
Ad 1000 ml ddH_2O

SP medium
8 g Nutrient Broth
0.25 g $MgSO_4$ x 7 H_2O
1 g KCl
Ad 1000 ml ddH_2O
After autoclaving, the following, sterile compounds were added:
1 ml 0.5 M $CaCl_2$
1 ml 10 mM $MnCl_2$
2 ml 2.2 mg ml^{-1} Ferric Ammonium Citrate (CAF)

2.3.4. Defined minimal media

C minimal medium
20 ml 5x C-salts
1 ml 5 mg ml^{-1} Tryptophan
1 ml 2.2 mg ml^{-1} CAF
1 ml III´ salts
Ad 100 ml ddH_2O

CE minimal medium
C minimal medium
2 ml 40% Potassium Glutamate (E)
Ad 100 ml ddH_2O

CSE minimal medium
C minimal medium
2 ml 40% Potassium Glutamate
2 ml 30% Sodium Succinate (S)
Ad 100 ml ddH_2O

2.3.5. Buffers and solutions

Buffer W
12.114 g Tris
8.77 g NaCl
0.3722 g Na_2EDTA x 2 H_2O
HCl to pH 8
Ad 1000 ml ddH_2O

Buffer E
0.027 g D-Desthiobiotin
Ad 50 ml Buffer W

Buffer Z
1.068 g Na_2HPO_4
0.552 g NaH_2PO_4
75 mg KCl
24.7 mg $MgSO_4$
Ad 50 ml ddH_2O
Directly before use: 175 µl β-Mercaptoethanol

LD Mix
100 mg Lysozyme
10 mg DNase I
Ad 10 ml ddH_2O

LD Buffer
4 ml Buffer Z
20 µl LD Mix

5x TBE Buffer
54 g Tris base (T)
27.5 g Boric acid (B)
20 ml 0.5 M EDTA pH 8.0 (E)
Ad 1000 ml ddH_2O

50x TAE Buffer
242 g Tris base (T)
57.1 ml Acetic acid (A)
100 ml 0.5 M EDTA pH 8.0 (E)
Ad 1000 ml ddH_2O

10x TBS Buffer
60 g Tris base (T)
90 g NaCl
Adjust pH to 9.2 with NaOH
Ad 1000 ml ddH_2O

TB Buffer
3.46 g PIPES
18.64 g KCl
Ad 940 ml ddH_2O
After autoclaving, the following, sterile compounds were added:
2.2 g $CaCL_2$ x H_2O
55 ml 1 M $MnCl_2$
Ad 60 ml ddH_2O

5x Laemmli Buffer
1.4 ml 1.5 M Tris HCl pH 6.8
5 ml Glycerol
0.5 g Sodium dodecyl sulfate (SDS)
1.6 ml β-Mercaptoethanol
0.02 g Bromophenol Blue
Ad 10 ml ddH_2O

Protein gels

Stacking gel
1.3 ml 30% Acrylamide-Bisacrylamide
1 ml 1.5 M Tris HCl pH 6.8
5.5 ml ddH_2O

80 µl 10% SDS
80µl 10% Ammonium persulfate (APS)
8 µl Tetramethylethylenediamide (TEMED)

12% Running gel
6 ml 30% Acrylamide-Bisacrylamide
3.8 ml 1 M Tris HCl pH 8.8
4.9 ml ddH_2O
150 µl 10% SDS
150 µl 10% APS
6 µl TEMED

Native EMSA 6% PAA gel
7 ml 30% Acrylamide-Bisacrylamide
3.5 ml 5X TBE
24.5 ml ddH_2O
175 µl 10% APS
52.5 µl TEMED

Coomassie staining solutions

Staining solution
5 g Coomassie Brilliant Blue R250
100 ml Acetic acid
450 ml Methanol
Ad 1000 ml ddH_2O

Destaining solution
100 ml Acetic acid
450 ml Methanol
Ad 1000 ml ddH_2O

Silver staining solutions

Fixing solution
50 ml Methanol
12 ml Acetic acid
100 µl 37% Formaldehyde
Ad 100 ml ddH_2O

Thiosulfate solution
20 mg $Na_2S_2O_3$ x 5 H_2O
Ad 100 ml ddH_2O

Impregnator
0.2 g $AgNO_3$
37 µl 37% Formaldehyde
Ad 100 ml ddH_2O

Developer
6 g $NaCO_3$
2 ml Thiosulfate solution
50 µl 37% Formaldehyde
Ad 100 ml ddH_2O

Stop solution
1.86 g EDTA
Ad 100 ml ddH_2O

Blotting solutions

Blotto
25 g Skim milk powder
100 ml 10x TBS
1 ml Tween 20
Ad 1000 ml ddH_2O

Transfer Buffer
15.1 g Tris base
72.1 g Glycine
750 ml MeOH
Ad 5000 ml ddH_2O

Buffer III
12.1 g Tris base
5.8 g NaCl
Adjust pH to 9.5 with NaOH
As 1000 ml ddH_2O

Miscellaneous

5x DNA loading dye
5 ml Glycerol
200 µl 50x TAE buffer
0.01 g Bromophenol Blue
0.01 g Xylencyanol
Ad 10 ml ddH_2O

2.4. Methods

2.4.1. Common techniques

Methods that represent state-of-the-art lab techniques, well-known and regularly performed by most members of the fields of molecular biology and microbiology, are summarized and referenced in the following Table 2.

2.4.2. Cultivation of bacteria

Unless otherwise stated, bacteria were grown at a temperature of 37°C, liquid cultures were placed on shakers and agitated at 200 rpm. Liquid *B. subtilis* cultures were inoculated from single clones on SP agar plates not older than three days, grown SP plates were stored at room temperature (RT). For experiments in minimal medium, unless otherwise stated, a single colony of *B. subtilis* from a complex medium plate was used to inoculate a complex medium preculture. A minimal medium preculture was then inoculated from this complex medium preculture. Finally, the main culture was inoculated from the minimal medium preculture. Growth in liquid media was measured by determination of the optical density at 600 nm (OD_{600}), using the Amersham Ultrospec 2100 pro photo spectrometer or the Synergy MC platereader (Commichau et al. 2015b; Sambrook et al. 1989).

2.4.3. Storage of bacteria

For storage over several days to weeks, plates with *E. coli* cells were stored at 4°C, those with *B. subtilis* cells were stored at RT in the dark. For long-term storage, cells were resuspended in medium containing 10% (v/v) DMSO and stored at -80°C (Commichau et al. 2015b).

2.4.4. Harvest and washing of cells

For volumes lower than 50 ml, the Kendro Biofuge primo R was used with 5000 rpm for 6 minutes. For volumes between 50 and 500 ml, Sorvall RC6 plus was used at 5000 rpm for 15 minutes. Lysozyme treated *B. subtilis* cells were centrifuged for 2 minutes at 13,000 rpm using the Kendro Biofuge primo R to pelletize the protoplasts and keep the cell wall fragments in solution. *E. coli* cells were harvested using the same centrifuges at 5000 rpm for 15 minutes.

Table 2: Common techniques used in this work.

Method (alphabetical order)	Reference
Absorption measurement	Sambrook et al. 1989
Coomassie staining of protein gels	Fazekas de St Groth et al. 1963
Ethidium bromide staining of DNA gels	Sambrook et al. 1989
Gel electrophoresis of DNA	Sambrook et al. 1989
Gel electrophoresis of proteins (SDS PAGE)	Laemmli 1970
Ligation of DNA fragments	Sambrook et al. 1989
Plasmid preparation from *E. coli*	Sambrook et al. 1989
Precipitation of nucleic acids	Sambrook et al. 1989
Protein quantification via Bradford assay	Bradford 1976
Sequencing according to the chain termination method	Sanger et al. 1977
Sequencing according to the Solexa/Illumina method	Bentley et al. 2008
Silver staining of protein gels	Merril et al. 1981

2.4.5. DNA manipulation and strain construction

DNA manipulation and strain construction was carried out as recently described (Commichau et al. 2015b; Rosenberg et al. 2016). Insertion and deletion cassettes were constructed using the Long Flanking Homology (LFH) PCR method (Wach 1996). Primers, templates and vectors used are described in the results part and listed in the appendix. PCR products and plasmid DNA up to a fragment length of 1000 bp were sequenced by Seqlab Sequence Laboratories Göttingen (Germany) using the Sanger sequencing method. Whole Genome Sequencing (WGS) was performed by the Göttingen Genomics Laboratories (G2L, Germany) according to the Illumina method.

2.4.6. Protein expression and purification

Recombinant expression in and purification of Strep-tagged proteins from *E. coli* were performed as recently described (Rosenberg et al. 2016; Commichau et al. 2015b). Briefly, genes encoding proteins of interest were amplified in a PCR reaction and cloned into the respective overexpression vector (pGP172, pGP574 or pET-SUMOadapt) in *E. coli* DH5α. Single clones were isolated and sequenced. Checked constructs were used to transform *E. coli* BL21(DE3), which then carried a plasmid with the gene encoding the protein of interest under a conditional promoter. Overexpression of recombinant proteins was performed in liquid LB medium at 37°C by addition of IPTG after the cells reached an OD_{600} of 0.6-0.9 for 1-3 hours. The ideal duration of induction was tested for each individual construct. Cultures were harvested by centrifugation, the pellet was washed in Buffer W and stored at -20°C for later use. For purification, pellets were resuspended in Buffer W and cells were disrupted using the French Press method with 18,000 psi using the French Pressure Cell Press. Cell debris was pelletized by centrifugation and the cell-free crude extract was passed through a Strep-Tactin Sepharose column, the column bed volume (CV) depended in the construct. The column was washed with 10 CV of Buffer W and proteins were eluted three times by addition of first 0.5 CV and then twice 1 CV of Buffer E. Expression and purification of the proteins were visualized using SDS PAGE and Coomassie straining. Protein amounts were measured using the Bradford assay. The used constructs, duration of induction and protein amounts are mentioned in the results part.

2.4.7. Bacillithiylation assay

To identify bacillithiylated proteins, a previously published protocol was applied (Chi et al. 2013). Strains were grown in 30 ml C Glc minimal medium with 0.1 µM PL to an OD_{600} of 0.4 at 37°C. Then the cultures were washed, split and resuspended in 30 ml C Glc minimal medium. One of the resulting cultures was supplemented with 0.1 µM PL. Both cultures were grown to an OD_{600} of 0.5 and 2 OD_{600}^{-1} ml of the cultures were centrifuged and the pellets were stored at -20°C. For analysis of the bacillithiylated proteins the pellets were resuspended in 45 µl LD Buffer and incubated for 1 hour at 37°C to digest the cell wall. Subsequently the samples were frozen at -20°C for 3 hours and thawed to lyse the cells. Protein amounts were measured using a Bradford assay and an equivalent of 15 µg protein was subjected to SDS-PAGE. The protein bands were then blotted onto a MeOH-activated PVDF membrane, using a semi dry procedure (60 minutes at 80 mA in Transfer Buffer), which subsequently was blocked with Blotto overnight. The membrane was incubated with a specific antibody (rabbit anti-BSH-tagged proteins, diluted 1:500 in Blotto, courtesy of Haike Antelmann) for 3 h (Chi et al. 2013). After washing with blotto and incubation with an anti-rabbit immunoglobulin G-alkaline phosphatase secondary antibody, the bacillithiylated proteins were visualized using a CDP detection system as described previously (Commichau et al. 2007).

2.4.6. Strep-tagged protein interaction experiment (SPINE)

Genes encoding proteins of interest were cloned into the respective expression vectors (pGP380 or pGP382) using DH5α and single colonies were isolated and sequenced. Checked plasmids were used to transform *B. subtilis* 168 which would then synthesize the Strep-tagged version of the protein of interest. A single clone of transformed *B. subtilis* 168 was used for the SPINE in liquid LB medium, which was carried out as previously described (Herzberg et al. 2007). Briefly, the cells carrying a plasmid

encoding the Strep-tagged protein of interest were used to inoculate a 500 ml LB culture, which was grown to an OD_{600} of 1. The culture was then split into two 250 ml cultures. One culture was treated with 4% paraformaldehyde/PBS to a final concentration of 0.6%, while the other was treated with the volume of PBS as control. After 20 minutes incubation at 37°C, both cultures were harvested by centrifugation, pellets were washed in Buffer W. Both pellets were used to purify proteins as described in 2.4.6. Purified and co-purified proteins were visualized using SDS PAGE and silver staining, protein bands were sequenced by our cooperation partners at the University of Greifswald, Dr. Elke Hammer and Prof. Uwe Völker (UV) using GC MS/MS.

2.4.7. Isothermal titration calorimetry (ITC)

ITC experiments were carried out with an ITC 200 microcalorimeter as previously described (Gundlach et al. 2015). All experiments were carried out with freshly purified and dialyzed protein. Exact buffer conditions and parameters are described in the figure legends.

2.4.8. Size exclusion chromatography coupled multi-angle light scattering (SEC-MALS)

SEC-MALS was performed as previously described (Rosenberg et al. 2015). Purified protein was analyzed in degassed buffer (20 mM Tris, 150 mM NaCl, 1 mM $MgCl_2$) using an Äkta system with a S75 Superdex 10/300GL column, a miniDawn Treos multiangle light scattering system and an Optilab T-rEX RI detector. Data was analyzed with ASTRA version 6.1 software (Wyatt Technology).

2.4.9. Bacterial two-hybrid assay (B2H)

B2Hs were carried out to visualize interactions between two proteins of interest as previously described (Rismondo et al. 2015). Briefly, the genes encoding the proteins of interest were cloned into the vectors carrying the reading frames encoding the T18 or T25 domain of the *Bordetella pertussis* adenylate cyclase, fusing them to the reading frame either 5`or 3`of the gene (pUT18, pUT18C, p25-N, and pKT25). Cloning was performed using *E. coli* XL1-blue. Always one checked T25-fused version of the first and one T18-fused version of the second gene encoding a protein of interest were used to co-transform *E. coli* BTH101. After transformation, the cells were dropped onto a plate containing X-Gal and cells containing plasmids encoding interacting proteins turned blue, because the domains of the adenylate cyclase were brought into close proximity and could produce cAMP, which would activate transcription of the reporter gene *lacZ*. As positive control, pKT25-Zip and pUT18C-Zip were used, because Zip is known to show a very strong self-interaction. The constructs used are mentioned in the results part.

2.4.10. Crosslinking experiment

Crosslinking experiments were carried out as described elsewhere (Rosenberg et al. 2015). In short, purified protein was incubated in Buffer W containing different amounts of glutardialdehyde (GAL) for 1 hour at RT, the reaction volume being 20 µl. The reaction was stopped by addition of 4 µl of 200 mM Glycine. Afterwards, 6 µl of 5x Laemmli Buffer were added, the sample was cooked at 95°C for 10 minutes and subjected to SDS PAGE with subsequent silver or Coomassie staining. Protein and crosslinker amounts and concentrations are depicted in the results part.

2.4.11. Electrophoretic mobility shift assay (EMSA)

EMSAs were carried out as described elsewhere (Rosenberg et al. 2016). DNA and protein amounts as well as used proteins are described in the results part.

2.4.12. β-galactosidase reporter (LacZ) assay

LacZ assays were carried out as described previously (Rosenberg et al. 2016). Briefly, strains carrying the constructs of interest were grown in minimal medium to an OD_{600} of 0.5-0.8 and then harvested by centrifugation. Pellets were stored at -20°C. For analysis, the pellets were resuspended in LD Buffer and lysed at 37°C for 1 hour. Cell debris was removed by centrifugation and the reaction with ONPG was performed at 28°C until the samples showed visible yellow coloration, which was followed by addition of Na_2CO_3 to stop the reaction. Absorption at 420 nm was measured and the β-

galactosidase activity was calculated according to the formula:

$$\frac{OD\ 420\ nm}{(\Delta T\ OD\ 595\ nm)}\ 2005.3475.$$

The OD 595 nm represents the absorption at 595 nm as measured in the Bradford assay with the remaining crude extract. Growth conditions, used strains and constructs are described in the results part.

2.4.13. Drop dilution assay

Drop dilution assays were carried out as described elsewhere (Rosenberg et al. 2017b). In short, single clones of the strains of interest were picked from fresh SP plates and used to inoculate the precultures as described above. After incubation, the cells were again washed twice and resuspended in C Glc to an OD_{600} of 1. From these cultures, serial dilutions were made at steps of 1 to 10. 5-10 µl of these dilutions were then dropped on minimal medium plates. For drop dilution assays under conditions in absence of vitamin B6, the cells from the preculture were washed in C Glc twice and incubated at 28 or 37°C for at least one hour to dilute the remaining vitamin B6. Strains and dilutions are depicted in the results part.

2.4.14. Coevolution of mutually dependent strains

Strains of interest were picked from fresh SP plates and inoculated together as cocultures and as control as single cultures. Cultures were incubated at 37°C and passaged in different intervals mentioned in the results part. To distinguish the strains, they were regularly plated on plates containing antibiotics and when needed they were isolated and genomic DNA was used for amplification of the constructs of interest by PCR and Sanger sequencing by Seqlab.

For coevolution on minimal medium plates, the strains were streaked out on plates either next to each other to allow diffusion of the metabolites though the agar, or they were plated as a mixed culture to allow direct cell-to-cell contact. When the strains were plated next to each other, a negative control with a stripe of medium cut out between the strains was done as well as a positive control with the exchanged metabolites externally applied to the medium. Plates were incubated at 37°C.

2.4.15. Fluorescence microscopy

Fluorescence microscopy was performed with a Zeiss Axioskop 40 with 100x magnification using a HXP 120 light source. YFP and GFP were visualized using filter sets F36-528 and 37, respectively. Cells were cultivated in the dark prior to analysis. Pictures were analyzed with Zeiss Axiophot software or ZEN.

3. Results

3.1. A toxic pathway

Vitamin B6 is thought to be essential to all known forms of life due to its involvement in many crucial and some essential enzymatic functions of the cell (Percudani, Peracchi 2003, 2009). However, vitamin B6 is also known to be toxic at concentrations above a certain threshold because it interacts with metabolites, which can lead to deamination and with proteins, partly inactivating them (Vermeersch et al. 2004; Dong, Fromm 1990; Ohsawa, Gualerzi 1981; Metzler, Snell 1952; Shanbhag, Martell 1991). Furthermore, it has been shown that 4HTP, an intermediate of the DXP-dependent pathway, is toxic and that *B. subtilis* can adapt to this by resistance mechanisms involving proteins of threonine biosynthesis (Commichau et al. 2015a; Rosenberg et al. 2016). Of course, these detrimental effects of the vitamin itself and of at least one intermediate of the pathway are one reason for the lack of an existing production strain for this valuable compound. Taken the fact that a profitable fermentative production would require the production of approximately 10 g l^{-1} vitamin B6 (25 mM), the product resistance is one of the major obstacles of fermentative production (Rosenberg et al. 2017a). In this work, the mechanisms of resistance are investigated. Ultimately, the understanding of the mechanisms of sensitivity should help us to uncover the so far unknown interactions of vitamin B6 and its biosynthetic pathways with the rest of the cell in both, heterologous and endogenous environments.

3.1.1. The 4-hydroxythreonine resistance protein ThrR

As described above and in the introduction (1.3), 4HTP is toxic to *B. subtilis* cells. This was found, when the DXP-dependent pathway was expressed and 4HTP was fed to the cells to enhance the production of PN. The resistant mutants (e.g. BV708) had detrimental mutations in the loci of the branched chain amino acid transporters BcaP and YbxG and an activating mutation in the promoter of the threonine biosynthetic operon consisting of *hom*, *thrB*, and *thrC* (Commichau et al. 2015a). Interestingly, similar mutations in the promoter region of the *hom* operon (P_{hom}) had been observed in a study that investigated suppressors overcoming isoleucine auxotrophy (Vapnek, Greer 1971b, 1971a). The so called *spr* pseudo-revertants overcame the lack of the threonine dehydratase IlvA by enhanced expression of the *hom* operon, leading to the unmasking of a minor threonine dehydratase activity of ThrC. Some of these mutations were located in P_{hom} and others were detrimental mutations in the putative regulatory protein encoding *thrR* gene. ThrR is an ACT-domain containing protein, which was therefore thought to be a regulatory protein with a possible small molecule effector (see 1.4.4). It was also previously shown that P_{hom} contained a CodY-responsive element, which could indicate an interesting competitive or synergistic regulation by these two (putative) regulators (Belitsky, Sonenshein 2013). Previous works in this lab had investigated, whether the protein ThrR was a regulator of the *hom* operon and found that it indeed bound to the promoters of *hom* and *thrD*, aspartokinase III (Rosenberg et al. 2016). Furthermore, the existing EMSAs and LacZ assays suggested that threonine was an effector of the ThrR activity, which would make sense, because this would establish a feedback inhibition loop leading to the inhibition of threonine synthetic genes when threonine is abundant. As part of this work, this regulatory relationship between threonine and other amino acids and ThrR and its regulatory activity on the *hom* operon was investigated.

It was known that ThrR can bind DNA suggesting that it would oligomerize in the manner that many DNA-binding proteins would do. To visualize this oligomerization in solution and *in vitro*, the purified protein was subjected to SEC-MALS and a crosslinker experiment. The ThrR protein was overexpressed in *E. coli* BL21 (DE3) from the existing plasmid pBP323 encoding the N-terminally strep-tagged version (Rosenberg et al. 2016). The cultures were grown to an OD_{600} of 0.8 and were induced by addition of IPTG for 3 hours at 37°C. Subsequently, the protein was purified by affinity chromatography using a strep-tactin Sepharose matrix and elution with desthiobiotin. Figure 5 shows that ThrR forms tetramers in solution and dimers *in vitro*, suggesting the existence of a dimer of dimers. It however remains unknown, which of the oligomeric states exists in presence or absence

of DNA binding. In the following, existing EMSAs of purified, strep-tagged ThrR with wildtype P_{hom} and addition of threonine were repeated and in contrast to the previous results they showed no effect of threonine on the DNA binding capability (Figure 6A). Furthermore, the suppressor mutations C26T and T9C (from *spr8* and BV708, respectively) in the promoter regions did show an influence on the expression of the *hom* operon and on the binding capability of ThrR in previous experiments. But again, in contrast to this the new data did not show any effect on the capability of ThrR to bind to its operator (Figure 6B).

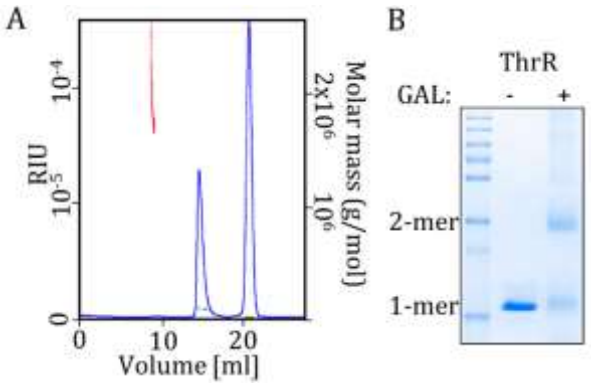

Figure 5: ThrR likely forms a dimer of dimers. (A) SEC-MALS was performed with purified strep-tagged ThrR. RIU stand for the differential refractive index units. The peak eluting at ~15 ml represents a size of ~80 kDa, which corresponds to a tetramer of the strep-tagged ThrR. No dimers were observed in this experiment. (B) The crosslinking experiment was carried out with the same batch of protein after SEC and showed a distinct band at ~18 kDa in the absence of GAL and an additional band at 35 kDa in presence of the crosslinker. This size corresponds to a dimer. No tetramers could be detected in this experiment, possibly due to the cooking step in the protocol. A 12% PAA, SDS gel with 1kb ladder was subjected to PAGE and strained with Coomassie as described in the methods section.

These data indicated that the regulation by ThrR might either not be dependent on threonine, or that the *in vitro* approach was not suitable to show this relation because an adapter protein or second effector was necessary to convey the regulatory effect. To assess whether ThrR was directly binding threonine, an ITC experiment was performed with ThrR in combination with threonine and its catabolic product 2-oxobutanoate (2-OB) (Figure 7, 2-OB not shown). These data showed no evidence for any interaction between the protein and its suspected effector threonine or 2-OB, leaving only the conclusion that the effector must be a different small molecule or a protein, which itself might bind threonine.

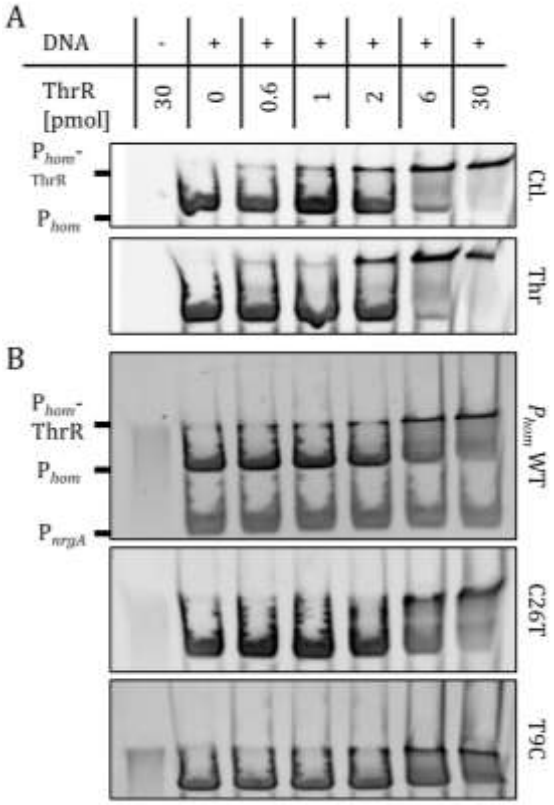

Figure 6: The DNA-binding ability of ThrR is not threonine-dependent. (A and B) EMSAs were carried out with purified, strep-tagged ThrR expressed from pBP323. In all conditions with DNA, P_{hom} (amplified with primers MT24/MT25) and P_{nrgA} (negative control, amplified with primers CD1/CD2) were added to a final amount of 1 pmol. The negative control was always added to the reactions and run on the same gel but was cut out to save space; no shift of the negative control was observed in any of the experiments conducted. The reaction volume was 20 µl. (A) P_{hom} shifts with addition of 6 pmol of purified protein, regardless of the mutations in the promoter sequence. (B) EMSAs in the presence and absence of 0.5% threonine (Thr). The control shifts at approximately 2 pmol ThrR, which is earlier than in A. This is likely due to the use of newly purified protein and promoter DNA batches and the error margin of the Nanodrop and Bradford quantification. However, the addition of threonine to the reaction mix had no effect on the shift of the DNA-ThrR complex.

This lead to the hypothesis that not threonine, but a compound up- or downstream in the biosynthetic pathway (see 1.4.4) was the effector of ThrR. First, the transcriptional activity of P_{hom} in the presence of members of the aspartate derived amino acids pathway (aspartate, homoserine, threonine, isoleucine, and valine) as well as other amino acids that might be regulatorily related to this pathway (serine, glycine, tryptophan, phenylalanine, lysine, cysteine, and methionine) was tested. To avoid toxic effects of the amino acids and by that a bias on the LacZ assay, the highest non-toxic concentrations of the amino acids for strain 168 were tested in growth experiments in CSE Glc medium (Figure S 1 and Figure S 2 ,Table 3).

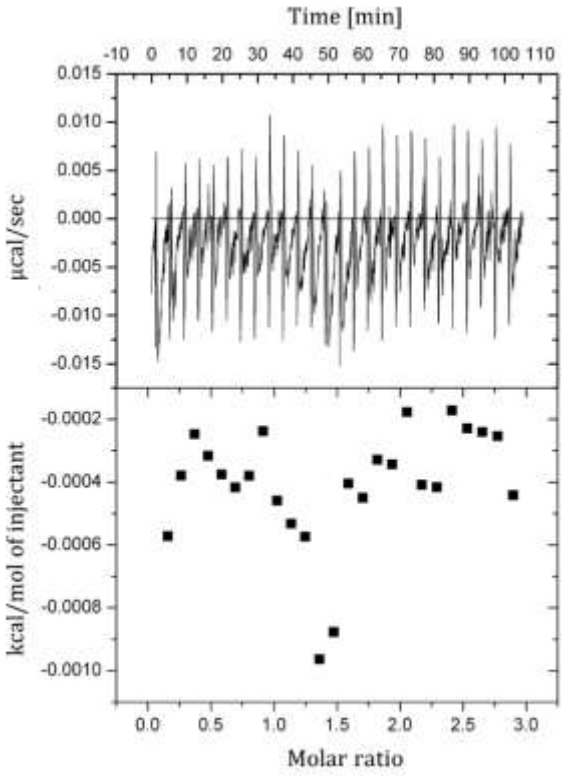

Figure 7: ITC of 10 µM ThrR with 150 µM threonine in reaction buffer (20 mM Tris, 150 mM NaCl, 1 mM $MgCl_2$). Injections of 14 µl of ligand solution with 1 µl s^{-1} and 300 seconds spacing at 20°C, a reference power of 12.5 µcal s^{-1} and 500 rpm stirring. The experiment showed no saturation curve and the amplitude of the peaks indicates no other than water-solvent interactions.

Notably, the threonine concentration that was used for previous LacZ assays was considerably higher than the identified highest non-toxic concentration (0.5% compared to 0.004%, respectively), which could explain the deviation of the results and is in good agreement with previous studies (Belitsky 2015; Lamb, Bott 1979a, 1979b). The identified concentrations were used to analyze the transcriptional activity of P_{hom} *in vivo* using LacZ assays. As depicted in (Figure 8A), many added amino acids had no strong effect on the transcriptional activity of P_{hom}. However, the transcriptional activity of P_{hom} was strongly inhibited by CAA, which acted as a positive control. Furthermore, homoserine and isoleucine had an inhibitory effect, lowering the activity to approximately 50%.

Table 3: Maximal non-toxic amino acid concentrations in CSE Glc medium.

Amino acid	Maximal non-toxic concentration [%]
Gly	0.5
Ser	0.008
Phe	>1.0
Trp	0.125
Tyr	0.063
Asp	>1.0
Val	>1.0
Thr	0.004
Thr*	>2
H-Ser	>1.0
Ile	>1.0
Lys	>1.0
Cys	0.5
Met	>1.0

*in combination with 0.005% Valine

Homoserine is a precursor of threonine and the product of Hom and Isoleucine is a downstream product of the same pathway, which can be won out of homoserine via threonine and 2-OB. Interestingly, the opposite effect was observed for lysine and cysteine, addition of which lead to a 2-3x upregulation of the operon. Lysine has previously been shown to induce *thrD*, which taken together with the new data could indicate a positive regulation of the complete aspartate derived amino acid pathway by lysine, diverting the aspartate derived metabolites from the diaminopimelate pathway into its direction. The regulation by cysteine, however, remains mysterious (see Figure 8). Next, the effect of ThrR and CodY on the regulation of P_{hom} was analyzed. Figure 8B shows that the deletion of *codY* alone lowered the transcriptional repression of P_{hom} by isoleucine. The deletion of *thrR* on the other hand lead to a deregulation of

P_{hom} in absence of any added amino acid (except glutamate) and in presence of homoserine and isoleucine (separate and in combination), cysteine, and threonine. The latter again showed no influence at all on the promoter activity, supporting the previous findings of this study.

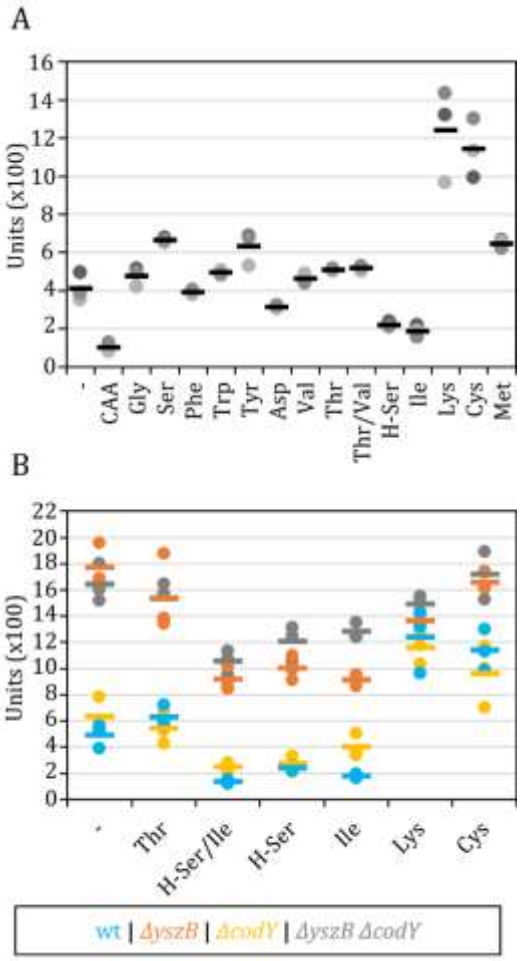

Figure 8: Activity of P_{hom} in wild type and mutant background with addition of amino acids. (A and B) Cells were grown in CSE Glc medium (containing glutamate) with addition of the named amino acids (three-letter-code, homoserine: H-Ser). Each dot represents one independent biological replicate, bars represent means of those. Wild type values of A and B are partly identical and from the same experiment. (A) The activity of P_{hom} is lower in the control condition with CAA, indicating a functional reporter system. Otherwise, homoserine and isoleucine inhibit activity while lysine and cystine enhance it. (B) P_{hom} is deregulated in the *thrR* mutant background, double mutants of *thrR* and *codY* behave the same except for conditions with isoleucine, where the mutations exhibit an additive effect. In the *codY* mutant background, repression by isoleucine is reduced.

However, the presence of isoleucine and/or homoserine still inhibited transcription in the *thrR codY* double mutant indicating additive inhibition by these regulators and an additional regulatory mechanism. Neither the deletion of *codY*, nor of *thrR* influenced the upregulation by lysine, indicating that its presence completely inhibited ThrR or it already activated the promoter to its maximum by a different mechanism. Furthermore, double deletion of *codY* and *thrR* had no additive effect on transcriptional regulation except for the condition with isoleucine. This and the lowered regulation in the *codY* single mutant suggest that the regulation by isoleucine is conveyed via CodY. Subsequently, the interaction of lysine and cysteine with ThrR was tested with the help of an ITC experiment. The experiment with lysine showed no interaction with ThrR (data not shown), while the experiments with cysteine showed ambiguous results depending on the absence or presence of a reducing agent (Figure S 3). Furthermore, the influence of isoleucine and homoserine on DNA binding of ThrR was tested in additional EMSAs, but also showed no influence. These investigations gave no evidence for the interaction of ThrR with any of the tested amino acids despite the obvious regulatory effects on the target.

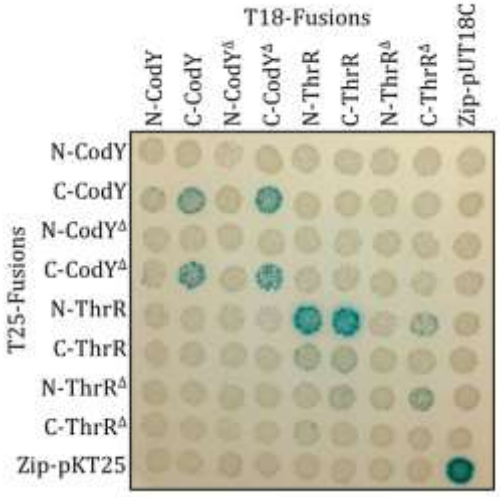

Figure 9: ThrR and CodY do not interact in a B2H. N- and C-CodY or ThrR mean that the adenylate cyclase domains T25 and T18 are fused to the N- or C-terminal end of the proteins, respectively. $ThrR^{\Delta}$ and $CodY^{\Delta}$ are variants lacking the DNA-binding domains. Only self-interactions could be observed, complementing previous findings of ThrR oligomerization.

As mentioned earlier, an alternative working hypothesis was that ThrR is not itself binding an effector but that it is regulated by a protein, which integrates the effector availability and passes the signal on. It had been described that P_{hom} is also bound by CodY, which made this protein a valid candidate for the interaction, although the LacZ assays suggested otherwise.

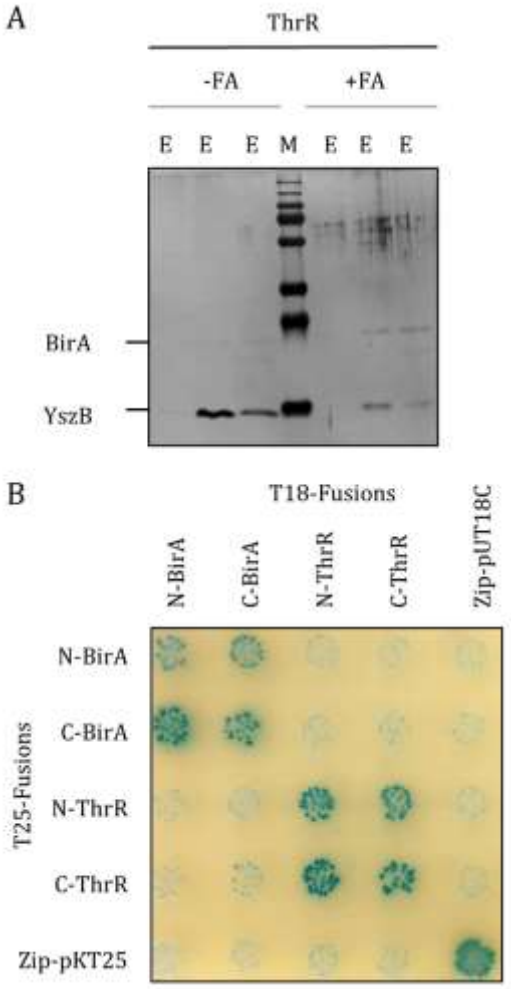

Figure 10: BirA could not be confirmed as a potential interaction partner. (A) A SPINE was performed with plasmid pBP620 in strain 168, the picture shows a silver stained SDS gel of the samples after cooking. Elution fractions one to three (E) were applied to the gel. The lower band at ~18 kDa corresponds to ThrR, the band at ~25 kDa was cut out and analyzed by cooperation partners in Greifswald and turned out to be BirA. (B) A B2H with BirA and ThrR. C- and N-BirA or ThrR stand for C- or N-terminally attached adenylate cyclase domains. Both proteins show strong self-interaction in this B2H but again no interaction with each other.

A B2H was performed investigating the interaction between CodY and ThrR. We tested the full-length proteins and truncated versions lacking the DNA-binding activity, to avoid polar effects and false positives in the host *E. coli* (Slack et al. 1995). In the process, plasmids pBP600 to pBP615 were constructed carrying the full-length and truncated versions of both proteins in the four B2H plasmid backbones pUT18, pUT18C, pKT25 and p25-N. As shown in Figure 9, both CodY and ThrR interact with themselves but not with each other in this assay. For CodY the self-interaction is independent from the truncation which is not surprising because the truncation is very small. In the case of ThrR, the self-interaction of the truncated version is strongly reduced, indicating an involvement of the DNA-binding domain, which has been completely removed. The self-interaction of ThrR complements the crosslinking and SEC-MALS data that showed a multimeric complex. In the following, an untargeted approach was chosen to take all possible interactions into account. A SPINE was performed with ThrR. In the process, the two genes encoding the full-length versions and the truncated CodY and ThrR were introduced into the SPINE vectors pGP380 and pGP382 yielding plasmids pBP616 to pBP623. Figure 10A shows the silver-stained SDS gel of the experiment with the N-terminally tagged version of ThrR. The band at ~25 kDa was cut out and the protein was analyzed via GC MS/MS by cooperation partners in Greifswald (Table 4). BirA was clearly the most abundant protein and also much more abundant in the samples without the crosslinker formaldehyde (FA), which could indicate a specific interaction between BirA and ThrR (Herzberg et al. 2007).

Table 4: Proteins in the investigated gel piece identified by mass spectrometry (Cooperation partner in Greifswald)

Accession Number	Mol. Weight	MS/MS Count	
		-PFA	+PFA
birA|BSU22440|	36 kDa	41	11
rok|BSU14240|	22 kDa	7	
rpsC|BSU01220|	24 kDa	5	6
yceC|BSU02890|	22 kDa	3	2
yceE|BSU02910|	21 kDa	2	2
atpD|BSU36810|	51 kDa	2	

The interaction between these two proteins was peculiar, since BirA is a trigger enzyme involved in biotin and not amino acid metabolism. It acts as the essential biotin ligase transferring the cofactor to the ACCase subunit AccB and at the same time as a repressor for the biotin biosynthetic *bio* operon in presence of a surplus of biotin. However, biotin is bound to lysine in its target proteins and lysine has been shown to regulate the target of ThrR above. This might indicate a role in the recycling of lysine from biotinylated proteins as has been described in *Enterococcus faecalis* (Koivusalo et al. 1963). A B2H to investigate the interaction between full-length BirA and ThrR was performed. Most plasmids for this study were present, however two versions of the *birA* plasmids were not available yet, so it was cloned into pUT18 and p25-N producing plasmids pBP624 and pBP625. The experiment yielded no evidence for an interaction between the two proteins (Figure 10B), but only strong self-interactions for both proteins.

To conclude, ThrR is a regulator of the threonine synthetic *hom* operon, but the mechanism of regulation and possible effectors remain unknown (see 4.2).

3.1.2. Adaptation to toxic amounts of pyridoxal

As previously introduced, vitamin B6 can be toxic due to its reactivity, which naturally poses a problem for an industrial production by fermentation. It is to date unknown, whether PL is as toxic as the phosphorylated PLP and whether the toxicity takes effect only inside the cell or also at the membrane or cell wall. To elucidate this, it is imperative to find the yet unknown importers and exporters of the B6 vitamers and the dedicated phosphatases, which ensure retention of the imported vitamers (see 4.5). It was hypothesized that cultivation of *B. subtilis* in presence of toxic PL amounts would lead to the emergence of suppressor mutants which mutated the unknown import systems for the B6 vitamers, the B6 kinase encoding *pdxK* gene or deregulate the unknown phosphatases. The identification of the unknown transporter systems could help the construction of a versatile production strain circumventing toxicity by either accomplishing product resistance or changing cultivation and production conditions to avoid accumulation of a toxic product. Here, the toxicity of vitamin B6 for *B. subtilis* and its mode of action were investigated.

First, the inhibitory concentration for growth of the *B. subtilis* wild type strain SP1 was determined.

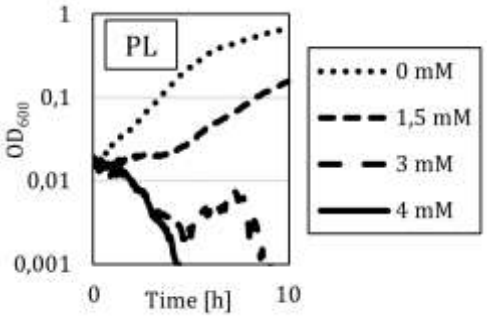

Figure 11: Growth of *B. subtilis* SP1 is inhibited by millimolar amounts of PL. Cells were grown in C Glc medium supplemented with no to 4 mM PL and incubated at 37°C with agitation. In absence of externally added PL, the cells proliferate normally, while already addition of 1.5 mM PL slows growth down and 3 mM PL inhibit growth completely. Values are means of technical triplicates.

As depicted in Figure 11, SP1 was grown in minimal medium containing different amounts of pyridoxal (PL). 1.5 mM PL were detrimental for growth of the strain, and 3 mM PL completely inhibited growth. Subsequently, different amounts of strain SP1 were spread on minimal medium with and without toxic amounts of PL. As shown in Figure 12, SP1 formed suppressor mutants resistant to 3 mM PL and which themselves and formed satellites. When two suppressors and a group of satellites were transferred to fresh minimal medium with 3 mM PL, the suppressors could grow normally but the satellites formed new suppressors. Moreover, the clones formed by the satellites predominantly grew in proximity of the suppressors (Figure 12). Two suppressor clones were picked, streaked out under the same conditions and they were designated as BP924 and BP925. To check, whether the resistant strains grew better than the SP1 wild type, a growth experiment in liquid minimal medium and a drop dilution experiment with and without toxic amounts of PL were carried out. In the experiment in liquid medium also PN was tested in order to verify the reports that PN, in contrast to PL, is not a toxic vitamer (Prunetti et al. 2016).

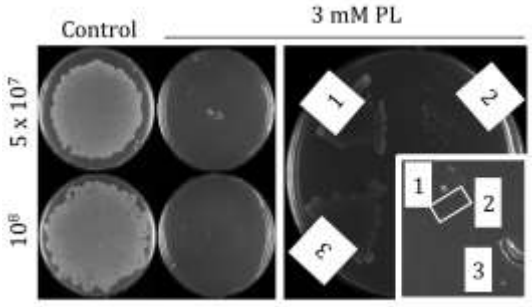

Figure 12: *B. subtilis* SP1 forms suppressor mutants on C Glc minimal medium containing 3 mM PL. Interestingly, satellite colonies arise around the suppressors. The suppressor mutants (1 and 3) can be restreaked on minimal medium containing 3 mM PL, but the satellite colonies (2) themselves form suppressors and can be fed by the suppressors. This indicates that the resistant clones secrete something that allows the satellites to grow. Numbers on the left depict plated cells. The inlay is a magnification of the plate with 3 mM PL and 5×10^7 cells, from where the suppressors were picked.

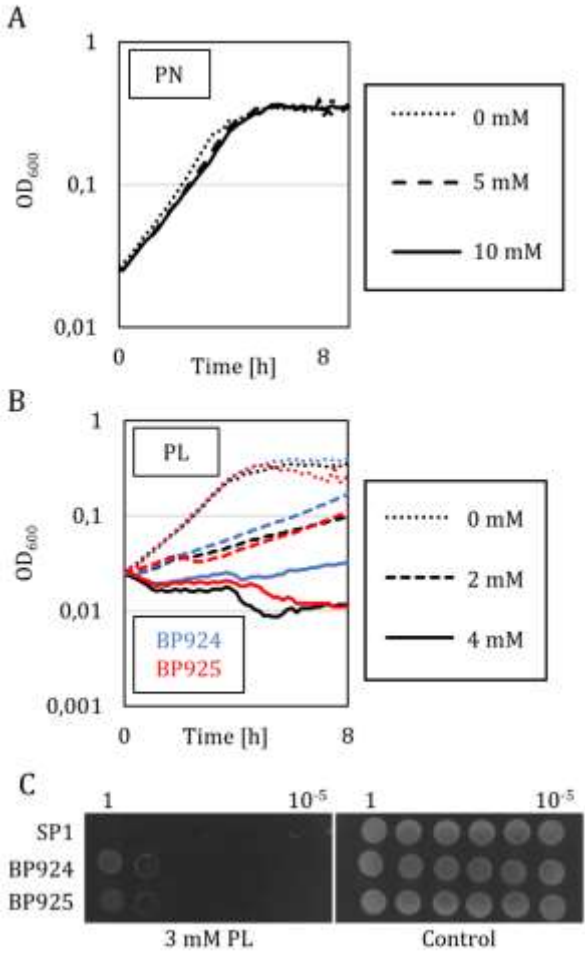

Figure 13: PL is toxic, while PN is not and the suppressor mutants are more resistant than SP1 on solid medium, but not in liquid medium. (A and B) Strains were grown in C Glc minimal medium with addition of different amounts of PN and PL. Values are means of technical triplicates. (A) PN has no effect on growth of SP1 up to a concentration of 10 mM, indicating that this compound is not toxic. (B) In liquid medium, no difference of growth is observable between the suppressor mutants BP924, BP925 and SP1 (black). All strains grow normally without PL, are impaired in growth with 2 mM and completely stop growth in presence of 4 mM PL. (A and B) Values are means of technical triplicates. (C) Drop dilution assay of SP1, BP924 and BP925 on solid C Glc medium with and without 3 mM PL. All strains grow the same on C Glc minimal medium but the suppressors BP924 and BP925 grow better on 3 mM PL. Cells were resuspended to an OD_{600} of 1 and serial dilutions were made in 10^{-1} steps, the highest dilution being 10^{-5}. 10 µl of each dilution was dropped onto the plate.

Figure 13 shows that PN indeed has no toxic effect even when present in concentrations up to 10 mM. Furthermore, the suppressor mutants grew better than the wildtype on solid minimal medium with 3 mM PL, but showed no enhanced resistance in liquid medium. This difference might be related to the ability to form satellites and the different diffusion conditions in liquid medium compared to solid agar plates. The genomic DNA of BP924 and BP925 was isolated and subjected to illumina sequencing by the Göttingen Genomic Laboratory (G2L). The strains acquired a point mutation in the promoter of the biotin biosynthetic operon (P_{bioW}) 96 bases upstream of the *bioW* start codon, in the sublancin 168 exporter encoding gene *sunT* and in a non-coding region 24 bases downstream of the tRNA (m7G46) methyltransferase encoding gene *trmB* (Table 5).

The mutation in *sunT* leads to a frame shift in the first third of the reading frame, very likely causing a non-functional protein. The expected consequence would be the lack of sublancin 168 production and an accumulation of SunA, the precursor of the lantibiotic. The mutation downstream of *trmB* might very well be inside the terminator region and could have an influence on the expression of *ytnP* or *malS*, which are both located downstream without additional terminators. Furthermore, according to expression data, a terminator sequence is located inside the *trmB* reading frame. This could mean that the mutation has influence also on the expression of *trmB* itself due to the strengthening or weakening of a premature termination during transcription. Lastly, the mutation in P_{bioW}, which has been found in both isolated suppressors indicated that the amount of biotin synthesizing proteins might have an influence on the resistance to PL.

Table 5: Mutations acquired by PL-resistant clones.

Gene	Position*	Sequence change
	BP924	
P_{bioW}	3,095,526	A96G
sunT	2,269,015	ΔT449 (Stop)
	BP925	
P_{bioW}	3,095,526	A96G
T_{trmB}	3,059,523	ΔT24

* in NC_000964 (Barbe et al. 2009)

To check the P_{bioW} and T_{trmB} mutations, the promoter and terminator sequences of the wild type SP1 and the suppressors were fused to the *lacZ* gene constructs to assess the influence on transcription. The promoter of the *bio* operon was introduced into the vector backbone pAC7, producing pBP627 and pBP630 with the wild type and mutated promoter sequence, respectively. The wild type and mutated putative terminator of *trmB* was introduced into pAC6 with addition of the constitutive promoter P_a 5' of the terminator sequence and the resulting plasmids were designated pBP628 and pBP631, respectively (Gundlach et al. 2017). The constructs were used to transform *B. subtilis* SP1 and recombination was confirmed with an amylase test on starch agar, resulting in strains BP968, BP969, BP971, and BP972. The LacZ assays revealed that the mutation downstream of *trmB* had no influence on the expression of the reporter. However, the mutation in P_{bioW} had a strong deregulatory effect (Figure 14). This indicates that a stronger expression of the biotin synthesizing enzymes has a protective effect. It was unclear whether this mutation was beneficial because the cells were lacking biotin under high PL stress or whether they needed the proteins for a different function, e.g. to bind large amounts of PL to lower the concentration of vitamers in solution. It was also possible that the *bio* operon was repressed by PL or that the repressor BirA was regulated by PL. To check the latter hypothesis, another reporter fusion was constructed with the *birA* promoter in pAC7, giving pBP629. The plasmid was also used to transform SP1, resulting in strain BP970. Strains BP968 to BP972 were grown in presence of low concentrations of PL and the highest non-toxic concentration of isoniazid (IA), a B6 antagonist that depletes vitamin B6 from the cytosol, and the β-galactosidase activity was measured. The toxicity of isoniazid was tested in a previous experiment (Scherr, Rafelson 1962) (Figure S 4). However, the activity of P_{birA} was very low and the addition of 10 µM PL or IA had no influence on the expression of any of the promoters (data not shown). It was unknown what effect toxic amounts of PL would have on the expression. This condition was tested as part of a master thesis by Oğuz Bolgi and showed, that the addition of toxic amounts of PL had a deregulatory effect on P_{birA} and an inhibitory effect on P_{bioW}, indicating that the expression of the repressor of the *bio* operon was enhanced under these condition, leading to a detrimental inhibition of biotin synthesis (Bolgi 2017).

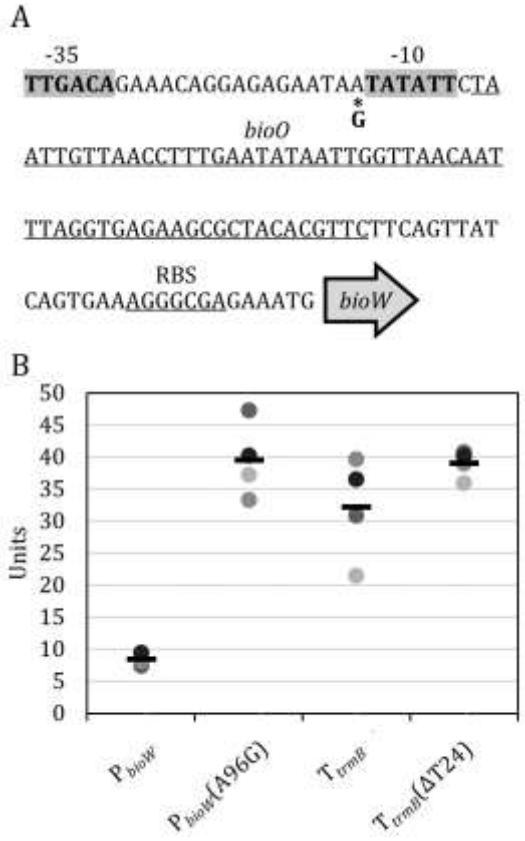

Figure 14: A mutation in the promoter region of *bioW* leads to deregulation of the promoter. (A) Scheme of the promoter sequence and annotated features. The -35 and -10 regions of P_{bioW} are shown bold and with grey background. The underlined bases are the putative BirA binding site (*bio* operator, *bioO*), which was determined by sequence similarity with the described *bioO* from *E. coli* (Bower et al. 1996) and the ribosomal binding site (RBS). The identified mutation is shown in bold with an asterisk. (B) β-Galactosidase activity assay with the wildtype and mutated sequences of P_{bioW} and T_{trmB}, showing that the mutation in the terminator region has no effect on expression of the marker gene, while the *bioW* promoter is approximately 4x more active in presence of mutation A96G. Dots are independent biological replicates, bars are the means of those.

Taken this hypothesis, external addition of biotin should also allow for growth with 3 mM PL. Therefore, SP1 was plated on minimal medium plates and drops of biotin and casamino

acids (CAA) were placed on the same plate. CAA served as a positive control because it has been previously described that PL interacted with amino acids, which should lower the amount of PL in solution and thus its toxicity (Choi 2015). Also as part of the mentioned master thesis, it could be shown that the external addition of CAA or biotin has a protective effect in solid and liquid medium with addition of toxic PL amounts, supporting the work hypothesis (Bolgi 2017).

Summarizing it can be said that *B. subtilis* can grow in presence of 3 mM PL with the external addition of either amino acids or biotin; or mutations deregulating the biotin synthesizing protein encoding genes. This connection between the two vitamins is completely novel and might indicate an unknown regulatory crosstalk worth investigating (see 4.4).

3.2. Optimization of B6 production by targeted evolution

As introduced and shown previously, the fermentative production of vitamin B6 was until now not possible due to obstacles mainly related to pathway and product toxicity. The reasons for this were still under investigation and even if we knew everything about the interference between host and heterologous pathways, the optimization would be a complicated genetic engineering process. Therefore, this task was given into the hands of evolution. To do so, an evolutionary pressure needed to be created forcing the strains to accumulate the desired mutations. The desired mutation targets were, of course, the genes conferring resistance to the metabolites and products of the pathway, but also the import and export, the channeling, and the modification of the related compounds. Little is known so far about B6 metabolism apart from the synthetic route and many targets of the cofactor (Rosenberg et al. 2017a). E.g. the bacterial transporters as well as related transcription factors have not been identified or characterized yet. Therefore, the desired approach should provide answers to two questions: (1) what do we need to do to optimize the production of vitamin B6, and (2) what are the yet unknown players in B6 metabolism and related pathways? The methods presented here were to prove the principle and concept of the feeding experiments and should be used to scale the process up, e.g. through targeted evolution in multiwell plates and selection for speed of growth.

3.2.1. Co-culture of mutually dependent B6 producers

As described previously, the existing production strains for vitamin B6 have reached obstacles as the unbalanced expression of the synthetic genes and the toxicity of metabolites and products (see 1.3). As an approach to optimize the expression of the pathway genes and the modification as well as transport of the metabolites and to find novel interaction partners of B6 metabolism, the existing production strain was divided into several strains expressing partial pathways.

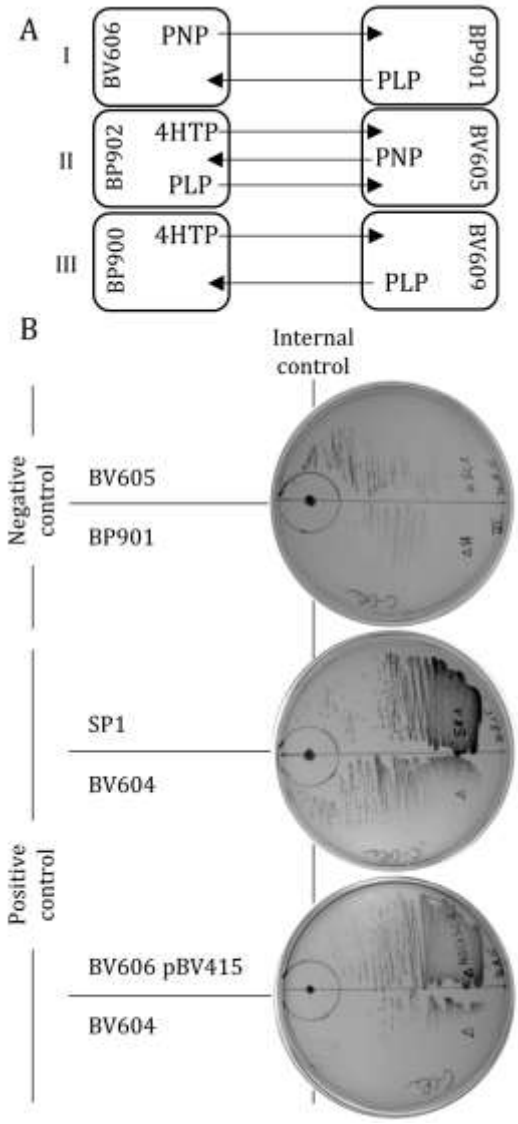

Figure 15: Experimental design and proof-of-concept of mutually dependent strains. (A) Combinations of mutually dependent strains exchanging the shown metabolites. All strains were constructed in a Δ*pdxST* background. No. I is a combination of BV606, which produces PN and BP901, which converts PNP to PLP. No. II is a combination of BP902, which produces 4HTP and can convert PNP into PLP and BV605, which can convert 4HTP to PNP. No. III is a combination of BP900, which can produce 4HTP and BV609, which can convert 4HTP to PLP. (B) Proof-of-concept. The strains were streaked out on C Glc minimal medium next to each other and 5 µl of a 10 mM PL solution was dropped onto one side of the plate as an internal positive control (black dot). The combination of BV605 and BV604 serves as a negative control and these strains only grow near to the drop of PL. Both the wild type SP1 and the production strain BV606 pBV415 can feed the auxotroph BV604.

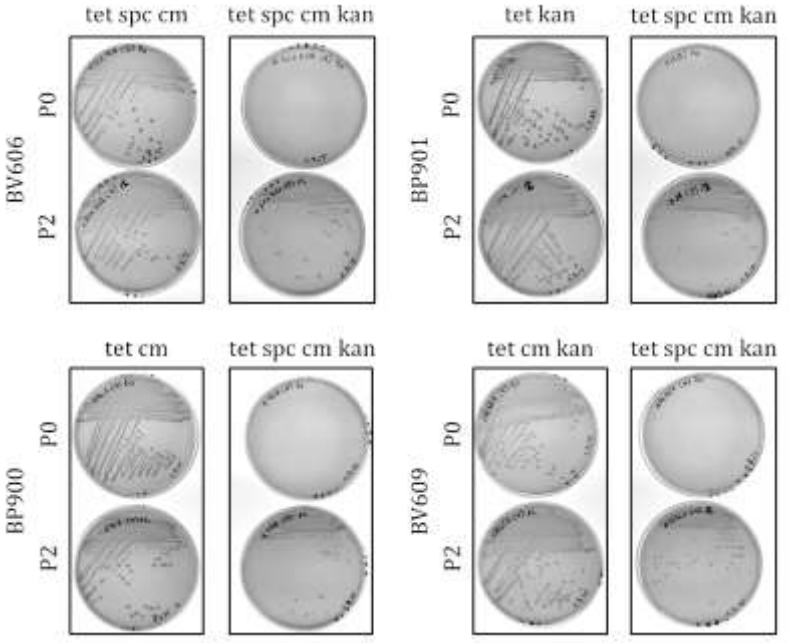

Figure 16: Co-cultured strains exchanged genetic information. The co-cultures were streaked out on SP plates containing antibiotics selecting for the specific strains and single clones were streaked on the SP plates shown in the figure, containing the depicted antibiotics (tetracycline, tet; spectinomycin, spc; chloramphenicol, cm; kanamycin, kan). When the cultures were streaked before the first passage, the strains could be separated from each other and could only grow on plates containing the antibiotics selecting for their respective resistance markers. After two passages however, the strains could not be separated anymore, and the clones could also grow on plates containing all antibiotics. This indicated that the strains acquired all B6 synthetic constructs recombining to BV606 pBV415 after only two passages.

These strains were all constructed in a Δ*pdxST* background making them B6 auxotrophs that could only live in coculture exchanging the metabolites and the product, PL. Figure 15A depicts the experimental design with the strain combinations exchanging different metabolites to thrive in minimal medium without external PL. The co-cultures of these strains were to be passaged to allow the strains to accumulate mutations beneficial for growth together. This way we would create evolutionary pressure towards the optimization of the pathway and possibly the transport of the metabolites. The strains were constructed using the existing production strain BV606 (Δ*pdxST amyE::SC1 sacB::SC2**) encoding the pathway genes *epd*, *pdxB*, *serC* (synthetic construct 2*, *SC2**), *pdxA*, and *pdxJ* (synthetic construct 1, *SC1*), and the plasmid pBV415 carrying *pdxH* and the kanamycin resistance gene *kan* (Commichau et al. 2014). The resulting strains were BP900 (Δ*pdxST sacB::SC2**), BP901 (Δ*pdxST* pBV415), and BP902 (Δ*pdxST sacB::SC2** pBV415). Furthermore the existing strains BV604 (Δ*pdxST*), BV605 (Δ*pdxST amyE::SC1*), BV609 (Δ*pdxST amyE::SC1* pBV415) were used. The different constructs were transferred into the SP1 background using genomic DNA and successful transformation was checked on antibiotic plates also ensuring that only the desired constructs were transferred. Initial proof-of-concept experiments showed that the strains could indeed feed each other and live in co-culture on plates (Figure 15B). In the following the combinations I and III were inoculated from cryo-cultures as co-cultures in LB complex medium to an OD_{600} of 0.1 (0.05 per strain) and the cultures were passaged to an OD_{600} of 0.1 when grown. The combination of BV605 and BP900 served as a negative control because this combination lacks *pdxH*, the gene encoding the pyridoxine oxidase. The combination of BV606 pBV415 and BV604 served as a positive control (Table 6).

Table 6: Growth of co-cultures in complex medium. Values are the optical density at 600 nm measured after growth overnight (P0 and P1) and after 8 hours (P2).

Culture	P0	P1	P2
I	1.3	2	1
III	1.2	2.1	1
Pos. Ctrl.	1.9	2.4	0.9
Neg. Ctrl.	1.6	1.5	0.2

All co-cultures grew to OD_{600} 1-2 overnight, indicating that the medium or the cells contained sufficient residual vitamin B6. Only in

P2 the negative control slowed down growth showing that the combination of mutually dependent strains allowed growth, while the combination of strains that lack one of the B6 biosynthetic steps could not do so.

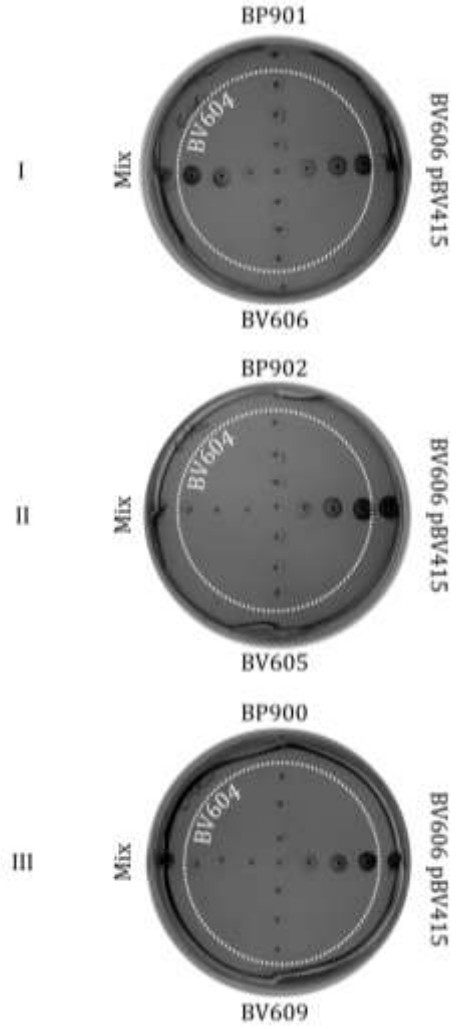

Figure 17: B6 production test on C Glc minimal medium plates. Precultures of the used strains were mixed prior to drops were applied to the medium. Combinations I-III were tested. BV604 was used as an indicator for B6 production (white circle). On all plates, the positive control BV606 pBV415 allowed growth of BV604. Combinations I and III also allowed growth of the same, I almost as strongly as the control. Combination II and the single strains did not allow for growth of BV604. 10 µl of the cultures were dropped onto the plate.

The cultures were streaked out on antibiotic medium to separate and single out the strains before passaging. These plates showed that the strains had very quickly (latest after two passages) exchanged genetic information and exchanged the genes necessary for *de novo* synthesis of vitamin B6 (Figure 16).

Additionally, the combinations I-III were dropped onto a plate with the auxotrophic BV604 to show the production of vitamin B6 on plate. BV606 pBV415 served as a positive control, the single strains as negative controls. The strains were grown in complex medium precultures overnight and then mixed before being dropped on the plate. As shown in Figure 17, combinations I and III could feed the auxotrophic strain BV604, while combination II and the single strains could not do so. To check, whether this feeding is also due to genetic exchange as shown in the experiments in liquid medium, mixed cultures were picked and streaked onto SP medium with antibiotics selecting for all antibiotic markers of the constructs. Figure 18 shows that combinations I and III both formed single clones that could grow on those plates, indicating that also on plate, the strains rapidly exchange the genes for *de novo* synthesis of vitamin B6.

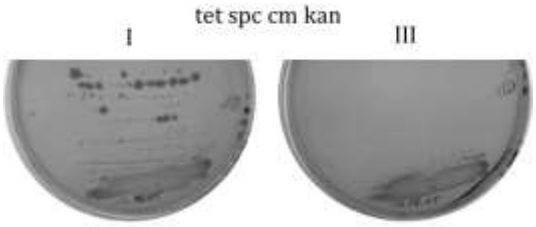

Figure 18: Growth of combinations I and III on SP medium selecting for clones that combined all B6 synthetic constructs by genetic exchange. Combination I showed clearly visible single clones, combination III showed several small single clones.

Therefore, the observed growth of the strains in co-culture is likely not due to successful feeding, but due to the acquisition of the whole recombinant pathway necessary for the *de novo* synthesis of PLP. This, admittedly predictable behavior showed that *B. subtilis* has impressive capabilities to exchange genetic information in very short time. This fact is further investigated and optimized for usage as a transformation method in 3.3. It also showed that this approach could only be successful when the strains were stripped of the capability to take up DNA, preventing the recombination of the constructs. Therefore, the *comG* operon was deleted in all

used strains by insertion of a phleomycin cassette. The cassette was first inserted into SP1 using LFH, resulting in strain BP914. The construct was amplified using primers JR126, JR127, JR129, and JR130 as well as CZ126 and CZ127. Furthermore, the plasmid based *pdxH* construct was replaced by a genomic construct (described in more detail in 3.5.2). The *pdxH* gene from *E. coli* DH5α in combination with P_a, a synthetic, constitutive promoter (Gundlach et al. 2017), was inserted into the *aprE* locus of SP1 via a marker-less LFH giving strain BP911.

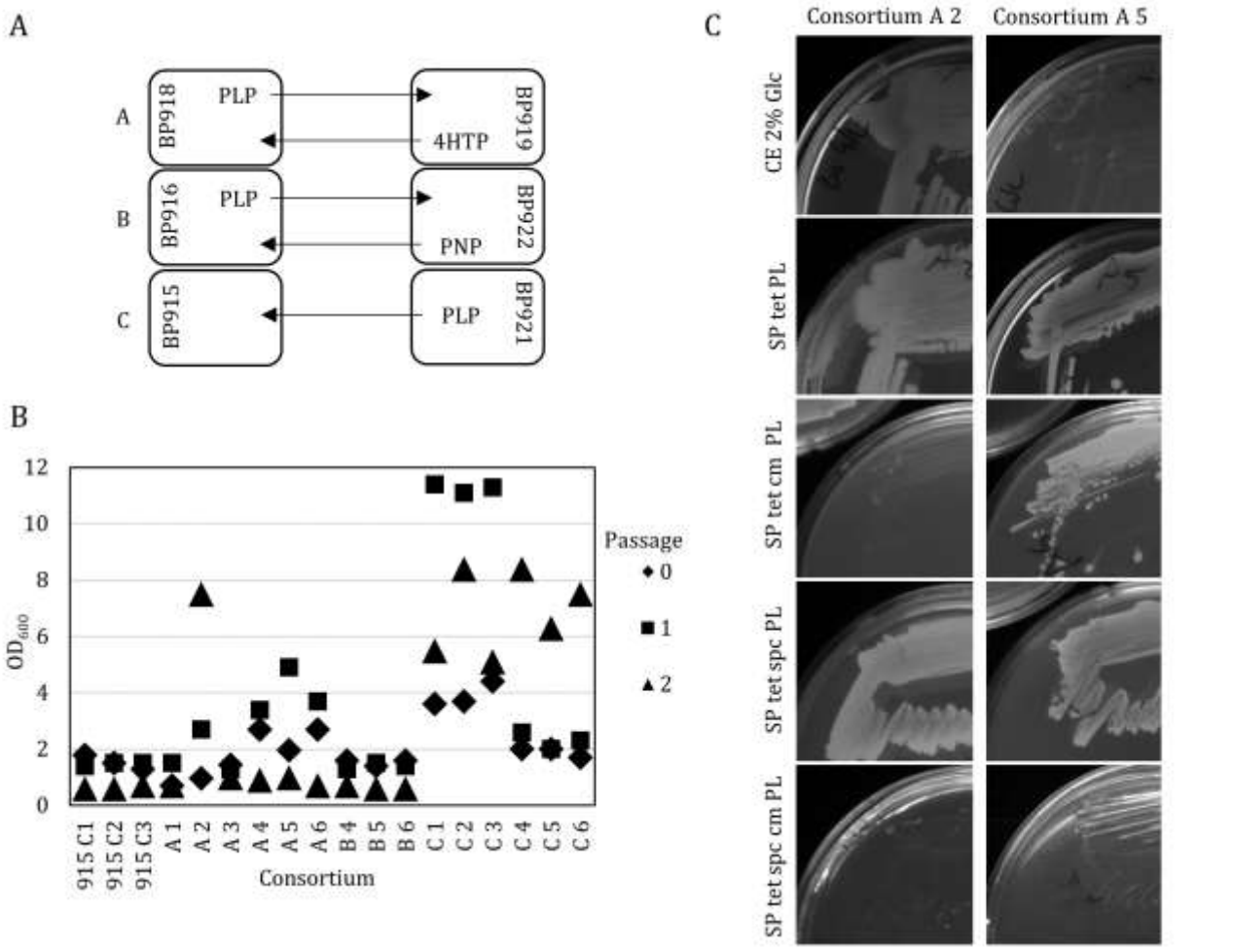

Figure 19: Non-competent consortia do not exchange genomic information but also cannot feed each other. (A) A scheme illustrating the strains in the different consortia and the metabolites they exchange. (B) OD_{600} of consortia and controls measured before passaging in a 24-hour interval. The consortia were inoculated as triplicates with 0.1 µM PL in passage 0, PL amount was halved with every passage. A 1-3 and C 1-3 originated from a previous experiment and were passaged twice before, BP915 served as negative control. The measured optical densities of the negative control as well as consortia A and B were lower with every passage except for A 2, which grew like the positive controls A 4-6. The positive controls C 1-3 reached optical densities around 4 to 12, C 4-6 reached higher optical densities with each passage. (C) Evaluation of consortium A 2 in comparison to A 5. Consortium A 2 could grow on C 2% Glc minimal medium independent of PL, while consortium A5 could not. The SP plates checking for antibiotic markers showed that neither consortium exchanged genetic information (no growth on SP tet spc cm PL, which allows growth only for strains with antibiotic markers of BP918 and BP919). Furthermore, consortium A 5 contains both strains BP918 (Δ*pdxST::tet amyE::SC1 spc aprE::pdxH* Δ*comG::phleo*), which could grow on SP tet spc PL, and BP919 (Δ*pdxST::tet sacB::SC2* cat* Δ*comG::phleo*), which was able to grow on SP tet cm PL. Consortium A 2 however, only consisted of BP918. No growth was observable on SP tet cm PL.

The successful integration was ensured by selection on C Glc minimal medium containing PN, which only allows growth in presence of PdxH. Due to the fact that the deletion of the *comG* operon would abolish the competence of the strains, the *pdxH* construct was first introduced yielding strains BP912 (Δ*pdxST amyE::SC1 aprE::pdxH*) and BP913 (Δ*pdxST amyE::SC1 sacB::SC2* aprE::pdxH*). Afterwards the deletion of *comG* was transferred to the target strains. The resulting strains were BP915 (Δ*pdxST* Δ*comG*), BP916 (Δ*pdxST aprE::pdxH* Δ*comG*), BP918 (Δ*pdxST amyE::SC1 aprE::pdxH* Δ*comG*), BP919 (Δ*pdxST sacB::SC2** Δ*comG*), BP921 (Δ*pdxST aprE::pdxH amyE::SC1 sacB::SC2** Δ*comG*), BP922 (Δ*pdxST amyE::SC1 sacB::SC2** Δ*comG*). The combinations for the new co-cultures in liquid medium are shown in Figure 19A.

Consortium A was made up of the 4HTP producer BP919 and BP918, which had the capability to convert 4HTP to PLP. Consortium B consisted of BP922, which could convert PNP to PLP and BP916, which was a PNP producer. Consortium C was a positive control consisting of the producer strain BP921 and the auxotrophic strain BP915. BP915 alone served as a negative control. Preliminary experiments showed that the consortia would not grow in minimal medium when they were washed in CE Glc before inoculation, which was thought to be because the strains would need more time to acquire mutations that allow feeding in co-culture. Therefore, the experiment was started with the addition of 0.1 µM PL. The amount of externally added PL was halved with every passage. In anticipation that one of the strains in the consortia would start growth before the other and might use up nutrients before mutations that allow co-feeding occur, the minimal medium was modified. It was decided to use CE Glc instead of C Glc minimal medium to ensure sufficient amino acid and nitrogen supply. Furthermore, 2% glucose were added to the medium instead of the usually applied 0.5% to make sure that carbon supply was not limiting growth of the strains. The CE Glc cultures were inoculated from LB precultures to an OD_{600} of 0.1 (in case of combinations 0.05 for each strain each) and passaged to an OD_{600} of 0.1 every day. Triplicates of each consortium were used. It must be noted that the experiment was performed with fresh consortia and consortia from a previous experiment. A 1-3 and C 1-3 have previously been passaged twice and were plated on SP medium supplemented with tetracycline and PL prior to this experiment (data not shown). As shown in Figure 19B, the overall OD_{600} measured after 24 hours lowered every day for the negative controls as well as for consortia A and B. However, the positive controls consortium C 1-3, which consisted of the strains BP921 and BP915 reached very high optical densities of 4 to 12 after one passage indicating that none of the nutrients was limiting growth and that they were well adapted to the medium. In contrast, the fresh consortia C 4-6 grew to higher OD_{600} with each passage. Furthermore, consortium A 2 also reached very high optical densities, while the other replicates behaved like B and C. This could have indicated that the strains in this consortium became able to feed each other. To check this, the well-grown replicate A2 was streaked on SP plates with antibiotics selecting for strains BP918 (SP tet spc PL) and BP919 (SP tet cm PL) as well as on plates checking genetic exchange (SP tet spc cm PL). The strains were also streaked onto CE 2% Glc. This was done to check whether the strains could grow independently of externally added PL. As depicted in Figure 19C, the strains did not exchange genetic information showing that the deletion of *comG* had the desired effect. Consortium A 5, which showed decreasing growth in the passaging experiment, also showed no growth on CE 2% Glc minimal medium. Consortium A 2 could grow on this medium however, indicating again that the strains could exchange metabolites. Interestingly, the antibiotic marker plates showed that consortium A 2 was no consortium anymore but only consisted of BP918. This indicated that BP918 formed suppressor mutants that could grow independent of BP919 in the absence of externally added B6 and completely overgrew the consortium. A fact, which has also been observed for other strains carrying *pdxJ*, *pdxA*, and *pdxH* (see 3.4.1). Therefore, consortium A was considered unsuitable for co-culturing.

The remaining consortium B showed decreasing growth in the experiment shown in Figure 19B, indicating that no feeding occurred. It was hypothesized that the toxicity of 4HTP might be a reason. Therefore, the experiment was repeated with the addition of CAA. Amino acids

have been shown to be an antidote of 4HTP toxicity (Commichau et al. 2014). As depicted in Figure 20, the growth of the consortium again decreased over the passages until it completely stopped after P3. Peculiarly, the positive control consortium C again reached a very high OD_{600}. It was checked how the distribution of the strains in consortium C 2 was by plating on SP containing PL and tetracycline and on SP with chloramphenicol. The former should allow growth of BP915 and BP921, the latter of only BP921.

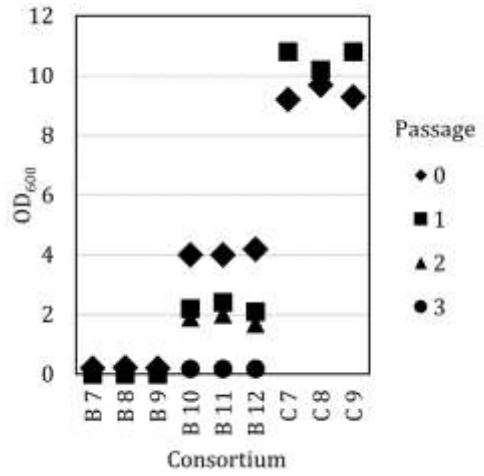

Figure 20: Consortium B was also not able to establish stable co-feeding. OD_{600} of consortia and controls measured before passaging in a 24-hour interval. The consortia were inoculated as triplicates. B 10-12 and C 7-9 were inoculated with 0.1 µM PL in passage 0, PL amount was halved with every passage. B 7-9 were cultivated without PL. C 7-9 again served as positive controls.

Before plating, samples of consortium C 2 were diluted 1 to 1,000,000 in 1X C salts. The experiment was carried out in triplicates and showed that the culture was made up almost exclusively of the producer strain BP921 (Table 7). The CFUs on the plates were almost identical, indicating that at least very most of the cells in the culture were of strain BP921. This hinted at inefficient feeding of PL in this consortium, because common knowledge about producer-consumer ("cooperator/cheater") relations indicated that the distribution should be exactly the opposite in the case of successful feeding (West et al. 2006).

Table 7: Composition of consortium C 2

Medium	SP tet PL			SP cm		
CFUs	81	70	83	66	64	120
Mean		78			83	

It was hypothesized that this was due to the absence of existing transport mechanisms for the heterologous products of the pathway and possibly inefficient secretion or uptake of PL from the medium (see 4.5). However, the passaged BP921 clones from the consortia might have acquired interesting mutations allowing for more rapid growth in minimal medium and more efficient production of vitamin B6. However, the passaged strains neither grew significantly better than the original strain, nor did they produce more vitamin B6 (Figure S 5). At this point it was decided that the co-feeding approach in liquid medium was not promising and was aborted in favor of a plate-based approach (see 3.2.2).

Taken together, when the DXP-dependent pathway was split into several parts and expressed in different strains of *B. subtilis* and these strains were cultured together, they rapidly exchanged the genetic information necessary to reestablish the complete biosynthetic pathway. This was an interesting outcome indicating that the combination of the pathway in one strain was favored over the division of labor between mutually dependent strains. When this problem was circumvented by deletion of the genes necessary to take up DNA from the medium, the strains did not exchange genetic information anymore. However, the strains also did not manage to establish a stable consortium, indicating that the necessary machinery is not present and not easily acquired by *B. subtilis* using the described approach (see 4.6).

3.2.2. Co-cultivation of strains exchanging B6 and amino acids

The previous approaches showed that strains expressing partial B6 pathways were not able to exchange the metabolites they were required to feed each other to form a stable consortium. It was hypothesized that the reason for this would be the absence of transport and modification

proteins and enzymes for the heterologous metabolites. Furthermore, some of the strains would form suppressors that could grow in absence of the other strain of the consortium leading to overgrowth. To circumvent these problems a consortium of strains exchanging endogenous products was designed (Figure 21A). The strains would exchange threonine and PL, for both of which transporters and enzymes should be present. Threonine is taken up by *B. subtilis* via the transporter SteT and possibly also by the isoleucine and valine transporter BcaP (Georgopoulos 1969; Reig et al. 2007; Belitsky 2015). For PL, the feeding has been shown during previous experiments (Figure 15). Furthermore, the deletion mutants should be stable since neither for the *pdxST*, nor the *thrC* mutant suppressors have been described previously to the best of knowledge. Strain BV604 was transformed using the genomic DNA of BKE32250 (Δ*trpC*) giving BP920 (Δ*pdxST* Δ*thrC*), whose genomic DNA was then transferred to BP913, producing BP962 (Δ*pdxST aprE::pdxH amyE::SC1 sacB::SC2** Δ*thrC*) (Boston Genetic Stock Center, Rudner, unpublished). BP962 was threonine auxotrophic but expressed the heterologous B6 pathway allowing it to feed PN and PL. The other strain in the consortium was constructed by transformation of BV604 with genomic DNA from BKE27910 (Δ*thrR*), giving BP908 (Δ*pdxST* Δ*thrR*) (Boston Genetic Stock Center) (Rosenberg et al. 2016). Strain BP908 is PL auxotrophic and lacks ThrR, which has been shown previously to inhibit threonine synthesis. It was therefore hypothesized that a *thrR* mutant would overproduce threonine. As shown in Figure 21B, the strains were streaked out on C Glc minimal medium plates next to each other. As negative control, a piece of agar was vertically cut out between the strains to prevent diffusion of PL or threonine through the agar. As a positive control for the different strains, the agar was cut out horizontally and threonine or PL were added to the different halves to allow growth of the different strains. The strains were streaked out as described and the plates were incubated at 37°C. The strains were able to grow without the addition of threonine or PL when the metabolites could diffuse through the agar. The negative control however, showed no growth indicating that the diffusion was required for growth under these conditions. Lastly, on the positive control plates, both strains could grow in presence of their respective auxotrophy marker and also allowed the other strain to grow under this circumstance. The strains were passaged when growth was observable by scraping of all cells off the agar and plating on fresh plates after resuspension in 1x C salts. Cells were always taken from the feeding plates and same amounts were applied to the three setups of feeding, negative control and positive control. The strains needed eight to 14 days to show growth, with no tendency to grow faster (Table 8). However, the qualitative determination of growth can be misleading because no quantitative measurements were performed.

Table 8: Days between passages of the BP962/ BP908 consortium.

Passage	0	1	2	3	4	5	6
Days	8	9	12	12	14	14	14

As shown in Figure 21B, growth of the strains was mostly observable in the central part of the plate where the strains were closest to each other, forming a "line of growth". This made sense, because at that position the concentrations of the exchanged metabolites would be highest. However, during some passages suppressor mutants of BP962 were observed that arose at a distinct range from the "line of growth". These suppressors, when streaked on C Glc in absence of BP908 or threonine, grew well and proved to be suppressors of threonine auxotrophy. Furthermore, they were streaked on SP plates containing erythromycin and lincomycin to check the presence of the *thrC* deletion cassette and grew on this selective medium (Figure 21C). The emergence of suppressor mutants that could grow independent of the consortium again made these strains unsuitable for the coculturing experiments and the possible up-scaling of the process. However, the suppressor mutants of the *thrC* deletion have not been observed previously to the best of our knowledge and might therefore be very interesting. As described above (3.1.1) (Vapnek, Greer 1971b, 1971a), cells with a deletion of *ilvA*, which encodes the enzyme directly downstream of ThrC in the isoleucine

biosynthetic pathway could overcome isoleucine auxotrophy by the overexpression of *thrC*, showing the potential of closely related enzymes to take over the function of each other. Therefore, it was hypothesized that the suppressor mutants might have acquired mutations in the operons related to threonine and isoleucine synthesis or their regulators, allowing threonine production by a mechanism analogous to the one of the *spr* suppressors. Three suppressors were isolated and designated as BP1005, BP1006 and BP1007. The loci of *thrR*, P_{hom}, P_{ilvA}, and *ilvA* were checked by sequencing, but no mutations could be observed (data not shown). This fact indicated that the mechanism of the suppressor mutations might be a different one than in the *spr* suppressors and might be worth investigation (see 4.7).

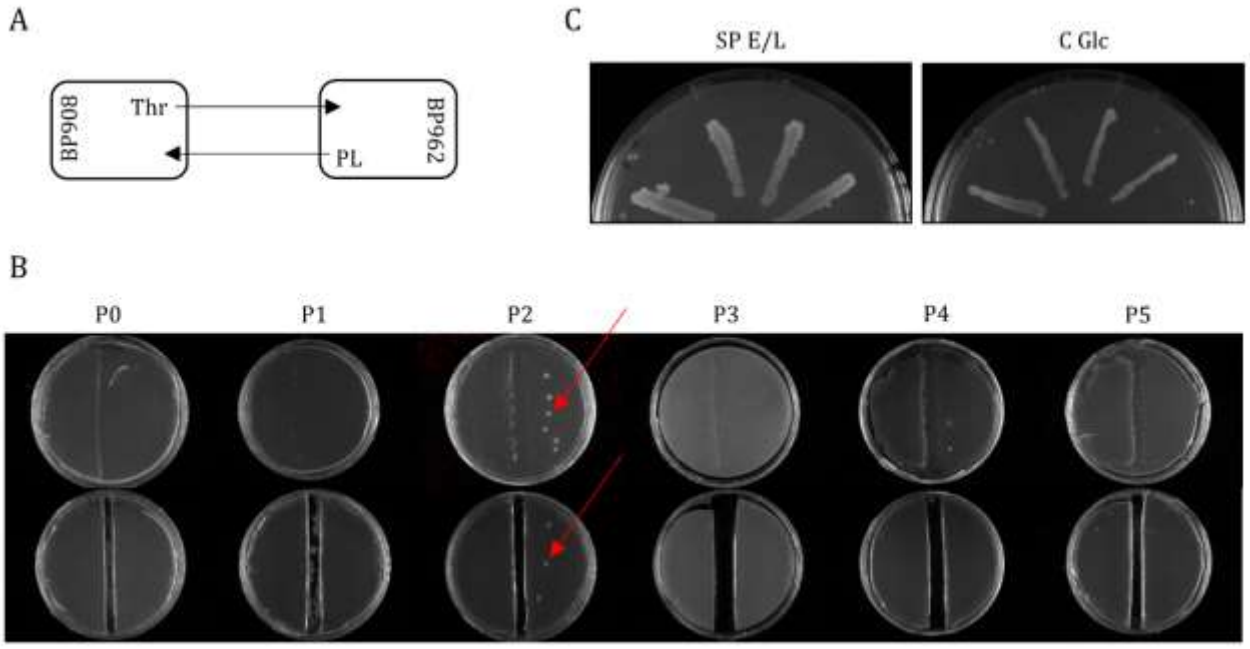

Figure 21: The consortium exchanging threonine and PL could thrive over several passages, but suppressors of threonine auxotrophy jeopardized the experiment. (A) Scheme illustrating the pair of strains exchanging threonine (Thr) and PL. (B) Pictures of the plates before passaging. Shown are the feeding plates (top) and the negative controls (bottom), BP908 is always located on the left-hand side, BP962 on the right-hand side. Suppressors allowing growth of BP962 on its own were observed starting with P2 (red arrows). (C) The suppressor mutants were streaked out on SP agar with erythromycin and lincomycin (E/L) to check whether they still carried the *thrC* deletion cassette. Furthermore, the clones were streaked on C Glc medium without addition of threonine to check for threonine prototrophy. The suppressors were able to grow on both media indicating that they acquired suppressor mutations bypassing the *thrC* deletion.

3.3. *B. subtilis* rapidly exchanges genetic information in coculture

As described in 3.2.1, co-cultured strains of *B. subtilis* rapidly exchanged genetic information, when selection favored this event. It was hypothesized that this behavior could be utilized to develop a streamlined an efficient transformation protocol using antibiotic markers. The observed exchange of cassettes containing B6 synthetic enzymes occurred most likely due to the evolutionary pressure to form one strain that comprises all the required genes. This setup was obviously the evolutionarily favored setup, as it was not possible to isolate consortia that would feed each other even after deleting the competence genes required for DNA uptake. It should be possible to replace the evolutionary pressure to combine the biosynthetic B6 pathway with the pressure to combine antibiotic markers. Therefore, two strains carrying different antibiotic markers in different loci were chosen to prove this concept. The two strains also carried different fluorescent markers in the same loci under an identical promoter. BP494 (168 *bglS::(hag-cfp)*) and BP496 (168 *amyE::(hag-yfp)*) were used to inoculate LB complex medium with 50% of the selective concentration of kanamycin and chloramphenicol in co-culture and single culture (Diethmaier et al. 2011). After incubation over night at 37°C, the different cultures were streaked out on SP complex medium plates with different combinations of the said antibiotics. As shown in Figure 22A, the strains exchanged genetic information over night and the resulting strain could grow on SP complex medium with both chloramphenicol and kanamycin, while the strains that were inoculated as single cultures could not. The genetic exchange was supposed to be visualized by fluorescence microscopy. However, under the used conditions, strain BP494 showed yellow fluorescence when excited with ultraviolet (UV) light and strain BP496 showed an extremely low YFP emission, making the strains unsuited for the experiment (data not shown). Alternatively, the genetic exchanges were supposed to be shown by a PCR checking for the sizes of the *bglS* and *amyE* loci. Figure 22B depicts the PCR product sizes amplified from the genomic DNA of single clones isolated from the plates. Curiously, they showed that the resulting strain carried both cassettes with the fluorescent and antibiotic markers. However, it also carried a wild type copy of the *bglS* locus. This indicated a very stable co-culture and a second single streak out should be sufficient to single out the transformed strain.

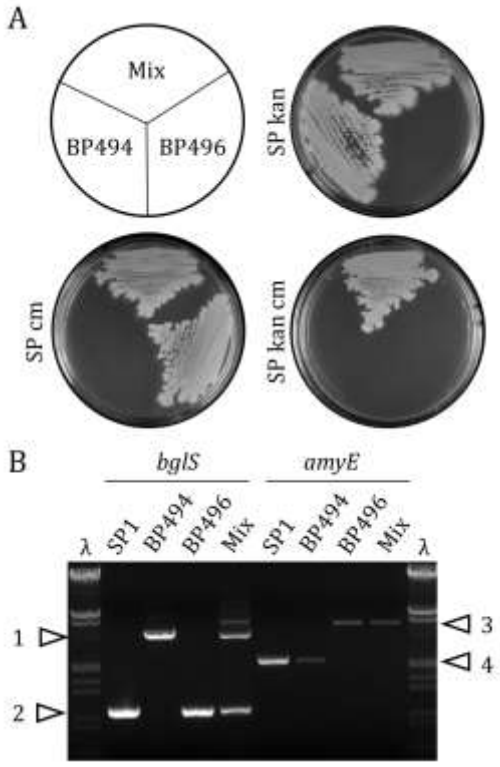

Figure 22: Two strains exchange genetic information over night as seen on plate and by PCR. (A) Plates selecting for growth of BP494 (SP kan), BP496 (SP cm) and for a strain carrying both markers (SP kan cm). Clearly, only the mixed culture could grow on plates with kanamycin and chloramphenicol. (B) Agarose gel with products of PCR reactions checking the size of the two loci used for integration of the kanamycin and chloramphenicol resistance genes. BP494 and BP496 show the expected bands for the loci, BP494 the wild type *amyE* (4) and engineered *bglS* locus (1), BP496 the other way around with the wildtype *bglS* (2) and the engineered *amyE* locus (3). The isolated clone from the mixed culture showed the product size for the engineered *amyE* locus. However, the PCR checking the *bglS* locus produced two products, one the size of the wild type locus and one of the engineered one.

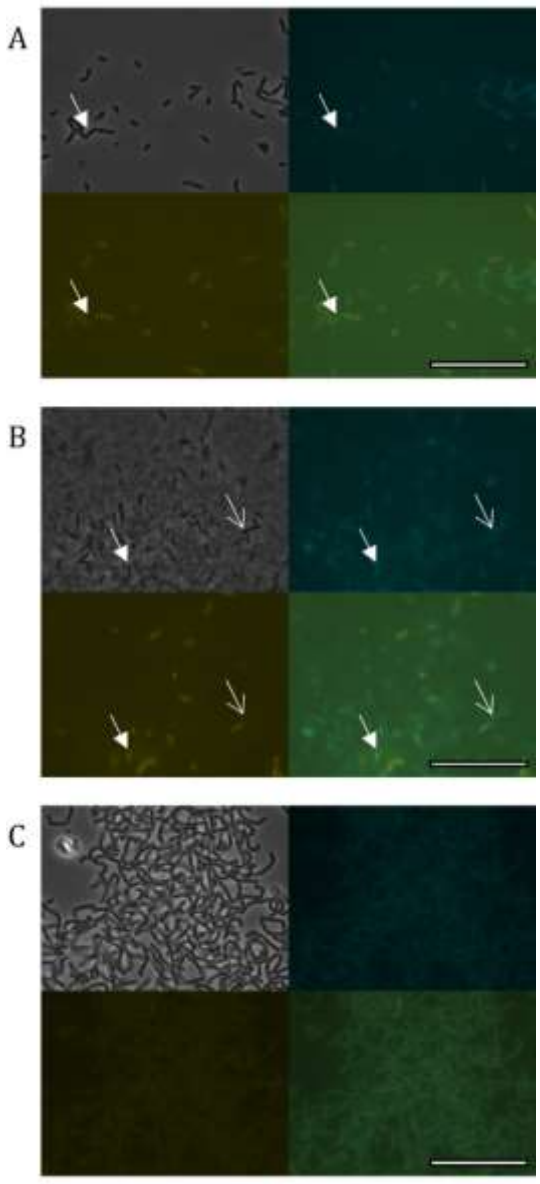

Figure 23: BP999 and BP1000 exchanged genetic information within 24 hours of co-cultivation as shown by fluorescence microscopy. (A) The two strains BP999 and BP1000 were mixed on the object tray and microscopic pictures were taken immediately afterwards. The strains are clearly distinguishable, even when close to each other in a group (arrow). (B) After 24 hours in culture, some cells show both fluorescence signals (solid arrow), while most of the cells were still distinguishable (arrow with thin head). (C) After 48 hours, no distinguishable cells could be observed, all cells express low signals of both fluorescence markers. Pictures are exemplary for the experiment. The YFP signal was detected with 1500 ms exposure. The CFP signal was detected with 500 ms exposure. Scale bars show 20 µm.

The missing distinguishability of the fluorescent signals was thought to be due to the used *hag* promoter, which is considered constitutively active but might be active dependent on the growth phase and differentially regulated under the complex medium conditions used (Katrin Gunka, personal communication). To optimize the visualization, new strains expressing the two fluorophores under the constitutive *gudB* promoter were constructed. For this, the existing plasmids pBP241 (pGP882 P_{gudB}-*cfp*) and pBP26 (pAC5 P_{gudB}-*yfp*) were used to transform SP1 (Gunka 2010). The resulting strains were BP999 (*lacA::*P_{gudB}-*cfp*) and BP1000 (*amyE::*P_{gudB}-*yfp*). The strains were used to inoculate a co-culture in LB complex medium with chloramphenicol and erythromycin/lincomycin. As controls, the strains were also used to inoculate single cultures with their respective selective antibiotic. As shown in Figure 23, the strains are distinguishable even when mixed. After 24 hours growth in the same medium with both antibiotics single cells with both fluorescent markers could be observed. After 48 hours, all cells showed both fluorescence signals, indicating that the original strains have been overgrown by the transformed strain.

This experiment showed the potential of this method to transform strains without the need to isolate DNA. This is, of course, only suitable for isogenic strains and as shown above, the selection of the transformed strains should be ensured by at least two rounds of single streak outs.

3.4. Reverse engineering of a recombinant B6 production strain

As described in previous chapters of this work and on several occasions in literature, the vitamin B6 synthetic pathways were to date poorly understood, had pleiotropic effects to the hosting organism and were difficult to optimize for feasible industrial application (see 1.3, 3.1 and 3.2). As also described above, other groups have previously observed that *E. coli* had an underground metabolism that allowed it to bypass the lack of PdxB (see 1.5 and 4.11) (Kim et al. 2010). Furthermore, is was shown that *B. subtilis* as well had the ability to bypass blocks of several biosynthetic pathways, e.g. for threonine, isoleucine and vitamin B6 (3.2.2, 3.1.1 and 3.2.1/3.4.1, respectively). The latter was observed in two independent experiments and like the bypass observed by Kim and colleagues since the suppressors originated from a strain that was lacking *pdxST* but carried the *S. meliloti* genes *pdxAJH* and *serC*. It was therefore considered likely that the mechanism by which these cells were enabled to grow would be analogous to the one known from *E. coli* (Also see 1.5 and 4.11). This hypothetical *B. subtilis* underground metabolism would be an invaluable asset to the further optimization of vitamin B6 production and could be utilized to engineer completely novel and potentially more productive fermentation strains. Furthermore, the identification of proteins of this underground metabolism could lead to the discovery of novel enzyme functions or regulatory networks related to vitamin B6.

3.4.1. B6 auxotrophs with a partial heterologous pathway can form suppressors

On several occasions, when B6 auxotrophic strains carrying a partial DXP-dependent pathway from *E. coli* consisting of *pdxA*, *pdxJ* and *pdxH* were cultivated on medium without vitamin B6, suppressor mutants arose. This was the case during the co-culturing experiments in 3.2.1 and in a reproduction of previous works by Commichau and colleagues. The strain BV609 was plated on C Glc medium with and without 4HT, which was known to be readily converted to PL by this strain (Commichau et al. 2015a). Interestingly, the strain formed suppressor mutants on the plate without 4HT, which could be passaged on minimal medium and continuously grew independently of vitamin B6 or 4HT. Importantly, literature and the work of Commichau and colleagues provided no evidence that wild type *B. subtilis* would need or synthesize 4HT or the downstream metabolites of the DXP-dependent pathway (KEGG)(Commichau et al. 2015a). These findings prompted us, as mentioned earlier in 3.4, to hypothesize that *B. subtilis*, like *E. coli* would contain an underground metabolism allowing it to rewire the partial heterologous pathway to its endogenous metabolism. To find out at which step the heterologous pathway got rewired to the endogenous metabolic network, the DXP-dependent pathway was reversely engineered starting with the enzyme catalyzing the last step, PNP oxidase PdxH. The B6 auxotrophic strain carrying only the *pdxH* gene from *E. coli* (BP911) and the strain with the *pdxA*, *pdxJ*, and *pdxH* genes (BP912) were already available. Additionally, a strain carrying *pdxJ* and *pdxH* was constructed by integration of a LFH cassette containing the *E. coli pdxJ* gene and the spectinomycin resistance gene *spc* into the *amyE* locus of BP911. The cassette was amplified using primers JR192 to JR197 and genomic DNA of BV606 as a template. The resulting strain was named BP965 (Δ*pdxST amyE::pdxJ aprE::pdxH*). The strains were streaked on C Glc medium with and without PL and BV604 (Δ*pdxST*) was used as a negative control. The plates were incubated at 37°C for five days. As shown in Figure 24A, the negative control and BP911 could not grow on minimal medium without PL and formed no suppressor mutants. The strains BP912 and BP965 however, formed suppressor mutants at frequencies of 5.7×10^{-6} and 2.2×10^{-6}, respectively. These findings indicated that the wild type strain SP1 did not have the capability to easily rewire its underground pathway to achieve production of vitamin B6 in absence of the partial heterologous pathway. To check whether also the *pdxJ* gene alone would be sufficient to form suppressors, strain BP1002 was constructed by transfer of *pdxJ* from BP965 to BV604 using genomic DNA. This strain did not form suppressor mutants on C Glc medium, showing that SP1 does not have the ability to easily acquire the PNP oxidase activity of PdxH by mutation (Figure S 6).

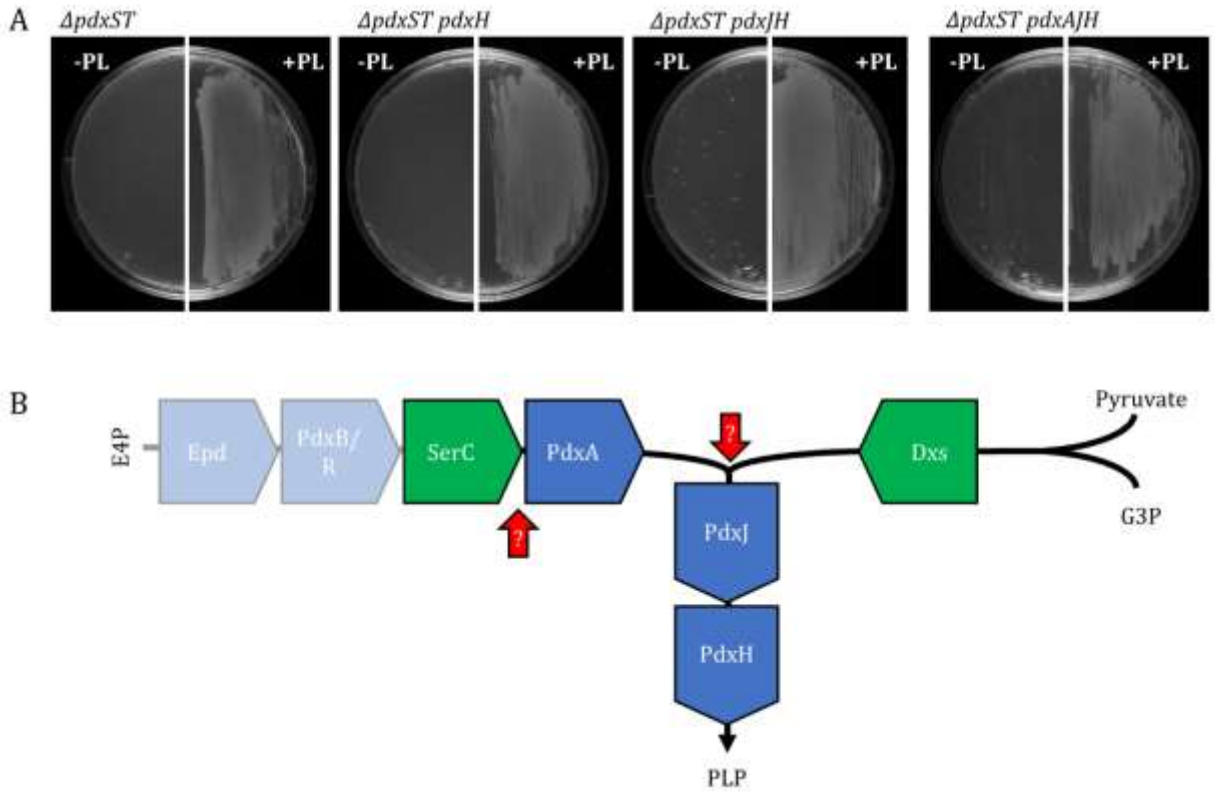

Figure 24: Cells expressing at least PdxJ and PdxH form suppressors on C Glc minimal medium. (A) C Glc minimal medium plates with and without PL. The strains BV604, BP911, BP965, and BP912 were streaked on these plates after cultures were washed in C Glc medium and hungered for three hours. Plates were incubated at 37°C, pictures were taken after four days. (B) Schematic of the DXP-dependent B6 production pathway in the heterologous environment. Green enzymes are endogenously present in *B. subtilis*, blue enzymes are from *S. meliloti*. The Experiment in (A) showed that the endogenous metabolism fed into the heterologous pathway at the positions indicated by the red arrow, upstream of PdxJ. 3-phosphohydroxy-1-aminoacetone (APA), the product of the previous enzyme PdxA, as well as 4HTP, the product of SerC, are not known to be produced by *B. subtilis* and might originate from underground metabolic activity.

SP1 required at least the *pdxJ* and *pdxH* genes to recruit its potential underground metabolism and to feed into the B6 synthesis at the level of *pdxJ*. Of the intermediates of the DXP-dependent pathway, only E4P and DXP are endogenously produced by *B. subtilis* to the best of knowledge. They might however have been produced in extremely low amounts by promiscuous gene activity, which was enhanced by the suppressors. Alternatively, or additionally, the suppressors might have acquired mutations that allowed enzymes with similar substrates or products to perform the required catalysis.

3.4.2. Bacillithiol interferes with the heterologous pathway

Four suppressor mutants of BV609 were isolated and the genomic DNA was extracted. The genomes of strains BP926, BP927, BP928, and BP929 were sequenced by cooperation partners in Göttingen (G2L). The results of the sequencing experiments are shown in Table 9. All suppressor mutants acquired detrimental mutations in the putative cysteine ligase encoding *bshC* gene. BshC was also known to be required for the synthesis of BSH and very likely catalyzes the last step in the synthesis pathway (see 1.4.5) (Gaballa et al. 2010). Furthermore, two of the strains (BP926 and BP927) acquired a point mutation in the gene *yqeT*, which encodes a putative ribosomal protein L11 methyltransferase belonging to the PrmA family. The mutation A461T lead to the exchange of phenylalanine at position 154 to tyrosine (F154Y).

Table 9: Mutations acquired by the BV609 suppressors on C Glc.

Strain	Locus	Coordinates*	Sequence change
BP926	*bshC*	1,582,406	C343T (Stop)
	yqeT	1,627,996	A461T (F154M)
BP927	*bshC*	1,582,406	C343T (Stop)
	yqeT	1,627,996	A461T (F154M)
BP928	*bshC*	1,582,406	C343T (Stop)
BP929	*bshC*	1,582,944	T881A (Stop)

* in NC_000964 (Barbe et al. 2009)

It was however hypothesized that this mutation was less important, because the strains carrying only the *bshC* mutation or both mutations all grew in a similar fashion (Figure 25). To find out whether the mutation of *bshC* alone was indeed sufficient for growth in minimal medium, the gene was knocked out by introduction of a LFH cassette carrying the chloramphenicol resistance marker gene *cat*. The cassette was constructed using primers JR131 to JR134, cat-fw and cat-rev. SP1 served as template for the homologous flanks and pGEM-cat for *cat*. The assembled cassette was introduced into BV604 yielding strain BP953 (Δ*pdxST* Δ*bshC*). The genomic DNA of this strain was used to transform BP911, BP912, BP965, and BP1002, producing strains BP956 (Δ*pdxST aprE::pdxH* Δ*bshC*), BP959 (Δ*pdxST amyE::SC1 aprE::pdxH* Δ*bshC*), BP978 (Δ*pdxST amyE::pdxJ aprE::pdxH* Δ*bshC*), and BP1003 (Δ*pdxST amyE::pdxJ* Δ*bshC*). The strains with the partial heterologous pathway and the deletion of *bshC* were used for a drop dilution assay on C Glc medium with and without PL. As shown in Figure 26, all strains could grow in presence of PL. On minimal medium without PL however, only BP978 was able to grow.

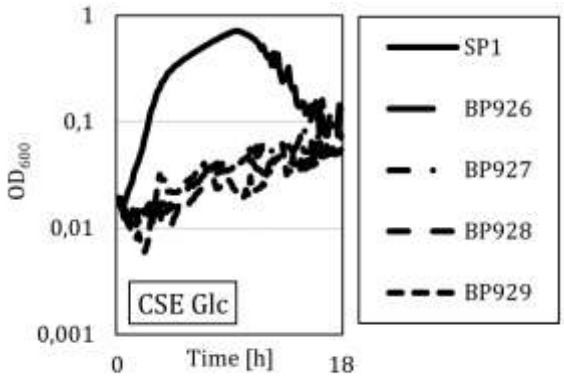

Figure 25: The suppressor mutants could grow in CSE Glc minimal medium but were much slower than the wild type SP1. The cells were grown in CSE Glc minimal medium at 37°C. Values are means of technical triplicates.

This showed that (I) only the strain encoding at least PdxJ and PdxH could benefit from the deletion of *bshC* and that (II) the deletion of *bshC* alone was sufficient to rewire the two enzymes to the endogenous metabolism. A question was, whether the deletion of the putative cysteine ligase encoding gene *bshC* was conveying the phenotype, or the absence of BSH. To investigate this, a different gene from the BSH biosynthesis pathway was to be deleted. As described in the introduction (see 1.4.5), two paralogs of *bshB* are present in *B. subtilis*, which was why *bshA* was chosen to be deleted. Fortunately, a construct with a *de facto* deletion of *bshA* was already available in the lab.

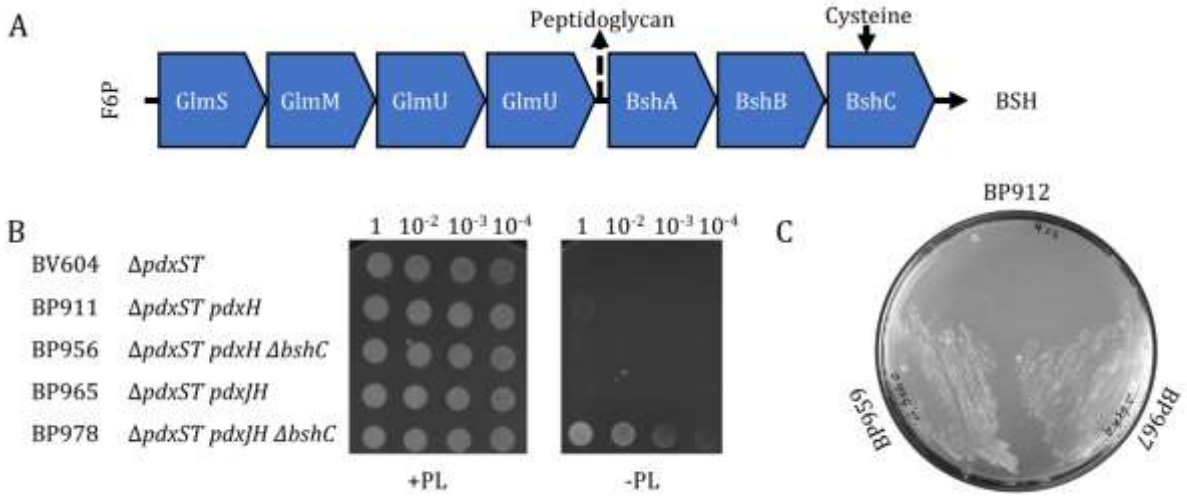

Figure 26: The absence of BSH is beneficial only to cells that express at least PdxJ and PdxH. (A) Schematic of the BSH biosynthetic pathway. BSH biosynthesis branches off from peptidoglycan synthesis and uses the precursor UDP-GlcNAc, which is converted to BSH by BshA, BshB1/2 and the putative cysteine ligase BshC. (B) Drop dilution assays with a B6-auxotrophic strain and isogenic variants expressing PdxH and PdxJ in presence or absence of BSH. Drops were placed on plates with and without PL after washing and hungering for three hours in C Glc minimal medium. All strains could grow on minimal medium with PL, but only the strain expressing PdxJ and PdxH in combination with a deletion of *bshC* could grow in absence of PL. Plates were incubated at 37°C, pictures were taken after two days of incubation. (C) The deletion of either *bshC* or *bshA* allows for growth of a strain expressing PdxAJH, indicating that the absence of BSH is key. The strains were streaked out on C Glc minimal medium after washing and hungering in C Glc minimal medium for three hours. (C) was not performed by the author but by Jan-Philipp Becker as part of his Bachelor studies.

The strain GP88 (*trpC2 bshA::pX2*) had been constructed to delete the gene without abolishing expression of the essential genes downstream of it (Landmann 2011). The construct was transferred to strain BP912 using the genomic DNA of GP88 and the integration was checked by selection for chloramphenicol resistance as part of a students work under supervision of the author (courtesy of Jan-Philipp Becker). The resulting strain BP967 (Δ*pdxST amyE::SC1 aprE::pdxH bshA::pX2*) was checked by sequencing of the *bshA* locus by the company Microsynth, confirming the replacement of the start codon of *bshA* with a stop codon (data not shown). Strain BP967 was streaked on C Glc minimal medium without PL and strains BP912 and BP959 were used as negative and positive controls, respectively. As shown in Figure 26C, the negative control BP912 (Δ*pdxST amyE::SC1 aprE::pdxH*) was not able to grow, while both BP967 and BP959 (Δ*pdxST amyE::SC1 aprE::pdxH ΔbshC*) formed distinguishable single colonies. This result could indicate that the absence of BSH and not BshC was the selected trait of the suppressor mutants. However, all suppressors had mutations in *bshC*, and none in *bshA*, raising the question whether strain BP967 might have acquired a mutation in *bshC* itself. Therefore, the locus was checked via sequencing by Microsynth and was shown to contain the intact wild type sequence (data not shown). It was also a question, whether (I) the absence of BSH was allowing the cells to grow with very low amounts of PL, which they produced with the heterologous proteins, or (II) BSH would interfere with the rewiring of the heterologous enzymes preventing any PL production. To find out which was the case, the auxotrophic strain BV604 and the isogenic strain in which *bshC* was deleted, BP953, were grown in minimal medium supplemented with different very low concentrations of PL. In the case that the absence of BSH benefits growth at very low PL concentrations, BP953 should grow better than BV604. As shown in Figure 27, the absence of BSH does not enhance growth in with limited PL, indicating that it indeed interferes with the rewiring of heterologous and endogenous metabolism.

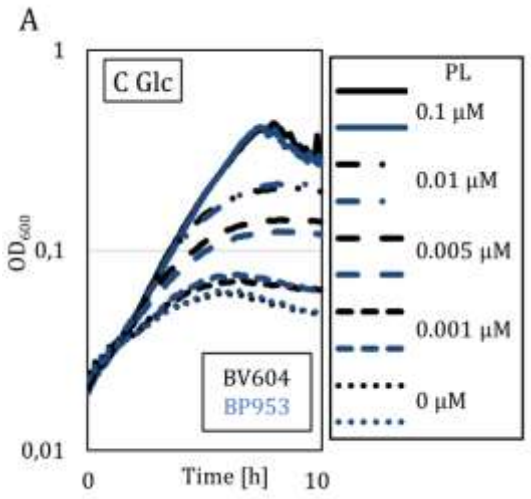

Figure 27: The absence of BSH does not enhance growth of *B. subtilis* with low concentrations of PL. The cultures were grown in C Glc minimal medium supplemented with different amounts of PL. The strains did not show any difference in growth neither at high, nor at very low amounts of PL. Values are means of technical triplicates.

Therefore, it could be confirmed that the absence of BSH, not of BshC is key to allow only the *B. subtilis* strains expressing a partial DXP-dependent pathway growth on minimal medium without PL.

3.4.3. Bacillithiylation is not the driving force for suppressor mutations

The connection between BSH and the utilization of the heterologous pathway remained enigmatic. As shown in 1.4.5, BSH is mainly involved in the response to oxidative stress and can protect exposed cysteine residues from irreversible oxidation when activated by the stress regulator Spx. It does so by S-bacillithiylation of the cysteine residues, which can be reversed by the specialized proteins BrxA and BrxB. In the process, BSH inactivates the proteins it protects, as known for *B. subtilis*' methionine synthase MetE (Gaballa et al. 2010; Chi et al. 2011). It is further known that vitamin B6 has an antioxidant property (see 1.2), which might have been impaired in the suppressor mutants BP926 to BP929, because they produced only minimal amounts of the vitamin. It was hypothesized that the low amounts of B6 would put the cells under chronic oxidative stress activating the oxidative stress response conveyed via Spx, leading to methionine auxotrophy due to the inactivation of MetE. In this case, the strain BP912 should be able to grow on minimal medium without PL in presence of methionine. To check this, strain BP912 (Δ*pdxST amyE::SC1 aprE::pdxH*) was plated on C Glc medium. As shown in Figure 28, BP912 could not grow on this medium and again formed suppressor mutants. The *bsh* loci of three of the suppressors on C Glc medium with only methionine, BP990, BP991 and BP992, were amplified and sequenced by the company Microsynth. Very interestingly, two of the suppressors were found to have frame-shift mutations in *bshC* and one of them in *bshA*, complementing the deletion experiment above (Table 10, Figure 26C).

Table 10: Status of the *bsh* loci of BP912 suppressors on minimal medium with methionine. All mutations lead to frameshifts or premature stop codons.

Strain	*bshA*	*bshB1*	*bshB2*	*bshC*
BP990	n.a.	n.a.	n.a.	ΔA463
BP991	n.a.	n.a.	n.a.	G482A
BP992	ΔT1077	wt	wt	wt

n.a.: not analyzed
wt: wild type sequence

Additionally, the bacillithiylation patterns of wildtype SP1, the B6 auxotroph BV604 (Δ*pdxST*), BP965 (Δ*pdxST amyE::pdxJ aprE::pdxH*), and BP978 (Δ*pdxST amyE::pdxJ aprE::pdxH* Δ*bshC*) were checked in a Bacillithiylation assay (Chi et al. 2013). The named strains were grown in C Glc minimal medium with PL to an OD_{600} of 0.4, were then washed in minimal medium without PL and split in two cultures: one with PL and one without; both cultures were diluted 1:1. The cultures were grown to an OD of 0.4 to 0.5. Same amounts of crude extracts were used for the bacillithiylation assay as described in the methods. As shown in Figure 28D, bacillithiylation could be observed in all strains but BP978, which was consistent with the expectations, because this strain lacked BshC and therefore the ability to synthesize BSH. For strains BV604 and BP965 a mild increase of the overall Bacillithiylation signal could be observed. However, compared to the signals published in previous works, the signal of bacillithiylated MetE was not strongly increased

(Chi et al. 2013). A reproduction with NaOCl as a positive control confirmed the trend, but was inconclusive concerning strain BP965 and therefore not shown. Based on this preliminary data, no evidence for toxic Bacillithiylation of MetE could be found.

The formation of suppressors on medium with methionine and the Bacillithiylation assay indicated that the inactivation of MetE and resulting methionine auxotrophy were not the causative pressure for the inactivation of BSH synthesis.

3.4.4. A link between amino acid starvation and B6 limitation

The investigation of a connection between S-Bacillithiylation and the utilization of the heterologous B6 biosynthesis pathway lead to no conclusive results. Another idea was that BP912 would be starved for cysteine in absence of sufficient PL. Therefore, the hypothesis was that absence of BSH biosynthesis was beneficial because the last step from malyl-glucosamine (GlcN-mal) and cysteine to Bacillithiol consumed cysteine. This hypothesis was checked by cultivation of BP912 on minimal medium plates and in liquid minimal medium, both supplemented with cystine. Cystine was chosen, because it proved to be more stable and less toxic to *B. subtilis* in preliminary experiments (Figure 28A). Additionally, the experiments were carried out with addition of casamino acids (CAA) and methionine. As shown in Figure 28C, the addition of cystine alone had no beneficial effect, neither in liquid, nor on solid minimal medium. So was the case for the addition of methionine alone and for the combination of methionine and cystine. CAA had a positive effect, however. As shown in Figure 28B, the addition of CAA to the minimal medium plate allowed growth of BP912 in a defined radius around the place where the paper plate was placed. In liquid medium (Figure 28E) the effect was also visible, but it also became clear that the positive growth effect was temporal. When methionine or cystine were applied in combination with CAA, however, growth in liquid medium was greatly enhanced. On solid medium, this effect was less prominent (Figure 28B, C and E). For the interpretation of this effect it should be considered, that CAA, which are won by hydrolysis of casein, contain only low amounts of cystine. It was possible that the combination of CAA and cystine or methionine (which can be converted to cysteine) was beneficial because it supplied the cells with all needed amino acids. Considering that vitamin B6 is required mainly for amino acid metabolism, this indicated a connection to B6 limitation in general and less to the utilization of the heterologous pathway. To date, only few publications had shown a direct link between B6 limitation and amino acid starvation, albeit this connection seemed obvious. It would therefore be very interesting to further investigate this connection and for the first time directly prove this effect of vitamin B6 deficiency. It was also tried to generate suppressors of the B6 auxotrophic strain BV604 under conditions with low amounts of amino acids. However, these experiments did not lead to viable suppressor mutants so far (not shown).

The starvation for cysteine or/and methionine alone did not seem to be the driving force for the suppressor mutations in the BSH synthesizing gene *bshC* observed in Figure 24. However, the addition of a complex mixture of amino acids consisting of CAA and sulfur-containing amino acids had a positive growth effect, which might be related to the introduction of the heterologous, DXP-dependent pathway or to the effects of B6-starvation in general and might be worth further investigation (see 4.8).

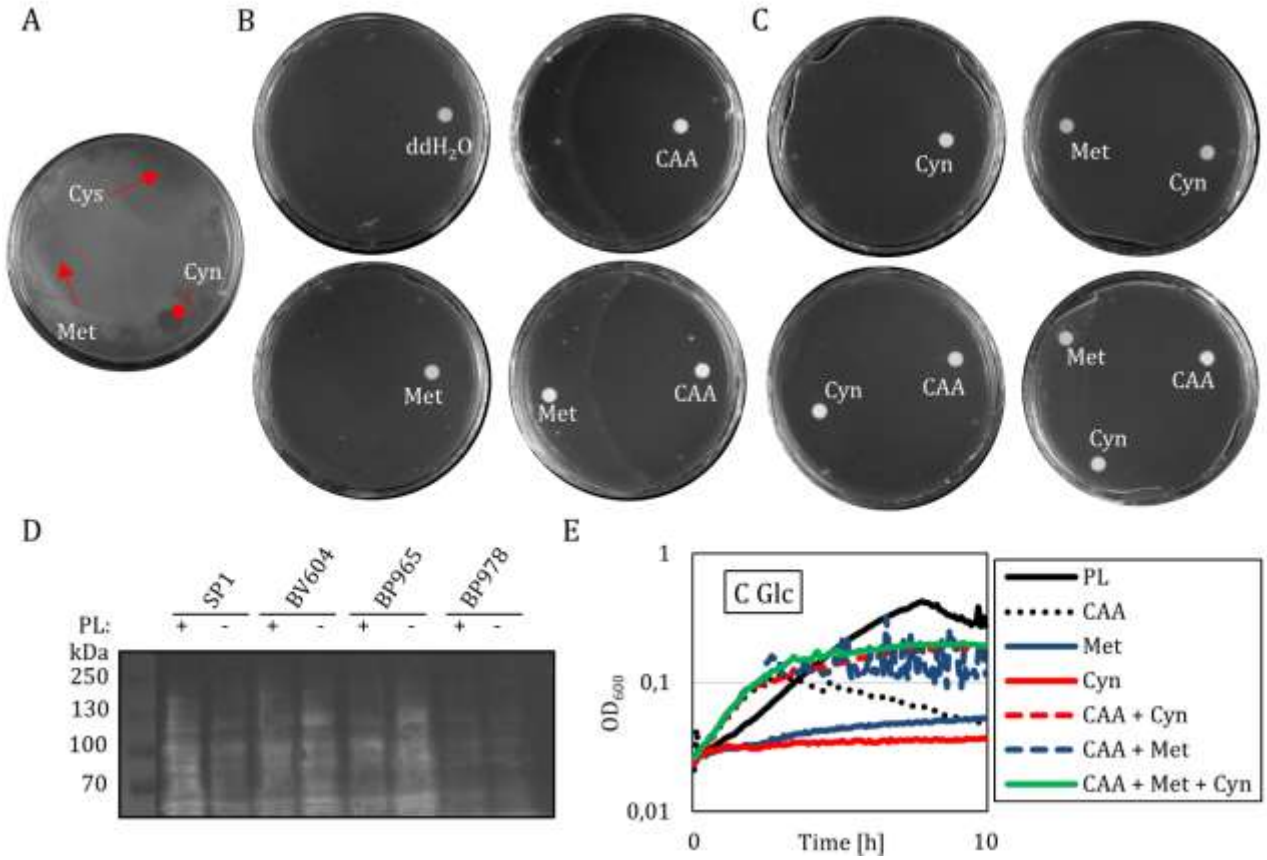

Figure 28: Amino acid starvation and PL limitation are connected but not related to the suppressor mutation of the BSH synthetic genes. (A) Positive control of BP912 streaked onto C Glc minimal medium with PL. Cysteine (Cys), methionine (Met) and cystine (Cyn) were dropped onto the plate to show toxic effects (0.1 mg each). Cysteine showed a relatively strong inhibitory halo, cystine only a very small one. (B) BP912 formed suppressors in presence of Met and CAA. CAA caused a halo where growth seems to be inhibited, but outside the halo growth seemed to be promoted. (C) BP912 formed suppressors in presence of Cyn alone and in combination with Met and CAA. CAA again showed an inhibitory halo and growth promotion at a greater distance. (A, B and C) Cells were washed and starved in C Glc minimal medium prior to plating. Plates were incubated at 37°C for four days. (A and B) 0.1 mg of substances were applied on paper discs. (D) Bacillithiylation assay of wild type SP1, B6 auxotroph BV604 and the strains BP965 and BP978. The BSH deficient strain BP978 shows a very weak signal, which is in agreement with the expectation. Strains BP965 and BP978 show a mildly increased bacillithiylation in absence of PL, but no strong, distinct band absent in SP1, indicating that bacillithiylation is not the reason for the inactivation of BSH synthesis. (E) BP912 could grow in C Glc minimal medium in presence of CAA in combination with sulfur-containing amino acids. Cells grew well in the control condition with PL, but barely in presence of only Cyn or Met. In presence of CAA, growth was considerably improved, but halted after only a few hours. In presence of CAA in combination with Met and/or Cyn, the cells proceeded, starting out faster than with PL but reaching only a lower final optical density. PL was added to a final concentration of 0.1 µM, CAA, Met and Cyn to 0.1%. Values are means of technical triplicates.

3.4.5. Overexpression of YtoQ reestablishes growth

The previously described approaches did not yield a conclusive explanation for the mechanism that lead to the detrimental suppressor mutations in the BSH biosynthesis genes *bshC* and *bshA*. It was therefore decided to choose an untargeted approach. The suppressor mutants BP926 to BP929 as well as the constructed strains BP959 and BP978 were able to grow in minimal medium but were much slower than the wildtype SP1 (Figure 25). This gave one the possibility to evolve these strains by continuous passaging in minimal medium selecting for faster growth. This experiment was carried out as part of a master thesis under supervision of the author (Yeak 2017). The strains BP965 (Δ*pdxST amyE::pdxJ aprE::pdxH*) and BP978 (Δ*pdxST amyE::pdxJ aprE::pdxH* Δ*bshC*) were used to inoculate C Glc minimal medium cultures which were incubated at 37°C until growth was visible. They were subsequently passaged to fresh medium. Every day, the cultures were harvested by centrifugation and the medium was replaced with fresh one to select for fast growing mutants rather than for a consortium of feeding and cheating cells. They were passaged over several weeks, until they grew over night. BP1032 and BP1036, which were retrieved from this experiment, grew almost as fast as the wild type. (For more detail, see Yeak 2017). Whole genome sequencing by our cooperation partners in Göttingen revealed that they acquired several mutations (G2L, Table 11).

Table 11: Mutations acquired by the evolved strains BP1032 and BP1036.

Strain	Locus	Coordinates*	Sequence change**
BP1036	P_{ytoQ}	3,054,693	C55A
	bshC	1,578,853	ΔA464 (Stop)
BP1032	*ytoQ-ytzE*	~3,054,200-~3,072,700	genom. ampl.
	glyQ	2,608,574	C76T (D26N)
	comEC	2,638,135	G1739T (A580E)

* in NC_000964 (Barbe et al. 2009)
**genom. ampl.: genomic amplification

Strain BP1036 was evolved from BP965 (Δ*pdxST amyE::pdxJ aprE::pdxH*) which encoded PdxJ and PdxH, BP1032 from the isogenic strain lacking *bshC*, BP978 (Δ*pdxST amyE::pdxJ aprE::pdxH* Δ*bshC*).

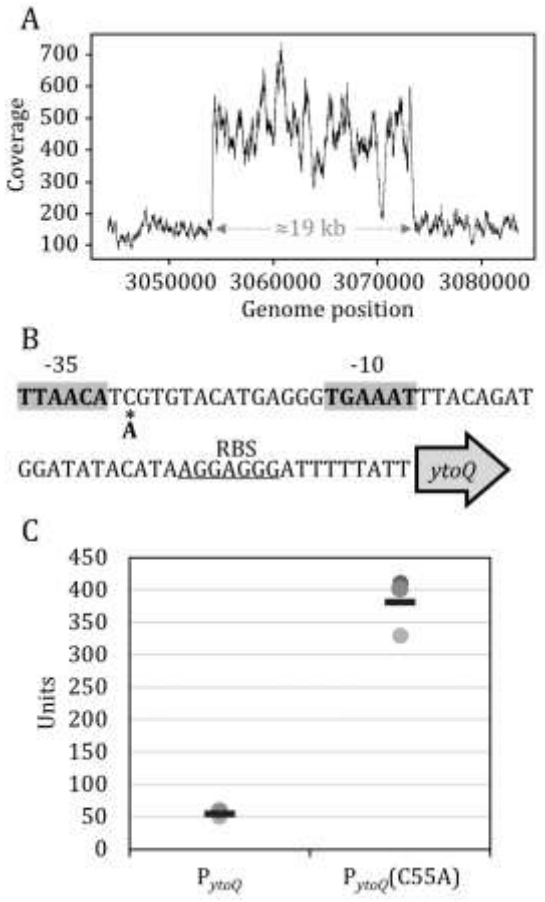

Figure 29: The evolved strains acquired genomic modifications that enhance the expression of the unknown gene *ytoQ*. (A) BP1032 showed an approximately three times amplification of a genomic region stretching from *ytoQ* to *ytzE*. (B) BP1032 acquired a mutation in the putative promoter region of *ytoQ*, 55 bp upstream of the start codon. Putative -35 and -10 regions are shown in bold with grey background. The mutated residue is shown in bold with an asterisk. The ribosomal binding site (RBS) is underlined. (C) LacZ assay with the wild type and mutated P_{ytoQ} constructs. The mutated promoter region as a 6-8x higher activity than the wild type. Dots represent independent biological replicates; bars show means of those.

Intriguingly, BP1036 also acquired a mutation in *bshC*, supporting the hypothesis that the absence of BSH is required for the rewiring of the endogenous metabolism to the heterologous pathway. Furthermore, BP1032 acquired

mutations in the essential glycyl-tRNA synthase encoding *glyQ* gene and in the competence-related channel encoding *comEC* gene. When the original strains of the two evolved strains were resequenced by Microsynth, it was found that already BP978 had acquired the mutation in *comEC*, possibly as a side effect of selection for transformants. The mutation in *glyQ* was very interesting, especially because the amino acid glycine and the substrate of PdxJ APA have a very similar molecular structure, possibly indicating an involvement of the tRNA synthase in the metabolic rewiring (see Figure 40 and 4.12). The mutation in *glyQ* is likely located on the dimerization interface and might alter oligomerization (structure 1J5W from PDB, Figure S 7 supplements) (Berman 2000). It is however unlikely that the enzyme has become inactive since it is essential in *B. subtilis* (Michna et al. 2016). However, both evolved strains harbored modifications concerning the unknown gene *ytoQ*. BP1032 had an increased coverage in an area covering a 19kb genomic region from *ytoQ* to *ytzE* of 434 compared to 151 in the rest of the genome (R script Code S 1). This indicated that the area has been amplified and is on average present in three copies in the genome of BP1032 (Figure 29A). The other strain had a point mutation 55 bases upstream of the start codon of *ytoQ* (C55A) in what might be the promoter region (Figure 29B). If the mutation in the promoter region was causing an up regulation, this could mean that overexpression of *ytoQ* was reestablishing wildtype growth of the strains in minimal medium without externally added PL. To test this hypothesis, first a LacZ construct with the intergenic region upstream of *ytoQ* was constructed. The intergenic region was amplified using primers JR268 and JR269 and was cloned into pAC7, using genomic DNA of BP1036 and SP1 as templates, producing pBP637 (P_{ytoQ}(C55A)-*lacZ*) and pBP638 (P_{ytoQ}-*lacZ*), respectively. The plasmids were used to transform the wildtype SP1 and integration was checked using an amylase test (not shown). The resulting strains BP996 (*amyE::*P_{ytoQ}(C55A)-*lacZ*) and BP997 (*amyE::*P_{ytoQ}-*lacZ*) were used to determine the promoter activity in a LacZ assay in C Glc minimal medium and as shown in Figure 29C, the mutated promoter region indeed showed a 6-8x higher activity than the wild type promoter region.

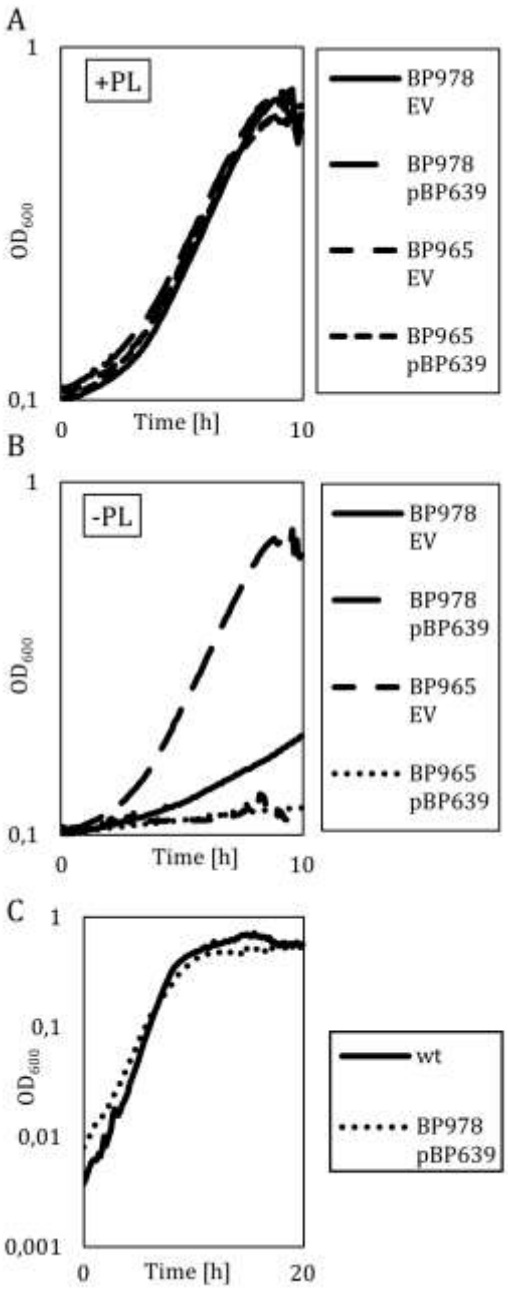

Figure 30: Overexpression of *ytoQ* in a strain with the partial DXP-dependent pathway and without BSH restored growth to wild type (wt) level. (A) Growth of BP965 and BP978 with the pBQ200 empty vector (EV) or the *ytoQ* overexpression construct pBP639 in presence of PL. (B) Same as (A) without added PL. (C) Growth of the wild type SP1 and BP978 pBP639 without added PL. (C) was not performed by the author but by Kah Yen Yeak. All values are means of technical triplicates. All experiments were carried out in C Glc minimal medium at 37°C.

Taken this strong evidence that overexpression of the unknown gene *ytoQ* could convey the

observed phenotype, an overexpression construct was designed. The gene was amplified from the SP1 genome using primers JR266 and JR267 and was cloned into pBQ200. The resulting plasmid pBP639 was used to transform the strains BP965 and BP978 in parallel with pBQ200 as empty vector control. Growth of the strains was compared in a platereader experiment in C Glc minimal medium with and without added PL. As shown in Figure 30A, both BP965 and BP978 could grow in presence of PL irrespective of the presence or absence of the *ytoQ* overexpression.

However, in absence of PL, only BP978 could grow. BP978 with the empty vector grew very slowly, as previously observed for the suppressors BP926 to BP929 (Figure 30B). In contrast BP978 was growing very rapidly when the *ytoQ* overexpression vector was present. Indeed, when BP978 pBP639 was compared to the wildtype strain SP1 by Kah Yen Yeak they grew in a very similar fashion (Figure 30C). Very interestingly, the overexpression of *ytoQ* was only beneficial for the strain lacking BSH. This is a similar outcome as in the case of BSH, where the deletion of *bshC* was only beneficial when the heterologous proteins PdxJ and PdxH were present in the same strain. It was hypothesized that the function of YtoQ is required for growth of the strains expressing PdxJ, PdxH and lacking BSH in minimal medium. The mutation of the promoter or the genomic amplification would then have facilitated the enzymatic or regulatory function of YtoQ to feed more of the unknown compound X into the heterologous pathway. To test this hypothesis a drop dilution assay was performed with *ytoQ* deletion strains. For that the existing mutant BKE29850 (Δ*ytoQ*) was used to isolate genomic DNA and for transformation of SP1 yielding BP1016 (Δ*ytoQ*). The genomic DNA of this strain was then used to generate BP1017 (*ΔpdxST ΔytoQ amyE::pdxJ aprE::pdxH*) and BP1018 (*ΔpdxST ΔbshC ΔytoQ amyE::pdxJ aprE::pdxH*). These strains were used for a drop dilution assay together with additional controls (Figure 31). All strains could grow on C Glc minimal medium in presence of PL, but without PL strains BP1017 and BP1018 were not able to grow. This showed that even in a strain with the partial heterologous pathway and without BSH, the absence of YtoQ is detrimental for growth. That indicated that the function of YtoQ was required for the rewiring of PdxJ and PdxH to endogenous metabolism. Furthermore, also in the drop dilution experiment, BP978 pBP639 grew like the wildtype, complementing the experiment in liquid medium.

In summary, B6 auxotrophic *B. subtilis* strains expressing *pdxJ* and *pdxH* from *E. coli* and lack BSH were evolved to grow like the wildtype in minimal medium without addition of vitamin B6. These strains acquired mutations that lead to an overexpression of the so far uncharacterized protein YtoQ. A reversely engineered strain with a plasmid-based overexpression construct of YtoQ could grow like the wildtype in minimal medium. Furthermore, it was shown that the unknown function of YtoQ was required for the growth of the strains expressing the partial DXP-dependent pathway.

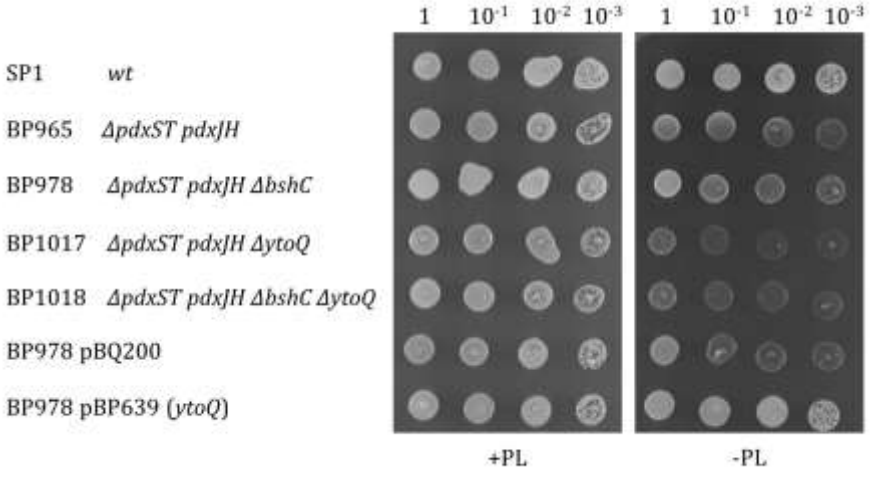

Figure 31: YtoQ is required for growth of the B6 auxotrophic strains expressing the partial DXP-dependent pathway. Drops were placed on plates with and without PL after washing and hungering for three hours in C Glc minimal medium. All strains could grow on minimal medium with PL, but only strains lacking *bshC* and expressing YtoQ could grow in absence of PL. Plates were incubated at 37°C, pictures were taken after two days of incubation.

3.4.6. YtoQ likely conveys the ability to synthesize APA

Figure 24 shows a scheme of the truncated pathway and shows at which points the cells were most likely feeding into it. Strains BP1032 and BP1036 only contain PdxJ and PdxH, so the only possible point where feeding occurs is upstream of PdxJ. The known substrates for PdxJ are APA and DXP, the latter being the product of the endogenous, essential enzyme Dxs. APA was not known to be produced by *B. subtilis* to the best of knowledge, however the current data hinted at the presence under the tested conditions. It was therefore hypothesized that this metabolite was produced by an enzymatic reaction of YtoQ or by an enzyme directly or transcriptionally regulated by YtoQ. A database search for YtoQ and similar proteins revealed that YtoQ is similar to nucleoside deoxyribosyl transferases (HHPred). Furthermore, according to KEGG database it contains in addition to the transferase domain a poorly conserved helix-turn-helix (HTH) motif, which might indicate DNA binding and thus a regulatory function. To check the potential DNA-binding capability, the online tool DP-bind was used with the known regulator ThrR as a control (Kuznetsov et al. 2006; Hwang et al. 2007). As shown in Figure 32, the DNA-binding prediction profile of YtoQ has similarity to the known DNA-binding protein ThrR, possibly indicating regulatory activity (see also Table S 1). However, EMSAs to show unspecific binding to plasmid DNA and PCR products were not able to show any DNA-binding (not shown). It was therefore proceeded with the search for the pathways that could possibly feed into the heterologous pathway. The substrates for a reaction producing APA would likely contain an amino group and would originate from central carbon metabolism or amino acid metabolism. It would also be possible that bases were substrates for a hypothetical reaction. As shown in Figure 24, the BSH synthesis shares a precursor with peptidoglycan synthesis, which is UDP-GlcNAc, an amino sugar bound to a base and as such a possible substrate for YtoQ, which is similar to a nucleoside deoxyribosyl transferase. The fact that the suppressor mutants all mutated BSH synthesis would make sense in the case that UDP-GlcNAc was the substrate for the required YtoQ function.

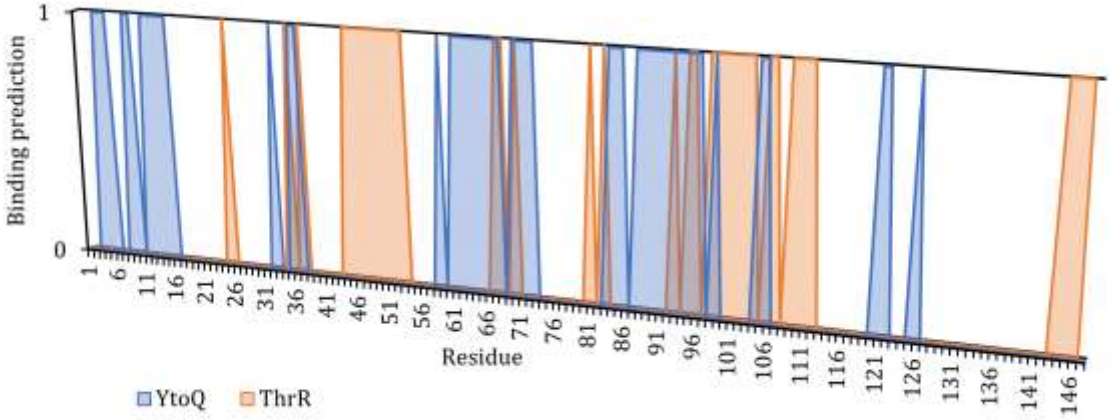

Figure 32: YtoQ has a DNA-binding prediction profile similar to a known regulator. The prediction was done with DP-bind (Kuznetsov et al. 2006; Hwang et al. 2007). The designators 1 and 0 mean predicted binding or no binding, respectively. The table this figure is based on can be found in the supplements. For more information visit http://lcg.rit.albany.edu/dp-bind/help.html.

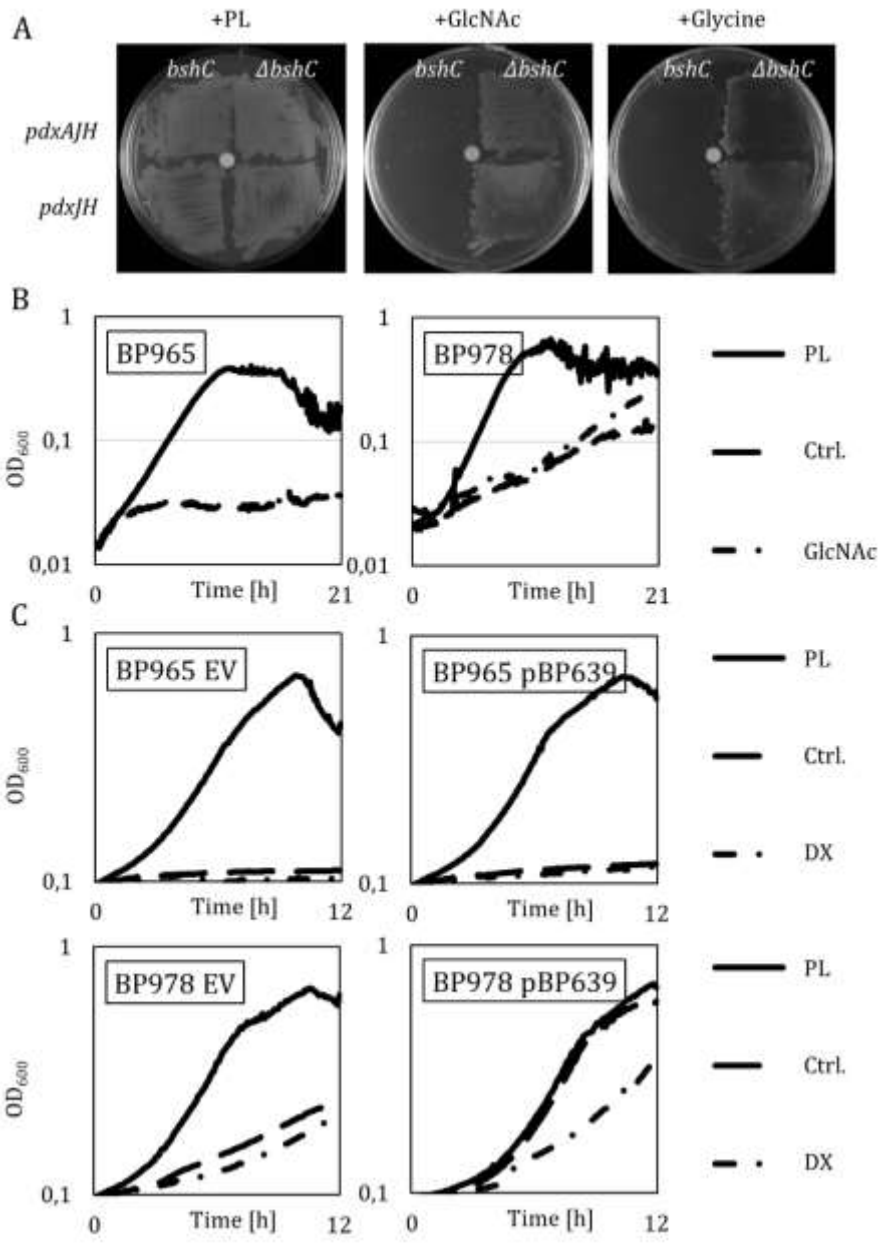

Figure 33: B6 auxotrophic strains with the partial DXP-dependent pathway formed suppressor mutants also on GlcNAc and glycine; GlcNAc and DX have different effects on the *bshC* deletion mutants. (A) Strains BP912 (*pdxAJH bshC*), BP959 (*pdxAJH* Δ*bshC*), BP965 (*pdxJH*), and BP978 (*pdxJH* Δ*bshC*) were streaked onto C Glc minimal medium with paper plates containing 0.1 µg of either GlcNAc or glycine, the positive control contained 2.4 µg PL. Plates were incubated at 37°C overnight. Strains able to produce BSH did form suppressors as on medium without additives and *bshC* deletion mutants grew better on GlcNAc than on glycine. (B) Growth of strains BP965 and BP978 with GlcNAc and glycine. BP978, which lacked BSH, showed enhanced growth in the late phase in presence of GlcNAc. (C) Comparison of BP965 and BP978 with the pBQ200 empty vector (EV) and the YtoQ overexpression plasmid pBP639 in presence of DX. DX had no effect on growth of strains producing BSH but a negative effect on *bshC* deletion strains, which was even stronger in combination with pBP639. (B and C) Cells were grown in C Glc minimal medium with PL as positive and without any additives as negative control (Ctrl.) at 37°C. Values are means of technical triplicates.

Furthermore, it has been mentioned above that glycine has a similar chemical structure as APA, basically lacking 'only' one methyl group at the position of the 2-hydroxy group and one of the evolved strains acquired a mutation in the essential glycyl-tRNA synthase in addition to the up-mutation of P_{ytoQ}. Glycine and GlcNAc were therefore fed to strains expressing the partial DXP-dependent pathway to check whether these compounds would allow growth like the *bshC* deletion mutants. As shown in Figure 33A, the strains did still form suppressors on this medium and did not grow like the *bshC* deletion strains. Glycine did not seem to have any effect on growth of neither BP912 (*ΔpdxST amyE::SC1 aprE::pdxH*) nor BP965 (*ΔpdxST amyE::pdxJ aprE::pdxH*) and also not the isogenic *bshC* deletion mutants BP959 or BP978, respectively. Peculiarly, GlcNAc showed an effect on BP959 (*ΔpdxST ΔbshC amyE::pdxJ aprE::pdxH*), which

grew considerably better on C Glc minimal medium supplemented with this compound than on the medium with glycine. This could have indicated that the absence of BSH and the overexpression of YtoQ would add up to accumulation of GlcNAc. To further investigate this relationship, an experiment in liquid medium was performed. For that different isogenic *bshC* deletion strains with and without the heterologous genes *pdxJ* and *pdxH* were grown in C Glc minimal medium supplemented with 1% GlcNAc. As shown in Figure 33B, in liquid medium a mild effect of 1% GlcNAc could be observed for BP978, which grew better in the later growth phases in presence of the compound compared to the condition without. However, growth was still considerably slower than in presence of PL. No effect could be observed for any of the other strains. These data give some hints that GlcNAc might be beneficial in absence of BSH, but together with the other data, no direct feeding into the pathway could be shown (see 4.10.1). To bring light to this relationship, metabolic flow analysis by metabolome assays and heavy isotope labelled metabolite experiments will be performed in the future.

Another idea was that APA would be produced by underground metabolism in sufficient amounts already and that YtoQ would produce DXP and that its overexpression would relieve the drainage of DXP from the essential thiamine and isoprenoid synthesis pathways. As previously described (see 1.2.1), in α- and γ-proteobacteria, Dxs and its product DXP are essential to several pathways: DXP-dependent B6 biosynthesis as well as thiamine and isoprenoid metabolism. In *B. subtilis*, Dxs is also essential, but only for the latter two functions. Therefore, it was hypothesized that DXP was depleted by the enzymatic activity of PdxJ and that YtoQ would replenish the DXP pool by its endogenous or a promiscuous activity. To test this hypothesis, strains BP965 and BP978 with the YtoQ overexpression plasmid pBP639 and the empty vector controls were grown in C Glc minimal medium supplemented with 0.5% of the dephosphorylated DX. As shown in Figure 33C, strain BP978 pBP639 again grew very well and there was barely any difference between the conditions with and without PL, supporting the previous experiments. The addition of DX had no effect on the BP965 variants and BP978 with the empty vector, but BP978 pBP639 grew considerably worse in the presence of DX. The reasons for this negative effect on growth were unknown.

In summary, the precursor of BSH synthesis GlcNAc and the dephosphorylated form of the substrate of PdxJ DX had some effect on the growth of the strains expressing the partial DXP-dependent B6 synthesis pathway indicating an involvement in the rewiring of the heterologous enzymes to endogenous metabolism. However, the substrate(s) for the reaction(s) feeding into the pathway remain unknown. Future metabolome analyses, heavy isotope labelling and *in vitro* analysis will shed light on the connection between the observations.

3.4.7. Purified YtoQ oligomerizes as dimers of dimers

The function of YtoQ remained widely unknown, but we could very reproducibly show that the unknown protein was required for the feeding into the partial DXP-dependent pathway. In addition to the genetic approaches to unravel the function of YtoQ, the protein was also analyzed biochemically and structurally. For this purpose, three *in vitro* constructs were designed. For uncomplicated purification and YtoQ, the encoding gene was cloned into the vectors for N- and C-terminal Strep-tagged expression, pGP172 and pGP574, respectively. Both vectors were used, because it was unknown, whether one of the tags would be detrimental for the catalytic or regulatory function. In addition to these plasmids *ytoQ* was also cloned into the vector pET-SUMOadapt, which adds a N-terminally located His-tag, but also the N-terminal SUMO tag, which should enhance solubility of the protein and can also be cut off, to use the untagged protein *in vitro*. For these constructs, the gene was amplified with the primer pairs JR296/297, JR298/299 and JR300/301 for the vectors pET-SUMOadapt, pGP172 and pGP574, respectively. The products were integrated into the vectors via restriction cloning and the plasmids were used to transform *E. coli* DH5α. The sequenced (Microsynth) plasmids were then isolated from the cells and used to transform the expression strain BL21 (DE3), which in contrast to DH5α was carrying the T7 polymerase, which was required for the expression, since all used

plasmids put the gene under control of a T7 promoter. Figure 34A shows the purification of the Strep-tagged protein variants. The purification of both, the N- and C-terminally Strep-tagged versions was very efficient with yields of approximately 3-5 mg from 500 ml culture. The purified protein was used for *in vitro* oligomerization analysis by a cross-linking experiment with glutardialdehyde (GAL). Figure 34B shows that the protein formed a strong dimer band in presence of the cross-linker, but also weaker bands corresponding to the size of a tri-, tetra- and hexamer. This indicated the capability of YtoQ to form relatively large oligomeric structures. This data is to be complemented by SEC-MALS in the future. Furthermore, the protein was handed to the structural biochemistry department for a crystallization assay, which yielded proteinous crystals awaiting further investigation (Figure 34C).

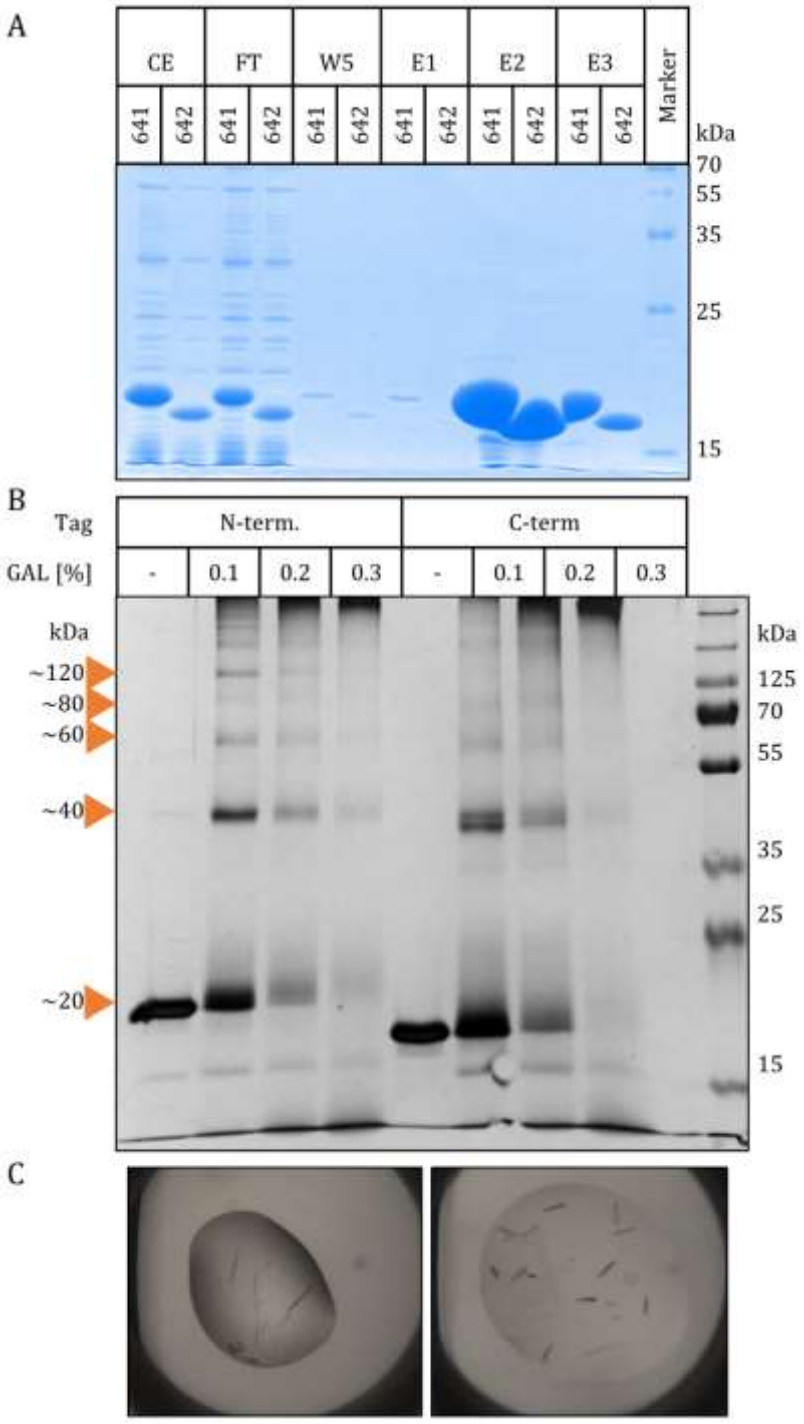

Figure 34: YtoQ was easily purified and formed oligomeric structures in a cross-linker experiment. (A) Plasmids pBP641 (641) and pBP642 (642) were used to overexpress YtoQ in *E. coli* BL21 (DE3). Induction was carried out for 1.5 h at 37°C in 500 ml LB medium. The Strep-tagged proteins were purified using Streptactin-sepharose columns and eluted with D-Desthiobiotin. 4 µl of crude extract (CE), flow through (FT), wash fraction 5 (W5) and the elution fractions 1-3 (E1-E3) were separated by an SDS-PAGE using a 12% polyacrylamide gel and visualized using a Coomassie staining. The yield of the N- and C-terminally tagged proteins was 10 and 6.8 mg ml^{-1}, or 5 and 3.4 mg total protein, respectively. (B) 50 pmol of the purified proteins were cross-linked *in vitro* using glutardialdehyde (GAL). Both the N- and C-terminally tagged YtoQ formed oligomers in this experiment. Bands corresponding to the sizes of dimers, trimers, tetramers and even larger complexes were visible (orange triangles for N-terminally tagged construct). The proteins were separated by an SDS-PAGE using a 12% polyacrylamide gel and visualized using silver staining. (C) Exemplary pictures of proteinous crystals, which formed at pH 4-7 with addition of either polyethylene glycol (3350 or 8000 g mol^{-1}), or methyl pentane diol.

3.5. Streamlining of the production pathway

As previously described (see 1.3), there have been many groups trying to establish a fermentative platform for vitamin B6 production. Furthermore, a major part of this work has been devoted to optimize the production strain designed and constructed by Commichau and colleagues (Commichau et al. 2014; Commichau et al. 2015a). However, to date none of the previous approaches led to the desired outcome of a strain producing sufficient B6 for industrial application. It was hypothesized that at least 10 g l^{-1} would have to be produced in a fermentative approach within a few days to compete against the currently used chemical synthesis (Rosenberg et al. 2017a). The DXP-independent pathway consists of only two enzymes, which form a heterotetracosamer (24-mer), rendering genetic engineering difficult due to the high possibility of polar effects in oligomerization and structural integrity of the complex (Rosenberg et al. 2017a). The DXP-dependent pathway on the other hand has been shown to produce toxic intermediates and to have possibly detrimental effects on other pathways in the host organism as shown for the tryptophan synthesis pathway (Rosenberg et al. 2017a; Commichau et al. 2015a). However, the approach by Commichau and colleagues was a brute force overproduction by strong and constitutive promoters, which did not consider the specific activities and possible accumulation of intermediates. It is imaginable that the strict streamlining of the pathway by modular and tightly controlled expression of the single enzymes might circumvent the accumulation of toxic intermediates and interference with endogenous metabolism.

The following chapter will assess the required enzyme amounts, provide a proof of concept and propose a strategy to construct a streamlined production pathway.

3.5.1. Assessment of the required enzyme amounts for streamlined production

For the streamlining of the DXP-dependent B6 synthesis pathway it was assumed that the initial substrate E4P was present in saturating amounts to allow v_{max} and k_{cat} to be true. It was furthermore assumed that all cofactors were present in saturating amounts. This way, the needed amounts of the enzymes could be calculated using the k_{cat} to assure that none of the intermediates would accumulate far above the k_M of the enzyme downstream. The results of this chapter are also summarized in Table 12.

Conversion of E4P to PE by Epd

There are two publications that characterized the activity of Epd from *E. coli* (Zhao et al. 1995; Boschi-Muller et al. 1997). Zhao and colleagues identified the k_M to be 960 µM and v_{max} 91.2 x10^3 U mg^{-1}, calculating the k_{cat} to be 200 s^{-1} per tetramer. This however is impossible, since the v_{max} together with the stated tetramer molecular weight of 132 kDa would add up to a k_{cat} of incredible 200,000 s^{-1}, which can be considered unlikely. It was therefore assumed that there was a mistake and the 200 s^{-1} were taken, as is also stated in uniport (http://www.uniprot.org/uniprot/P0A9B6). Boschi-Muller and colleagues determined the k_{cat} to be 20 s^{-1} of a unit they did not state. However, the experimental data they presented hinted at a turnover of approximately 9 s^{-1} per aptamer (specific activity of 14.7 U mg^{-1} multiplied by M of 37.170 kDa).

Concluding, one mole Epd would produce 10-50 moles of PE from E4P per second. For this theoretical approach, the ideal conditions will be considered and the turnover of 50 s^{-1} will be taken as given.

Conversion of PE to HPAKB by PdxB

Rudolph and colleagues have investigated this enzyme and determined the k_M to be 2.9 µM and the k_{cat} 1.4 s^{-1} (Rudolph et al. 2010). Under ideal conditions one mole of this enzyme would generate 1.4 moles of HPAKB from 1.4 moles of PE. This turnover per aptamer of enzyme is at least 7x slower than of Epd and would already lead to a significant accumulation of PE without streamlining. Indeed, on several occasions mutant carrying detrimental mutations in *epd* were observed, when the pathway was dispensable (Commichau et al. 2014)(and data not shown).

Conversion of HPAKB to 4HTP by SerC

SerC is one of the genes of the DXP-dependent B6 biosynthesis pathway, which is also present

in the Gram-positive *B. subtilis* and has an essential function in the serine synthesis. Therefore, the heterologous SerC from *E. coli* should serve as a dedicated enzyme for the recombinant DXP-dependent pathway in the rationally designed strain. SerC was characterized by Drewke and colleagues. However, they were only able to measure the reverse reaction from 4HTP to HPAKB with a k_M of 110 µM and a k_{kat} of 0.15 s^{-1} (Drewke et al. 1996). One mole of PdxB will therefore likely produce 0.15 moles of 4HTP from 0.15 moles of HPAKP.

Conversion of 4HTP to APA by PdxA

The conversion of 4HTP to APA is only semi-enzymatic. The first step from 4HTP to AOPB is carried out by PdxA and the second step to APA is a spontaneous decarboxylation. Again, two groups analyzed this enzyme and came to similar kinetics. Cane and colleagues determined the k_M to be 85 µM and the k_{cat} as 1.66 s^{-1} (Cane et al. 1998; Cane et al. 1999). A third publication determined the k_{cat} to be 1.4 s^{-1} (Laber et al. 1999). All of these publications from the nineties assumed that the PdxA dimers would contain two active enters, however according to more recent structural analyses, every dimer only contains one catalytic center which is made up of residues from both aptamers, doubling the k_{cat} (Sivaraman et al. 2003). The turnover per mole of enzyme does not change because of this, which is why one mole of PdxA will ideally produce 1.66 moles of APA and CO_2 from 1.66 moles of 4HTP per second.

Conversion of APA and DXP to PNP by PdxJ

This reaction also has been investigated by Cane and colleagues, who did the kinetics for DXP and the k_M turned out to be 26.9 µM and the k_{cat} 0.07 s^{-1} (Cane et al. 1999). Therefore, one mole of PdxJ will under perfect circumstances produce 0.07 moles of PN from 0.07 moles of APA and DXP.

Oxidation of PNP to PLP by PdxH

The last reaction in the PLP synthesis pathway is carried out by PdxH, which has been determined to have a k_M of 2 µM and a k_{cat} of 0.76 s^{-1} (Zhao, Winkler 1995). Furthermore it was shown that PdxH is feedback-inhibited by PLP (Zhao, Winkler 1995). Under ideal conditions one mole of PdxH will produce 0.76 moles of PLP and H_2O_2 from 0.76 moles of PNP and O_2 per second, which is at least 10x faster than the reaction upstream.

Table 12: Estimation of relative enzyme amounts required to produce 10 g l^{-1} PNP within 48 hours.

Enzyme	Turnover per aptamer [s^{-1}] [a]	Relative to Epd	Amt. needed for 10 g l^{-1} PNP [µM]	M [kDa]	Amt. needed for 10 g l^{-1} PNP [g l^{-1}]	% of PDW at OD_{600} = 10
Epd	50	1	0,0046	37,14	0,0002	0.02
PdxB	1.4	36	0,1659	41,2	0,0068	0.85
SerC	0.15[c]	333	1,5484	39,6	0,0613	7.66
PdxA	1.66	30	0,1399	35,9	0,0050	0.63
PdxJ	0.07	714	3,3181	26,2	0,0869	10.87
PdxH	0.76	66	0,3056	25,55	0,0078	0.98
Dxs	0.8	63	0,2903	67,44	0,0196	2.45

[a] highest reported value

[c] reverse reaction was measured

3.5.2. The insertion of a genomic *pdxH* construct enhanced growth

As shown above, the enzymes of the recombinant DXP-dependent B6 synthesis pathway are required in very different amounts. To show an effect on the pathway as a proof of principle, the last reaction from PNP to PLP by the PNP oxidase PdxH was streamlined by insertion of a genomic *pdxH* construct replacing the plasmid based construct. This should lower the gene dose and therefore the relative enzyme amount in the cell. Furthermore, this should lower the amount of produced PLP and the accumulation of non-toxic PNP. However, the gene dose of the genomic construct is identical with the inserted SC1 containing *pdxJ* and since PdxH is approximately 10x faster than PdxJ, the streamlining was still not perfect. However, in the case we saw an effect, this would already have been strong evidence for the potential of streamlining. Strains carrying the genomic *pdxH* construct was already mentioned above (see 3.2.2). The *pdxH* gene was amplified from *E. coli* DH5α with primers JR75 and JR78 and the constitutively active promoter P_a and a synthetic, perfect RBS were placed in front of the *pdxH* start codon with JR75 (Gundlach et al. 2017). The flanking regions were amplified with JR74, JR76, JR77, and JR79. The *pdxH* gene and the flanks were fused by PCR and the resulting LFH construct was used to transform the B6 auxotrophic strain BV604 (Δ*pdxST*). The successful integration of the marker-less construct was checked by selection on plates containing 1 µM PN, which only allowed growth in presence of a functional PLP oxidase. The resulting strain BP911 (Δ*pdxST aprE::pdxH*) was transformed using the genomic DNA of BV411-1 (*amyE::SC1*), resulting in strain BP912 (Δ*pdxST aprE::pdxH amyE::SC1*) (Commichau et al. 2014). Strain BP912 was subsequently transformed with genomic DNA from BV412-1 (*sacB::SC2**) resulting in strain BP913 (Δ*pdxST aprE::pdxH amyE::SC1 sacB::SC2**) (Commichau et al. 2014). This strain was lacking the endogenous B6 synthesis genes *pdxST* but contained the complete DXP-dependent synthesis pathway in single copy as part of genomic constructs. BP913 was compared to the wildtype strain SP1 and the previously published strain BV606 (Δ*pdxST amyE::SC1 sacB::SC2**) pBV415, which carried the plasmid-based *pdxH* construct (Commichau et al. 2014).

As shown in Figure 35, strains BP913, BV606 pBV415 and SP1 were compared in liquid C Glc minimal medium. Strain BP913 grew almost like the wildtype, while BV606 pBV415 grew markedly slower. These data showed that even a small change in the amount of one of the enzymes could have an influence on the production strain and how promising the streamlining of the pathway could be.

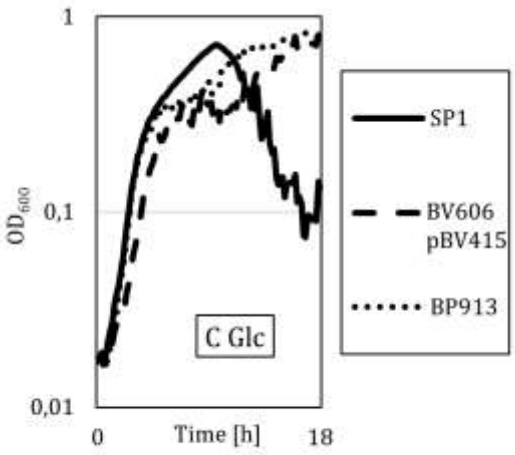

Figure 35: The genomic integration of *pdxH* and lower gene dosage have a slight growth effect on the heterologous strain. Strains were grown in C Glc minimal medium at 37°C. Values are means of technical triplicates.

3.5.3. Rational design of a vitamin B6 production strain

Considering that the different enzymes of the recombinant pathway from Epd to PdxH catalyze their reactions at different speed, it might be useful to adjust the amounts of the enzymes in the production strain as has been shown above Figure 35. In case of Epd, for example, the turnover per mol is at least 7 times faster than of PdxB. This means that PE will accumulate when the two enzymes are present in the same amounts (meaning that expression of both is controlled by one or the same promoter). If PdxB was expressed 7 times stronger than Epd, less PE would accumulate, however. Of course, this is an idealized view on how kinetics work. All these enzymes have been tested in their respective host organisms or i*n vitro* and never in a recombinant *B. subtilis* strain. Furthermore, we know little about possible feedback mechanisms, which might lead to a higher or lower activity of certain

enzymes in the pathway (as known for PdxH Zhao, Winkler 1995). To hypothesize which protein amounts are needed in the final cell or culture, it is needed to know the amount of total protein in living bacterial cells. Delgado and colleagues showed that growing *E. coli* cells consist of 70% water and that 50-55% of the dry weight is made up by proteins, and it is assumed that this data would also be true for *B. subtilis* (Delgado et al. 2013). A fermenter with a volume of 1 l with a culture with the OD_{600} of 10 will contain approximately $8x10^{12}$ cells. When each cell has a dry weight of approximately 200 fg during stationary phase (Delgado et al. 2013), the total bacterial dry mass in the fermenter is (8×10^{12} cells x 200×10^{-15} g = 1600×10^{-3} g =) 1.6 g and the protein weight is 0.8 g. To calculate the needed amount of the pathway proteins, first an amount of B6 to be produced with the strain is defined. For an industrially interesting amount, 10 g l^{-1} in a 48h fermentation process, which would be 0.041 moles per liter of PLP (M=247.142 g mol^{-1}) or 0.040 mol l^{-1} of PNP (M=249.158 g mol^{-1}) was previously defined (Rosenberg et al. 2017a). For fermentation it would be more useful to produce PNP to circumvent the toxic effects of PLP, which is why the following calculations were done for PNP. The fermentation time of 48 hours is equal to 172800 seconds. By dividing the needed amount of PNP molecules by the time in seconds, one can calculate the needed minimal turnover per second, corresponding to the lowest possible number of catalyzed reactions by the slowest enzyme that still allows to reach the targeted amount within 48 hours: 0.040 mol l^{-1}/172800 s = 2.32×10^{-7} mol l^{-1} s^{-1}. From the turnover and the molecular weight of each enzyme, it can be calculated which amount of enzyme is needed and together with the reported dry weight of the cells, what percentage of the protein dry weight (PDW) this makes up (Table 12). First, the needed minimal turnover is divided by the turnover of each individual enzyme to calculate the required amount in mol l^{-1}. Then this is multiplied with the molecular weight in g mol^{-1} yielding the g l^{-1} needed for 10 g l^{-1} PNP within 48 hours. With the given data from Delgado and colleagues the approximate percentage of the PDW is calculated by dividing the needed grams per liter of each enzyme by the PDW (0.8 g l^{-1}) (Table 12). These amounts are then compared to the proteome of *B. subtilis*. According to a 2D-Gel analysis by Eymann and colleagues, the most abundant protein is TufA, which makes up 5.4% of the total protein detected on the gel, followed by IlvC (2.5%), Hag (2.3%), RplL (2%), and MetE (1.9%) (Eymann et al. 2004). The most abundant B6-synthetic gene, PdxS (YaaD) is ranked at place 99 and makes up 0.2% of the total protein. Considering that not all proteins are in the *pI* range that was chosen and that there must be a certain number of proteins not detected, the percentage might be even lower than stated. Looking at the recombinant pathway, the required amounts of some of the enzymes are relatively high. The required amount of PdxJ and SerC, for example, is higher than the amount of the most abundant protein in the normal *B. subtilis* cell. This, of course, is a serious problem. Workarounds could be a considerably higher OD_{600}, and/or longer fermentation times in combination with a lower expression of the pathway genes.

A recent publication presented promoter and RBS libraries that can modulate expression over a large range which can modulate GFP expression from nM to mM concentrations in exponential and stationary phase (Guiziou et al. 2016). This is perfect to modulate the expression of the single enzymes in the B6 pathway in fed-batch or single batch fermentation (Table 13). The weakest promoter was chosen for the enzyme that was required in the lowest amounts, Epd. For the other enzymes promoters with the desired relative strength were chosen (Table 13). The strength of the promoters was determined by Guiziou et al. using GFP expression, which can be used to predict the amounts of protein that will be produced by the promoters (Guiziou et al. 2016). The predicted PNP amounts produced by this setup (if the *E. coli* proteins were expressed like GFP) is ~50 times higher than planned. To adjust the expression to fit the required amounts RBS R2-8 fits perfectly. It almost exactly modulates the expression by a factor of 0.02 and could be used to achieve the desired enzyme amounts (Table 13). For better comparability, Guiziou and colleagues designed the constructs with a standardized transcription start sequence (TSS) from P_{fbaA} (GGAGAAAA), which should also be used in the rationally designed strain to ensure the desired relative

expression. This way the new synthetic constructs will look as shown in Table S 2.

Table 13: Possible promoter combinations according to estimations.

Enzyme	Turnover relative to Epd	Promoter with desired strength	relative strength	GFP conc. [µM]	Amt. needed for 10g l^{-1} PNP [µM]	relative amount needed/GFP
Epd	1	PS22	1,00	0,29	0,0046	0,0160
PdxB	36	PV7	36,00	10,45	0,1659	0,0159
SerC	333	P_{ymdA}	309,00	89,70	1,5484	0,0173
PdxA	30	PY13	28,24	8,19	0,1399	0,0171
PdxJ	714	PY4	723,67	209,87	3,3181	0,0158
PdxH	66	PS10	63,28	18,35	0,3056	0,0167
Dxs	63	PS10	63,28	18,35	0,2903	0,0158

4. Discussion

4.1. The multifaceted regulation of the *hom* operon

As described in the introduction, the amino acid-dependent regulation of the *hom* operon and of the gene encoding aspartokinase III, *thrD* has been investigated in previous studies, showing that aspartokinase III is induced by lysine and at the same time inhibited by a combination of lysine and threonine (see 1.4.4, Kobashi et al. 2001; Graves, Switzer 1990). Both the *hom* operon and *thrD* are also believed to be inhibited in presence of branched chain amino acids by the regulator CodY, which binds to a conserved recognition site in their promoters (Belitsky, Sonenshein 2013; Sonenshein 2005). The exact regulatory effects of different amino acids on the *hom* operon are shown above and in Rosenberg et al. 2016. Interestingly, no regulation by threonine, the product of the enzymatic pathway encoded in this operon could be observed. The same holds true for most other tested amino acids. The operon is only repressed by isoleucine and homoserine and only the effect of isoleucine is CodY-dependent (Figure 8). A study by Kriel et al. showed that when ppGpp synthesis was induced in presence of CodY, the *hom* operon was strongly activated, which was also the fact in a ppGpp *codY* double mutant. Complementarily, in a conditional ppGpp mutant in presence of CodY and with abundant GTP (which enhances CodY activity, Brinsmade, Sonenshein 2011), the operon was almost completely shut down (Kriel et al. 2014). Taken the fact that this dissertation finds only evidence for CodY-dependent regulation of *hom* through isoleucine, the inhibition observed by Kriel et al. most likely is conveyed through this amino acid. However, the same study also found that the *hom* operon is regulated under the same conditions in a CodY-independent manner, as shown by the lack of activation in a ppGpp *codY* double mutant with abundant GTP (Kriel et al. 2014). The latter observation can be partially explained by the inhibition through ThrR presented in this work. Figure 8 shows that ThrR inhibits transcription of the *hom* operon constitutively and in presence of most amino acids except for lysine and cysteine, which both activate transcription to the level of a *thrR* mutant. However, even the *codY thrR* double mutants are regulated by homoserine and isoleucine, which indicates that this regulation is conveyed by yet another mechanism, which might be GTP-dependent, as described by Kriel and colleagues. An explanation for a non-proteinous GTP-dependent regulation of transcription during stringent response has been elaborated before the cited Kriel study. It is known that transcription initiation during stringent response in *B. subtilis* is controlled by the purines at positions +1 and +2 of the transcript, called 'stringent transcription control' (Tojo et al. 2010). This distinguishes *B. subtilis* from *E. coli*, where ppGpp inhibits initiation of transcription by RNA-polymerase and allows the ppGpp-independent upregulation of transcripts starting with adenines during GTP limitation in stringent response. The *hom* operon starts with two adenines as described in the publication this work is part of (Rosenberg et al. 2016), which explains why it is not upregulated when GTP is abundant although CodY and ppGpp are absent (Kriel et al. 2014).

Another possibility is that homoserine and isoleucine have influence on the glutamine-dependent transcriptional regulator TnrA, which has been shown to inhibit the *hom* operon during nitrogen limitation (Mirouze et al. 2015). However, the used CSE minimal medium contains glutamate and ammonium, which should lead to absence of TnrA repression (Wray et al. 2001; Gunka, Commichau 2012).

Summarizing, the *hom* operon is regulated by at least four mechanisms being (I) CodY-dependent inhibition of transcription in presence of isoleucine and GTP, (II) the inhibition by ThrR in absence of cysteine and lysine, (III) the N-dependent inhibition by TnrA, and (IV) stringent transcription control, a GTP-dependent mechanism.

4.2. The regulation of the *hom* operon by ThrR

The data presented in 3.1.1 is part of a work which shows that the previously uncharacterized ACT-domain containing protein ThrR is a transcriptional regulator of the *thrD* and *hom* operons encoding aspartokinase III, homoserine dehydrogenase, homoserine kinase and threonine synthase and therefore a major part of the threonine and isoleucine synthesis pathway (Figure 8). This became

visible only because several publications over more than three decades reported several phenotypes. In the 70's Vapnek and Greer reported the capability of threonine synthase ThrC to take over the function of threonine dehydratase IlvA by deregulation of the *hom* operon, in 1986 Parsot showed that the two enzymes had a common ancestor (and are indeed 25% identical) and in the project this work is a part of, a new mutation in the unknown gene *thrR* revealed that the *hom* operon is actually repressed by the encoded regulator ThrR (Vapnek, Greer 1971a, 1971b; Parsot 1986; Rosenberg et al. 2016). However, it could take another decade until the effector of ThrR will be found. ThrR contains a weakly conserved helix-turn-helix (HTH) motif from residue 27 to 56 and a well conserved aspartate kinase-chorismate mutase-tyrA (ACT) domain from residue 70 to 141 (KEGG). ACT domains are known to have regulatory effects and often bind small molecule ligands, which are mostly amino acids (Pfam PF01842 , Liberles et al. 2005). For example the regulation of aspartokinase III by lysine is conveyed by an ACT domain (Kobashi et al. 2001, uniprot). Therefore, the initial hypothesis was that ThrR would be an amino acid-dependent regulator. However, although DNA-binding and cysteine and lysine-dependent regulation of *hom* by ThrR could be shown, no interaction between ThrR and these amino acids could be proven by ITC measurements (data not shown for lysine, for cysteine see Figure S 3). In the case of cysteine, the ITC measurements are difficult to interpret. In absence of a reductive agent, there is a very strong but unstable signal indicating a binding ratio of 1:1 but in presence of DTT, no strong signal could be observed. This might hint at a different exothermic reaction than the interaction between cysteine and ThrR as for example an oxidation of cysteine to cystine or cysteine sulfonic acid (Poole 2015). On the other hand, DTT might lead to an inactivation of ThrR. Future experiments as for example NMR might shed light on the interaction between ThrR and cysteine (Otting 1993). So long, no amino acid seems to interact directly with ThrR. However, the regulation by isoleucine, homoserine, cysteine and especially lysine is obvious. An explanation for this divergence might be an indirect regulation of ThrR by the amino acids as it is the case for TnrA (Wray et al. 2001). ACT domains have been shown to take part in dimerization (Grant 2006) and might therefore also take part in the interaction between proteins. The applied techniques to identify possible proteinous interaction partners show no reproducible results, however. The possible interaction with CodY was ruled out by a B2H and SPINE and so was the interaction between BirA and ThrR. The fact that BirA was found in the SPINE is likely due to a cross-contamination.

Summarizing, ThrR is a novel regulator of the *hom* and *thrD* operons, but the mechanism by which it is regulated itself remains unknown.

4.3. Update on the regulation of aspartate-derived amino acids

In *B. subtilis*, isoleucine, threonine, lysine and methionine all derive from aspartate and therefore are called the aspartate-derived amino acids. The regulation of the pathways leading to the different amino acids are very differentially regulated by transcriptional inhibition and activation and allosteric feedback inhibition (see 1.4.4). Figure 36 shows a scheme for the regulation of these pathways dependent on the abundance of different amino acids. The differential regulation starts with the three aspartokinases I, II and III, DapG, LysC and ThrD, respectively. DapG is known to be inhibited by DAP and therefore thought to be the designated enzyme for initiation of the pathway leading to lysine and peptidoglycan (Graves, Switzer 1990). The same holds true for LysC, which is transcriptionally repressed by lysine (Grundy et al. 2003; Kochhar, Paulus 1996). ThrD is inhibited by threonine and lysine in combination, indicating a more general role than the other two feedback-inhibited aspartokinases (Kobashi et al. 2001). At the same time, ThrD is transcriptionally activated by lysine and so are Hom, ThrB and ThrC, the threonine synthetic enzymes (Graves, Switzer 1990; Rosenberg et al. 2016). This indicates that the aspartokinases function like a sorter, distributing metabolites to either of the pathways, suggesting possible channeling mechanisms. In the case that lysine, threonine and DAP are all equally abundant, all aspartokinases will be inactive, DapG is inhibited by DAP, LysC will be repressed by lysine and ThrD will be inactivated by lysine and threonine but presumably very abundant due to

transcriptional activation by lysine. This might be an insurance that the cell will have enough aspartokinase to swiftly start producing ASA if needed. In the situation that lysine and DAP are abundant, but threonine is limited, lysine and DAP inhibit DapG and repress LysC, but ThrD is still active due to the lack of inhibition by threonine. When threonine is abundant but DAP and lysine are scarce, Hom is feedback inhibited and ASA is funneled into the DAP pathway (Yeggy, Stahly 1980; Parsot, Cohen 1988; Gutiérrez-Preciado et al. 2009). Furthermore, homoserine and isoleucine will most likely also be abundant under these circumstances, leading to transcriptional inhibition of Hom, ThrB and ThrC. Additionally, the transcriptional activation of these three enzymes by cysteine shown in this work guarantees threonine synthesis when cysteine is abundant, by pulling homoserine into threonine synthesis. This mechanism is very elegant, since the aspartokinases will be inhibited once the other aspartate-derived amino acids are abundant and do not need to be inhibited also by cysteine.

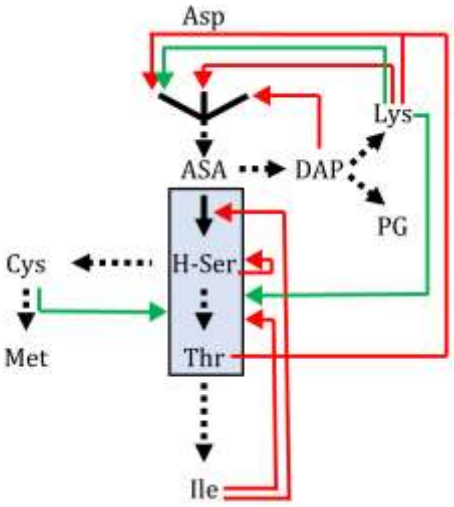

Figure 36: Scheme illustrating the regulation of the aspartate derived amino acid pathways. Amino acids are three-letter-code; ASA, L-aspartate-4-semialdehyde, H-Ser, homoserine; PG, peptidoglycan; DAP, diaminopimelate. Blue box, part of the Thr pathway encoded in the *hom* operon. Red and green lines show inhibitory and activating regulation, respectively. Dotted lines show metabolic pathways containing more than one enzyme.

In summary, the synthesis of aspartate-derived amino acids is a very sophisticated, fine-tuned tree of pathways originating from three sorter-like aspartokinases.

4.4. Biotin as a vitamin B6 antidote

It has been previously shown and demonstrated here that exogenous PL is toxic to cells above a certain threshold, which for *B. subtilis* is approximately 3 mM (Figure 11, Vermeersch et al. 2004; Dong, Fromm 1990; Ohsawa, Gualerzi 1981; Metzler, Snell 1952; Shanbhag, Martell 1991). The reasons for this toxicity are most likely inactivation of proteins (Vermeersch et al. 2004; Dong, Fromm 1990) and non-enzymatic deamination of metabolites as shown for amino acids (Metzler, Snell 1952; Shanbhag, Martell 1991; Keller et al. 2015). In 3.1.2 it is shown that *B. subtilis* can form suppressor mutants that are able to live in presence of toxic amounts of PL on solid medium. Interestingly, the suppressors also allow growth of neighboring cells (Figure 12) and are not able to grow in liquid medium with toxic PL amounts (Figure 13B). This might indicate that the acquired mutations lead to the secretion of either (I) a beneficial metabolite (antidote) or (II) a PL(P)-degrading or -modifying enzyme. Both mechanisms would have stronger effect in a more stable and diffusion restricted environment like agar plates, because the factor conveying resistance does not diffuse away as fast and the toxic compound does not get replenished as fast as in liquid medium, fitting the observation in Figure 13. Possibility (I) could be explained by the need for amino acids, which are depleted by deamination through PL. In this case the secretion of amino acids could be beneficial by interaction with PL resulting in a lower concentration of free vitamers. Indeed, external addition of casamino acids allows growth of *B. subtilis* in presence of toxic PL amounts (Bolgi 2017). A mechanism like (II) is commonly known from β-lactamases, which are secreted into the medium, degrade β-lactam antibiotics and lead to the formation of satellite colonies, which themselves do not necessarily encode the resistance (Medaney et al. 2016). However, the suppressor mutants acquired genomic alterations leading to the up-regulation of the biotin synthesis operon (see Table 5 and Figure 14). Furthermore, additional results from this lab could show that the external addition of biotin improved growth in presence of toxic amounts of PL (Bolgi 2017). Therefore, this

resistance is caused by the accumulation and seemingly also secretion of another B vitamin, biotin. Biotin and PLP are metabolically connected, because both 8-amino-7-oxoonanoate synthase BioF and 7,8-diaminopelargonic acid aminotransferase BioA are PLP-dependent enzymes (Mann, Ploux 2011). Apart from that, the two vitamins have quite different functions. Vitamin B6 is involved mainly in the amino acid-related metabolism, while vitamin B7 is involved in the synthesis of fatty acids by biotinylation of the acetyl-CoA carboxylase subunit AccB and in pyruvate metabolism through the biotin-dependent pyruvate carboxylase PycA (Cronan, Waldrop 2002; Valle 2017). Despite these differences, there are currently three working hypotheses for the effect of biotin, some of which have already been tested:

1) Biotin and PL or PLP could interact directly. This way biotin would lower the amount of free vitamers thereby lowering the probability of harmful interactions. The biochemical basis for this could be an interaction of pyridoxal with the secondary amines of biotin or the primary amino groups of the precursor 7,8-diamino-onanoate. These compounds and PL might form a Schiff base and leave a great percentage of the interaction partners in an intermediate state, because non-enzymatic deamination is inefficient at pH<9 (Shanbhag, Martell 1991). However, biotin and PL could not be shown to interact directly in ITC measurements, hinting that PL might not be able to interact with secondary amines (Bolgi 2017). It might however be possible that external addition of biotin leads to an accumulation of its precursors, which could then interact with PL. This hypothesis remains to be tested.

2) PLP might compete with biotin for the biotinylation site of AccB and BirA. It is known that PLP interacts with lysine residues in PLP-dependent enzymes, which is also true for biotin in biotinylated enzymes (Toney 2005; Waldrop et al. 1994). Furthermore, the toxic effect of exogenously added PL has been accounted to the modification of lysins in DNA topoisomerase and initiation factors (Vermeersch et al. 2004; Ohsawa, Gualerzi 1981). This could mean that the higher amount of biotin would ensure that the biotinylation sites are saturated and less prone to binding and modification by PLP. Others tried to pinpoint this interaction by overexpression of AccB in *B. subtilis* in order to achieve higher tolerance to exogenous PL but the experiments gave no evidence for a beneficial effect of AccB overexpression (Bolgi 2017). For future experiments, BirA and PycA should also be considered, because also this enzyme might be inhibited by PLP.

3) Lastly, PLP might by an unknown mechanism inhibit the expression of the *bio* operon, depleting the essential vitamin biotin. Interestingly, experiments showed that this is indeed the case. In presence of high amounts of exogenous PL, the biotin operon is inhibited (Bolgi 2017). Under the same conditions, the operon containing the trigger enzyme BirA encoding *birA* gene was upregulated (Bolgi 2017). It seems therefore plausible that toxic PL amounts cause regulatory biotin auxotrophy in *B. subtilis*. This relationship should be proven by BirA-independent, constitutive expression of the *bio* operon in the future. However, in the presence of toxic PL concentrations, the biotin concentration should drop according to our observations, which is in contrast to the present understanding, that a lower biotin concentration leads to derepression of the *bio* operon, because BirA inhibits the *bio* operon in the monomeric, biotin-bound state (Wang, Beckett 2017). The fact that the cells anyhow acquired deregulating mutations in the promoter of the operon indicate that this mechanism does not work under the tested conditions. Therefore, it should be evaluated whether PLP inhibits BirA dimerization and enhances DNA binding, creating a pressure to deregulate the *bio* operon.

4.5. The search for bacterial vitamin B6 transporters

To date, no bacterial vitamin B6 transporter has been identified, although the capability to take up B6 vitamers has been proven as summarized in the following. It was shown a longer time ago that only non-phosphorylated vitamers can be taken up by *E. coli* and kinetic measurements hint at facilitated rather than simple diffusion (Yamada et al. 1977). However, one study showed that PN was possibly diffusing through membranes of guinea pig jejunum (Yoshida et al. 1981). Again on the contrary, vitamin B6 is transported and retained by phosphorylation in

E. coli as shown by competition experiments (Yamada, Furukawa 1981). The first transporter was quite recently identified in *Saccharomyces cerevisiae*. Tpn1p of the purine-cytosine permease family is a high affinity PN:proton symporter (Stolz, Vielreicher 2003). This transporter has 23% identity with *B. subtilis*' uncharacterized putative purine-cytosine permease YxlA with a query cover of 86% (BLAST, Altschul et al. 1997). Shortly after Tpn1p, the pyridoxine kinase PdxK was found in *B. subtilis*, which is a homolog of PdxK from *E. coli* and is very likely involved in the retainment of imported B6 vitamers (Park et al. 2004). The novel transporter Bsu1p of the major facilitator family was found in *Schizosaccharomyces pombe*, which is not a homolog of Tpn1p. It is also a proton symporter and is most likely a homolog of the human transporter (Stolz et al. 2005; Hediger et al. 2013). This transporter has 24% identity with Bcr from *E. coli* with a query cover of 79% and very low similarity with YdgK from *B. subtilis* (BLAST, Altschul et al. 1997). PUP1 (not to be confused with PUP1 from yeast) is a vitamin B6 proton symporter from *Arabidopsis thaliana*, which is not homologous to the previously known transporters and has no homologs in prokaryotes or archaea according to pBLAST (Szydlowski et al. 2013; Altschul et al. 1997). Additionally, NUP1, a homolog of PUP1, was found in *Nicotiana tabacum* (Kato et al. 2015).

As mentioned above, Tpn1p has quite some similarity to a protein from *B. subtilis*, the putative purine-cytosine permease YxlA. YxlA together with the known allantoin permease PucI is part of the Nucleobase:cation symporter-1 (NCS1) family, which is known to function as proton symporters for nucleobases and vitamin B6 (Pfam, Finn et al. 2016). *B. subtilis* contains also three transporters of the NCS2 family, PbuX, PucJ and PucK. The similarity of these transporters makes all of them strong candidates for putative vitamin B6 transporters of *B. subtilis* worth investigation in the future. However, the fact that no transporters were found to be mutated in the PL-resistant strains indicates that *B. subtilis* either encodes several or no dedicated B6 transporters at all. It could also be interesting to follow a bioinformatic approach, comparing the expression of the B6-synthetic *pdxST* genes to known transporter genes. The synthetic genes should be needed when the cofactor is scarce in the environment, while the transporter is dispensable and on the opposite, when there is plenty of the cofactor in the environment, the synthesis genes are dispensable, and the transporters are required. Therefore, the dedicated transporters, if there are any, should be reversely regulated and it should be possible to analyze existing transcriptomic and proteomic data to find candidates. There is an example for a transporter, whose expression is negatively corelating with *pdxST*. The genes encoding the Na^+/H^+ antiporter system KhtSTU, which is involved in the detoxification of methylglyoxal (MG) (Subtiwiki, Michna et al. 2016; Chandrangsu et al. 2014).

4.6. *B. subtilis* is not able to feed intermediates of B6 synthesis

In 3.2, the incapability of consortium B to form a stable coexistence of the strains showed that strains expressing partial B6 biosynthesis pathways were not able to feed each other the intermediates PN and/or PL. We know however, that PL producing strains can feed auxotrophs as shown for example in Figure 15 and Figure 17. Therefore, the vitamer that cannot be fed must be PN. There are two possible reasons for this. (I) The export of PNP requires dephosphorylation and the import successful phosphorylation. The enzymes responsible for these processes might be inactive under the used conditions or specific for PLP in *B. subtilis*, or (II) the production of PNP is feedback-inhibited and the product never reaches a titer sufficient to sustain growth of both strains.

The first possibility might be explained by the fact that some phosphatases are inhibited by inorganic phosphate and the used C Glc minimal medium is rich in phosphate (Huang et al. 2012; Tazoe et al. 2005; Martin-Verstraete et al. 1990). Furthermore, a high phosphate concentration changes the equilibrium in favor of phosphorylated compounds thus preventing phosphatase activity and export of vitamers. Additionally, the unknown phosphatase of *B. subtilis* might also be specific to PLP, forcing PNP to be retained inside the cell. The phosphatase PdxP from *S. meliloti* catalyzes hydrolysis of PLP and PNP but not of PMP, for example. Since *E. coli* and *B. subtilis* share no homologs of this phosphatase, it is completely unknown, which

specificities their phosphatases could have (EggNOG, Huerta-Cepas et al. 2016). The second possibility seems realistic because it is known that PdxH and Dxs are subject to feedback-inhibition. PdxH from *E. coli* is inhibited by its product PLP and Dxs from several organisms including *B. subtilis* by isopentenyl pyrophosphate and dimethylallyl pyrophosphate (for *B. subtilis* this was only shown for the latter), which are both products of the isoprenoid synthesis pathway (Zhao, Winkler 1995; Banerjee et al. 2013; Kudoh et al. 2017). For vitamin B6 synthesis intermediates and for other enzymes feedback inhibition has not been described so far but remains imaginable.

Summarizing, the incapability of strains expressing partial DXP-dependent pathways to exchange intermediates of the pathway could be due to the unknown specificities and feedback-inhibitory interactions of the synthesizing and modifying enzymes. It does not seem to be beneficial to the cell to produce a useless vitamer (PN) in huge quantities to feed it to another strain. Therefore, the ability to achieve a stable consortium would likely need to be engineered.

4.7. An alternative route for threonine and isoleucine biosynthesis in *B. subtilis*

As described in 3.2.2, a *thrC* deletion strain carrying the heterologous DXP-dependent vitamin B6 synthesis pathway formed suppressor mutants that could grow on C Glc minimal medium without external addition of threonine (Figure 21). This kind of suppressor mutant has not been described previously according to thorough literature research. The mechanism by which these mutants overcome the absence of the threonine synthase is so far unknown. It has previously and above been described that mutants lacking threonine dehydratase IlvA could overcome isoleucine auxotrophy by overexpression of the *hom* operon leading to higher amount of ThrC, which has a minor threonine dehydratase activity (Vapnek, Greer 1971a, 1971b). Furthermore, threonine synthase, threonine dehydratase and D-serine dehydratase have been shown to have a common ancestor and that they are carrying out their reaction by a conserved mechanism (Parsot 1986). It was hypothesized that the current suppressors would overcome lack of threonine synthase by overexpression of IlvA, possibly utilizing a minor threonine synthase activity. However, the promoter of *ilvA* (P_{ilvA}) was not mutated. Another possibility was that enzymes upstream of ThrC could take over the threonine synthase activity and that this would be achieved by deregulation of the *hom* operon. Therefore, the sequence encoding the described regulator of *hom*, ThrR and the sequence of the promoter of *hom* (P_{hom}) were checked, but also turned out to be the wildtype alleles. Additionally, *B. subtilis* can catabolize threonine to glycine via threonine dehydrogenase Tdh and 2-amino-3-ketobutyrate CoA ligase Kbl and both reactions of this catabolic pathway are theoretically reversible, which proposes a possible mechanism for a bypass of ThrC (Figure 37 and Figure 40; Schmidt et al. 2001; Higashi et al. 2005; Raj et al. 2014). The suppressor mutations might lead to an accumulation of glycine raising the reaction equilibrium concentration and/or a deregulation of the operon of *tdh* and *kbl* leading to an inversion of the metabolic flux and production of threonine. Future investigations should check these possibilities and also take into account the D-serine deaminase SdsA, which could also show a minor threonine synthase activity due to its mechanistic similarity (Parsot 1986).

Furthermore, other bacteria including *E. coli* possess the capability to degrade threonine directly to glycine by activity of threonine aldolase LtaE (which is also part of a serendipitous B6 synthesis pathway in *E. coli*) (Liu et al. 1998; Kim et al. 2010). This reaction is in theory also reversible. *B. subtilis* does not encode any homolog of this enzyme, although it is universally present in all domains of life and even other bacilli do encode it (Pfam, Finn et al. 2016). It would however be imaginable that *B. subtilis* acquired this function by mutation. It was furthermore found that 2-isopropylmalate synthase LeuA is 40% identical and homologous to (R)-citramalate synthase CimA from *Methanocaldococcus jannaschii* (2.3.1.182) whose activity is not thought to be existent in *B. subtilis* (BLAST, Altschul et al. 1997). (R)-citramalate synthase feeds into an alternative acetyl-CoA and pyruvate-derived isoleucine synthesis pathway and might thereby bypass the *thrC* deletion in the case that the reaction

from 2-OB to threonine is reversible *in vivo*, which might be the case when the given pathway is strongly deregulated and 2-OB accumulates (Charon et al. 1974; Howell et al. 1999; Risso et al. 2008). Lastly, although it is unlikely that the enzymes of the DXP-dependent vitamin B6 synthesis pathway are able to convey threonine synthase activity, this possibility should be ruled out by knockouts of the heterologous constructs or crossing with the *thrC* deletion mutant BP920 (Δ*pdxST* Δ*thrC*) with selection for threonine prototrophy and checks for the absence of the heterologous constructs.

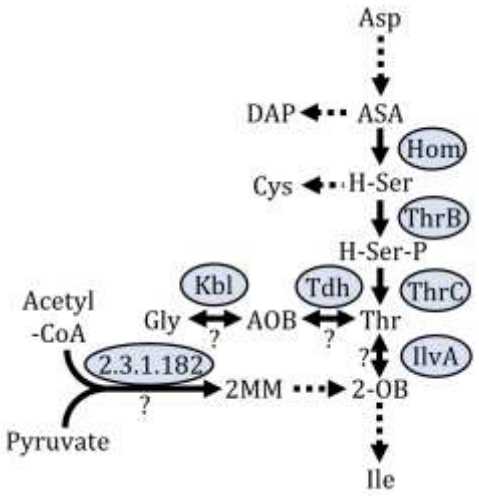

Figure 37: Scheme showing possible ThrC bypasses in *B. subtilis*. Amino acids are three-letter-code; ASA, L-aspartate-4-semialdehyde, H-Ser(-P), homoserine (phosphate); DAP, diaminopimelate; 2-OB, 2-oxobutyrate; 2MM, (R)-2-methylmalate; AOB, 2-amino-3-oxobutyrate. Protein shown in circles, dotted lines depict pathways carried out by more than one enzyme. Reactions that have not yet been shown to exist or be reversible in *B. subtilis* are depicted with question marks.

Summarizing, there are several possible routes the isolated *thrC* suppressor mutants might take to bypass threonine synthase (Figure 37) and future experiments should elucidate this potential alternative route for synthesis of isoleucine and threonine. It is strongly advised to further investigate these suppressors and possibly determine the whole genome sequence.

4.8. Vitamin B6 starvation is connected to amino acid limitation

Vitamin B6 is a cofactor in many amino acid-related reactions (see 1.2). It is therefore a logical consequence that lack of this essential cofactor is accompanied by disturbances in amino acid metabolism. However, only little is known about the effects of vitamin B6 starvation on the cellular level. It has been shown decades ago that isoleucine and threonine prolong growth during PN starvation (Dempsey, Sims 1972). Furthermore, B6 limitation in humans leads to accumulation of intermediates of cysteine and methionine homeostasis (Dalto, Matte 2017). The presented results show that vitamin B6 auxotrophic strains expressing the heterologous partial DXP-dependent pathway can grow at least for some hours in presence of CAA in combination with methionine or cysteine (Figure 28). It however remains unknown, whether this effect is due to feeding into the heterologous pathway or starvation for amino acids. Other work in this lab showed that addition of complex amino acids did not allow for growth of vitamin B6 auxotrophic strains suggesting that the growth promoting effect is indeed due to feeding into the pathway (Yeak 2017). Future research should elucidate the connection between B6 starvation and amino acid limitation.

4.9. Adaptation to the introduction of a heterologous pathway

This work tried to find novel ways to ship around the current obstacles in the widely unknown waters of fermentative vitamin B6 production (see 1.3). In many ways the heterologous introduction of production pathways into host organisms is equivalent to horizontal gene transfer (HGT). In both cases genes are acquired that did not coevolve with the host metabolism (at least in the recent past). This can mean that substrates, cofactors, export systems or detoxification mechanisms required for use of function encoded by the new genes are absent. To use the pathway encoded by the newly acquired genes, the host must adapt, and the adaptations can be categorized into (I) adaptation to interactions between acquired and host metabolism and (II) rewiring of the acquired pathway to the host metabolism (Figure 38).

(I) Other reports previously highlighted the fact that horizontal transfer of elements (genes, mobile elements, plasmids) conveying a trait is not necessarily sufficient and needs further refinement because the new trait might be costly or interactions between host metabolism and the heterologous or xenologous element can be detrimental (Carroll, Marx 2013; Michener et al. 2014; Rand et al. 2017). It can in fact be expected that many beneficial mutations that occur after acquisition are restoring the metabolic state before the event, rather than rewiring or optimizing the newly acquired element. This, among other adaptations, has been observed when the pathway for the use of methanol of *Methylobacterium* was replaced with a heterologous pathway (Carroll, Marx 2013). The newly acquired pathway was functional but depleted the cellular NADPH which was countered by mutations deregulating the *pntAB* genes which encode transhydrogenases capable of replenishing the NADPH pool. In case the new element is not required for growth, the adaptation could also lead to inactivation, for example when the DXP-dependent vitamin B6 pathway was introduced into B6 prototrophic *B. subtilis* the gene encoding the first enzyme of the pathway, *epd*, was inactivated by mutation in several cases (Commichau et al. 2014). This lead to inactivation of the unnecessary and obviously costly heterologous pathway.

(II) The actual rewiring of the new pathway to the host metabolism can happen in several different ways, for example through regulatory rewiring, activation of pathways supporting the new function, or inactivation of pathways that inhibit it. This was observed, when the previously described beneficial but costly pathway for use of methanol as a carbon source was expressed from a plasmid in *Methylobacterium* lacking the endogenous methanol catabolic pathway (Chou, Marx 2012). The engineered cells initially grew worse than the wildtype on medium containing methanol but acquired mutations that optimized the gene dosage or expression of the recombinant pathway.

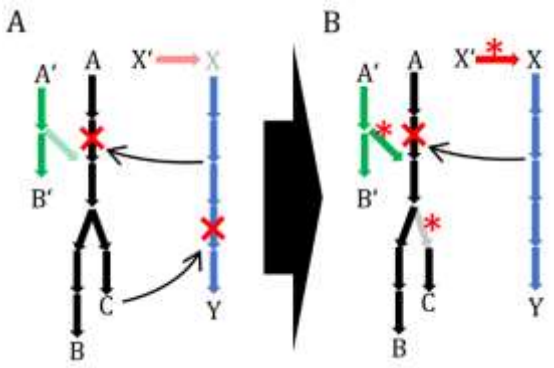

Figure 38: Scheme showing some possible host-heterologous interactions and possibilities to circumvent these. (A) Situation directly after introduction of the heterologous element (blue) conveying the trait of conversion of X to Y. Endogenous pathways shown in red, green and black. The host does not endogenously produce X, but a similar compound X'. One intermediate of the heterologous pathway interferes with synthesis of A and B. C allosterically inhibits one of the heterologous enzymes. (B) Situation after adaptation. The host acquired mutations unmasking underground metabolic activities leading to the conversion of X to X' and an activity bypassing the inhibited enzyme in the pathway synthesizing A and B. The host furthermore inactivated the pathway synthesizing C, thus allowing production of Y.

Furthermore, mutations optimizing and rewiring the new pathway have been observed in an *E. coli* strain that has been engineered to utilize levulinic acid as a carbon source (Rand et al. 2017). Laboratory evolution lead to deregulatory mutations in the heterologous construct and the functional deletion of *fadE* and *atoC*, rerouting metabolic flux to rewire the newly acquired pathway.

In summary, overexpression of a heterologous pathway is regularly not sufficient to reach ideal function of the foreign enzymes in the new host and genetic optimization by engineering approaches or adaptive evolution are required to overcome detrimental effects and rewire the new pathway to host metabolism.

4.10. Adaptation of *B. subtilis* to a partial DXP-dependent vitamin B6 synthesis pathway

As part of this work, the DXP-dependent vitamin B6 synthesis pathway from *E. coli* was reversely engineered in a B6 auxotrophic *B. subtilis* strain. The introduction of only the genes encoding the

last two enzymes of the heterologous pathway PdxJ and PdxH, allowed the isolation of suppressor mutants that could grow on minimal medium without external addition of vitamin B6 (Figure 24). The precursors for the reaction catalyzed by PdxJ are DXP and APA. The first is also present in *B. subtilis* and produced by Dxs as a precursor for thiamine and isoprenoid synthesis. The latter is not known to be present in *B. subtilis*, although similar compounds exist (Figure 40, KEGG). However, the fact that the suppressors can grow means either that *B. subtilis* is able to produce this compound, or that the ability was acquired by the suppressor mutants. The following part of this discussion will evaluate the current knowledge and propose future strategies to determine how the suppressors are able to utilize the partial DXP-dependent pathway.

4.10.1. A mysterious connection

All suppressor mutants inactivated biosynthesis of BSH (Table 9 and Table 10). BSH has been shown to be present in *B. subtilis*, *S. aureus* and *D. radiodurans* and is important for management of the redox potential of the cell, coping with oxidative stress and detoxification of formaldehyde (FA), fosfomycin as well as MG (Newton et al. 2009; Chandrangsu et al. 2014; Chandrangsu et al. 2017). According to its function as a protectant under oxidative stress conditions, the genes encoding its synthetic pathway are regulated by the oxidative stress regulator Spx (see 1.4.5). The isolated, BSH-deficient suppressor mutants could grow on minimal medium but were much slower than the wildtype (Figure 25). It was speculated that absence of BSH would allow the cells to grow independently or at least in presence of extremely low amounts of vitamin B6. However, only cells encoding PdxJ and PdxH could profit from the absence of BSH, but not for example cells only encoding PdxH or B6 auxotrophs grown under B6 limitation (Figure 26 and Figure 27). That clearly indicates that the deletion of BSH synthetic genes was directly related to the partial heterologous DXP-dependent B6 pathway and not to endogenous B6 metabolism. This raised the question how the mutation aided B6 synthesis. BSH is synthesized from UDP-GlcNAc in three steps by L-malic acid transferase BshA, GlcN-mal deacetylase BshB and the putative cysteine ligase BshC (Gaballa et al. 2010; Upton et al. 2012; Fang et al. 2013; VanDuinen et al. 2015; Winchell et al. 2016). The synthesis consumes UDP-GlcNAc, malic acid and cysteine and cells deficient in BSH accumulate the GlcNAc-related precursors (Gaballa et al. 2010). It is therefore tempting to speculate that a GlcNAc derivative is feeding into the reaction catalyzed by PdxJ. However, addition of GlcNAc, which should readily be converted to UDP-GlcNAc and enter the BSH pathway by action of the GlcNAc phosphotransferase NagP and GlcNAc-1P uridyltransferase GlmU, was not beneficial for growth of strains expressing the DXP-dependent pathway in presence of BSH (Figure 33). GlcNAc should be taken up also in presence of glucose, because in contrast to the GlcNAc utilization enzymes NagAB, neither the transporter NagP, nor GlmU are under control of carbon catabolite repression, possibly because even in presence of glucose, recycling of cell wall components might be beneficial (Blencke et al. 2003; Plumbridge 2015). Interestingly, GlcNAc had a beneficial effect for strains lacking BSH (Figure 33). BSH might have a regulatory effect on the peptidoglycan synthetic pathway, hinting at a feedback-regulatory mechanism distributing the UDP-GlcNAc precursor to peptidoglycan or BSH synthesis. In this case presence of BSH would inhibit the metabolic flux towards BSH synthesis and absence would cause too much of the produced or scavenged UDP-GlcNAc to be funneled away from peptidoglycan synthesis. External addition of GlcNAc could then alleviate the growth defect caused by lack of cell wall precursors through a higher equilibrium concentration. In fact, BshA is feedback inhibited by BSH, supporting this hypothesis (Upton et al. 2012). Another explanation is that UDP-GlcNAc indeed feeds into the partial DXP-dependent B6 synthesis pathway, but that the unknown GlcNAc-derived precursor cannot be utilized by PdxJ in presence of BSH because of the feedback inhibition of BshA. This would imply that not UDP-GlcNAc but one of the BSH synthesis intermediates feeds into the pathway. This however is not the case, because deletion of *bshA* has the same, beneficial effect as deletion of *bshC* (Figure 26). Taken together, the accumulating precursors of BSH do not seem to directly feed into the pathway in the suppressor mutants.

Cells deficient in low molecular weight (LMW) thiols like BSH are also thought to accumulate MG (Choi et al. 2008; Chandrangsu et al. 2014). The major detoxification mechanism of MG in *B. subtilis* involves a BSH-bound intermediate, which activates proton import leading to acidification of the cytosol and consequently prohibition of nucleophilic damage by MG (Chandrangsu et al. 2014). The precursor of MG dihydroxyacetone phosphate (DPA), has a very similar structure like the substrate of PdxJ APA (Figure 40), proposing a possible feeding into the pathway. In this case, the deletion of BSH could lead to accumulation of MG, either by induction of oxidative stress due to the absence of this redox buffer, or by lack of detoxification of MG. However, for the conducted experiments C Glc was used which is a minimal medium containing high amounts (100 mM) of phosphate and moderate amounts of glucose. Under these conditions, MG synthesis by MgsA should be inhibited due to allosteric inhibition through phosphate (Martin-Verstraete et al. 1990; Landmann et al. 2011; Falahati et al. 2013). In comparison, the media used in the publications stating MG accumulation used either LB complex medium or HL5, which contain an unknown but low amount and 4 mM phosphate, respectively (Choi et al. 2008; Chandrangsu et al. 2014; Rich axenic medium (HL5) 2008). It can therefore be assumed that the accumulation of MG is not the mechanism by which the suppressors feed into the pathway under the given conditions. To be completely sure about this, MG feeding experiments should be performed with the B6 auxotrophic strains expressing *pdxJ* and *pdxH* to check whether they grow like the isogenic BSH deficient strains.

It was also checked, whether the deletion of BSH would lead to an increased pool of sulfur containing amino acids (Figure 28). It is known, that under oxidative stress conditions, BSH is reversely inactivating the methionine synthase MetE protecting it from irreversible inactivation by oxidation of a vulnerable cysteine residue (Chi et al. 2011; Chi et al. 2013). This bacillithiylation leads to methionine auxotrophy, which is relieved when oxidative stress is removed. It was hypothesized that a B6 auxotrophic strain expressing the partial DXP-dependent pathway would suffer from vitamin B6 limitation, which might induce oxidative stress, because vitamin B6 is a reductant in eukaryotes (Bilski et al. 2000; Jain, Lim 2001). The lack of the putative bacterial antioxidant vitamin B6 would lead to activation of the Spx-mediated oxidative stress response and activation of many genes including *bshC* and the operon encoding BshA, BshB1, MgsA, and BirA. In consequence, MetE would be inactivated, and the cells would starve for methionine and the inactivation of BSH synthesis by mutation of *bshA* or *bshC* would relieve this auxotrophy. However, experiments on medium with methionine showed that the addition of methionine does not allow growth and that the suppressor mutants arising on this medium also abolished BSH synthesis (Figure 28 and Table 10). Furthermore, no evidence for oxidative stress induced bacillithiylation could be observed (Figure 28). An alternative hypothesis was that the limitation of vitamin B6 would lead to the limitation of cysteine and that the deletion of the gene encoding the putative cysteine ligase BshC could relieve this limitation. Indeed, it has been shown that in humans, vitamin B6 limitation leads to accumulation of intermediates from cysteine metabolism, because most of these use PLP as a cofactor (Dalto, Matte 2017). However, a B6 auxotrophic strain expressing the partial DXP-dependent pathway could also not grow on plates with cysteine, also in combination with methionine. Therefore, the mutations abolishing BSH synthesis were not selected by limitation of sulfur-containing amino acids, which is in good agreement with data indicating that in bacteria threonine and isoleucine are the first amino acids to be limited under vitamin B6 starvation conditions (Dempsey, Sims 1972).

Two other hypotheses are that the introduction of the recombinant genes leads to oxidative stress, because PdxH produces H_2O_2, explaining why the deletion of BSH synthesis genes only benefits strains expressing the recombinant genes (Zhao, Winkler 1995). Or the heterologous proteins PdxJ and PdxH, which both contain cysteines, could be inhibited by BSH directly. However, no bacillithiylated proteins of that size were found in an bacillithiylation assay (data not shown).

Summarizing, there seems to be a connection between the expression of the partial heterologous DXP-dependent vitamin B6 synthesis pathway and the oxidative stress

response in *B. subtilis*. The exact mechanism by which they are connected could not yet be elucidated, but it has been shown that the presence of BSH is detrimental for the function of the heterologous pathway in *B. subtilis*. Future experiments should elucidate, whether MG has a beneficial effect on the growth in presence of PdxJ and PdxH and whether the investigated strains are activating the Spx-mediated stress response.

4.10.2. The unknown protein YtoQ

The strains expressing the partial heterologous pathway and with deletion of *bshC* can grow in absence of externally added vitamin B6, but do so much slower than the wildtype (Figure 25). Therefore, the strains were evolved in minimal medium, which gave rise to two evolved strains capable to grow as fast as the wildtype (Figure 30, Yeak 2017). These strains carried, among others, mutations leading to a deregulation or higher gene dosage of *ytoQ*. Reverse engineering of the suppressor mutants by overexpression of the encoded, unknown protein YtoQ showed that growth like wildtype can be ascribed to the *ytoQ*-related mutations and deletion of *ytoQ* lead to inability of these strains to grow in absence of vitamin B6 (Figure 30 and Figure 31). Thus, YtoQ is needed for growth of B6 auxotrophic strains with the partial heterologous pathway and its overexpression allows growth like wildtype.

The gene *ytoQ* encodes an unknown protein of the YtoQ family (IPR019884) containing a nucleoside deoxyribosyl transferase domain (PF11071) and a weakly conserved helix-turn-helix motif (KEGG, Pfam, Interpro, Table 11, Finn et al. 2016; Finn et al. 2017). This family of proteins has distant similarity to the nucleoside deoxyribosyl transferase family (COG3613), members of which are known to be able to add, release, and interchange bases of nucleosides and are thought to be a part of a pyrimidine salvage pathway (EC 2.4.2.6) (Macnutt 1952; Porter et al. 1995; Finn et al. 2017). It is to date unknown, what function members of the YtoQ family have but according to the features, YtoQ might be a regulatory protein, an enzyme, or both, allowing for a plethora of possible effects on the suppressor mutants. The following part of the discussion will deal with what is known about YtoQ and try to draw some conclusions.

In *Lactococcus lactis*, a very distant *ytoQ* relative of the COG3613 family is regulated by a Spx homolog under envelope stress, suggesting a similar function of YtoQ family proteins, since *ytoQ* is regulated by Spx in *B. subtilis* (Veiga et al. 2007; Zuber et al. 2011). Another member of this distantly related family from *Rattus norvegicus* has been implied to be involved in tumorigenesis (Ghiorghi et al. 2007). The closer relatives of YtoQ are even less characterized. Proteins containing domains of the same nucleoside deoxyribosyl transferase family as YtoQ (PF11071) are found in some eukaryotes and many bacteria, mostly in firmicutes (mainly bacillacae), α- and γ-proteobacteria (but not enterobacteria) but also some actinobacteria (Pfam, Finn et al. 2016). A STRING database search showed that its neighborhood with *ytpQ* and *ytpR* is conserved among bacillacae and the neighborhood with *ytpR* also in proteobacteria (STRING, Zuber et al. 2011). Importantly, both *ytoQ* (as earlier mentioned) and the divergently orientated tricistronic operon containing *ytpP*, *ytpQ* and *ytpR* are under control of the oxidative stress regulator Spx in *B. subtilis* (Zuber et al. 2011).

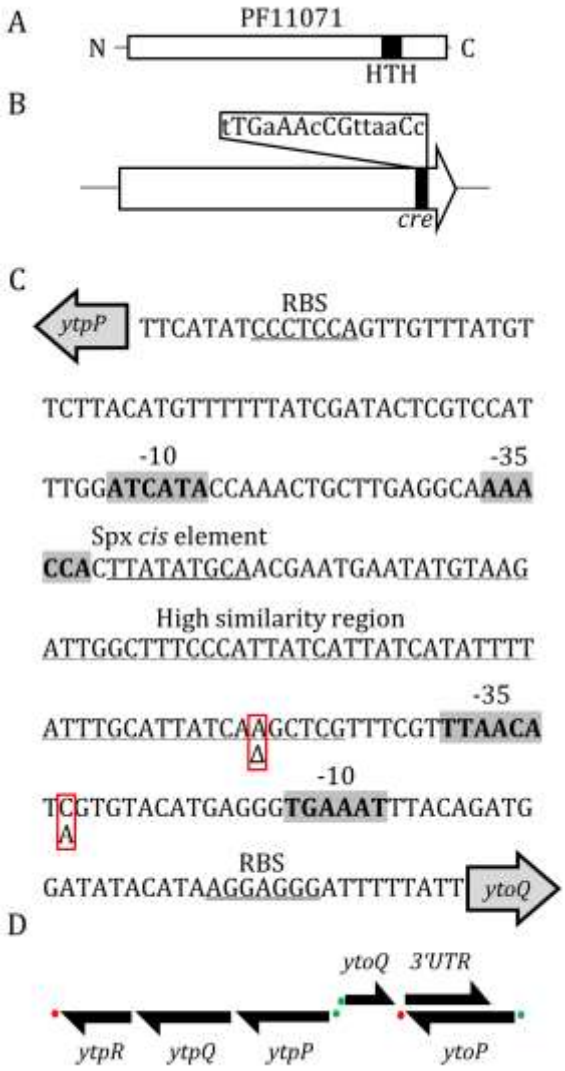

Figure 39: (A) Scheme of the protein YtoQ, which is mainly made up by the nucleoside deoxyribosyl transferase domain (PF11071) containing a HTH motif. (B) The gene contains a *cre* element close to its stop codon. Capitalized letters are conserved among *cre* elements (Fujihara et al. 2002). (C) The intergenic region upstream of *ytoQ* contains an annotated Spx *cis* element and a region bearing high similarity to regions upstream of *trxA* and *trxB* (dotted line) (nBLAST, Zuber et al. 2011; Altschul et al. 1997). The mutations leading to deregulation of *ytoQ* are shown in red squares. Ribosomal binding sites (RBS) are underlined, promoter regions are highlighted in grey and bold letters (Zuber et al. 2011; Rosenberg et al. 2017b). (D) Chromosomal context of *ytoQ*. Green and red dots depict promoters and terminators, respectively.

YtpP shows similarity to thioredoxins, YtpQ has been shown to be involved in iron homeostasis and YtpR is similar to a subunit of the phenylalanine-tRNA synthetase, suggesting an involvement in oxidative stress management (Subtiwiki, Zuber et al. 2011; Michna et al. 2016). Interestingly, an additional deregulating mutation in the intergenic region has been isolated by others in this lab from a B6 auxotrophic strain with the partial heterologous pathway evolved on solid minimal medium. This strain carries a base deletion upstream of the *ytoQ* promoter (nBLAST, Altschul et al. 1997; Yeak 2017). The fact that YtpP shows similarity to thioredoxin gave reason to compare the intergenic region between *ytoP* and *ytoQ* to the regions upstream of the two known thioredoxins from *B. subtilis*, *trxA* and *trxB*. Apparently, this new mutation is in a region with high similarity with the promoter regions of the Spx-regulated *trxA* (63%) and *trxB* (56%) genes, which gives a further hint that YtoQ is involved in the Spx-mediated oxidative stress response (Figure 39). Downstream of *ytoQ* a quite long 3'-UTR (729 bp) has been annotated, which overlaps with the convergently transcribed *ytoP*, encoding a protein similar to glutamyl aminopeptidase (Subtiwiki, Michna et al. 2016). This head-to-head orientation without an annotated terminator of the *B. subtilis ytoQ* transcript suggests a regulatory function of the 3'-UTR and a possible regulatory tandem between *ytoP* and *ytoQ*. Indeed, the transcriptomics data of *ytpP* shows a little dip at the very 3'-end and the amount of the *ytoQ* 3'-UTR transcripts decreases strongly when reaching the *ytpP* ORF (Nicolas et al. 2012). Interestingly, in a Rho-deficient mutant, the *ytoQ* transcript is not terminated at all and *ytpP* transcription is almost absent, showing that the transcription of *ytoQ* indeed has an influence on the convergently expressed gene *ytpP*. The hypothetical regulatory tandem between *ytoQ* and *ytoP* does not seem to be well conserved. YtoP cooccurs with YtpP and YtpR in most phylae of bacteria and even some eukaryotes, but the cooccurrence with YtoQ is limited to *Halomonas*, some Rhizobiales, Rhodobacteracae and Bacillacae according to STRING. There are also organisms encoding only YtoQ or YtoP (e.g. *E. coli* only encodes YtoP). However, STRING database and Pfam show contradictory results when searching conservation.

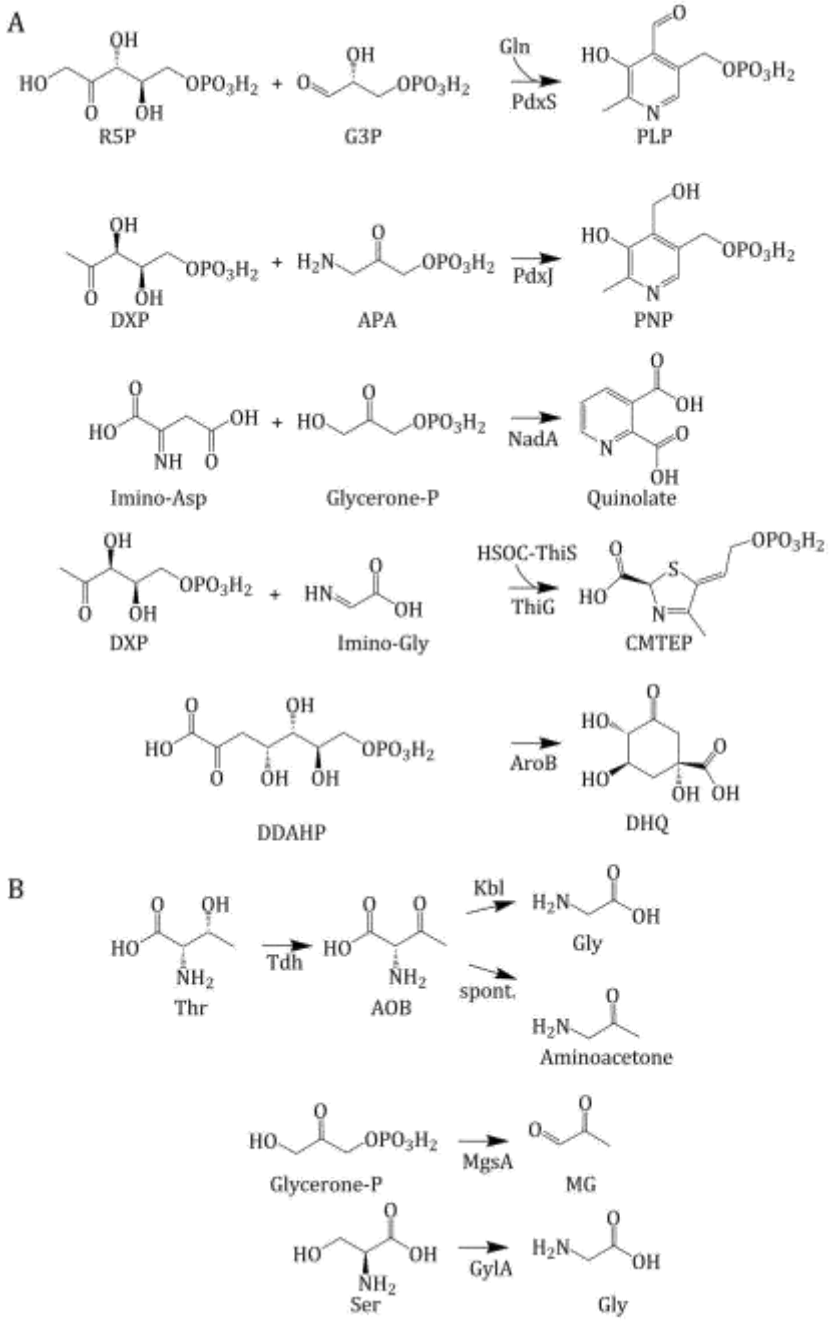

Figure 40: Examples for reactions with similarity to vitamin B6 synthesis (at the top of A). Side products were omitted for clarity. (A) Reactions using similar substrates and/or performing similar cyclization. (B) Reactions using substrates or leading to products with similarity to 1-amino-3-phosphohydroxy-acetone (APA). Amino acids are three-letter-code; R5P, ribulose-5-phosphate; G3P, glyceraldehyde-3-phosphate; PLP, pyridoxal phosphate; DXP, 1-deoxyxylulose-5-phosphate; PNP, pyridoxine phosphate; CMTEP, 2-[(2R-5Z)-2-carbohyxy-4-methylthiazol-5 (2H)-ylidene] ethyl phosphate; DDAHP, 2-dehydro-3-deoxy-D-arabino-heptonate-7-phosphate; DHQ, 3-dehydroquinate; AOB, 2-amino-3-oxobutyrate; MG, methylglyoxal.

For example, Pfam annotates a nucleoside deoxyribosyl transferase domain containing protein in *S. aureus* and a pBLAST search revealed a very similar and annotated YtoQ family protein in this genus, but STRING does not annotate a YtoQ homolog, thus I am recommending professional caution when using those databases and interpreting the presented results. However, the databases and BLAST agree on all other, arbitrarily tested organisms (Pfam, BLAST, STRING, Altschul et al. 1997; Finn et al. 2016). The *ytoQ* gene is furthermore subject to transfer-messenger RNA (tmRNA) -dependent termination close to its STOP codon under physiological conditions (LB, 37°C) (Fujihara et al. 2002). *B. subtilis* uses tmRNA to rescue ribosomes from stalled transcription and this mechanism has been connected to CCR and CcpA-dependent transcriptional inhibition in previous studies (Fujihara et al. 2002; Ujiie et al. 2009; Himeno et al. 2014). Interestingly, *ytoQ* also contains a *cre* site and it has been hypothesized that the tmRNA-dependent termination rescues the ribosomes from the CcpA roadblock at the *cre* site (see 1.4.3, Fujihara et al. 2002). It is not exactly known,

whether the tmRNA-dependent termination leads to a functional protein, because the isolated, custom tmRNA-tagged peptide had a similar size as the full-length protein, but the *cre* element is located well in front of the stop codon at bases 392-406 of 444bp (Figure 39).

Summarizing, the current knowledge about YtoQ connects it to the Spx-mediated oxidative stress response, pyrimidine salvage pathways, regulation of the unknown gene *ytoP*, and CCR. Distant relatives of YtoQ have been shown to be nucleoside deoxyribosyl transferases, but the enzymatic or regulatory nature of members of the YtoQ family of proteins is so far completely unknown.

4.11. Comparison to similar studies in *E. coli*

It has been shown in this work that growth of B6 auxotrophic *B. subtilis* cells in minimal medium without externally added vitamin B6 could be reestablished to wildtype level by introduction of the last two enzymes of the heterologous DXP-dependent pathway from *E. coli* with only two genomic alterations abolishing BSH synthesis and deregulating *ytoQ* (Figure 24 and Figure 30). As shown in Figure 41, the strains have managed to efficiently feed into the DXP-dependent pathway at the reaction of APA and DXP to PNP, mediated by PdxJ. The mechanism by which feeding occurs is still unknown, but previous studies have investigated a very similar situation with a truncated DXP-dependent pathway in *E. coli* (Kim et al. 2010; Kim, Copley 2012; Oberhardt et al. 2016).

In one study, the absence of the 4-phosphoerytronate dehydrogenase PdxB was overcome by three serendipitous pathways, which were identified of a plasmid collection with overexpression constructs of ORFs from *E. coli* (Kim et al. 2010; Kim, Copley 2012). One of the pathways was described in detail, where metabolites from serine synthesis were diverted by activity of the enzymes SerA, YeaB, LtaE and ThrB, converting 3-Phosphoglycerate to 4HTP in four enzymatic and one non-enzymatic steps. This pathway includes the condensation of glycine and glycolaldehyde to 4HT by LtaE, which has no homolog in *B. subtilis* and the addition of glycine to the medium had no observably beneficial effect in the presented work (BLAST, Figure 33, Altschul et al. 1997). Some of the enzymes involved in this serendipitous pathway have homologs in *B. subtilis* however, suggesting a similar mechanism in the suppressors presented in this work (Table 14). Interestingly, the observed suppressor mutations (Table 9 and Table 11) show no apparent connection to this or the other pathways described in the study, although it must be said that the *E. coli* strains with the serendipitous pathways have not been sequenced according to the publications and therefore might have acquired undocumented similar mutations (Kim et al. 2010; Kim, Copley 2012). Furthermore, this pathway feeds into the DXP-dependent pathway at the level of 4HTP and the presented *B. subtilis* formed suppressor mutants without PdxA, which would decarboxylate 4HTP. It could also be shown, that strains lacking PdxA could not utilize 4HTP also in presence of PdxJ and PdxH, indicating that *B. subtilis* has no endogenous 4HTP decarboxylase activity (data not shown). For the same reason, the remaining pathway consisting of HisB, Php and YjbQ feeding into B6 synthesis at the level of SerC, is no candidate for feeding in the presented mutants.

Table 14: *E. coli* genes found by Kim et al. and homologs in *B. subtilis* SP1.

E. coli	*B. subtilis**
hisB	*hisB*
php	none
yjbQ	*yugU*
yeaB	none
thrB	*thrB*
ltaE	none
folB	*folB*
aroB	*aroB*

*according to pBLAST and Pfam, (Altschul et al. 1997; Finn et al. 2016)

Another serendipitous pathway observed by Kim et al. was mediated by activity of AroB and it could be shown that overexpression of *B. subtilis aroB* gene from plasmid pBP632 could also enhance growth of the B6 auxotrophic strains expressing *pdxJH* and lacking BSH synthesis (and not the ones producing BSH) (data not shown). However, the growth promoting effect was much weaker than by overexpression of *ytoQ*, which is why this lead was not further followed. It shows however, that AroB could also be involved in the rewiring of

the pathway in *B. subtilis* and could be worth investigation in the future.

In the other study, ThiG has been shown to bypass PdxB in *E. coli*. It is suspected to be able to catalyze a reaction like PdxS from *B. subtilis* and was found in a bioinformatic screen for enzymes that are predicted to catalyze reactions similar to PLP-synthetic enzymes with a tool predicting promiscuous activities (Oberhardt et al. 2016). The precursors and exact reaction mechanisms remain to be elucidated but interestingly, the reactions catalyzed by ThiG and PdxJ very similar and one substrate (DXP) is identical, making ThiG an interesting candidate for future investigations (Figure 40). However, the fact that B6 auxotrophic *B. subtilis* strains did not form suppressors in absence of the partial DXP-dependent pathway despite presence of ThiG suggests that this enzyme is not able to take over the function of PdxS in this organism (Figure 26).

Other possible routes organisms could take to feed into the DXP-dependent pathway have been described by previous works. For example, proteins of 2-oxoglutarate and iron-dependent dioxygenases family (PF10014) which are able to hydroxylate free amino acids, including threonine to 4HT, have been found in several organisms (Smirnov et al. 2012). 4HT could then be phosphorylated either by ThrB or enzymes of the novel DUF1537 containing enzyme family and enter the DXP-dependent pathway at the level of PdxA. However, proteins of the 2-oxoglutarate and iron-dependent dioxygenases family are not present in *B. subtilis* SP1, although other species of *B. subtilis* supposedly have a protein containing such a domain (Pfam and BLAST, Altschul et al. 1997; Finn et al. 2016). Furthermore, as mentioned earlier, *B. subtilis* has no endogenous 4HTP decarboxylase activity and the strains in this work are lacking PdxA and could thus also not utilize the product of these interesting enzymes. Lastly, one point mutation leading to change of glycine to serine is enough to allow PdxJ to take over function of PdxH (Man et al. 1996). However, our strains were not able to form suppressors when only PdxJ was present (Figure S 6).

Therefore, previous approaches in other organisms show some resemblance to the case presented in this work but the comparability is very limited. Connections could only be observed for AroB, overexpression of which however showed a much weaker growth promoting effect than overexpression of YtoQ.

4.12. A model how feeding into the partial heterologous pathway occurs

The presented and discussed work shows that vitamin B6 auxotrophic *B. subtilis* expressing a truncated DXP-dependent pathway can grow in absence of vitamin B6 by feeding into the heterologous pathway. It can only do so, when BSH is absent and the unknown protein YtoQ present. Furthermore, data suggests that the feeding occurs at the reaction of APA and DXP to PNP, which is catalyzed by PdxJ. As previously discussed, *B. subtilis* is not known to produce APA, but some acetone derivatives and similar two-carbon compounds do exist as intermediates of other reactions (Figure 40). Thus, *B. subtilis* either produces APA using its underground metabolism, or it utilizes different metabolites to produce PNP via PdxJ. PdxJ is provably involved in PLP synthesis by these strains, because those isogenic variants expressing only PdxH were not able to form suppressor mutants on minimal medium and did also not benefit from inactivation of BSH synthesis (Figure 26). It has furthermore been shown that the unknown activity of YtoQ is required for growth of these strains, because those variants lacking YtoQ were not able to grow on minimal medium despite the absence of BSH synthesis (Figure 31). It can therefore be inferred that a novel, non-native pathway involving YtoQ, PdxJ and PdxH has been established in the investigated strains (Figure 41).

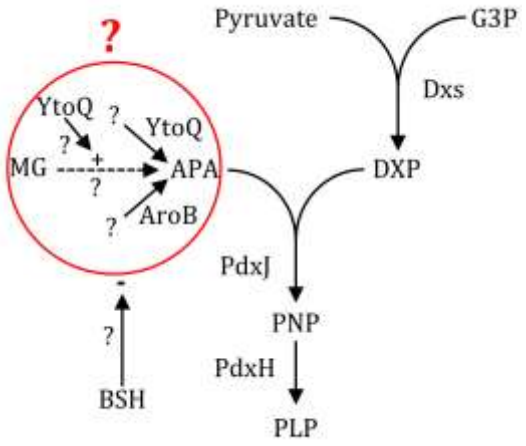

Figure 41: Scheme illustrating how *B. subtilis* could feed into the heterologous pathway. The known reactions of the PLP producing pathway are catalyzed by Dxs, PdxJ and PdxH. The unknown reactions are shown in a red cycle. YtoQ could activate enzymes that catalyze to the synthesis of 1-amino-3-phosphohydroxy-acetone (APA) from methylglyoxal (MG, as an example) or any other of the similar compounds. YtoQ and/or AroB could also directly catalyze a reaction producing APA. Bacillithiol (BSH) is by an unknown mechanism detrimental for feeding into the pathway. PNP, pyridoxine phosphate; PLP, pyridoxal phosphate; DXP, 1-deoxyxylulose-5-phosphate; G3P, glyceraldehyde-3-phosphate.

It is thereby unknown, how YtoQ mediates the feeding into the pathway. It could well do so as an enzyme producing APA directly, or producing a precursor that feeds into an underground reaction producing the compound. YtoQ could also regulate other enzymes. Either it inhibits enzymes catalyzing reactions that compete for substrates of the heterologous pathway, or it activates enzymes feeding into it. What we know, is that YtoQ is required and beneficial for the pathway. Furthermore, BSH must by some mechanism interfere with the pathway, but all tested hypotheses were rejected, which is why it can only be said that BSH needs to be absent for the heterologous pathway to be rewired. Therefore, *B. subtilis* is most likely producing APA via an unknown pathway involving YtoQ or/and AroB, which is inhibited by BSH by an unknown mechanism and this way feeds into the heterologous vitamin B6 pathway consisting of PdxJ and PdxH (Figure 41).

4.13. Unearthing underground metabolism

The ability of organisms to carry out enzymatic functions they probably do not require under 'normal' growth conditions has been described above and can be described as a second, invisible level of plasticity. It is called underground metabolism, because these reactions are usually very difficult to identify and are mostly found as accidentally observations. Underground metabolism consists of uncharacterized enzymes with unknown functions, reactions that need to be activated via mutation (of the specific enzyme or a regulatory sequence) and promiscuous enzyme activity. For several decades many groups in academia and industry have spent a lot of time and money to optimize synthetic pathways to produce valuable goods. In the process, heterologous expression systems and alternative routes for the utilization of carbon sources, the production of amino acid and fatty acid synthesis among others have been observed and most of these recombinant systems lead to adaptive mutations in the host genome, some of which have been discussed above (Chou, Marx 2012; Michener et al. 2014; Mizukami et al. 2014; Rand et al. 2017; Bracher et al. 2017; Jeschek et al. 2017). These findings were made independently and as parts of completely different projects. If all these efforts were orchestrated to unravel the underground potential of microbiological species, the understanding of host-heterologous pathway interactions would greatly profit from this; opening completely new horizons for strain design. It is hereby suggested to apply the approach presented in this work to other pathways. Several, non-homologous, convergent pathways are known so far and it is likely that more will be discovered in the future (Dairi et al. 2011). This knowledge presents a huge potential for the design of 'custom metabolisms' for heterologous engineering. To utilize the pathways, one must understand the mechanisms by which they can be rewired in a heterologous environment and which functional redundancies exist in the underground metabolism.

This work presents a ubiquitously applicable reverse engineering approach to achieve this. Reverse engineering of heterologous pathways

not only reveals the deleterious interactions between heterologous and host pathways but also the potential of the underground metabolism to rewire the partial pathways. This way completely new, hybrid pathways can be established that can have superior traits compared to the original ones. Due to the hybrid nature, the endogenous parts of the pathways have coevolved with the host metabolism, allowing detoxification mechanisms, export systems and cofactors to be properly regulated in concert with the production pathway. On the other hand, the heterologous parts of the pathway did not coevolve with the host and are therefore less likely to be feedback-inhibited by other pathways or pleiotropic regulators. Therefore, hybrid pathways would ideally combine the best of heterologous and host pathways.

4.13.1. Known redundant and degenerate pathways

As mentioned earlier, a recent review has dealt with the occurrence of non-homologous or homologous but mechanistically different pathways (Dairi et al. 2011). The following section will summarize these and some additional pathways and go into details for some of them. The most obvious and already explained example is vitamin B6 synthesis with the DXP-independent and the DXP-dependent pathways (see 1.2.1). Others are:

Isoleucine

Different pathways in archaea and bacteria as described above (see 4.7 ,Risso et al. 2008).

Mevalonate

Two pathways, one in most bacteria using G3P and pyruvate, which is also present in *B. subtilis* and *E. coli* (Rohmer et al. 1993), another in eukaryotes using Acetyl-CoA which is also present in some bacteria including the pathogens *S. aureus* and *Streptococcus pneumoniae* (Wilding et al. 2000; Goldstein, Brown 1990). Some organisms contain both pathways (Seto et al. 1996). The pathways have also already been subject to metabolic engineering for improved production of IPP as a precursor for biofuels (Kang et al. 2016). The endogenous MEP and the heterologous mevalonate (MVP) pathway have already been expressed for industrial application in *E. coli* (Martin et al. 2003; Yang, Guo 2014).

3-Dehydroquinate (DHQ)

The shikimate (SHK) pathway has been subject to intense efforts to achieve more efficient production of aromatic compounds by fermentation, including the optimization of carbon flux from central carbon metabolism and the optimized expression of the enzymes of the pathway (Liu et al. 2014; Gu et al. 2017b; Yu et al. 2016; Liu et al. 2016; Gottlieb et al. 2014; Gosset 2009; Papagianni 2012). Therefore, the existence of two alternative synthetic routes and the chance for reverse engineering as proposed in this work might be worth realizing. DHQ is part of the SHK pathway, which is present only in microorganisms and plants and absent in animals (Herrmann, Weaver 1999). DHQ is metabolized to chorismite in five steps, which are universally conserved among the organisms containing this pathway. After chorismate (from Latin `*fork*`) the pathway branches into the tryptophan, folate, terpenoid, menaquinone, and phenylalanine pathways. In most organisms the SHK pathway is initialized by the synthesis of DHQ by the 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase AroA and the 3-dehydroquinate synthase AroB from E4P and PEP (Herrmann, Weaver 1999). The synthesis of DHQ follows a different path in a few archaea like the marine methanogenic *Methanocaldococcus jannaschii* and the marine halophilic *Halobacterium salinarum* who do not contain any homologs of the *aroAB* genes. Instead, these organisms produce DHQ from fructose-1-6-bisphposphate (F16P) or fructose-1-phosphate (F1P). From either of these substrates they can form the compound 6-Deoxy-5-ketofructose 1-phosphate by an unique activity of fructose-bisphosphate aldolase Fba (EC 2.2.1.11) which is then further metabolized by the 2-amino-3,7-dideoxy-D-threo-hept-6-ulosonate synthase AroA' and the 3-dehydroquinate synthase II AroB' (White 2004; Gulko et al. 2014). This alternative pathway gives the possibility to alter the initial step of the shikimate pathway and allows for a reverse engineering approach as proposed in this work. Indeed, previous researchers already proposed a reverse engineering approach to unravel the last mysteries of this industrially extremely valuable pathway, concerning mainly the

phenylalanine branch (Báez-Viveros et al. 2007).

Menaquinone

Two pathways for synthesis of menaquinone are known, a CoA-dependent pathway and the futalosine pathway, both starting from chorismate (Nowicka, Kruk 2010). The CoA-dependent pathway was found first in *E. coli* and has been summarized elsewhere (Meganathan, Kwon 2009). Briefly, it starts with the formation of isochorismate by isochorismate synthase MenF. Then the 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate synthase MenD and the O-succinyl-benzoate synthase MenC metabolize it further until MenE attaches CoA to yield succinyl-benzoyl-CoA. The naphthoate synthase MenB further forms 1,4-dihydroxy-2-naphtoyl-CoA. The elimination of CoA is not well described but according to subtiwiki, MenB can also catalyze this reaction (Michna et al. 2016, subtiwiki). However, in *E. coli*, the hydrolysis of CoA is achieved by action of 1,4-dihydroxy-2-naphthoyl-CoA hydrolase MenI, which happens to be 53% identical with *B. subtilis* YuxO (Chen et al. 2013; Altschul et al. 1997, BLASTp). 1,4-dihydroxy-2-naphthoate octaprenyl transferase MenA and SAM-dependent menaquinone biosynthesis methyltransferase MenH then form menaquinone. The alternative futalosine pathway was found in *Streptomyces coelicolor* after bioinformatic studies revealed that several organisms, including *Helicobacter pylori* and *Campylobacter jejuni* were producing menaquinone but lacking the *men* genes (Hiratsuka et al. 2008). This pathway itself has two alternative routes, one including futalosine and the other skipping the name-giving compound. The initial step is carried out by the chorismate dehydratase MqnA forms 3-[(1-carbocyvinyl)oxy]benzoate, which is further metabolized by aminodeoxyfutalosine synthase MqnE to form 6-amino-6-deoxy-futalosine (Mahanta et al. 2013). From here either futalosine is formed by aminodeoxyfutalosine deaminases (Goble et al. 2013), which is then further metabolized to dehypoxanthine futalosine by MqnB, or futalosine is skipped by activity of methylthioadenosine nucleosidase (Kim et al. 2014). Dehypoxanthine futalosine is subsequently cyclized by action of the cyclic dehypoxanthinyl futalosine synthase MqnC (Cooper et al. 2013; Hiratsuka et al. 2008) and further metabolized to 1,4-dihydroxy-6-naphtoate (different to R-2-naphtoate in the CoA-dependent pathway) by action of 1,4-dihydroxy-6-naphthoate synthase MqnD (Hiratsuka et al. 2008). The final prenylation and methylation to form menaquinone has not been described in detail but is thought to be catalyzed by homologs of CtaO/B and MenG, respectively (Cooper et al. 2013; Altschul et al. 1997, BLASTp).

Spermidine

Also for spermidine, two non-homologous pathways are known, to both of which arginine is the initial substrate. The pathway discovered first, the SAM-dependent pathway is found in all eukaryotes, many archaea and many bacteria, including *E. coli* and *B. subtilis*. Here, Arginine is decarboxylated by arginine decarboxylase SpeA yielding agmatine, which is subsequently hydrolyzed by agmatinase SpeB forming putrescine (Wu, Morris 1973; Satishchandran, Boyle 1986; Carvajal et al. 1999). Then, an aminopropyl group from S-adenosyl-methionamine is transferred by spermidine synthase SpeE (Bowman et al. 1973). The latter substrate of this reaction originates from SAM and is formed by decarboxylation through S-adenosyl-methionine decarboxylase SpeD (Anton, Kutny 1987). *E. coli* furthermore has the ability to produce putrescene from ornithine by activity of ornithine decarboxylase SpeF, which is not present in *B. subtilis* (Applebaum et al. 1975). It was observed that many organisms including the pathogen *Vibrio cholerae*, do not contain the *spe* genes but instead homologs of the genes encoding *sym*-norspermidine, but anyhow produced spermidine. It was found that these enzymes encoded by these genes are able to produce both *sym*-norspermidine and spermidine and were part of an alternative polyamine pathway in organisms of the genus *Vibrio*, which is however present in many species (Lee et al. 2009). This L-aspartate-4-semialdehyde (ASA)-dependent pathway starts at putrescene, which is fused to ASA by carboxynorspermidine synthase CANSDH to form carboxyspermidine (Nakao et al. 1991) and then decarboxylated to spermidine by carboxynorspermidine decarboxylase CANSDC (Nakao et al. 1990).

Lysine

The lysine synthesis pathways (and analogously the ornithine pathways) are extremely diverse and several variants have been evolved to form the same compound. There are two major lysine synthesis routes, (I) the DAP and the aminoadipate (AA) route. The DAP route is again divided into 4 different pathways present in different organisms. All of these pathways originate in ASA, which is hydrolyzed and cyclized together with pyruvate by the 4-hydroxy-tetrahydrodipicolinate synthase DapA (Yugari, Gilvarg 1965; Blickling et al. 1997). In the next step, L-tetrahydrodipicolinate (TDC) is formed by 4-hydroxy-tetrahydrodipicolinate reductase DapB (Farkas, Gilvarg 1965; Devenish et al. 2010). From here, the different routes all originate.

The one present in *B. subtilis* is mostly limited to *Bacilli*. TDC N-acetyltransferase YkuQ forms N-acetyl-L-2-amino-6-oxopimelate, which is further metabolized to N-acetyl-DAP by tetrahydrodipicolinate N-acetyltransferase PatA. In the next step, DapI deacetylates its substrate to DAP, which is then transformed to m-DAP by the diaminopimelate epimerase DapF (Chatterjee, White 1982).

The alternative pathway in most other bacteria, which was also identified first is very similar to the pathway from *Bacilli*, but utilizes succinylation instead of acetylation. Here 2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-succinyltransferase DapD forms N-succinyl-L2-amino-6-oxopimelate from TDC, which is subsequently metabolized to N-succinyl-DAP by N-succinyldiaminopimelate aminotransferase ArgD (Simms et al. 1984; Ledwidge, Blanchard 1999). In the last unique step, DAP is formed by succinyl-diaminopimelate desuccinylase DapE (Kindler, Gilvarg 1960).

Two more alternatives exist in the DAP route, one is present in several bacteria including *Bacilli* and *Corynebacteria* and bypasses the need of any addition of acetyl or succinyl by use of the spontaneous decyclization of TDC to L2-amino-6-oxopimelate and subsequent NADPH-dependent amination to m-DAP by diaminopimelate dehydrogenase DAPDH (Misono et al. 1979). Another alternative is present in plants and cyanobacteria and forms DAP directly from TDC by action of the LL-diaminopimelate aminotransferase (Hudson et al. 2006).

The AA route originates from 2-oxoglutarate and Acetyl-CoA, which are metabolized to L-2-Aminoadipate in several enzymatic steps. At this compound the route divides into two different pathways, the fungal LysW pathway and the bacterial pathway. Fungi utilize LysW as a carrier protein for the reactions catalyzing the biosynthesis of lysine, which is also the case for ornithine synthesis, which will be described below. AA is bound to LysW by action of the ligase LysX and subsequently the substrate is phosphorylated by the LysW-gamma-L-alpha-aminoadipate kinase LysZ. In the following the phosphorylated LysW-AA is reduced to LysW-aminoadipate-6-semialdehyde by LysY and the transaminase LysJ converts it to LysW-lysine, which is cleaved from the carrier protein by LysK (Ouchi et al. 2013).

Ornithine

There are two non-homologous synthesis pathways for ornithine, which are widely analogous to the lysine synthesis pathways. There is the acetylation pathway in bacteria and the LysW pathway in fungi and archaea, which both start with glutamate as the initial substrate. The pathway from bacteria starts by acetylation of glutamate by N-acetylglutamate synthase ArgA (Maas et al. 1953) with subsequent phosphorylation, reduction, amination and deacetylation to ornithine by ArgBCDE, respectively (Gil et al. 1999; Baich, Vogel 1962; Ledwidge, Blanchard 1999; Meinnel et al. 1992). The alternative pathway from fungi is homologous to the lysine pathway described above with the enzymes ArgXBCDE as homologs of LysXZYJ, respectively (Ouchi et al. 2013).

The summarized pathways are all suitable for the suggested approach to reversely engineer them in a heterologous environment to unearth unknown host-heterologous interactions and underground metabolic activities as exemplified in Figure 38 and Figure 41.

5. Summary

Fermentative production of valuable goods is an economically competitive and ecologically sustainable alternative to chemical synthesis. Several previous studies have been dedicated to the establishment of vitamin B6 production strains, but none was successful to compete with chemical synthesis. Fermentative production of vitamin B6 is hampered by toxic intermediates, a toxic end-product and insufficient knowledge about vitamin B6 homeostasis and related proteins and pathways. This work is dedicated to the identification of deleterious interactions between the heterologous production pathway and the host metabolism, and proposes and applies an approach to design novel, hybrid production pathways combining host underground metabolism and partial heterologous pathways. Mutant derivatives of the Gram-positive model bacterium *Bacillus subtilis*, which are able to grow in presence of toxic amounts of pyridoxal 5'-phosphate are shown to carry mutations leading to deregulation of the biotin biosynthesis operon. Furthermore, the inhibitor of the threonine synthesis operon ThrR, which is also involved in resistance to the vitamin B6 synthesis intermediate 4-hydroxy-threonine phosphate, is characterized. In order to optimize an existing B6 production strain, targeted evolution is applied leading to a novel, non-native biosynthesis pathway of endogenous underground and heterologous enzyme activities. This approach is regarded to be ubiquitously applicable in different species and for other products and the characterization of the host-heterologous interferences is of great value for future projects for fermentative B6 production.

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7. Appendix

7.1. Materials

Table 15: Chemicals used in this work

Chemical	Manufacturer
2-oxobutanoate	Roth, Germany
Acrylamide	Roth, Germany
Agar	Roth, Germany
Agarose	Peqlab, Germany
Ammonium iron (III) citrate	Sigma-Aldrich, Germany
Ammonium Peroxydisulfate	Roth, Germany
Antibiotics	Sigma, Germany
Bromphenol blue	Serva, Germany
BSA	MBI Fermentas, Germany
Casamino acids	BD, Germany
Coomassie Brilliant Blue, G250	Roth, Germany
Desthiobiotin	IBA, Germany
D-Glucose	Merck, Germany
dNTPs	Roche Diagnostics, Germany
EDTA	Roth, Germany
Ethidium bromide	Roth, Germany
Ethidium bromide	Merck, Germany
Formaldehyde (37%)	Roth, Germany
Glutardialdehyde	Roth, Germany
Glutardialdehyde	Sigma Aldrich, USA
Glycerol	Merck, Germany
HCl	ChemSolute, Germany
Iron- (III)-Chloride	AppliChem, Germany
Isopropyl ß-D-1- thiogalactopyranoside	Peqlap, Germany
L-amino acids	Roth, Germany
Magnesium chloride	AppliChem, Germany

Chemical	Manufacturer
Methanol	AppliChem, Germany
Nutrient Broth	Merck, Germany
o-Nitrophenyl-β-D-Galactopyranosid	Sigma-Aldrich, Germany
Paraformaldehyde	Roth, Germany
Potassium chloride	AppliChem, Germany
RotiQuant	Roth, Germany
Silver nitrate	Roth, Germany
Skim milk powder	Oxoid, Germany
Sodium chloride	Roth, Germany
Sodium Dodecyl Sulfate	Roth, Germany
ß-Mercaptoethanol	Roth, Germany
Strep-Tactin Sepharose	IBA, Germany
Tetramethylethylenediamine (TEMED)	Roth, Germany
Tris (hydroxymethyl)aminomethane	Roth, Germany
Tryptone	Oxoid, Germany
Tween20	Sigma-Aldrich, Germany
X-Gal	Roth, Germany
Yeast extract	Oxoid, U.K.

Table 16:Auxilalary materials used in this work

Material	Manufacturer
96-Well-plates	Sarstedt, Germany
Centrifuge cups	Beckmann, Germany
Cuvettes (microlitre, plastic)	Greiner, Germany
Dialysis tube	Roth, Germany
Eppendorf tubes	Greiner, Germany
Erlenmeyer flasks (100 – 2000 ml)	Duran, Germany
Falcon tubes	Sarstedt, Germany
Gene amp reaction tubes (PCR)	Perkin Elmer, Germany

Material	Manufacturer
Glas pipette	Brand, Germany
Microlitre pipettes (1 µl, 2 µl, 20 µl, 200 µl, 1000 µl, 5 ml)	Eppendorf, Germany
Petri dishes	Greiner, Germany
Pipette tips	Greiner, Germany
Poly-Prep Chromatography columns	Bio-Rad, Germany
Polyvinylidendifluorid-Membran (PVDF)	Bio-Rad, Germany
Syringes	Terumo, Belgium
VivaSpin Turbo 15 concentrator	Sartorius, Germany

Table 17: Instruments used in this work

Machine or component	Manufacturer
Äkta	GE Healthcare
Biofuge primo R	Kendro, Germany
CDP detection system	Roche, Switzerland
Fiberlite F9/F40 rotors	Thermo Fischer Scientific, Germany
Filter set 37	Zeiss, Germany
Filter set F36-528	AHF Analysetechnik, Germany
French pressure cell press	Spectronic Instruments, G. Heinemann, Germany
Gel electrophoresis apparatus	PeqLab, Germany
HXP 120 light source	Zeiss, Germany
Incubator Innova R44	New Brunswick, Germany
ITC 200 microcalorimeter	MicroCal Inc., USA
LabCycler	SensorQuest, Germany
miniDawn Treos multiangle light scattering system	Wyatt Technology Europe, Germany
Mini-Protean III System	Bio-Rad, Germany
Nanodrop ND-1000	Thermoscientific, Germany
Optilab T-rEX RI detector	Wyatt Technology Europe, Germany

Machine or component	Manufacturer
pH meter Calimatic	Knick, Germany
Refrigerated centrifuge	Kendro, Germany
S75 Superdex 10/300GL	GE Healthcare
Scale	Sartorius, Germany
Special accuracy weighing machine	Sartorius, Germany
Spectral photometer Ultraspec 2100 pro	Amersham, Germany
Standard power pack	Bio-Rad, Germany
Sterile Bench Hera Safe	Thermo Scientific, Germany
Synergy MX Platereader	BioTek Instruments, USA
Thermocycler	Biometra, Germany
Thermomixer compact	Eppendorf, Germany
TS Sorvall WX ultraseries centrifuge/RC 6+	Beckmann Coulter, Germany
Ultracentrifuge, Sorvall Ultra Pro 80	Thermo Scientific, Germany
Ultrospec 2100 pro photometer	Amersham, Germany
UV Transilluminator 2000	Bio-Rad, Germany
Water desalination plant	Millepore, Germany
Zeiss Axioskop 40	Zeiss, Germany

Table 18: Kits used in this work

Kit or component	Manufacturer
DNeasy Tissue Kit (250)	Qiagen, Germany
GeneRuler DNA Ladder Mix	MBI Fermentas, Germany
Lambda DNA *Eco*RI + *Hind*III	MBI Fermentas, Germany
NucleoSpin Plasmid-Kit	Macherey-Nagel, Germany
Prestained Protein Marker (PageRuler)	MBI Fermentas, Germany
QIAquick PCR-Purification Kit	Qiagen, Germany

Table 19: Enzymes and antibodies

Enzyme	Manufacturer
Anti-rabbit immunoglobulin G-alkaline phosphatase secondary antibody	Promega, USA
FastAP	MBI Fermentas, Germany
Lysozoyme	Merck, Germany
PfuS-DNA polymerase	own purification
Phusion DNA polymerase	Finnzymes, Finland
Restriction nucleases	NEB Biolabs, Germany
RNase A	MBI Fermentas, Germany
T4-DNA ligase	MBI Fermentas, Germany
Tag-DNA polymerase	Roche Diagnostics, Germany

Table 20: *B. subtilis* Strains used in this work

Strain	Genotype	Reference
168	*trpC2*	Laboratory Collection
BKE27910	*ΔthrR::ermC*	Rudner, unpubl. Boston Genetic Stock Center
BKE29850	*ΔytoQ::ermC*	Rudner, unpubl. Boston Genetic Stock Center
BKE32250	*ΔthrC::ermC*	Rudner, unpubl. Boston Genetic Stock Center
BP494	*trpC2 bglS::(hag-cfp kan)*	Diethmaier et al. 2011
BP496	*trpC2 amyE::(hag-yfp cat)*	Diethmaier et al. 2011
BP555	*trpC2 codY::spc amyE::(P_{hom}-lacZ cat)*	Rosenberg et al. 2016
BP556	*trpC2 ΔthrR::ermC codY::spc amyE::(P_{hom}-lacZ cat)*	Rosenberg et al. 2016
BP557	*trpC2 ΔthrR::ermC amyE::(P_{hom}-lacZ cat)*	Rosenberg et al. 2016
BP558	*trpC2 amyE::(P_{hom}-lacZ cat)*	Rosenberg et al. 2016
BP559	*trpC2 amyE::(P_{hom}(C26T)-lacZ cat)*	Rosenberg et al. 2016
BP560	*trpC2 amyE::(P_{hom}(T9C)-lacZ cat)*	Rosenberg et al. 2016
BP900	*ΔpdxST::tet sacB::SC2* cat*	This work
BP901	*ΔpdxST::tet pBV415*	This work
BP902	*ΔpdxST::tet sacB::SC2* cat pBV415*	This work
BP908	*ΔpdxST::tet ΔthrR::ermC*	This work
BP911	*ΔpdxST::tet aprE:pdxH*	This work

Strain	Genotype	Reference
BP912	*ΔpdxST::tet aprE::pdxH amyE::SC1 spc*	This work
BP913	*ΔpdxST::tet aprE::pdxH amyE::SC1 spc sacB::SC2* cat*	This work
BP914	*ΔcomG::phleo*	This work
BP915	*ΔpdxST::tet ΔcomG::phleo*	This work
BP916	*ΔpdxST::tet aprE::pdxH ΔcomG::phleo*	This work
BP917	*ΔpdxST::tet amyE::SC1 spc ΔcomG::phleo*	This work
BP918	*ΔpdxST::tet aprE::pdxH amyE::SC1 spc ΔcomG::phleo*	This work
BP919	*ΔpdxST::tet sacB::SC2* cat ΔcomG::phleo*	This work
BP920	*ΔpdxST::tet ΔthrC::ermC*	This work
BP921	*ΔpdxST::tet aprE::pdxH amyE::SC1 spc sacB::SC2* cat ΔcomG::phleo*	This work
BP922	*ΔpdxST::tet amyE::SC1 spc sacB::SC2* cat ΔcomG::phleo*	This work
BP924	P_{bioW}*(A96G) sunT(ΔT449)*	This work
BP925	P_{bioW}*(A96G)* T_{trmB}*(ΔT24)*	This work
BP926	*bshC(C343T) yqeT(A461T)*	This work
BP927	*bshC(C343T) yqeT(A461T)*	This work
BP928	*bshC(C343T)*	This work
BP929	*bshC(T881A)*	This work
BP953	*ΔpdxST::tet ΔbshC::cat*	This work
BP956	*ΔpdxST::tet aprE::pdxH ΔbshC::cat*	This work
BP959	*ΔpdxST::tet amyE::SC1 spc aprE::pdxH ΔbshC::cat*	This work
BP962	*ΔpdxST::tet aprE::pdxH amyE::SC1 spc sacB::SC2* cat ΔthrC::ermC*	This work
BP965	*ΔpdxST::tet aprE::pdxH amyE::pdxJ spc*	This work
BP967	*ΔpdxST::tet amyE::SC1 spc aprE::pdxH bshA::pX2 cat*	This work
BP968	*amyE::(*P_{bioW}*-lacZ kan)*	This work
BP969	*amyE::(*T_{trmB}*-lacZ cat)*	This work
BP970	*amyE::(*P_{birA}*-lacZ kan)*	This work
BP971	*amyE::(*P_{bioW}(A96G)*-lacZ kan)*	This work
BP972	*amyE::(*T_{trmB}(ΔT449)*-lacZ cat)*	This work

Strain	Genotype	Reference
BP978	*ΔpdxST::tet amyE::pdxJ spc aprE::pdxH ΔbshC::cat*	This work
BP990	*Suppressor of BP912 with methionine*	This work
BP991	*Suppressor of BP912 with methionine*	This work
BP992	*Suppressor of BP912 with methionine*	This work
BP996	*amyE::P_{ytoQ}(C55A)-lacZ*	This work
BP997	*amyE::P_{ytoQ}-lacZ*	This work
BP999	*lacA::P_{gudB}-cfp ermC*	This work
BP1000	*amyE::P_{gudB}-yfp cm*	This work
BP1002	*ΔpdxST::tet amyE::pdxJ spc*	This work
BP1003	*ΔpdxST::tet amyE::pdxJ spc ΔbshC::cat*	This work
BP1005	*Suppressor of BP962 without threonine*	This work
BP1006	*Suppressor of BP962 without threonine*	This work
BP1007	*Suppressor of BP962 without threonine*	This work
BP1016	*ΔytoQ::ermC*	This work
BP1017	*ΔpdxST::tet ΔytoQ::ermC ΔbshC::cat amyE::pdxJ spc aprE::pdxH*	This work
BP1018	*ΔpdxST::tet ΔytoQ::ermC amyE::pdxJ spc aprE::pdxH*	This work
BP1032	*glyQ(C76T) comEC(G1739T) bshC::cat [ytoQ-ytzE]$^{ampl.}$*	Rosenberg et al. 2017b
BP1036	*P_{ytoQ}(C55A) bshC(ΔA464)*	Rosenberg et al. 2017b
BV411-1	*amyE::SC1 spc*	Commichau et al. 2014
BV412-1	*sacB::SC2* cat*	Commichau et al. 2014
BV604	*ΔpdxST::tet*	Commichau et al. 2014
BV605	*ΔpdxST::tet amyE::SC1 spc*	Commichau et al. 2014
BV606	*ΔpdxST::tet amyE::SC1 spc sacB::SC2* cat*	Commichau et al. 2014
BV609	*ΔpdxST::tet amyE::SC1 spc pBV415*	Commichau et al. 2014
BV708	*HT^{R}-1 (bcaP(A347G) ybxG(T907C)) P_{hom}(T9C)*	Commichau et al. 2015a
GP88	*trpC2 bshA::pX2 cat*	Landmann 2011
GP349	*spr8 (trpC2 ilvA2 P_{hom}(C26T))*	Rosenberg et al. 2016
SP1	*trpC^{+}*	Commichau et al. 2014

$^{ampl.}$ Genomic amplification

Table 21: *E. coli* strains used in this work

Strain	Genotype	Reference
DH5α	*recA1 endA1 gyrA96 thi hsdR17 rK- mK+ relA1 supE44 Φ80 ΔlacZ ΔM15 Δ(lacZYA-arg)U169*	Sambrook et al. 1989
BL21 (DE3)	*B(832)-derivate F- lon ompT hsdS(rB mB) gal dcm[DE3]*	Sambrook et al. 1989
BTH101	*F- cya-99' araD139 galE15 galK16 rpsL1 (str^R) hsdR2 mcrA1 mcrB1*	Karimova et al. 1998

Table 22: Plasmids used in this work

Plasmid	Relevant feature	Reference
p25-N	*MCS-T25*	Karimova et al. 1998
pAC6	transcriptional fusion to *lacZ cat*	Stülke et al. 1997
pAC7	translational fusion to *lacZ cat*	Weinrauch et al. 1991
pBHA12	*MCS kan*	Commichau et al. 2014
pBP241	*P_{gudB}-cfp*	Gunka 2010
pBP26	*P_{gudB}-yfp*	Gunka 2010
pBP323	*pGP172-thrR*	Rosenberg et al. 2016
pBP600	*pUT18C-codY*	This work
pBP601	*pUT18-codY*	This work
pBP602	*pKT25-codY*	This work
pBP603	*p25-N-codY*	This work
pBP604	*pUT18C-codY^Δ*	This work
pBP605	*pUT18-codY^Δ*	This work
pBP606	*pKT25-codY^Δ*	This work
pBP607	*p25-N-codY^Δ*	This work
pBP608	*pUT18C-thrR*	This work
pBP609	*pUT18-thrR*	This work
pBP610	*pKT25-thrR*	This work
pBP611	*p25-N-thrR*	This work
pBP612	*pUT18C-thrR^Δ*	This work
pBP613	*pUT18-thrR^Δ*	This work
pBP614	*pKT25-thrR^Δ*	This work
pBP615	*p25-N-thrR^Δ*	This work
pBP616	*pGP380-codY*	This work
pBP617	*pGP380-codY^Δ*	This work
pBP618	*pGP382-codY*	This work
pBP619	*pGP382-codY^Δ*	This work
pBP620	*pGP380-thrR*	This work
pBP621	*pGP380-thrR^Δ*	This work
pBP622	*pGP382-thrR*	This work
pBP623	*pGP382-thrR^Δ*	This work

Plasmid	Relevant feature	Reference
pBP624	*pUT18-birA*	This work
pBP625	*p25-N-birA*	This work
pBP627	*pAC7-*P_{bioW}	This work
pBP628	*pAC6-*P_{alf2}*-*T_{trmB}	This work
pBP629	*pAC7-*P_{birA}	This work
pBP630	*pAC7-*P_{bioW}(A96G)	This work
pBP631	*pAC6-*P_{alf2}*-*T_{trmB}(ΔT24)	This work
pBP637	*pAC7-*P_{ytoQ}(C55A)	Rosenberg et al. 2017b
pBP638	*pAC7-*P_{ytoQ}	Rosenberg et al. 2017b
pBP639	*pBQ200-ytoQ*	Rosenberg et al. 2017b
pBP640	*pET-SUMOadapt-ytoQ*	This work
pBP641	*pGP172-ytoQ*	This work
pBP642	*pGP574-ytoQ*	This work
pBQ200	P_{deg}*-MCS*	Martin-Verstraete et al. 1994
pBV415	*pdxH kan*	Commichau et al. 2014
pET-SUMOadapt	SUMO-His-MCS	Mossessova, Lima 2000
pGP172	*Strep-MCS*	Merzbacher et al. 2004
pGP380	*Strep-MCS*	Herzberg et al. 2007
pGP382	*MCS-Strep*	Herzberg et al. 2007
pGP574	*MCS-Strep*	Schilling et al. 2006
pKT25	*T25-MCS*	Karimova et al. 1998
pUT18	*MCS-T18*	Karimova et al. 1998
pUT18C	*T18-MCS*	Karimova et al. 1998

Table 23: Primers used in this work

Name	**Description**	**Sequence**
cat-check fw	*cat* check down-fragment	CTAATGTCACTAACCTGCCC
cat-check rev	*cat* check up-fragment	GTCTGCTTTCTTCATTAGAATCAATCC
cat-fw (kan)	*cat* gene with *kan* tags (Template: pGEM-cm)	CAGCGAACCATTTGAGGTGATAGGCGGCAA TAGTTACCCTTATTATCAAG
cat-rev (kan)	*cat* gene with *kan* tags (Template: pGEM-cm)	CGATACAAATTCCTCGTAGGCGCTCGGCCAG CGTGGACCGGCGAGGCTAGTTACCC
cat-rev (kan) w/o Terminator	*cat* gene with *kan* tags and without terminator (Template: pGEM-cm)	CGATACAAATTCCTCGTAGGCGCTCGGTTAT AAAAGCCAGTCATTAGGCCTATC
CD1	P_{nrgA} fw	AAAGAATTCTCCTTCCTTTCCATCCCTCG
CD2	P_{nrgA} rev	CATAAAAACTGGATCCCCCATTTGCAT
CZ68	*ermC* gene with *kan* tags and without terminator (Template: pDG646 and pDG647)	CGATACAAATTCCTCGTAGGCGCTCGGTTAC TTATTAAATAATTTATAGCTATTG
CZ126	*phleo* gene with *kan* tags (Template: pDG148) fw	CAGCGAACCATTTGAGGTGATAGGGAACGA TGACCTCTAATAATTG
CZ127	*phleo* gene with *kan* tags (Template: pDG148) rev	CGATACAAATTCCTCGTAGGCGCTCGGGTAG TATTTTTTGAGAAGATCAC

Name	Description	Sequence
HOMF131	Seq primer P_{hom} fw	CATCCATTTTCCTTCGCTTC
HOMR1151	Seq primer P_{hom} rev	TCAGGAACGATATGCCATCA
JR59	Seq primer SC1 *pdxJ* fw	AGATCTATCCACGCTGTGTAAAAATTTT
JR60	Seq primer SC1 *pdxA* rev	TGTATTACTAGAAAATAACATAGTAAAACG GACA
JR61	Seq primer SC1 *pdxA* fw	CACATCCCAGAACTTGCGGA
JR62	Seq primer SC1 *pdxJ* rev	ACACTGACAGTGTTATGTCCGG
JR63	Seq primer SC2 serC fw	GTGCCTGCAGGAATAAAATTCCC
JR64	Seq primer SC2 serC rev	GTTGTTCCTCGATAGAGTCCTCG
JR65	Seq primer SC2 *pdxR* fw	AAACTTCAGATATGGAAGCACTTATGC
JR66	Seq primer SC2 *pdxR* fw	CGGTGTTGGCTTACGGCA
JR67	Seq primer SC2 *serC* rev	ATGCGTCCGAACCCGTTT
JR68	Seq primer SC2 *epd* fw	ATGAGACGGATTAAACACGCATTC
JR69	Seq primer SC2 *epd* rev	TATAAAAAAATACCCTCCTAGTACAGGAGG
JR74	*aprE::pdxH* UP fw	CACTCCTTTTCATTTATATCGTAACCGAA
JR75	*aprE::pdxH pdxH* fw	TGATGATGATTGTCAAGTGAAGGCGCGCTA TGCTACAATACAGCTTGGAAATGGATCTCT AGGAGGTTAGAGCTCATGTCTGATAACGAC GAATTGCAG
JR76	*aprE::pdxH* DOWN fw	TGATGATCTTTCTACTATGGAAAAGGGTTA
JR77	*aprE::pdxH* DOWN rev	GCTATCAAGTATCGCTTGACTATAACATTT
JR78	*aprE::pdxH pdxH* rev	TTGATTAACCCTTTTCCATAGTAGAAAGAT CATCATCAGGGTGCAAGACGATCAAT
JR79	*aprE::pdxH* UP rev	GCCTTCACTTGACAATCATCATCAGATTAAC GTTAACGCAAACAACAAGC
JR80	Seq primer *sacB* locus fw	CAGGTACCATTTGCCGTTCATTT
JR81	Seq primer *sacB* locus rev	GTCGGCGAAACTTCTATTGACAG
JR82	Seq primer *amyE* locus rev	CTATTTGGCTTTTCCCCGGGA
JR83	Seq primer *amyE* locus fw	CAATCATACCACCAGTGATTATGCC
JR92	*aprE::pdxH* check fw	GATATGGTTTTCACATGGACTGAAACA
JR93	*aprE::pdxH* check rev	GGTTTTGTGTTTGTGCTTTCGC
JR94	Seq primer *amyE* locus fw 2	TATGAACAAAAAAGAAACCATCATTGATGG
JR95	Seq primer *amyE* locus rev 2	AGGAAGCGGAAGAATGAAGTAAGA
JR96	Seq primer *tetB* rev	GAAAATATAAACTAATGTTTCATTTTGCCA CTTG
JR97	Seq primer *tetB* fw	GGTTCCCTTGGGGAGAAGAAG
JR98	Seq primer *yqeT* fw	ATACGCTGTGACACCTACTAACTG
JR99	Seq primer *yqeT* rev	TGGTAGTTTTGAAATGGTCCGAATTAAG
JR100	Seq primer *yllA* fw	TTTTTATATGGCAGCATCAAAGCATTG
JR101	Seq primer *yllA* rev	CTTTCGCATCGATGGTATGCTG
JR102	Seq primer *yllA* fw2	AAGACCACGATTTGGATGAGATTAATTT
JR103	Seq primer *yllA* rv2	ATCACCGCCGTAAACTCTTCC
JR108	Cody for B2H *PstI* fw	AAACTGCAGGATGGCTTTATTACAAAAAAC AAGAATTATTAACTC
JR109	CodY for B2H *KpnI* rev NO STOP	TTTGGTACCCGATGAGATTTTAGATTTTCT AATTCAATTAGGAATTTGT

Name	Description	Sequence
JR110	CodY for pGP380 *BamHI* fw	AAAGGATCCATGGCTTTATTACAAAAAACA AGAATTATTAACTC
JR111	CodY for pGP382 SD *BamHI* fw	AAAGGATCCAAAGGAGGAAACAATCATGGC TTTATTACAAAAAACAAGAATTATTAACTC
JR112	CodY for pGP380 *PstI* rev	TTTCTGCAGTTAATGAGATTTTAGATTTTC TAATTCAATTAGGAATT
JR113	CodY for pGP382 *PstI* rev NO STOP	TTTCTGCAGATGAGATTTTAGATTTTCTAA TTCAATTAGGAATTTGT
JR114	CodY$^{\Delta}$ for pGP380 *PstI* rev STOP	TTTCTGCAGTTAAATTAGGAATTTGTTGTT TAGTACCTTGATATAAGT
JR115	CodY$^{\Delta}$ for pGP380 *PstI* rev NO STOP	TTTCTGCAGGAATTAGGAATTTGTTGTTTA GTACCTTGATATAAGT
JR116	ThrR for pGP380 *BamHI* fw	AAAGGATCCATGAAAGAGGAGACATTTTAT CTTGTCC
JR117	ThrR for pGP382 SD *BamHI* fw	AAAGGATCCAAAGGAGGAAACAATCATGAA AGAGGAGACATTTTATCTTGTCC
JR118	ThrR for pGP380 *PstI* rev	TTTCTGCAGTTATGCACCTGAACCTAATATT TCAACC
JR119	ThrR for pGP382 *PstI* rev NO STOP	TTTCTGCAGTGCACCTGAACCTAATATTTCA ACCT
JR120	ThrR$^{\Delta}$ for pGP380 *BamHI* fw	AAAGGATCCAAATACAGGGATGCTGTTTTT CCATT
JR121	ThrR$^{\Delta}$ for pGP382 SD *BamHI* fw	AAAGGATCCAAAGGAGGAAACAATCAAATA CAGGGATGCTGTTTTTCCATT
JR122	ThrR for B2H *XbaI* fw	AAATCTAGAGATGAAAGAGGAGACATTTTA TCTTGTCC
JR123	ThrR$^{\Delta}$ for B2H *XbaI* fw	AAATCTAGAGAAATACAGGGATGCTGTTTT TCCATT
JR124	ThrR for B2H *KpnI* rev NO STOP	TTTGGTACCCGTGCACCTGAACCTAATATTT CAACCT
JR125	CodY$^{\Delta}$ for B2H *KpnI* rev NO STOP	TTTGGTACCCGAATTAGGAATTTGTTGTTT AGTACCTTGATATAAGT
JR126	*comG::phleo* UP fw	AATCGCTCATTTTGTTCGTTTAAAAGAAT
JR127	*comG::phleo* DOWN rev	CGAATTTGAAGGGGAGCTTAACAAT
JR128	*comG::phleo* check rev	AGCGCTAAAAAAATTGAAATGGCG
JR129	*comG::phleo* UP rev	CCTATCACCTCAAATGGTTCGCTGCTATATG GACGGGTTTCTTATTACATTCAT
JR130	*comG::phleo* DOWN fw	GCGCCTACGAGGAATTTGTATCGCGCCTTTC ACCTATATCCATTGC
JR131	*yllA::cat* UP fw	GGAAGACGAATATATTATTTTTGCAAAACG G
JR132	*yllA::cat* UP rev	CCTATCACCTCAAATGGTTCGCTGCTTTGCT AAAACAATGATTGAAATGATTTTATGT
JR133	*yllA::cat* DOWN fw	CGAGCGCCTACGAGGAATTTGTATCGGCAA GAACGGATTTGGAATATCATGT
JR134	*yllA::cat* DOWN rev	CAGAATGAGCTGTCCTTTATAATCGG
JR135	*yllA::cat* check fw	AGTCTGAAGGAATCCGGCTTTATAA
JR142	cat::yllA check rev	AATCAAATAAAATGCCGTCTACTTCTGT
JR154	Seq primer *thrR* rev	GCAAAATCAACTTCGCCTGCA
JR155	Seq primer *thrR* fw	CGATGGAAAATGAAGAAGTGCCAT
JR184	Seq primer *bioO* fw	CGGGACATTTCGAAACAGCAG

Name	Description	Sequence
JR185	Seq primer *bioO* rev	TCCGCCGGATATATGCTTTCC
JR186	Seq primer *sunT* fw	CAAGAAAATAAGACATTCGAAGCCCTA
JR187	Seq primer *sunT* rev	CGAAGATGCACCTTATTAAGACCATAC
JR188	Seq primer T_{trmB} fw	GGATCGATTCATTTTAAAACGGACAAC
JR189	Seq primer T_{trmB} rev	ATGATGATATTGAGCCCATCCTTTTG
JR192	*amyE::pdxJ spc* KI *pdxJ* fw	ACCAGTGATTATGCCGCGATTTAAGCCGTTTCTCCACAAGCT
JR193	*amyE::pdxJ spc* KI *pdxJ* rev	CCTGATCTTCAAATAAAGCACTCCCCAAGGGTTTATTGTTTTCTAAAATCTGATTACCAATTAGAATG
JR194	*amyE::pdxJ spc* UP rev	ATCGCGGCATAATCACTGGT
JR195	*amyE::pdxJ spc* UP fw	GCATTATGTTTGAATTTCCGTTTAAAGAATG
JR196	*amyE::pdxJ spc* DOWN fw	GGGAGTGCTTTATTTGAAGATCAGG
JR197	*amyE::pdxJ spc* DOWN rev	CTTTGATGTCTTTTTGTTTGTGAAGTATTTC
JR198	*amyE::pdxJ spc* check fw	GTAAAAGTGCGGGAGGAAGGT
JR199	*amyE::pdxJ spc* check rev	AACAAAACCCGCTCCGATTAAAG
JR200	*bioW* promoter for *lacZ* fusion fw *EcoRI*	AAAGAATTCGACTGTAAAAAGAAATCGAAAAAGACCG
JR201	*bioW* promoter for *lacZ* fusion rev *BamHI*	TTTGGATCCATCATTTCTCGCCCTTTCACTGA
JR202	*trmB* terminator with P_{alf2} for *lacZ* fw *EcoRI*	AAAGAATTCATTCTTGTCAAGTGAAGGCGCGCTATGCTATAATACAGCTTGGAATGGGGCTGAGGTTGAATGGAGAA
JR203	*trmB* terminator for *lacZ* rev *BamHI*	TTTGGATCCCGTCAAGCCAGGTCAACGT
JR204	*birA* operon promoter for *lacZ* fw *EcoRI*	AAAGAATTCCACACAAAACCCCTTTTCCTTCC
JR205	*birA* operon promoter for *lacZ* rev *BamHI*	TTTGGATTCACTGAACGAAACCTCCTATCATCC
JR206	Seq primer *bshB2* fw	CTTCAACTTTGTCTGGAATCCGTC
JR207	Seq primer *bshB2* rev	TTGGCTTGAAAACAGAAGAAGGC
JR208	Seq primer *bshB2* with promoter rev	AACGGCTGCCACTGTATCAA
JR209	P_{gudB} rev with perfect SD *BamHI*	TTTGGATCCCTCCTCCTAATTCGAATCTTCTGTTTCTCACATGCTCCCTTTC
JR210	*amyE::PgudB-cfp ermC cfp* rev	GCTTTCCAATACACGAAAGATATGTCATTACTTATAAAGTTCGTCCATGCCAAGTGTAATG
JR211	*amyE::PgudB-cfp ermC ermC* fw	CATATCTTTCGTGTATTGGAAAGCGATCCTTTAACTCTGGCAACCC
JR212	*amyE::PgudB-cfp* and *aprE::PgudB-yfp ermC* P_{gudB} fw	ACACGGGTAATGCCAAGTGTTTGCAAAAGTCATTCAGCTTTCAGAAAGCTTACAGCGAATC
JR213	*amyE::PgudB-cfp ermC* UP rev	ACTTTTGCAAACACTTGGCATTACCCGTGTGGGAGTGCTTTATTTGAAGATCAGG
JR214	*amyE::PgudB-cfp ermC* DOWN fw	CGCCTACGAGGAATTTGTATCGATCGCGGCATAATCACTGGT
JR215	*aprE::PgudB-yfp cat* UP rev	ACTTTTGCAAACACTTGGCATTACCCGTGTTCTTTCTACTATGGAAAAGGGTTAATCAAC
JR216	*aprE::PgudB-yfp cat cat* rev	GCTTTCCAATACACGAAAGATATGTCATTACTTGTACAGCTCGTCCAT

Name	Description	Sequence
JR217	*aprE::PgudB-yfp cat cat* fw	CATATCTTTCGTGTATTGGAAAGCCGGCAATAGTTACCCTTATTATCAAGATAAG
JR218	*aprE::PgudB-yfp cat* DOWN fw	GCGAGCGCCTACGAGGAATTTGTATCGCGTTAACGCAAACAACAAGCTG
JR219	Seq primer *bshC* with promoter fw	TGAAAGCTCCTGAATGCCGTAT
JR252	*metE* for *lacZ EcoRI* fw	AAAGAATTCTCGCAGAAAAAGCAGAGCAATC
JR256	*aroB* for pBQ200 *BamHI* fw	AAAGGATCCAGGAGGACAAACATGAAGACACTGCATGTTCAAACTG
JR257	*aroB* for pBQ200 *PstI* rev	TTTCTGCAGTCATGATGTCTCCTCCAATCGC
JR260	*ytoQ::kan* UP fw	TTGTCTCTGCGGTATGATCGTC
JR261	*ytoQ::kan* UP rev	CCTATCACCTCAAATGGTTCGCTGGTAATCGGGAGCTTCAGTGATTTTG
JR262	*ytoQ::kan* DOWN fw	CGAGCGCCTACGAGGAATTTGTATCGCTCCACCATCCTTTAAAAGAGCTTT
JR263	*ytoQ::kan* DOWN rev	TGAAATTCAAACAGATAACGGGCC
JR264	*ytoQ::kan* check fw	GTTCAAAATCCGGCTTGCGT
JR265	*ytoQ::kan* check rev	AGCGCTTTTCCAAACACTGAC
JR266	*ytoQ* for pBQ200 *BamHI* fw	AAAGGATCCAGGAGGACAAACATGGAGTTTATCGTCTATTTAGCAGGA
JR267	*ytoQ* for pBQ200 *PstI* rev	TTTCTCCAGTTATTCTGTTTCAAATAGATAGGAAAGGGC
JR268	P_{ytoQ} for pAC7 *EcoRI* fw	AAAGAATTCAGACCAATCATCCTTTACCGCTT
JR269	P_{ytoQ} in pAC7 *BamHI* rev	TTTGGATCCATAATAAAAATCCCTCCTTATGTATATCCATCTG
JR276	Seq primer *glyQ* fw	TTTTTTCTTTAAGCCAGCCTGTCAG
JR277	Seq primer *glyQ* fw	AAAGCGCTTTTTAGATCAAATGGAAAG
JR278	Seq primer *comEC* fw	AGCACGCGGATAGAATGTCTC
JR279	Seq primer *comEC* rev	TCTATCCTCTTCTATTTTTCAGCAGGTT
JR280	Seq primer P_{ytoQ} fw	GGGTGTCTTTTTCTCTGTCAAACA
JR281	Seq primer P_{ytoQ} rev	GCAGACAGTGGATGCGATTAAAG
JR294	T_{trmB} for *lacZ* (pAC6) P_{alf2} *BamHI* fw	AAAGAATTCATTCTTGTCAAGTGAAGGCGCGCTATGCTATAATACAGCTTGGAATGCGTCCAAAAAGTAAAAGACAGCG
JR296	*ytoQ* for pET-SUMOadapt *BsaI* fw	AAAGGTCTCATGGTATGGAGTTTATCGTCTATTTAGCAGGA
JR297	*ytoQ* for pET-SUMOadapt *XhoI* rev	TTTCTCGAGTTATTCTGTTTCAAATAGATAGGAAAGGGC
JR298	*ytoQ* for pGP172 *SacI* fw	AAAGAGCTCGATGGAGTTTATCGTCTATTTAGCAGGA
JR299	*ytoQ* for pGP172 *BamHI* rev	TTTGGATCCTTATTCTGTTTCAAATAGATAGGAAAGGGC
JR300	*ytoQ* for pGP574 *SacI* fw	AAAgagctcATGGAGTTTATCGTCTATTTAGCAGGA
JR301	*ytoQ* for pGP574 *BamHI* NO STOP rev	TTTGGATCCTTCTGTTTCAAATAGATAGGAAAGGGC
JR302	*ytoQ* for pGP380 *BamHI* fw	AAAGGATCCATGGAGTTTATCGTCTATTTAGCAGGA

Name	Description	Sequence
kan-check fw	*kan* check down-fragment	CATCCGCAACTGTCCATACTCTG
kan-check rev	*kan* check up-fragment	CTGCCTCCTCATCCTCTTCATCC
kan-fw	*kan* gene (Template: pDG780)	CAGCGAACCATTTGAGGTGATAGG
kan-rev	*kan* gene (Template: pDG780)	CGATACAAATTCCTCGTAGGCGCTCGG
kan-rev w/o Terminator	*kan* gene without terminator (Template: pDG780)	TTACTAAAACAATTCATCCAGTAAAATAT
mls-check fw	*mls* check down-fragment	CCTTAAAACATGCAGGAATTGACG
mls-check rev	*mls* check up-fragment	GTTTTGGTCGTAGAGCACACGG
mls-fw (kan)	*ermC* gene with *kan* tags (Template: pDG646 and pDG647)	CAGCGAACCATTTGAGGTGATAGGGATCCT TTAACTCTGGCAACCCTC
mls-rev (kan)	*ermC* gene with *kan* tags (Template: pDG646 and pDG647)	CGATACAAATTCCTCGTAGGCGCTCGGGCCG ACTGCGCAAAAGACATAATCG
MT24	P_{hom} fw	AAAAGAATTCATGAATATGCGGGCGCAGAA GCT
MT25	P_{hom} rev	AAAAGGATCCTTCAAAAAAACTCCACCTTTC TTTTGATTGTCC
p25-N fw	Seq primer p25-N fw	GCTTCCGGCTCGTATGTTGTGTG
p25-N rev	Seq primer p25-N rev	GCCATCGAGTACGGCTGCGG
pKT25 fw	Seq primer pKT25 fw	CGACTCGGCGCGCAGTTCG
pKT25 rev	Seq primer pKT25 rev	GCAAGGCGATTAAGTTGGGTAACGC
pUT18 fw	Seq primer pUT18 fw	CTTCCGGCTCGTATGTTGTGTGG
pUT18 rev	Seq primer pUT18 rev	GCCGGCGCGAGCGATTTTCC
pUT18C fw	Seq primer pUT18C fw	GAAGTTCTCGCCGGATGTACTGG
pUT18C rev	Seq primer pUT18C rev	CGGGTGTTGGCGGGTGTCG
pWH844 fw	Seq primer pWH844 MCS fw	TATGAGAGGATCGCATCACCAT
spec-check fw	*kan* check down-fragment	GTTATCTTGGAGAGAATATTGAATGGAC
spec-check rev	*kan* check up-fragment	CGTATGTATTCAAATATATCCTCCTCAC
spec-fw (kan)	*spc* gene with *kan* tag (Template: pDG1726)	CAGCGAACCATTTGAGGTGATAGGGACTGG CTCGCTAATAACGTAACGTGACTGGCAAGA G
spec-rev (kan)	*spc* gene with *kan* tags (Template: pDG1726)	CGATACAAATTCCTCGTAGGCGCTCGGCGTA GCGAGGGCAAGGGTTTATTGTTTTCTAAAA TCTG
T7-Prom.	Seq primer out of the T7 promoter	TAATACGACTCACTATAGGG
T7-Term.	Seq primer out of the T7 terminator	GCTAGTTATTGCTCAGCGG
tc-check-fw	*tet* check down-fragment	CGGCTACATTGGTGGGATACTTGTTG
tc-check-rev	*tet* check up-fragment	CATCGGTCATAAAATCCGTAATGC
tc-fwd2 (kan)	*tet* gene with *kan* tags (Template: pDG1514)	CAGCGAACCATTTGAGGTGATAGGGCTTAT CAACGTAGTAAGCGTGG
tc-rev (kan)	*tet* gene with *kan* tags (Template: pDG1514)	CGATACAAATTCCTCGTAGGCGCTCGGGAAC TCTCTCCCAAAGTTGATCCC

7.2. Supplementary information

7.2.1. Supplementary figures

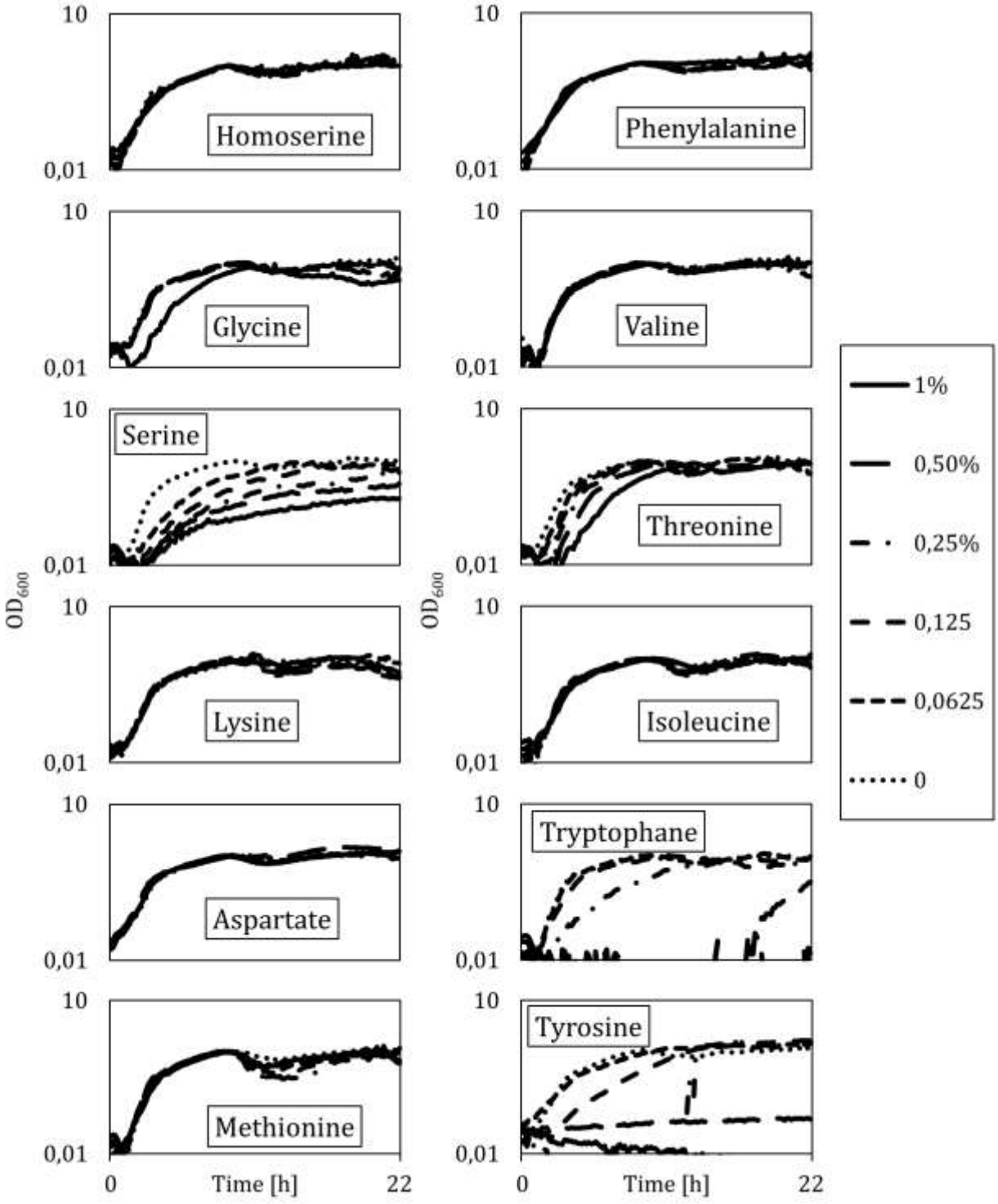

Figure S 1: Maximal non-toxic concentrations for *B. subtilis* 168 in CSE Glc minimal medium.

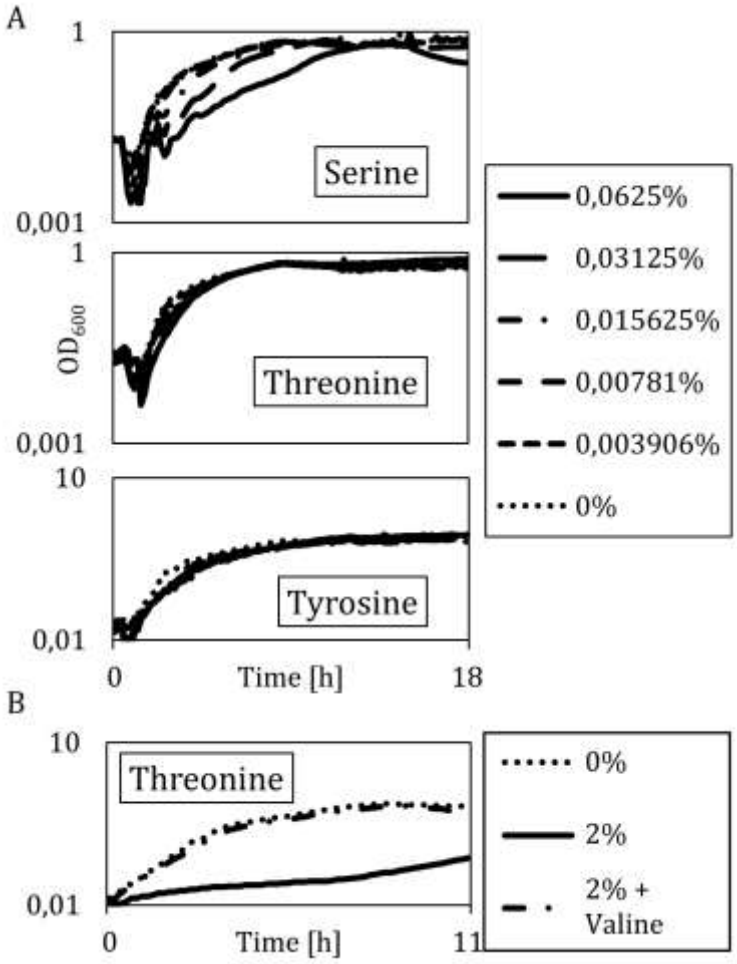

Figure S 2: (A) Maximal non-toxic concentrations for *B. subtilis* 168 of serine, threonine and tyrosine in CSE Glc minimal medium. (B) Growth in presence of 2% threonine with and without addition of 0.005% valine.

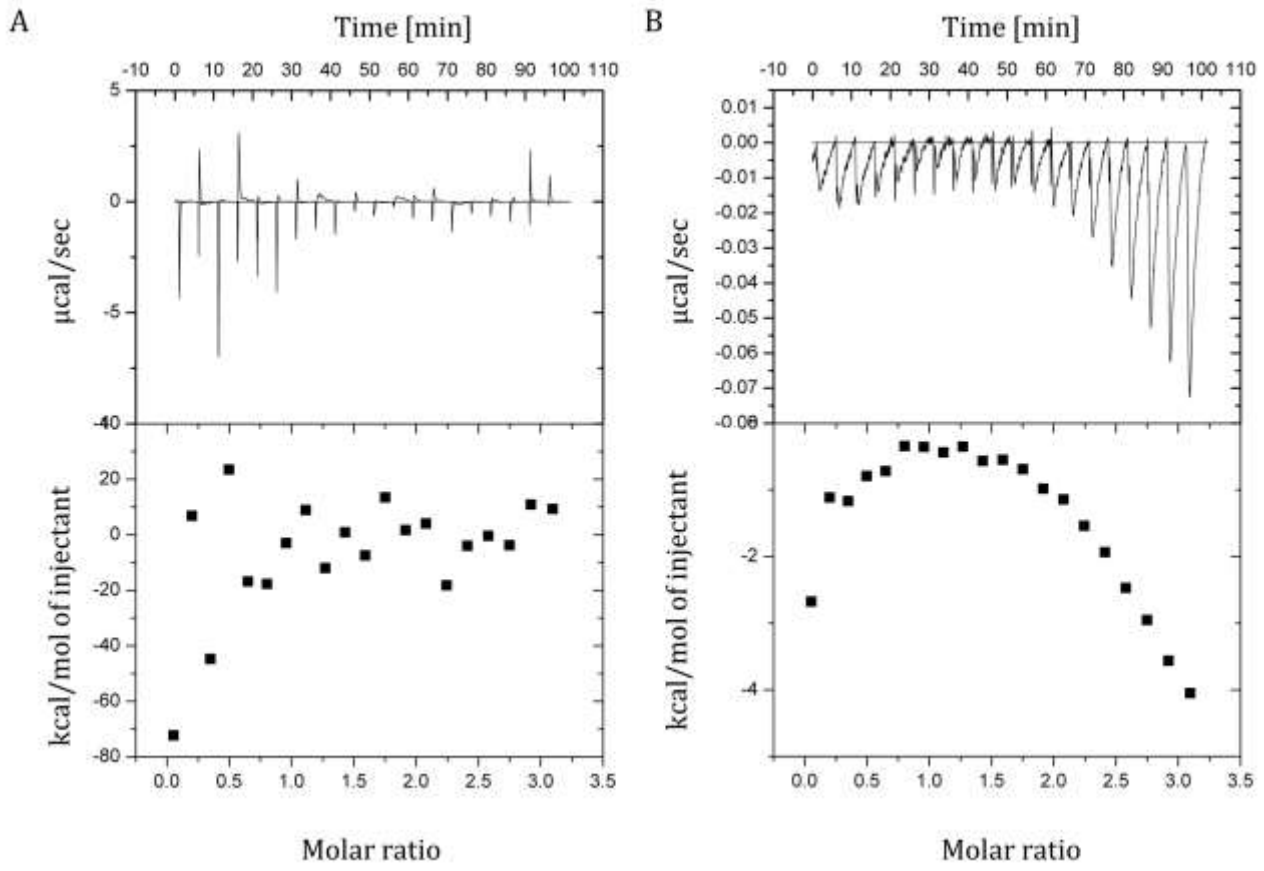

Figure S 3: ITC of ThrR with cysteine in absence (A) and presence (B) of DTT. 10 µM ThrR with 150 µM Cysteine in reaction buffer (20 mM Tris, 150 mM NaCl, 1 mM $MgCl_2$ with and without 1mM DTT). Injections of 14 µl of ligand solution with 1 µl s^{-1} and 300 seconds spacing at 20°C, a reference power of 12.5 µcal s^{-1} and 500 rpm stirring.

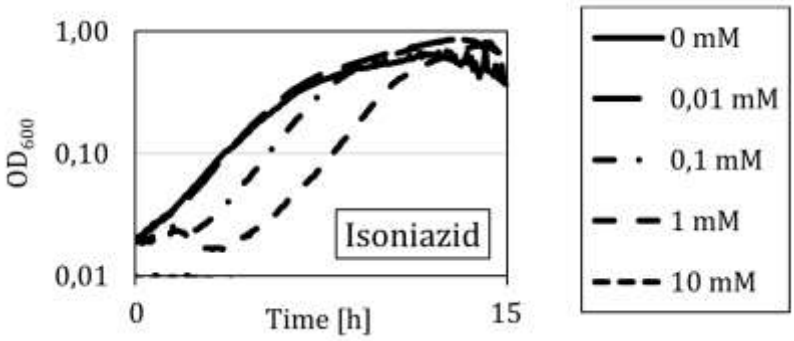

Figure S 4: Analysis of growth of *B. subtilis* 168 in C Glc minimal medium in presence of different concentrations of isoniazid to determine the highest non-toxic concentration.

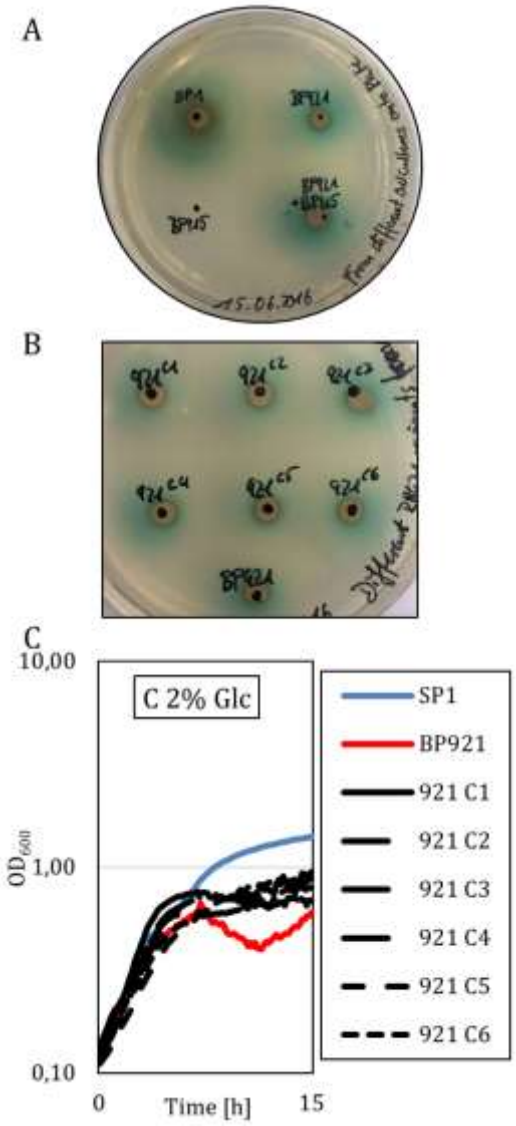

Figure S 5: Analysis of evolved BP921 from consortium C. Passaged BP921 does not produce more B6 or grow better. (A and B) 10 µl of a well-grown culture were dropped onto indicator plates containing strain BP905 and X-Gal. Compared to wild type SP1, evolved BP921 and consortia produce less vitamin B6 and evolved BP921 do not produce considerably more B6 than the original strain. (C) The evolved BP921 strains do not grow considerably better than the original strain in C Glc minimal medium (2% glucose).

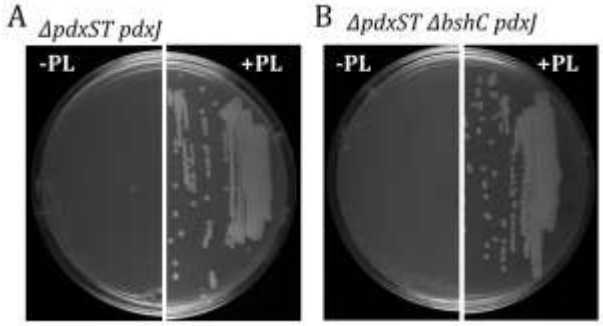

Figure S 6: The introduction of *pdxJ* alone was not sufficient for the B6 auxotrophic strain to form suppressor mutants on C Glc minimal medium. The deletion of *bshC* in this background also did not allow growth in absence of PL. Strains were streaked on C Glc minimal medium after washing them twice in C Glc and hungering for at least 2 hours.

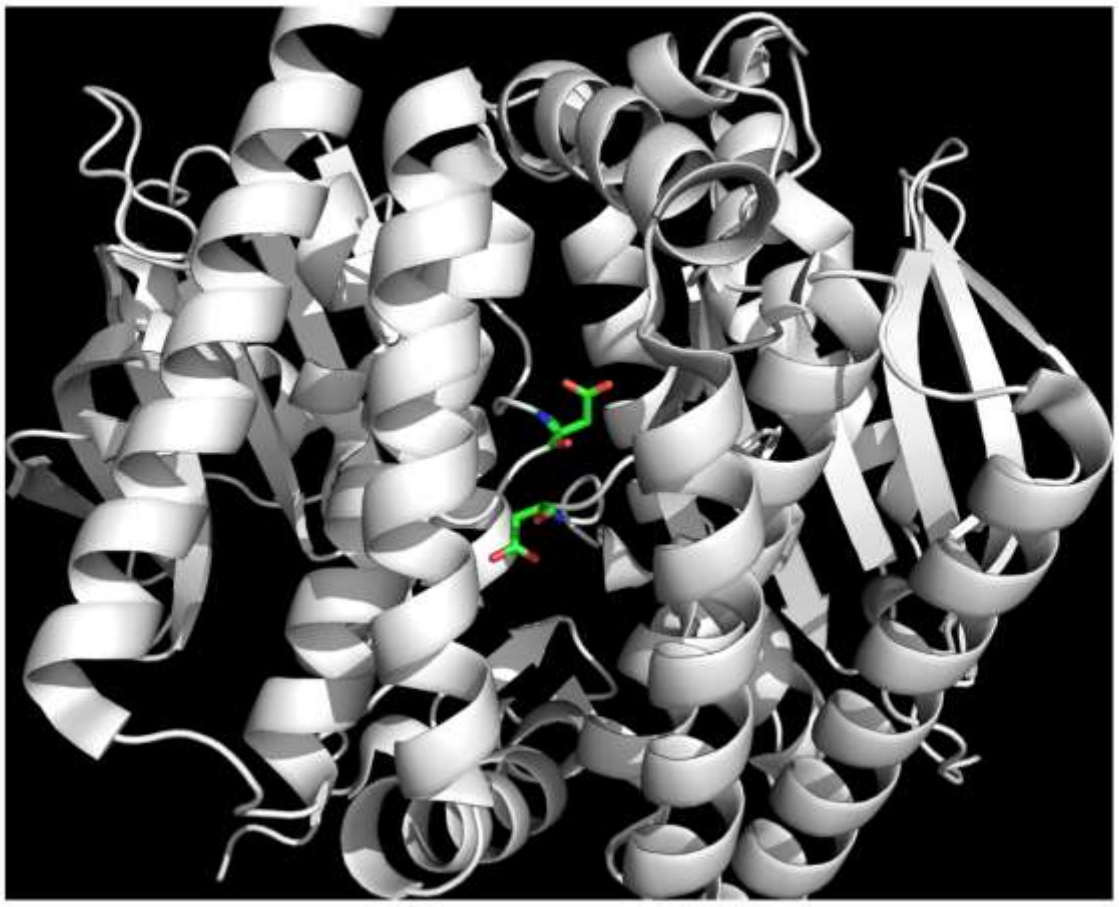

Figure S 7: Structure of GlyQ (1J5W) with the highlighted residue D26.

7.2.2. Supplementary tables

Table S 1: YtoQ DP-bind DNA binding prediction.

POS	RES	S_LBL	S_PRB	K_LBL	K_PRB	P_LBL	P_PRB	MAJ_CON	STR_CON
1	M	0	0,8055	0	0,7329	1	0,5137	0	NA
2	E	0	0,6848	0	0,8028	0	0,6743	0	0
3	F	0	0,7537	0	0,5745	0	0,5412	0	0
4	I	1	0,7125	1	0,8085	1	0,8083	1	1
5	V	0	0,5303	1	0,5804	1	0,7198	1	NA
6	Y	1	0,8573	1	0,8128	1	0,8145	1	1
7	L	1	0,535	0	0,7716	0	0,5471	0	NA
8	A	0	0,6397	0	0,5711	0	0,5569	0	0
9	G	1	0,6537	1	0,6279	1	0,5382	1	1
10	E	1	0,6219	1	0,7174	0	0,7986	1	NA
11	I	0	0,5236	0	0,6207	0	0,565	0	0
12	H	1	0,8011	1	0,5798	1	0,7291	1	1
13	S	0	0,576	1	0,5052	1	0,5598	1	NA
14	N	1	0,7157	1	0,6068	1	0,5976	1	1
15	W	1	0,7868	1	0,8002	1	0,7641	1	1

POS	RES	S_LBL	S_PRB	K_LBL	K_PRB	P_LBL	P_PRB	MAJ_CON	STR_CON
16	R	1	0,5844	1	0,5731	1	0,5642	1	1
17	E	0	0,858	0	0,8825	0	0,679	0	0
18	E	0	0,6843	0	0,8267	0	0,7795	0	0
19	I	0	0,7665	0	0,799	0	0,8574	0	0
20	K	0	0,7938	0	0,8551	0	0,7866	0	0
21	E	0	0,7976	0	0,9159	0	0,7913	0	0
22	K	0	0,6896	0	0,794	0	0,5957	0	0
23	T	0	0,7595	0	0,7554	0	0,7509	0	0
24	K	0	0,6853	0	0,8846	0	0,6688	0	0
25	S	0	0,6782	0	0,5673	0	0,8576	0	0
26	L	0	0,8014	0	0,776	0	0,9319	0	0
27	K	0	0,8925	0	0,8563	0	0,8709	0	0
28	L	0	0,948	0	0,9584	0	0,9177	0	0
29	P	0	0,8792	0	0,8107	0	0,8606	0	0
30	I	0	0,7375	0	0,8419	0	0,7282	0	0
31	T	0	0,7052	0	0,5921	0	0,6279	0	0
32	F	0	0,6398	0	0,655	0	0,6263	0	0
33	V	1	0,5619	1	0,5663	1	0,6527	1	1
34	G	0	0,6222	0	0,5069	0	0,5828	0	0
35	P	0	0,7357	0	0,7459	0	0,6567	0	0
36	M	1	0,56	1	0,5997	1	0,5716	1	1
37	E	1	0,5414	0	0,6924	1	0,5565	1	NA
38	N	0	0,7898	0	0,6354	0	0,5529	0	0
39	H	0	0,5642	0	0,5107	0	0,5184	0	0
40	D	0	0,8625	0	0,824	0	0,9382	0	0
41	R	0	0,5158	0	0,5008	0	0,5285	0	0
42	S	0	0,7402	0	0,7473	0	0,7026	0	0
43	D	0	0,8293	0	0,8288	0	0,8046	0	0
44	N	0	0,8004	0	0,8344	0	0,9145	0	0
45	I	0	0,9607	0	0,9759	0	0,9717	0	0
46	G	0	0,9014	0	0,8004	0	0,8836	0	0
47	E	0	0,9509	0	0,9352	0	0,9455	0	0
48	E	0	0,9558	0	0,9754	0	0,9771	0	0
49	I	0	0,9588	0	0,9645	0	0,9524	0	0
50	M	0	0,9428	0	0,936	0	0,9182	0	0
51	G	0	0,9177	0	0,9323	0	0,8578	0	0
52	V	0	0,9757	0	0,9876	0	0,888	0	0
53	Q	0	0,889	0	0,8705	0	0,8391	0	0
54	P	0	0,8293	0	0,8458	0	0,8667	0	0
55	N	0	0,8448	0	0,9484	0	0,839	0	0
56	A	0	0,6082	0	0,5767	1	0,5422	0	NA
57	V	0	0,773	0	0,7662	0	0,6558	0	0
58	L	0	0,5255	0	0,551	1	0,5666	0	NA
59	K	1	0,7463	1	0,8442	1	0,8615	1	1
60	D	0	0,7253	0	0,7061	1	0,5061	0	NA

POS	RES	S_LBL	S_PRB	K_LBL	K_PRB	P_LBL	P_PRB	MAJ_CON	STR_CON
61	D	1	0,5511	0	0,528	1	0,7631	1	NA
62	K	1	0,8204	1	0,59	1	0,8021	1	1
63	A	1	0,5485	1	0,6072	0	0,5628	1	NA
64	S	1	0,648	1	0,6318	1	0,6467	1	1
65	D	1	0,9133	1	0,8902	1	0,7797	1	1
66	I	0	0,5667	1	0,6823	1	0,5039	1	NA
67	N	1	0,8726	1	0,7594	1	0,6684	1	1
68	N	1	0,7827	1	0,837	1	0,6738	1	1
69	F	0	0,7117	0	0,6907	0	0,534	0	0
70	R	1	0,9405	1	0,98	1	0,8554	1	1
71	T	1	0,8683	1	0,8802	1	0,8194	1	1
72	A	1	0,8006	1	0,7164	1	0,7161	1	1
73	V	1	0,6487	1	0,7011	1	0,579	1	1
74	L	0	0,777	0	0,8149	0	0,7799	0	0
75	M	0	0,8738	0	0,9083	0	0,7069	0	0
76	N	0	0,7265	0	0,8144	0	0,8155	0	0
77	K	0	0,8663	0	0,8564	0	0,7291	0	0
78	A	0	0,8734	0	0,8982	0	0,8856	0	0
79	D	0	0,9265	0	0,8349	0	0,9078	0	0
80	F	0	0,9263	0	0,8929	0	0,8731	0	0
81	V	0	0,8991	0	0,9058	0	0,8754	0	0
82	I	0	0,611	0	0,5641	0	0,6699	0	0
83	A	0	0,6902	0	0,5545	0	0,7634	0	0
84	L	1	0,7333	1	0,6213	1	0,5486	1	1
85	F	1	0,5809	0	0,5234	1	0,577	1	NA
86	G	1	0,5234	1	0,5225	1	0,5006	1	1
87	E	0	0,8222	0	0,8037	0	0,6757	0	0
88	K	1	0,915	1	0,9422	1	0,7908	1	1
89	Y	1	0,5915	1	0,6806	1	0,6839	1	1
90	K	1	0,8549	1	0,8065	1	0,8073	1	1
91	Q	1	0,8926	1	0,9332	1	0,7772	1	1
92	W	1	0,8498	1	0,906	1	0,8529	1	1
93	N	1	0,9456	1	0,8975	1	0,8683	1	1
94	T	1	0,732	1	0,7217	1	0,5418	1	1
95	A	1	0,632	1	0,7391	1	0,5745	1	1
96	M	0	0,5126	1	0,5031	1	0,6727	1	NA
97	D	1	0,7336	1	0,7317	1	0,5863	1	1
98	A	1	0,6217	0	0,6535	0	0,5298	0	NA
99	S	1	0,5326	1	0,5735	1	0,6376	1	1
100	Y	0	0,6292	0	0,602	1	0,6042	0	NA
101	A	1	0,5407	0	0,5372	0	0,7088	0	NA
102	I	0	0,6975	0	0,7385	0	0,6235	0	0
103	A	1	0,5348	0	0,5114	0	0,608	0	NA
104	K	1	0,5252	0	0,563	0	0,6021	0	NA
105	G	1	0,7363	1	0,764	1	0,5944	1	1

POS	RES	S_LBL	S_PRB	K_LBL	K_PRB	P_LBL	P_PRB	MAJ_CON	STR_CON
106	K	1	0,6172	1	0,6799	1	0,7453	1	1
107	P	0	0,7802	0	0,719	0	0,5065	0	0
108	L	0	0,5553	0	0,6494	0	0,5114	0	0
109	I	0	0,8781	0	0,8428	0	0,8247	0	0
110	I	0	0,7554	0	0,908	0	0,8048	0	0
111	I	0	0,9434	0	0,9617	0	0,9009	0	0
112	R	0	0,6968	0	0,5702	0	0,6479	0	0
113	P	0	0,9617	0	0,954	0	0,9268	0	0
114	E	0	0,8748	0	0,9279	0	0,9228	0	0
115	S	0	0,7988	0	0,8312	0	0,8756	0	0
116	L	0	0,8578	0	0,8409	0	0,8364	0	0
117	H	0	0,8899	0	0,8608	0	0,8701	0	0
118	H	0	0,6618	0	0,6533	0	0,5225	0	0
119	P	0	0,6765	0	0,7172	0	0,7118	0	0
120	L	0	0,8015	0	0,5711	0	0,8443	0	0
121	K	1	0,799	1	0,705	1	0,5282	1	1
122	E	1	0,6528	1	0,6221	0	0,6241	1	NA
123	L	0	0,8666	0	0,8515	0	0,8183	0	0
124	S	0	0,549	0	0,6091	0	0,7198	0	0
125	N	0	0,5345	0	0,7904	0	0,6812	0	0
126	K	1	0,6902	1	0,6475	1	0,5575	1	1
127	A	0	0,7296	0	0,5836	0	0,6026	0	0
128	N	0	0,6803	0	0,8241	0	0,5659	0	0
129	I	0	0,7173	0	0,8686	0	0,7313	0	0
130	T	0	0,667	0	0,7016	0	0,5417	0	0
131	V	0	0,88	0	0,9408	0	0,7858	0	0
132	E	0	0,842	0	0,8842	0	0,6727	0	0
133	T	0	0,9297	0	0,9738	0	0,8731	0	0
134	V	0	0,9321	0	0,9392	0	0,8113	0	0
135	N	0	0,88	0	0,7893	0	0,8415	0	0
136	Q	0	0,9067	0	0,9516	0	0,9153	0	0
137	A	0	0,9875	0	0,9838	0	0,9526	0	0
138	I	0	0,9669	0	0,9313	0	0,9491	0	0
139	K	0	0,797	0	0,8896	0	0,827	0	0
140	A	0	0,9508	0	0,8407	0	0,9005	0	0
141	L	0	0,8902	0	0,922	0	0,8052	0	0
142	S	0	0,8124	0	0,6801	0	0,6246	0	0
143	Y	0	0,9006	0	0,918	0	0,6337	0	0
144	L	0	0,8977	0	0,8613	0	0,7558	0	0
145	F	0	0,9036	0	0,9548	0	0,791	0	0
146	E	0	0,8514	0	0,8815	0	0,6599	0	0
147	T	0	0,9505	0	0,9461	0	0,8635	0	0
148	E	0	0,9251	0	0,9519	0	0,9202	0	0

Table S 2: Sequences of the rationally designed constructs.

Enzyme	Construct sequence (promoter,TSS, RBS, start codon)
Epd	actgcgtcaatacacgttgacactcttttgagaatatgttctattatcagggagaaaaggtggaacggtgatgatcgacatg
PdxB	aattttgtcaaaataattttattgacaacgtcttattaacgttgataccggttaaattttatttgacaaaaatgggctcgtgttgaaca ataaatgtggagaaaaggtggaacggtgatgatcgacatg
SerC	gttaagatggcaagcttgacaagtatttccgacacatttacaatgaagttggagaaaaggtggaacggtgatgatcgacatg
PdxA	gttaagatggcaagcttgacaagtatttccaacggatttacaatgaagttggagaaaaggtggaacggtgatgatcgacatg
PdxJ	gttaagatggcaagcttgacaagtatttccgacacatttataatgaagttggagaaaaggtggaacggtgatgatcgacatg
PdxH	actgcgtcaatacacgttgacactcttttgatgctttgttaaattatcagggagaaaaggtggaacggtgatgatcgacatg
Dxs	actgcgtcaatacacgttgacactcttttgatgctttgttaaattatcagggagaaaaggtggaacggtgatgatcgacatg

7.2.3. Supplementary code

Code S 1: R script for the determination of the amplification.

```
#read data
data1 <- read.csv(choose.files(), header=T)
#extract amplification
amp <- data1[3093515:3112936, ]
#extract rest
rst <- data1[c(1:3093514,3112937:4277542), ]
#remove zeros
nz <- rst[rst$Coverage!=0, ]
#check whether zeros are correct
zero <- rst[rst$Coverage==0, ]
summary(zero) #only zeros
summary(nz) #no zeros
#calculate means
meanrest <- mean(nz$Coverage)
meanamp <- mean(amp$Coverage)
#calculate fold change
foldamp <- meanamp/meanrest
#make dataset for plot
amp.plot <- data1[3083515:3122936, ]
#make plot
xaxis <- seq(length=39422, from=3044174, by=1)
plot(xaxis, amp.plot$Coverage, type="l", ylab="Coverage", xlab="Genome position")
plot
```